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Assay of urinary 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ by gas chromatography–tandem mass spectrometry

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ABSTRACT

Prostacyclin (PGI_2), an important determinant of cardiovascular biology, is produced from arachidonic acid by endothelial cells. Measurement of its stable urinary metabolite, 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ (PGI_2-M), is the approach of choice to assess variations of the endogenous synthesis of PGI_2 that occur in response to dietary, pharmacological and pathological alterations. We developed a relatively simple stable isotope dilution assay for PGI_2-M which involves solid-phase extraction of 10 ml of urine with Chem Elut disposable columns, water/solvent partitioning from basic and acidic environments with ethyl acetate and methylene chloride, derivatization to 1-pentafluorobenzyl ester followed by TLC, methoximation and trimethylsilylation. Quantification was achieved, for the first time for PGI_2-M, by capillary GC–electron capture negative ion MS–MS with a triple quadrupole mass spectrometer operated in the negative ion detection mode with methane as moderating gas. The mean inter-assay R.S.D., determined on 12 different urine samples, was 5.1 (range 0.4% to 10.5%). The excretion of PGI_2-M in 34 healthy male subjects (age 26 to 57) was 156.2 ± 65.2 (mean ± S.D.) ng/24 h.

INTRODUCTION

Prostacyclin (PGI_2) is a member of the arachidonic acid cascade through the cyclooxygenase pathway [1,2]. Besides being a major endogenous vasodilator, PGI_2 is a powerful inhibitor of platelet aggregation and an important regulator of vascular cholesterol content [3–5]. The amount of endogenously synthesized prostacyclin relative to the synthetic level of its antagonist, thromboxane A_2 (TXA_2), is considered a useful indicator of platelet activity and atherothrombotic tendency [6,7].

The synthesis of prostacyclin can be altered by dietary manipulations and by certain drugs. The need to quantify these changes in clinical and pharmacological studies prompted several investigators to develop analytical procedures based on the measurement of urinary 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ (PGI_2-M), the major metabolite of PGI_2 (Fig. 1), in 24-h urine [8–13]. Here we present a reliable and relatively simple method that utilizes, for the first time in the analysis of PGI_2-M, tandem mass spectrometry. We decided to develop this method after two representative published procedures [11,13] not based on immunoaffinity extraction (*vide infra*), in our hands, proved to be unsatisfactory.

EXPERIMENTAL

Materials

We purchased the following materials from the indicated vendors: Chem Elut CE1020 cartridges, Varian Associates (Harbor City, CA, USA); pentafluorobenzyl bromide (PFBBr) and

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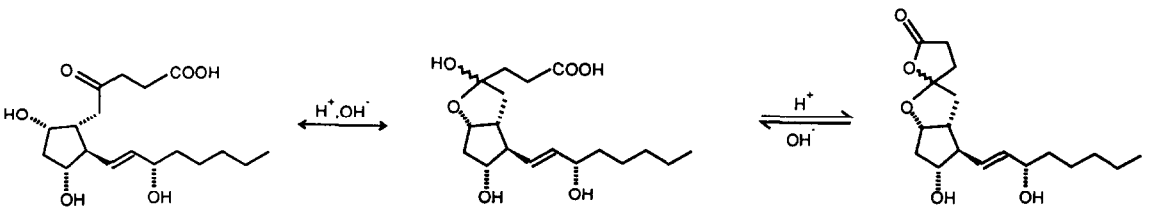

Fig. 1. 2,3-Dinor-6-oxo prostaglandin $F_{1\alpha}$ in the free acid, hemiketal and lactone forms.

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Pierce Chemical Co. (Rockford, IL, USA); methoxylamine hydrochloride, Aldrich Chemical Co. (Milwaukee, WI, USA); silica gel G 5 cm × 20 cm TLC plates, 500 μm thickness, Analtech (Newark, DE, USA). 2,3-Dinor-6-oxo prostaglandin $F_{1\alpha}$ and the internal standard, 2,3-dinor-6-oxo [19,19,20,20-$^2H_4$]prostaglandin $F_{1\alpha}$, were gifts from Dr. John Pike, The Upjohn Company (Kalamazoo, MI, USA). Solvents were analytical grade and distilled in glass. All analytical operations were carried out in silanized glassware.

Urine samples

Urine samples were collected in ice-cold silanized glass bottles. After the 24-h collections were completed, measured portions were stored at −22°C until analyzed.

Analytical procedure

All the analyses were done in duplicate as described below.

Twenty milliliters of urine was diluted with 10 ml of water, spiked with 10 ng of 2,3-dinor-6-oxo [19,19,20,20-$^2H_4$]$PGF_{1\alpha}$ internal standard and acidified to pH 3 with dilute hydrochloric acid. After standing 1 hour at room temperature, equal volumes of acidified urine (15 ml each aliquot) were loaded onto two Chem Elut CE1020 cartridges. After 3–5 min, 40 ml of methylene chloride–ethyl acetate (4:1, v/v) were passed through each column. Each residue obtained from solvent evaporation was treated with 150 μl of methanol, 4 ml of alkalized (NaOH) water (pH 11–12) and the pH was adjusted to 10 if necessary to ensure complete delactonization. After standing for 15 min, the solutions were shaken with 5 ml of ethyl acetate for 60 s. The top phases obtained after centrifuging were discarded and the pH of the lower phases was adjusted to 3 with 0.1 *M* hydrochloric acid. After 15 min the acidified solutions were shaken with 5 ml of methylene chloride for 60 s, then centrifuged at 500 *g* for 2 min. The top phases were discarded; the lower (organic) phases were evaporated, and the residues were treated with 3 drops of triethylamine–pyridine–water (1:10:10, v/v/v) to bring about opening of the lactone ring. After stirring, the solutions were heated at 55°C for 30 min, then treated with 3 drops of ethanol and evaporated to dryness under a stream of dry nitrogen. The residues were treated with 20 μl of diisopropylethylamine and 20 μl of 35% penta-fluorobenzyl bromide in acetonitrile and heated at 40°C for 30 min.

The residues from solvent evaporation were dissolved in 25 μl of methanol and placed on two silica gel G TLC plates. The plates were developed for 45 min (15–16 cm) with ethyl acetate–2,2,4-trimethylpentane (4:1, v/v) saturated with 0.1% aqueous acetic acid. Two-cm bands were scraped off where indicated by a reference plate (R_F *ca.* 0.3). Silica was extracted with 3 ml of 10% methanol in ethyl acetate, the solutions were evaporated to dryness and the residues were treated with 3 drops of a saturated solution of methoxylamine hydrochloride in pyridine. After heating at 40°C for 60 min, the crystalline residues obtained after thorough evaporation of pyridine were extracted 3 times with diethyl ether. The ether extracts were evaporated, the residues were treated with 20 μl of BSTFA in pyridine (1:1, v/v), and heated at 40°C for 15 min. The dry residues obtained after evaporation were dissolved in 20 μl of 5% BSTFA–pyridine (1:1, v/v) in 2,2,4-tri-

methylpentane and were thus ready for injection into the GC–MS system. Typically, 2-μl portions at a time were injected.

Gas chromatography–tandem mass spectrometry

Gas chromatography was done with a Varian 3400 instrument operated in the splitless mode with a 30 m × 0.25 mm I.D. DB-1 (J. and W. Scientific, Rancho Cordova, CA, USA) capillary column, phase thickness 0.25 μm. Injector temperature was 250°C. The oven was kept at 100°C for 1.0 min after injection, then it was heated to 300°C at a rate of 27°/min and held at 300°C for 10 min. The chromatograph was coupled to a Finnigan-MAT TSQ-70B mass spectrometer operated in the negative ion detection mode with methane used as moderating gas. The interface temperature was 300°C and the ion source temperature 150°C. Methane was supplied at a pressure of 932 Pa, argon collision cell pressure 0.13 Pa, collision energy 35 eV, electron energy 70 eV, emission current 0.2 mA. For MS–MS analysis we used the pair of daughter fragments at *m/z* 240 and *m/z* 244 [$P^- - (3 \times TMSOH + CH_3OH + CO_2)$] of the parent ions at *m/z* 586 and *m/z* 590 ($[M\text{-}PFB]^-$).

RESULTS

Mass spectra

Fig. 2 shows the daughter ion mass spectra of the two isomeric (*syn* and *anti*) derivatives of 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$.

Standard curves

Standard curves were developed by injecting increasing amounts (0–1 ng) of 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ in the presence of a constant amount (0.5 ng) of 2,3-dinor-6-oxo [19,19,20,20-$^2H_4$]prostaglandin $F_{1\alpha}$ internal standard, and by plotting the peak-area ratio analyte/internal standard against the amount of analyte injected. A typical linear regression curve followed the equation $y = 0.921x + 0.035$ ($r = 0.9977$).

Precision

The precision of the method was assessed by analyzing four identical 10-ml samples of urine. The mean concentration was 139.6 ± 4.4 (S.D.) pg/ml and the R.S.D. was 3.2%. When an identical test was conducted with urine from another donor, the mean concentration was 206.7 ± 22.8 (S.D.) and the R.S.D. was 11.0%. Precision was further tested by duplicate analysis of 12 urine samples from 12 different donors. The mean inter-assay R.S.D. was 5.1% (range 0.4 to 10.5%) (Table I). The lower quantification limit of the method is 50 pg/10 ml of urine.

Validation of accuracy

Accuracy of the assay system was validated by determining nanograms of PGI_2-M recovered *vs.* nanograms of PGI_2-M added before extraction. In a typical experiment, 40 ml of urine was spiked with 20 ng of the internal standard and then divided into four 10-ml portions. Increasing amounts (0, 100, 300, 500 pg/ml) of unlabeled analyte were added and all four aliquots were analyzed as described above. Results are shown in Table II.

Effect of fish oil ingestion on 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ excretion

The mean 24-h excretion rate of 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ during a 72-h period was measured in five healthy male adults before and after administration of 15 g/day of an anchovy oil supplement for ten weeks [14]. Before ingestion of the oil the excretion rates were 87, 203, 164, 202, and 164 ng/24 h; after fish oil ingestion they dropped to 76, 117, 152, 144, and 108 ng/24 h, respectively ($P = 0.0001$).

DISCUSSION

Efforts to develop a sensitive and reliable method for the gas chromatographic–mass spectrometric quantification of PGI_2-M in human urine have lasted more than a decade [8–13]. With a view toward studying the effect of dietary manipulations on the endogenous synthesis of prostacyclin, we evaluated two published procedures [11,13]. Those two methods, in our hands, could not consistently produce R.S.D.s low enough to meet reliability criteria and/or acceptable recovery values. This prompted us to develop the procedure described here.

The initial step consists of urine extraction

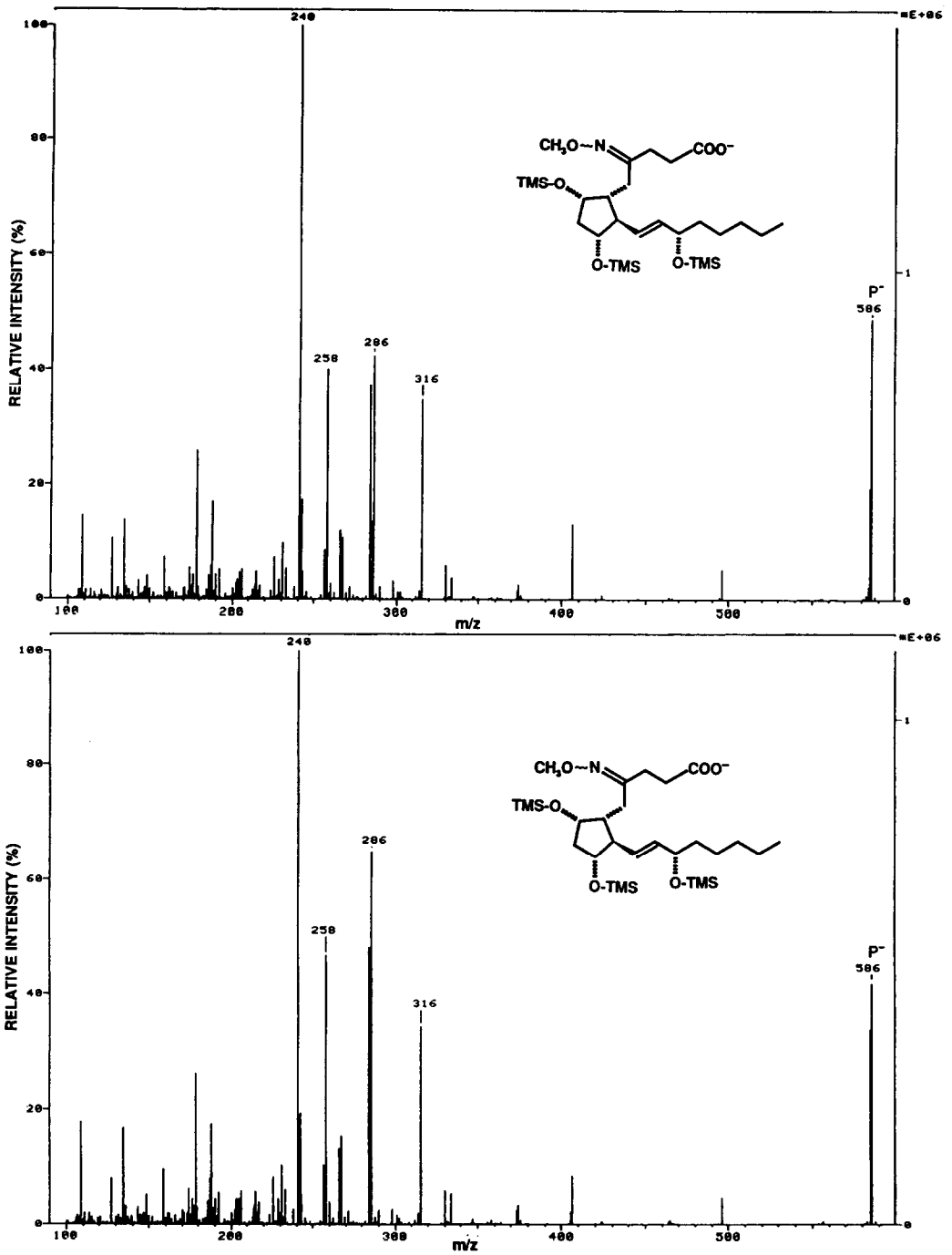

Fig. 2. Daughter ion mass spectra of the $[M\text{-}PFB]^-$ parent ion $P^-(m/z\ 586)$ of the major (upper panel) and minor (lower panel) (*syn*/*anti*) isomers of derivatized 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$.

TABLE I

ASSAY REPRODUCIBILITY WITH URINE SAMPLES FROM TWELVE SUBJECTS AND DUPLICATE DETERMINATIONS

Subject	Concentration (pg/ml)		R.S.D. (%)
	Individual values	Mean ± S.D.	
1	78.5 78.9	78.7 ± 0.3	0.4
2	186.1 179.0	182.6 ± 5.0	2.7
3	87.0 81.3	84.2 ± 4.0	4.8
4	210.0 198.0	204.0 ± 8.5	4.2
5	230.0 267.0	248.5 ± 26.2	10.5
6	230.0 224.0	227.0 ± 4.2	1.9
7	162.8 164.2	163.5 ± 1.0	0.6
8	94.3 87.8	91.1 ± 4.6	5.0
9	64.9 56.1	60.5 ± 6.2	10.3
10	95.5 91.5	93.5 ± 2.8	3.0
11	157.8 152.1	154.9 ± 4.0	2.6
12	61.5 62.8	62.1 ± 0.9	1.5
Mean			5.1

with Chem Elut which was first introduced by Martineau and Falardeau [11] (Chem Elut was then called Clin Elut). The remainder of the assay diverges from all published procedures, and for the first time we introduced the use of tandem mass spectrometry for the quantification of PGI_2-M. The ready lactonization of the analyte at low pH (Fig. 1) was utilized to pursue removal of matrix components by means of two consecutive extractions, one at pH 10 (ethyl acetate) and one at pH 3 (methylene chloride). Fig. 3 shows typical daughter ion chromatograms of derivatized endogenous 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ and of its tetradeuterated analog obtained from a urine extract.

The R.S.D. between two or more determinations is usually below 5% (Table I). Occasionally, however, it reaches considerably higher values for no apparent reason. We did not observe a similar phenomenon in the analysis of other eicosanoids. The unique problem with PGI_2-M might be related to the formation of a new asymmetric center at C_6 when the hemiketal is formed (Fig. 1). We recommend routine duplicate determinations to help avoid the consequences of this potential pitfall. The dual peaks shown in both panels of Fig. 3 correspond to the two isomeric (*syn/anti*) methoxime derivatives. For purposes of quantification, the peak areas of either the minor or the major isomers from the $^2H_0$ and the $^2H_4$ species can be used. The sums of the areas can also be used, and in all cases quite similar results are usually obtained.

The quantities of unlabeled analyte used to spike the urine in our recovery experiments were based on realistic expectations about the amount of PGI_2-M that would be present in most urine

TABLE II

RECOVERY EXPERIMENT CONDUCTED WITH FOUR 10-ml URINE SAMPLES

PGI_2-M added (ng)	Found (mean ± S.D.) (ng)	Recovery (ng)	Relative recovery (%)
0	1.23 ± 0.04	–	–
1	1.95 ± 0.01	0.72	72.0
3	3.91 ± 0.14	2.68	89.3
5	5.93 ± 0.01	4.70	94.0
Mean relative recovery			85.1

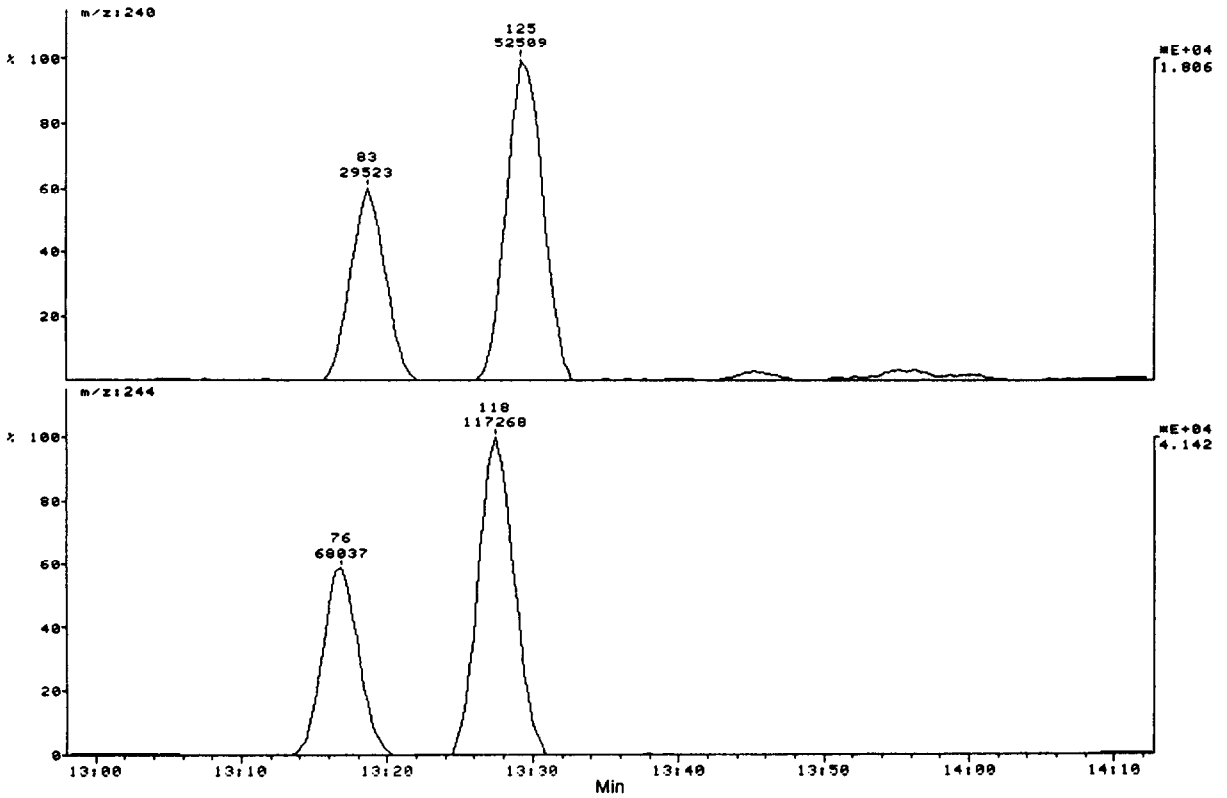

Fig. 3. Typical daughter ion chromatograms of derivatized endogenous PGI_2-M (upper trace, m/z 240) and [$^2H_4$]PGI_2-M (lower trace, m/z 244) from a urine extract.

samples (Table II). Under these conditions, we were not consistently able to run successful recovery experiments by simple electron capture negative ion chemical ionization mass spectrometry using the $[M\text{-}PFB]^-$ ion. This circumstance compelled us to introduce the tandem mass spectrometry approach. Chiabrando *et al.* [12] demonstrated the superior specificity of immunoaffinity extraction over solid-phase or solvent extraction in the analysis of several eicosanoids with favorable implications in the overall reliability of the assays. However, when the immunoaffinity technology is not available, tandem mass spectrometry appears to be a necessity.

Electron impact tandem mass spectrometry was used (for the first time in prostanoid analysis) by Schweer *et al.* [15] for the quantification of the nonenzymatic hydration product of prostacyclin, 6-oxo prostaglandin $F_{1\alpha}$. Very recently Blair *et al.* [16] described a procedure for the analysis of PGI_2-M by gas chromatography–electron capture negative ion chemical ionization mass spectrometry utilizing ^{18}O-2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ as internal standard. The inter-assay R.S.D., based on six determinations, was reported as 9%.

Although our procedure consists of several steps, up to twenty samples per working day can be prepared for GC–MS analysis by a single, relatively unskilled operator. The procedure is a suitable tool for clinical studies which are often associated with high sample volumes. The 2,3-dinor-6-oxo prostaglandin $F_{1\alpha}$ excretion rate in 34 healthy male subjects (age 26 to 57) was 156.2 ± 65.2 ng/24 h (mean ± S.D.) as determined in 72-h urine pools.

REFERENCES

1 S. Fischer, *Adv. Lipid Res.*, 23 (1989) 169.
2 W.L. Smith, *Biochem J.*, 259 (1989) 315.

3 G.J. Dusting, S. Moncada and J.R. Vane, *Prostaglandins*, 13 (1977) 3.
4 R.R. Gorman, *Fed. Proc.*, 38 (1979) 83.
5 K.B. Pomerantz and D.P. Hajjar, *Arteriosclerosis*, 9 (1989) 413.
6 J. Dyerberg and K.A. Jorgensen, *Prog. Lipid Res.*, 21 (1982) 255.
7 S. Fischer and P.C. Weber, *Prostaglandins*, 32 (1986) 235.
8 P. Falardeau, J.A. Oates and A.R. Brash, *Anal. Biochem.*, 115 (1981) 359.
9 O. Vesterqvist and K. Gréen, *Prostaglandins*, 28 (1984) 139.
10 C. Fischer and C.O. Meese, *Biomed. Mass Spectrom.*, 12 (1985) 399.
11 A. Martineau and P. Falardeau, *J. Chromatogr.*, 417 (1987) 1.
12 C. Chiabrando, V. Pinciroli, A. Campoleoni, A. Benigni, A. Piccinelli and R. Fanelli, *J. Chromatogr.*, 495 (1989) 1.
13 C. Weber, M. Höller, J. Beetens, F. de Clerck and F. Tegtmeier, *J. Chromatogr.*, 562 (1991) 599.
14 A. Ferretti, J.T. Judd, R. Ballard-Barbash, P.P. Nair, P.R. Taylor and B.A. Clevidence, *Lipids*, 26 (1991) 500.
15 H. Schweer, H.W. Seyberth and R. Schubert, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 611.
16 I.A. Blair, C. Prakash, M.A. Phillips, R.M. Dougherty and J.M. Iacono, *Am. J. Clin. Nutr.*, 57 (1993) 154.