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ANALYSIS OF GENETICALLY MODIFIED CANOLA VARIETIES BY ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRIC AND FLAME IONIZATION DETECTION

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ABSTRACT

Canola oil triacylglycerols from genetically modified canola lines were conclusively identified by reverse phase HPLC coupled with atmospheric pressure chemical ionization mass spectrometric (APCI-MS) detection. APCI-MS is a soft ionization technique, which gave simple spectra for triacylglycerols. Spectral identification of the triacylglycerols was based on the diacylglycerol fragments and on the protonated molecular ion $[M+H]^+$, except trisaturates which gave no $[M+H]^+$. Triacylglycerols were identified and quantitated in normal, high stearic acid and high lauric acid canola varieties by the RP-HPLC/APCI-MS technique. The LC/APCI-MS identification of canola oil triacylglycerols allowed their quantitation by reverse phase HPLC coupled with a commercial flame ionization detector (FID). There was agreement between fatty acid composition obtained by LC/APCI-MS and LC-FID. However, the triacylglycerol resolution obtained by LC/APCI-MS, was superior to LC-FID in the qualitative identification of triacylglycerols present in amounts even below one percent. The oils of the modified canola varieties, compared to typical canola oil, contained increased content of triacylglycerols known to be more

oxidatively stable like stearoyloleoyllinoleoyl, distearoyllinoleoyl, stearoyldilinoleoyl and distearoyloleoyl glycerols in high stearic acid canola oil and dilauroyllinoleoyl, dilauroyloleoyl and lauroyldilinoleoyl glycerols in high lauric acid canola oil. These oils contained fewer linolenate-containing triacylglycerols known to decrease oxidative stability. The LC/APCI-MS technique gave better resolution of, and quantitation of triacylglycerols in the canola oils, than the LC/FID. However, the LC/FID system gave satisfactory analyses suitable for many research programs, like the development of genetically modified canola varieties with oils of improved oxidative stability.

INTRODUCTION

Recently, some research has been directed toward the improvement, through plant genetic manipulation, of the functional properties of vegetable oils (VGO) for food uses (i.e. frying oils, salad oils, structured fats for margarine basestocks, confectionary products, baking shortenings) by altering the fatty acid (FA) composition and the triacylglycerol (TAG) composition.[1-9] Some of the plants, which are being modified by genetic manipulation, include soybean, canola, sunflower and flax. Other research has been directed toward the correlation of oxidative stability of VGO with TAG composition.[10-12] Understanding VGO oxidative stability is important to develop methods for protection of VGO shelf life and nutritional safety.[13,14] Therefore, it is important for these studies of VGO properties to have a facile method for identification and quantitation of TAG in the complex oil matrix.

Previously, for our VGO oxidative stabiltiy studies of soybean and high oleic acid canola oils from new plant varieties, we used reverse phase high performance liquid chromatography (RP-HPLC) coupled with flame ionization detection (FID) for qualitative and quantitative analysis of the oil TAG.[10,12] The TAG were tentatively identified by correlation of their theoretical carbon number with HPLC retention time.[15] The accuracy of the TAG identification and quantitation were supported by good agreement between FA composition, calculated from TAG composition, obtained by RP-HPLC-FID and experimental FA composition obtained by gas chromatography with FID of the methyl esters of the transmethylated oils.[16]

Recently, our oxidative stability studies have focused on the TAG mixtures obtained from high stearic (16-32 % stearic acid) and lauric (11-32 % lauric acid) canola oils from new genetically modified canola varieties. These oils contained many previously unidentified TAG, some of which coeluted by RP-HPLC and therefore, were not amenable to analysis by our RP-HPLC-FID technique.

Obviously, mass spectrometry, which has the capability of selective ion monitoring, would appear useful for RP-HPLC-MS analysis of co-eluting TAG. However, many MS techniques for TAG require collection of TAG HPLC fractions or, because of the interface between the HPLC column and the MS, have the potential for thermal degradation of the TAG or poor sensitivity for detection of minor TAG.[17] Furthermore, some MS ionization techniques, like electron impact ionization, produce complex spectra for TAG. Electrospray ionization (ESI) mass spectrometric analysis provides a soft ionization method for production of simple spectra with molecular ion information for polar complex molecules.[18] However, for non-polar compounds like TAG, the addition of ionic modifiers to the TAG solvent is required for ESI-MS analysis.[18,19] Moreover, ESI spectra show only molecular ions with no fragmentation into diacylglycerols (DAG), which are required for identification of TAG with identical molecular weights.[19]

We have recently developed satisfactory analytical methodology for qualitative and quantitative analysis of TAG by RP-HPLC, coupled with a quadrupole MS equipped with an atmospheric pressure chemical ionization interface.[17,19,20] The APCI interface, a soft ionization technique, proved suitable for non-polar compounds like the TAG. The resultant simple spectra showed only protonated TAG molecular ion $[M+H]^+$ and the required DAG fragments in sufficient abundance to conclusively identify even minor TAG.

We report here an analytical study, using RP-HPLC/APCI-MS, for qualitative and quantitative analysis of TAG in the complex TAG mixtures from high lauric and high stearic acid containing canola oils (CNO) with widely varying fatty acid composition. The TAG composition and resultant FA composition obtained by RP-HPLC/APCI-MS were compared to the results obtained by LC-FID. The FA compositions calculated from the TAG compositions were compared to the FA composition experimentally determined using GC-FID.

MATERIALS

The seeds from experimental high lauric and high stearic canola varieties were obtained from Calgene Inc. (Davis, CA). Solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or EM Science (Gibbstown, NJ). Solvents were HPLC grade or the highest available quality and were used without further purification. The crude oils (4.5-6.0 g) were obtained by extraction of 15 g canola seeds in duplicate using a previously described sonification-hexane extraction procedure.[12] Solid-phase extraction for purification of the crude CNO TAG to avoid interference by non-TAG components during RP-HPLC/APCI-MS, was performed by a reported procedure to give 0.9-1.0 g chromatographed oil per 1.2 g crude CNO.[12]

METHODS

Liquid Chromatography (APCI-MS)

The HPLC pump was an LDC 4100 MS (Thermo Separation Products, Shaumburg, IL) quaternary pump system with membrane degasser. The columns used were an Adsorbosphere C_{18} (Alltech Associates, Deerfield, IL), 25 cm x 4.6 mm, 5 µm (12 % carbon load) in series with an Adsorbosphere UHS C_{18} 25 cm x 4.6 mm, 10 µm (30 % carbon load). The flow rate throughout was 1. mL/min. A gradient solvent program with propionitrile (PrCN), dichloromethane (DCM) and acetonitrile (ACN) was used to separate the canola oil TGs. The gradient used was as follows: initial - PrCN/DCM/ACN (45:20:35, v/v/v); linear from 15 to 20 min. to PrCN/DCM/ACN (45:25:30, v/v/v), held until 35 min.; linear from 35 to 40 min. to PrCN/DCM/ACN (45:30:25, v/v/v), held until 95 min. This combination was used to reduce the undesirably high amount of PrCN adducts formed by this solvent in the absence of other solvents.

The quantitative analysis by RP-HPLC with flame ionization detection, used a linear gradient of ACN/DCM 70:30 to 40:60 v/v over 120 minutes. Columns and conditions were as previously described.[21]

The evaporative light scattering detector (ELSD) was an ELSD MKIII (Varex, Burtonsville, MD). The drift tube was set at 140 °C, the gas flow was 2.0 standard liters per minute. High purity N_2 was used as the nebulizer gas.

Mass Spectrometry

A Finnigan MAT (San Jose, CA) SSQ 710C mass spectrometer fitted with an atmospheric pressure chemical ionization source, was used to acquire mass spectral data. LC column eluent was split so that 600 µL/min was diverted to the APCI inlet, while 400 µL/min went to an evaporative light scattering detector. The APCI vaporizer was operated at 400 °C and the capillary heater was operated at 265 °C. The corona voltage was set at 6.0 µA throughout. High purity nitrogen was used for the sheath and auxillary gases, which were set to 55 psi and 5 mL/min respectively. Spectra were obtained from 400 amu to 1000 amu or 1100 amu with a scan time of 1.75 to 2.0 sec. Chromatograms were processed using five-point smoothing for graphical output, but no smoothing was applied during quantitation of extracted ion chromatograms.

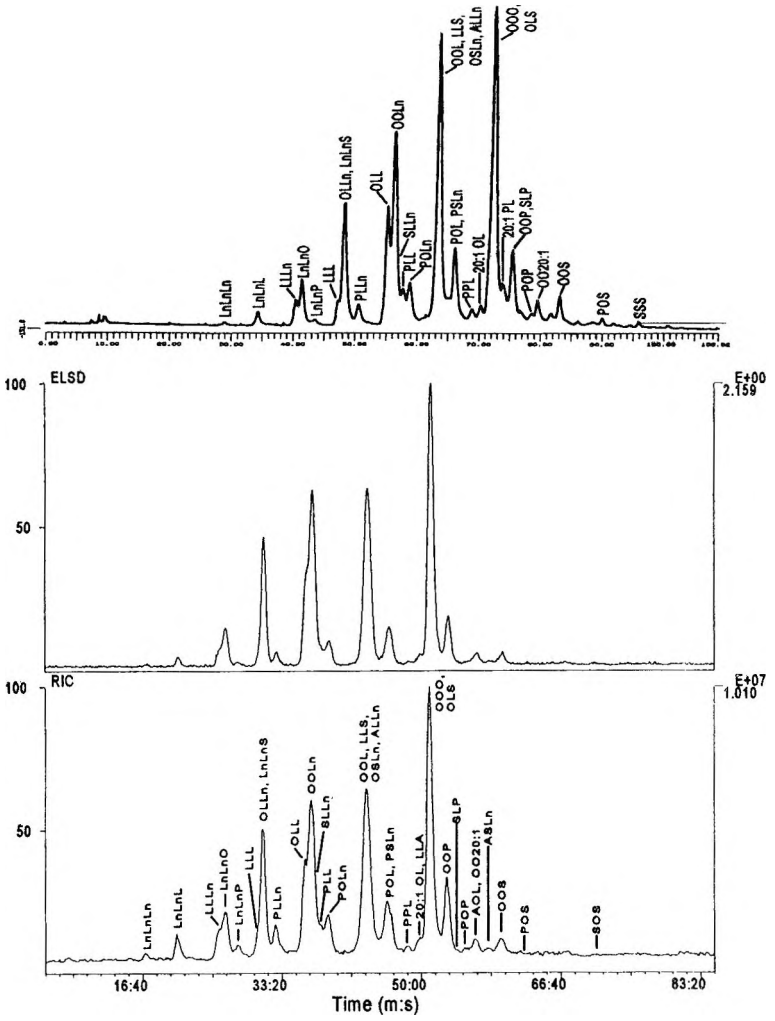

Figure 1. Normal canola oil: reverse phase high performance liquid chromatography/flame ionization detection chromatogram (top), reverse phase high performance liquid chromatography/evaporative light scattering detector chromatogram (middle), reverse phase high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometric chromatogram (bottom). Liquid chromatographic conditions in text. Abbreviations: Ln=linolenic; L=linoleic; O=oleic; S=stearic; P=palmitic; A=arachidic.

Gas Chromatography

Canola oil TAG were transmethylated according to the method of Glass[22] by reaction with 3 mL of 0.5 N potassium hydroxide in methanol at 50 °C for 15 min. Fatty acid methyl esters (FAMEs) were analyzed using a Varian 3400 (Palo Alto, CA) gas chromatograph equipped with a Supelco (Belefonte Park, PA) SP2380 30 m x 0.25 mm i.d. capillary column. The conditions were: inlet temperature = 240 °C; detector temperature = 280 °C; initial column temperature = 150 °C; initial time = 35 minutes; column temperature increased to 210 °C at 3 °C/min.

RESULTS

Normal Canola Oil

The RP-HPLC/APCI-MS reconstructed ion chromatogram, ELSD chromatogram and RP-HPLC/FID chromatogram are shown in Figure 1. Identification of components was accomplished using the LC/ACPI-MS spectral data. Figure 1 illustrates the chromatographic overlaps which occurred among many of the major TAG components. The LC-FID chromatogram did not have sufficient resolution to allow quantitation of all of the components identified by APCI-MS. The LC/APCI-MS data did allow the resolution of most overlaps by the use of extracted ion chromatograms (EICs), such as those shown in Figure 2. Numerous other TAG species were identified in the canola oil than are labeled in Figure 1, because of space limitations. Many TAG containing gadoleic (20:1) and arachidic (20:0) acids were qualitatively identified but not quantitated.

Areas under peaks associated with diglyceride and protonated triglyceride ions from each triglyceride component were obtained by integration of the extracted ion chromatograms. The integrated areas under these diglyceride and protonated triglyceride peaks were added together to obtain the total integrated areas for each TAG component. A percent composition was calculated from the total area for each TAG, divided by the total area obtained for all TAG components. In the cases where unresolvable DAG fragment overlaps occurred, the areas of the overlapped peaks were apportioned according to their statistically predicted percent composition.

The TAG percent composition obtained from the LC/APCI-MS integrated areas is given in Table 1, column 2. The percent composition obtained from integration of the LC/FID chromatogram is given in Table 1, column 4.

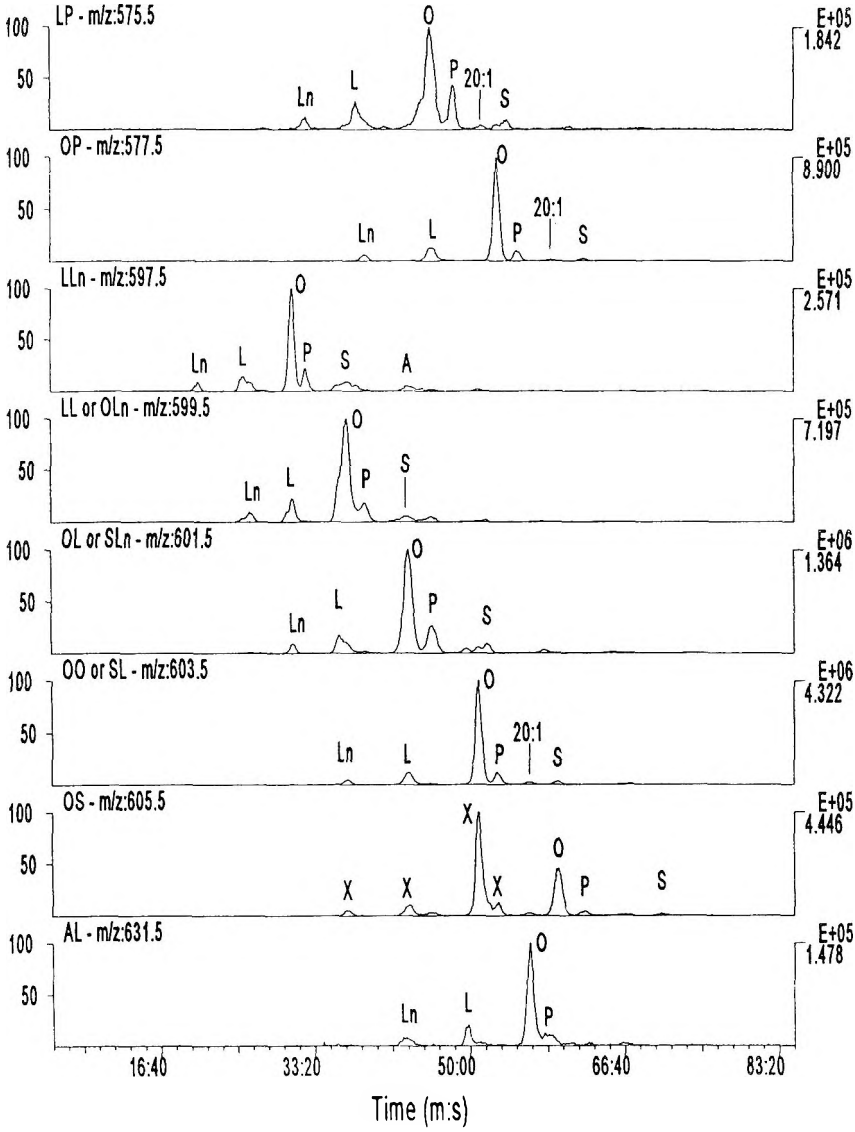

Figure 2. Normal canola oil: extracted ion chromatograms of diacylglycerol fragments. Diacylglycerol identity and mass indicated on the left in each frame. Chromatograms are labeled with the fatty acid which, combined with the diacylglycerol, make up triacylglycerol species. Abbreviations: X=carbon13 isotopic peak; fatty acids as in Figure 1.

Table 1

Normal Canola Oil TAG Composition by LC/MS and LC/FID

TAG	Raw LC/MS	GC-ADJ. %	LC-FID
OOO	22.0	23.7	23.3
OOL	17.1	18.5	19.3
OOLn	12.1	14.1	16.2
LnLO	8.5	9.9	9.0
LLO	7.1	7.9	4.5
LnLnO	3.5	4.3	3.7
OOP	6.7	4.1	4.8
PLO	5.6	3.6	5.2
PLnO	3.5	2.4	3.1
LLLn	1.8	2.1	0.9
OOS	2.0	1.6	1.1
PLnL	1.8	1.3	1.3
LOS	1.4	1.2	1.5
LLP	1.0	0.7	1.4
LLL	0.6	0.6	0.7
LnOS	0.7	0.6	
LnLnP	0.7	0.5	0.4
PoPoLn	0.1	0.3	
LnLS	0.4	0.3	
PLS	0.5	0.2	0.2
PPO	0.5	0.2	
PPL	0.5	0.2	0.2
PoPO	0.1	0.2	
LnLnL	0.1	0.2	0.9
POS	0.4	0.2	0.1
PoLnL	0.1	0.1	
LnLnS	0.1	0.1	
LLS	0.1	0.1	
PLnS	0.2	0.1	
PPLn	0.2	0.1	
SSL	0.1	0.1	
SSO	0.1	0.1	0.1
SSLn	0.1	0.1	
PoPL		0.1	
LnLnLn	0.0		0.2
PPP	0.1		1.0

Table 1 (continued)

Normal Canola Oil TAG Composition by LC/MS and LC/FID

TAG	Raw LC/MS	GC-ADJ. %	LC-FID
PPS			0.3
SSS			0.1
SSP			0.2
Sum	100.0	100.0	100.0

Table 2

Normal Canola Oil Fatty Acid Composition

FA	Raw LC/MS %	Adj. LC/MS %	LC-FID %	GC-FID %
Po	0.1	0.4	0.0	0.3
P	7.8	4.8	7.0	5.1
Ln	12.8	14.0	13.8	14.3
L	19.3	19.6	17.8	19.9
O	57.7	59.5	60.1	58.7
S	2.2	1.6	1.4	1.6
Sum	100.0	100.0	100.0	100.0
AARE %[1]	26.5	3.8	11.1	0.0

[1] Average absolute relative error = sum of the absolute value of relative error, compared to GC-FID, for each FA divided by the number of FA's (n=6).

Fatty acid compositions were calculated from the TAG compositions determined by LC-FID and by LC/APCI-MS. These are given in columns 2 and 4, respectively, of Table 2. The fatty acid composition of the CNO was determined as the fatty acid methyl esters by gas chromatography with flame ionization detection (GC-FID). The fatty acid composition obtained by GC with FID detection, represented a weight percent so, each FA weight percent, was divided by the molecular weight of each FAME to determine a molar percent composition, given in Table 2, column 5. To improve the agreement of the FA composition calculated from LC/APCI-MS data with the FA composition obtained by GC-FID, response factors were calculated for each FA, from the ratio of the FA percent by LC/APCI-MS, to the FA percent by GC-FID. The

response factors obtained for each FA were then multiplied together to produce response factors for each TAG. The FA response factors (normalized to the smallest) which were used to calculate the TAG response factors were: Po = 3.60190; P = 1.00000; Ln = 1.83201; L = 1.70928; O = 1.69596; S = 1.25385. The TAG response factors derived from the GC-FID data, were applied to the uncorrected LC/MS data to produce the TAG percent composition given in Table 1, column 3, referred to as 'GC-Adjusted %'. The FA composition, which resulted from the adjusted LC/MS data, is given in Table 2, column 3. The last row of Table 2 lists the average absolute relative error (AARE) for each result set, compared to GC-FID. The average absolute relative error was obtained by totaling the absolute value of the relative error for each FA and dividing by the number of FA, to give a measure of the average error associated with the FA composition. The AARE of the FA composition, determined by application of GC-FID response factors to the APCI-MS data, was smaller than the error obtained by LC-FID or by uncorrected APCI-MS. Application of the simply-derived response factors to the raw TAG composition, allowed calculation of a corrected TAG composition which had a FA composition which is in excellent agreement with the FA composition determined experimentally.

The results obtained for this study differed somewhat from the TAG and FA compositions reported previously for other canola oils.[12,15]

This was hardly surprising considering the amount of variation in the compositions of the varieties of CNO.

High Lauric Acid Canola Oil

Chromatograms of the separation of TAG in high lauric canola oil, obtained by LC/APCI-MS, ELSD, and FID, are given in Figure 3. The identifications shown in the chromatogram obtained by LC/FID were only possible because of the mass spectral data from the LC/APCI-MS. Standards (chromatographic or quantitative) were not available for the numerous lauric and myristic acid-containing TAG present in this oil sample, so identification by LC-FID alone, based on calculated equivalent carbon number, was not reliable. The extracted ion chromatograms shown in Figure 4, and others not shown, allowed identification of the major and minor TAG components given in Table 3, as well as nearly sixty minor components not listed in Table 3. Many of the minor components (< 0.2%) were included in the percent composition under the label 'Other', while others were identified qualitatively, but not, quantitatively. Identification of a TAG, which was present at a low level, is typified by MMO, present at 0.2%. MMO displayed a distinct oleoyl peak in the EIC, Figure 4, for 'MM' (m/z: 495.4±.5) and a noticeable myristoyl peak in the EIC for 'MO' (m/z:549.5±.5). The peaks in the two EICs occurred at the same time chromatographically, confirming the presence of MMO.

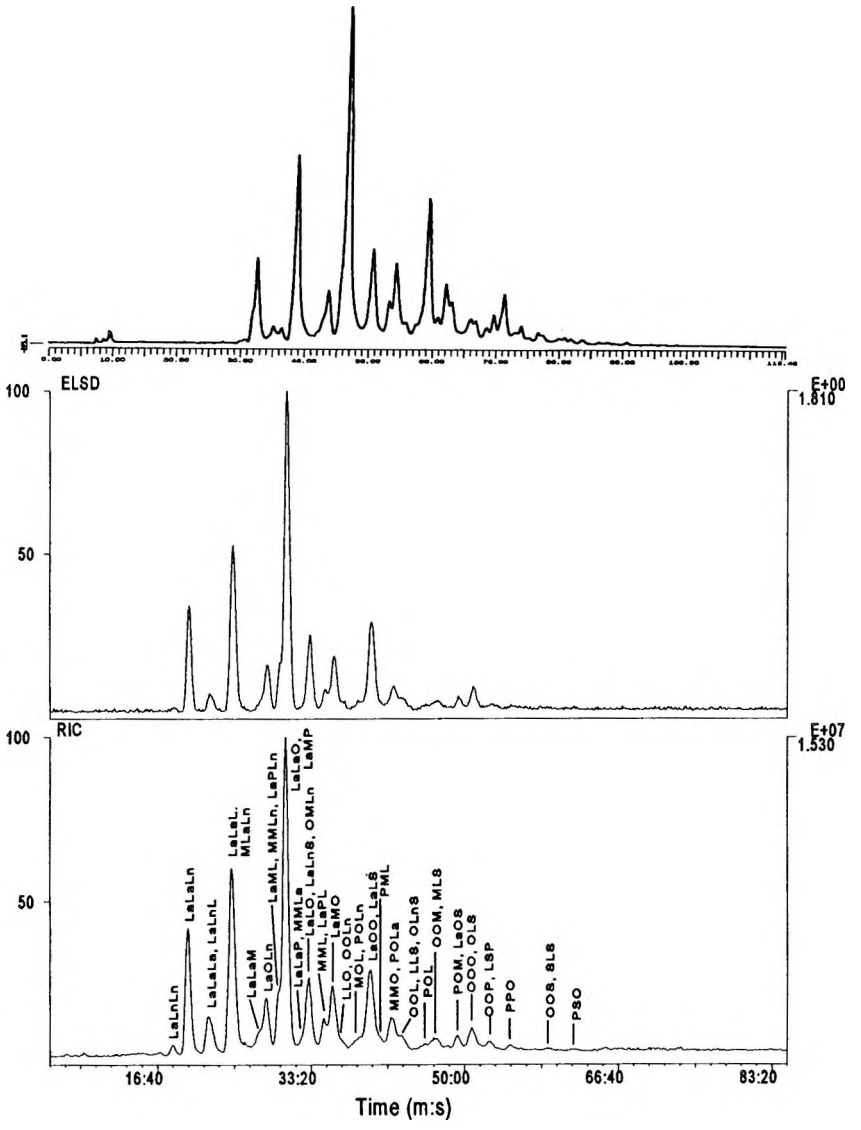

Figure 3. High lauric acid canola oil: reverse phase high performance liquid chromatography/flame ionization detection chromatogram (top), reverse phase high performance liquid chromatography/evaporative light scattering detector chromatogram (middle), reverse phase high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometric chromatogram (bottom). Liquid chromatographic conditions in text. Abbreviations: La=lauric; M=myristic; other fatty acids as in Figure 1.

Table 3

High Lauric Acid TAG Percent Composition

Triglyceride			Raw	Adj.	LC-FID%
La	La	O	25.4	21.2	27.6
La	O	O	8.4	11.2	11.2
La	La	L	15.2	10.8	14.2
La	La	Ln	8.8	7.5	7.4
La	M	O	5.0	5.1	5.6
La	L	O	3.8	4.3	6.6
La	P	O	2.8	3.3	3.6
La	Ln	O	2.4	3.3	4.0
O	O	O	1.5	3.2	3.3
La	M	L	3.1	2.7	2.0
L	O	O	1.2	2.1	2.1
La	P	L	1.9	1.9	
La	L	L	1.7	1.7	
La	M	Ln	1.6	1.6	
La	La	La	2.6	1.4	
La	Ln	L	1.1	1.3	2.3
P	O	O	0.6	1.2	0.7
M	O	O	0.7	1.2	1.3
La	P	Ln	0.9	1.0	
L	L	O	0.6	1.0	
La	O	S	0.7	0.9	1.4
M	L	O	0.6	0.8	
Ln	O	O	0.4	0.8	
P	L	O	0.5	0.8	
L	O	S	0.4	0.6	
La	Ln	Ln	0.4	0.6	0.1
La	O	20:1	0.7	0.6	
La	L	S	0.6	0.6	
M	Ln	O	0.3	0.5	
Ln	L	O	0.3	0.5	
O	O	S	0.2	0.4	
La	La	M	0.6	0.4	
P	Ln	O	0.2	0.3	
M	P	O	0.2	0.3	
M	L	L	0.2	0.3	
La	La	P	0.3	0.2	
M	M	O	0.2	0.2	

Table 3 (continued)

High Lauric Acid TAG Percent Composition

Triglyceride			Raw	Adj.	LC-FID%
P	Ln	L	0.1	0.2	
La	L	20:1	0.3	0.2	
P	P	O	0.1	0.2	
M	Ln	L	0.1	0.2	
M	P	L	0.1	0.2	
P	L	L	0.1	0.2	
	Other		3.3	3.6	6.6
	Sum		100.0	100.0	100.0

Table 4

High Lauric Acid Canola Oil Fatty Acid Composition

FA	Raw LC/MS %	Adj. LC/MS	LC/FID %	GC-FID %
La	48.6	41.9	48.0	38.7
M	4.7	5.0	3.2	4.6
Po	0.2	0.2	0.0	0.2
P	2.8	3.6	1.7	3.2
Ln	6.1	6.8	4.9	7.9
L	11.8	11.5	9.7	12.8
O	24.1	29.4	31.7	30.8
S	1.0	1.2	0.9	1.1
20:1	0.7	0.6	0.0	0.6
A	0.0	0.0	0.0	0.3
Sum	100.0	100.0	100.0	100.0
AARE%	15.3	10.1	20.6	0.0

A peak, at the same retention time in the EIC corresponding to the protonated triglyceride mass, also added support for this identification. Each TAG was identified based on concurrent appearance of peaks in several EICs at the same retention time. The TAG percent compositions obtained by LC/APCI-MS and LC-FID are shown in Table 3. It was clear, that significantly fewer TAG were identified by LC-FID than by LC/APCI-MS. The FA composition

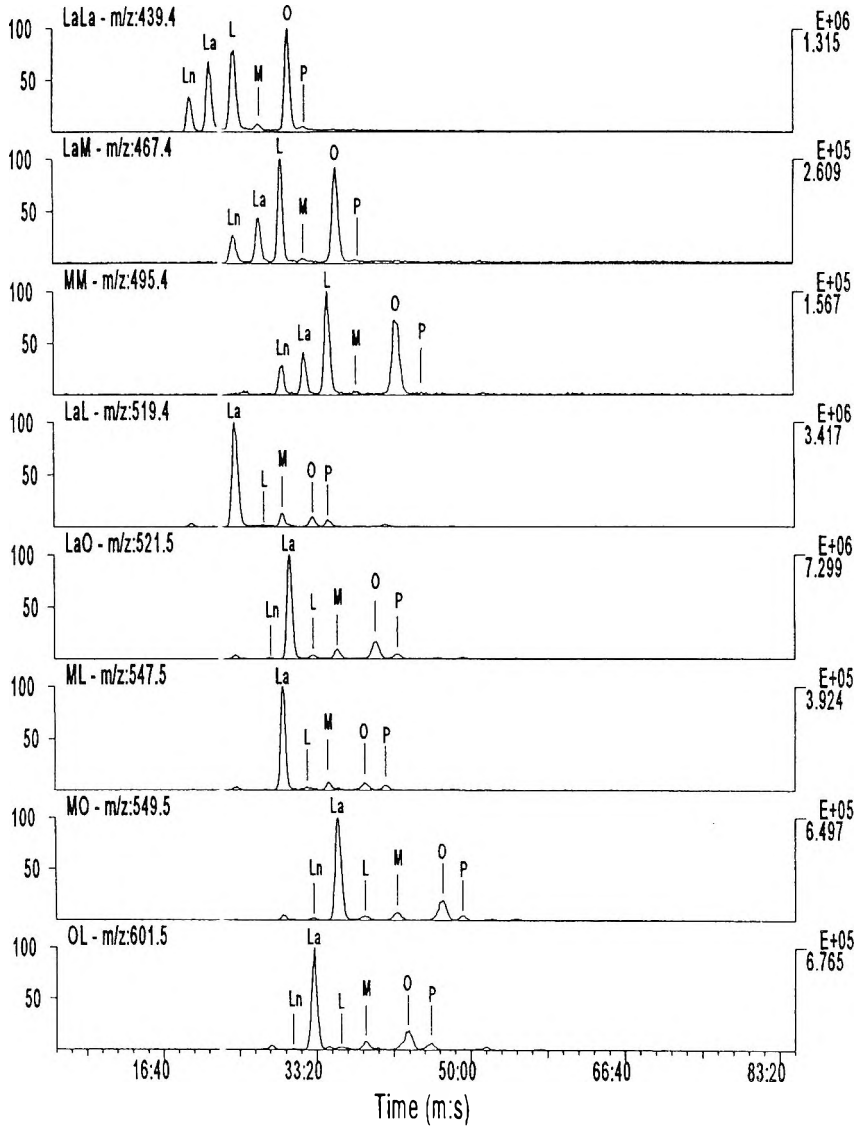

Figure 4. High lauric acid canola oil: extracted ion chromatograms of diacylglycerol fragments. Diacylglycerol identity and mass indicated on the left in each frame. Chromatograms are labeled with the fatty acid which, combined with the diacylglycerol, make up triacylglycerol species. Abbreviations as in Figure 3.

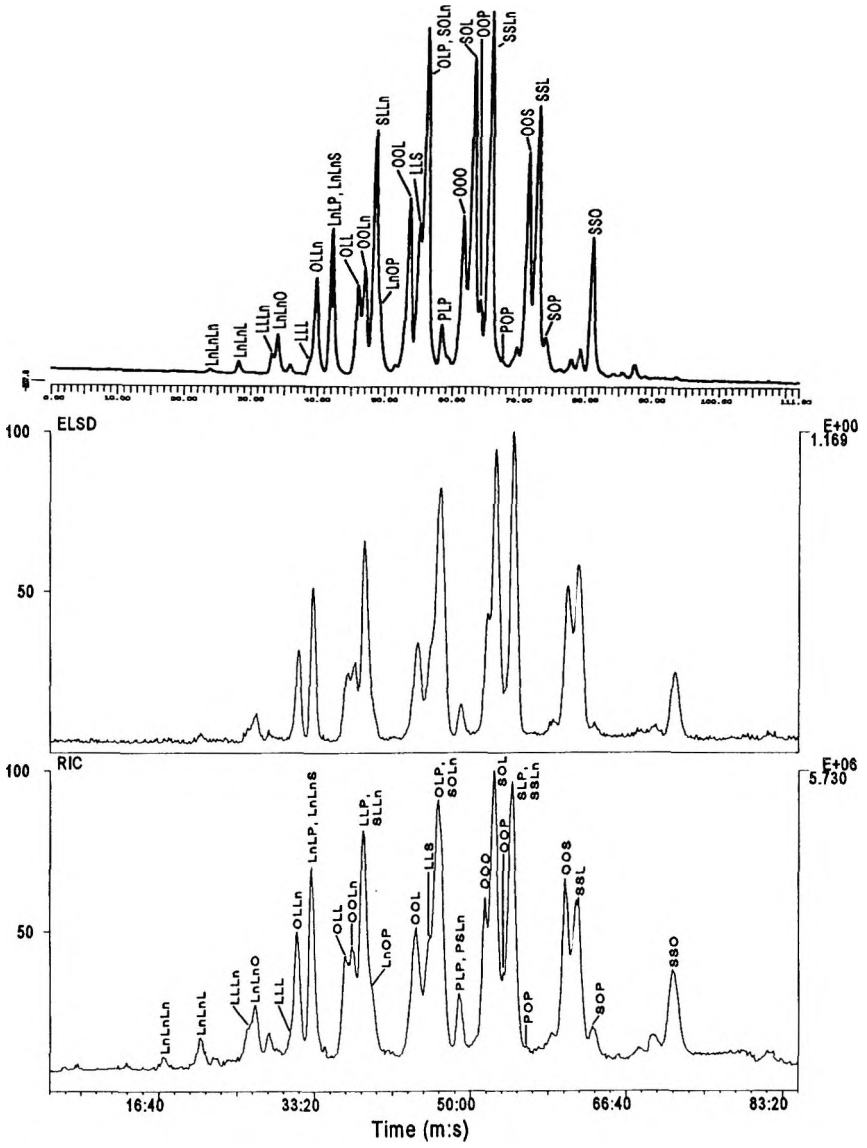

Figure 5. High stearic acid canola oil: reverse phase high performance liquid chromatography/flame ionization detection chromatogram (top), reverse phase high performance liquid chromatography/evaporative light scattering detector chromatogram (middle), reverse phase high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometric chromatogram (bottom). Liquid chromatographic conditions in text. Abbreviations as in Figure 1.

determined by calibrated GC-FID, and the FA compositions calculated from the TAG compositions, are given in Table 4. As with the normal canola oil, response factors were calculated for each TAG from the FA composition determined by GC-FID. The normalized FA response factors, calculated as the ratio of the GC-FID percent to the LC/APCI-MS percent, were: La = 1.00862; M = 1.24213; Po = 1.15609; P = 1.43088; Ln = 1.64047; L = 1.37635; O = 1.61944; S = 1.40003; 20:1 = 1.00000. The TAG composition obtained by application of the TAG response factors derived form the FA response factors is givn in column 3 of Table 3. The FA composition, which resulted from the GC-adjusted TAG composition, is given in column 3 of Table 4. The FA composition, obtained from the GC-adjusted TAG composition, showed good agreement with the FA composition obtained by GC-FID, and the least AARE compared to the FA composition calculated from the raw LC/APCI-MS, or the LC-FID TAG compositions. The GC-adjusted FA composition had a 10.1 % average relative error, compared to the composition determined by GC-FID. The FA composition calculated from the raw LC/APCI-MS TAG composition, showed better agreement (AARE = 15.3%) with the GC-FID results than did the FA composition calculated from the LC-FID TAG composition (AARE=20.6%).

High Stearic Canola Oil

Chromatograms of high stearic acid-containing canola oil obtained by LC/APCI-MS, ELSD and LC-FID are shown in Figure 5. Extracted ion chromatograms for high stearic acid canola oil are shown in Figure 6. When compared to the normal canola oil chromatograms in Figure 1, the chromatograms of the high stearic acid canola oil distinctly show more large peaks eluting at longer retention times. This was expected for TAG containing mono- and unsaturates, in this chromatographic system. Larger peaks for oleic and stearic acid- containing TAG indicated the larger amounts of these TAG present in this canola variety. The TAG composition determined from the LC/APCI-MS and by LC-FID are given in Table 5. Comparison of this composition to that of normal canola oil in Table 1, showed the increased amounts of oleic and stearic acid-containg TAG achieved in this genetically modified variety. Among the most abundant TAG, Table 5 showed larger amounts of TAG which contain the diacylglycerols OS (oleoylsteroylglycerol), OO (dioleoylglycerol or diolein), and SS (distearoylglycerol or distearin) than normal canola. The fatty acid composition obtained by GC-FID is given in Table 6, column 5. The FA compositions calculated from the LC/APCI-MS and LC-FID data are given in columns 2 and 4 of Table 6, respectively. The LC/APCI-MS TAG composition resulted in a FA composition which had 72.5 % average absolute relative error per FA. Most of this average relative error came from two of the FA, Po and 20:1, present at below 0.5 %. Excluding these two FA from calculation of the average relative error, led to an average absolute

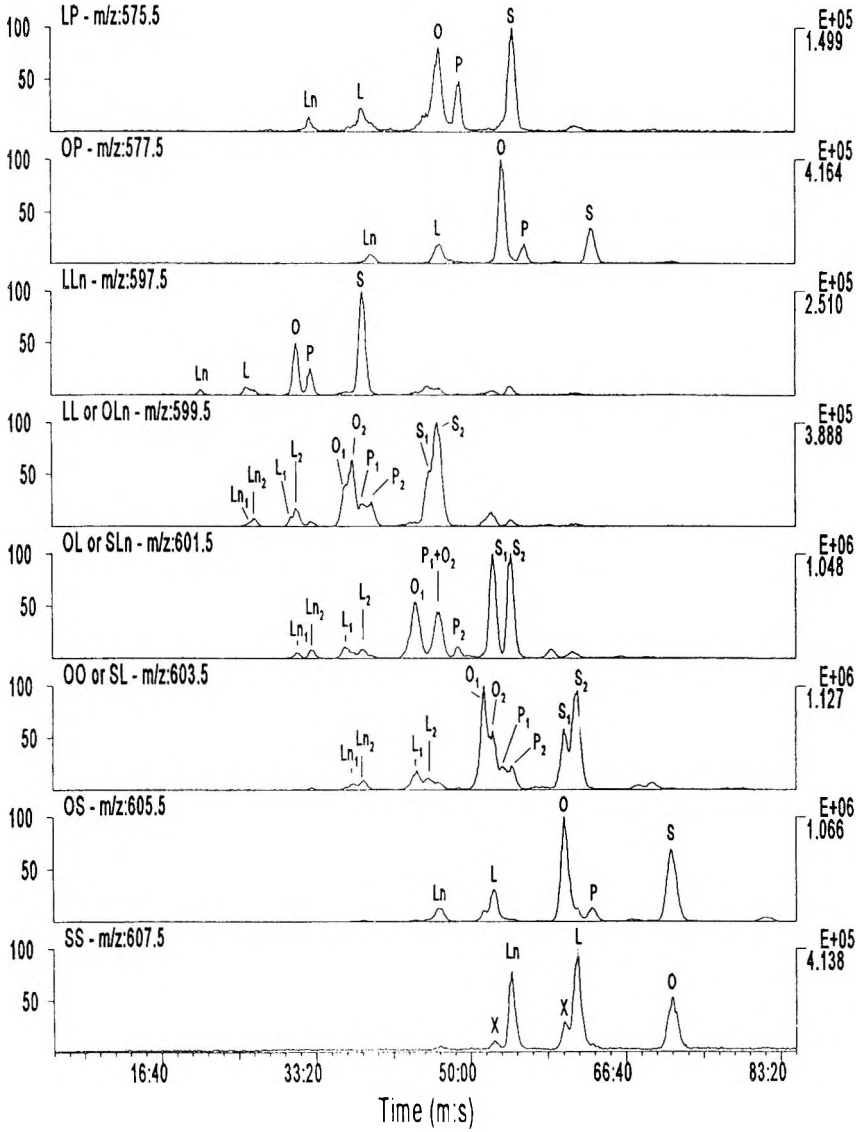

Figure 6. High stearic acid canola oil: extracted ion chromatograms of diacylglycerol fragments. Diacylglycerol identity and mass indicated on the left in each frame. Chromatograms are labeled with the fatty acid which, combined with the diacylglycerol, make up triacylglycerol species. Abbreviations as in Figure 1.

relative error of 11.7 % per FA, much closer to that given by the LC-FID data. The TAG composition determined by LC-FID resulted in a FA composition which had an average relative error of 13.2 % per FA. As with the other canola oils, response factors were calculated for each TAG from the FA composition determined by GC FID. The normalized FA response factors, calculated as the ratio of the GC-FID percent to the LC/APCI-MS percent, were: Po = 1.00000; P = 1.51979; Ln = 2.10244; L = 1.81170; O = 1.70925; S = 1.94573; 20:1 = 0.34708; A = 1.48562. The TAG composition which resulted from application of TAG response factors derived from the FA response factors, is given in column 3 of Table 5. The TAG composition, thus obtained, resulted in a calculated FA composition which was in excellent agreement with the FA composition obtained by GC-FID. The FA composition from the GC-adjusted TAG composition, had only 3.3 % average relative error per FA. If the error of the FA 20:1, present at below 0.1%, is omitted, then the average relative error for the FA composition is 0.7 % AARE per FA, representing excellent agreement with the FA composition obtained by GC-FID.

DISCUSSION

The chromatograms for normal canola oil were in good agreement with the LC-FID chromatogram published earlier,[12] although we were able to identify more TAG species than was previously possible. The extracted ion chromatograms shown in Figures 2, 4 and 6, were examples of the ability of the APCI-MS technique to qualitatively identify TAG species present even in low quantities. The capability for qualitative identification which LC/APCI-MS allowed, was of utmost importance in analysis of samples containing numerous components for which no commercially available standards were available, such as the high lauric canola oil. Even if chromatographic standards for each TAG were available, LC-FID still was incapable of providing the resolution possible with the LC/APCI-MS system. The ability to differentiate coeluting masses, allowed the LC/APCI-MS system to unambiguously identify many more species than was possible with the LC-FID system alone.

Calculation of the FA composition from the TAG composition gave us the ability to compare results obtained by APCI-MS and LC-FID to results obtained by calibrated GC-FID. We found that the uncorrected LC/APCI-MS composition obtained for a TAG mixture gave a FA composition which had an average relative error which was larger than, or similar to, the error exhibited by the FA composition calculated from the LC-FID results. In the case of all three oil samples studied here, we found that response factors could be calculated in a simple, straightforward manner. These gave TAG compositions for which the calculated FA compositions were in excellent agreement with the experimentally determined FA compositions. The FA compositions calculated

Table 5

High Stearic Acid Canola Oil TAG Composition

Triglyceride			Raw LC/MS	Adj. LC/MS	LC-FID%
L	O	S	14.2	14.0	11.1
Ln	O	S	9.8	11.2	11.6
O	O	S	9.2	8.5	7.8
Ln	S	S	4.8	6.2	12.7
Ln	L	S	5.0	6.1	2.4
O	S	S	4.9	5.1	5.5
L	S	S	4.4	5.0	11.8
Ln	L	O	4.3	4.6	1.3
L	O	O	4.9	4.2	4.1
Ln	O	O	3.6	3.6	6.6
Ln	Ln	S	2.2	3.1	2.3
L	L	S	2.6	2.7	3.1
L	L	O	2.6	2.4	1.4
P	L	S	2.0	1.7	0.0
O	O	O	2.0	1.7	3.9
Ln	Ln	O	1.3	1.6	0.7
P	O	O	2.2	1.6	2.4
P	Ln	O	1.7	1.6	1.4
P	L	O	1.6	1.3	1.1
P	O	S	1.5	1.2	1.4
P	Ln	S	1.2	1.2	0.0
Ln	L	L	0.9	1.0	0.2
Ln	S	A	0.9	0.9	0.0
P	Ln	L	0.9	0.9	0.6
L	S	A	1.0	0.8	0.0
Ln	Ln	L	0.6	0.7	0.2
Ln	O	A	0.8	0.7	0.0
L	O	A	1.0	0.7	0.0
Ln	L	A	0.7	0.6	0.0
O	S	A	0.7	0.5	0.0
P	L	L	0.6	0.5	0.0
P	Ln	Ln	0.5	0.5	0.2
O	O	A	0.7	0.5	0.0
Ln	Ln	A	0.4	0.4	0.0
L	L	L	0.4	0.4	0.6
Ln	Ln	Ln	0.2	0.3	0.1

(continued)

Table 5 (continued)

High Stearic Acid Canola Oil TAG Composition

Triglyceride			Raw LC/MS	Adj. LC/MS	LC-FID%
L	L	A	0.3	0.2	0.0
P	P	L	0.3	0.2	1.0
P	P	O	0.3	0.2	0.2
	Other		3.2	1.4	4.1
	Sum		100.0	100.0	100.0

Table 6

High Stearic Acid Canola Oil Fatty Acid Composition

FA	Raw LC/MS %	GC-Adj. %	LC-FID %	GC-FID %
Po	0.4	0.2	0.0	0.2
P	4.8	4.0	5.8	4.0
Ln	15.5	17.6	15.0	17.8
L	18.9	18.6	15.0	18.7
O	31.0	29.1	29.8	29.0
S	26.4	28.3	34.4	28.1
20:1	0.5	0.1	0.0	0.1
A	2.5	2.0	0.0	2.0
Sum	100.0	100.0	100.0	100.0
AARE%	72.5	3.3	13.2	0.0

after application of GC-FID-derived response factors showed significantly less average relative error than the uncorrected data. The effectiveness of this approach was shown by the FA composition determined for the high lauric canola oil, Table 4. Both the LC/APCI-MS data and the LC-FID data resulted in percentages of lauric acid which were ~10 % too high. This clearly indicated an over-response for lauric acid when using these detectors. This over-response was expected for the LC/APCI-MS system, in which lower molecular weight TAG were propagated through the system with more efficiency. The LC-FID detector, which depended on the carbon content of the TAG, should have given more response for those TAG which contained longer carbon chains, rather than shorter ones. In all cases, the FA composition calculated from the GC-Adjusted

LC/APCI-MS TAG composition agreed with the experimentally determined FA composition better than the FA compositions calculated from the raw LC/APCI-MS or LC-FID.

The TAG and FA results for the oils clearly displayed the changes in composition which were desired for these oils. For instance, the high lauric canola oil clearly contained numerous lauric acid-containing TAG which had a distinct effect on the physical characteristics of the oil. We found that, not only was the high lauric acid variety high in the desired lauric acid, but myristic acid was a major product of this genetic modification, as well. The APCI-MS EICs for the high lauric canola oil indicate that myristic acid behaves very similarly to lauric acid in the formation of TAG from FA. Furthermore, from the EICs we determined that there was a definite tendency for lauric acid and myristic acid to appear together as LaLa, LaM, or MM, even though myristic acid is present in a lower percentage than other FA. The different appearance of the EICs for these three diacylglycerols compared to the EICs for the other DAG revealed a preference for the combination of these FA. The LaLa, LaM, and MM diglycerides form triglycerides primarily with linoleic and oleic acids.

The TAG and FA compositions for high stearic canola oil clearly indicated success in the formation of more stearic acid-containing TAG. The FA percent rose from 1.6 % stearic acid in normal canola oil to 28.1 % in high stearic canola oil. The oleic acid percent was still high, as in normal canola oil, so that the most abundant TAG contained the OS, OO, and SS diacylglycerols. TAG containing these DAG are known to be more oxidatelvely stable than TAG which have a higher unsaturation or polyunsaturation content. The higher stearic content also changed the physical characteristics of the canola oil. The higher content of saturates in this CNO would mean a higher melting point, different mouthfeel, and, as mentioned, greater oxidative stability.

It has been demonstrated that LC/APCI-MS was an excellent tool for unambiguous qualitative identification of numerous TAG species, for which no standards were available and which occurred chromatographically overlapped with other TAG components. It has also been demonstrated that, using the FAME composition determined by GC-FID, TAG response factors were calculated from LC/APCI-MS data which allowed quantitation of TAG components with less average relative error than was obtained by LC-FID results. The relative error in the FA composition obtained by APCI-MS was as low as 0.7 % per FA when minor FA were ignored. It was found, that LC-FID was sufficient to identify the major TAG components of the canola oils when the TAG identities were already known, and that quantitation using LC-FID gave a FA composition which had 11 % to 20 % average relative error compared to the FA composition obtained by calibrated GC-FID. We have shown, that the changes in TAG composition which were induced by genetic modification can be qualitatively and quantitatively analyzed regardless of the

availability of standards. This facile and straightforward method of analysis represents an important technique to complement other analytical and organoleptic methods for seed oil analysis.

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LIQUID CHROMATOGRAPHIC ESTIMATION OF OCTANOL/WATER PARTITION COEFFICIENTS WITH A HIGH EFFICIENCY, NONPOROUS, ULTRASMALL PARTICLE SIZE REVERSE PHASE STATIONARY PHASE

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ABSTRACT

HPLC retention data, obtained over a range of organic modifier fractions (ϕ) in binary mobile phases, can be used to estimate a compounds octanol/water partition coefficient (log $P_{o/w}$). Such a determination is facilitated by a high efficiency, low capacity column. The utility of a nonporous, ultrasmall particles size (1.5 μm), C_{18} stationary phase capable of such performance was assessed using aqueous mobile phases containing either acetonitrile or methanol. In general, compounds were effectively eluted from this stationary phase at lower values of ϕ than with more typical stationary phases, thus increasing the accuracy and speed with which log K_w, the capacity factor in a 100% aqueous mobile phase, could be determined. Values of log K_w obtained for seventeen model compounds were highly correlated with the compound's log $P_{o/w}$. Comparison of retention trends in the methanol and acetonitrile mobile phases suggests differences in the retention mechanisms for both organic modifiers.

INTRODUCTION

The dependance of analyte retention on the organic modifier level in binary mobile phases in reverse phase HPLC is of interest for several reasons.[1] While such information has chromatographic significance (e.g., by providing insight into retention mechanisms and allowing for the optimization of chromatographic separations), an important use of this information is in the estimation of octanol-water partition coefficients (log $P_{o/w}$). The use of log $P_{o/w}$ as an indicator of a compounds lipophilic nature is well established and has become a standard method for modeling biological, environmental and physicochemical processes.[1-8] While log $P_{o/w}$ may be determined by calculation with traditional shake flask experiments and via chromatographic retention data, the use of HPLC retention data offers the benefits of accuracy, consistency, specificity, relative ease of determination and a broader dynamic range.

In the HPLC approach, a compound's log $P_{o/w}$ is related to its capacity factor obtained with a 100% aqueous mobile phase (K_w) via a Collander-type expression:

$$\log P_{o/w} = a(\log K_w) + b \qquad (1)$$

While direct measurement of K_w is desirable, it is not practically possible for many, even modestly, lipophilic compounds using conventional C_{18} or C_8 stationary phases and column configurations due to strong retention. In such instances, log K_w is obtained by extrapolation of plots of capacity factor (k') versus volume fraction (ϕ) of organic solvent in a binary water-organic mobile to $\phi = 0$:

$$\log k' = \log K_w + S\phi \qquad (2)$$

While equation 2 usually provides a reasonable fit to experimental retention data over a limited range in ϕ, both concave and convex deviations in plots of log k' versus ϕ have been observed, especially for lipophilic compounds which require high ϕ mobile phases for their effective elution.[1,5,9] Curvature of the log k' versus ϕ plots results in an inaccurate determination of log K_w which in turn is manifested in poor log $P_{o/w}$ versus log K_w correlations.

It is anticipated that the accuracy of such correlations would, thus, be enhanced by using a reverse phase column which offers high efficiency coupled with reduced chromatographic capacity. In such a situation, high efficiency separations, effected with lower ϕ mobile phases, could be used to facilitate the

Table 1

Marker Compounds Used and their Partition Coefficients

Marker Compound	Abbreviation	log $P_{o/w}$
Acetanilide	AD	1.46
Dimethyl phthalate	DMP	1.95
Acetophenone	AP	1.63
2-phenyl-2-propanol	PP	1.90
Methyl paraben (*)	MTPB	1.96
p-toluic acid	MBH	2.34
Diethyl phthalate	DEP	2.95
Ethyl paraben (*)	ETPB	2.47
4-ethyl benzoic acid	EBH	2.97
Propyl paraben (*)	PRPB	3.04
Butyl paraben (*)	BUPB	3.57
4-tert-butyl benzoic acid	BBH	3.78
Dipropyl phthalate	DPP	3.84
Antracene	ANTH	4.50
Dibutyl phthalate	DBP	4.72
Hexachlorobenzene	PCB	5.44
Dioctyl phthalate	DOP	7.88

(*) p-hydroxy benzoic acid, n-ester. In general the partition coefficients were obtained from the LOGKOW© data (Sangster Laboratories, first edition).

direct measurement of log K_w or, at least, allow for more accurate log K_w extrapolations. High efficiencies are necessary to ensure that an adequate interaction is achieved between the analyte and the stationary phase. In addition to the potentially increased log $P_{o/w}$ model accuracy, the retention data may be obtained more quickly with the high efficiency, low capacity LC column configuration.

Recently, a high efficiency, low capacity "fast" LC column based on nonporous, 1.5 μm C_{18} bonded silica microspheres has become commercially available. Such a stationary phase, available in a 33 by 4.6 mm column geometry, has been documented to produce high efficiency separations of low molecular weight analytes at much lower values of ϕ and in much shorter total analysis times than can be obtained with more conventional C_{18} stationary phases and column configurations.[10] In the study reported herein, the retention properties of seventeen marker compounds were determined over a wide range

of ϕ values in aqueous mobile phases containing either acetonitrile or methanol. The nature of the log k' versus ϕ relationship for this column type was established and the applicability of the data for the determination of log K_w and log $P_{o/w}$ was assessed.

EXPERIMENTAL

Materials

The marker compounds used and their log $P_{o/w}$ values, obtained from a computerized database,[11] are identified in Table 1. These compounds were chosen based on their high detectability (thereby allowing for good peak shapes due to small injection masses), their wide range in partition coefficients, and, for several analytes, their acid functionality. Individual stock solutions of each analyte were prepared by dissolving reagent grade reference materials in an appropriate strength binary solvent. Single analyte working standards were prepared at a concentration of $\approx$ 3 mg/L by diluting the stocks in 0.03 M phosphoric acid. Reagents used to prepare the mobile phases and other analytical solutions were reagent, or analytical grade, as appropriate. Water was obtained from a Barnstead NANOpureII water polishing system.

Analytical System

The column used was obtained from Micra Scientific (Northbrook, IL) and consisted of NPS RP-18, 1.5 µm stationary phase in a 33 by 4.6 mm stainless steel column. Binary methanol/0.03 M phosphoric acid or acetonitrile/0.03 M phosphoric acid mobile phases were used. The addition of acid was necessary to ensure that the acidic analytes were eluted in their protonated, uncharged form.

The chromatographic system consisted of a Kratos Spectroflow 400 pump, a Micrometics 723 autosampler (coupled with an electronically actuated Rheodyne 7010 valve), a Kratos Spectroflow 757 UV detector, a strip chart recorder and a Hewlett Packard HP 3357 LAS computer data collection system. A minimum length of 0.007" i.d. PEEK tubing was used to connect the injector and column, while the column and detector was connected directly using an Alltech Direct-Connect™ low dead volume connector. Minimizing extra-column system void volume is essential to maintaining the columns efficiency.

Table 2A

Capacity Factor Data for the Various Model Analytes

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k′)	% Organic	log (k′)
AD	0	0.757	*0*	*0.707*
	5	*0.403*	*5*	*0.061*
	10	*0.167*	*10*	*-0.234*
	15	*-0.019*	----	----
DMP	0	1.828	0	1.801
	5	*1.296*	*5*	*0.907*
	10	*0.969*	*10*	*0.489*
	15	*0.658*	*15*	*-0.35*
	20	*0.451*	----	----
AP	0	1.191	0	1.160
	5	*0.801*	*5*	*0.456*
	10	*0.557*	*10*	*0.152*
	15	*0356*	*15*	*-0.025*
MTPB	0	1.060	0	1.041
	5	*0.711*	*5*	*0.398*
	10	*0.464*	*10*	*0.061*
	15	*0.246*	*15*	*-0.160*
PP	0	1.142	0	1.096
	5	*0.821*	*5*	*0.533*
	10	*0.613*	*10*	*0.212*
	15	*0.411*	*15*	*-0.006*
	20	*0.279*	20	-0.122
MBH	0	1.297	0	1.258
	5	*1.105*	*5*	*0.759*
	10	*0.819*	*10*	*0.428*
	15	*0.620*	*15*	*0.165*
	20	*0.483*	20	0.017
DEP	*5*	*2.167*	*5*	*1.720*
	10	*1.785*	*10*	*1.230*
	15	*1.423*	*15*	*0.831*
	20	*1.153*	*20*	*0.529*
	25	*0.867*	*25*	*0.188*
	30	*0.607*	----	----
	35	*0.330*	----	----

(continued)

Table 2A (continued)

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k′)	% Organic	log (k′)
ETPB	0	1.574	0	1.761
	5	*1.170*	*5*	*0.880*
	10	*0.919*	*10*	*0.493*
	15	*0.668*	*15*	*0.196*
	20	*0.499*	20	-0.007

Note: Data in *talics* were used to generate the log K_w calculation plots.

Table 2B

Capacity Factor Data for the Various Model Analytes

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k′)	% Organic	log (k′)
EBH	0	1.807	1.760	
	5	*1.508*	*5*	*1.248*
	10	*1.284*	*10*	*0.898*
	15	*1.056*	*15*	*0.524*
	20	*0.896*	*20*	*0.288*
	25	*0.701*	----	----
	30	*0.496*	----	----
PRPB	----	----	0	2.081
	5	*1.721*	*5*	*1.408*
	10	*1.424*	*10*	*0.993*
	15	*1.156*	*15*	*0.588*
	20	*01946*	*20*	*0.288*
	25	*0.716*	----	----
	30	*0.495*	----	----
BUPB	*5*	*2.225*	*5*	*1.921*
	10	*1.937*	*10*	*1.479*
	15	*1.641*	*15*	*1.033*
	20	*1.419*	*20*	*0.734*

(continued)

Table 2B (continued)

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k′)	% Organic	log (k′)
BUPB	*25*	*1.150*	*25*	*0.294*
	30	*0.893*	----	----
	35	*0.635*	----	----
BBH	----	----	*5*	*2.025*
	10	*2.045*	*10*	*1.619*
	15	*1.775*	*15*	*1.188*
	20	*1.573*	*20*	*0.880*
	25	*1.325*	*25*	*0.410*
	30	*0.904*	*30*	*0.139*
	35	*0.806*	----	----
	40	*0.635*	----	----
DPP	----	----	*10*	*2.104*
	----	----	*15*	*1.613*
	20	*1.970*	*20*	*1.283*
	25	*1.628*	*25*	*0.844*
	30	*1.321*	*30*	*0.536*
	35	*0.994*	*35*	*0.260*
	40	*0.707*	----	----

Note: Data in ***italics*** were used to generate the log K_w calculation plots.

Table 2C

Capacity Factor Data for the Various Model Analytes

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k′)	% Organic	log (k′)
ANTH	----	----	*15*	*2.238*
	----	----	*20*	*1.928*
	25	*2.310*	*25*	*1.140*
	30	*2.043*	*30*	*0.814*
	35	*1.764*	*35*	*0.446*
	40	*1.509*	*40*	*0.284*

(continued)

Table 2C (continued)

Analyte	Elution Behavior			
	In Methanol		In Acetonitrile	
	% Organic	log (k')	% Organic	log (k')
	45	***1.218***	----	----
	50	***0.944***	----	----
	55	***0.727***	----	----
DBP	----	----	***20***	***2.029***
	----	----	***25***	***1.528***
	30	***2.104***	***30***	***1.140***
	35	***1.703***	***35***	***0.796***
	40	***1.369***	***40***	***0.513***
	45	***1.024***	***45***	***0.220***
	50	***0.702***	----	----
	55	***0.453***	----	----
PCB	----	----	***25***	***2.061***
	----	----	***30***	***1.685***
	----	----	***35***	***1.344***
	40	***2.124***	***40***	***1.049***
	45	***1.838***	***45***	***0.705***
	50	***1.552***	***50***	***0.472***
	55	***1.337***	----	----
	60	***1.066***	----	----
	65	0.739	----	----
DOP	----	----	***40***	***2.234***
	----	----	***45***	***1.763***
	55	***2.349***	----	----
	60	***1.887***	----	----
	65	***1.337***	----	----

Note: Data in ***italics*** were used to generate the log K_w calculation plots.

Procedure

Each model solute was injected in replicate in each appropriate mobile phase. The mobile phases were used and the model compounds injected in random order. While specific model compounds were injected into all mobile phases in which they possessed reasonable retention, the mobile phases used for each compound were limited at low ϕ by reasonable elution times (k' values less

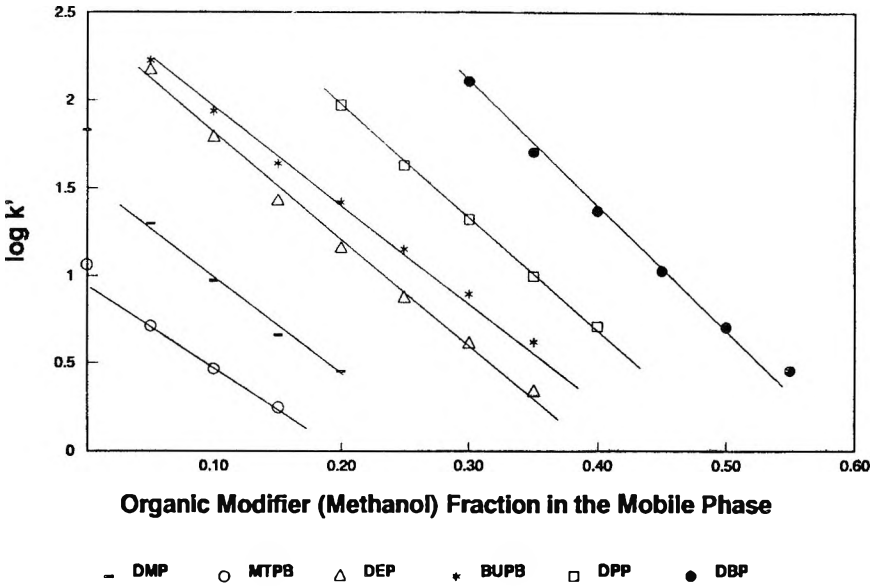

Figure 1. Plots of log k' versus organic modifier fraction (ϕ) for several model compounds using methanol as the organic modifier. In general the plots are quite linear and are roughly co-linear.

than 200) and at high ϕ by the need to obtain an effective column/compound interaction (k' values greater than 1). Mobile phase flow rate was 1 mL/min, sample injection size was 10 µL and the detection wavelength was 215 nm.

RESULTS AND DISCUSSION

The capacity factor versus mobile phase composition data obtained is summarized in Tables 2A through 2C. For nine of the models compounds, log K_w could be measured directly. For the other model compounds, the lowest mobile phase ϕ which could be used to obtain log K_w via extrapolation was typically 0.2 or less. Only in the case of the most strongly retained analyte (DOP) was the extrapolation performed with a minimum measured ϕ greater than 0.5.

Representative plots of log k' versus ϕ are shown in Figures 1 and 2. In most cases, the linear correlation between these two variables was excellent, with the best fit linear models exhibiting correlation coefficients (r^2) of 0.99 or

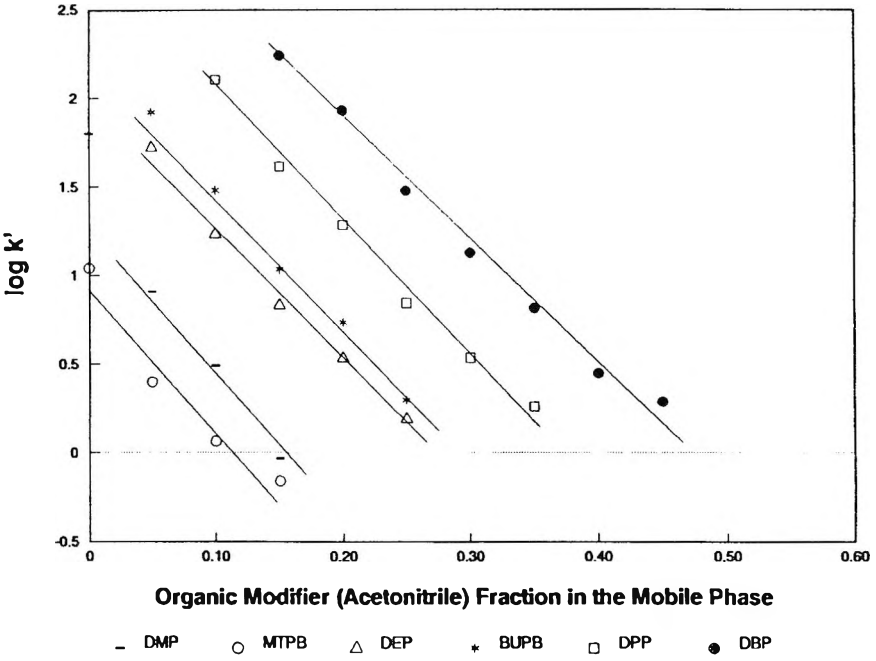

Figure 2. Plots of log k' versus organic modifier content (ϕ) for several model compounds using acetonitrile as the organic modifier. In general the plots are quite linear and roughly co-linear. The log k' obtained at equivalent values of ϕ is smaller in acetonitrile than in methanol, establishing acetonitrile as the stronger mobile phase modifier.

greater. For most analytes, however, some concavity in the plots were observed between ϕ values of 0.05 and 0.00 for both the acetonitrile and methanol mobile phases. For this reason, measured values of log k' at $\phi = 0.00$ were not used in the linear regression analysis.

The plots of log k' versus ϕ for a given organic modifier were strikingly co-linear. For methanol, the mean and standard deviation of the slopes from these plots for all 17 model compounds were 0.051 and 0.009. For acetonitrile, the mean and standard deviation of the slopes were 0.068 and 0.011. The co-linearity of the plots reflects the structural similarities among the model compounds used and suggests that the general retention mechanism was consistent among all the model compounds. The stronger eluting power of acetonitrile versus methanol is reflected in the larger mean slope for this organic

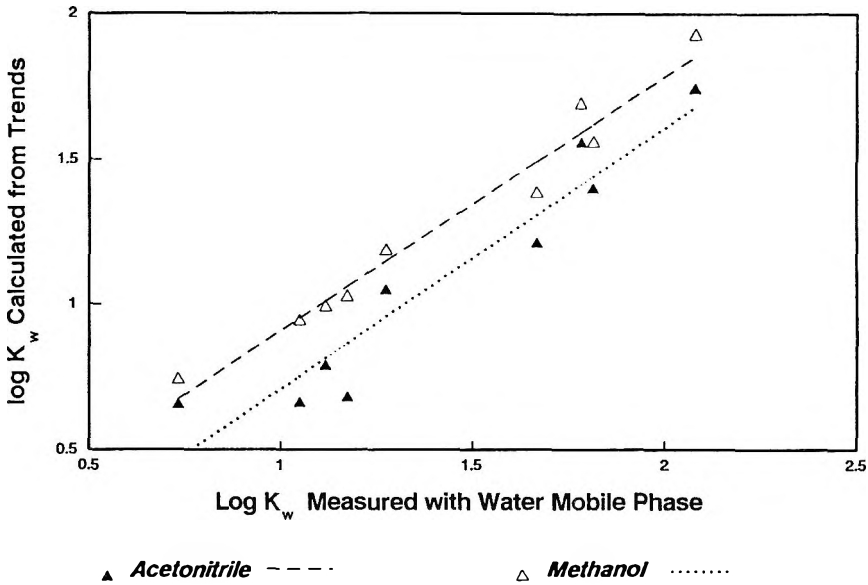

Figure 3. Plots of log K_w actually measured versus log K_w calculated using either methanol or acetonitrile as the mobile phase organic modifier. In general the calculated and measured values are linearly related; however the measured values are usually larger than the calculated ones due to concave curvature in the log k' versus ϕ plots.

modifier and is re-enforced by an examination of retention data in Table 2A through 2C. For every model compound studied, the ϕ required to produce a given k' is lower for acetonitrile than for methanol.

The effectiveness of the log K_w extrapolations can be addressed in two manners. Figure 3 represents the comparison between measured and calculated values of log K_w. The linear model used for the comparison was

$$\log K_w \text{ (calculated)} = a\,[\log K_w \text{ (measured)}] + b \qquad (3)$$

which, ideally, should exhibit a slope of 1.0, an intercept of 0.0 and a correlation coefficient of 1.0. For acetonitrile, pertinent curve fit data included the following: slope, 0.90 (0.11); intercept, -0.19 (0.13); r^2, 0.91; where the number in () is the standard error. For methanol, the pertinent curve fit data included slope, 0.87 (0.06); intercept, 0.03 (0.07); r^2, 0.97. While, in general, the correlations are good, the less than unit slopes reflect the generally concave nature of the log k' versus ϕ plots at low values of ϕ. That is, extrapolation of

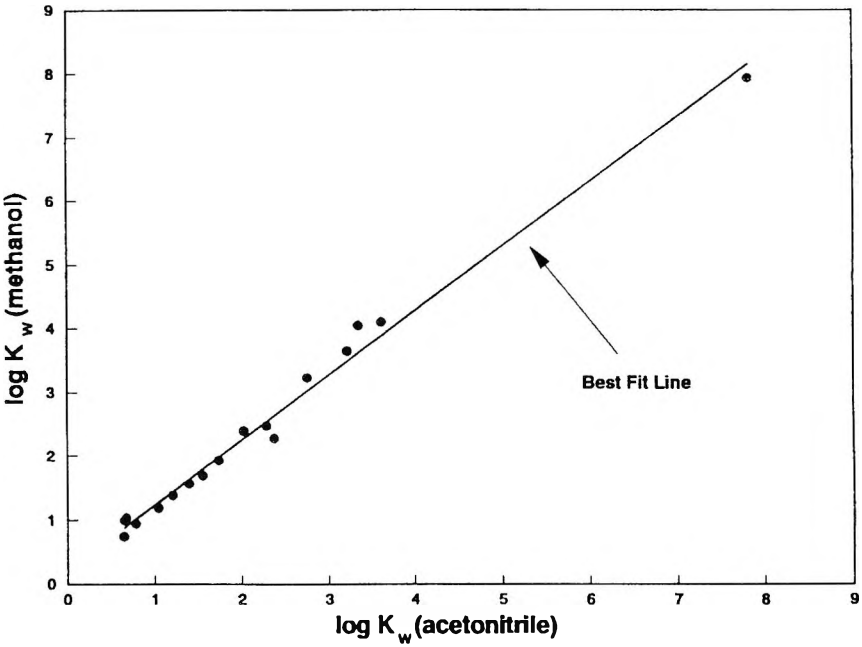

Figure 4. Plot of log K_w calculated, methanol versus acetonitrile as mobile phase additives. Within the regression and experimental errors, the log K_w values obtained with both modifiers are equivalent.

the log k' data to $\phi = 0$ generally produced a log K_w estimate which was lower than the corresponding measured value.

In a recent analysis of retention trends in HPLC, Valko, Snyder and Glajch observe that the reliability of extrapolated log K_w values can be assessed by comparing such values obtained from two different organic modifiers.[1] The point here is that log K_w is an analyte/column property which is organic modifier independent. The relationship between log K_w extrapolated from the data for all the model solutes using both organic modifiers is shown in Figure 4. In general, an excellent linear correlation is observed. Linear regression curve fit parameters for the model

$$\log K_w \text{ (methanol)} = a[\log K_w \text{ (acetonitrile)}] + b \qquad (4)$$

include slope, 1.02 (0.03); intercept, 0.22 (0.20); correlation coefficient (r^2), 0.99. Thus within the precision of the experimental measurements, the expected

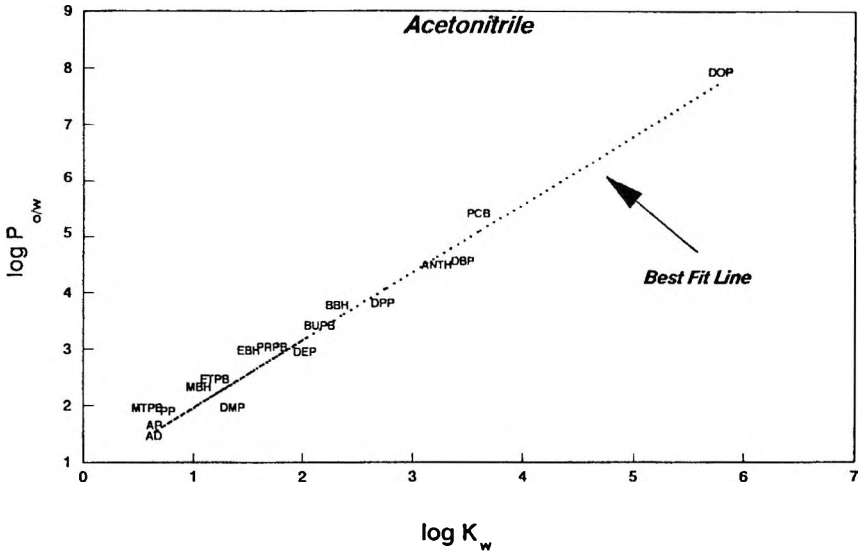

Figure 5. Plot of log $P_{o/w}$ versus log K_w for acetonitrile as the organic modifier. The two variables are linearly correlated, with a correlation coefficient (r^2) of 0.977. The random distribution of the data around the best fit line suggests that the model contains no significant compound class bias.

1 to 1 relationship between log K_w extrapolated from both organic modifiers is observed. The ultimate goal of this research is to examine the relationship between the extrapolated log K_w data and available log $P_{o/w}$ data specifically per equation 1. Plots of log $P_{o/w}$ versus log K_w are shown in Figures 5 and 6; the corresponding linear regression equations are as follows:

For acetonitrile, $\log P_{o/w} = 1.18 (\log K_w) + 0.87$, $r^2 = 0.977$

For methanol, $\log P_{o/w} = 0.90 (\log K_w) + 1.11$, $r^2 = 0.961$

Standard errors for the curve fit parameters included slope, 0.05 and intercept, 0.25 for acetonitrile and slope, 0.05 and intercept, 0.34 for methanol. As illustrated by the Figures and confirmed by the correlation coefficients, the partition coefficient models are quite accurate for the model compounds used. Examination of these figures indicates that the distribution of the data around the best fit line is essentially random and thus that the models contain no significant compound class bias.

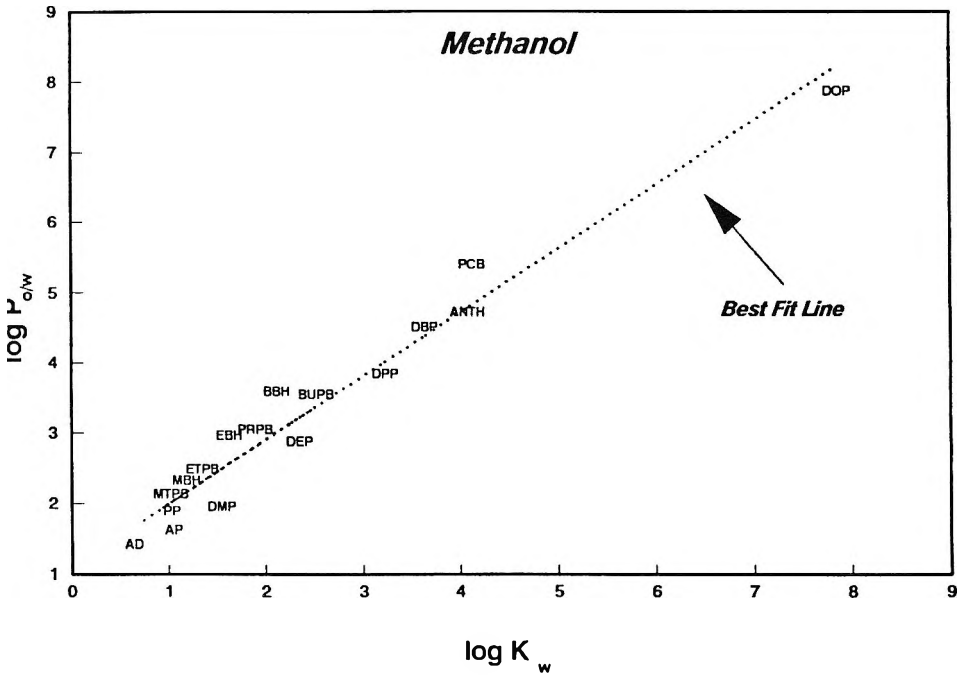

Figure 6. Plot of log $P_{o/w}$ versus log K_w for methanol as the organic modifier. The two variables are linearly correlated, with a correlation coefficient (r^2) of 0.961. The random distribution of the data around the best fit line suggests that the model contains no significant compound class bias.

As observed by Braumann, Weber and Grimme,[2] the magnitude of the slope and intercept of the Collander-like expressions are significant. The model's intercept provides a measure of the hydrophobicity of the stationary phase used versus octanol; the similar positive values obtained indicate that the stationary phase used was significantly more hydrophobic than octanol. These authors attributed this behavior to the solvation of the C_{18} stationary phase ligands by the organic modifier and free energy effects associated with water-organic mixtures relative to pure water.

The slopes of the regression equations are a measure of the solvent system's sensitivity to changes in the hydrophobicity of the model compounds. While both organic modifiers exhibit sensitivities to compound hydrophobicity which are similar to octanol (slopes near 1.0), it is clear that acetonitrile is more sensitive to solute nature than is methanol. Thus acetonitrile is the more

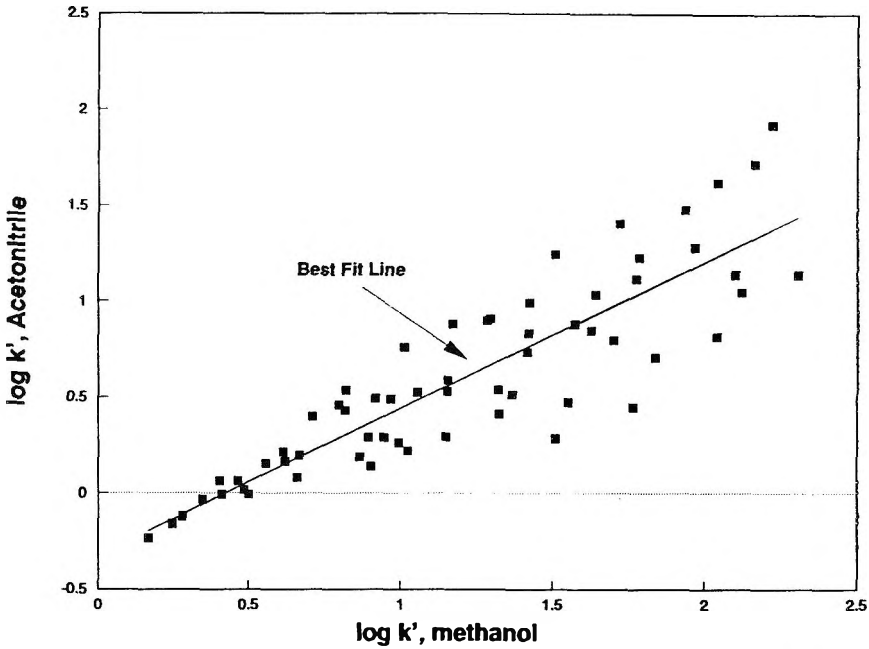

Figure 7. Plot of log k' measured for various compounds at similar values of ϕ in either methanol or acetonitrile containing mobile phases. While the log k' values for the two organic modifiers are directly related, the scatter in the data suggests that the retention mechanism in both organic modifiers is markedly different.

discriminating organic modifier and its use may facilitate the effective separation of mixtures of compounds. The two organic modifiers used are expected to have significantly different analyte retention profiles due to their marked chemical differences.[2,4,5,6,10]

Methanol, with both hydrogen donor and acceptor character, changes the ordering of water molecules in the mobile phase to only a limited extent and, thus, will not effect the mobile phases interaction potential with polar solutes. Acetonitrile, a weak hydrogen bond acceptor, will exert a more marked effect on the structure of the mobile phase and, hence, have a greater impact on the energetics of the solvophobic effect. Acetonitrile, with its high dipole moment,[5] may also participate in selective dipole-dipole interactions with certain solutes. Additionally, the participation of residual silanols in the retention process is more pronounced in organic modifiers other than methanol.[5,12]

Table 3

Comparison of Capacity Factors Obtained with Acetonitrile vs Methanol; Data Sorted by Analyte

Analyte	ϕ Range	Regression Model Parameters		
		Slope	Intercept	r^2
DMP	0.05 - 0.15	1.47	-0.98	0.994
AP	0.05 - 0.15	1.08	-0.42	0.991
PP	0.05 - 0.20	1.21	-0.49	0.986
MTPB	0.05 - 0.15	1.20	-0.47	0.993
MBH	0.05 - 0.20	1.39	-0.68	0.992
DEP	0.05 - 0.25	1.17	-0.68	0.992
ETPB	0.05 - 0.20	1.31	-0.68	0.996
EBH	0.05 - 0.20	1.58	-1.31	1.000
PRPB	0.05 - 0.20	1.45	-1.08	1.000
BUPB	0.05 - 0.25	1.50	-1.42	0.999
BBH	0.10 - 0.30	1.31	-1.17	0.961
DPP	0.20 - 0.35	1.05	-0.81	0.991
ANTH	0.25 - 0.40	1.10	-1.42	0.982
DBP	0.30 - 0.45	-0.85	-0.65	1.000
PCB	0.40 - 0.50	1.01	-1.11	0.988
Overall	0.05 - 0.50	0.76 (0.05)	-0.32 (0.24)	0.772

Note: The above data represents all values for ϕ for which there were log k' data for both methanol and acetonitrile. Analytes which had only two such data points and, thus, were not subjected to regression analysis included AN and DOP. Data in () are the standard error of regression. The regression model used was:

log k' (acetonitrile) = a[log k' (methanol)] + b.

A comparison of all log k' data for analytes whose retention was measured in both acetonitrile and methanol containing mobile phases with the same ϕ value is shown in Figure 7 (representing 61 data pairs). While in general, the log k' values for both organic modifiers are directly related, linear regression analysis of the entire dataset produces a poor correlation (r^2 = 0.77). The poor correlation does not result from the differing behavior of individual compounds as mobile phase ϕ changes; as shown in Table 3, the linear correlation betweenlog k' in acetonitrile versus that in methanol at constant ϕ is excellent for each individual compound studied.

Table 4

Comparison of Capacity Factors Obtained with Acetonitrile Versus Methanol; Data Sorted by Mobile Phase Composition (ϕ)

Mobile Phase Composition (ϕ)	Number of Compounds	Regression Model Parameters		
		Slope	Intercept	r^2
0.05	11	0.97	-0.29	0.991
0.10	12	0.96	-0.38	0.995
0.15	11	0.85	-0.39	0.977
0.20	10	0.83	-0.42	0.990
0.25	5	0.71	-0.46	0.948
0.30	4	0.70	-0.45	0.914
0.35	3	0.46	-0.18	0.516
0.40	3	0.87	-0.84	0.794
0.05 - 0.20	46	0.95	-0.40	0.953
0.25 - 0.50	15	0.68	-0.47	0.84

Note: The above data represents all values for ϕ for which there were log k' data for both methanol and acetonitrile. Mobile phases with values of ϕ greater than 0.50 had only two such datapoints and thus were not subjected to regression analysis. The regression model used was:

$$\log k' \text{ (acetonitrile)} = a\,[\log k' \text{ (methanol)}] + b$$

Considering data for each of the studied compounds separately, log k' in the acetonitrile and methanol containing mobile phases are linearly correlated and, the retention characteristics in a mobile phase containing one of the modifiers, can be used to accurately estimate the retention characteristics in a mobile phase containing the other modifier at the same volume fraction ϕ.

Differences in the slope and intercepts of the log k' comparisons in Table 3 suggest part of the non-linearity observed in Figure 7 results from different solute/solvent interactions in the mobile phases containing the two organic modifiers. Table 4, which sorts the log k' acetonitrile versus methanol dataset as a function of ϕ, provides an insight into the dominant driving force for the non-linearity of the relationship shown in Figure 7. As shown in Table 4 and Figure 8, the relationship between log k' in acetonitrile versus log k' in methanol is highly linear over two distinct ranges of ϕ; ϕ values between 0.05 and 0.20 and ϕ values greater than 0.25. While this discontinuity in relative retention behavior at a ϕ value of approximately 0.25 is a clear indication of a changing

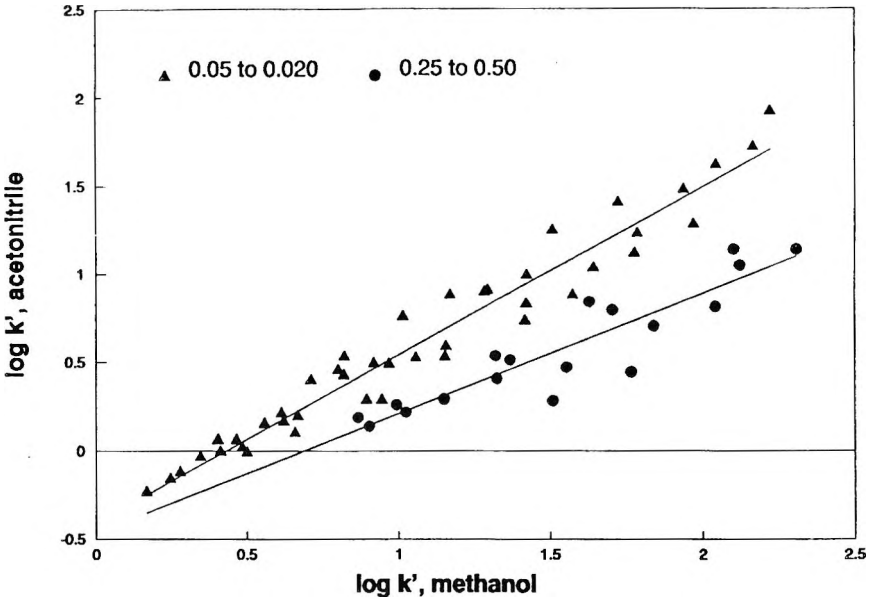

Figure 8. Data from Figure 7 sorted into two subsets; those for ϕ less than 0.25 and those for ϕ of 0.25 or greater. The clear breakdown of the data into two subsets as a function of ϕ suggests that the markedly different retention mechanisms for methanol versus acetonitrile mobile phases occurs above a ϕ value of 0.25. The somewhat poorer correlation of the data above ϕ of 0.25 suggests that compound/solvent interactions play a more significant role in defining the relative retention characteristics of methanol versus acetonitrile at the higher organic modifier volume fractions.

retention mechanism in one of the mobile phase types, the nature of this change in mechanism was not examined in detail in this study. It is suggested, however, that the influence of compound identity on the relative retention behavior of all compounds studied in the methanol or acetonitrile mobile phases is small at ϕ, less than 0.25 as evidenced by the excellent linear correlation for this data subset. However, the poorer correlation above $\phi = 0.25$ suggests that compound/solvent specific interactions play a more important role in defining the relative retention characteristics of the compounds in mobile phases prepared with organic modifiers present at high volume fractions.

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ANALYSIS OF CARCINOGENIC HETEROCYCLIC AMINES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation and determination of mutagenic and carcinogenic heterocyclic amines by a reversed phase isocratic HPLC method is here in described. Five of them, namely 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) were encountered in this study. Separation was accomplished on a reverse phase HPLC column of Supelcosil LC-8 equipped with a Supelguard LC-8 precolumn, at a flow rate of 2.0 mL/min. IQ, MeIQx and 4,8-DiMeIQx were resolved using 15% (v/v) acetonitrile in 50 mM triethylamine-phosphate buffer, pH 3.2, whereas 4,7,8-TriMeIQx and PhIP using 30% (v/v) acetonitrile in the same buffer. Detection of the eluted heterocyclic amines was performed at 263 nm for IQ, MeIQx and 4,8-DiMeIQx and at 254 nm for 4,7,8-TriMeIQx and PhIP.

Reproducibility tests measuring peak areas gave a relative standard deviation of 1.8-4.4%. The calibration graphs for all five amines injected into the column were linear up to approximately 2.0 μmol and the detection limits (signal-to-noise ratio 2:1) ranged from 10 to 30 pmol. The high sensitivity of the proposed method permits the accurate and reproducible determination in mixtures containing only a few ng/mL of such heterocyclic amines

INTRODUCTION

Heterocyclic amines belong to a group of compounds which contain three aromatic rings with one or more nitrogen atoms in their ring system. Usually they are found in the urine of healthy individuals eating a normal diet containing heat-processed food, such as broiled meat or grilled chicken.[1,2] Heterocyclic amines can be synthesized in heat-processed food from amino acids, protein pyrolysates and carbohydrates. During the last decade, it has been recognized that some members of aromatic amines, including the family of heterocyclic amines, may induce cancer in humans.[3]

There are five types of such amines isolated from cooked food: aminoimidazoquinoline, aminoimidazoquinoxaline, aminoimidazopyridine, aminodiazopyridoindole and aminopyridoimidazole. Compound 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), which belongs to the first type, 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethyl-imidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx), belonging to aminoimidazoquinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) that belongs to aminoimidazopyridine group, constitute heterocyclic amines that are usually found in various cooked foods.[4,5] These amines are able also to induce mutagenesis and carcinogenesis when fed to mice and rodents.[6] The chemical structures of these mutagenic amines are shown in Figure 1.

The necessity for the development of analytical methods with high sensitivity and selectivity, so that mutagenic and carcinogenic heterocyclic amines reliably and accurately can be identified and determined, is obvious.

Several analytical methods, such as enzyme-linked immunosorbent assay (ELISA),[7] GC-MS[8,9] and HPLC-MS,[10,11] have been recently developed for this purpose. However, they either require very expensive equipment or, are of low selectivity, for the determination of individual heterocyclic amines. of selective determination for most of these compounds.

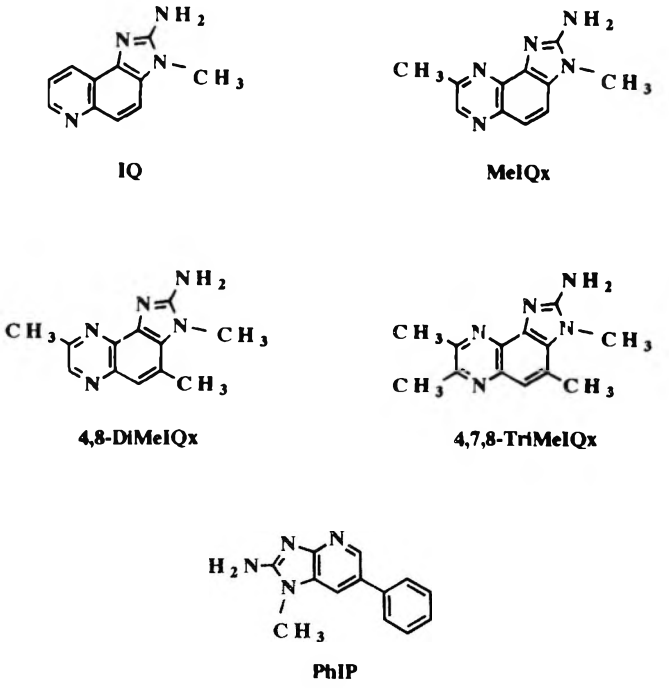

Figure 1. Chemical structures of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f] quinoxaline (4,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f] quinoxaline (4,7,8-TriMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) encountered in this report.

Direct HPLC methods using UV[12] or electrochemical detection[13] offered the advantage. In the case of HPLC-UV technique, the gradient elution causes an increased baseline elevation during chromatography leading, to our experience, in difficult interpretation of the results when this method is applied on a routine basis.

In this paper, we report on an isocratic highly sensitive HPLC method by which the five most important heterocyclic amines (IQ, MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx and PhIP) are completely resolved from each other and, at the same time, are easily determined in the daily laboratory practice.

EXPERIMENTAL

Apparatus and Chemicals

For the determination of heterocyclic amines, a LDC system with a LDC III pump, a UV-vis detector LDC 1204A set at 263 or 254 nm with 8-μL flow cell and with a 50-μL loop injector was used. The analytical column is a Supelcosil LC-8, 5-μm, 250 x 4.6 mm I.D., stainless steel (Supelco) equipped with a Supelguard column, 20 x 4.6 mm I.D. (Supelco).

Studied compounds, as well as, unknown mixtures of known amounts of heterocyclic amines were prepared and generously offered to be tested by Dr. M. Rabache (C.N.A.M.-Conservatoire National des Arts et Metiers, Biochimie Industrielle et Agro-alimentaire, Paris, France). HPLC-grade acetonitrile, obtained from Merck (Darmstadt, Germany) and glass-distilled water constitute the eluant components. All other solvents and chemicals used were of analytical reagent grade.

Chromatographic Conditions

The mobile phase used for the separation of the heterocyclic amines IQ, MeIQx and 4,8-DiMeIQx, designated as eluant 1, was 15% (v/v) acetonitrile in triethylamine-phosphate buffer, pH 3.2 and for the determination of 4,7,8-TriMeIQx and PhIP, designated as eluant 2, was 30% (v/v) acetonitrile in the same buffer. Triethylamine-phosphate buffer was prepared by diluting 1.4-mL triethylamine (Fluka, No. 90340) to 1000-mL of HPLC-grade water, followed by exact adjustment to pH 3.2 with 10% (v/v) phosphoric acid (Merck, No. 573). The flow rate for both determinations was 2.0 mL/min and the pressure approximately 1300 psi. The detection of IQ, MeIQx and 4,8-DiMeIQx was performed at 263 nm, whereas of 4,7,8-TriMeIQx and PhIP at 254 nm. The separation was performed at room temperature. Eluants used were degassed by vacuum filtration through a 0.2-μm membrane filter followed by agitation in an ultrasonic bath.

System Suitability

The column was equilibrated with each eluant separately at a flow rate of 2.0 mL/min. Once a stable baseline was obtained, the standard solutions were injected into the column and the peaks appeared over the increased retention time.

Table 1

High Performance Liquid Chromatographic Characteristics of Heterocyclic Amines

Compound	Retention Time/min (t_R)	Resolution (R_s)
IQ	5.2 ± 0.15[a]	1.8
MeIQx	7.8 ± 0.20[a]	2.3
4,8-DiMeIQx	13.4 ± 0.35[a]	--
4,7,8-TriMeIQx	4.8 ± 0.20[b]	--
PhIP	10.1 ± 0.40[b]	2.1

[a, b] Values obtained using eluant 1 and eluant 2, respectively.

The resolution factors, R_s, are calculated between the chromatographic peak of 4,8-DiMeIQx and each individual peak of IQ and MeIQx and between the peak of 4,7,8-TriMeIQx and PhIP from the equation: $R_s = (t_2-t_1)/(W_1+W_2)$, where t_2 and t_1 are the retention times of the two peaks, while W_1, W_2 are the peak widths at the half height of the respective peaks. The resolution factors, R_s, are more than 1.8 in all cases, indicating complete separation between 4,8-DiMeIQx and IQ, MeIQx, as well as between 4,7,8-TriMeIQx and PhIP as illustrated in Table 1.

Selectivity

Chromatographic analysis of the various heterocyclic amines which were provided in methanolic solution and kept at 2-4°C until use, showed the presence of low amounts of impurities, which do not have any endogenous interference at the retention times of the separated amines. This fact indicates that the proposed method can be used in the determination of the five heterocyclic amines, avoiding the use of an internal standard.

Detection Limit

The detection limits for each heterocyclic amine is estimated as the quantity of these substances producing a signal of the peak height twice the

baseline noise. The minimum detectable amount in pmol injected into the column was estimated to be 10 pmol for 4,8-DiMeIQx and approximately 30 pmol for IQ, MeIQx, 4,7,8-TriMeIQx and PhIP.

Standard Calibration Graphs

Heterocyclic amines were dissolved in methanol and accurately weighed by the provider. Stock solutions of approximately 10 μg per g of solution were used in the present work. Standard solutions were prepared by serial dilutions of the stock solutions. Aliquots of 5 to 50 μL that were accurately weighed by us (N=12), were taken for HPLC analysis. Calibration curves were constructed by plotting the peak areas of heterocyclic amines against their concentrations expressed as ng/mL. Calibration graphs were evaluated for their linearity according to the standard method of van Trijp and Roos.

RESULTS AND DISCUSSION

Retention times of the heterocyclic amines tested, are reproducible under the chromatographic conditions used with a relative standard deviation of less than 0.8%. The mobile phases used enable good column performances for a long period of time. On the other hand, all five heterocyclic amines used are stable for at least several months when kept in methanolic solutions at 2-4°C, provided exclusion of light.

Repeated chromatographies of the heterocyclic amines under the separation conditions described, showed the presence of low amounts of impurities in all preparations. Since the detected impurities did not interfere with the determination of the amines tested, no further purification of the preparations was needed.

Increasing the concentration of the organic modifier, acetonitrile, from 15% to 30% (v/v) results in higher and more reproducible peaks for the more retarded 4,7,8-TriMeIQx and PhIP, improving the sensitivity and accuracy of the described method for these two amines. As shown in Figure 2, the best resolution among the heterocyclic amines IQ, MeIQx and 4,8-DiMeIQx is obtained using 15% (v/v) acetonitrile (Fig. 2a). In higher acetonitrile concentration (30%), 4,7,8-TriMeIQx is completely resolved from PhIP, whereas MeIQx is coeluted with IQ (Fig. 2b).

Evaluation of the method's quality parameters (including linearity, detection limit and precision) was carried out using methanolic solutions for all of the described derivatives, under optimum separation conditions and

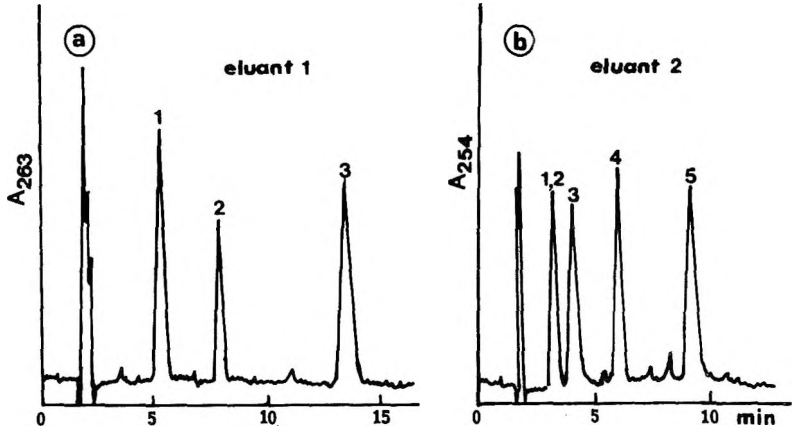

Figure 2. Typical HPLC chromatograms of IQ, MeIQx and 4.8-DiMeIQx (a) and 4,7,8-TriMeIQx and PhIP (b) under elution conditions described in the experimental section. IQ, MeIQx and 4.8-DiMeIQx were detected at 263 nm and 4,7,8-TriMeIQx and PhIP at 254 nm. Eluant 1= 15% (v/v) acetonitrile in triethylamine-phosphate buffer, pH 3.2, and eluant 2= 30% (v/v) acetonitrile in the same buffer. Flow rate was 2.0 mL/min.

concentrations ranging from 0.005 to 10 μg/mL. Calibration graphs obtained by plotting peak area versus concentration (ng/mL), exhibit excellent linearity to all heterocyclic amines tested (Fig. 3). The correlation coefficients of linearity graphs range from 0.9994-0.9999 (N=7-13) for MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx and PhIP, and 0.9957 (N=16) for IQ. Twelve 50 ng of consecutive analyses (10-μL injection from 5.0 μg/mL solution) of each heterocyclic amine were used to determine the precision of the method. The relative standard deviations (R.S.D., %) obtained were in the range of 1.8-4.4%. The detection limits, based on the signal-to-noise ratio of 2:1, ranged from 10 to 30 pmol, according to the retention times.

In order to test the applicability of the proposed analytical method, unknown mixtures, containing unknown heterocyclic amines at the level of 150 to 300 ng/g of solution, were prepared and provided by Dr. Rabache's laboratory, as previously mentioned. As shown in Fig. 4, the detected compounds were IQ, 4,8-DiMeIQx and PhIP. The estimated amounts after a two day analysis in triplicates were 217.0 ± 9.3, 214.75 ± 12.1 and 247.35 ± 5.8 ng/g, respectively. Obtained results show an excellent agreement with target values of 209.4, 202.5 and 257.0 for IQ, 4,8-DiMeIQx and PhIP, respectively.

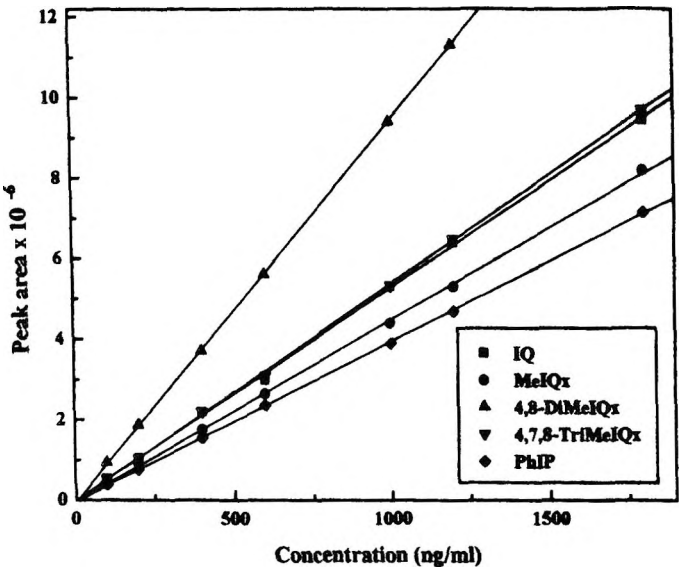

Figure 3. Linearity graphs of detector response, as peak areas vs concentration of heterocyclic amines in ng/mL. The detector response corresponds to the following equations: IQ, y= 2763.6 + 10281.5 x (sd= 95856.8); MeIQx, y= -16335.1+ 710.0 x (sd=14004.1); 4,7-DiMeIQx, y= -329592.7 + 14305.2 x (sd=852746.7); 4,7,8-TriMeIQx, y= -45012.5 + 3908.3 x (sd=215007.7) and PhIP, y= -233307.5 + 7257.8 x (sd=257854.5).

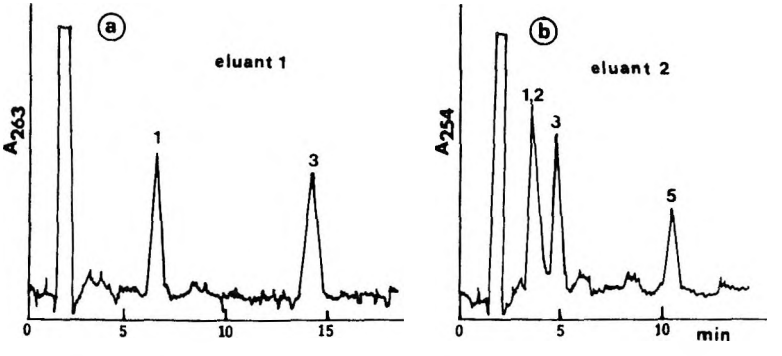

Figure 4. Analysis of mixtures containing unknown heterocyclic amines using eluant 1 (a) and eluant 2 (b). Compounds detected in chromatogram (a) are IQ (peak 1) and 4,8-DiMeIQx (peak 3) and in chromatogram (b) are IQ and/or MeIQx (peak 1, 2), 4,8-DiMeIQx (peak 3) and PhIP (peak 5). Eluants and conditions as in Fig.1. The injected amounts as estimated from the chromatogram and the linearity plots were 10.5 ng for IQ, 10.1 ng for 4,7-DiMeIQx and 12.85 ng for PhIP.

CONCLUSIONS

Identification of the type and chemical analysis of mutagenic and carcinogenic heterocyclic amines present in heat-processed food, are of great value in estimating the risk of these compounds to human beings. Description of a simple reversed phase isocratic HPLC-UV method for the separation and determination of five heterocyclic amines is presented in this report. Tests for linearity, sensitivity, precision and applicability of the method show, that this procedure can be reliably applied for the accurate and reproducible analysis of these constituents. Analysis of heterocyclic amines in heat-processed food can be performed after a successful extraction and clean-up procedure, according to the methods established by Galceran et al.[13] and Gross et al.[14]

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. III. THE EFFECT OF A COMPETING BASE, AND SEPARATIONS WITH AN ION PAIRING AGENT

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ABSTRACT

The effect of tertiary butyl ammonium phosphate as a competing base has been investigated in the reverse phase separation of twenty-two sulphonamides (SFA) and three commonly used dihydrofolate reductase inhibitors (DHFR). At the concentrations of t-butyl ammonium phosphate examined, the retention of the DHFR was dramatically reduced, but did not aid the separation. The effects on the SFA were inconsistent with the known $pK_{a,1}$ data and suggested either more complex mechanisms of interaction with the stationary phase or some doubt regarding the $pK_{a,1}$ values.

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Heptane sulphonic acid (HPSA) was tested as an ion pairing agent. Large effects were observed for the three DHFR but only a few of the SFA. Again, the results were inconsistent with the known $pK_{a,1}$ data. Low concentrations of HPSA were found to be most useful, and slightly different concentrations had significantly different effects in several parts of the chromatogram. Thus, 0.001 M phosphate buffers around pH 2.9 were modified with 0.5 and 1.0 mM HPSA and combined with previously determined MEOH gradients. Sulphamoxole and sulphamethizole were inseparable, but all other pairs of compounds had $R_s \geq 0.9$. The result is clearly superior to any previous HPLC separation .

INTRODUCTION

In previous papers on the reverse phase retention behaviour of twenty-two sulphonamides(SFA) and the three commonly used dihydrofolate reductase inhibitors(DHFR), we have demonstrated[1,2] that most combinations of the drugs are separable. In the context of a total separation of the 25 drugs in a reasonable analysis time, there are two commonly recurring difficulties. Sulphathiazole(ST, usually eluting as the eighth sulphonamide) and sulpha-pyridine(SP, 9) were frequently not separated. The other group of drugs that were difficult to separate under the majority of conditions investigated, were sulphameter(SM, 11), sulphamoxole(SAM, 12), sulphamethazine(SMAZ, 13), diaveridine(DVD, 14) and sulphamethizole(SMIZ, 15). These five compounds were generally tightly bunched, usually included more than one coelution and often overlapped with the next compounds to elute.

In methanol modified mobile phases[1], the optimum pH[3,4] was confirmed to lie between 2.7 and 3. Twenty peaks were commonly discernable, and in the best case, all but two compounds could be distinguished. SAM(12) and SMAZ(13) were coincident, and, ST(8) and SP(9) were only partly resolved. However, the other 21 drugs and the commonly observed hydrolysis product (of SAM) and the one usually hidden were both clearly resolved. Although no results were shown for acetate, separations in these buffers were inferior to those in phosphate. For multiple drug analyses, there were several generalisations that could be made. Firstly, at low phosphate concentrations (0.001 M) the DHFR are eluted much earlier. This removes DVD from the crowded central section of the chromatogram (although there is still one multiple elution in the middle). With an appropriate gradient, the last 10 drugs elute almost perfectly. On the other hand, the front of the chromatogram is not as good under these conditions. Secondly, at higher phosphate concentrations (0.01-0.1 M), the front end of the chromatogram is improved at the expense of the back end. ST and SP show some degree of separation. Thirdly, higher pHs

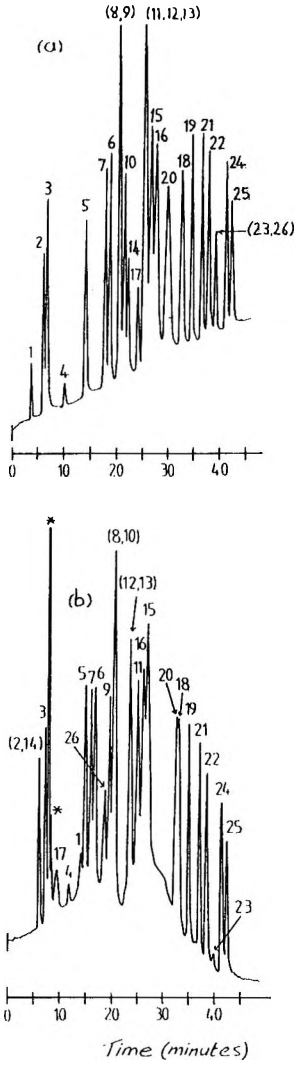

Figure 1. Complex ternary gradient and flow programmed elution (see Table 1) at pH 2.80. Chromatogram (a) was obtained without TBA and (b) was obtained with 0.1 g/L of TBA added to the aqueous buffer. The compounds are: **(1)**SNAC, **(2)**SG, **(3)**SAN, **(4)**SAM hydrolysis product, **(5)**SAC, **(6)**SDZ, **(7)**SISM, **(8)**ST, **(9)**SP, **(10)**SMRZ, **(11)**SM, **(12)**SAM, **(13)**SMAZ, **(14)**DVD, **(15)**SMIZ, **(16)**SMP, **(17)**TMP, **(18)**SCP, **(19)**SMOX, **(20)**SST, **(21)**SISX, **(22)**SB, **(23)**PST, **(24)**SDIM, **(25)**SQ, **(26)**PYR.

are generally unfavourable and lead to excessively congested chromatograms. However, there is one exception. Due to fortuitous differences in the pK_a,[2] values, the most difficult to separate group (11-15) are baseline resolved from each other at pH 6.5.

In acetonitrile modified solutions,[2] separations are not quite as good, but some minor selectivity differences do occur. Attempts to incorporate the beneficial differences into MEOH gradients were unsuccessful, apparently due to complex chromatographic behaviour. Returning to MEOH modified mobile phases, flow programming was successful in the resolution of the first 13 compounds, including the seldom separated ST and SP ($R_s \approx 1.1$)[5].

Separation of all 25 drugs simultaneously has not been achieved (Fig. 1). Hence, we have investigated other solution variables. Although the stationary phase was end-capped and the peaks did not show excessive tailing, the effect of a competing base is sometimes unpredictable, such that advantageous selectivities may arise. Hence, in this paper we examine this effect, and finally, ion pairing.

EXPERIMENTAL

With the exception of the laboratory reagent tetrabutyl ammomium phosphate (TBA; Sigma, St. Louis, MO) and the reagent grade heptane sulphonic acid (HPSA; Aldrich, Milwaukee, WI), all chemicals, equipment and experimental methods were as previously described.[1,2] The pH was set at 2.80 (unless otherwise specified) and 0.001 M phosphate buffer was used in all experiments. In the case of TBA (0.1 g/L) and HPSA, the additional component was added to the aqueous buffer before pH adjustment. The column oven was set to 33 Celcius for all experiments.

The full names of the DHFR, their abbreviations and pK_a values are given in the results and discussion section where they are required and the equivalent data for the SFA is given in Table 2. The structures of all of the drugs have been listed previously.[6]

RESULTS AND DISCUSSION

The Effect of a Competing Base

The gradient used to examine the effect is given in Table 1. It is a complex ternary gradient involving both MEOH and ACN, and flow programming. Its choice is not significant. It happened to be the last of such

Table 1

Gradient Program Used for the Chromatograms Shown in Figure 1

Time (Minutes)	% 0.001 M Phosphate	% MeOH	% ACN	Flow Rate (mL/Min)
0	100	0	0	1.0
5	95	0	5	0.7
9.5	87	5	8	1.0
15	81	8	11	1.0
20	75	10	15	0.8
25	73	11	16	0.6
30	71	12	17	0.2
35	67	15	18	1.0
45	76	0	24	1.0
50	70	0	30	1.0

runs done at the time. Figure 1(a) is the reference chromatogram and Figure 1(b) shows the result of the addition of 0.1 g/L of TBA to the aqueous phase. The peaks marked with an asterisk in Figure 1(b) are impurities in the TBA.

In spite of using an end-capped stationary phase with good peak symmmetry indicating minimal evidence of mixed retention mechanisms, the retention times of the DHFR are dramatically reduced in the presence of the competing base. Taking sulphanilic acid (SA, 1) as an indication of t_m (Figure 1(a), ~4 minutes), the amount of retention (t_R') is reduced from 18 to 2 minutes for diaveridine (DVD, compound number 14), 20 to 5½ minutes for trimethoprim (TMP, 17) and 35 to 14½ minutes for pyrimethamine (PYR, 26). This clearly indicates that the principle retention mechanism for these drugs involves interaction with the surface silanols. As the three DHFR are all 2,4-diaminopyrimidines it would be expected that their ion exchange, ion-dipole and/or dipole-dipole interactions with silanol groups would be similar. The reductions in retention caused by the addition of the TBA are of similar magnitudes (16, 14½ and 20½ minutes) as expected. With $pK_{a,1}$ values close to 1.3,[7] $pK_{a,2}$ = 6.6 and 7.0 for TMP and PYR, respectively, and each pK_a refering to a deprotonation of a pyrimidine ring nitrogen, at the mobile phase pH of 2.80, the DHFR carry an average charge around +1.03. Hence, ion exchange is possible at underivatised surface silanol sites. This is consistent with the possible 'adsorption' of the DHFR on the walls of untreated silica capillaries in CZE (at pH 2.1)[7], and the adsorption of other cationic species on silica at low pH[8,9]. A full discussion of this and alternative possible interactions was given.[7]

Table 2

Changes in Retention for the Sulphonamides in the Presence of TBA

Sulphonamide (Abbreviation)	Number	Change in Retention (minutes)	$pK_{a,1}$	$pK_{a,2}$	Charge at pH 2.80
Sulphamethoxypyridazine (SMP)	16	-2	n/a	6.7	---
Sulphadiazine (SDZ)	6	-2	2.0	6.5	+.14
Sulphisomidine (SISM)	7	-2	n/a	n/a	---
Sulphamoxole (SAM)	12	-1½	n/a	n/a	---
Sulphamethazine (SMAZ)	13	-1½	2.4	7.4	+.28
Sulphapyridine (SP)	9	-1	2.6	8.4	+.39
Sulphamerazine (SMRZ)	10	-1	2.3	7.0	+.24
Sulphameter (SM)	11	-½	n/a	6.8	---
Sulphaguanidine (SG)	2	0	n/a	11.3	---
Sulphanilamide (SAN)	3	0	2.4	10.4	+.28
Sulphathiazole (ST)	8	0	n/a	7.2	---
Sulphamethizole (SMIZ)	15	0	n/a	5.4	---
Sulphachloropyridazine (SCP)	18	0	n/a	5.5	---
Sulphamethoxazole (SMOX)	19	0	n/a	5.6	---
Sulphisoxazole (SISX)	21	0	1.5	5.1	+.05
Sulphabenzamide (SB)	22	0	1.8	4.6	+.09, -.02
Phytalyl sulphathiazole (PST)	23	0	2.9 (ca)	7.2 (ca)	-.44
Sulphadimethoxine (SDIM)	24	0	n/a	6.2	---
Sulphaquinoxaline (SQ)	25	0	n/a	5.5	---
Sulphacetamide (SAC)	5	+1	1.8	5.4	+.09
SAM hydrolysis product (HP)	4	+1½	n/a	n/a	---
Succinyl sulphathiazole (SST)	20	+2	4.2 (ca)	7.2 (ca)	-.04
Sulphanilic acid (SNAC)	1	+10	n/a	3.2	-1, +.7

Some of the cationic SFA also display reduced retention in the presence of the TBA. Table 2 (column 3) lists the SFA in order from the greatest reduction in retention to the least. All of the losses in retention are small, and where pK_a data is available, it is clear that the compounds experiencing reduced retention due to the presence of TBA are all partly in the form of the positively charged conjugate acid. However, there appears not to be any correlation between the exact magnitudes of the losses of retention and the average charges (last column). Furthermore, SAN has the same $pK_{a,1}$ (and therefore positive charge) as SMAZ but is unaffected by the TBA presence. The effect of the competing base is certainly a marginal effect on the SFA, with the majority having exactly the same retention times in the presence of TBA as they had in its absence. With the exception of SNAC, SST, HP and PST, all of the SFA are 4-aminobenzenesolphonamides and at low pH are partly protonated on the 4-amino substituent. As this protonation is so far removed from the point of substitution on the amide that distinguishes the molecules, it is not suprising that

the $pK_{a,1}$ values fall in such a small band, and that the size of the effect of substitution is not highly variable. (Those SFA for which a $pK_{a,1}$ is not available (n/a) would be expected to have values in the same range.) With the exception of SAN, it appears that the more positively charged SFA are affected by the presence of TBA, and by implication undergo small, but significant, interactions with surface silanols, whilst those with smaller positive charges do not (SISX, SB and SAC). This indicates that the nature of the SFA-silanol interaction may be ion exchange.

At the bottom of the third column of Table 2 there are four SFA which experience increases in retention in the presence of TBA. This is presumed to be due to 'ion pairing' and is an effect often observed in the presence of competing bases.[10] For this to happen, a negative charge is required on the analyte. SNAC, which is a relatively strong acid, exists as a zwitterion below the pH range for the deprotonation of the positively charged 4-amino group ($pK_{a,2}$ = 3.2).[11] Thus, at pH 2.8, SNAC is 30% anionic and retention is dramatically increased from ~0 to 10 minutes. SST is only 4% anionic and the increase in retention is only 2 minutes. PST is the other SFA which would be expected to 'ion pair' with TBA. No effect is observed. It should be noted that, for SST and PST the $pK_{a,1}$ and $pK_{a,2}$ values are assumed to be the same as for the component parts and the values relevent to the calculation of the charge at pH 2.8 ($pK_{a,1}$) are most likely to be different. From the pH dependence of retention in methanolic mobile phases[1], the observed inflection points indicate $pK_{a,1}$ values of ~4.5 for SST and ~3.75 for PST. These values would greatly decrease the negative charges on both of these SFA, but do not rectify the inverse correlation between charge and the extent of increased retention for SST and PST. Worse still, the revised $pK_{a,2}$ value of ~4.5 for SST would reduce the charge to -.02, in which case any significant 'ion pairing' might be suprising.

Overall then, whilst it does seem clear that TBA exerts the conventional effect of a competing base and negatively charged analytes may be 'ion paired' with it, the lack of quantitative correlation in each case and the exceptions, indicate that the retention mechanisms and perhaps their mutual modification may be much more complex. These factors also bring into question the very basis for the above analysis, which was, that the identical retention times for half of the SFA in the absence and presence of TBA could be assumed to be due to the absence of both of these effects. Perhaps it is a balancing of multiple, more complex retention mechanisms that leads to the zero net effects and specific molecular characteristics that lead to the unexpected imbalances that provide the exceptions.

In terms of the attempted separation, there were three coelutions containing seven compounds in the absence of TBA, whereas there were four coelutions containing eight compounds in the presence of TBA. The separation

Table 3

Ternary Gradient Used to Generate the Data Shown in Figures 2 and 3 The Ion Pairing Agent Heptane Sulphonate was Included in the Aqueous Phase.

Time (minutes)	% 0.001 M Phosphate	% MeOH	% ACN
0	100	0	0
0.01	95	5	0
7	92	8	0
7.01	96	0	4
21	96	0	4
26	84	16	0
31	82	18	0
36	70	30	0
60	70	30	0
60.01	50	0	50
90	50	0	50
110	0	0	100

of compound 11 from 12 & 13 and the removal of SNAC(1) from the vicinity of the solvent front were advantages obtained by the inclusion of TBA in the mobile phase. Overall, there was little net gain, but the highly selective nature of the effects of adding the competing base make it a potentially useful adjunct to separation in appropriate circumstances.

Ion Pairing

Ion pairing (IP) is the term used to describe enhanced retention as the result of the addition to the mobile phase of a large ion of opposite charge to the molecular ions to be separated. The ion pairing agent (IPA) used is generally a bulky, buried charge alkyl ammonium ion of the general form $R_1R_2R_3R_4N^+$ for the increased retention of molecular anions. TBA is typical.

For molecular cations, a dispersed charge anion with a non polar tail such as alkyl sulphates or sulphonates are generally utilised. In this study, heptane sulphonate (HPS^-) was used. These IPA are chosen because they have a great deal of non polar character and will be trapped in equilibrium concentrations in association with the non polar stationary phase. Due to the requirement for electroneutrality, the HPS^- counter ions will also be present in the modified

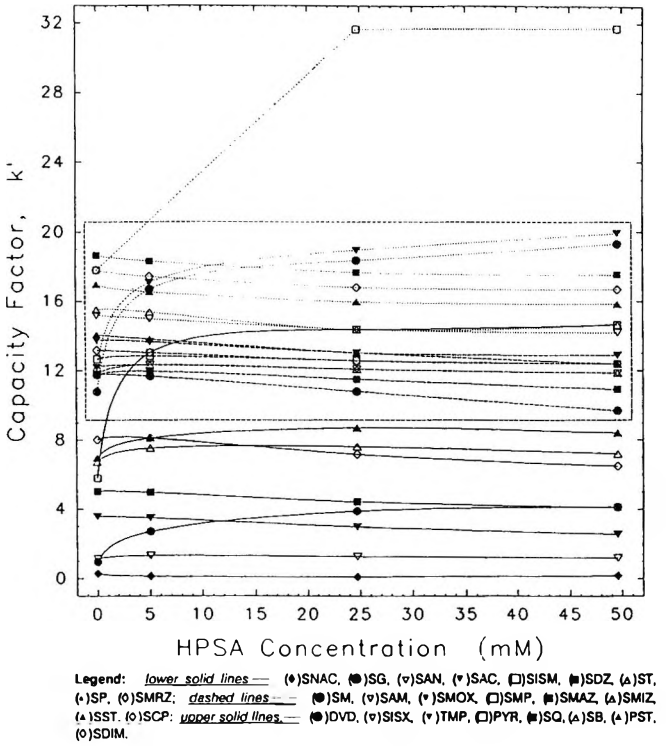

Figure 2. Plots of k' vs the concentration of HPSA for the 22 SFA and 3 DHFR compounds. The gradient used is given in Table 3 and the pH was 2.80.

mobile phase making the process of retention enhancement more one of ion exchange than IP. However, that is the common terminology and we will use it without further qualification.

Table 3 is the (ternary) gradient used for the initial examination of the effect of the IPA. HPSA was added to the aqueous buffer phase (only) prior to pH adjustment. Figure 2 shows the variation of the capacity factor, k', versus the concentration of HPSA. The crowded (boxed) central section is enlarged in Figure 3. Again, the data are not straight forward. SNAC, SST and PST have net negative charges (Table 2) and would therefore be expected to experience some repulsion from the stationary phase modified by the presence of HPS^-. All are progressively less well retained as the $[HPS^-]$ increases, but PST and SST display very similar behaviour in spite of appearing to have very different magnitudes of charge. Moreover, most of the SFA exhibit the same decreases in

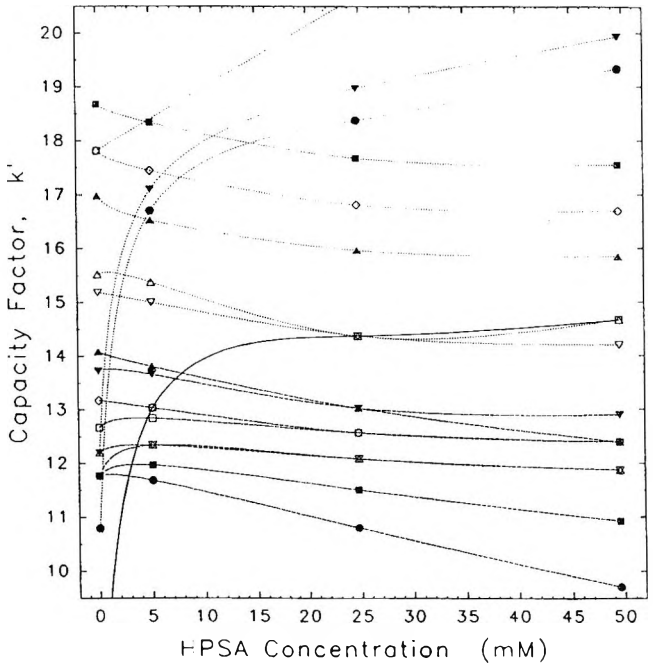

Figure 3. Enlargement of the boxed area in Figure 2 showing plots of k' vs the concentration of HPSA for the SFA and DHFR. All conditions and legends are as in Figure 2.

retention as [HPS$^-$] increases in spite of their positive charges (see Table 3 and the discussion in the previous section). We are unable to explain these results. Large increases in retention of the DHFR are observed as [HPS$^-$] increases, as expected. A proportionally larger increase occurs for SISM, but unfortunately there is no $pK_{a,1}$ data in this case. A high value is indicated. This is also true for SG. SP with the largest $pK_{a,1}$ value and positive charge (of the SFA) has increased retention, but SMAZ and SMRZ - with similar charges - do not.

With several compounds displaying each different type of observed behaviour, there are multiple crossovers in the k' vs. [HPSA] plot. As was noted in the ACN work[2], this provides good separations of some pairs that have been hitherto difficult or impossible under most or all circumstances (for example, SG and SAN, and, SP and ST); but it also provides a multitude of new coelutions and the fusion of several k' vs. [HPSA] plots.

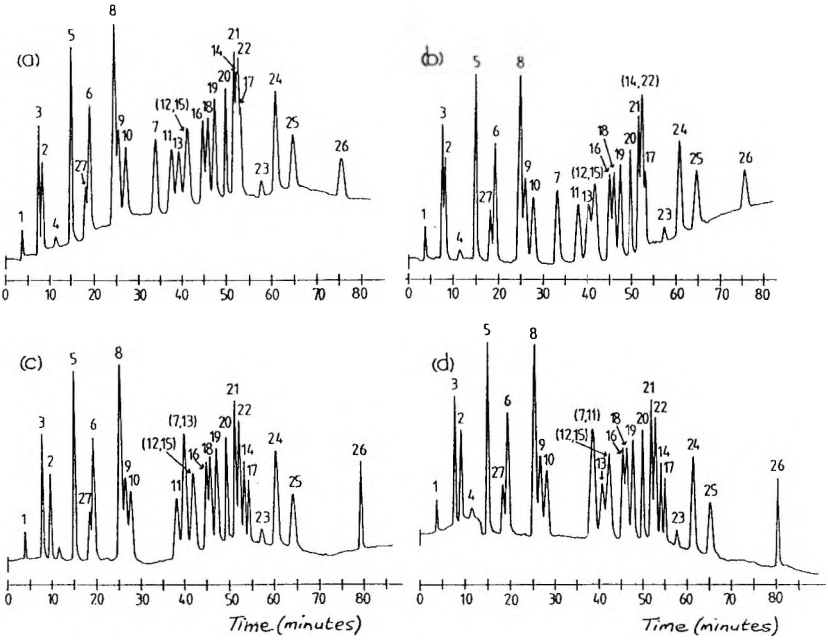

Figure 4. Chromatograms obtained using the binary MEOH gradient in Table 4. The aqueous buffers were (a) pH 2.86 and (b) pH 2.97, and were both 0.5 mM with respect to HPSA. (c) pH 2.85 and (d) pH 2.96 were 1.0 mM with respect to HPSA. The compounds are numbered as in Figure 1. **27** is the second SAM hydrolysis product.

Table 4

Binary Gradient Used to Generate the Chromatograms Shown in Figures 4(a) - (d).

Time (minutes)	% 0.001 M Phosphate + HPSA	% MeOH
0	100	0
3	92	8
30	88	12
35	82	18
40	70	30
45	70	30
55	40	60

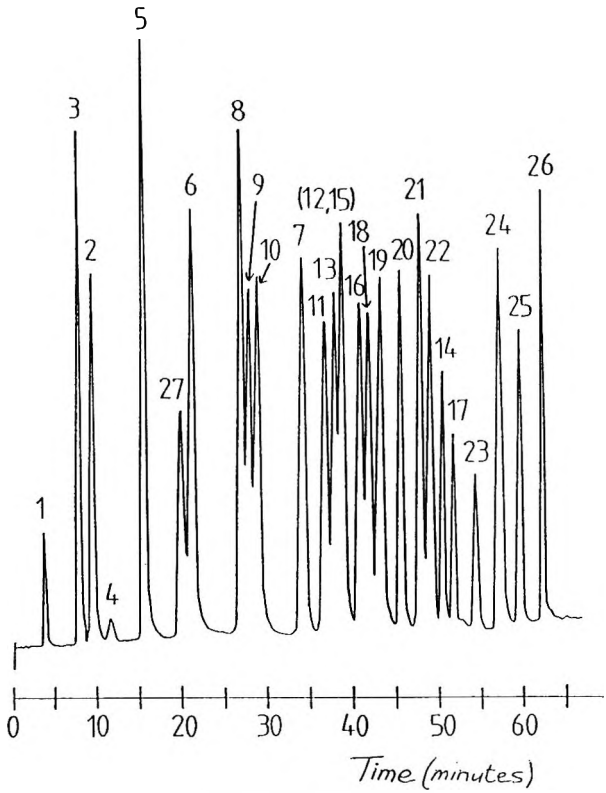

Figure 5. The chromatogram obtained using the solvent program given in Table 5. Alternative concentrations of 0.5 and 1.0 mM HPSA are used in various parts of the program. The numbering of the compounds is as in Figure 1. **27** is the second SAM hydrolysis product.

For example, the SISX and SB plots merge over much of the middle of the [HPSA] range and SISM, which is generally well removed from SISX and SB, is also coeluted. Unfortunately, several such occurences can be seen over the [HPSA] range examined and runs at intermediate [HPSA]s were generally disappointing.

However, at low [HPSA] few coelutions remained. In 1 mM HPSA there were only two and the crowded central section of the chromatogram contained many largely resolved peaks. Thus, the net result approached the best previously achieved. Hence, further low [HPSA] conditions were investigated. Table 4 gives a previously utilised binary (MEOH) gradient into which HPSA

Table 5

Gradient Used to Generate the Chromatogram Shown in Figure 5

Time (minutes)	% 0.001 M Phosphate + 1.0 mM HPSA	% MeOH	% 0.001 M Phosphate + 0.5 mM HPSA
0	100	0	0
0.01	95	5	0
8	93	7	0
8.01	0	7	93
20	0	11	89
20.01	89	11	0
30	84	16	0
40	70	30	0
45	65	35	0
50	30	70	0

was introduced. Figures 4(a)-(d) show the chromatograms: (a) and (b) employed 0.5 mM HPSA in the aqueous phase and had exactly measured pHs of 2.86 and 2.97, respectively, whilst (c) and (d) employed 1.0 mM HPSA in the aqueous phase and had exactly measured pHs of 2.85 and 2.98, respectively. Due to the high mobility of SISM(7) in the chromatograms - both with respect to [HPSA] and pH - it is clear that the group of compounds 7-15 are better eluted in 0.5 mM HPSA. On the other hand, the SAN(3) & SG(2) pair and the group of compounds 14, 21, 22 & 17 are far better separated in 1.0 mM HPSA, and, ST(8) & SP(9) are also better resolved, albiet at some loss of resolution between SP(9) & SMRZ(10). This loss is minimised at the slightly higher pH. The separation of the second SAM hydrolysis product(27) from SDZ(6) was also favoured by the slightly higher pH.

Consequently, further chromatograms were run at the slightly higher pH, using gradients modified to incorporate the benefits of both the 0.5 and 1.0 mM HPSA concentrations. The results were as expected, and Table 5 gives the final gradient in which the MEOH concentrations are increased to bring the total run times back from about 80 minutes to under 65. The chromatogram is given in Figure 5.

Unfortunately, one coelution remains in the crowded central section. Otherwise, the least resolved pairs are (27 & 6) and (8 & 9), $R_s \approx 1.0$; (9 & 10), (13 & 12/15) and (16 & 18), $R_s \approx 0.9$.[5] Overall, it is clearly the best SFA separation obtained to date.

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HIGH SPEED HPLC DETERMINATION OF BIS(PIVALOYLOXYMETHYL)-PMEA AND ITS DEGRADATION PRODUCTS, MONO(POM)-PMEA AND PMEA

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ABSTRACT

A high-speed HPLC gradient method for the determination of the antiviral ester prodrug bis(POM)-PMEA and its degradation products mono(POM)-PMEA and PMEA, is described. Perfusion chromatography enabled us to increase the flow rate up to 4 mL/min, resulting in high sample turn-over. Using a gradient system, the three compounds were separated from each other and the sample matrix within six minutes, while column equilibration was obtained in less than 3 minutes. Tetrabutylammonium was used as a counterion for PMEA. Notwithstanding the high flow rate, excellent reproducibility was obtained: the intraday as well as the interday precision, expressed as the relative standard deviation, for concentrations ranging from 5 to 50 μM PMEA and bis(POM)-PMEA, was lower than 5%. The applicability of the method is demonstrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA.

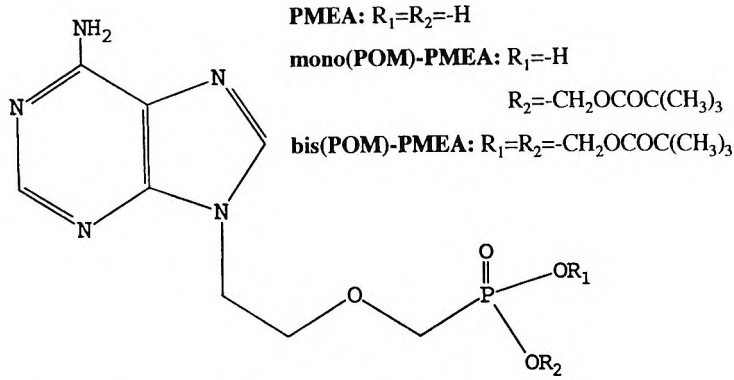

Figure 1. Structure of PMEA and bis(POM)-PMEA.

INTRODUCTION

9-(2-phosphonylmethoxyethyl)adenine (PMEA) is an acyclic nucleoside phosphonate analogue (Figure 1), which shows promising antiviral activity against herpes-, retro- and hepadnaviruses.[1-4] Unfortunately, oral bioavailability is limited by low permeability through biological membranes. Therefore, several ester prodrugs have been developed which show enhanced membrane permeability.[5-7] The bis(pivaloyloxymethyl) ester of PMEA (bis(POM)-PMEA, Figure 1) is such an oral prodrug of PMEA.[8] It is presently in clinical trials in patients infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV). Bis(POM)-PMEA is converted to mono(POM)-PMEA by enzymatic and chemical degradation, and further, to PMEA by enzyme-catalysed ester hydrolysis.[6] Concentrations of bis(POM)-PMEA and its degradation products can be measured using an HPLC system with UV detection (260 nm). The difference in polarity of the compounds to be determined, requires the use of a gradient system. Disadvantages of a gradient method using a conventional column are a relatively long analysis time, followed by extended column re-equilibration. As pharmacokinetic studies, as well as stability studies, yield a large number of samples to be analysed, a long analysis time results in long apparatus occupation times.

In this study, a high-speed HPLC method is presented for the simultaneous determination of bis(POM)-PMEA, mono(POM)-PMEA and PMEA by using perfusion chromatography. The applicability of the analysis method described is illustrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA.

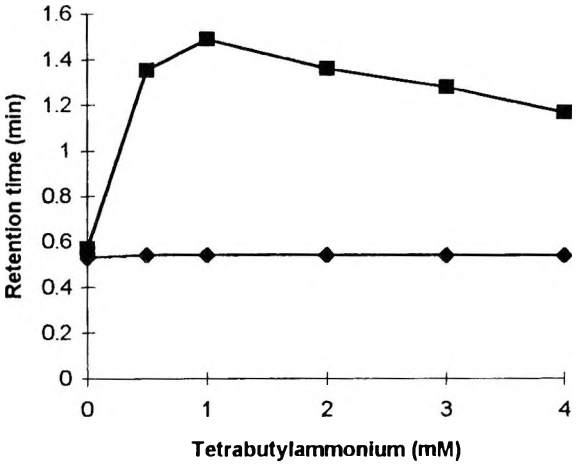

Figure 2. Effect of the concentration of tetrabutylammonium in the mobile phase on the retention of PMEA (potassium phosphate 2 mM, pH 9.0, flow rate 3 mL/min) (◆ : solvent peak; ■: PMEA).

MATERIALS AND METHODS

Reagents

Bis(POM)-PMEA was synthesized at Gilead Sciences. PMEA was kindly provided by Dr. A. Holý (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic). Acetonitrile (far UV grade) was purchased from BDH (Poole, England). Tetrabutylammonium dihydrogen phosphate (99 %) and potassium dihydrogen phosphate (Suprapur) were from Fluka (Neu-Ulm, Switzerland) and Merck (Darmstadt, Germany), respectively.

Chromatographic Conditions

The HPLC system was equipped with a Waters (Millford, Mass.) Model 600 Controller, Model 717 plus auto-injector and a Model 480 Lambda Max UV detector (260 nm). Chromatograms were recorded and processed using the Maxima 820 computer programme (Waters). The column used was a 4.6-mm diameter x 100 mm length (1.7 mL column bed volume) POROS© R1/H (PerSeptive Biosystems, Cambridge, MA). The packing consisted of 10 µm

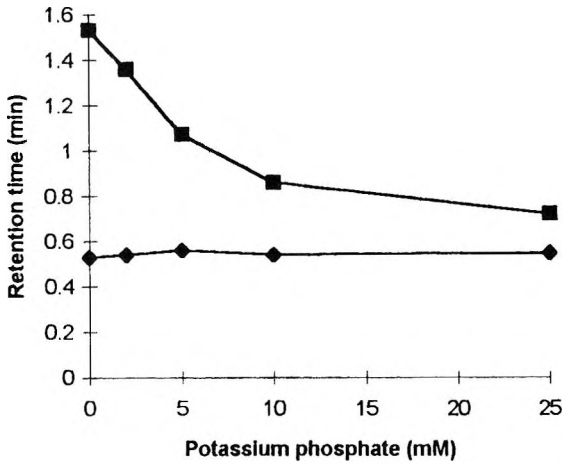

Figure 3. Effect of the concentration of potassium phosphate in the mobile phase on the retention of PMEA (tetrabutylammonium 2 mM, pH 9.0, flow rate 3 mL/min) (◆ : solvent peak; ■ : PMEA).

flow-through particles of cross-linked poly(styrene-divinylbenzene). Mobile phase A was an aqueous buffer containing 1 mM phosphate and 0.5 mM tetrabutylammonium (pH adjusted to 8.0 with NH_3 5%). Mobile phase B contained 30 % acetonitrile and 70 % water. Separation was carried out with an initial isocratic stage of 1 min in mobile phase A and a linear gradient from 3 to 90 % mobile phase B over 3.6 min, followed by an isocratic stage of 0.8 min and a return to initial conditions over 0.8 min. Column re-equilibration in solvent A took 2.8 min. The flow rate of the mobile phase varied between 3 and 4 mL/min. Both mobile phases were filtered and degassed continuously by He sparging. Samples were stored at a temperature of 4 °C in the autosampler, while the separation was carried out at ambient temperature. The injection volume amounted to 25 µL. System back pressure in mobile phase A was approximately 600 psi.

Stock Solutions and Calculations

Solutions of PMEA and bis(POM)-PMEA were made up in an isotonic buffer solution (pH as mentioned in Figure legends). Concentrations of PMEA and bis(POM)-PMEA were determined using a calibration curve made up by standards, in which concentration was plotted against peak area. No internal

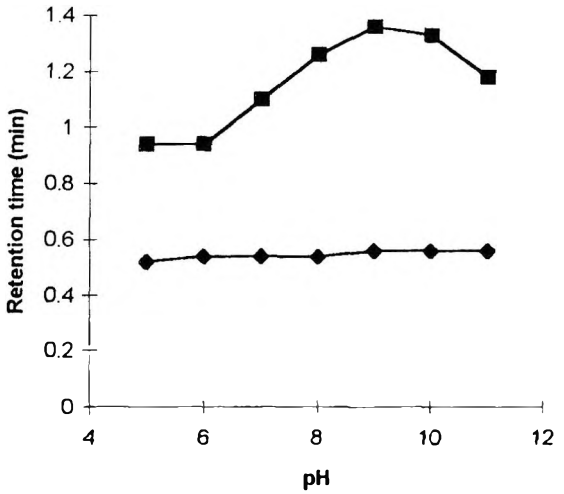

Figure 4. Effect of the pH of the mobile phase on the retention of PMEA (potassium phosphate 2 mM, tetrabutylammonium 2 mM, flow rate 3 mL/min)(◆: solvent peak; ■ : PMEA).

standard was used. Concentrations of mono(POM)-PMEA were determined using the calibration curve of bis(POM)-PMEA, because at the time of method development no mono(POM)-PMEA was available. This was justified because no change in UV response could be observed when bis(POM)-PMEA was gradually degraded into mono(POM)-PMEA.

RESULTS AND DISCUSSION

PMEA offers great promise as antiviral agent because of its broad-spectrum activity against retro-, herpes- and hepadnaviruses. However, like other acyclic nucleoside phosphonates, PMEA shows low cellular uptake and poor oral bioavailability. Therefore, prodrugs have been developed with improved cellular uptake and oral bioavailability. This prodrug approach has yielded bis(POM)-PMEA. This compound had originally been selected as the lead compound out of a series of prodrugs because of its permeability characteristics across Caco-2 monolayers,[9] which is an in vitro model for the intestinal mucosa.[10] Oral bis(POM)-PMEA has proved effective in suppressing murine retroviral infections in SCID mice;[11] following oral administration of bis(POM)-PMEA, only PMEA, but not bis(POM)-PMEA, was recovered from plasma, which suggests that, following uptake by the intestinal mucosa, bis(POM)-PMEA was readily cleaved to free PMEA (oral bioavalability being

53 %). Indeed, in aqueous solution, bis(POM)-PMEA is chemically degraded to mono(POM)-PMEA, while, in biological systems, bis(POM)-PMEA is first degraded to mono(POM)-PMEA and further on to PMEA by enzyme-catalysed ester hydrolysis.

Determination of the concentrations of these compounds is usually performed by HPLC.[11] Polarity differences between the parent compound and its metabolites necessitate the use of a gradient system. Disadvantage of a conventional column when using a gradient, are the relatively long analysis time and extended re-equilibration after returning to the initial conditions. To increase sample turn-over, a high speed HPLC gradient method was developed using perfusion chromatography. This enabled us to reduce analysis time without loosing resolution or reproducibility by increasing the flow rate of the mobile phase. High flow rates are made possible by the bimodal pore structure of the packing material, which is a base matrix of poly(styrene-divinylbenzene). Through-pores of 6000-8000 Å allow the mobile phase to flow through the packing material, while diffusion is possible in smaller pores (800-1500 Å) that are connected to them.[12] The presence of the pores allows high speed analysis, without a dramatic increase in backpressure.

In a first set of experiments, the effect of various separation parameters, relating to both mobile phase and sample were investigated. The retention of PMEA (pKa_1 = 2.0; pKa_2 = 6.8) on the column, was strongly dependent on the concentration of tetrabutylammonium as a counterion for PMEA, the concentration of phosphate ions and the pH of the mobile phase. The influence of different experimental conditions on the retention of PMEA was investigated in the isocratic mode at a flow rate of 3 mL/min. Increasing the concentration of the counterion resulted in a satisfactory separation at 0.5 mM, reaching a maximum retention at a concentration of 1 mM (Figure 2; other parameters: 2 mM phosphate, pH 9.0). Potassium phosphate reduced the retention of PMEA in the presence of counterion, which indicated that a low capacity buffer had to be used (Figure 3; other conditions: tetrabutylammonium 2 mM, pH 9.0). As the column material allowed an increase of the pH of the mobile phase to 14, the pH effect could be studied over a broad pH range.

Optimum separation from the sample matrix was obtained at pH 9.0 (Figure 4; other conditions: tetrabutylammonium 2 mM, phosphate 2 mM). However, this high pH could not be used for the simultaneous determination of PMEA and its parent compound bis(POM)-PMEA when using a gradient system, because on column degradation of bis(POM)-PMEA was observed at this high pH. Based on these observations, mobile phase A was composed of 0.5 mM counterion, 1 mM phosphate and a pH of 8.0. After an isocratic stage of 1 min at the initial conditions (3 mL/min), the gradient was started as illustrated in Figure 5 (upper panel). Perfusion chromatography enabled us to

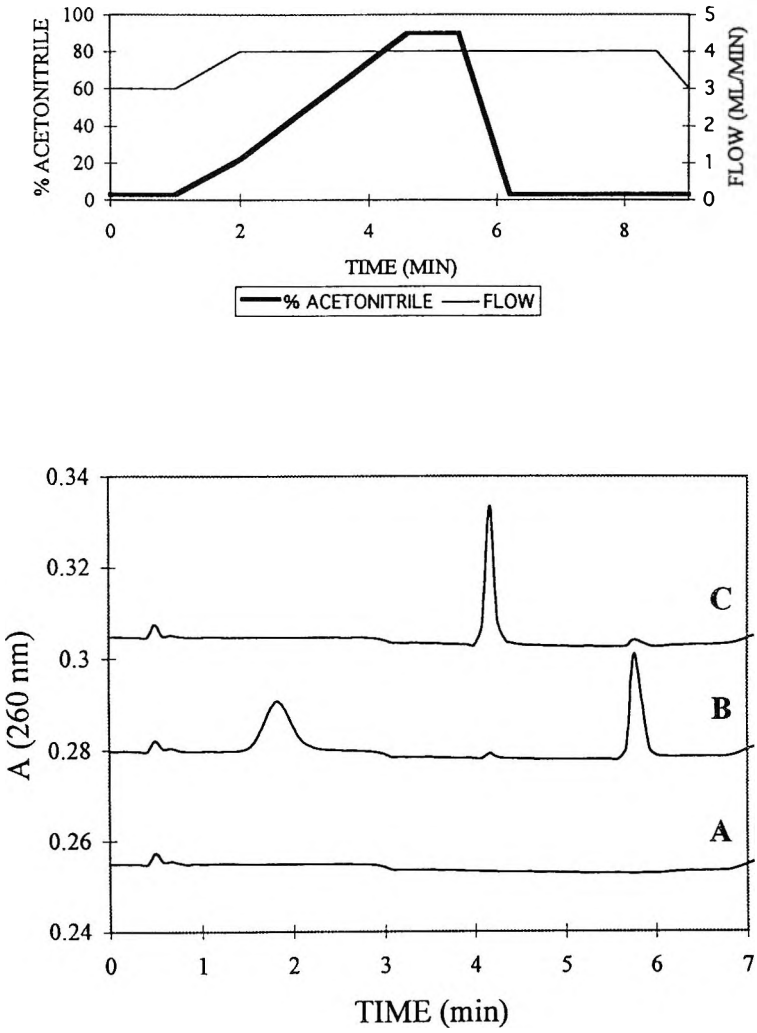

Figure 5. Top panel: Time course of the gradient and flow rate. Lower panel: chromatograms of a blank sample (A), a mixed PMEA / bis(POM)-PMEA sample (B), and a mono(POM)-PMEA sample (C) (PMEA, rt = 1.8 min; mono(POM)-PMEA, rt = 4.1 min; bis(POM)-PMEA, rt = 5.7 min).

Table 1

Reproducibility of PMEA Analysis

Concentration Added (μM)	Concentration Found ± SD	RSD*
Intraday variation (n=5)		
50	50.2 ± 0.3	0.55
40	40.1 ± 0.3	0.84
30	29.8 ± 0.4	1.33
20	20.1 ± 0.2	1.16
10	9.9 ± 0.2	2.09
5	5.4 ± 0.2	3.44
Interday variation (n=7)		
50	50.5 ± 0.7	1.31
40	40.0 ± 0.6	1.38
30	29.8 ± 0.9	2.87
20	19.8 ± 0.5	2.25
10	10.3 ± 0.3	3.25
5	5.4 ± 0.2	3.82

* relative standard deviation

increase the flow rate during the gradient to 4 mL/min. PMEA, mono(POM)-PMEA and bis(POM)-PMEA were well separated from each other and the sample matrix within six minutes, while column equilibration was obtained in less than three minutes. Typical chromatograms recorded under optimized conditions are given in Figure 5 (lower panel). All samples used in this study were made up in isotonic buffers. PMEA as well as bis(POM)-PMEA showed linearity over a range of 5 to 50 μM (r^2>0.999). Detection limits were 2.5 μM PMEA and 0.625 μM bis(POM)-PMEA, corresponding to absolute amounts of approximately 60 and 15 pmol. Intraday and interday precision were determined as shown in Tables 1 and 2.

The applicability of the method is demonstrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA. Aqueous solutions of bis(POM)-PMEA are known to be unstable, with rapid hydrolysis of one of the pivaloyl ester bounds, followed by spontaneous elimination of

Table 2

Reproducibility of bis(POM)-PMEA Analysis

Concentration added (μM)	Concentration Found ± SD	RSD*
Intraday variation (n=5)		
50	49.7 ± 0.4	0.77
40	39.6 ± 0.3	0.72
30	29.5 ± 0.2	0.69
20	19.6 ± 0.2	0.77
10	9.7 ± 0.1	1.08
5	5.0 ± 0.1	1.33
Interday variation (n=7)		
50	50.4 ± 1.1	2.20
40	39.9 ±1.2	2.92
30	30.0 ± 0.9	3.00
20	20.0 ± 0.4	2.04
10	10.3 ± 0.2	1.43
5	5.4 ± 0.3	4.99

* = relative standard deviation.

formaldehyde, yielding mono(POM)-PMEA. The ester hydrolysis was monitored by a decline in parent compound and the appearance of mono(POM)-PMEA. Concentrations of mono(POM)-PMEA were determined using the calibration graph of bis (POM)-PMEA. This was justified because no change in UV response (260 nm) could be observed when bis(POM)-PMEA was gradually degraded into mono(POM)-PMEA (data not shown). Chemical degradation of bis(POM)-PMEA as a function of time is displayed in Figure 6. The stability of aqueous bis(POM)-PMEA solutions at different pH values is illustrated in Figure 7. Bis(POM)-PMEA was shown to be stable for at least one day at pH 3. At pH 7.4, a 40 % decline of bis(POM)-PMEA was observed after an eight-hour period. An analogous experiment at varying temperatures of the aqueous solution (pH 7.4) revealed that even at 4°C, a decline of 5 % was observed after 24 hr (Figure 8). Therefore, all samples in further studies (usually pH 7.4) were

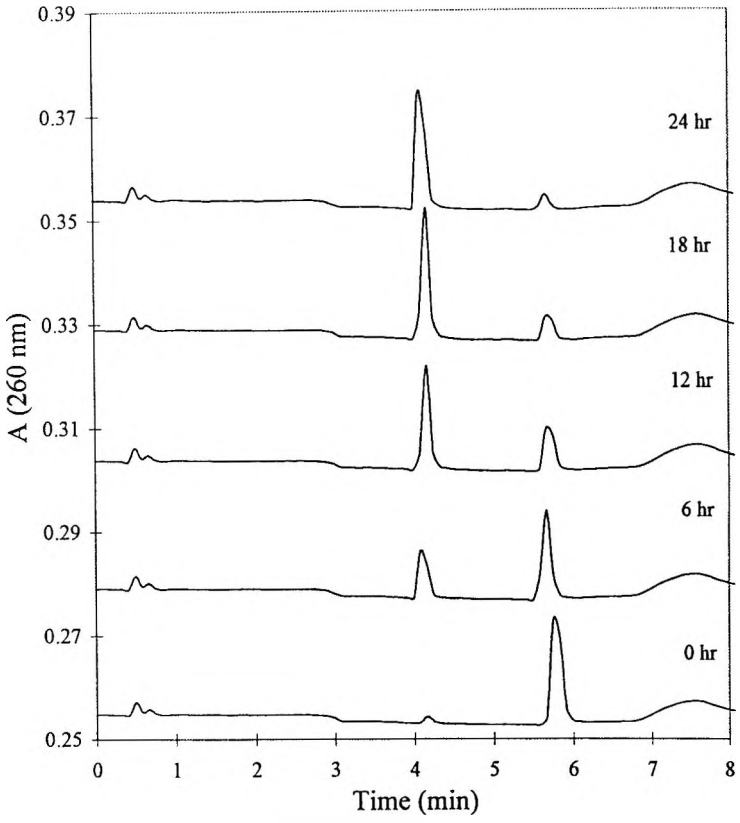

Figure 6. Chromatograms displaying the chemical degradation of bis(POM)-PMEA at a concentration of 50 μM (37°C, pH 7.4) as a function of time (mono(POM)-PMEA, rt = 4.1 min; bis(POM)-PMEA, rt = 5.7 min).

stabilized by adding 200 μL HCL 0.05 N to 800 μL sample, thus adjusting the pH to 3.3. Peak splitting of PMEA was observed if 50-μL samples were injected; therefore, the injection volume was restricted to 25 μL. The formation of PMEA was not observed in this study; the appearance of PMEA in *in vivo* or in *in vitro* studies results from enzyme-catalysed ester hydrolysis. As the back pressure was only 600 psi, it may even be possible to further increase the flow rate of the mobile phase. In conclusion, the high speed HPLC gradient method presented in this study offers sufficient sensitivity for the quality control of bis(POM)-PMEA preparations.

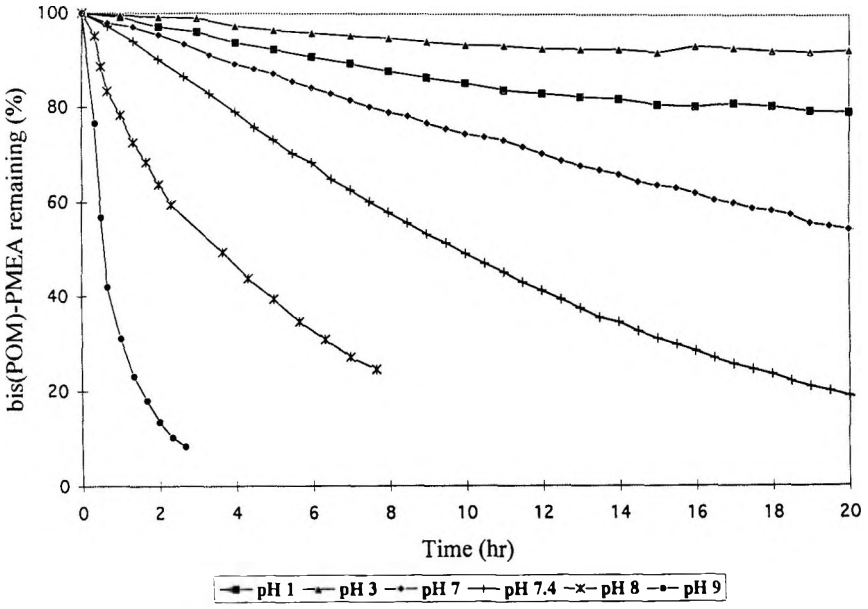

Figure 7. Influence of pH on the degradation of bis(POM)-PMEA

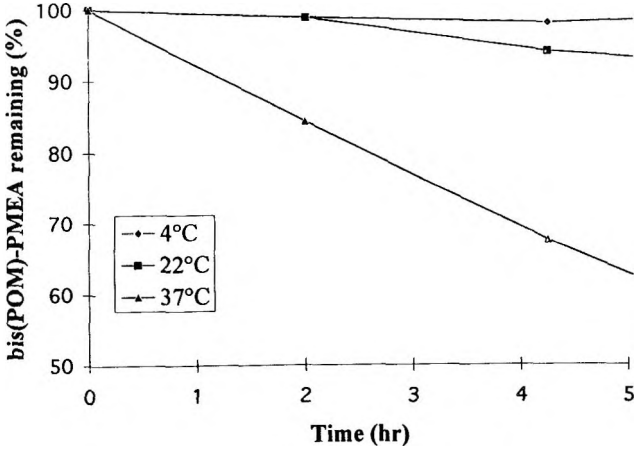

Figure 8. Influence of temperature on the degradation of bis(POM)-PMEA.

The short analysis time allows to increase the sample turn-over and to decrease the apparatus occupation time. However, the method may not provide the sensitivity needed for the analysis of samples obtained from pharmacokinetic studies *in vivo*.

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HPLC DETERMINATION OF PROPOFOL-THIOPENTAL SODIUM AND PROPOFOL-ONDANSETRON MIXTURES

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ABSTRACT

High performance liquid chromatography procedures have been developed for the assay of propofol-thiopental sodium and propofol-ondansetron mixtures. The separation and quantitation of propofol-thiopental sodium were performed on a stable bond phenyl column at ambient temperature using a mobile phase of 55:45 v/v aqueous 0.01 M monobasic postassium phosphate pH 4 - acetonitrile at a flow rate of 1 mL/min, with detection set at 235 nm. The separation was achieved within 20 min. Propofol and thiopental sodium were linear in the 12.7 - 38 and 31.4 - 94 μg/mL ranges, respectively. Accuracy and precision were in the range 0.2 - 2.6 and 0.2 - 3.2%, respectively, for the two analytes and the limits of detection for propofol and thiopental sodium were 1210 and 317 ng/mL, respectively, based on a signal to noise ratio of 2 and a 20 μL injection. The separation and quantitation of the propofol-ondansetron mixture was achieved on a 10 μm particle size phenyl column.,using a mobile phase of 50:50 v/v aqueous 0.01 M monobasic potassium phosphate pH 4 - acetonitrile at a flow rate of 1 mL/min, with detection set at 268 nm. The separation was achieved within 15 min. Propofol and

ondansetron were linear in the 2.5 - 37.5 and 0.48 - 7.2 μg/mL ranges, respectively. Accuracy and precision were in the range 0.4 - 2,4 and 0.2 - 0.6%, respectively, for the two analytes and the limits of detection for propofol and ondansetron were 117 and 61 ng/mL, respectively, based on a signal to noise ratio of 2 and a 20 μL injection.

INTRODUCTION

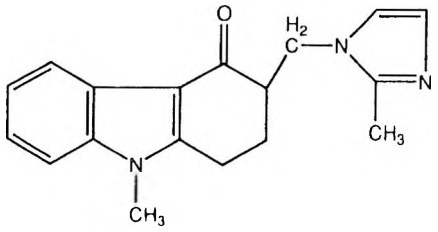
Mixtures of propofol-thiopental sodium (Mixture A) and propofol-ondansetron (Mixture B) are administered as perioperative injections in U.S. hospitals. Interest in this laboratory, in the stability and compatibility of each drug mixture over time, required the development of HPLC methods. A search of the literature indicated that HPLC methods were not available to assay each analyte in Mixture A or Mixture B, concurrently in a single injection.

Propofol has been analyzed primarily by gas chromatographic methods.[1-3] The assays are based on liquid-liquid extraction clean-up procedures and were used for determining drug levels in plasma. An HPTLC assay was reported to determine the sorption of propofol in infusion containers.[4] A comparison of HPLC to second-derivative UV spectroscopy, was reported for a propofol oil in water emulsion dosage form.[5] Other HPLC procedures were based on reverse phase chromatography and used UV, electrochemical, and fluorescence detection to determine propofol levels in serum or plasma samples.[6-8]

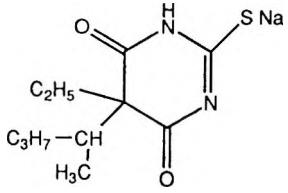
Thiopental sodium has been analyzed by a variety of methods. The official USP 23 method is based on UV spectrophotometry at 304 nm.[9] Other procedures utilize stripping voltammetry, HPLC, GC and micellar electrokinetic chromatography using chemically modified cyclodextrins, to determine thiopental sodium in a myriad of samples.[10-14]

Ondansetron has been assayed by high performance thin layer chromatography (HPTLC), HPLC methods and radioimmunoassay methods. The HPTLC method was developed especially for plasma samples, but the sample output was low and the equipment is not generally available in most laboratories.[15] The HPLC assays used either a silica column with an aqueous-organic mobile phase, or a cyanopropyl column operated in the reverse phase mode.[16-17]

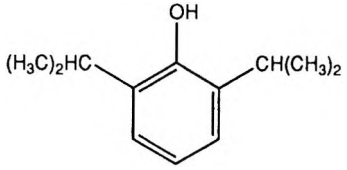
Detection of the analyte was either by UV at 305 nm, or radiochemical detection. The radioimmunoassay was combined with sample cleanup, using a cyanopropyl solid phase extraction cartridge to provide a subnanogram per mL determination of ondansetron.[18]



ONDANSETRON



THIOPENTAL SODIUM



PROPOFOL

Figure 1. Chemical Structures of Compounds Studied.

In this paper, isocratic HPLC assays are presented for the simultaneous analysis of propofol and thiopental sodium (Mixture A), and propofol and ondansetron (Mixture B) mixtures. Both mixtures were separated on phenyl columns using aqueous phosphate buffer pH 4 - acetonitrile eluents. Each separation was achieved within 15-20 min with sensitivity generally in the lower ng to lower μg/mL range for all 3 analytes.

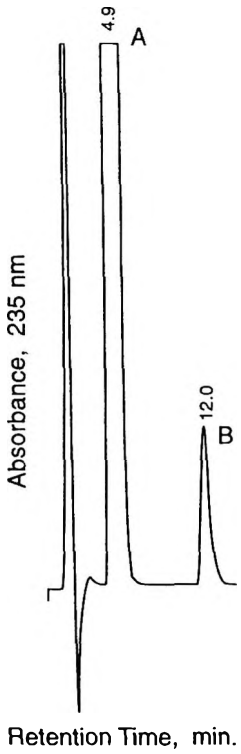


Figure 2 Typical HPLC Chromatogram of thiopental sodium (A) and propofol (B) on a phenyl column with 55:45 v/v 0.01 M phosphate buffer pH 4 - acetonitrile. See Experimental Section for assay conditions.

EXPERIMENTAL

Reagents and Chemicals

The structural formulae of the compounds studied are shown in Figure 1. Thiopental Sodium was a gift from Abbott Laboratories (North Chicago, IL) and propofol was obtained from Stuart Pharmaceuticals (Wilmington, DE 19897). Ondansetron hydrochloride (Lot AWS 332A) was a gift from Glaxo, Inc. (Research Triangle Park, NC 27709). Acetonitrile (J.T. Baker, Phillipsburg, NJ 08865) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, Ga 30076). Monobasic potassium phosphate and concentrated phosphoric acid were Baker analyzed reagents.

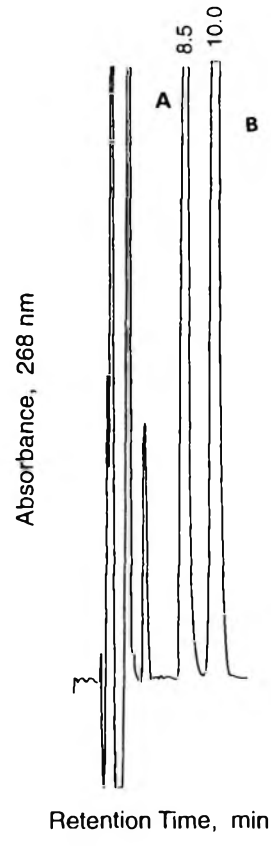


Figure 3 Typical HPLC Chromatogram of ondansetron (A) and propofol (B) on a phenyl column with 50:50 v/v 0.01 M phosphate buffer pH 4 - acetonitrile. See Experimental Section for assay conditions.

Instrumentation

The chromatographic separations were performed on an HPLC system consisting of a Waters Model 501 pump (Milford, MA 01757), an Alcott Model 728 autosampler (Norcross, GA 30093) equipped with a 20 µL loop, a Beckman Model 163 variable wavelength UV-VIS detector (Fullerton, CA 92634) and a Hewlett-Packard Model 3395 integrator (Avondale, PA 19311). Separation of Mixture A was achieved on a 150 mm phenyl column (Zorbax SB, 4.6 mm i.d., 5 µm particle size, MacMod Analytical, Chadds Ford, PA 19317). The mobile phase consisted of 55:45 v/v 0.01 M aqueous monobasic potassium phosphate

pH 4.0 (adjusted with 10% phosphoric acid)-acetonitrile. The separation of Mixture B was accomplished on a 300 mm phenyl column (T-Bondapak, 4.6 mm i.d., 10 μm particle size, Waters, Milford, MA 01757). The mobile phase consisted of 50:50 v/v 0.01 M aqueous monobasic potassium phosphate pH 4.0 (adjusted with 10% phosphoric acid)-acetonitrile. The mobile phases were filtered through a 0.45 μm nylon 66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was set at 1.0 mL/min for both mixtures and the detector was set at 235 nm for Mixture A and 268 nm for Mixture B.

Preparation of Standard Solutions

A combined standard solution containing propofol and thiopental sodium was prepared by accurately weighing 0.38 mg propofol and 1.03 mg thiopental sodium in a 10 mL volumetric flask. Another standard solution containing propofol and ondansetron was prepared by accurately weighing 0.38 mg propofol and 0.083 mg ondansetron hydrochloride in a 10 mL volumetric flask. Methanol was added to each mixture and the flasks were shaken vigorously for 2 min, followed by methanol to volume. Dilutions of the combined propofol-thiopental sodium and propofol-ondansetron standard solutions gave solutions in the 12.7 - 38 μg/mL and 31.4 - 94 μg/mL range for propofol-thiopental sodium and 2.5 - 37.5 μg/mL and 0.48 - 7.2 μg/mL range for propofol-ondansetron, respectively. Additional dilutions of Mixture A and Mixture B standard solution were prepared in methanol, to serve as spiked samples for each analyte to determine accuracy and precision. Quantitation was based on linear regression analyis of analyte peak height versus analyte concentration in μg/mL.

RESULTS AND DISCUSSION

There were no reports in the literature describing separations of propofol-thiopental sodium and propofol-ondansetron mixtures. Initial studies to develop HPLC methods for each mixture using isocratic conditions, involved the use of underivatized silica, phenyl, octyl, deactivated octyl and octadecyl columns, with various mobile phases containing methanol-aqueous phosphate buffers and/or acetonitrile-aqueous phosphate buffer at 1 mL/min. The best resolution of the analytes in Mixtures A was obtained on a 150 mm stable bond 5 μm phenyl column, using 55:45 v/v phosphate buffer pH 4 - acetonitrile. Mixture B was best resolved on a 300 mm 10 μm phenyl column using 50:50 v/v phosphate buffer pH 4 - acetonitrile.

Table 1

Analytical Figures of Merit for Propofol-Thiopental Sodium and Propofol-Ondansetron Mixtures

Mixture	r^{2a}	System Suitability	LOD[c] ng/mL	k	Theoretical Plates[d]	Tailing Factor	Rs
A							
Propofol	0.9996	1.43	1210	6.6	1039	1.1	6.0
Thiopental Sodium	0.9999	1.13	317	2.1	482	1.1	
B							
Propofol	0.9999	0.48	117	2.8	2755	1.1	4.0
Ondansetron	0.9999	0.65	61	1.7	1322	1.3	

[a] Range examined from 12.7 - 38 μg/mL propofol (n=6) and 31.4 - 94 μg/mL thiopental sodium for Mixture A at 235 nm and 2.5 - 37.5 μg/mL propofol and 0.48 - 7.2 μg/mL ondansetron for Mixture B at 268 nm.
[b] Mean RSD% of 6 replicate injections at 25.3 μg/mL propofol and 62.7 μg/mL thiopental sodium for Mixture A at 235 nm and 25 μg/mL propofol and 4.8 μg/mL ondansetron for Mixture B at 268 nm.
[c] Limit of Detection, S/N = 2.
[d] Calculated at $N=16 (tr/w)^2$.
[e] calculated at 5% peak height

The columns also allowed the separation of methylparaben (preservative found in most commercial injections) from the analytes. Typical chromatograms showing the separation of each mixture are shown in Figures 2 and 3.

From an earlier study in our lab, it was shown that propofol and thiopental sodium absorb strongly at 235 nm in an acetonitrile - phosphate buffer system. It was also determined, that propofol and ondansetron absorb around 268 nm in essentially an identical mobile phase. Therefore, 235 and 268 nm were selected as the detection wavelengths for Mixture A and B, respectively, since they provided good accuracy and precision data for the two component mixtures.

Table 2

Accuracy and Precision Using Samples With Added Drug

Mixture	Concn Added μg/mL	Conc Found μg/mL[a]	Percent Error	RSD%
A				
Propofol	16.55	16.12 ± 0.51	2.6	3.2
	29.72	29.47± 0.39	1.1	1.3
Thiopental	43.34	43.72 ± 0.18	0.9	0.4
Sodium	78.01	77.24 ± 0.13	1.0	0.2
B				
Propofol	12.10	12.05 ± 0.03	0.4	0.3
	29.10	29.05 ± 0.10	0.2	0.3
Ondansetron	2.40	2.35 ± 0.02	2.1	0.9
	5.76	5.62 ± 0.01	2.4	0.2

[a] Mean ± standard deviation based on n = 3.

The HPLC method for Mixture A showed concentration versus absorbance linearity for propofol-thiopental sodium in the 12.7 - 38 μg/mL and 31.4 - 94 μg/mL ranges, respectively, at 235 nm. Table 1 gives the analytical figures of merit for each of the analytes in Mixture A. The HPLC method for Mixture B showed concentration versus absorbance linearity for propofol-ondansetron in the 2.5 - 37.5 μg/mL and 0.48 - 7.2 μg/mL ranges, respectively, at 268nm. Table 1 also gives the analytical figures of merit for each of the analytes in Mixture B.

A photodiode array detector (Model 990, Waters Associates, Milford, MA 01757) was used to verify that none of the degradation products of the analytes, in either Mixture A or B (analyzed under their respective analytical conditions), interfered with the quantitation of each drug at 235 or 268 nm.

These experiments were performed on solutions of each drug, in 0.9% sodium chloride injection after they has been degraded for 1-6 hr at ambient temperature and 45-60°C with 0.1 N hydrochloric acid, 0.05 N sodium hydroxide, and 3-30% hydrogen peroxide solutions.

Percent error and precision of the methods were evaluated using spiked samples containing each analyte. The results for mixtures A and B are shown in Table 2. The results indicate, that the procedures give acceptable accuracy and precision for the analytes in both mixtures.

Intraday variabilities for propofol-thiopental sodium (Mixture A) expressed as % RSD, were 1.13 and 1.43% (n=6), respectively. Interday variabilities of the assay for propofol and thiopental sodium were 0.82 and 0.66% (n=18 over 3 days), respectively. Intraday variabilities for propofol-ondansetron (Mixture B) expressed as % RSD, were 0.48 and 0.65% (n=6), respectively. Interday variabilities of the assay were 0.36 and 0.59% (n=18 over 3 days) for propofol and ondansetron, respectively.

In summary, a 5 μm stable bond phenyl column and a 10 μm phenyl column with aqueous phosphate buffer pH 4-acetonitrile mobile phases, were shown to be suitable for the separation and quantitation of a propofol-thiopental sodium mixture (A) and a propofol-ondansetron mixture (B). This study suggests that the HPLC methods developed, herein, can be used to investigate the chemical stability of these analyte mixtures.

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HPLC ANALYSIS OF ANTHRAQUINONE DERIVATIVES IN MADDER ROOT (*Rubia tinctorum*) AND ITS CELL CULTURES

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ABSTRACT

A sensitive and reproducible RP-HPLC method was developed for the characterization of madder root and its cell cultures extracts and for the determination of anthraquinone derivatives as glycosides (ruberythric acid, lucidin primveroside) and aglycones (alizarin, lucidin, purpurin) in them.

The fingerprint chromatographic patterns of the extracts were obtained on C_{18} silica column, using gradient with acetonitrile-water-acidic buffer eluent system. For quantitative measurements, the anthraquinone derivatives were separated by isocratic elution in the same type of mobile phase. The chief components were identified and quantitatively determined.

The natural plant and its cell suspension cultures were compared to each other. The preparative fractionation of the extracts was achieved by gel chromatography, HPLC and selective extractions with solvent series, solid-phase extraction techniques and SFE.

INTRODUCTION

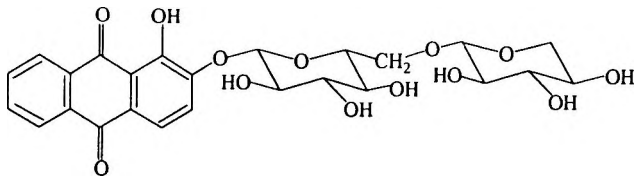
The natural plant derived food colors tend to be frequently used, and the need is growing for heat- and light-resistant natural substances. Madder pigments seem to satisfy these requirements.

The natural dyes obtained from rubiaceous plants have been highly esteemed, since ancient times in the East and West, because of the excellent dyeing qualities and light fastness. In recent years, however, rubiaceous plants have found little use in dyeing since the advent of chemical dyes. Some compounds of pharmacological interest, were found in them with hopeful applications. So, their plant cell cultures are a potential source for production phytochemicals of high economic value.

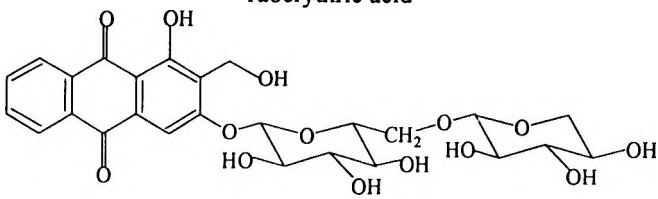
In Rubia species, many biologically active compounds, mostly secondary plant products, metabolites, are found, for example: anthraquinones, hydroxyanthraquinones, their derivatives and glycosides, naphthohydroquinones, naphthoquinones, their derivatives and glycosides, naphthoquinone dimers, as well as peptides. The formulas of our measured components are given in Fig. 1.

Rubia tinctorum (madder root), the source of natural dye, produces anthraquinone pigments and secondary metabolites in the roots and, also, in the cultured cells, one of them being alizarin[4]. The herbal drugs, consisting of crude Rubia extracts, have some compounds of pharmacological interest with the activity of dissolving bladder and kidney stones (especially Ca-oxalate and Ca-phosphate) in the urinary tract. This effect has been clinically tested and some medicines are produced: Cystenal® by SPOFA (Praha, Czechoslovakia), Rubia Teep® tablets from Madaus (Köln, FRG). All parts of Rubia tinctorum contain pigment complexes of different quantity and rather different composition, but the underground organs (rhizome and roots) have the highest pigment content.[22]

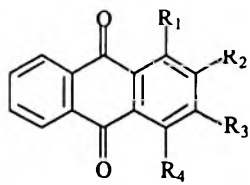
It has been shown, that Rubia tinctorum produces lucidin, in addition to alizarin, and that these hydroxyanthraquinones are present as glycosides which decompose in rat to the genotoxic hydroxyanthraquinones lucidin and



ruberythric acid



lucidin primveroside



	R_1	R_2	R_3	R_4
anthraquinone	H	H	H	H
2-hydroxy-anthraquinone	H	OH	H	H
anthraqumone-1-methylether	OCH_3	H	H	H
1-hydroxy-2-methyl-anthraquinone	OH	CH_3	H	H
alizarin	OH	OH	H	H
alizarin-2-methylether	OH	OCH_3	H	H
anthragallol	OH	OH	OH	H
lucidin	OH	CH_2OH	OH	H
purpurin	OH	OH	H	OH

Figure 1. Formulas of the measured compounds.

1-hydroxy-anthraquinone.[2,3,5,6,13,27,28,30,33] Alizarin, produced in cell cultures, can be used as an indicator for the production of the anthraquinone metabolites in cultured Rubia cells.

Many methods have been reported for the separation of the naturally occuring free anthraquinone aglycones. These have been based on paper chromatography,[8,23] thin-layer chromatography,[15,17] centrifugal partition chromatography,[7] counter-current chromatography,[35] low-pressure column chromatography,[21,27] and high performance liquid chromatography (HPLC).[11,14,16,18-20,25,29,31,32]

The adsorption of anthraquinone pigments on Amberlite XAD-2 resin was described previously, but the method was not suitable for routine use with cell and tissue culture extracts.[29] Column purification, however, was practical and C_{18} cartridges proved to be effective in the HPLC determination of anthraquinone in pulping liquors.[11]

Recently, some HPLC systems have been elaborated for analysis of madder extracts. Odake et al.[12] applied Nucleosil-508 (4.6×250 mm) column and methanol with 0.1% phosphoric acid was the solvent.

According to Westendorf et al.[28] a Merck- and a Spectra Physics HPLC-system were applied for analysis of Rubia extracts with acetonitrile gradient in 0.1% acetic acid.

Quercia[14] offered for HPLC determination of some anthraquinone glycosides: Permaphase ODS or Micropack Si 10 (250×2 mm) columns with various mixtures of methanol/water (for RP-HPLC) or of methanol/n-pentane (for NP-HPLC).

MATERIALS

Chemicals were obtained from the following sources: alizarin from Merck (Darmstadt, FRG), ruberythric acid (depur, referred in this paper as glycoside mixture) from Carl Roth GmbH & Co. (Karlsruhe, FRG) and Rubia Teep from Madaus (Köln, FRG). Lucidin was synthesized according to the method described by Murti et al.[10]

The other derivatives were synthesized in our lab.[1,26] A pure, authentic, ruberythric acid sample was synthesized by Cs. Weber[26] in our lab, according to a modified method of Zemplén and Bognár.[34] The plant cell cultures were prepared and treated in the laboratory of Department of Plant Anatomy, Eötvös University.

EXPERIMENTAL

Sample Preparation

Dried and powdered material (10 mg), obtained from R. tinctorum cell culture, was suspended in 2.5 mL of 80% (v/v) ethanol,[15] sonicated for 5 min (Branson 2200 sonicator) and soaked at 80°C for 0-10 h. The extract was separated by centrifugation, the residue was mixed with 1.5 mL of 80% (v/v) ethanol, incubated for 4 h at 80°C and centrifuged again. The combined supernatants were evaporated to dryness and the residue was dissolved in 1 mL of 80% (v/v) ethanol and used as the crude extract in further HPLC studies.

Purification of the Crude Extracts

The crude extract (1 mL) was diluted tenfold with water and passed dropwise through preactivated SPE cartridges (Bond Elut LRC C_8 1 cc; Analytichem, Harbor City, CA, USA), which were then washed with 2 mL of water, followed by 1 mL of methanol-water (30:70, v/v). After drying the cartridges with air, the fraction containing the anthraquinones was eluted from the tube with 1 mL of methanol-water (80:20, v/v).

HPLC of the Extracts

a.) Chromatography was performed using a Beckman 342 HPLC system, equipped with a Beckman 114 M solvent-delivery module, a Beckman 420 controller, a Beckman 165 variable-wavelength detector and an Altex 210 loop injector (20 μL loop volume). The ratiograms (254/280 nm) were recorded with a BBC Goertz Metrawatt SE-120 two-channel recorder (BBC) and chromatograms (254 nm, quantitation signal), with a Merck-Hitachi-2000 chromato-integrator. The ratio threshold was set at 2%. The components were separated on an ODS Hypersil (5 μm particle size) reverse phase column (125×4 mm I.D.) (Shandon Southern Products, Runcorn, UK). The isocratic elution of components was accomplished using methanol-5% acetic acid (pH 3.0) (70/30) at a flow-rate of 1.0 mL/min. Peaks were identified by comparing their retention times, ratiogram plots and on-line detection of the UV spectra with those of standards

b.) Separations were performed on a Knauer system consisting of two pumps Model 64 with analytical pumphead, a gradient programmer Model 50B, an injection valve with 20 μL sample loop and an UV spectral photometer

(or RI detector) with analytical flow cell (Knauer GmbH, Bad Homburg, FRG). Column effluents were monitored at 254 or 280 nm. The columns were: 250×4 mm Superpac PEP S C_2/C_{18} RP 5 μm (Pharmacia LKB, Uppsala, Sweden); 250×4 mm BST-Nucleosil C_{18} RP, 5 μm (BST, Budapest, Hungary); 300×4 mm Polygosil 60-10NH_2 (Macherey Nagel, Düren, Germany); 250×4 mm MOS-Hypersil C_8 RP 6 μm (Shandon Southern Products, Runcorn, UK); 250×4 mm BST Rutin 10 C_{18} Pep 5 μm (BST, Budapest, Hungary); 4.6×250 mm Beckman Ultrasphere ODS 5 μm (USA) (Fig. 2). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Budapest, Hungary). The chromatograph was operated isocratically and gradient with flow rates between 0.8 and 2 mL/min. Peaks were identified with standard addition (co-injection) using more eluent systems isocratically and with gradient elution, too.

Eluent systems were:

Isocratic:

acetonitrile - 0.05 M phosphate buffer, pH 4.5 55:45 (v/v) for alizarin;
acetonitrile - 0.05 M acetate buffer, pH 4.5 43:57 (v/v) for lucidin measurements.
acetonitrile - water 82.5:17.5 (v/v) for carbohydrate-determinations
acetonitrile - 0.02 M acetate buffer, pH 4
43:57 (v/v) for hydroxyanthraquinones
22:78 (v/v) for anthraquinone glycosides

Gradient:

1. Eluent A:
acetonitrile - 0.05 M phosphate buffer, pH 4.5 - methoxy-ethanol 15:80:5 (v/v/v)
Eluent B:
acetonitrile - 0.05 M phosphate buffer, pH 4.5 - methoxy-ethanol 80:15:5 (v/v/v)
Gradient: linear, 20→90% B in A over 40 min;

2. Eluent A:
acetonitrile - 0.02 M acetate buffer, pH 4 15:85 (v/v)
Eluent B:
acetonitrile - 0.02 M acetate buffer, pH 4 85:15 (v/v)
Gradient: linear, 10%B→90%B or 20%B→90% B in A over 40 min.

For preparative separations ammonium acetate buffer was used in place of phosphate one. Samples were prepared with methanol extraction. KNAUER system was applied for preparative HPLC with preparative pumpheads, flow cell and injector. The column was 120×16 mm KNAUER Lichroprep RP18 15-25 μm (Knauer GmbH, Bad Homburg, FRG).

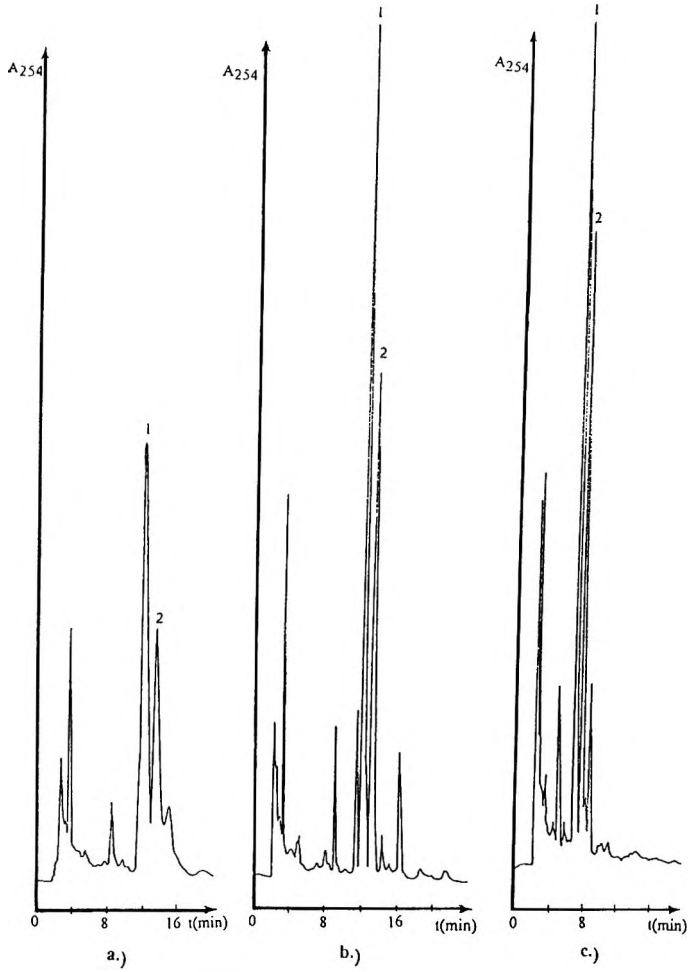


Figure 2. Chromatographic pattern of aqueous extract of Rubia tinctorum cell suspension culture (RF 413) on three different columns. Columns: a.) BST Rutin 10 C_{18} Pep (250×4.6 mm) b.) Beckman Ultrasphere ODS 5μ (250×4 mm) c.) MOS Hypersil C_8 RP 5 μm (250×4 mm). The other conditions were the same. Eluent: acetonitrile: 0.02M ammonium acetate buffer pH 4 22:78 (v/v). Injection volume: 20μL. Detection: 254 nm. Flow rate: 1 mL/min. Peaks: 1. lucidin primveroside, 2. ruberythric acid.

c.) Pharmacia LKB-HPLC system was used sometimes with Superpac PEP S C_2/C_{18} RP 5 μm, 250×4 mm (Pharmacia LKB, Uppsala, Sweden) column in the eluent systems above.

Table 1

TLC R_F Values of Rubia Components

Compounds	R_F Values		
	S1	S2	S3
anthraquinone	0.95	0.80	0.84
2-hydroxy-anthraquinone	0.84	0.75	0.87
anthraquinone-1-methylether	0.91	0.82	0.80
alizarin	0.82	---	0.77
alizarin-2-methylether	0.87	0.87	0.81
anthragallol	0.65	---	---
lucidin	0.89	0.80	0.66
purpurin	0.78	---	---

S1: -eluent: benzene:ethanol (80:20); Kieselgel 60 (Merck)
S2: -eluent: benzene:ethanol (80:20; Kieselgel 60 (Merck) desact.
S3: -eluent: benzene:ethyl acetate-methanol (40:30:5);[36,37] Kieselgel G (Merck)

PREPARATIVE PURIFICATION

5 g of lyophilized, fermented, washed bio-mass was treated with methanol in Soxhlet-extractor. The extract was evaporated in vacuo to dryness. The extraction-step was repeated with dichlorometane. The material was 0.16 g after removal of the dichlorometane and the residue (1.61 g) was extracted with water. The water solutions were lyophilized (or evaporated). 0.30 g of crude product was dissolved in 10 mL of water and applied into a column (42×2 cm) of Dowex 50 (H^+ form). After washing (H_2O) the column was eluted with methanol. 20 mg substance was obtained. It was fractionized further by GPC on Sephadex LH-20 column (65×2 cm) with methanol (Fig. 3). Four chief fractions with different colour bands were collected and evaporated in vacuo to dryness, respectively. The eluates were analyzed by HPLC.

RESULTS AND DISCUSSION

The analysis has been started with TLC studies of Rubia extracts (Table 1). The efficiency of TLC separations were not enough for our studies with Rubia extracts because of great number of components.

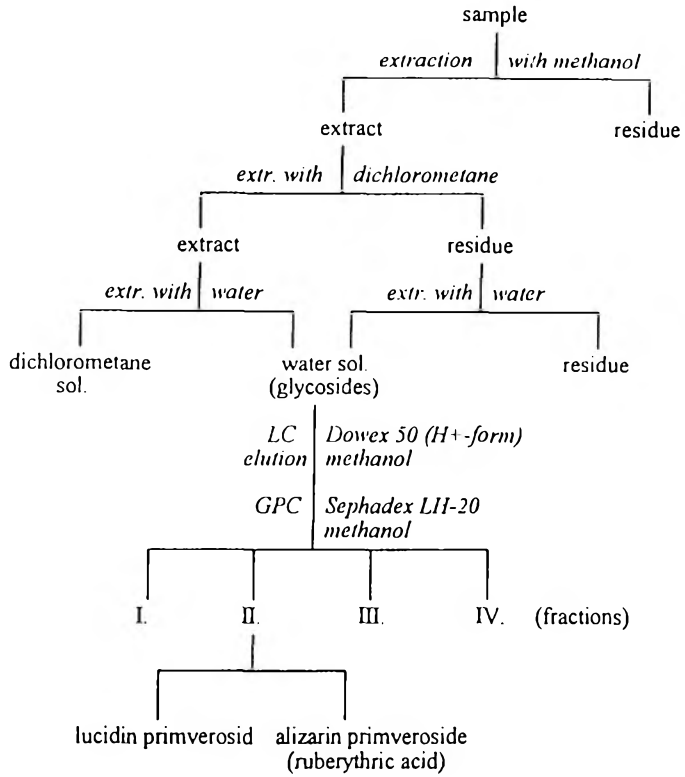


Figure 3. Flow chart. Extractions and column liquid chromatography for isolation of anthraquinones and their glycosides.

The analysis of the intact roots and the cell suspension culture was developed, and then carried out in our laboratory by HPLC. After optimisation alizarin, lucidin, purpurin, anthraquinones, their derivatives, and ruberythric acid were detected in the intact roots as well as in the cell suspension culture (Table 2). The pharmacological products (as Cystenal®) show similar chromatographic pattern, as previous ones.

We have developed a gradient HPLC methoc for „finger-print" chromatographic analysis of different Rubia extracts and cultures. This system is efficient for measurement of both anthraquinone aglycones and its glycosides, too (Fig. 4). At first, isocratic methods were applied for the extraction and purification of alizarin from Rubia tinctorum plant cells.

Table 2

(HPLC) Chromatographic Data of Rubia Components

Compound	Gradient System, t_R (retention time) min	Isocratic System, t_R min	k' (t_0=2.1 min)	eluent I:II (v/v)
anthraquinone	28.2	26.0	11.38	43:57
2-hydroxy-anthraquinone	20.1	9.8	3.67	43:57
1-hydroxy-2-methyl-anthraquinone	37.1	23.4	10.14	57:43
alizarin	20.4	10.5	4.00	43:57
alizarin-2-methylether	26.9	24.0	10.38	43:57
anthragallol	16.7	6.4	2.05	43:57
lucidin	18.1	7.7	2.67	43:57
purpurin	25.6	18.2	7.67	43:57
ruberythric acid	8.1	14.1	5.70	22:78
lucidin primveroside	7.7	13.1	5.24	22:78

Eluent: I: acetonitrile II: 0.02 M ammonium acetate buffer
Column: Beckman Ultrasphere ODS 5μm (250×4 mm)
Flow rate: 1 mL/min

For clean up of the samples, Soxhlet-extraction and solid phase extraction were applied. Isolation and subsequent purification using solid-phase extraction (SPE) with C_8 cartridges, following HPLC analysis and UV detection, provided a fast, sensitive and easy method for the determination of free alizarin.

Our results show that the pigments of Rubia tinctorum could be produced in large scale plant cell culture for industrial purposes. According to the analytical results, it is possible to optimize the production toward alizarin or ruberythric acid.

Acetic acid or acidic buffer (phosphate, acetate pH 4-5) in the mobile phase, affected the peak shape of the anthraquinones, the strongest effect being with alizarin. The washing and elution process with SPE removed most of the impurities having short or long retention times, lowering the detection limit in the HPLC analysis. It is possible, that some of the impurities removed by SPE and unknown components in the purified eluate, are other anthraquinones (free

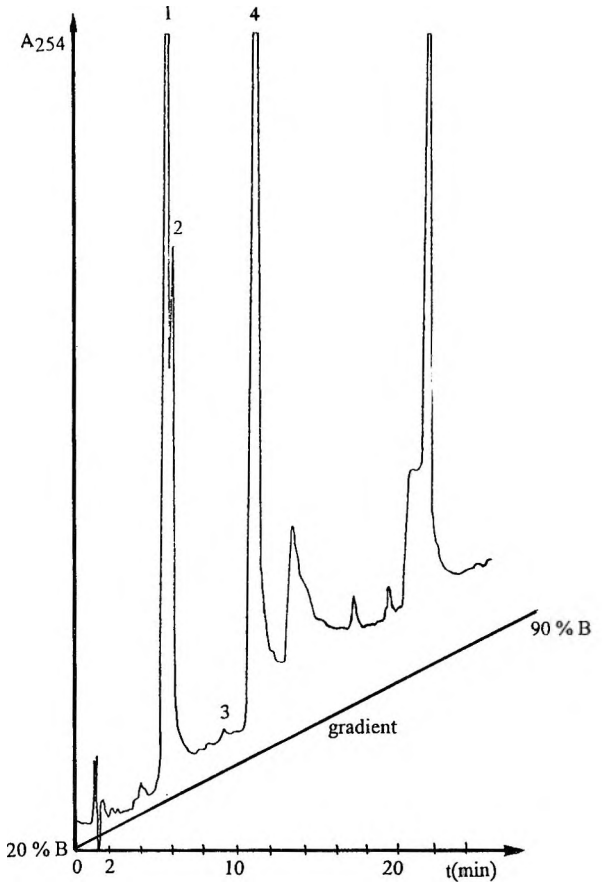


Figure 4. Chromatographic pattern of Rubia tinctorum root extract (with methanol). Column: Superpac PEP S C_2/C_{18} RP 5μm (250×4 mm). Eluent: A: acetonitrile : 0.02 M ammonium acetate buffer pH 4 15:85, B: acetonitrile : 0.02M ammonium acetate buffer pH 4 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. lucidin primveroside, 2. ruberythric acid, 3. lucidin, 4. alizarin.

or glycosidic), as they are known to be synthesized in R. tinctorum.[4] Stepwise development of the SPE cartridges seems to be useful for the purification of other anthraquinones, but if the cartridge size or sample type is changed then the SPE process should be optimized again.

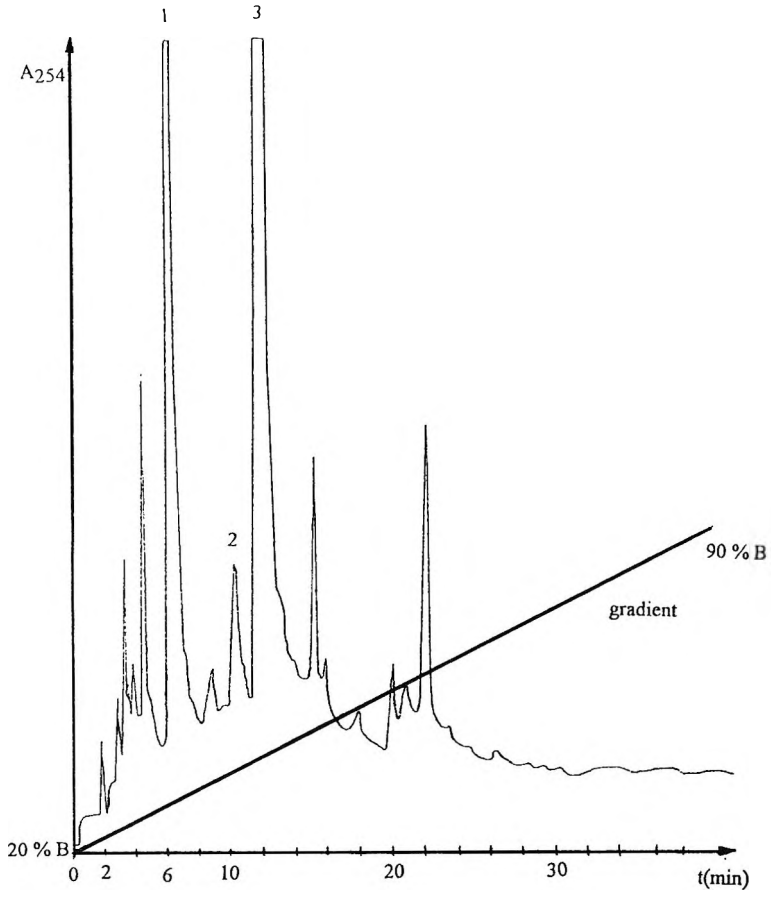


Figure 5. Chromatographic pattern of extract (with methanol) of Rubia tinctorum cell suspension culture (25/2). Column: Superpac PEP S C_2/C_{18} RP 5 μm (250×4 mm). Eluent: A: acetonitrile: 0.02 M ammonium acetate buffer pH 4 15:85, B: acetonitrile: 0.02 M ammonium acetate buffer pH 4, 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. ruberythric acid, 2. lucidin, 3. alizarin.

This isocratic method is useful for studies with alizarin and other anthraquinones produced by R. tinctorum and, should also, facilitate the further isolation, identification and determination of their glycosides, too. Non-glycosidic forms of anthraquinone derivatives can be used as marker molecules in further studies of anthraquinone biosynthesis in cultured R. tinctorum, e.g.,

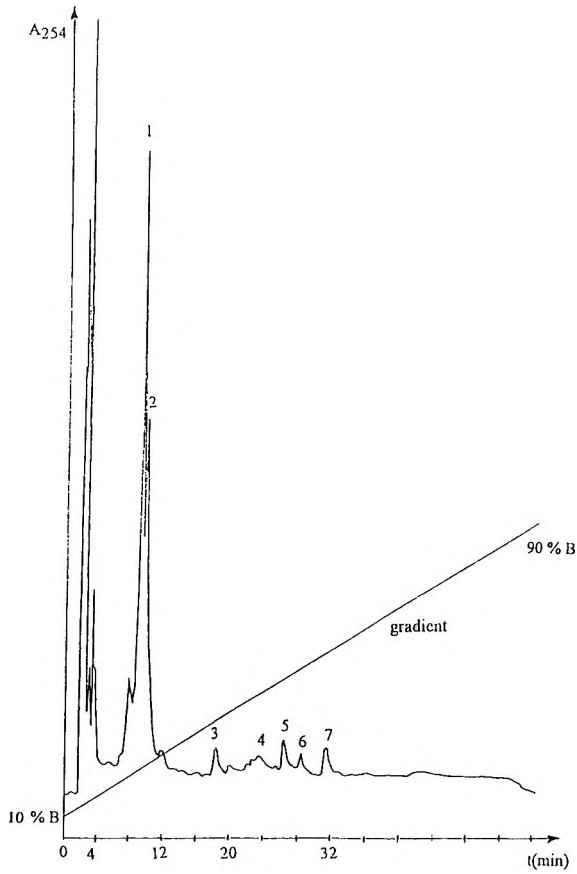


Figure 6. Chromatographic pattern of extract (with methanol) of Rubia tinctorum cell suspension culture (RF 413). Column: MOS-Hypersil C_8 RP 6μm (250×4 mm). Eluent: A: acetonitrile: 0.02 M ammonium acetate buffer pH 4, 15:85, B: acetonitrile: 0.02 M ammonium acetate buffer pH 4 85:15, linear gradient 20% B → 90% B at 40 min. Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.0 mL/min. Peaks: 1. lucidin primveroside, 2. ruberythric acid, 3.-7. hyroxy anthraquinones.

alizarin, produced in cell cultures, can be used as an indicator for the production of the anthraquinone metabolites in cultured Rubia cells. For chromatographic identification of the peaks the standard additions method was used at different chromatographic conditions.

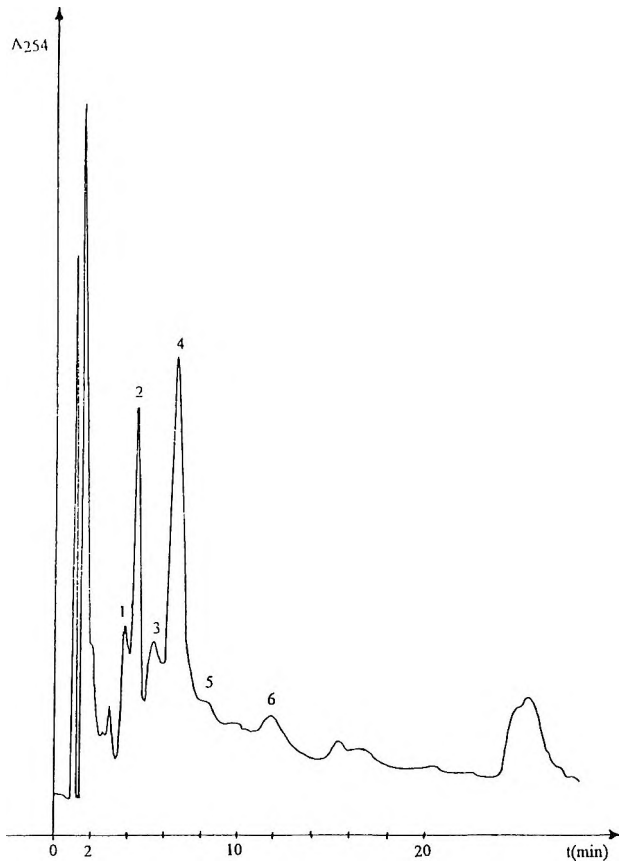


Figure 7. Chromatographic pattern of supercritical fluid extract of Rubia tinctorum cell suspension culture (84). Column: Superpac PEP S C_2/C_{18} RP 5 μm (250×4 mm). Eluent: acetonitrile:0.02 M ammonium acetate buffer pH 4, 43:57 (v/v). Injection volume: 20 μL. Detection: 254 nm. Flow rate: 1.5 mL/min. Identified peaks: 1. lucidin, 2. anthragallol, 3. alizarin, 4. 2-hydroxy-anthraquinone, 5. alizarin-2-methylether, 6. anthraquinone.

The results from the extraction of alizarin with ethanol at 80°C at different times, indicate that incubation for 10 h was needed to achieve the maximum recovery for cultured plant cell suspension material. The typical free alizarin content was 2 mg/g in dry Rubia tinctorum material, with a range of 0.4-4 mg/g depending on the culture.

Linear calibration graphs (based on the peak heights in mm) with good correlation ($r^2 > 0.999$), were obtained for alizarin (range 78-10 000 ng/mL) and anthraquinone (156-20 000 ng/mL), the first value of the range showing the detection limit at a signal-to-noise ratio of 3.

The precision of the whole assay was 1.5% (from identical plant cell culture samples) and the recovery from the SPE step was estimated to be more than 99% (n=3) for alizarin.

Our optimized HPLC measurements were applied for

- qualitative characterization („finger print") of extracts (Fig. 4-7),
- identification of components in extracts (Fig. 4-7),
- quantitative determinations of chief components (as alizarin, lucidin, ruberythric acid etc.) (Table 3),
- comparison of natural plant and cell cultures (Fig. 4-6),
- indication of anthraquinones biosynthesis via their measurements,
- structure determination of components in extracts,
- semipreparative and preparative fractionation, separation and purification of components,
- control of pharmaceutical products containing extracts,
- checking the purity of authentic standard samples,
- characterization of commercial ruberythric acid,
- qualitative comparison of different fractionation methods (as Soxhlet, LC, SFE etc.) (Fig. 4-7) and
- purity control of chemically synthesized anthraquinone products.

Preparative HPLC was performed on RP C_{18} preparative columns; methanol or acetonitrile, ammonium acetate buffer (pH 4)/water gradient system. Fractions were collected, checked by anal. HPLC, evaporated or lyophilized, then crystallized (e.g. from hot water to yield ruberythric acid). The structure of isolated compound was identified by UV, IR, NMR and MS data.

Table 3

Anthraquinone Constituents (content %)

	Alizarin	Lucidin	Ruberythric Acid	Lucidin Primveroside
madder root	0.15 - 0.17	$0.2x10^{-3}$ -$9.0x10^{-3}$	4 -5	2 - 3
cell culture homogenates	0.03 - 0.19	$0.9x10^{-3}$ -$9.6x10^{-3}$	3.8 - 5	0.08 - 0.1

After preparative GPC fractionation (on Sephadex LH-20 column) we have found, that the first fraction contains more compounds, second fraction has two, very similar ones (very well crystallizable yellow needles) (Fig. 3), the chief part of third fraction was found to be alizarin, the fourth fraction contains purpurin .

For structure determination the fraction II. was hydrolyzed with dilute (5%) hydrochloric acid: glucose and xylose (HPLC identification: Polygosil 60-10NH_2 column; eluent: acetonitrile-water 82.5:17.5 (v/v); flow rate: 1.5 mL/min; det.: RI) were obtained in addition to alizarin and lucidin. The MS, IR, UV data were in good agreement with those of ruberythric acid.

Suzuki and coworkers[24] isolated from their homogenate on very similar manner lucidin-ethylether, pseudopurpurin, alizarin, purpurin and ruberythric acid. Data of this ruberythric acid was identical with those of authentic ruberythric acid isolated from roots by the method of Hill and Richter[27] based on lead complex precipitation.

In order to elucidate the biogenesis of anthraquinones in madder, Burnett and Thompson[4] isolated nineteen anthraquinones, all substituted in one benzenoid ring only, from mature plants of R. tinctorum by extraction with solvent-series of different polarity.

Their method was applied for fractionation of cell culture extract, too. The comparison of fractions could be checked by HPLC.

In order to replace traditional solvent extraction method such as Soxhlet one, nowadays there is a better way: supercritical fluid extraction using carbon dioxide. Madder root and cell culture homogenates were treated in a preliminary experiment with CO_2 according to SFE. SFE experiments were performed by B. Simándy, Technical University, Budapest. The extracts were studied by HPLC. It was found, that the chromatographic patterns have

changed: carbon dioxide guaranted a substantial extraction (yield 6.7 %) for apolar compounds, as aglycones (2-hydroxy-anthraquinone, anthragallol etc) (Fig. 7).

We have found, that the authentic ruberythric acid (depur, produced from Carl Roth GmbH & Co. (Karlsruhe, FRG) contains two substances. Westendorf found, similarly, that it contained, in addition to alizarin primveroside, substantial amounts of lucidin primveroside too.

The anthraquinones were identified qualitatively and quantitatively on the basis of cochromatography with authentic compounds and comparison of their hydrolysis products.

Rubia Teep® was also analysed by HPLC and the glycosides ruberythric acid and lucidin primeveroside, as well as the free aglycons alizarin and lucidin, were observed. Small amount of lucidin was detected (present in free form and as the glycoside derivative) in each tablet of Rubia Teep®. Its HPLC pattern demonstrates two peeks: one is ruberythric acid, the second was identified as lucidinprimveroside.

These two compounds were found in our root extracts and cultured cells too. Their purity were characterized by HPLC, TLC and ^{1}H-NMR spectroscopy.

Our results demonstrated very well the sensitivity and efficiency of HPLC methods for chemical characterization of madder root and its cell cultures.

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A NEW CHEMICALLY DEACTIVATED SILICA-BASED REVERSE PHASE/ION EXCHANGE SUPPORT

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ABSTRACT

A new base-deactivated reverse phase chromatographic medium is described. Basic ligands have been incorporated into the silica matrix to interact with the free silanol groups, to supress their activity. Depending on the mobile phase, the support exhibits hydrophobic and/or ion exchange behavior and is stable between pH 1.5 - 7.5. Basic, acidic, polar, or apolar analytes can be separated with excellent selectivity and peak shape. Different selectivities were observed when the pH of the mobile phase was varied above and below the pK_a's of acidic analytes. Good separations were also observed for basic compounds, under chromatographic conditions where the bases were free or protonated, respectively.

INTRODUCTION

Silica-based reverse phase chromatographic media permit the separation of many organic compounds with reduced plate heights of 2 and asymmetries of 1.0. Other analytes, mainly strong organic bases and polar compounds, exhibit excessive tailing that is related to the interaction of these compounds with the

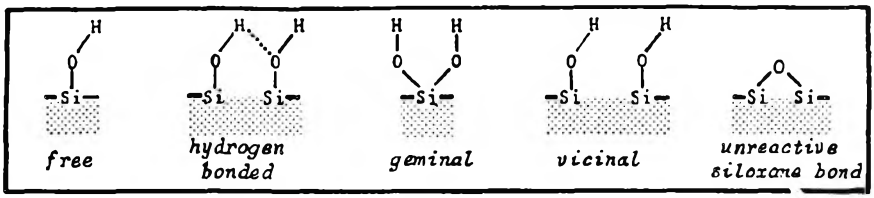


Figure 1. Configurations of the surface silanol groups.

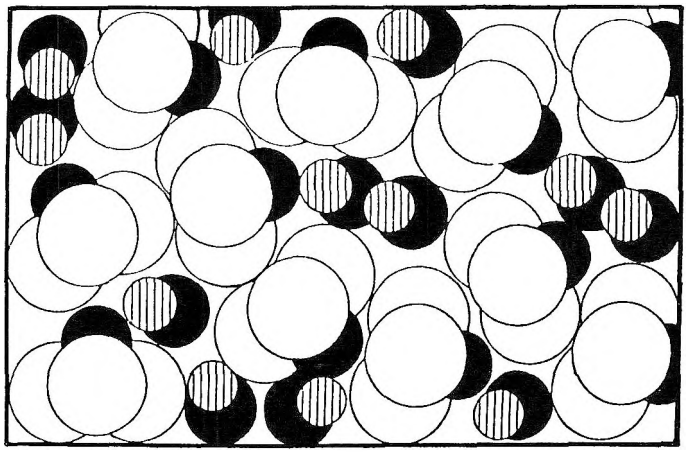


Figure 2. Silica surface with maximum coverage of trimethylsilyl (TMS) molecules. Circles represent projection of the TMS-methyl groups (open), silanol hydrogen atoms (hatched), and oxygen atoms of both (dark).

free silanols on the surface of the silica. To overcome this problem, a new base-deactivated reverse phase medium that minimizes the activity of the residual silanol groups was developed. On a hydrated silica surface, there are about 4.8 silanol groups/nm^2 or 8 $\mu mole/m^2$. Most of these silanols are hydrogen bonded to neighboring silanol groups, as shown in Figure 1. Other silanols are free as a result of unfavorable spatial configurations that prevent the formation of such bonds. These *free silanol* groups are more acidic and tend to associate more strongly with silanophilic analytes than do *hydrogen bonded* or self-associated silanols.

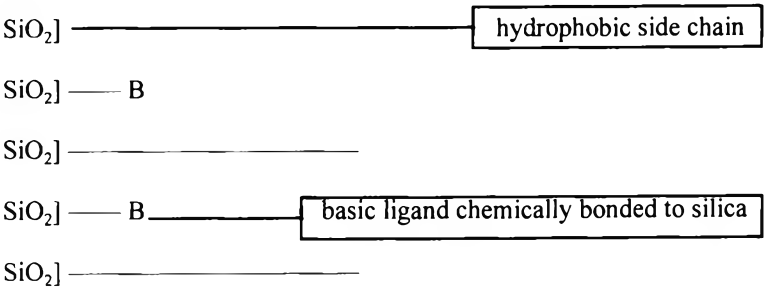
Reverse phase, silica-based, supports are synthesized by substituting the —OH of the silanol groups with dimethylalkylsilyl moieties to yield a brush-type reverse phase. The smallest of these moieties, trimethylsilyl, binds to the

surface at the 4 μmole/m^2 level, while the larger C_{18} homolog binds at the 3.2 μmole/m^2 level. This leaves about half of the 8 μmole/m^2 of the available silanol groups unchanged.[1]

It should be noted, also, that the population of **free** *silanols* on the surface of silylated silica increases as some of the remaining silanols (which were hydrogen-bonded before silylation) become more isolated and prevented from forming intra-hydrogen bonds (Fig. 2).

In reverse phase supports introduced a few years ago, bulky diisopropylalkylsilyl, instead of dimethylalkylsilyl moieties, were substituted for the —OH silanol-groups in an attempt to minimize band broadening and peak tailing, as well as increase the chemical stability of the bonded ligands.[2,3] However, though the diisopropylalkylsilyl ligands blocked access to some free silanols, others were still accessible, interacting with silanophilic analytes and resulting in broadened bands and tailing peaks for these compounds. A different approach to minimize the reactivity of the residual silanols in reverse phase supports was recently described.[4] In this approach, basic/polar ligands were chemically incorporated into the silica matrix. This approach was used to synthesize the ProTec-C_8 medium. Here, amine-type ligands were incorporated into the silica matrix of a C_8 bonded phase, resulting in a reverse phase support with ion-exchange character as shown in Scheme 1. These ligands form hydrogen bonds and ion pairs with the free silanols, internally suppressing (deactivating) their activity and enhancing the chromatography of basic, acidic, and polar analyses.

Scheme 1



In the presence of buffers between pH 2 - 7, the bonded basic ligands (—B) are in their conjugated acid form (—BH^+A^-, where A^- represents the buffer counter ion held on the stationary phase). When aqueous salts have no

buffer capacity, i.e., $NaClO_4$ at pH 7.0, the basic ligands stay predominantly free with only a small fraction protonated ($—BH^+OH^-$), causing a sharp *local* pH gradient between the basic surface and the bulk of the mobile phase.

As we shall later show, the ionic strength of the buffer is important for efficient and reproducible separations.

EXPERIMENTAL

Instruments

The chromatographic system consisted of a Model 300 pump (Gynkotek, Germerine bei Munchen, Germany); a Model 7125 Rheodyne injector fitted with a 20μL loop; a Spectroflow 759A Detector (Applied Biosystems, Mountain View, CA) and a ProTec-C_8, 150 mm x 4.6 mm column (ES Industries, Berlin, NJ).

Eluant flow rate was 1.0 mL/min and the eluate was monitored at 254 nm, except where otherwise indicated.

Chemicals

HPLC grade acetonitrile, methanol, and water were obtained from J. T. Baker (Phillipsburg, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI).

Sample Preparation

Samples were dissolved in mobile phase at concentrations such that detector signals were about 100 mV full scale.

RESULTS AND DISCUSSION

Column Conditioning Study

Because the ProTec series columns have, in part, ion exchange character, the ion exchange sites of the stationary phase are ion-paired with counter-ions

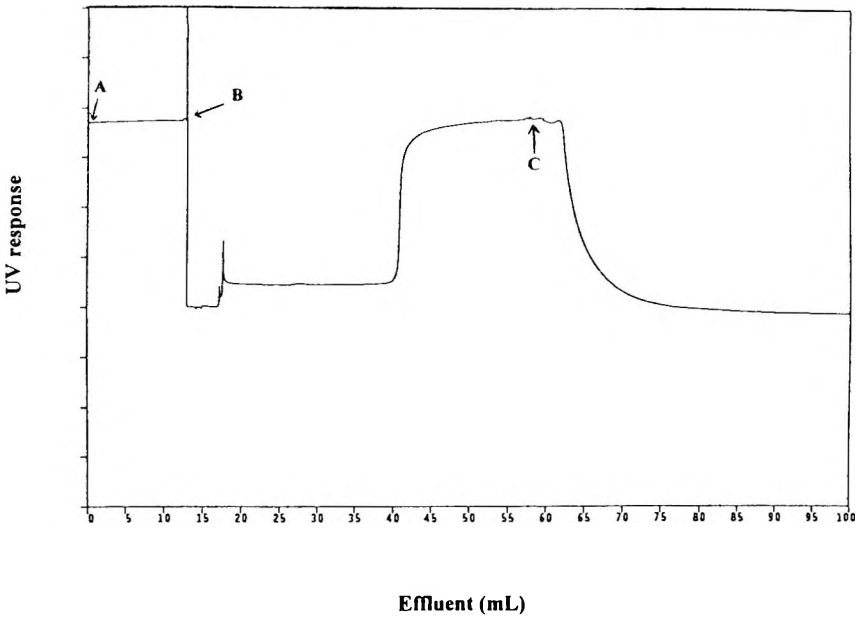


Figure 3. The breakthrough curve for triethylamine displacing phosphate ions. Column initially conditioned with 20:80 (v/v) methanol-50 mM K_2HPO_4, pH 7.0 at 1.0 mL/min until a steady baseline was obtained. Detector: 220 nm. A: Column bypassed and system washed with 80:20:0.2 (v/v) acetonitrile-water-triethylamine mobile phase. B: Column and system washed with triethylamine-containing mobile-phase. C: start column and system wash with 80:20 acetonitrile-water mobile phase.

of the buffer. When a new mobile phase buffer is used, the column must be reconditioned to replace the counter ions from the previous mobile phase buffer that were ion-paired to the basic ligands with counter-ions from the new buffer.

A series of experiments was performed to determine the amount of phosphate and trifluoroacetate (from trifluoroacetic acid) bound to the column. For each experiment, the column and system were washed to assure that only phosphate, triethylamine, or trifluoroacetate were breaking through. The chromatograms shown in Figures 3 and 4, respectively, show that about 400 μmole of triethylamine (TEA, equivalent to about the same quantity of phosphate, assuming a 1:1 triethylamine-phosphate ion-pair) were required to release the phosphate ions and about 1040 μmole of trifluoroacetate ions were ion paired to the protonated basic ligands.

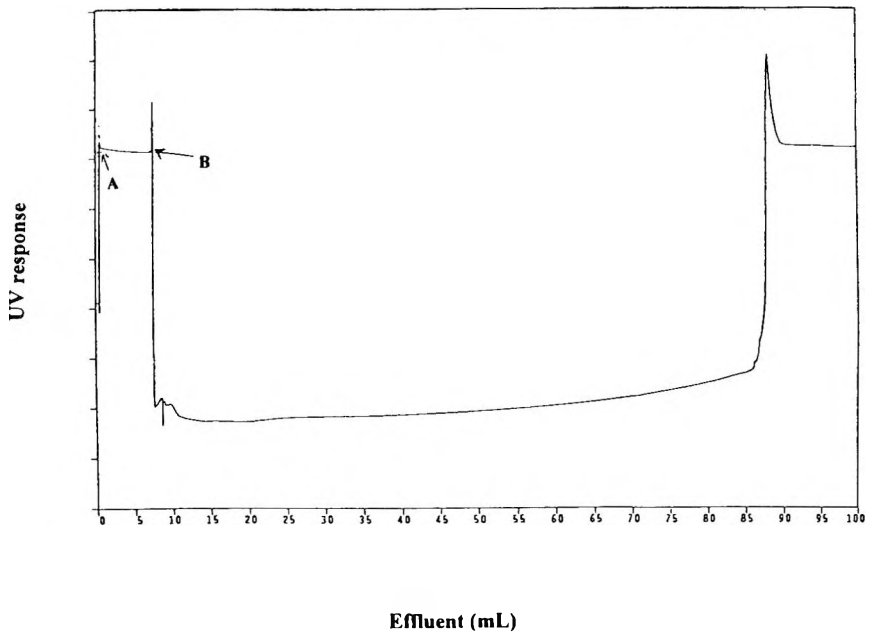


Figure 4. Trifluoroacetic acid breakthrough curve. Detector: 220 nm; Column washed with an 80:20 (v/v) acetonitrile-water mobile phase until a steady baseline was obtained. A: column bypassed and system washed with a 40:60:0.1 (v/v) acetonitrile-water-trifluoroacetic acid mobile phase. B: column and system washed with trifluoroacetic acid-containing mobile phase.

The reverse phase and ion-exchange behavior of the ProTec-C_8 column for the separation of basic, acidic and polar and apolar compounds was characterized in a series of experiments. As shown in Figure 5A, strong organic bases, such as cyclobenzaprine and amitriptyline, were separated as free bases with good resolution and peak shape using a mobile phase consisting of 50:50 (v/v) acetonitrile-50 mM $NaClO_4$.

Both compounds, which were injected as their hydrochloride salts, were converted, on column, by ion-exchange, into free bases by the basic ligands that are an integral part of the ProTec-C_8 medium. The better resolution of these compounds as free bases is due, in part, to the enhanced hydrophobic partitioning of the free bases compared to that of their conjugated acid forms in a buffered pH 7.0 mobile phase, as shown in Figure 5B.

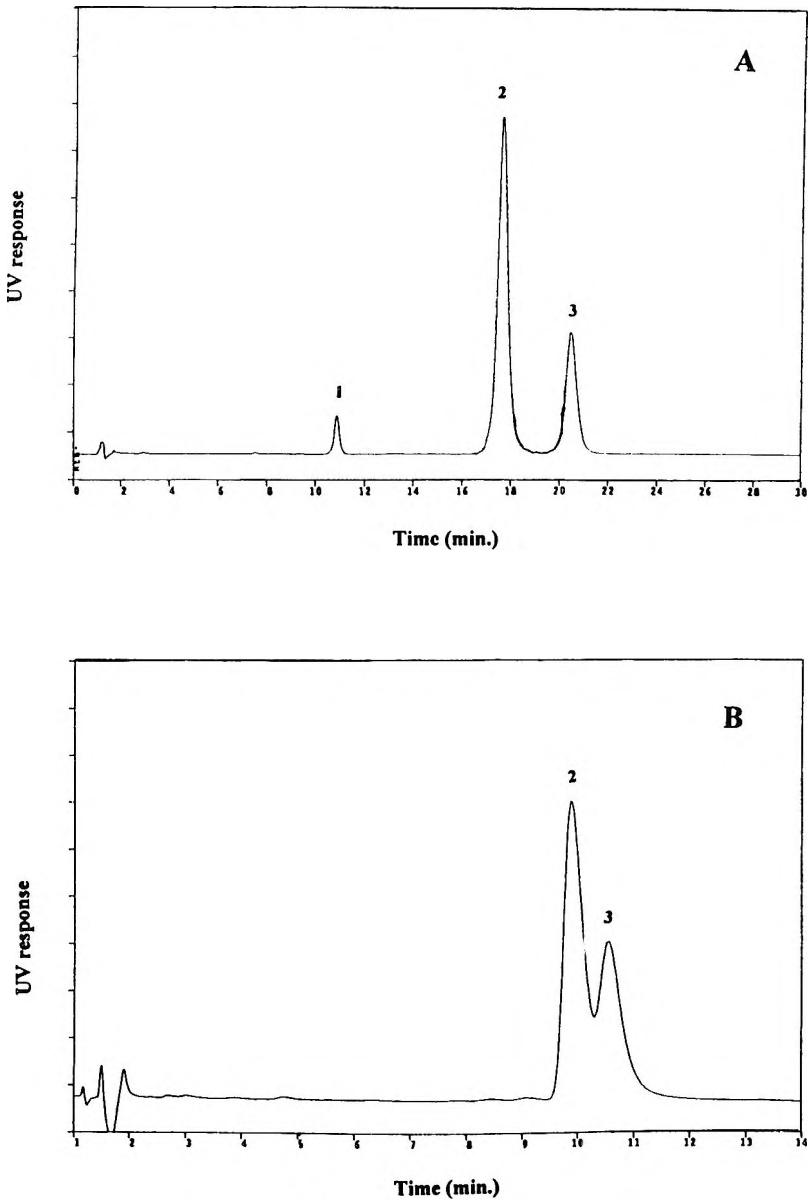


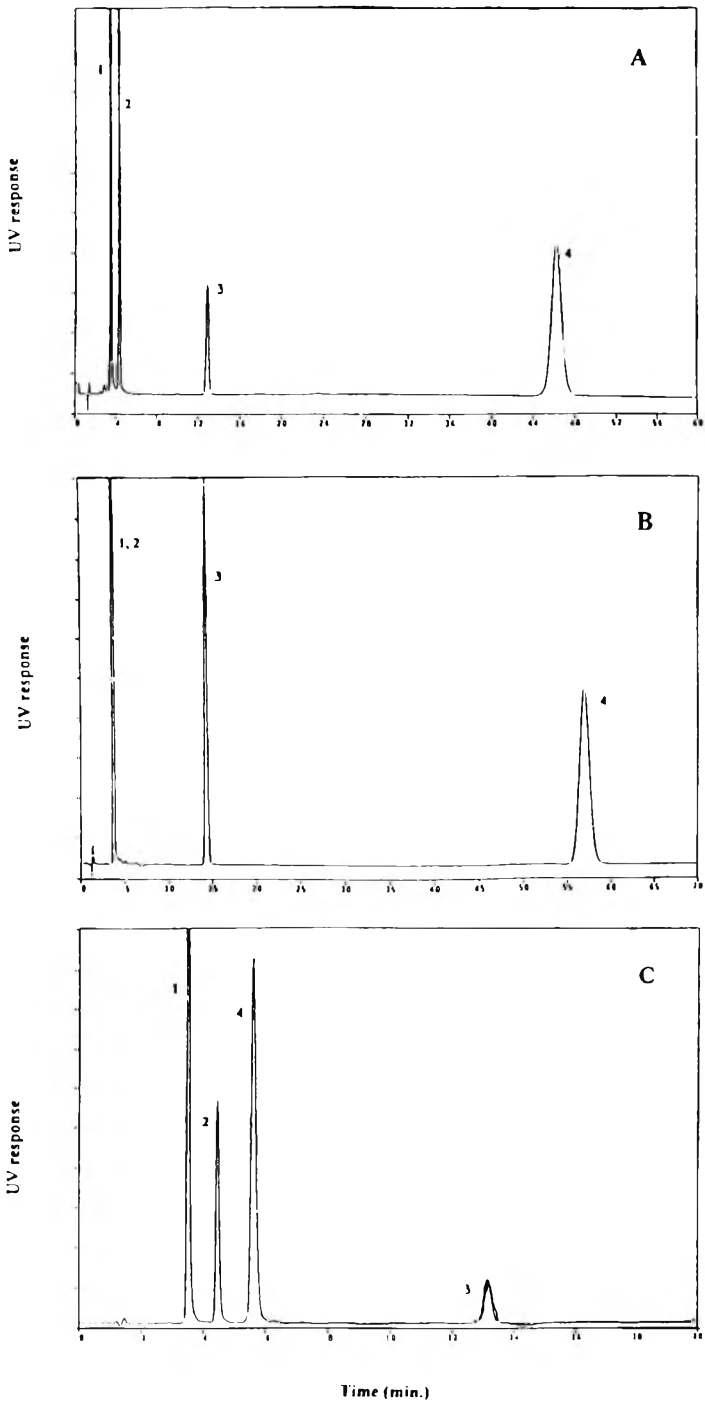
Figure 5. Separation of free bases and their conjugated acid forms in neutral unbuffered and buffered mobile phases. Mobile phase A: 50:50 (v/v) acetonitrile-50 mM $NaClO_4$; mobile phase B: 40:60 (v/v) acetonitrile-50 mM K_2HPO_4, pH 7.0. 1 = naphthalene (internal standard), 2 = cyclobenzaprine, 3 = amitryptyline.

Since the free basic ligands are gradually converted to their conjugated-acid form by ion exchanging the hydrogen chloride from the basic analytes, the column should be periodically washed with a 0.02% TEA-containing mobile phase to remove the accumulated HCl. In addition, when aqueous mobile phases without buffer capacity are used, the dissolved carbonic acid from ambient air accumulates on the column and slowly decreases the retention times of cationic compounds (results not shown). In this study, an amino guard column was placed before the injector to remove dissolved carbonic acid when $NaClO_4$-containing mobile phases were used.

Since the basic ligands bonded to silica matrix suppress the activity of the free silanols, it is expected that triethylamine will control only pH and not further suppress the silanophilic activity of these free silanols, and the separation will depend on hydrophobic interactions between the analyte, support, and mobile phase. To illustrate this, a mixture of typical weak and strong bases and polar and apolar compounds was separated using unbuffered, buffered pH 7.0, and basic mobile phases. The four test compounds: aniline (PK_a 4.63),[5] amitriptyline (pK_a 9.4),[6] phenol (pK_a 9.95),[7] and toluene (used as an indicator of chromatographic performance because it does not exhibit band broadening due to interactions with free silanols) were separated using a 40:60 (v/v) acetonitrile-water mobile phase on a new column that was not exposed to a buffer solution as shown in Figure 6A. Separations similar to that shown in Figure 6A were also obtained with a mobile phase of 40:60 (v/v) acetonitrile-water on a column that was previously exposed to different buffers and pre-washed with a TEA-containing mobile phase (results not shown). Using the high pH TEA-containing mobile phase, phenol is converted to its phenolate form and co-elutes with aniline as shown in Figure 6B.

Under the conditions used in Figures 6A and 6B, the basic analytes, aniline and amitriptyline, elute as free bases. In the presence of a buffer at pH 7.0, aniline is a free base and its retention is unaffected by a change in the conditions of the mobile phase, as shown in Figure 6A-C. On the other hand, in the presence of a pH 7.0 buffer, amitriptyline is protonated and the 10-fold difference in retention times using the basic and buffered pH 7.0 mobile phases is a consequence of the greater retentivity of the free base, as shown in Figures 6A and 6C, respectively.

Figure 6 (right). Chromatograms of weak and strong base and polar and apolar compound test mixture. Mobile phase A: 40:60 (v/v) acetonitrile-water; mobile phase B:40:60:0.02 (v/v) acetonitrile-water-triethylamine; mobile phase C: 40:60 (v/v) acetonitrile 50 mM K_2HPO_4 /KH_2PO_4, pH 7.0. 1 = aniline, 2 = phenol, 3 = toluene, 4 = amitriptyline.


A
1
2
3
4
UV response
B
1, 2
3
4
UV response
C
1
2
3
4
UV response
Time (min.)

Effect of Ionic Strength

In the presence of a buffer at pH 7.0, most of the basic ligands bonded to the medium are protonated. A rapid decrease in the ionic strength of the buffer, KH_2PO_4/K_2HPO_4 (pH 7.0), from 50 mM to 2 mM, causes the positively charged amitriptyline to elute close to the void volume (k' ~0.7) in an ion-exclusion mechanism caused by the repulsion between the positively charged analyte and the positively charged ligands under low ionic strength conditions.[8,9] The retention times of the neutral analytes (aniline, phenol and toluene) are not affected.

After extensively washing the column with the 2 mM buffer, the retention time of amitriptyline gradually increased because of a gradual decrease in the charge density on the surface of the medium (results not shown). This behavior illustrates how decreasing the ionic strength of the buffer can be useful in enhancing selectivity by changing the chromatographic behavior of interfering protonated analytes.

Using acidic mobile phases (0.1% TFA), organic acids and phenolic compounds are efficiently separated. At low pH (pH~1.5), the common organic acids are protonated and are retained on the column, mostly due to hydrophobic interactions with the stationary phase. They elute in symmetrical bands with the later eluting compounds showing better peak symmetry (see Fig. 7A). Salicylic acid, which is a stronger acid than the regular carboxylic acids, has an unusually long retention time compared to its retention on other base-deactivated reverse phase columns due to mixed ion-exchange/ hydrophobic interactions [k' = 5.6 with a 30:70:0.1 (v/v) AcCN:H_2O:TFA].

The ion-exchange sites of the ProTec-C_8 column bind carboxylate groups of organic acids and 'prevent' their elution by low ionic-strength buffers. As a result mobile phases containing buffers at pH's above the pK_a's of organic acids will produce substantially longer retention times than on conventional reverse phase columns. Using a 30/70 (v/v) acetonitrile-50 mM ammonium acetate mobile phase (adjusted to pH 5.0 with NH_4OH) resulted in very poor peak shapes and long retention times, i.e., benzoic acid eluted after 60 minutes (results not shown).

When the ammonium acetate concentration was increased to 200 mM, the separation shown in Fig. 7B was obtained. Thus, when separating a mixture containing organic acids, after the separation of the basic, polar or nonpolar compounds is achieved, an ionic-strength gradient can be used to elute/separate acids in a mobile phase with the same organic solvent strength.

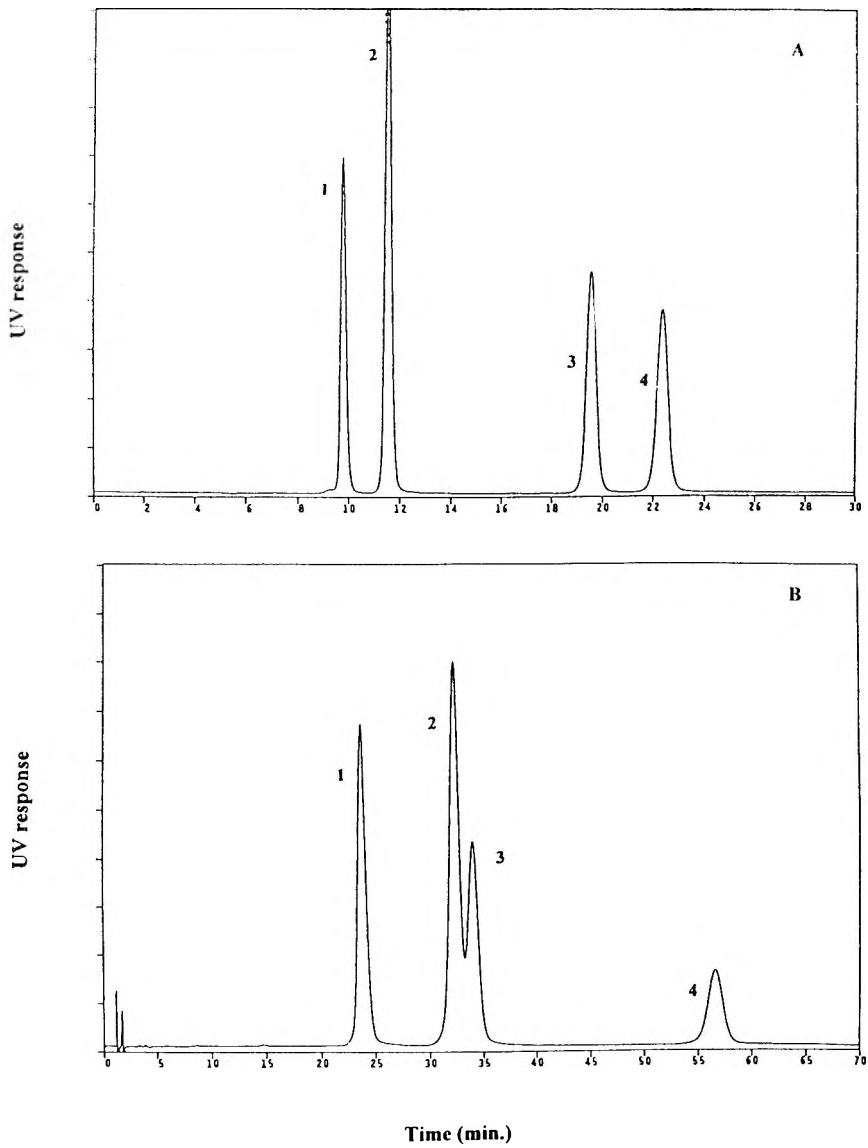


Figure 7. Separation of organic acids below and above their pK_a values. Mobile phase A: 20:80:0.1 (v/v) acetonitrile-water-trifluoroacetic acid; mobile phase B: 30:70 (v/v) acetonitrile-water containing 200 mM ammonium acetate and adjusted to pH 5.0 with NH_4OH. 1 = phenylacetic acid, 2 = benzoic acid. 3 = o-toluic acid, 4 = p-toluic acid.

Table 1

Retention Times, Plate Numbers and Peak Asymmetries for Toluene and Naphthalene for Column Stability Study

	Toluene			Naphthalene		
Injection Time (h)	R_t* (min)	Number of Plates	Asymmetry	R_t* (min)	Number of Plates	Asymmetry
50:50:0.1 (v/v) Acetonitrile-Water-Trifluoroacetic Acid at 30°C:						
1	5.76	14,300	1.25	7.40	15,400	1.10
17	5.63	14,400	0.99	7.27	15,200	1.20
23	5.64	14,400	1.08	7.27	15,200	0.94
40	5.68	15,500	1.21	7.36	16,000	1.26
66	5.72	14,100	0.98	7.43	14,800	0.94
40:60:0.1 (v/v) Water-Methanol-Trifluoroacetic Acid at 30°C:						
17	6.65	12,200	1.14	10.24	12,700	1.07
47	6.70	12,200	1.11	10.35	12,700	1.01
63	6.75	11,500	1.08	10.48	12,000	0.97
50:50:0.02 (v/v) Acetonitrile-Water-Triethylamine at 30°C:						
1	6.88	15,700	0.98	9.48	16,000	1.04
18	6.92	14,800	1.14	9.64	15,300	0.86
65	7.03	12,600	0.83	9.93	12,100	0.75
87	7.07	12,200	0.80	10.02	11,900	0.77

* Column washed between injections at 0.3 mL/min. Flow rate for each injection was 1.0 mL/min.

Stability of the ProTec-C_8 Medium

The particular chemistry utilized for the synthesis of the ProTec-C_8 medium allows the use of mobile phases containing 0.1% (13 mM) trifluoroacetic acid or other strong acids such as methanesulfonic acid of similar molarities. The results presented in Table 1 show that, after long exposure to mobile phases containing strong organic acids, separation efficiency and peak asymmetry are relatively unchanged, indicating good stability of the support. After long use with a basic mobile phase, i.e., 50:50:0.02 (v/v)

acetonitrile:water:TEA at 30°C, retention times increase and the number of theoretical plates and peak symmetry decrease. This indicates that the silica matrix slowly dissolves, but the ligand-silica surface bond is stable.

CONCLUSIONS

The new base-deactivated ProTec-C_8 medium incorporates basic groups in the bonded phase to internally suppress the activity of the free silanol groups. Using buffers at pH 7 0, the bonded ligands are in their cationic form and strong organic bases migrate through the column in their ionic forms. In buffers whose ionic strength is greater than 20 mM, the charge density of the anionic counter ions suppresses the coulombic repulsion between the positively charged analytes and basic ligands, resulting in hydrophobic retention of the analytes. When the counter ion concentration is decreased — when the ionic strength is decreased from 50 mM to 2 mM — the resulting increase in repulsive force decreases the retention of positively charged analyses. This can be useful for the resolution between positively charged and neutral analytes that were similarly retained using the higher buffer concentration. On the other hand, at pH 5.0, acidic analytes, for example, migrate through the column as carboxylate ions and bind to the basic ligands. As a result, their separation is enhanced with the use of an ionic strength concentration gradient.

Conditions where the basic groups of the ProTec-C_8 medium is free permit the on-column conversion of strongly basic analytes to their free base form. Separation of these free bases allows greater selectivity than was obtained for the separation of the same bases in their conjugated-acid form. Moreover, the stability of the medium at $pH_{app.}$ 1.5 allows acidic analytes to be separated under conditions in which their dissociation is suppressed.

The chromatographic characteristics of the ProTec-C_8 medium provides the analyst with a flexible chromatographic tool that can be used over a wide pH range for the enhanced separation of acids, bases and polar analytes. This unique medium allows for an ion-exchange contribution to reverse phase chromatography to enhance separation and selectivity.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ERYTHROMYCIN

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ABSTRACT

Liquid chromatographic analysis can provide a simpler and superior alternative to microbial assay of antibiotics. A number of liquid chromatographic methods for the analysis of erythromycin have been published in the past 17 years. However, many of these methods are complex and lack the selectivity needed for the assay of erythromycin in the presence of erythromycin derivatives and impurities. We examined several C_{18} based stationary phase columns and developed a significantly improved C_{18} liquid chromatographic method for the assay of erythromycin. In comparison to a recently published polymer (poly(styrene-divinylbenzene)) stationary phase LC method, our method is simpler, more rugged, faster, and more sensitive. The developed method has been successfully used for the analysis of erythromycin in commercial bulk samples. The chromatographic assay results correlate with microbiological assay.

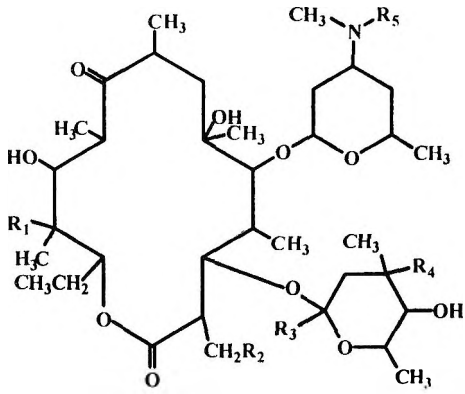
INTRODUCTION

Erythromycin is a widely used broad spectrum macrolide antibiotic. Erythromycin A (EA) is the main component of commercial bulk erythromycin. Erythromycin B (EB), erythromycin C (EC), erythromycin E (EE), N-demethyl erythromycin A (NDEA), erythromycin A enol ether (EEEA), anhydroerythromycin A (AE), and other erythromycin derivatives can be present as impurities. The antimicrobial activity of bulk erythromycin is due mainly to EA, EB & EC forms. Chemical structures of Erythromycin A and related substances are shown in Figure 1.

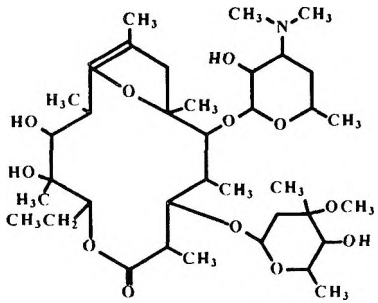
Several methods for the assay of Erythromycin A and related substances in biological samples have been published.[1-7] However, these methods are either not rugged or not suitable for the assay of erythromycin in both bulk and pharmaceutical dosage forms. In the last few years, several attempts were made to develop LC methods suitable for the assay of erythromycin in bulk and solid dosage forms.[8-12] In general, these methods lack the resolution needed for the separation of several related substances, such as EE, and common erythromycin impurities. Because of the presence of EE in commercial products and due to its low antimicrobial activity, a method capable of the separation of EE from other components is desired.[13] A column-switching technique was used by Cachet et al.[14] to separate EA from potential impurities. They succeeded in separating EE from EA, but NDEA was not separated from EE. Nilsson et al.[15] used a polymeric packing material and an alkaline mobile phase for the determination of erythromycin in plasma.

Recently,[16] a method utilizing poly (styrene-divinylbenzene) stationary phase, capable of the separation of EE and EA, was developed and adopted by the European Pharmacopoeia.[17] This European pharmacopoeial method is complex, delicate, and does not provide baseline separation of erythromycin isomers and related substances (such as EEEA), within a reasonable time. In short, the method succeeds in improving the selectivity at the expense of the simplicity and ruggedness needed for the routine assay of erythromycin.

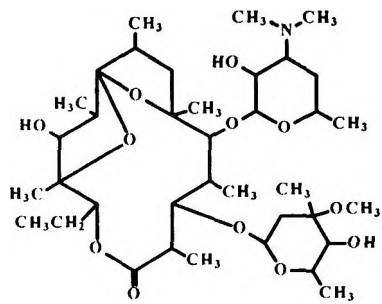
In this manuscript we are presenting a C_{18} based method that is simple, sensitive, rugged, and able to separate and assay erythromycin and most related substances commonly found in commercial samples. DryLab software[18] was used as a tool in method optimization. Computer chromatographic optimization software programs, such as DryLab, have been commercially available for a few years. In addition to speeding up chromatographic method development, these programs can be also utilized to assure the reliability, ruggedness and reproducibility of the separation.



	R_1	R_2	R_3	R_4	R_5
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3
Erythromycin B (EB)	H	H	H	OCH_3	CH_3
Erythromycin C (EC)	OH	H	H	OH	CH_3
Erythromycin E (EE)	OH	—O—		OCH_3	CH_3
N-demethylerythromycin A (NDEA)	OH	H	H	OCH_3	H



Erythromycin A enol ether (EEEA)



Anhydroerythromycin A (AE)

Figure 1. Chemical Structures of Erythromycin A and related substances.

Table 1

Gradient System for the Erythromycin Assay

Time (min.)	%A[a]	% B[b]
0.0	78.0	22.0
39.0	26.0	74.0
47.0	26.0	74.0

[a] Mobile phase A (10% CH_3CN) was prepared by mixing 69 mL stock ammonium phosphate buffer (0.20 M, pH = 6.5), 60 mL stock tetrabutylammonium sulfate (0.20 M, pH = 6.5), and about 250 mL Milli-Q water. This was followed by the addition of 100 mL acetonitrile, diluting to 1 L with milli-Q water, mixing well and filtering through a 0.45 μm nylon membrane filter.

[b] Mobile phase B (50% CH_3CN) was prepared as in "A" with the only exception being the use of 500 mL acetonitrile in the mobile phase preparation.

EXPERIMENTAL

Chemicals and Reagents

USP erythromycin reference standard (RS) was used throughout the study. Other erythromycin standards (EB, EC, EE, NDEA, AE, and EEEA) were obtained from Abbott Laboratories, North Chicago, Illinois. In addition, Abbott Laboratories kindly provided samples of ten different batches of bulk erythromycin along with the relevant analytical data. Two bulk erythromycin samples were provided by the UPJOHN Company (Kalamazoo, Michigan). Several samples of bulk erythromycin were purchased from commercially available sources as identified in Table 4. All erythromycin samples were used without further treatment.

Ammonium hydrogen phosphate, ammonium hydroxide, and tetrabutylammonium hydrogen sulfate were purchased from different sources of the highest available purity and, were used without additional purification. Acetonitrile and methanol were HPLC grade and water was deionized and filtered through a Milli-Q™ water purification system (Millipore, New Bedford, MA).

Solutions

Stock 0.20 M ammonium phosphate buffer was made by dissolving the calculated amount of $(NH_4)H_2PO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter. Stock 0.20 M tetrabutylammonium sulfate (mobile phase additive) was made by dissolving the calculated amount of $(C_4H_9)_4NHSO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter.

Chromatographic Conditions

The HPLC system used in this investigation consisted of Spectra-Physics SP 8800 pump, Spectra-Physics SP 8880 autosampler, Spectra FOCUS Forward Optical Scanning detector set at 205 nm (unless specified otherwise), COMPAQ DESKPRO XL 5100 computer, and PC1000 System Software™ (Ver. 3.0). Two Prodigy 5 ODS-2 250 x 4.6 mm I.D. columns (Phenomenex, Torrance, CA) were used in the study. The gradient profile and mobile phase composition are described in Table 1. In all experiments, the concentrations of both the buffer and tetrabutylammonium sulfate were maintained constant in both mobile phases and the only difference between mobile phase A and B is the percentage of acetonitrile (10 vs 50, respectively). The gradient delay volume was determined to be 5.5 mL and no equilibration time was needed between injections. The mobile phase flow rate was set at 1.3 mL/min. The column temperature was controlled at 45°C with a block column heater (Jones Chromatography, Lakewood, CO) and the sample injection volume was 50 μL.

Samples were prepared by dissolving the weighed amount in a solvent made by mixing equal volumes of the mobile phases (50%A-50%B) to give a final concentration of 5-10 mg/mL. Samples were placed in an ultrasonic bath for about 5 minutes to enhance dissolution. In developmental experiments, different sets of chromatographic conditions were optimized, selected and employed (as in Figures 2 & 3).

RESULTS AND DISCUSSION

Developmental Experiments

The initial objective was to develop a simple and rugged HPLC method, preferably an isocratic reverse phase procedure, for the assay of erythromycin and most common isomers and impurities found in commercial bulk products.

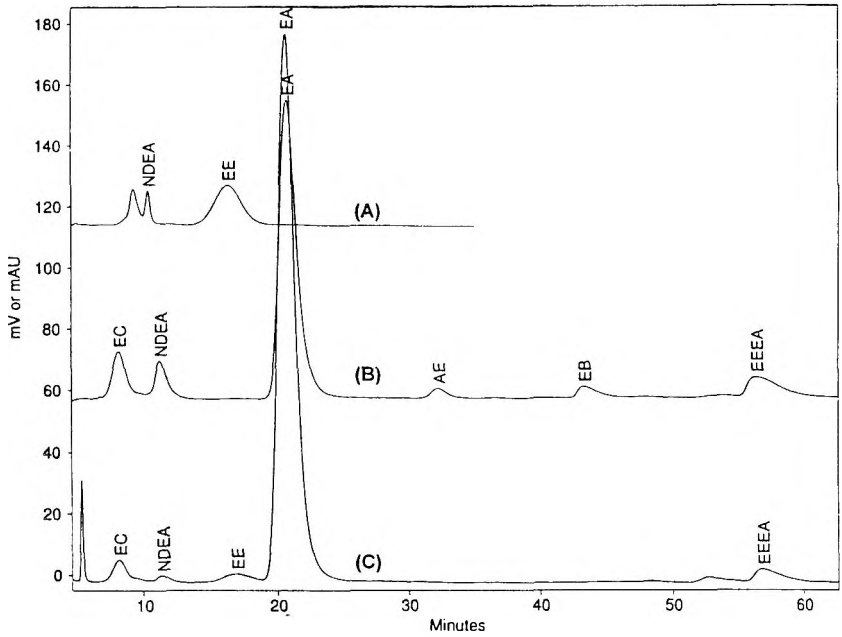


Figure 2. Typical chromatograms (monitored at 205 nm) obtained for (A) standard erythromycin E (EE), (B) a standard mixture of EC, NDEA, EA, AE, EB & EEEA, and (C) a commercial bulk erythromycin sample. Chromatographic conditions: mobile phase was made by mixing 255 mL CH_3CN, 60 mL of tetrabutylammonium sulfate (0.20 M, pH 6.5), 60 mL of ammonium phosphate buffer (0.20 M, pH 6.5) and diluting to 1.0 L with Milli-Q water; flow rate was set at 1.5 mL/min. and temperature was controlled at 45 °C.

After thorough review of the recent literature,[10-14] several commercially available C_{18} columns suitable for the assay of basic compounds, were examined.

Many of the tested C_{18} columns failed to provide baseline separation of NDEA, EE, and EA. Using a Zorbax RX-C_{18} (250 x 4.6 mm I.D.), we succeeded in developing a simple isocratic method capable of the separation of EA, EB, EC, EE, NDEA, AE, and EEEA. Figure 2 provides an illustration of the chromatographic separation achieved and a summary of chromatographic conditions. This method has distinct advantages over the recently adopted European Pharmacopoeial method.[17] The method is simpler and more sensitive.

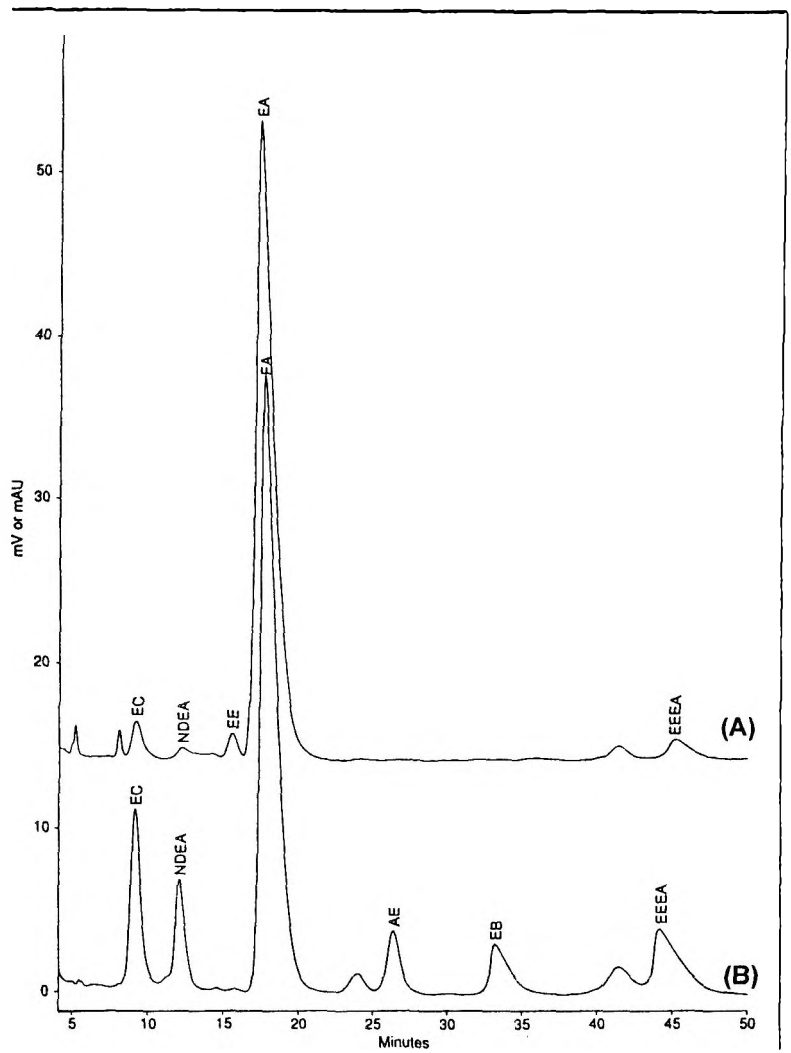


Figure 3. Typical chromatograms (monitored at 215 nm) obtained for (A) a commercial bulk erythromycin sample and (B) a standard mixture of EC, NDEA, EA, AE, EB & EEEA. Chromatographic conditions: mobile phase was made by mixing 500 mL CH_3OH, 100 mL of tetrabutylammonium sulfate (0.20 M, pH 6.5), 125 mL of ammonium phosphate buffer (0.20 M, pH 6.5) and diluting to 1.0 L with Milli-Q water; flow rate was set at 1.5 mL/min. and temperature was controlled at 45 °C.

Major components (including EEEA) are eluted in a reasonable time (less than 60 minutes) and peaks are more symmetrical. However, the EE peak and the late eluted peaks are broad, as expected, in a long isocratic run. In addition, the column life span under the assay conditions is short .

In order to improve method ruggedness and to overcome these disadvantages, another attempt was made utilizing the same Zorbax RX-C18 column. Methanol replaced acetonitrile as the organic modifier and both the buffer and tetrabutylammonium sulfate concentrations were adjusted for optimum separation. Typical chromatograms obtained are illustrated in Figure 3. Compared to acetonitrile, methanol has a similar effect on the system selectivity but the resolution between EE and EA is improved. Also, the use of methanol results in shortening the analysis time (50 minutes versus 60 minutes).

We had an initial success in applying this improved methanol method in the assay of erythromycin in bulk, as well as, finished solid dosage forms. However, the method shared most of the problems observed previously (acetonitrile method) including broad peaks (especially EEEA) and the column packing material instability under the employed chromatographic conditions. At this point, it was apparent that a different approach in C_{18} column selection was needed, aiming at enhancing the stability of the packing material without sacrificing the selectivity of the chromatographic system.

Gradient Method Development

Recently, several articles and application notes have been published, emphasizing the effect of high purity silica on the improvement of the resolution and peak shapes for basic compounds. New generation columns are made from high purity reagents and use improved bonding and packaging technologies . For example, Prodigy™ columns are made using high purity silica and manufacturer data strongly suggest greater stability (> 1000 hours) over a wide pH range (2.0 - 9.0).[19]

Kirkland et al.[20] have suggested recently that some silica-based C_{18} packings can be used for long periods at higher pH, without significant changes in packing material stability. A Prodigy™ column was selected for this investigation.

It has been shown, that the use of quaternary ammonium salts improve the quality of separation of basic compounds on C_{18} reverse phase chromatography, in addition to shortening analysis time possibly by preventing undesirable retardation effects commonly found in C_{18} columns.[10,12,21,22]

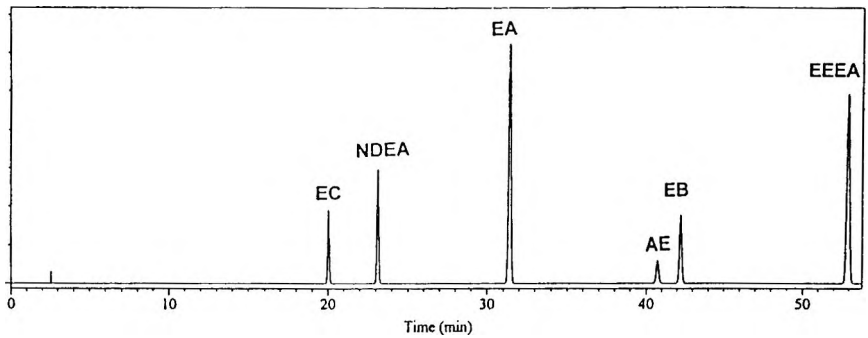


Figure 4. An optimized DryLab chromatogram of a standard erythromycin mixture(EA, EB, EC, NDEA, AE, and EEEA). Chromatographic conditions of preliminary gradient runs are described in Table II. t_0=2.56 min (estimated from preliminary runs), minimum resolution 4.89, and run time 54 min.

Table 2

Chromatographic Conditions used to Generate Data Needed for DryLab Method Optimization[a]

Time (min.)	%A[b]	% B[b]
0.0	80.0	20.0
t_G[c]	20.0	80.0

[a] HPLC system and Column were the same as described in the experimental section. The gradient delay volume was determined to be 5.5 mL, the temperature was controlled at 45 °C and flow rate was set at 1.0 mL/min.

[b] Mobile phases A and B are the same as described in Table 1.

[c] the gradient time (t_{G1}) for the first run was 50.0 minutes, whereas the gradient time for the second run (t_{G2}) was 100.0 minutes.

Tetrabutylammonium sulfate was the quaternary ammonium salt used in this study. Also, from the literature and our previous experience, it seemed that a phosphate buffer at pH 6.5 provides the optimum buffer for the separation without accelerating the degradation of erythromycin.

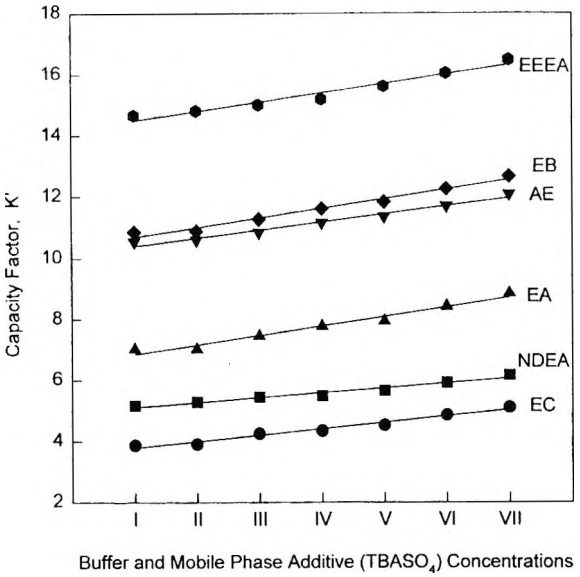


Figure 5. Effect of varying the concentrations of buffer (ammonium Phosphate, pH 6.5) and mobile phase additive (tetrabutylammonium sulfate, pH 6.5) on the separation of erythromycin isomers and related substances . The gradient profile (Table 1) was employed and the temperature was controlled at 45°C.

Mobile Phase	Buffer Conc., mM	Mobile Phase Additive Conc., mM
I	6.0	6.0
II	6.0	12.0
III	12.0	6.0
IV	12.0	12.0
V	12.0	24.0
VI	24.0	12.0
VII	24.0	24.0

DryLab software[18] was one of the tools used in method optimization. Two gradient runs of a standard mixture (made by mixing EA, EB, EC, AE, NDEA, and EEEA) were carried out under the chromatographic conditions described in Table 2 and, the data obtained was used by DryLab to generate a computer optimized separation (Figure 4). Chromatographic parameters generated by DryLab were then optimized further to produce the developed gradient assay

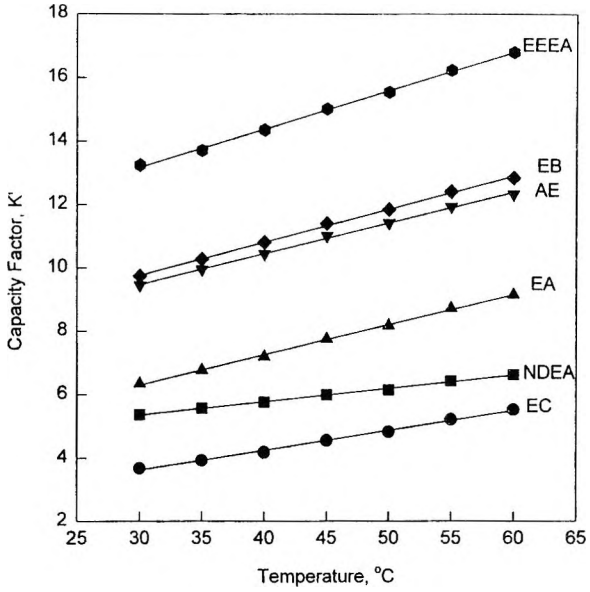


Figure 6. Effect of temperature on the separation of erythromycin isomers and related substances. Details of chromatographic conditions are described in the experimental section and Table 1. The concentrations of both the buffer and tetrabutylammonium sulfate were 12.0 mM.

method (as described in the experimental section and Table 1). Limited success was obtained in the conversion of the gradient method to an isocratic procedure. The isocratic procedure has some undesirable features such as: broad peaks, longer analysis time, and a noticeable decrease in sensitivity.

In order to confirm the ruggedness of the developed method, the effects of temperature, buffer concentration and the concentration of the mobile phase additive (tetrabutylammonium sulfate) on the developed gradient method (as described in the experimental section and Table 1) were investigated. The effect of varying the concentrations of both the buffer (ammonium phosphate, pH 6.5) and the mobile phase additive (tetrabutylammonium sulfate, pH 6.5) on the system selectivity was examined (chromatographic runs I-VII). Figure 5 summarizes the effect of varying the concentrations of the buffer and tetrabutylammonium sulfate on the separation of erythromycin and related substances. Erythromycin E (EE) was not used in the preparation of the

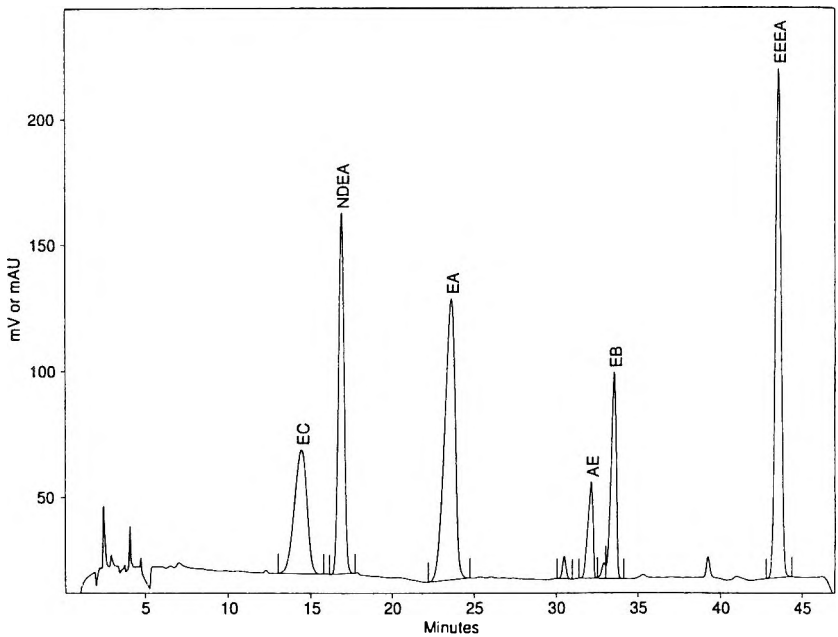


Figure 7. Typical chromatogram of a standard mixture containing EC, NDEA, EA, AE, EB, and EEEA. Details of chromatographic conditions are described in the experimental section and Table1.

standard mixture because only a very small amount (<1 mg) was available and it was used only as an HPLC marker (as in Figure 2). Mobile phase composition IV (12.0 mM buffer and 12.0 mM tetrabutylammonium sulfate) was selected. It provides optimum resolution and increasing the concentration of either component does not enhance the separation significantly.

From Figure 5, it is evident that a slight variation in the concentration of either the buffer or the additive (tetrabutylammonium sulfate) does not affect the selectivity. The effect of temperature on the separation of erythromycin isomers and related substances was also investigated (Figure 6). In order to optimize the separation of erythromycin and foster the stability of the column packing material, a problem encountered in our earlier experiments, the temperature was controlled at 45 °C.

Figure 7 provides an illustration of a typical chromatogram of a standard mixture analyzed, using the developed gradient method, as described in the experimental section and Table 1, whereas Figure 8 is a chromatogram of a

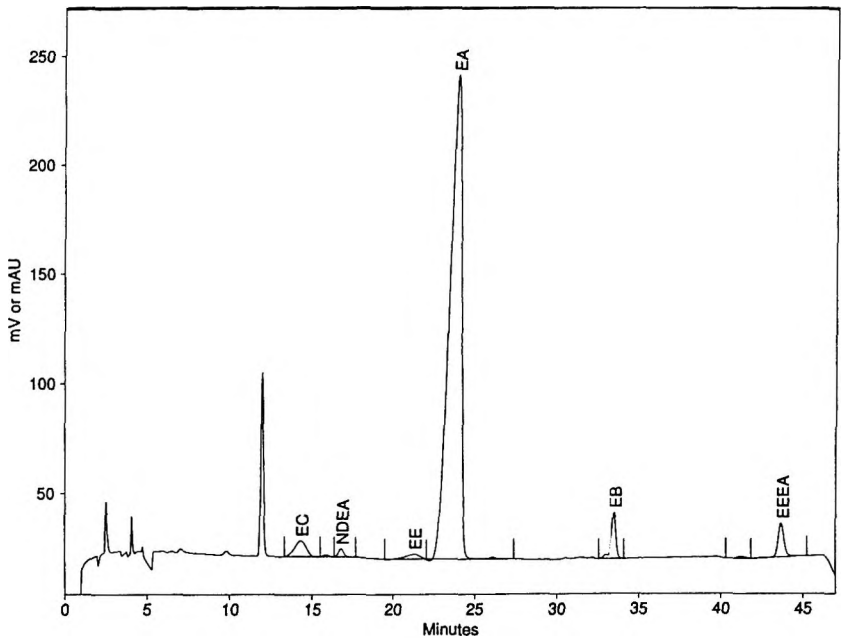


Figure 8.Typical chromatogram (monitored at 205 nm) of a bulk erythromycin sample (10.00 mg/mL) analyzed using the developed method. Details of chromatographic conditions are described in the experimental section and Table 1.

Table 3

HPLC Method Performance, Repeatability (n=5)

Components Found in a Bulk Erythromycin Sample	Retention Time, min. (% RSD)	Peak Area x 10^{-5} (% RSD)	Peak Ht x 10^{-3} (% RSD)
EC	14.4 (0.27)	3.50 (1.41)	7.24 (0.79)
NDEA	16.9 (0.18)	0.87 (2.15)	3.84 (1.45)
EE	21.4 (0.34)	1.68 (5.60)	2.54 (2.43)
EA	24.1 (0.25)	114.0 (0.37)	222.1 (0.28)
AE	32.1 (0.16)	0.30 (5.27)	2.01 (5.07)
EB	33.6 (0.17)	4.17 (0.48)	21.19 (0.43)
EEEA	43.8 (0.17)	3.53 (2.71)	14.79 (1.77)

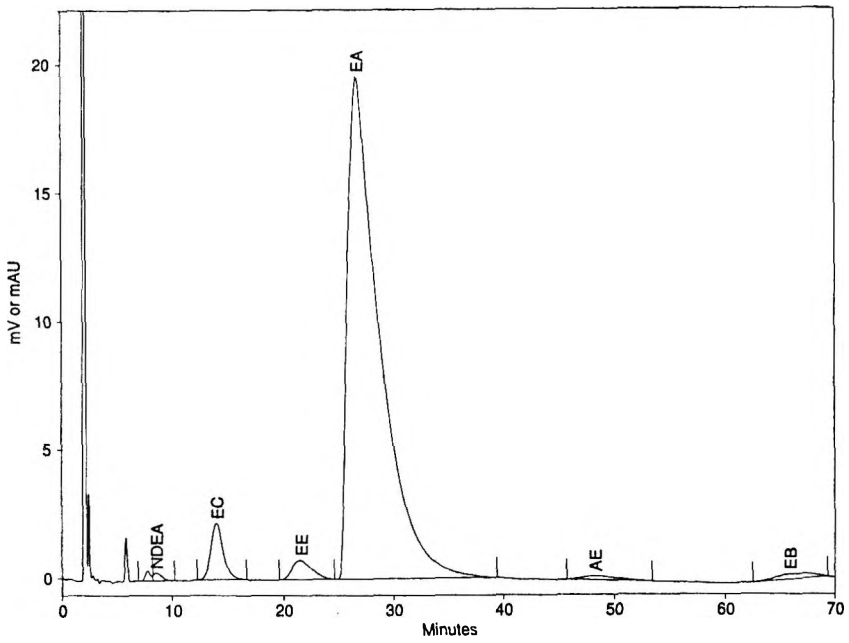


Figure 9. Typical chromatogram (monitored at 215 nm) of a bulk erythromycin sample (same sample as in Figure 8 at a concentration of 7.70 mg/mL) analyzed using the European Pharmacopoeial Method (17). EEEA was not observed due to its longer retention (greater than 80 minutes).

commercial bulk erythromycin sample. The result of method performance repeatability test (Table 3) illustrates the high precision obtained. In another test for system ruggedness, packing materia batch-to-batch evaluation was performed by using two Prodigy columns: column 1 (batch 17M2; serial number 103575) and column 2 (batch 19M; serial number 113448). The chromatography obtained in both cases was nearly identical and there was no apparent difference in system selectivity.

The same commercial sample was analyzed using the recently adopted European Pharmacopoeial method,[16,17] in which a Polymer Lab 250 x 4.6 mm I.D. PLRP-S column (1000 Å, 8 μ) was used at a flow rate of 1.0 mL/min (Figure 9). Due to high backup pressure and baseline noise, the flow rate was reduced from 2.0 mL/min as specified by the European Pharmacopoeia. Others involved in the validation of the European Pharmacopoeial method experienced

Table 4

HPLC Assay of Commercial Bulk Erythromycin Products

Commercial Bulk Products[a]	% Erythromycin[b,c] (% RSD)
Boehringer Manneheim, Lot No. 13742321-21	91.2 (0.34)
UPJOHN, Lot No. 306AA	96.7 (0.16)
UPJOHN (Micronized), Lot No. 159MH	96.4 (0.24)
Pharma-Tek ERYTHRO-R_x Lot No. ZJ4E	94.1 (0.18)
ICN Biomedicals, Lot No. 50148	101 (0.19)
Fluka BiomChemika, Lot No. 333594/1	95.7 (0.12)
Spectrum, Lot No. KC122	96.9 (0.45)
SIGMA, Lot No. 31H0577	97.2 (0.22)
SIGMA, Lot No. 61H03496	99.0 (0.42)

[a] Samples were dissolved in a solvent made of 50%A and 50%B (as described in the experimental section) at concentrations of 5-10 mg/mL.
[b] Average of 3 runs.
[c] Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on EA response factor.

the same difficulty.[23] Both the European Pharmacopoeial method and our developed method, succeed in the separation of erythromycin isomers and many of its impurities. Comparing Figures 8 and 9, the enhancement in sensitivity (ten fold), peak symmetry, selectivity, and resolution the developed gradient method provides should be noted. Also, the developed method is simpler and has a shorter anlaysis time.

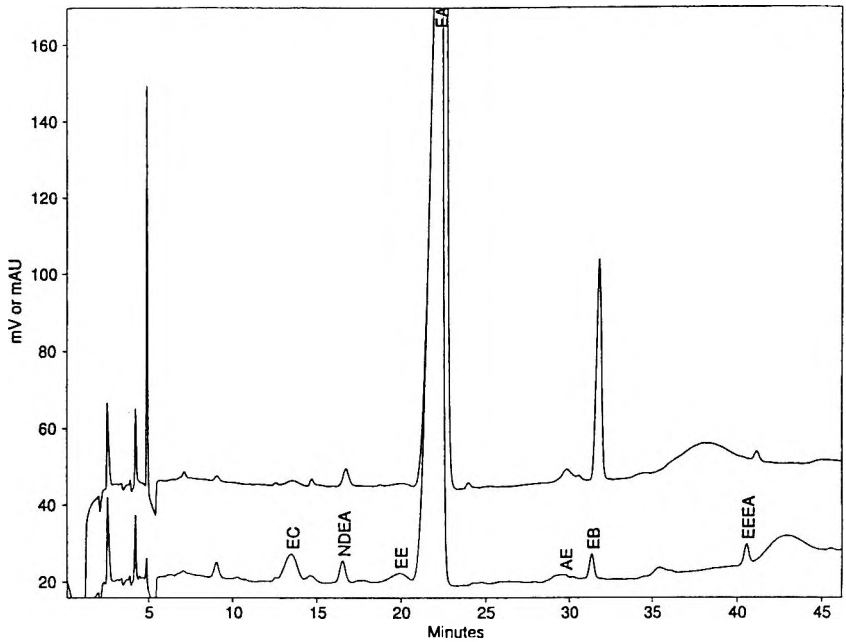


Figure 10. An illustration of detected differences in chromatographic patterns distinguishing two major United States bulk erythromycin manufacturers. Details of chromatographic conditions are described in the experimental section and Table 1.

Stability of Erythromycin in Sample Solutions

Recent literature addressed the stability of erythromycin solutions.[24,25] Erythromycin solutions were found to be relatively stable in neutral media (pH 6-8)[24] in both methanol and acetonitrile solutions.[25] However, acetonitrile solutions were more stable.[25] Since the solvents used in these studies[24,25] were different from the sample solutions (50%A - 50%B) used in this investigation, the stability of erythromycin in sample solutions was tested both at room temperature and 45.0 °C in order to accelerate any possible degradation. Erythromycin solutions were stable at room temperature for more than 48 hours and a degradation product with similar retention to EB was produced in measurable amounts after 5 days. At 45.0 °C, solutions were stable up to 4 hours, after which chromatographic interferences with EB was noticeable. In summary, erythromycin solutions are stable at room temperature many hours longer than needed under the described analysis conditions and the production of degradation product(s) should not affect the accuracy of the assay.

Table 5

HPLC Assay of Ten Different Lots of Abbott's Bulk Erythromycin

Lot Number	Pharm. Eur. (17)[a,b]	USP Bioassay[a]	HPLC Assay[b,c]
1	91.4%	90.6%	92.8%
2	93.4%	94.2%	90.2%
3	93.0%	96.1%	86.6%
4	91.5%	93.4%	88.1%
5	90.9%	91.8%	93.5%
6	89.8%	91.9%	90.8%
7	94.7%	93.2%	94.8%
8	90.9%	92.6%	93.2%
9	91.4%	93.6%	90.8%
10	89.2%	92.1%	89.3%

[a] Analytical data was provided by Abbott Laboratories.
[b] Calculated as the sum of erythromycins A, B, and C.
[c] Average of 3 runs.

Assay of Bulk Erythromycin Samples

Several commercial samples of bulk erythromycin were obtained and assayed using the developed method. The results of the assay are summarized in Table 4. The percentage of erythromycin in these samples was calculated as the sum of EA, EB, and EC. The absorptivities of erythromycin isomers and related substances in the sample solution (50%A - 50%B) at 205 nm were determined and, it was found that the relative absorptivity of EB/EA = 0.88 and EC/EA = 1.2. In spite of these differences, the concentrations of both EB and EC were calculated using the response factor of EA.

The ability of the developed method in the separation of erythromycin and its common impurities, coupled with the enhanced sensitivity especially for late eluted peaks (such as EB & EEEA), makes this method a useful technique in distinguishing different sources of bulk erythromycin.

Figure 10 provides an illustration of detected differences in chromatographic patterns of two major United States bulk erythromycin manufacturers. Chromatographic fingerprinting is becoming a useful tool in the identification of bulk pharmaceutical sources and in fraud investigations.[26]

Ten different samples, representing ten different batches, were obtained from Abbott Laboratories (North Chicago, Illinois) and analyzed using the developed gradient method. The results of their assay using this HPLC method, the European Pharmacopoeial method, and USP bioassay procedure are summarized in Table 5. The results in Table 5 illustrates the good correlation obtained for most batches between the developed method, with both the USP bioassay and the European Pharmacopoeial assay procedure.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF MULTIPLE SULFONAMIDE RESIDUES IN CHICKEN MUSCLE USING PRE-COLUMN DERIVATIZATION AND FLUORESCENCE DETECTION

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ABSTRACT

A rapid liquid chromatographic (LC) method is described for the quantitation of sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline residues in chicken muscle. The sulfonamides are extracted with chloroform, partitioned into hydrochloric acid, and submitted to pre-column derivatization with fluorescamine. LC analysis of the fluorescent derivatives is performed on a C_{18} column using a mobile phase of acetonitrile /20 mM phosphate buffer pH 4, (34/66, v/v), containing 20 mM octanesulfonate sodium salt. Owing to the sensitivity and selectivity of the fluorescence detection, residue

levels of as low as 3 ng/g for sulfadiazine, 4 ng/g for sulfamethazine, 9 ng/g for sulfadimethoxine, and 40 ng/g for sulfaquinoxaline could be readily determined in chicken muscle. Overall recoveries were found to be 77.7±4.8% for sulfadiazine, 84.6±4.2% for sulfamethazine, 92.3±4.1% for sulfadimethoxine, and 82.5±7.0% for sulfaquinoxaline. The linearity of the method was quite acceptable in the range examined (5-100 ng/g for sulfadiazine and sulfamethazine, 15-150 ng/g for sulfadimethoxine, and 50-300 ng/g for sulfaquinoxaline).

INTRODUCTION

Sulfonamides are widely used in food-producing animals for the prevention and treatment of diseases. However, improper use of these antibacterials can leave residues in edible animal products. Owing to the potential impact of such residues on human health, maximum residue levels that oscillate between 0 and 100 ppb have been established,[1-3] whereas food surveys for monitoring of violatile sulfonamide residues are often made.[4]

Large-scale screening applications require multiresidue methods that are rapid, accurate, selective, and provide low detection limits. Several multiresidue LC methods for analyzing sulfonamides in animal tissues have been reported, but most are based on ultraviolet detection which does not have sufficient selectivity to eliminate matrix interferences without resorting to time-, labor-, and material-intensive sample purification.[5-9] Post-column derivatization and detection at 450 nm has been also applied for enhancing the selectivity of the analysis, but this approach still requires significant handling for sample cleanup,[10] or for setting up and optimizing the rather expensive equipment needed.[11]

Pre-column derivatization with fluorescamine and fluorescence detection has been suggested,[12] in the past, as a very efficient means of increasing the selectivity and sensitivity of sulfonamides analysis by LC. This most promising approach, which eliminates extensive clean-up and/or chromatographic separation of the fluorescent derivatives from the excess reagent, has been recently adopted in the development of rapid LC methods for the determination of multiple sulfonamide residues in animal tissues.[13,14]

These methods give useful information for trace analysis of sulfonamide residues, but conflict each other as far as the optimum pre-column derivatization conditions and some performance characteristics concern.

The objectives of this study were a re-examination of the conditions that influence the pre-column derivatization with fluorescamine, and the establishment of the most suitable LC conditions for separation of the fluorescent derivatives in order to develop an optimized LC multiresidue method for rapid, sensitive, accurate, and precise quantitation of some sulfonamides, representative of those commonly used in a commercial basis, in chicken muscle.

EXPERIMENTAL

Instrumentation

LC was carried out on a Gilson system consisting of a Model 802 manometric module, a Model 302 piston pump, a Model 121 fluorometric detector, a Model TC 831 HPLC-Technology column oven (Macclesfield, UK), and a model N1 variable-span recorder (Villiers-le-Bel, France). Injections were made using a Rheodyne 7125 sample injector equipped with a 20-μL loop.

Homogenization of the samples was performed using an Ultra-Turrax (Janke & Kunkel, GmbH, Germany) high speed blender, and centrifugation using a Centra-MP4 IEC centrifuge (Needham Heights, MA, USA).

Reagents

Analytical-grade reagents including chloroform, hydrochloric acid, phosphoric acid, sodium dihydrogen phosphate, and octanesulfonate sodium salt, and HPLC-grade methanol and acetonitrile were obtained from Merck AG (Darmstadt, Germany). Sodium acetate Suprapur-grade was purchased from Merck-Schuchard (Munchen, Germany), whereas, HPLC-grade water was produced using a Milli-Q (Millipore, Bedford, MA, USA) purification system.

Fluorescamine reagent (0.1%) was prepared by dissolving 10 mg of Fluram (Sigma, St. Louis, MO, USA) in 10 mL of acetonitrile. The reagent was prepared fresh weekly and stored in the dark at 4 °C when not in use.

Standard sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline compounds were all purchased from Sigma (St. Louis, MO, USA). Stock solutions of the individual sulfonamides (100 μg/mL) were prepared by weighing ca 10 mg of each and dissolving in and diluting to 100 mL with methanol. Aliquots of these stock solutions were appropriately diluted with 3 N hydrochloric acid to give standard working solutions for the calibration

curve study in the range 4.8-300 ng/mL. Spiking solutions for the precision and accuracy study were also prepared from stock solutions by diluting with 0.03 M sodium dihydrogen phosphate (range 0.3-18 μg/mL).

Extraction Procedure

The extraction procedure used in this investigation was based on that reported by Takeda and Akiyama.[13] A 3-g ground tissue sample was mixed with 30 mL chloroform to be further homogenized for 2 min. Following centrifugation for 5 min at 3000g, the supernatant was filtered through filter paper, and a 10-mL aliquot was added to 1 mL 3 N hydrochloric acid. The two-phase system was vortexed for 1 min, and centrifuged for 5 min at 2000g. The aqueous layer was collected and a 250-μl volume was submitted to the derivatization procedure.

Derivatization Procedure

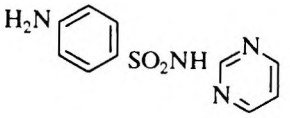
The collected aqueous extract was mixed with an equal volume (250-μL) of 3.8 M sodium acetate solution to be further mixed, under vortexing, with 100 μL of fluorescamine reagent. The reaction was left to proceed for 20 min at ambient temperature before a 20-μL aliquot, which was equivalent to 0.0083 g of the sample, be injected into the LC system.

Derivatization of standard sulfonamides was also performed using 250-μL volumes of each of the working solutions and mixing them with 250 μL 3.8 M sodium acetate and 100 μL of fluorescamine reagent as above.

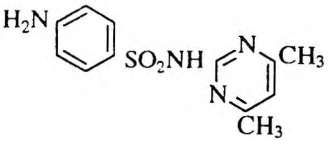
LC Analysis

LC analysis was performed at 30 °C on a reverse phase Hichrom column, 250x4.6 mm, packed with Nucleosil 120 C_{18}, 5 μm, whereas a mobile phase consisting of acetonitrile/20 mM sodium dihydrogen phosphate, pH 4, (34/66, v/v) and containing 20 mM octanesulfonate sodium salt was used to elute, isocratically, the fluorescent sulfonamide derivatives. The mobile phase was passed through 0.45 μm filter before use, degassed using helium, and delivered at a rate of 1.2 mL/min.

Detection was made using an excitation wavelength of 405 nm and an emission wavelength of 495 nm. Recordings were made at a chart speed of 2 mm/min and a 0.02 RFU sensitivity setting.



Sulfadiazine



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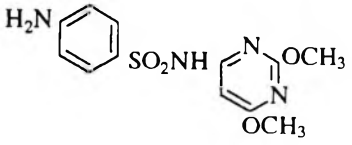
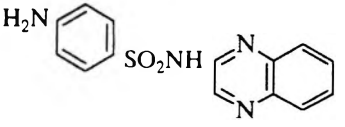
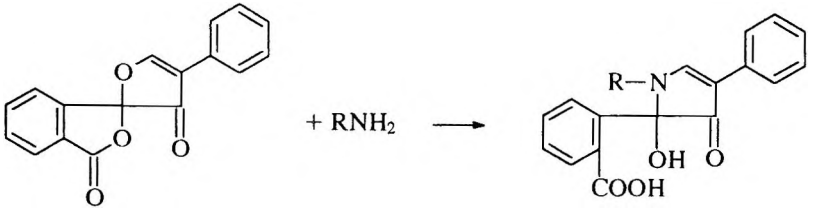


Sulfadimethoxine



Sulfaquinoxaline



Fluorescamine Sulfonamide derivative

Figure 1. Chemical structures of investigated sulfonamides and their reaction with fluorescamine.

Calculation

Standard calibration curves were constructed by derivatization and LC processing five replicates of each of the four series of the working solutions of standard sulfonamides. The recorded peak heights, y (mm), were plotted versus the quantity, x (ng), of each sulfonamide injected, and the slope (b), intercept (a), and least square fit of each calibration curve were computed according to the equation $y=a+bx$. The concentration (ppb) of each sulfonamide in tissue samples was determined by reference to corresponding calibration curve and multiplication by appropriate dilution factor.

Table 1

Peak Heights (mm) of Sulfadiazine (SDZ), Sulfamethazine (SMZ), Sulfadimethoxine (SDX), and Sulfaquinoxaline (SQX) Derivatives with Fluorescamine as a Function of the Reaction Time and the Derivatizing Reagent Concentration

	0.02% Fluorescamine				0.05% Fluorescamine				0.10% Fluorescamine			
Reaction Time, Min	SDZ	SMZ	SDX	SQX	SDZ	SMZ	SDX	SQX	SDZ	SMZ	SDX	SQX
5	35.8	46.4	46.5	35.4	64.3	64.3	85.5	61.0	89.9	75.2	115.7	86.1
10	43.2	51.0	61.0	49.2	77.0	68.7	108.9	80.0	96.0	75.0	131.2	93.2
20									107.7	79.7	141.4	100.8
35	65.9	59.0	106.0	75.0	79.3	64.7	120.5	82.9	96.9	75.4	139.2	100.1
40	70.1	61.4	102.0	75.3	76.6	62.1	128.7	90.2	79.3	63.3	139.3	93.0
90	52.9	47.1	94.3	65.3								
125					72.7	56.6	106.5	73.1	83.8	62.9	124.7	88.2

RESULTS AND DISCUSSION

Optimization of Fluorescamine Derivatization

Fluorescamine, a fluorogenic reagent specific for primary amines, reacts directly with sulfonamides at ambient temperature to form pyrrolinone derivatives (Fig. 1), which upon excitation at 405 nm emit strong fluorescence at 495 nm.[12] Separation of any excess reagent, that might interfere with the quantitation of the fluorescent derivatives is not required, since fluorescamine and its hydrolysis products, unlike other fluorogenic reagents, are nonfluorescent.[15] These properties have made the fluorescamine reaction attractive for the identification and quantitation of many compounds, notably those of biological importance.[16] Analytical parameters for optimal reaction conditions of sulfonamides with fluorescamine prior to LC can be found in recent literature; however, complete agreement as to the exact pH value, time, and amount of fluorescamine needed for optimal reactivity does not exist.[13,14]

Using serially diluted sodium acetate solution for the derivatization reaction, optimum reactivity was found in the pH range 3.0-3.4 (3.5-3.8 M sodium acetate solution), decreasing at both lower and higher pH values. It was further observed, that the purity of the sodium acetate reagent could become the critical parameter in the LC analysis of the produced fluorescent derivatives; considerable interferences appeared when not highly purified sodium acetate was used.

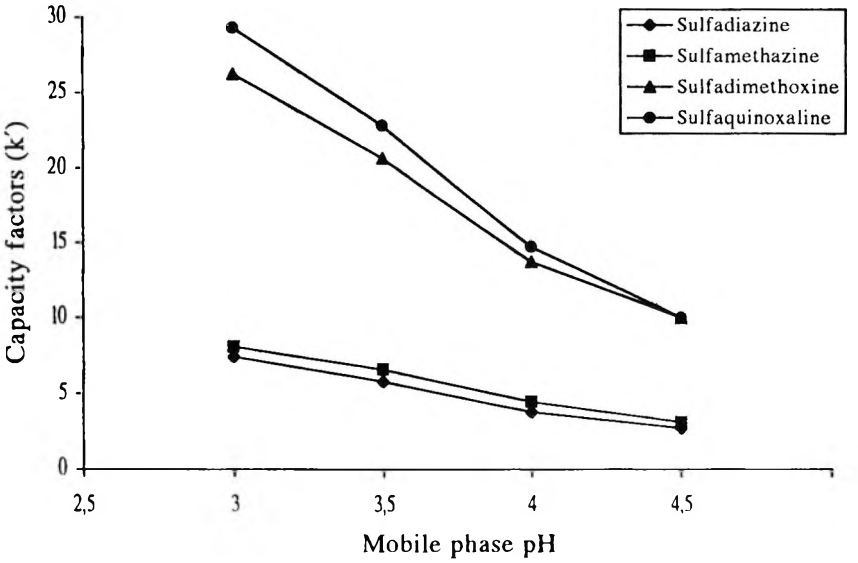


Figure 2. Capacity factors (k') of fluorescamine derivatives of sulfonamides versus mobile phase pH. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer (34:66, v/v); column, 250x4.6 mm, C_{18} 5 µm; temperature, 30 °C; flow rate, 1.2 mL/min; wavelength, ex 405 nm em 495 nm.

These results lend support to previous findings[13] indicating that the fluorescence yield at pH 3.75 is lower than that at 3.0, but oppose others suggesting optimum reactivity at pH 3.6.[14]

Using a 3.8 M sodium acetate solution to attain a pH value of 3.4 for the derivatization mixture, the reaction time and the amount of fluorescamine needed for optimal derivatization were also investigated. Table 1 shows the effect of reaction time on peak heights of the produced sulfonamide derivatives under three different concentrations of fluorescamine reagent. It becomes evident that the reaction yield largely depends on both the reaction time and the concentration of the fluorescamine reagent used. Optimal reactivity for all sulfonamides was observed after 20-min reaction using the 0.1% fluorescamine reagent. When the reaction was left to proceed for less than 5 min, as in the method of Tsai and Kondo[14] where it proceeds for only 1 min, the yield was low, even using 0.1% fluorescamine reagent. On the other hand, when the reaction was carried out with 0.02% fluorescamine, as in the procedure of Takeda and Akiyama,[13] the yield was very low, even if the reaction was left to proceed longer than 20 min.

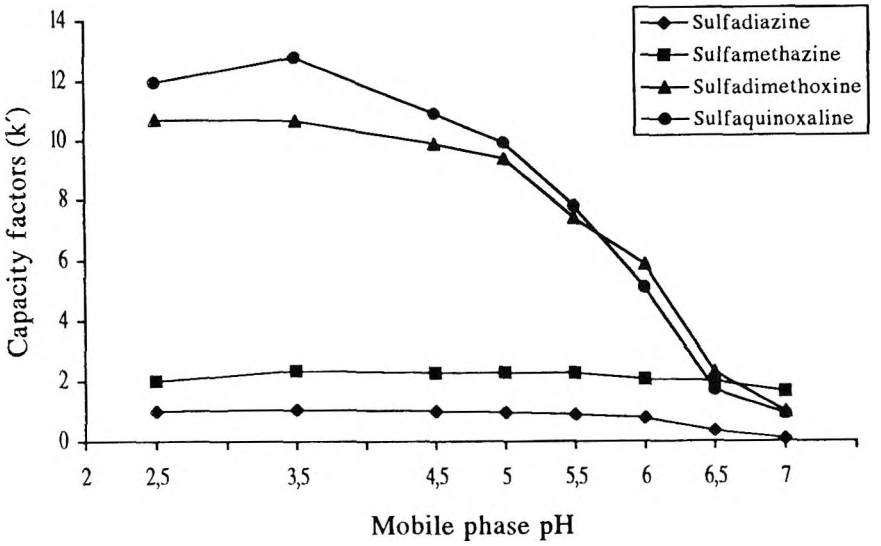


Figure 3. Capacity factors (k') of underivatized sulfonamides versus mobile phase pH. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer (18:82, v/v); column, 250x4.6 mm, C_{18} 5 μm; temperature, 35 °C; flow rate, 1.0 mL/min; wavelength, 272 nm.

LC Analysis

Literature LC conditions have described reverse phase separations of the fluorescamine derivatives of sulfonamides, using mobile phases of either acetonitrile-2% acetic acid, pH 2.5, at 55 °C or acetonitrile-10 mM phosphate buffer, pH 5.3, at ambient temperature. None of these conditions was found to be the most suitable to our LC system; the former ones caused a gradual baseline rise during each run unless column temperature had been set below 40 °C, a temperature where the compounds eluted too late, while the latter resulted in chromatograms with small but undesirable peak tailing.

Initial experiments towards optimizing LC conditions were made using acetonitrile/20 mM potassium dihydrogen phosphate (34/66, v/v) mobile phases of varying pH. LC runs were all performed at 30 °C, a temperature slightly higher than the ambient temperature, to avoid decreasing the fluorescence intensity of the sulfonamide derivatives due to elevated temperature. In Fig. 2 the observed capacity factors, k', of the derivatives of sulfonamides as a function of mobile phase pH are presented. The larger, apolar derivatives of sulfadimethoxine and sulfaquinoxaline gave the highest k' values. On the other hand, slight changes in the mobile phase pH had great impact on k' values of all

compounds, the retention behavior being mainly governed by the ionization state and polarity; all k′ values lowered with increasing pH from 3 to 4.5, a behavior accounted for by the presence of the carboxylic group which should be in a dissociated form in the derivatized molecules. Contribution to this ionization state by other groups should be excluded at this pH range, since the acidic NH linkage adjacent to the sulphonyl group has a pK_a ranging from 6.0 for sulfadimethoxine to 7.6 for sulfamethazine.[11] Additional support to these literature values may be given by Fig. 3 where the capacity factors of underivatized sulfonamides versus mobile phase pH are presented.

Fig. 2 shows that the sulfonamide derivatives remain well resolved when the pH of the mobile phase is less than 4.5; however, the retention of the late eluted compounds is high at these conditions. Attempts to decrease their retention by increasing the concentration of acetonitrile in the mobile phase resulted in loss of resolution. Considering, finally, the ionization state of the analytes, the use of octanesulfonate, a negatively charged ion-pairing reagent, for decreasing their retention was examined. The retention of such charged solutes has, in many instances, been decreased by addition to the mobile phase of an anionic ion-pair reagent, the decrease being the result of reduced availability of the octadecylsilica surface which becomes negatively charged and displaces similarly charged ions reducing, thus, their retention.[17]

The dependence of the retention of sulfonamide derivatives on the concentration of octanesulfonate reagent was investigated at pH 4.0 using mobile phases containing 0-20 mM of the ion-pair reagent. Fig. 4 shows that, the addition of octanesulfonate markedly decreases the retention of all analytes without affecting their resolution. The decrease was higher with 20 mM of octanesulfonate, a concentration which was finally selected as peak shapes were also substantially improved.

Calibration Curves and Detection Limits

In Fig. 5 a chromatogram of a mixture of standard sulfonamides, derivatized according to the described conditions, is presented. Chromatographic peaks are well resolved, the retention times being 8.5, 9.5, 22.0 and 24.0 min for sulfadiazine, sulfamethazine, sulfadimethoxine, and sulfaquinoxaline derivatives, respectively. The linearity of the fluorescence intensity of the fluorescamine derivatives was evaluated in the concentration range of 0.04-0.83 ng/20 μL for sulfadiazine and sulfamethazine, 0.12-1.25 ng/20 μL for sulfadimethoxine, and 0.42-2.5 ng/20 μL for sulfaquinoxaline. Regression analysis of the data, obtained by running five replicates of the derivatized standard working solutions, showed the response to be linear in the range examined for each sulfonamide (y=0.8+213.9x, r=0.9997 for sulfadiazine;

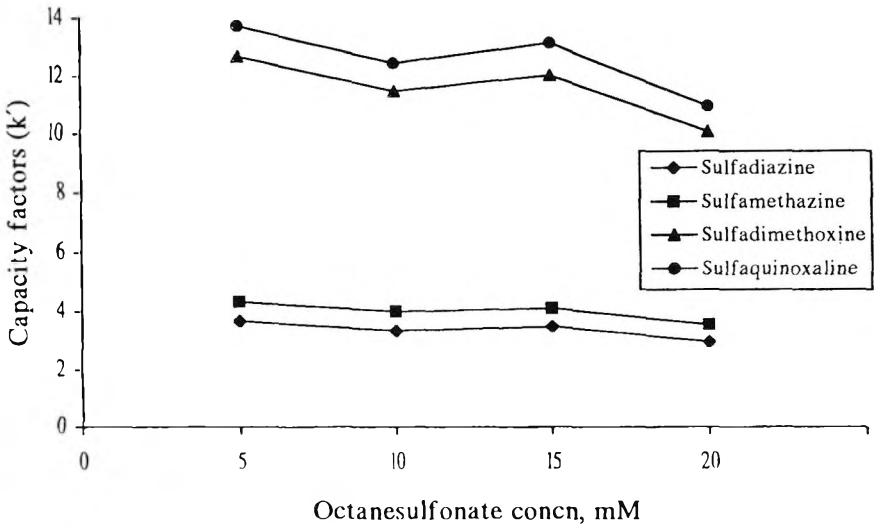


Figure 4. Capacity factors (k') of fluorescamine derivatives of sulfonamides versus concentration of octanesulfonate in the mobile phase. Conditions: mobile phase, acetonitrile-0.02 M phosphate buffer, pH 4, (34:66, v/v) in presence of octanesulfonate; other LC conditions as in Fig. 2.

y=0.7+158.0x, r=0.9998 for sulfamethazine; y=1.7+55.9x, r=0.9989 for sulfadimethoxine; y=0.8+15.1x, r=0.9995 for sulfaquinoxaline, where *y* represents peak height in mm and *x* the sulfonamide quantity in ng, relative to the fluorescamine derivative injected). In standard solutions, with a 20-μL injection volume, 2.4, 3.4, 8.0 and 33.5 ng/mL were the lowest concentrations that could be detected. These correspond to residue concentrations of as low as 3 ng/g for sulfadiazine, 4 ng/g for sulfamethazine, 9 ng/g for sulfadimethoxine, and 40 ng/g for sulfaquinoxaline, which could be readily determined in chicken tissue (peak to noise ratio, 3/1) due to absence of any interfering peaks in sample chromatograms (Fig. 5). The detection limits achieved are much better than those reported by other workers, but cannot reach those (0.003-0.006 ng/g) stated by Takeda and Akiyama[13] which, however, have been incorrectly calculated.

Precision and Accuracy

To evaluate the accuracy and the precision of the method, series of 3-g tissue samples were spiked with standard sulfonamides (50 μL of 0.3-18 μg/mL spiking solutions) at 5 fortification levels ranging from 5 to 100 ng/g for

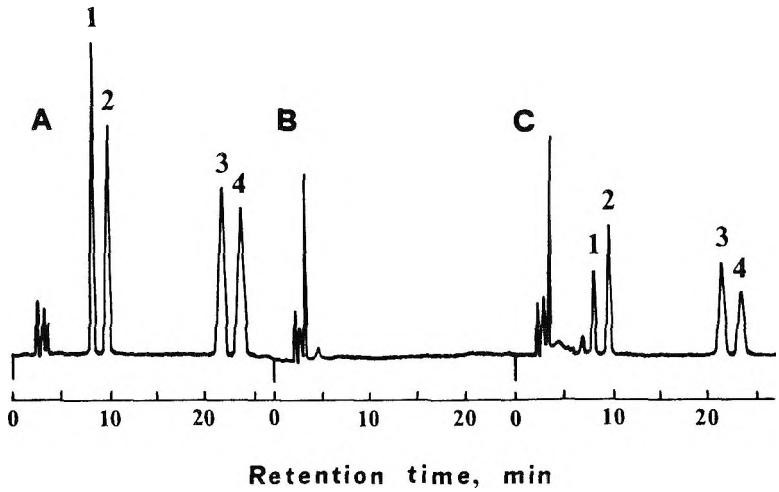


Figure 5. Typical chromatograms of (A) standard solution containing 30 ng/mL sulfadiazine (1) and sulfamethazine (2), 50 ng/mL sulfadimethoxine (3), and 200 ng/mL sulfaquinoxaline (4), relative to the fluorescamine derivative injected, (B) a blank muscle tissue sample, (C) muscle tissue sample fortified with 10 ppb of sulfadiazine, 20 ppb of sulfamethazine, 30 ppb of sulfadimethoxine, and 100 ppb of sulfaquinoxaline. LC conditions: mobile phase, acetonitrile-0.02 M phosphate buffer, pH 4, (34:66, v/v) containing 20 mM octanesulfonate sodium salt; column, 250x4.6 mm, C_{18} 5 µm; temperature, 30 °C; flow rate, 1.2 mL/min; wavelength, ex 405 nm em 495 nm; recorder sensitivity, 0.02 RFU; chart speed, 2 mm/min; injection volume, 20 µL.

sulfadiazine and sulfamethazine, 15 to 150 ng/g for sulfadimethoxine, and 50 to 300 ng/g for sulfaquinoxaline. Five replicates were analyzed at each fortification level. Least-squares and regression analysis of the data presented in Tables 2 and 3, showed that the relationship between "added" and "found" was adequately described by a linear regression for each of the four sulfonamides tested, (y=0.32+0.777x, r=0.9956 for sulfadiazine; y=1.50+0.846x, r=0.9966 for sulfamethazine; y=1.49+0.825x, r=0.9913 for sulfadimethoxine; y=0.28+0.923x, r=0.9958 for sulfaquinoxaline).

Therefore, the slopes of these regression lines could be used as estimates of the overall recovery for sulfadiazine (77.7±4.8%), sulfamethazine (84.6±4.2%), sulfadimethoxine (92.3±4.1%), and sulfaquinoxaline (82.5±7.0%) determination in chicken muscle.

Table 2

Precision and Accuracy Data for the Determination of Sulfadiazine and Sulfamethazine Residues in Chicken Muscle

	Sulfadiazine		Sulfamethazine	
Sulfonamide Added, ppb	**Mean Concn.[a] Found, ppb**	**Mean Rec.[b], %**	**Mean Concn.[a] Found, ppb**	**Mean Rec.[b], %**
5.0	4.5 ± 0.31	89.5 (6.9)	4.8 ± 0.05	96.7 (1.0)
15.0	11.7 ± 0.29	77.8 (2.5)	13.4 ± 0.71	89.3 (5.3)
35.0	27.6 ± 0.68	78.9 (2.5)	33.1 ± 1.07	94.6 (3.2)
70.0	54.4 ± 2.26	77.7 (4.2)	54.8 ± 2.24	85.4 (3.7)
100.0	78.4 ± 5.39	78.4 (6.9)	86.0 ± 3.83	86.0 (4.4)

[a] Mean of 5 replicates ± SD.
[b] Values in parenthesis represent RSD %.

Table 3

Precision and Accuracy Data for the Determination of Sulfadimethoxine and Sulfaquinoxaline Residues in Chicken Muscle

	Sulfadimethoxine		Sulfaquinoxaline	
Sulfonamide Added, ppb	**Mean Concn.[a] Found, ppb**	**Mean Rec.[b], %**	**Mean Concn.[a] Found, ppb**	**Mean Rec.[b], %**
15.0	13.2 ± 0.86	87.9 (6.9)		
30.0	27.8 ± 0.86	92.5 (3.1)		
50.0			37.9 ± 1.83	75.8 (4.8)
70.0	66.0 ± 1.98	94.3 (3.0)		
100.0	94.0 ± 5.13	94.0 (5.5)	77.8 ± 6.02	77.8 (7.7)
150.0	137.3 ± 2.14	91.5 (5.2)	140.1 ± 1.46	93.4 (1.0)
200.0			165.7 ± 5.6	82.8 (3.4)
300.0			243.0 ± 9.72	81.0 (4.0)

[a] Mean of 5 replicates ± SD.
[b] Values in parenthesis represent RSD%.

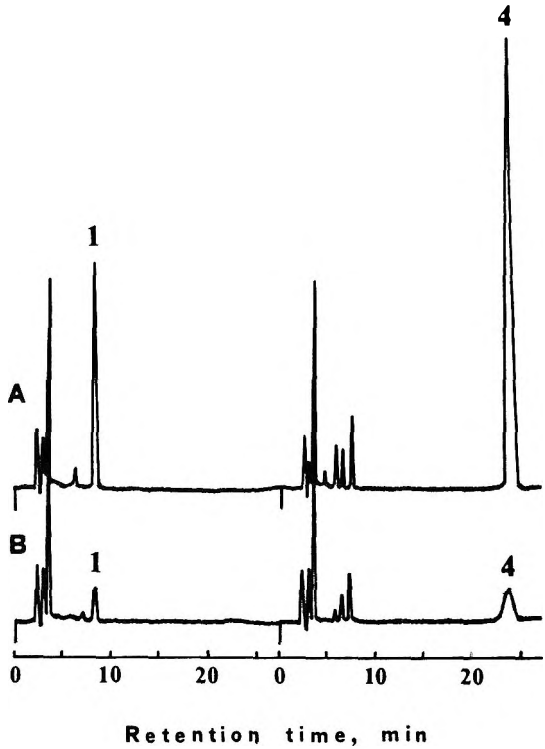


Figure 6. Chromatograms of muscle tissue samples from sulfadiazine (1) or sulfaquinoxaline (4) medicated broilers 48 h (A) and 96 h (B) after drug withdrawal; LC conditions as in Fig. 5.

Application

To validate the method with real samples, a trial was undertaken to quantitate residues in muscle tissue of four broilers, each two administered with water sulfadiazine at 80 mg/L and sulfaquinoxaline at 45 mg/L for 5 days and 3 days, respectively. Analysis data showed that both compounds could be detected in muscle tissue, 48 h (26.9 ng/g for sulfadiazine; 733.9 ng/g for sulfaquinoxaline) and 96 h (3.8 ng/g for sulfadiazine; 53.9 ng/g for sulfaquinoxaline), after drug withdrawal (Fig. 6). Extractable metabolites could be seen in muscle tissue from sulfaquinoxaline-medicated broilers; their chromatographic behavior indicated a substantial increase in polarity over the parent compound. When the method was further applied to liver and kidney samples, significant residue levels could also be found (Fig. 7).

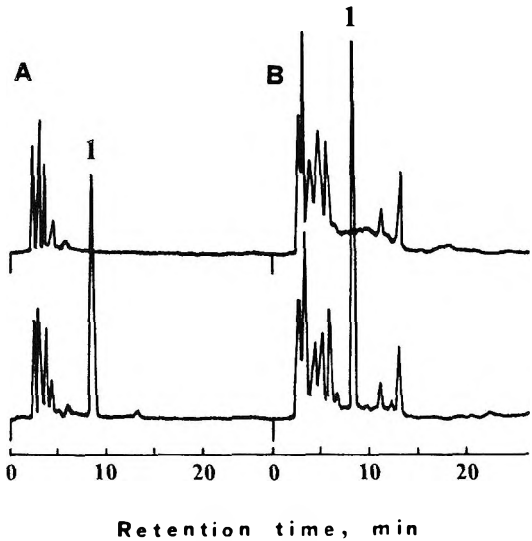


Figure 7. Chromatograms of kidney (A) and liver (B) samples from sulfadiazine (1) medicated or not broilers; LC conditions as in Fig. 5.

CONCLUSION

The results of the present study suggest that pre-column derivatization with fluorescamine may be an efficient and reliable means for enhancing the sensitivity and selectivity of the detection of multiple sulfonamide residues in chicken tissues.

The precision of the measurement is not affected due to the pre-column derivatization, whereas, there are considerable savings in terms of time-, labor- and material-requirements, compared to classical procedures. Considering that the derivatization step is easily amenable to automation through use of suitable LC autosamplers, the method may be proved in the future particularly useful for regulatory purposes.

ACKNOWLEDGMENT

The project was financially supported by the General Secretariat of Research and Technology of the Greek Ministry of Industry and Energy.

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J. LIQ. CHROM. & REL. TECHNOL., 19(14), 2365–2366 (1996)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

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The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

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Details of this course may be obtained from Dr. Jack Cazes, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA. Tel: (609) 424-3505; FAX: (609) 751-8724; E-Mail: jcazes@voicenet.com.

ANNOUNCEMENT

FREDERICK CONFERENCE ON CAPILLARY ELECTROPHORESIS

October 21-23, 1996

Hood College
Frederick, Maryland

The focus of this premier conference is the scientific exchange and dissemination of the technical aspects of *Capillary Electrophoresis*. Discussion of established methodologies, as well as creative and innovative approaches and techniques, are encouraged.

The format of the meeting will include oral and poster presentations by individual conference participants. An optional definitive CE course will be presented. Invited speakers will provide expert overviews of the basic aspects and applications of CE and MECC, including

- ✓ Instrument and column design
- ✓ Detection
- ✓ Optimization
- ✓ Factors which influence mobility, selectivity and resolution.

Also, the application of CE to the separation of small ions, as well as large biomolecules, with emphasis on clinical, pharmaceutical and biomedical applications will be discussed. Plenary lectures will be given by distinguished invited speakers.

There will be no charge for conference registration. Further information may be obtained from Margaret Fanning, Conference Coordinator, SAIC Frederick, NCI-FCRDC, P. O. Box B, Frederick, MD 21702-1201, USA. Tel: (301) 846-5865; FAX: (301) 846-5866.

J. LIQ. CHROM. & REL. TECHNOL., 19(14), 2367 (1996)

MEETING REPORT

PITTCON® '96

The 47th edition of the Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy was held in Chicago's McCormick Place Convention Center on March 3-8, 1996. President John Sember reports that the conference and its exposition and technical program were among the most successful ever. Attendance was the largest in history, exceeding the 1990 New York attendance and verifying the need for a northern city in the PittCon rotation.

Statistics for the 1996 Conference

Registration

Conferees, 16,947 Exhibitors, 17,132 Total, 34,079

Exposition

Exhibiting companies, 1,116 Total booths, 2,949

Technical Program

Invited presentations, 199 Contributed presentations, 1,148
Poster presentations, 562 Total presentations, 1,909

Philanthropic Efforts

Grant for development of an early childhood science exhibit at the Chicago Children's Museum.
Establishment of a Microscale Chemistry Training Center for the Chicago area at Roosevelt University.
Science workshops for teachers at both the elementary school and secondary school levels.
Grants and awards for Chicago area teachers.

Future Conference Sites and Dates

Atlanta, GA	March 16-21, 1997
New Orleans, LA	March 1-6, 1998
Orlando, FL	March 7-12, 1999
Chicago, IL	March 5-10, 2000

J. LIQ. CHROM. & REL. TECHNOL., 19(14), 2369–2373 (1996)

THE BOOK CORNER

CELL ELECTROPHORESIS, Johann Bauer, editor, CRC Press, Inc., Boca Raton, FL., 1994, 328 pages.

The contents of this book are divided into sixteen chapters. The first two chapters deal with mathematical theory contributing to free flow electrophoresis and numerical description of zone electrophoresis in continuous flow electrophoretic devices. As such, the book has many useful equations. Chapters three through six emphasize the improvements of the method. Chapter three describes the crescent distortion which can be avoided by applying an external electric field to control electroosmosis and by moving the chamber wall mechanically to eliminate pressure gradient.

Chapter four compares the quantitative effects of cell loading and unloading procedures on the quality of cell separation by five electrophoresis methods. Chapter five deals with the use of membranes, and their types, in cell electrophoresis. Chapter six elucidates the cell separation by electrophoresis and double antibody tagging, quantitation of antibody tagging by micro-electrophoresis and the study of the effect of presence of antigen on the electrophoretic mobility and proliferating response of T-cells in continuous flow electrophoresis.

Chapters seven through nine discuss the buffers and their effects on cells. Chapter seven concludes that organic zwitterionic buffers offer significant advantages over conventional buffers for improvement of resolution of vial cells in zonal electrophoresis. Chapter eight discusses the possible mechanisms of the effect of low ionic strength solution on cells in cell electrophoresis. Chapter nine emphasizes the vital activity of cells during preparation and in the process of electrophoresis which is affected by many factors influencing the electrical state of the cell surfaces. Chapters ten through fourteen cover the importance of the determination of the negative surface charge density of the cells. Chapter ten emphasizes the simultaneous measurement of cell electrophoretic mobility (EPM) and sedimentation velocity (SV) in heterogenous cell systems, which may be helpful for studying the relationship between cell electrokinetic properties and those properties correlating with their sedimentation velocities.

Chapter eleven elucidates the effects of Lewis acid-base equilibria and electrostatic forces on cell interactions and surface hydrophilicity. Chapter twelve deals with the endothelium cell functions and how they are directly or indirectly influenced by electrical charges on the cell surface.

The electrical surface properties of endothelial cells and their measurement with electrophoresis are presented. Chapter thirteen concentrates on the macrophage electrophoretic mobility test used for the immunological detection of cancer cells. Other applications include the study of biocompatibility and body fluid investigation. Chapter fourteen uses the negative surface charge densities and electrophoretic mobility (EPM) values of many kinds of animal and human cells to draw a correlation between the EPM values of a cell and its biochemical parameters which may have correlation with cancer cell line.

Chapter fifteen deals with the use of microgravity electrophoresis for the isolation of live cells that can be used for transplant or the seeding of commercial bioreactors to produce valuable pharmaceuticals in earth-based plants. Other applications include protein and macro-molecular separation using cell electrophoresis.

Chapter sixteen is an interesting one which deals with the determination of resolution and throughput of electrophoresis machines which can be promoted in microgravity, and in the design of a biotechnological laboratory for space conditions to ensure that each of the different tools and methods works well in micro-gravity.

This is an interesting book with many illustrations. The volume is useful for those working in cell electrophoresis, to whom the book is intended.

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CAPILLARY ELECTROPHORESIS, Dale R. Baker, Editor, John Wiley & Sons, Inc., New York, NY., 1995, 244 pages.

Capillary Electrophoresis is a volume in the Techniques in Analytical Chemistry Series which promotes the following objectives: (a) to provide the reader with overviews of methods of analysis that include a basic introduction to principles, but emphasize such practical issues as technique selection, sample preparation, measurement procedures, data analysis, quality control, and quality

assurance; (b) to give the reader a sense of the capabilities and limitations of each technique, and a feel for its applicability to specific problems; and (c) to communicate practical information in a readable, comprehensible style.

The author, Dr. Baker, states, in the preface, that he wrote the book so it will be used as: (a) a primary source of information for someone who is just starting to use capillary electrophoresis, and has no experience in high-performance liquid chromatography or slab gel electrophoresis; (b) a reference book for someone with experience in capillary electrophoresis; and (c) a reference for a short course or as a textbook for a longer course on capillary electrophoresis.

The above objectives and goals are met in Capillary Electrophoresis. I believe this book will be an excellent text for a short one- to two-day course, or included in a separation course at a university. Dr. Baker should be congratulated for a well organized and written book. It is clear, simple and uncluttered with unnecessary examples and equations. The reader, especially the beginner, will benefit immensely from this book. His simple but practical explanation of electroosmotic flow, citing the flow of a river and rowboats, is a joy to read and one which I use in my teachings. The book is also a good reference to the established practitioner. I recommend it to all those interested in this fast moving and useful microseparation technique.

The book is divided into seven chapters. Chapter one describes the development of capillary electrophoresis, and provides an overview of modern electrophoresis in comparison with other separation techniques. Chapter two involves the principles of separation with electrophoresis and the effect of electroosmotic flow and electrophoretic mobility on the separation, while Chapter three discusses the modes of capillary electrophoresis. In Chapter four, emphasis is on the instrumentation for capillary electrophoresis. Chapter five describes the guidelines used in the development of optimization procedures for capillary electrophoresis. Chapter six emphasizes the qualitative and quantitative aspects of capillary electrophoresis. Chapter seven covers the applications of capillary electrophoresis to contemporary chemical problems.

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LIQUID CHROMATOGRAPHY CALENDAR

1996

AUGUST 18 - 23: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

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

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

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

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

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

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

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

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

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

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

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

SEPTEMBER 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd International Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 17 - 20: 10th International Symposium on Cap[illary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. Contact: J.A. Brown, FACSS, 198 Thomas Johnson Dr., Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 17 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

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

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000; FAX: (703) 684-7349.

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090; FAX: (708) 634-4595.

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200, 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

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

NOVEMBER 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

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

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S., Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.ord.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128; Email: miscmtgs@acs.org.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

1998

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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Author(s)' names and full addresses
Abstract
Text Discussion
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F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

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MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed boldface, in capital letters, centered.

Secondary headings, if any, should be placed flush with the left margin, and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The **first line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES**, in boldface type, should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

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

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

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2. All text should be **typed single-spaced**.

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