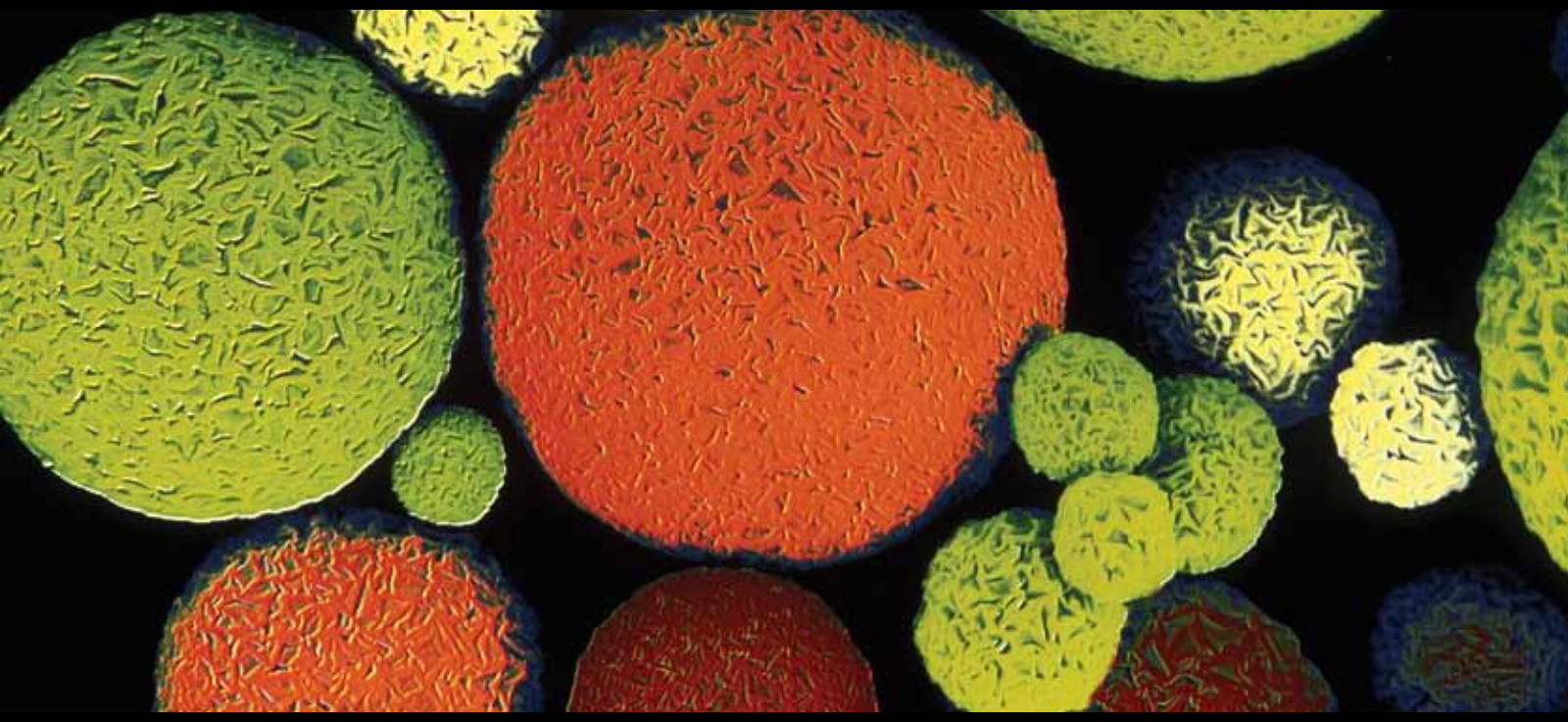
Chromatography Research International

Micellar Liquid Chromatography: Recent Advances and Applications

Guest Editors: Samuel Carda-Broch, Josep Esteve-Romero, Maria Rambla-Alegre, Maria Jose Ruiz-Angel, Alain Berthod, and Devasish Bose



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Editorial Board

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 573690, 2 pages
doi:10.1155/2012/573690

Editorial

Micellar Liquid Chromatography: Recent Advances and Applications

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Received 5 April 2012; Accepted 5 April 2012

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic (RPLC) mode with a mobile phase consisting in an aqueous solution of surfactant above its critical micellar concentration (CMC). The idea of using pure micellar solutions as mobile phases in RPLC is very attractive given their lower cost, less toxicity, and poorer environmental impact. In practice, however, the addition of a small amount of organic solvent to the micellar solution is needed to achieve retention in practical time windows and to improve peak efficiency and resolution. MLC also provides a solution to the direct injection of real samples (physiological or food) by solubilising proteins. The possibility of directly injecting samples into the chromatograph simplifies and expedites treatment, which confers analytical procedures of greater accuracy and a lower cost. Fundamental studies into MLC have served to develop the technique and to establish its theoretical basis, without which its later use in diverse applications would be impossible. In this special issue on MLC, we have invited a few papers that address such issues.

A paper by M. Rambla-Alegre describes the basics of MLC, highlighting the particularities of this technique over the RPLC. The partitioning equilibria are described in detail. It also introduces the term of submicellar liquid chromatography. Another paper by the same author lies on the description of the retention in MLC. The complexity of the mixtures of compounds studied and the relevant modification of their chromatographic behavior when changing the mobile phase composition requires the use of computer-assisted simulations in MLC to follow the modifications in the chromatograms in detail. These simulations can be done with sound reliability thanks to the use of chemometrics tools. The most frequently used empirical and mechanistic models that describe the retention behavior of compounds are revised. The modeling of peak shape and the strategies to measure the peak resolution are also discussed.

A paper by N. Memon et al. studies the selectivity of a non ionic surfactant (Brij-35) in MLC separation of positional isomers. The effect of surfactant and organic solvent concentration on the separation of some selected isomers is studied and evaluated in terms of Linear Solvation energy Relationship (LSER). Non ionic MLC offers different mode of interaction than hydro-organic or ionic micellar liquid chromatography. Besides basicity, dipolarizability and excess molar refraction are responsible for fine tuning of separation. This new face of non-ionic MLC opens field for many applications in separation of positional isomers.

In the paper by A. U. Kulikov an MLC method was developed and validated according to ICH guidelines for the determination of sesquiterpenic acids in root and rhizome extract from *Valeriana officinalis* and valerian dry hydroalcoholic extract. The proponed method does not require gradient elution which is widely used in reversed-phase HPLC methods. This is one of the main advantages of MLC that allows separating compounds with different

hydrophobicity in a single run without the gradient elution. This is an application that denotes the usefulness of this technique.

In the paper by M.-L. Chin-Chen another applied work that determines the levels of the biogenic amine spermine in anchovy sauce after derivatization with 3,5-dinitrobenzoyl chloride is introduced. The suggested methodology was found useful in routine analysis of spermine in fish sauce samples. This methodology was validated in terms of linearity, sensitivity, limits of detection and quantification, accuracy, precision and recovery, following the FDA guidelines. Direct injection of samples (after filtration) was used, thus avoiding any tedious extraction and purification step. This is another interesting advantage of the MLC technique.

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 564243, 8 pages
doi:10.1155/2012/564243

Research Article

Micellar LC Separation of Sesquiterpenic Acids and Their Determination in *Valeriana officinalis* L. Root and Extracts

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Received 28 November 2011; Accepted 17 January 2012

Academic Editor: Samuel Carda-Broch

A simple micellar liquid chromatography (MLC) method was developed and validated according to ICH Guidelines for the determination of sesquiterpenic acids (valerenic, hydroxyvalerenic, and acetoxyvalerenic acids) in root and rhizome extract from *Valeriana officinalis* L. and valerian dry hydroalcoholic extract. Samples were analyzed on Nucleosil C18 column (150 mm×4.6 mm, 5 μm) using an isocratic mobile phase which consisted of Brij 35 (5% (w/v) aqueous solution; pH 2.3 ± 0.1 by phosphoric acid) and 1-butanol (6% (v/v)); UV detection was at 220 nm. Micellar mobile phase using allows to fully separate valerenic acids within 25 minutes. Linearity for hydroxyvalerenic, acetoxyvalerenic, and valerenic acids was 1.9–27.9, 4.2–63.0, and 6.1–91·3 μg.mL^{-1}, and limit of detection was 0.14, 0.037, and 0.09 μg·mL^{-1}, respectively. Intraday and interday precisions were not less than 2% for all investigated compounds. The proposed method was found to be reproducible and convenient for quantitative analysis of sesquiterpenic acids in valerian root and related preparations.

1. Introduction

Valeriana officinalis L. is the most common species of the genus *Valeriana* that is used for its medicinal properties [1]. The root and rhizome of the *valerian* plant is used medicinally for its sedative properties with indications including nervous tension, insomnia, anxiety, and stress. *Valerian* is also considered to have antispasmodic, anticonvulsant, and antidepressant effects.

The roots and rhizomes of *Valeriana officinalis* contain two main groups of constituents: sesquiterpenes of the volatile oil (valerenic acid and its derivatives, valeranone, valeranal, and kessyl esters) and valepotriates (valtrate, didrovaltrate, acevaltrate, and isovaleroxyhydroxyvaltrate), in addition to other constituents such as flavonoids, triterpenes, lignans, and alkaloids [2, 3].

Valerian is available in a variety of formulations, including tablets, capsules, liquid, teas, and tinctures. Products may contain whole herb and/or a proprietary blend or may also be combined with other herbal supplements (e.g., lemon balm, hops, kava, St. John's worth, etc.). *Valerian* root and some commercial products (drugs) are standardized according to the content of valerenic acid, but concentrations vary among products.

Valerenic acids are sesquiterpenes based on the dual ring valerane structure with the main representatives being valerenic acid ((2E)-3-[(4S,7R,7aR)-3,7-dimethyl-2,4,5,6,7,7a-hexahydro-1H-inden-4-yl]-2-methylacrylic acid; $C_{15}H_{22}O_2$), acetoxyvalerenic acid, and hydroxyvalerenic acid. Chemical structures of these acids are presented in Figure 1.

Several methods, adopting different analytical techniques, have been reported to analyze sesquiterpenic acids: TLC [4–6], SFC [7], and HPLC-UV, which is the most commonly used method.

Bos et al. [8, 9] simultaneously analyzed valepotriates (valtrate, isovaltrate, acevaltrate, and didrovaltrate), valerenic acids (valerenic, hydroxyvalerenic, and acetoxyvalerenic), baldrinal and homobaldrinal by HPLC-DAD using LiChrospher 100 RP-18 column, and gradient elution of acetonitrile-water mobile phases. Total analysis time was 30 min.

Gobbeto and Lolla [10] proposed the HPLC method for the determination of valerenic acids in *valerian* extracts using Hypersil ODS column and gradient elution with acetonitrile

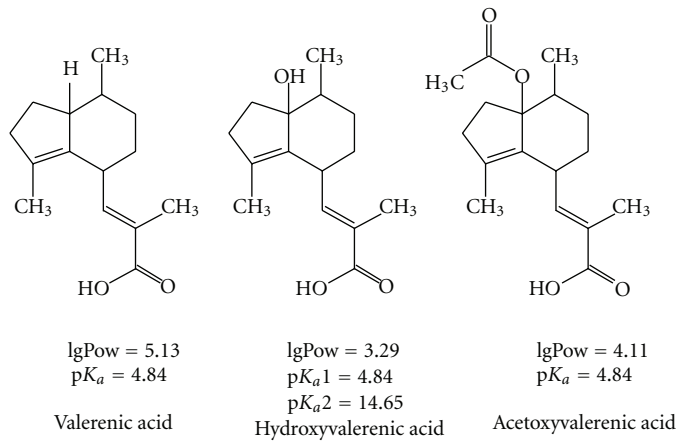

Figure 1: Sesquiterpenic acids chemical structures, calculated values of logarithm of partition constant in 1-octanol-water system lgPow, and protonization constants (pK_a).

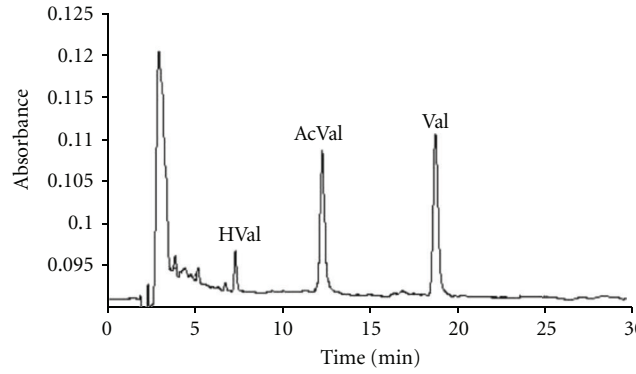

Figure 2: Chromatogram of sesquiterpenic acids separation obtained by approved RP HPLC method. Kromasil C18 chromatographic column; linear gradient elution with 5 g·L^{-1} aqueous H_3PO_4-acetonitrile mobile phases; flow rate 1.5 mL·min^{-1}; UV 220 nm. HVal: hydroxyvalerenic acid, AcVal: acetoxyvalerenic acid, and Val: valerenic acid.

containing 0.1% phosphoric acid-acetonitrile mobile phase; UV detection at 220 nm; analysis time was 40 min.

Bicchi et al. [7] developed supercritical fluid chromatography method with UV detection for the determination of valerenic acids and valepotriates in *valerian* extracts (analysis time is 20 min) and compared results, obtained during *valerian* extracts analysis that were determined by SFC and HPLC methods.

European Pharmacopoeia [11] for the analysis of valerenic acids in *valerian* root end extracts proposed HPLC-UV method with C18 column using an acetonitrile-phosphoric acid aqueous solution gradient elution. Detection at 220 nm and total analysis time is about 30 min (Figure 2).

As we can see, all LC methods required gradient elution techniques for separation and assay of sesquiterpenic acids in raw plant materials and drugs.

It was already described that micellar liquid chromatography (MLC) is an alternative for RP HPLC method [12, 13]. MLC is successfully applied to the analysis of drugs [14, 15] and biological fluids [16], and nowadays this method is widely used for the analysis of raw plant materials and plant containing drugs [17, 18]. One of MLC advantages that made this method preferable to HPLC is the simultaneous separation of both ionic and nonionic compounds, substances with different hydrophobicity without needing gradient elution.

The aim of this work is an investigation of possibility to separate valerenic acids using MLC method, to validate method according ICH validation requirements (specificity, accuracy, precision, and linearity), and an application of the developed method for the rapid sesquiterpenic acid analysis in raw materials (roots and rhizomes of *Valeriana officinalis*) and *valerian* dry extracts.

2. Experimental

2.1. Reagents and Materials. Acetoxyvalerenic acid (AcVal), hydroxyvalerenic acid (HVal), and valerenic acid (Val) were isolated from *Valeriana officinalis* L. in Plant Material Department of the Scientific and Expert Pharmacopoeial Centre, Ukraine, as described in [19]. Chemical structure was confirmed using IR, ^{1}H, and ^{13}C NMR spectra (in the State Scientific Institution "Institute for Single Crystals" of the National Academy of Sciences of Ukraine). Chromatographic purity was determined by RP HPLC method [11]; those were more than 98.5% for all cases.

Valerian standardized dry extract EP CRS (0.38% $C_{15}H_{22}O_2$ (valerenic acid) was from European Pharmacopoeia (Strasbourg, France).

1-butanol (1-BuOH), concentrated phosphoric acid, polyethylene glycol dodecyl ether (Brij 35), methanol (HPLC grade), and other chemicals were purchased from Fluka Chemie (Buchs, Switzerland). Double-distilled water was used in all experiments.

Fragmented underground parts of *Valeriana officinalis* L. were from "Sumyfitofarmacia Ltd," Ukraine; *valerian* dry extracts were from "AIM Ltd," Ukraine.

2.2. Apparatus. The chromatographic measurements were carried out with Hewlett Packard equipment (Agilent Technologies, Waldbronn, Germany) consisting of a Series 1050 pump, a Series 1050 spectrophotometric detector with the variable wavelength, and a Series 3395 integrator. The analytical column was the reversed-phase Nucleosil C18 (150 mm $\times$ 4.6 mm, 5 μm, Macherey-Nagel, Germany). The pH values were determined with a Beckman Φ-200 pH meter (Beckman Instruments, Fullerton, CA, USA) and Paratrode electrode (Metrohm AG, Herisau, Switzerland).

For the determination of peak purity, the Waters 2695 Separation Module (Waters, Milford, MA, USA) with Waters 996 Photodiode Array Detector (Waters) was used.

2.3. Standard Preparation. A stock standard solution was prepared by weighting an amount of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in a 100 mL volumetric flask, dissolving it in methanol to obtain concentrations of investigated compounds 0.0465 mg·mL^{-1}, 0.105 mg·mL^{-1}, and 0.152 mg·mL^{-1}, respectively. The solution was stored in a dark place at the temperature 5°C during 7 days.

Working standard solutions (used for linearity evaluation, evaluation of precision, repeatability, and accuracy of the MLC method) were prepared by dilution of the stock standard solution with a micellar mobile phase to obtain solutions with different concentrations within the range of interest.

2.4. Sample Preparation

Valerian Root. Extract 1.50 g of powdered underground parts of *Valeriana officinalis* (1 mm) with 20 mL of methanol and heat on a water bath under a reflux condenser for 30 min. Allow to cool and filter through a paper filter into a 50 mL volumetric flask. Add another 20 mL of methanol, heat on a water bath under a reflux condenser for 15 min, cool, filter into the same 50 mL volumetric flask, and dilute to 50.0 mL with methanol [11].

Valerian Dry Extract. Place about 0.5 g of *valerian* dry extract in a 25 mL volumetric flask, add 15 mL methanol, mix, and sonicate during 10 min. Then the solution is diluted to 25.0 mL with methanol and mix [11].

All samples must be filtered through a 45 μm HPLC filter (DynaCard HPLC filter, Microgon, USA) before analysis.

2.5. Chromatographic Separation. A 5% (w/v) aqueous solution of Brij 35 with 6% (v/v) of 1-butanol, adjusted to pH 2.3 $\pm$ 0.1 by phosphoric acid, was prepared and used as mobile phase. The flow rate was set at 1.0 mL·min^{-1}, and the injection volume was 10 μL. The chromatographic runs were carried out at 40.0 $\pm$ 0.1°C. Detection wavelength was chosen at 220 nm.

2.6. Data Sources, Software, and Processing. The statistical analyses were performed with Microsoft Excel (2007, Microsoft Corporation, http://office.microsoft.com/). The values of lgPow and pK_a were calculated using ACDLabs 10.0 (2010, http://www.ACDLabs.com/) and ChemBioDraw 11.0 (2010, Cambridgesoft, http://www.cambridgesoft.com/).

3. Results and Discussion

Nonionic surfactants, unfortunately, are not widely used for micellar liquid chromatographic separation of compounds with different hydrophobicity. It was not found any recommendation in the literature about an approximate nonionic surfactant concentration, which is required for different hydrophobicity substances separation, as described for sodium dodecyl sulfate as micellar-forming agent [12]. In [20–22] it was found that concentration of nonionic surfactants to be micellar eluents should be sufficiently large—5–10% (w/v).

Figure 1 provides the chemical structures and some physical-chemical characteristics of investigated compounds: hydrophobicity as lgPow (logarithms of partition constant in 1-octanol-water system) and pKa values. Hydrophobicity of compounds is not quite different and ranges from 3 for hydroxyvalerenic acid to 5 for valerenic acid. Wang et al. [20] separated quinazoline derivatives with hydrophobicity 2–4 using 0.06 M Brij 35 (6% w/v) as a micellar mobile phase.

Preliminary experiment for valerenic acids separation showed that its retention in approximately 0.06 M Brij 35 mobile phase was about 75 min, and peaks asymmetries of investigated compounds were high.

According to pK_a values, at pH value below 4, the predominant form of compounds in solution is molecular forms—all ionizable groups are fully protonated, and at pH, above 6 compounds are negatively charged. A mobile phase for acid separation should be acidified. Preliminary experiments have shown that when acidified 0.06 M Brij 35 containing micellar mobile phase used for separation of the investigated compounds, peak symmetry is lower than 2; it is appropriate to the assay determination.

It was investigated retention time, peak symmetry, and resolution for different micellar mobile phases to choose an optimal micellar mobile phase composition. Brij 35 concentrations in mobile phases were varying from 2% to 8% (w/v) with a step of 1%, and micellar mobile phase organic modifier (1-bithanol) was varying from 1% to 10% (v/v) with the same step. The mobile phase pH in all cases was about 2.3 $\pm$ 0.1.

The optimization procedure [23, 24] that was used for choosing the optimal mobile phase composition showed that

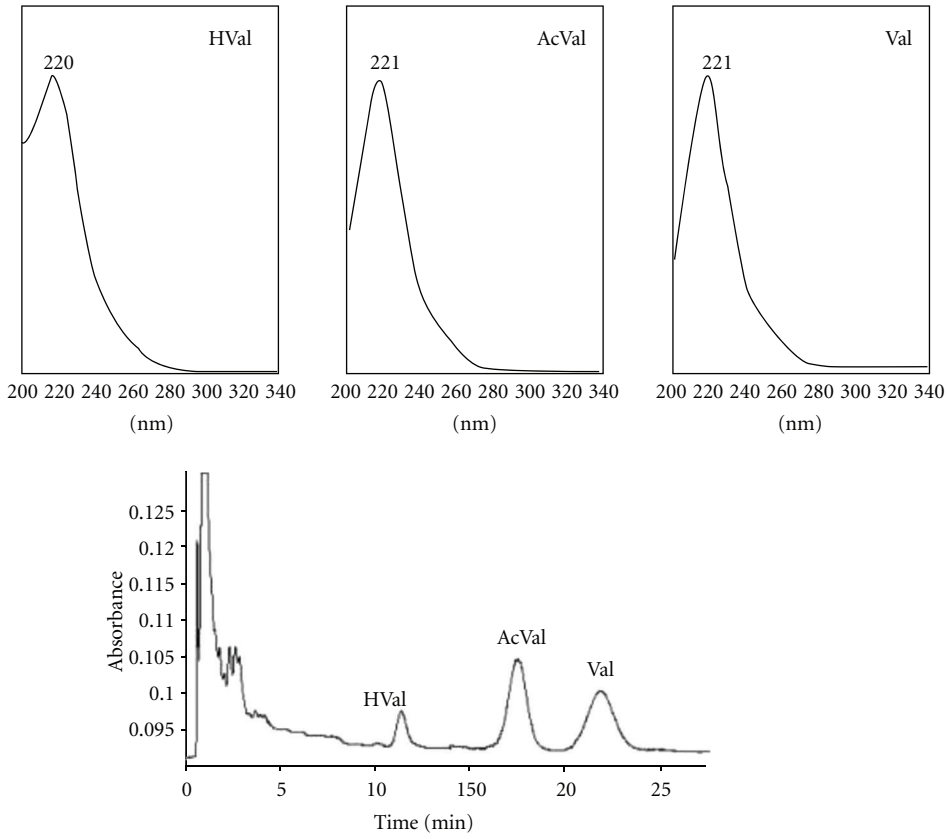

Figure 3: Micellar LC separation of sesquiterpenic acids during selectivity determination. HVal: hydroxyvalerenic acid, AcVal: acetoxyvalerenic acid, and Val: valerenic acid.

for all micellar mobile phases were obtained good chromatographic characteristics: sesquiterpenic acid resolution was more than 1.5, and peak symmetry was 1.5–2. Lowest retention time (12 min) was obtained when 8% (w/v) of Brij 35 and 10% (v/v) of 1-BuOH as micellar mobile phase components were used. Unfortunately, this micellar mobile phase has high viscosity, and column input pressure was unacceptable: 350–370 bar whereas chromatograph pump upper pressure limit is 400 bar. When a mobile phase with low viscosity (2% (w/v) of Brij 35 and 1% (v/v) of 1-BuOH) was used for sesquiterpenic acids separation, total analysis time was about 90 min.

Optimum between micellar mobile phase viscosity (relatively low viscosity) and analysis time (till 25 min) was found, when Brij 35 concentration of 5% (w/v) and 1-BuOH volume fraction of 6% (v/v) were chosen.

In addition, the efficiency of the separation and peak asymmetry can be improved by using high temperatures due to faster mass transfer of solutes between mobile and stationary phases [15], and for reducing of mobile phase viscosity. A column temperature 40°C was chosen, because higher temperature (50 and 60°C) had not exerted significant influence on the chromatographic behavior of investigated compounds.

3.1. Validation of the MLC Method. The procedures and parameters used for the validation of the chromatographic method developed in this study are those described in [25, 26].

3.1.1. Specificity (Selectivity). The specificity of the method was determined by analyzing *valerian*-standardized dry extract EP CRS and methanolic extract of *valerian* root. Methanolic extract was analyzed without and with addition of investigated compounds; increasing peak areas indicate the presence of valerenic acids in the chromatogram. Selectivity of the MLC method was assessed by the comparison of the spectrum extracted from the chromatogram of *valerian*-standardized dry extract and methanolic extract obtained using photodiode array detector; peak purity was also determined.

Figure 3 shows a chromatogram and UV spectra obtained for *valerian*-standardized dry extract, which contains (see Figure 2) hydroxyvalerenic, acetoxyvalerenic, and valerenic acids. The same spectra were obtained for *valerian* methanolic extract. The peaks purity was more than 98.5% in all cases.

3.1.2. Linearity. A linear plot was obtained from six different concentrations of working standard solutions using four replicate injections [25, 26]. The regression line was calculated as $Y = a + bC$, where C was the analyte concentration

Table 1: Characteristic parameters of the calibration equations for the proposed MLC method for sesquiterpenic acids determination.

Parameters	Hydroxyvalerenic acid	Acetoxyvalerenic acid	Valerenic acid
Calibration range ($\mu g \cdot mL^{-1}$)	1.9–27.9	4.2–63.0	6.1–91.2
Detection limit ($\mu g \cdot mL^{-1}$)	0.037	0.09	0.14
Quantitation limit ($\mu g \cdot mL^{-1}$)	0.11	0.27	0.42
Parameters of regression equation $Y = a + b \cdot C$, where C is the concentration of compound in $\mu g \cdot mL^{-1}$, Y is the peak area			
Intercept (a)	$-5.5 \cdot 10^3$	$-7.8 \cdot 10^4$	$-1.6 \cdot 10^4$
SD of the intercept (S_a)	$0.3 \cdot 10^3$	$0.6 \cdot 10^4$	$0.1 \cdot 10^4$
Slope (b)	$27.1 \cdot 10^3$	$21.3 \cdot 10^4$	$23.8 \cdot 10^4$
SD of the slope (S_b)	$0.2 \cdot 10^3$	$0.2 \cdot 10^4$	$0.2 \cdot 10^4$
RSD of the slope, %	0.74	0.94	0.84
Correlation coefficient (r)	0.9995	0.9994	0.9995

($\mu g\, mL^{-1}$), and y was the peak area. The calibration plot was obtained by linear least-squares regression.

Calibration parameters, such as slope, intercept, and correlation coefficient are provided in Table 1. The correlation coefficient (r) was close to unity; hence, there was a linear relationship between amount of analyte and detector signal. The systematic deviations between experimental and predicted responses were not observed.

Since the coefficient of correlation is not suitable as a general acceptance criterion to the linearity performance of an analytical procedure [27], the relative standard error of slope was used as a parameter with respect to precision of the regression. This parameter should be comparable to the relative standard deviation obtained in precision studies within the given concentrations range. Relative standard error of slopes was obtained to be less than RSD of precision.

3.1.3. Limit of Detection and Quantitation. The limits of detection (LOD) and quantitation (LOQ) were calculated in accordance with the 3.3 s/m and 10 s/m criteria, respectively, where s is the standard deviation of the peak area for the sample, and m is the slope of the calibration curve, determined from linearity [25]. Both values were calculated and presented in Table 1.

3.1.4. Accuracy. The accuracy of the proposed method was tested in some ways. First, samples of known concentration (working standard solutions) were analyzed, and measured values were compared with the true value. The results obtained from determination of accuracy, expressed as percentage recovery, are summarized in Table 2. Accuracy criterion for the assay method is that the mean recovery will be $100 \pm 2\%$ for each concentration over the investigation range [28]. The recovery of the proposed method was good.

Second, this study was performed by addition of known amounts of studied compounds to a solution of *valerian* dry extract (standard addition method). The resulting mixtures were analyzed by proposed MLC method, and the obtained results were compared with the expected results. The excellent recoveries of standard addition method (Table 2) suggested good accuracy of the proposed method.

Third, accuracy of MLC method was determined by comparing results obtained by proposed method with results from the validated method using reversed-phase HPLC [11]. MLC and RP-HPLC results for sesquiterpenic acids assay were compared. Statistically analysis of the results obtained for both methods using variance ratio F-test shows that there is no significant difference between these results. The calculated F values were less than those of the theoretical values at 95% confidence level (see data from Table 3).

3.1.5. Precision. The precision as intraday and interday reproducibility, expressed as RSD %, was characterized by the spread of data from replicate determinations.

For the intraday reproducibility, that is, repeatability, we performed nine determinations covering the specified range of the method. Working standard solutions were analyzed (four replicates each). Interday precision of the method was checked on three different days by preparing and analyzing working standard solutions (four replicate injections) under the same conditions.

The results obtained from these analyses are listed in Table 4 as mean recovery. The table shows that there is no significant difference between assay results either within days or between days, implying that the reproducibility of MLC method was good. In all instances, the % R.S.D. values were less than 2%—criterion for an intra-assay precision [28].

3.2. Analysis of Plant Material and Drug. Sesquiterpenic acids (valerenic, acetoxyvalerenic, and hydroxyvalerenic acids) were quantified using developed MLC method in the *valerian* root and rhizomes and *valerian* dry extract (chromatograms of investigated compounds are presented in Figure 4). Fragmented underground parts of *Valeriana officinalis* L. cultivated in 2008–2011 years were from "Sumyfitofarmacia Ltd," Ukraine; *valerian* dry hydroalcoholic extracts obtained from above-mentioned *valerian* root were from "AIM Ltd," Ukraine.

The percentage of valerenic acid, acetoxyvalerenic acid, and hydroxyvalerenic acid, in root and rhizomes was, according to [11] not less than 0.17% of sesquiterpenic acids expressed as valerenic acid, calculated with the reference to

Table 2: Accuracy results of MLC method: analysis of sesquiterpenic acid mixtures and standard addition technique.

Compound	Added ($\mu g \cdot mL^{-1}$)	As found* ($\mu g \cdot mL^{-1}$)	% Recovery	Δ	Added, $\mu g \cdot mL^{-1}$	As found*, $\mu g \cdot mL^{-1}$ with addition	As found*, $\mu g \cdot mL^{-1}$ without addition	Recovery, %
Hydroxyvalerenic acid	1.90	1.88	99.0	−1.0	0	—	2.10	
	9.30	9.25	99.5	−0.5	1.90	4.20	2.20	104.8
	27.9	27.8	99.6	−0.4	5.60	7.74	2.14	102.1
					9.3	11.4	2.13	101.7
	Mean, %R.S.D.		99.4, 0.8				Mean, %	102.9
Acetoxyvalerenic acid	4.20	4.19	99.8	−0.2	0	—	33.4	
	21.0	20.85	99.3	−0.7	12.6	46.3	33.7	102.1
	63.0	63.1	100.1	+0.1	21.0	54.2	33.2	99.4
					29.4	63.5	34.1	102.1
	Mean, %R.S.D.		99.7, 1.1				Mean, %	101.2
Valerenic acid	6.10	6.07	99.5	−0.5	0	—	50.7	
	30.4	30.5	100.3	+0.3	18.2	69.4	51.2	101.0
	91.2	91.4	100.2	+0.2	30.4	80.7	50.3	99.2
					42.6	93.6	51.0	100.6
	Mean, %R.S.D.		100.0, 1.1				Mean, %	100.3

* Mean value of the four determinations.

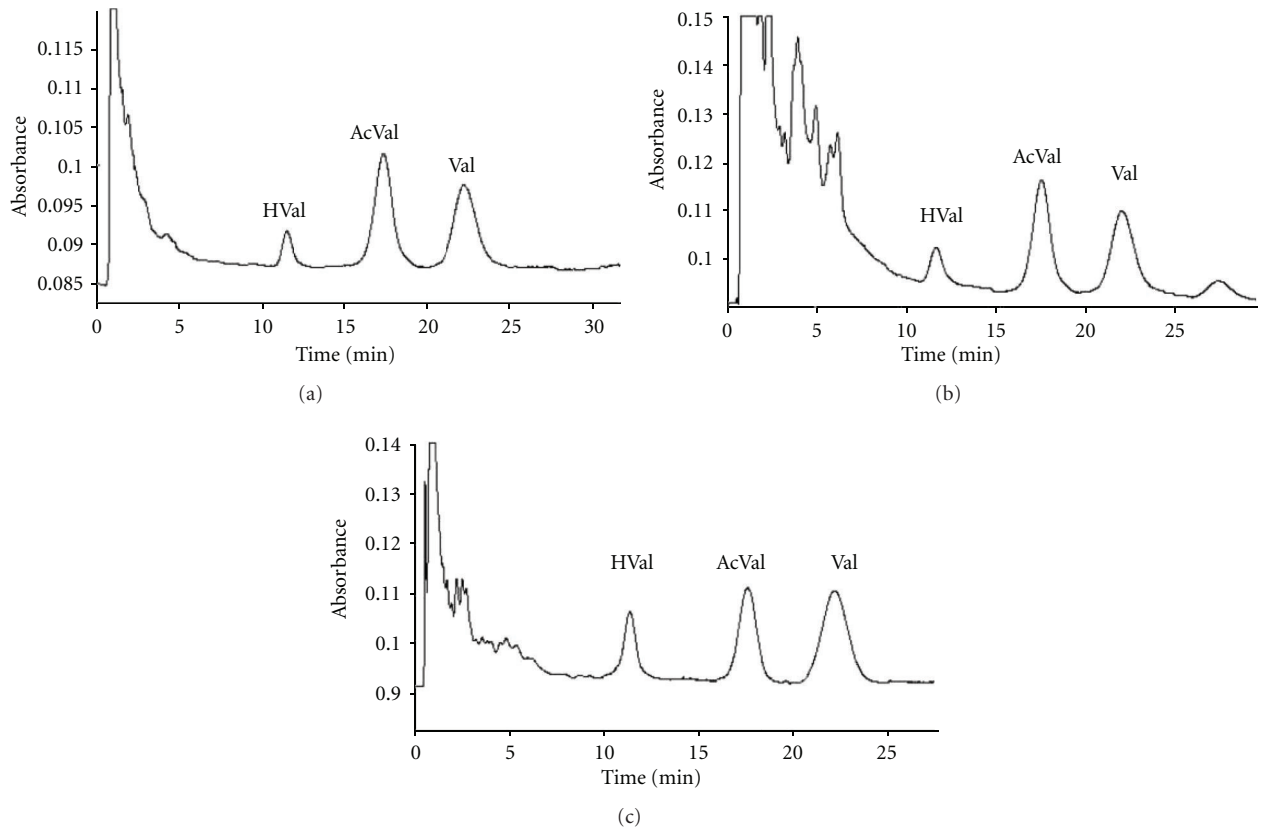

Figure 4: Chromatograms obtained during *Valeriana officinalis* L. root and extract analysis. HVal: hydroxyvalerenic acid, AcVal: acetoxyvalerenic acid, and Val: valerenic acid. (a) valerenic acids standard solution; (b) methanolic extract of *Valeriana officinalis* L. root and rhizome; (c) chromatogram of *valerian* hydroalcoholic dry extract.

Table 3: Results from sesquiterpenic acids assay in various samples using RP-HPLC and MLC methods and verification of the variance of significance between two methods.

MLC*				HPLC*				Δ				Calculated F values**		
HVal, %	AcVal, %	Val, %	Sum, %	HVal, %	AcVal, %	Val, %	Sum, %	HVal, %	AcVal, %	Val, %	Sum, %	HVal	AcVal	Val
Valerian root and rhizome (3 samples)														
0.005	0.073	0.088	0.166	0.006	0.070	0.087	0.163	−0.001	−0.003	+0.001	+0.003	3.152	4.261	2.154
0.001	0.071	0.084	0.156	0.001	0.069	0.082	0.152	0	+0.002	+0.002	+0.004	1.555	3.644	2.233
0.002	0.082	0.098	0.182	0.001	0.085	0.099	0.185	+0.001	−0.003	−0.001	−0.003	4.176	3.954	3.771
Valerian dry extract (3 samples)														
0.031	0.142	0.178	0.351	0.033	0.145	0.177	0.355	−0.002	−0.003	+0.001	−0.004	3.244	1.547	3.334
0.045	0.133	0.201	0.379	0.047	0.132	0.204	0.383	+0.002	+0.001	−0.003	−0.004	3.672	3.674	1.780
0.028	0.186	0.214	0.428	0.030	0.188	0.213	0.431	−0.002	−0.002	+0.001	−0.003	2.966	2.425	2.655

HVal: hydroxyvalerenic acid, AcVal: acetoxyvalerenic acid, Val: valerenic acid, and Sum: total contents of valerenic acids.
*Mean value of the four determinations
**Tabulated F value is 6.38.

Table 4: Intraday and inter-day precision data.

Analyte	Target concentration (100%), μg/mL	Inter-day variation* (% ± R.S.D)	Inter-day variation (% ± R.S.D)
Hydroxyvalerenic acid	1.90	101.3 ± 1.1	101.7 ± 1.8
	9.30	100.9 ± 0.9	100.3 ± 1.1
	27.9	99.9 ± 0.7	100.2 ± 0.9
Acetoxyvalerenic acid	4.20	100.7 ± 0.5	100.2 ± 1.1
	21.0	100.1 ± 0.2	100.5 ± 0.8
	63.0	99.8 ± 0.3	100.1 ± 0.5
Valerenic acid	6.10	99.5 ± 0.9	100.2 ± 1.4
	30.4	100.8 ± 1.0	100.5 ± 1.8
	91.2	100.3 ± 0.5	100.9 ± 1.1

* Mean value of the four determinations.

the dried drugs. Ukrainian Pharmacopoeia [29] required plants that grown in Ukraine and Russia regions, sum of sesquiterpenic acids not less than 0.10% (as valerenic acid, calculated with the reference to the dried drugs).

Valerian dry hydroalcoholic extract should content minimum 0.25% of sesquiterpenic acids, expressed as valerenic acid (dry extract) [11].

Table 3 provides the results of the determination of sesquiterpenic acids in various analytical subjects, which was obtained using micellar liquid chromatographic method. As can be seen from Table 3, not all raw materials satisfied the European Pharmacopoeia [11] requirements for *valerian* root, but all results fulfilled requirements of Ukrainian Pharmacopoeia. All results for *valerian* dry extract analysis comply with requirements of European Pharmacopoeia [11].

4. Conclusions

A simple and reliable MLC method for the simultaneous determination of sesquiterpenic acids (valerenic, acetoxyvalerenic, and hydroxyvalerenic acids) in *valerian* root and rhizomes and *valerian* dry extract was developed. Chromatographic method with using micellar mobile phase does not require gradient elution which is widely used in reversed-phase HPLC methods. That is the main advantage of MLC that allows separating compounds with different hydrophobicity in a single run without the gradient elution.

The method was completely validated showing satisfactory data for all the parameters tested. This method is also ecofriendly for its low concentration of organic solvent, as compared to other analytical techniques.

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 421909, 6 pages
doi:10.1155/2012/421909

Research Article

Micellar Liquid Chromatography Determination of Spermine in Fish Sauce after Derivatization with 3,5-Dinitrobenzoyl Chloride

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Received 29 November 2011; Revised 14 January 2012; Accepted 17 January 2012

Academic Editor: Maria Jose Ruiz-Angel

A practical liquid chromatographic method has been developed for the selective determination of the levels of spermine in anchovy sauce after derivatization with 3,5 dinitrobenzoyl chloride. The micellar liquid chromatographic separation proposed here uses a C18 column (125 × 4.6 mm), followed by detection of spermine derivative at 260 nm. Elution of the analyte was performed using a mobile phase of 0.15 M SDS-4% (v/v) 1-pentanol-pH 7 running under isocratic mode at 25°C. Validation parameters were linearity (2–100 μg/mL, $R^2 > 0.999$), detection and quantification limits (0.4 and 1.2 μg/mL, resp.), precision (less than 3.6%), accuracy (93.3–101.1%), and robustness (less than 4.8%). These results are in agreement with the requirements of the FDA guidelines. The proposed method was successfully applied to the monitorization of spermine formation in unsalted and salted fish sauce samples. The suggested methodology was found useful in routine analysis of spermine in fish sauce samples.

1. Introduction

Biogenic amines are biological metabolites present in foods either as natural products or after fermentation, decay microbial contamination, decomposition, or putrefaction processes [1]. They are largely responsible for the foul odour of putrefying flesh, as well as contributing to the odour of such processes as bad breath and bacterial vaginosis. Biogenic amines are also involved in local immune responses, neurotransmission, and chemotaxis of white blood cells. The consumption of an excess of biogenic amines, known as histaminic intoxication, is mainly related to heart, gastrointestinal, and skin diseases, as well as headache [1–3]. Food containing considerable amounts of these amines include alcoholic beverages, beef, chocolate, cheeses, fish, pork, and poultry. Biogenic amines can also be found in semen and some microalgae, together with related molecules like spermine and spermidine. In fact, spermine is formed from spermidine and can be found in a wide variety of organisms and tissues, as it is an essential growth factor in some bacteria. Thus, its detection and quantification is useful to assess the degree of bacterial contamination, mainly caused by incorrect handling or stocking conditions (freezer at −18°C), in fish flesh, or derivates as fish sauce. Then the determination of spermine is of the utmost importance to assure that the fish sauce can be eaten without health risk [4, 5].

Several analytical methods have been developed for the determination of spermine. Among them, HPLC with UV-visible absorbance detection using 3,5-dinitrobenzoyl chloride (DNBZ-Cl) as a chromophore has provided excellent results for quantification of biogenic amines in complex food samples. Derivatization reaction is quantitative, quite fast (less than 5 min), and reproducible. The high stability and sensitivity of the obtained derivative makes it an excellent choice for the analysis of spermine [2, 6].

In these methods, the analytes have to undergo a previous extraction step in a suitable organic solvent, evaporation to dryness and redissolution in order to purify and preconcentrate the derivatized amines [6]. However, it introduces the risk of contamination and losing of the sample, improving the possibility of error and even increasing the analysis time [7–9]. Moreover, chromatographic conditions result in either

insufficient separation, prolonged analysis times (higher than 1 hour) [6, 10] or need mobile phase running under a gradient program. This requires a stabilization time between two injections, lengthening analysis time and making difficult the analysis of a wide amount of samples [11]. These problems can be avoided by the use of micellar liquid chromatography (MLC), which usually allows direct injection of samples (after filtration), without needing any extraction and purification step [12–16]. Moreover, micellar mobile phases are less toxic, nonflammable, biodegradable, and relatively inexpensive in comparison to aqueous-organic solvents. MLC has proved to be a useful technique in the determination of a wide range of compounds in low time using mobile phases under isocratic conditions, by optimizing separation parameters [12, 13] including food samples [14–16].

The aim of this work was to develop a fast, simple, and selective procedure for the determination of spermine by MLC using a C18 column and UV detection. This analyte was derivatized with DNBZ-Cl to increase sensitivity, and directly injected in the chromatographic system. The suggested methodology was validated in terms of linearity, sensitivity, limits of detection and quantification, accuracy, precision, and recovery, following the FDA guideline [17]. Finally, the method was applied to the study of the anchovy sauce degradation by means of the determination of spermine depending on the storage treatment.

2. Experimental

2.1. Apparatus and Instrumentation. The pH was measured with a Crison GLP 22 (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and ultrasonification unit were from Selecta (Barcelona). The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, a thermostated autosampler, and column compartment. The dead time was determined as the mean value of the first significant deviation from the baseline in the chromatograms of the analyte. The signals were acquired by a PC computer connected to the chromatograph through an HP Chemstation.

2.2. Chemicals and Reagents. Spermine and 3,5-dinitrobenzoyl chloride (98% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) (99% pure) was from Merck (Darmstadt, Germany); acetonitrile, 1-propanol, 2-propanol, 1-butanol, and 1-pentanol were from Scharlab (Barcelona), sodium dihydrogen phosphate, HCl, and NaOH were from Panreac (Barcelona). All solutions were prepared in ultrapure water (Millipore, S.A.S. Molsheim, France). Mobile phases and samples were filtered through nylon membranes (Millex-HN, Millipore, Bedford, MA, USA). Spermine was dissolved in 0.1 M HCl to provide final concentrations of 100 μg/mL.

2.3. Derivatization of Biogenic Amines with 3,5-Dinitrobenzoyl Chloride. Derivatising reagent 5 mM 3,5-dinitrobenzoyl chloride (DNBZ-Cl) was dissolved in acetonitrile. Aliquots (400 μL) of spermine (SP) standards, 1 M NaOH (1200 μL), 2-propanol (700 μL) and 3,5-DNBZ-Cl (2100 μL) were mixed in a reaction tube. After 3 min of shaking at 25°C, 2 M HCl (1000 μL) was added to stop the reaction. Finally, after 1 min of shaking, derivatized sample was injected into the chromatographic system. Under these conditions, the formed derivative was $(DNBZ)_4SP$ [6]. The fish sauce medium does not affect the derivatization reaction, because the conditions were strongly changed by the addition of organic alcohol and sodium hydroxide. Some matrix compounds are precipitated in 2-propanol/NaOH media, and others are solubilized in the SDS medium [2, 6].

2.4. Chromatographic Conditions. Derivatized spermine elution was performed in a reversed-phase C18 column (125 × 4.6 mm, 5 μm particle size) from Scharlab thermostated at 25°C. Mobile phase was a 0.15 M SDS-4% (v/v) 1-pentanol-NaH_2PO_4 0.01 M solution at pH 7, running under isocratic mode. Flowrate, injection volume, and UV wavelength were 1 mL/min, 20 μL and 260 nm, respectively. Samples were thermostated at +15°C in the autosampler module of the HPLC system to avoid decomposition of the spermine derivative [7]. Under these conditions, the retention time was 11.5 min. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. B.01.03).

2.5. Sample Preparation. Fish sauce samples of anchovies (*Eugraulidea* spp.) were obtained from a supermarket. A part of the anchovies was mixed with common salt in a relation of 75/25 w/w (a well-known treatment to avoid food spoilage) and another portion was untreated. In both cases, samples were stored at +4°C. For the analyses of fish sauces, 1 g was mixed with 0.5 mL of ethanol that was topped up to 10 mL with 0.1 M SDS. Samples were derivatized as detailed in Section 2.3 and then they were injected directly into the chromatographic system without any other treatment then filtration.

Also, spiked samples were prepared by adding the appropriate volume of spermine standard solution (dissolved in 0.1 M HCl) to 1 g of sample and then the mixture was vigorously shaken to favour homogenization and stored for one day at +4°C to favour the contact between the analyte and the sample, as well as solvent evaporation [18, 19]. Then the spiked sample was mixed with 0.5 mL of ethanol and topped up to 10 mL with 0.1 M SDS solution. An aliquot of the sample (400 μL) was derivatized as explained in Section 2.3, filtered and directly injected into the chromatograph [2, 6].

3. Results and Discussion

3.1. Optimization Strategy and Mobile Phase Selection. Mobile phases were aqueous solutions of SDS buffered at pH 7 with phosphate buffer and the stationary phase was a C18 column [2, 6]. The four pK_a values of spermine lie in the interval 8–11 [20], so its retention behavior will not vary in the whole pH range of the C18 column used (2.5–7.5). In addition, working at pH 7 increases the life of the column.

Table 1: Intra- and interday precision (RSD, %) and accuracy (recovery, %) for spermine (*added concentrations expressed in μg/mL).

	Intraday precision[a] (RSD, %)			Intraday accuracy[a] (%)	
*2	10	20	2	10	20
3.6	2.5	1.8	93.3	97.4	99.0
	Interday precision[b] (RSD, %)			Interday accuracy[b] (%)	
2.5	1.8	0.7	96.2	101.0	99.5

[a] $n = 9$; [b] $n = 5$.

On the other hand, it should be highlighted that spermine is a polar compound, but the derivative $(DNBZ)_4SP$ is quite hydrophobic [6]. Initially, the chromatographic parameters for the spermine derivative were obtained using a pure micellar mobile phase of SDS. Several mobile phases containing 0.05, 0.1, and 0.15 M SDS were tested. At these three SDS concentrations, the retention times were found too high. Instead of increasing SDS concentration, the use of an aqueous solution of 0.15 M SDS with a small amount of organic solvent was envisaged to reduce the retention time. Different amounts of 1-propanol and 1-butanol were tested and the retention time was reduced, but it remains very high (more than 60 min), and with an irregular peak shape. Thus, 1-pentanol was tested, and finally a mobile phase of 0.15 M SDS-4% (v/v) 1-pentanol allowed the elution of spermine derivative in an adequate retention time (nearly 11.5 min). The chromatographic parameters for spermine derivative in this mobile phase were k (retention factor) = 9.75; N (efficiency) = 4050 theoretical plates, and B/A (asymmetry factor) = 1.0. Their calculation was performed as in [21].

The possible interference of other compounds in spermine analysis was also studied. A blank of each matrix was performed by analyzing aliquots of unsalted and salted anchovy sauce free of spermine. The front shows a considerable maximal band height and width and no other peak was observed, indicating that all the substances elute at the dead time (Figure 1(a)). In fact, hydrophobic substances present in the matrices are introduced in micelles, thus avoiding their precipitation and allowing their fast elution. Moreover, the addition of 1-pentanol in the mobile phase improves its elution strength, reducing even more the retention time of matrix compounds. Samples of unsalted and salted anchovy sauce were spiked with 10 μg/mL of spermine. As can be seen in Figure 1(b), the chromatographic peak of spermine elutes far from the dead time and without overlapping with any other substances of the unsalted anchovy sauce matrix. The same result was found for the salted anchovy sauce samples. The selectivity of the analytical method has been assessed; it has been optimized by the separation of spermine from other interferences of the matrix. Other biogenic amines do not interfere in the determination of the spermine since they elute earlier [2, 7].

3.2. Method Validation. The whole validation was performed by spiking samples with spermine, following the Food and Drug Administration (FDA) guideline [17]. Unsalted and salted anchovy sauce samples were studied. Since results were found similar in both cases, only the obtained values for unsalted fish sauce are presented and discussed.

Calibration curves were constructed using the areas of the chromatographic peaks (nine replicates) obtained at seven different concentrations of spermine, in the 2–100 μg/mL range. To study the variability of the calibration parameters, the curves were obtained during 5 days over a period of two months for a different set of standards, and then the average value was considered. Results were similar in the two matrices studied. The slope and intercept were determined by the method of least square linear regression analysis, taking the absorbance in arbitrary units and the concentration in μg/mL. Limits of detection (LOD, *3s criterion*) and quantification (LOQ, *10s criterion*) for derivatized spermine using the proposed method ($n = 10$) were determined. The obtained parameters were slope, 0.15 ± 0.01; intercept, 0.02 ± 0.03; determination coefficient (R^2), 0.999; LOD, 0.4 μg/mL; and LOQ, 1.2 μg/mL.

The intra- and interday precision and accuracy of spermine were determined by analysis of spiked samples at 2, 10, and 20 μg/mL. The intraday values were determined by assaying test solutions nine times on the same day, and interday value was the average of nine measurements of intra-day values taken on 5 days over a 3-month period. The precision was taken as the RSD (%) of the obtained areas, whereas the accuracy was the ratio between the calculated value of the recovered spermine and the spiked one (%). The results obtained were between 0.7–3.6% for precision and 93.3–101% for accuracy (Table 1). Both were in agreement with FDA guideline, which indicates that precision should be less than 15% and accuracy between 80 and 120% [17].

Robustness of the method was examined by replicate injections ($n = 6$) of spermine spiked sample of unsalted anchovy sample at 10 μg/mL under small changes in the chromatographic parameters (SDS concentration, percentage of 1-pentanol, pH and flow rate). Insignificant differences in peak areas (<4.8%) and less variability in retention time (<3.5%) were observed (Table 2). These results indicate that the selected factors remain unaffected by small variations in these parameters.

Stability studies indicated that the degradation of biogenic amines derivatizated with DNBZ-Cl took place in 12 h when kept in the fridge and in three hours at room temperature. These results were confirmed by the displacement of the peaks in chromatograms. The biogenic amine samples and the derivatizing reagent DNBZ-Cl were stable for three days in the fridge and four months when kept in a freezer.

3.3. Analysis of Food Samples. Unsalted and salted anchovy sauces (*Engraulidae* spp.) were analyzed in different days in

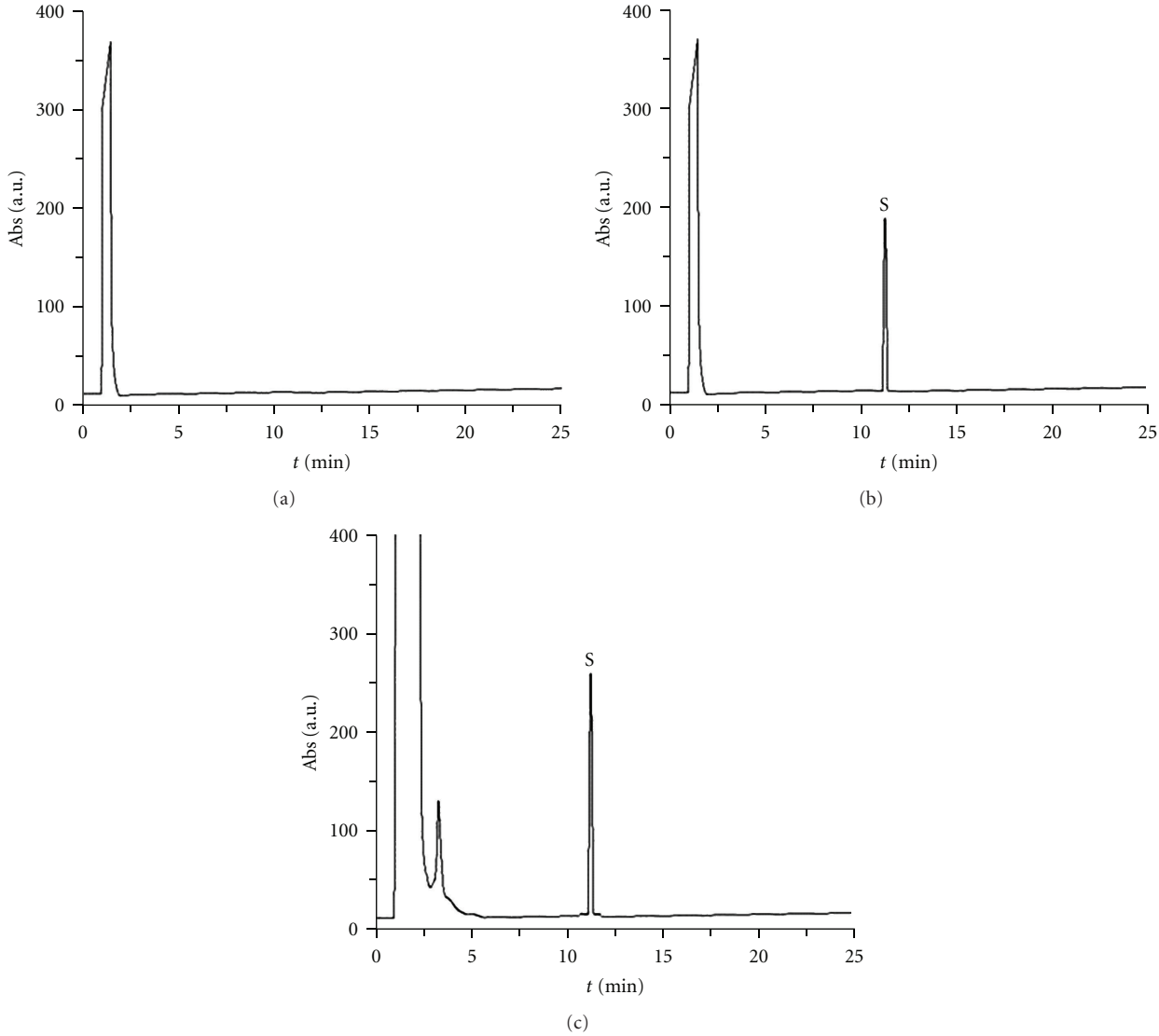

Figure 1: Chromatograms obtained by the analysis of unsalted anchovy sauce samples following the proposed methodology: (a) blank, (b) spiked with 10 μg/mL spermine, and (c) a real sample containing 15 μg/mL of spermine after 5-days storage. Chromatographic conditions: 0.15 M SDS-4% (v/v) 1-pentanol-pH 7.

Table 2: Evaluation of the MLC method robustness.

Changes in the parameters	Level	Retention time (min) (RSD, %)	Area (RSD, %)
SDS (M)	0.145–0.155	11.6 ± 0.3 (2.6)	1.72 ± 0.05 (2.9)
1-Pentanol (%)	3.9–4.1	11.8 ± 0.4 (3.4)	1.68 ± 0.08 (4.8)
pH	6.9–7.1	11.5 ± 0.2 (1.7)	1.66 ± 0.04 (2.4)
Flow (mL/min)	0.95–1.05	11.3 ± 0.4 (3.5)	1.70 ± 0.05 (2.0)

$n = 6$.

order to evaluate the microbacterial contamination depending on the storage conditions, by means of the amount or spermine. Figure 1(c) shows the chromatogram obtained by analysis of a real sample of unsalted anchovy sauce after 5 days of storage, where 15 μg/mL were detected without interferences. The amount of spermine after 1 month of storage was evaluated. Unsalted sample contained more than 100 μg/mL of spermine, indicating a high microbacterial contamination (Figure 2). The unsalted anchovy sauce is spoilt and is unable to eat. However, the salted sample shows no microbacterial contamination (spermine level under LOD) and can be eaten without risk. These results indicate that salting is an efficient method to prevent microbial contamination during anchovy sauce storage.

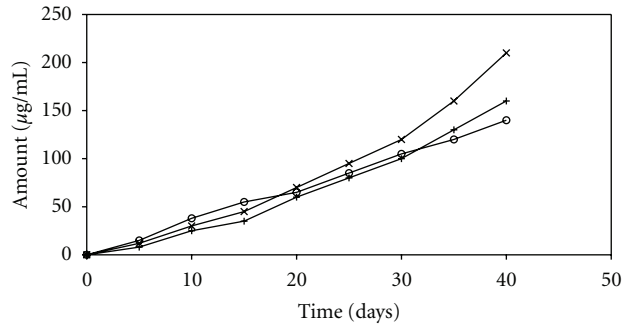

Figure 2: Amount found of spermine in three samples of unsalted anchovy sauce stored at +4°C and analyzed by the proposed methodology in different days.

4. Conclusions

In conclusion, the results indicate that the micellar liquid chromatography procedure here developed can be used for the analysis of spermine, with analysis times below 12 min. The analytical method is sensitive enough to be applied for quality control and routine analyses. It is a simple, rapid, effective, and alternative method and does not require any extraction step: the sample is directly injected after the derivatization procedure. The reagent 3,5-dinitrobenzoyl chloride was found to be highly suitable for the analysis of spermine with a very simple method and fast reaction at room temperature and is therefore recommended for use in pollution surveys, to study the degree of microbial contamination, and in the routine practice of food-quality control.

Acknowledgments

This work was supported by Fundació Caixa Castelló-Bancaixa P1-1B2006-12 projects and MEC CTQ 200764473/BQU. M.-L. Chin-Chen also thanks the Foundation for its grant.

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 402635, 5 pages
doi:10.1155/2012/402635

Research Article

Retention Behaviour in Micellar Liquid Chromatography

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Received 30 November 2011; Accepted 11 January 2012

Academic Editor: Samuel Carda-Broch

Retention in micellar liquid chromatography is highly reproducible and can be modelled using empirical or mechanistic models with great accuracy to predict the retention changes when the mobile phase composition varies (surfactant and organic solvent concentrations), thus facilitating the optimisation of separation conditions. In addition, the different equilibria inside the column among the solute, the mobile phase, and the modified stationary phase by monomers of surfactant have been exhaustively studied. In a sequential strategy, the retention of the solutes is not known a priori, and each set of mobile phases is designed by taking into account the retention observed with previous eluents. By contrast, in an interpretative strategy, the experiments are designed before the optimization process and used to fit a model that will allow the prediction of the retention of each solute. This strategy is more efficient and reliable. The sequential strategy will be inadequate when several local and/or secondary maxima exist, as frequently occurs in chromatography, and may not give the best maximum, that is to say, the optimum. More often than not, the complexity of the mixtures of compounds studied and the relevant modification of their chromatographic behaviour when changing the mobile phase composition requires the use of computer-assisted simulations in MLC to follow the modifications in the chromatograms in detail. These simulations can be done with sound reliability thanks to the use of chemometrics tools.

1. Introduction

Most reported procedures for the determination of compounds in MLC make use of micellar mobile phases containing an organic modifier, usually a short-chain alcohol or acetonitrile. These modifiers increase the elution strength and often improve the shape of the chromatographic peaks. The modifiers solvate the bonded stationary phase and reduce the amount of surfactant adsorbed, the effect being larger with increasing concentration and hydrophobicity of the alcohol. Selection of the pH in the mobile phase is also often extremely important for the resolution of complex mixtures, owing to the side acid-base reactions of many solutes. Other variables to be considered are temperature and ionic strength [1].

The chromatographer is concerned with the achievement of the optimum mobile phase that permits the separation of the composition in a mixture, in the minimum time. This task may be really difficult when two or more variables are involved in the optimisation process. The optimisation strategy may be sequential or interpretative. In a sequential strategy, the retention of the solutes is not known *a priori*, and each set of mobile phases is designed by taking into account the retention observed with previous eluents. In contrast, in an interpretative strategy, the experiments are designed before the optimisation process and used to fit a model that will permit the prediction of the retention of each solute. This strategy may be much more efficient and reliable. A sequential strategy will be inadequate when several local (or secondary) maxima exist (as occurs in chromatography) and may not give the best maximum, that is, the optimum.

The necessity for an adequateexperimental design becomes especially important when dealing with forms of liquid chromatography suitable for the simultaneous analysis of ionic and nonionic compounds, such as MLC, where several variables should be controlled (e.g., type and concentration of surfactant and organic modifier, pH, temperature, and ionic strength). The method development strategy must provide the chromatographer with an answer to which variables should be used, and how to set up initial experiments to search the appropriate variable space in an effective way.

The separation process in a micellar chromatographic system requires a structured approach in the development of practical applications. Ideally, the resolution of complex mixtures should be made and optimized in a short time, with minimal consumption of reagents.

2. Empirical Models

Retention in a hybrid micellar mobile system can be modelled using a procedure that utilizes the retention data of only five mobile phases [2]: four measurements at the corners of the selected two-dimensional variable space, defined by the concentrations of surfactant and modifier, and the fifth in the centre. The chromatographic data obtained (retention factor, efficiency, and asymmetry factor) are used to fit some equations. The models used for the description of retention behaviour are summarized as in Section 2.1.

2.1. Empirical and Mechanistic Models Used in MLC

Empirical Models. k is the retention factor for a given mobile phase composition; $[M]$ is the concentration of surfactant forming micelles (total concentration of surfactant minus the critical micellar concentration, CMC); φ is the volume fraction of the organic solvent; c_0, c_1, c_2, c_3, and c_{11} are fitting coefficients:

$$\log k = c_0 + c_1[M] + c_2\varphi, \tag{1}$$

$$\frac{1}{k} = c_0 + c_1[M], \tag{2}$$

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi, \tag{3}$$

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi + c_3[M]\varphi, \tag{4}$$

$$\frac{1}{k} = c_0 + c_1[M] + c_2\varphi + c_3[M]\varphi + c_{11}\varphi^2. \tag{5}$$

Mechanistic Models. K_{AS} is the product of the solute- (A) stationary (S) partition coefficient by the phase ratio, and K_{AM} the solute-micelle (M) association constant; K_{AD} and K_{MD} measure the relative variation produced in the concentration of solute in bulk water and micelle, respectively, in presence of modifier, taking the pure micellar solution as a reference; K_{AD2} corresponds to a quadratic hyperbolic variation in K_{AS} and K_{AM} with φ:

$$k = \frac{K_{AS}}{1 + K_{AM}[M]}, \tag{6}$$

$$k = \frac{K_{AS}(1/(1 + K_{AD}\varphi))}{1 + K_{AM}((1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))[M]}, \tag{7}$$

$$k = \frac{K_{AS}(1/(1 + K_{AD1}\varphi + K_{AD2}\varphi^2))}{1 + K_{AM}((1 + K_{MD}\varphi)/(1 + K_{AD1}\varphi + K_{AD2}\varphi^2))[M]}, \tag{8}$$

$$k = \frac{K_{AS}((1 + K_{SD}\varphi)/(1 + K_{AD}\varphi))}{1 + K_{AM}((1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))[M]}, \tag{9}$$

$$k = \frac{K_{AS}(1/(1+K_{AD1}\varphi+K_{AD2}\varphi^2))}{1+K_{AM}((1+K_{MD1}\varphi+K_{MD2}\varphi^2)/(1+K_{AD1}\varphi+K_{AD2}\varphi^2))[M]}. \tag{10}$$

Equations (1)–(5) correspond to empirical models. In (1), it is assumed that solute retention is linearly related to the mobile phase variables within a selected portion of the space and fitted to a separate logarithmic linear function. However, the predictions obtained with (1) are not accurate enough. Retention in micellar mobile phases with a fixed amount or without organic solvent [3] has been extensively proved to be described by the hyperbolic relationship shown in (2). To model the retention of a solute at varying concentrations of both surfactant and modifier [4], (3) can be used and has yielded acceptable results for small ranges of concentrations of surfactant and modifier, but an interaction between both factors, $[M]$ and φ, exists for larger domains.

The capability of a series of empirical equations to describe the retention behaviour for any surfactant and organic solvent content has been studied [5–7]. Several models were considered where $\log k$ or $1/k$ values were related to the micellar concentration and volume fraction of organic modifier. Logarithmic models usually yielded poorer results. Equation (4) is the simplest equation giving good predictions for both polar and moderately polar compounds, such as amino acids, sulfonamides, β-blockers, and diuretics. Equation (4) yields linear plots of $1/k$ versus φ at fixed concentrations of surfactant. However, for highly hydrophobic compounds, such as steroids or polycyclic aromatic hydrocarbons, the plots are nonlinear. An additional term is required to achieve more accurate descriptions, as shown in (5). The parameters in (4) and (5) should be obtained by fitting the data in experimental designs with at least four and five mobile phases, respectively. However, at least one additional measurement should be taken to check the accuracy of the fittings.

3. Mechanistic Models

The parameters of the empirical models of MLC are related to physicochemical constants that describe the interactions of the solutes with the three environments involved in micellar mobile phases: bulk water, micelles, and the stationary phase. A better understanding of the retention mechanism in micellar systems is provided by these models.

The mechanistic models are based on (2), which is the classical equation proposed for micellar mobile phases at a fixed volume fraction of organic modifier [2]. Equation (2) can be written as (6), which relates the retention of a solute to the concentration of monomers of surfactant in the form of micelles.

For hybrid micellar mobile phases, (6) can be expressed as (7), while (5) gives rise to (8) [8], which may suggest an

excessive dependence of the retention on the organic solvent concentration and produce high errors when an extrapolation is made in a region of high concentration of modifier.

As a result, (9) was proposed as an alternative model for highly hydrophobic solutes, and it takes into account the additional change in the concentration of solute associated with the stationary phase produced by the presence of modifier [7]. In (9), the constants K_{MD} and K_{AD} account for the displacement of the water-micelle equilibrium, whereas K_{SD} and K_{AD} describe the modification of the water-stationary phase equilibrium. These changes are caused by the decrease in the polarity of water and the modification of the interactions of solute with micelles and stationary phase, when a modifier is added. Equation (9) provides an accurate description of the retention of solutes of a wide range of polarities, when they are eluted with hybrid mobile phases of SDS and alcohol (propanol, butanol or pentanol) [8]. For acetonitrile and tetrahydrofuran [9, 10], (10) fits better.

4. Peak Shape Modelling

The major drawback of applying MLC to practical separations is still the low chromatographic efficiency, which is caused by resistance to mass transfer in the processes involving micelles and a surfactant-modified stationary phase. This is especially important since the increase in micelle concentration causes a decrease in plate number, resulting in a varying efficiency over the variable space. Thus, it is very important to include the expected peak shape in the expression of the chromatographic quality. The complexity of the chromatographic process does not allow the use of simple equations to describe peak profiles. The best peak-profile predictions are achieved using a Gaussian equation where the standard deviation depends polynomially on the distance to the peak time (polynomial modified Gaussian model) [11]:

$$h(t) = H_0 e^{-0.5((t-t_R)/(s_0+s_1(t-t_R)+s_2(t-t_R)^2+\cdots))^2}, \quad (11)$$

where $h(t)$ is the predicted signal at time t, H_0 the maximal peak height, t_R the retention time, and the coefficients s_i are related to the width and asymmetry of the chromatographic peak. For a given solute and mobile phase, t_R and s_i are ideally invariable, whereas H_0 depends on the concentration. Better descriptions of peak profiles are achieved by increasing the degree of the polynomial. The use of a larger number of coefficients improves the fittings but decreases the practical application of the model. A linear standard deviation in (11) approximates real peak profiles satisfactorily. The linear equation is also useful for simulating chromatograms.

5. Strategies to Measure the Peak Resolution

The simulation of chromatograms requires predictions of peak retention and peak profile that are as accurate as possible. Computer optimization attempts to mimic the methodology followed by an experienced chromatographer so as to reduce the time and the effort required. A software application was developed to assist the chromatographer in the selection of the optimal composition of the mobile phase in MLC [12]. This program allows chromatograms to be simulated quickly and therefore enables the changes in the chromatograms to be observed as the user simulates variations in the composition of the mobile phase. The resolution surfaces and ease of simulation can be applied in a straightforward manner to select the best composition of the mobile phase.

Different measurements of diverse complexity have been proposed to depict chromatographic performance. Optimization criteria based on calculation of an individual or elementary resolution measurement, r_i, for the least resolved peak or peak pair is a very widely used procedure in chromatographic practice, because of its simplicity:

$$R = \mathrm{MIN}(r_i) \quad 1 \le i \le p, \quad (12)$$

where p is the number of peaks or peak pairs, and R is the global resolution.

This criterion is reasonable, but it considers the resolution of only one peak or peak pair, and is insensitive to the remaining peaks. In many cases, a practically identical resolution of the worst peak can be obtained, while the resolution of the other peaks can be improved. The product of peak resolutions solves this drawback, since it optimizes the resolution of all peaks in the chromatogram.

The normalized-by-mean product is conventionally applied. Section 5.1 shows (13), the global resolution functions, and elementary resolution criteria. This treatment normalizes the resolution approximately, using the mean r_i of all the peaks in the chromatogram instead of the extreme values.

The unnormalized product of (14) seems to be a better alternative, although it can be used only with intrinsically normalized resolution measurements. This product varies from 0 (complete overlapping between at least two peaks) to 1 (full resolution of every peak in the chromatogram).

After the selection of the global resolution function, the appropriate elementary resolution criterion (Section 5.1) should be decided. Some criteria have been based on conventional elementary measurements, such as the modified selectivity in (15), peak-to-valley measurements in (16), and overlapping fraction measurements in (17).

In (16), the valley between two consecutive peaks can be measured at the time giving the largest possible distance, measured orthogonally. If the valley is observed orthogonally, this point is obvious even when there is substantial overlap.

The criterion of overlapping fractions takes into account not only positions but also peak profiles; it isolates the contribution of each component in a mixture, associating a value to each individual peak, which is not affected by the identity of its neighboring peaks, and the intrinsic normalisation facilitates understanding of the information obtained in the optimization process.

5.1. Global Resolution Functions and Elemental Resolution Criteria Used in MLC

Resolution Function. k_i and k_{i+1} ($k_{i+1} > k_i$) are the retention factors of two neighboring peaks, and $\alpha_{i;i+1}$ is the selectivity; h_1 represents the height of the signal at a specific time depicting the valley location, and h_2 is an interpolated height, measured at that time, from baseline to the line obtained by joining the maximums of the two neighboring peaks; o_i' is the area under a given peak overlapped by the chromatogram yielded by the remaining peaks, and o_i the total area of the peak:

$$R = \frac{\prod_{i=1}^{p} r_i}{\left[\sum_{i=1}^{p} r_i/p\right]^p}, \tag{13}$$

$$R = \prod_{i=1}^{p} r_i, \tag{14}$$

$$r_{i,i+1} = 1 - \frac{k_i}{k_{i+1}}, \tag{15}$$

$$r_{i,i+1} = 1 - \frac{h_1}{h_2}, \tag{16}$$

$$r_i = 1 - \frac{o_i'}{o_i}. \tag{17}$$

6. Conclusions

The development of a micellar analytical procedure for the determination of solutes requires optimization of the column type, the type and concentration of the surfactant and organic modifier, pH, flow rate, and temperature. In addition, the optimization strategy can be sequential or interpretive. In a sequential strategy, a set of mobile phases is designed by taking into account the retention observed with previous eluents (the retention of the solutes is not known a priori). When the experiments are designed before the optimization process and used to fit a model that will allow the retention of each solute to be predicted, the strategy is called interpretative, and, of the two, it is more efficient and reliable. The need for an adequate experimental design becomes especially important when dealing with forms of liquid chromatography suitable for the simultaneous analysis of ionic and nonionic compounds, such as MLC, in which several factors should be controlled. The strategy used to develop the method must provide answers to questions concerning the variables to be used as well as the set-up of the initial experiments that will yield an effective search of the appropriate variable space. Retention in a hybrid micellar mobile system can be modeled using a procedure that utilizes the retention data of only five mobile phases: four measurements at the corners of the selected two-dimensional variable space, defined by the concentrations of surfactant and modifier, and the fifth in the center. The chromatographic data obtained (retention factor, efficiency, and asymmetry factor) are used to fit some equations. Thus, computer optimization permits to mimic the methodology followed by an experienced chromatographer, reducing the time and the effort required.

Acknowledgment

The author wishes to thank the MEC for the FPU grant.

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 898520, 6 pages
doi:10.1155/2012/898520

Research Article

Basic Principles of MLC

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Received 30 November 2011; Accepted 11 January 2012

Academic Editor: Samuel Carda-Broch

Micellar liquid chromatography (MLC) is an efficient alternative to conventional reversed-phase liquid chromatography with hydro-organic mobile phases. Almost three decades of experience have resulted in an increasing production of analytical applications. Current concern about the environment also reveals MLC as an interesting technique for "green" chemistry because it uses mobile phases containing 90% or more water. These micellar mobile phases have a low toxicity and are not producing hazardous wastes. The stationary phase is modified with an approximately constant amount of surfactant monomers, and the solubilising capability of the mobile phase is altered by the presence of micelles, giving rise to a great variety of interactions (hydrophobic, ionic, and steric) with major implications in retention and selectivity. From its beginnings in 1980, the technique has evolved up to becoming in a real alternative in some instances (and a complement in others) to classical RPLC with aqueous-organic mixtures, owing to its peculiar features and unique advantages. The addition of an organic solvent to the mobile phase was, however, soon suggested in order to enhance the low efficiencies and weak elution strength associated with the mobile phases that contained only micelles.

1. Introduction

Micellar liquid chromatography (MLC), which uses mobile phases containing a surfactant above its critical micellar concentration (CMC), is an alternative to conventional reversed-phase liquid chromatography and provides a solution to the direct injection of physiological or food samples by solubilising proteins (that are eluted together or shortly after the solvent front) [1–3]. The possibility of directly injecting samples into the chromatograph simplifies and expedites treatment, which confers analytical procedures greater accuracy and a lower cost.

The versatility of MLC is due to the wide variety of interactions that are established among the eluted solutes, the stationary phase, the aqueous phase and micelles. Their eluent characteristics allow the analysis of compounds with a wide range of polarities.

The presence of a surfactant not only modifies the interactions established inside the column but also reduces the necessary amount of organic solvent in the mobile phase, which can be recycled due to low evaporation. These characteristics are genuinely interesting given current concerns about reducing organic contaminant residues in laboratories.

MLC shares the basic components of reversed-phase liquid chromatographic (RPLC) systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudophase) and the surrounding bulk water or aqueous-organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC. On the other hand, the stationary phase is modified by the adsorption of surfactant monomers, creating a structure similar to an open micelle, and reducing silanophilic interactions. With nonionic surfactants, only the polarity of the stationary phase changes, whereas with ionic surfactants, a net charge (positive or negative) appears on its surface with major implications.

On the other hand, a new micellar chromatographic mode has been recently described: high submicellar chromatography [4, 5], where the surfactant forms micelles and

organic solvent content is high. This mode opens up a range of possibilities to new applications in this chromatographic technique and complements low submicellar chromatography (also known as ion pair chromatography) where the number of free molecules of the surfactant in the mobile phase is insignificant, but sufficient to cover the stationary phase.

It should be highlighted that the fundamental studies into MLC have served to develop the technique and to establish its theoretical basis, without which its later use in diverse applications would be impossible.

2. Particularities of the Micellar Mobile Phase

Micelles provide hydrophobic and electrostatic (for ionic surfactants) sites of interaction. In the micelles, three sites of solubilisation can be identified: the core (hydrophobic), the surface (hydrophilic), and the palisade layer (the region between the surfactant head groups and the core). Solutes associated to micelles experience a microenvironment that is different from that of bulk solvent [6].

Although pure micellar mobile phases are sometimes used, most separations in MLC are performed with hybrid micellar mobile phases in a buffered medium that contains micelles, surfactant monomers, molecules of organic solvent, and water. The organic solvent decreases the polarity of the aqueous solution and alters the micelle structure. Although the separation mode is still predominantly micellar in nature, the micelle is perturbed by the organic solvent. This can change micellar parameters, such as the CMC and surfactant aggregation number. A high percentage of organic solvent can disrupt the micelle structure. The maximal allowable concentration depends on the type of organic solvent and surfactant.

2.1. Critical Micellar Concentration. A suitable surfactant for MLC should have a low CMC. A high CMC would imply operating at high surfactant concentration, which would result in viscous solutions, giving undesirable high system pressure and background noise in UV detectors. The selection is often limited to the following surfactants: the anionic sodium dodecyl sulphate (SDS), the cationic cetyltrimethylammonium bromide (CTAB), and the nonionic Brij-35, whose main characteristics are summarized in Table 1. The CMC values of these surfactants in pure water are low enough for MLC. It should also be taken into account that the CMC is strongly affected by the presence of an organic solvent. The changes are related to the modification of the structure of the micelle, which also induces, at least partially, the reduced retention in MLC [7]. Recently, some novel ionic liquid-based surfactants like 1-hexadecyl-3-butylimidazolium bromide have been used in MLC [8, 9].

2.2. Krafft Point. The Krafft point is defined for ionic surfactants as the temperature at which the solubility of a surfactant monomer becomes equal to the CMC [12]. Below the Krafft point temperature, the solubility is quite low and the solution appears to contain no micelles. Chromatographic work in MLC should be conducted above this temperature to avoid surfactant precipitation. This means that the Krafft point should be well below room temperature. The Krafft point for SDS and CTAB is around 15°C and 20–25°C, respectively [13, 14].

Table 1: Characteristics of the most common surfactants in MLC[a].

Surfactant	Molecular weight (g/mol)	CMC (mol/L)	R[b] (nm)	V[c] (L/mol)
SDS	288.4	8.2×10^{-3}	2.5	0.246
CTAB	364.5	9×10^{-4}	3.2	0.364
Brij-35	1198 (avg.)	9×10^{-5}		

[a]References [10] and [11]; [b]Micellar radius; [c]Molar volume.

Non-ionic surfactants also have a specific temperature, that if exceeded, phase separation occurs, which is called the cloud point [11, 15]. Chromatographic work with these surfactants should be conducted below this temperature (e.g., Brij-35, is ca. 100°C for aqueous 1–6% solutions, whereas for Triton X–100 this value is 64°C).

2.3. pH of the Mobile Phase. MLC employs the same packing materials as classical RPLC, which for conventional columns have a limited working pH range of 2.5–7.5. Appropriate pH values depend on the nature of the analytes and the surfactant selected. The pH of the micellar mobile phase is commonly fixed with phosphoric or citric acid buffers [2, 3]. For mobile phases containing SDS, potassium salts are not recommended as potassium dodecyl sulphate presents a high Krafft point and precipitates from aqueous solutions at room temperature [2].

2.4. Organic Solvents: Types and Concentration. The selection of the appropriate organic solvent modifier in MLC should consider the polarities of the analytes. For polar compounds, sufficiently short retention times (below 20 min) are obtained with 1-propanol, 2-propanol, or acetonitrile. For nonpolar compounds or compounds with high affinity for the surfactant adsorbed on the stationary phase, stronger solvents as 1-butanol or 1-pentanol are needed [16]. However, it should be noted that the two latter alcohols give rise to microemulsion formation at sufficiently high concentration [17]. In practice, the amount of organic solvent that can be added is limited by its solubility. It should be noted that at high organic solvent concentration, the micelles disaggregate and the mobile phase contains only free surfactant molecules. The organic solvent contents that preserve the integrity of micelles are below 15% for propanol and acetonitrile, 10% for butanol, and 6% for pentanol [18]. These contents are low in comparison with those needed in classical RPLC. The lower organic solvent consumption results in reduced cost and toxicity, which may become prominent for "green chemistry". Also, the stabilization of the organic solvent in the micellar media decreases the risk of evaporation. This means that micellar mobile phases can be preserved in the laboratory for a long time without significant changes in their composition.

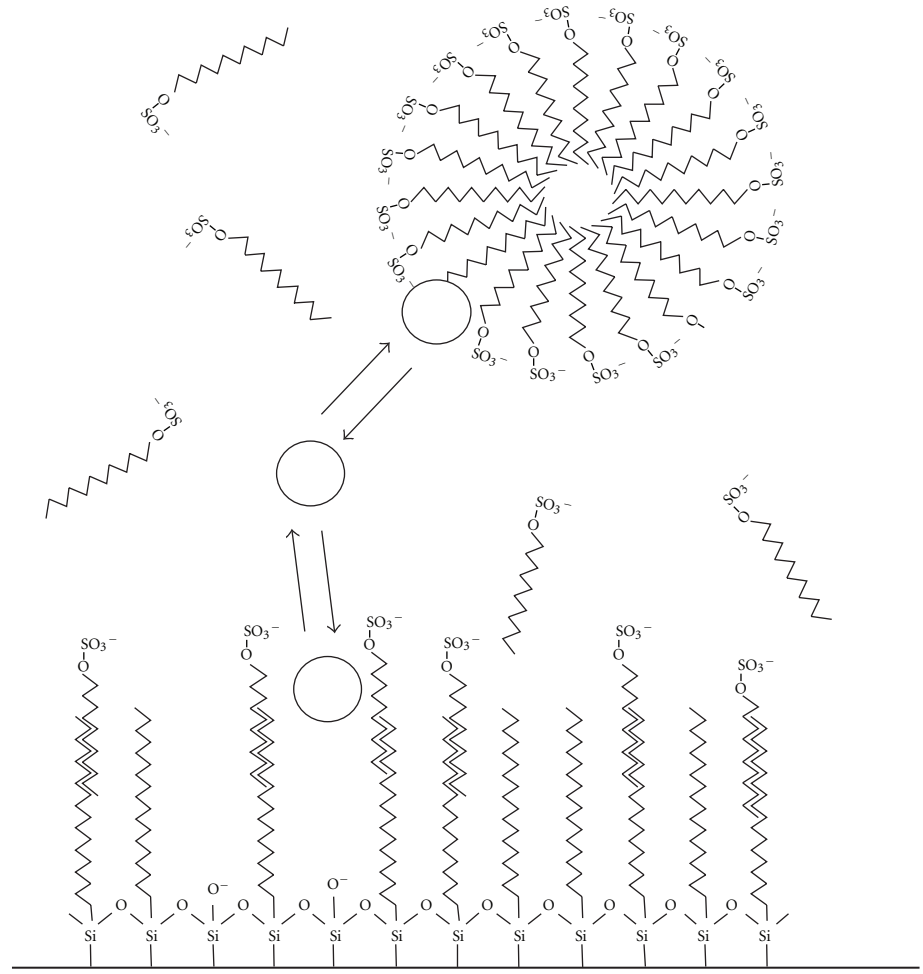

Figure 1: Solute environment in a chromatographic system using octadecyl-bonded phase, and mobile phase containing the anionic SDS. Equilibria between bulk solvent, micelle, and surfactant-modified stationary phase are depicted (reproduced with permission from [22].)

3. Modified Stationary Phase

3.1. Surfactant Adsorption. The alkyl-bonded C18 is the stationary phase most widely used in MLC, but other columns can be selected (e.g., C8 and cyanopropyl). Alkyl-bonded phase columns are strongly modified when SDS, CTAB, or Brij-35 is incorporated into the mobile phase.

Surfactant adsorption on the porous RPLC packing affects drastically the chromatographic retention, owing to the change of diverse surface properties of the stationary phase (e.g., polarity, structure, pore volume, and surface area). Surfactant molecules coat the stationary phase pores, reducing appreciably their volume [19].

Ionic compounds are frequently added to micellar mobile phases for pH buffering and, eventually, ionic strength adjustment. Salt addition may change the amount of adsorbed ionic surfactant due to the reduction of both electrostatic repulsion and surfactant CMC, and the enhancement of hydrophobic interactions [20].

Surfactant coating masks the bonded-stationary phase. This means that a full similar coating would render the stationary phases all similar. Solid-state nuclear magnetic resonance studies for the most common used surfactant, SDS, reveal that the hydrophobic tail was found to be associated with the C18 alkyl-chain bonded to the silica stationary phase, the sulphate head group oriented away from the surface (Figure 1) [21]. This creates a negatively charged hydrophilic layer affecting the penetration depth of solutes into the bonded phase.

3.2. Presence of an Organic Solvent in the Mobile Phase. Organic solvents are added to micellar mobile phases to improve peak efficiencies and reduce retention times, giving rise to the so-called hybrid micellar mobile phases. Competition between alcohols and surfactant molecules for adsorption sites on the stationary phase explains the linear reduction in the amount of adsorbed surfactant with increasing concentration of alcohol in the mobile phase. Mobile phases rich in organic solvent can sweep completely the adsorbed surfactant molecules from the bonded phase.

4. Care of the Chromatographic System in MLC

4.1. Mobile Phase Saturation. Pure and hybrid micellar solutions contain high amounts of water (usually more than 90% v/v) and are able to dissolve small amounts of silica, which could produce serious column damage. This is especially critical at 30°C and/or pH 6. For this reason, a saturating short column packed with 10 μm bare silica, or alternatively, the same packing as the analytical column, should be placed after the pump and before the injection valve to reduce pressure build-up.

4.2. Column Conditioning. A column for MLC is generally stored in 100% methanol. Before starting column conditioning, the solvent should be replaced by 100% water. For this operation, a low flow rate ($\leq$0.5 mL/min) should be selected at the beginning because of the high viscosity of the methanol-water mixture. Once the pressure decreases, the flow-rate may be raised. At least 30 column volumes of water are required to assure complete organic solvent removing. Now, the system is ready to be flushed with the micellar mobile phase. Different studies of column coating through surfactant breakthrough patterns have revealed that most surfactant adsorbs in less than one hour on the bonded-stationary phase [8, 20].

4.3. Mobile Phase Flushing. The micellar mobile phase should be continuously flushed through the system. If the chromatographic system is stopped during several hours, the micellar solution should not stay in contact with the bonded-silica-based stationary phase to avoid surfactant precipitation. A static micellar mobile phase can also produce crystals around the pump plungers and seals. Such crystals may obstruct the system producing plugged connecting tubing and frits, seal failure, or scratched pistons. A micellar mobile phase can be kept inside the chromatographic system overnight if the pump is not off. This avoids daily cleaning and re-equilibration. To reduce the cost, the mobile phase can be recycled, reducing the flow-rate to a minimal value (often 0.1–0.25 mL/min). However, it should be noted that in case of energy supply failure, column damage can occur. Mobile phase recycling is possible because of the low evaporation risk of organic solvents in hybrid micellar eluents. For the same reason, the micellar mobile phase can be recycled during the analysis, as long as a low number of injections are made.

4.4. Column Cleaning. In general, regeneration can be appropriately performed with methanol, where most surfactants are highly soluble [23]. The cleaning protocol comprises a two-step procedure that takes about half an hour.

(i) First, the micellar mobile phase should be replaced by 100% pure water, by rinsing the chromatographic system with 10 to 20 column volumes of pure water. This step is necessary to avoid salt crystallization provoked by a brutal change from a buffered micellar mobile phase to 100% methanol.

(ii) Next, water will be replaced by 100% methanol to remove the adsorbed surfactant on the stationary phase. The same caution commented under "column conditioning" about the initial use of a low flow-rate should be followed. To assure complete surfactant desorption, at least 10 column volumes of methanol should be passed through the column.

5. Solute-Micelle and Solute-Stationary Phase Interactions

The unique capabilities of micellar mobile phases are attributed to the ability of micelles to selectively compartmentalise and organise solutes at the molecular level. However, the association of the surfactant monomers to the bonded phase has deep implications with regard to retention and selectivity. The chromatographic behaviour in an RPLC system of a solute eluted with a mobile phase containing a surfactant above the CMC can be explained by considering three phases: stationary phase, bulk solvent, and micellar pseudophase. Figure 1 illustrates the three-phase model. Solutes are separated on the basis of their differential partitioning between bulk solvent and micelles in the mobile phase or surfactant-coated stationary phase. For water-insoluble species, partitioning can also occur via direct transfer of solutes between the micellar pseudophase and the modified stationary phase (Figure 2).

The partitioning equilibria in MLC can be described by three coefficients: P_{WS} (partition between aqueous solvent and stationary phase), P_{WM} (between aqueous solvent and micelles), and P_{MS} (between micelles and stationary phase). The coefficients P_{WS} and P_{WM} account for the solute affinity to the stationary phase and micelles, respectively, and have opposite effects on solute retention: as P_{WS} increases, the retention increases, whereas as P_{WM} increases, the retention is reduced due to the stronger association to micelles.

The retention behaviour depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with non-ionic and ionic surfactants and charged solutes eluted with non-ionic surfactants will only be affected by nonpolar, dipole-dipole, and proton donor-acceptor interactions [24]. Besides these interactions, charged solutes will interact electrostatically with ionic surfactants (i.e., with the charged surfactant layer on the stationary phase and the charged outer layer of micelles). In any case, the steric factor can also be important.

With ionic surfactants, two situations are possible according to the charges of solute and surfactant: repulsion or attraction (by both surfactant-modified stationary phase and micelles). In the case of electrostatic repulsion, charged solutes cannot be retained by the stationary phase and elute at the dead volume, unless significant hydrophobic interaction with the modified bonded layer exists. In contrast, combined electrostatic attraction and hydrophobic interactions with the modified stationary phase may give rise to strong retention in MLC. Mixtures of polar and nonpolar solutes can be resolved, provided that an appropriate surfactant is chosen.

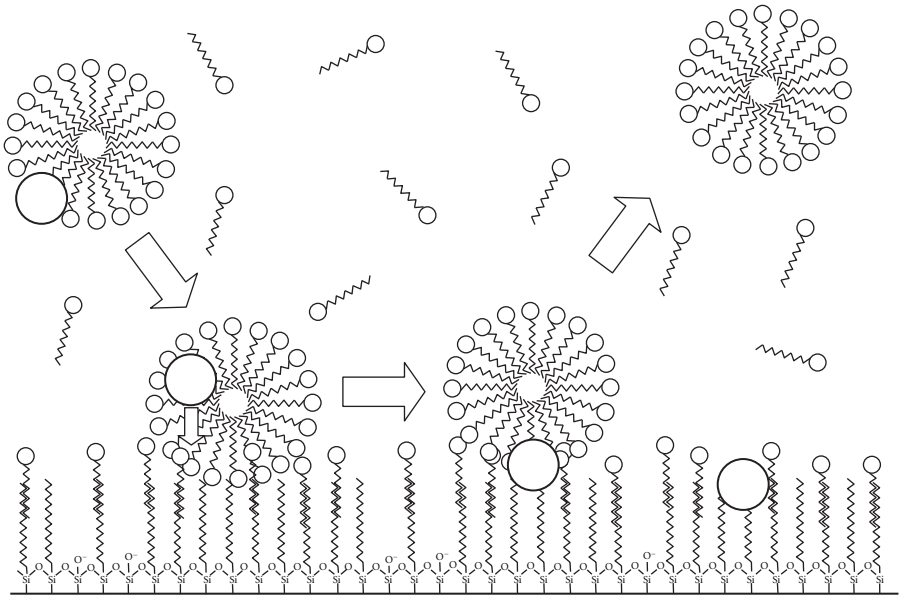

Figure 2: Direct transfer of highly hydrophobic solutes between micelle and surfactant-modified stationary phase (reproduced with permission from [22].)

6. Submicellar Liquid Chromatography: A New Mode in MLC

Depending on the concentration level of surfactant and organic solvent, two micellar chromatographic modes have been recently described [4, 5, 25], each one with particular characteristics.

6.1. Low Submicellar Chromatography. In this chromatographic mode, the stationary phase is coated with SDS, acquiring thus a negative charge. Cationic solutes can interact hydrophobically with the uncovered alkyl-bonded layer, or with the adsorbed surfactant monomers through electrostatic attraction. As long as the adsorbed amount of surfactant does not reach the maximal capacity of the column, surfactant coating on the stationary phase increases with its concentration in the mobile phase. This derives in larger retention times.

Under submicellar conditions at low surfactant concentration, the amount of free surfactant molecules in the mobile phase is negligible. This indicates that an ion-exchange retention mechanism is dominant and ion-pair formation with the surfactant in the mobile phase is practically inexistent. The addition of an organic solvent to the mobile phase increases the elution strength as a result of the decreased mobile phase polarity, and the competition between organic solvent and surfactant molecules for adsorption sites, which reduces the amount of surfactant adsorbed on the stationary phase.

6.2. High Submicellar Chromatography. The surfactant is at a concentration where micelles are formed in water, and the organic solvent content is high. This prevents the formation of micelles. Consequently, only surfactant monomers exist in the mobile phase, which are dissolved in the hydro-organic medium. The retention mechanism dominant in this region depends on the amount of surfactant that has been swept off the alkyl-bonded phase by the organic solvent and the existence of micelles. As long as a certain amount of surfactant remains adsorbed, and micelles exist, the retention mechanism will be the typical of the micellar mode. When micelle disaggregation occurs, a submicellar situation is achieved where ion-pair interactions with surfactant monomers in the bulk mobile phase will replace those with micelles.

7. Conclusions

The addition of a surfactant to the mobile phase in RPLC changes the chromatographic behaviour with aqueous-organic mixtures. In MLC, neutral and charged surfactants are used, and the surfactant concentration exceeds the CMC, which has major implications in both stationary and mobile phases. The stationary phase is modified, but now the adsorption reaches saturation or shows relatively small changes with mobile phase composition. In this way, a stable stationary phase is obtained (in a reversible process) with features remarkably different from those of the underlying bonded phase. This has a deep impact on solute interactions. Not less important is the fact that above the CMC, surfactant monomers aggregate to form micelles, which show particular solubilising properties, remarkably different from those of aqueous-organic mixtures. Highly hydrophobic solutes are removed effectively from the stationary phase transported by the micelles. The presence of surfactant associated to either stationary phase or mobile phase in RPLC implies a change in retention mechanisms, which affects the retention and selectivity.

The variety of interactions found in MLC does not exist in any homogeneous aqueousorganic mobile phase. Owing to the amphiphilic nature of surfactants, solutes can associate with both micelles and the surfactant-coated stationary phase through a combination of electrostatic, hydrophobic, and steric interactions. For this reason, micellar mobile phases are compatible with a wide range of solutes (ionic to water-insoluble). The main strength of MLC lies precisely in the capability of performing and controlling the separation of mixtures of cationic, anionic, and uncharged polar and nonpolar solutes, with isocratic elution.

Acknowledgment

M. Rambla-Alegre wishes to thank the MEC for the FPU grant.

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Hindawi Publishing Corporation
Chromatography Research International
Volume 2012, Article ID 458153, 6 pages
doi:10.1155/2012/458153

Research Article

Selectivity of Brij-35 in Micellar Liquid Chromatographic Separation of Positional Isomers

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Received 19 September 2011; Revised 31 October 2011; Accepted 14 November 2011

Academic Editor: Maria Jose Ruiz-Angel

Implementation of Brij-35, a nonionic surfactant, as a mobile phase for separation of positional isomers is investigated. Chromolith C-18 SpeedROD is used as a stationary phase. The effect of surfactant and organic modifier (propanol) concentration on the separation of some selected isomers is studied and evaluated in terms of linear solvation energy relationship (LSER). Shape selectivity is assessed by α value of sorbic and benzoic acid, which is found to be 1.339 by using mobile phase composed of 0.5% aqueous solutions of Brij-35 and propanol in 9 : 1. Isomers of parabens, nitroanilines, nitrophenols, and quinolinols are successfully separated using mobile phases composed of various percentages of surfactant and propanol. System constants for nonionic MLC using LSER analysis show that hydrogen bond basicity and dipolarity may be major contributors to selectivity, while excess molar refraction helps fine-tuning the separation which also imparts unique selectivity to nonionic surfactants as compared to ionic ones.

1. Introduction

Use of surfactants as mobile phases in HPLC above critical micellar concentration (cmc) gave birth to a new branch of chromatography, now known as micellar liquid chromatography (MLC) [1]. Since its first use for separation of PAHs in 1980 [2], a number of publications, books, and monographs appeared in the literature covering the applications and characterization of MLC systems [3–5].

The most commonly used surfactant in liquid chromatography and in overall analytical chemistry is sodium dodecylsulfate (SDS) [6], an anionic surfactant as compared to other cationic and nonionic surfactants [7]. In nonionic surfactants, Brij-35 (polyoxyethylene dodecyl ether) has found applications in liquid chromatography as it is non-UV absorbing, which is a drawback of other nonionic surfactants having aromatic ring into their structure.

Brij-35 is reported as mobile phase for the separation of a number of compounds [7]. In a previous report [8] by our group, we reported that isomers of propyl-parabens could be better separated by Brij-35 as compared to SDS. Takayanagi et al. [9, 10] have also reported the separation of various positional isomers using Brij-35 as a surfactant in capillary electrophoresis. Vlasenko et al. [11] have studied the dissociation constants of hydroxybenzoic acids and parabens and found that addition of Brij-35 alters the dissociation of these compounds. Inspite of the number of publications on MLC using non-ionic surfactants as mobile phases, none of them is specifically focused on separation of isomers.

Separation of positional isomers is of importance from pharmaceutical perspective, due to differences in their complexation, toxicity, and reactivity in biological systems. Positional isomers cannot normally be separated through reverse phase HPLC with ODS columns commonly available in the laboratories with methanol/water or acetonitrile/water as mobile phases [12]. The objective of current study is to evaluate if Brij-35 could be used as mobile phase for separation of positional isomers and to find out the interaction responsible for selectivity in MLC using Brij-35. Here, we have investigated nonionic surfactants as mobile phase for separation of positional isomers using Chromolith C-18 column. The organic modifier (propanol) and surfactant (Brij-35) concentration on separation of some

Table 1: Retention time and resolution data for various positional isomers using hydro-organic and hybrid surfactant mobile phase composition.

Analyte	Solvent composition	$^*t_R$ (R)	Solvent composition	$^*t_R$ (R)
2-nitroaniline	1 : 1 MeOH/water	2.87	88 : 12	4.57
3-nitroaniline		2.28 (1.99)	Brij35 (2% aq. soln.)/propanol	3.7 (2.56)
4-nitroaniline		1.99 (1.14)		3.43 (0.92)
2-quinolinol	1 : 1MeOH/water	2.2	99 : 1	2.53
4-quinolinol		1.67 (1.53)	Brij-35 (1.5% aq. soln.)/propanol	1.78 (1.629)
o-cresol	7 : 3 MeOH/water	2.03	90 : 10	1.69
p-cresol		1.96	Brij35 (1.75% aq. soln.)/propanol	1.65
m-cresol		1.96		1.65
m-nitrophenol	7 : 3 MeOH/water	2.27	88 : 12	1.83
p-nitrophenol		2.07 (0.453)	Brij-35 (2% aq. soln.)/propanol	1.62 (1.35)
Dexamethazone	7 : 3 MeOH/water	2.49	99 : 1	4.24
Betamethasone		2.52	Brij-35 (1.5% aq. soln.)/propanol	4.04
Propylparaben	7 : 3 MeOH/water	2.3	96 : 4	7.33
iso-propylparaben		2.21 (0.32)	Brij-35 (1.75% aq. soln.)/propanol	6.47 (0.809)
Bezoic acid	4 : 6 MeOH/water	1.78	90 : 10	4.92
Sorbic acid		2.24 (0.64)	Brij-35 (1.5% aq. soln.)/propanol	3.88 (1.73)

$^*t_R$ is retention time in minutes; in parenthesis "R" is resolution.

positional isomers is studied. The findings are discussed and selectivity is accessed by using benzoic acid/sorbic acid selectivity ratio and reported in terms of LSER parameters, which then are correlated to understand the system selectivity.

2. Experimental

2.1. Instrumentation. A Hitachi 6010 liquid chromatograph fitted with a Hitachi L-4200 variable wavelength UV-Vis detector, a Rheodyne 7125 injector, and a Chromolith performance RP-18 e, 100 mm × 4.6 mm i.d. column (E. Merck, Darmstadt, Germany) was used. CSW32 software (Data Apex) was used for data acquisition and integration. The λ 220 nm (quinolinols), 254 nm (Methazones, parabens, cresols, and nitrophenols), and 320 nm (nitroanilines) were set, and flow rate was 1.0 mL/min for all experiments except nitrophenols and quinolinols, which is 2.0 mL/min.

2.2. Materials and Reagents. Brij-35 was purchased from Sumito Corporation, Tokyo, Japan, prepared in Millipore water and used without degassing and filtration; propanol (Fluka, Sigma-Aldrich, Buchs, St.Gallen, Switzerland) was mixed within system depending on percentage required. Benzoic acid, quinolinols, nitroanilines, parabens (Fluka, Sigma-Aldrich, Buchs, St.Gallen, Switzerland), sorbic acid (Merck, Frankfurt, Germany), beta-and dexamethasone (Glaxo SmithKline, Dungarvan, Ireland) and cresol (Riedel-Hansen, Sigma-Aldrich, Seelze, Lower Saxony, Germany) were used as received their stock solutions were prepared in propanol, and working solutions were diluted with running mobile phase. HPLC grade methanol was purchased from Fisher Scientific, Loughborough, UK.

3. Results and Discussion

3.1. Separation of Isomers. Table 1 shows the retention time and resolution data for various isomers including benzoic acid and sorbic acid for hydro-organic and hybrid non-ionic micellar mobile phase. It could be clearly observed that same mobile phase is not optimum for all separations and for most solutes studied here; separation is also possible with hydro-organic mobile phases but micellar mobile phase offers better resolution.

These separations were studied with mobile phase composed of aqueous solution of surfactant with concentration (1-2%) and modifier in the ratio 1–20% to the surfactant at pH 3 (adjusted with phosphoric acid). All the solutes were studied for 1.25, 1.5, 1.75, and 2% Brij-35 aqueous solution prepared in millipore water with various percentages of propanol. It was found that the increase in surfactant concentration can reduce the retention time but does not affect separation appreciably. So, for all pair of analytes, surfactant that provides better resolution was selected. Addition of organic modifier (1-propanol) imparts interesting changes in retention times and resolution. Figure 1 shows the effect of propanol concentration on retention times of isomers of nitroanilines, quinolinols, and parabens.

Propanol decreases the retention times in all cases, and more difference in retention time is seen at small

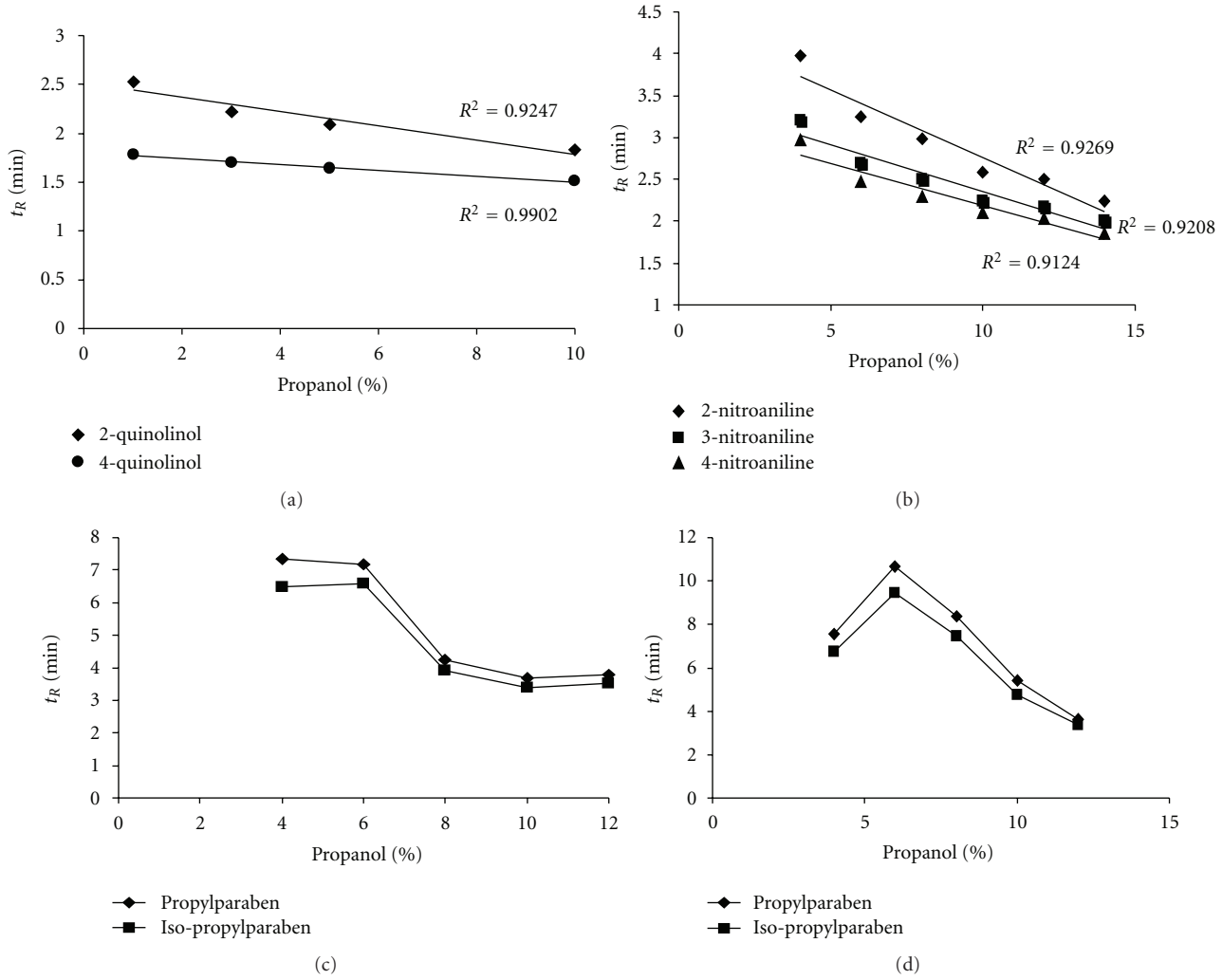

Figure 1: Effect of propanol concentration on retention of isomers of (a) quinolinol (Brij-35 1.5% aq. Soln.), (b) nitroanilines (Brij-35 1.75% aq. Soln.), (c) parabenes (Brij-35 1.5% aq. Soln.), and (d) parabens (Brij-35 1.75% aq. Soln.).

organic modifier content but resolution is better at higher concentrations. The decrease in retention is linear for quinolinols (Figure 1(a)) and nitroanilines (Figure 1(b)) though for nitroanilines not as good as for quinolinols. For parabens, a visible shift in retention time trend at 6% propanol (Figures 1(c) and 1(d)) is seen which is also observed with nitroanilines (visible when Figure 1(b) is expanded) and other compounds (data not shown). These findings are different in the previously reported data [7] that up to 20% propanol concentration micelles exist which may still be valid that this trend may be due to stripping of adsorbed surfactant from stationary phase at increasing propanol concentration. This study indicates that retention behaviour in MLC changes at 6% propanol with non-ionic Brij-35 surfactant when hybrid micellar mobile phase in HPLC with C-18 columns is used.

Better separation of isomers is observed at higher propanol concentration in most cases studied here as relatively sharp peaks appeared as compared to those at low propanol content (Figure 2). This may be due to decreased

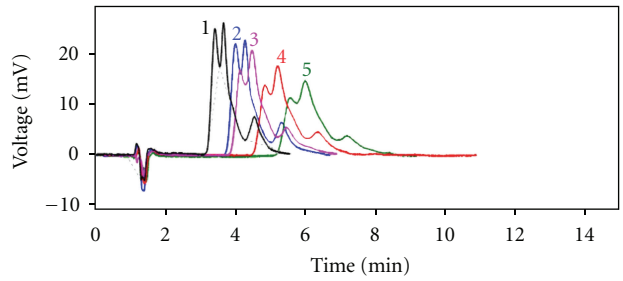

Figure 2: Effect of propanol concentration on sharpness of peaks of isomers of nitroanilines. Ratio of Brij-35 (2% aq. Soln.) to propanol (1) 88 : 12, (2) 90 : 10, (3) 92 : 08, (4) 94 : 06, (5) 96 : 04.

amount of adsorbed surfactant on C-18 stationary phase, which helps better mass transfer between stationary and mobile phase hence reduce diffusion of analyte.

It is also interesting to observe that o- and m-nitroanilines are better resolved with methanol/water while

m- and p-nitroanilines with hybrid surfactant system. Cresols could not be separated with either system; for rest of the studied isomers, better separations are obvious using hybrid surfactant mobile phases.

Selectivity of system was assessed by calculating α value of benzoic acid/sorbic acid $\alpha_{BA/S}$. Highest $\alpha_{BA/S}$ value of 1.40 was observed with Brij35 (1.5% aqueous solution)/propanol in the ratio of 90 : 10, while with 40 : 60 methanol/water the factor was 0.74. Also the elution order was reversed; with surfactant containing mobile phase sorbic acid elutes first, while with hydro-organic benzoic acid appears first.

3.2. Interactions Responsible for Separation of Isomers. In traditional hydro-organic chromatography, selectivity for separation of isomers is correlated with molecular order within stationary phase. Selectivity is enhanced with increasing hydrocarbon stationary phase, increased chain length, and decreased temperature [17]. Unique selectivity is observed with micellar liquid chromatography as compared to hydro-organic chromatography, that is, retention factor has linear relationship with homologous series in MLC in contrast to LC which is logarithmic. The phenomenon is explained on the basis of absence of free energy change in MLC for the formation of cavity in mobile phase for incorporation of solute with increasing hydrophobicity [18]. The linear increase in "k" is also ascribed for larger number of compounds eluted per unit time [14, 18].

In MLC, not only mobile phase contributes to the separation but surfactant modified stationary phase also plays a role. It is obvious in early reports on MLC that surfactants adsorb on stationary phases consequently modify their properties. Ionic surfactants adsorb on ODS surfaces and reaches plateau when surfactant-modified mobile phases are run. For nonionic surfactants, no plateau is observed and adsorption of surfactant is continuous with micellar mobile even above the cmc [19]. Quiñons-Torrelo et al. [20] have reported that nonionic surfactant impart interesting changes in polarity of stationary phase and termed the type of chromatography as Micellar Biopartitioning Chromatography (MBC). The same author further reported that this cannot afford separation of enantiomers, while in our work we can partially separate enantiomers of steroids; this shows that statement is valid for MBC only.

Linear solvation energy relation (LSER) is used to understand the types and relative strengths of chemical interactions that control retention and selectivity in various separation techniques [21]. LSER equation as proposed by Abraham is $\log k' = c + eE + sS + aA + bB + vV$, where k is retention factor, c is constant, E, S, A, B, and V are solute descriptors independent of mobile and stationary phase used. E is solute excess molar refraction, S is dipolarity/polarizability, A and B are acidity and basicity and V is MsGowan's characteristic molecular volume. The lower case letters c, e, s, a, b, and v are the system constant reflecting the difference in solute interactions between mobile and stationary phase [22].

LSER has also been applied to MLC [13], where additional solute descriptors are added in the equation to explain the behaviour of ionic surfactants. In a recent review by Ruiz-Ángel [14], it is demonstrated that hydrophobicity coefficient "v" varies in narrow range with change in micellar mobile phase composition hence hydrophobic interactions are scarcely affected. LSER studies of MBC has shown to have pronounce effect by hydrophobicity as given by high coefficients for "v" [20]. This type of effect can also be observed in a report by Mutelet et al. [15], where v values for Brij-35 (0.08 M), SDS (0.1 M), and CTAB (0.01 M), with 15% isopropanol as additive are 1.69 ± 0.13, 0.75 ± 0.08, and 0.56 ± 0.08, respectively.

Nonetheless, characterization of separation of isomers is explained on the basis of hydrophobicity value v since most of the positional isomers have identical v value for example, 2-, 3-, 4-nitroaniline. Berthod et al. [22] have discussed the utility of Abraham's LSER model to characterize chiral recognition behaviour of teicoplanin aglycon chiral phases. Appreciably different patterns were found for chiral phases as compared to others, while it is worth noting in the report that e and s parameters besides b and v also play a role in fine tuning of separation mechanism.

The system constants using LSER approach are system specific, that is, applicable to given stationary and mobile phase. As for non-ionic liquid chromatography, system constants are already reported so to understand the isomer recognition mechanism of MLC Linear Solvation Energy Relationship parameters for various systems reported in the literature gathered (Table 2).

Systems listed in Table 2 are included here on the basis of columns and mobile phases used; ODS column is most commonly used while polar-endcapped and polar embedded columns were included bearing in the mind that non-ionic surfactants that modify the surface of ODS column may have any similarity. TAG columns are chiral selectors with multiple interactions which also could provide clue, and monolith column is the part of current study and is most relevant. The LSER parameter; "e" is maximum for TAG phases with MeOH as mobile phase, 0 with monolithic columns with 10% MeOH and negative for Brij-35, "s" is negative for all systems except TAG phases with MeOH, while "a" and "b" are negative and "v" is positive throughout. Table 2 shows that excess molar refraction "e" is a discriminating factor for non-ionic micellar liquid chromatography, which is negatively related to log k value. Such types of interactions are also reported by Quina et al. [16] while characterizing incorporation of non-ionic solutes in aqueous micelles using LSER analysis [23–25] and Altomare et al. [26] for estimating partitioning parameters of non-ionic surfactants. The "e" value for Brij-35 surfactant is 1.63 as compared to 0.76 for CTAB and 0.32 for SDS. The author has attributed this to polyoxyethylene head groups in Brij-35 that contribute to the polarizability of the micellar solubilization sites. Also the hydrogen bond basicity of micellar solubilization site is enhanced by the presence of ether oxygen of the polyoxyethylene headgroup. Jason et al. [23] have highlighted similar finding for other non-ionic surfactants, where molar excess refraction and basicity are major contributors to partitioning as compared to previous work on other systems. To check if similar factors contribute here, available molecular descriptors for some of the isomers are compiled in Table 3. It could be observed from Table 3 that dipolarizability is a major

Table 2: LSER system constants for various stationary and mobile phases reported in the literature.

Column	Mobile phase	System parameters					Ref.
		e	*s*	*a*	*b*	*v*	
ODS	50% ACN	0.181	−0.527	−0.417	−1.646	1.492	[13]
Polar Endcapped	50% ACN	0.173	−0.444	−0.396	−1.261	1.323	
Polar embedded	50% ACN	0.146	−0.339	−0.079	−1.581	1.320	
TAG	20% ACN	0.345	−0.001	−0.315	−1.202	1.349	[14]
TAG	25% MeOH	0.478	0.267	−0.493	−1.211	1.496	
Monolith C-18	50% ACN	0.07	−0.33	−0.52	−1.55	1.49	[15]
Monolith C-18	50% MeOH	0.30	−0.67	−0.41	−1.77	2.10	
Monolith C-18	10% MeOH	0	−0.51	−0.26	−2.18	3.83	
Monolith C-18	0.04 M Brij-35*	−0.165	−0.087	−0.191	−1.284	1.080	[16]

* Mobile phase is 0.04 M Brij-35 with 0.01 M sodium dihydrogen phosphate at pH 7.4 adjusted with NaOH at 40°C.

Table 3: Abraham's LSER molecular descriptors for some of the isomers included in current study.

Analyte	*A* (acidity)	*B* (basicity)	*S* (dipolarity/dipolarizability)	*E* (excess molar refraction)
4-nitroaniline	0.46	0.35	1.93	1.22
3-nitroaniline	0.4	0.36	1.71	1.2
2-nitroaniline	0.3	0.36	1.37	1.18
o-cresol	0.52	0.30	0.86	0.84
m-cresol	0.57	0.34	0.88	0.82
p-cresol	0.57	0.31	0.87	0.82
p-nitrophenol	0.82	0.26	1.72	1.07
m-nitrophenol	0.79	0.23	1.57	1.05

contributor while molar excess refraction also varies for three isomers. For cresols, no appreciable separation was observed. Only, o-cresol was little separated and this separation may be attributed to the combined effect of "*S*" and "*E*". For p- and m-dinitrophenols, "*B*" and "*S*" are major contributors.

4. Conclusions

Non-ionic micellar liquid chromatography offers different mode of interaction than hydro-organic or ionic micellar liquid chromatography. Better separation of positional isomers is possible with Brij-35/propanol hybrid mobile phase. Besides basicity, dipolarizability and excess molar refraction are responsible for fine-tuning of separation. This new face of non-ionic MLC opens field for many applications in separation of positional isomers.

Acknowledgment

Corresponding author is thankful to Professor Dr. Willie L. Hinze of Department of Chemistry, Wake Forest University, Winston, Salem, NC, USA, for his suggestions and discussion in using LSER for characterization of molecular interactions.

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