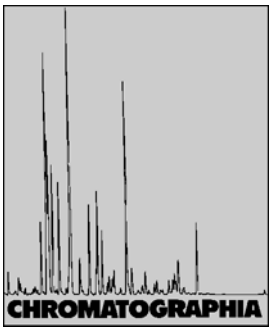
Assay of Xanthene Dyes in Lipsticks by Inverse Supercritical Fluid Extraction and HPLC



2001, *53*, 490–494

S. Scalia* / S. Simeoni

Department of Pharmaceutical Sciences, University of Ferrara, via Fossato di Mortara 17, 44100 Ferrara, Italy; E-Mail: sls@unife.it

Key Words

Column liquid chromatography
Supercritical fluid extraction
Xanthene dyes
Lipstick

Summary

An inverse supercritical fluid extraction (SFE) procedure has been developed for the efficient isolation of six of the most common xanthene dyes (fluorescein; 4′,5′-dibromofluorescein; 2′,4′,5′,7′-tetrabromofluorescein; 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein; 4′,5′-diiodofluorescein and 2′,4′,5′,7′-tetraiodofluorescein) from lipstick matrices. A rapid high-performance liquid chromatographic method is also presented for the separation of the six dyes on a cyanopropyl-bonded silica column eluted with acetonitrile-methanol-acetate buffer. Extraction of the lipstick with supercritical CO_2 at 350 atm and 40 °C for 10 min exclusively removed the matrix components, whereas the xanthene colorants remained in the extraction vessel. After treatment with CO_2, the target analytes were quantitatively recovered (> 96.8%) by dispersion of the sample in ethanol under sonication. The inverse SFE method was compared with conventional liquid-liquid extraction on commercial lipstick products. The two methods produced comparable recoveries. However, inverse SFE was superior in terms of precision and speed of analysis and gave cleaner extracts.

Introduction

In recent years increasing attention has been focused on the widespread use of synthetic dyes, especially in food and cosmetics, because of their potential toxicity [1, 2]. The European Economic Community (EEC) Directive on cosmetics [3] includes a list of the authorized coloring agents with the permitted application areas. For some of the colorants, purity requirements and maximum allowed concentrations are also specified [3]. Xanthene dyes are extensively employed as colorants in lipstick preparations [4] and hence they are likely to come in contact with the mouth. Consequently, the quality control assay of these coloring additives in the finished products is particularly important. Different chromatographic methods have been described for the analysis of the xanthene dyes such as thin-layer chromatography [5], capillary electrophoresis [6] and high-performance liquid chromatography (HPLC) [4, 7–12]. The latter technique employing the ion-pair separation mode represents the method of choice [7–11]. Because of the complex lipstick matrix [4, 9, 11], the HPLC determination of the xanthene dyes requires cumbersome sample pretreatments including heating, sequential liquid-liquid extractions, solvent evaporation and solid-phase extraction [4, 8, 9, 11]. These procedures are time-consuming, labour-intensive and use large volumes of toxic solvents.

Supercritical fluid extraction (SFE) exhibits several desirable properties for the isolation of target analytes from complex matrices [13]. Since supercritical fluids combine liquid-like solvating capabilities with almost gas-like transport properties, enhanced mass-transfer and faster extractions are achieved [13–15].Another attractive property is that the dissolving power of a supercritical fluid can be tuned by simply changing the applied pressure and/or temperature [13]. Carbon dioxide, the most commonly used supercritical fluid, has the additional advantages of being non-flammable, fairly non-toxic and chemically inert. The main limitation of this technique is that supercritical CO_2 does not possess the solvent strength necessary to efficiently extract polar compounds. In order to enhance the solvating power of CO_2, the addition of modifiers has been described [13, 16]. An alternative approach has been recently reported [17, 18] based on the removal by SFE of the lipophilic matrix from the CO_2-insoluble analyte. This process has been referred to as "inverse SFE" [17, 18].

0009-5893/00/02 490-05 $ 03.00/0

This study describes the systematic development of an inverse SFE procedure for the isolation of the six most common [4, 9, 11] xanthene dyes (fluorescein; 4′,5′-dibromofluorescein; 2′,4′,5′,7′-tetrabromofluorescein; 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein; 4′,5′-diiodofluorescein and 2′,4′,5′,7′-tetraiodofluorescein) from lipstick preparations. An improved HPLC method for the separation and quantitation of the six colorants is also reported. The proposed procedure is applicable to the assay of commercial lipstick products.

Experimental

Reagents

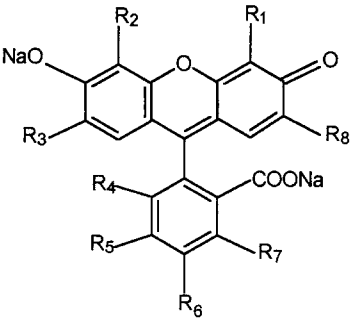
The xanthene dyes, referred to by their Colour Index (CI) numbers [19] were purchased from Aldrich Chimica (Milan, Italy).The following colorants were used in this study (see Figure 1): fluorescein (CI 45350); 4′,5′-dibromofluorescein (CI 45370); 2′,4′,5′,7′-tetrabromofluorescein (CI 45380); 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein (CI 45410); 4′,5′-diiodofluorescein (CI 45425); 2′,4′,5′,7′-tetraiodofluorescein (CI 45430). The azo dye Pigment Red 57 (CI 15850) was a gift from Micys Company (Casatenovo, Lecco, Italy). All other chemicals were of analytical grade (Sigma, St. Louis, MO, USA). Instrument-grade liquid carbon dioxide supplied in cylinders with a dip tube was from Sapio (Monza, Italy). Hydromatrix (diatomaceous earths) was provided by Applied Separations (Allentown, PA, USA). Methanol, acetonitrile of HPLC-grade were obtained from Merck (Darmstadt, Germany).Commercial lipstick samples were from different suppliers.

High-Performance Liquid Chromatography

The HPLC apparatus comprised a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 5 µL sample loop (Rheodyne, Cotati, Ca, USA) and a Model 975-UV variable wavelength UV-vis detector (Jasco, Tokyo, Japan). The column eluent was monitored at 495 nm for CI 45350 and at 525 nm for the other xanthene derivatives using the wavelength time-programming capability of the detector. Data acquisition and processing were accomplished with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were effected with a Model 80365 syringe (Hamilton, Bonaduz, Switzerland). Separations were performed on a 5 µm Zorbax SB-CN column (250 × 4.6 mm i. d.; Stepbio, Bologna, Italy) fitted with a guard column (5 µm particles, 4 × 2mm i. d.; Phenomenex, Torrance, CA, USA) and eluted isocratically with aqueous sodium acetate (0.02 M, pH 4.5)-acetonitrile-methanol (55:35:10, *v/v*).The mobile phase was filtered through 0.2 µm nylon filters (Alltech Italia, Sedriano, Milan). Chromatography was carried out at ambient temperature, at a flow-rate of 0.8 mL min^{-1} . Other columns used in this study included a Hypersil BDS C_{18} (5 µm particles, 150 × 4.6 mm i. d.; Hypersil, Runcorn, UK), a Hypersil BDS Phenyl (5 µm particles, 150 × 4.6 mm i. d.; Hypersil) and a Luna C_{18} (5 µm particles, 150 × 4.6 mm i. d.; Phenomenex). The identity of the separated peaks was assigned by co-chromatography with the authentic standards. Quantification was carried out by integration of the peak areas using an external standard method.

Sample Preparation

Supercritical fluid extractions were performed with a Spe-ed SFE system (Model 7010/680 atm; Applied Separations) which consists of an air-driven pump to deliver the CO_2 to the extraction cell (5 mL stainless steel vessel with 2 µm frits at either ends) housed within a temperature-controlled oven. The CO_2 pump head was cooled by means of circulating water at 4 °C (Dese Lab, Padua, Italy). The outlet of the extraction cell was connected to a thermally-controlled variable restrictor which maintains supercritical pressure conditions in the system. The lipstick product (ca. 0.2 g) was accurately weighed and cut into small pieces. The sample was mixed with Hydromatrix and loaded into the extraction cell. A plug of polypropylene wool was inserted into the cell at both ends. Extractions were carried out in the dynamic (continual flow) mode for 10 min at 40 °C and at a pressure of 350 atm.The restrictor was maintained at 80 °C and the measured flow rate for the supercritical fluid was 2.5 L min^{-1} of expanded gas. As the CO_2 evaporated at the restrictor outlet due to decompression, the CO_2-extractable material was collected in an empty glass vial fitted with a septum and a needle



Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
CI 45350	H	H	H	H	H	H	H	H
CI 45370	Br	Br	H	H	H	H	H	H
CI 45380	Br	Br	Br	H	H	H	H	Br
CI 45410	Br	Br	Br	Cl	Cl	Cl	Cl	Br
CI 45425	I	I	H	H	H	H	H	H
CI 45430	I	I	I	H	H	H	H	I

Figure 1. Chemical structures of the investigated xanthene dyes.

vent. Following CO_2 treatment, the sample was transferred from the extraction vessel into a flask and dispersed in ethanol (25 mL) under sonication (5 min). The sonication was repeated with fresh solvent and the combined ethanol fractions were adjusted to a known volume (50 mL). A portion of the resulting suspension was filtered (0.2 µm nylon filters; Alltech Italia) and analysed by HPLC.

Assay Validation

A lipstick test sample was prepared in the laboratory by adding each xanthene dye at a level of 0.1% (*w/w*) to the formulation components (petrolatum, candelilla wax, beeswax, carnauba wax, stearyl alcohol, tocopheryl acetate, octyl palmitate, butylated hydroxyanisole). The percentage recoveries were calculated by comparing the peak areas of the xanthene derivatives extracted from the test sample by inverse SFE with those obtained by direct injection of equivalent concentrations of the analytes dissolved in ethanol.

Calibration curves of peak area versus concentration were generated with placebo extracts spiked with known amounts of the examined xanthene dyes in the concentration range 0.4 – 100 µg mL^{-1}.

The chromatographic precision was evaluated by repeated analyses (n=5) of the same sample solution from a lipstick.The method precision was calculated by inverse SFE pretreatment and HPLC

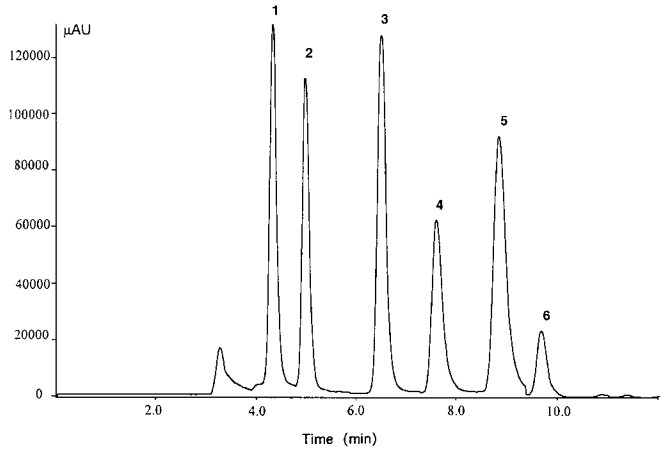


Figure 2. HPLC separation of a standard mixture of xanthene dyes. Chromatographic conditions as described in Experimental. Detection, 0 min, 525 nm; 9.3 min, 495 nm. Peaks: **1** = CI 45410, **2** = CI 45380, **3** = CI 45430, **4** = CI 45370, **5** = CI 45425, **6** = CI 45350.

assay of independent samples (n = 5) from the same lipstick product.

Results and Discussion

Chromatography

The first objective of this study was to develop an isocratic HPLC method for the rapid and efficient separation of the six major xanthene dyes used in cosmetics [4, 9, 11]. Several HPLC columns and solvent systems were tested in order to optimize the resolution of these coloring agents. Preliminary experiments were carried out on a C_{18} column (Hypersil BDS C_{18}) with 40% acetonitrile in phosphate buffer (pH 3.0) as the eluent. Under these conditions, co-elution of CI 45370 and CI 45380 was observed, which is a major drawback since these two coloring additives are frequently used concurrently in cosmetics [9]. In addition, rapid decomposition of CI 45350, CI 45370 and CI 45425 occurred during elution, which was traced to the low pH of the aqueous portion of the mobile phase. Given the instability of xanthene derivatives in acidic media [7], it is surprising that HPLC analyses of these colorants using acetonitrile-water buffered at pH 3 have been described in the literature [4, 8]. Good stability for the xanthene dyes is attained by raising the phosphate buffer pH from 3.0 to 6.8. However, under these conditions, the compounds eluted as little retained and unresolved peaks. Replacing sodium phosphate (pH 3) by sodium acetate (pH 4.5) as buffering ion in the mobile phase minimized the on-column degradation of CI 45350, CI 45370 and CI 45425, but did not afford satisfactory separation for the two pairs of compounds, CI 45370/CI 45380 and CI 45350/CI 45410. Using methanol instead of acetonitrile as the organic modifier in combination with the acetate eluent, produced lower column efficiency and failed to resolve the triplet CI 45370, CI 45380 and CI 45425. A C_{18} packing from a different manufacturer (Luna C_{18}) and a phenyl column (Hypersil BDS Phenyl) were also tested in conjunction with the acetonitrile-acetate buffer mobile phase. However, overlapping of several component peaks (CI 45370/CI 45380 and CI 45425/CI 45430) was observed. Interestingly, the chromatography of the xanthene colorants on a cyanopropyl packing showed a marked improvement in selectivity. Complete separation of the six dyes was attained with this stationary phase, under the elution conditions optimized for the C_{18} sorbent. Moreover, it was found that the addition of a third component to the mobile phase (acetonitrile-methanol-acetate buffer) produced more efficient resolution of the foregoing compounds with a shorter analysis time (Figure 2). The use of a cyanopropyl column for the chromatographic analysis of xanthene colorants has not been reported before. The separation presented in Figure 2 is at least twice as fast as those typically obtained by the HPLC procedures reported in the literature [4, 7–12]. In addition, no deterioration of the quality of resolution was noticed over more than 3 months of continuous use. Hence, the proposed chromatographic system overcomes the inherent disadvantages of the currently used ion-pairing methods [7, 9–11] namely, rapid deterioration of column performance and slow mobile phase equilibration [9, 20].

SFE

Because of the complex matrix, the HPLC assay of xanthene dyes in lipsticks requires a preliminary sample clean-up to remove miscellaneous lipidic material which interferes with the subsequent chromatographic analysis [4, 9, 11]. In the present work, the potential use of SFE for the isolation of xanthene dyes from the lipstick lipophilic base was examined. As a first step, the recovery of CI 45350, CI 45370, CI 45380, CI 45410, CI 45425 and CI 45430 added to a relatively inert matrix (filter paper) was evaluated to acquire information on analyte solubilities in the supercritical fluid. Preliminary experiments were performed for 15 min with CO_2 at 40 °C and at pressures ranging from 200 to 400 atm. No detectable levels of the dyes were recovered from the filter paper by the supercritical fluid even at the highest value of pressure and hence solvating power. This is in accordance with the results described in a previous publication [12] which demonstrated that pure CO_2 failed to extract CI 45350 and CI 45410 from sand or Na_2SO_4. In order to increase the solubility and extractability of the xanthene derivatives in CO_2, the addition of polar modifiers (e. g., methanol, butylamine) to the supercritical fluid has been described [12].

In the present study a different strategy was investigated, based on the use of supercritical CO_2 to remove only the matrix components leaving the analyte unextracted (inverse SFE). This process requires the compound of interest to be insoluble in the supercritical fluid [17, 18] and, in fact, this is the case for the polar xanthene colorants, as demonstrated by the data reported above. In addition, for the successful application of inverse SFE the sample matrix must be readily extractable by the supercritical fluid [17, 18]. This criterion is fulfilled by lipstick preparations which are predominantly mixtures of waxes and oils [21], with typically high solubilities in supercritical CO_2 [22]. In order to determine the optimum conditions for the removal of this lipidic material, a lipstick sample spiked with the six xanthene dyes at 0.1%, w/w was smeared on filter-paper and subjected to 15 min extractions at 40 °C, varying the CO_2 pres-

Table I. Conditions for sample processing after supercritical CO_2 extraction.

Support	Hydromatrix
Elution solvent	ethanol
Solvent volume	25 mL (× 2)
Sonication time	5 min (× 2)
Sonication steps	2

sure over the range 200–350 atm. Increasing the pressure from 200 to 300 atm enhanced the matrix extraction yields (expressed as weight of extract divided by weight of the starting sample) from 27.5% to 70.2%. No significant improvement in the extraction efficiency was observed at higher pressure (350 atm). However, all further experiments were performed at 350 atm since in just 10 min this produced a percentage removal of the matrix components (73.7%) comparable to that achieved by operating for 15 min at 300 atm. Another important step in the development of the inverse-SFE method is the recovery of the non-CO_2 extractable components. Several parameters affecting the transfer of the xanthene dyes from the extraction vessel for analysis were examined (Table I). Initially, filter paper was used as a support for the lipstick. After treatment with CO_2 (350 atm for 10 min), the sample-loaded filter paper was removed from the vessel and subjected to 15 min sonication in methanol (two 25 mL aliquots). Under these conditions, 76.1–87.1% of the spiked colorants were recovered. An increase in the extraction efficiency to 85.6–88.3% was obtained by mixing the lipstick sample with Hydromatrix instead of smearing it on filter paper, and consequently a Hydromatrix support was used for all subsequent experiments. Next, procedures involving different liquid solvents (i.e., methanol, ethanol, acetonitrile or acetone) and sonication times (7, 10 and 15 min) were explored. The highest recovery values (96.8–101.0%) for the spiked xanthene dyes were produced by two sequential 5 min sonications in ethanol of the remaining material after SFE extraction. Comparable results were achieved using methanol instead of ethanol. However, the latter solvent was preferred because of its lower toxicity. The final conditions arrived at for the post-CO_2 extraction work-up are summarized in Table I.

Applying the optimized inverse-SFE procedure to the assay of the spiked lipstick sample, the six xanthene dyes were determined with relative standard deviation (RSD) values in the ranges 1.75–2.29% and 4.80–6.75% for the chromatographic and the method precision, respectively. Calibration curves for each colorant were linear over the range 0.4–0.6 μg mL^{-1} and 80–100 μg mL^{-1}, with correlation coefficients greater than 0.997.

Table II. Levels of xanthene dyes extracted from lipsticks, using inverse SFE compared with conventional liquid-liquid extraction [4, 8, 9].

Sample	Concentration[a] (% *w/w*)			
	CI 45370	CI 45380	CI 45410	CI 45430
Lipstick 1				
Liquid-liquid extraction			0.514 (6.8)	
Inverse SFE			0.520 (4.9)	
Lipstick 2				
Liquid-liquid extraction		0.209 (4.9)		
Inverse SFE		0.217 (4.2)		
Lipstick 3				
Liquid-liquid extraction				0.079 (6.9)
Inverse SFE				0.077 (3.3)
Lipstick 4				
Liquid-liquid extraction		0.012 (12.4)		
Inverse SFE		0.012 (6.7)		
Lipstick 5				
Liquid-liquid extraction	0.095 (11.8)		0.066 (6.1)	
Inverse SFE	0.096 (4.3)		0.072 (5.7)	

[a] Each value is the mean (RSD) of at least 5 determinations.

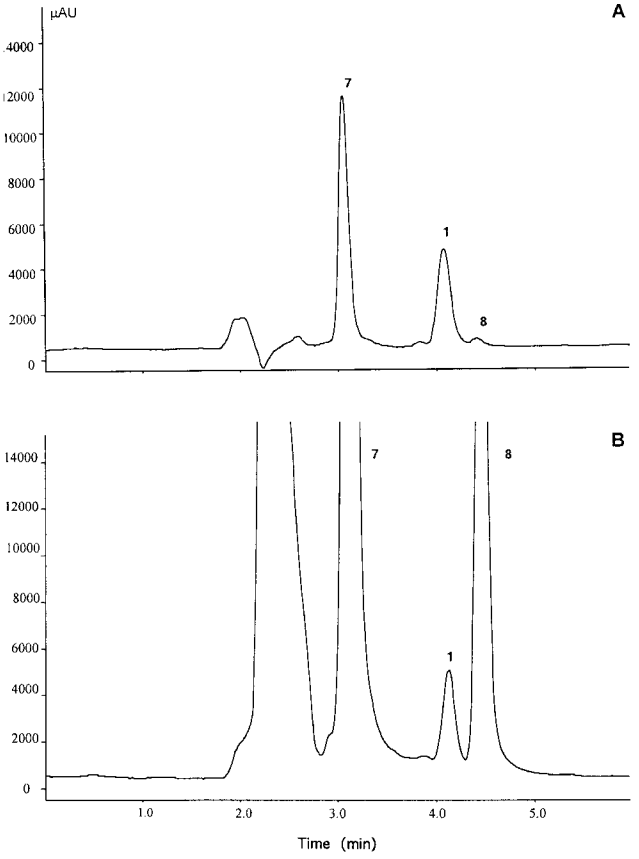


Figure 3. HPLC chromatograms of a lipstick product purified (A) by inverse SFE or (B) by the method reported in the literature [4, 8, 9].Conditions and peak identification as in Figure 2, except for detection wavelength, 260 nm; **7** = CI 15850, **8** = propyl p-hydroxybenzoate.

Additional validation of the proposed inverse SFE method was performed by quantitative comparison, on the same lipstick preparation, with a previously reported liquid-liquid extraction procedure based on dispersion of the product in phosphoric acid-dimethylformamide fol-

lowed by heating, filtration, sequential extractions with hexane and dichloromethane and concentration [4, 8, 9]. Five different commercial products were assayed and the xanthene dye levels measured by HPLC are presented in Table II. The obtained results demonstrated that inverse SFE produced recoveries comparable to those attained by conventional liquid-liquid extraction but achieved improved precision (see Table II). Representative HPLC traces, recorded at 260 nm [7], of a lipstick product processed by the inverse SFE method described here and by the liquid-liquid extraction procedure reported in the literature [4, 8, 9] are shown in Figures 3A and 3B. The chromatograms clearly indicate that the inverse SFE technique (Figure 3A) afforded a more effective purification of the lipstick matrix compared to conventional sample clean-up (Figure 3B), as illustrated by the major reduction of peaks from co-extracted formulation ingredients (e. g., propyl p-hydroxybenzoate, CI 15850). Moreover, sample preparation by inverse SFE is more rapid (taking less than 25 min to perform) and labour-saving than the published techniques [4, 8, 9, 11]. Although the isolation of the xanthene dyes from the Hydromatrix-dispersed sample after supercritical CO_2 extraction requires the use of ethanol, this organic solvent is less toxic than those (dimethylformamide, hexane, dichloromethane, chloroform, acetonitrile, tetrahydrofuran) currently employed in the purification schemes reported in the literature [4, 8, 9, 11].

Conclusions

A rapid procedure has been developed for the isolation of the xanthene colorants from lipstick matrices using inverse SFE followed by HPLC assay on a cyano column. This analysis scheme exhibits several advantages over the previously reported methods [4, 8, 9, 11] including milder extraction conditions, reduction of the sample-handling steps and of the use of hazardous solvents. In addition, the isocratic feature of the chromatographic system enhances the simplicity and reproducibility of the method and makes it preferable to the currently adopted gradient elution techniques [4, 8–12]. Because of the features outlined above, the proposed procedure is suitable for routine quality control analyses of xanthene dyes in lipsticks.

Acknowledgements

The authors thank Micys Company (Casatenovo, Lecco, Italy) for the provision of several lipstick samples.

References

[1] Haveland-Smith, R.B.; Combe, R.D. *Food Cosmet. Toxicol.* **1980**, *18*, 215–221.
[2] Sigman, C.C.; Papa, P.A.; Doeltz, M.K.; Perry, L.R.; Twhigg, A.M.; Helmes, C.T. *J. Environ. Sci. Health* **1985**, *A20*, 427–484.
[3] European Economic Community Council Directive 76/768, Annex IV, *EC Official J.* **1976**, No. L262.
[4] Gagliardi, L.; Amato, A.; Cavazzutti, G.; Tonelli, D.; Montanarella, L. *J. Chromatogr.* **1988**, *448*, 296–300.
[5] Sjöberg, A.-M.; Olkkonen, C. *J. Chromatogr.* **1985**, *318*, 149–154.
[6] Li, Q.X.; Alcantara-Licudine, J.P.; Li, L.-P. *J. Chromatogr. Sci.* **1997**, *35*, 573–577.
[7] Kobayashi, F.; Ozawa, N.; Hanai, J.; Isobe, M.; Watabe, T. *Anal. Chem.* **1986**, *58*, 3048–3051.
[8] Gagliardi, L.; Cavazzutti, G.; Amato, A.; Basili, A.; Tonelli, D. *J. Chromatogr.* **1987**, *394*, 345–352.
[9] Wegener, J.W.; Klamer, J.C.; Govers, H.; Brinkman, U.A.Th. *Chromatographia* **1987**, *24*, 865–875.
[10] Van Liedekerke, B.M.; De Leenheer, A.P. *J. Chromatogr.* **1990**, *528*, 155–162.
[11] Rastogi, S.C.; Barwick, V.J.; Carter, S.V. *Chromatographia* **1997**, *45*, 215–228.
[12] Alcantara-Licudine, J.P.; Kawate, M.K.; Li, Q.X. *J. Agric. Food Chem.* **1997**, *45*, 766–773.
[13] Hawthorne, S.B. *Anal. Chem.* **1990**, *62*, 633A–642A.
[14] Scalia, S.; Ruberto, G.; Bonina, F. *J. Pharm. Sci.* **1995**, *84*, 433–436.
[15] Scalia, S.; Williams, J.R.; Shim, J.H., Law, B.; Morgan, E.D. *Chromatographia* **1998**, *48*, 785–789.
[16] Scalia, S. *J. Chromatogr. A* **2000**, *870*, 199–205.
[17] Messer, D.C.; Taylor, L.T. *Anal. Chem.* **1994**, *66*, 1591–1592.
[18] Moore, W.N.; Taylor, L.T. *J. Pharm. Biomed. Anal.* **1994**, *12*, 1227–1232.
[19] Colour Index, Third Edition, Society of Dyers and Colorists, Bradford, **1971**.
[20] Dolan, J.W. *LC GC Int.* **1996**, *9*, 400–403.
[21] Motitschke, L. In *Cosmetics and Toiletries*: Umbach, W., Ed, Ellis Horwood, New York, **1991**, p 85.
[22] Lembke, P.; Engelhardt, H. *Chromatographia* **1993**, *35*, 509–516.

Received: Nov 30, 2000
Revised manuscript received: Jan 22, 2001
Accepted: Jan 30, 2001