Journal of Chromatography, 627 (1992) 287–293
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 667

Short Communication

Determination of oxytetracycline in marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicularia plana*) by high-performance liquid chromatography using solid-phase extraction

Hervé Pouliquen, Djénéba Keita and Louis Pinault

Service de Pharmacie et Toxicologie, École Nationale Vétérinaire de Nantes, C.P. 3013, 44087 Nantes Cedex 03 (France)

(First received July 14th, 1992; revised manuscript received September 29th, 1992)

ABSTRACT

A reversed-phase high-performance liquid chromatographic method was developed to detect oxytetracycline (OTC) in three species of marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicularia plana*). Shellfish tissues were analysed after solid-phase extraction by using a mobile phase containing acetonitrile and 0.02 *M* orthophosphoric acid solution. The linearity and precision of the method were checked over the concentration range 0.100–1.500 μg/g. The limits of detection and determination of OTC were 0.040 and 0.100 μg/g, respectively. The recovery of OTC from spiked shellfish tissues was 79.8%. The OTC concentration in oyster (*Crassostrea gigas*) spiked at 0.500 μg/g and stored at −20°C was stable for 6 weeks. The method was applied to a 21-day experimental study performed with oysters.

INTRODUCTION

Because of its wide antibacterial spectrum and high potency, oxytetracycline (OTC) is a common antibiotic used against bacterial infections in fish farming. The drug is administered to fish mixed with feed at a dosage rate of 50–100 mg per kg of biomass per day for 8–10 days. The first sign of an infectious and systemic disease in fish is usually a reduced feed intake. Further, OTC is very poorly absorbed through the intestinal tract of fish [1]. Therefore, a large part of the medicated feed presumably reaches the environment of the fish farms.

The environmental effects of OTC used in marine aquaculture are largely unknown. The development of fish farming in salt marshes along the French Atlantic coastline has been shown to be responsible for the pollution of sea water and sediment by OTC [2,3]. There is no published information on residues of OTC in marine shellfish. Hence an adequate method for the determination of OTC in marine shellfish is needed.

The commonly used bacterial bioassay methods for determining OTC are less sensitive and less specific than high-performance liquid chromatographic (HPLC) methods [4,5]. Several methods using HPLC with C_{18} and C_8 columns for the determination of OTC in fish plasma and tissues have been

Correspondence to: H. Pouliquen, Service de Pharmacie et Toxicologie, École Nationale Vétérinaire de Nantes, C.P. 3013, 44087 Nantes Cedex 03, France.

0021-9673/92/$05.00

described [4–15]. No analyses have been undertaken for marine shellfish.

The aim of this study was to develop a simple, rapid and accurate HPLC method for the determination of OTC in three species of marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicularia plana*) and to apply this method to an experimental study performed with *Crassostrea gigas*.

EXPERIMENTAL

Chemicals and reagents

Citric acid monohydrate, oxalic acid dihydrate, phosphoric acid and disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$) were supplied by Merck (Darmstadt, Germany), perchloric acid and disodium hydrogenphosphate dodecahydrate by Prolabo (Paris, France), HPLC-grade acetonitrile by BDH Chemicals (Toronto, Canada), HPLC-grade methanol by Carlo Erba (Milan, Italy) and oxytetracycline hydrochloride (91% purity) by Pfizer (Amboise, France). All other reagents were of analytical-reagent grade.

McIlvaine buffer (pH 3.0) consisted of 0.1 *M* citric acid–0.2 *M* disodium hydrogenphosphate (79.45:20.55, v/v); 0.1 *M* Na_2EDTA McIlvaine buffer (pH 4.0) [16] was prepared by dissolving 37.224 g of $Na_2EDTA \cdot 2H_2O$ in water, adjusting the pH to 4.0 with McIlvaine buffer (pH 3.0) and diluting to 1 l with water.

HPLC apparatus

The analyses were performed on a Varian (Palo Alto, CA, USA) Model 5000 chromatograph equipped with a Valco injection valve. The analytical column was 5-μm LiChrospher 100 RP-18E (125 × 4.6 mm I.D.) (Merck) equipped with a 5-μm LiChrospher 100 RP-18E guard column (4 × 4.6 mm I.D.). The detector was a Vari-Chrom UV 50 (Varian). Peak heights were calculated on a D-2500 integrator (Merck). The data were handled with a Deskpro 386/s Model 40 3.5 computer (Compaq, Houston, TX, USA) equipped with the HPLC Manager Software System (Merck).

Operating conditions

The mobile phase was acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3 (24:76, v/v) [17]. The mixture was filtered with a Millipore (Bedford, MA, USA) HPLC solvent filtration system and Nalgene 47-mm, 0.20-μm nylon filters (Nalge, Rochester, NY, USA) and then sonicated for 15 min. The chromatographic experiments were performed at room temperature (24–25°C). The flow-rate was 1.2 ml/min and the UV detector was set at 355 nm and 0.01 a.u.f.s. The sample volume injected on to the column was 50 μl. The guard column was changed at intervals of 150–200 sample injections. The new columns were conditioned prior to use by flushing with acetonitrile–water [75:25 (v/v) for 2 h, 50:50 (v/v) for 2 h, 40:60 (v/v) for 2 h, 30:70 (v/v) for 2 h] and mobile phase (for 5 h) at a flow-rate of 0.2 ml/min. The columns were reconditioned for 2 h after each day of operation with acetonitrile–water (50:50, v/v) at a flow-rate of 0.2 ml/min.

Preparation of standard solutions

A stock standard solution of OTC in methanol (1 mg/ml) was prepared and was stable for 1 month when stored at −20°C. A stock standard solution (100 μg/ml) in methanol was also prepared immediately before use. Working standard solutions were prepared by diluting aliquots of the latter stock standard solution with 0.1 *M* perchloric acid. All these solutions were protected from light throughout the analyses because of the instability of OTC to light. A 50-μl aliquot of the standard solutions (0.500 and 1.000 μg/ml) was injected on to the HPLC column at the beginning of each day of operation. The calibration graph was based on peak-height measurements.

Sample preparation

The sample preparation was adapted from that used by Oka *et al.* [16] for the determination of tetracycline residues in animal liver. Shellfish tissues (*Crassostrea gigas, Ruditapes philippinarum* or *Scrobicularia plana*) were homogenized using a high-speed blender (Ultra-Turrax; Bioblock, Illkirch, France). A 1-g amount of the homogenate was transferred into a polypropylene tube (8 ml) and extracted three times with 4, 4 and 2 ml of 0.1 *M* Na_2EDTA McIlvaine buffer (pH 4.0). After centrifugation at 3500 *g* for 10 min at 4°C in a Jouan (Saint Herblain, France) Model MR 1822 centrifuge, the combined supernatants were filtered through 90-mm Whatman No. 541 filter-paper

(Whatman, Maidstone, UK) and then concentrated by passing through 3-ml Bond-Elut solid-phase extraction cartridges (Analytichem International, Harbor City, CA, USA). Before use, the cartridges were activated with methanol (4 ml) and water (4 ml). After the samples had been passed, OTC was eluted with 2 ml of 0.01 *M* methanolic oxalic acid solution and collected in a 3-ml flask. The eluate was evaporated to dryness under nitrogen in a water-bath at 30°C and reconstituted to 1.0 ml in 0.1 *M* perchloric acid. The sample was then centrifuged at 13 000 *g* for 10 min at 4°C in a Jouan Model MR 1822 centrifuge. The supernatant was filtered through an Analypore MC 25-mm, 0.45-μm filter (OSI, Paris, France). A 50-μl aliquot was injected on to the HPLC column. All the aforementioned steps were conducted in subdued light.

Method of validation

Overall validation of the method was performed using oyster (*Crassostrea gigas*). A calibration graph was obtained daily by spiking oysters at four concentrations of OTC (0.100, 0.250, 0.500 and 1.500 μg/g) and analysing two replicates on three consecutive days. The precision of the method was tested by spiking oysters at a concentration of 0.500 μg/g with OTC and analysing six replicates on two consecutive days. The extraction recoveries of OTC were determined for three consecutive days by comparing the peak heights from the analyses of oysters spiked at 0.100, 0.250, 0.5000 and 1.500 μg/g with those resulting from direct injection of standard solutions in 0.1 *M* perchloric acid. A study of stability was peformed by storing at −20°C oyster samples spiked at a concentration of 0.500 μg/g with OTC and analysing them during a 6-week period.

A simplified validation of the method was performed using *Ruditapes philippinarum* and *Scrobicularia plana*. The calibration curve, precision and extraction recoveries of OTC were determined as previously, except the analyses only lasted one day.

Experimental conditions and device

An experimental study was performed in order to estimate the contamination of simulated oyster farms polluted by a seawater effluent; this effluent contained OTC and was released by a fish farm model.

A raceway, consisting of a long, rectangular tank (10 × 1 × 1 m) used in intensive fish breeding (seabass and sea-bream), simulated a fish farm located in a salt marsh. The shellfish farms were simulated in four tanks (2 × 2 × 0.6 m), A, B, C and T. All the tanks were run through by the water of the raceway, except tank T, which was the control tank. All the tanks were supplied with an 8-cm sediment layer and 42 oysters (*Crassostrea gigas*). The seawater flows were 6.7 l/min in the raceway and 2.2 l/min in the four tanks. The daily changes of sea water in the raceway and in the tanks were 128% and 182%, respectively. All the tanks were exposed to natural sunlight so that the nights were about 10 h long. The average temperature, pH and salt content of the sea water were 14.5°C, 8.64 and 33.1‰, respectively.

OTC was added in the raceway at a dosage rate of 45 g per day for 7 days. The medicated feed containing OTC was prepared each morning and was distributed in the raceway at 9 a.m. and 5 p.m. The experiment continued for 21 days and was divided into two periods: a 7-day "treatment" period during which OTC was distributed in the raceway and a 14-day "post-treatment" period.

During the 21 days of the experiment, three oysters were removed from each tank on each sampling day and then stored at −20°C until such time as the analysis was carried out. The method described previously was used for determining the OTC residues in oyster from the four tanks. A mixture of the three oysters picked from each tank on each sampling day was used for determining each OTC concentration.

RESULTS AND DISCUSSION

Reversed-phase HPLC has often been used for determining tetracycline antibiotics in fish plasma and tissues. In our experiment, a LiChrospher 100-RP 18E prepacked column was also used as a reversed-phase column in order to determine OTC in shellfish tissues. When using acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3 (24:76, v/v) as the mobile phase, the chromatograms were free from intefering peaks. However, baseline drift and severe peak tailing were consistently noted. These effects are due to the presence of either residual free silanol groups or metal ion contamination in the base silica of the reversed phase [18]. Chelating

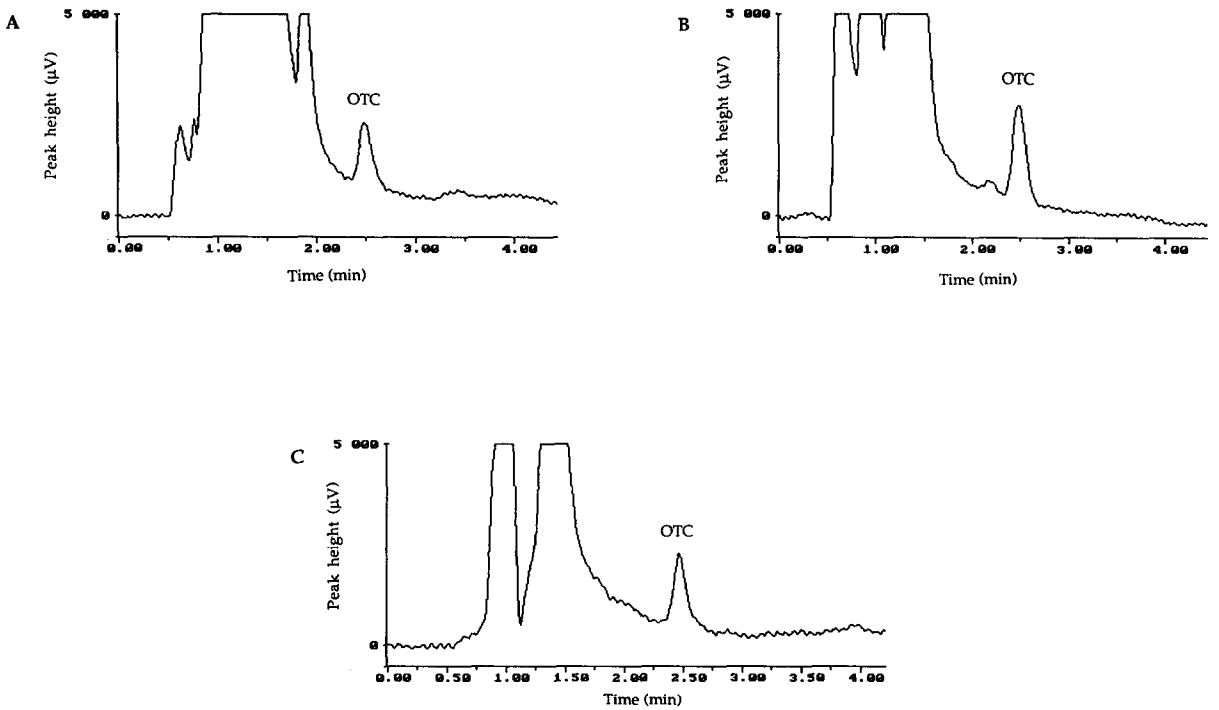

Fig. 1. HPLC of shellfish tissues. (A) *Crassostrea gigas* sample containing OTC at a concentration of 0.475 μg/g; (B) *Ruditapes philippinarum* sample containing OTC at a concentration of 0.660 μg/g; (C) *Scrobicularia plana* sample containing OTC at a concentration of 0.455 μg/g. Conditions: mobile phase, acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3 (24:76, v/v); column, 125 × 4 mm I.D., C_{18} (5 μm); flow-rate, 1.2 ml/min; wavelength, 355 nm; recorder sensitivity, 0.01 a.u.f.s.; injection volume, 50 μl.

agents, such as EDTA, were added to the mobile phase in order to block the reactive sites of the silica. Their presence did not completely eliminate peak tailing. It was found that, by recycling after each day of operation acetonitrile–water (50:50, v/v) through the HPLC column for 2 h, the peak response was consistently high and neither tailing nor baseline drift were evident.

The tetracycline antibiotics have a tendency to combine with proteins and to form chelate com-

TABLE I

LINEARITY AND REGRESSION DATA FOR THE CALIBRAITON GRAPHS OBTAINED FROM *CRASSOSTREA GIGAS*, *RUDITAPES PHILIPPINARUM* AND *SCROBICULARIA PLANA* TISSUES SPIKED WITH OXYTETRACYCLINE FROM 0.100 TO 1.500 μg/g

$y = ax + b$; y = peak height (μV); x = oxytetracycline concentration (μg/g); a = slope; b = intercept.

Species	Slope	Intercept	Correlation coefficient	Variance	$F_{2,4}$ linearity
Crassostrea gigas					
Day A	2769.76	98.01	0.997	7676.04	0.241
Day B	2624.74	134.59	0.999	2208.74	0.829
Day C	2648.10	124.37	1.000	600.40	0.489
Ruditapes philippinarum	2799.73	109.66	1.000	774.30	0.349
Scrobicularia plana	2654.09	146.98	1.000	925.77	0.948

TABLE II

CALCULATION OF THE LIMIT OF DETERMINATION OF OTC IN OYSTER (*CRASSOSTREA GIGAS*) (0.100 μg/g)

Day	Peak height (μV)
A	383.00
B	366.00
C	375.00
D	400.00
E	302.00
Mean	365.20
Standard deviation (S.D.)	37.47
$T = 362.00/(37.47/\sqrt{5}) = 21.60$	
($t_4 = 2.77$ at $P = 0.10$)[a]	
3 × S.D. = 112.41 μV	

[a] Student's *t*-test with 4 degrees of freedom.

plexes with metal ions [16,18]. Therefore, it is difficult to extract these antibiotics from animal tissues. In our experiments, the efficiency of the extraction of OTC from shellfish tissues with 1 *M* hydrochloric acid, 50% trichloracetic acid and 0.1 *M* Na_2EDTA McIlvaine buffer (pH 4.0) was examined. The extracting solvent giving the highest recovery of OTC from shellfish tissues was 0.1 *M* Na_2EDTA McIlvaine buffer (pH 4.0) [16].

No endogenous material interfered with the separation and determination of the OTC. No changes in retention times were noted with continuous column use. OTC was eluted in 2.50 min. Typical chromatograms of shellfish tissues are shown in Fig. 1.

A statistical test of linearity was performed for each calibration graph separately, using a weighted analysis of variance (ANOVA) [19,20]. All the calibration graphs showed linearity in the range examined (0.100–1.500 μg/g) at the 0.05 level (Table I). A good correlation was obtained between concentrations and peak heights: all the correlation coefficients were between 0.997 and 1.000 (Table I).

The limits of detection and determination of OTC in shellfish tissues were 0.040 and 0.100 μg/g (precision ± 10%), respectively. This concentration of 0.100 μg/g was accepted as the limit of determination because a *t*-test with four degrees of freedom showed that this is significantly different from zero and the mean repsonse was greater than three standard deviations. An example of the calculation of the limit of determination in oysters is shown in Table II.

The relative standard deviations (R.S.D.s) of within-day precision for shellfish tissues spiked with OTC at 0.500 μg/g were 2.5%, 3.7% and 1.8% (n = 6) for *Crassostrea gigas*, *Ruditapes philippinarum* and *Scrobicularia plana*, respectively. The R.S.D. of between-day precision for oyster tissues spiked with OTC at 0.500 μg/g was 2.2% (n = 6 per day). In oysters, an ANOVA showed that there was no significant difference between the within- and between-day precisions at the 0.05 level.

OTC was recovered from shellfish tissues using the solid-phase extraction procedure described. The mean recoveries (n = 12) of OTC were 79.4% (R.S.D. = 2.6%) from *Crassostrea gigas*, 79.7% (R.S.D. = 3.5%) from *Ruditapes philippinarum* and 80.4% (R.S.D. = 2.1%) from *Scrobicularia plana* over the concentration range 0.100–1.500 μg/g. No significant difference was apparent between the three species of shellfish at the 0.05 level.

A study of the stability of OTC in oyster samples spiked at a concentration of 0.500 μg/g and stored at −20°C was performed. An average chart was constructed using the recovery results from the validation; its limits were determined using the principles based on control charts for individuals [19,20]. With this chart, there was a basis for monitoring the degradation of OTC in oysters stored at −20°C. The results are shown in Fig. 2. All the recoveries of

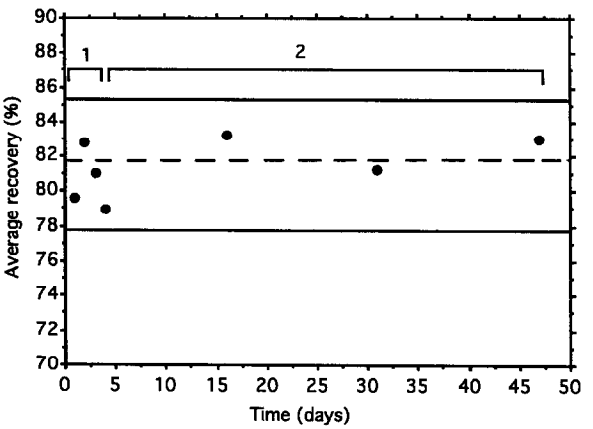

Fig. 2. Average chart of the recovery of OTC from oyster (*Crassostrea gigas*) spiked at 0.500 μg/g and stored at −20°C for 6 weeks. 1 = Validation; 2 = stability study.

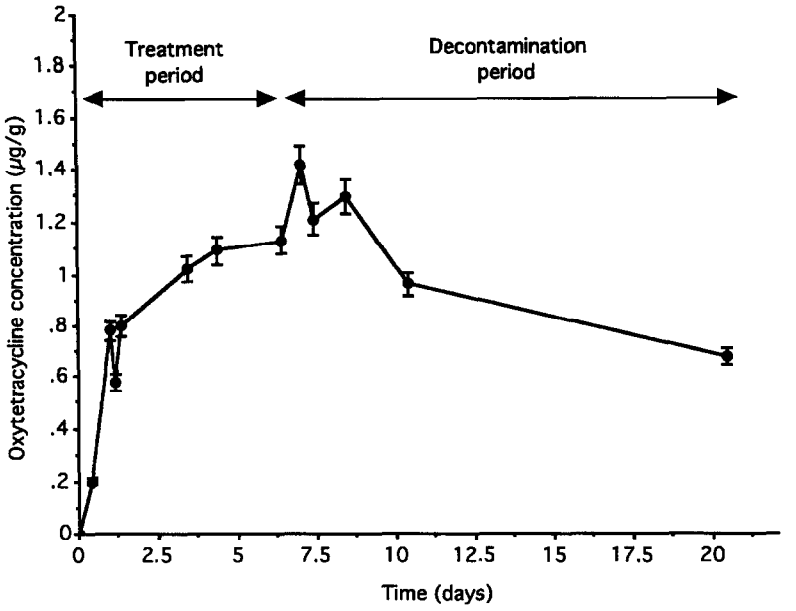

Fig. 3. Uptake and decline of OTC in oyster tissues (*Crassostrea gigas*) from tanks A, B and C after administration of medicated feed in the raceway. Each data point represents the average concentration of the samples assayed from oysters from the three tanks on the sampling day ± standard deviation. For further details, see text.

OTC from the oyster samples stored at −20°C fall within the average limits. Moreover, there was no significant decrease in the OTC recovery from oysters maintained at −20°C for 6 weeks.

The results of the experimental study are shown in Fig. 3. The OTC concentrations in oysters increased to reach a peak 7 days after the beginning of the antibiotic treatment and then decreased slowly. Fourteen days after the end of the antibiotic treatment, the OTC concentration in oysters was still high (average 0.680 μg/g). The OTC concentrations in oysters from tank T were always below the limit of detection of the method.

The main results of the procedure that allowed the efficacy of the analytical protocol to be ascertained showed the method to have good specificity, sensitivity, precision and accuracy. Because the clean-up procedure only involved filtration, centrifugation and solid-phase extraction, the method is simple, rapid and not too expensive. An analyst familiar with the method could easily process fifteen samples a day. In conclusion, the proposed HPLC method is an efficient and reliable means for pharmacokinetic and residue studies on OTC in marine shellfish.

ACKNOWLEDGEMENTS

We thank Ms. Michaëlle Larhantec for technical assistance. This research was supported by a grant from the Regional Council of Pays-de-la-Loire and the General Councils of Loire-Atlantique and Vendée and conducted with the experimental facilities of the Ifremer Aqualive Laboratory (Noirmoutier, France) in collaboration with the Department of Aquaculture of the National Veterinary School of Nantes (France).

REFERENCES

1 J. P. Cravedi, G. Choubert and G. Delous, *Aquaculture*, 60 (1987) 133–141.
2 H. Pouliquen, H. Le Bris and L. Pinault, *Aquaculture*, in press.
3 H. Pouliquen, H. Le Bris and L. Pinault, *Mar. Ecol. Prog. Ser.*, in press.
4 Y. Onji, M. Uno and K. Tanigawa, *J. Assoc. Off. Anal. Chem.*, 64 (1984) 1135–1137.
5 I. Nordlander, H. Johnsson and B. Osterdahl, *Food Addit. Contam.*, 4 (1987) 291–296.
6 H. Björklund, *J. Chromatogr.*, 432 (1988) 381–387.
7 J. Murray, A. MacGill and R. Hardy, *Food Addit. Contam.*, 5 (1988) 77–83.

8 A. Rogstad, V. Hormazabal and M. Yndestad, *J. Chromatogr.*, 11 (1988) 2337–2347.
9 K. Einvindvik and K. E. Rasmussen, *J. Liq. Chromatogr.*, 12 (1989) 3061–3071.
10 B. Iversen, A. Aanesrud and A. K. Kolstad, *J. Chromatogr.*, 493 (1989) 217–221.
11 R. Ueno, K. Uno, S. S. Kubota and Y. Horiguchi, *Nippon Suisan Gakkaishi*, 55 (1989) 1273–1276.
12 A. R. Long, L. S. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 864–867.
13 T. Agasoster and K. E. Rasmussen, *J. Chromatogr.*, 570 (1991) 99–107.
14 R. G. Aoyama, K. M. McErlane and H. Erber, *J. Chromatogr.*, 588 (1991) 181–186.
15 W. H. H. Farrington, J. Tarbin, J. Bygrave and G. Shearer, *Food Addit. Contam.*, 8 (1991) 55–64.
16 H. Oka, H. Matsumoto, K. Uno, K. Harada, S. Kadowaki and M. Suzuki, *J. Chromatogr.*, 325 (1985) 265–274.
17 D. J. Fletouris, J. E. Psomas and N. A. Botsoglou, *J. Agric. Food Chem.*, 38 (1990) 1913–1917.
18 J. H. Knox and J. Jurand, *J. Chromatogr.*, 186 (1979) 763–782.
19 J. R. Lang and S. Bolton, *J. Pharm. Biomed. Anal.*, 9 (1991) 357–361.
20 J. R. Lang and S. Bolton, *J. Pharm. Biomed. Anal.*, 9 (1991) 435–442.