*Analusis*, **1999**, *27*, 186-193


# High performance liquid chromatography of fatty acids as naphthacyl derivatives

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**Abstract.** A procedure for the separation of fatty acids, after their derivatization as fatty acid naphthacyl esters, by reverse-phase high performance liquid chromatography is described. A reproducible resolution (50 min), shorter than former HPLC analyses, of a standard mixture of fatty acid naphthacyl esters (25 fatty acids; C7:0 – C22:6 *n* – 3), was achieved by a ternary elution gradient of methanol-acetonitrile-water. Compared with gas chromatography, HPLC analysis of fatty acid naphthacyl esters showed similar percentages of molar distribution of long-chain fatty acids (≥14 carbons). The separation was monotered by UV absorbance at 246 nm, which was highly sensitive (the detection limit was about 0.1 ng), did not destroy the fatty acid naphthacyl esters and thus allowed individual recovery for further analysis (specific radioactivity determination or positional isomer identification). This preparative HPLC method provides, therefore, a useful process for the study of fatty acid metabolism in biological systems.

**Key words.** Fatty acids – fatty acid naphthacyl esters – high performance liquid chromatography – fatty acid metabolism.

## Introduction

Studying fatty acid metabolism in living cells requires not only analytical methods leading to the separation and quantification of each fatty acid, but also preparative methods allowing the recovery of each eluted fatty acid. Numerous fatty acid separation systems based on chromatographic methods (gas chromatography, high performance liquid chromatography) have been developed in the past years [1]. However, none of them seem to be both resolutive and preparative enough to investigate the metabolic bioconversion of fatty acids with physiological significance (biosynthesis, elongation, desaturation, retroconversion and β-oxidation).

Gas chromatography (GC) has been the method of choice for separation and quantification of fatty acids for a long time, usually after their conversion to methyl esters [2,3]. Indeed the use of a capillary column coupled to a flame ionization detector (FID) provides high resolution for fatty acid analysis in complex mixtures, including separation of some positional and geometrical isomers. However, there are several inherent limits to GC methods. First of all, short-chain fatty acid methyl esters are volatile and may be lost on refluxing the esterification medium [1]. Secondly, problems originate from possible thermal degradation and structural modification of unsaturated fatty acids during their conversion to methyl esters [4,5]. Thirdly, the main limit to GC is that FID detection destroys the fatty acids and does not allow the recovery of any separated material for further analysis.

A great advantage of high performance liquid chromatography (HPLC) methods over GC methods is that the resolved fatty acids are not destroyed during their detection, which enables further analyses to be performed. Recently, several HPLC systems for the separation of fatty acids have been reported, especially with biological applications [6,7].

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Received December 08, 1998; revised February 26, 1999; accepted February 26, 1999.



Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:1999165

To enhance the detectability of eluting fatty acids, which are only weakly chromophoric, derivatization with chromophoric groups have been used [8], including methyl esters [9], nitrobenzyl [10], benzyl [11], naphthaliminoethyl esters [12], or phenacyl [13], phenylazophenacyl [14] and naphthacyl esters [15]. The more commonly used derivatization with 2-bromoacetophenone enhanced the sensitivity enough to allow the detection of nanograms of fatty acids in complex biological samples [6,7,13,16,17]. However, compared with GC methods, HPLC methods still suffer from a lower resolving power.

We report here a new HPLC method for fatty acid separation, identification and quantification, which can also lead to the recovery of each eluted fatty acid. Two main new improvements are presented allowing a shorter resolution (50 min) than previous HPLC analyses [16,17], for all components of a complex mixture of fatty acids, from C7:0 to C22:6 *n* – 3. Firstly, a little-known chromophore, 2-bromo-2'-acetonaphthone [13,15], was employed for the synthesis of fatty acid naphthacyl esters. These naphthacyl derivatives were shown to be detected at 246 nm, which provided high sensitivity (the detection limit was found to be about 0.1 ng of fatty acid per injection). Secondly, a three-component solvent system of methanol - acetonitrile - water with successive linear gradients was used. Using this elution system, a reproducible and quantitative resolution of fatty acid naphthacyl esters was achieved by reverse-phase HPLC on a $C_{18}$ column. Moreover, because of the non-destructive UV detection of naphthacyl derivatives, this method has the major advantage of being preparative. Resolved fatty acid naphthacyl esters can then be individually recovered for further investigation: determination of specific fatty acid radioactivity or identification of unsaturated fatty acid positional isomers. Applied to the study of fatty acid metabolism in biological systems, this preparative method provides therefore a useful means for the investigation of the metabolic conversion of fatty acids.

## Materials and methods

### Reagents and chemicals

Fatty acid standards and boron trifluoride (14% w/w in methanol) were purchased from Sigma (Saint Quentin Fallavier, France). Dicarboxylic acid standards were provided by Fluka (Saint Quentin Fallavier, France). [1-$^{14}$C]-fatty acids were purchased from Amersham (Les Ulis, France) and DuPont NEN (Le Blanc Mesnil, France). 2-bromo-2'-acetonaphthone was obtained from Aldrich (Saint Quentin Fallavier, France). As the presence of impurities was indicated in the commercial derivatization reagent [18], we recrystallized 2-bromo-2'-acetonaphthone from hexane. This purification step was an effective way of decreasing the impurities previously detected on the chromatogram. High purity reagents for HPLC application were purchased from Fisher (Elancourt, France). Other reagents and chemicals were obtained from Sigma (Saint Quentin Fallavier, France) and Merck (Darmstadt, Germany).

### Fatty acid naphthacyl ester preparation

Fatty acid standards (1 – 5 µmoles) or fatty acids released by saponification from a biological lipid extract and dissolved in hexane (2 mL) were first dried under a stream of nitrogen. They were then converted to fatty acid naphthacyl esters according to the method of Wood and Lee [13] and adapted in our laboratory to naphthacyl ester derivatization: the derivatization procedure was started in screw-capped tubes by adding 20 µmoles 2-bromo-2'-acetonaphthone (500 µL of a 10 mg/mL solution in acetone) and 50 µmoles triethylamine (500 µL of a 10 mg/mL solution in acetone) to the fatty acid extract. After 15 min in a boiling water-bath, 33 µmoles acetic acid (1 mL of a 2 mg/mL solution in acetone) were added for an additional 10 min period at 100 °C, to react with the remaining excess of free 2-bromo-2'-acetonaphthone. After evaporation under a stream of nitrogen, naphthacyl derivatives were dissolved with 200 µL of a mixture of methanol/dichloromethane (3:1 v/v) and filtered (0.45 µm polytetrafluoroethylene membranes).

### Fatty acid naphthacyl ester separation on HPLC

Fatty acid naphthacyl esters (20 µL of the derivative solution) were separated on HPLC (Alliance integrated system, Waters, Saint Quentin en Yvelines, France) using a Nova-Pak $C_{18}$ column (4 µm, 4.6 × 250 mm, Waters) and a guard column (Nova Pak $C_{18}$; 4 µm, 3.9 × 20 mm, Waters). The column temperature was maintained at 30 °C. Elution was performed at a programmed flow-rate of 1 mL/min with a gradient of methanol/acetonitrile/water starting at 80:10:10 (v/v/v), increasing first linearly to 86:10:4 (v/v/v) in 30 min, then increasing linearly to 90:10:0 (v/v/v) in 10 min, holding at 90:10:0 (v/v/v) for 5 min and returning to the initial conditions in 5 min.

### Fatty acid naphthacyl ester detection, identification and collection

Elution of naphthacyl derivatives was monotered by UV absorbance (Tunable absorbance detector 486, Waters) at 246 nm (which is the maximum absorbance for naphthacyl esters). Preliminary identification of fatty acid naphthacyl esters was based upon retention times obtained for naphthacyl esters prepared from fatty acid standards. Each eluted, detected and identified naphthacyl ester could be recovered with a fraction collector (Waters).

### Gas chromatography

Fatty acids were methylated with 1 mL boron trifluoride (14% w/w in methanol) at 70 °C for 10 min [19] and then extracted twice with 4 mL and 2 mL pentane. Fatty acid

methyl ester analysis was performed by gas chromatography, as previously described [20], using a GIRA CG181 chromatograph (GIRA, Morlaas, France) with a split injector (1:10) at 250 °C, a bonded silica capillary column (30 m × 0.25 mm I.D., DB 225, J & W Scientific, Folsom, CA) with a stationary phase of (50% cyanopropylphenyl)-methylpolysiloxane (0.25 µm film thickness). Helium was used as gas vector (30 cm/s) under isothermal conditions (190 °C) with a flame ionization detector at 250 °C (hydrogen and air at 1 bar). Margaric acid (C17:0) was used as internal standard.

### *Monounsaturated fatty acid double bond position determination*

Individual recovery of each monounsaturated fatty acid naphthacyl ester was performed after separation by the HPLC procedure described previously. The solvents were dried under a stream of nitrogen. Each monounsaturated fatty acid naphthacyl ester was then submitted to oxidative cleavage of the double bond. This method was initially described by Von Rudloff [21] and adapted in our laboratory to naphthacyl derivatives according to the following procedure. The oxidative reaction was initiated by addition of 4 mL of a mixture of permanganate-periodate (10 mM $NaIO_4$ and 2.5 mM $KMnO_4$ in water), 2 mL $Na_2CO_3$ (0.1 M in water) and 3 mL t-butanol. After 6 h shaking at room temperature, the reaction was stopped by addition of 2 mL sodium metabisulfite (0.25 M $Na_2S_2O_5$ in water). The monocarboxylic and dicarboxylic acid products were acidified, extracted by addition of diethylether, and converted again to naphthacyl esters as previously described. Mono and dicarboxylic acid naphthacyl esters were then separated on HPLC using the same column as previously described. The separation was performed by elution (1 mL/min) with an isocratic mixture of acetonitrile/water 50:50 (v/v) for 10 min which was then increased linearly to 100:0 (v/v) for 40 min. Preliminary identification of mono and dicarboxylic acid naphthacyl esters was based upon retention times obtained by standards analysis.

### *Separation and identification of polyunsaturated fatty acid positional isomers*

Naphthacyl derivatives of a mixture of C18:3 positional isomers (mixture of C18:3 *n* – 3 and C18:3 *n* – 6) were not separated by the HPLC procedure described previously. In order to separate these isomers, the single C18:3 peak obtained from the chromatography was collected, and the solvents were dried. The mixture was then dissolved in dichloromethane and analyzed with a silver loaded cation exchange column (Chromspher Lipids, 5 µm, 250 × 4.6 mm, Chrompack, Les Ulis, France). Column temperature was maintained at 30 °C, and fatty acid naphthacyl esters were eluted with an isocratic solution of hexane/acetonitrile 99:1 (v/v) at a flow rate of 1 mL/min and detected at 246 nm.

## Results and discussion

### *Fatty acid naphthacyl ester preparation*

Although the esterification reaction leading to the synthesis of fatty acid naphthacyl esters was an equilibrium (Fig. 1), complete conversion of fatty acids to naphthacyl esters was achieved in 15 min by adding triethylamine to the reaction solution. The complete conversion to naphthacyl derivatives was ascertained by using commercial [1-$^{14}$C]-fatty acids (radiolabeled C14:0, C16:0, C18:0, C18:1 *n* – 9 and C18:2 *n* – 6 were used). After the derivatization procedure, HPLC analysis of the sample containing one of these [1-$^{14}$C]-fatty acid naphthacyl esters showed that all the injected radioactivity was recovered in a single peak eluted at the expected retention time (Fig. 3). However, when lower derivatization time and temperature were investigated, a second peak of radioactivity was detected, corresponding to the non-derivatized fatty acid (with a lower retention time than that of the same fatty acid as naphthacyl ester).

2-bromo-2'-acetonaphthone has already been used to prepare naphthacyl derivatives [13,15], but 2-bromoacetophenone has often been preferred to prepare fatty acid phenacyl esters, because of shorter retention times [6,7,13,16,17]. In our study, naphthacyl ester separation resulted in an improved resolution of the fatty acids (see the section describing the separation profile). Moreover, detection of naphthacyl derivatives was shown to be highly sensitive (see the next section). These two improvements led us to choose this chromophore. Miller et al. [22] reported a potassium fluoride assisted derivatization of fatty acids to phenacyl esters. We found that this catalytic reagent was not necessary for the preparation of naphthacyl derivatives. Finally, the fatty acid naphthacyl esters prepared in this way exhibited good stability: there was no detectable decomposition by keeping the injection mixture below –20 °C for two months.

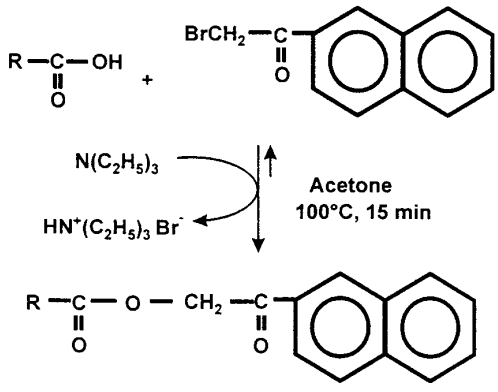


**Fig. 1.** Free fatty acid derivatization reaction as fatty acid naphthacyl esters.

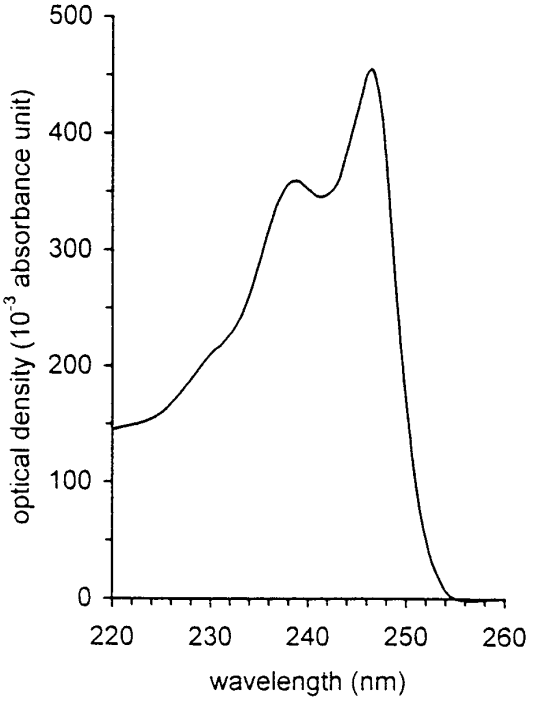


**Fig. 2.** Margaric acid (C17:0) naphthacyl ester UV absorbance spectrum in the ternary elution mixture described as a function of time in materials and methods. The absorbance spectrum between 220 and 260 nm was obtained using a spectrophotometer Uvikon 810 (Kontron Instruments, Saint Quentin en Yvelines, France).

### *Fatty acid naphthacyl ester absorbance spectrum and sensitivity*

Margaric acid (C17:0) naphthacyl ester was prepared as previously described and analyzed by HPLC. After the collection of the eluted peak (Fig. 3), margaric acid (C17:0) naphthacyl ester absorbance spectrum (Fig. 2) was then measured in the three-component solvent mixture described as a function of time in materials and methods. This spectrum displayed a maximum absorbance at 246 nm. Moreover, we found that the fatty acyl chain and the solvent composition (which depended on the elution gradient) have little effect on this maximum absorbance value (data not shown). Detection at 246 nm was then applied to all the naphthacyl derivatives. Using the conditions described here, we injected a standard sample that contained 0.1 ng of each of four fatty acids (C8:0, C12:0, C17:0 and C22:0), which allowed the detection of all components. The limit of detection (assuming a signal to noise ratio of 3) was then found to be about 0.1 ng of fatty acid per injection, which was more than 10 times less than previous values reported for fatty acid phenacyl esters [6,13,17] and fatty acid naphthacyl esters [15].

The use of variable-wavelength detectors allows detection at 246 nm. This possibility was extremely important with naphthacyl derivatives, because detection at 254 nm dramatically decreased the sensitivity but also the resolution of the adjacent peaks C14:0, C22:6 ($n$ – 3), C16:1 ($n$ – 7) and C20:4 ($n$ – 6).

### *Fatty acid naphthacyl ester separation profile*

Figure 3 shows the naphthacyl derivative separation profile obtained using our method, by analyzing a complex mixture of standards of fatty acids. Fatty acid naphthacyl ester retention time increased with increasing fatty acid carbon chain length (saturated fatty acids were eluted from C7:0 up to C22:0), and with a decreasing number of double bonds (C18:2 was for instance eluted before $C_{18}$:1 which appeared itself before C18:0). The increase of the retention time resulting from a two-carbon increase in the fatty acid chain length was within the same range as the decrease in the retention time resulting from the addition of one double bond. Consequently, critical pairs of fatty acids (like C14:0–C16:1 or C16:0-C18:1) had similar retention time. These critical pairs were however well-resolved by our three-component solvent system. Compared with previous studies [13,16] using binary eluents (methanol - water or acetonitrile - water) to resolve phenacyl esters, a ternary eluent of methanol - acetonitrile - water greatly increased the resolution efficiency for both phenacyl [17] and naphthacyl (our results) derivatives separation. This improvement was well-illustrated by the separation of the adjacent peaks C18:3, C14:0, C22:6, C16:1 and C20:4. In addition, the separation of oleic acid (cis-C18:1 $n$ – 9) and elaidic acid (trans-C18:1 $n$ – 9) showed that geometrical isomers of unsaturated fatty acid could be analyzed. The retention time of naphthacyl derivatives depended also on the position of the double bonds: positional isomers of eicosatrienoic acid (C20:3 $n$ – 6 and $n$ – 9) were separated but not those of octadecenoic acid (C18:1 $n$ – 9 and $n$ – 7) and octadecatrienoic acid (C18:3 $n$ – 6 and $n$ – 3). Using the conditions described here, a single 50 min analysis allowed the separation, the identification and if necessary the individual recovery of C7:0 through C22:6 $n$ – 3 fatty acid naphthacyl esters. Naphthacyl derivative elution therefore depended on the fatty acid molecular structure, including carbon chain length, degree of unsaturation, position and geometrical configuration of double bonds.

These results may be compared to those already reported in the literature. Some good HPLC separation of fatty acids as phenacyl derivatives have been reported by Borch [16], Wood and Lee [13], and Hanis et al. [17]. The main advantage of our new method consists of reducing the analysis time and complexity. Indeed first results obtained by Borch [16] required very long column (90 cm) and up to 4 h for analysis at a flow-rate of 2 mL/min with a binary eluent of acetonitrile - water. In the more recent work of Hanis et al. [17], phenacyl ester separation still required 80 min to resolve a mixture of 22 fatty acid standards, from C6:0 to C22:6 $n$ – 3. Thus, our method showed that in combination (i) the use of prepared naphthacyl derivatives having a highly sensitive detection at 246 nm, (ii) the choice of a

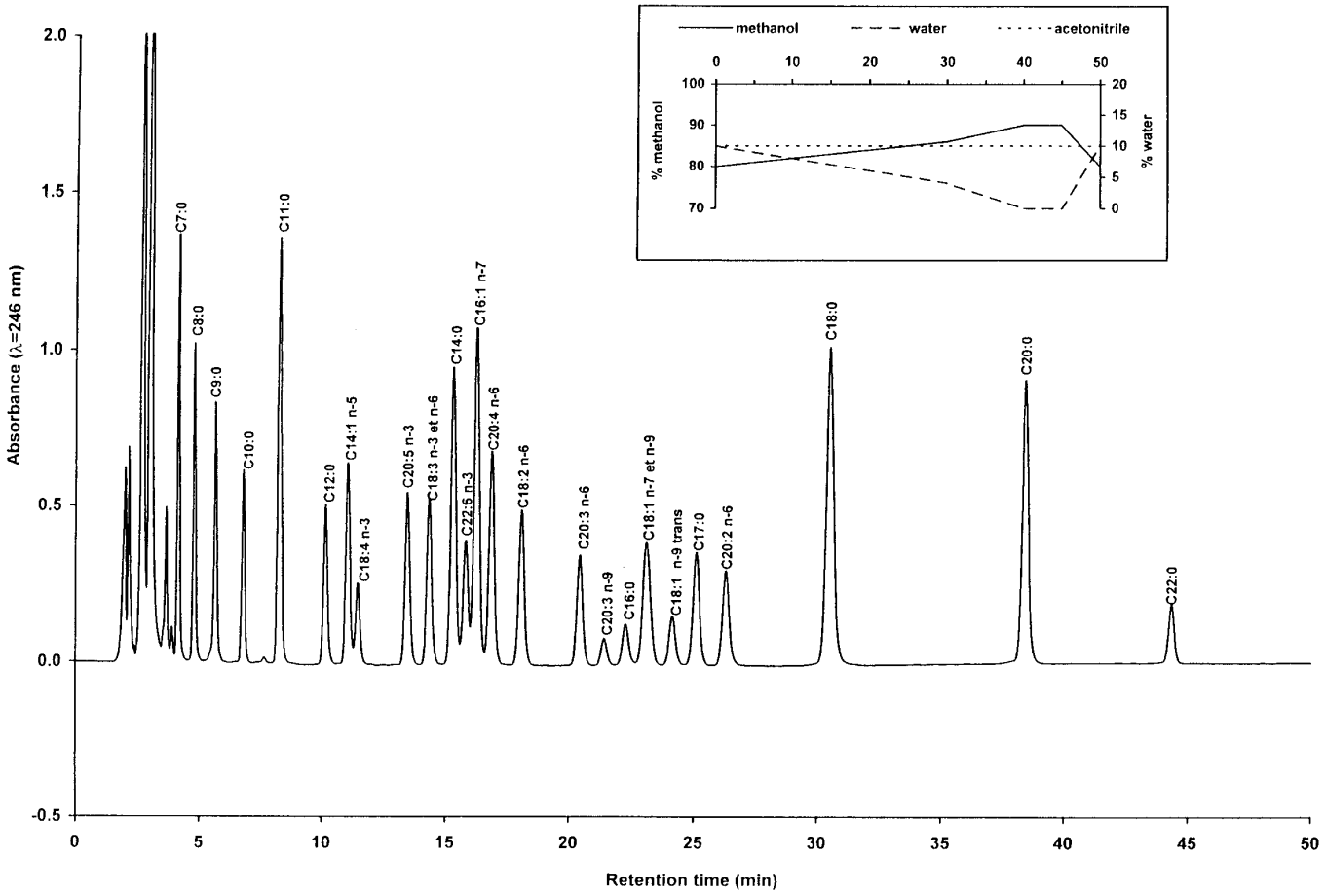


**Fig. 3.** HPLC separation profile of standards of fatty acid naphthacyl esters. Total amount of injected fatty acids was 0.3 µmoles. Peaks appear in the following order (trans isomers are specified): C7:0 heptanoic acid; C8:0 caprylic acid; C9:0 pelargonic acid; C10:0 capric acid; C11:0 undecanoic acid; C12:0 lauric acid; C14:1 *n* – 5 myristoleic acid; C18:4 *n* – 3 stearidonic acid; C20:5 *n* – 3 eicosapentaenoic acid; C18:3 *n* – 3 and *n* – 6 α-linolenic and γ-linolenic acids; C14:0 myristic acid; C22:6 *n* – 3 docosahexaenoic acid; C16:1 *n* – 7 palmitoleic acid; C20:4 *n* – 6 arachidonic acid; C18:2 *n* – 6 linoleic acid; C20:3 *n* – 6 8,11,14 eicosatrienoic acid; C20:3 *n* – 9 5,8,11 eicosatrienoic acid; C16:0 palmitic acid; C18:1 *n* – 9 and *n* – 7 oleic and cis-vaccenic acids; C18:1 *n* – 9 trans elaidic acid; C20:2 *n* – 6 eicosadienoic acid; C17:0 margaric acid; C18:0 stearic acid; C20:0 arachidic acid; C22:0 behenic acid. In the insert the ternary elution profile (methanol - acetonitrile - water) is presented as a function of time.

ternary eluent varying with successive linear gradients, (iii) the use of quality equipment (quality column, regulated column temperature, optimal flow control) may result in an improvement of the chromatographic performance to resolve physiologically important fatty acids, without decreasing the highly reproducibility of the retention times (retention time variation did not exceed 1% by using the same column for 4 months).

### *Quantitative analysis of fatty acids*

A calibration assay was first realized by analyzing different standard solutions of a mixture of caprylic acid (C8:0), lauric acid (C12:0), margaric acid (C17:0), oleic acid (C18:1 *n* – 9) and linoleic acid (C18:2 *n* – 6), and by plotting the injected amount of each fatty acid (from 2 pmol up to 10 nmol) against the obtained peak area of the derivatives. Table I showed that, for each analyzed fatty acid, the peak area was closely related to the amount of the injected fatty acid, even with the small amounts used (2 pmol). Identical linear regression equations were obtained for each of the fatty acids, even for caprylic acid which indicated that there was no loss of this short-chain fatty acid during the evaporation of hexane (see materials and methods). The linear concentration range was wide, running from 2 pmol up to 10 nmol with good correlation coefficients. The limit of quantification (assuming a signal to noise ratio of 10) was then found to be about 0.5 ng of fatty acid per injection. In addition, reproducibility of fatty acid quantitative determinations by the whole procedure (derivatization and separation) was calculated from repeated analyses (*n* = 11) and

showed that a given amount of each fatty acid could be determined with a coefficient of variation which did not exceed 6%.

Thereafter, the validation of the fatty acid quantitative analysis was performed by comparing the values obtained with our HPLC method and with GC (gas chromatography) method on a fatty acid sample extracted from cultured rat hepatocytes. Rat hepatocyte lipids were first extracted and saponified. After acidification, fatty acids were extracted and analyzed as methyl esters by GC, which is well-described as being a quantitative method [2,3], and as naphthacyl esters by HPLC, with margaric acid as internal standard. The comparative results are presented in Table II. Values obtained in fatty acid molar percentage were nearly identical. Thus, Table II supports the view that HPLC analysis of long-chain fatty acid (≥C14:0) naphthacyl esters is a quantitative method, as has already been proved for phenacyl esters [13,17].

### *Applications to the study of fatty acid metabolism*

This HPLC separation, identification and quantification procedure of fatty acids as naphthacyl derivatives has all the classical applications (more often routine determination of fatty acid composition) of previous chromatographic methods. Moreover, a number of new investigations on fatty acid metabolism are also possible.

Primarily, the described method can be readily applied, as previous GC methods, to the determination of fatty acid composition of lipid samples with various origins (lipids or lipid species from various biological structures or fats). Moreover, because of the non-destructive UV detection of naphthacyl derivatives, this method has the major advantage of being preparative. Resolved fatty acid naphthacyl esters can then be individually collected for further investigation. For preparative purposes, free fatty acids can, for instance, be recovered after saponification for 30 min at 70 °C by 1 mL of 2 M KOH in ethanol, and extraction with diethylether. The collection procedure is also especially useful for the determination of specific fatty acid radioactivity during studies on the metabolic conversion of labelled fatty acids in biological systems. By using $^{14}C$- or $^{3}H$-labelled fatty acids as precursors, the percentage distribution of radioactivity among fatty acids can be readily measured by collection of the peaks of interest followed by liquid scintillation counting in the eluting solvents. As reported by Narce et al. [23] in a study on HPLC separation of radiolabelled polyunsaturated fatty acid methyl esters, the presence of a slight amount of water in the elution mixture did not hamper fatty acid naphthacyl ester radioactivity measurement, after the addition of scintillation liquid on the collected sample. Using these conditions and using radiolabelled fatty acids, studies on the biological utilization of a precursor, and studies on a specific metabolic way such as the fatty acid biosynthesis, the elongation, the desaturation or the retroconversion and β-oxidation, can be considered. Several authors have already emphasized the interest of HPLC methods coupled to radioactive labeling to investigate the metabolism of some fatty acids in organs such as the liver [23], the retina [6,7] or in cellular models such as cultured hepatocytes [24].

In addition to individual fatty acid specific radioactivity measurement, the recovery of resolved fatty acid naphthacyl esters offers the opportunity to undertake structural studies. Compared to some GC methods, one disadvantage of HPLC methods is in the difficult separation of unsaturated fatty acid positional isomers. Some of these fatty acid positional isomers indeed remain unseparated as naphthacyl derivatives, but also as phenacyl derivatives. For instance, positional isomers of eicosatrienoic acid (C20:3 $n-6$ and $n-9$) were separated using our method, but those of octadecenoic acid (C18:1 $n-9$ and $n-7$) and octadecatrienoic acid (C18:3 $n-3$ and $n-6$) were eluted in a single peak. The recovery of this single peak allows further analyses to determine the precise structure (for instance the double bond position), and if necessary the ratio of each isomer in the mixture. Accordingly, after the collection of a mixture of positional isomers of an unsaturated fatty acid naphthacyl ester, we now report on two applications to separate them.

The first application leads to the determination of the double bond position on monounsaturated fatty acids. Small amounts of a monounsaturated fatty acid naphthacyl ester positional mixture were submitted to an oxidative cleavage [21]. The resulting carboxylic acid oxidation products were converted again to naphthacyl derivatives and separated by a second HPLC procedure (Fig. 4). Knowing the chain

**Table I.** Parameters of the calibration for the quantification of several fatty acid naphthacyl esters. Injected amounts of each fatty acid were from 2 pmoles to 10 nmoles.

| *Fatty acid* | *Linear regression between the injected amount of the fatty acid (nmol) and the obtained peak area (μV sec)* | *Correlation coefficient [r]* |
|---|---|---|
| C8:0 | $2.48\ 10^6$ | 0.9996 |
| C12:0 | $2.53\ 10^6$ | 0.9998 |
| C17:0 | $2.38\ 10^6$ | 0.9999 |
| C18:1 $n-9$ | $2.75\ 10^6$ | 0.9994 |
| C18:2 $n-6$ | $2.39\ 10^6$ | 0.9980 |

**Table II.** Compared quantification (in molar percentage) of fatty acids extracted from rat hepatocytes by high performance liquid chromatography (HPLC) and gas chromatography (GC). Values are calculated using margaric acid (C17:0) as internal standard. Three different fatty acid extracts from the same hepatocyte culture were analyzed twice as methyl esters by GC and as naphthacyl esters by HPLC.

| *Fatty acid* | *GC* | *HPLC* |
|---|---|---|
| | *(in molar percentage)* | |
| C14:0 | 0.46 ± 0.16 | 0.66 ± 0.16 |
| C16:0 | 23.57 ± 0.45 | 23.43 ± 0.51 |
| C16:0 *n* – 7 | 3.05 ± 0.25 | 3.49 ± 0.19 |
| C18:0 | 16.18 ± 0.47 | 15.05 ± 1.42 |
| C18:1 *n* – 9 | 15.30 ± 0.31 | 14.44 ± 0.91 |
| C18:2 *n* – 6 | 18.19 ± 0.39 | 18.13 ± 0.06 |
| C20:3 *n* – 6 | 0.70 ± 0.24 | 0.44 ± 0.10 |
| C20:4 *n* – 6 | 16.34 ± 0.18 | 16.13 ± 0.21 |
| C22:6 *n* – 3 | 6.20 ± 0.30 | 8.21 ± 0.30 |
| Sum of fatty acids | 100% | 100% |

length of the original monounsaturated fatty acid and that of the mono and dicarboxylic acids produced, the double bond position could be assigned. Longmuir et al. [25] showed the advantage of HPLC analysis of oxidative cleavage products of unsaturated fatty acids, after their conversion to phenacyl esters. Furthermore, we demonstrate that oxidative cleavage can be done directly on the collected monounsaturated fatty acid naphthacyl ester. In addition, the percentage distribution of radioactivity among mono and dicarboxylic acid oxidation products from an original radiolabelled monounsaturated fatty acid can be readily established as previously described. This procedure can be easily applied for all the monoenes, but seems to be more difficult with polyunsaturated fatty acids.

The second application leads to the separation of polyunsaturated fatty acid naphthacyl ester positional isomers. We report here the separation of octadecatrienoic acid positional isomers. After the collection of a mixture of C18:3 naphthacyl esters (C18:3 *n* – 3 and *n* – 6), this mixture was

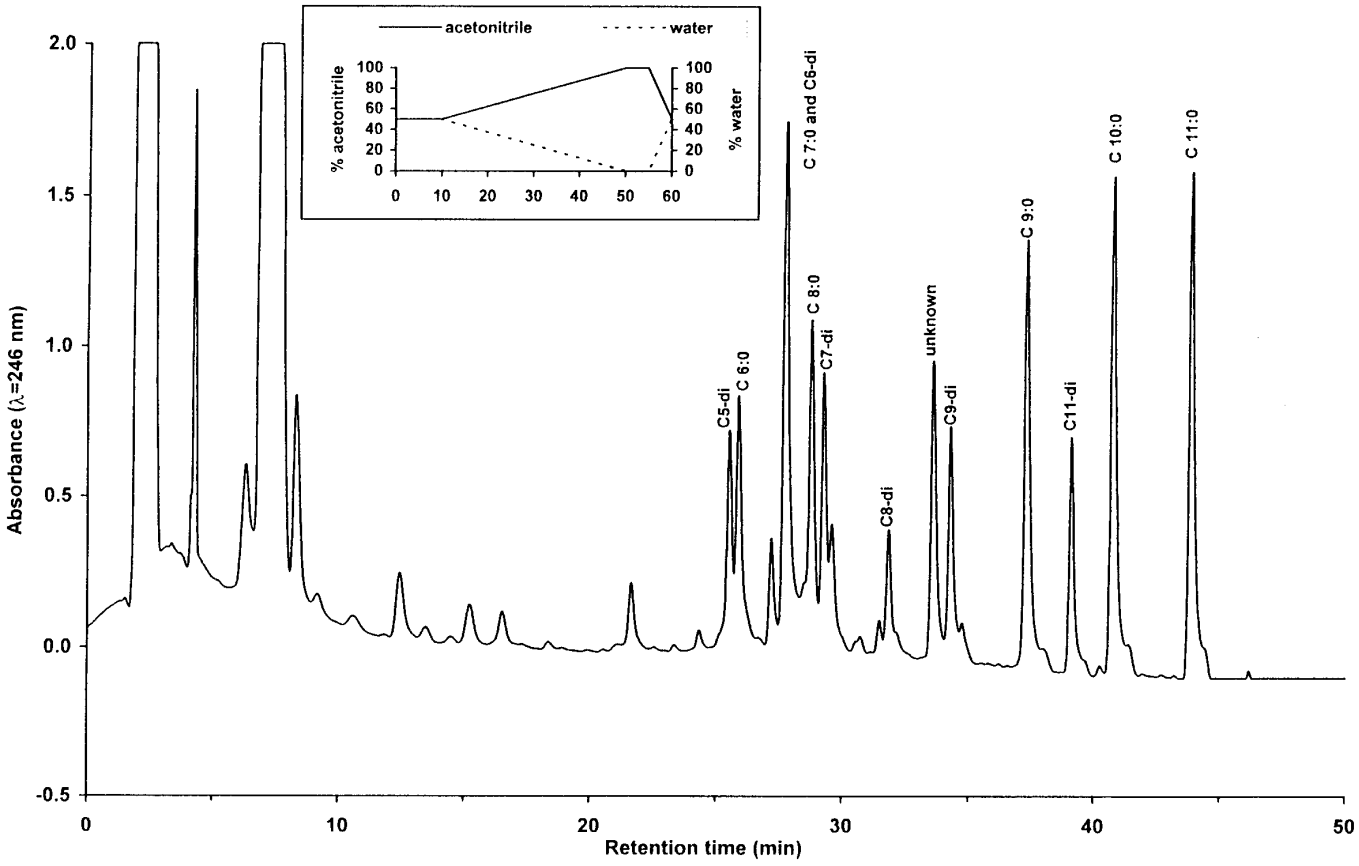


**Fig 4.** HPLC separation profile of standards of mono and dicarboxylic acid naphthacyl esters. Peaks appear in the following order (dicarboxylic acids are specified "di"): C5-di glutaric acid; C6:0 caproic acid; C7:0 and C6-di heptanoic and adipic acids; C8:0 caprylic acid; C7-di pimelic acid; C8-di octanedioic acid; C9-di azelaic acid; C9:0 pelargonic acid; C11-di undecanedioic acid; C10:0 capric acid; C11:0 undecanoic acid. In the insert the binary elution profile (acetonitrile - water) is presented as a function of time.

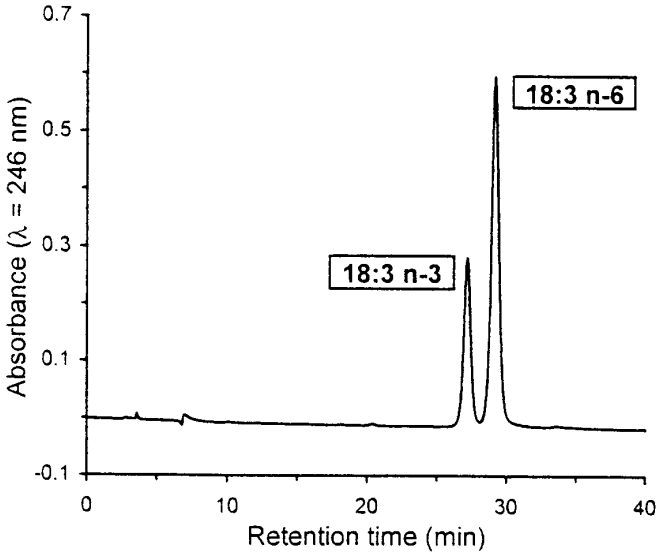


**Fig 5.** HPLC separation profile of the positional isomers of octadecatrienoic acid (α-linolenic acid C18:3 *n* – 3 and γ-linolenic acid C18:3 *n* – 6) as their naphthacyl derivatives on a silver loaded cation exchange column.

directly resolved by a silver loaded cation exchange column (Fig. 5). The column used in the present paper was already used by Sehat et al. [26] to resolve complex mixtures of conjugated octadecadienoic acid (C18:2) isomers. Dobson et al. [27] showed that silver ion chromatographic methods were of particular interest for the separation of fatty acids by thin-layer chromatography or high performance liquid chromatography.

All these procedures are particularly useful for studying the products and rates of fatty acid desaturation [20]. The separation of fatty acids leads to the characterization and measurement of desaturation rate [28], by collection and determination of the containing substrate and product radioactivity. The two additional applications lead to the identification of the involved desaturase, by identifying the position of the introduced double bond.

## Conclusion

The method reported in this paper demonstrates the increasing capacity of HPLC to solve problems related to fatty acid analysis. Separation is more efficient than former HPLC analyses, the number of resolved fatty acids has increased, the analysis time is reduced and further investigation is possible due to the collection of the separated molecules. The described procedures will enable further extensive studies of fatty acid metabolism in biological systems.

## Acknowledgments

The authors are indebted to ARILAIT RECHERCHES (France) for constructive scientific discussion and financial support.

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