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ENANTIORESOLUTION OF SUBSTITUTED 2-METHOXY-6-OXO-1,4,5,6-TETRAHYDRO-PYRIDINE-3-CARBONITRILES ON MACROCYCLIC ANTIBIOTIC AND CYCLODEXTRIN STATIONARY PHASES

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ABSTRACT

Nine different 4-, or 5- substituted racemic pyridones were synthesized and resolved by reversed phase LC. Seven of the compounds showed complete or partial resolution on the vancomycin bonded phase column, while five compounds each were separated on both the teicoplanin and the β-cyclodextrin chiral stationary phase (CSPs). No enantioselective separations were obtained on α- or γ-cyclodextrin stationary phases. The antineoplastic agent methotrexate also was resolved. Structural factors that significantly altered enantioselectivity included: changing the pyridone substituent from the 4 to the 5 position or vice versa, changing the size of the substituent, changing the degree of unsaturation of the substituent and changing the nature and length of the substituent "tether". The enantioselectivity of the two related macrocyclic antibiotic CSPs are somewhat similar but not identical. This provides a highly useful optimization approach for these columns. Frequently, when partial enantioresolution is obtained on one antibiotic CSP, a complete resolution is obtained on the related column using

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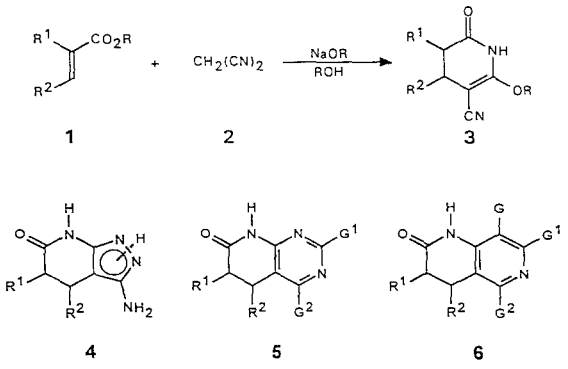
identical elution conditions. It is apparent that these separations (and CSPs) are highly complementary to each other.

INTRODUCTION

The synthesis of 2-methoxy-4-dimethoxymethyl-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitrile was reported in 1978 by Victory and Diago (1,2). The reaction of an α, β-unsaturated ester (see structure **1** below) and malononitrile (compound **2** below) provides a straight-forward route to the related 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles (structure **3** below) (3). Since α,β-unsaturated esters, **1**, can have a wide range of substituents, they are versatile starting materials and have been used in the general synthesis of pyrazolo[3,4-b]pyridines (structure **4** below) (4), pyrido[2,3,-d]pyrimidines (structure **5** below) (5-10), and 1,6-naphthyridines (structure **6** below) (11,12). When there is a substituent (R^1 or R^2) other than hydrogen on the α, β-unsaturated ester, a stereogenic-center is created upon addition to the nitrile (see compounds **3**,**4**,**5** and **6** below). These products are racemic and must be resolved if any stereoselective studies are to be done. A series of racemic pyridones related to compound **3** were synthesized, but not yet resolved by any analytical or preparative techniques.



Scheme 1

Recently macrocyclic antibiotics have been proposed as novel chiral selectors in LC, TLC, CE, foam flotation, etc. (13-18). Both ansa compounds (13,14) and particularly oligophenolic glycopeptides (13,16-18) have been used to successfully resolve a large number of enantiomers. We found that vancomycin, teicoplanin and ristocetin A make excellent LC chiral stationary phases when covalently attached to a silica gel support (13,19). In this work, we examine the chromatographic resolution of several racemic 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles and the compound methotrexate on both the macrocyclic antibiotic stationary phases and the more traditional β-cyclodextrin bonded phase.

EXPERIMENTAL

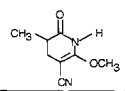
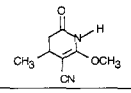
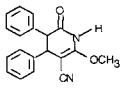
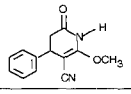
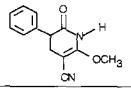
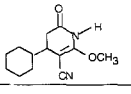
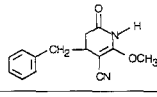
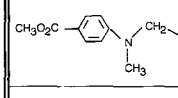
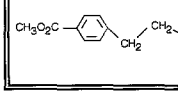
Chemicals

All of the racemic substituted pyridone compounds used in this study were synthesized by Dr. J. I. Borrell and Associates of the Institut Químic de Sarrià, Barcelona, Spain. Methotrexate and its (+)-enantiomer were purchased from Sigma (St. Louis, MO). Teicoplanin was the generous gift of Marian Merrill Dow (Kansas City, MO). All HPLC solvents (acetonitrile, methanol, triethylamine, glacial acetic acid, methyl-*tert*-butyl-ether) were obtained from Fisher Scientific (Pittsburgh, PA). The doubly filtered distilled water was used to prepare triethylammonium acetate buffer.

Methods

The HPLC was performed with a Waters model 590 HPLC with a 745B data module and a UV detector with a fixed wavelength of 280 nm or a Shimadzu LC6A with a variable wavelength SPD6A UV-detector (254 nm) and a CR601 Chromatopac recorder. All separations were carried out at a flow rate of 1.0 ml/min and at room temperature (~ 22°C). Mobile phase compositions are listed in Table I. The vancomycin-bonded stationary phase was prepared according to the recent work of Armstrong (13). It is now available commercially as the

Table I. Chromatographic Data for the Enantioresolution of 4 or 5 Substituted 2-Methoxy-6-oxa-1,4,5,6-tetrahydropyridine-3-carbonitriles.

Compound	Code	k'[a]	α	R_s	Mobile Phase[b]	Column[c]
	L_{Me}	0.85	1.23	0.14	A	Van
	L_C	2.37 0.51 2.30	1.14 1.06 1.34	1.90 0.60 1.56	A A D	β Van Tei
	L_{PhPh}[d]	3.62[d] 1.91[d]	8.50[d] 1.91[d]	9.35[d] 2.52[d]	A E	β Tei
	L_{Ph}	4.00 0.53 3.31	1.08 1.14 1.38	1.25 1.50 2.21	A C D	β Van Tei
	L_A	3.30 1.18	1.12 1.12	1.66 0.90	A A	β Van
	L_{CH}	0.59 4.10	1.13 1.44	0.91 1.72	C D	Van Tei
	L_{BZ}	6.37 3.00	1.10 1.20	1.70 0.96	A D	β Tei
	L_{M1}	1.43	2.19	5.40	A	Van
	L_{M2}	2.40 1.46	1.38 1.23	2.50 1.45	C A	Van Van

[a] Capacity factor for the first eluted enantiomer.

[b] Mobile phases are: A=90% buffer I/10% acetonitrile; B=495/5/1/1 (acetonitrile/methanol/acetic acid/triethylamine by volume); C=4ml methyl-*tert*-butyl ether/200 ml mixture of 90% buffer I and 10% methanol; D=90% buffer II/10% methanol; E: 70% buffer II/30% methanol. Buffer I contains 1% triethylammonium acetate, pH 7.0. Buffer II contains 1% triethylammonium acetate, pH 4.1.

[c] Column β, Van and Tei stand for β-cyclodextrin, vancomycin and teicoplanin bonded stationary phases, respectively.

[d] This compound has two stereogenic centers and consists of two pairs of enantiomers. While the diastereomeric separation was adequate on all columns, only a partial resolution of the enantiomers was obtained. In the case of the β-cyclodextrin column the second pair of enantiomers was partially resolved ($R_s \cong 0.6$); while in the case of the teicoplanin column, the first pair of enantiomers was partially resolved. ($R_s \cong 0.5$).

ERRATUM

S. Chen, Y. Liu, D. W. Armstrong, J. I. Borrell, B. Martinez-Taipel, J. L. Matallana: "Enantioresolution of Substituted 2-Methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles on Macrocyclic Antibiotic and Cyclodextrin Stationary Phases," which was published in Journal of Liquid Chromatography, **18**, 1495-1507 (1995)

The separation data for compound L_{M1} in Table 1 of the above cited paper should be as follows:

$$\alpha = 1.20,\ R_S = 0.6$$

instead of:

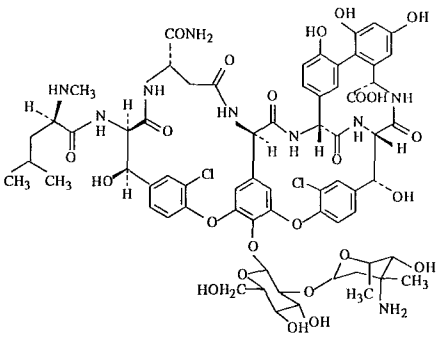
$$\alpha = 2.19,\ R_S = 5.40$$

CHIROBIOTIC V column from Advanced Separation Technologies (Whippany, NJ). The teicoplanin column was prepared in the same way as previously reported for vancomycin (13). The β-cyclodextrin bonded stationary phase (Cyclobond I, 250 x 4.6 mm i.d.) was obtained from Advanced Separation Technologies as were the γ- and α-cyclodextrin bonded phase columns. The 1%, pH = 4.0 ~ 7.0 triethylammonium acetate buffer was prepared by dissolving 10 ml of HPLC grade triethylamine in HPLC grade water and diluting it to a volume of 1 liter. Glacial acetic acid was used to adjust the solution to the desired pH.

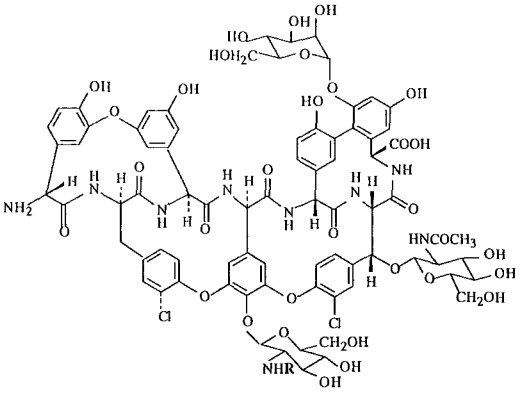
RESULTS AND DISCUSSION

A series of pyridones (based on 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitrile) were synthesized with various substituents at either the 4 or 5 positions. All of these compounds are racemic mixtures. They also are considered precursors for potential antineoplastic agents. Given current regulatory concerns (20) and the fact that enantiomers often produce difficult biological responses (21,22), it was necessary to have an analytical means to monitor individual enantiomers. Other secondary considerations were that the method be compatible with water containing solvents (in view of the synthetic process and work-up, as well as future *in vitro* and *in vivo* studies) and that analogous preparative scale separations be feasible. To our knowledge these racemic substituted pyridones have not been resolved previously by any means.

Recently vancomycin (a chiral, macrocyclic, oligophenolic, glycopeptide antibiotic) was attached to 5μ silica gel and subsequently shown to be an effective chiral stationary phase (CSP) (13). Native vancomycin is ionizable and has a molecular weight of 1449. The aglycone portion of the molecule consists of three small, fused macrocyclic rings. Together they form a "basket-shaped moiety" (13,15). A freely rotating disaccharide is attached to the "basket". More extensive details on the chemical and physical properties of vancomycin and its molecular recognition properties have been published (15). Teicoplanin A_2 also is a chiral, macrocyclic, oligophenolic glycopeptide antibiotic that is related to



Vancomycin



Teicoplanin A_2

A_2-1: R=(Z)-4-decanoic acid; A_2-2: R=8-methylnonanoic acid;
A_2-3: R=n-decanoic acid; A_2-4: R=8-methyldecanoic acid;
A_2-5: R=9-methyldecanoic acid.

Figure 1. Structures of the two oligophenolic, glycopeptide, macrocyclic antibiotics used in this study. Note that the aglycone portion of vancomycin consists of three fused rings while that of the teicoplanin A_2 consists of four fused rings. Both contain attached sugar moieties that are free to rotate. The teicoplanin A_2 also has a hydrophobic "tail" group attached to one of the sugars.

vancomycin (23,24). It differs from vancomycin in that the aglycone "basket" contains four rather than three small, fused macrocyclic rings; and that there are three attached monosaccharides. In addition there is a C_9 or C_{10} hydrocarbon "tail" attached to one of the monosaccharides (23,24). Hence, teicoplanin is a mixture of very closely related compounds with molecular weights between 1876 and 1892. Teicoplanin A_2 was attached to a 5μ silica gel support in a manner identical to that for vancomycin (19). The structures of vancomycin and teicoplanin are shown in Figure 1.

Both vancomycin and teicoplanin CSPs are multimodal in that they can be used in the reversed phase mode, normal phase mode and the polar organic mode (*e.g.*, 90-99% acetonitrile plus minor additives) and give different enantioselectivities in each (13, 25). The enantioresolution capabilities of the two new antibiotic-based CSPs in the reversed phase mode were compared to the more traditional cyclodextrin columns. Table I summarizes the enantioseparation data for the nine racemic, substituted pyridone compounds. Also, listed are the "code symbols" for each compound. The best resolution was obtained in the reversed phase mode in all cases. A partial resolution for L_{CH} was obtained (data not included) using a polar organic mobile phase, but, in general, this approach was not effective for these compounds. In all but a single case (i.e., L_A) the antibiotic-based LC columns produced the highest enantioselectivities (αs). The β-cyclodextrin column gave the best resolution in three cases (i.e., L_A, L_C and L_{BZ}) largely because of efficiency considerations. No enantioresolutions were observed with either the α-, or γ-cyclodextrin columns.

Apparently hydrophobic association can be important for both the antibiotic and cyclodextrin-type of chiral selectors. This is well established for cyclodextrins in the reversed phase mode where inclusion complexation is involved (26-28). In contrast, the individual macrocyclic rings of vancomycin and teicoplanin are too small to include the compounds in this study. However when the smaller rings are fused, they form a "basket-like" structure with hydrophobic and hydrophilic regions (Figure 1). It was previously shown that

the retention on a vancomycin column shows typical reversed-phase behavior (i.e., high retention with highly aqueous mobile phases and decreasing retention as the organic modifier is increased)(13). The compounds in this study show analogous retention behavior, which is indicative of hydrophobic association. However, it should be noted that only in rare cases are hydrophobic interactions alone sufficient for chiral recognition (29). Most often additional interactions are needed, including: hydrogen bonding, steric repulsion, dipole stacking, π-π associations, and so forth.

As shown in Table I, relatively small changes in a group's substitution position, degree of saturation and chain length can affect enantioselectivity. For example the only difference between L_{ME} and L_C (Table I) is that the methyl substituent on the pyridone ring is in the 4 position in one case and the 5 position in the other. However the 4-methyl compound (L_C) is baseline resolved with both the teicoplanin and β-cyclodextrin columns, while the 5-methyl analogue (L_{ME}) is not resolved by either column. There are several other related examples where small structural changes can completely change the enantioselectivities (i.e., L_{Ph} vs L_A, L_{Ph} vs L_{CH}, L_{Ph} vs L_{BZ}, L_A vs L_{M2} and L_{M1} vs L_{M2} in Table I).

From an analytical point of view, it is interesting to compare separations on the two related macrocyclic antibiotic columns. In some cases a compound was adequately resolved on both columns (Figure 2A). However, more often a partial resolution was obtained with one macrocyclic antibiotic while complete resolution was obtained on the other, related CSP (Figures 2B and C). This behavior was observed not only for the compounds in this study but for a variety of other types of analytes as well (Figure 3). This provides a very interesting and useful means for optimizing an enantiomeric separation that does not exist with other columns. Apparently the structurally related oligophenolic, glycopeptide antibiotics have somewhat similar, but not identical enantioselectivities. Hence, if no more than a partial resolution can be obtained on one CSP, it is highly likely that a complete separation can be obtained on a near "relative" CSP using the

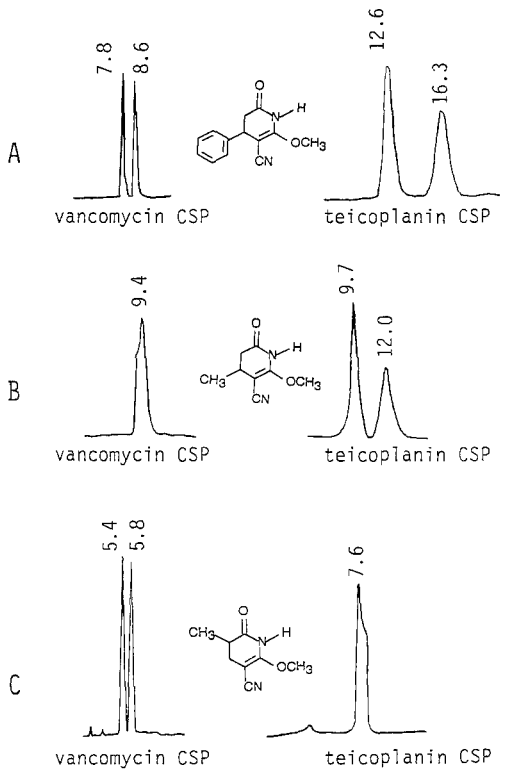


Figure 2. Enantiomeric separations of three racemic substituted pyridones that illustrate the complementary enantioselectivities of the two related antibiotic chiral stationary phases. All three racemates (A, B and C) were separated under identical conditions (i.e., mobile phase = 10:90, methanol: 1% triethylammonium acetate buffer, pH 4.1 at a flow rate of 1.0 ml/min). In each case the columns consisted of either a 25 cm x 0.44 cm (i.d.) vancomycin or teicoplanin bonded phase material (5 μ silica support).

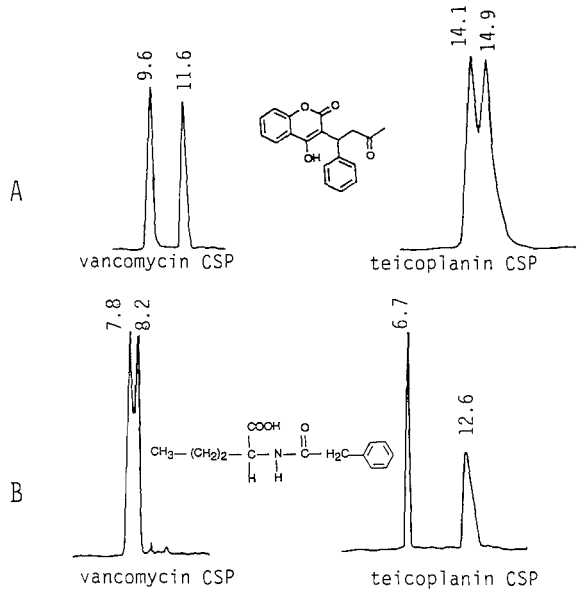


Figure 3. Chromatograms illustrating that the "principle of complementary separations" for the macrocyclic antibiotic CSPs applies to a variety of different compounds. Part "A" shows the separation of racemic warfarin on a vancomycin vs a teicoplanin CSP. In both cases the mobile phases consisted of 10:90, acetonitrile: 1% triethylammonium acetate buffer, pH 4.1; and a flow rate of 1.0 ml/min. Part "B" shows the separation of racemic N-CBZ-norvaline. In both cases the mobile phase consisted of 20:80, methanol: triethylammonium acetate buffer, pH 4.1; and a flow rate of 1.0 ml/min.

same mobile phase conditions (Figures 2 and 3). This "principle of complementary separations" can be useful when a baseline resolution must be found quickly.

As stated previously, the substituted pyridones resolved in this study can be used in the synthesis of active agents for cancer chemotherapy. One of the better known compounds of this genre is methotrexate, which is produced as a racemate

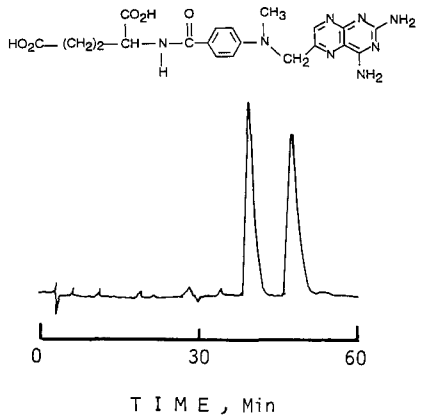


Figure 4. Enantiomeric resolution of methotrexate on a 25 cm x 0.44 cm (i.d.) Cyclobond I column. The mobile phase was 7.5:92.5, acetonitrile: 1% triethylammonium acetate buffer, pH 7.0; at a flow rate of 1.0 ml/min.

(30-33). The resolution of methotrexate is shown in Figure 4. Apparently the type of columns used in this study can be used to resolve both the synthetic intermediates and the final products for this class of compounds.

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LIQUID CHROMATOGRAPHIC SEPARATION OF GEOMETRICAL ISOMERS USING SPHERICAL CARBON PACKINGS PREPARED FROM SPHERICAL CELLULOSE PARTICLES

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ABSTRACT

The packings of spherical carbon beads were prepared by graphitizing the spherical cellulose particles. A types of carbon spherical packings were obtained by various degrees of graphitization. These packings were characterized by examining their chromatographic properties. The higher the degree of graphitization is, the higher the π-electron recognition became, although there was no change in the selection for a methylene group. The mechanism for recognizing the π-electron and the steric selectivity is described and discussed.

INTRODUCTION

In 1984, we developed a gel which could easily be prepared from cellulose (1, 2) and this gel is currently available as Cellulofine (Chisso Co., Ltd.) for a size-exclusion chromatography (SEC). The sphering and reticulating of the cellulose material is easily

carried out by the *suspension evaporation* method. In addition, this packing is much harder than the standard dextran gels and it has a high flow rate resistance, which is a property which is currently being marked for in the use with aqueous SEC.

On the other hand, we prepared new graphitized carbon microbeads ($d_p < 5$ μm) from spherical cellulose particles without cohesion and conglutination among the microbeads (3). They show no excessive retention even for electron-rich compounds or hydrophobic compounds, and they do not contain residual silica, metal impurities and silanol groups because the packings are prepared from particles composed solely of cellulose. We previously reported that the specific separation of geometrical isomers has been achieved with poly(γ-methyl L-glutamate) (PMG) spheres (4) and silica-supported comb-shaped polymers (5). The retention mechanism of the PMG spheres is based on the formation of a molecular slit which is derived from the rigidity of the PMG main chain. The retention mechanism of silica-supported comb-shaped polymers is related to the highly oriented structures of an immobilized phase.

On the other hand, the carbon packings obtained can separate geometrical isomers by the charge-transfer interaction mechanism. The excellent selection process for geometrical isomers seems to be related to the surface property derived from the layered structures of the surface graphite. This paper gives some results on the study of the relationship between the chromatographic properties and the structure or surface properties of types of carbon packings, differing on the graphitization degree.

EXPERIMENTAL

Preparation of graphitized carbon microbeads

Spherical cellulose particles (Cellulofine GC-15 uf) the starting material, had a mean particle diameter of 4.77 μm, an exclusion limit of Da. 6200 and porosity 73 % in aqueous SEC. Using this cellulose spherical particles as a starting material, the carbon microbead was prepared by the method previously reported without conglutination among the microbeads due to adsorbed water and coal tar. The microbeads carbonized at 1000 °C were heated up to 2800 °C at heating rate of 10 °C min^{-1}, and in order to vary the degree of graphitization, respectively allowed to stand for 1.0, 2.5 and 4.5 h at the temperature and cooled. According to these procedures, the three kinds of carbon packings were prepared.

Chromatography

A slurry of 2.5 g of the beads in 25 ml of dioxane-paraffin (20 : 80) was packed into a stainless steel column (50~150 x 4.6 mm I. D.). As a reference column, Inertsil ODS (150 x 4.6 mm I. D.) were purchased from GL Science Co., Ltd.. TONEN Carbonex were also purchased. The chromatograph included a JASCO 880-PU pump, an UV-VIS Shimadzu

photodiode array SPD-M6A and a Shodex refract monitor SE-51. A 5 ml portion of the sample dissolved in an eluent (1.0 mg ml^{-1}) was injected through a Rheodyne Model 7125 injector. All chromatography was carried out at 25°C.

Other measurements

Surface area analysis of the particles was carried out by Brunau-Emmet-Teller (BET) method using a Shimadzu 2205 measurement of surface area analysis. Scanning electron micrographs of the beads were obtained using a JEOL JSM-T20, and X-ray diffraction was carried out using a Geiger flex RAD-IB.

RESULTS AND DISCUSSION

Characterization

TABLE 1 lists the abbreviations and the results of the elemental analysis of the microbeads obtained in each process. All of the carbon contents reached almost 100 %.

FIGURE 1 shows the X-ray diffraction patterns of the carbon microbeads obtained. The diffuse reflections for Carbon-0 indicate that their structures are turbostratic, that is, the graphite-like layer in the structure is small two-dimensionally, in addition, it is disordered three-dimensionally. In terms of the heating treatment at 2800 °C, the hk0 and 00l reflections increase as the hkl reflection becomes sharp. In addition, the d_{001} reflection gradually appeared. This indicated that the degree of graphitization increased, that is, the sheets of the graphite-like layers expanded two-dimensionally. The d_{002} reflection for Carbon-1.0, -2.5 and -4.5 was split into two reflections at 25.9° and 26.3°. The d_{002} reflection at 26.3° should be associated with the needle-like structure, as reported by Knox et. al.[6] In addition, the

TABLE 1 Abbreviations and elemental analysis of beads obtained in each process

Time of graphitization (h)	Beads	Elemental analysis	
		C%	H%
	Starting materials (Cellulose)	43.0	6.3
0	Carbon-0	96.3	0.6
1.0	Carbon-1.0	99.7	-
2.5	Carbon-2.5	99.6	-
4.5	Carbon-4.5	99.8	-

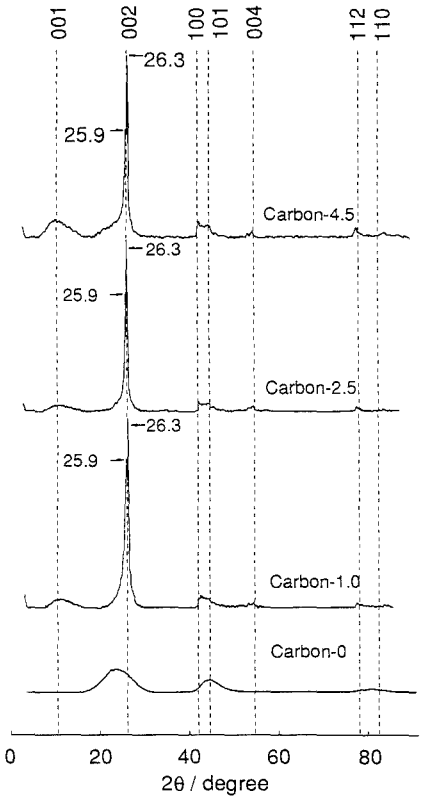

FIGURE 1 X-ray diffractgrams of Carbon-0, -1.0, -2.5 and -4.5.

longer the time of graphitization is, the bigger the increase of the d_{002} reflection is at 26.3° than at 25.9°. This suggests that the Warren structure of the two-dimensional turbostratic graphites, with no layer registration, are converted into three-dimensional graphites with layer registration (6).

FIGURE 2 shows the scanning electron micrographs. In spite of the heat-treatment at high temperatures, microbeads, after carbonization or graphitization, maitained a completely spherical shape. The amount of the needle-like structure increased in the order of Carbon-0, Carbon-1.0, -2.5 and -4.5 with the progress of graphitization. This corresponded to the results of the X-ray diffraction patterns, as shown in FIGURE 1. The surface area of the

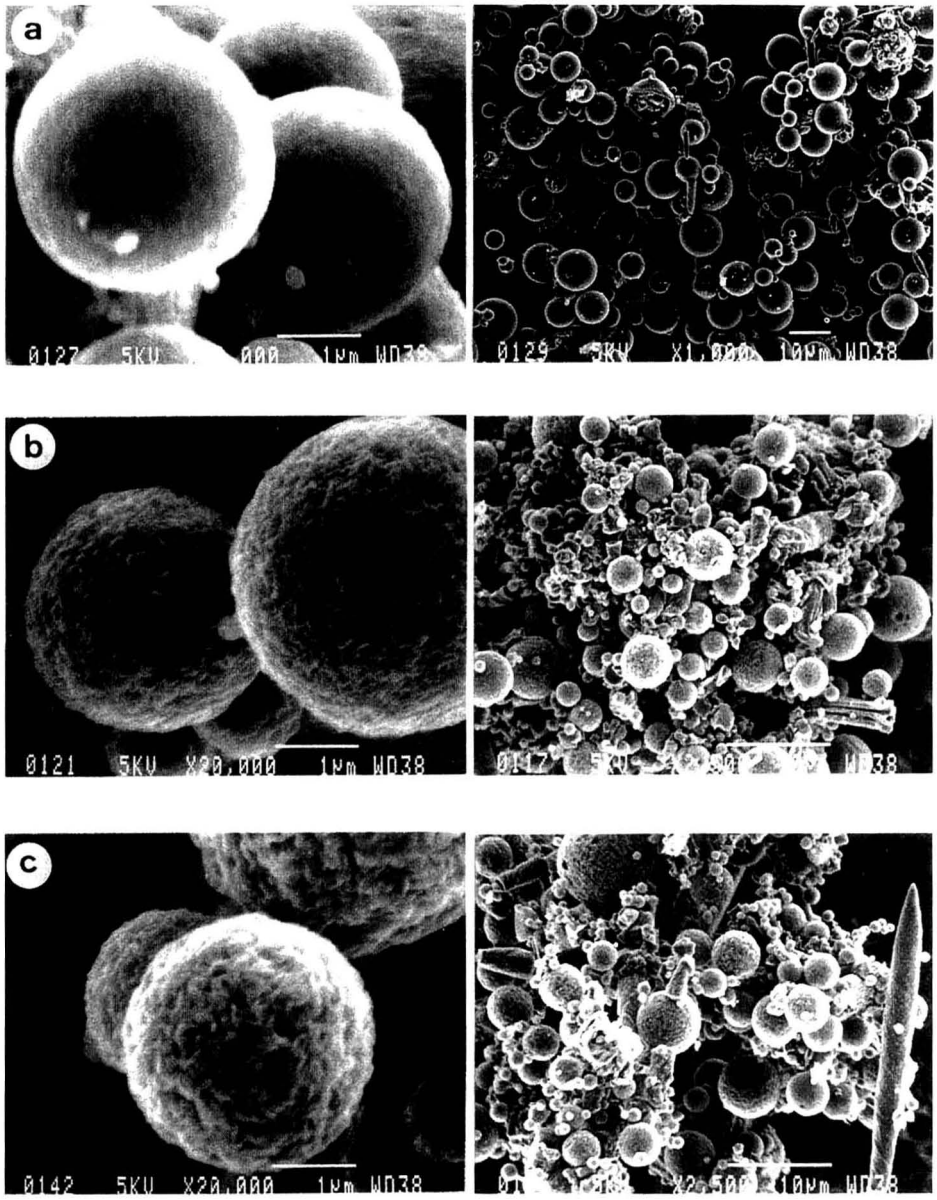

FIGURE 2 Scanning electron micrographs of Carbon-1.0 (a), Carbon-2.5 (b) and Carbon-4.5 (c).

microbeads obtained in each process was measured by the BET method. The microbead surface areas were 216, 2.6, 2.8 and 5.8 ($m^2\ g^{-1}$), respectively for Carbon-0, Carbon-1.0, Carbon-2.5 and Carbon-4.5.

Chromatography

Charge-transfer interaction

Non-graphitized carbon, the Carbon-0, showed the adsorption for all solutes owing to turbostratic structural carbon surface of the high surface area. In contrast, as shown in FIGURE 3, a close correlation between the k' value and the carbon number of methyl alkanates was observed similarly in the, Carbon-1.0, Carbon-2.5 and Carbon-4.5 columns. The retention capacity factors of all the graphitized carbon packing types increased with the increase of the alkyl chain lengths. The longer the time of graphitization is in the Carbon-1.0, -2.5 and -4.5 columns, the more the retentions for methyl alkanates increases. The capacity and separation factors for each series of solutes with all of the carbon packing types are listed in TABLE 2. Although the retention of methyl alkanates increased, no change in the separation factors occurred. Accordingly, these results seem to be related to the surface area of the carbon packings. In contrast, the separation factors, $k'_{p\text{-hydroxyalkylbenzoate}}/k'_{alkylbenzoate}$ and $k'_{alkylbenzoate}/k'_{alkylbenzene}$ gradually increased, with the progress of the graphitization degree in the Carbon-1.0, Carbon-2.5 and Carbon-4.5 columns. These attributes are derived from the increase of the charge-transfer interaction between the lone pair electrons of the solute and the π-electron of the carbon surface, because the degree of graphitization increased, that is, the sheets of the graphite-like layers expanded two-dimensionally, in addition, the π-electron seemed to transfer regularly, as the two-dimensional turbostratic graphites are converted into three-dimensional graphites.

Steric selectivity

As shown in FIGURE 4 and 5, the carbon packings gave a excellent mixture of geometrical isomers which were separated, although the ODS showed a low selectivity process for geometrical isomers. The specificity of the carbon packings was emphasized by examining the relationship between their separation factors and surface properties.

TABLE 3 lists the separation factors of the *E*- and *Z*- isomers of the stilbene for Carbon-1.0, 2.5, -and 4.5. The larger retention of the *E*-isomer can be explained by the fact that the *E*-isomer, which is more planar (7), has a larger area for contact with the carbon phase than the *Z*-isomer, as shown in FIGURE 6. This assumption can be explained by the significant preference for the planar compound, triphenylene than for a non-planar compound, *o*-terphenyl, as shown in TABLE 3. In addition, this interaction is derived from the π-electron of the sheets of the graphite-like layers, which can work as electron-accepting

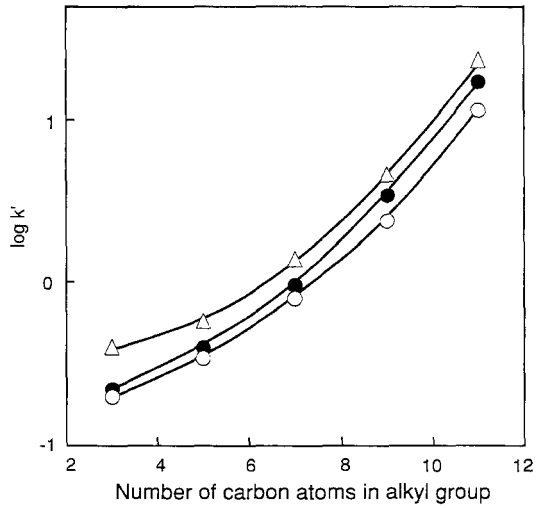

FIGURE 3 Relationships between the capacity and carbon number of methyl alkanoates for Carbon-1.0 (a), -2.5 (b) and -4.5 (c). Solute: methyl butanoate, methyl hexanoate, methyl octanoate, methyl decanoate, methyl dodecanoate. Eluent: acetonitrile-water (60 : 40).

TABLE 2 Selectivity of solutes with Carbon-1.0, -2.5 and -4.5

Solute	Carbon-1.0		Carbon-2.5		Carbon-4.5	
	k'	α	k'	α	k'	α
Methyl *n*-Decanoate	2.4		3.4		4.7	
		4.7		4.9		4.9
Methyl *n*-Dodecanoate	11.3		16.8		22.9	
n-Butylbenzene	1.3		1.3		1.9	
n-Butylbenzoate	1.7	1.4	2.0	1.8	3.0	2.3
		1.0		1.2		1.5
n-Butyl *p*-hydroxybenzoate	1.8		2.3		4.3	

Eluent: acetonitrile-H_2O (60 : 40)

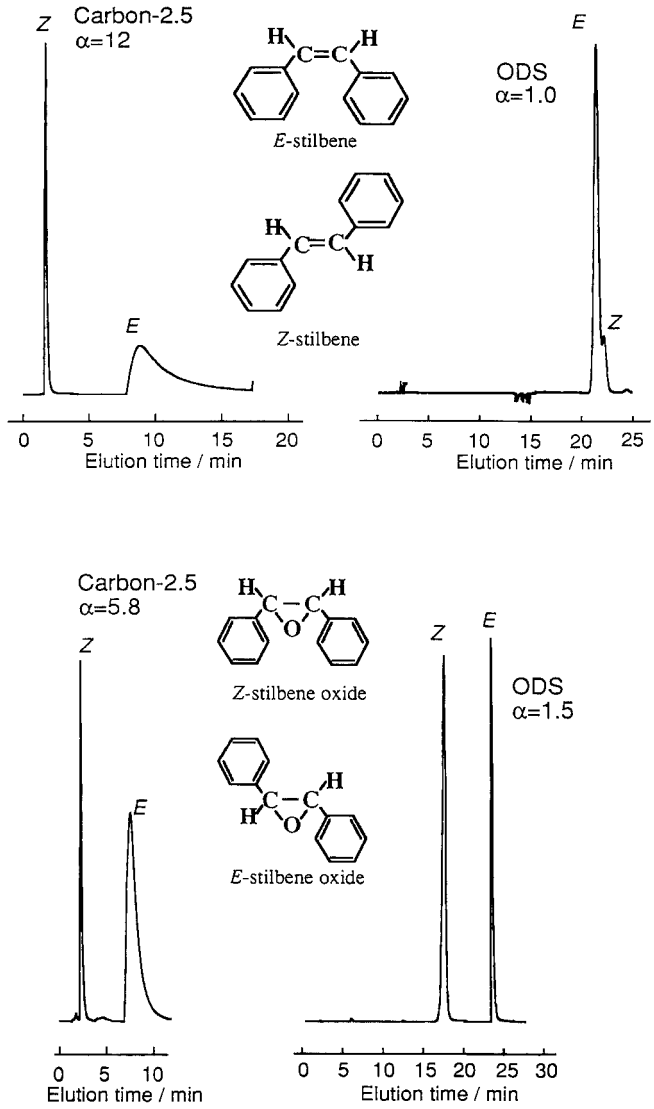

FIGURE 4 Chromatograms of *E* / *Z*-stilbene and *E* / *Z*-stilbene oxide isomers with Carbon-2.5 and ODS. Eluent, acetonitrile for Carbon-2.5, acetonitrile-water (50 : 50) for ODS in the cases of *E* / *Z*-stilbene, acetonitrile-water (60 : 40) in the cases of *E* / *Z*-stilbene oxide, respectively. Flow rate, 0.5 ml min^{-1}.

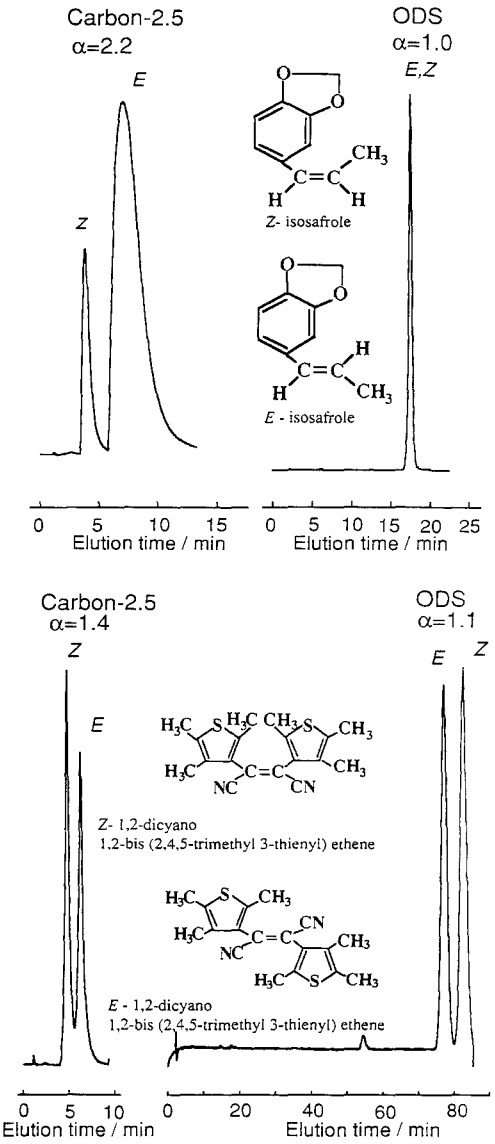

FIGURE 5 Chromatograms of *E* / *Z*-isosafrole and *E* / *Z*-1,2-dicyano 1,2-bis (2,4,5-trimethyl 3-thienyl) ethene isomers with Carbon-2.5 and ODS. Eluent, acetonitrile-water (60 : 40), Flow rate, 0.5 ml min^{-1}.

TABLE 3 Selectivity of *E* / *Z*- isomers for each column

Solute	Carbon-1.0		Carbon-2.5		Carbon-4.5		ODS	
	k'	α	k'	α	k'	α	k'	α
Z-Stilbene	1.2[a]		0.6[a]		1.7[a]		8.3[b]	
		4.5		12				1.1
E-Stilbene	5.5[a]		7.0[a]		adsorption[a]		8.1[b]	
o-Terphenyl	0.1[c]		0.1[c]		0.2[c]			
		80.4		259		318		
Triphenylene	9.2[c]		19.0[c]		63.2[c]			
Z-1,4-Dichloro 2-butene	1.3[d]		1.6[d]		2.1[d]		5.0[b]	
		1.0		1.1		1.1		1.0
E-1,4-Dichloro 2-butene	1.4[d]		1.8[d]		2.3[d]		5.0[b]	

a: acetonitrile, b: acetonitrile-H_2O (50 : 50), c: tetrahydrofuran, d: acetonitrile-H_2O (30 : 70)

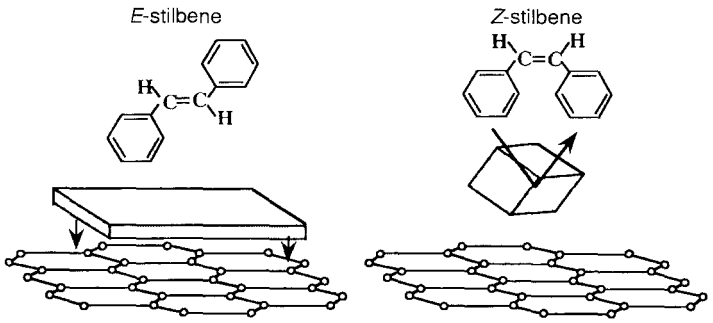

FIGURE 6 Schematic illustration of adsorption of planer and non-planer compounds on the surface of carbon beads.

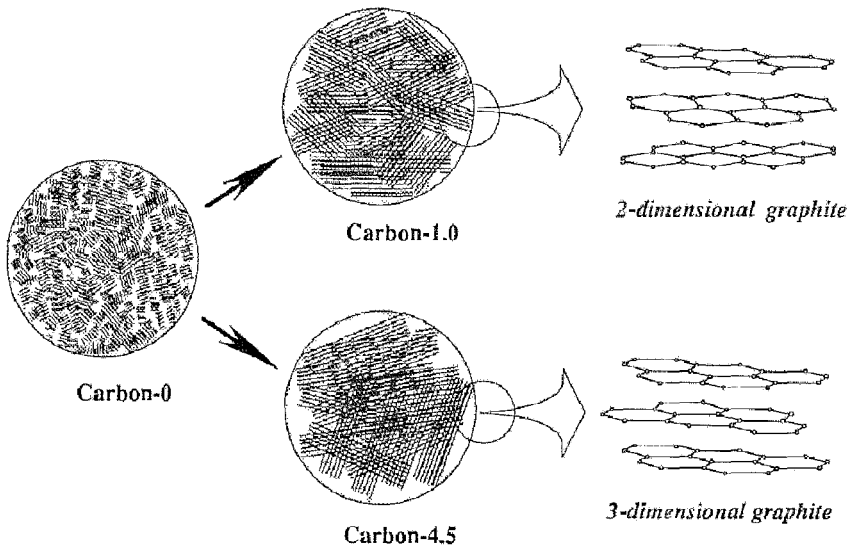

FIGURE 7 Schematic illustration of structural changes of graphite-like layer with progressing of graphitization.

groups. This assumption is supported by the following facts, from calculating the energy level of the HOMO (highest occupied molecular orbital) in stilbene, we found that the *E*-isomer is more of an electron donating isomer (-8.63 eV) than the Z-isomer (-9.06 eV), as calculated by one of our members (8). However, the carbon packings showed almost no selectivity for geometrical isomers of 1,4-dichloro-2-butene, although their retention increased with the progress of graphitization. Geometrical isomerism of 1,4-dichloro-2-butene for 1,4-dichloro-2-butene which had non-conjugating substituent groups provided no difference in the energy level of the HOMOs (*E*-isomer: -10.035 eV Z-isomer: -10.102 eV) (9). This indicated the the contribution of the steric effect, that is, the contribution of the slit-like structure (10) of surface was small. According to these results, the separation mechanism of the geometrical isomers was derived mainly from the π- electron of the carbon bead surface.

In conclusion, it was assumed that the carbon packings which were obtained that their sheets of graphite-like layers expanded two-dimensionally, and that the Warren structure of two-dimensional turbostratic graphites with no change in layer registration were converted into three-dimensional graphites with layer registration, that is, a pyrolytic deposition of carbon needles takes place, presumably from organic vapors which were released just above 1000 °C, with the progress of graphitization. Therefore, we assume that the carbon packings

obtained in our study developed structural changes when graphitizing occurred, as schematically illustrated in FIGURE 7. It is considered that the increase in the charge-transfer interaction between the lone pair of electrons from the solute and the π-electron from the carbon beads surface are derived from these structural changes. As the results electron-rich compounds are retained more on the carbon beads surface.

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CHIRAL CHROMATOGRAPHIC DISCRIMINATION ABILITY OF A CELLULOSE 3,5-DIMETHYL-PHENYLCARBAMATE/10-UNDECENOATE MIXED DERIVATIVE FIXED ON SEVERAL CHROMATOGRAPHIC MATRICES#

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ABSTRACT

The properties as chiral selector in HPLC chiral stationary phases (CSPs) of a cellulose derivative bearing simultaneously 3,5-dimethylphenylamino carbonyl and 10-undecenoyl groups are described. This polysaccharide is reticulated (or bonded) on chromatographic supports such as silica gel, previously treated or not, graphite or alumina. The chiral stationary phases thus obtained are resistant to the usual solvents used in liquid chromatography and can be used on normal or reversed phase conditions.

Presented at the 5th International Symposium on Chiral Discrimination (Stockholm, October 1994).

The results obtained on the resolution of racemic compounds with these stationary phases depend on the nature of the support. The best results were obtained with the chiral stationary phase in which the cellulose derivative is bonded on allyl silica gel.

INTRODUCTION

Lactose, starch and cellulose are the oldest chiral chromatographic supports used in the resolution of racemic compounds[1]. Regarding polysaccharide derivatives, the first use of cellulose acetate as stationary phase was reported in 1967[2]. However, the development of polysaccharide derivatives in chiral HPLC was marked by the studies of Hesse and Hagel, published in 1973, on cellulose triacetate[3]. These authors show that the secondary structure of the polymer has an important role in the enantioselectivity of the stationary phase.

Since 1984 Okamoto et al. described the preparation of chiral stationary phases (CSPs) for HPLC constituted by macroporous γ-aminopropylsilica gel on which phenylcarbamates of cellulose and other polysaccharides had been adsorbed[4,5]. These CSPs are now widely used because of their ability in the resolution of a very large range of racemic compounds, and they are commercially available[6]. Nevertheless, the chiral selector in these CSPs is soluble in a number of organic solvents. This solubility limits the choice of eluant.

Okamoto and co-workers described the preparation of stationary phases in which the phenylcarbamates of cellulose are not absorbed but bonded to silica gel[7,8]. Although the optical resolving power of these CSPs is, in certain cases, slightly lower than that of the same cellulose derivatives absorbed on silica gel, their enantioselectivity is still good enough. In spite of the advantage of their stability in the presence of solvents, these bonded CSPs are not commercially available. This is probably a consequence of the relative complexity of the preparation described for these CSPs.

In this study the chromatographic behavior of CSPs whose chiral selector is a cellulose derivative, which is resistant to solvents usually used in liquid chromatography, is described. This property has been obtained from a cellulose derivative in which the glucose units bear 3,5-

dimethylphenylaminocarbonyl and 10-undecenoyl groups at the same time. This compound can undergo reticulation on chromatographic supports (silica gel, modified silica gel, graphite, alumina). When the matrix used is allyl silica gel this reticulation results in the covalent bonding of the chiral selector to the matrix surface. The characteristics and the performances of the resulting CSPs are discussed.

EXPERIMENTAL SECTION

Elemental analyses were performed by the Service Central de Microanalyses du CNRS (Vernaison, France). The chromatographic experiments were performed on an HPLC system consisting of a Waters 600E pump, a Waters 717 auto sampler (Millipore, Milford, MA, USA) and equipped with a Waters 996 photo diode array detector and a Perkin-Elmer 241LC polarimetric detector (Perkin-Elmer, Uberlingen, Germany). The chiral stationary phases were packed into stainless-steel tubes (150 x 4.6 mm ID) by the slurry method. The volume of sample injected was 1 ml. The flow-rate of the pump was 1 ml/min. The detection wavelength was 254 nm. The void volume in normal phase conditions was determined using tri-tert-butylbenzene.

Cellulose Derivative and Chiral Stationary Phases

The chiral selector and the stationary phases have been prepared as it is indicated on Figure 1[9]. The elemental analysis for the cellulose derivative was C 67.37, H 7.62, N 3.78%. This analysis corresponds to a cellulose derivative having 1 10-undecenoyl group and 1.6 3,5-dimethylphenylaminocarbonyl groups for each glucose unity. The same result was obtained from the H^1-NMR data.

RESULTS AND DISCUSSION

The cellulose derivative was fixed on allyl silica gel (**A**), "end capped" silica gel (**B**), non-treated silica gel (**C**), alumina (**D**) and graphite (**E**) in a

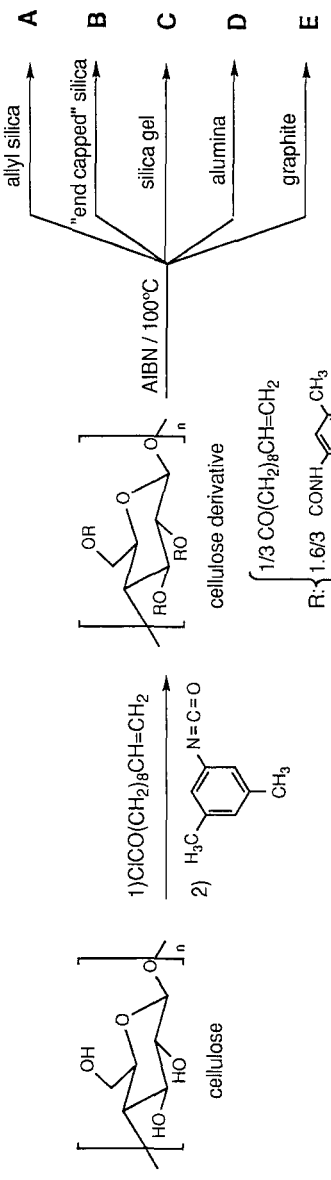

FIGURE 1
Preparation of Chiral Stationary Phases

radical reaction by means of the C-C double bonds on the undecenoyl group. The fixation process took place either by polymerization of the cellulose derivative with groups able to react on the matrix surface (allyl, in CSP **A**), or by reticulation of the 10-undecenoyl groups of the cellulose derivative itself on the matrix surface. The fixation resulted in the insolubilization of the cellulose derivative, which was soluble in chloroform before the treatment but was not soluble, even in boiling chloroform, after the reaction.

The elemental analyses of the resulting CSPs, all of which were obtained under the same experimental conditions, are given in Table 1. The nitrogen percentages, and therefore the amount of chiral selector fixed by stationary phase unit mass is almost the same for all supports and independent of the matrix.

In Tables 2 to 4, several chromatographic results obtained with these CSPs are presented. As a result of the fixation process, these CSPs were eluted with heptane/chloroform mixtures (Table 2). Moreover, the same column can be used on reverse and normal phase conditions, and changed from one to the other with the only precaution of using perfectly miscible solvents . However, all CSPs showed better enantioselectivity in normal phase conditions than in reversed phase conditions. CSPs **B** (end capped silica gel as a matrix) and **E** (graphite as a matrix) showed high retention times against several racemic compounds in normal phase conditions. However, the selectivity did not improve when they were used in reverse phase (ACN/water, ACN/$NaClO_4$, ...). Generally, in the resolution of racemic compounds **1** to **9**, the enantioselectivity was slightly

TABLE 1
Elemental Analyses of the Chiral Supports

CSP	%C	%H	%N
A	15.58	1.80	0.94
B	12.65	2.14	0.71
C	12.84	2.02	0.90
D	12.44	1.83	0.84
E	93.19	1.36	0.85

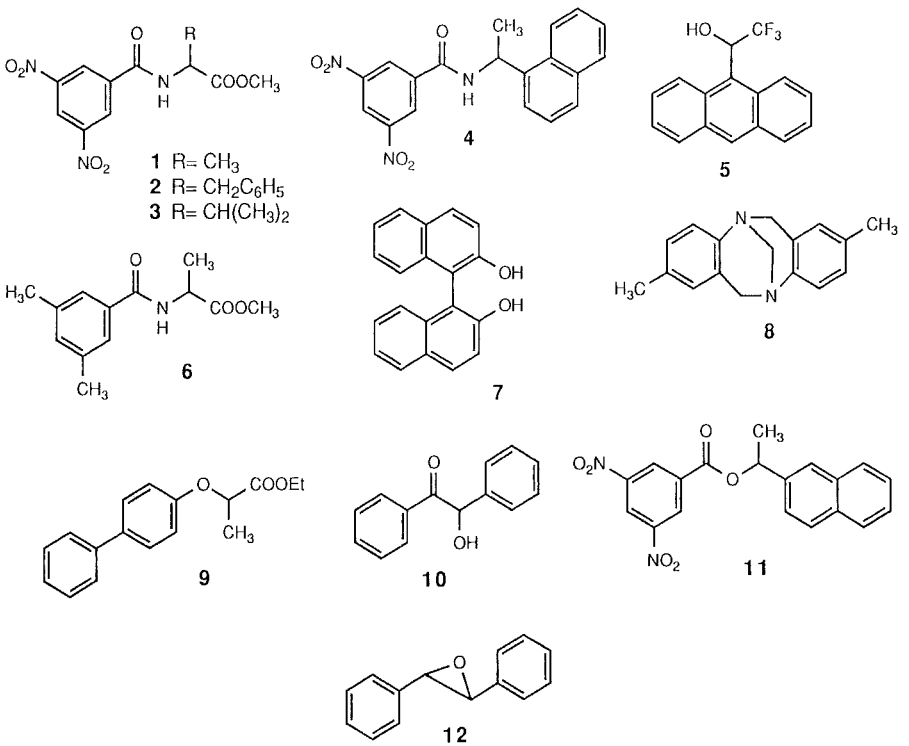

FIGURE 2
Chemical Structures of Racemic Test Compounds

TABLE 2
Chromatographic Results Obtained with Chiral Stationary Phases **B**, **C**, **D** and **E**.

RACEMIC COMPOUNDS	B		C		D		E		mobile phase Heptane/Cl_3CH
	k'_1	α	k'_1	α	k'_1	α	k'_1	α	
1	1.81	1.15	5.64	1.05	3.45	1.00	4.04	1.14	25:75
2	0.98	1.12	2.24	1.00	1.85	1.00	3.52	1.00	25:75
3	0.76	1.20	1.79	1.09	1.33	1.00	1.83	1.18	25:75
4	3.88	1.27	>15*	-	7.53	1.19	>15*	-	50:50
5	1.77	1.99	>15*	-	5.52	1.76	>15*	-	50:50
6	1.31	1.00	2.91	1.00	2.12	1.12	1.14	1.00	75:25
7	0.97	1.18	3.69	1.05	>15*	-	1.44	1.21	50:50
8	2.94	1.00	2.53	1.00	2.76#	1.17	1.19#	1.18	75:25
9	0.72	1.17	1.06	1.13	1.31	1.25	1.22	1.11	90:10

k'_1, Capacity factor for the first eluted enantiomer; α, selectivity factor. Column: 15 cm x 0.46; flow rate: 1ml/min.
* When 100% chloroform was used as mobile fase. # Mobile phase: 90:10 Heptane/Cl_3CH.

TABLE 3
Chromatographic Results Obtained with Chiral Stationary Phase **A**

RACEMIC COMPOUNDS	Heptane/Chloroform			Heptane/2-Propanol			Acetonitrile/Water	
	k'1	α	Rs	k'1	α	Rs	k'1	α
1	2.27[a]	1.23	1.33	12.68[a]	1.12	1.00	5.98[a]	1.00
2	1.35[a]	1.22	1.06	8.91[b]	1.20	1.48	4.78[b]	1.00
3	1.00[a]	1.26	1.12	4.61[b]	1.00	-	12.19[a]	1.00
4	1.47[a]	1.23	1.19	6.12[b]	1.28	1.88	8.94[b]	1.08
5	1.30[a]	2.05	4.61	0.87[b]	1.73	2.97	5.08[b]	1.12
6	2.01[b]	1.10	-	2.42[a]	1.24	1.74	4.29[a]	1.09
7	1.54[c]	1.24	1.49	2.96[a]	1.16	1.21	13.58[a]	1.00
8	4.12[b]	1.00	-	1.10[b]	1.12	-	12.77[a]	1.04
9	1.09[d]	1.18	-	0.65[a]	1.25	1.20	6.18[b]	1.00
10	5.54[d]	1.32	1.23	2.03[a]	1.22	1.70	5.58[a]	1.08
11	7.55[d]	1.09	-	5.42[a]	1.07	-	17.89[b]	1.00
12	1.00[d]	1.75	2.06	0.64[a]	1.27	1.30	6.63[b]	1.00
	a) 25:75 Heptane/Cl_3CH b) 75:25 Heptane/Cl_3CH c) 50:50 Heptane/Cl_3CH d) 90:10 Heptane/Cl_3CH			a) 90:10 Heptane/2-PrOH b) 80:20 Heptane/2-PrOH			a) 40:60 ACN/H_2O b) 60:40 ACN/H_2O	

k'_1, Capacity factor for the first eluted enantiomer; α, selectivity factor; Rs, resolution. Column: 15 cm x 0.46; flow rate: 1ml/min.

TABLE 4
Chromatographic Results Obtained with Chiral Stationary Phase **A**

RACEMIC COMPOUNDS	Heptane/2-Propanol			Acetonitrile/Water			Acetonitrile/Phosphate buffer	
	k'1	α	Rs	k'1	α	Rs	k'1	α
TEMAZEPAM	12.88[a]	1.13	-	5.92[a]	1.17	1.60		
LORMETAZEPAM	16.89[a]	1.00	-	7.12[a]	1.27	2.48		
OXAZEPAM	9.90[a]	1.27	1.76	3.88[a]	1.25	2.19		
LORAZEPAM	9.84[a]	1.64	3.48	3.76[a]	1.28	2.43		
WARFARIN	4.85[a]	1.81	3.03	2.97[a]	1.17	-		
VERAPAMIL	2.21[b]	1.00	-	5.38[b]	1.03	-	7.28[a]	1.00
BENDROFLUMETIAZIDE	8.34[b]	1.17	-				4.24[a]	1.00
METOPROLOL	1.61[b]	1.24	1.22	4.91[c]	1.00	-	0.90[a]	1.13
ATENOLOL	9.78[b]	1.14	-	0.74[c]	1.00	-	0.11[a]	1.00
PROPANOLOL	1.91[b]	1.18	-	2.39[b]	1.04	-	2.99[a]	1.00
NADOLOL	3.43[b]	1.36	1.25	1.21[c]	1.00	-	0.50[b]	1.00
PINDOLOL	5.51[b]	2.84	5.35	6.83[c]	1.36	1.30	2.60[b]	1.23
NAPROXEN	4.48[c]	1.16	1.31				5.39[b]	1.00
IBUPROFEN	1.19[c]	1.09	-				12.09[b]	1.00
	a) 90:10 Hept./2-PrOH b) 80:20:0.1 Hept./2-PrOH/DEA c) 98:2:0.1 Hept./2-PrOH/TFA			a) 40:60 ACN/H_2O b) 40:60 ACN/$NaClO_4$ 0.1M c) 20:80 ACN/$NaClO_4$ 0.1M b,c) flow rate: 0.5 ml/min			a) 40:60 ACN/Phosphate buffer 0.03M pH: 5.8 b) 20:80 ACN/Phosphate buffer 0.03M pH: 5.8	

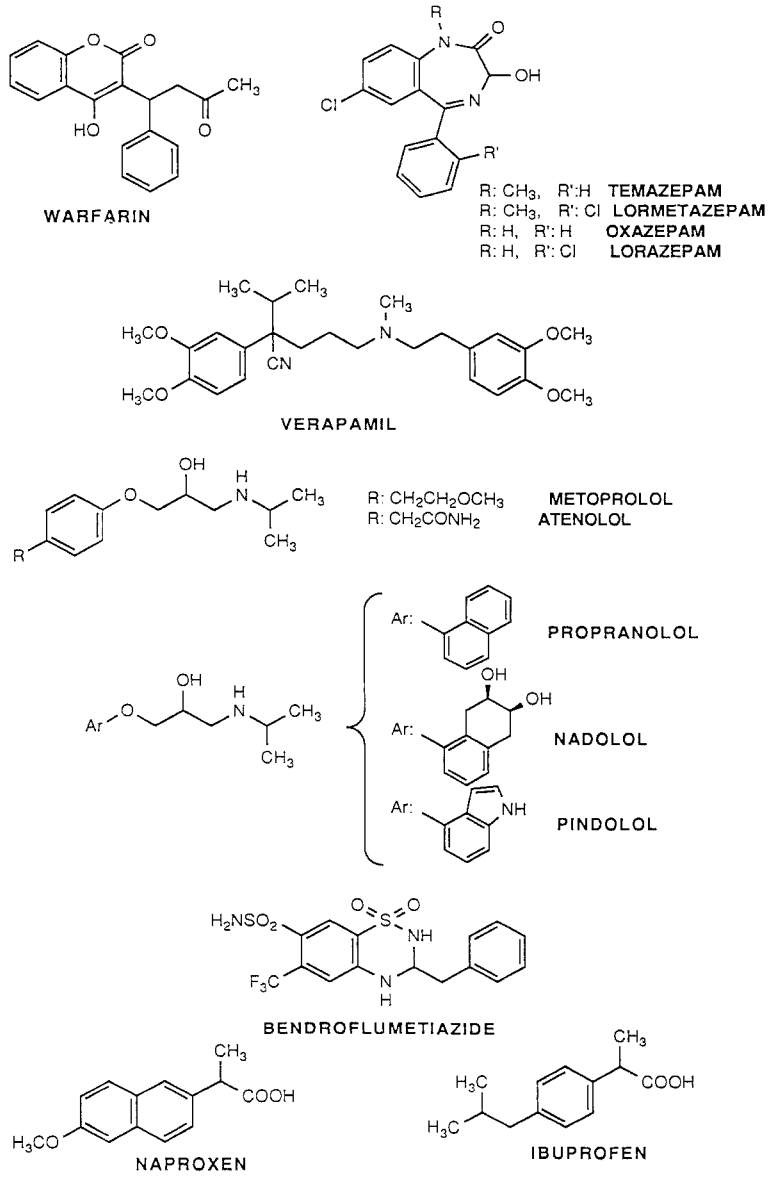

FIGURE 3
Chemical Structures of Racemic Drugs (Table 4)

better when heptane/chloroform was used as mobile phase rather than heptane/2-propanol The best results were obtained with CSP **A** (Table 3).

In Table 4, the resolution of several racemic drugs including benzodiazepines, aminoalcohols and arylpropionic acids, under normal (heptane/2-propanol) and reverse phase conditions (ACN/water, ACN/$NaClO_4$, ACN/phosphate buffer), is presented.

CONCLUSION

The method described here for the preparation of polysaccharide chiral stationary phases allows the fixation of the chiral selector, a 3,5-dimethylphenyl/10-undecenoyl mixed derivative of cellulose, on all kinds of chromatographic matrices. The resulting CSPs are resistant to usual HPLC solvents and conditions.

Although several variables have not yet been optimized, such as the nature of the starting polysaccharide derivative, the ratio of reagents or the order of introduction of these to the polysaccharide (these will be the object of forthcoming papers), the results presented here are promising.

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STEREOSELECTIVE BIOANALYSIS OF OXCARBAZEPINE AND THE ENANTIOMERS OF ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The simultaneous liquid chromatographic determination of oxcarbazepine and the enantiomers of its metabolites 10,11-dihydro-10-hydroxycarbamazepine and trans-10,11-dihydroxycarbamazepine in spiked human plasma is described. The compounds are subjected to solid phase extraction before chromatography. The separation of the analytes is achieved using chiralcel OD column coupled on line with chiralcel ODH column and a mobile phase consisting of *n*-hexane-ethanol (70/30, v/v). The compound were detected by ultraviolet absorbance at 220 nm. The limit of quantification for each compound was 5 ng/ml.

INTRODUCTION

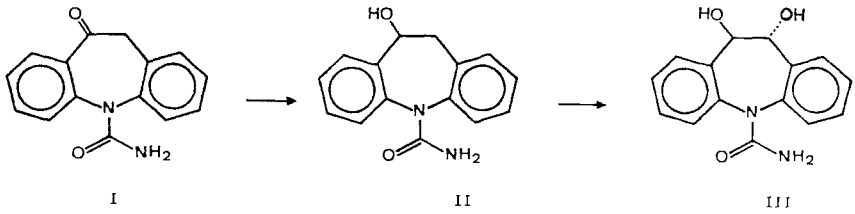
Oxcarbazepine (OXC) (10,11-dihydro-10-oxo-5*H* dibenz (*b,f*) azepine-5-carboxamide) is an antiepileptic drug structurally related to carbamazepine (CBZ). Unlike CBZ, which is metabolized by an oxidation, OXC appears in the human blood in

trace amounts and undergoes rapid reduction to the 10-oxo function to the chiral 10-hydroxy derivative (10,11-dihydro-10-hydroxycarbamazepine, MHC or GP47779) which is eliminated in the urine as unchanged and as glucuronide conjugate. A small amount of MHC is converted to the chiral trans-10,11 dihydroxyderivative (trans-10,11-dihydroxycarbamazepine, DHC or CGP10000) (Fig.1) [1-3]. Conversely, in animal species such as rat, guinea-pig, rabbit, dog and rhesus, the metabolic pattern is different from that seen in man. Indeed, preliminary studied showed plasma concentration of the parent compound higher than those of the metabolite [4].

Recently, it has been shown that in humans the metabolism of OXC is stereospecific. Indeed, after an oral dose of 600 mg oxcarbazepine,the area under the plasma concentration-time curve (AUC) of the (+)-S-MHC represented 81% of the total AUC, whereas that of the (-)-R-MHC represented only 19% [5].

High-performance liquid chromatographic (HPLC) methods with ultraviolet detection have been developed for the determination of oxcarbazepine and the sum of both enantiomers of MHC and DHC in plasma after administration of the parent drug [6-9] but only Flesch et al. reported an HPLC method for the measurement of the two enantiomers of MHC in plasma samples [5]. Neither oxcarbazepine, nor the enantiomers of DHC were detected at that time, although the authors admitted the co-elution of the standards of these latter analytes with the enantiomers of MHD.

In this paper we describe an enantioselective HPLC method to determine oxcarbazepine simultaneously with the enantiomers of the monohydroxylated and dihydroxylated metabolites using solid phase extraction and a spectrophotometric detection.



I II III

Figure 1. Structures of oxcarbazepine (I), racemic 10,11-dihydro-10-hydroxy-carbamazepine (II), racemic 10,11-dihydro-10,11-trans-dihydroxy-carbamazepine (III).

The method was applied to human blank serum spiked with oxcarbazepine, its metabolites and carbamazepine as an internal standard.

MATERIAL AND METHODS

Chemicals

Oxcarbazepine, racemic MHC, (+)-S and (-)-R-enantiomers of MHC, racemic DHC and carbamazepine were obtained from Ciba-Geigy (Basle, Switzerland). It was not possible to obtain or characterize the two enantiomers of DHC, which were simply named as the first (DHC enantiomer n°1) and the second enantiomer (DHC enantiomer n°2) depending on their HPLC retention times.

Extrelut-1 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were of analytical reagent grade.

Chromatographic instrumentation and conditions

The HPLC system consisted of a Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L4200 UV-VIS detector set to 220 nm and a Merck-Hitachi D2000 chromato-integrator (Bracco,

Milan, Italy). The columns used, from Daicel, Inc. (Schilling, Milan, Italy) were Chiralcel OD and Chiralcel ODH, both 25 cm x 4.6 mm i.d. and 10 um particle size. The Chiralcel OD column was coupled on line with the Chiralcel ODH column, which was heated at 40°C.

Resolution of the substances was achieved with n-hexane-ethanol (70/30, v/v) as the mobile phase at a flow rate of 0.9 ml/min. The mobile phase was left to equilibrate at least 2 hours before injections.

Solutions and sample preparation

Solutions of stock reference standards of OXC, racemic MHC, (+)-S and (-)-R-enantiomers of MHC, racemic DHC and CBZ (1 mg/ml, 10 ug/ml and 1 ug/ml) were prepared in ethanol and stored below 0°C. Dilutions were made fresh daily for each analysis. Serum standards were prepared daily by adding known amounts of the stock standards to blank human serum.
A 1 ml aliquot of spiked serum, with 100 ul of carbamazepine as internal standard (2 ug/ml ethanolic solution) added, was vortex-shaken for 30 sec and transferred to an Extrelut-1 glass column, which was preconditioned with 5 ml of dichloromethane-isopropyl alcohol (9/1, v/v) just before extraction and dried under nitrogen. After 10 min, the analytes were eluted under gravity with 5 ml of dichloromethane-isopropyl alcohol (9/1, v/v). The organic phase was evaporated to dryness under a stream of nitrogen and redissolved in 100 ul of HPLC mobile phase. A 20 ul volume was injected into the HPLC column.

Calibration, analytical recovery and precision

Spiked sera carried through the entire procedure were used to create calibration curves and to determine analytical

recoveries, intra-day and inter-day variabilites. The linearity of the calibration curves was studied in the range of 5-2000 ng/ml for each analyte. Analytical recoveries were perfomed at three different concentrations (10, 100, and 500 ng/ml for each substance) with 5 samples for each concentration. The same concentrations were used to test the analytical imprecision, performing analyses of serum samples for up to six days.

Drugs Interferences

Several drugs commonly administered to epileptic patients were examined for their possible interference with the determination of oxcarbazepine and its metabolites. The substances tested were: phenobarbital, diphenylhydantoin, valproic acid, carbamazepine.

One microgram of each drug was added to blank serum and to serum spiked with OXC, MHC and DHC carried through extraction procedure and analyzed by HPLC.

RESULTS AND DISCUSSION

The chiral separation of the enantiomers of MHC and DHC, and oxcarbazepine was achieved only using an on line-coupled two column system as shown in Fig. 2a.

Indeed, when Chiralcel OD column was used alone, with a mobile phase consisting of *n*-hexane-2-propanol, the peak of the second enantiomer of DHC completely overlapped that of oxcarbazepine (Fig.2b).

Modifications of the mobile phase and the addition of an achiral silica column did not improve the separation.

On the other hand, when Chiralcel ODH was used alone with a

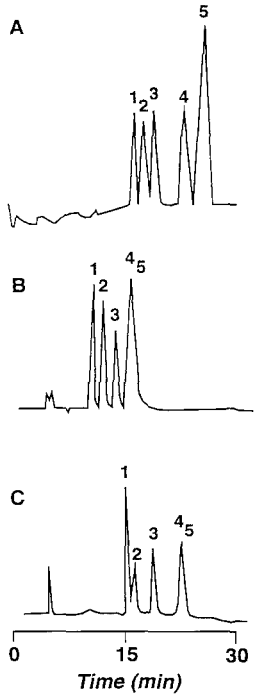


Figure 2. Chromatographic separation of oxcarbazepine and its metabolites using: a) Chiralcel OD column coupled on line with Chiralcel ODH column; b) Chiralcel OD column ; c) Chiralcel ODH column.

mobile phase of n-hexane-2-propanol (88:12), not only the second enantiomer of DHC overlapped oxcarbazepine, but also the (-)-R isomer of MHC was poorly separated with the first enantiomer of DHC (Fig.2c).

The on-line coupling of Chiralcel OD and ODH column had a beneficial effect on the separation. Furthermore, when the ODH column was heated at 40°C both the tailing factors and peak widths decreased with an improving of the peak shape. Baseline resolution was achieved using ethanol instead of 2 propanol in the mobile phase. No loss of resolution was observed after more

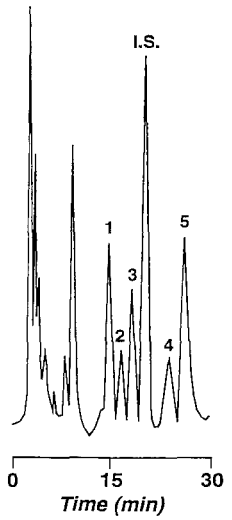


Figure 3. Chromatogram of extract of serum sample.
Peaks: 1= 1.9 ug/ml (-)-R-MHC; 2= 1.1 ug/ml first enantiomer of DHC; 3= 1.9 ug/ml (+)-S-MHC; 2 ug/ml carbamazepine (I.S.); 4= 1.1 ug/ml second enantiomer of DHC; 5= 1.1 ug/ml oxcarbazepine.

than 100 chromatographic runs over a period of five months.

None of the other antiepileptic drugs, but phenobarbital which coeluted with oxcarbazepine, interfered with the assay. Carbamazepine, which was extracted with a good recovery and eluted between the (+)-S-MHC and the second enantiomer of DHC (retention time= 21.9 min), was chosen as internal standard of the assay when determing the analytes in spiked serum (Fig.3).

The analytical recoveries of all the analytes, and the intra-day and inter-day variabilities are shown in Table 1.

The detection limit (signal-to-noise ratio of 3) and the linearity of the method are shown in Table 2.

The calibration curves were linear over the range 5-2000 ng/ml for OXC and its metabolites with correlation coefficients always higher than 0.99.

TABLE 1

Recovery and Variability

Concentration (ng/ml)	Recovery (mean ±S.D.) (%)	Variability (%)	
		Intraday	Interday
Oxcarbazepine			
10	93.3 ± 1.1	1.1	1.8
100	94.1 ± 1.2	1.1	2.3
500	94.1 ± 1.4	1.6	2.4
(-)-R-MHC			
10	98.2 ± 1.0	1.1	1.4
100	98.4 ± 1.5	1.7	1.9
500	98.7 ± 1.7	1.7	2.1
(+)-S-MHC			
10	98.1 ± 1.0	1.2	1.8
100	98.4 ± 1.5	1.3	1.9
500	98.6 ± 1.6	1.5	1.9
DHC enantiomer n° 1			
10	89.5 ± 2.1	2.3	3.1
100	90.1 ± 2.3	2.6	3.3
500	91.8 ± 2.5	2.9	3.7
DHC enantiomer n° 2			
10	89.7 ± 2.0	2.2	2.9
100	90.1 ± 2.5	2.8	3.1
500	92.0 ± 2.5	2.8	3.4

TABLE 2

Detection Limit and Linearity

Compound	Retention time (min)	Detection limit (ng/ml)	Linearity
Oxcarbazepine	28.9	5	y=3.5x + 0.25
(-)-R-MHC	15.4	5	y=1.7x + 0.67
(+)-S-MHC	19.0	5	y=1.8x + 0.11
DHC enantiomer n° 1	17.6	5	y=1.5x - 0.13
DHC enantiomer n° 2	26.2	5	y=1.2x - 0.07

In conclusion, the above results demonstrate that this HPLC method permits quick and simple extraction and simultaneous determination of OXC and the enantiomers of its metabolites MHC and DHC. The development of this enantiospecific assay could be of great help in pharmacokinetic and pharmacodynamic studies of OXC both in human and animal models, taking into account the different metabolism in these species.

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NON-SIZE EXCLUSION EFFECTS DURING GEL PERMEATION CHROMATOGRAPHY OF MILK PROTEIN HYDROLYSATES ON AN FPLC SUPEROSE 12 COLUMN

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ABSTRACT

Hydrophobic peptides and aromatic amino acids adsorbed to the matrix of a Superose 12 FPLC column during gel permeation chromatography (GPC) of milk protein hydrolysates. The adsorption phenomenon was most obvious when hydrolysates were prepared using enzyme mixtures which contained exopeptidases. The elution areas of peaks for tryptophan and tyrosine from a Superose 12 column was linear with concentration in the range 0 to 1 μmol. ml^{-1}. Amino acid analysis confirmed that a strongly adsorbed peak in the elution profile, measured by absorbance at 280 nm, of an extensively hydrolysed rennet casein contained tryptophan. Individual hydrophobic peptides, used as molecular weight markers, also interacted with the column, eluting later than expected. Nitrate in a general water supply used for the production of a whey protein hydrolysate on a pilot-scale was responsible for the appearance of an additional peak in the elution profiles from a Superose 12 column, as measured by absorbance at 214 nm.

INTRODUCTION

Proteins and peptides can be separated according to differences in molecular size using gel permeation chromatography (GPC), otherwise referred to as size (steric) exclusion chromatography (SEC) or gel filtration. Theoretical and practical aspects of GPC have been discussed (Cooper and Matzinger, 1977; Pharmacia, 1979; Yau *et al.*,

1979). A variety of gel permeation media and pre-packed columns covering a wide fractionation range are available (Pfannkoch *et al.*, 1980). The Sephadex range, G15 to G50 (Pharmacia), has been used to estimate molecular weight profiles of hydrolysed casein (Hernandez and Asenjo, 1982; Umetso *et al.*, 1983), casein-derived peptides in cheese (Reville and Fox, 1978), whey protein hydrolysates (Kuehler and Stine, 1974; Monti and Jost, 1977) and soy protein hydrolysates (Vallejo-Cordoba *et al.*, 1986). High performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) have now largely replaced the traditional low-pressure columns which were time-consuming and cumbersome to use. Developments in the analysis of milk proteins and peptides by HPLC and FPLC have been reviewed (Gonzalez-Llano *et al.*, 1990). The Toya Soda (TSK) range of HPLC gel permeation columns was discussed by Kato (1984). TSK 2000 SW columns were used to monitor proteolysis in cheese (Lemieux *et al.*, 1989) and to study casein hydrolysates (Mahmoud *et al.*, 1992; Lemieux and Amiot, 1990) and Biosil TSK 20 and Biogel TSK 125 columns (BioRad, Munich, Germany) were used for molecular weight determination of peptides (Richter *et al.*, 1983).

A column of Superose 12 gel (a cross-linked agarose-based matrix suitable for operation at low pressures) was used in an FPLC system to analyse whey proteins (Andrews *et al.*, 1986), milk protein hydrolysates (Thibault, 1990; Chobert *et al.*, 1988a, b) and to monitor proteolysis in cheese (Wilkinson *et al.*, 1992).

Proteins and peptides should, ideally, not interact with the column matrix during GPC. However, in practice, such conditions are difficult to achieve. Examples of non-size exclusion effects include ionic and hydrophobic interactions between GPC media and proteins, peptides and/or amino acids (Gelotte, 1960; Porath, 1960; Eaker and Porath, 1967; Belew *et al.*, 1978; Tchorbanov *et al.*, 1991; Ilieu and Tchorbanov, 1992; Wallace, 1992). Inorganic ions also interact with GPC media (Neddermeyer and Rogers, 1968; Yoza, 1973; Sinibaldi and Lederer, 1975). A useful discussion on non-size exclusion effects was presented by Barth (1980).

Non-size exclusion phenomena are often ignored in reports on size analysis of peptides in protein hydrolysates. Tryptophan and tryptophan-containing peptides were adsorbed onto polyacrylamide Biogel P2 during chromatography of protein hydrolysates and fermentation media (Tchorbanov *et al.*, 1991). A technique for quantifying tryptophan, exploiting this phenomenon, was described by Ilieu and Tchorbanov (1992).

This chapter describes anomalous elution patterns observed during chromatography of milk protein hydrolysates on a Pharmacia FPLC Superose 12 column and, in particular, interactions of amino acids, peptides, $NaNO_3$ and $NaNO_2$ with the gel matrix.

MATERIALS AND METHODS

Gel permeation chromatography

Chromatography was performed using a fast protein liquid chromatograph (FPLC) fitted with a Superose 12 gel permeation column, two ultraviolet single-path monitors (UV-1) set to operate at 214 or 280 nm (in that order), an autosampler (ACT 100) and fraction collector (FRAC 100) from Pharmacia LKB Biotechnology, Uppsala, Sweden. The analogue outputs of the UV-1 monitors were interfaced with a Minichrom data handling package (V.G. Data Systems, Altrincham, Manchester, UK) for data collection and peak integration. The elution buffer (pH 7.0) was 0.1 M Tris-HCl - 0.1 M NaCl - 10% methanol, at a flow rate of 0.5 ml min^{-1}. Protein hydrolysates were diluted to the equivalent of 0.25% protein (N x 6.38) in the elution buffer and 100 µl applied to the column. When fractions were recovered, 6 mg protein, disolved in 200 µl of elution buffer, was applied to the column and 1 ml fractions collected.

Calibration standards

The calibration standards used were obtained from Sigma Chemical Co. Ltd., Gillingham, Dorset, UK; Serva Feinbiochemica, GmbH and Co., Heidelberg, Germany; Pharmacia LKB Biotechnology, Uppsala, Sweden or BDH Laboratory Supplies, Merck Ltd., Poole, Dorset, UK.

Amino acid analysis

Amino acid analysis was performed on a Beckman 6300 autoanalyser (Beckman Instruments Ltd., High Wycombe, UK) using a cation exchange column, (Na form, 12 cm x 4 mm i.d.). A standard amino acid mixture

(Sigma, A-9781) and a tryptophan standard (Sigma, T-1029) were used to calibrate the column. Norleucine (Sigma, N-8513) was used as internal standard. Samples and standards were diluted in 0.2 M sodium citrate buffer, pH 2.2; 50 µl were applied to the column and eluted with sodium citrate buffers as described in Table 1. Amino acids were post-column derivitized with ninhydrin and detected by absorbance at 570 and 440 nm. Data collection and integration was with a V.G. Minichrom system, as described above.

Protein hydrolysates

The milk protein hydrolysates were prepared as described by O'Callaghan (1994).

Protein determination

The nitrogen content of protein solutions was determined in duplicate by the micro-Kjeldahl procedure (AOAC, 1980) and converted to protein by multiplying by 6.38.

RESULTS

Elution pattern of standard proteins, peptides and amino acids

Protein-containing eluates from chromatographic columns are generally detected by measuring absorbance at 280 nm which relies on the absorbance of the aromatic residues, tryptophan and tyrosine (Sober, 1968). However, in the case of small peptides, detection at 280 nm may be unsuitable, as all peptides may not contain these aromatic residues; therefore, wavelengths in the range 205-220 nm are more suitable for peptides as the amide bond between residues absorbs in this region (Segal, 1976).

The elution data for all the standards, their molecular weights (M_r) and elution volumes (V_e), as measured by absorbance at 214 and 280 nm, are shown in Table 2. The void volume of the column (V_o) was estimated to be 7.7 ml (see elution volume for blue dextran) and the

TABLE 1

Elution programme for analysis of amino acids on the Beckman 6300 autoanalyser using a cation exchange column (Na form, 12 cm x 4 mm i.d.).

(a) Buffer (Na citrate) changes

Conc., M	pH	Time, min
0.2	3.28	0-17.8
0.2	4.25	17.8-32.0
1.0	6.40	32-62.0
0.2	3.28	62-75*

(b) Temperature changes

Temp. °C	
47	0-11
70	11-28
77	28-60
47	60-70

*Prior to regeneration, the column was washed for 1 min with 1 M NaOH

total column volume (*Vf*) to be 19.5 ml (see elution volume for NaN_3 and average elution volume of the non-aromatic amino acids).

The elution plots (log_{10} molecular weight *vs* elution time, min), as measured by absorbance at 214 and 280 nm are shown in Figure 1. For the sake of clarity, all the points are not shown in the 214 nm plot as the elution time of some of the low molecular weight standards overlapped. All the protein standards eluted as single peaks in order of decreasing molecular weight, as expected; however, some peptides and aromatic amino acids showed anomalous elution patterns. Angiotensinogen, a hydrophobic plasma peptide (M_r, 1759) consisting of 14 residues (Mahler and Cordes, 1968), eluted as two peaks of equal absorbance at 20 ml and 22 ml at 280 nm but when assayed at 214 nm, the peak at 22 ml dominated. Actinomycin, an antibiotic (M_r, 1280) from *Streptomyces spp.* which is structurally a complicated molecule possessing two short chains (5 residues each) linked to a dicarboxylic acid derivative of a phenoxazone (Stryer, 1988), had similar elution patterns at both wavelengths, i.e., a major peak at 22.2 ml, preceded by

TABLE 2

Elution volume, ml, as determined by absorbance at 280 and 214 nm, of molecular weight standards used to determine the elution behaviour from a Superose 12 FPLC gel permeation column. The source, molecular weight, daltons, and $\log_{10}$ molecular weight of standards are shown

No.	Standard	Source	Code	Molecular weight (daltons)	$\log_{10}$	Elution volume, ml 280 nm	214 nm
1	Blue dextran	Pharmacia	17-0442-01	2,000, 000	6.301	7.87	7.69
2	Ferritin	Serva	21318	455, 000	5.658	10.93	10.93
3	IgG	Sigma	I-5506	150, 000	5.176	12.08	12.07
4	BSA	Pharmacia	17-0442-01	67, 500	4.829	13.00	13.00
5	Ovalbumin	Pharmacia	17-0442-01	43, 000	4.633	13.92	13.77
6	β-Lactoglubulin	Sigma	L-0130	36, 000	4.556	14.35	14.23
7	α-Lactalbumin	Sigma	L-6010	14, 400	4.158	15.58	15.47
8	Cytochrome C	Serva	18020	12, 300	4.090	16.20	16.07
9	Insulin chain B	Serva	52190	3, 494	3.543	18.00	17.87
10	Angiotensinogen	Serva	51295	1, 759	3.245	19.83/22.56*	18.58/22.13*
11	Actinomycin	Serva	10708	1, 280	3.107	19.77/22.95*	18.93/22.75*
12	Pz-Pro-Leu-Gly-Pro-D-Arg	Serva	52268	777	2.890	24.72	24.58
13	Suc-Ala-Ala-Ala-$_p$NA	Serva	51053	451	2.654	22.57	22.42
14	Riboflavin	Sigma	R-450	377	2.576	24.27	24.13
15	DL-Tryptophan	Sigma	T-0129	204	2.310	28.65	28.50
16	L-Tyrosine	BDH	37156	181	2.258	21.75	21.65
17	L-Arginine	Sigma	A-5131	174	2.241	-	20.00
18	DL-Phenylalanine	BDH	2496090	165	2.218	-	20.38
19	L-Histidine	Sigma	H-8000	155	2.190	-	19.41
20	L-Lysine	BDH	C010980	146	2.164	-	19.50
21	L-Aspartic acid	Sigma	A-9256	133	2.124	-	18.77
22	L-Leucine	Sigma	L-8000	131	2.118	-	19.80
23	L-Isoleucine	Sigma	I-2752	131	2.118	-	19.40
24	L-Cysteine	Sigma	C-7880	121	2.069	-	19.80
25	L-Valine	Sigma	V-0500	117	2.062	-	19.41
26	Proline	Sigma	P-0380	115	2.061	-	19.10
27	Serine	Sigma	S-4500	105	2.022	-	19.40
28	Alanine	Sigma	A-7627	89	1.950	-	19.37
29	$NaNO_3$	BDH	10255	85	1.929	-	22.22
30	Glycine	BDH	10119	75	1.876	-	19.41
31	NaN_3	BDH	10369	56	1.748	-	19.50

*indicates that more than one peak was detected.

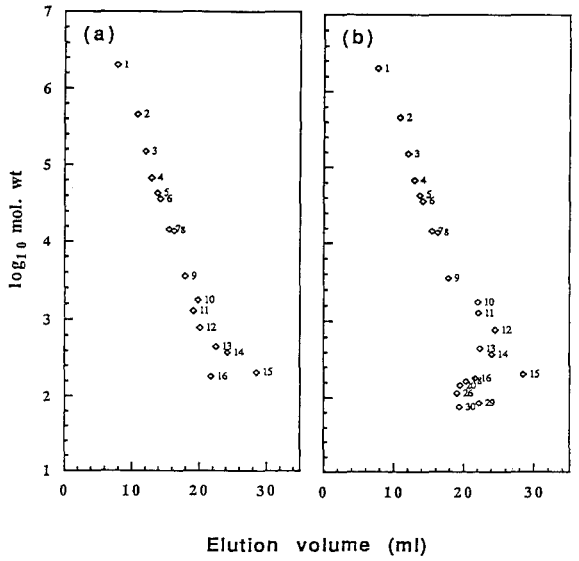

FIGURE 1. Elution pattern of standard proteins, peptides, amino acids and inorganic salts from an FPLC Superose 12 gel permeation column (Pharmacia) assayed by absorbance at: a) 280 and b) 214 nm. The elution buffer (pH 7.0) was 0.1M Tris-HCl - 0.1M NaCl - 10% methanol; flow rate, 0.5 ml min^{-1}. Standards are identified in Table 2.

a broad skewed peak at 19 ml. The synthetic peptides, PZ-Pro-Leu-Gly-Pro-D-Arg (M_r, 777) and Suc-Ala-Ala-Ala (M_r, 451) eluted as single peaks at 25 and 22 ml, respectively. The hydrophobic nature of these peptides was assumed to be responsible for their interaction with the column and they were excluded from the calibration plots used to estimate molecular size distribution of peptides in protein hydrolysates (Fig. 2). Wallace (1990) showed that single peptides also eluted later than expected from a Sephadex G-25 column. The aromatic amino acids, tryptophan (Trp) and tyrosine (Tyr) were adsorbed strongly on the Superose 12 column, eluting at volumes of 28.5 and 22 ml, respectively. Phenylalanine (Phe) showed little interaction with the column, eluting close to V_t at 20.35 ml. Of all the amino acids, Phe, Tyr and Trp are the most hydrophobic, with side chain hydrophobicities of 11.1, 12 and 12.5 kJ.mol^{-1}, respectively (Fennema, 1985), which corresponded to the order in which they eluted from the column. Tyr

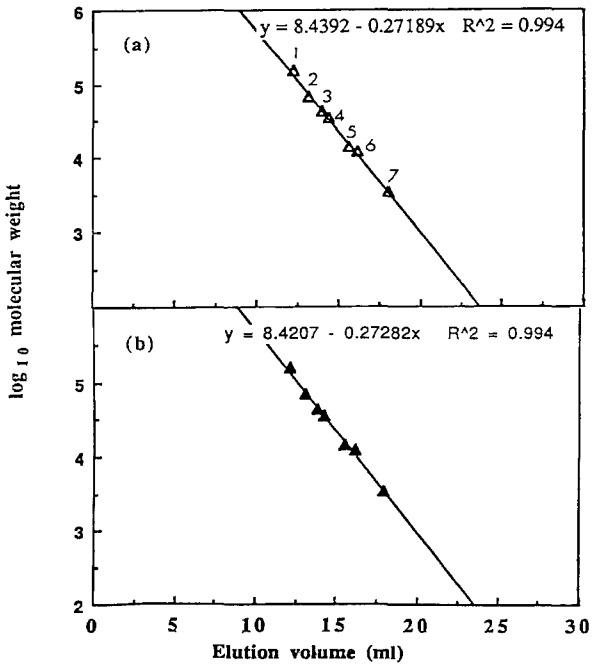

FIGURE 2. Calibration plots for the FPLC Superose 12 column, determined by absorbance at: a) 280 and b) 214 nm, used to estimate molecular size distribution of peptides in protein hydrolysates; 1, IgG (150,000); 2, BSA (67,500); 3, ovalbumin (43,000); 4, b-lactoglobulin (38,000); 5, a-lactalbumin (14,700); 6, cytochrome C (12,300); 7, insulin chain B (3,494).

was less interactive than Trp. All other amino acids eluted close to V_t, including Leu and Ile, which have side chain hydrophobicities of 10.1 and 12.4 kJ. mol-1, respectively. The aromatic side chains of Phe, Tyr and Trp are most likely to be responsible for interaction with the gel matrix.

Calibration plots for estimating molecular weight profiles of peptides in protein hydrolysates

Calibration plots were prepared using elution data for the seven standard proteins, ranging in molecular weights from 150, 000 to

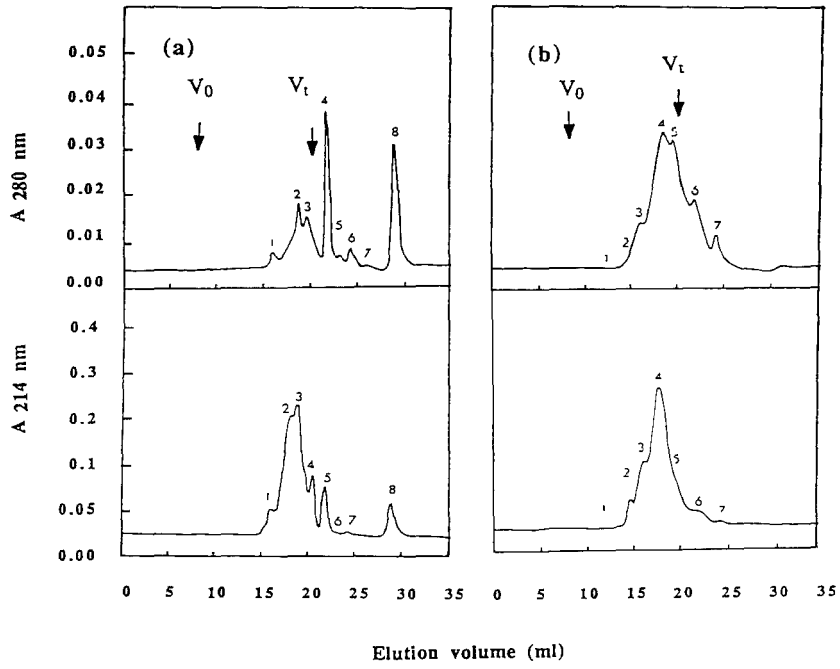

FIGURE 3. Elution profiles of casein hydrolysates from an FPLC Superose 12 column assayed by absorbance at: a) 280 and b) 214 nm. Elution conditions were as in Fig. 1. Sample volume: 100 µl of 0.25%, w/v, protein solution; (a) rennet casein hydrolysed with a mixed enzyme preparation, BN2001 (DH, 23%); (b) rennet casein hydrolysed with a specific proteinase preparation, Profix (Papain, DH, 10%).

3,494 daltons and are shown in Fig. 2. The elution volumes of the adsorbing, low molecular weight peptides were not included in the calibration plots because of their anomalous elution pattern.

The regression equations for the calibration plots had high r^2 values (0.994) and from the regression lines, the lower end of the fractionation range was estimated to be approx. 1000 daltons.

Hydrophobic interactions during GPC of protein hydrolysates

During GPC of milk protein hydrolysates, a number of peaks were observed to elute after V_t, in particular when the hydrolysates were prepared using enzyme mixtures containing exopeptidases (Chapters 3

to 5). Figure 3 shows the elution profiles, as measured by absorbance at 280 and 214 nm, of two casein hydrolysates, one prepared using a mixed enzyme preparation (BN2001, Imperial Biotechnology, London, UK) which contains pancreatic exopeptidases and the other prepared using a single proteinase, papain (Profix, Quest-Biocon, Cork, Ireland). In the hydrolysate prepared using the mixed enzyme preparation (Fig. 3a) two major peaks (4 and 8) and some minor peaks eluted after V_t (which corresponded to peak 3). However, in the hydrolysate produced with papain (Fig. 3b) there was little adsorption since little material eluted after V_t.

Isolation and identification of material present in adsorbed peak 8 from a chromatogram of casein hydrolysate, prepared using proteinase BN2001, on Superose 12

Casein hydrolysate (6 mg) prepared using the mixed proteolytic enzyme preparation, BN2001, was applied to the Superose 12 FPLC column and eluted as described previously. The elution profile was monitored at 280 nm and the eluate corresponding to peak 8 collected. Some of the eluate (100 μl) was rechromatographed on the same column; a tryptophan solution (100 μl) was also chromatographed on the column under similar conditions. The elution profile of the casein hydrolysate, the rechromatographed peak and tryptophan are shown in Fig. 4. The adsorbed peak (8) and the tryptophan standard had the same elution volume (28.5 ml). A sample (50 μl) of eluate corresponding to peak 8, a standard Sigma amino acid mixture and a tryptophan standard were analysed separately on a Beckman 6300 amino acid analyser. The chromatogram for the standard Sigma amino acid mixture is shown in Fig. 5a. Peaks 1 to 20 were identified based on retention times for individual amino acids (Table 3). Peak 18 corresponded to Trp which eluted as a broad peak. This is attributed to its bulky nature which causes a molecular sieving effect during ion exchange chromatography (Bech-Anderson, 1992, National Institute of Animal Science, Tjele, Denmark, personal communication). Peak 19 was identified as ammonia and appeared as a contaminant in all chromatograms, including blank runs. Its appearance has been attributed to absorption of atmospheric ammonia by the acidic citrate buffers (Brown, 1991, Beckman Instruments, High Wycombe, UK). Norleucine (peak 13) was included as an internal standard in the dilution buffer.

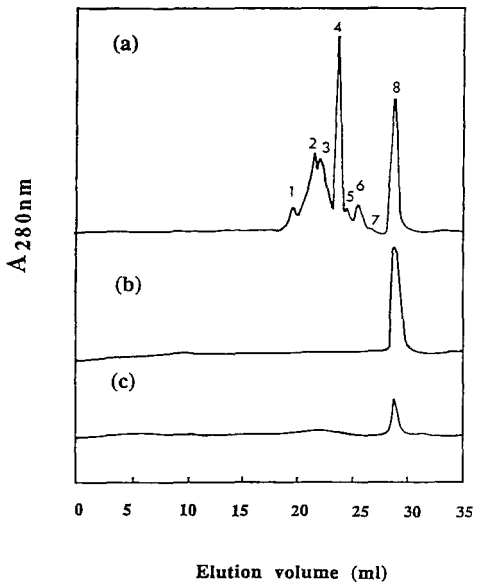

FIGURE 4. Chromatograms from the Superose 12 FPLC column, assayed by absorbance at 280 nm, of a) BN2001/casein hydrolysate; b) tryptophan standard, 80 μmol. ml-1; c) eluate (100 μl) corresponding to adsorbed peak 8 reapplied to column.

The ninhydrin derivative of proline (peak 5) absorbed weakly at 570 nm and was detected separately at 440 nm. Changes in baseline absorbances were due to buffer and temperature changes during the elution programme (Table 2). Figure 5b shows the chromatogram of the Trp standard. The chromatogram of the eluate containing the material in the 'adsorbed' peak (8) from the FPLC column showed four ninhydrin-positive components (Fig. 5c), two of which corresponded to peaks 13 and 19, i.e., norleucine and ammonia, respectively; one of the other ninhydrin-positive compounds corresponded to Trp (peak 19). The elution profile obtained for a 1:1 mixture of the FPLC buffer and dilution buffer containing the internal standard showed two peaks, one corresponding to norleucine (19), the other (T) to Tris-hydroxymethylamine from the FPLC buffer. These results confirm that peak 8 in the FPLC chromatogram (Figs. 3 and 4) contained tryptophan which interacts hydrophobically with the Agarose moiety

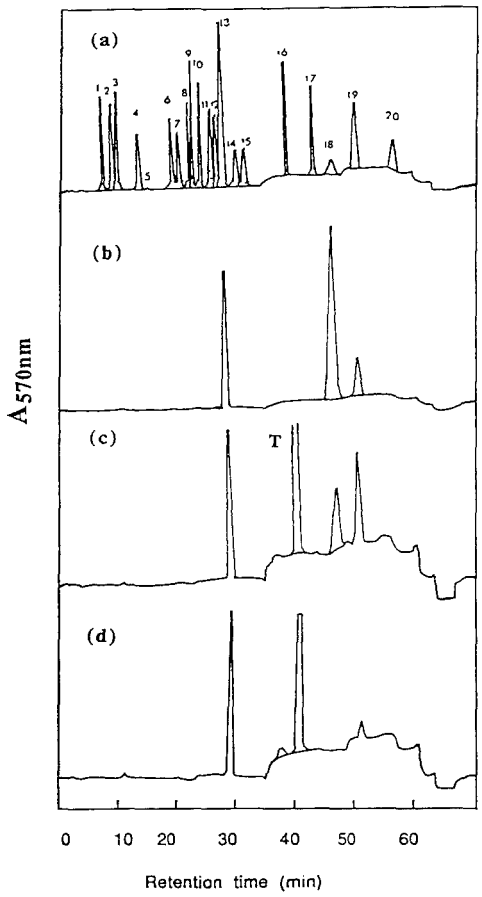

FIGURE 5. Chromatograms of amino acids obtained from a Beckman 6300 AAA assayed by absorbance at 570 nm after post-column derivitisation with ninhydrin. Elution conditions are in Table 1 and peaks were identified according to retention times of individual amino acids (Table 3). a) standard amino acid mixture, 25 nmoles ml^{-1}; b) tryptophan standard, 125 nmoles ml^{-1}; c) adsorbed peak 8 from the Superose 12 FPLC column; d) FPLC elution buffer (pH 7.0), Tris-HCl - 0.1M NaCl - 10% methanol.
The peak labelled 'T' corresponds to Tris-hydroxymethylamine

TABLE 3

Identification of ninhydrin-positive compounds in a standard amino acid mixture (Sigma A-9781), analysed on a Beckman 6300 autoanalyser (cation exchange column, Na form, 12 cm x 4 mm i.d.), as shown in the chromatogram in Figure 5a

Peak No.	Amino acid	Abbreviation	retention time (min)
1	Aspartic acid	Asp	8.80
2	Threonine	Thr	10.45
3	Serine	Ser	11.38
4	Glutamic acid	Glu	14.92
5	Proline	Pro	16.34
6	Glycine	Gly	20.53
7	Alanine	Ala	21.83
8	Cysteine	Cys	23.51
9	Valine	Val	24.03
10	Methionine	Met	25.37
11	Isoleucine	Ile	27.08
12	Leucine	Leu	28.00
13	Norleucine[1]	Nor	29.15
14	Tyrosine	Tyr	31.39
15	Phenylalanine	Phe	32.94
16	Histidine	His	39.86
17	Lysine	Lys	44.33
18	Tryptophan	Trp	47.41
19	Ammonia	NH_3	51.72
20	Arginine	Arg	58.17

1. Norleucine was used as internal standard.

of Superose 12. Tryptophan has also been observed to bind to the polyacrylamide gel medium, Biogel P2, during chromatography of commercial protein hydrolysates (soy and casein) and fermentation media (Tchorbanov *et al.*, 1991; Ilieu and Tcharbanov, 1992).

Adsorption of inorganic salts to the Superose 12 matrix

During elution of a whey protein hydrolysate, produced on a pilot-scale, from the Superose 12 column, an additional peak (V_e ~22 ml) was observed after V_t in the elution profile assayed at 214 nm (Fig. 6c), which was not evident in the chromatograms of laboratory-prepared hydrolysates assayed at 214 nm (Fig. 6b) or in the chromatogram of any hydrolysate assayed at 280 nm. It was assumed initially that this adsorbed peak was due to an additional peptide. Analysis of the chromatograms of the materials (WPC, enzymes, neutralising agents, etc) used in the preparation of the hydrolysate showed no peak eluting in this position. When whey protein hydrolysates prepared in the laboratory under various conditions were chromatographed on the Superose 12 column this additional peak was absent. In the pilot-scale process used for the production of whey protein hydrolysates, a general water supply was used to prepare the whey solution and when 100 µl of this water were chromatographed on the FPLC column, a peak with an elution volume corresponding to that of the additional peak (V_e, ~22 ml) was evident (Fig. 6d). Furthermore, the material responsible for this additional peak was removed by passing the water through an anion exchange resin, while passage through a cation exchanger or activated carbon did not remove the material, suggesting that the material adsorbing on the column was anionic.

When aqueous solutions (0.2%, w/v) of $MgCl_2$, Na_2HPO_4, K_2SO_4, Na_2SO_4, KCl, NaCl and $CaCO_3$ were chromatographed on the Superos 12 column, no absorbance at 214 nm in the elution region of interest was observed. However, when solutions of $NaNO_3$ and $NaNO_2$ (0.2%, w/v) were chromatographed on the column a large absorbance at 214 nm in the region of 22 ml elution was evident, which corresponded in elution volume to the 'additional' peak seen in the pilot-scale whey protein hydrolysate and in the general water supply (Fig. 6d). Analysis on the general water supply showed that it contained a high level of nitrate (22 $mg.kg^{-1}$ NO_3). After deionisation, NO_3 levels in the water were neglible and when this deionised water was used, the FPLC

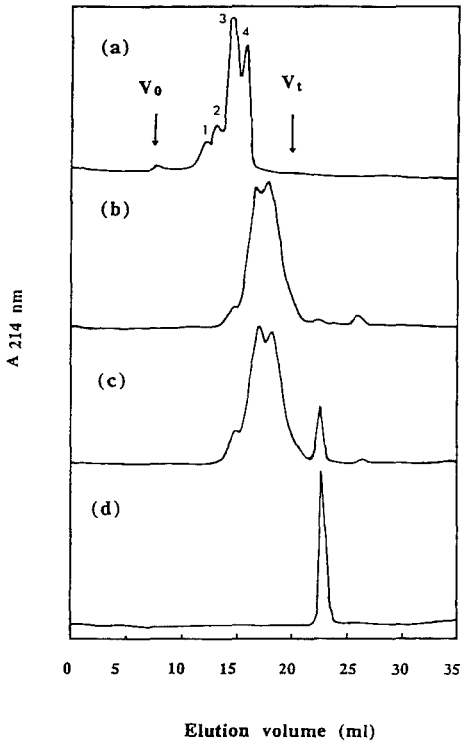

FIGURE 6. Elution profiles of whey protein hydrolysates from an FPLC Superose 12 column, assayed by absorbance at 214 nm. Elution conditions were as in Fig. 1. Sample volume, 100 µl of 0.25% (w/v) protein solution; (a) whey protein concentrate; 1, IgG; 2, BSA; 3, b-Lg, 4, a-La; (b) laboratory-prepared whey protein hydrolysate; (c) pilot-scale whey protein hydrolysate showing an additional peak at 22 ml; (d) 100 µl of general water supply used to prepare pilot-scale hydrolysate, $NaNO_3$ (20 $mg.kg^{-1}$) eluted in exactly the same position. The void volume (V_0) and total column volumn (V_t) are indicated by arrows.

chromatograms of later pilot-scale hydrolysates were similar to those of laboratory-produced hydrolysates.

Quantitative adsorption of Tyrosine, Tryptophan and NaNO3 to the Superose 12 column matrix

When standard solutions (0-1 µmole ml^{-1}) of tyrosine and tryptophan were chromatographed on the Superose 12 column and the

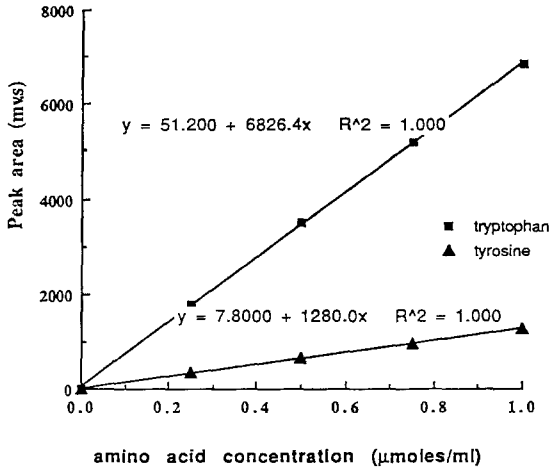

FIGURE 7. Relationship between peak area (mv.s) and concentration of applied tryptophan and tyrosine standard solutions (0-1 µmol. ml^{-1}), assayed by absorbance at 280 nm, of eluate from the FPLC Superose 12 column. Regression analysis showed $r^2 = 1.00$

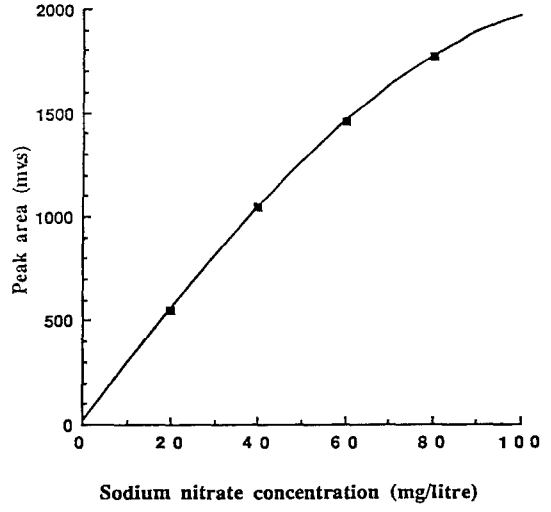

FIGURE 8. Relationship between peak area (mv.s) and concentration of applied $NaNO_3$ solutions (0-80 $mg.l^{-1}$), assayed by absorbance at 214 nm, of eluate from the FPLC Superose 12 column. Regression analysis showed $r^2 = 1.00$

absorbance at 280 nm measured, a linear relationship (r^2 =1 .00) was found between peak area, as measured in millivolt seconds (mv.s), and the concentration of applied amino acid solutions (Fig. 7). When standard solutions of $NaNO_3$ (0-80 $mg.kg^{-1}$) were chromatographed on the column, a curvilinear response (r^2 =1.00) was observed between peak area (mv.s), assayed by absorbance at 214 nm, and concentration of applied solutions (Fig. 8).

DISCUSSION

When reporting on the molecular size distribution of protein hydrolysates, as determined by gel permeation chromatography, it is necessary to keep in mind that elution is not necessarily governed by differences in molecular size only. The gross shape of the molecule and its physicochemical characteristics, especially net charge and hydrophobicity, may also play a role in separation. Hydrophobic amino acids and pure peptides containing hydrophobic residues interacted with the Agarose matrix of the Superose 12 column. Gelotte (1960) was one of the first to observe the phenomenon of hydrophobic interaction of aromatic and heterocyclic compounds with Sephadex. Wallace (1990) found a poor relationship between molecular weight and elution volume of pure peptides, which eluted later than expected from a Sephadex G-25 column.

During GPC of milk protein hydrolysates on a Superose 12 FPLC column, a number of peaks eluted after V_t, one of which contained the aromatic amino acid, tryptophan. The elution of hydrophobic amino acids from the Superose 12 column was in the order of increasing hydrophobicity, i.e., Phe, Tyr and Trp. Adsorption of tryptophan to Biogel-P2 was observed previously during GPC of commercial protein hydrolysates and fermentation media (Tchorbanov *et al.*, 1991; and Ilieu and Tcharbanov, 1992). These authors proposed that the adsorption of tryptophan to the Biogel P2 medium could be exploited for quantifying tryptophan in extensively hydrolysed protein samples. The adsorption of tryptophan and tyrosine on Superose 12 matrix was also quantitative and can be exploited to monitor exopeptidase activity during enzymatic hydrolysis of milk proteins (Chapter 5). The inorganic salts, $NaNO_3$ and $NaNO_2$, also interacted with the Superose 12 column matrix, giving rise to peaks when assayed at 214 nm but not at 280 nm.

A salt exclusion effect was observed previously with Sephadex (Neddermeyer and Rogers, 1960) where anions were excluded from the

gel bed and eluted earlier than expected. This 'ion exclusion' effect arises when the surface of a gel matrix has a net charge and solutes of similar charge become excluded from the pores because of electrostatic repulsion. However, this type of interaction does not explain the elution of NO_3^- and NO_2^- from the Superose 12 column as these ions eluted later than expected from the column. An 'ion inclusion effect' was reported by Stenlund (1976) during gel chromatography, and was explained on the basis of a Donnan membrane equilibrium between ions and the gel surface.

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AUTHENTICATION OF L-ASCORBYL-2-SULFATE IN SALMONID GASTRIC TISSUE: HPLC/ELECTRO-SPRAY IONIZATION MASS SPECTROSCOPIC VERIFICATION

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ABSTRACT

New findings establish the intact transfer of the L-ascorbyl-2-sulfate ester across the stomach membrane in salmon. In addition, measurement of the ester has been confirmed in a commercial fish diet. The procedure used a non ion-pairing C_{18} reverse phase column for the separation of L-ascorbyl-2-sulfate (AS) from coho salmon gastric tissue. The AS fraction was eluted from the column with 0.1 M ammonium acetate pH 5.0 and rechromatographed to ascertain purity. When chromatographed under the same conditions, the collected AS fraction had the same retention time as the purified standard of L-ascorbyl-2-sulfate dipotassium salt. The purified AS fraction was then confirmed as to structure by Electrospray Ionization Mass Spectrometry . The AS fraction gave the same Electrospray Ionization Mass Spectrometry results as the standard AS when subjected to mass spectrometry-mass spectrometry (MSMS) analysis.

INTRODUCTION

A stable form of vitamin C has been sought for moist and dry feed by the aquaculture and animal industries. They needed a form of C that would maintain activity during high temperature processing and storage. Several products had been synthesized and were available to feed manufacturers. Previously, one of these products (AS) has been identified as an extractive in fish liver (1, 2 and 3).

After discovery of AS in 1971 (4) and the synthesis of AS (5), there was keen interest in utilizing this product as a source of vitamin C in animal feed. However, before the product could be fully evaluated in the intact animal, there needed to be an unequivocal method for identification of the vitamin C form (AS) in biological tissue. Most HPLC methods of identification have been fraught with co-elution problems (6, 7). This co-elution problem had made it difficult to quantify the AS in tissue and feeds, leading to skepticism by feeders. It was important for investigators to validate specifically what was being measured and the exact amount.

The purpose of this work was to establish credibility in the identification of AS in fish food and in tissue so that studies of the product could move forward.

METHODS AND MATERIALS

Equipment:

The analytical column used was an Alltima (Alltech Associates) C_{18} reverse-phase column, 250 mm x 4.6 mm and a 5 µm particle size. A hand-packed Alltima guard column was used to protect the analytical column.

The HPLC equipment used was a Perkin-Elmer Corporation (P E) (Norwalk, CT) model 250 isocratic pump with an in line PE solvent filter, a PE model 290 UV/Vis detector, set at 254 mm, and a Bioanalytical Systems Inc. (BAS) electro-chemical detector, set at +0.72 volts. The data system was linked to a PE Nelson model 950 interface and an Epson III+ computer. The software used was the PE Nelson 2100.

The HPLC method and conditions were as previously described (7).
Briefly, the column was run at room temperature (25°C) with a mobile phase of 0.10 M ammonium acetate pH 5.0 and a flow rate of 0.75 ml/min. The column pressure ranged from 2000 to 2200 psi., depending upon the column age, condition, and nature of pre-column filters.

Tissue was homogenized with a Brinkmann Polytron homogenizer and centrifuged with an Eppendorf Minifuge (Brinkmann Instrument Co., Westbury, N.Y.).

The AS and AA standards were obtained from Pfizer, Inc., Groton, CT and Hoffmann La Roche Co., Nutley, N.J. respectively.

Mass Spectroscopy:

Samples were analyzed on a triple-quadrupole Sciex API III instrument (PE/SCIEX Thornhill, Ontario, Canada). Samples were infused into the electrospray source via a 50 micron i.d. fused silica transfer line using a Harvard Apparatus pump at 1-3 uL/min. Positive ion mass spectroscopy (MS) and mass spectroscopy-mass spectroscopy (MS/MS) were run with an orifice voltage of 70 to 80 volts. Negative ion electrospray MS and MS/MS used an

orifice voltage from -70 to -90 volts. The interface temperature was maintained at 52° C. For tandem mass spectrometry (MS/MS), precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.20 daltons (Da) and 1ms/step. In order to select a mass from Q1 for MS/MS studies, parent ion transmission was maximized by reducing the resolution of Q1 to transmit a 2 to 3 m/z window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart. Spectra were collected and analyzed using proprietary software from Sciex Corporation.

The mass spectrometer was calibrated to eight different poly (propyleneglycol) masses.

Solvent and Tissue Preparation:

The solvent system for the mobile phase (0.10 M ammonium acetate pH 5.0) was made as follows: 0.1 M reagent grade acetic acid was titrated to pH 5.0 with isothermally distilled ammonium hydroxide.

Tissue extracts were made as follows: The 5% trichloroacetic acid (TCA) extract was made by homogenizing 1 vol. of tissue to 4 vols. of glass distilled water for 30 sec. While homogenizing, 5 vols. of 10% TCA were added, and homogenizing was continued for another 60 sec. This extract was centrifuged and supernatant solution was decanted and filtered through a 0.45 mm syringe filter. The filtered solution was used for the HPLC determination.

HPLC Peak Collection:

The AS retention time was 3.23 minutes when using the ammonium acetate (0.1 M and pH 5.0) mobile phase (AS shown chromatographed with AA in Figure 1). The AS peak was collected at the outlet from the ultraviolet (UV) detector with a time frame of 3.0 to 3.5 minutes (vol. = 375 µl). This peak was rechromatographed to establish purity and quantity.

Tissue Collection:

Each coho salmon smolt (weighing about 400 grams)was force-fed AS in the following manner: the smolt was anesthetized with Tricaine Methane Sulfonate (MS 222) until it could be handled without struggling; then a polished-end glass tube (3.5 mm ID) was inserted through the mouth into the stomach. A #13 gelatin capsule was filled with 75 mg of AS mixed with crystalline serum albumin (3+1 parts respectively). This small capsule was forced through the glass tube with a 6 in. (Puritan) applicator stick. The glass tube was removed and the fish was returned to the tank. In order to make sure the capsule was not regurgitated, the fish had to be observed carefully for about 3 minutes .

After 24 hours, the fish was sacrificed and the stomach removed. The stomach cavity was opened and washed clear of any solids or absorbed substances, after which the portion from

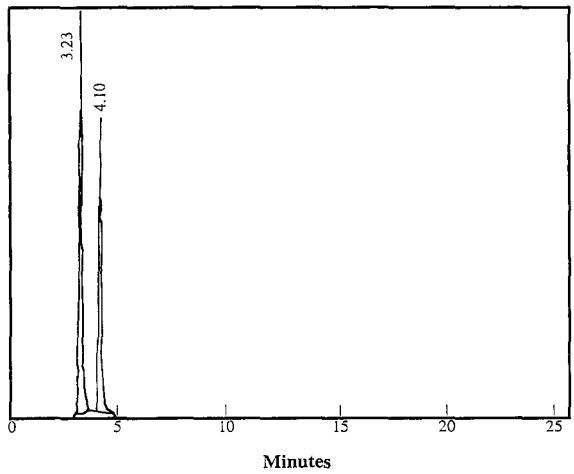


Figure 1 HPLC standards, L-ascorbyl-2-sulfate (3.23 min.) and ascorbic acid (4.10 min.) from a C18 Altima column using a mobile phase of 0.1 M ammonium acetate pH 5.0. Retention time in minutes

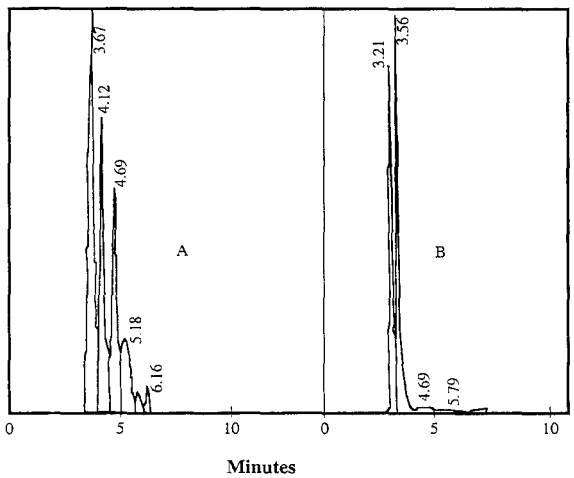


Figure 2 A A typical HPLC spectrum of a 5 % TCA gastric tissue extract from a control fish, fed a normal commercial fish diet containing ascorbic acid (AA).

Figure 2 B A typical HPLC spectrum of a 5 % TCA gastric tissue extract from a fish force-fed L-ascorbyl-2-sulfate (AS). Note the absence of ascorbic acid in the force-fed fish gastric tissue extract. The 3.56 min. peak represents an unknown that is enhanced in the presence of AS.

the esophagus to the large intestine was excised. The excised tissue was washed 10 times with 50 volumes of glass distilled water. The final rinse was saved and checked for any residual AS. The residual AS found in the rinse water represented 0.35 % of that found in the tissue. The gastric tissue was extracted with TCA as described above and injected into the HPLC.

RESULTS AND DISCUSSION

HPLC Data:

Figure 1 shows a typical HPLC computer profile of AA and AS standards using the mobile phase and other conditions listed above. These standard profiles are shown for comparison purposes with Figures 2 A and 2 B below. In Figure 1, the AS had a retention time (RT) of 3.23 minutes and AA had a RT of 4.10 minutes at medium to high concentrations. These retention times did not vary under the prescribed conditions stated above except when the concentrations were high or very low. For example, when the concentration approached the detection limits of 2 ng and 1 ng for AS and AA, respectively, the RT's were 3.17 and 4.10 minutes (7). At high concentrations they shifted to 3.29 and 4.16, respectively. Therefore, at the standard concentrations of 500 and 200 ng of AS and AA they are as seen in Figure 1. It was found that when the retention times were earlier, the column was not equilibrated.

Figure 2 A illustrates a typical HPLC profile of a 5% TCA extract of coho stomach tissue from a control fish. The tissue is from a fish that has been fed a commercial fish diet containing AA as the vitamin C source. The AA peak is clearly discernible at the retention time of 4.12 minutes. Attention is brought to the fact that there is no indication of a peak at 3.2 minutes, the retention time for AS in this system. Figure 2 B is a typical profile of a similar gastric tissue extract from an experimental fish force-fed AS as described above. The tissue extract was taken at 24 hours post feeding time. Minimization of the other peaks in this profile is due to the high concentration of AS found in the gut tissue extract. The AS peak represents 2.043 µg whereas the AA seen in Figure 2 A represents one tenth of that amount, 0.255 µg.

Note the two large and sharp peaks in Figure 2 B at RT's 3.21 (AS) and 3.56 minutes. The latter peak, which varies from 3.54 to 3.67 minutes is found in both experimental and control fish extracts. It is possibly a tissue matrix peak or carrier in nature since this peak is enhanced whenever AS is fed to the fish. Figures 3 A and 3 B are computer enlargements of Figures 2 A and 2 B. In the TCA extractives of the control fish seen in the 3 A enlargement there are no indications that would affirm the presence of AS. Figure 3 B enlargement defines AS presence in the TCA gastric extract . By way of contrast, Figure 3 C illustrates the change in the control fish profile of 2 A when 200 ng of AS standard is added to the 5% TCA gut extract.

Figure 4 A, an enlargement, was made in order to show the smaller peaks occurring after the peaks of interest (similar to a 5% TCA gut extract of a fish force-fed AS as seen in Figure 2

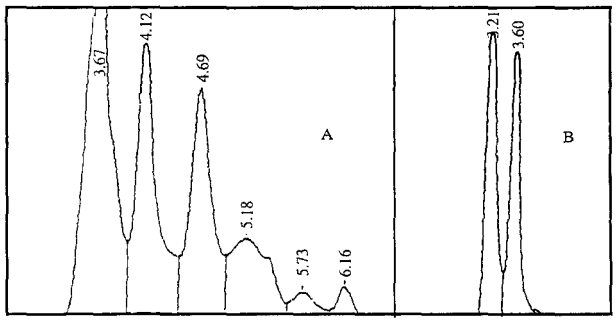


Figure 3 (A) and (B) are computer enlargements of figure 2 A and 2 B, more clearly detailing the peaks of interest.

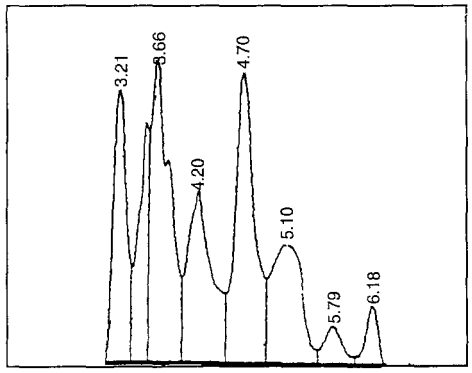


Figure 3 (C) This is a profile of the 5 % TCA extract from gut tissue of the coho salmon on a commercial diet containing AA as the vitamin C source to which is added 200 nano grams of AS. (Also a computer enlargement)

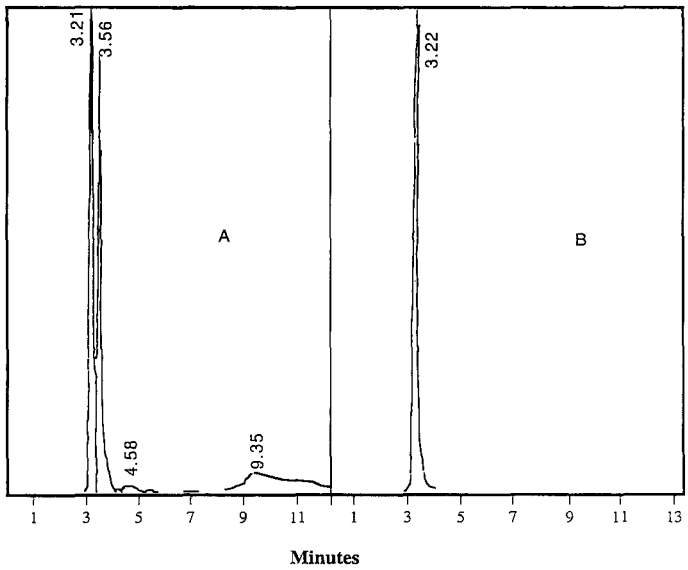


Figure 4 (A) A similar HPLC spectrum of a 5% TCA gastric tissue extract from a fish force-fed AS. 4 (B) is the spectrum of the eluted first peak from the extract at RT of 3.21 shown in 4 (A).

B). Note that there is no AA peak in this stomach extract, since there is no peak appearing between 3.89 and 4.59 where AA should be retained. Figure 4 B is the HPLC profile of the eluted AS peak seen at RT of 3.21 in Figure 4 A. The eluted UV detector peak from the 5% TCA gut extract was collected from 3.0 to 3.5 minutes (as described above). Note its relative purity . The eluted peak amount represents 0. 214 μg . The collected fraction is homogeneous as determined by the HPLC profile.

Previously it has been hypothesized that AS may be desulfated in fish gut. These new findings indicate that AS may be transported intact across the gastric membrane rather than being desulfated to AA by stomach acid and/or enzymes.

Figure 5 is the electro-chemical (EC) profile of the 5 % TCA gut extract from the force-fed fish seen in the 4 A UV profile . The spectrum is obtained from the tandem electrochemical detector connected to the outlet from the UV detector. There is a lag time of 0.3 minutes between the UV and EC detector spectrum before the spectrum of the latter appears. At RT 3.95 minutes, the EC peak is the oxidation of the UV peak (with a RT of 3.6) that is seen in Figures 3 A, B, and C. This peak is oxidizable at a glassy carbon electrode with a potential of +0.72 volts; and it is also enhanced when AS is present, posing an interesting transport possibility.

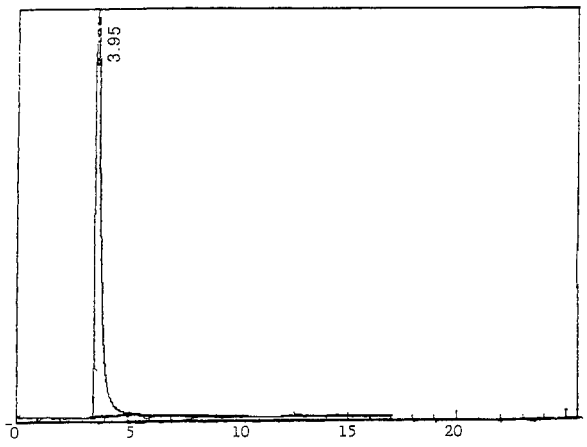


Figure 5 This electro-chemical detector spectrum at RT of 3.95 minutes, is the oxidation of the 3.5 minute UV detector peak of the 5% TCA gut extract from the force-fed fish seen in Figure 4 A.

Figure 6 A is a UV spectrum of a 5% TCA extract from a commercial diet supplemented with AS (50 mg/100g). Note that the AS retention time (3.29 min.) is characteristic of a high concentration, as previously described. The RT difference is in hundreds of a second. The dietary AS peak amount represents 1.32 µg. The AA peak is very small since it represents a residual AA of 20 µg/g from storage diet. The AS peak calculates to a dietary concentration of 52.50 mg/100 g as determined by standard addition, representing a recovery of 105 %.

Figure 6 B is the spectrum of the eluted AS peak from the diet extract shown in Figure 6 A. The separation of the AS peak from a diet extract is very difficult because there is a contaminating peak (appearing to the left of the major peak).

Mass Spectroscopy Data:

Figure 7 illustrates a typical MS spectrum of standard L-ascorbyl-2-sulfate, Astos standard. The solubilized form of L-ascorbic acid-2-sulfate has a calculated mass of 255 Da. Note that the major base peak of the standard has a m/z of 255.2 Da. This mass peak was used for the MS/MS analysis. Figure 8 is the MS spectrum of the eluted peak from the HPLC run of the coho gut extract . It is basically identical to the Astos standard seen in Figure 7. The change in Argon density may have caused the difference in the mass peak intensity at 227 Da and 241

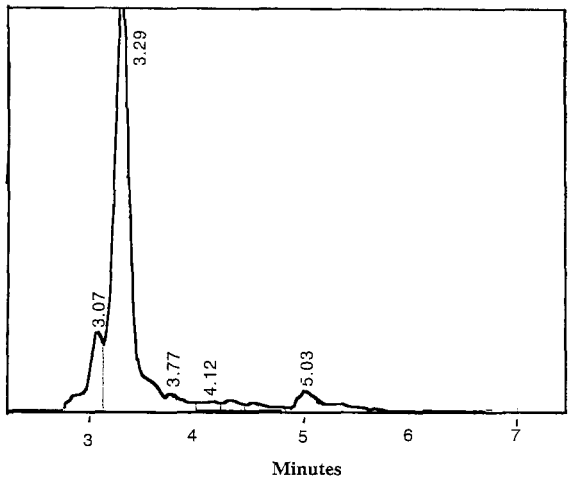


Figure 6 (A) An HPLC spectrum of the 5% TCA extract from a commercial diet containing internal AS standard (at RT of 3.29 min.) and a small quantity of AA at RT 4.12 minutes. Expanded spectrum.

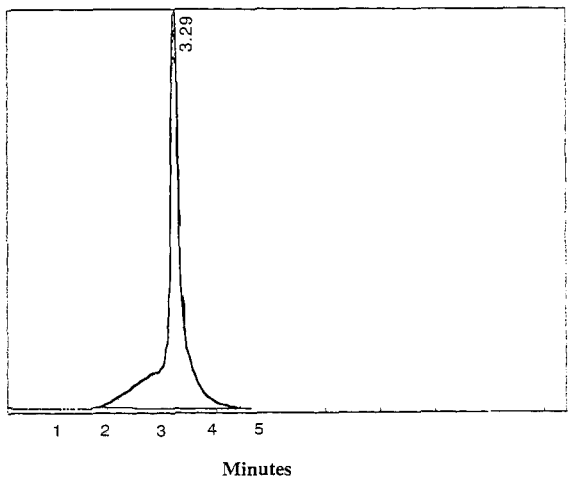


Figure 6 (B) is the spectrum of the eluted AS peak at RT of 3.29 min. from the commercial diet extract shown in Figure 6 (A) above. Expanded spectrum.

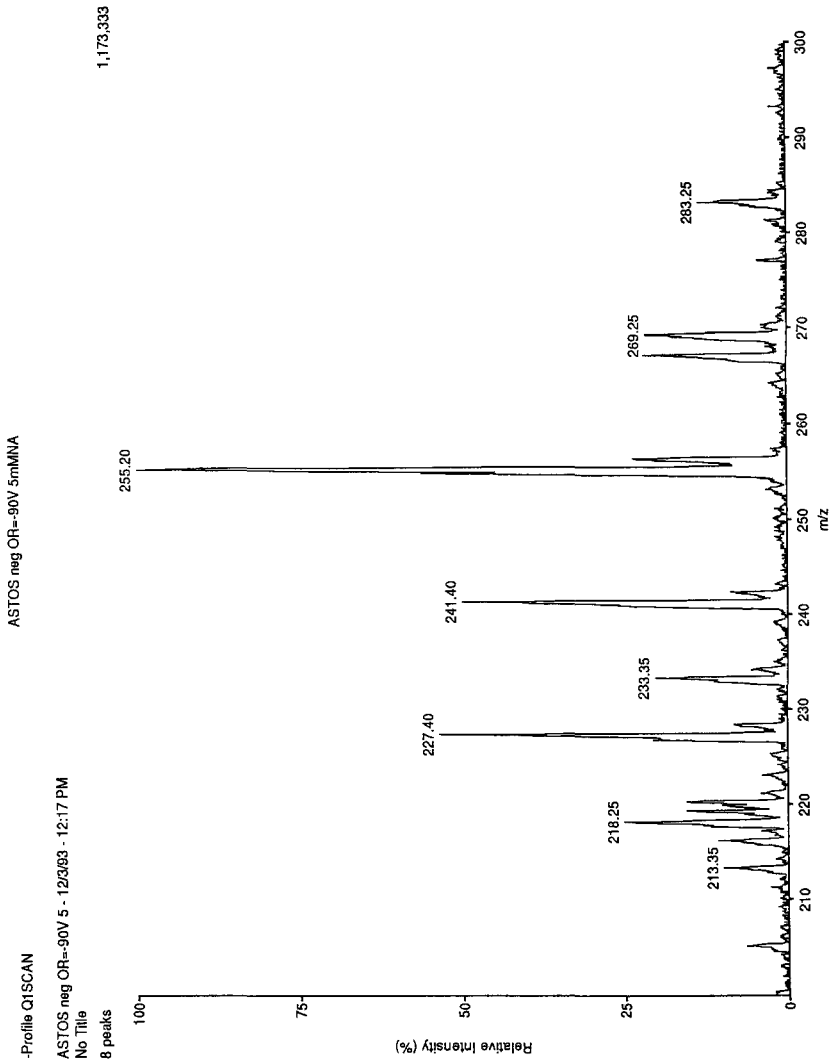


Figure 7 An Electro-Spray ionization mass spectrum of L-ascorbyl-2-sulfate (Astos) standard. X axis = m/z and Y axis = relative intensity

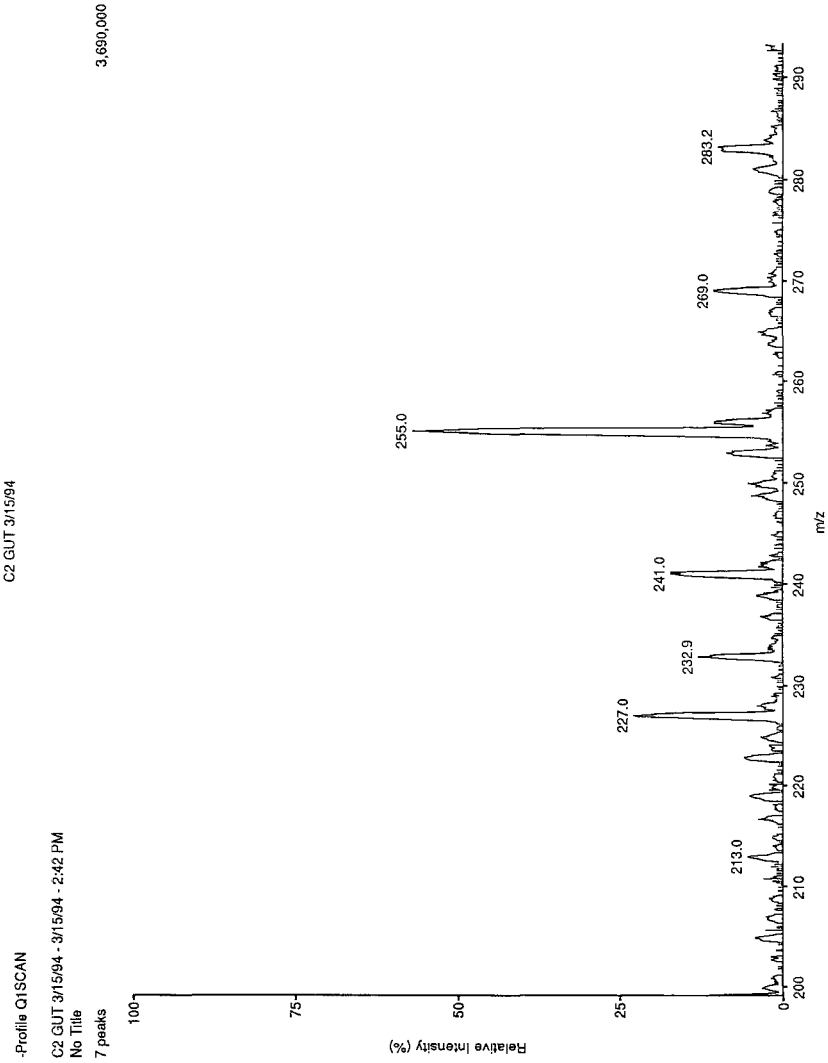


Figure 8 An Electro-Spray ionization mass spectrum of AS eluted from 5% TCA gut extract HPLC run shown in Figure 4 (B) above. The difference in mass peak intensity at 227 is related to argon density difference.

Da. Argon densities effect the intensities of the daughter ions. For example in higher argon density the smaller daughter ions predominate; whereas with less argon density, the converse is true.

When running a mass spectrometer MS/MS program , the instrument focuses exclusively upon the base peak of interest. All other mass peaks are filtered out. Most peaks in a mass spectrum appear at integral mass numbers. However multiplied charged ions can appear at fractional masses, i.e. 59.1 vs 59, as seen in Figure 10. These fractional numbers can also be caused by differences in carbon C_{12} to C_{13} ratios. Figure 9 depicts the ion fragments produced when the MS / MS program is performed on the base peak 255 of standard AS.

It should be emphasized that in MS/MS, only unimolecular reactions are important in electron ionization. In the electron interaction with the parent molecule, the first result is the formation of the molecular ions by injection of another electron. Then upon further degradation, part of the molecular ions will fragment into ions such as seen in the spectrum. Table 1 lists the likely ion fragments from the MS/MS data of the base peak of 255.2 (shown in Figure 7 for standard Astos). The table shows formula weights of the fragments and their most probable structures. Note, these structures exist only in unstable gaseous forms. Unequivocal proof for establishing the identity of an unknown would be for its MS/MS fragments to compare with those of the standard compound in Figure 9. Figure 10 includes figure 9 and shows this comparison. It offers convincing evidence that the Astos standard is the same as the eluted peak from the AS force-fed coho. Any minor differences in mass fragments between the two can be due to argon gas density differences and the differences discussed above.

Figure 11 is an MS spectrum of the 3.25 min. RT peak eluted from an HPLC run of a 5% TCA dietary extract. The diet was supplemented with AS. Note the similarity of the eluted dietary extract MS to that of standard AS shown in Figure 7. The fragment peaks of interest are 227, 233, 241, 255, 269 and 283 as seen in the Astos run. Figure 11, the MS spectra of the eluted dietary AS peak, illustrates and confirms the elution of AS from the diet matrix. A co-eluted impurity from the diet may have caused the unknown fragments that are not the same as AS standard. However, this mass spectrometer fragmentation offers strong evidence that the 3.25 min. HPLC peak is AS.

Figure 12 is the MS spectrum of the eluted HPLC peak (with a RT of 3.5 min. seen in Figure 2 B). This eluate was from the HPLC run of the 5% TCA gut extraction from the AS force-fed-fish. Note the fragment peaks are identical to the Astos spectra (227, 233, 241, 255, 269, and 283). The other different fragment peaks with masses of 213, 222.8, 239, 249.9, and 281 may be associated with the " carrier" substance that has a retention time of 3.5 to 3.6 min. This peak is found as well in the spectra of the control fish TCA extract (not force-fed AS or containing AS) seen in Figure 2 A.

The finding of this RT peak with its AS component is consistent with the fact that the 3.56 minute retention time peak is enhanced when fish are fed AS—a finding that may offer indirect evidence of an AS carrier found in fish gastric tissue. It is possible that this apparently -bound AS fraction may account, in part, for the low reported uptakes of AS in fish (8). The

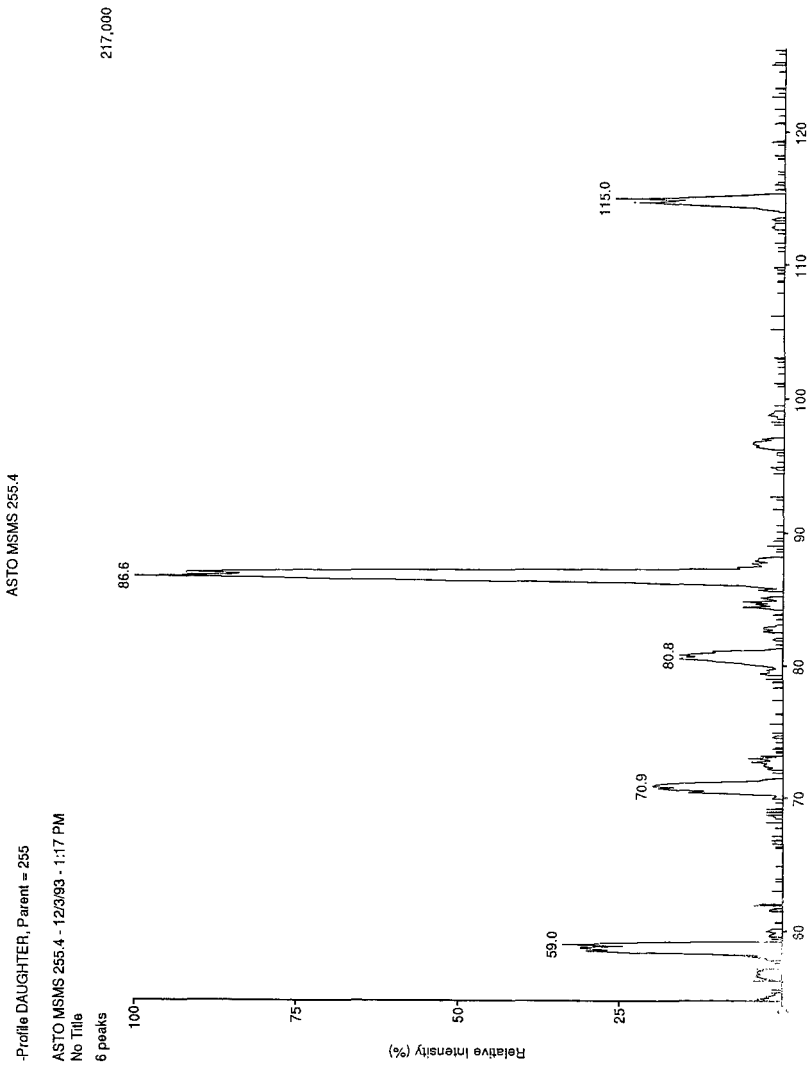


Figure 9 An Electro-Spray ionization mass spectrum of a MS/MS fragmentation of L-ascorbyl-2-sulfate standard.

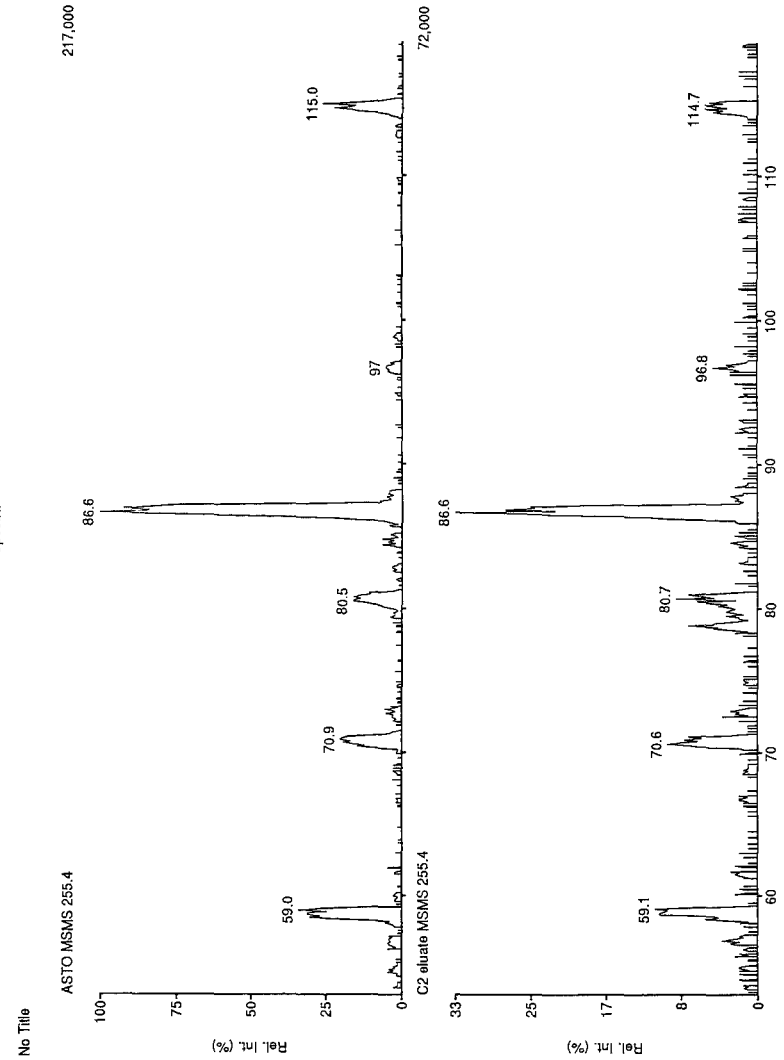
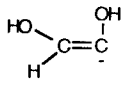
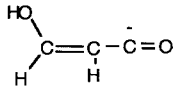
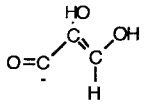
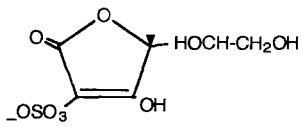


Figure 10 An MS/MS spectral comparison of standard AS (top).and the eluted peak at RT of 3.22 from the 5% TCA gastric extract of fish force-fed AS.

TABLE 1
Summary of fragments resulting from the MS/MS fragmentation of Astos (AS) standard. Order of fragments are from top to bottom with calculated atomic masses and proposed structures. Fragments are referenced to the parent compound L-ascorbic acid-2-sulfate shown at bottom of table.

Mass Number	Formula	Structure
59.0.	$C_2H_3O_2$-	
70.9	$C_3H_3O_2$-	
80.8	SO_3-	
86.6	C3H3O3-	
115.0	Unknown	
255	$C_6H_7O_9S^-$	

final proof for this eluted "carrier's" identity requires the more rigorous chemical identification that is being pursued.

During the study three separate coho 5 % TCA gut extracts were made. Two MS spectra are shown in Figure 13 and a third in Figure 8. They compare favorably with the standard AS seen in Figure 7. These three extracts were made in the manner described in the Methods section but were made at different time periods. The first one was made on 12/3/93, the second one on 2/1/94 and the third one on 3/15/94. As a result, the argon gas densities and sensitivities may vary slightly but the characteristic mass fragments are the same as Astos standard AS, 227, 233, 241, 255, 269, and 283.

In conclusion, the basic purpose of the mass spectrometer is to convert a sample into measurable products that are indicative of the original molecule (9). The work reported here is convincing evidence that the original molecule AS and the eluted tissue components do fragment

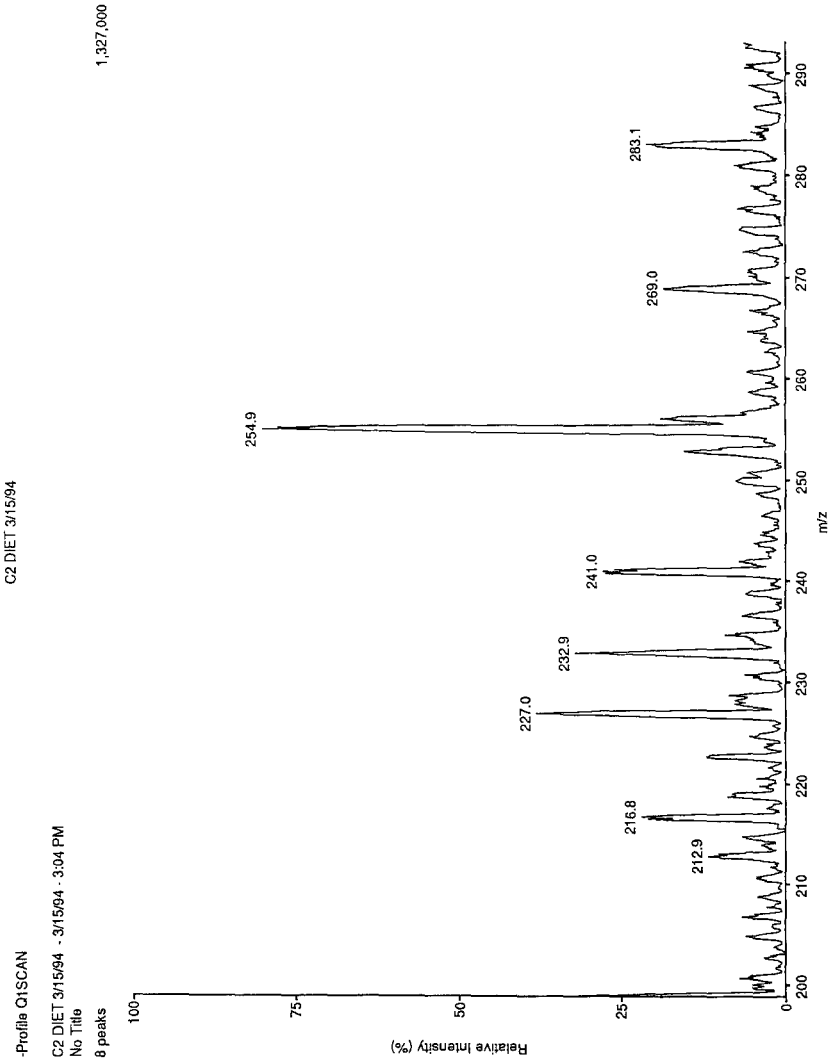


Figure 11 An Electro-Spray ionization mass spectrum of the AS peak eluted from a HPLC run of a 5% TCA extract from the commercial diet extract containing AS (Eluate seen in Figure 6 B).

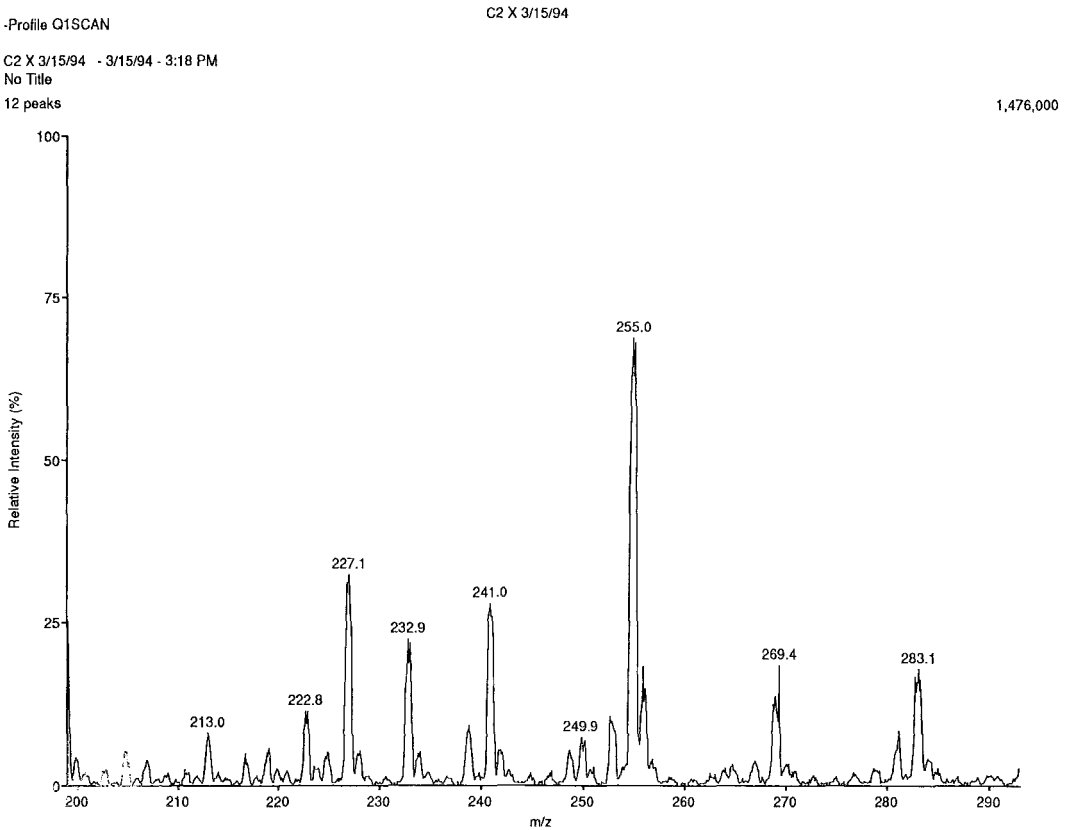


Figure 12 The An Electro-Spray ionization mass spectrum of the eluted HPLC peak (with the retention time of 3.5 min.) shown in figure 2 B.

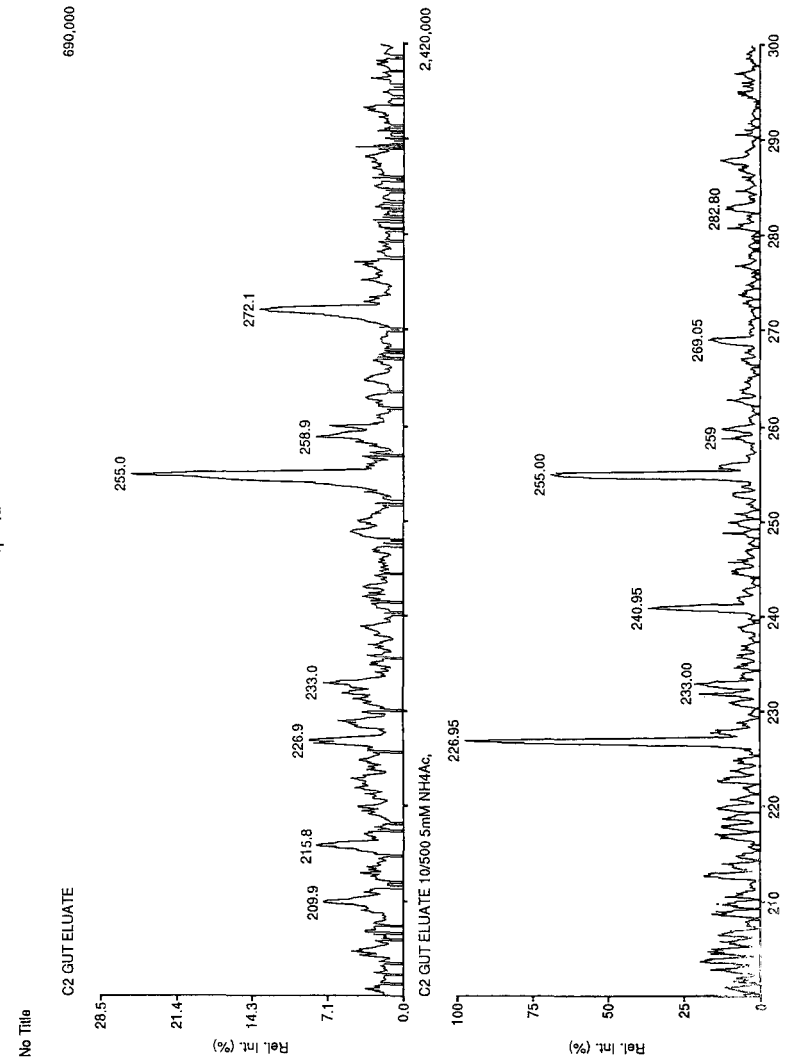


Figure 13 These Electro-Spray ionization mass spectra represent two additional eluted peaks from HPLC runs of 5% TCA gastric tissue extracts from fish force-fed AS. To be compared with Figure 8.

in an identical manner. The real value of this study is threefold: 1) By using the sensitive method of Electrospray Ionization Mass Spectrometry , to verify the existence of L-ascorbyl-2-sulfate in tissue, and to confirm earlier indications that relied upon a less precise HPLC identification; 2) to report to investigators and nutritionists that the previously documented HPLC methods are confirmed as viable analytical tools; and 3) to provide confidence to the aquaculture and animal industries that AS is confirmed in tissue as well as in diets.

ACKNOWLEDGEMENT

This confirmation study was supported by funding from Pfizer Inc., Food Science Group, New York, New York. The Electrospray Ionization Mass Spectrometry was made possible through a collaboration with Drs. K. Walsh and L. Ericsson in the Department of Biochemistry at the University of Washington Medical School, Seattle, Washington. Drs. Walsh and Ericsson have also reviewed this manuscript.

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RPHPLC METHOD FOR THE ASSAY OF 2 METHYL-4,5-TRIMETHYLENE-4-ISOTHIAZOLIN-3-ONE IN WATER-OIL EMULSIONS

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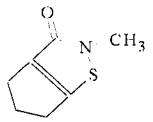
ABSTRACT

An HPLC procedure was developed for the determination of 2 Methyl 4,5-trimethylene-4-isothothiazolin-3-one in water-oil emulsions. The analytical procedure consists of the extraction in the presence of orthophosphoric acid and elution of MTI from Hypersil ODS column with acetonitrile solution of H_3PO_4 - water solution of H_3PO_4 as a mobile phase.

INTRODUCTION

Promexal W50 is a brand new preservative, from "Proxel" biocides family, for use in aqueous systems such as paints, water-oil emulsions, synthetic polymer emulsions. The main component of Promexal W50 is 2 Methyl 4,5-trimethylene-4-isothothiazolin-

3-one (MTI) patented by ICI. It prevents growth of deteriogenic species of microorganisms.



The quantity of MTI required to provide protection depends on a number of factors: susceptibility of the substrate to attack, the exposure conditions, and the product composition. The antimicrobial activity of MTI may be affected in the presence of some oxidizing and reducing agents, for example, persalts, sulphites. MTI is active only when it is present in sufficient concentrations, hence the need for an accurate and precise technique for routine analyses. The minimum inhibitory concentrations of Promexal W50 for bacteria, fungi and yeasts are between 1-40, 40-80 and 40-80 respectively [1].

An HPLC method for the assay of MTI in Promexal W50 was developed [2]. However this method can not be used directly in determination of MTI in water-oil emulsions because of the presence of interferent compounds found in the sample matrix. This creates the need of extraction.

EXPERIMENTAL

Reagents

Acetonitrile of analytical grade was obtained from Reachim (Russia) and orthophosphoric acid (85%

w/w H_3PO_4) was supplied by POCh (Poland). High purity water was obtained through HP 661A Water Purifier and MTI (purity 94.2% w/w) was purchased from ICI (UK). Samples of four emulsions with unknown MTI concentrations based on anionic-nonionic emulsifier with corrosion inhibitor (derivatives of diethanoloamine and boric acid) were from the Surfactants Department of our Institute.

Apparatus

The HPLC system consisted of a Hewlett-Packard HP 1050 liquid chromatograph equipped with an automatic solvent degasser, a Rheodyne 7125 injection valve with a 25 µl loop, and a Knauer Variable Wavelength Monitor. A personal computer and Grams/386 for Chromatography software (Galactic, Salem, NH, U.S.A.) were used for data collection and quantification of peak areas. Chromatography was carried out at a temperature of 40°C on a 200x2.1 mm steel column packed with Hypersil ODS (d_p=5µm) (Hewlett Packard) with a guard column 20x2.1 mm filled with the same material. A reciprocating shaker, type 327 (Premed, Poland) and a micro-centrifuge, type 320 (14000 rpm) (Mechanika Precyzyjna, Poland) were applied. In the preliminary studies a Hewlett-Packard HP 1090 liquid chromatograph equipped with DAD detector, and the same Rheodyne 7125 injection valve with a 25 µl loop and the same chromatographic column were used for

choosing the optimum of the gradient conditions and determination of the maximum absorption wavelength.

Procedure

2 g of H_3PO_4 was added to 20 g of a water-oil emulsion. The mixture was vigorously shaken for 20 min. Then, 2 mL of the mixture was centrifuged for 15 min at 14000 g.

20 µL of aqueous, bottom phase of the separated mixture was chromatographed under the following gradient conditions:

F = 0.3 mL/min

Gradient program:

t (min)	A (%)	B (%)
0	90	10
10	90	10
11	50	50

A = 45 mM of H_3PO_4 in H_2O,

B = 45 mM of H_3PO_4 in CH_3CN.

RESULTS AND DISCUSSION

Using DAD detector installed at HP 1090 model it was found that maximum absorption occurred at a wavelength of 270 nm (Fig.1). For the routine analysis a Knauer Variable Wavelength Monitor set at 270 nm was used.

A typical HPLC chromatogram for the MTI determination in water- oil emulsions is presented in Fig.2.

In the present study 4 samples of emulsions were analyzed for MTI content. The results are shown in Table 1.

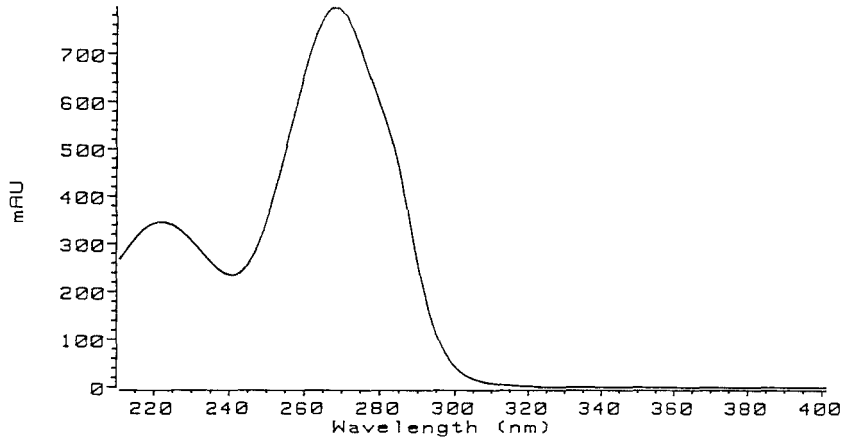


Figure 1.
UV spectra of MTI.

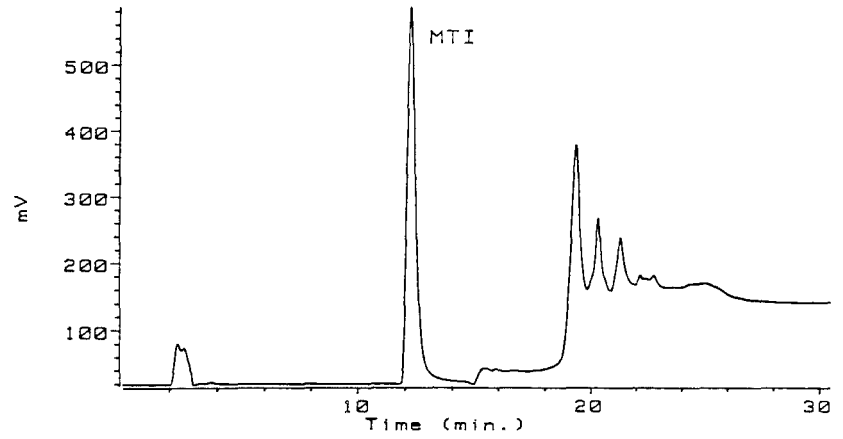


Figure 2.
RP-HPLC chromatogram of the bottom phase of a separated mixture of water-oil emulsion. Conditions of the analysis are described in the text.

Table 1. Values for the assay of MTI in water-oil emulsions.

Emulsion No	MTI, ppm (mean ± SD, n=4)
1	12.74±0.60
2	5.55±0.07
3	1.01±0.02
4	0.54±0.01

The quantitation was based on a calibration by series of dilutions from primary standard. The dependence between the peak area and the amount of MTI was determined. The regression analysis of triplicate calibration data has shown linear relationship in the 0.02 to 21.71 ppm range of MTI, with the r^2=0.9997, a=0.68 (including origin).

To estimate the efficiency of the recovery the emulsion samples were spiked with 1.5 and 14.9 µg/mL of MTI. The recovery was 96.8 and 99.2%, respectively. The yields of extraction are the mean of three replicate experiments. Reproducibility was found to be very good, and the limit of detection was estimated to be 0.01 ppm.

CONCLUSION

The method described here takes less than 50 min to perform and permits the assay of MTI at the ppm level in water-oil emulsions. No deterioration of the HPLC column was observed over several weeks of

continuous use. Because of the simple sample preparation, good accuracy and precision, the method is well suited to routine quality control analyses of commercial emulsions.

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QUANTITATIVE ANALYSIS OF FLUVOXAMINE MALEATE IN TABLET FORMULATIONS BY HPLC

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ABSTRACT

A rapid, specific and reliable high performance liquid chromatographic assay of flavoxamine maleate in tablets has been developed. Reversed-phase chromatography was conducted using a mobile phase of 0.05M ammonium acetate and acetonitrile, (40% v/v) and detection at 240nm. The recovery and coefficient of variation from six placebo tablets containing 100 mg of fluvoxamine maleate were 100.56% and 0.439% respectively. Relicate regression analyses of three standard plots in the concentration range of 0.5 - 12 mcg/ml obtained on three different days gave a correlation coefficient > 0.9997 and the coefficient of variation of the slopes < 0.1%. The assay was precise within day and between days as indicated by ANOVA test. The recoveries from 10 replicate tablets of two commercial tablets (50,

100mg) was 99.2, 101.8 and 100.3% of the label amount and their coefficients of variation were 1.854 and 1.119%.

INTRODUCTION

Fluvoxamine is a specific 5-hydroxy tryptamine inhibitor (1,2), which belongs to antidepressants. Very few methods have been developed for the determination of Fluvoxamine Maleate in tablets, spectrophotometric (3) and Hplc method (4). The Hplc method (4) developed by Hagga et al, the minimum detectable amount of drug was 15 mcg/ml which is considered a relatively very high concentration. The purpose of this study was to develop a simple and direct HPLC assay for the quantitation of Fluvoxamine Maleate in tablet formulations.

EXPERIMENTAL

Chemicals and Reagents : Fluvoxamine maleate(5) and propyl paraben(6) were used without further purification. Acetonitrile(7), methanol(7) and water were HPLC grade. All other chemicals and reagents were USP or ACS quality and were used as received.

Instrumentation : A water HPLC systems(8) was used consisting of the following components : Model 45 pump, the WISP model 710 B autosampler, the model 481 UV detector set at 273 nm at 0.02 AUFS, the model 730 data system. Chromatographic separation was

accomplished using C18 column, 8 mm i.d. x 10cm u Bonda Pack C18 column with 10 um packing.

Chromatographic Conditions : The eluting medium consisting of 40% v/v of 0.05M ammonium acetate and acetonitrile at pH 5.2 with glacial acetic acid, was prepared and degassed by bubbling helium gas for 5 min prior to use. Column equilibrium with the eluting solvent was established by pumping the mobile phase at a rate of 0.2ml/min overnight. The flow rate was set at 0.8 ml/min during analysis. The chromatogram was recorded and integrated at a speed of 0.2 cm/min.

Internal Standard : Stock solution of propyl paraben containing 10 mg in 100ml methanol was prepared weekly and stored at 4°C.

Standard Solution of Fluvoxamine Maleate : A stock solution of fluvoxamine maleate was prepared by dissolving 10 mg of fluvoxamine maleate in 10 ml of methanol. Nine aliquots equivalent to 0.5, 1, 2, 4, 6, 8, 10 and 12 mcg of fluvoxamine were added to 1 ml volumetric flasks. After an aliquot of the internal standard equivalent to 5 mcg was added, the flasks were brought to volume by acetonitrile and thoroughly mixed. Twenty uL of the standard solutions was injected onto the column for analysis.

The peak area ratio of the drug : internal standard were plotted against the standard fluvoxamine concentrations. Least square linear regression analysis was performed to determine the slope, y intercept, and the correlation coefficients of the standard plots.

Sample Preparation : Individual tablets were pulverized using a morter and pestle, and completely transferred to 100 ml volumetric flask. The volume was adjusted with methanol and the flask was mechanically

shaken for five min. Five ml of the solution was removed into a centrifuge tube and centrifuged at 3000 r.p.m for 5 min. Fifty uL was transferred to a one ml volumetric flask containing 50 uL of propyl paraben stock solution, and diluted to the volume with acetonitrile. Twenty uL was loaded into the sample loop for chromatography. Ten replicate commercial tablets of fluvoxamine were analyzed for statistical evaluation of the assay.

Quantitation : The amount of fluvoxamine per tablet was determined from the following equation :

$$Q = [\ R/A + B\ ]\ X \text{ Dilution factor}$$

were Q is the mg fluvoxamine per tablet, R is the peak area ratio (drug / internal standard), A is the slope of the calibration curve and B is the y-intercept.

Recovery of fluvoxamine from the fabricated placebo tablets : The reference tablets containing 100 mg of fluvoxamine and 50 mg each of starch and lactose were prepared and subjected to the described HPLC assay to measure the accuracy and precision.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram obtained following analysis of fluvoxamine in tablets. Using the chromatographic conditions described, fluvoxamine and propyl paraben were well separated and their retention times were 9 and 7 min, respectively. For both compounds sharp and symmetrical peaks were obtained with good

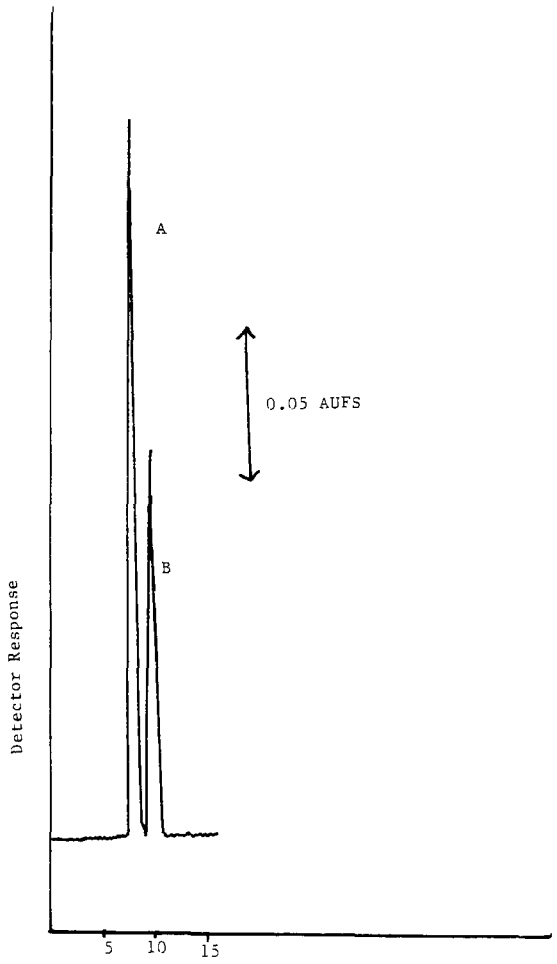

Figure 1. Chromatogram of Fluvoxamine Maleate Tablet
Key A-Propylparaben, B-Fluvoxamine Maleate

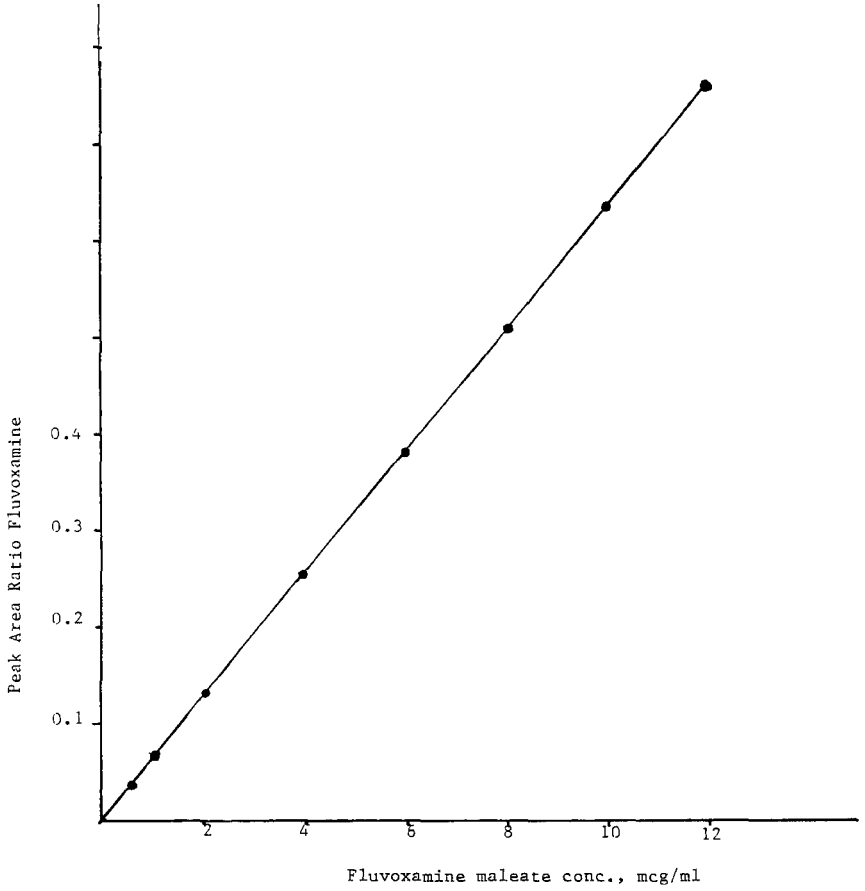

Figure 2. Standard Calibration Plot of Fluvoxamine Maleate.

baseline resolution and minimal tailing, thus facilitating accurate measurement of the peak area ratio. No interfering peaks were found in the chromatogram due to tablet excipients. Figure 2 shows a calibration plot for the peak area ratio of varying amounts of fluvoxamine (0.5 - 12 mcg/ml) to a constant amount of propyl paraben

Table 1

Regression Analyses of the Three Standard plots of Fluvoxamine

Standard[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1.	0.06229	0.00333	0.99985
2.	0.6234	0.00353	0.99972
3.	0.6224	0.00346	0.99980

a) obtained in 3 different days.

b) the mean of 3 determinations at each drug concentration.

(20 mcg/ml). The plots wer linear (r = 0.99985) and the regression analysis of the data gave the slope and intercept as :

$$Y = 0.06229\ x + 0.003335$$

were Y and x are the peak area ratio and fluvoxamine concentration, respectively. Three replicate analyses of fluvoxamine at concentration of 0.5-12 mcg/ml were performed at three different days over one week period. The results of this evaluation are summarized in table 1. The average correlation coefficient was higher than 0.9997 and the coefficient of variation of the slopes of the three lines was < 0.1%. Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F=3.5$, $P > 0.01$). The similarities in the slopes and the high correlation coefficients indicate that the assay possesses excellent reporducibility and linearity.

Table II

Analysis of Variance for Intra- and Inter day Precision

Day/Assay	1	2	3	4	5	6
1	100.2	10.6	100.3	100.4	101.1	100.2
2	100.8	99.6	98.4	100	100.2	101.1
3	100.4	99.2	99.2	99.6	99.9	99.8
4	99.8	98.6	101.4	98.8	99.8	99.6

Mean 99.95 mg

ANOVA TEST

Source of variation	DF	Sum of squares	Mean of squares	F ratio	P
Within day	5	2.2021	0.44042	0.7322	0.05
Between day	3	2.4346	0.81153	1.349	0.05
Error	15	9.0229	0.6015	0.6168	
Total	23	13.6596			

Thus, the method should be accurate and precise within the assay day as well as between assay days.

Precision and accuracy : Six placebo tablets containing 50 mg each of lactose and starch and 100 mg of fluvoxamine were assayed for four consecutive days for intra and interday precision studies. The average recovery shown in Table II was 99.95 mg (100.08%) with the

Table III

Recoveries From Spiked Placebo Tablets

Fluvoxamine Maleate mg added	mg Recovered	% Recovery
100	100.1	100.1
100	100.9	100.9
100	100.5	100.5
100	99.9	99.9
100	101.1	101.1
100	100.9	100.9
Mean	100.56	100.56
SD	0.442	0.442
CV	0.439	0.439

coefficient of variation of 0.886%. Estimates of day to day and within day precision were calculated by ANOVA test. The calculated F values, $F_{0.05}$ (5, 15) = 0.73 and $F_{0.05}$ (3, 15) = 1.349 were smaller than the table values $F_{0.05}$ (5, 15) = 2.44 and $F_{0.05}$ (3, 15) = 2.24 respectively. Thus it was concluded that there was no significant difference for the assay which was tested within day and between days.

Recovery : Table III shows the average recovery for the placebo tablets containing 100 mg fluvoxamine and 50 mg each of lactose and starch. The average recovery was 100.56 and its relative standard deviation was 0.442.

Table IV

Analysis of Dosage Form of Fluvoxamine

Sample	n[a]	Mean % Recovery	SD	% CV
A	10	99.2	1.84	1.854
B	10	101.8	1.14	1.119

a) Number of replicates

Analysis of Fluvoxamine tablets : Table IV present the results obtained from analysis of fluvoxamine (50, 100 mg) commercially available. The mean percent recoveries were 99.2, 101.8.

The stability indicating nature of the assay has not been demonstrated in this study, since no sign of degradation was observed by TLC after subjecting the drug solution(pH3 and 9) at 70°C for 2 hrs which was also evident from the absence of any additional peaks in the chromatograms.

Conclusion : The HPLC method developed in this study has the advantages of simplicity, precision and convenience. It also allows for the direct determination of fluvoxamine. Therefore, the method should be useful for routine analytical and quality control assay of fluvoxamine in dosage forms.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF TERBUTALINE FOR PREFORMULATION STUDIES

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ABSTRACT

A reversed phase, stability indicating high performance liquid chromatographic (HPLC) assay was developed for the evaluation of terbutaline (TL) as a potential candidate for transdermal drug delivery. TL was quantitated in the permeation diffusate and human skin extract samples. The isocratic mobile phase was 30 % acetonitrile in pH 5.6 phosphate buffer. The effluent was monitored at 225 nm. Propranolol (PP, 2.5 μg/ml) was used as the internal standard. Baseline separation of TL and PP was attained with a cyano column. The linear range for the calibration curve was established at 5-15 μg/ml. The assay was reproducible with low inter-day and intra-day variation of the slopes of the calibration curves (3.5 and 4.2 % for diffusate samples and 5 and 5.5 % for the human skin extract samples respectively). The lowest detectable quantity was 0.1 μg/ml with a signal noise ratio of 4. The application of the assay in various preformulation studies was demonstrated.

* To whom correspondence should be addressed

INTRODUCTION

Terbutaline (1-(3,5-dihydroxyphenyl)-2-tertiarybutyl amino ethanol is is a beta-2 selective bronchodilator. Its structure is shown in Figure 1. It is indicated for the long term treatment of obstructive airway diseases and in the treatment of bronchospasm. It is the only selective beta-2 bronchodilator used parenterally in the emergency treatment of asthmaticus. It is also indicated as a tocolytic agent. It is currently delivered by the oral, inhalation and subcutaneous route. It has a bioavailability of 14 $\pm$ 2 %, a clearance of 3.4 $\pm$ 0.6 $ml.min^{-1}$ and a volume of distribution of 1.8 $\pm$ 0.3 liters/kg. The low bioavailability can be overcome by the noninvasive transdermal route of drug administration. The advantages of transdermal route of drug administration are well documented (1). This route increases bioavailability by bypassing the hepatic first pass metabolism. It provides prolonged duration of action. It improves patient compliance by eliminating multiple dosing. In addition the dosage can easily be discontinued if toxic side effects occur. Thus a transdermal dosage form of terbutaline would be of great advantage. A number of preformulation studies are required to evaluate the potential of transdermal delivery of TL. These include partition coefficient, solubility studies, pH stability and extent of drug permeation through the skin. In addition determination of the amount of drug retained in

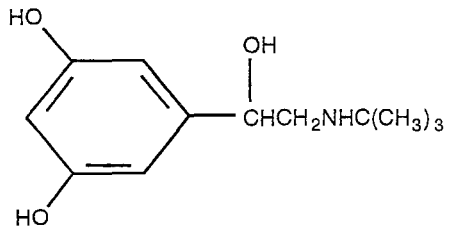


Figure 1: Chemical Structure of Terbutaline

the skin also provides useful information as to the depot effect of the drug or in elucidating the mechanism of skin penetration enhancers. The prerequisite for all these studies is a sensitive, stability indicating assay method. There a number of assay methods reported in literature for the quantitation of TL in plasma samples and solutions (2-9). These include coupled column, GCMS, electrochemical detection. None of these methods is specific for the quantitation of the drug in the skin. The goal of this study is to develop a simple, sensitive, stability indicating HPLC assay which can be used in all the preformulation studies.

MATERIALS

All chemicals and solvents were purchased from Sigma Chemical CO. (St. Louis, MO). The human cadaver skin (abdominal area, 65 white male) was obtained from the local hospital.

METHODS

High Performance Liquid Chromatography

The liquid chromatograph (Consta-Metric I) was from Laboratories Data Control, Riviera Beach, FL. It has a 100 μl injection sample loop (Rheodyne, Berkeley, CA) and a variable wavelength ultra violet detector (Spectro Monitor III, Laboratories Data Control). The cyano column used (Sphersorb, 4.6 X 250 mm, 5 μm particle size) was from AllTech. The isocratic mobile phase was 30 % acetonitrile in pH 5.6 buffer. The effluent was monitored at 225 nm. The flow rate was 1.4 ml/min. Propranolol HCl was used as the internal standard at a concentration of 2.5 μg/ml.

Degraded Samples from Extreme pH and Heat Conditions

A 15 μg/ml TL solution was boiled for 3 minutes in 0.1 Normal HCl or 0.1 Normal NaOH to obtain the extreme acidic and alkaline conditions respectively. The solutions were analyzed by the developed HPLC assay.

Calibration Curves

Calibration curves were constructed in the range of 5-15 μg/ml for diffusate and skin extract samples. TL and PP stock solutions in deionized water (100 μg/ml) were prepared. Appropriate dilutions were

made either with the diffusate or the skin extract solution to obtain standard solutions of cocentration 5, 7, 10, 12 and 15 μg/ml and an internal standard concentration of 2.5 μg/ml.

Extraction Efficiency of TL From the Skin (10)

The slope of the calibration curve (obtained by adding known amount of TL and PP to 400 mg of skin and homogenizing the samples) was determined (X). The same amounts of TL and PP were then added to the blank skin extract and its slope of the calibration curve obtained was calculated. (Y). The extraction efficiency of the TL was calculated from the equation:

Extraction efficiency (%) = (X/Y) X 100

Inter and Intra Day Variations of the Slopes of Calibration Curves

The slopes of six calibration curves (in saline phosphate buffer) constructed on the same day (inter-day) were determined and the coefficient of variation among them was calculated. Similarly the intra-day variation was determined by evaluating the slopes of six calibration curves constructed on different days over a period of 8 months. The inter and intra day variation was also determined for the calibration curves constructed with human skin extract homogenate. Approximately 300 mg of skin was weighed and the appropriate

amount of internal standard and drug solution was added to it. The membrane was then homogenized with methanol. (with 5 ml of methanol four times). The homogenate was combined filtered, evaporated to dryness, diluted with the mobile phase and quantitated by the developed assay.

In vitro Skin Permeation Study (11)

To evaluate the extent of TL permeation through the human skin, Franz diffusion cells (FDC 108 Series with FDC 128 manifold and FDC 127 magnetic bar, Crown Glass CO., Somerville, N.J.). The receptor chamber was filled with saline phosphate buffer (pH 7.4) to simulate the physiological pH. A circulating water bath maintained the cell at 37°C. The defatted human cadaver skin was placed between the donor and the receptor chambers of the difusion cell with the stratum corneum facing outward and the dermis facing the buffer solution. 200 μl of the test solution (TL in propylene glycol with or without penetration enhancer) was applied to the stratum corneum. Aliquot samples were taken at predetermined time intervals and analyzed for drug content by the developed HPLC assay. An equal volume of buffer solution was always added back to the receptor chamber to ensure contact between the buffer and the skin.

Skin Permeation Data Analysis(11)

A plot of the amount of drug permeated vs time was constructed. The slope of the linear phase of the profile yielded the flux (J) of the drug. The X-intercept of the linear phase was the lag time (T). "C" was the concentration of the drug solution added to the skin. The thickness of the memebrane (d) was assumed to be 50 μm. The diffusion coefficient (D), permeatbility coefficient (Kp), partition coefficient between the solution and the skin (Km) was calculated from the follwing equations:

$D/d^2 = 1/6T$

$Kp = J/C$

$Km*d = Kp/(D/d^2)$

Reservoir Effect Study

The amount of drug retained in the skin at the end of the skin permeation study provide insight into the mechanism of skin enhancer. The drug exposed skin was washed thrice with 3 ml of water to remove any residual drug. The skin was then homogenized with 5 ml of methanol using a polytron homogenizer. The homogenate was filtered and the process was repeated thrice with the residue. The filtrates (15 ml) was combined, evaporated to dryness. The residue was reconstituted with the mobile phase, internal standard solution added and suitably diluted and analyzed for drug content.

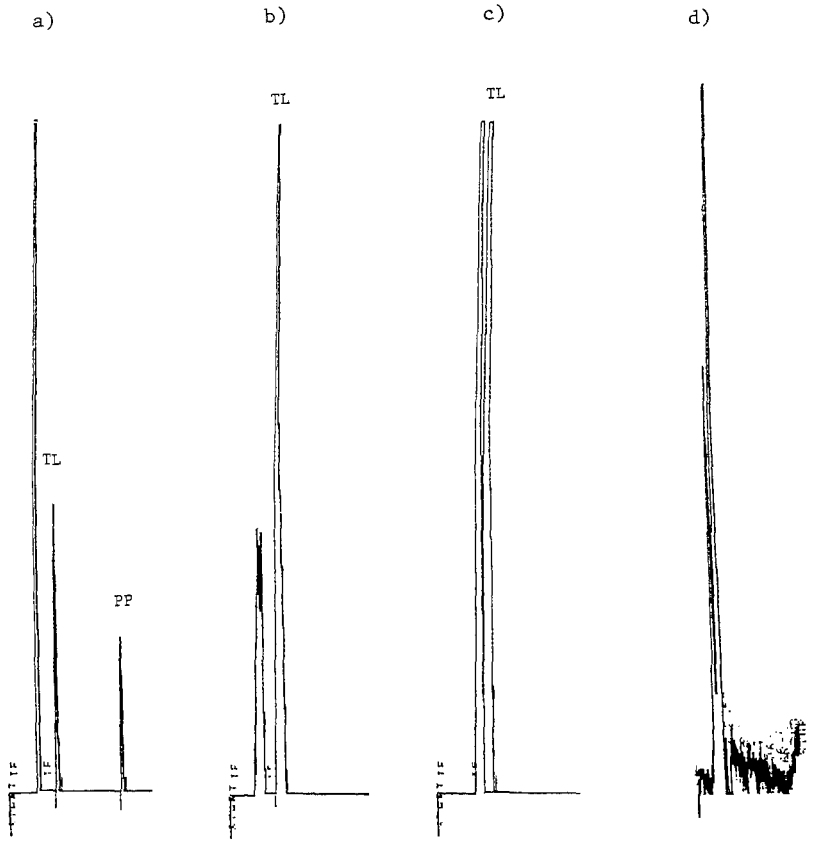
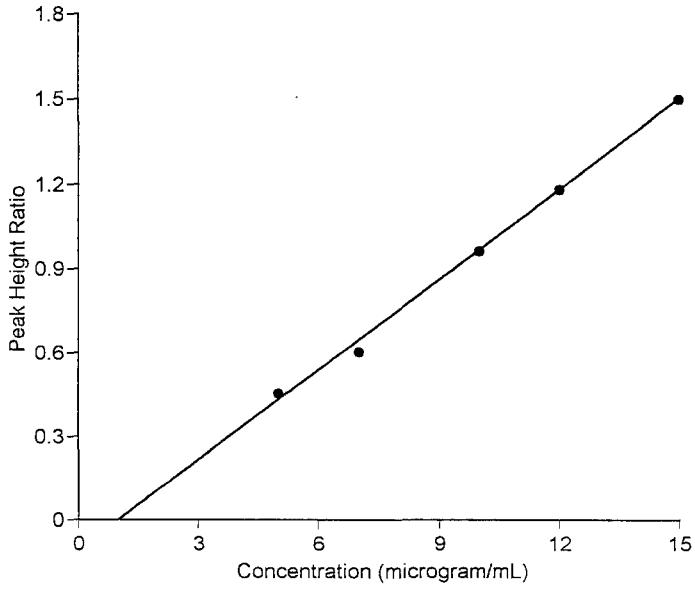


Figure 2: Typical Chromatograms of terbutaline a) in diffusate samples b) base degraded sample c) acid degraded sample d) blank skin extract

RESULTS AND DISCUSSION

Baseline separation of TL from the internal standard PP was obtained with the cyano column and the mobile phase used. Typical chromatogram for TL and PP in the diffusate are shown in Figure 2a.



Figrue 3: Calibration curve of terbutaline in the diffusate medium

The retention times of TL and PP were 3.3 and 7.9 minutes respectively. The calibration curve was linear in the range of 5-15 μg/ml with a $r^2 > 0.98$ (Figure 3).

Forced Degraded Samples

Baseline separation of TL was obtained for the base-degraded sample (Figure 2b) and acid-degraded sample (Figure 2c), demonstrating the stability of the assay under those conditions of degradation. There was no interference from the blank skin extract at

the retention times of either the drug or the internal standard (Figure 2d).

Assay Sensitivity

The sensitivity of the assay was determined to be 0.1 μg/ml when 5 μl of the sample was injected. A higher sensitivity can be attained by increasing the volume of the injection.

Linearity

The calibration curves were linear in the range of 5-15 μg/ml for the diffusate and skin extract samples. The regression coefficient was 0.98 and 0.96 for the diffusate and skin extract samples respectively.

Inter and Intra Day Variation

The inter and intra-day variation was 3.5 and 4.2 % respectively for the diffusate standard curves. The corresponding values for standard curves constructed with human skin extract samples were 5 and 5.5 % respectively.

Extraction Efficiency

The extraction efficiency of TL from the human skin was calculated to be 92 $\pm$ 11.5 %. This suggests that both the drug and the internal standard were well extracted from the skin.

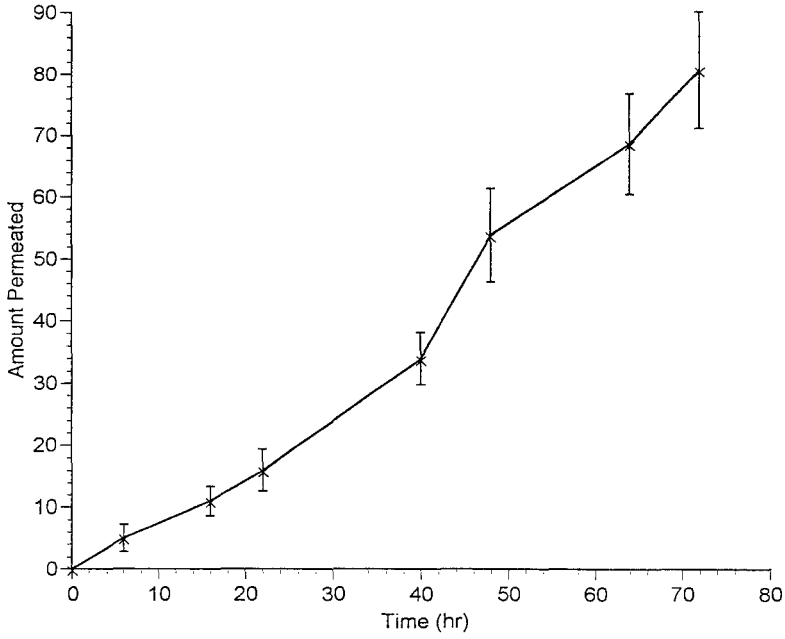


Figure 4: Permeation profile of terbutaline through the human cadaver skin

Table 1: Terbutaline Permeation Parameters through the Human Cadaver Skin

Flux(J) $\mu g/cm^2.hr$	Lag-time (T) hr	Permeability Coeff (Kp) cm/hr	Partiton Coeff (Km*d) cm	Diffusion Coeff. (D/d^2) cm^2/hr
1.3 ± 0.1	6.6 ± 2.2	0.34 ± .03	13.3 ± 4.5	.03 ± .02

Permeation Study

The permeation profile of terbutaline through the human cadaver skin was shown in Figure 4. The TL flux through the human skin was 1.2 $\mu g/cm^2.hr$. The permeation parameters were reported in Table I. Approximately 3.8 % of the dose was retained in the skin. Based on the preliminary studies it appears that terbutaline is a good candidate for transdermal delivery.

HPLC Assay Application

The developed HPLC assay was useful in the skin permeation study. The assay was also useful in quantitation of TL in the pH-stability study, solubility studies, partition coefficient study and in the evaluation of the rabbit skin as a suitable model for the human skin (12). The assay was also useful in screening of various skin penetration enhancers.

ACKNOWLEDGEMENTS

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DETERMINATION OF BENZO(a)PYRENE IN TOTAL PARTICULATE MATTER OF VIRGINIA AND BLACK TOBACCO SMOKE BY HPLC WITH FLUORIMETRIC DETECTION

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ABSTRACT

A method for the determination of the benzo(a)pyrene by RP-HPLC with fluorimetric detection in total particulate matter of Virginia and blak tobacco smoke is developed. The total particulate matter was collected on glass fibre filters, which were ultrasonically extracted with cyclohexane. A fraction of the resulting extract was cleaned up on Sep-Pak Vac Si cartridges and the PAHs were eluted with mehylene chloride. The eluate was evaporated to drynees and the residue was dissolved in methanol. The mean recoveries in the extraction and clean-up steps were 100.1% and 49.2%, respectively, with relative standard deviations of 3.8% and 2.4%, respectively, in the concentration range 20-140 ng. The quantification is achieved by means of external standard method.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have been the subject of intensive analytical investigation in studies relating to tobacco smoke (1, 2), air (3-5), water (6, 7) and food (8, 9) because of the carcinogenic activity of some members of this family. Benzo(a)pyrene (B(a)p), a carcinogenic polycyclic aromatic hydrocarbon, has received the most attention. In recent years, a number of methods for the quantitative analysis of B(a)p have been published, which employed improved techniques such as GC and HPLC and laborious procedures (10, 11). The determination of B(a)p in cigarette smoke is a major analytical problem, because the particulate matrix of cigarette smoke contains thousands of PAHs or their derivatives (12) and the absolute deliveries of B(a)p are not large, usually 6-30 ng B(a)p/cigarette (cig.)(13).

The isolation of the B(a)p fraction from the extract of total particulate matter of cigarette smoke must involve a solid phase extraction using a cartridge (12, 14) or HPLC system with a semipreparative column (12, 13). The first method is much cheaper, simpler and faster, but the second once is more efficient in terms of recovery and precision. Reversed phase high performance liquid chromatography (RP-HPLC) coupled with a fluorescence detector offers

good selectivity and sensitivity for the determination of PAHs, due to the specific columns used (5, 15).

In this paper we report the development and optimization of a fast, cheap, simple and precise method for the determination of B(a)p in total particulate matter of Virginia and black tobacco smoke by HPLC with fluorimetric detection.

EXPERIMENTAL

Apparatus and material

The chromatographic system consisted of the following components: a high pressure gradient Milton Roy CM 4000 pump, a Rheodyne 7125 injection valve with a 20 μl loop, a Perkin-Elmer LS 30 luminescence spectrometer and a Milton Roy CI 4100 integrator. A P-Selecta Precisterm bath was used to maintain the column temperature at 22°C. The column was an Ultraspher 5 μm particle size (125 mmx 4 mm I.D.) from Merck. A P-Selecta ultrasonic bath was used to extract PAHs from the filters. For the clean-up of the extract a Sep-Pak Vac Si, 500 mg (6 ml) cartridge from Millipore were used. A Visiprep vacuum manifold system from

Supelco was also used. Solvents used to prepare the mobile phase and organic extracts from the tobacco smoke particulate samples were filtered through 0.45 μm pore size nylon Lida membrane filters.

Reagents

Stock solutions of the PAHs with concentrations in the range $(10-1.0)x10^{-4}M$ were prepared by dissolving the solids (from Sigma) in methanol; more dilute solutions were prepared by diluting with this solvent. HPLC purity acetonitrile, methanol and methylene chloride from Carlo Erba were used. Water was purified with a Milli-Q system from Millipore. Other chemicals used were of analytical reagent grade.

Cigarettes

Both commercial Virginia and black tobacco cigarette brands used in this study were purchased in a Madrid tobacco nist's. The cigarettes are 85 mm in length, 8 mm in diameter, and filtered.

Procedures

Tobacco smoke particulate sampling and sample preparation

The Virginia and black tobacco smoke particulate was collected on glass fibre filters of 4 cm diameter using a smoking machine. The filters, containing the smoke particulate matter of five cigarettes, were treated with 10 ml of cyclohexane in the ultrasonic bath for thirty minutes. A 2 ml extract aliquot was injected into a cartridge, which had previously been conditioned with 15 ml of cyclohexane. The cartridge containing the extract aliquot was dried under a compressed air stream. The B(a)p was eluted with 2 ml of methylene chloride. This elution fraction was evaporated to small volume, transferred to 1.0 ml volumetric flask and then evaporated to dryness by means of the vacuum manifold system. The B(a)p residue was dissolved in 1 ml of methanol and determined by the chromatographic procedure by injecting 20 μl.

Chromatographic analysis

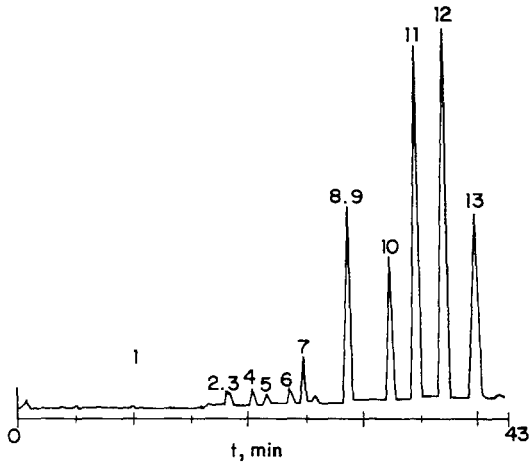
Once the column had been conditioned with the mobile phase, calibration graphs at five concentration levels were prepared from solutions containing B(a)p in the range

0.001-0.04 ng/μl. The mobile phase gradient was prepared by mixing water (A) and acetonitrile (B) and was degassed with helium. The gradient profile for the desorption of the analytes from the analytical column was: A-B (55-45) (0 min), which was held constant for 5 min, and subsequently changed linearly to 85% B in 25 min. The separations were carried out at 22°C, at a flow-rate of 1.0 ml/min. Detection was carried out fluorimetrically at the optimal excitation and emission wavelengths (295 and 405 nm) of B(a)p. The areas of the peaks were used to quantify B(a)p.

RESULTS AND DISCUSSION

Chromatographic and analytical characteristics

The chromatographic parameters were selected after a previous systematic study. Figure 1 shows the separation of B(a)p from the 12 PAHs using the optimized procedure which is specified in the experimental section. The B(a)p concentrations tested were in the range 0.001-0.04 ng/μl. Linearity was found with a regression coefficient of 0.999. The relative standard deviation (RSD, %) at concentration level of 0.02 ng/μl in the middle of the linear range studied was 5.3% (n=4) and the detection limit (DL = 3 S/N) was 24 ng/l.



PAH	c, ng/μl
1-Naphthalene	0.88
2-Acenaphthene	0.44
3-Fluorene	0.20
4-Phenanthrene	0.13
5-Anthracene	0.11
6-Fluoranthene	0.05
7-Pyrene	0.06
8-Benzo(e)anthracene	0.08
9-Chrysene	0.16
10-Benzo(e)pyrene	0.13
11-Benzo(a)pyrene	0.04
12-Dibenzo(ah)anthracene	0.10
13-Benzo(ghi)perylene	0.08

FIGURE 1. HPLC chromatogram of a standard mixture of 13 PAHs. Conditions: Ultraspher (125 x 4 mm, 5 μm) column; temperature, 22°C; acetonitrile/water programming from 55:45 (held for 5 min), to 85:15 at 30 min; flow rate, 1 ml/min; injection volume, 20 μl; fluorimetric detection, 295 nm excitation and 405 nm emission wavelengths.

Determination of B(a)p in tobacco smoke particulate

1. Optimization of extracting agent and recovery study

Three solvents (acetonitrile, methylene chloride and cyclohexane) were evaluated as media for extracting B(a)p from filters containing tobacco smoke particulate. Cyclohexane was chosen as the extractant for B(a)p from mainstream smoke condensate because acetonitrile and methylene chloride extract more interfering material.

The B(a)p recovery study was carried-out on twelve filters containing of B(a)p in the range 20-140 ng, applying the experimental procedure without the clean-up step. The results of the analysis are shown in Table I. The mean B(a)p recovery was 100.1% and the relative standard deviation was 3.8%. The B(a)p was not detected in the filter used as a blank.

2. Clean-up step recovery study

Acetonitrile, methylene chloride and cyclohexane were also tested for eluting the B(a)p. The methylene chloride

TABLE I.

Recovery of B(a)p standard in the extraction step.

B(a)p Amount added, ng	%Mean Recovery	n	%RSD
-	-	1	-
20	95.8	3	5.7
60	104.2	3	2.4
100	102.0	3	3.0
140	98.2	3	4.2
Average	100.1		3.8

n measure number

gave lowest interferences. Different elutant volumes were also tested , but volumes above 2 ml give higher chromatographic interferences.

The B(a)p recovery study was carried out on twelve filters containing B(a)p in the range 20-140 ng by applying the experimental procedure. Table II shows the results obtained. The mean B(a)p recovery was 49.2% and the relative standard deviation was 2.4%. B(a)p was not detected in the extract of the blank.

TABLE II.

Recovery of B(a)p standard in the clean-up step.

B(a)p Amount added, ng	%Mean Recovery	n	%RSD
-	-	1	-
20	48.4	3	4.2
60	50.5	3	3.7
100	48.0	3	1.0
140	49.8	3	5.1
Average	49.2		2.4

n measure number

3. B(a)p identification and quantification

Excitation and emission spectra and retention time were used to identify of B(a)p. Excitation and emission spectra corresponding to the chromatographic peaks of sample and standard solution were registered; the flow rate was stopped when the fraction of B(a)p was in detector's flow cell. The spectra are shown in Figures 2 and 3. A Perkin-Elmer LS 30 fluorimetric detector was used. This instrument can be used either as a spectrofluorimeter or as an LC detector. The comparison of spectra confirms the presence of B(a)p in the samples.

The proposed method was applied to determine the delivery of B(a)p in particulate matter of Virginia and black tobacco smoke.

Figures 4 and 5 compares the chromatograms of Virginia (Figure 4) and black (Figure 5) tobacco samples. Both profiles are remarkably similar in their general features. Quantitatively, chromatogram 5 (black tobacco) is more intense than chromatogram 4 (Virginia tobacco).

Table III compares the B(a)p deliveries of the Virginia and black tobacco when smoked. Of particular interest is the delivery of B(a)p from the black tobacco,

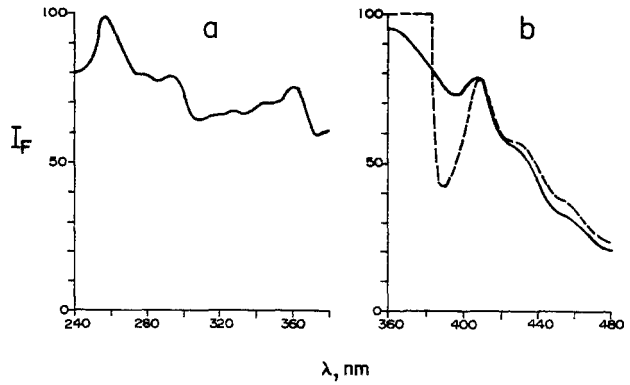


FIGURE 2. Excitation and emission spectra of B(a)p chromatographic peak of a sample; **a** = excitation (λ_{em} = 405 nm), **b** = emission (λ_{ex} = ——— 295, ---- 363 nm).

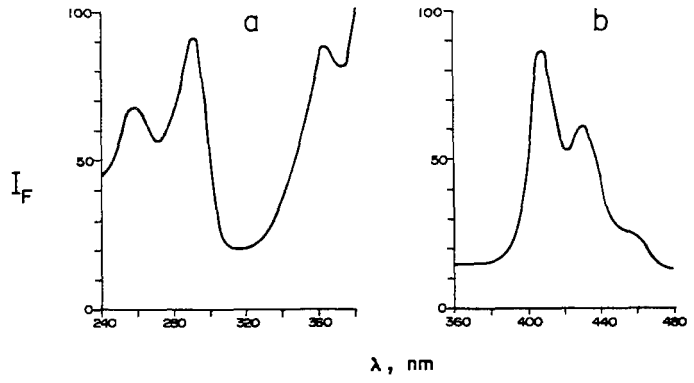


FIGURE 3. Excitation and emission espectra of B(a)p chromatographic peak of a standard solution; **a** = excitation (λ_{em} = 405 nm), **b** = emission (λ_{ex} = 295 nm).

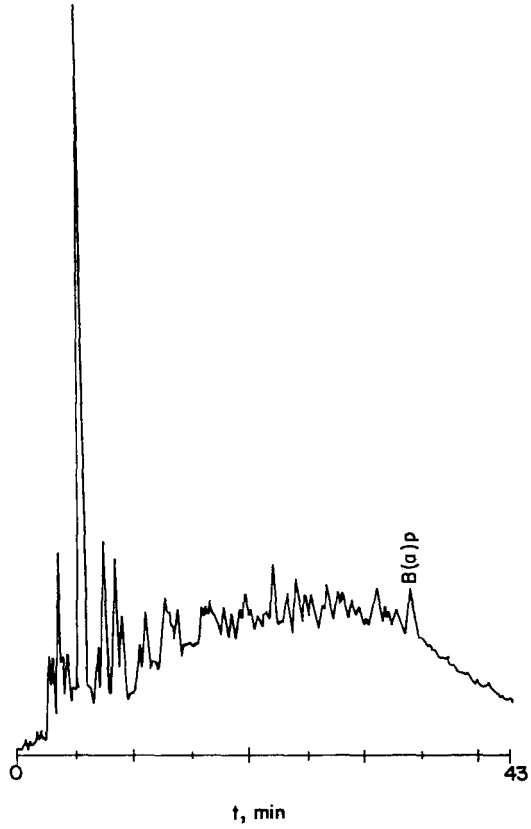


FIGURE 4. HPLC chromatogram of a Virginia tobacco sample. Conditions: see figure 1.

which is one and a half times that of the Virginia tobacco. The values reported exhibit a precision (expressed as relative standard deviation) of approximately 5.2-6.3%. The mean value obtained for the B(a)p delivery from a Virginia tobacco, 68.6 ng/5 cig. (5.2%, relative standard deviation, n=6), is in the range of that described for the commercial

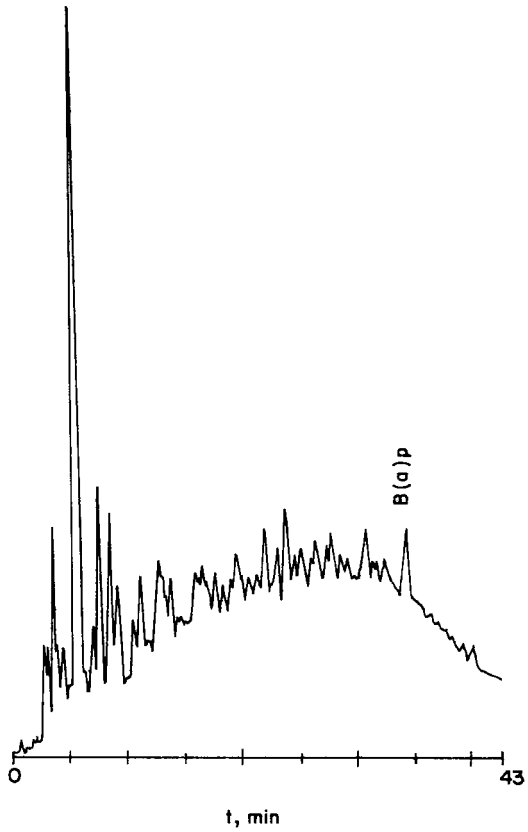


FIGURE 5. HPLC chromatogram of a black tobacco sample. Conditions: see figure 1.

tobacco cigarettes and from a black tobacco was 105.6 ng/5 cig. (6.3%, relative standard deviation, n=4). No references were found in the literature for the amount of B(a)p in black tobacco.

TABLE III.

Determination of B(a)p in Virginia and black tobacco.

Sample	Amount of B(a)p (ng/5 cigarettes)	
	Virginia tobacco	Black tobacco
1	73.9	100.7
2	68.6	101.3
3	68.6	105.4
4	69.4	115.1
5	62.7	-
6	68.4	-
$\bar{x}$	68.6	105.6
%RSD	5.2	6.3

CONCLUSIONS

The proposed method involves an extraction and a clean-up step for the determination of B(a)p in Virginia and black tobaco smoke. The optimized extraction and clean-up processes proved to be adequate for this type of samples the recoveries being 100.1% and 49.2%, respectively. The recovery in the clean-up step can be increased, but many interferences arise.

The B(a)p deliveries from the smokes of commercial cigarettes can be determined precisely, quickly and cheaply by applying our analytical procedure.

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HPLC METHOD FOR THE STUDY OF ANAEROBIC DEGRADATION OF POLYETHYLENE GLYCOLS

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ABSTRACT

This paper describes a rapid gradient reversed-phase high-performance liquid chromatography method for the characterisation of PEGs, HO-(CH2-CH2-O)n-H, in water. This method allows the routine analysis of a mixture of these compounds, in the range n=1-50 (up to PEG 1500) in unesterified samples, at room temperature. The method has been used for the study of anaerobic digestion of PEG-200 and PEG-300 in an one litre laboratory digester.

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INTRODUCTION

Polyethylene Glycols are used in the production of surfactants, lubrificants and cosmetics (1-3). They are, therefore, present in waste or surface waters as catabolic products of these compounds (4). Many polar compounds cannot be eliminated by various treatment steps such as: biochemical, chemical and physico-chemical tratment (soil filtration, ozone treatment or activated carbon processes) (5,6). The recalcitrant nature of PEGs by aerobic degradation led researchers to investigate the biodegrability under anaerobic conditions (1,7-9). The information regarding its biodegradation is scarce and conradictory (2,8). The study of degradation process is based mainly on the bacterial growth observation (8), chemical oxigen demand (COD) (1) or GC analysis of the final products (2,7,9). The anaerobic degradation mechanism in medium with PEGs as the sole source of carbon and energy was not established. In this field, the determination of the highest size polimer of PEGs, that are biodegradable, as well as the bacteria with a higher degrading capacity are very important.

The direct analysis of the PEGs' molecular distribution in an anaerobic digestor is very useful for studying the fundamental aspects of this process. The GC or GC/MS methods have a very limited applicability with these problems, taking into account that these compounds are polar and thermolabile (6). A newly established HPLC method would be suitable to solve the many analytical aspects.

The use of a refractive index detector (10,11) is limited to isocratic methods and therefore to a limitated range of polymers. The reported gradient elution methods are using detection to 185nm (12), which is a inaccesible value for many detectors, or the use of the coupled columns (13).

Other HPLC methods are working with high temperature (14), derivatizated samples (15,16) or a mass spectrometer as a detector (17). The use of mass spectrometer can improve the detection limit with two orders (18) but higher than room temperature is used and therefore can produce thermal degradation of the polymers.

The size-exclusion chromatography (SEC) methods gave separation of PEGs into approximate groups (19).

We have found that the use of the nitric acid (12) for solvent absorbance equalisation at 190 nm, 2-36% acetonitrile gradient and the use of a short RP-C18 column (15 cm) makes possible the rapid separation of PEGs up to 1500 (n=1-50) at room temperature. The detection limit is 0.5 ug and the elution time 40 min. This method has been used in the study of laboratory anaerobic digesters.

EXPERIMENTAL

All chemicals and solvents were of analytical grade. For sample filtration, a syringe filter of type nylon, 0.45um, 25mm in diameter was used.

The PEGs used in this experiment were from: Panreac, 08110 Barcelona, Spain (PEG-200, PEG-300, PEG-400 and PEG-1500) and Merck-Schuchardt, 8011 Hohenbrunn bei München, Germany (PEG-600 and PEG-1000).

A Gilson HPLC system was used (Gilson Medical Electronics, Middelton, WI 53562 USA) equipped with: Pump 305(A) and 306(B), Manometric Module 805, Dinamic Mixer 811C and an 20ul Injector (Reodyne, Cotati POB 996, California 94931, USA).

A Dinamax UV-1 Absorbance Detector (Rainin Instrument CO, Mack Road, Woburn, MA 101801, USA)

equipped with a dual-pathlength flowcell (9mm and 1mm lightpaths) set at 190nm was used. The output of the detector was monitored by a Macinntosh Classic II Computer (Aplle Computer, Inc. California USA).

The analyses were made using a 15cm, 4.6mm id, Nucleosil 5u, C-18, 120A column (Tecnocroma, 08190 Barcelona, Spain) at room temperature. The acetonitrile in water gradient was used, as shown in Table 1. As an absorbance equalisation for water to 190 nm we used 3ppm nitric acid.

RESULTS AND DISCUSSION

The resolution. In Figure 1 the separation of the PEGs polymers with n between 1 and 50 are shown. This sample was obtained by a mixture of PEG-200, 300, 400, 600, 1000 and 1500, 50 mMolar each. The work conditions are as in Experimental. Only for Ethyleneglycol (EG) which is eluting at 1 min., very close to dead time (0.7 min.), the retention factor (capacity factor) is lower than 1. All other are higher, which is an important condition for good reproductability (20). As was shown (21,22), the retention factor for adjacent oligomers decreases with the increase of mobile phase strength which is equivalent to resolution decreasing.

In Table 2, column 2, 3 and 4 respectively, the range of n, the value of n corresponding to major polimer (nm) for every PEG and the resolution calculated near nm (Rm) are shown. The ranges of n were calculated taking into account all peaks with the area higher than 5% from a major polymer.

Up to PEG-600, the resolution is better than 1.5, which is a minimum value for complete separation of the adjacent peaks (23). For PEG-1000 the valley hight (the

Table 1. Gradient of Acetonitrile in Mobile Phase

time(min.)	A(%)	B(%)
0	96	4
10	70	30
41	28	72

A=1mM Phosphoric acid, B=1mM Phosphoric acid+Acetonitrile (1:1), Flow 1.5 ml/min

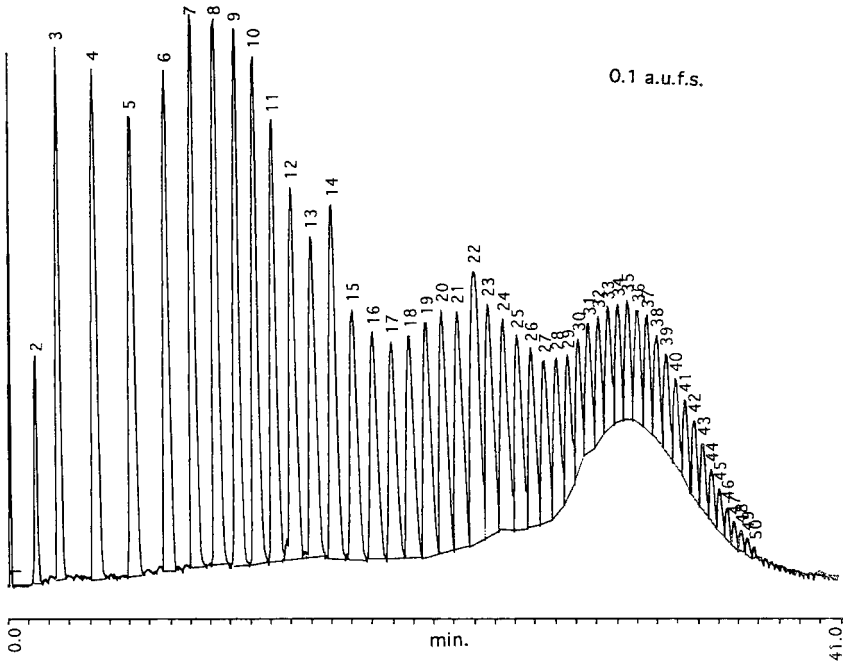

FIGURE 1. The separation of PEGs polymers, n = 1-50. (50 mM PEG-200, 300, 400, 600, 1000 and 1500). Conditions as in Experimental

Table 2. Range of n, n for major polymer (nm), Resolution close to nm (Rm) and Estimated Detection Limit for the first peak (DL)1 and for the first six peaks (DL)6.

PEG	range n	nm	Rm	(DL)1	(DL)6
TEEG	4	4(100%)	-	0.5	-
PEG-200	2-8	4(30.2%)	3.34	1.65	10.86
PEG-300	2-11	6(18.3%)	2.68	2.75	6.2
PEG-400	4-15	9(16.4%)	2.03	3.00	6.3
PEG-600	7-20	13(13.3%)	1.60	3.75	6.7
PEG-1000	14-32	23(9.2%)	1.00	5.5	6.8
PEG-1500	25-50	34(7.5%)	0.41	6.6	8.5

ratio of the valley level to the peak height relative to the base line) is smaller than 15% and for PEG-1500, it is around 50%. Therefore the study of individual polymers can be made with good precision, up to PEG-1000, and with medium precision for PEG-1500.

In Figure 2 the chromatogram obtained for PEG-1000 is shown.

Detection Limit (DL). The Detection Limit was tested with Tetraethyleneglycol (TEEG), n = 4, M = 194. For a ratio signal/noise 3.5 we found a value of 0.5 ug/injection equivalent to 25 ppm (w/v) TEEG in water (Injection = 20 ul).

Taking into account the polymer distribution as an n function, a Detection Limit for comerccial PEGs as a Detection Limit for major polymer, (DL)1, and as Detection Limit for the first six polymers, (DL)6, were evaluated (Table 2, column 5 and 6 respectively). In comparison with early reported results, the (DL)6 is better than using refractive index detector (40 ug, PEG-

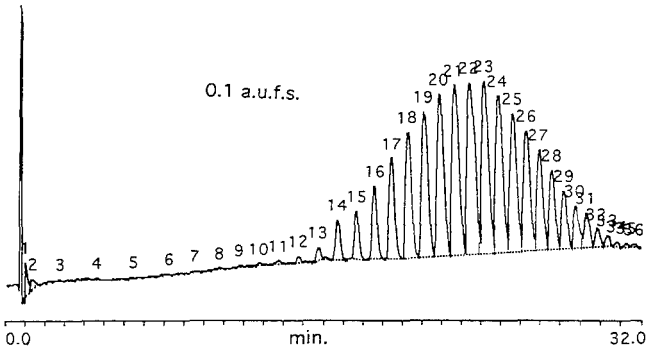

FIGURE 2. The PEG-1000 (10 mM) chromatogram. Conditions as in Experimental

400) (10,11) but is less (but comparable) than using methods involving sample derivatization (1 ul PEG-600) (16).

The linearity. Because the feed digestor is made with PEGs of 1% (w/v) concentration, the range of individual polymers is 0 - 0.1% (w/v) and corresponds with 0 - 20 ug/injection. Therefore, we have tested the liniarity with TEEG (n = 4) in the range 0-24 ug/injection. The calibration curve is shown in Figure 3.

The relation between the response (peak area) and quantity injected is given by the equation:

$$A = 7790.94 \times q + 375.05 \qquad (1)$$

where: A is peak area (uV x sec.) and q is the TEEG quantity injected (ug).

The correlation coeficient (r2) was 0.9982. The medium R.S.D. for differente concentrationes calculated with this calibration curve was around 3.00%

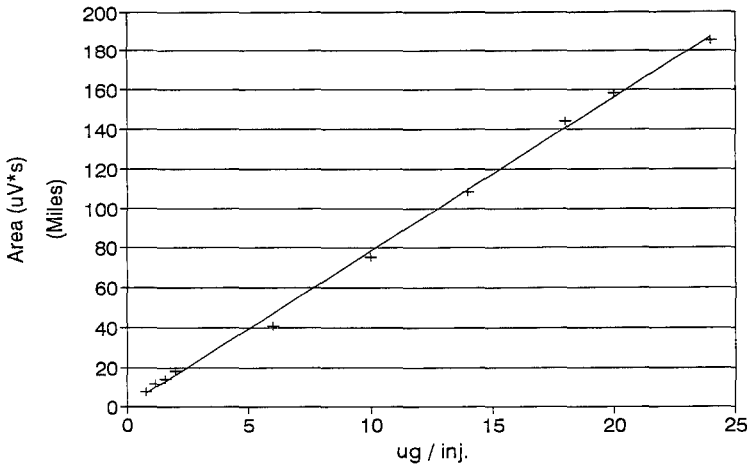

FIGURE 3. The calibration curve obtained for TEEG (n = 4, M =194)

The degradation of PEGs in an anaerobic digester. We have been studying the degradation of the PEGs as a sole source of carbon and energy in a laboratory stirring anaerobic digestor with the semicontinuous feed (daily) with PEG-200 and PEG-300 respectively of 1% concentration (w/v). The anaerobic bacteria were methanogenic cultures obtained from a municipal sludges digester. The volume of the digester was of 1 litre and was maintained at a constante temperature (36 C).

In order to determine the degradation process, an assesement was made of the molecular distribution of the PEGs for digestor samples, 24 h after feeding, and then compared with the initial configuration.

The degradation rate (r) for every polymer was calculed as:

$$r(\%) = (Qi-Qf)x100/Qi \qquad (2)$$

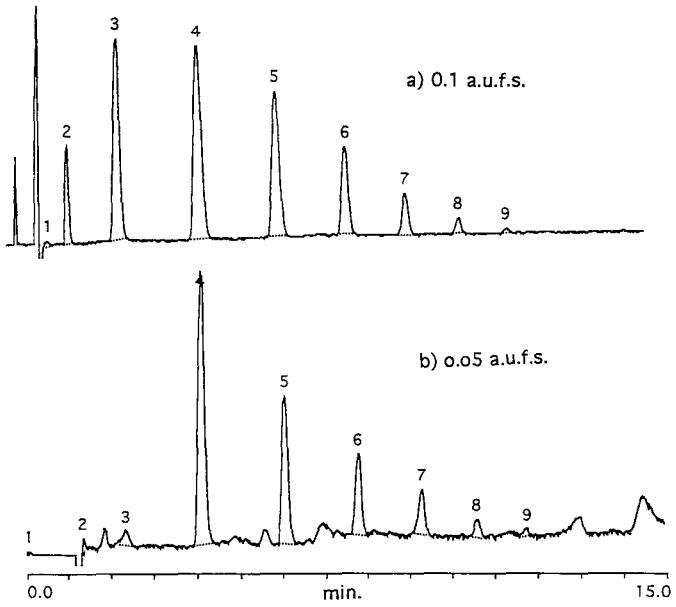

FIGURE 4. PEG-200 chromatogram. (a) initial, (b) from the anaerobic digestor. Conditions as in Experimental

where Qi and Qf are the initial and final quantity of polymer and were calculated from the peak area. R is a degradation rate for a correspondent PEG and was calculed in the same way from the sum of polymers.

An other parameter measured was the volume of gas results by anaerobic degradation (CH_4 + CO_2) which is also a indicator about degradation rate. When the gas production were smaller as 0.5 l a new quantity of bacterial culture was inoculated. In the Figure 4 the chromatogram for initial PEG-200 (a) and for a sample collected from the anaerobic digestor (b) at ten days after bacterial inoculation is shown.

The daily degradation rate r and R and gas production for PEG-200 from different days is presented

Table 3. Polymeric degradation rate, r, Total degradation rate, R, and Gas production (l) (daily) for PEG-200

n	r(%)				
	day 1	day 4	day 7	day 8	day 10
2	>96.48	>96.48	>96.48	>96.48	>96.48
3	97.26±0.41	98.72±0.18	97.69±0.06	>99.13	>99.13
4	52.73±2.36	58.80±0.41	81.29±0.62	87.34±0.02	91.96±0.52
5	59.67±2.01	76.70±0.40	92.40±0.26	95.07±0.02	96.27±0.54
6	60.79±2.11	71.17±0.34	92.90±0.34	94.63±0.04	95.74±0.63
7	58.47±3.02	68.46±0.91	89.980.76	88.50±0.02	93.18±0.90
8	57.87±3.10	65.32±2.6	79.08±2.20	83.81±0.02	>86.48
R(%)	68.71±1.56	76.01±0.21	89.89±0.75	93.49±0.06	95.49±0.57
Gas(l)	0.270±0.003	0.450±0.004	0.670±0.007	0.670±0.007	0.650±0.01

Table 4.Polymeric degradation rate, r, total degradation rate, R, and gas production (l) (daily) for PEG-300.

n	r(%)				
	day 1	day 4	day 7	day 8	day 10
2	>80.14	>80.14	>80.14	>80.14	>80.14
3	>95.58	>95.58	>95.58	>95.58	>95.58
4	46.67±2.18	58.95±1.57	93.60±1.72	93.78±0.83	>98.07
5	84.51±1.23	91.83±0.78	98.70±0.18	98.64±0.45	>98.67
6	81.94±0.50	90.87±0.94	98.58±0.47	98.48±0.37	>98.88
7	74.18±1.16	80.85±1.14	94.67±0.20	96.87±0.02	>98.88
8	72.69±1.01	78.51±0.15	91.12±1.09	95.75±0.88	>98.47
9	68.21±2.50	68.95±1.22	87.32±1.38	91.92±1.33	>97.56
10	70.67±2.30	61.80±0.53	79.89±6.4	91.59±1.21	>95.15
11	71.52±8.35	63.66±4.08	87.14±2.51	88.20±1.36	>89.39
R(%)	74.53±3.25	80.60±0.11	94.25±0.13	95.75±0.41	>98.5
Gas	0.250±0.002	0.430±0.004	0.680±0.007	0.710±0.007	0.680±0.01

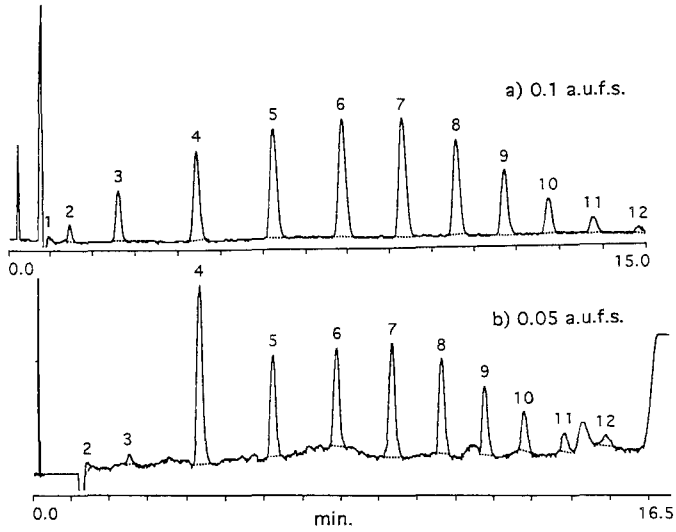

FIGURE 5. PEG-300 chromatogram. (a) initial, (b) from the anaerobic digestor. Conditions as in Experimental

in Table 3. The same data for PEG-300 are shown in Table 4.

In Figure 5 the chromatogram for initial PEG-300 (a) and a sample colected from digestor 13 days after bacterial inoculation (b) are shown.

Ten days after bacterial inoculation (100 ml, 5g/l suspension solids) the daily degradation ratio R for PEG-200 was changed from 68.71% to 95.49%. The degradation of PEG-300 was similar but with a degradation range of 74.00% to higer than 98.5%. Taking into account that a degradation rate higer than 98.5% is out of detection limit, the degradation of the PEG-300 after 10 days can be complete.

For every PEG, a linear corelation between the quantity of a PEG degraded and the volume of gas eliminated was observed (Table 3 and 4).

We found that daily degradation rate for PEG-200 and PEG-300 depending on polymeric length. The elution time for $\underline{n}$ = 1, in the digestor samples is superimposed with the elution time corresponding to acetic acid and ethyl alchol present as degradation products (7). Therefore the EG ($\underline{n}$ = 1) was not measured. For $\underline{n}$ = 2 and n = 3 the degradation is very strong, about 100%. This is in acordance with the data of Chemical Oxigen Demand (COD) reported for Diethyleneglicol (DEG), Triethyleneglycol (TEG) and PEG-400 (1). For n = 4, in both situations we found a lower rate relative to the polymers with n > 4. As a result of this, the polymeric distribution for the samples colected from the digestor are very diferrent in comparison to the initial PEGs (Figure 4 and 5).

For PEG-200 the polymeric degradation rate for $\underline{n}$ >4 are very similary. In the situation of the PEG-300 the polymers $\underline{n}$ = 5 and $\underline{n}$ = 6 have the degradation rate higher than $\underline{n}$ > 7.

The order of dgradation rate, $\underline{r}$, observed for diferrent values of $\underline{n}$ are:

PEG-200: 2 = 3 >> 5,6,7,8,9 > 4
PEG-300: 2 = 3 >> 5,6 > 7,8,9,10,11 > 4

These results can be suported by the exitence of two types of bacterium strain, one degrading short oligomers (up TEG, n=3) and the other one degrading higher polymers, with depolimerization action out of the cell (2), in contrast with the hypothesis regarding the penetration of the cytoplasmic membrane by longer polymers to the bacterium cell (8,24). It must be noticed the smaller r value for n = 4 polymer. Experiments are in progress in our laboratory to examine the degradation of higher PEGs.

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TRACE ANALYSIS OF ETHANOLAMINE IN WATER USING ION PAIR CHROMATOGRAPHY WITH ON-LINE PRECONCENTRATION

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ABSTRACT

A simple and rapid method for the determination of ethanolamine in water was developed. The problem of ethanolamine preconcentration was solved by adding octane-1-sulfonic acid (OSA) to the water sample and ion pair was sorbed on the precolumn C18. After flushing ion pair of ethanolamine was desorbed to the analytical column with lower concentration of OSA in mobile phase. Electrochemical detection was used. The limit of the detection at 0.88 V and signal/noise ratio of 5 was about 2 ppb. The mean relative standard deviation was 10% at 10 ppb level.

INTRODUCTION

The sources which discharge ethanolamine into the waste water are varied a numerous. These sources include industrial processes of manufacture of dyes, drugs, plastics and detergents. Ethanolamine has been found to be toxic to most organisms, therefore, legal requirements of many countries are increasing, making it necessary to determine etanolamine at very low level (0.5 mg/litre) [1]. Environmental samples can

have very complex matrices, therefore detection and separation methods are preferred for the determination of ethanolamine.

The widely used method is gas-liquid chromatography of derivate of ethanolamine with flame ionization detection [1,2]. Ion exchange liquid chromatography with photometric or fluorometric detection after derivatization provides an alternative which avoids the necessity for producing a volatile derivate [3-5]. However, the reaction of derivatization and work-up required are time-consuming, and the formation of derivates can be complicated, giving rise to erratic results.

For this reason, we have investigated the development of a sensitive method for the determination of ethanolamine. The method takes advantage of the well-known reaction with octane-1-sulfonic acid (OSA) in mobile phase which proceeds ion pair. The goal of this study is to optimise of composition of mobile phase as well as to introduce a selective enrichment step for ethanolamine from water. The use of solid-phase extraction for this purpose is growing rapidly in popularity in comparison to liquid/liquid extraction or other concentration techniques. The advantage include the speed and selectivity of the solid-phase extraction.

EXPERIMENTAL

The chromatographic system with on-line preconcentration consisted of two pumps (Waters Model 501), a chromatographic column (0.32 x 15 cm) packed with 5 μm particles Separon SGX C18 (Tessek, Prague, Czech republic), an electrochemical detector (Waters Model 460) and a preconcentration column (0.32 x 3 cm) packed with 5 μm particles Separon C18 (Tessek, Prague, Czech republic). The scheme of the LC system is shown in Figure 1. All solvents and the modifiers should be as clear as possible. The flow rate was only 0.5 ml/min because the lower flow rate has the lower noise level at the electrochemical detection.

Before introduction of the sample the preconcentration column was washed with 0.01 M OSA solution. The concentration of OSA in the water sample was adjusted to 0.01 M by addition of solid OSA. The sample is then injected via a loop (0.5 ml) and pumped through the preconcentration column. After flushing ion pair of ethanolamine is desorbed to the analytical column with 0.00076 M OSA in 34% methanol - water. Electrochemical detection was performed with ampermetric detector using carbon electrode. The applied potential with respect to argento-chloride electrode (as reference) was +0.88V.

River water was filtered over a 0.5 μm membrane filter (Millipore type AA). For the recovery experiment the river water was spiked with ethanolamine up to

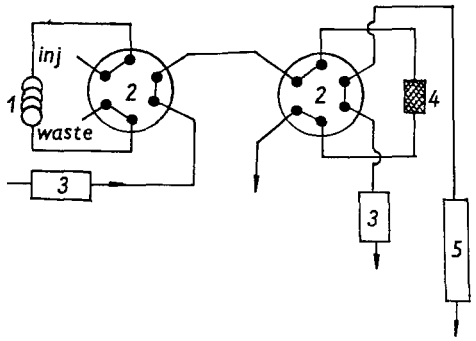

Fig.1. Schematic diagram of the LC apparatus. 1-injection loop (0.5 ml); 2-valve; 3-pump; 4-preconcetration precolumn; 5-analytical column.

concentration 10 ppb. Water solutions for calibration experiments were made starting from 2 ppb to 200 ppb. Preconcentration of 0.5 ml was found possible on 3 x 0.32 cm I.D. preconcentration precolumn without breakthrough.

The quantitative evaluation was based on the regression analysis where the dependence between the area of peak of ethanolamine and the quantity was determined. The linearity range was between 2 and 200 ppb of ethanolamine.

RESULTS AND DISCUSSION

Reversed ion-pair liquid chromatography has become a well established method for the separation of ionic compounds, in which the retention can be regulated by the nature and concentration of organic modifier and ion pair reagent as well as by a competing ion with the same charge as that of the analyte.

To find the optimal composition of mobile phase (contents of methanol and OSA) with the minimal number of measurements, Doehlert matrix design [6] was used. The retention of ethanolamine in ion-pairing reversed phase chromatography was modelled via changing the volume percentage of methanol and concentration of ion-pairing agent octylsulfonic acid in the mobile phase. To fit the second-order polynomial of eq. 1, Doehlert matrix design was employed.

$$\ln(k) = a_0 + a_1 \cdot X_1 + a_2 \cdot X_2 + a_{11} \cdot X_1^2 + a_{22} \cdot X_2^2 + a_{12} \cdot X_1 \cdot X_2 \qquad (1)$$

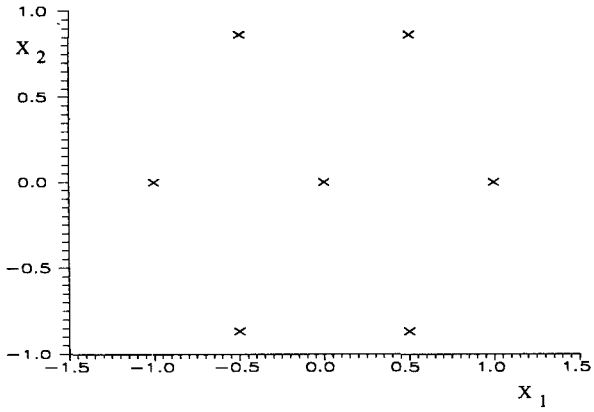

Fig. 2. Doehlert matrix design for two factors X_1, X_2. The experimental points in our procedure are listed in Tab.1.

Tab. 1. Doehlert matrix design for modelling of ethanolamine retention.

Point	Variables in coded units		Variables in original scale	
	X_1	X_2	φ(MeOH) volume fraction	c(OSA) [mol\L]
1	1	0	0.30	0.0010
2	-1	0	0.30	0.0002
3	0.5	0.866	0.45	0.0008
4	-0.5	0.866	0.45	0.0004
5	0.5	-0.866	0.15	0.0008
6	-0.5	-0.866	0.15	0.0004
7	0	0	0.30	0.0006

Tab.2. Analysis of classical residuals.

Point	Response measured $y_{exp[i]}$ {ln(k)}	Prediction calculated $y_{calc[i]}$ {ln(k)}	Standard deviation $\sigma(y_{calc[i]})$ [x10^{-8}]	Classical residual [x10^{-8}]
1	1.4400	1.4400	3.9151	2.1653
2	1.3600	1.3600	4.2887	2.0238
3	0.9900	0.9900	3.9151	0.4506
4	2.2700	2.2700	3.8254	2.1535
5	2.4300	2.4300	3.6325	0.4495
6	0.6800	0.6800	2.8954	2.1367
7	0.0700	0.0700	2.5588	0.9876

Residual sum of squares = $1.8393.10^{-15}$

where k is the capacity factor, X_1 is the volume fraction of methanol in the mobile phase, X_2 is the concentration of ion-pairing agent in the mobile phase and a_0, a_1, a_2, a_{11}, a_{22} are parameters of regression function.

Doehlert described how to generate the designs up to at least ten factors [7]. An experiment in two factors (X_1, X_2) may be thought as a point in two-dimensional space. The two factors Doehlert design requires seven experiments (Fig.2).

The results of regression using model in eq.1. are shown in Tab.2. The analysis of residuals shows a very good fit of experimental data. According to eq. 1. the contour plot (Fig. 2) can be constructed.

The contour plot of dependence ln (k) = f {φ(MeOH); c (OSA) } according to eq. 1 is shown in Fig.3. The contour of 1.3 (capacity ratio 3.7) was most available because the symmetrical shape of ethanolamine peak was achieved. From this point of view the concentration of OSA was 0.00076 M in 34% methanol - water. As it can be seen from Fig.3 the value of capacity ratio of ethanolamine is very high at low concentration of methanol and higher concentration of OSA in mobile phase using C18 column. This phenomena was applied to preconcentration of ethanolamine in the precolumn and from this reason OSA must be added to the water sample before injection.

Figs. 4 and 5 show the chromatograms after preconcentration of 0.5 ml spiked and unspiked river sample. It was not observed the river water contained a trace amount of ethanolamine. From this reason, the river water was spiked with ethanolamine such

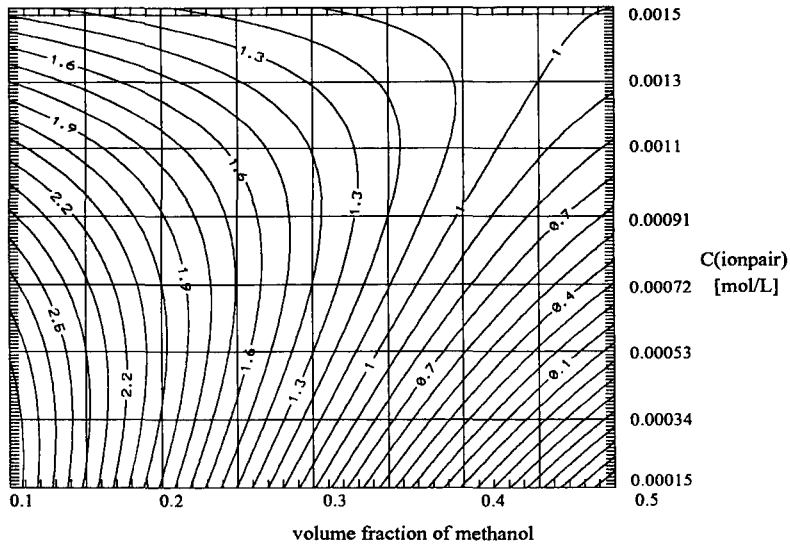

Fig.3. The contour plot of dependence ln(k) = f {φ(MeOH); c (OSA)} according to eq.1.

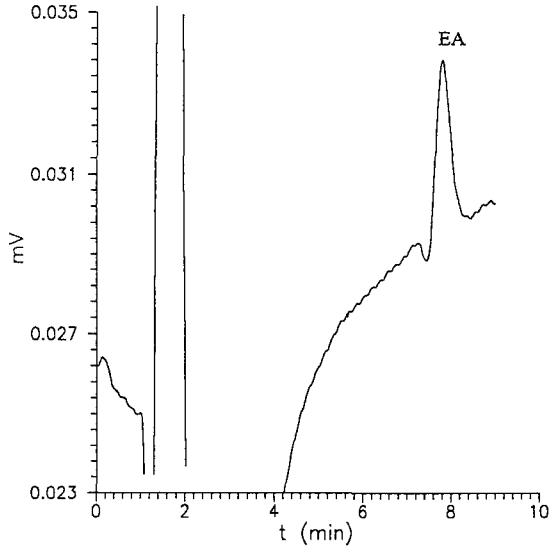

Fig.4. Chromatogram of the spiked river water (10 ppb) after on-line preconcentration (sampling volume 0.5 ml). Chromatographic column Separon SGX C18. Flow rate was 0.5 ml/min., mobile phase - see experimental part, ampermetric detection at +0.88V.
EA - ethanolamine

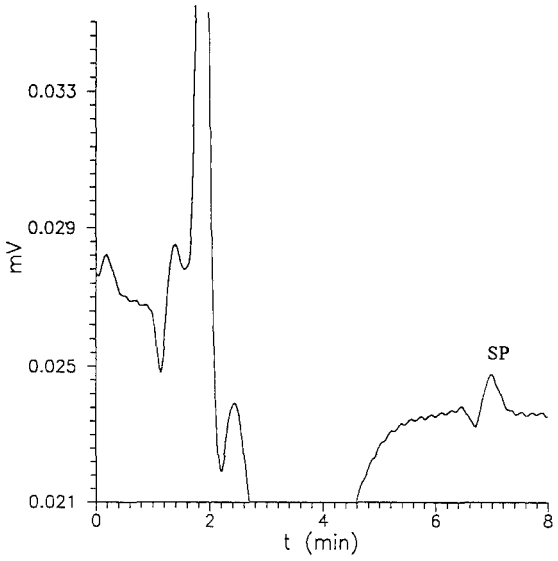

Fig.5. Chromatogram of the unspiked river water after on-line preconcentration (sampling volume 0.5 ml). For the chromatographic conditions see Fig 4.
SP - solvent peak

that the concentration became 5 ppb to demonstrate the possibility of the method. After the experiments the difference between the peak areas of the unspiked and spiked chromatograms was used to calculate the concentration of ethanolamine. The recovery of solid phase extraction was about 75%.

The selectivity of the preconcentration system allows the LC analysis to be performed within about 15 minutes with a detection limit of 5 ppb. Lowest limit has been determined using a peak area integrator signal-to-noise ratio of five. Triplicate injection of ethanolamine (10 ppb) yielded the relative standard deviation of about 10 %.

The results of this study clearly indicate that OSA is a useful ion pairing agent for chromatographic separation of ethanolamine. The work also demonstrates the effectivity of Doehlert matrix design for the optimization of mobile phase composition in ion pair chromatography.

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PURIFICATION OF THE MAIN GINSENOSIDES FROM A FRENCH CROP OF *PANAX QUINQUEFOLIUM* L.

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ABSTRACT

Active components of *Panax quinquefolium* L. roots (French growing) have been separated by centrifugal partition chromatography (CPC). Five pure ginsenosides, namely Rb1, Re, Rd, Gypenoside XVII, and F11, have been isolated in one step, using a biphasic mixture of ethyl acetate / 1-butanol / water and gradient elution. Part of the effluent was monitored with an evaporative light scattering detector (ELSD), for direct control of the collected fractions, allowing an easy detection of these molecules with low or no absorbance in the UV. Isolated ginsenosides have been identified by comparison with standards and by ^{1}H and ^{13}C NMR studies.

INTRODUCTION

American ginseng *Panax quinquefolium* L. originates from the northern region of the United States and grows in several countries, *e.g.* Canada and north of China[1]. Its growth has been recently introduced in France. Tonic properties of its extracts are used in food chemistry (tonic water, dietetic..) and make it comparable to the Korean ginseng *Panax ginseng* Meyer. Ginsenosides (triterpenoic saponosides) composition for both species is close[2], but ginsenosides Rd, Re, and especially Rb (Rb1 and Rb2), are known to be more abundant in *Panax quinquefolium* L. . Rb1 could be the active compound for anti-stress properties, and has a beneficial influence against Alzheimer's disease[3]. Ginsenosides from root extracts of *Panax quinquefolium* L. have been fractionated by various chromatographic methods, including reverse phase HPLC and droplet countercurrent chromatography (DCCC)[4]; several operations were needed in HPLC and poor yields were obtained, whereas DCCC is time consuming and restricted to few biphasic systems. In both case, detection at 202-207 nm was not easy or in some case, was impossible.

Centrifugal partition chromatography (CPC), combined with evaporative light scattering detection (ELSD), appears to be a suitable method for quantitative separation of these polar molecules, and we report here a typical separation of the main ginsenosides of *Panax quinquefolium* L. which we achieved in our laboratory.

EXPERIMENTAL

Preparation of the extract

Panax quinquefolium L. roots came from a two year and three month old growing (COOPAL, Picardie, France). 466 g of ground root were heated in 6 liters of refluxing aqueous methanol (1/4) (v/v). The aqueous-alcoholic extract was concentrated under reduced pressure, then diluted with 1 liter of water and extracted 4 times with 1 liter of water saturated butanol (4 x 250 mL). The butanolic solution yielded 49.45 g (10.6%) of crude extract after evaporation of the solvent. 16 g of this extract were diluted with 150 mL of water and dialyzed against water in a

Visking PM (6000-8000) tube (Polylabo, Strasbourg, France) for 72 hours. After lyophilization, 8.6 g of purified ginsenosides were obtained, the final yield being of 5.73 %.

Apparatus

A Series 1000 HPCPC (Sanki Eng. Limited, Nagaokakyo, Kyoto, Japan) was used[5]. It is a bench top CPC (30 x 45 x 45 cm, ≈ 60 kg); the column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 240 mL. The column is connected to the injector and the detector through two high pressure rotary seals. A 4-port valve included in the series 1000 allows the HPCPC to be operated in either the descending or ascending mode. The HPCPC was connected to a solvent delivery pump Techlab economy 2/ED (Techlab, D-38173 Erkerode, Germany), supplied with solvents through a gradient generator ISCO model 2360 with preparative options (ISCO, Inc., Lincoln, NB, USA). A flow splitter with a restriction was installed at the outlet of the HPCPC, the main line (90% of the flow) going to a fraction collector Pharmacia type Super Frac (Pharmacia, Uppsala, Sweden), the other line (10% of the flow) going to an evaporative light scattering detector Varex type ELSD II A (Varex, Barstonville, MD, USA). The temperature of the nebulizer was set at 118.5°C. *Panax quinquefolium* L. extracts were injected through a Rheodyne model 7125 injector with a 5 mL sample loop.

Biphasic system

The ternary system ethyl acetate (EtOAc) / 1-butanol (1-BuOH) / water (H_2O) has been used. The ternary diagram corresponding to this system is shown on Figure 1. This system is favorable for a gradient run in the normal phase mode, *i.e.* if the lower, water rich phase, is used as the stationary phase[6]. The polarity of the mobile phase can then be modified by changing the ratio of BuOH to EtOAc, which does not result in significant modification of the stationary phase composition, as shown by the orientation of the tie-lines. A similar gradient elution has been used by M. Vanhaelen *et al.* [7] with the ternary system EtOAc / 2-BuOH / H_2O, to purify several flavonoids from a commercial extract of *Ginkgo biloba* leaves. Initial composition of

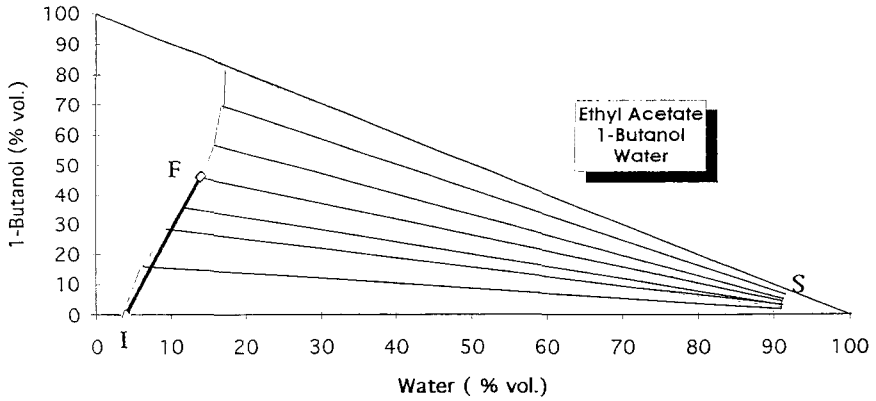


Figure 1 Ternay system ethyl acetate / 1-butanol / water. Composition of the liquid phases used to generate a gradient of butanol in ethyl acetate on a water-rich liquid stationary phase :

I : initial mobile phase
F : final mobile phase
S : stationary phase.

the mobile phase we were using was EtOAc / 1-BuOH / H_2O, 95 / 1 / 4 (v/v/v)(I on Fig. 1), and final composition was EtOAc / 1-BuOH / H_2O, 40 / 46 / 14 (F on Fig. 1) The gradient was linear with a duration of 4 hours; the flow rate was 4 mL/min and the rotational speed was 1200 rpm. With these experimental conditions, the mobile phase volume in the HPCPC was ≈ 60 mL, *i.e.* a retention of the stationary phase of about 75%. Back pressure was 29 to 34 bars,

Analysis of the collected fractions :

160 x 8 mL fractions were collected. Each fraction was analyzed by TLC on Whatman K6F plates (Whatman, Maidstone, England); the mobile phase for TLC was a mixture of EtOAc / 1-BuOH / H_2O, 1 / 4 / 3; spots were visualized by H_2SO_4 spray and heat (100ºC for 10 min). Identical fractions were pooled and evaporated

to dryness. They were characterized by comparison with standards of Rg1, Re, Rc, Rd, Rb1, Rb2 and Rf (Extrasynthese, 69730 Genay, France). Fractions containing only one spot were then analyzed by NMR. Spectra were recorded in pyridine with a Bruker model AC300 (Bruker, Wissembourg, France), at 300 MHz for ^{1}H NMR, and 75 MHz for ^{13}C NMR. Spectra were compared to data found in the literature, and to spectra of standards.

Solvents

All solvents were pure grade for analysis and came from SDS (113124 Peypin, France).

RESULTS AND DISCUSSION

The polarity range of the system EtOAc / 1-BuOH / H_2O is very useful for the fractionation of ginsenosides found in *Panax quinquefolium* L.. Ginsenosides partition in favor of the lower aqueous phase (stationary) for EtOAc rich biphasic systems (*i.e.* partition coefficients are large), while they partition in favor of the organic upper phase (mobile) for BuOH rich biphasic systems (*i.e.* their partition coefficients are small). In this case, an increase of the BuOH content in the mobile phase will allow elution of ginsenosides of increasing polarities, according to a normal phase mode of chromatography. Five pure compounds have been obtained by using the gradient of Fig. 1, after injection of 210 mg of the extract (in 5 mL of stationary phase) : the major compound of *Panax quinquefolium* L., Rb1 (32 mg, 0.87 %), and four other ginsenosides, Rd (18 mg, 0.49 %), Re (13 mg, 0.35 %), gypenoside XVII (9 mg, 0.24 %)[8], and F11 (5 mg, 0.13 %)[9]. A CPC chromatogram is shown in Figure 2, and the structure of ginsenosides in Figure 3. Fraction 12-17 (9 mg) contains impure Rg1, fraction 87-93 (10 mg) is a mixture of Re and Rc, fraction 94-109 (12 mg) is a mixture of Rc and Rb2; these mixtures were identified by comparison with standards in TLC. The CPC run is fully reproducible using the same experimental settings and gradient. As can be seen in Fig. 2, elution of ginsenosides in CPC follows an order similar to that found in TLC, except for Re. Difference in polarities of ginsenosides is mainly due to their various sugar moieties. F11 has only one chain with two sugars and is eluted first, then Rd and gypenoside XVII which contain three

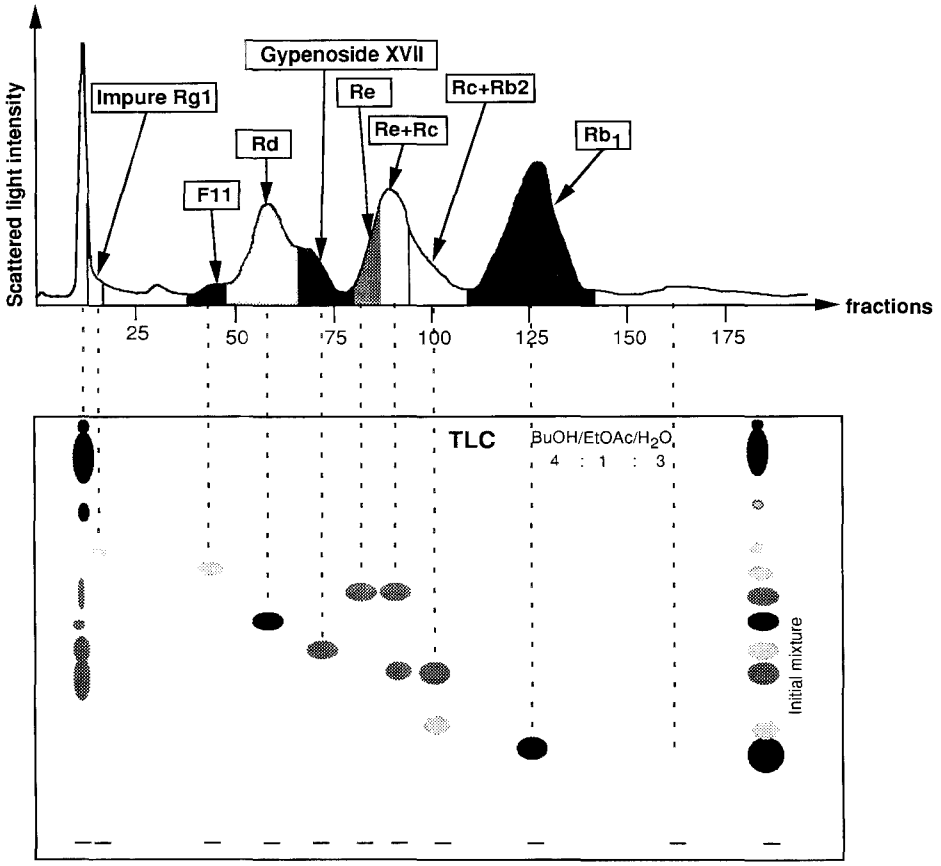
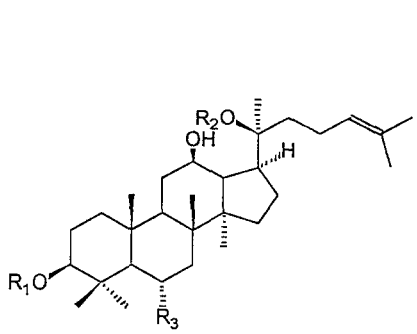
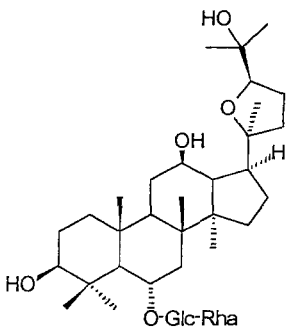


Figure 2 CPC separation of ginsenosides from *Panax quinquefolium* L. gradient elution with the ethyl acetate / 1-butanol / water system; stationary phase : water saturated with ethyl acetate and butanol (S on Fig.1). gradient duration : 4 hours. Ascending mode, flow rate : 4 ml/min rotational speed : 1200 rpm; back pressure : 29 to 34 bars volume of the mobile phase in the HPCPC : 60 ml.





F 11

	R_1	R_2	R_3
Gypenoside XVII	Glc	Glc	H
Rd	Glc-Glc	Glc-Glc	H
Rc	Glc-Glc	Ara(f)-^{6}Glc	H
Rb_1	Glc-Glc	Glc-Glc	H
Rb_2	Glc-Glc	Ara(p)-^{6}Glc	H
Re	H	Glc	O-Glc-Rha

Figure 3 Chemical structures of the ginsenosides found in *Panax quinquefolium* L.

sugars, then Rc and Rb1 which contain four sugars. Results found in the literature show that Rb1, Rd and Re are the main ginsenosides of *Panax quinquefolium* L. [2,8], which is in agreement with our results. Stated another way, F11 and gypenoside XVII, which are said to be negligible[8], are found to be up to 7% in the extract studied in our laboratory.

CONCLUSION

Centrifugal partition chromatography is well suited to the purification of polar compounds such as ginsenosides, and gradient runs allow purification of several

compounds in only one step. Scaling up our results should be easy by use of centrifugal partition chromatographs of larger capacities. *Panax quinquefolium* L. growing in France proves to be rich in ginsenosides from two year old, maximum quantities being expected for four year old growings[1]. The high content of Rb1 (>15 % of the mixture), and its fast purification by CPC makes *Panax quinquefolium* L. a valuable raw material, if its potential therapeutic interest is confirmed.

ACKNOWLEDGMENTS

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SEPARATION OF THE TWO MAJOR ANTHOCYANINS FROM CHAMPAGNE VINTAGE BY-PRODUCTS BY GRADIENT ELUTION CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Centrifugal Partition Chromatography (CPC) was applied, for the first time, to the preparative scale separation of anthocyanins. The two major anthocyanins from Champagne vintage by-products, malvidin 3-glucoside and peonidin 3-glucoside, were isolated in one step, using gradient elution with the TFA-acidified ternary solvent system ethyl acetate / 1-butanol / water.

INTRODUCTION

Anthocyanins are natural water-soluble pigments which are responsible for the pink, orange, red, purple, blue... colors of flowers, fruits and vegetables [1-2]. They

are used in a complex mixture form as natural colorants and for their P-vitamin properties in food and pharmaceutical industries [3]. They have a neutralizing effect against toxic free radicals and, therefore, have a protective activity towards collagen [4].

Since the elaboration of Champagne white wines requires mostly red grapes, anthocyanins need to be expelled from wine making; within the context of agroresource valorization of Champagne-Ardenne region, it appeared interesting to further purify these anthocyanins present in vintage by- products.

Anthocyanins are polar and fragile heterosides which belong to the class of flavonoids. In aqueous media, these polyphenolic compounds lead to several resonance forms which are pH-dependent. The most stable form is the flavylium cation predominant under acid conditions (pH 1-3). Separation and isolation techniques must be carried out in mild conditions and in acidic media [2], and the main chromatographic methods to isolate these compounds [5] are paper chromatography, ion exchange chromatography on resins or Sephadex, reversed phase HPLC, and droplet counter current chromatography (DCCC) [6]. Irreversible adsorption and degradation of these polar molecules are often encountered on solid sorbents, while DCCC, which does not have these disadvantages, turns out to be time consuming and restricted in the choice of solvents .

It seemed interesting to apply, for the first time, the centrifugal partition chromatography (CPC) for quantitative purification of anthocyanosides.

We report, in this paper, our results on isolation of two major anthocyanins, malvidin 3-glucoside and peonidin 3-glucoside, from by-products of Champagne vintage (Pinot noir wine plants).

MATERIAL AND METHODS

Extraction

The by-products from Champagne vintage (1.015 kg), which contain wetted red grape skins, seeds and stalks, were collected in October, 1993, and macerated during 5

days in MeOH-AcOH (99:1 , 1.45 L). After filtration the aqueous methanolic solution was concentrated to 0.25 L under reduced pressure at 28°C. This solution was then extracted with 1-BuOH (4 x 0.2 L) saturated with water. The organic solvent was evaporated *in vacuum* at 65°C and the residue was lyophilized to give the anthocyanic extract (14.29 g; yield = 1.4 %).

Fractionation by CPC

A Series 1000 HPCPC (Sanki Eng. Limited, Nagaokakyo, Kyoto, Japan) was used [7]. It is a bench top CPC (30 x 45 x 45 cm, ≈ 60 kg); the column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 240 mL. The column is connected to the injector and the detector through two high pressure rotary seals. A 4-port valve included in the series 1000 allows the HPCPC to be operated in either the descending or ascending mode. The HPCPC was connected to a solvent delivery pump Techlab economy 2/ED (Techlab, D-38173 Erkerode, Germany), supplied with solvents through a gradient generator ISCO model 2360 with preparative options (ISCO, Inc., Lincoln. NB, USA).

Detection was performed with a UV/visible detector ISCO type V^4, set at 540 nm. Fractions were collected with a collector model Superfrac manufactured by Pharmacia (Pharmacia, Uppsala, Sweden). Sample injections were carried out by a Rheodyne injection valve type 7125 (Altech Associates, Inc., Deerfield, Illinois USA) through a 5 mL sample loop.

The solvent system ethyl acetate / 1-butanol / water (EtOAc/1-BuOH/H_2O) has been used for this separation. Figure 1 shows the ternary diagram corresponding to this solvent system.

The initial mobile phase was EtOAc/1-BuOH/H_2O 77/15/8 (V/V/V) (Point I, Fig 1), the final mobile phase was EtOAc/1-BuOH/H_2O 40/46/14 (V/V/V) (Point F, Fig 1) and the stationary phase was water saturated with EtOAc and 1-BuOH; all phases were acidified with trifluoroacetic acid (0.8%) . The gradient was linear with a duration of 3 hours, the flow rate being 3 ml/min in the ascending mode and the rotational speed varied between 1300 rpm and 1500 rpm. With these conditions, the volume of the

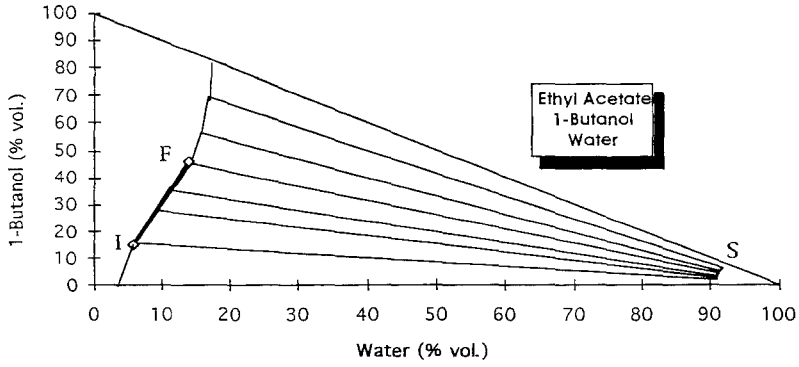

Figure 1 Ternay system ethyl acetate / 1-butanol / water
Composition of the liquid phases used to generate a gradient of butanol in ethyl acetate on a water-rich liquid stationary phase : I : initial mobile phase
F : final mobile phase
S : stationary phase

mobile phase in the CPC "column" was around 78 mL, corresponding to a retention of the stationary phase of 67%, and the pressure drop was 35 to 47 bars.

Identification of anthocyanins

Composition of the fractions was determined by TLC on cellulose F plates (Merck, Darmstadt, Germany), using a mixture of 1-BuOH/AcOH/H_2O (61:10.5: 28.5) as the mobile phase. The pure isolated products were compared by TLC with authentic samples of malvidin-3-glucoside and peonidin-3-glucoside (Extrasynthese, Genay, France). They were also analyzed by ^1H NMR spectroscopy in CD_3OD acidified with 0.3% of TFA. Spectra were recorded at 300 MHz on a Bruker AC 300 spectrometer (Bruker, Wissemburg, France) and compared with spectra obtained from commercial samples and with data found in the literature [8].

Reagents

All chemicals were analytical grade (SDS, 13124 Peypin, France)

RESULTS AND DISCUSSION

Considering the diversity of the compounds present in our extract, gradient elution turned out to be necessary to isolate anthocyanins. This method requires a biphasic ternary system, the ternary diagram of which possesses converging tie-lines. Because of the glycosidic structures of anthocyanins, we selected the biphasic solvent system EtOAc/1-BuOH/H_2O, which we used already in our laboratory for isolation of ginsenosides from *Panax quinquefolium* L. [9]. With a similar ternary system (EtOAc/2-BuOH/H_2O), Vanhaelen *et al.* obtained good fractionation of flavonoids from a commercial extract of *Ginkgo biloba* leaves [10]. Our system looks very favorable to run gradients in the normal phase mode, using the polar water rich phase as the stationary phase, and varying the polarity of the mobile phase by changing the ratio of 1-BuOH to EtOAc, while the composition of the stationary phase remains relatively constant, *i.e.* water saturated in EtOAc and 1-BuOH. [11].

Partition tests of our anthocyanic extracts in systems EtOAc/1-BuOH/H_2O of various compositions directed us to start our gradient with more polar conditions than those previously used to purify the ginsenosides from *Panax quinquefolium* L. [9]; we also acidified all phases, in order to preserve the anthocyanin stability.

Figure 2 shows the CPC chromatogram, recorded at 540 nm, obtained after injection of 600 mg of the anthocyanin extract (in 5 mL of stationary phase). 100 x 9 mL fractions were collected and further analyzed by TLC. Identical fractions 54 to 68 were pooled, evaporated to dryness, and then analyzed by ^1H NMR : they contained 15 mg (2.5 % of the extract) of pure peonidin 3-glucoside. Similarly, we found that fractions 71 to 89 yielded 20 mg (3.33% of the extract) of pure malvidin 3-glucoside. The intermediate fractions (69-70, 2 mg, 0.33%) contained a mixture of these two compounds. Thus, we obtained 35 mg of pure anthocyanins, which corresponds to 0.81 % of the starting material (grape skins, seeds and stalks). The remains (563 mg) was a mixture of minor anthocyanins, and other compounds such as sugars, flavonoids and tannins.

The observed elution sequence is in conformity with the normal phase elution mode which was used. Peonidin 3-glucoside, which does not have a methoxyl group in 3' position (Fig 2) is less polar than malvidin 3-glucoside, and is eluted first. The same order of elution is also observed on TLC plates.

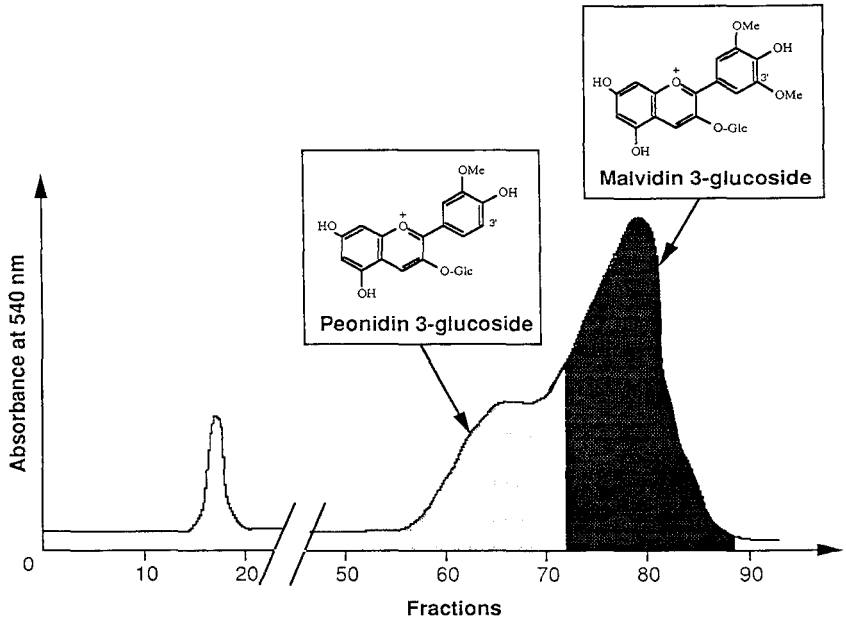

Figure 2 CPC chromatogram of anthocyanins from Champagne vintage by-products. gradient elution with the ethyl acetate / 1-butanol / water system; stationary phase : water saturated with ethyl acetate and butanol (S on Fig.1);all phases were acidified with 0.8% of trifluoroacetic acid.
Gradient duration : 3 hours.
Ascending mode, flow rate : 3 mL/min.
Rotational speed : 1300-1500 rpm; back pressure : 35 to 47 bars.
Volume of the mobile phase in the HPCPC : 78 mL.
Detection UV/Vis at 540 nm.
Sample : 650 mg of a lyophilized extract of anthocyanins in 5 mL of stationary phase.

CONCLUSION

Centrifugal Partition Chromatography is an effective tool for the separation and purification of biologically active polar and fragile molecules, extracted from plants. These kind of compounds can hardly be isolated by conventional column chromatography because of strong adsorption and decomposition. Moreover, the introduction of certain innovations like the use of mobile phase gradient offers more

possibilities. The two major anthocyanins from vintage by products have been isolated, in a pure state and with good yields. As anthocyanins are generally used in a complex mixture form in food and pharmaceutical industries, it seems interesting to get pure products in largest quantities. In this perspective we think of transposing our method on an apparatus with a larger capacity.

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TLC SEPARATION OF TRANSITION METAL IONS AND THEIR QUANTITATIVE ESTIMATION BY ATOMIC ABSORPTION SPECTROSCOPY

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ABSTRACT

Separation of an eight component mixture of transition metal ions has been studied on plain and impregnated silica gel layers. The solvent systems used were pyridine-benzene-acetic acid-water (6:5:8:4,v/v) on 1,10-phenanthroline (1%), n-butanol-benzene-formic acid (5:10:9,v/v) on DMG (1%) and pyridine-benzene-acetic acid-water (5:5:4:1,v/v) on EDTA (2%) impregnated silica gel layers. ß-naphthol (0.1% in methanol) has been developed as a new locating reagent. The percentage recovery of these metal ions in quantitative estimation by AAS ranged between 65.2 to 89.9.

INTRODUCTION

Separation and identification of transition metal ions is of great importance in chemical, biochemical,

biological, industrial and environmental sciences. The transition metal ions are found in various alloys in solid state and in the form of solution in various parts of the body and a cross section of these metals exists as free ions and their complexes in the blood stream. Various transition metal ions are toxic and produce various types of diseases in the body (1,2). TLC has long been used for the separation and identification of metal ions on the various adsorbent layers (3). Impregnation of TLC layers has been used for the better and reproducible separation of various compounds (4-8) but there are only few reports available on the separation of transition metal ions on impregnated layers (3). In view of the importance of metal ions and practical utility of impregnated TLC plates, attempts were made to separate and identify Cr(III), Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) metal ions on different impregnated silica gel lagers using three new solvent systems. The present paper describes the TLC separation and quantitative estimation by atomic absorbtion

spectroscopy of these metal ions. In addition to this, ß-napthol (0.1% in methanol) was developed as a new locating reagent for these metal ions.

EXPERIMENTAL

The metal salts were obtained from E. Merck, India,. Silica gel G (size, 10-40 μ) with calcium sulphate (13%) as binder, impurities of chloride, iron and lead (0.02% each), and showing a pH 7.0 in a 10% aq. suspension, was from E. Merck India. All other reagents and solvents used were of A.R. grade and purchased from E. Merck, India. Perkin Elmer (U.S.A.) Atomic Absorbtion Spectrophotometer model 3100 was used.

Preparation of TLC plates and solution of metal ions:

TLC plates (20cm x 20cm x0.5mm) coated with plain slica gel G were prepared by spreading a slurry of silica gel G (50g) in double distilled water (100 mL) with the help of Stahl type applicator. Impregnated silica gel layers were prepared by spreading a slurry of silica gel G (50 g) in 70%

methanol (100 mL), containing 1,10-phenanthroline (1%), DMG (1%) and EDTA (2%) respectively. The plates were dried overnight at 60±2^{0}C in an oven. The solutions of different metal salts (10^{-3} M) were prepared in double distilled water.

Development of Chromatograms:

The solutions of metal ions were applied on plain and impregnated silica gel plates at 50 ng level using a 25 μL Hamilton syringe. The chromatograms were developed upto 10 cm at 25 ± 2^{0}C in pyridine-benzene-acetic acid-water (6:5:8:;4,v/v), n-butanol-banzene-formic acid (5:10:9,v/v) and pyridine-benzene-acetic acid-water (5:5:4:1,v/v) solvent systens in paper lined rectangular glass chambers which were pre-equilibrated with the solvent systems for 10-20 minutes. The plates were then dried in an oven at 60 ± 2^{0}C for 30 minutes. The plates were sprayed with ß-napthol (0.1% in methanol) and further heated for 20 minutes. The spots of these metal ions were located as redish-yellow in color.

Quantitative Estimation by AAS:

The standard solutions of each metal ions (1 ppm to 4 ppm) were prepared in double distilled water for the calibration of atomic absorption spectrophotometer. The studied metal ions separated in TLC were erased along with silica gel G from the plates. These metal ions were extracted from silica gel G in double distilled water (10 mL) individually and separately. The concentration of each metal ion was determined by atomic absorbtion spectrophotometer.

RESULTS AND DISCUSSION

The hR_f values of metal ions on plain and various impregnated silica gel layers are given in table-1. The results are an average of atleast three identical runs with a standard deviation of ± 0.40 to ± 0.50 on plain and impregnated layers respectively. The resolution of these metal ions was calculated and confirmed by usual method (4). In order to optimize the separation conditions, variation in the concentrations of impregnating reagents and in the

TABLE 1

hR_f Values of Transition Metal Ions on Plain and Impregnated Silica Gel Layers

Sl. No.	Metal Ion	A			B	C	D
		I	II	III	I	II	III
1	Cr (III)	38	88	13	24	37	43
2	Mn (II)	54T	82T	41T	28	34	57
3	Fe (II)	47	77	20	37	30	50
4	Co (II)	90	66	83	57	68	21
5	Ni (II)	50T	54T	67T	61	53	28
6	Cu (II)	47T	60T	56	68	47	47
7	Zn (II)	50T	64T	58T	33	56	24
8	Cd (II)	54	75	61	43	64	38

T : Tailing

A : Plain silica gel layers, B: 1,10-phenanthroline (1%) impregnated silica gel layer, C: DMG (1%) impregnated silica gel layer, and D: EDTA (2%) silica gel layer.

Solvent systems :

I : Pyridine-benzene-acetic acid-water (6:5:8:4,v/v)
II : n-Butanol-benzene formic acid (5:10:9,v/v)
III: Pyridine-benzene-acetic acid-water (5:5:4:1,v/v)

Room temperature : 25 ± 2°C.
Solvent Front : 10 cm.
Detection : ß-Napthol (0.1% in methanol).

composition of solvent systems was carried out. As a result of extensive experimentation the best solvent systems and impregnating reagents were selected and reported herewith.

It is clear from table-1 that only four to five metal ions got separated from an eight component mixture, with broad spots, on plain silica gel plates. Contrary to this, all the eight metal ions got separated on impregnated silica gel layers with compact spots. It is a well known fact that the impregnating reagent selected e.g, 1,10-phenanthroline, DMG and EDTA are very good chelating agents and have a very good tendency to form chelates with transition metal ions immediately. Therefore, the transition metal ions should form chelates with the reported impregnating reagents on impregnated silica gel layers and got separated, due to the different migration behaviour of these chelates on the impregnated plates than on the plain plates. Thus it must be the combined effect of partition and adsorption phenomenon of these chelates which should

be responsible for the resolution of these metal ions. This fact was verified by preparing the chelates of these metal ions with the reported impregnating reagents and separating them on plain silica gel plates using the same solvent systems. The results were in good agreement with those on impregnated silica gel layers.

The results of the quantitative estimation of these metal ions on impregnated TLC plate are given in table-2. It is clear from table-2 that percentage recovery of these metal ions varies between 62.2 to 89.9. It is interesting to note that none of the metal ion was hundred percent recovered. This fact suggests that some amount of the metal ions diffuse on TLC plates and remains adsorbed on the silica gel layer even after extraction. The difference in the recovery of transition metal ions may be due to the difference in their diffusion and adsorption capacities on silica gel layers. This observation is verified by the fact that the percentage recovery of these metal ions is greater on EDTA impregnated plates

TABLE 2

Quantitative Estimation of Transition Metal Ions on Impregnated Silica Gel Layers by Atomic Absorbtion Spectrophotometer.

Sl. No.	Metal Ion	λmax (nm)	E	B		C		D	
				F	G	F	G	F	G
1	Cr (III)	357.9	10	6.52	65.2	6.62	66.2	6.91	69.1
2	Mn (II)	279.5	10T	7.67	76.7	8.02	80.2	8.35	83.5
3	Fe (II)	248.3	10	8.38	83.8	8.63	86.3	8.99	89.9
4	Co (II)	240.7	10	6.70	67.0	7.02	70.2	7.45	74.5
5	Ni (II)	232.0	10	8.15	81.5	8.67	86.7	8.84	88.4
6	Cu (II)	324.8	10	7.17	71.7	7.67	76.7	7.90	79.0
7	Zn (II)	213.9	10	7.83	78.3	8.01	80.1	8.30	83.0
8	Cd (II)	228.8	10	6.32	63.2	6.92	69.2	7.10	71.0

B : 1, 10-Phenanthroline (1%) impregnated silica gel layer.
C : DMG (1%) impregnated silica gel layer.
D : EDTA (2%) impregnated silica gel layer.
E : Amount of transition metal ions spotted in μg.
F : Amount of transition metal ions recovered in μg.
G : Percentage of transition metal ions recovered .

in comparison to DMG and 1,10-phenanthoraline impregnated plates. EDTA is a stronger chelating ligand than DMG and 1,10-phenanthroline which form chelates with transition metal ions quickly leaving very poor amount for diffusion and adsorption on silica gel layers and therefore gives greater percentage recovery.

Thus, the reported chromatographic system can be considered as sucessful, reliable and reproducible method for the separation and identification of reported transition metal ions and can be used for the separation of these metal ions from unknown samples.

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LIQUID CHROMATOGRAPHY CALENDAR

1995

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 5 - 8: 5th Symposium on Our Environment / 1st Asia-Pacific Workshop on Pesticides, Singapore. Contact: The Secretariat, 5th Symp on our Environment, Chem Dept, National University of Singapore, Kent Ridge, Republic of Singapore 0511.

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.

JULY 23 - 28: 35th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency, Denver, Colorado. Contact: Patricia Sulik, Rocky Mt. Instrum. Labs, 456 S. Link Lane, Ft. Collins, CO 80524, USA.

JUNE 25 - 28: Method Development in HPLC, Virginia Tech, Blacksburg, Virginia. Contact: Dr. H. McNair, Chem Dept, Virginia Tech, Blacksburg, VA 24061-0212, USA

AUGUST 13 - 17: ICFIA'95: International Conference on Flow Injection Analysis, Seattle, Washington. Contact: Prof. G. D. Christian, Dept of Chemistry BG-10, University of Washington, Seattle, WA 98195, USA.

AUGUST 14 - 19: 35th IUPAC Congress, Istanbul, Turkey. Contact: Prof. A. R. Berkem, 35th IUPAC Congress, Halaskargazi Cad. No. 53, D.8, 80230 Istanbul, Turkey.

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

AUGUST 20 - 25: ACS sponsored Symposium on Saponins: Chemistry and Biological Activity, Chicago, Illinois. Contact: G. R. waller, Oklahoma State University, Dept of Chem & Molecular Biology, Stillwater, OK 74078, 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.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

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.

1996

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.

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

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.

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

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

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

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

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

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

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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Utah Biomedical Test Laboratory
520 Wakara Way
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1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18 1979-1998 (1985)

Book:

1. L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley & Sons, Inc., New York, 1979

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

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1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

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