Journal of Chromatography, 445 (1988) 189–197
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 517

DIRECT, SENSITIVE AND SELECTIVE DETECTION OF FREE FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN ION-PAIR EXTRACTION AND ABSORBANCE DETECTION

JAMES F. LAWRENCE* and CLAUDETTE F. CHARBONNEAU
Food Research Division, Food Directorate, Health Protection Branch, Ottawa, Ontario K1A 0L2 (Canada)
(First received February 10th, 1988; revised manuscript received March 31st, 1988)

SUMMARY

Free fatty acids (C_8–C_{18}) are separated by reversed-phase liquid chromatography and detected using a simple post-column dynamic extraction system in which the acids are extracted as ion pairs with chloroform from the aqueous acetonitrile (gradient: 79–99% acetonitrile) mobile phase after the post-column addition of aqueous Methylene Blue solution. The chloroform phase containing the ion pairs is monitored with an absorbance detector at 651 nm. The detection limits ranged from 26 to 83 ng, depending upon the acid, with coefficients of variation of 1.2–14%. Application of the method to butter and margarine samples permitted detection of free fatty acids down to 35 ppm and in orange juice, down to 0.5 ppm using only an organic solvent extraction without further sample clean-up for isolation of the fatty acids.

INTRODUCTION

Fatty acids are naturally present in food and often need to be quantitated as indicators of rancidity[1], freshness[2] or adulteration[3]. They are also functional groups of many surface active agents presently used as food additives. Emulsifiers and surfactants with fatty acid moieties are used in a considerable number of food preparations[4].

Most methods of determination of fatty acids involve preparation of their methyl esters or other derivatives[5–10] before gas chromatographic (GC) or liquid chromatographic (LC) separation. Total fatty acids can be determined by direct colorimetric determination of cupric acetate–pyridine complexes in sample extracts[11–13]. Solvent extraction flow injection analysis (SEFIA)[12] of the same complexes has also been evaluated.

The poor ultraviolet absorptivity at 215 or 192 nm[10] or refractive index detection[14] makes the direct LC separation and quantitation of fatty acids in food extracts difficult due to interferences particularly if the acids are present only at parts per million (ppm) levels.

0021-9673/88/$03.50 © 1988 Elsevier Science Publishers B.V.

We have developed a post-column ion-pair extraction technique that enables the detection of fatty acids in nanogram quantities after separation by LC. The method is based on the principle that fatty acids can be extracted from aqueous solutions as neutral ion pairs with the dye Methylene Blue. The acids are detected by absorbance of the dye associated with the ion pair. This same approach was successfully applied to the direct determination of the artificial sweeteners cyclamate, saccharin and acesulfam K in diet beverages using the dye Methyl Violet 2B[15]. The additive, sodium dioctylsulfosuccinate, was also easily detected in beverage powders by applying a similar approach[16].

The present paper describes a post-column extraction system optimized specifically for free fatty acids in the range of C_{10}–C_{18} and applies the method to the separation and selective detection of them in orange juice, margarine and butter samples using an LC mobile phase consisting predominantly of acetonitrile.

EXPERIMENTAL

Reagents

All solvents were distilled in glass. The water for the mobile phase and the dye solution was Milli-Q® (Millipore, Bedford, MA, U.S.A.). Methylene Blue (Aldrich), sodium phosphate dibasic 12-hydrate (Baker) and orthophosphoric acid (Aldrich) were analytical reagent grade and used as received.

The C_8–C_{18} fatty acids were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions of each acid were prepared individualy in methanol and diluted as required with mobile phase (79% aqueous acetonitrile, pH 4.0).

Liquid chromatography

Separation of the fatty acids was accomplished using two Beckman Model 114 M pumps controlled by a Beckman 421 A controller (Beckman, Fullerton, CA, U.S.A.). The mobile phase gradient commenced from 79 to 87% aqueous acetonitrile from 0 to 10 min, increased to 99% acetonitrile from 10 to 15 min, maintained isocratically from 15 to 20 min, then returned to 79% acetonitrile from 20 to 22 min and equilibrated for 6 min before the next run. All mobile phases were adjusted to pH 4.0 with 5% orthophosphoric acid before addition of acetonitrile, then degassed. Mobile phase flow-rate was 0.8 ml/min. An autoinjector (Micromeritics Model 725) fitted with a 10-μl loop was used for injection of the samples onto a Spherisorb ODS-2 column (150 × 4.6 mm I.D., 5 μm) (HPLC Technology, Maccles-field, U.K.).

Post-column ion-pair extractor

The post-column extractor was assembled as shown in Fig. 1. All components were of stainless steel except the phase separator. The dye was mixed with mobile phase at mixing tee A (Valco, $\frac{1}{16}$-in. zero dead-volume). The effluent flowed through a 45 cm × 0.5 mm I.D. × $\frac{1}{16}$ in. O.D. tubing to mixing tee B (Valco) where it was mixed with chloroform. The two-phase mixture flowed through a 50 cm × 1.0 mm I.D. × $\frac{1}{16}$ in. O.D. tubing which enabled the extraction of the fatty acid–Methylene Blue ion pair into the chloroform phase. The phases were separated by gravity (and aided by preferential wetting) in the phase separator which was constructed from a chemically inert CTFE polymer tee (Hamilton, $\frac{1}{16}$ in. bore). The phase separator and

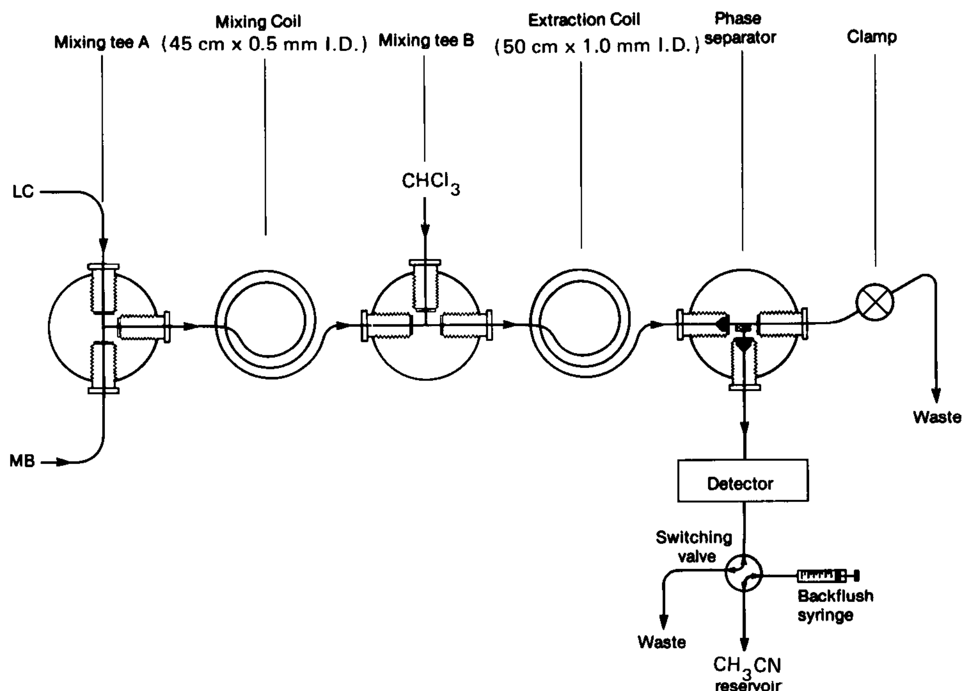

Fig. 1. Diagram of post-column extraction system. Details are described in the text. MB = Methylene Blue

the end of the extraction tubing were modified to prevent aqueous segments from entering the detector stream. These modifications are shown in Fig. 2. A 1-cm portion of the end of the extraction tubing was cut out as shown in the figure to leave a thin length of the metal wall on the upper side of the tubing to act as a hydrophilic surface for wetting by the aqueous phase. This aided separation of the two phases and has been discussed elsewhere[17]. The port leading to the detector was drilled out to $\frac{1}{8}$ in. and filled with a 20-μm polyethylene filter disc (removed from a disposable SPE column, Baker, or equivalent), taking care to keep the upper edge of the disc flush with the bottom wall of the horizontal ports. The stainless-steel tubing to the detector and the end of the extraction tubing were secured in place as shown in the figure using flange free ferrules and nuts (Alltech, Arlington Heights, IL, U.S.A.). The port leading to waste was connected with 2-mm I.D. PTFE tubing using a standard flanged fitting.

The Methylene Blue solution (2 mg/l in 0.02 *M* disodium hydrogenphosphate) was pumped at 2.0 ml/min while the chloroform flow as 1.0 ml/min. All mobile phases and reagent solutions were degassed daily before use. Flow through the detector was adjusted to 0.3–0.5 ml/min by applying a back-pressure on the waste line by means of a screw-clamp. In order to minimize pulsations from the post-column reagent addition, a coiled flattened tube pulse-dampener (Alltech) was installed on each of the two pumps (Waters Model 6000A for the dye and a Beckman Model 110A for the chloroform). In addition an old 250 × 4.6 mm I.D. LiChrosorb C_{18}

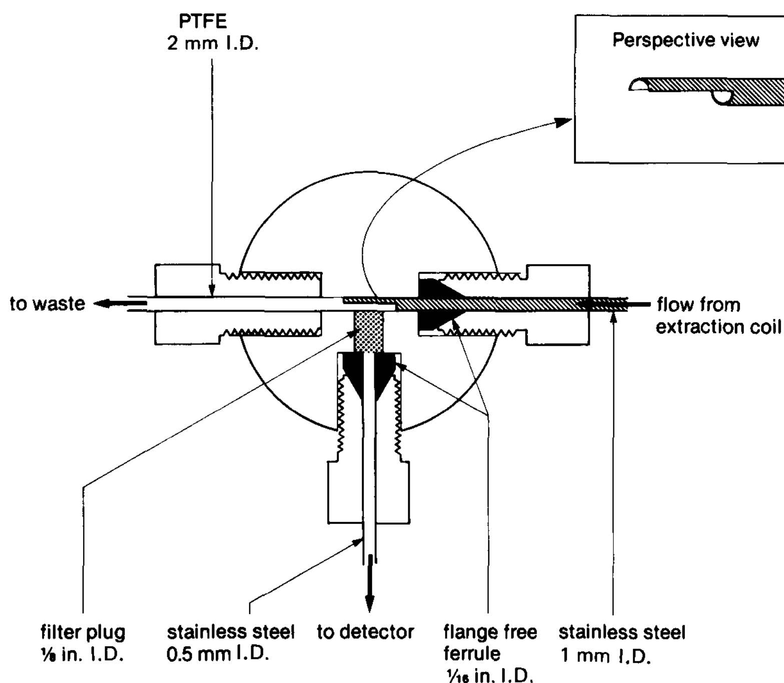

Fig. 2. Diagram of $\frac{1}{16}$ in. CTFE phase separator tee. Other components as described in text.

column was installed after the pulse dampener on the chloroform pump in order to further minimize pulses. The chloroform flow was always the first to be turned on and the last to be turned off each day to ensure that no aqueous phase enters the detector.

The ion pairs were detected with a variable-wavelength detector (Micromeritics Model 788) set to 651 nm. The detector cell was rinsed regularly throughout the day (after every two or three injections) with 2-3 ml acetonitrile by attaching a 5-ml syringe to the detector outlet via a four-port valve.

Sample preparation

Orange juice. Volumes of 10 ml of fresh or reconstituted frozen juice were mixed with 70 ml water and 8 g sodium chloride in a 250-ml centrifuge tube. After the salt had dissolved, the mixture was extracted with three 50-ml portions of dichloromethane by shaking 1 min for each extraction. The tube was centrifuged at 400 *g* for 5 min to aid phase separation and the dichloromethane extracts were collected in a 250-ml round bottom flask. The solution was evaporated to dryness on a flash evaporator at 40°C. The residue was dissolved with five 3-ml portions of acetonitrile, warming the flask in a 40°C water bath to help solubilize the fat. The rinses were transferred to a 15-ml graduated centrifuge tube and evaporated to exactly 1-ml under nitrogen in a 40°C water bath. The solution was filtered through a 0.45-μm filter disc (Millex-HV, Millipore) before injection.

Butter and margarine. A 1-g sample of butter or margarine was weighed in a

15-ml centrifuge tube and warmed in a 40°C water bath until the fat melted. A 10-ml volume of acetonitrile was added and the mixture strirred on a Vortex mixer (Scientific Industries) for 5 min. A 1-ml portion was filtered through a 0.45 μm disc (Millex-HV, Millipore) before injection.

RESULTS AND DISCUSSION

Post-column extractor

The construction of the extraction system from stainless steel posed no problems. In fact for ion-pair extractions where the analyte is extracted from an aqueous phase for measurement in an organic phase, stainless steel or glass would be preferred since it has been shown earlier that with these hydrophilic materials band broadening is less affected by increasing extraction tubing length[18]. The phase separator was modified to accept stainless-steel tubing as shown in Fig. 2. With the modifications no problems were encountered with aqueous phase entry into the detctor and the overall system funcitoned very well throughout the study.

A number of operational parameters were studied in order to optimize the post-column extraction system for fatty acids. The main problem was to design the system so that it could handle mobile phases consisting predominantly of acetonitrile, a solvent which is miscible in both water and chloroform. The single pump reagent/organic solvent addition as described earlier[15] was not possible, thus a second pump was required to deliver the aqueous dye solution at a rate that would yield two phases when mixed with the acetonitrile from the mobile phase and the chloroform extractant. The optimum conditions in the present work were: mobile phase, 0.8 ml/min; aqueous dye, 2.0 ml/min; and chloroform, 1.0 ml/min with a maximum flow of the organic phase through the detector of 0.5 ml/min. A 50-cm extraction coil (1 mm I.D.) was found to be satisfactory for extraction of the ion pairs. Longer tube lengths did not improve the extraction while a 25-cm coil led to a decreased efficiency. The concentration and pH of the dye solution were selected to provide optimum formation and extraction of the fatty acid ion pairs.

One problem associated specifically with the system as set up for fatty acids was the need to routinely backflush the detector cell with acetonitrile to remove deposits which caused fatty acid adsorption and thus loss of resolution and sensitivity. It is possible that excess Methylene Blue adsorbs into the quartz windows of the cell and retains the fatty acids as they pass through. In the beginning of the study when the acetonitrile washing was not carried out routinely, the cell required washing with 4 ml of 1 *M* sodium hydroxide to return the detector to its optimum performance. Dilute nitric or hydrochloric acid washes failed to remove the deposit. This effect was not studied with other detectors. However, it was not observed when the same detector was used for post-column ion-pair extraction detection of other compounds under different post-column and chromatography conditions[15,16].

Separation and detection of fatty acids

Fig. 3 shows the chromatographic separation of ten fatty acids ranging from C_8 to C_{18} using the post-column extraction system. Good separation was achieved for the *cis* and *trans* isomers of $C_{18:2}$ (linoleic and linolelaidic acids) and for the other C_{18} congeners. The separation is somewhat improved over that obtained earlier[10,19]

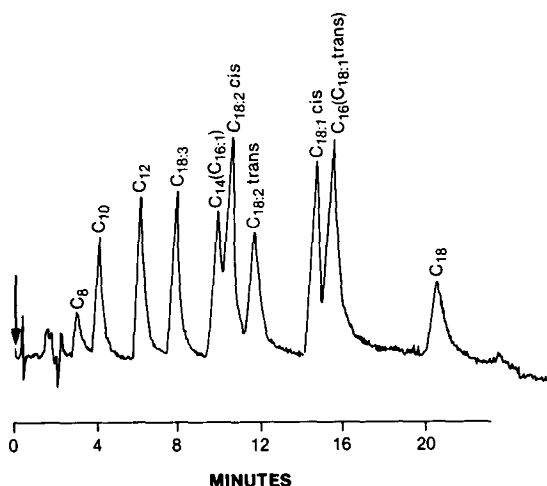

Fig. 3. Chromatogram of fatty acids at 0.64 a.u.f.s. and 651 nm. (200–300 ng injected). Spherisorb ODS-2. Conditions as described in text.

and similar to other work which employed two columns in series[5,6]. Table I lists retention times, calculated detection limits and coefficients of variation for repetitive injections of fatty acids. It can be seen that with the exception of C_8 and C_{18} fatty acids the detection limits ranged from 26 to 44 ng (three times peak-to-peak baseline noise). The detection limit for C_8 acid was worse due to a lower extraction efficiency.

TABLE I

ESTIMATED DETECTION LIMITS AND REPEATABILITY OF FATTY ACIDS

Fatty acid	*Retention time (min)*	*Detection limit (ng)**	*Coefficient of variation (%)***
C_8 Caprylic	3.0	83	13.9
C_{10} Capric	4.0	28	8.4
C_{12} Lauric	6.0	26	9.1
$C_{18:3}$ Linolenic (*cis,cis,cis*, 9, 12, 15)	7.3	38	2.7
C_{14} Myristic	9.1***	33	2.4
$C_{16:1}$ Palmitoleic	9.1***	44	2.4
$C_{18:2}$ Linoleic (*cis,cis*, 9, 12)	9.7	39	1.2
$C_{18:2}$ Linolelaidic (*trans, trans*, 9, 12)	10.8	42	3.1
$C_{18:1}$ *cis*-Oleic	13.8	41	4.0
C_{16} Palmitic	14.5	32	4.7
$C_{18:1}$ *trans*-Elaidic	14.5	60	ND§
C_{18} Stearic	20.5	64	9.3

* Defined as three times the peak-to-peak noise level at 0.64 a.u.f.s. at 651 nm.

** For $n=3$ repetitive injections of standards (200–300 ng of each acid) with an acetonitrile wash of the cell between injections.

*** Elute together, but detection limit determined separately.

§ ND = Not determined.

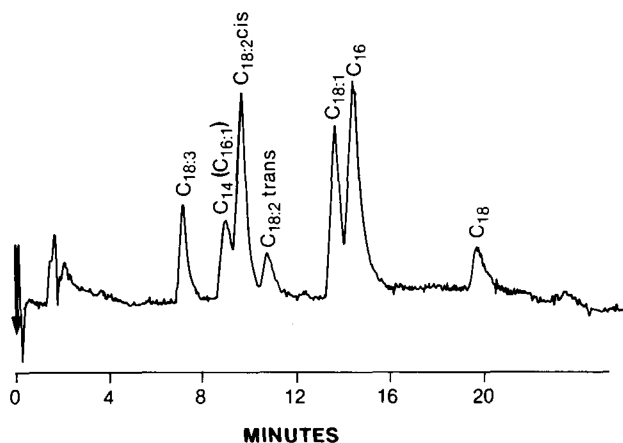

Fig. 4. Chromatogram of free fatty acids in orange juice. C_{14}–C_{18} fatty acids, 0.7–2.3 ppm.

A similar effect was noted for alkylsulfonic acids where ion pair extractability was related to alkyl chain length[20]. The linear range for the fatty acids extended from the detection limit to over 1 μg per injection. This range could be extended to higher levels if the Methylene Blue concentration were increased. However, this would lead to increased background noise resulting in poorer detection limits.

Sample analysis

Fig. 4 shows a chromatogram of an orange juice sample extracted as described in the Experimental section. Although only an organic solvent extraction was employed for clean-up, the chromatograms look as clean as standards. This demon-

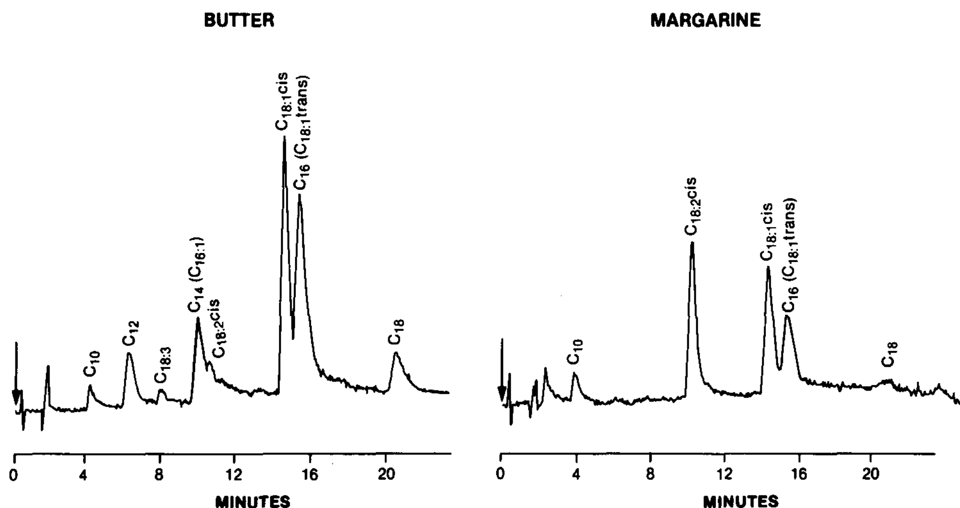

Fig. 5. Chromatogram of 34–586 ppm of free fatty acids (C_{10}–C_{18}) in butter, and 35–256 ppm of the acids in margarine.

strates the excellent selectivity of the detection system for these compounds. The levels of free fatty acids found in two different juice samples ranged from 0.7 to 2.3 ppm of C_{14}–C_{18} acids and were generally in the same relative proportions as found elsewhere for other orange juice samples using a GC technique after forming the methyl esters[21]. The apparent presence of 18:2 (*trans*) was somewhat surprising. However, low levels of *trans* fatty acids have been reported in orange juice[22]. The minimum detectable level of fatty acids in orange juice was estimated to be about 0.5 ppm depending upon the acid. This could be improved by concentration of the final sample extract. Recovery from spiked orange juice averaged 60% (range, 44–73%, depending upon the acid) at a level of about 5 ppm.

Fig. 5 shows chromatograms of butter and margarine samples extracted with acetonitrile as described in the experimental section. Again, the chromatograms are very clean showing only the responses of the free fatty acids. The levels in the butter ranged from 34 to 586 ppm for acids with chain lengths of C_{10}–C_{18} and from 35 to 421 ppm for the same acids in two different samples of margarine. The detection limit for these samples was about 35 ppm but could be improved by concentrating the sample extract. Certain acid pairs such as palmitic (C_{16}) and elaidic ($C_{18:1}$ *trans*) as well as myristic (C_{14}) and palmitoleic ($C_{16:1}$) could not be separated with the chromatographic conditions used and thus could not be individually quantitated. However, the levels found in the butter and margarine samples compare well to results found by other techniques[1,14]. The peak eluting at about 4 min in the margarine sample appears to be capric (C_{10}) acid which is unusual in the absence of C_{12} or C_{14} fatty acids. Confirmation of its identity was not attempted.

CONCLUSION

The post-column ion-pair extraction technique was found to be well suited to the detection of fatty acids separated by LC. The selectivity of the system appears to be excellent compared to either direct low wavelength absorption or refractive index detection enabling the determination of C_{10}–C_{18} free fatty acids at low ppm levels in both predominantly aqueous (orange juice) or fatty (butter, margarine) samples. The method should also be easily applicable to total fatty acids in biological samples after saponification and extraction.

REFERENCES

1 A. H. Woo and R. C. Lindsay, *J. Dairy Sci.*, 63 (1980) 1058.
2 S. Nagy and H. E. Nordby, *J. Agric. Food Chem.*, 18 (1970) 593.
3 R. G. Mears and A. J. Shenton, *J. Food Technol.*, 8 (1973) 357.
4 *Monographs for Emulsifiers for Foods*, European Food Emulsifier Manufacturers Association, Brussels, 2nd ed. 1985.
5 R. A. Miller, N. E. Bussell and C. Ricketts, *J. Liq. Chromatogr.*, 1 (1978) 291.
6 H. C. Jordi, *J. Liq. Chromatogr.*, 1 (1978) 215.
7 H. D. Durst, M. Milano, E. J. Kikta, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
8 J. W. King, E. C. Adams and B. A. Bidlingmeyer, *J. Liq. Chromatogr.*, 5 (1982) 275.
9 R. F. Arrendale, G. W. Chapman and O. T. Chortyk, *J. Agric. Food Chem.*, 31 (1983) 1334.
10 M. I. Aveldano, M. Van Rollins and L. A. Horrocks, *J. Lipid. Res.*, 24 (1983) 83.
11 R. R. Lowry and I. J. Tinsley, *J. Am. Oil Chem. Soc.*, 53 (1976) 470.
12 J. S. Canham and G. E. Pacey, *J. Am. Oil Chem. Soc.*, 64 (1987) 1004.

13 D. Y. Kwon and J. S. Rhee, *J. Am. Oil Chem. Soc.*, 63 (1986) 89.
14 A. G. Bailie, Jr., T. D. Wilson, R. K. O'Brien, J. M. Beebe and J. D. Stuart, *J. Chromatogr. Sci.*, 20 (1982) 466.
15 J. F. Lawrence, *Analyst (London)*, 112 (1987) 879.
16 J. F. Lawrence, *J. Assoc. Off. Anal Chem.*, 70 (1987) 15.
17 C. de Ruiter, J. H. Wolf, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta*, 192 (1987) 267.
18 J. F. Lawrence, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 185 (1979) 473.
19 A. W. Reed, H. C. Deeth and D. E. Clegg, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 718.
20 C. P. Terweij-Groen, J. C. Kraak, W. M. A. Niessen, J. F. Lawrence, C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Int. J. Environ. Anal. Chem.*, 9 (1981) 45.
21 J. B. Stack, F. L. Joe, D. G. Cunningham, T. Fazio and J. A. G. Roach, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 551.
22 H. E. Nordby and S. Nagy, *Phytochemistry*, 8 (1969) 2027.