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Determination of underivatized long chain fatty acids using RP-HPLC with capacitively coupled contactless conductivity detection

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method with capacitively coupled contactless conductivity detector (C^4D) has been developed for the separation and the simultaneous determination of five underivatized long chain fatty acids (FAs), namely myristic, palmitic, stearic, oleic, and linoleic acids. An isocratic elution mode using methanol/1 mM sodium acetate (78:22, v/v) as mobile phase with a flow rate of 0.6 mL min^{-1} was used. The separation was effected by using a Hypersil ODS C_{18} analytical column (250 mm × 4.6 mm × 5 μm) and was operated at 45 °C. Calibration curves of the five FAs were well correlated ($r^2 > 0.999$) within the range of 5– 200 μg mL^{-1} for stearic acid, and 2–200 μg mL^{-1} for the other FAs. The proposed method was tested on four vegetable oils, i.e., pumpkin, soybean, rice bran and palm olein oils; good agreement was found with the standard gas chromatographic (GC) method. The proposed method offers distinct advantages over the official GC method, especially in terms of simplicity, faster separation times and sensitivity.

1. Introduction

Fatty acids (FAs) are widely found in nature (e.g., food products, vegetable oils, and living organisms) and are vital as nutritional substances and metabolites in living organisms. They form the basic components of most naturally occurring lipids in both animals and plants. The diversity of the chain length, degree of unsaturation, geometry and position of double bonds, as well as the presence of other groups, render their composition the most definitive characteristic of these lipids and their origin [1]. Analysis of FAs is important in the control of technical products, in medical diagnostics, cancer research and in the testing of purity, origin, and shelf-life studies of food products, and in biodiesel projects [2].

The most commonly used method for the analysis of FAs involves the determination of the corresponding methyl esters using capillary gas chromatography (GC) with flame ionization detector (FID) [2]. A derivatization procedure is mandatory to increase the volatility and overcome adsorption of the polar functional groups to the GC column [3]. In general, GC provides excellent separation and quantification together with acceptable sensitivity. However, there is a growing interest in the use of high-performance liquid chromatography (HPLC) for studying FAs. The major advantages of HPLC over GC are the lower temperatures required during the analysis (reduce the risk of isomerisation of double bonds) and the possibility of collecting fractions for further analysis. Apart from that, HPLC is considered more flexible as the retention characteristics can be easily modified by varying the composition of mobile phase.

HPLC analysis of the FAs is complicated as these analytes are neither UV nor fluorescence-active. Thus, derivatization procedures allow the "tagging" of chromophore or fluorophore to the analyte, rendering them to be detected using UV–vis or fluorescence detector [4–8].

While the derivatization procedure used to increase the HPLC sensitivity markedly (especially for fluorescence detection), the derivatization process itself, however, is not preferred. Inherent problems include not only the longer analysis time required, but also the possibility of inaccurate results due to incomplete or unstable reaction with the derivatization compounds; unselective labelling that leads to interfering by-products; and the expensive and unstable nature of some derivatization reagents. There is thus, a strong emphasis lately on developing alternative methods to the traditional HPLC methods that require UV and fluorescence [4–8] for detection. HPLC methods for the determination of underivatized FAs involving detectors such as mass spectrometer [3,9], chemiluminesce [1,10], electrochemical [1], refractive index [11], and evaporative light scattering detectors (ELSD) [2,12] have been reported.

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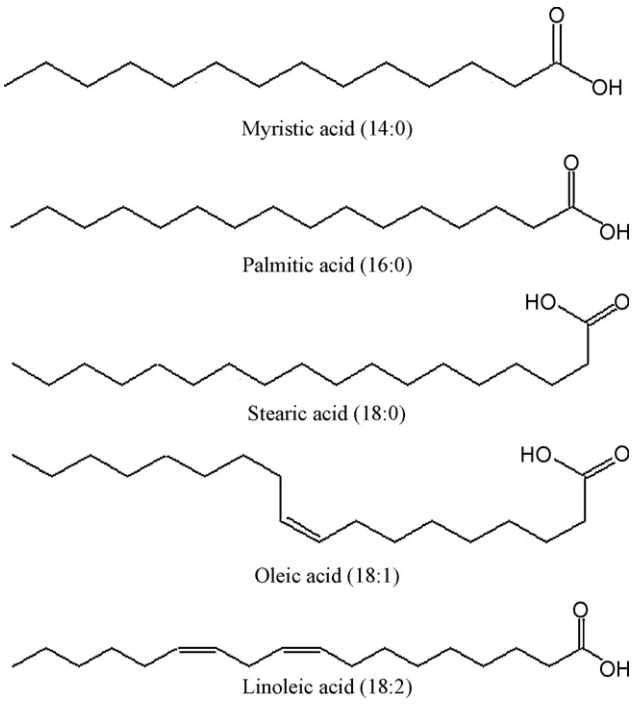

Fig. 1. Structures of fatty acids (FAs) studied.

Capacitively coupled contactless conductivity (C^4D) detector, originally developed for capillary electrophoresis (CE), and was used for HPLC for the first time in 2006 [13]. The capability for detecting small organic molecules and non-choromophoric compounds offer unique advantages over other detectors. Moreover it is cheap and requires low maintenance [14].

The separation of short-chain FAs (acetic, propionic and butyric acids) using ion-chromatography with chemical suppression and conductivity detection was achieved within 10 min. The method exhibits good linearity and limit of detection and is comparable to other techniques [15].

CE with indirect UV and conductivity detection was reported for the determination of long chain FAs in drying oils that were used as binding media [16]. A CE–C^4D method has been reported [17]. The method offered good separation for nine saturated FAs, but the separation of palmitic and oleic acid was not achieved [17].

In this work, a reversed-phase HPLC method for the determination of the common FAs myristic (MA), palmitic (PA), stearic (SA), oleic (OA) and linoleic (LA) acids (Fig. 1) using C^4D detection is described. Key factors affecting the separation (e.g., mobile phase composition, buffer concentration, column temperature, detector frequency) and detection conditions were investigated. The adopted conditions were applied for the analysis of FAs in a few types of vegetable oils (palm olein, soybean, rice bran, and pumpkin oils) after the saponification. For comparison, the oils were transesterified using boron trifluoride-methanol and was analysed using the conventional GC–FID method.

2. Experimental

2.1. Chemicals and reagents

Lauric acid (99%), myristic acid (99%), palmitic acid (≥99%), stearic acid (≥99%), oleic acid (99%), linoleic acid (99%), and sodium hydroxide were purchased from Sigma–Aldrich (Supelco, MO, USA). Methanol (HPLC grade), sodium acetate, hexane and boron trifluoride were purchased from Merck (Merck, Darmstadt, Germany). Ultra pure water (resistivity, 18.2 MΩ cm^{-1}) was used throughout for the preparation of solutions and the mobile phase. Vegetable oil samples were purchased from local supermarkets.

2.2. Instrumentation

2.2.1. HPLC–C^4D

A Waters-Alliance (model 2695) HPLC system (Waters, MA, USA), equipped with C^4D (EDAQ, Denistone East, Australia) was used. Connection between the HPLC and the C^4D detector was made by using a fused silica capillary (Supelco, PA, USA) with 250 μm I.D. and 360 μm O.D. The chromatographic separation was performed on Hypersil ODS C_{18} analytical column (250 mm × 4.6 mm × 5 μm) (Thermo Fisher Scientific, MA, USA) operated at 45 °C. Initially, other columns such as Chromolith RP-18e 100 mm × 4.6 mm, Chromolith RP-18e 100 mm × 3 mm (Merck, Darmstadt, Germany), Inertsil ODS-3 C_{18}, 250 mm × 4.6 mm × 3 μm (GL Sciences, Tokyo, Japan) were also tried. The adopted chromatographic conditions were: mobile phase, methanol: 1 mM sodium acetate 78:22 (v/v); flow rate, 0.6 mL min^{-1}; injection volume, 20 μL. The C^4D was employed at peak-to-peak amplitude of 100 V and the frequency was 100 kHz. The data were processed using licensed PowerChrom v2 software (EDAQ, Denistone East, Australia).

2.2.2. GC–FID

GC analysis was performed using a Clarus 500 gas chromatograph unit from PerkinElmer (PerkinElmer, CT, USA), with a Supelcowax 10 fused silica capillary column of 30 m × 0.32 mm I.D., film thickness 0.25 μm from Supelco (Supelco, PA, USA). The chromatographic conditions were performed according to the Association of Oil Chemists Society (AOCS) procedures (official method Ce 1e-91). The oven temperature was programmed as follows: 80 °C (hold for 2 min) at 20 °C min^{-1} to 125 °C (hold for 1 min) then at 3 °C min^{-1} to 220 °C (hold for 5 min). The injector and the FID were operated at 240 °C. Nitrogen was used as a carrier gas at flow rate of 1 mL min^{-1} and using a split ratio of 10. Chromatographic data were processed using TotalChrom Workstation version 6.3.1 software (PerkinElmer, CT, USA).

2.3. Preparation of standards

2.3.1. Preparation of standards for HPLC–C^4D

A stock solution (1000 μg mL^{-1}) of the five FAs was prepared in methanol and was stored at 4 °C. Working solutions were prepared fresh every day in methanol.

2.3.2. Preparation of standards for GC–FID

A stock solution (1000 μg mL^{-1}) of six FAs including lauric acid (internal standard) was dissolved in boron trifluoride in methanol (7%), and was heated (80 °C) with continuous stirring (60 min). The transesterified mixture was stored at 4 °C. Working solutions were prepared fresh every day in methanol. Hexane (1 mL) was added to the working standard (1 mL) in 5 mL vials and was vortexed (15 min). The upper layer was separated and injected into the GC column.

2.4. Sample preparation

Prior to the HPLC analysis, a saponification process was carried out by mixing oil (100 mg) and 2 M sodium methanoate (2 mL) in a vial (5 mL). The vial was tightly capped and was heated for 5 min at 60 °C. The mixture was diluted to 100 mL using methanol. The sample was further diluted to a suitable ratio using methanol before HPLC injection.

Before the GC–FID analysis, the oil was transesterified according to the method reported by Eras et al. [19]. Oil (30 mg) was dissolved in methanol (1.5 mL), spiked with the internal standard

and 14% boron trifluoride in methanol (1.5 mL) and transferred to a 15 mL vial. The vial was capped and was heated at 80 °C with stirring for 60 min. Water (2.5 mL) and hexane (2.5 mL) were added to the cooled mixture. The mixture was vortexed for 15 min and the upper layer was separated and injected into the GC column.

2.5. Optimization C^4D

Many variables (e.g., sodium acetate concentration and mobile phase composition) can affect the performance of the C^4D detector. Since different concentrations of sodium acetate (0.1–10 mM) were investigated, the mobile phase composition was also investigated over the range of 78–83% methanol. The amplitude was fixed at 100 V, while the frequency was optimized from 50 to 500 kHz.

3. Results and discussion

In order to separate the FAs several parameters were considered. Initially, different columns (Chromolith RP-18e 100 mm × 4.6 mm, Chromolith RP-18e 100 mm × 3 mm, Inertsil ODS-3 C_{18}, 250 mm × 4.6 mm × 3 μm, and Hypersil ODS C_{18}, 250 mm × 4.6 mm × 5 μm) were investigated for the separation of the FAs. Most of these columns do not result in the satisfactory separation of OA and PA. Furthermore, tailing or fronting was observed for some peaks [13]. However, more promising results were obtained when the Hypersil ODS C_{18} column was used as it resulted in not only good separation among the FAs but also the peaks were more symmetrical. Thus this column was used.

Since the C^4D detector response is very much influenced by variation in the mobile phase composition, isocratic elution was applied. Gradient elution was attempted by some authors, but sloping of baseline was observed [18]. Nevertheless, this problem was overcame by the authors using suitable softwares [18].

3.1. Optimization of chromatographic conditions

3.1.1. Selection of mobile phase

An important strategy in the method development involving C^4D detector is that it is important for the analytes to be charged under the operated conditions; moreover the mobile phase should have low conductivity as possibly can [18]. As the long chain FAs are weak acids (pK_a around 4.7), a basic mobile phase will ensure that the FAs will be in the ionised form and at the same time possess low conductivity [18]. Acetic acid and its salts are frequently used as HPLC mobile phase with organic solvents due to its low conductivity and can provide suitable pH environments to ensure that the analytes are in the ionized form [13,18]. It had been suggested that buffers with specific conductivity less than 1 mS cm^{-1} are able to provide the necessary background conductivity [18,20]. Sodium acetate seems to fit these criteria and was thus selected. Different concentrations of sodium acetate (0.1–10 mM) were investigated under the optimum frequency and amplitude for each concentration of sodium acetate. The best sensitivity and separation was achieved using 1 mM sodium acetate for all the FAs. As the concentrations of sodium acetate decrease (≤10 mM), peaks areas were found to increase due to the decrease of the conductivity of the mobile phase. However, when the concentration of sodium acetate was less than 1 mM the sensitivity start to decrease due to the environment that do not favour the ionization of the FAs. Peak fronting was also observed when less than 1 mM sodium acetate was present (Fig. 2).

Methanol and acetonitrile are among the most widely used mobile phase components in HPLC separations. Using methanol resulted in better sensitivity than acetonitrile, thus methanol was used. The effect of varying methanol composition 78–83% was also investigated. Slightly higher peak area for the FAs was achieved using 81% methanol, but 78% methanol was chosen as it offers better separation between PA and OA, and thus this composition was used for the remaining studies.

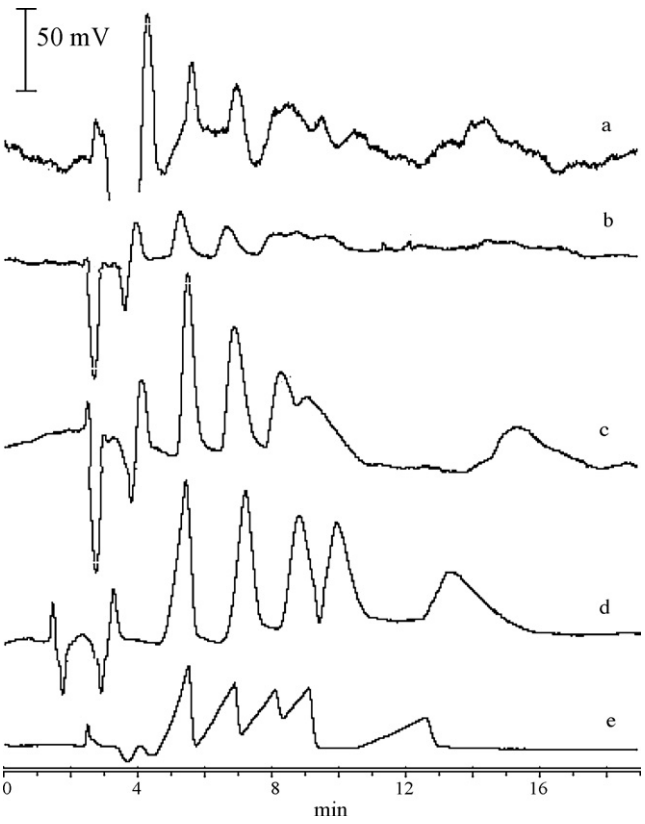

Fig. 2. Influence of sodium acetate concentration on the separation of five FAs using HPLC–C^4D method. Column, Hypersil ODS 250 mm × 4.6 mm × 5 μm; flow rate 0.6 mL min^{-1}; mobile phase composition 78% methanol, 22% sodium acetate. Concentration of FAs; 80 μg mL^{-1}, sodium acetate concentration: (a) 10 mM, (b) 5 mM, (c) 2.5 mM, (d) 1 mM, and (e) 0.1 mM.

3.1.2. Selection of detector conditions

The detector should be operated at its optimum frequency, which is as low as possible to minimize the effect of stray capacitance [18]. The detector amplitude was fixed at 100 V, while the frequency was varied from 50 to 500 kHz. The highest signals of the FAs were found using 100 kHz, thus 100 kHz was used. Furthermore, more stable baseline was found. Typical chromatogram when operated under the adopted conditions is shown in Fig. 3. The FAs are all separated in about 15 min.

3.2. Method validation

3.2.1. Linearity of calibration

Linearity was checked by preparing standard solutions of FAs at eight different concentrations using the stock solution. Each solution was injected thrice. The method showed a good linearity over 5–200 μg mL^{-1} for SA, and 2–200 μg mL^{-1} for the other four FAs (Table 1).

3.2.2. Limit of detection (LOD)

The LOD was estimated at a signal-to-noise ratio of 3. The HPLC–C^4D method exhibited significantly lower LOD values for the five FAs (0.1–0.6 μg mL^{-1}) (Table 1) compared to the GC–FID (0.7–0.8 μg mL^{-1}) and the reported methods using HPLC–ELSD (1.5–5 μg mL^{-1}), and CE–C^4D (10.5–12.3 μg mL^{-1}) [2,17]. The low LOD was attributed to the low baseline noise of the detector for the selected mobile phase and frequency used.

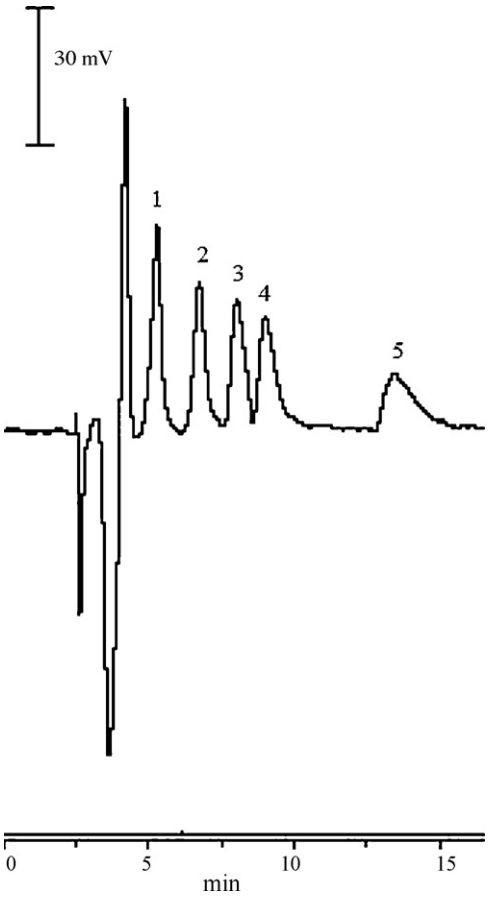

Fig. 3. Typical chromatogram for the separation of standard FAs using HPLC–C^4D method operated under the adopted conditions. Column, Hypersil ODS 250 mm × 4.6 mm × 5 μm; flow rate 0.6 mL min^{-1}; mobile phase composition 78% methanol, 22% 1 mM sodium acetate. Concentration of FAs, 40 μg mL^{-1}. Peak description: (1) myristic acid, (2) linoleic acid, (3) palmitic acid, (4) oleic acid, (5) stearic acid.

3.3. Intra and inter-day precision

The intra-day precision was studied by repeatedly ($n = 3$) injecting three standards (5, 100, 200 μg mL^{-1}), while the inter-day studies were done by injecting the standards on four different days within a week. Good precision as reflected in the relative standard deviation (≤1.87%) data for peak areas was found (Table 2).

Table 1
Comparison of calibration curves and limits of detection for the five FA standards using C^4D-HPLC and GC–FID[a].

Fatty acid	Linear range (μg mL^{-1})	Regression equations	r^2	LOD (μg mL^{-1})
Myristic acid	2–200 (10–150)	$y = 3.2386x - 3.1564$ ($y = 0.0073x + 0.0478$)	0.9999 (0.9894)	0.6 (0.8)
Palmitic acid	2–200 (10–150)	$y = 1.6776x + 2.6400$ ($y = 0.0076x + 0.1249$)	0.9991 (0.9758)	0.1 (0.7)
Stearic acid	5–200 (10–150)	$y = 2.5823x - 13.8500$ ($y = 0.0076x + 0.0605$)	0.9995 (0.9831)	0.1 (0.7)
Oleic acid	2–200 (10–150)	$y = 1.6882x + 4.4503$ ($y = 0.0073x + 0.0496$)	0.9991 (0.9899)	0.1 (0.8)
Linoleic acid	2–200 (10–150)	$y = 2.5695x - 0.0587$ ($y = 0.0081x + 0.0375$)	0.9997 (0.9849)	0.1 (0.7)

[a] Figures in parenthesis.

Table 2
Intra and inter-day precision (RSD %) of the five FAs using HPLC–C^4D method (based on peak area).

	Intra-day $n = 9$ (RSD %)			Inter-day $n = 36$ (RSD %)		
Concentration (μg mL^{-1})	5	100	200	5	100	200
Fatty acid						
Myristic acid	0.54	0.53	0.94	1.03	1.08	1.30
Palmitic acid	1.32	1.28	1.48	1.77	1.34	1.87
Stearic acid	0.65	0.83	0.45	0.73	1.02	0.82
Oleic acid	1.06	1.26	1.79	1.27	1.51	1.49
Linoleic acid	0.60	0.73	0.68	1.13	1.20	1.03

Table 3
Comparison of FA compositions (mg/100 mg) of tested oils using the HPLC–C^4D and GC–FID[a] methods.

Fatty acid	Soybean oil	Palm olein oil	Pumpkin oil	Rice bran oil
Myristic acid	ND (ND)	1.5 ± 0.0 (1.5 ± 0.2)	ND (ND)	ND (ND)
Linoleic acid	47.3 ± 0.2 (47.3 ± 2.5)	10.5 ± 0.1 (9.5 ± 0.3)	39.4 ± 0.4 (36.7 ± 4.2)	28.2 ± 0.4 (26.6 ± 1.2)
Palmitic acid	9.3 ± 0.0 (10.9 ± 0.5)	38.7 ± 0.2 (37.7 ± 2.5)	10.0 ± 0.1 (9.3 ± 0.5)	18.3 ± 0.2 (17.7 ± 0.7)
Oleic acid	24.5 ± 0.2 (23.6 ± 1.3)	42.6 ± 0.1 (43.9 ± 2.1)	36.1 ± 0.2 (31.5 ± 4.3)	43.2 ± 0.1 (40.9 ± 1.6)
Stearic acid	5.2 ± 0.0 (5.1 ± 0.2)	5.5 ± 0.0 (4.8 ± 0.6)	5.8 ± 0.0 (5.9 ± 0.8)	3.7 ± 0.0 (ND)

[a] Figures in parenthesis.

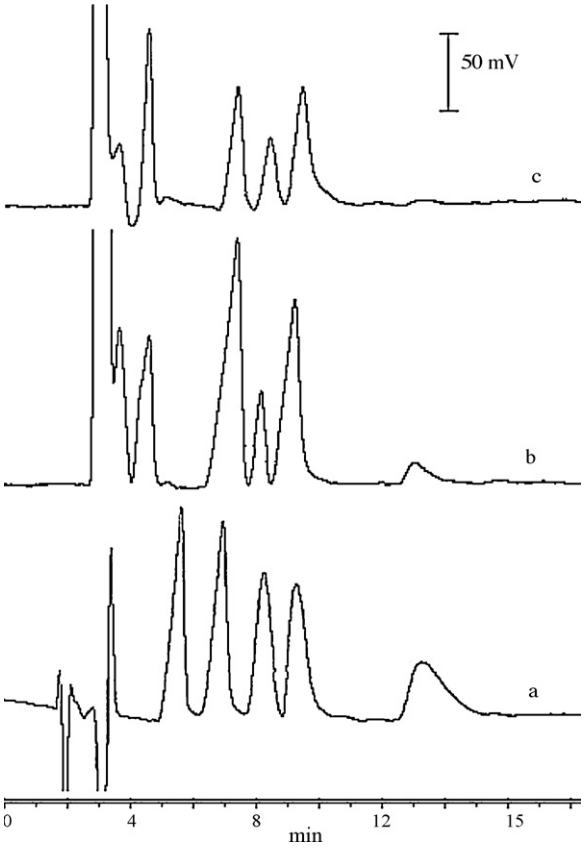

Fig. 4. Determination of five FAs in vegetable oil samples using the HPLC–C^4D method. Column, Hypersil ODS 250 mm × 4.6 mm × 5 μm; flow rate 0.6 mL min^{-1}; mobile phase composition 78% methanol, 22% 1 mM sodium acetate. Peak description: (1) myristic acid, (2) linoleic acid, (3) palmitic acid, (4) oleic acid, (5) stearic acid. (a) 80 μg mL^{-1} standard mixture, (b) pumpkin oil sample and (c) rice bran oil sample.

3.4. Analysis of vegetable oil samples

The proposed method was applied to the analysis of FAs in soybean (*Glycine max* L.), rice bran (*Oryza sativa* L.), pumpkin seed (*Cucurbita pepo* L.) and palm olein oil (*Arecaceae Elae*) oil samples. MA was found only in palm olein oil, consistent with literature values [21]. Soybean and palm olein oils were selected as they are among the most widely used vegetable oils whereas pumpkin oil and rice bran oils were selected as they are commonly added in functional foods. The levels of the FAs were in good agreement with the GC–FID method (Table 3). The tailing of the stearic acid peak does not seem to affect the results. It can be readily seen that the HPLC–C^4D method gave better reproducibility than the GC–FID method. Typical chromatograms are shown in Fig. 4. The separation time was significantly shorter (∼15 min) compared to the GC–FID method (∼40 min).

4. Conclusion

An alternative analytical method for the determination of underivatized FAs using reversed-phase HPLC using C^4D detection was developed using methanol/1 mM sodium acetate (78:22, v/v) as mobile phase. Under the adopted conditions, separation of the analytes was achieved in about 15 min. The method was superior to the standard GC–FID method both in terms of speed and sensitivity. Furthermore, the GC–FID method requires the mandatory liquid–liquid extraction step and derivatization to methyl esters prior to the analysis. Good agreement of the levels of FAs was found between the proposed method and the GC–FID method when applied to the analysis of soybean, palm olein, pumpkin and rice bran oils.

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