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Utilization of high-performance liquid chromatography as an enrichment step for the determination of cyclic fatty acid monomers in heated fats and biological samples

J.L. Sebedio*, J. Prevost, E. Ribot and A. Grandgirard

INRA, Station de Recherches sur la Qualité des Aliments de l'Homme, Unité de Nutrition Lipidique, 17 Rue Sully, 21034 Dijon Cedex (France)

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ABSTRACT

A method was developed to determine traces of cyclic fatty acid monomers (CFAM) in oils and animal tissues. This method is a combination of some techniques developed earlier but with the enrichment step being achieved by high-performance liquid chromatography (HPLC) instead of urea inclusion. After transformation of the lipids into methyl esters, the latter were hydrogenated after addition of an internal standard (methyl heptadecanoate or ethyl hexadecanoate). The mixture was enriched in CFAM by HPLC on a semi-preparative C_{18} reversed-phase column using acetonitrile–acetone (90:10, v/v) at 4 ml/min. The enriched fraction containing the CFAM and the internal standard was then analysed by gas chromatography on a polar column (cyanosilicone phase). This method was developed using known mixtures of CFAM isolated from both heated sunflower and linseed oils. Small amounts of CFAM (50 μg/g of sample) were determined with good reproducibility without any loss during the HPLC enrichment step and with no modification of the relative proportions of the CFAM in the mixture. This method can be applied to either heated fats and oils or biological samples (heart cell culture) that contain only traces of CFAM. Ethyl hexadecanoate (16:0 ethyl ester) can be used as an internal standard for samples containing small amounts of 17:0.

INTRODUCTION

Among the components of oils that are formed during deep-fat frying [1,2] are cyclic fatty acid monomers (CFAM), which have shown potential toxicity in some cases [3–7]. The structures of these cyclic fatty acids are different if they are formed from linoleic rather than linolenic acids [8–10]. Those formed from linoleic acid are mainly C_{18} monounsaturated acids having a five-carbon α-disubstituted ring, whereas those arising from linolenic acid are a mixture of C_{18} diunsaturated fatty acids having a five- or a six-carbon α-disubsituted ring. The rat has been widely used as a model to study the potential toxicity of heated fats, and we have recently shown that heart cell in culture is a good model to follow the incorporation of CFAM in phospholipids [11]. However, the amounts incorporated into the phospholipids of tissues are small, and it has been very difficult to determine the CFAM very precisely with existing analytical methods [7]. All the methods are based on gas chromatography of the totally hydrogenated fatty acid methyl esters [7,12]. After hydrogenation, the sample to be analysed usually consists of a mixture of straight-chain saturates (mainly 16:0, 18:0, 20:0 and 22:0) and the hydrogenated CFAM. There are basically two methods for their determination. One is a direct GC analysis of the hydrogenated sample [12,13] and the other is the use of an enrichment step prior to

* Corresponding author.

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GC analysis. This enrichment step is usually carried out by low-temperature crystallization [14] or by urea adduction [15]. Both methods (with or without enrichment steps) present major drawbacks, and Gere *et al.* [16] have shown that discrepancies exist when determining CFAM using the different methods.

Methods using an enrichment step are frequently used [7]. However, urea adduction or low-temperature crystallization does not completely eliminate 18:0 so that some CFAM are eluted in the tailing 18:0 peak and, further, the utilization of urea for enrichment in CFAM results in a loss of about 20% of the cyclic fatty acid during complexation [7]. The problem could be partly resolved by using phenanthrene as an internal standard instead of methyl heptadecanoate (17:0). Phenanthrene would behave in a similar way to cyclic monomers [16]. Further, we have found that the utilization of urea for small sample size brings out many impurities which can be detected during GC analyses. A clean-up procedure was developed by Rojo and Perkins [17] in order to remove interfering substances that co-elute with the CFAM during GC analysis.

We have therefore developed a method to determine small amounts of CFAM in oils and biological samples using a different enrichment step. This method is a combination of hydrogenation of the total methyl esters on PtO_2, enrichment in CFAM using high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column followed by analysis by gas chromatography coupled with mass spectrometry (GC–MS). This method is very reproducible for amounts of CFAM as low as 50 μg/g of lipid sample.

EXPERIMENTAL

Standards

Methyl octadecanoate and heptadecanoate and ethyl hexadecanoate were purchased from Sigma Chimie (La Verpillère, France).

Isolation of cyclic fatty acid monomers

Two types of CFAM were isolated using methods described elsewhere [8]. Those arising from linoleic acid were isolated from sunflower oil heated at 275°C for 12 h under nitrogen, whereas those arising from linolenic acid were prepared from linseed oil heated under the same conditions. Briefly, the heated oils were saponified and converted into fatty acid methyl esters using sulphuric acid as catalyst. The total fatty acid methyl esters were fractionated by column chromatography and the non-polar fraction was submitted to urea fractionation as described previously [18]. For CFAM isolated from the heated sunflower oil, the non-adduct fraction which contained a mixture of CFAM and 18:2 $n-6$ ($n-6$ represents the position of ethylenic bonds on the carbon chain) was further purified by preparative liquid chromatography as described previously [8] using a reversed-phase column (Waters, Milford, MA, USA) (30 cm × 5.7 cm I.D.) and acetonitrile–water (90:10) as the eluent at a flow-rate of 150 ml/min.

Hydrogenation of CFAM

Hydrogenation was affected using PtO_2 (Merck, Darmstadt, Germany) as catalyst [12] in 10 ml of chloroform–methanol (2:1, v/v) as solvent and with a hydrogen pressure of 3–4 bar. The reaction was allowed to proceed for 4 h. The catalyst was removed by filtration.

High-performance liquid chromatography

HPLC analyses were carried out on a C_{18} reversed-phase column (Merck, LiChrosorb) (25 cm × 7 mm I.D., particle size 5 μm) using a Waters R 410 refractive index detector. The sample (up to 40 mg) was dissolved in acetone. The solvent systems used were either acetonitrile–acetone (90:10, v/v), pure acetonitrile or pure methanol at 4 ml/min, depending on the separation tested. A Nova Pak C_{18} column (Waters) (10 cm × 8 mm I.D., particle size 4 μm) was used for smaller amounts (up to 1 mg) with the same solvent systems tested at 1.6 ml/min.

Gas chromatography

GC analyses were effected on a Intersmat IGC 120 FL chromatograph (Delsi, Argenteuil, France) fitted with a flame ionization detector and a Ross injector. The analyses were performed on capillary columns coated with CP-Sil

84 at 180°C (Chrompack, Middelburg, Netherlands) (50 m × 0.25 mm I.D., film thickness 0.2 μm). All quantitative analyses were carried out using a Spectra-Physics (San Jose, CA, USA) Chromjet integrator.

Gas chromatography–mass spectrometry

All GC–MS analyses were performed using a DB-Wax column (J&W Scientific, Folsom, CA, USA) (30 m × 0.25 mm I.D., film thickness 0.5 μm) and a Hewlett-Packard (Palo Alto, CA, USA) HP-5970 mass-selective detector. The chromatographic conditions were similar to those already published for the analyses of CFAM [10]. The temperature was programmed from 50 to 200°C at 20°C/min, held at 200°C for 25 min, then programmed from 200 to 220°C and held at 220°C until completion of the analyses. Splitless injection was used in all instances, and the injection port was maintained at 240°C.

Reproducibility of the method

The reproducibility of the method was checked by preparing a solution that contained 30 mg of 18:0, 1.5 μg of CFAM isolated from a heated linseed oil and 1.5 μg of 17:0 as internal standard. A similar mixture containing the CFAM isolated from a heated sunflower oil was also studied. Each sample was run five times through the HPLC system, followed by GC analysis, using 17:0 as an internal standard, to determine the reproducibility of the method.

Determination of CFAM in spiked rat liver lipids

Liver lipids of Wistar rats were extracted according to the method Folch *et al.* [19] and converted into the methyl esters [20]. Two identical mixtures were then prepared. CFAM (2.5 μg) isolated from heated linseed oil and 16:0 EE (EE = ethyl ester) (2.5 μg) were added to the liver lipid methyl esters (50 mg). The mixtures were totally hydrogenated. One hydrogenated mixture was submitted to HPLC fractionation and the other to urea adduction [18], and the non-urea adduct fraction which contained the CFAM was analysed by GC on a CP-Sil 84 column (see above).

Heart cell culture

The culture medium was Ham's F10 basal medium supplemented with 10% foetal bovine serum (Seromed, Munich, Germany) and 10% human serum (CTS, Dijon, France). CFAM in ethanol was added to the medium using lipid-free bovine serum albumin (fraction V) (Sigma, St. Louis, MO, USA) at 37°C, at a CFAM-to-albumin molar ratio of 6:1 [21]. The medium containing the CFAM was then sterilized by filtration (Millex GS, 0.22 μm; Millipore, Milford, MA, USA).

Primary cultures of rat ventricular cells were prepared as described previously [22]. The hearts from 2–4-day-old rats were aseptically removed, minced and washed three times in a cold Saline G solution and once again in the same solution for 10 min at 30°C in a shaking water-bath. The fragments were then submitted to a seven-step trypsinization process. The supernatants of the last six proteolytic treatments were pooled and diluted in culture medium. The muscle to non-muscle cell ratio was increased by a two-step (30- and 150-min) selective adhesion procedure. The final cell suspension was diluted to $4 \cdot 10^5$ cells/ml in culture medium and seeded in 60-mm plastic Petri dishes (5 ml per dish). Cultures were maintained at 37°C in a humidified atmosphere (5% CO_2, 19% O_2, 76% N_2). The culture medium was renewed 24 h after seeding and thereafter every 48 h.

Cells were incubated in the CFAM-containing solutions (2.5, 5 or 10 mg/l) for 2 days. Cells were then harvested by scraping with a rubber "policeman" and pelleted by centrifugation.

Total lipids were extracted from the medium according to the method of Folch *et al.* [19] slightly modified [11].

Phospholipids were separated from non-phosphorus lipids using a Sep-Pak silica cartridge (Waters) as described by Juaneda and Rocquelin [23]. The lipids were converted into methyl esters using BF_3–MeOH according to Morrison and Smith [20].

RESULTS AND DISCUSSION

An ideal method should permit the CFAM to be determined after total elimination of the 18:0

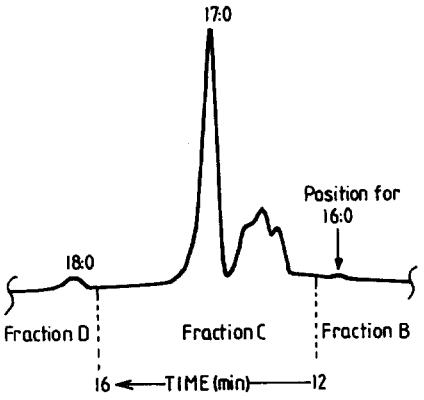

Fig. 1. HPLC fractionation of a mixture of 17:0 and hydrogenated CFAM isolated from a heated linseed oil. Eluent: acetonitrile–acetone (90:10, v/v) at 4 ml/min.

as some CFAM have retention times close to that of 18:0 on either polar or non-polar phases [15,24]. Unfortunately, this is not the case when using urea adduction as the enrichment step [24,25]. This elimination of 18:0 should also be done without any selective loss of CFAM and the method should allow verification of the compounds detected as CFAM and not artefacts having similar retention times. The present method is a combination of esterification, total hydrogenation, isolation of the CFAM by HPLC and GC or GC–MS analyses of the isolated fraction.

The HPLC procedure was developed in order first to isolate in the same fraction both the internal standard and the CFAM, and second to separate them from the major straight-chain saturated fatty acid methyl esters formed after hydrogenation such as methyl hexadecanoate

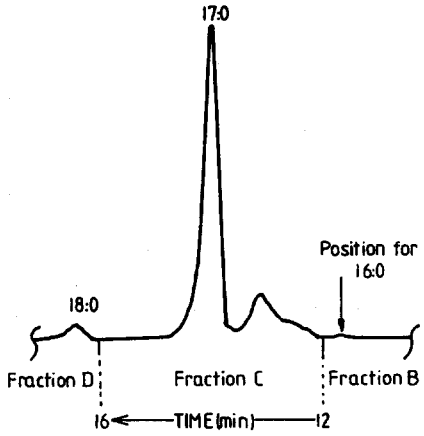

Fig. 2. HPLC fractionation of a mixture of 17:0 and hydrogenated CFAM isolated from a heated sunflower oil. Eluent: acetonitrile–acetone (90:10, v/v) at 4 ml/min.

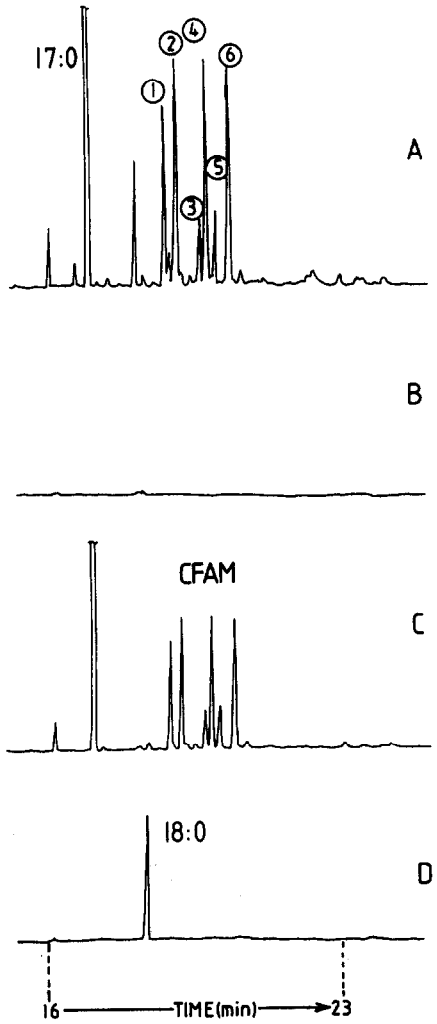

Fig. 3. GC analyses (CP-Sil 84) of (A) a mixture of 17:0 and hydrogenated CFAM isolated from a heated linseed oil and (B, C and D) of the fraction collected by HPLC (Fig. 1). For peak identification (1–6) see CFAM from linseed oil in Table I.

(16:0) and 18:0. This was possible (Figs. 1 and 2) using a C_{18} reversed-phase (semi-preparative) column and acetonitrile–acetone (90:10, v/v) as eluent at 4 ml/min. Both the CFAM isolated from linseed and sunflower oils [8] have the same retention volume under these experimental conditions. This is very important as these are representative of CFAM formed from the two major polyunsaturated fatty acids of vegetable oils, *i.e.*, linolenic and linoleic acids. The fraction collected between 16:0 and 18:0 would therefore contain both the internal standard and the CFAM. Of all the solvents tested, acetonitrile–acetone (90:10, v/v) gave a good compromise between total separation and a short analysis time. The separation can also be effected using analytical columns using the same solvent mixture at 1.6 ml/min; 1 mg would be the maximum amount injected. However, for GC–MS studies it is often necessary to have appreciable amounts of CFAM, so it is better to carry out the separation on a semi-preparative column where the maximum amount of sample injected would be between 25 and 30 mg.

In this method, the internal standard (17:0) is added to the sample before esterification and hydrogenation. Any loss of material during these steps would not affect the determination. It is also possible to use a triglyceride as an internal standard (triheptadecanoin). For samples that may contain appreciable amounts of 17:0, it is possible to use ethyl hexadecanoate (16:0 EE). It is necessary to add it after esterification and just prior to hydrogenation in order to avoid its conversion into the methyl ester. For the HPLC fractionation, 16:0 EE has a retention volume between those of CFAM and 17:0.

In order to verify that the GC profile of CFAM was not modified after the HPLC step, a mixture of CFAM isolated from a heated linseed oil and of 17:0 as internal standard was fractionated by HPLC. The mixture was first analysed by GC (Fig. 3A) and by GC–MS (Table I). Peak identifications were made by comparison of their mass spectra and GC retention times on CP-Sil 84 and DB-Wax columns with those of authentic standards synthesized previously [26]. This sample was submitted to HPLC and three fractions (B, C and D, Fig. 1) were collected and further analysed by GC (Fig. 3). The first fraction was collected from the solvent peak to the end of the retention volume of 16:0 (fraction B). The second fraction was collected from the end of the retention volume of 16:0 to the beginning of the elution of 18:0 (fraction C), and the third fraction (D) corresponded to the elution of 18:0. Fraction B (Fig. 3) did not contain appreciable amounts of any known fatty acids, whereas fraction C (Fig. 3) contained the CFAM and the internal standard and fraction D (Fig. 3)

TABLE I

MAJOR HYDROGENATED CFAM IDENTIFIED BY GC–MS IN HEATED SUNFLOWER AND LINSEED OILS (275°C, 12 h)

Sample	Peak No.	Component	Configuration
Heated linseed oil (Fig. 3)	1	Methyl 9-(2′-butylcyclopentyl)nonanoate	*trans*
	2	Methyl 10-(2′-propylcyclopentyl)decanoate	*trans*
	3	Methyl 9-(2′-butylcyclopentyl)nonanoate	*cis*
	4	Methyl 9-(2′-propylcyclohexyl)nonanoate	*trans*
	5	Methyl 10-(2′-propylcyclopentyl)decanoate	*cis*
	6	Methyl 9-(2′-propylcyclohexyl)nonanoate	*cis*
Heated sunflower oil (Fig. 4)	1	Methyl 7-(2′-hexylcyclopentyl)heptanoate + methyl 4-(2′-nonylcyclopentyl)butanoate	*trans* *trans*
	2	Methyl 9-(2′-butylcyclopentyl)nonanoate	*trans*
	3	Methyl 7-(2′-hexylcyclopentyl)heptanoate	*cis*
	5	Methyl 9-(2′-butylcyclopentyl)nonanoate + ?	*cis*

contained the 18:0 without any detectable amount of CFAM.

A similar experiment was carried out with the CFAM isolated from a heated sunflower oil (Table I, Fig. 2). The determination of the structures of CFAM 4, 6, 7 and 8 for sunflower oil are still under investigation. The GC analyses of the total CFAM mixture and the internal standard and also the three fractions collected are shown in Fig. 4. Fraction D contained only 18:0 and fraction C contained the CFAM and

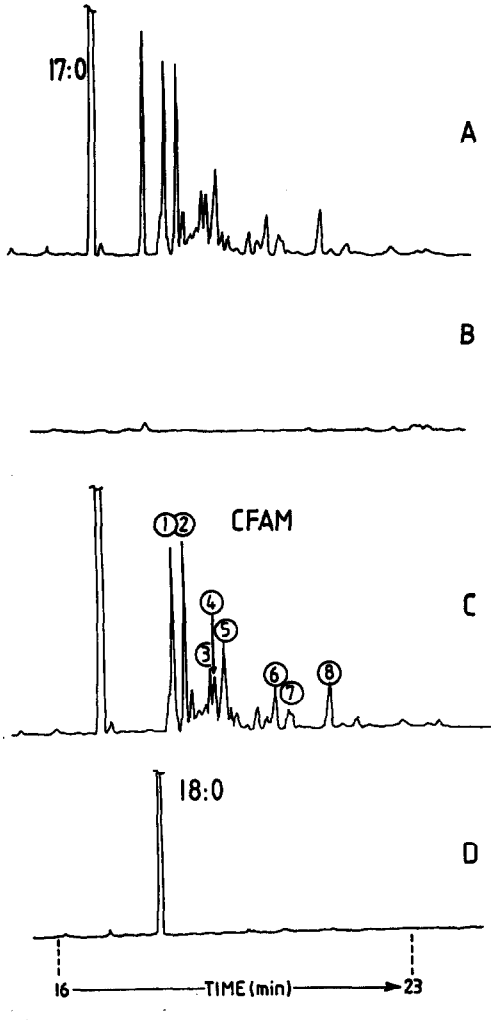

Fig. 4. GC analyses (CP-Sil 84) of (A) a mixture of 17:0 and hydrogenated CFAM isolated from a heated sunflower oil and (B, C and D) of the fraction collected by HPLC (Fig. 2). For peak identification (1–8) see CFAM from sunflower oil in Table I.

17:0. Fraction B showed only trace amounts of components that did not seem to be any known fatty acids. Each fraction was also analysed by GC–MS in order to confirm the structures of the different fatty acids. The relative proportions of the major CFAM obtained while collecting the CFAM fractions were not modified by HPLC (Table II). The data in Table II represent an average of two HPLC runs. Only very small differences were observed.

The results of the reproducibility tests described under Experimental are reported in Table III. Before HPLC, the CFAM represented 50 μg/g of the total sample. After HPLC the values ranged from 43 to 51 μg/g for the CFAM isolated from linseed oil and from 46 to 57 μg/g for those isolated from sunflower oil. The mean values were 49.2 ± 4.4 and 50.0 ± 3.6 μg/g, respectively, which indicated that the method was very reproducible at this low CFAM level. The reason why the reproducibility of the method was checked on a sample containing 50 μg of a mixture of CFAM per gram of sample is that this value seems to be much lower (5–10 times) than the level usually detected in refined commercial oils [15,27,28]. An attempt was made to determine lower concentrations of CFAM in oils. For CFAM isolated from a heated linseed oil (four major and two minor peaks, Fig. 3), one can determine levels as low as 10 μg/g of oil. However, at this level, the relative proportions of the different isomers changed slightly from one quantification to another, and 50 μg/g of such a mixture (six components) should be considered as the minimum optimum level of total CFAM mixture that can be analysed with good reproducibility.

The purpose of this study was to compare the results obtained using HPLC as the enrichment step with those obtained using urea inclusion and to apply this method to biological samples. Fig. 5 shows parts of the GC analyses of the CFAM fraction which was used to spike the sample of rat liver lipids (Fig. 5A), the isolated HPLC fraction (Fig. 5B) and the non-urea adduct fraction (NUAF) (Fig. 5C). The same CFAM profile was obtained when HPLC was used, whereas the GC analysis of the NUAF showed a different profile. GC–MS analysis of this fraction

TABLE II

RELATIVE PROPORTIONS OF CFAM ISOLATED FROM HEATED LINSEED OIL (FIG. 3) AND SUNFLOWER OIL (FIG. 4) BEFORE AND AFTER HPLC RECOVERY

Average of two determinations (HPLC followed by GC).

Sample	Stage	Peak No. (Figs. 3 and 4)							
		1	2	3	4	5	6	7	8
Heated linseed oil	Before HPLC	15.8	22.4	7.0	22.3	9.4	23.1		
	After HPLC	15.8	22.3	6.5	22.5	9.3	23.6		
Heated sunflower oil	Before HPLC	25.5	19.8	7.7	9.2	17.4	7.6	5.1	7.7
	After HPLC	25.9	20.5	8.2	8.5	16.7	7.7	5.1	7.4

also showed the presence of impurities. Further, one could speculate whether preferential urea inclusion of some CFAM could occur when dealing with such small amounts of CFAM (2.5 μg). GC quantitative analysis of the HPLC fraction using 16:0 EE as an internal standard gave 52.6 μg of CFAM per gram of sample, compared with 50.0 μg/g in the mixture prior to fractionation. The HPLC fractionation method was far superior to urea adduction, especially for samples that may contain only traces of CFAM. GC–MS analysis of the fraction collected by HPLC gave spectra similar to those published previously [10].

This method was applied to the determination of CFAM in heart cell cultures. Heart cell cultures have been used in our laboratory to study the biological effects of CFAM [29]. In order to compare the effects of CFAM arising from linoleic acid with those arising from linolenic acid, cultured rat cardiomyocytes were treated with solutions containing CFAM at concentrations of 2.5, 5 and 10 mg/l as described under Experimental. After separation of the non-phosphorus lipids and the phospholipids, the amount of CFAM was determined using the described method. Each result (Table IV) represents the average of three separate cultures (about 100 Petri dishes each). These data show that small amounts of CFAM can be determined in this type of biological sample and that these CFAM can be incorporated into the membrane lipids. Our continuing research will permit the effects of such an incorporation to be investigated [29].

The major advantages of this method over those published previously are the purity of the CFAM fraction, the rapidity and the sensitivity. Also, there is no loss of product during the isolation step (HPLC) and no modification of the ratio of the different types of cyclic fatty acids. We should also emphasize that the analysis of the isolated CFAM fractions should be carried out by GC–MS instead of GC to verify that there are no contaminants in the isolated sample, as already mentioned by Rojo and Perkins [30].

TABLE III

DETERMINATION OF CFAM (μg/g OF SAMPLE) ISOLATED FROM HEATED SUNFLOWER AND LINSEED OILS BEFORE AND AFTER HPLC FRACTIONATION (FIVE INDEPENDENT DETERMINATIONS)

Stage	Heated linseed oil	Heated sunflower oil
Before HPLC	50	50
After HPLC	44, 58, 50, 43, 51 (mean ±S.D. = 49.3 ± 5.4)	46, 46, 49, 52, 57 (mean ±S.D. = 50.0 ± 4.6)

Fig. 5. Parts of the GC analyses (CP-Sil 84) of (A) a mixture of 17:0 and hydrogenated CFAM isolated from a heated linseed oil, (B) the CFAM fraction collected by HPLC of the methyl esters of a spiked lipid extract of rat liver and (C) the non-urea adduct fraction of the same sample.

In the biological field, this method is applicable to animal tissues where the amounts of CFAM incorporated are small.

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TABLE IV

DETERMINATION OF CFAM IN PHOSPHOLIPIDS (PL) AND NON-PHOSPHORUS LIPIDS (NPL) OF CARDIOMYOCYTES INCUBATED IN CFAM-CONTAINING MEDIUM

Results are expressed as μg esters/mg proteins. Average of three separate cultures.

Sample	Concentration of CFAM medium (mg/l)	PL	NPL
Heated linseed oil	2.5	0.5 ± 0.08	0.6 ± 0.05
	5	0.8 ± 0.01	1.1 ± 0.17
	10	1.7 ± 0.14	2.3 ± 0.44
Heated sunflower oil	2.5	1.0 ± 0.07	1.5 ± 0.10
	5	1.5 ± 0.18	3.2 ± 0.11
	10	2.8 ± 0.19	702 ± 1.04

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