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Analysis of phytosterols in extra-virgin olive oil by nano-liquid chromatography

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ABSTRACT

In this work the applicability of nano-liquid chromatography (nano-LC) was evaluated for the determination of phytosterols in extra-virgin olive oil samples. These compounds represent a minor part of lipids in vegetable oils, but their quantification can be useful to establish oil origin and to reveal intentional adulterations. The analysis of five main sterols, specifically brassicasterol, stigmasterol, campesterol, cholesterol and β-sitosterol, was performed in a laboratory-assembled nano-LC system coupled with a UV detector. The separation of all compounds was obtained in about 20 min, employing a capillary column packed with a C18-RP (sub-2 μm particles) stationary phase for 15 cm. Methanol only was used as mobile phase. The simple method developed and optimized was validated in terms of repeatability, linearity, limit of detection and limit of quantification (0.78 and 1.56 μg/mL, respectively) achieving good results. After this, it was applied to the determination of phytosterols in extra-virgin olive oil samples. Isolation of phytosterols was obtained by solid-phase extraction, after saponification and liquid–liquid extraction of the unsaponified fraction with diethyl ether. Recovery tests were performed and values between 90% and 103%, with RSDs within 5%, were obtained. Moreover the nano-LC system was coupled with a mass spectrometer for an accurate identification of phytosterols.

1. Introduction

Vegetable oils are mainly composed by triacylglycerols, but minor essential compounds (2–5%), such as fatty alcohols, tocopherols, sterols, wax esters, phenolic compounds, etc. are also present [1,2]. The qualitative and quantitative occurrence of minor components in these oils may vary depending on the type of plant used for their production. In addition, using the same type of plant, their quantity may be very different because it depends on several parameters, like the geographical origin, cultivar, harvesting period and ripening of fruit (considering i.e. olive oil) [1,3,4]. Among the several vegetable oils used, extra-virgin olive oil (EVOO), obtained only from mechanical pressing of ripe olive fruits without any further refining process, is considered the most vintage vegetable oil due to its organoleptic properties as well as to its beneficial effects on human health [5–7].

The list of minor essential compounds present in these oils includes phytosterols which are the main constituents of the non-saponificable fraction of lipids in olive oil. They can be present as free or esterified structures with sugar or fatty acid moieties, etc. [8].

In the last years, several studies demonstrated that phytosterols exhibit anti-inflammatory, anti-pyretic, antibacterial, antifungal, antineoplastic activities [6,7]. More recently, reduced blood cholesterol level was also observed [9,10]. As a consequence, the dietary intake of plant sterols, as part of the normal diet, or as a supplement (nutraceutical food) has increased. A large number of commercialized products, declare to contain these compounds.

Considering the business behind EVOOs and nutraceutical food, it can be easily explained all fraud related to adulteration and sophistication that are constantly being made, such as mixing oils of low quality with olive oil.

Therefore phytosterols represent a marker to be monitored in EVOOs for their characterization. In this context, qualitative and quantitative analysis of phytosterols can be used to assess the degree of purity of the oil and the absence of other vegetable oils.

Analytical methods so far used for the analysis of these compounds include gas chromatography (GC) [11–13] and high-performance liquid chromatography (HPLC) with UV or evaporative light scattering detection [14]. In several studies sterols have been identified by coupling GC, and more recently HPLC, with mass spectrometry (MS) [2,14–16]. The major disadvantages of GC, which are recommended by official methods, are the requirement of both thermally stable columns and chemical derivatization prior analyses. Few reports, dealing with the separation of these compounds by capillary zone electrophoresis (CZE) or capillary electrochromatography (CEC), are also available [17–19].

The use of miniaturized techniques appear very useful in food analysis, especially in quality control or to highlight contamination and/or adulteration. They offer several advantages over classical techniques and among these, it is worth mentioning the following: short analysis time, high efficiency, reduced consumption of both

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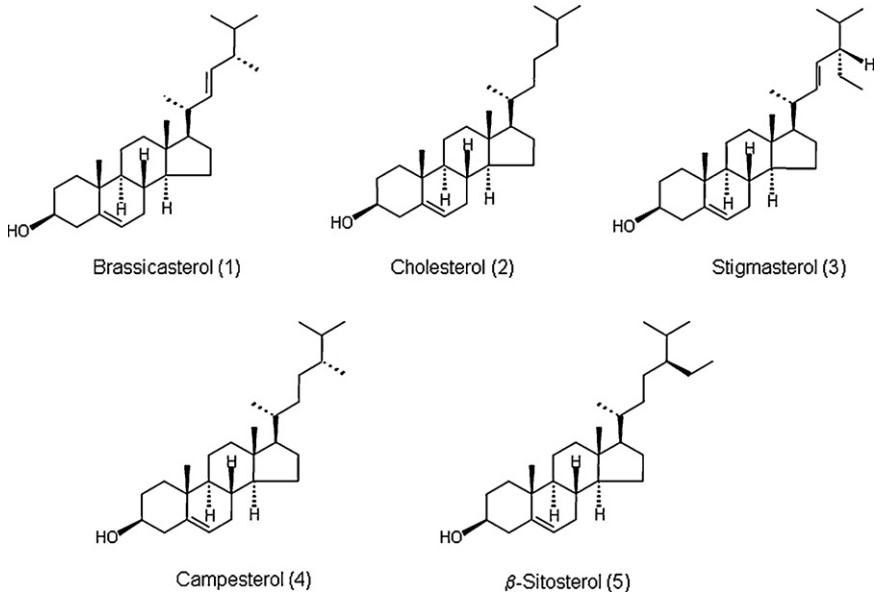

Fig. 1. Structures of studied sterols.

mobile and stationary phase, easily coupling with MS, etc. [20–23].

Recently miniaturization was also introduced in HPLC setting up capillary/nano-liquid chromatography (cLC and nano-LC), which are increasingly utilized in the analytical field [24].

Although both cLC and nano-LC have already been employed in food analysis [24–30], to the best of our knowledge they have not been applied to the analysis of phytosterols in EVOO.

In this study the applicability of nano-LC for the quantification of some sterols, namely β-sitosterol, brassicasterol, cholesterol, stigmasterol and campesterol, in the EVOO samples was evaluated (for their chemical structure see Fig. 1). These molecules were selected considering that in crude olive oil the most abundant phytosterol is β-sitosterol (even more than 90% of total amount of phytosterols), followed by campesterol and stigmasterol [2,31]. Cholesterol is also present, but at very low concentrations (less than 0.1%). Therefore higher quantity of this compound may indicate the addition of animal fats. After all, the presence of brassicasterol (at trace levels in EVOO) is used as marker to uncover adulteration with rapeseed oil.

In order to obtain a good separation of all analytes different stationary and mobile phases were considered. The optimized method was validated, in terms of repeatability, linearity, limit of detection (LOD) and quantification (LOQ) and afterwards it was applied to the analysis of some phytosterols present in extracts of extra-virgin olive oil. The nano-LC system was also coupled with mass spectrometry for certain identification of the separated compounds.

2. Experimental

2.1. Chemicals

Methanol (MeOH), ethanol (EtOH), diethyl ether and chloroform were purchased from Carlo Erba (Milan, Italy). Each solvent was of HPLC grade. Potassium hydroxide was obtained from Fluka (Buchs, Switzerland). β-Sitosterol (5-stigmasten-3β-ol, MW 414.70), campesterol (24α-methyl-5-cholesten-3β-ol, MW 400.69), stigmasterol (5,22-stigmastadien-3β-ol, MW 412.70), cholesterol (5-cholesten-3β-ol, Mw 386.66) and 6-ketocholestanol (5a-cholestan-3b-ol-6-one) were from Sigma (St. Louis, MO, USA). Brassicasterol (5,22-cholestadien-24β-methyl-3β-ol, MW 398.66) was from Steraloids (London, UK). Each standard compound was dissolved in MeOH and stock solutions of 1 mg/mL were stored at −21 °C. Before analyses, samples were diluted 10 times with MeOH.

Ultrapure water was obtained by a Milli-Q system, Millipore/Waters (Milford, MA, USA). Commercial EVOO sample was bought in a supermarket in Rome.

2.2. Instrumentation

Nano-LC experiments were performed in a laboratory assembled instrument. It was composed by a Spectra System P2000 conventional gradient (HPLC) pump, a Spectra System SCM1000 vacuum membrane degasser and a UV–vis on-column Spectra System UV 1000 detector, each one purchased from Thermo Separation Products (San José, CA, USA). The splitting device, required to reduce the μ- to nano-flow rate, consisted of a stainless steel tee (VICI Valco, Houston, TX, USA). One access of the tee was connected to the pump with a polyether ether ketone (PEEK) (tube (50 cm × 130 μm); the second entry to the waste through a fused silica capillary (50 cm × 50 μm I.D.). Finally a stainless steel tube (3 cm × 500 μm I.D.) connected the injection valve to the third entrance of the tee. Analytes were detected at 195 nm and data were collected by Spectra System Software PC1000 (Fremont, CA, USA). Samples were introduced into the column by a nano injector valve Sepaserve (Munster, Germany), injecting for 10 s and washing the loop immediately after with the mobile phase. Nano-LC optimized experiments were performed using hydride-based RP-C18 sub-2 μm (kindly donated by Professor J.J. Pesek, Department of Chemistry, San José State University, San José, CA, USA) and eluting with MeOH as mobile phase at an estimated flow rate of about 330 nL/min.

A PerkinElmer series 10 LC pump (Palo Alto, CA, USA) was used for packing the capillaries as previously published [28,32].

For the identification and characterization of analytes with MS, the nano-LC system was coupled with an LCQ quadrupole ion-trap mass spectrometer, Thermo-Finnigan (S. José, CA, USA) controlled by XcaliburTM 1.3 software (Thermo-Finnigan).

The end of the column was joined to the probe through a nano-electrospray ionization (nano-ESI) source (Thermo-Finnigan). Emitter tips were prepared in our laboratory shaping a fused silica capillary (10.5 cm × 25 μm I.D. × 375 μm O.D.) supplied by Composite Metal Services (Ilkley, UK) with sand paper on a rotating disk. The tip was washed with water and methanol before use. It was set

on the nano-ESI interface and positioned at 1–2 mm from the MS orifice.

The analytes were detected in the positive ion mode setting the capillary voltage at 8 V. The ion-spray voltage and the capillary temperature were at 2.0 kV and 200 °C, respectively. The acquisition was realized in full scan mode in the m/z range 150–500. In order to enhance the ionization of the studied compounds, the mobile phase (100% MeOH) was supplemented with the appropriate amount of ammonium acetate salt (final concentration was 15 mM).

2.3. Sample preparation

The sample preparation was carried out considering previously published methods with some simple modifications [12,33]. Briefly, about 1 g of EVOO was added to 10 mL of 2 M KOH ethanolic solution. The mixture was heated under reflux at 80 °C until saponification took place (transparent solution). Afterwards 10 mL of distilled water was added and the solution was cooled at room temperature. After adding to this mixture 10 mL of diethyl ether, the solution was vortex-mixed. The two phases obtained by centrifuging the mixture at 3000 rpm for 15 min (ALC 4236 Centrifuge, ALC, Milan, Italy) were separately collected and treated as follows. The aqueous phase was treated twice with 10 mL diethyl ether (each time). The three fractions of organic solvent were joined and washed with distilled water (10 mL × time) until neutral pH of washing water was observed.

Diethyl ether solution was then distilled and dried by a rotary evaporator at reduced pressure. The residue (non-saponificable material) was dissolved in chloroform (2 mL) and passed through a Sep-Pak Vac C18 6 mL cartridge from Waters (Milford, MA, USA). The column cartridge was previously activated by washing with MeOH and with water, and then dried. The sterols fraction was eluted with 15 mL mixture of 5% (v/v) MeOH in chloroform. The eluate was dried with nitrogen and then dissolved with 1 mL of MeOH. Before injection samples were filtered and further diluted 10 times with MeOH.

3. Results and discussion

3.1. Optimization of nano-LC phytosterols separation

In recent years there has been a growing interest on sterols derived from plants because of the possible beneficial effects on human health. Phytosterols can be found in vegetable oils and/or in the so called "novel food", i.e., yogurt as food ingredients. Hence their determination is becoming a need for at least two reasons: to detect adulteration of vegetable products (especially oils) and to verify their presence in novel food.

Some important phytosterols of plant origin, namely brassicasterol, cholesterol, stigmasterol, campesterol and β-sitosterol were selected and their separation studied by using nano-LC. The chromatographic separation was optimized with the aim to achieve optimum resolution in the shortest analysis time.

For these purposes, some stationary phases and mobile phase compositions were evaluated, always working in reverse-phase mode.

RP-C8, -Phenyl, -Cholesteryl, -CN and RP-C18 stationary phases, of different particles size (5, 3 and sub-2 μm), were examined in order to obtain the complete resolution of all selected compounds. For each stationary phase, a capillary column (15 cm packed length × 100 μm I.D.) was packed. Various mobile phase mixtures, composed by different ratios of MeOH-water, acetonitrile (ACN)-water or MeOH–ACN-water, were selected. In some instances also a step gradient mode was considered. Unfortunately, even working with an initial mobile phase with very low elution power (high amount of water), no increased selectivity was observed. Among all the examined conditions, finest results, in terms of resolution and analysis time, were obtained with a column packed with RP-C18 sub-2 μm particles, operating in isocratic elution mode (100% MeOH as mobile phase). In these conditions, a partial resolution of campesterol–stigmasterol (R_s = 1.05), difficult to achieve with HPLC [10], was obtained. This limited separability is due to their closely related hydrophobicity. In fact, in the stigmasterol molecule, the effect of ethyl group in position 24 (enhanced hydrophobic interactions) is counterbalanced by the double bond in position 22 (decreased hydrophobic interactions), so that its resultant hydrophobicity is very similar to the campesterol one [34]. Consequently, the highest selectivity of sub-2 μm particles stationary phase towards the studied compounds can be explained considering its larger surface area available for hydrophobic interactions than the other phases.

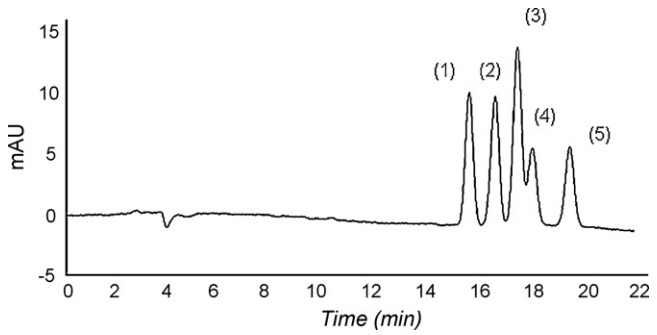

Fig. 2. Nano-LC separation system of (1) brassicasterol, (2) cholesterol, (3) stigmasterol, (4) campesterol, (5) β-sitosterol. Experimental conditions: capillary column, 100 μm I.D., 25 cm total length, 15 cm packed length, 20 cm effective length; stationary phase, RP C18 sub-2 μm particle size; mobile phase, 100% MeOH; detection wavelength, 195 nm. Concentration of each standard compound: 0.1 mg/mL.

Experiments carried out using a longer column (25 cm), allowed the baseline separation of all studied compounds. However too long analysis time (about 60 min) was recorded (results not shown).

Fig. 2 shows the chromatographic separation of the studied compounds obtained using the experimental conditions above mentioned with the shortest column. The addition of small amounts of formic acid was also performed in order to increase separation efficiency, as reported in literature [34], but without any significant improvement.

3.2. Validation data

The intra-day and the inter-day precision of the method were evaluated by injecting the standard mixture six times on the same day and in three different days. The calculated values of relative standard deviations (RSDs) for retention times and peak areas were in the range 0.93–1.18% and 3.22–4.15%, respectively (see Table 1).

The LOD and LOQ were determined as a signal-to-noise ratio (S/N) 3:1 and 10:1, respectively. The noise was calculated by measuring its own height. A LOD of 0.78 μg/mL and a LOQ of 1.56 μg/mL were found for all phytosterols. The linearity of the optimized

Table 1
Precision data retention time and peak area of studied analytes.

Analytes	Intraday precision (n = 6) RSD (%)		Interday precision (n = 3) RSD (%)	
	t_R	Peak area	t_R	Peak area
Brassicasterol	1.07	4.15	3.91	4.14
Cholesterol	1.05	3.57	3.68	3.73
Stigmasterol	0.93	3.22	3.48	3.70
Campesterol	1.13	4.60	2.61	4.63
β-Sitosterol	1.18	3.91	1.74	3.96

Table 2
Recovery data and RSD of studied phytosterols in EVOO samples.

Analytes	Phytosterol recovery (%, $n = 3$)					
	10 μg/mL		50 μg/mL		300 μg/mL	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
Brassicasterol	92.33	3.52	94.87	3.76	–	–
Cholesterol	90.14	3.89	93.33	4.03	–	–
Stigmasterol	102.56	4.76	99.67	2.97	–	–
Campesterol	103.24	2.98	101.34	3.54	–	–
β-Sitosterol	–	–	–	–	93.72	3.44

method was assessed in the concentration range between LOQ value and a concentration of 0.10 mg/mL for brassicasterol, cholesterol, stigmasterol and campesterol while for β-sitosterol in the range 0.1–2.0 mg/mL. The higher concentration range used for the last compound has been selected considering its abundance in extra-virgin olive oil (more than the 90% of phytosterols). Seven points of different concentration were obtained by diluting the more concentrated solution. For each point the injection of proper standard mixture was repeated three times. Good linearity was observed for analytes studied with acceptable correlation coefficients R^2, in the range of 0.991–0.996, without the employment of an internal standard.

3.3. Analysis of extra-virgin olive oil and recovery study

The method was applied to the analysis of EVOO real samples, specifically a commercial product and a home-made Algerian one.

The oil samples were subjected to saponification and then to a solid-phase extraction (SPE) procedure, as specified in the Section 2.

Although phytosterols fraction is usually obtained by using thin-layer chromatography (TLC), recently some studies concerning the use of more practical SPE procedures have been reported [12,33,34]. Therefore in this study we selected the SPE procedure. The procedure was validated spiking-oil samples with standard mixture of phytosterols before extraction. Considering the less abundant sterols (brassicasterol, cholesterol, stigmasterol and campesterol), two different spiking concentration levels were selected in the linear range studied for calibration curves (10 and 50 μg/mL). The concentration of β-sitosterol added was 300 μg/mL. Good recovery values were obtained. Recovery (R) was evaluated considering the following formula:

$$R = \frac{SS - NS}{C_S} \times 100 \quad (1)$$

where SS is the value obtained from spiked sample, NS from not spiked sample, C_S the amount added.

Each spiked sample was assessed in duplicate and then injected three times. The average values obtained are reported in Table 2. The sterol amount of commercial and home-made extra-virgin olive oil is reported in Table 3. As it can be seen, in both EVOO samples the amount of β-sitosterol was predominant. Brassicasterol was present only at trace level in the home made sample. Any difference in the amount of phytosterols between commercial and home made EVOO samples can be ascribed, as specified in the introduction, to several parameters. Unfortunately we don't have data concerning the cultivar, hastening period, etc. of the analyzed oils, but in any case the values obtained are in good agreement with data reported in the literature [16,31].

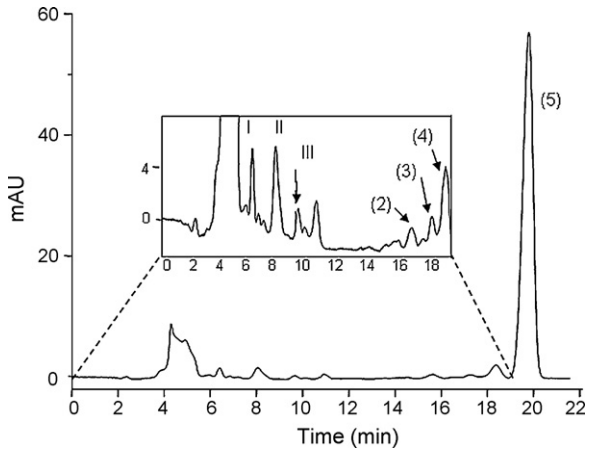

Fig. 3. Analysis of sterols in commercial EVOO sample (not spiked sample). (I) δ-tocopherol, (II) γ-tocopherol, (III) α-tocopherol; (1) brassicasterol, (2) cholesterol, (3) stigmasterol, (4) campesterol, (5) β-sitosterol. Experimental condition as in Fig. 2. In the frame, magnification of the same chromatogram to better show minor components detected.

Fig. 3 reports the chromatogram of the commercial EVOO sample extract. The peak identification was done by comparing retention times. Furthermore, samples were spiked with standard solutions of phytosterols to confirm the identity of the peaks.

In the chromatograms of real sample, other peaks were recorded. Since tocopherols are components of the unsaponifiable fraction of EVOO [4], the real sample was spiked with standard solutions of α-, γ- and δ-tocopherols. The presence of these compounds in the EVOO analysed was confirmed. Studies to verify the recovery of tocopherols with the employed sample preparation procedure were not performed, however these data suggest the potential use of the developed nano-LC method for the simultaneous assay of other minor components.

3.4. Peak characterization by using MS coupled with nano-LC

One of the most valuable advantages of nano-LC is its easy hyphenation with MS. In fact the nano-flow rate of this chromatographic system is highly compatible with the MS. The nano-LC/MS coupling is performed through different nano-spray interfaces. Among them, ESI is usually employed. The use of MS detector allows unambiguously identifying and characterizing analytes, also improving the sensitivity. Because of their physical-chemical properties, phytosterols , exhibit quite poor ionization with the soft ESI. For this reason atmospheric-pressure chemical ionization (APCI) is preferred when a "standard" chromatographic system (LC or HPLC) has to be coupled with MS for the analysis of such compounds. Nevertheless in some cases, it has been reported that phytosterols were analyzed using ESI interface [2,35].

Thus, to further support our results, in terms of identification, we coupled the nano-LC system with the ion-trap MS detector, by means of a nano-ESI interface. Nano-LC experiments were carried out using the same experimental conditions optimized with UV

Table 3
Sterol content in EVOO samples.

EVOO sample	Brassicasterol		Cholesterol		Stigmasterol		Campesterol		β-Sitosterol		Total sterols (mg/kg)
	(mg/kg)	RSD (%)	(mg/kg)	RSD (%)	(mg/kg)	RSD (%)	(mg/kg)	RSD (%)	(mg/kg)	RSD (%)	
Commercial product	<LOQ	–	2.03	2.03	2.55	3.22	8.40	3.32	1270.45	3.62	1283.43
Algerian home-made	2.50	1.95	1.42	1.99	1.83	2.42	6.24	3.25	1853.15	4.15	1865.14

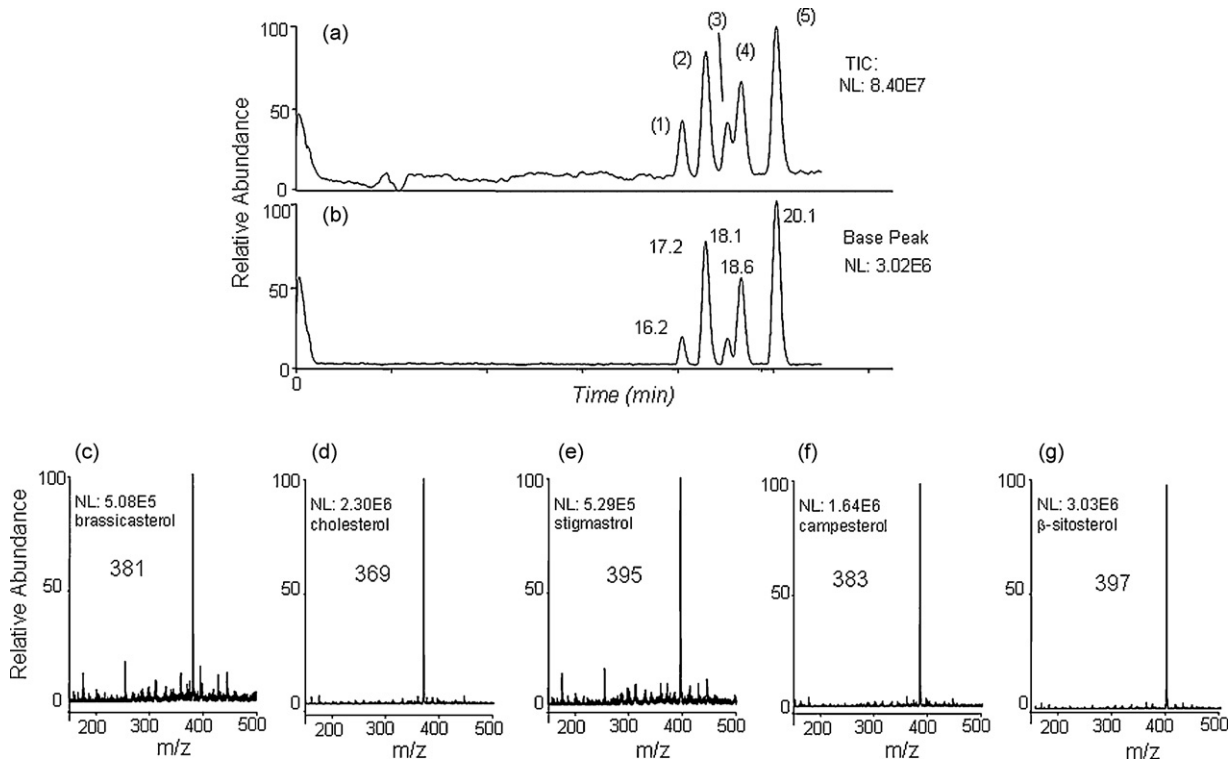

Fig. 4. Nano-LC separation with on-line ESI-MS detection of (1) brassicasterol, (2) cholesterol, (3) stigmasterol, (4) campesterol and (5) β-sitosterol. (a) Total ion chromatogram (TIC), (b) base peak chromatogram and (c–g) full scan mass spectra of the studied phytosterols. Experimental conditions as reported in Fig. 2 with the same mobile phase containing 15 mM ammonium acetate.

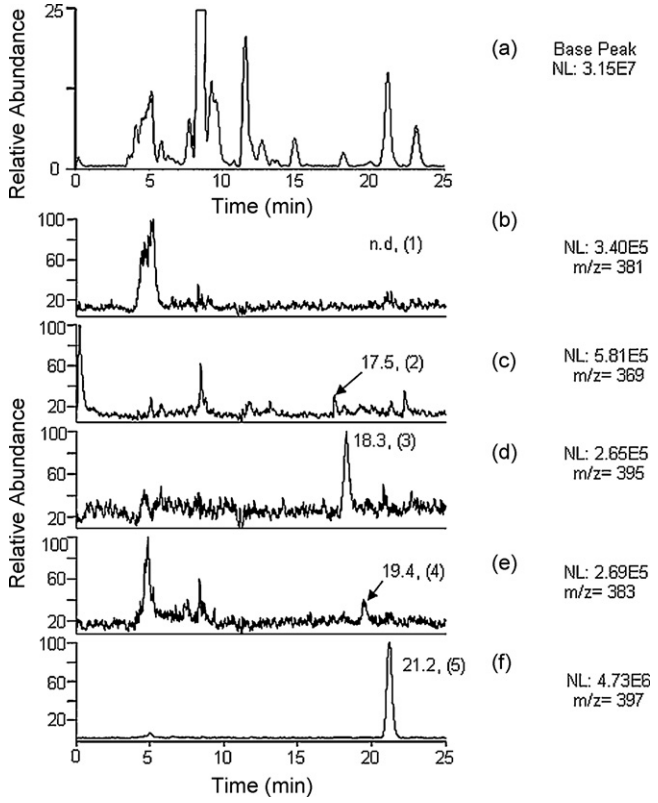

Fig. 5. Nano-LC separation with on-line ESI-MS detection of phytosterols present in an extracted commercial EVOO sample. (a) Base peak chromatogram, (b–f) selected mass track *m/z* 381, 369, 395, 383 and 397, respectively. For experimental conditions, see Fig. 4.

detection only modifying the mobile phase composition adding ammonium acetate for improving analytes ionization in the MS.

The total ion (Fig. 4a) and base peak (Fig. 4b) chromatograms of a standard mixture containing the five studied phytosterols and the selected mass-track (Fig. 4c–g) are depicted in Fig. 4. Fig. 5 instead shows the chromatogram obtained analyzing the extracted sample of the commercial EVOO. The extracted ion chromatograms confirmed the presence of all phytosterols observed with UV detection.

The recorded spectra show, for all studied compounds, a main signal of good sensitivity, corresponding to the fragment-ion resulting from loss of one water molecule, $[M+H-H_2O]^+$ [35,36]. Comparing the results reported in Fig. 2 with those of Fig. 4 concerning the analysis of a standard mixture of phytosterols, it is worth noting that the signal response of analytes are quite different due to the absorbing properties and ionisation of these compounds. With the aim to verify the possibility to further increase the sensitivity of the method, the standard mixture at LOD value concentrations was injected. Unfortunately it was not possible to detect any peak (data not shown). From the discussed data it can be observed that although nano-LC/MS with an ESI interface could not be used for improving the sensitivity in the analysis of phytosterols in EVOO samples, this tool offered interesting potentiality for the assessment of the identity of studied compounds.

4. Conclusions

As shown in this paper, a simple and rapid analytical method for the analysis of important food constituents was developed. A laboratory assembled nano-LC system was used for chromatographic experiments carried out in a packed fused silica capillary containing RP18 stationary phase. Phytosterols were eluted using an isocratic mode. The optimized method was validated and applied to the analysis of sterols in EVOO samples. The on-line UV detector was used for quantitation, while an ion-trap mass spectrometer was coupled

with the nano-LC apparatus for mass determination in order to identify separated compounds. The studied miniaturized method can be useful for both qualitative and quantitative determination of sterols after sample treatment with good sensitivity, precision and in short analysis time (20 min).

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