

Quantification of Underivatized Fatty Acids From Vegetable Oils by HPLC with UV Detection

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Abstract

We propose a chromatographic method for the separation of saturated and unsaturated fatty acids by a high-performance liquid chromatography system, equipped with a photo diode array detector. Central to the method is the use of an appropriate mobile phase composed of acetonitrile, methanol, and *n*-hexane in ratio 90:8:2 acidified with 0.2% acetic acid, which allows the detection of fatty acids without a preliminary derivatization with chromophores or fluorescent dyes. Calibration on solutions of standards mixtures gives a quantification limit (at a wavelength of 208 nm) of 0.232, 0.093, 0.039, 0.056, 0.068, 0.004, 0.0005, 0.067 mg/mL for the myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and erucic acids, respectively. The method, applied to different vegetable oils (olive, sunflower, soybean, and palm) was able to distinguish the main fatty acids and quantify their amount. Data reliability was tested by comparing our results (on the relative percentages of some fatty acids in the olive oil) with those obtained by gas chromatographic analysis. Differences of the order of 0.3%, 0.6%, 2%, and 6% were observed for the oleic, linoleic, palmitic, and linolenic acids. Although less accurate, our method proved to be a simple alternative to standard gas chromatographic technique, as it can be applied even using a simple UV detector.

Introduction

Vegetable oils contain a large number of compounds that can be classified into unsaponifiable and saponifiable fraction. The latter, accounting for about 98% of the oil, is mostly constituted from fatty acids forming esters with glycerol to produce mono-, di-, and tri-glycerides. The fatty acids composition of oils varies considerably according to the vegetable matrix (olive, sunflower, corn, palm, hazelnut, soybean, etc.) and even varies for the same vegetable matrix when considering variety, local factors, climatic condition, harvest time, extraction processes from fruits or seeds, etc. (1–7). Indeed, identification and quantitative determination of fatty acids composition is one of the most used tools for monitoring the authenticity of edible oils (8,9).

Two different approaches are commonly used for determining the oil acidic components: (i) the direct study of triglycerides by high-performance liquid chromatography (HPLC) (2,3,7,8,

10,11); (ii) the determination by gas chromatography (GC) of fatty acids methyl esters obtained by triglycerides hydrolysis (7,8,10,12). The latter procedure is of particular interest also for the study of fatty acids present in other food products, living organisms, and biological matrices (13,14).

The direct determination of fatty acids by HPLC is made difficult by their poor absorbance in visible-UV region and by the absence of chromophores or fluorescent groups. However, HPLC offers some advantages with respect to the GC method, for example, sample recovery or reduced risk of isomerization of unsaturated acids. This has prompted many researchers to develop efficient HPLC methods based on the conversion of fatty acids in methyl ester form or on their derivatization with chromophores or fluorescent dyes (15–20). Alternative HPLC methods that require no derivatization reactions were also proposed. In these cases, major attention was paid to the detection that could be based on electrochemical reaction of quinone added to the mobile phase (21), or evaporative light scattering (22), or refractive index (23) change. Some qualitative results on the determination of underivatized fatty acids by UV detection can be found in a paper by Hein and Isengard (24). In that work, three HPLC methods based on different mobile phases and detectors were applied to adequately treated samples of some vegetables oils. The authors observed that the accuracy of the detection based on UV absorption was not comparable with that based on the refractive index change; moreover, the method was not able to determine the saturated fatty acids. No quantitative results on the detectable unsaturated fatty acids were reported.

The present work introduces a chromatographic protocol that allows for the separation and quantification of some underivatized fatty acids (both saturated and unsaturated) by using a HPLC with a photo diode array (PDA) detector. Although this detector is able to record the sample spectrum in the 190–900 nm wavelength (λ) range, our study was mainly limited to a single λ value because our aim was to find a protocol that could be easily applied even when using a simple UV detector. The latter represents a standard detector system for many HPLC instruments, but its use for fatty acid determination is limited by the low molar absorbance coefficient of samples and by the large detection cut-off imposed by the absorbance of the mobile phase (25). By testing trials on mixtures of fatty acid standards, we found that a mobile phase composed of acetonitrile, methanol, and *n*-hexane in the ratio 90:8:2, acidified with acetic acid (0.2% w/w) provided a convenient and fast separation medium.

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We applied our method to the determination of the acidic component of different vegetable oils (olive, soybean, sunflower, palm) after hydrolysis of their triglycerides. Table I shows the typical percentage of fatty acids in the chosen oils (26). The relative percentage of fatty acids changes logically. For example, the oleic acid is the major component of the olive oil (56–84%), but its amount decreases in favor of the palmitic acid in the case of the palm oil as well as in favor of the linoleic acid in the case of sunflower and soybean oils. This set of oils was therefore appropriate for testing the method.

The chromatographic data were analyzed by a simple calibration procedure and by partial least square (PLS) analysis (27). The latter is a multiple linear regression method that allows to build a linear relationship between the observed data and a set of predictor variables and to make reasonable predictions on new observations. To the purpose of validating the procedure as a whole, results relative to the olive oil were compared with those obtained using official methods for the determination of fatty acids (8).

Materials and Methods

Olive oil samples were kindly supplied by Sicilian oil producers in the frame of a European project and subjected to the lawful analyses prescribed for their classification as extra virgin oils. The seed oils were bought at the local market. All samples were stored in the dark at 23°C.

Acetonitrile, methanol, *n*-hexane of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid was provided by Merck (Darmstadt, Germany). Low boiling petroleum ether, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and erucic acids were purchased from Fluka.

Sample preparation

Standard acids, stored at 4°C before use, were solubilized in methanol at room temperature and filtered through a 0.22- μ m Millipore (Billerica, MA) filter before HPLC injection. Mixtures of different standards were prepared at concentration similar to those present in the oil samples.

Aliquots of 5 g of oil were hydrolyzed with 50 mL of 0.5 M NaOH at 100°C for 1 h. After cooling, the dispersion was

extracted by adding 50 mL of petroleum ether two times in order to remove the unsaponifiable fraction. The remaining dispersion was acidified with 1 M HCl to pH 2.9, and then free fatty acids were extracted by adding 50 mL petroleum ether two times. The organic solvent was removed at 40°C under a gentle stream of N₂. Finally, the acids were recovered with 4 mL of methanol, opportunely diluted and filtered through 0.22 μ m Millipore filters before HPLC injection.

HPLC system and conditions

The HPLC system was a Shimadzu LC-2010 AT Prominence equipped with a UV-vis photodiode array detector (SPD-M20A), an on-line Degasser filter (DGU-20A5), and a 20- μ L sample loop.

A Supelco (Bellefonte, PA) Discovery reversed phase HS-C₁₈ (250 mm \times 4.6 mm i.d., particle size 5 μ m) column was used. A C₁₈ column of the same producer was preliminarily tested and discarded because its lower hydrophobicity resulted in a poor selectivity. Different mobile phases and flow rates were tested on mixture of fatty acid standards to optimize the separation conditions. Initially, acetonitrile and water in the ratio 90:10 were chosen as organic and polar solvent, respectively, at a flow rate of 1 mL/min. These conditions provided poor selectivity and too large retention times. Then, we tried to use a mixture of methanol and *n*-hexane in the ratio 80:20 at 0.7 or 1 mL/min flow rate. A faster separation was obtained, but the selectivity did not improve. The best resolution was instead obtained with a mixture of acetonitrile, methanol, and *n*-hexane in the ratio 90:8:2 at flow rate of 1 mL/min. All mixtures tested were acidified with 0.2% acetic to stabilize the fatty acids in their associated form. Experiments were carried out at room temperature.

Quantitative determination

Calibration curves of eight acids were obtained by triplicate measurements on methanolic solutions of single acid standards at increasing concentration. Each calibration curve was found by using the simple equation:

$$A = m \times C \quad \text{Eq. 1}$$

where *C* is the fatty acid concentration and *A* is the peak area, calculated by HPLC software.

Calibration data allowed to evaluate the minimal amount of each fatty acid that we were able to quantify. This limit of quantification (LOQ) was estimated using the formula (28):

$$\text{LOQ} = 3t(v, \alpha)\sigma_y/m \quad \text{Eq. 2}$$

where *m* is the slope of the calibration plot for a single standard as determined by equation 1, *v* = (*N* - 1) is the number of degrees of freedom, with *N* being the number of points in the linear regression, and α is the significance level, a quantity complementary to the confidence level, (α = 5 %), *t* (*v*, α) is the Student *t*-distribution, and

$$\sigma_y = [\sum_i (A_i - m \times C_i)^2 / v]^{1/2}$$

is the residual standard deviation of the linear fit.

The analysis of chromatograms of combined standard mix-

Table I. Relative Content of Fatty Acids in Oils from Different Vegetable Matrices (26)

Acid	Olive (% w/w)	Sunflower (% w/w)	Soybean (% w/w)	Palm (% w/w)
Myristic	0.0–0.1	max 0.1	max 0.1	max 0.1
Palmitic	7.0–20.0	5.0–8.0	9.0–13.0	43.0–48.0
Palmitoleic	0.3–3.5	max 0.5	max 0.3	max 0.3
Stearic	1.0–4.0	2.5–7.0	3.0–5.0	4.5–5.5
Oleic	56.0–84.0	13.0–40.0	19.0–30.0	35.0–45.0
Linoleic	3.0–21.0	40.0–74.0	48.0–58.0	8.5–11.0
Linolenic	0.2–1.5	max 0.3	5.0–9.0	max 0.4
Erucic	0.0	0.0	0.0	0.0

Fatty Acid	Conc. linearity range (mg × mL ⁻¹)	(m ± σ _m)10 ⁶ (min × mL × mg ⁻¹)	LOQ (mg × mL ⁻¹)
Myristic (C14:0)	1.4–11.4	0.27 ± 0.005	0.232
Palmitic (C16:0)	0.2–1.5	0.25 ± 0.03	0.093
Palmitoleic (C16:1)	0.22–1.1	2.20 ± 0.07	0.039
Stearic (C18:0)	0.55–1.28	0.21 ± 0.02	0.056
Oleic (C18:1)	0.43–2.8	2.20 ± 0.18	0.068
Linoleic (C18:2)	0.023–0.5	19.8 ± 1.8	0.004
Linolenic (C18:3)	0.002–0.091	32.7 ± 0.1	0.0005
Erucic (C22:1)	0.64–2.78	2.10 ± 0.07	0.067

tures and oil samples required to resolve eventually overlapping peaks, as those observed for linoleic, myristic, and palmitoleic fatty acids. Peak areas were evaluated in this case by using partial least squares (PLS) linear regression (29). The database for PLS analysis was built starting from the chromatographic data relative to several mixtures of the linoleic, myristic, and palmitoleic acids standards at various concentration ratios. In applying the PLS method to chromatographic data, we noted that the accuracy of PLS results could be improved if eventual small shifts in the retention time (i.e., 10 s) were taken into account. For this reason, we used as input factors the integrals of the chromatographic spectra over a time interval larger than the overlap zone. More precisely, indicating by $A(t, \lambda)$ the chromatographic absorbance as a function of the retention time t and wavelength λ , the data were analyzed by using the equation:

$$I(\lambda) = \int_{t_0}^{t_1} A(t, \lambda) \times dt \quad \text{Eq. 3}$$

where t_0 and t_1 are respectively the initial and the final values of the chosen interval time.

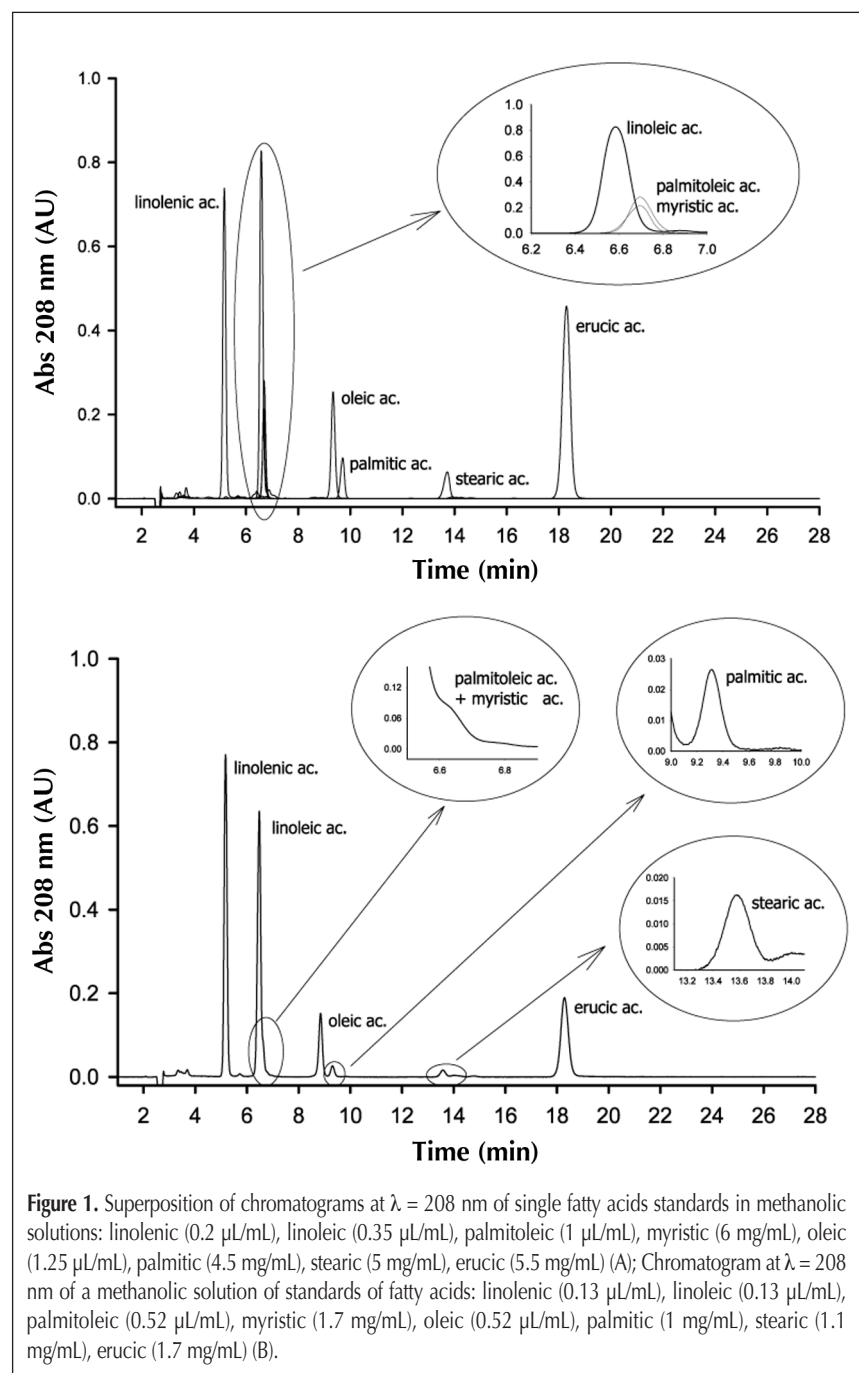
The wavelength range from 207 to 240 nm was included in the analysis by sampling 28 values uniformly spaced. Indeed, the different dependence on wavelength of the molar extinction coefficient of the fatty acids studied here allows considerable expansion of the PLS database.

The $I(\lambda)$ signals relative to 11 mixtures were analyzed by the cross-validation method. Each set was used in turn as the test set while keeping the remaining ones as calibration data. The procedure provided an average residual error for the concentration value of the three fatty acids studied.

Successively, the PLS method was applied to estimate the amount of the same fatty acids in some vegetable oils.

Results and Discussion

The first step in setting an HPLC method appropriate for fatty acids quantification was searching for the most convenient chromatographic conditions. Different mobile phases and flow rates, as described in the previous section, were previously tested on standard mixtures to obtain a well-resolved separation of the fatty acid peaks. The best results were obtained with a mixture composed of acetonitrile, methanol, and *n*-hexane in the ratio 90:8:2, acidified with 0.2% acetic acid and a flow rate of 1 mL/min. These conditions were used to measure the calibration curves of individual fatty acid standards listed in Table I. Erucic acid, which should be totally absent in



the chosen oils, was also included, because its presence is a mark of adulteration with rape-seed oil.

Replicate injections of single standards at different concentrations were used to obtain calibration curves as reported in the previous section. The UV detection wavelength was set at 208 nm. The retention time of acids was observed to depend on the chain length, polarity, and number of double bonds. Table II shows the analytical HPLC parameters obtained. The *m* parameter [and limit of quantitation (LOQ)] values show the different sensitivity of the UV detection for the analyzed acids. The sensitivity is highest for linolenic and linoleic acids and lowest for stearic, palmitic, and myristic. It should be noted that LOQ values in Table II are about an order of magnitude larger than those obtained for methyl esterified acids by using HPLC with evaporative light scattering (30) or refractive index (31) detection.

Although this resolution loss may be critical for detection of fatty acids present in very small amount, it is not serious for the determination of the main acidic components, as discussed in the following.

Figure 1A shows the superposition of the chromatograms of

single standards. At the chosen wavelength, the absorbance of the mobile phase allows an adequate detection. The retention time increases with decreasing the polarity and number of double bonds, and with increasing the length of the alkyl chain. Myristic, palmitoleic, and linoleic acids overlap in the interval between 6.2–7.2 min, as highlighted by the inset in Figure 1A. In fact, these acids are known to be very difficult to separate, and for such reason they are usually referred to as critical pairs (16,19).

In order to verify if interactions between the different acids could modify the chromatographic profile, mixtures containing eight acids standards were analyzed. Figure 1B shows that the chromatogram of the mixture is very similar to that of Figure 1A obtained by superimposing the individual acids signal. To resolve the overlapping peaks of linoleic, palmitoleic, and myristic acids and obtain their quantitative determination, the PLS method was used (27,29). We tested PLS reliability by analyzing the results of HPLC measurements on 11 mixtures of three standards at different concentrations. We obtained an average residual error of 9.3%, 11.1%, and 13.0% for linoleic, myristic, and palmitoleic acid, respectively.

The capability of a qualitative and quantitative determination

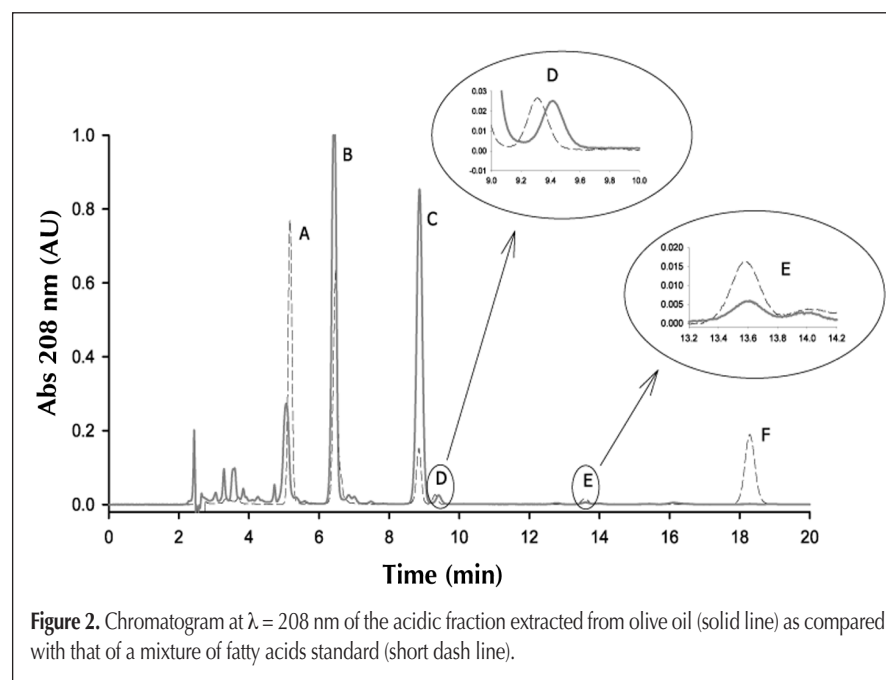


Figure 2. Chromatogram at $\lambda = 208$ nm of the acidic fraction extracted from olive oil (solid line) as compared with that of a mixture of fatty acids standard (short dash line).

of fatty acids by this chromatographic method was tested by studying oils from different vegetable matrices. The acidic fraction, obtained from hydrolysis of triglycerides, was analyzed by HPLC. Figure 2 shows the chromatogram of the acidic components extracted from a sample of olive oil in comparison with that obtained for a mixture of acids standards. Small shifts in retention time were observed, probably due to matrix effects. In the case of olive oil, it was possible to quantify the linolenic (peak A), oleic (peak C), palmitic (peak D) acids by using the calibration curves. The stearic acid (peak E) is present at a concentration lower than LOQ, so allowing just a qualitative determination. The erucic acid peak is absent as expected for olive oil samples. PLS analysis was applied to obtain information about peak B, which was thought to be the convolution of linoleic, palmitoleic, and myristic acid. PLS was able to quantify the

Table III. Comparison of Fatty Acids Content by HPLC and GC

No	Oleic acid		Palmitic acid			Linolenic acid			Linoleic acid			
	HPLC		HPLC		GC	HPLC		GC	HPLC		GC	
	mg/mL	% w/w	mg/mL	% w/w		mg/mL	% w/w		mg/mL	% w/w		% w/w
1	3.4*	82.4	83.9	0.4 [†]	9.9	8.8	0.04 [‡]	1.0	0.4	0.27 [§]	6.5	6.9
2	3.0*	80.5	80.8	0.4 [†]	11.8	11.6	0.04 [‡]	0.9	0.5	0.25 [§]	6.6	7.1
3	1.9*	71.6	74.3	0.5 [†]	17.2	16.2	0.03 [‡]	1.3	0.3	0.27 [§]	10.7	9.3
4	4.5*	75.3	77.4	1.0 [†]	16.8	14.7	0.08 [‡]	1.3	0.6	0.40 [§]	6.5	7.3
5	2.3*	79.0	80.5	0.4 [†]	12.8	12.6	0.03 [‡]	0.8	0.4	0.21 [§]	7.2	6.9

* a RSD < 0.3%

[†] RSD < 2%

[‡] RSD < 6%

[§] RSD < 0.6%

linoleic acid only. This might be expected in the case of olive oil, where the combination of extinction coefficient and typical relative concentration of the three fatty acids falling in the same chromatogram region can make unfeasible a quantitative determination of the minor components*.

In fact, the assumption that only linoleic acid is present and the straightforward calculation of the peak area by the HPLC software gave results similar to those obtained through the PLS analysis. However, it must be noted that PLS analysis can be valuable in resolving fatty acid composition of matrices (32) in which linoleic and myristic or palmitic acid are present in a more favorable ratio.

To the purpose of validating the proposed HPLC protocol, results obtained from five samples of different olive oils were compared with those obtained by GC analysis, as shown in Table III. Note that the comparison is limited to the four fatty acids that we can quantify with our proposed method. For each olive oil sample and for each fatty acid, we report: the HPLC-measured absolute concentration (first column); the concentration relative to the total amount of the four acids, measured with GC and HPLC (second and third column). A good agreement is obtained for oleic, linoleic, and palmitic acids. The values fall inside the range stated by the International Olive Oil Council (33) and the European Commission (8), as reported in Table I. Results for linolenic acids are affected by a systematic error. The incorrect determination of this acid can be ascribed to its low content and/or to the presence of impurity in the HPLC peak associated to linolenic acid. This region of chromatogram is, in fact, particularly crowded as shown in Figure 2 (peak A).

Results on the chosen set of vegetable oils are summarized in Figure 3, which shows on a ternary plot the relative percentages of linoleic, palmitic, and oleic acid in each sample. These three acids were chosen as the most representative components of the oils considered. Further, they present the major variations with the vegetable matrix, allowing an easy discrimination between them. This can be appreciated by looking at the figure where each type of oil is seen to occupy a well-distinguished region. Results are in good agreement with the literature data (see Table I).

Conclusions

This study introduces and tests a chromatographic protocol for the separation and qualitative/quantitative determination of underivatized fatty acids by HPLC with a photodiode array (PDA) detector. The detection was accomplished at $\lambda = 208$ nm, the optimal wavelength to maximize the signal of acids. Central to the protocol is the choice of a mobile phase that provides a good

selectivity and a no too large cut-off of absorbance at wavelength below 208 nm. These conditions were met by using a mixture of acetonitrile, methanol, and *n*-hexane in the ratio 90:8:2, acidified with acetic acid (0.2%, w/w).

The procedure was applied to the determination of the acidic components of some vegetable oils after hydrolysis of the triglycerides. The quantitative determination was carried out using a simple calibration procedure and PLS method. Results obtained for olive oils showed a good agreement with those obtained using the standard procedure for the determination of fatty acids based on GC. Finally, an easy discrimination between oils of different vegetable origin was obtained by studying the relative percentage of the most significant acids.

On the whole, it is interesting to observe that although the proposed HPLC procedure do not provide high sensibility, it offers the great advantage of its simplicity: (i) no gradient was used, either of concentration or flow; (ii) the mobile phase can be prepared before the analysis without a sophisticated mixing system; (iii) the detection can be performed with a simple UV detector; (iv) preliminary derivatization of fatty acids is not required.

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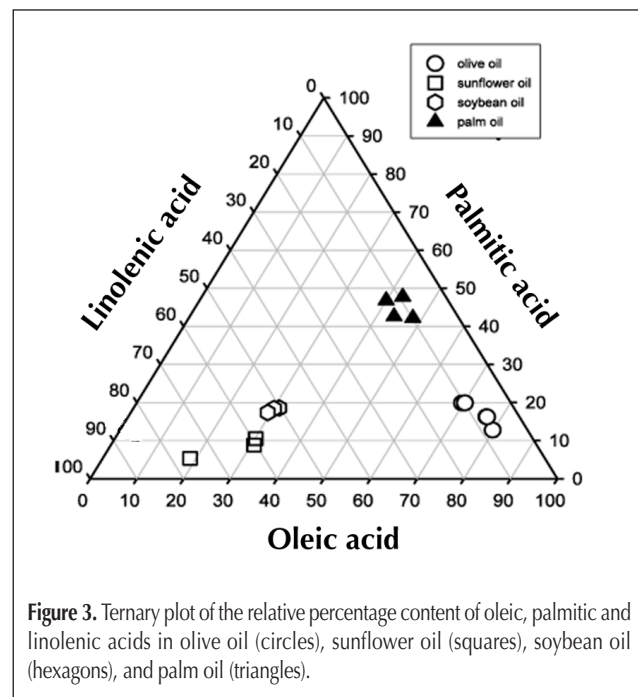


Figure 3. Ternary plot of the relative percentage content of oleic, palmitic and linolenic acids in olive oil (circles), sunflower oil (squares), soybean oil (hexagons), and palm oil (triangles).

* By considering that the fatty acids constitute about the 95% of vegetable oils (24), the concentration (C_{FA}) of a given acid can be estimated by $C_{FA} = 0.95 \times P_{FA} \times 0.91^{-1}$, where P_{FA} is the relative fatty acid percentage reported in Table I, and 0.91 g/mL is the oil density. Thus, linoleic, myristic, and palmitoleic acid concentration in the olive oil are 31–220, 0–1, and 3–36 mg/mL, respectively. To measure the absorbance of linoleic acid inside its linearity range (Table II), the sample must be diluted 60 ÷ 400 times. Upon dilution, the concentration of palmitoleic acid may fall below the detection limit depending on its relative amount with respect linoleic acid. The myristic acid should be below its LOQ value even in undiluted olive oil.

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