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Separation and determination of sterols in olive oil by HPLC-MS

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Abstract

This study presents the first liquid chromatographic method for the identification and quantification of seven phytosterols in olive oil. Sterols were identified and quantified by liquid chromatography with mass spectrometry detection in positive APCI (atmospheric pressure chemical ionisation) mode. The samples were saponified by refluxing with 2 N ethanolic KOH, and the non-saponificable fraction was extracted with diethyl ether. This fraction was subjected to thin layer chromatography (TLC) on silica gel plates and then the band of sterols was isolated and extracted with methanol. Sterols were quantified by LC-MS, on a Waters Atlantis 5 μm $dC_{18}2{,}1 \times 150$ mm column with a gradient of acetonitrile/water (0.01% acetic acid) at a flow of 0.5 mL min^{-1}; column temperature 30 °C. The method presents values between 123 and 677 ng mL^{-1} for detection limits, with relative standard deviations between 4.0% and 5.4% at a concentration of 5 mg L^{-1} for each sterol. Sterol contents were determined in a virgin olive oil, a refined olive oil, an olive-pomace oil and a crude olive-pomace oil.
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1. Introduction

Vegetable oils are mainly made up of triacylglycerols and a complex mixture of minor compounds (2–5%) with a wide range of chemical structures. These minor constituents show a broad qualitative and quantitative composition, depending on the vegetal species.

Sterols are nutritionally important lipids that need to be routinely determined in foods. In olive oil, content and composition of sterols can vary due to the agronomic and climatic conditions, fruit or seed quality, oil extraction and refining procedures and storage conditions. Plant sterols, also called phytosterols, make up the greatest proportion of the non-saponificable fraction of lipids. Their composition depends on the kind of olive oil.

Chemical structures of these sterols are similar to cholesterol. In crude olive oil the predominant phytosterols are sitosterol (90%) and stigmasterol. Compositional analysis of the sterol fraction of olive oil can be used to assess the degree of purity of the oil and the absence of other plant oils. This determination also permits characterization of the type of olive oil in question: extra virgin, virgin, refined, etc (European Union Commission, 2003).

Phytosterols have also been recognized as cancer preventive biologically active substances together with other secondary plant products. The growing interest in the physiological properties of phytosterols (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000) means it is very important to obtain accurate quantitative data on their occurrence in food matrices.

The methods for minor constituent determination usually require isolation and several procedures of separation, identification and quantitation. The analyses are usually based on the official methods developed for determining

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total sterols in fats and oils. Official methods (AOCS Official methods, 1991, chap. 6-91; European Union Commission, 1991; International Standard Office, 1999) for isolation of the total sterols from olive oils involve saponification of the lipids, extraction of the non-saponificable matter with diethyl ether and washing of the extract with water, separation by thin layer chromatography (TLC) on silica gel plates and derivatisation of the sterols and subsequent chromatographic analysis. The conventional method for quantifying sterols and triterpenic alcohols involves capillary GC analysis with FID, of the fraction isolated by TLC, as trimethylsilyl derivatives (AOCS Official methods, 1991, chap. 6-91; European Union Commission, 1991; International Standard Office, 1999).

Gas chromatography (GC) with different detectors (Alonso, Fontecha, Lozada, & Juarez, 1997; Dutta & Normen, 1998; Jekel, Vaessen, & Schothorst, 1998; Kamm, Dionisi, Hischenhuber, Schmarr, & Engel, 2002; Lalas & Tsaknis, 2002; Lechner, Reiter, & Lorbeer, 1999; Phillips, Ruggio, & Bailey, 1999; Verleyen et al., 2001) and with mass spectrometry (MS) (Bruni et al., 2002; Cercaci, Rodriguez-Estrada, & Lercker, 2003; Jeong & Lachance, 2001; Medvedovici, David, & Sandra, 1997; Ng & Hupe, 1998; Van Boven, Daenens, Maes, & Cokelaere, 1997), are the most usual techniques for sterol determination.

High performance liquid chromatography (HPLC) with different detection systems has also been used to determine and quantify major plant sterols in foods (Breinhölder, Mosca, & Lindner, 2002; Holen, 1985; Indyk, 1990; Warner & Mounts, 1990). A few publications exist regarding the identification by LC-MS of some sterols in soybean oil (Careri, Elviri, & Mangia, 2001) and in edible seaweed (Sánchez-Machado, López-Hernández, Paseiro-Losada, & López-Cervantes, 2004) but there are no publications on the analysis of sterols in olive oil by HPLC-MS.

Therefore in this work the applicability of LC-MS with atmospheric pressure chemical ionisation (APCI) was evaluated for the characterization of the sterol fraction in the olive oil sample. The objective of this study was to accomplish the complete separation of a complex mixture of sterols by LC and their identification by mass spectrometry. A good separation of cholesterol, stigmasterol, β-sitosterol, sitostanol, fucosterol, erythrodiol and uvaol was achieved.

2. Materials and methods

2.1. Reagents, stock solutions and reference compounds

Cholesterol was acquired from Riedel-de Haën (Germany), β-sitosterol, erythrodiol, and stigmasterol was obtained from Fluka (Switzerland) and sitostanol, uvaol and fucosterol from Sigma (Germany). Stocks solutions containing 400 μg mL^{-1} of phytosterols were prepared in HPLC-grade methanol and stored in the dark at 4 °C for at least 2 months. The final stock solution concentration was calculated taking into account the purity of commercial standards. Working standard solutions were prepared from these solutions and diluted with methanol prior to analysis. HPLC-grade ethanol, methanol, acetonitrile, diethyl ether and chloroform were purchased from Panreac (Spain). Potassium hydroxide and sodium sulphate anhydrous were also purchased from Sigma, and Silicagel 60 TLC plates (20×20) from Merck (Spain). Water was deionized by using a Milli-Q system (Millipore, Bedford, USA).

2.2. Instrumentation

The LC system was an Alliance 2695 equipped with autosampler, degasser, and heater column purchased from Waters (Milford, MA, USA). The mass spectrometer system was a ZQ 2000 single quadrupole purchased from Waters-Micromass (Manchester, UK) and was used with an APCI interface. Data were collected by MassLynx 4.0 software on a personal computer. The compounds were separated by a 5-μm Atlantis dC_{18} 150×2.1-mm i.d. from Waters with a gradient of acetonitrile/water (0.01% acetic acid) at a flow of 0.5 mL min^{-1}.

2.3. Sample preparation

Four olive oil and pomace olive oil samples of different geographical origin were obtained in March of 2005. The method for the saponification, the isolation of the non-saponificable fraction and the separation of sterols of this fraction in these oil samples was according to the method described by the official Journal of the European Communities (European Union Commission, 1991).

The samples (5 g of olive oil) were saponified by refluxing with 50 mL of ethanolic solution of KOH 2 M for 1 h. After cooling at room temperature, 100 mL of water were added. After phase separation in a separation funnel, the aqueous phase was washed three times with diethyl ether. Finally, the diethyl fractions were collected and washed with water and dried with sodium sulphate anhydrous, filtered and then evaporated to dryness using a rotary evaporator at reduced pressure, and the residue (the non-saponificable material) was dissolved in chloroform (1 mL).

The extract (300 μL) was subjected to thin layer chromatography (TLC) on silica gel plates yielding different separated bands corresponding to the different classes of minor components. Then the band of sterols was isolated and extracted with methanol (25 mL) and subsequent liquid chromatographic analysis.

2.4. LC-MS method

Sterols in the non-saponificable fractions of the olive oil samples were analyzed by LC-MS. The compounds were isolated with a gradient of acetonitrile/water (0.01% acetic acid) at a flow of 0.5 mL min^{-1}. The gradient LC conditions were: the mobile phase was water (acetic acid 0.01%) (Phase A) and acetonitrile (Phase B) and the solvent

Table 1
Characteristic fragment-ions observed in the ESI and APCI mass spectra of sterols in positive mode

m/z	Ion $[M-H_2O]^+$		Ion $[M+H–H_2O]^+$		Ion $[M+H]^+$		Ion $[M+Na]^+$		Ion $[2M+H]^+$	
	ESI	APCI	ESI	APCI	ESI	APCI	ESI	APCI	ESI	APCI
Cholesterol	–	–	369	369	–	–	409	–	–	–
Stigmasterol	–	394	–	395	–	–	–	–	–	–
β-Sitosterol	–	396	–	397	415	–	–	–	852	–
Sitostanol	–	398	–	399	–	–	–	–	–	–
Fucosterol	–	394	395	395	413	–	–	–	–	–
Erythrodiol	–	424	–	425	–	443	465	–	908	–
Uvaol	–	424	–	425	–	443	465	–	908	–

gradient changed according to the following conditions: from 0 to 2 min, 30% (A):70% (B) to 0% (A):100% (B); this value was maintained for 28 min and then, from 30 to 31 min, 0% (A):100% (B) to 30% (A):70% (B) using this percentage during 4 min, and the run was ended. The column temperature was set to 30 °C during all the running and the injection volume was 10 μL. Analytes were detected with an APCI probe in the positive mode (probe tip at 450 °C). The temperature source was 120 °C; the flows for desolvation and cone gas were 350 L h^{-1} and 50 L h^{-1}, respectively; the corona was set to 21.6 μA. Detection was done in SIM mode at *m/z* 369.20, 395.30, 397.30, 399.30 and 425.30.

3. Results and discussion

3.1. Characterization of sterols in mass spectrometry by ESI and APCI

Mass spectra were acquired using direct infusion by FIA (flow injection analysis) at 10 μL min^{-1} and 100 μL min^{-1}, for ESI and APCI mode, respectively, of each standard in full scan mode at a concentration of 20 and 50 mg L^{-1} in methanol for ESI and APCI mode, respectively. ESI and APCI was used as ionisation sources, both in negative and positive mode changing the cone voltage between 20 and 60 V, in a range of *m/z* between 70 and 1000 but only effective results were obtained in positive mode. As can be seen in Table 1 the ESI source is not applicable for the determination of sterols because no sufficient fragmentation occurs for these compounds at these conditions. Table 1 shows the main fragments observed for each source in positive mode.

To confirm the ion selection the positive APCI mode was finally selected to identify and quantify the sterols. The most abundant signal corresponded to a protonated fragment-ion $[M+H–H_2O]^+$, which was chosen for the identification.

3.2. Variables in the HPLC-MS methodology

3.2.1. Mass spectometer variables

After detection, ionization and fragmentation conditions for the APCI source in positive mode were optimised for the sterols by continuous flow injection of 10 μL of pure standard solutions at 50 mg L^{-1} in methanol. Table 2 summarises the optimal MS conditions.

3.2.2. HPLC variables

Different mobile phases were tested in order to separate the target compounds. We assayed different gradients of water (acetic acid 0.01%) (Phase A)/acetonitrile (Phase B) at a constant flow of 0.5 mL min^{-1}. In the Table 3 we show detailed the gradient proved.

Table 2
Mass spectometer conditions for the LC-MS method

Voltages	Corona	21.6 μA
	Cone	20 V
	Extractor	5 V
	RF Lens	0.5 V
Temperatures	Source	120 °C
	Probe temp	450 °C
Gas flow	Desolvation	350 L h^{-1}
	Cone	50 L h^{-1}
Analyser	LM Resolution	15
	HM Resolution	15
	Ion Energy	0.5
	Multiplier	650

Table 3
Description of different gradients proved

Time	Gradient 1		Gradient 2		Gradient 3		Gradient 4	
	Phase A	Phase B	Phase A	Phase B	Phase A	Phase B	Phase A	Phase B
0	30	70	40	60	50	50	60	40
2	0	100	0	100	0	100	0	100
30	0	100	0	100	0	100	0	100
31	30	70	40	60	50	50	60	40
35	30	70	40	60	50	50	60	40

For the four gradients used the resolution between sterols was very similar but shorter times were obtained using gradient 1. The chromatogram of seven analites is shown in Fig. 1. In order to identify the sterols mass spectra of standards were used.

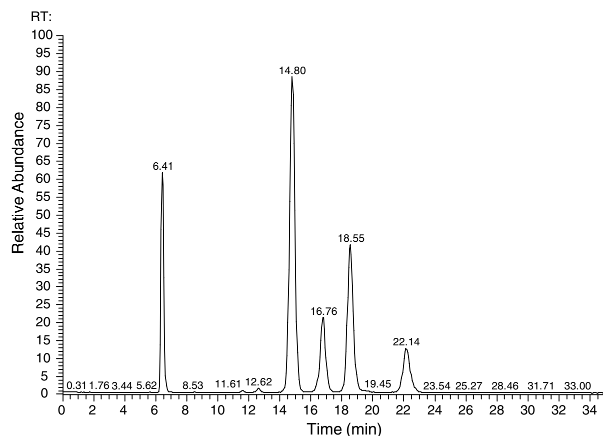

Fig. 1. HPLC-MS chromatogram of a standard solution of sterols (10 mg L^{-1}) with gradient 1. Time scale in minutes: erythrodiol and uvaol (6.41 min), cholesterol (14.80 min), fucosterol (14.80 min), stigmasterol (16.76 min), β-sitosterol (18.55 min), sitostanol (22.14 min).

3.3. Analytical parameters

Analytical performance characteristics of the proposed method were evaluated. Detection in selected-ion monitoring (SIM) at m/z 369.20 (for cholesterol), 395.30 (for fucosterol and stigmasterol), 397.30 (for β-sitosterol), 399.30 (for sitostanol) and 425.30 (for erythrodiol and uvaol) was used to improve the characteristics of the analytical parameters. Calibration curves for the different sterols were obtained from standard solutions at different concentration levels, selected as representative of the range of concentrations in the samples. Ranges for each sterol were between 0.05 and 20 mg L^{-1} for each sterol and between 0.05 and 200 mg L^{-1} for β-sitosterol. Linear calibration graphs were constructed by least-squares regression of concentration versus peak height of the calibration standards. Calibration curves obtained for sterols showed good linearity in the range tested with regression coefficients (r) higher than 0.999 in all cases. Detection (LODs) and quantitation (LOQs) limits were determined as the lowest concentration level that yielded a signal-to-noise (S/N) ratio of 3 and 10, respectively. These values vary between 123 and 677 ng mL^{-1} for detection limits and 500 and 2515 ng mL^{-1} for quantitation limits. Repeatability of the

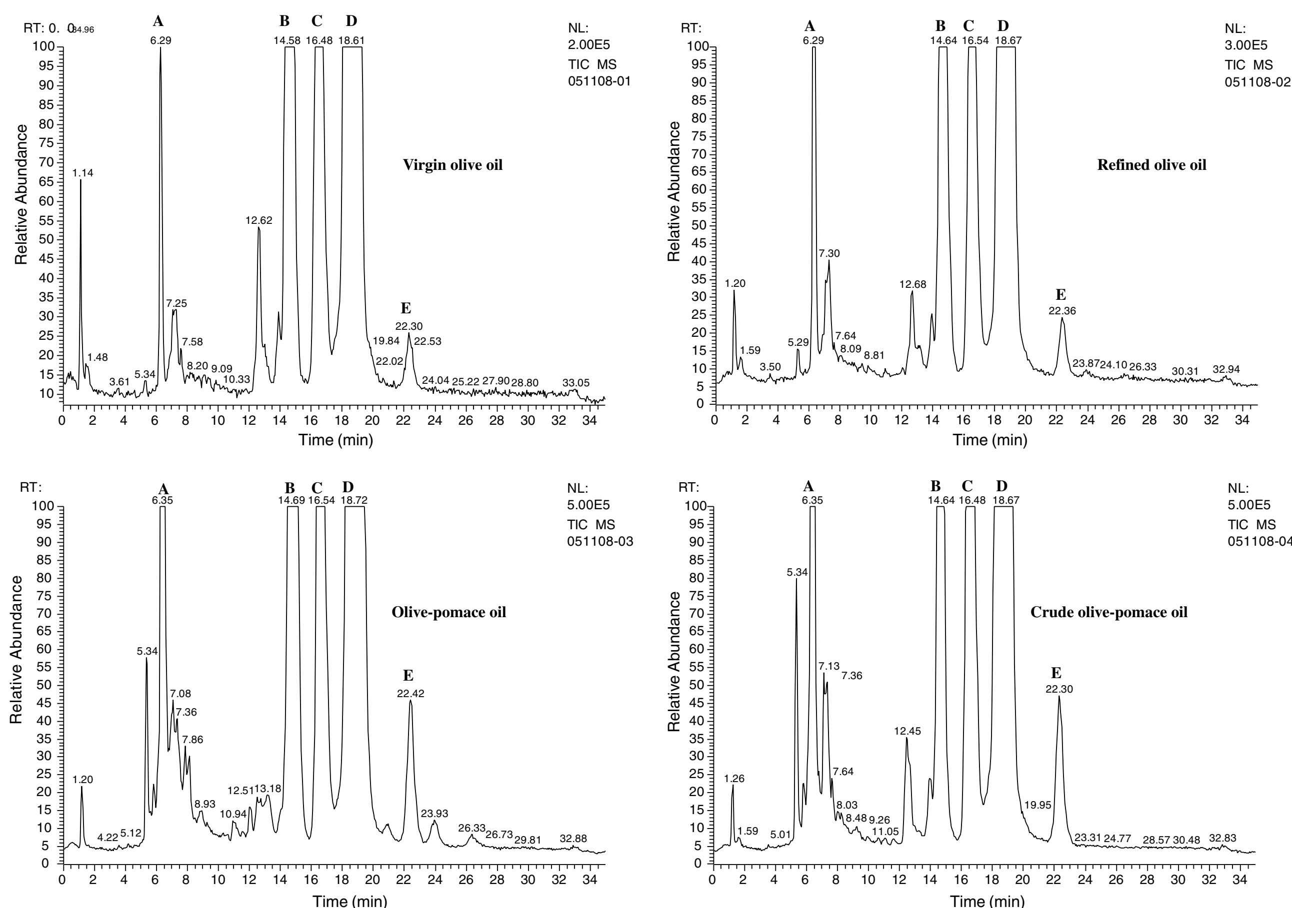

Fig. 2. LC-APCI-MS extracted ion chromatograms of oil extracts. Time scale in minutes. Erythrodiol and uvaol m/z 425.30 (A), cholesterol m/z 369.20 and fucosterol m/z 395.30 (B), stigmasterol m/z 395.30 (C), β-sitosterol m/z 397.30 (D), sitostanol m/z 397.30 (E).

Table 4
Sterol contents (mg kg^{-1}) of the different olive oils analyzed

	mg sterol/kg oil					
	Cholesterol	Stigmasterol	β-Sitosterol	Sitostanol	Fucosterol	Erythrodiol–uvaol
Virgin olive oil	2.0	5.3	666.8	4.4	95.0	12.9
Refined olive oil	2.0	7.0	898.3	7.7	74.5	47.7
Olive-pomace oil	2.6	20.8	2266.2	38.9	119.7	628.7
Crude olive-pomace oil	2.9	27.8	2972.3	44.9	170.9	689.1

HPLC-MS method was evaluated by performing ten repetitive analyses of 5 mg L^{-1} of each sterol, which gave an RSD between 4.0% and 5.4% showing a good precision.

3.4. Sample analysis

Sterol contents in the non-saponificable fractions of a virgin olive oil, a refined olive oil, an olive-pomace oil and a crude olive-pomace oil samples were determined by HPLC-MS. Virgin olive oils are the oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decanting, centrifuging and filtration. Refined olive oil is an olive oil obtained from virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure. Olive-pomace oil is the oil obtained by treating olive pomace with solvents or other physical treatments, to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds. This oil consists of a blend of refined olive-pomace oil and virgin olive oils (Codex Standard for Olive Oils & Olive Pomace Oils, 2003).

Samples of virgin olive oil and refined olive oil were obtained in Albolote (Granada), olive-pomace oil and crude olive pomace-oil were obtained in Pinos Puente (Granada) in March 2005. The fatty substance is saponified with potassium hydroxide in ethanolic solution and the unsaponifiables are then extracted with ethyl ether. The sterol fraction is separated from the unsaponifiable extract by chromatography on a basic silica gel plate. Then the band of sterols was isolated and extracted with methanol as at described in 2.3. The injection volume for the isolation of the compounds was 10 μL of methanolic extracts. All of the analyses were carried out at the optimal conditions mentioned in 2.4. The following compounds were isolated and quantified: cholesterol, stigmasterol, β-sitosterol, sitostanol, fucosterol, erythrodiol and uvaol. The chromatograms of four samples are shown in Fig. 2.

Results of sterol contents expressed in mg for each sterol per kg of olive oil are shown in Table 4.

4. Conclusions

LC-MS with atmospheric pressure chemical ionisation (APCI) was evaluated for the first time to identify and characterize sterol fraction in the olive oil sample. A sufficient separation of cholesterol, stigmasterol, β-sitosterol, sitostanol, fucosterol, erythrodiol and uvaol was achieved and the method has been satisfactorily applied to different real samples.

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