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Separation of picrocrocin, *cis-trans*-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection

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Abstract

High-performance liquid chromatography with photodiode-array detection was used to separate picrocrocin (bitter-tasting component, glucoside of safranal), *cis/trans*-crocins (carotenoids, glucosyl esters of crocetin) and safranal (flavour, monoterpene aldeyde) of saffron. All components of pure red Greek saffron were extracted from dried stigma with 50% methanol. These compounds were detected, separated collected and identified simultaneously using a Merck LiChroCART 125-4 Superspher 100 RP-18 (4 μm) column and as mobile phase a linear gradient from 20% to 100% acetonitrile in water in 20 min with a detection wavelength at 308 nm.

1. Introduction

Saffron, in filaments, is the dried, dark-red stigmata of *Crocus sativus* L. flowers [1]. It is used both as a spice for flavouring and colouring food preparations and as a drug in medicine. It has also been used as a perfume. Foods that have been spiced with saffron include cream and cottage cheese, bouillabaisse, chicken, rice and paella [2].

Crocus sativus L. plants are now cultivated in various parts of the world such as India, Iran, China, Spain and Greece. In Greece, cultivation takes place in the region of Macedonia, Krokos Kozanis area. It seems probable that the village of Krokos derived its name from this plant. In ancient Greek "kroki" meant a string or filament (Iliad IX and XII). A definite identification of *Crocus sativus* L. dates from about 1700–1600 BC in the form of a fresco painting in the palace of Minos at Knossos in Crete. Another fresco, dated about 1500 BC, is at Akrotini on the Island of Thera (Santorini) [2].

The colouring components of saffron are crocins, which are unusual water-soluble carotenoids (glucosyl esters of crocetin). The major component is a digentiobiosyl ester of crocetin [$C_{44}H_{64}O_{24}$, 8,8′-diapo-ψ,ψ′-carotenedioic acid bis(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester]. Safranal ($C_{10}H_{14}O$, 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), one of the main components responsible for the aroma of saffron, is a monoterpene aldehyde. The principal bitter-tasting substance is picrocrocin, a

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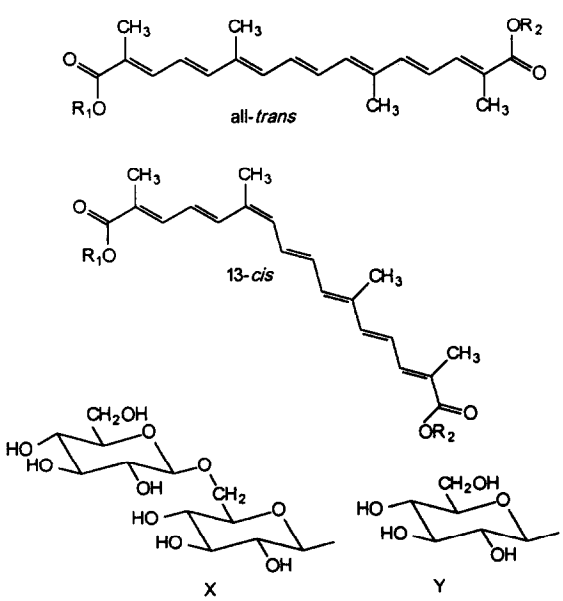
glucoside of safranal ($C_{16}H_{26}O_7$, 4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) [3–10]. Structures of the compounds are shown in Fig. 1.

One stigma of saffron weighs about 2 mg and each flower has three stigmata; 150 000 flowers must be carefully picked one by one to obtain 1 kg of spice. At present, the price on the international market is *ca.* US$ 900–1000/kg. The small retail packets of saffron, seldom over 2 g and often only 0.25 g, cost US$ 10–15/g.

Dietary factors play a significant role in both cancer promotion and prevention. Carotenoids are one of the most common dietary compounds that have been studied as cancer-preventive agents [11,12]. Recently, extracts from natural products and saffron have also been shown to exhibit anticancer activity [13–15].

High-performance liquid chromatography (HPLC) has been shown to be the most efficient technique for the analysis of sensitive compounds in complex extracts of natural products. Photodiode-array (PDA) detection is useful for the determination of *cis/trans* isomers. The aim of this work was to separate the components of saffron using HPLC–PDA for the determination of quality or impurities.



Crocins (CRCs): Glucosyl esters of Crocetin
A-Crocin: R_1=R_2=β-D-gentiobiosyl (X)
B-Crocin: R_1= β-D-gentiobiosyl (X), R_2= β-D-glucosyl (Y)
C-Crocin: R_1= β-D-gentiobiosyl (X), R_2= H
D-Crocin: R_1=R_2= β-D-glucosyl (Y)
E-Crocin: R_1= β-D-glucosyl (Y), R_2= H

Dimethylcrocetin: R_1=R_2=Me
Crocetin: R_1=R_2=H

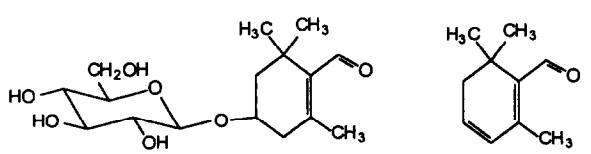


Fig. 1. Structures of saffron components.

2. Experimental

2.1. Plant materials

Stigmata of pure red Greek saffron were kindly supplied by the Cooperative of Saffron, Krokos Kozanis. The flowers were hand picked and the stigmata were separated from the petals and the stamens and dried, in October 1992.

2.2. Sample preparation and standards

Careful attention was paid during the extraction and preparation of saffron components. All solvents were pure and specifically ethers were free from peroxide or contained the antioxidant butylated hydroxytoluene (BHT).

Extraction of the dried saffron intact stigma

For the determination of saffron components, 1 ml of methanol and 1 ml of water were added to one intact stigma saffron, followed by maceration for about 24 h in the dark at ambient temperature with occasional stirring [1,8].

Extraction of saffron in a Soxhlet extractor

Saffron (20 g) was successively and exhaustively extracted with light petroleum (b.p. 40–60°C),

diethyl ether (0.03% BHT) and methanol in a Soxhlet extractor in the dark. The light petroleum extract contained non-glucoside carotenoids and lipids. The diethyl ether extract contained lipids and picrocrocin. The methanol extract contained the glucoside carotenoids of saffron (crocins-CRCs) [3]. All solutions were evaporated to dryness in the dark at 25°C using a Rota Vapor (Buchi, Flawil, Switzerland).

Solution of saffron methanolic extracts in a Soxhlet extractor

For analytical purposes, 1.5 mg of dried methanol extract of saffron from a Soxhlet extractor were dissolved in 2 ml of methanol–water (50:50).

Compounds

Crocetin (CRT), pyridine salt, *ca.* 95%, from saffron was obtained from Sigma (St. Louis, MO, USA) and crocetin (CRT) free acid was produced from a dried methanol extract of saffron in a Soxhlet extractor, in basic (1% KOH) and acidic (1% HCl) solution. Dimethylcrocetin (DMCRT) was also produced from a methanol solution of saffron in basic (1% KOH) solution.

Picrocrocin (PCRC) was purified from the dried diethyl ether extract of saffron in a Soxhlet extractor [3]. It was then diluted with methanol and passed through a 0.45-μm filter (Millipore, Bedford, MA, USA) and purified using HPLC. Safranal (SFRN) was also extracted from saffron using three different extraction methods: (a) steam distillation (SD) under normal pressure (100 g of saffron), (b) micro-steam distillation (MSD) (10 g of saffron) and (c) vacuum headspace (VHS) (20 g of saffron) [16,17], and was purified using HPLC.

Picrocrocin and safranal are very sensitive compounds and produce oxidized and decarboxylated derivatives which may be obtained enzymatically or non-enzymatically during flower growth and the processing and storage of saffron. Further, it should be noted that the method of isolation of the fragrance is a more important step. Artifacts are produced by the SD and MSD extraction procedures. The VHS method for the isolation of safranal from stigmata is rapid and uncomplicated. The volatile components are distilled at low temperature, thus providing the fragrance concentrate form.

2.3. Equipment

UV–visible spectra were obtained using a Philips (Amsterdam, Netherlands) PU8700 UV–Vis spectrophotometer.

HPLC instrumentation

(a) A Waters (Milford, MA, USA) sample injector (20-μl loop), two Model 510 pumps, a Model 680 automated gradient controller, a Model 991 PDA detector interfaced with an NEC power mate 386/25 personal computer, Waters 991-3D chromatogram measuring software (PDA) and a Model 5200 printer–plotter were used. A Merck (Darmstadt, Germany) LiChroCART 125-4 Superspher 100 RP-18 (4 μm) column was employed. The mobile phase was a linear gradient from 20 to 100% acetonitrile in water in 20 min, with an elution profile from 0 to 30 min. The solvent flow-rate was 0.5 ml/min and the column pressure was 4.97 MPa. Detection was performed simultaneously at 220–500 nm.

(b) A Waters Model U6K universal injector (1-ml loop), two Model 510 pump, a Model 680 automated gradient controller, a μBondapak C_{18} (10 μm, 125 Å) column (300 × 7.8 mm I.D.), a UV spectrophotometric detector, and a Model 740 integrated data module system were used, together with an LKB Model 2112 fraction collector. The mobile phase was a linear gradient from 20 to 100% acetonitrile in water in 20 min, with an elution profile from 0 to 25 min. The solvent flow-rate was 5 ml/min and the column pressure 12.76 MPa. Detection was performed at 250, 308 and 328 nm. Fractions were collected automatically by the fraction collector (50 drops per tube or 1.6 ml per tube).

Two Hamilton (Reno, NV, USA) straight-edged needle syringes (25 and 250 μl) were used to inject the samples on to the column. Acetonitrile was of HPLC grade from Aldrich (St. Louis, MO, USA) and water was purified using a

Milli-Q+ system from Millipore (Bedford, MA, USA). All solvents were filtered through a 0.45-μm Millipore filter before use.

3. Results and discussion

3.1. UV–visible spectra

Picrocrocin had a UV–visible spectrum with a broad band at 250 nm and a shoulder at 350 nm. Safranal had an absorption maximum at 308 nm. It is consistent with the presence of a cyclic conjugated aldehyde.

The UV–visible spectra of aqueous saffron solutions had three absorption bands. The first at 256 nm corresponds to glycoside bonds of crocins, the second at 323 nm is attributed mainly to the presence of *cis* double bonds in the polyene conjugated system of crocins, characteristic of the absorption for all the mono-*cis*-carotenoids, and the third band between 400 and 500 nm, with λ_{max} at 440 nm, is characteristic of most all-*trans*-carotenoids [7,18,19]. However, shifts in λ_{max} and the molar absorptivity are readily induced by changing the solvent [18]; thus, in pure methanol saffron showed four peaks at 253, 319, 430 and 455 nm ($\lambda_{max} = 430$ nm), while methanol–water (50:50) resulted in three peaks at 256, 323 and 437 nm ($\lambda_{max} = 437$ nm).

3.2. High-performance liquid chromatography

Fig. 2 shows the results for (A) a methanol–water (50:50) extract of dried saffron intact stigma, (B) a methanol–water (50:50) saffron extract obtained in a Soxhlet extractor and (C) a mixture of methanol–water (50:50) extract of dried saffron intact stigma, crocetin and dimethylcrocetin detected at 440 nm. Peaks 3, 4, 5, 6 and 9 belong to the *trans*-crocins and peaks 7 and 8 to the *cis*-crocins. Peaks 11 and 12 can be identified as crocetin and dimethylcrocetin, respectively.

A comparison between profiles A and B shows that the second extract contains more *cis* isomers than the first. In addition, three minor peaks appeared, which belong to carotenoid derivatives, with retention times of 14.4, 16.8 and 26.3 min. The last peak is consistent with the presence of dimethylcrocetin (peak 12) (Fig. 2B).

The isomerization from the *trans* to the *cis* form and the production of dimethylcrocetin and the other minor unknown peaks are greatly dependent on the extraction method. In fact, the conjugated double bond systems of the saffron carotenoids are very sensitive especially to light, heat, oxygen and acids, and glycoside bonds are readily hydrolysed by alkaline and acidic reactions.

Crocins as all carotenoids are stable only in their natural plant cell environment, and are subject to considerable degradation once isolated. Common causes of pigment loss include heat-induced isomerization from the *trans* to the *cis* form, epoxidation in the presence of oxygen or peroxide, acidic degradation of naturally occurring epoxides to furanoid oxides and photosensitized degradation catalysed by metals [18–21].

Fig. 2 also showed that the retention times for *trans*/*cis*-crocins are between 8.5 and 15 min, for *trans*/*cis*-crocetin 20 and 20.5 min (broad peaks, co-eluted) and for *cis*/*trans*-dimethylcrocetin 25.8 and 26.3 min. These results contradict those of Sujata *et al.* [10]. They used similar conditions but the peaks characterized as crocetins (crocetin 1–4), in fact, correspond to *cis*,*trans*-crocins because the retention time of crocetin (pyridine salt and free acid form), used as a standard, is much longer than those of crocins (glycosyl esters of crocetin).

Fig. 3 shows the chromatographic profiles of a methanol–water (50:50) extract of the dried saffron intact stigma, which were obtained simultaneously at three different wavelengths, (A) 250, (B) 308 and (C) 440 nm. Peaks 1 and 10 with retention times of 5.5 and 18.5 min, respectively, were identified as picrocrocin and safranal from the UV–visible spectrum and confirmed by comparison with the chromatographically separated compounds. Peak 2 with a retention time of 6.5 min had a UV–visible spectrum similar to that of picrocrocin with two absorption bands,

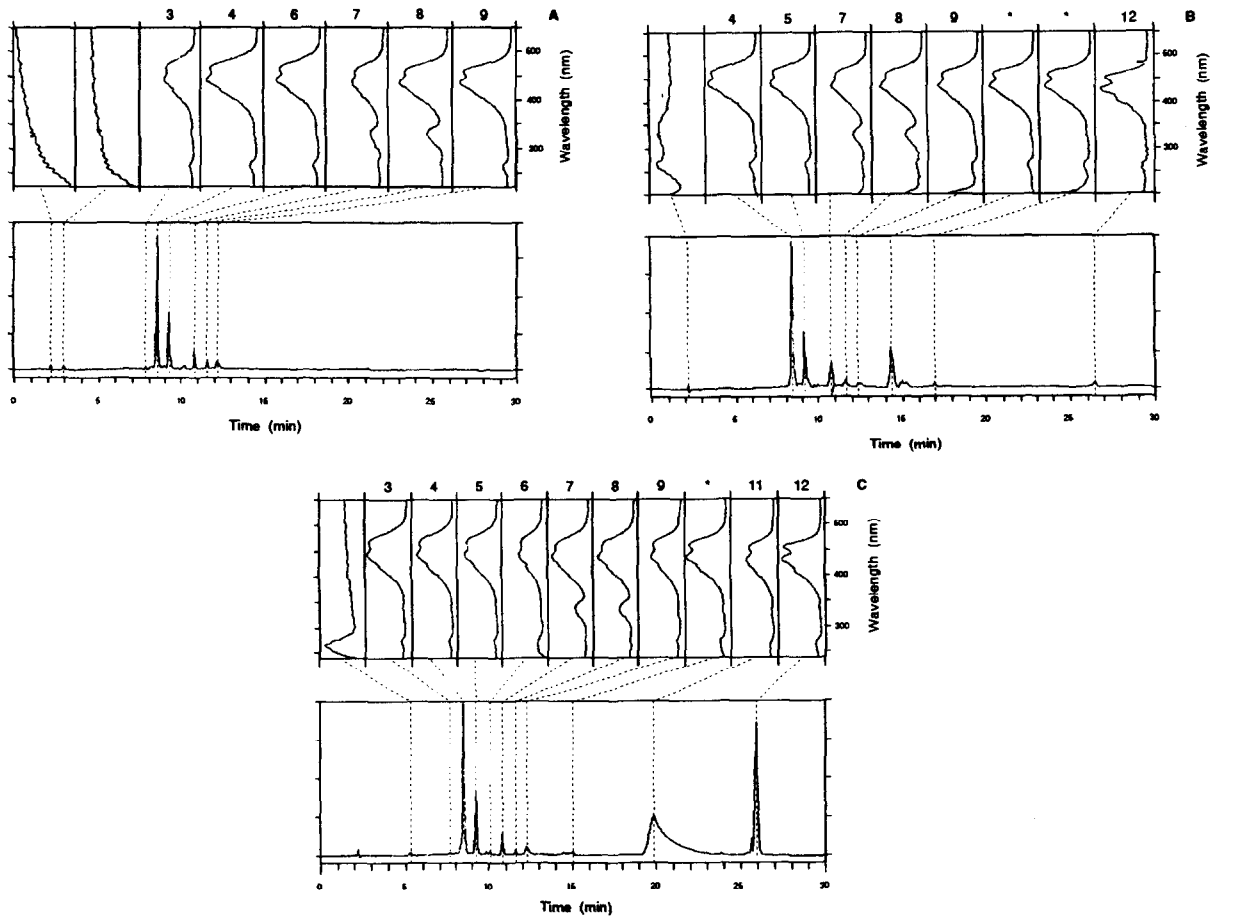


Fig. 2. Chromatograms of (A) a methanol–water (50:50) extract of dried saffron intact stigma, (B) a methanol–water (50:50) saffron extract obtained in a Soxhlet extractor and (C) a mixture of methanol–water (50:50) extracts of dried saffron intact stigma, CRT and DMCRT detected at 440 nm. Column, Merck LiChroCART 125-4 Superspher 100 RP-18 (4 μm); mobile phase, linear gradient from 20 to 100% acetonitrile in water in 20 min, with an elution profile from 0 to 30 min; solvent flow-rate, 0.5 ml/min; column pressure, 4.97 MPa. Peaks: 3–6 and 9 = *trans*-crocins; 7 and 8 = *cis*-crocins; 11 = crocetin; 12 = dimethylcrocetin.

the first at 265 nm and the second at 345 nm, but it is not picrocrocin.

From these profiles, it can be seen that detection at 308 nm is the most appropriate wavelength because all the main constituents of saffron are recorded and especially safranal, for which Sujata *et al.* [10] suggested that it was better to carry out another isocratic analysis separately.

Finally, the main factor that can limit complete resolution in the chromatographic separation of natural products on non-end-capped reversed-phase C_{18} columns is the deposition of lipid materials on the columns (Fig. 3A, retention time 22 min) [19]. In order to avoid these interferences from lipid materials, the columns were frequently washed with methanol, dichloromethane and acetone.

4. Conclusions

The HPLC profile of the methanol–water (50:50) extract of dried stigma of saffron, using a

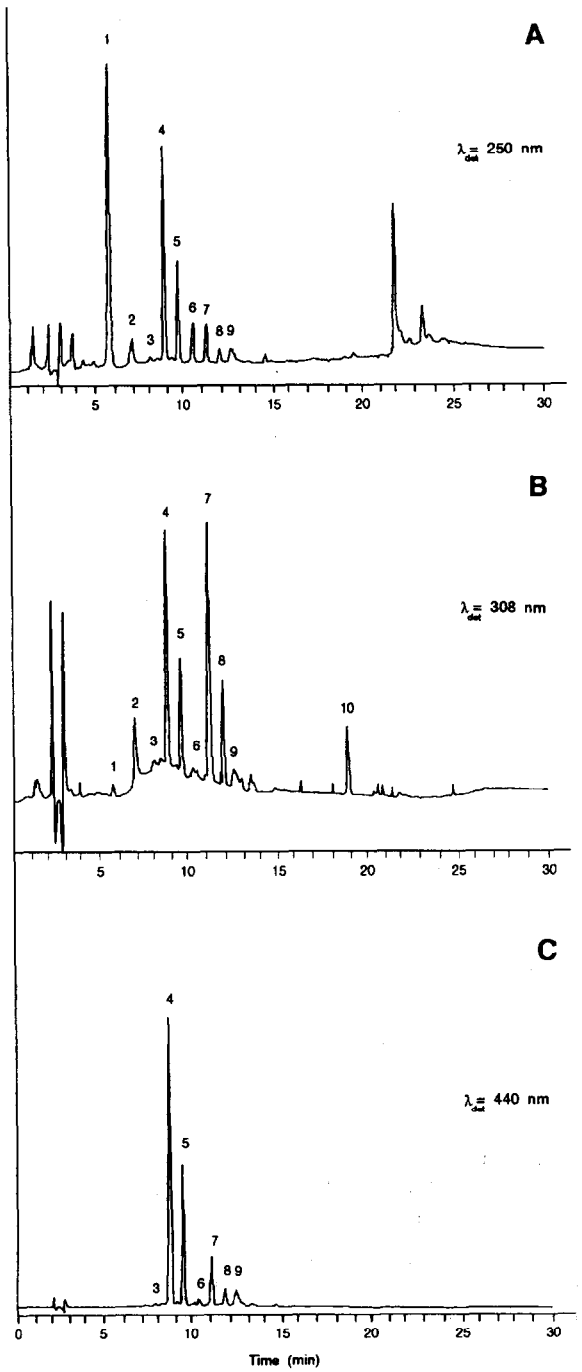


Fig. 3. Chromatograms of a methanol–water (50:50) extract of dried saffron intact stigma recorded at (A) 250, (B) 308 and (C) 440 nm. Conditions as in Fig. 2. Peaks: 1 = picrocrocin; 2 = unidentified; 3–6 and 9 = *trans*-crocins; 7 and 8 = *cis*-crocins; 10 = safranal.

Merck, LiChroCART 125-4 Superspher 100 RP-18 (4 μm) column and a linear gradient from 20 to 100% acetonitrile in water in 20 min with detection at 308 nm is useful for the determination of quality and impurities, because it gives information about all sensitive components (picrocrocin, *cis*/*trans*-crocins and safranal) of saffron.

5. Acknowledgements

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6. References

[1] *International Standard, Saffron —Specification, ISO 3632*, International Organization for Standardization, Geneva, 1980.
[2] D. Basker and M. Negbi, *Econ. Bot.*, 37 (1983) 228.
[3] R. Kuhn and A. Winterstein, *Ber. Dtsch. Chem. Ges.*, 67 (1934) 344.
[4] N.S. Zarghami and D.E. Heinz, *Phytochemistry*, 10 (1971) 2755.
[5] H. Pfander and F. Wittwer, *Helv. Chim. Acta*, 58, 6 (1975) 1608.
[6] H. Pfander and M. Rychener, *J. Chromatogr.*, 234 (1982) 443.
[7] G. Speranza, G. Dada, P. Manitto, D. Monti and P. Grammatica, *Gazz. Chim. Ital.*, 114 (1984) 189.
[8] D. Basker and M. Negbi, *J. Assoc. Publ. Anal.*, 3 (1985) 65.
[9] H. Himeno and K. Sano, *Agric. Biol. Chem.*, 51 (1987) 2395.
[10] V. Sujata, G.A. Ravishankar and V.L. Venkataraman, *J. Chromatogr.*, 624 (1992) 497.
[11] K.A. Steinmetz, J.D. Potter and A.R. Foloson, *Cancer Res.*, 53 (1993) 536.
[12] W. Stahl, A.S. Sundquist, M. Hanusch, W. Schwarz and H. Sies, *Clin. Chem.*, 39 (1993) 810.
[13] S.C. Nair, B. Pannikar and K.R. Panikkar, *Cancer Lett.*, 57 (1991) 109.

[14] H. Morjani, P. Tarantilis, M. Polissiou and M. Manfait, *Anticancer Res.*, 10 (1990) 1398.
[15] P. Tarantilis, M. Polissiou, H. Morjani, P. Avot, A. Bel Jebbar and M. Manfait, *Anticancer Res.*, 12 (1992) 1889.
[16] M. Frutos, J. Sanz and I. Martinez-Castro, *Chromatographia*, 25 (1988) 861.
[17] W. Rodel and M. Petrzika, *J. High Resolut. Chromatogr.*, 14 (1991) 771.
[18] A.G.H. Lea, in R. Macrae (Editor), *HPLC in Food Analysis*, Academic Press, London, 2nd ed., 1988, Ch. 8, p. 277.
[19] C.A. O'Neil and S.J. Schwartz, *J. Chromatogr.*, 624 (1992) 235.
[20] R.C. Mordi, *Chem. Ind. (London)*, (1993) 79.
[21] E. Lesellier, A. Tchapla, C. Marty and A. Lebert, *J. Chromatogr.*, 633 (1993) 9.