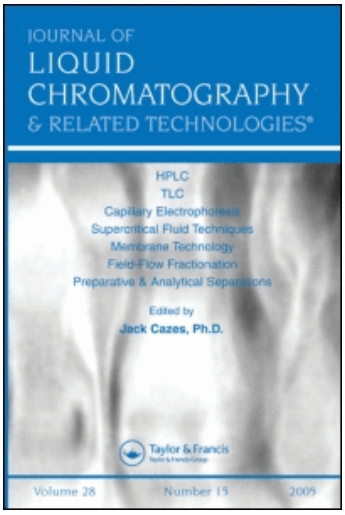

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Laugel, C. , Baillet, A. and Ferrier, D.(1998) 'Improved HPLC Determination of the *Centella asiatica* Terpenes: Analysis in a Multiple Emulsion, Influence of the Surfactants on the Retention', Journal of Liquid Chromatography & Related Technologies, 21: 9, 1333 — 1345
To link to this Article: DOI: 10.1080/10826079808005881
URL: http://dx.doi.org/10.1080/10826079808005881

J. LIQ. CHROM. & REL. TECHNOL., 21(9), 1333–1345 (1998)

IMPROVED HPLC DETERMINATION OF THE *CENTELLA ASIATICA* TERPENES: ANALYSIS IN A MULTIPLE EMULSION, INFLUENCE OF THE SURFACTANTS ON THE RETENTION

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ABSTRACT

High performance liquid chromatographic methods were established for the determination of the three terpenic compounds of *Centella Asiatica.* The samples were analyzed with a Spherisorb ODS-2 reversed phase column and detected at UV 206 nm.

Regression equations were derived showing linear relationships (individual correlation coefficients ranged between 0.998 - 1.000).

This quick and simple method has been used for evaluating the interaction of *Centella Asiatica* terpenes with surfactants. By comparing the elution volume of terpenes injected onto the surfactant-free column (V_e) with that of terpenes injected onto the surfactant-doped column (V), an association constant (K_a) for the terpene-surfactant complex was determined.

INTRODUCTION

Centella Asiatica is a herbaceous plant of the Umbelliferae family. Its active principle is a triterpenic derivative, from which is obtained a glycolic extract, which is widely used topically in dermatology[1] to promote the epithelialization of wounds and ulcers and as an anticellulitic and vasotonic compound.[2]

The commercial extract of *Centella Asiatica* is composed of madecassic acid, asiatic acid and asiaticoside. Madecassic acid differs from asiatic acid by one hydroxyl function. Asiaticoside results from an esterification of the carboxylic function by three glucosyl residues (Figure 1).

Several methods have been reported for its determination by titrimetric analysis,[3] gas chromatography/mass spectrometry,[4,5] high-speed counter-current chromatography,[6] high-performance liquid chromatography.[7] The titrimetric analysis is non-selective, non-specific, and lacks precision and accuracy. The gas chromatography/mass spectrometry and the high-speed counter-current chromatography are performant, but not easily usable in the routine analyses. The RP-HPLC proposed by Inamdar et coll.[7] used a gradient elution mode on, an octadecyl silica of 10 μm particle size. This method is quite poor in resolution and accuracy.

In the first part of this paper, we describe a reversed-phase liquid chromatographic method which presents a better resolution per unit of time than the reported analytical method. Analysis time, precision, and accuracy are improved. Moreover, the attempted specificity was investigated by applying the technic to other terpenes with a similar chemical structure.

Firstly, the method was developed to assay the three constituents of *Centella Asiatica*, mainly for the determination of small amounts of the extract within multiple emulsions.

Secondly, it was applied to study the physico-chemical interaction between *Centella Asiatica* compounds and surfactants. The understanding of such interactions is crucial to the development of surfactants using a new topical formulations. This is of particular interest for multiple emulsions for which the chemical structure of surfactants modifies the yield of encapsulation and the release of active molecules. Partition coefficient and binding constants are determined, and correlations are established between capacity factors and partition coefficient for the terpenes studied.

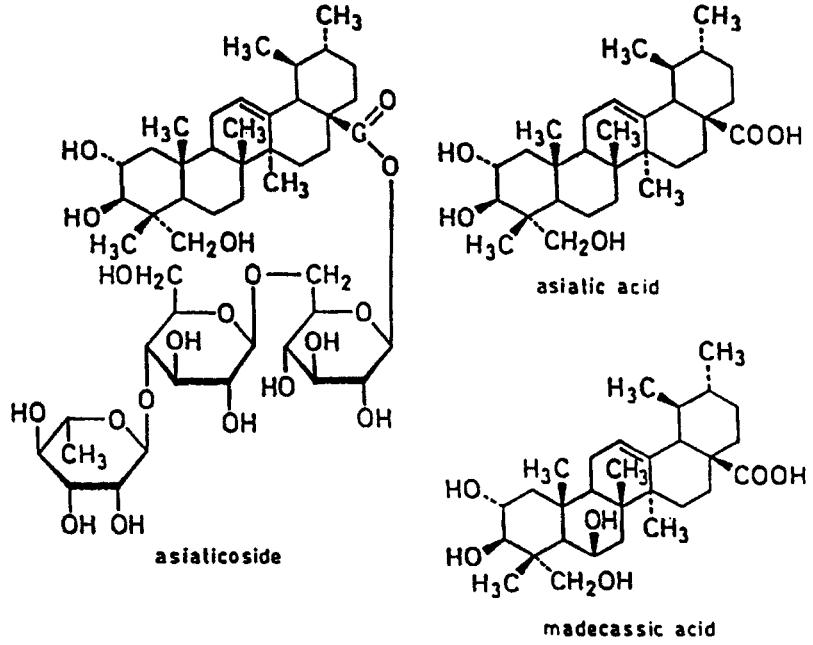


Figure 1. Chemical structures of the terpenes.

EXPERIMENTAL

HPLC Analysis

Instrumentation

Chromatographic measurements were made with a Jasco PU 980 pump (Prolabo, Paris, France) equipped with a Rheodyne model 7125 injection valve with a 20 μL loop. UV detection at 206 nm (maximum absorbance of three terpenes in the mobile phase) was effected with a Shimadzu SPD-2A UV spectrophotometer (Touzart et Matignon, Vitry-sur-Seine, France). The flow rate was set at 1 mL/min. The chromatograms were recorded using a Hewlett-Packard Model 3395 integrator. An Spherisorb ODS-2 reversed phase C_{18} column (5 mm, 250 x 4 mm I.D.) was used and a Spherisorb ODS-2 reversed phase C_{18} guard column (5 μm, 10 x 4 mm I.D.). The flow rate was 1 mL/min. The mobile phase was filtered through a 0.22 μm Millipore filter under vacuum.

Chemicals

Methanol, acetonitrile, tertahydrofuran, acetic acid obtained from Prolabo, were of HPLC grade. Paraffin oil was obtained from Cooperation pharmaceutique française (Melun, France), Ultra-high quality water was obtained from a Milli-Q plus 185 system (Millipore, St-Quentin-en-Yvelines, France). The extract of *Centella Asiatica* was obtained from Indena, Milan, Italy.

Sample treatment for chromatographic analysis

Samples of multiple emulsion were diluted with paraffin oil (1:1). The suspension was decanted for one hour. The supernatant was pipetted and then clarified by dilution with tetrahydrofuran (1:2).

RESULTS AND DISCUSSION

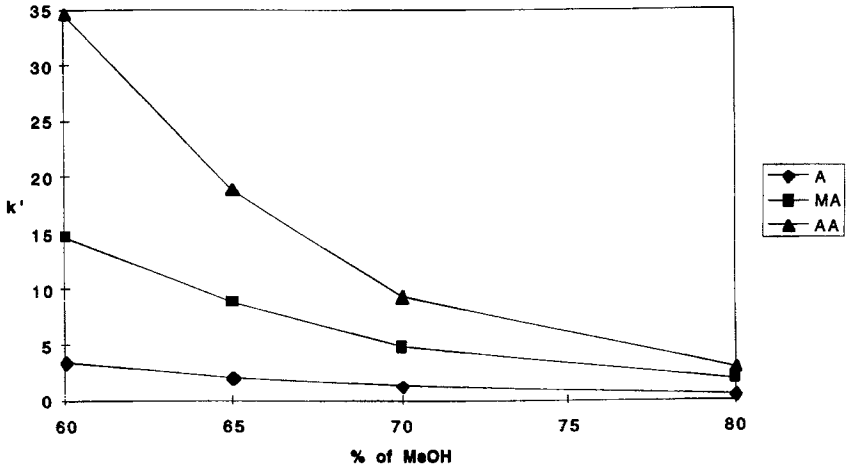
Chromatographic Parameter Optimization

Asiatic acid, madecassic acid, and asiaticoside are terpenes with an ursane skeleton (Figure 1). Madecassic acid differs from asiatic acid by one hydroxyl function. Asiaticoside results from an esterification of the carboxylic function by three glucosyl residues. The chromatographic analysis of these terpenes were complicated by the main properties of these compounds, i.e. a lipophilic character (for both acids), a high molecular weight, a poor solubility in aqueous medium, and a weak detectability in the UV (206 nm).

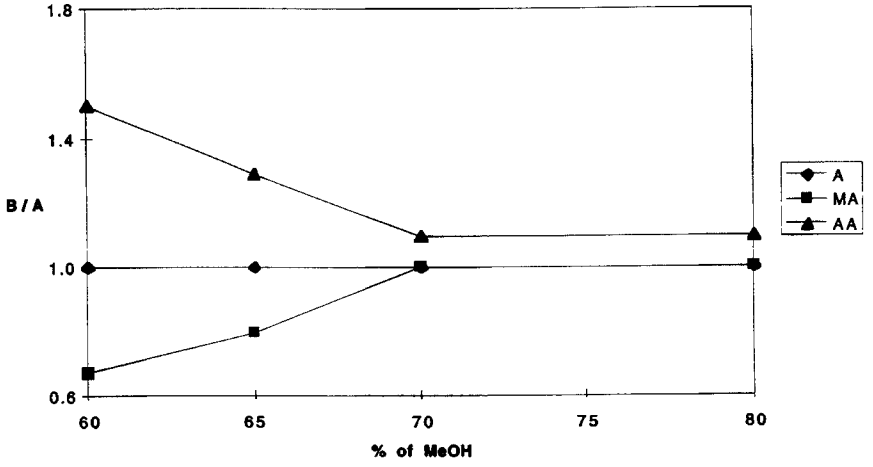
The optimization of the chromatographic conditions was achieved by considering two parameters : the capacity factor k' and the asymmetry factor B/A. This latter was calculated at 10% of the peak height using the ratio of the widths of the rear and front sides of the peak. To accomplish the optimal separation of the *Centella Asiatica*, different experimental variables like the composition and the pH of the mobile phase were attempted.

Composition of the mobile phase

According to their solubility evaluated in the most currently used solvents in RP-HPLC, we have chosen mixture of methanol and water as mobile phase. The objective of these experiments was to obtain both a capacity factor near 1 for the first compound and an acceptable capacity factor for the last (corresponding to an analysis time not more than 45 min).



(a)



(b)

Figure 2. Plot of k' (a) and B/A (b) vs percentage of methanol in the mobile phase. Chromatographic conditions : Spherisorb ODS 2 (A : asiaticoside, MA: madecassic acid, AA : asiatic acid).

Table 1

Influence of the Buffer Nature on the k' and B/A

Buffer	k'			B/A		
	A	MA	AA	A	MA	AA
Without buffer	2.10	8.33	17.30	1.0	0.8	1.3
Formate	2.04	10.10	22.10	1.0	1.0	1.2
Phospate	1.92	9.59	20.09	1.0	0.9	1.2
Acetate	2.05	9.90	20.40	1.0	1.0	1.0

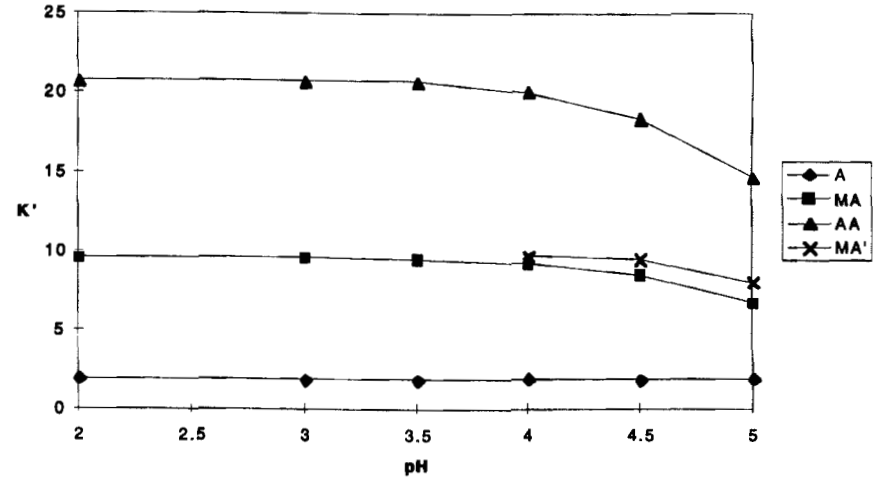
Four percentages of methanol were investigated (Figure 2). The capacity factor was decreased with increasing the methanol percentage. This elution order was asiaticoside, madecassic acid, asiatic acid, according to their polarity. The smaller k' value (for the asiaticoside) should be set at k' = 1. For percentage of methanol higher than 65%, the k' values were too small for a valid analysis. Moreover, the B/A value of the both acids was not 1. These preliminary experiments showed that the retention time of the first compound was to small and the one of the last was to high.

Two solutions were possible : the used of a gradient elution or the addition of acetonitrile. At last, 5% of acetonitrile was added to the mobile phase (methanol/water 60/40), for improving these two parameters (Table 1).

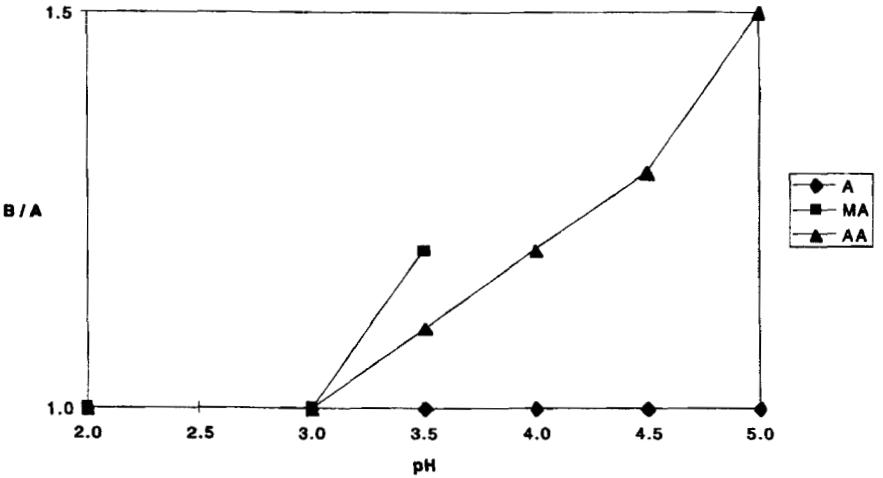
Influence of pH

To determine the influence of the pH, we compared B/A and k' in presence of six levels of pH with acetate buffer (Fig. 3). The pH level both influences the degree of ionization of the two acids and the hydrogen interactions between the hydroxyl groups of glusosyl residues and silanols of the stationary phase. After pH = 4, the chromatographic peak of madecassic acid was split into two. Therefore, the optimum separation measured throught the resolution factor of each pair of peaks, was obtained by using the acetate buffer at pH = 3 and a mobile phase MeOH/ACN/H_2O (60/5/35).

As expected, the presence of an acidic buffer decreased the peak asymmetry. Particularly for the madecassic acid and asiatic acid, the introduction of the buffer in the mobile phase allowed to obtain the protonated form for both compounds.



(a)



(b)

Figure 3. Plot of k' (a) and B/A (b) vs pH of the mobile phase. Chromatographic conditions : Spherisorb ODS 2 Methanol/Acetonitrile/Eau (60/5/35) (A : asiaticoside, MA : madecassic acid, AA : asiatic acid).

Table 2

Validation Parameters of the Chromatographic Method

Compounds	Retention Time	Linearity (r^2)	Limit of Detection
Asiaticoside	1.99 min	1.000	$1.6.10^{-6}$ g/L
Madecassic Acid	9.82 min	0.998	$9.5.10^{-5}$ g/L
Asiatic Acid	20.33 min	0.999	$7.8.10^{-5}$ g/L

Nature of the buffer

Formate, phosphate, acetate buffer were tested at a 0.05 M (pH = 3). Their influence on k' and B/A values was summarized in Table 1. With acetic acid, the k' and B/A were best (B/A = 1 for the three compounds, and k' were not so high).

Validation of the analytical method

This technique was then validated in the further purpose of a quantitative determination of *Centella Asiatica* in cosmetic preparations in which the actual percentage is near 1%. The calibration curve was determined by injecting the solutions of the concentrations ranging from 0.5 to 12 mg/L for each. The corresponding calibration graphs exhibited excellent linearity and regression analysis showed correlation coefficient greater than 0.998 with intercept values that did not deviate significantly from origin for all the three constituents. The plot of residuals (e_i) vs. concentration shows an uniform variance and a within group error proportional to the concentration. This errors is about 2%.

The injection of the emulsion without terpenes shows that the small peaks generated by those compounds does not interfere with the three terpene peaks. The limit of detection has been defined as the lowest concentration of the one compound that our analytical method can detect with a signal to noise ratio 3:1 (Table 2). The repeatability and the reproducibility were verified and R.S.D. was about 2%.

This method was available for the assay of the *Centella Asiatica* incorporated within a multiple emulsion (Fig. 4). This method was more resolutive and accurate than that described by Inamda (linearity for concentrations ranging from 0.5-7.5 mg/mL).

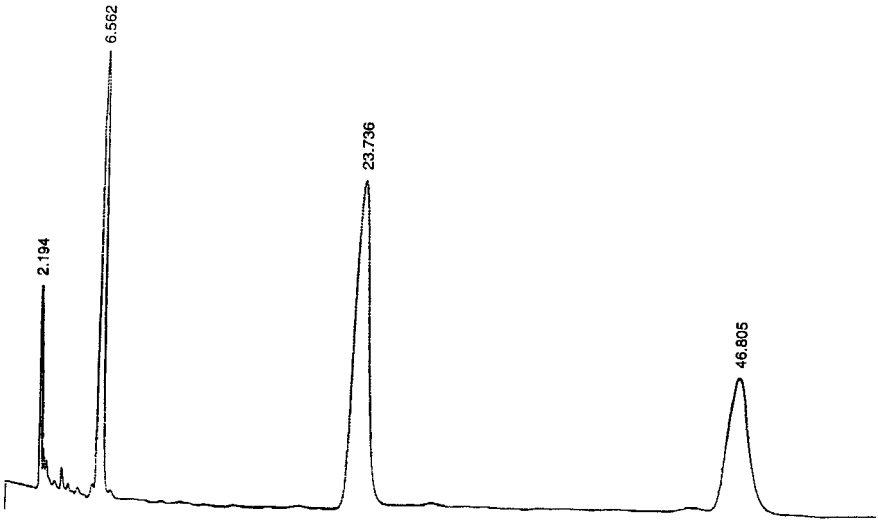


Figure 4. Chromatogram of the terpenes. Chromatographic conditions : Spherisorb ODS 2 Methanol/Acetonitrile/Eau (60/5/35) Acetate buffer 0.05M (pH=3).

Table 3

Capacity Factors of Different Terpenes Obtained by the Chromatographic Analysis

Compounds	Capacity Factor
Asiaticoside	1.99
Glycyrrhetinique Acid	4.92
Hederagenine	7.66
Madecassic Acid	9.82
Oleanolique Acid	14.66
Asiatic Acid	20.33

The chromatographic analysis of other terpenes

In this part, this developed analytical method was applied to other terpenes with a similar structure. The k' were presented in Table 3 and the chemical structure in Figure 5. This method was appropriated to separate compounds with a similar chemical structure (Figure 6).

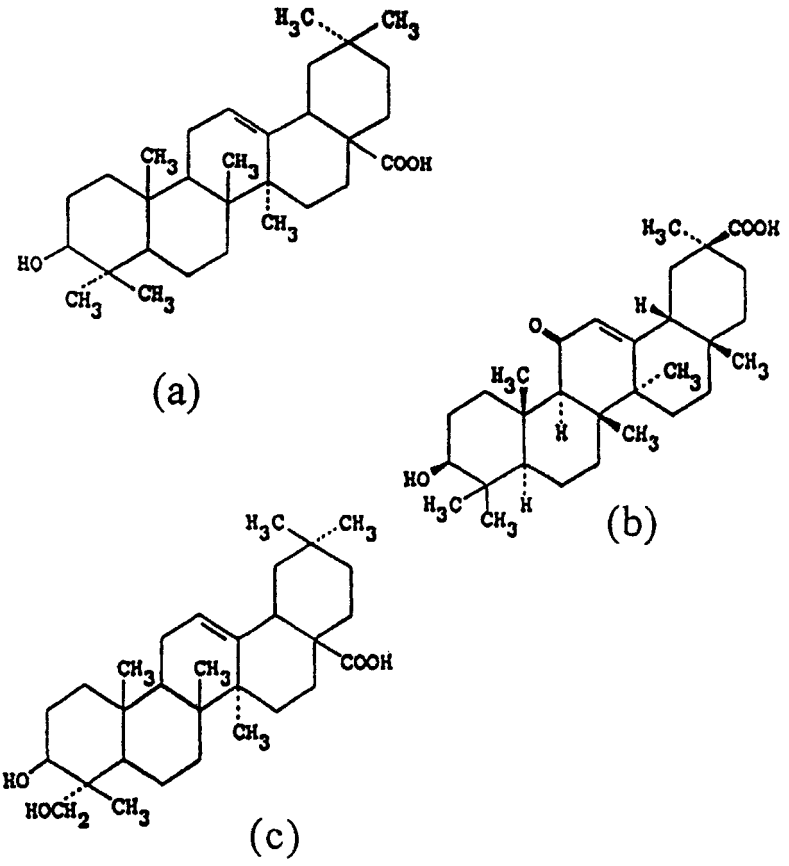


Figure 5. Chemical structure of oleanolique acid (a), glycyrrhetinique acid (b) and hederagenine (c).

Association Constants of the Three Terpenes with Surfactants

Terpenes-surfactant association constants are of great interest for the understanding of terpenes release from O/W/O multiple emulsions. A way to assess the values of these constants is to study the chromatographic terpene behavior in micellar chromatography using a mobile phase containing the studied surfactants. The presence of the surfactants modified the retention behavior of the terpenes. In this method, the surfactants were employed at critical micelle concentration (CMC).[8] Solute molecules (as terpenes) can interact with both the stationary phase and the micellar phase.

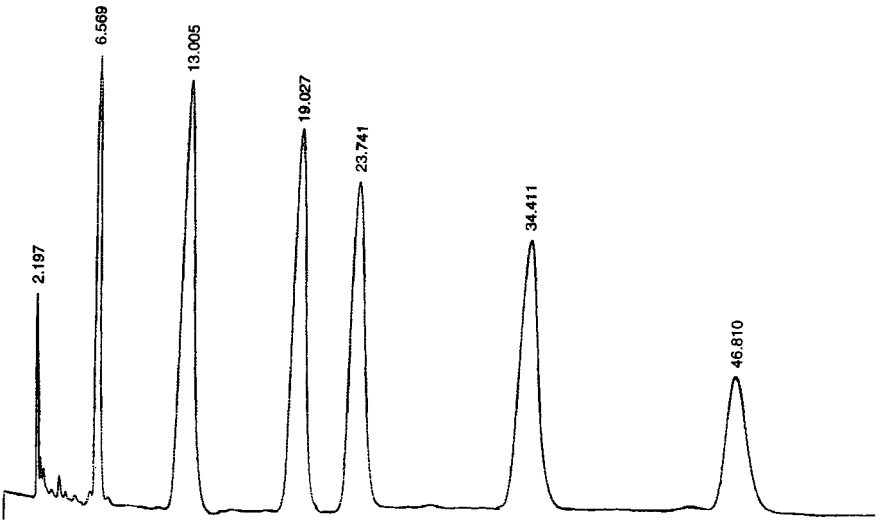


Figure 6. Chromatogram of six terpenes. Chromatographic conditions: Spherisorb ODS 2 Methanol/Acetonitrile/Eau (60/5/35) Acetate buffer 0.05M (pH=3).

The partition coefficient involved that of a solute between the mobile phase and the stationary phase, between the mobile phase and the surfactants, and directly between the surfactants and the stationary phase. Several equations have been developed relating the retention parameters with the surfactant concentration, with the partition coefficients of solutes between the phases, and with the solute-surfactant bond constant. These such interactions are very useful for micellar reversed-phase liquid chromatography.

Our calculations were based on the reference,[9] where :

$$K_a = \frac{1}{[S]_t} \times \frac{(V - V_e)}{(V_e - V_0)} \qquad (1)$$

where : - K_a : is the association constant of terpene/surfactant complex
- $[S]_t$: is the total concentration of the surfactants (M)

$-\frac{(V - V_e)}{(V_e - V_0)}$: is the affinity factor introduced to correct the small variations in V_e from day to day.

Table 4

Association Constant of Terpenes/Surfactans Complexes*

Compounds	Arlacel 989	Tween 60	Synperonic PF/F127
Asiaticoside	0.508	0.518	0.524
Madecassic Acid	0.106	0.108	0.112
Asiatic Acid	0.050	0.070	0.100

* n = 3.

-V_e : the elution volume of terpene without surfactant (mL)
-V : the elution volume of terpene with surfactant (mL)
-V_0 : the void volume (mL).

This developed method is quick and simple to perform and gives association constants for the three terpenes and surfactants. The data presented in Table 4 can be used to calculate (K_a), between terpenes and surfactants.

The association constants of terpene/surfactant complex were determined and are listed in Table 4. The retention times of terpenes injected onto a C_{18} column free of surfactant and onto the same column doped with surfactant increases with the quantity of surfactant dopant increases.

Whatever, the surfactant association constant was much greater for asiaticoside than those for madecassic acid and asiatic acid. The acids are more lipophilic than the osidic derivative. The order of elution of the selected compound was not changed with the surfactant and with change in the micellized surfactant concentration in the mobile phase. On modifying the surfactant concentration, the selectivity was modified, increasing or decreasing, depending on the type of solute and of surfactant. Relatively higher retention are obtained with Arlacel than the other.

The influence of Arlacel 989 was more important than Synperonic PE/F127 and Tween 60. Also, the polarity of surfactant influenced the retention of the terpenic compound. More the surfactant was lipophilic, more the retention time was increasing.

In conclusion, this method was available for the association constant determination of the terpenic compounds. The association constant allowed to predict the behavior of the terpenes in the multiple emulsion itself.

CONCLUSION

This work described a reversed-phase liquid chromatographic method which is superior to the reported analytical method for the analysis of terpenic compounds. This method is precise, accurate, and rapid and usable for other terpenes.

In this article, we have improved a simple methodology to study solute-surfactant associations in solution. The proposed model is accurate for solute-surfactant associations.

REFERENCES

1. F. Bonte, M. Dummies, C. Chaudagne, A. Meybeck, Planta Med., **60**, 133-135 (1994).

2. G. P. Montecchio, A. Samaden, S. Carbone, M. Vigotti, S. Siragusa, F. Piovella, Haematologica, **76**, 256-259 (1991).

3. C. Castellani, A. Marai, P. Vacchi, Boll. Chim. Farm., **120**, 570-575 (1981).

4. W. R. Rush, G. R. Murray, D. J. M. Graham, Eur. J. Drug Metab. Pharmacokinet., **18**, 323-326 (1993).

5. M. Holeman, E. Theron, R. Pinel, Parfums Cosmetiques Arômes, **120**, 52-55 (1994).

6. B. Diallo, R. Vanhaelen-Fastre, M. Vanhaelen, J. of Chromatogr., **558**, 446-450 (1991).

7. P. K. Inamdar, R. D. Yeole, A. B. Ghogare, N. J. de Souza, J. of Chromatogr., **742**, 127-130 (1996).

8. V. Gonzalez, M. A. Rodriguez-Delgado, M. J. Sanchez, F. Garcia-Montelongo, Chromatographia, **34**, 627-635 (1992).

9. B. J. Backes, S. D. Rychnovsky, Anal. Biochem., **205**, 96-99 (1992).

Received July 30, 1997
Accepted August 9, 1997
Manuscript 4557