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Characterization of the alcoholic fraction of vegetable oils by derivatization with diphenic anhydride followed by high-performance liquid chromatography with spectrophotometric and mass spectrometric detection

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ABSTRACT

Aliphatic and triterpene alcohols present in vegetable oils have been identified and determined by HPLC using UV–vis and MS detection after previous derivatization with diphenic anhydride. The alcoholic fraction was obtained by saponification, extraction and TLC (according to the European Union official procedure). Derivatization was performed in tetrahydrofuran in the presence of suspended grinded urea, which increases the reaction rate and yield. Derivatized extracts were chromatographed on a C8 column using gradient elution with acetonitrile/water mixtures containing 0.1% acetic acid, with UV–vis followed by negative-ion mode MS detection. Using linear discriminant analysis of the HPLC-MS data (extracted ion chromatograms), oil samples belonging to seven botanical origins (hazelnut, sunflower, corn, extra virgin olive, soybean, peanut and grapeseed) were correctly classified with excellent resolution among all the categories.

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1. Introduction

Vegetable oils are mainly constituted by triacylglycerols, also containing an unsaponifiable fraction which amounts to 1–3%. Among other compounds, this fraction contains aliphatic and triterpene alcohols. The nature and quantitative distribution of these components are characteristic of the original lipid source [1,2,3], thus being useful for the identification of the biological origin of the lipid matrix from which they were extracted. In fact, the unsaponifiable minor components have been employed as a fingerprint of most vegetable oils [4]. Moreover, in the same species, content and composition of these components can vary due to the environmental conditions, fruit or seed quality, oil extraction system and refining process [3]. For these reasons, the determination of these minor components is of great value in establishing the oil genuineness and quality [3,5], having also a marked influence on typicality, flavour, aroma and shelf-life [6].

Vegetable oils contain linear alcohols [7], which are constituted by primary fatty alcohols having generally 20–32 carbon atoms in the alkyl chain. Triterpene alcohols, also known as 4,4′-dimethylsterols, have a steroid structure and are present at different levels in all vegetable lipids [7]. Other alcohols, as phytol, are generated as artifacts of lipid saponification [7].

Long chain aliphatic and triterpene alcohols in vegetable oils have been normally analyzed by GC with flame ionization detection (FID) [8–23] and with MS detection [8,9,12,13,22–24]. Their contents, established by GC-FID, jointly with the contents of other minor components, have been used to distinguish virgin olive oils according to their genetic variety [14–18,20], geographical origin [21] and maturity stage [21], and to detect olive oil adulteration with hazelnut oil [19,22,25–26]. The differences in the contents of these alcohols in olive seed, pulp and fruit oils have been also described [17].

On the other hand, HPLC has been scarcely used to analyze the alcoholic fraction of oils [27]. Due to the low absorbance of these compounds in the UV, Cortesi et al. [27] have derivatized aliphatic and triterpene alcohols with 3,5-dinitrobenzoyl chloride. By using MS with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), fatty alcohols are not detected [28,29]. Along the last years, APCI-MS has gained interest in sterol characterization [30–33].

The derivatization of fatty alcohols with symmetric cyclic anhydrides, including maleic [28] and phthalic [29,34,35] anhydrides, previous to RP-HPLC has been proposed. Fatty alcohol derivatization with these anhydrides is speeded up by suspending grinded urea in the reaction medium [28,29].

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Table 1
Botanical origin, number of samples and brand of the vegetable oil samples used in this work.

Origin	Number of samples	Brand
Hazelnut	4	Guinama
Sunflower	2	Koipesol
	2	Hacendado
Corn	2	Guinama
Corn germ	1	Guinama
	1	Hacendado
Extra virgen olive	1	Carbonell
	1	Grupo Hojiblanca
	1	Borges
	1	Torrereal
Soybean	2	Guinama
	2	Biolasi
Peanut	4	Guinama
Grapeseed	4	Guinama

In this work, an RP-HPLC method to characterize the alcoholic fraction extracted from oils with different botanical origins has been developed. Previous to HPLC separation, the alcoholic fraction was esterified with diphenic anhydride. Esterification was carried out in tetrahydrofuran (THF), and was shown to be speeded up in the presence of urea. Both UV–vis and MS detection were implemented. The application of linear discriminant analysis (LDA) to the resulting alcohol data profiles obtained by HPLC-MS was used to classify oils from seven different botanical origins.

2. Experimental

2.1. Reagents and samples

The following analytical grade reagents were used: ammonium hydroxide, acetic acid, methanol (MeOH), ethanol, acetonitrile (ACN), THF, anhydrous sodium sulphate (Sharlau, Barcelona, Spain), urea (99.5%, Fluka, Buchs, Switzerland), diethyl ether, chloroform (J.T. Baker, Deventer, The Netherlands), KOH (Probus, Barcelona, Spain), *n*-hexane (Riedel-de Haën, Seelze, Germany), 2,7-dichlorofluorescein (Sigma, St. Louis, MO, USA) and diphenic anhydride (98%, Aldrich, Milwaukee, WI, USA). The following linear alcohols were used as standards: 1-hexadecanol (C16, Sigma–Aldrich), 1-octadecanol (C18, Fluka), 1-docosanol (C22), 1-tetracosanol (C24) and 1-hexacosanol (C26) (Sigma). Glass plates for thin-layer chromatography (TLC), coated with silica gel without fluorescent indicador (0.25 mm plate thickness, Merck, Darmstadt, Germany) were used. Deionized water (Barnstead deionizer, Sybron, Boston, MA, USA) was also used. The vegetable oils employed in this study (Table 1) were either purchased at the local market or kindly donated by the manufacturers. The botanical origin and quality grade of all the samples were guaranteed by the suppliers.

2.2. Instrumentation and working conditions

A 1100 series liquid chromatograph provided with a quaternary pump and UV–vis diode array detection (Agilent Technologies, Waldbronn, Germany), was used. Separation was carried out with a C8 fused-core type column (Ascentis-Express, 2.7 μm, 15 cm × 4.6 mm I.D., Supelco, Bellefonte, PA, USA). Mobile phases were prepared by mixing ACN and water, both containing 0.1% acetic acid. Elution was performed isocratically with 90% ACN for 25 min, followed by a linear gradient from 90% to 100% ACN for 10 min, and by isocratic elution with 100% ACN for 10 more min. UV–vis detection was performed at 200 ± 10 nm (360 ± 60 nm as reference). In all cases, 40 μL was injected, being the flow rate 1 mL min^{-1}.

The liquid chromatograph was also coupled (in series with the UV–vis detector) to the ESI source of an HP 1100 series ion trap mass spectrometer (ITMS) (Agilent). The ITMS working conditions were: nebulizer gas pressure, 0.24 MPa (35 psi); drying gas flow, 7 L min^{-1} at 300 °C; capillary voltage, 2.5 kV; voltages of skimmers 1 and 2, −41.0 and −6.0 V, respectively. Nitrogen was used as nebulizer and drying gas (Gaslab NG LCMS 20 generator, Equcien, Madrid, Spain). The mass spectrometer was scanned within the m/z 300–800 range in the negative-ion mode. The ion trap target mass was set at m/z 605 ($[M-H]^-$ peak of the diphenic hemiester of C26). Maximum loading of the ion trap was 3×10^4 counts, and maximum collection time was 300 ms. To enhance sensitivity in the detection of the $[M-H]^-$ ions of the hemiesters, the pH of the eluate was increased. For this purpose, a T union located after the UV–vis detector and before the ESI source, and an auxiliary isocratic HPLC pump set at 0.1 mL min^{-1}, were used to mix the eluate with a 0.01 M NH_3 stream. Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) were smoothed using a gaussian filter set at 9 points.

2.3. Sample preparation and data treatment

The alcoholic fraction of vegetable oils was obtained following the procedure established by the European Union [36]. Accordingly, 5 g oil was saponified by refluxing with 2 M ethanolic KOH for 20 min; 50 mL distilled water was added and the non-saponifiable fraction was extracted three times with diethyl ether. The extracts were combined and washed in a separatory funnel with distilled water (50 mL each time) until neutral reaction. The organic layer was dried with anhydrous sodium sulphate, filtered and evaporated to dryness using a rotatory evaporator. The remaining unsaponifiables were dissolved in 2 mL chloroform and the alcoholic fraction was separated by TLC using a plate-developing chamber containing 60:40 (v/v) hexane/diethyl ether. After TLC separation, the silica plate was sprayed lightly and uniformly with 2,7-dichlorofluorescein. The two bands containing, respectively, the aliphatic and triterpene alcohols were removed from the plate using a spatula. Both bands were jointly suspended in 4 mL THF and introduced in a screw-cap tube (15 mL, 15 cm long) also containing 0.45 g diphenic anhydride and 0.25 g finely grinded urea. The tube was shaken and introduced in a thermostatic bath at 60 °C for 120 min. After cooling, 2 mL of a 2:1 (v/v) MeOH/water mixture containing 0.1 M NH_3 was added. The suspension was sonicated for 15 min and passed through a 0.45 μm pore-size nylon filter (Albet, Barcelona, Spain). The solution was immediately injected in the chromatograph or stored in a freezer.

All the alcoholic extracts were injected at least three times. The peak area of each alcohol derivative was measured from the smoothed EIC, 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, USA).

3. Results and discussion

3.1. Optimization of the esterification procedure

The esterification reaction is illustrated in Fig. 1. The resulting hemiester provides not only a chromophore group with a large molar absorptivity, but also a negative charge, which further

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