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# Fast Separation and Determination of Sterols in Vegetable Oils by Ultraperformance Liquid Chromatography with Atmospheric Pressure Chemical Ionization Mass Spectrometry Detection

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A method for the determination of sterols in vegetable oils by ultraperformance liquid chromatography (UPLC) with atmospheric pressure chemical ionization mass spectrometry detection has been developed. The separation of sterols was optimized in terms of mobile phase composition, column temperature and flow rate. The optimal conditions were achieved using an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) with a mobile phase consistent of acetonitrile/water (0.01% acetic acid) using a linear gradient, at a flow rate of 0.8 mL $min^{-1}$ and column temperature of 10 °C, giving a total analysis time below 5 min. The determination was performed in selective ion recording mode. The limits of detection were in all cases below 0.07 μg $mL^{-1}$, with relative standard deviation values of retention times and peak areas below 0.4 and 5%, respectively. The content of main sterols present in several vegetable oils with different botanical origins was also established.

**KEYWORDS: Botanical origin; mass spectrometry; sterols; ultraperformance liquid chromatography; vegetable oils**

## INTRODUCTION

Phytosterols (also called plant sterols) are bioactive components occurring in vegetable oils, constituting the greatest proportion of the unsaponifiable fraction, which represents a total of 1−3% (*1*). Phytosterols have been received particular attention not only regarding their nutritional value but also due to their capacity to decrease the cardiovascular risk of coronary heart disease (*2*), and to reduce blood cholesterol levels, showing anti-inflammatory, antibacterial, and antioxidant activities (*3*).

The nature and quantitative distribution of sterols are characteristic of the original lipid source (*4*−*6*), which could be useful for the identification of the botanical origin of vegetable oils. In fact, the unsaponifiable minor components have been employed as a fingerprint of most vegetable oils (*7*). 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 (*6*). For these reasons, the determination of these minor components is of great value in establishing the oil genuineness and quality (*6*, *8*), having also a marked influence on typicality, flavor, aroma and shelf life (*9*).

Official methods for analysis of sterols involve saponification of the oil, extraction of the unsaponifiable fraction with diethyl ether and isolation of the sterol fraction by thin layer chromatography (TLC). Quantification of the silanized sterol fraction is commonly performed by GC with flame ionization detection (FID) (*10*−*15*), but GC with MS detection is also used (*10*, *14*, *16*, *17*). The major disadvantage of GC is the requirement of both thermally stable columns and chemical derivatization before analysis. For this reason, other methods such as capillary electrochromatography with UV−vis detection (*18*, *19*), direct infusion MS (*20*) and HPLC−MS (*21*−*23*) have been also developed to determine sterols in vegetable oils.

In the past few years, separation science has been revolutionized with the introduction of ultraperformance liquid chromatography (UPLC). UPLC enables the use of columns packed with 1−2 μm size range, delivering mobile phases at pressures up to 1000 bar (*24*−*28*). According to the Van Deemter equation, when the particle size is lower than 2.5 μm, there is a significant gain in efficiency with increasing flow rates (*25*). Thus, the main advantages of UPLC are (i) a reduction in the analysis time, (ii) an increase in the signal-to-noise ratio and (iii) an enhancement in peak resolution (*26*, *27*). In order to address the very narrow peaks produced by UPLC, a high data capture rate detector is necessary; for this reason, MS detection has been commonly used for UPLC−MS coupling (*25*). The potential of this technique has been tested in different food products (*25*, *28*), such as phytosterols in different food materials (*28*), phenolic compounds in olive oil (*29*) and in olive cakes (*30*).

In this work, the applicability of UPLC−atmospheric pressure chemical ionization (APCI)−MS to characterize the sterol fraction of vegetable oils with different botanical origins has been

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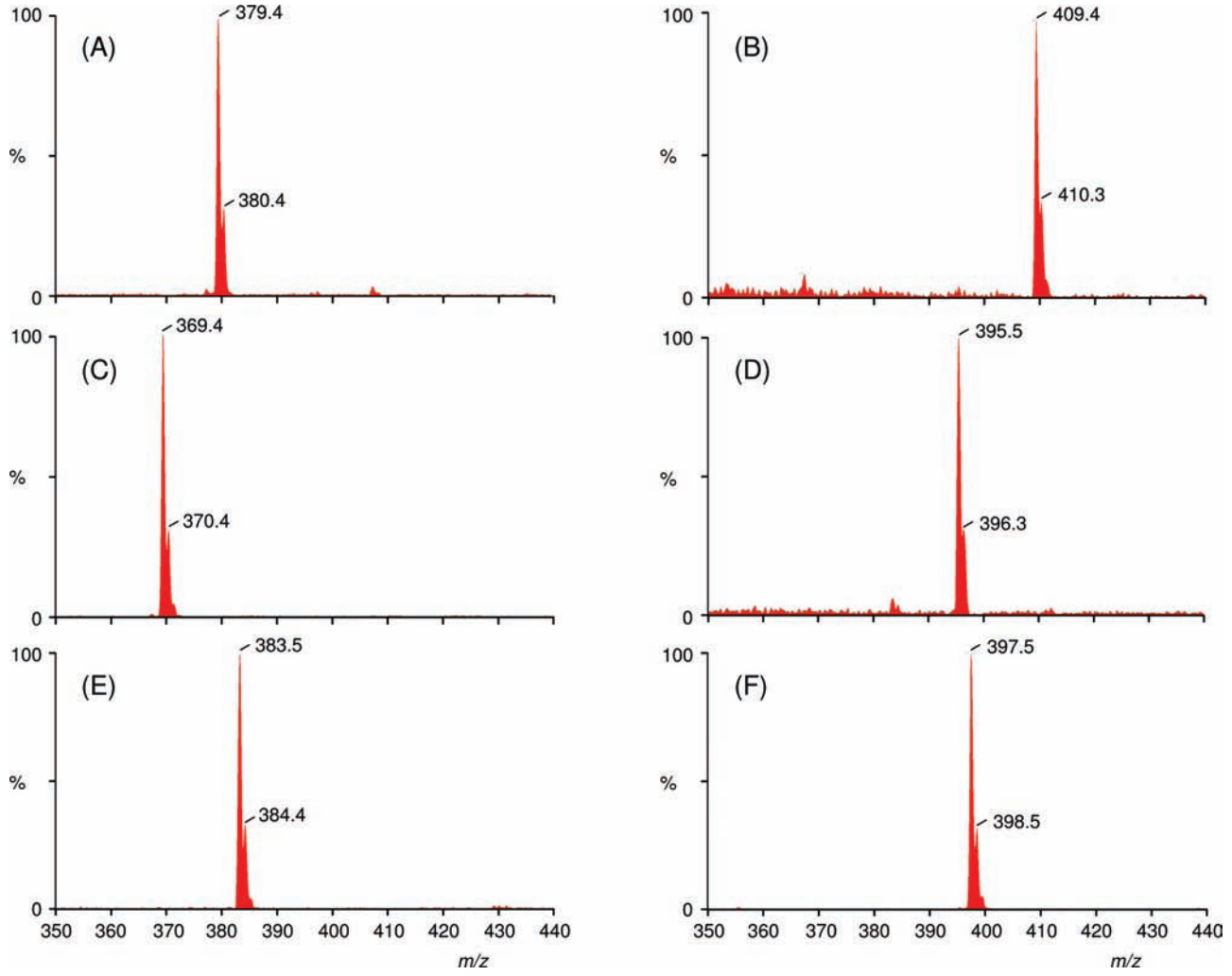


**Figure 1.** Full scan UPLC−MS spectra of the six sterol standards used in this study. Peak identification: (**A**) ergosterol; (**B**) lanosterol; (**C**) cholesterol; (**D**) stigmasterol; (**E**) campesterol; (**F**) $\beta$-sitosterol.

demonstrated. The sterol separation was optimized in terms of mobile phase composition, column temperature and flow rate. The developed method was also applied to the identification and determination of the main sterols present in several vegetable oils.

## EXPERIMENTAL PROCEDURES

**Reagents and Samples.** The following analytical grade reagents were used: ethanol, 2-propanol, acetic acid, acetonitrile (ACN), anhydrous sodium sulfate (Scharlau, Barcelona, Spain), diethyl ether, chloroform (J.T. Baker, Deventer, The Netherlands), potassium hydroxide (KOH, Probus, Barcelona), *n*-hexane (Riedel-de Haën, Seelze, Germany), and 2,7-dichlorofluorescein (Sigma, St. Louis, MO). Glass plates for TLC, coated with silica gel without fluorescent indicator (0.25 mm plate thickness, Merck, Darmstadt, Germany), were used. Deionized water (Barnstead deionizer, Sybron, Boston, MA) was also employed. The sterols used as standards were $\beta$-sitosterol (mixture containing 75% $\beta$-sitosterol and 10% campesterol), ergosterol, stigmasterol (Acros Organics, Morris Plains, NJ), cholesterol (Aldrich, Milwaukee, WI) and lanosterol (Maybridge Chemical Co., Cornwall, England). A total of 32 vegetable oils were employed in this study, 4 for each botanical origin. These samples, which were either purchased in the local market or kindly donated by the manufacturers, were the following: avocado (Guinama and Marnys), corn (Guinama, Asua and Crystal), olive (Carbonell, Grupo Hojiblanca, Borges and Torrereal), hazelnut (Guinama, Percheron and Flumen), grapeseed (Guinama, Paul Corcelet, Pons and Coosur), peanut (Guinama, Bellsola, Apsara Vital and Maurel), soybean (Coosur, Guinama, Biolasi and Sojola) and sunflower (Koipesol, Hacendado and Coosur). In all cases, the botanical origin and quality grade of all the samples were guaranteed by the suppliers.

**Instrumentation and Working Conditions.** An AcQuity ultra-performance liquid chromatograph using a binary pump system (Waters, Mildford, MA) was used. The UPLC was coupled to the APCI ion source of a SQD mass spectrometer (Waters Corporation), which was used as detection system. Separation was carried out with an AcQuity UPLC BEH C18 column (50 × 2.1 mm, 1.7 $\mu$m, Waters). Mobile phases were prepared by mixing ACN:acetic acid (100:0.01, v/v) (phase A) with water:acetic acid (100:0.01, v/v) (phase B). Elution was performed using a linear gradient from 80 to 100% A for 0.5 min followed by an isocratic elution with 100% A for 4.5 more min. The column temperature was kept at 10 °C, and the flow rate was 0.8 mL $min^{-1}$. The injection volume was 15 $\mu$L.

Ionization was performed in APCI positive-ion mode, and data were collected in the selected ion recording (SIR) mode. Optimization of the source parameters was performed automatically by the Waters Intellistar software (Waters). The ionization source parameters were as follows: corona, 4 kV; source temperature, 120 °C; desolvation gas temperature, 400 °C, with a flow rate of 750 L/h. Nitrogen, supplied by a gas generator (Dominick Hunter generator, Gateshead, England), was used as desolvation gas. The individual cone voltage for each sterol standard was evaluated between 10 and 60 V, by infusing 1 $\mu$g $mL^{-1}$ of each compound to obtain the best instrumental conditions, which corresponded to 30 V to each of them. All chromatograms were smoothed using a mean algorithm set at window 3 and number 5. The software used was MassLynx 4.1 (Waters).

**Sample Preparation.** The sterol fraction of vegetable oils was obtained following the procedure established by the Official Journal of

the European Union (*31*). Briefly, the oil samples were saponified by refluxing with 2 N ethanolic KOH solution, and the nonsaponificable fraction was extracted with diethyl ether. The sterol fraction was isolated using TLC and recovered from the plates with diethyl ether. A rotatory evaporator was used to remove the solvent, and the residue was dissolved in 500 $\mu$L of 2-propanol and stored at −20 °C in amber vials. These solutions were diluted with the mobile phase and injected. All the sterol extracts were injected three times. The peak area of each sterol was measured from the smoothed SIR.

## RESULTS AND DISCUSSION

**Optimization of Sterol Separation.** In order to optimize sterol separation in terms of mobile phase composition, column temperature and flow rate, a test mixture composed of six sterol standards (*ca.* 50 $\mu$g mL$^{-1}$, except for $\beta$-sitosterol and campesterol that were *ca.* 38 and 5 $\mu$g mL$^{-1}$, respectively) was used. According to literature (*21*), mixtures of mobile phases A and B were tried in gradient elution mode, using a constant column temperature (30 °C) and flow rate (0.5 mL min$^{-1}$). For each sterol standard, two SIR channels, which corresponded to the $[M + H]^+$ and $[M + H - H_2O]^+$ ions, were monitored. However, and as previously reported (*20*, *21*, *23*), the $[M + H - H_2O]^+$ peaks showed higher intensities than the respective $[M + H]^+$ peaks. For this reason, the intensities obtained at the SIR channels of the $[M + H - H_2O]^+$ peaks (379.5, 369.5, 383.5, 395.5, 397.5, and 409.5 for ergosterol, cholesterol, campesterol, stigmasterol, $\beta$-sitosterol and lanosterol, respectively) were used for identification and quantification. The APCI mass spectra showing the $[M + H - H_2O]^+$ peaks of the six sterol standards are depicted in **Figure 1**. In all the gradient elutions tested, stigmasterol and campesterol peaks overlapped. This result was in agreement with previous reports (*28*). A linear gradient from 80 to 100% A for 0.5 min, followed by an isocratic elution with 100% A, was selected as the best compromise between analysis time and separation (**Figure 2B**). Under this gradient elution and using a flow rate of 0.5 mL min$^{-1}$, the influence of column temperature was evaluated. As shown in **Figure 2**, the chromatographic behavior of sterols was affected by changing the temperature of the column from 10 to 40 °C. At 40 °C (**Figure 2A**), lanosterol and cholesterol peaks overlapped. When the temperature was decreased from 30 to 10 °C (**Figure 2B−D**), a slight decrease in efficiency values jointly with an increase in analysis time was observed. However, the global resolution (measured as the geometrical mean of the resolution between the consecutive sterol pairs) slightly improved (from 1.18 to 1.30). For this reason, a column temperature of 10 °C was selected for further studies. Next, the influence of the flow rate on sterol separation was also studied (see **Figure 3**). When the flow rate was increased from 0.4 to 0.8 mL min$^{-1}$ (**Figure 3A**,**B**), higher efficiency values were obtained whereas the global resolution decreased from 1.47 to 1.28; a decrease of both parameters was observed when flow rate was increased up to 1.2 mL min$^{-1}$ (**Figure 3C**). As a result, a flow rate of 0.8 mL min$^{-1}$ was selected as the best compromise between efficiency, resolution and analysis time.

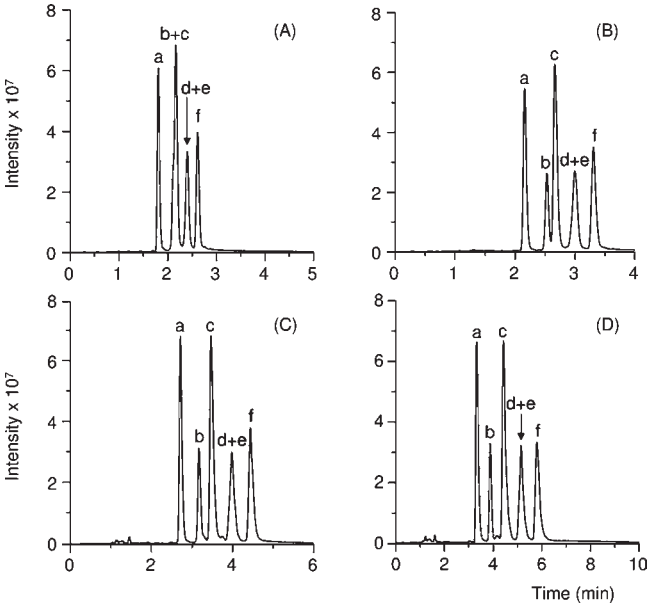


**Figure 2.** Influence of the column temperature on the separation of sterols: (**A**) 40 °C, (**B**) 30 °C, (**C**) 20 °C and (**D**) 10 °C. Chromatographic conditions: linear gradient from 80 to 100% A for 0.5 min followed by isocratic elution with 100% A for 4.5 more min using a flow rate of 0.5 mL min$^{-1}$. Peak identification as in **Figure 1**.

**Quantitation Studies and Application to Vegetable Oils.** External calibration curves were constructed by injecting six standard solutions of each solute within its linearity range (0.5−50 $\mu$g mL$^{-1}$, except for $\beta$-sitosterol that ranged up to 250 $\mu$g mL$^{-1}$). Straight lines with $R^2 > 0.998$ were obtained. Other analytical figures of merit are given in **Table 1**. Precision was determined by studying the intra- and interday repeatabilities of peak areas and retention times obtained by injecting the same 1 $\mu$g mL$^{-1}$ solution for all analytes, 10 times per day during 3 days. In all cases, the relative standard deviation values were lower than 5 and 0.4% for peak areas and retention times, respectively. The relative sensitivities of sterols (with respect to $\beta$-sitosterol) gave values comprised between 0.9 and 1.2, except for lanosterol that provided a value of 0.4. This behavior was due to differences in sterol structures, which was in agreement with previous studies (*28*). The limits of detection, which were estimated for a signal-to-noise ratio of 3, were comprised between 0.03 and 0.07 $\mu$g mL$^{-1}$, whereas the limits

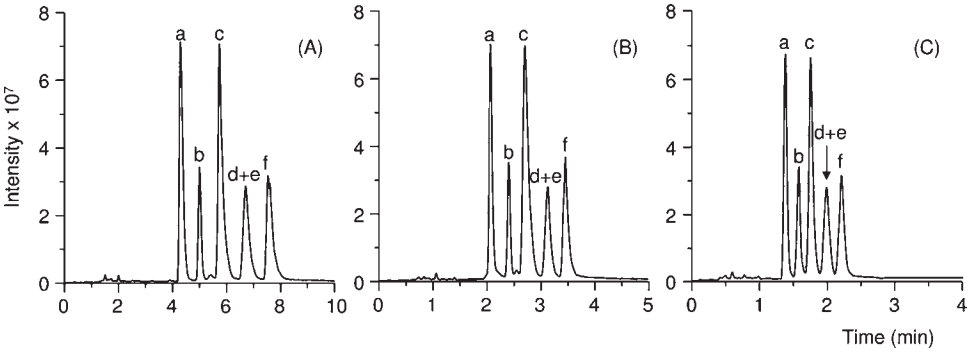


**Figure 3.** Influence of flow rate on the separation of sterols: (**A**) 0.4, (**B**) 0.8 and (**C**) 1.2 mL min$^{-1}$. Chromatographic conditions: temperature 10 °C; gradient elution and peak identification as in **Figure 2**.

**Table 1.** Analytical Figures of Merit for the UPLC−MS Method for the Determination of Sterols

| | repeatability, % | | | | | | |
|---|---|---|---|---|---|---|---|
| | intraday[a] | | interday[b] | | LOD | LOQ | rel |
| analyte | area | $t_R$ | area | $t_R$ | ($\mu$g mL$^{-1}$) | ($\mu$g mL$^{-1}$) | sensitivity[c] |
| ergosterol | 2.8 | 0.08 | 3.6 | 0.25 | 0.04 | 0.13 | 1.1 |
| lanosterol | 2.7 | 0.08 | 3.2 | 0.27 | 0.07 | 0.25 | 0.4 |
| cholesterol | 2.7 | 0.10 | 4.1 | 0.30 | 0.03 | 0.10 | 1.2 |
| stigmasterol | 2.6 | 0.11 | 3.7 | 0.30 | 0.04 | 0.13 | 0.9 |
| campesterol | 2.9 | 0.11 | 3.8 | 0.31 | 0.03 | 0.10 | 1.2 |
| $\beta$-sitosterol | 2.4 | 0.12 | 5.0 | 0.40 | 0.04 | 0.13 | 1.0 |

[a] For a sterol concentration of 1 $\mu$g mL$^{-1}$ ($n$ = 10). [b] For a sterol concentration of 1 $\mu$g mL$^{-1}$ (3 days). [c] As the ratio of the slopes of calibration curves of sterols (respect to $\beta$-sitosterol).

**Table 2.** Ions Observed in the APCI Mass Spectra of the Oil Samples with Their Corresponding Retention Times ($t_R$)

| peak no. | analyte | $t_R$ (min) | ion $[M + H - H_2O]^+$ ($m/z$) |
|---|---|---|---|
| 1 | erythrodiol | 1.4 | 425.5 |
| 2 | uvaol | 1.4 | 425.5 |
| 3 | ergosterol | 2.1 | 379.5 |
| 4 | brassicasterol | 2.2 | 381.5 |
| 5 | $\Delta^5$-avenasterol | 2.4 | 395.5 |
| 6 | cholesterol | 2.7 | 369.5 |
| 7 | campesterol | 3.1 | 383.5 |
| 8 | campestanol | 3.1 | 385.5 |
| 9 | stigmasterol | 3.1 | 395.5 |
| 10 | clerosterol | 3.1 | 395.5 |
| 11 | $\Delta^{5,24}$-stigmastadienol | 3.3 | 395.5 |
| 12 | $\beta$-sitosterol | 3.5 | 397.5 |
| 13 | $\Delta^7$-stigmastenol | 3.5 | 397.5 |
| 14 | sitostanol | 4.5 | 399.5 |

of quantification, based on a signal-to-noise ratio of 10, ranged from 0.10 to 0.25 $\mu$g mL$^{-1}$. These values were higher than those reported by Lu et al. (*28*) working with an UPLC system using the same stationary phase but a triple-quadrupole mass spectrometer, whereas they were lower than those obtained using a conventional HPLC system with a single-quadrupole instrument (*21*, *22*). In any case, a substantial reduction in the analysis time was achieved with the proposed method (between 4- and 10-fold lower than those found in the literature (*21*−*23*)).

On the other hand, GC methods using FID or MS detection (*10*−*14*, *16*, *17*, *31*) showed a superior resolution (especially in some peak pairs as $\beta$-sitosterol/$\Delta^7$-stigmastenol and stigmasterol/clerosterol), but longer analysis times (25−30 min) were obtained in comparison to the proposed method (5 min). In spite of these overlappings, satisfactory results were achieved in terms of resolution/analysis time ratio. Besides, an additional advantage of the recommended method is that it avoids the derivatization step, which is time-consuming and could be a source of errors (*28*).

The optimized method was applied to the analysis of oil samples. In addition to the SIR channels of standards, additional signals (see **Table 2**) were also recorded according to the $[M + H - H_2O]^+$ values of other expected sterol compounds in vegetable oil samples (*19*, *21*, *23*). The TIC and SIRs of an extra virgin olive and hazelnut extracts are shown in **Figure 4**. The main sterols found in the samples, with their corresponding retention time and $[M + H - H_2O]^+$ ions, are summarized in **Table 2**. To quantify the main sterols found in samples, those not available as standards were estimated as follows: the saturated sterols (campestanol and sitostanol) were estimated using the calibration curve of lanosterol, which showed a similar sensitivity to that reported in literature for this type of compounds (*28*). Due to the structural resemblance between erythrodiol + uvaol and lanosterol, these solutes were also

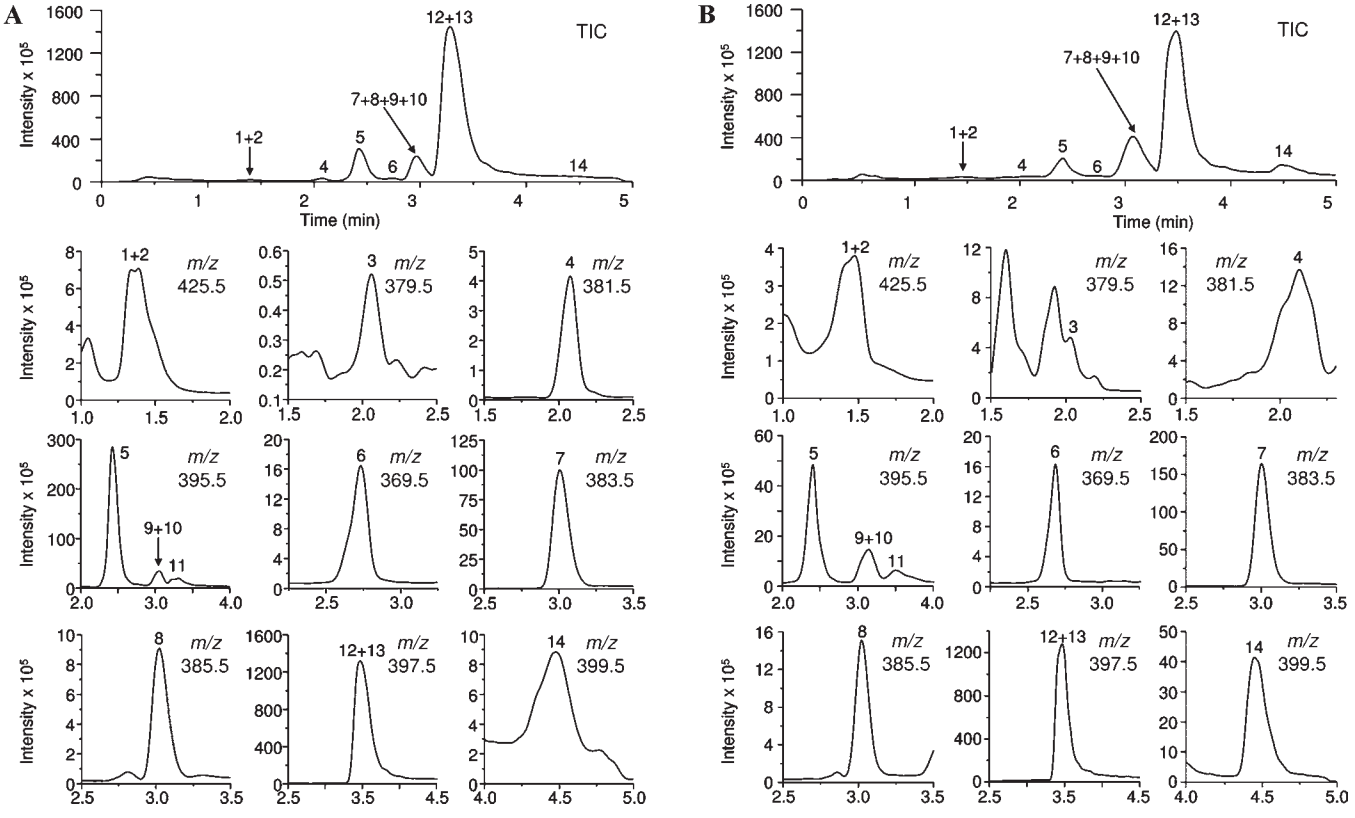


**Figure 4.** TIC and SIRs of (**A**) extra virgin olive and (**B**) hazelnut oil extracts. Chromatographic conditions: gradient elution as in **Figure 2**; column temperature, 10 °C; flow rate, 0.8 mL min$^{-1}$. Peak identification as indicated in **Table 1**.

**Table 3.** Proportions of Sterols Found in Total Sterol Fraction (%)

| sterol | avocado | corn | extra virgin olive | grapeseed | hazelnut | peanut | soybean | sunflower |
|---|---|---|---|---|---|---|---|---|
| erythrodiol + uvaol | 0.05−0.17 | 0.27−0.45 | 0.53−0.90 | 0.01−0.07 | 0.16−0.19 | 0.30−0.35 | 0.10−0.15 | 0.25−0.35 |
| ergosterol | 0.01−0.02 | 0.01−0.02 | 0.01−0.02 | 0.00−0.02 | 0.25−0.34 | 0.50−0.90 | 0.01−0.05 | 0.03−0.05 |
| brassicasterol | 0.25−0.37 | 0.15−0.18 | 0.11−0.18 | 0.10−0.20 | 0.30−0.40 | 0.10−0.18 | 0.28−0.30 | 0.19−0.23 |
| $\Delta^5$-avenasterol | 17.20−18.15 | 3.95−5.75 | 11.00−13.00 | 2.50−2.80 | 1.90−2.10 | 9.18−11.32 | 3.50−4.00 | 6.45−9.05 |
| cholesterol | 0.22−0.62 | 0.31−0.53 | 0.32−0.47 | 0.54−0.78 | 0.30−0.40 | 0.68−2.10 | 0.80−0.90 | 0.78−0.83 |
| campesterol | 4.50−6.30 | 19.00−19.75 | 2.74−3.13 | 12.82−13.14 | 4.50−5.00 | 16.47−18.20 | 17.00−19.00 | 10.20−10.89 |
| campestanol | 0.31−0.41 | 0.83−0.91 | 0.24−0.32 | 0.58−0.62 | 0.20−0.23 | 0.50−0.74 | 0.60−0.80 | 0.43−0.53 |
| stigmasterol + clerosterol | 0.44−0.75 | 6.54−8.08 | 1.51−3.00 | 13.94−15.54 | 0.90−1.00 | 10.30−11.93 | 18.40−19.10 | 8.94−9.37 |
| $\Delta^{5,24}$-stigmastadienol | nd[a] | 0.63−0.70 | 0.80−0.91 | nd | 0.95−1.03 | 0.49−0.51 | nd | 0.62−0.68 |
| $\beta$-sitosterol + stigmastenol | 72.67−76.54 | 61.33−66.21 | 77.69−82.44 | 64.39−67.27 | 87.01−88.54 | 53.27−61.03 | 54.70−58.61 | 67.56−71.76 |
| sitostanol | 0.48−0.54 | 2.10−2.30 | 0.30−0.38 | 2.24−2.44 | 2.00−2.30 | 0.45−0.50 | 0.70−1.00 | 0.35−0.46 |

[a] Not detected.

estimated using the lanosterol calibration curve. The other sterols (brassicasterol, $\Delta^5$-avenasterol and $\Delta^{5,24}$-stigmastadienol) were estimated using the $\beta$-sitosterol calibration curve.

The quantitative results of sterols in the vegetable oils analyzed are shown in **Table 3**. The range values were obtained from the mean value of the three injections of every sample belonging to each category. In general, the levels of sterols found in these samples are in good agreement with data reported in literature (*14*, *18*, *21*, *22*, *32*). As it can be seen, $\beta$-sitosterol was the main sterol in all the analyzed oils. Soybean oil contained large quantities of campesterol and stigmasterol + clerosterol, whereas corn oil had less amounts of these latter sterols. The contents of these compounds found in extra virgin olive oil were quite low. Then, the presence of stigmasterol peak could be used as adulteration marker in extra virgin olive oil. The highest contents of $\Delta^5$-avenasterol were found in avocado followed by extra virgin olive and peanut oils. This sterol was found in much low amount in hazelnut than in extra virgin olive oil; however, sitostanol showed a larger content in hazelnut (see **Figure 4A**,**B**). Thus, these peaks could be used to detect the challenging adulteration of extra virgin olive oil with hazelnut. On the other hand, hazelnut and peanut oils showed large ergosterol contents. These high amounts could indicate a fungal activity in the raw material, which also indicates the quality of these oils (*33*).

In conclusion, this method provides a fast and reliable protocol for the separation and identification of sterols in vegetable oils by UPLC−APCI−MS. The separation of sterols could be achieved in less than 5 min, providing narrow peaks with good peak symmetry. Although several peaks in the samples were not completely resolved, the high selectivity provided by the SIR acquisition of MS instrument made it possible to separate most of analytes. The present procedure is of great interest for the routine quality control or adulteration purposes in vegetable oil samples.

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