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Capillary Electrophoresis of Free Fatty Acids by Indirect Ultraviolet Detection: Application to the Classification of Vegetable Oils According to Their Botanical Origin

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ABSTRACT: A method for the determination of fatty acids in vegetable oils by capillary electrophoresis with indirect UV—vis detection has been developed. The separation of fatty acids was optimized in terms of Brij surfactant nature and concentration and organic modifier (2-propanol) percentage. The optimal background electrolyte consisted of 10 mM *p*-hydroxybenzoate, 5 mM Tris at pH 8.8, 80 mM Brij 98, 40% acetonitrile, and 10% 2-propanol. Under these conditions, vegetable oils from five botanical origins (avocado, corn, extra virgin olive, hazelnut, and soybean) were analyzed and the fatty acid contents established. Linear discriminant analysis (LDA) models were constructed using fatty acid peak areas as predictors. An excellent resolution among all category pairs was obtained, and all samples were correctly classified with assignment probabilities of >95%.

KEYWORDS: botanical origin, CE, fatty acid, linear discriminant analysis, vegetable oils

INTRODUCTION

Authentication of edible quality oils is of great importance from the viewpoints of commercial value and health impact. The organoleptic properties, high nutritional value, and health benefits of quality oils are related to the presence of many components, such as fatty acids, the concentration profiles of which differ according to fruit variety. A relevant aspect of oil authenticity is the adulteration of quality oils by mixing them with oils of lower quality. Then, the evaluation of fatty acid profiles could be an excellent tool to assess oil authenticity.

Traditionally, analysis of fatty acids has been performed spectroscopically[1−3] and chromatographically.[4−8] The chromatographic technique most widely applied to determine fatty acid profiles of lipids has been gas chromatography,[4,5] in which long-chain fatty acids are analyzed as methyl or trimethylsilyl esters in polar columns. On the other hand, high-performance liquid chromatography (HPLC) has been also used to determine fatty acids in lipid matrices, where several UV-absorbing derivatives have been usually employed, such as phenacyl[9] or naphthacyl[10] esters and 2-nitrophenylhydrazides.[11] However, derivatization reactions often produce incomplete conversion of the analyte and undesirable interfering side products.

In the past decade, capillary electrophoresis (CE) has been proposed as an interesting alternative for the analysis of underivatized long-chain fatty acids.[12−25] However, one of the major concerns in analyzing fatty acids by CE has been their limited solubility in aqueous electrolyte systems. To solve this problem, CE separation has been described by using background electrolytes (BGEs) containing organic solvents, such as methanol,[15,16] ethanol,[26] acetonitrile (ACN),[22,25,27] 1-octanol,[25,27] and methylformamidedioxane.[19] In addition, the use of additives to the BGE, such as cyclodextrins[16,22,24,26] or surfactants (sodium dodecyl sulfate[24,26] and polyoxyethylene 23 lauryl ether (Brij 35)[19,21,25,27,28] among others), has been described to modify selectivity on analyte separation. On the other hand, fatty acids do not possess strong chromophores in their structures, which makes difficult their sensitive detection in direct photometric mode. Then, direct UV or fluorescence detection was only employed when a previous derivatization step was performed, although the use of indirect UV and indirect fluorescence detection[29] was preferred. The chromophoric agents used include *p*-anisate,[15,16] diethylbarbiturate,[30] adenosine monophosphate,[19] dodecylbenzenesulfonate,[19,21,25,27,28] and *p*-hydroxybenzoate,[14] among others.

In this work, a CE method with an alkaline buffer in the presence of an anionic chromophore (*p*-hydroxybenzoate) for the indirect UV detection of fatty acids was developed. The separation of fatty acids was optimized in terms of Brij surfactant nature and concentration and organic modifier (2-propanol) percentage. The fatty acid content present in different vegetable oil samples was obtained. Moreover, the fatty acid profiles observed were used to construct linear discriminant analysis (LDA) models to classify oil samples according to their botanical origin.

MATERIALS AND METHODS

Reagents and Samples. The following analytical grade reagents were used: acetonitrile (ACN), methanol, ethanol, 1-propanol, 2-propanol (Scharlau, Barcelona, Spain); tris(hydroxymethyl)aminomethane (Tris, Fluka, Buchs, Switzerland); polyethylene glycol dodecyl ether (Brij 30, $C_{12}EO_4$; EO = number of ethoxylate groups), polyoxyethylene 23 lauryl ether (Brij 35, $C_{12}EO_{23}$), polyoxyethylene (20) oleyl ether (Brij 98,

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Table 1. Botanical Origin, Number of Samples, and Brands of Vegetable Oil Samples Used in this Work

origin	no. of samples	brand
avocado	2	Guinama
	2	Marnys
	2	Serra Vita
corn	2	Guinama
	1	Asua
	1	Crystal
	1	Gloria
	1	Mazola
extra virgin olive	1	Borges
	1	Carbonell
	1	Coosur
	1	Grupo Hojiblanca
	1	Romanico Torrereal
	1	Torrereal
hazelnut	3	Guinama
	3	Percheron
soybean	2	Coosur
	2	Guinama
	1	Biolasi
	1	Coppini

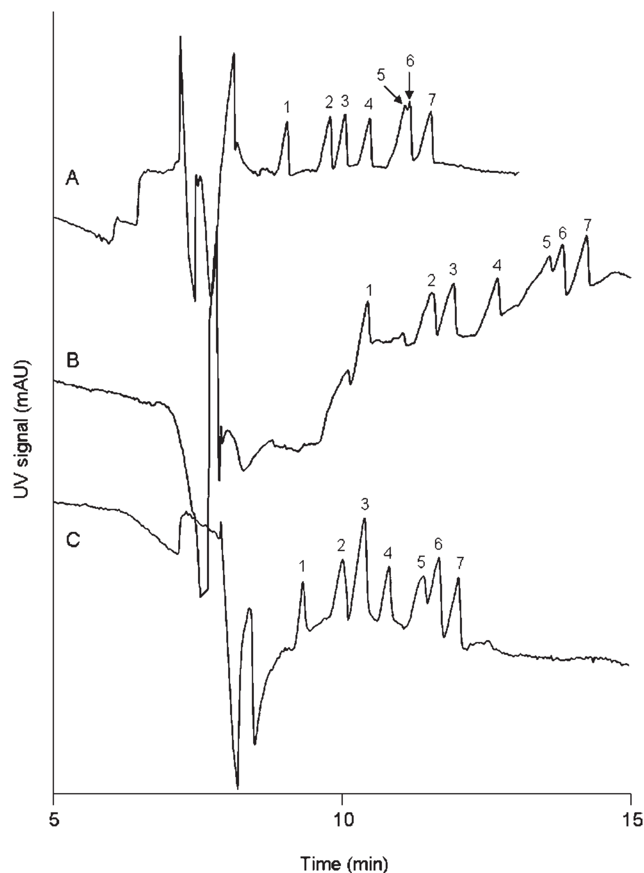

Figure 1. Influence of Brij surfactant nature on the separation of fatty acid standards: Brij 35 (A), Brij 30 (B), and Brij 98 (C) in a BGE composed of 10 mM *p*-hydroxybenzoate, 5 mM Tris at pH 8.8, 40 mM Brij, 40% ACN, and 10% 2-propanol. Working conditions: injection at 50 mbar × 3 s; separation at 25 kV at 45 °C; indirect detection at 254 nm. Peaks: 1, C18:0; 2, C18:1; 3, C16:0; 4, C18:2; 5, C16:1; 6, C18:3; 7, C14:0.

$C_{18}EO_{20}$), sodium *p*-hydroxybenzoate (Sigma-Aldrich, St. Louis, MO); sodium hydroxide (NaOH) and ammonia (NH_3; Panreac, Barcelona, Spain). Deionized water (Barnstead deionizer, Sybron, Boston, MA) was also used. The fatty acids used as standards were myristic (C14:0), palmitoleic (C16:1), palmitic (C16:0), linolenic (C18:3), linoleic (C18:2), oleic (C18:1), and stearic (C18:0) (Sigma-Aldrich). Individual stock solutions of the fatty acid standards (7 mM) were prepared in a MeOH/1-propanol 85:15 (v/v) mixture containing 40 mM NH_4OH. The vegetable oils employed in this study and their commercial brands are shown in Table 1. Monovarietal extra virgin olive oil (EVOO) samples (Hojiblanca, Arbequina, and Picual, the three most important varieties in Spanish production) were from four different geographical areas of Spain. Other vegetable oil samples from different areas of Europe and South America were also used. Most samples were bought in the Spanish market, except for hazelnut oil (Percheron, France), corn oils from Crystal, Gloria, and Mazola (Mexico), and peanut oil (Coppini, Italy). The genetic variety of the olive oils and the botanical and geographical origin of all samples were guaranteed by the suppliers.

Instrumentation and Procedures. An HP^{3D} CE system (Agilent, Waldbronn, Germany) provided with a diode array spectrophotometric detector and uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 80.5 cm length (72 cm effective length) × 50 μm id (375 μm o.d.) were used. New capillaries were successively flushed with 1 and 0.1 M NaOH and water at 60 °C for 10 min each. Daily, before use, the capillary was successively rinsed with 2-propanol, water, and 0.1 M NaOH for 5 min each, followed by the BGE for 10 min more. Between runs, the capillary was flushed with the BGE for 5 min. Hydrodynamic injections at 50 mbar × 3 s were performed. Separations were performed at 25 kV at 45 °C. Indirect detection was done at 254 nm. The BGEs were prepared weekly and stored at 4 °C. Before injection, all solutions were filtered through 0.45 μm pore size nylon filters (Albet, Barcelona, Spain). The optimal BGE consisted of 10 mM *p*-hydroxybenzoate, 5 mM Tris at pH 8.8, 80 mM Brij 98, 40% ACN, and 10% 2-propanol.

Sample Preparation, Data Treatment, and Statistical Analysis. Oil samples (400 mg) were saponified by refluxing at 75−80 °C for 25 min with 0.5 M ethanolic NaOH. After saponification, samples were 1:20 diluted with MeOH and directly injected or stored at 4 °C until their use. All samples were injected three times. The peak area of each compound was measured, and a data matrix was constructed using the areas of all the peaks as original variables. After normalization of the variables, statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL). LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained.[31] This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of λ_w approaching zero are obtained with well-resolved categories, whereas overlapped categories made λ_w approach one. Up to $N - 1$ discriminant vectors are constructed by LDA, with N being the lowest value for either the number of predictors or the number of categories. The selection of the predictors to be included in the LDA models was performed using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the

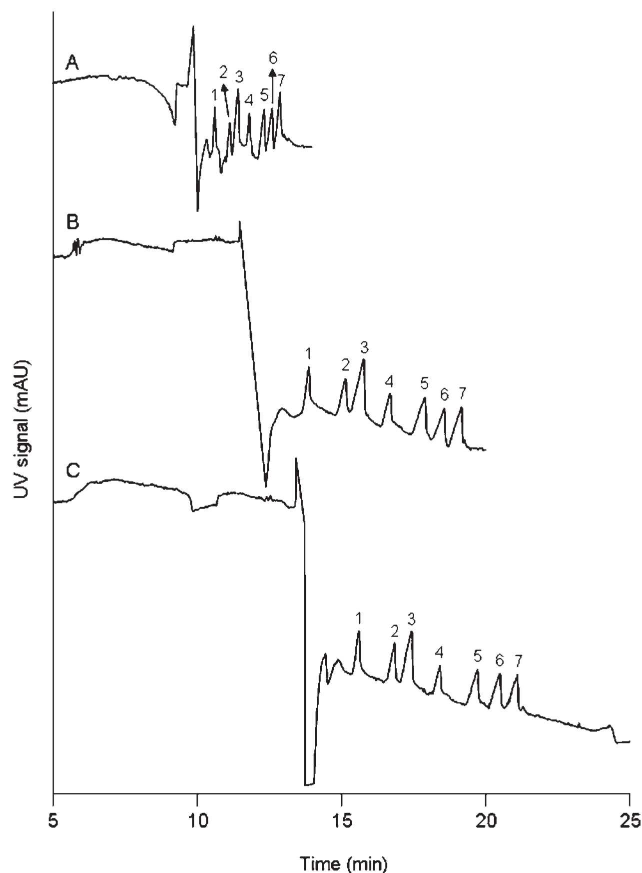

Figure 2. Influence of Brij 98 concentration in the BGE on the separation of fatty acid standards: 60 mM (A); 80 mM (B); 100 mM (C). Other working conditions were as in Figure 1.

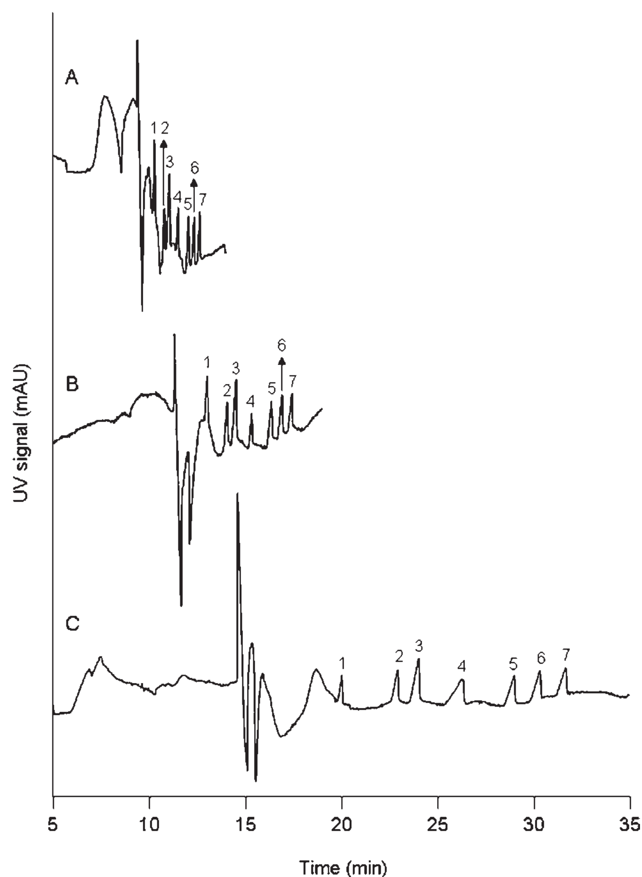

Figure 3. Effect of 2-propanol percentage in the BGE on the separation of fatty acid standards: 5% (A); 7% (B); 15%. BGE composed of 10 mM *p*-hydroxybenzoate, 5 mM Tris at pH 8.8, 80 mM Brij 98, and 45, 43, and 35% ACN, respectively. Other working conditions were as in Figure 1.

reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or F test. However, the entrance of a new predictor modifies the significance of those predictors that are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The default probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

RESULTS AND DISCUSSION

Optimization of BGE. To optimize fatty acid separation in terms of BGE, a test mixture containing the seven fatty acid standards described in the Reagents and Samples section was used. The initial BGE composition was adapted from the literature,[14] which contained 10 mM *p*-hydroxybenzoate, 5 mM Tris, 40 mM Brij 35, and 50% ACN. However, under these conditions, the injection of most oil samples led to a disruption in current, probably due to the poor solubility of fatty acids in the BGE. Then, and in order to enhance the solubility of these compounds, 10% 2-propanol was initially added to the BGE, but keeping the organic percentage constant (40% ACN). The fatty acid separation obtained with this BGE is shown in Figure 1A. As observed, all peaks were separated except the C16:1/C18:3 peak pair. Several studies[19,22,25,27,28] have demonstrated that the use of Brij 35 as nonionic surfactant helps to solubilize fatty acids, leading also to an increase in solvophobic interactions with analytes, which can improve peak resolution. However, to our knowledge, there are no reports about the influence of Brij series surfactants on the separation of fatty acids. For this purpose, fatty acid separation using Brij 30 and Brij 98 was evaluated. These surfactants were selected due to differences in both alkyl chain length and number of EO groups. Thus, for Brij 30 ($C_{12}EO_4$) the hydrophilic group is quite smaller than that of Brij 35 ($C_{12}EO_{23}$), whereas the alkyl chain length was the same. For Brij 98 ($C_{18}EO_{20}$), the hydrophilic group is slightly smaller than that of Brij 35 but the alkyl chain length increased from 12 to 18 carbon atoms. Figure 1 shows the influence of the nature of Brij surfactant on the separation of fatty acids under the same concentration of Brij (40 mM). As observed, a reduction in the EO units of Brij surfactant from 23 (Brij 35, Figure 1A) to 4 (Brij 30, Figure 1B) led to an increase in analysis time jointly with an incipient separation of C16:1/C18:3 peaks. On the other hand, when the alkyl chain length increased from 12 (Brij 35, Figure 1A) to 18 (Brij 98, Figure 1C), an increase in resolution of C16:1/C18:3 peaks was observed with a concomitant increase in analysis time. This migration behavior can be explained by taking into account the solvophobic interactions between the alkyl chain of the surfactant and the polyoxyethylene moieties with the analytes. As evidenced in Figure 1, the hydrophobicity of polyoxyethylene moiety has a stronger effect than the hydrophobicity of the alkyl chain length. At the sight of these results, Brij 98 was

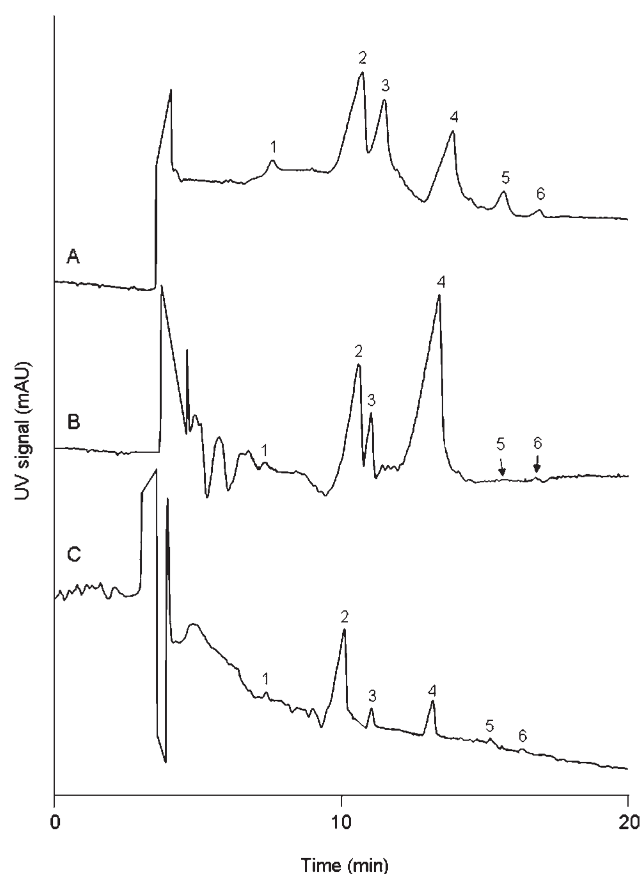

Figure 4. Representative electropherograms of avocado (A), corn (B), and hazelnut (C) oil samples. BGE composed of 10 mM *p*-hydroxybenzoate, 5 mM Tris at pH 8.8, 80 mM Brij 98, 40% ACN, and 10% 2-propanol. Other working conditions were as in Figure 1.

selected for the following studies as the best compromise between resolution and analysis time.

Next, Brij 98 concentration was varied between 40 and 100 mM. The results obtained are shown in Figure 2. As observed, an increase in the Brij 98 content led to longer migration times. This behavior could be explained by taking into account both electroosmotic flow (EOF) changes (due to an increase in the BGE viscosity) and the solvophobic interactions between the analytes and the surfactant. On the other hand, an increase in peak resolution was observed up to 80 mM Brij 98, whereas a slight improvement in peak resolution was achieved at expenses of longer analysis time at 100 mM; then, a concentration of 80 mM was selected for further studies.

Finally, the percentage of 2-propanol was also optimized. Its percentage was varied between 5 and 15%. As evidenced in Figure 3, the addition of 2-propanol led to a progressive increase in migration times of fatty acids, which was due to the EOF reduction caused by 2-propanol (solvent with higher viscosity and lower dielectric constant than ACN). On the other hand, when the content of 2-propanol was increased, an improvement in peak resolution was obtained. Then, a percentage of 10% was selected as the best compromise between resolution and analysis time.

Characterization of the Fatty Acid Profiles of Vegetable Oils. The optimal method was applied to the analysis of vegetable oil samples. Representative electropherograms of avocado (A), corn (B), and hazelnut (C) oil samples are shown in Figure 4. As observed, the myristic acid (C14:0) peak was not observed in any sample; thus, this peak was not used in the following statistical treatments. On the other hand, different fatty acid fingerprints were obtained for the oils; then, fatty acid should be related with the different botanical origins of the oil samples. The differences observed in oil fingerprints were enhanced when chemometric analysis of the data was performed.

Table 2. Migration Time (t_m) and Peak Area (A) Repeatabilities[a] of the CE Method

compd	t_m (%)	A (%)	compd	t_m (%)	A (%)
C14:0	1.5; 1.8	2.0; 2.5	C18:1	1.8; 2.6	2.9; 3.5
C16:0	1.7; 2.2	2.4; 2.7	C18:2	1.8; 2.8	3.1; 3.7
C16:1	1.6; 2.3	2.4; 2.8	C18:3	1.9; 2.9	3.3; 3.7
C18:0	1.8; 2.5	2.8; 3.2			

[a] As intra- and interday relative standard deviations.

Table 3. Percentages of Fatty Acids Found in Vegetable Oils

fatty acid	avocado	corn	extra virgin olive	hazelnut	soybean
C14:0	<LOD	<LOD	<LOD	<LOD	<LOD
C16:0	19.8	10.8	11.9	7.1	6.8
C16:1	6.1	<LOD	<LOD	0.6	<LOD
C18:0	1.2	2.3	2.2	1.8	4.8
C18:1	55.7	25.1	71.7	75.0	19.2
C18:2	16.3	61.0	14.3	15.0	68.1
C18:3	0.9	0.8	0.8	0.5	1.1

Quantitation Studies. External calibration curves were constructed by injecting six standard solutions of each fatty acid between 0.1 and 5 mM. Straight lines with $r^2 > 0.998$ were obtained. Other analytical parameters of interest are given in Table 2. Precision was determined by studying the intra- and interday repeatabilities of migration times and peak areas obtained by injecting the same 0.5 mM solution for all analytes 10 times per day during 3 days. In all cases, the relative standard deviation values were lower than 2.9 and 3.7% for migration times and peak areas, respectively. The limits of detection (LOD) were obtained for signal-to-noise ratio of 3, giving values of ca. 0.020 mM. These values were similar to those reported in the literature.[14,32]

The quantitative results of fatty acids found in the vegetable oils analyzed are shown in Table 3. In general, the levels of these compounds found in the samples are in good agreement with data reported in the literature.[33−36] Corn and soybean oils showed large quantities of linoleic acid (>50%) compared to those found in EVOOs. For this reason, the C18:2 peak has been used as an adulteration marker for detecting these seed oils in EVOOs at contents >21%.[33,34] Consequently, two binary mixtures containing 25% corn or soybean oil with EVOO were prepared and injected. An electropherogram showing the EVOO spiked with 25% soybean oil is shown in Figure 5B. Thus, the presence of this seed oil could be easily evidenced by an increase in the C18:2 peak area when compared with that obtained for the pure EVOO (Figure 5A). A similar increase was evidenced when the EVOO sample was spiked with 25% corn oil (data not shown).

Normalization of the Variables and Construction of LDA Models. To reduce the variability associated with sources of variance that can affect the sum of the areas of all peaks, normalized rather than absolute peak areas were used. To normalize the variables, the area of each peak was divided by each one of the

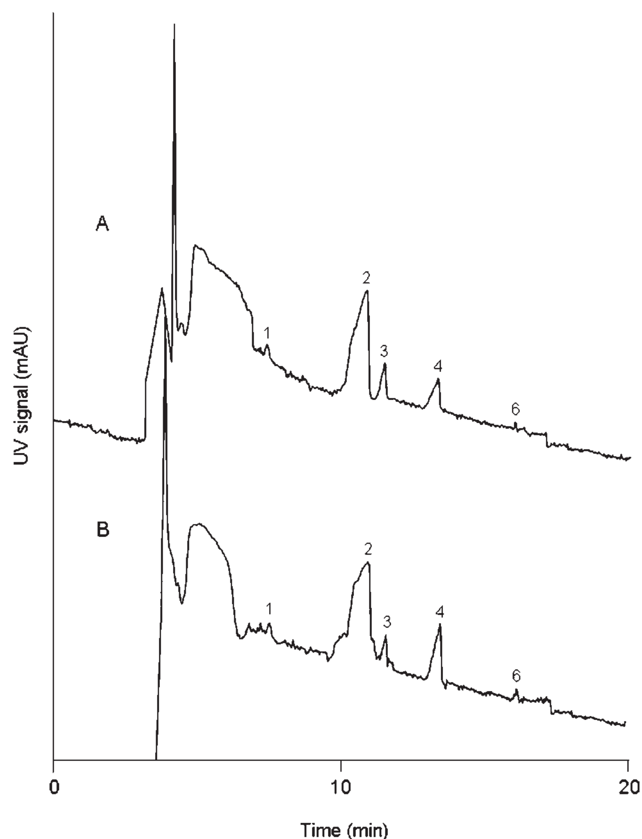

Figure 5. Electropherograms of (A) an EVOO sample and (B) an EVOO sample spiked with 25% soybean oil. Peak identification and working conditions were as in Figure 4.

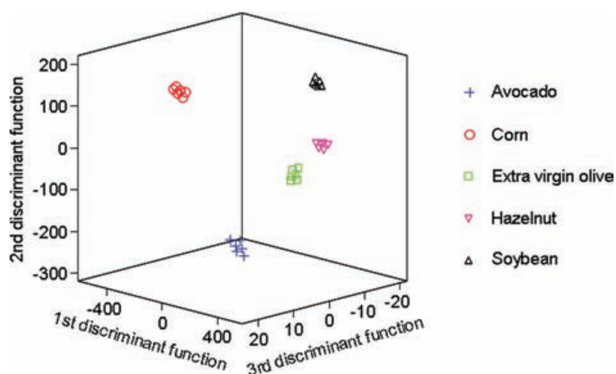

Figure 6. Score plot on an oblique plane of the 3-D space defined by the three first discriminant functions of the LDA model constructed to classify vegetable oils according to their botanical origin.

areas of the other five peaks; in this way, and taking into account that each pair of peaks should be considered only once, $(6 \times 5)/2 =$ 15 nonredundant peak ratios were obtained to be used as predictors. Using the normalized variables, LDA models capable of classifying the vegetable oil samples according to their respective botanical origin were constructed. From the 30 samples of Table 1, a matrix containing 90 objects (which correspond to the three replicates of each sample) and 15 predictors was constructed. A response column, containing the five categories corresponding to the five botanical origins of the oils, was added to this matrix.

Table 4. Predictors Selected and Corresponding Standardized Coefficients of the Discriminant Functions f_1-f_4 of the LDA Model Constructed To Predict the Botanical Origin of Vegetable Oils

predictor[a]	f_1	f_2	f_3	f_4
C16:1/C18:2	−2.24	−3.94	0.76	0.04
C16:0/C18:3	1.24	2.98	0.07	0.34
C16:0/C18:1	14.41	−7.01	−0.44	−0.14
C18:3/C18:2	−1.39	7.55	−0.12	1.07
C18:3/C18:1	16.89	−5.55	0.02	−0.83
C18:2/C18:1	−24.77	9.06	1.04	0.31

[a] Ratios of fatty acid peak areas.

This matrix was used as an evaluation set. To construct the LDA training matrix, only the means of the three replicates of each sample were included (30 objects); in this way, the internal dispersion of the categories was reduced, which was important to reduce the number of variables selected by the SPSS stepwise algorithm during model construction. To classify the vegetable oils according to the five botanical origins of Table 1, an LDA model was constructed. An excellent resolution between all category pairs was obtained (Figure 6, $\lambda_w = 0.074$). The variables selected by the SPSS stepwise algorithm, and the corresponding model standardized coefficients, showing the predictors with large discriminant capabilities, are given in Table 4. According to this table, the main fatty acid ratios selected by the algorithm to construct the LDA model corresponded to C18:2/C18:1, C18:3/C18:1, and C16:0/C18:1. All the points of the training set were correctly classified by leave-one-out validation. The evaluation set, containing the 30 original data points, was used to check the prediction capability of the model. Using a 95% probability, all the objects were correctly classified; thus, the prediction capability was 100%.

This classification model is compared with another classification approach previously published in the literature for the classification of vegetable oils according to their botanical origin using amino acid profiles established by HPLC-UV−vis.[37] A lower λ_w value was obtained with the proposed model (0.074 vs 0.393 for the model constructed with amino acid profiles), being the categories resolved in the latter higher than those discriminated in the present work (7 vs 5 categories). On the other hand, a total of 6 predictors was selected by the LDA model constructed in the current work, which was lower than those selected by the model constructed with amino acid profiles (13 predictors). Taking into account all these aspects, a better model has been obtained in the present work.

In conclusion, the developed method provides a reliable protocol for the separation and determination of fatty acids in vegetable oils by CE with indirect photometric detection. The feasibility of classifying vegetable oils according to their botanical origin by using fatty acid profiles established by CE has been also demonstrated. Additionally, the method allows the detection of adulterations of contents >25% low-cost edible oils, such as soybean or corn in EVOO. To improve the sensitivity, further work is now in progress by the use of extended-path length capillaries.

The developed methodology is a promising alternative to the traditional GC method with the advantages of simplicity and lower consumption of reagents. Thus, the present procedure is of great interest for routine quality control or adulteration purposes in vegetable oil samples.

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