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Journal of Chromatography A, 935 (2001) 249–257

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Liquid chromatography–UV determination and liquid chromatography–atmospheric pressure chemical ionization mass spectrometric characterization of sitosterol and stigmasterol in soybean oil

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Abstract

A narrow-bore HPLC–UV method was developed for the analysis of two of the more abundant naturally occurring phytosterols in vegetable oils: sitosterol and stigmasterol. The method enabled detection of the compounds at a concentration of 0.42 μg/ml and quantitation at concentrations of 0.52 and 0.54 μg/ml for sitosterol and stigmasterol, respectively. An excellent linearity was determined over two orders of concentration magnitude (r^2 0.999–1.000) and verified by applying the Mandel fitting test ($p>0.099$) and the lack-of-fit test ($p>0.057$) performed at the 95% confidence level. A good intra-day precision ranging from 0.15 to 1.16% was calculated at two concentration levels (2 and 100 μg/ml). The inter-day reproducibility was verified on 3 different days by performing an homoscedasticity test and analysis of variance. A solid-phase extraction method was developed on silica cartridges for the isolation of phytosterols from soybean oil providing recovery values of 101±9 and 106±7% for sitosterol and stigmasterol, respectively. Good accuracy of the method was statistically demonstrated since no matrix effect was found for both the analytes. The developed method was applied to the quantitative assay of phytosterols in a soybean oil sample (61±5 mg/100 g of stigmasterol and 118±4 mg/100 g sitosterol). The HPLC–atmospheric pressure chemical ionization MS technique enabled the identification of stigmasterol, sitosterol and campesterol in the oil sample.

Keywords: Oils; Soybean; Validation; Food analysis; Sitosterol; Stigmasterol; Phytosterols; Sterols; Vitamins

1. Introduction

Sterols make up the greatest proportion of the unsaponifiable fraction of lipids. Plant fats and oils contain phytosterols as naturally occurring constituents, which are present in pure or esterified form, or conjugated as glycosides. Their composition is characteristic of the plant species and has been widely described before [1]. The predominant phytosterol is sitosterol (≈90%); minor components are campesterol, stigmasterol, Δ7-avenasterol and brassicasterol.

In the course of studies of the protective mechanisms exerted by foods of vegetable origin, phytosterols have been recognized as cancer preventive biological-active substances together with other secondary plant products such as carotenoids, flavonoids and phytoestrogens [2]. In animals, sitosterol has been shown to exhibit anti-inflammatory, anti-neo-

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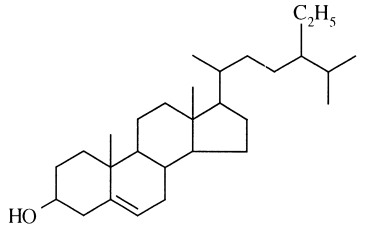
0021-9673/01/$ – see front matter
PII: S0021-9673(01)01079-2

plastic, anti-pyretic and immuno-modulating activity [3].

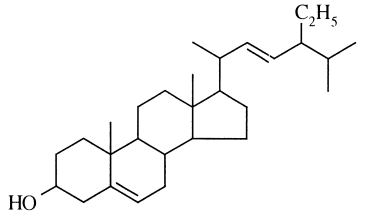
In addition, recently a cholesterol-lowering efficacy of dietary phytosterols has been described [4]. In particular, it has been shown that ingestion of plant sterols results in a favorable reduction of plasma total cholesterol in human subjects at a level between 0.5 and 26%. On the other hand, sitosterol has been reported to exhibit oestrogenic activity towards livestock or induce vitellogenin in male fish [5].

For the determination of phytosterols in plant materials, analysis is usually performed by capillary gas chromatography (GC) [6–9] and GC–mass spectrometry (MS) [10–12], which necessitate cumbersome sample preparation. Using GC with flame ionization detection (FID), validated methods including quality control check analyses have been recently published for the precise determination of sterols in diet samples [13] and in human serum [14]. To our knowledge, high-performance liquid chromatography (HPLC) has been proposed only in the past, without any validation of the method for the quantification of plant sterols [15,16].

Due to the growing interest in the physiological properties of phytosterols, it is very important to obtain accurate quantitative data in the determination of these nutritionally significant lipids both in food and in biological samples. In a research program dealing with the development of new rapid and sensitive methods by means of HPLC and HPLC–MS for the analysis of naturally occurring oestrogens in food [17], we developed and validated a simple and accurate method for the extraction, purification and determination of sitosterol and stigmasterol in sterol-rich food items such as soybean oil (Fig. 1). Solid-phase extraction (SPE) on silica cartridges was used for purification of the unsaponifiable fraction and determination of plant sterols was carried out using HPLC on a narrow-bore HPLC column and spectrophotometric UV detection. Further, the applicability of HPLC–MS with atmospheric pressure chemical ionization (APCI) was evaluated for the characterization of sterol fraction in the soybean sample.



Sitosterol (MW. 414.7)



Stigmasterol (MW. 412.7)

Fig. 1. Chemical structures of phytosterols investigated. MW= Molecular mass.

2. Experimental

2.1. Reagents and materials

HPLC-grade acetonitrile, hexane, diethyl ether and methanol were purchased from Carlo Erba (Milan, Italy). Ethyl acetate (analysis grade) and potassium hydroxide were from Carlo Erba; sodium sulfate anydrous (99% purity) was from Janssen (Geel, Belgium).

Sitosterol (60% purity) and stigmasterol (95% purity) were purchased from Fluka (Buchs, Switzerland). 6-Ketocholestanol (5α-cholestan-3β-ol-6-one, purity >98%) and vitamin E (DL-α-tocopherol, purity >98%) were obtained from Sigma (St. Louis, MO, USA).

Stock solutions containing 500 μg/ml 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.

2.2. Instrumentation

2.2.1. HPLC–UV system

A HP 1050 pump (Hewlett-Packard, Palo Alto,

CA, USA) equipped with a HP 1050 autosampler and a spectrophotometric UV–Vis variable-wavelength detector HP 1050 (Hewlett-Packard) with micro cell (2 μl) was used. Chromatographic separation was carried out using a C_8 narrow-bore column (150×2.1 mm, 5 μm) (Supelco, Bellefonte, PA, USA) under isocratic conditions with a mixture of acetonitrile–water (86:14, v/v) as the mobile phase at the flow-rate of 0.3 ml/min. The operative wavelength was set at 208 nm. Other detector parameters were set as follows: output voltage 1 V, output range 0.01–0.1 AU (absorbance units), offset 50 mV and sampling rate 30 Hz. Injection volume was 1 μl.

2.2.2. HPLC–MS system

An Alliance 2690 (Waters, Milford, MA, USA) liquid chromatograph equipped with a 120-vial capacity sample management system was used. A Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) was used with an APCI interface. Interface parameters were set as follows: nebulizer temperature, 400°C; source temperature 130°C; corona discharge, 2.5 μA; cone voltage (OR), 20 V; cone gas flow (N_2, 99.999% purity), 160 l/h; desolvation gas flow (N_2, 99.999% purity), 630 l/hr. HPLC–MS determinations were performed by operating the mass spectrometer in the positive ion (PI) mode. Full-scan mass spectra were acquired in the 300–450 u range using a step size of 0.1 u and a scan time of 0.5 s; the resolution of quadrupole was tuned to unit resolution. Chromatographic separation was obtained on a C_8 narrow-bore column (150×2.1 mm, 5 μm) (Supelco) under isocratic conditions with an acetonitrile–water (86:14, v/v) mixture at a flow-rate of 0.3 ml/min. Injection volume was 1 μl. For data acquisition and processing MassLynx v3.4 software was used.

2.3. Sample preparation

2.3.1. Sterol extraction

A 200-mg amount of soybean oil was added to 250 μl of a solution of 6-ketocholestanol (1260 μg/ml) in hexane as internal standard and 2 ml of 2 *M* KOH in methanol in a 50-ml round-bottomed flask. The mixture was heated under reflux at 90°C for 1 h. After cooling at room temperature, 4 ml of water and 4 ml of ethyl acetate were added to the mixture and vortex-mixed. After phase separation, the aqueous phase was washed three times with diethyl ether. Finally, the diethyl ether solution was dried on sodium sulfate anhydrous, filtered and then dried with a nitrogen stream. Sample extract was stored at −18°C until analysis.

2.3.2. SPE procedure

Silica gel (packing 500 mg/6 ml, Altech, Milan, Italy) SPE tubes were used after conditioning with 15 ml of hexane. Sample dissolved in 5 ml hexane–ethyl acetate (95:5, v/v) mixture was added to the SPE cartridge at a flow-rate of 1 ml/min. This solvent mixture (5 ml) was also used to perform the washing step, followed by elution with 6 ml of hexane–ethyl acetate (60:40, v/v). The eluate was dried under a nitrogen stream and resolved in 1 ml of hexane. The extracts were filtered on a PTFE membrane filter (0.45 μm) and diluted with hexane (1:2) before HPLC injection. Three replicated injections were performed for each sample.

2.4. Validation procedure

The detection limit (L_D) and the quantitation limit (L_Q) were expressed as signals based on the mean blank (x_b) and the standard deviation (s_b) of the blank responses as follows:

$$L_D = x_b + 2ts_b \quad L_Q = x_b + 10s_b$$

where t is a constant of the t-Student distribution (one-sided) dependent from the confidence level and the degree of freedom ($\nu = n-1$, n = number of measurements). Ten blank measurements were performed to calculate x_b and s_b. L_D and L_Q were converted from signal domain to concentration domain [limits of detection (LOD) and quantitation (LOQ), respectively] using a calibration function calculated in the 0.8–2.8 μg/ml concentration range [18]. In order to satisfy basic requirements such as homoscedasticity and linearity, the Bartlett test and linearity tests (the lack-of-fit test and the Mandel fitting test [19]) were performed at the 95% significance level.

Linearity was studied over two orders of magnitude of concentrations in the 0.7–70 μg/ml range. Six equispaced concentration levels were chosen and

three replicated injections were performed at each point. As in the case of LOD and LOQ calculation, the homoscedasticity test, the lack-of-fit test and the Mandel fitting test were run.

Precision was calculated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was calculated in terms of RSD% ($n=5$) on two concentration levels (2 and 100 μg/ml) for each analyte. The inter-day reproducibility was checked on 3 different days at the same concentration levels as for intra-day repeatability; for this purpose, a homoscedasticity test and analysis of variance (ANOVA) were performed on the replicated measurements ($n=15$) at the 95% significance level.

All statistical analyses and tests were carried out by using the statistical package SPSS v. 8.0 for Windows (SPSS, Italy).

2.5. Matrix effect

In order to investigate matrix effect, first the calibration function of the fundamental analytical procedure was determined:

$$y = a_c + b_c x_c$$

Analytical calibration procedure was performed on the unspiked and spiked samples following the standard addition method. The analytical results x_f were then calculated using the found signal values y_f and the analysis function, i.e., the calibration function solved for x:

$$x_f = y_f - a_c/b_c$$

By plotting the ''found concentrations'' (x_f) versus the original calibration concentrations (x_c), the recovery curve was calculated, which is mathematically described by the recovery function (linear regression line):

$$x_f = a_f + b_f x_c$$

In the ideal case, the recovery function results in a line with the intercept $a_f=0$ and the slope $b_f=1$ as well as a residual standard deviation which corresponds to the standard process deviation of the fundamental analytical procedure [19].

3. Results and discussion

3.1. LC–UV and SPE method development

In a first step a HPLC–UV method for the separation of sitosterol and stigmasterol was developed operating under normal-phase partition chromatography (NPLC) a CN stationary phase and mixtures of heptane and *n*-propanol (from 100 to 95%) as mobile phases. Since no selectivity was observed under NPLC, the use of a reversed-phase (RP) partition mechanism was considered. Initially, separation was carried out on a C_{18} column using different mobile phase mixtures made up of methanol, water and acetonitrile. Hydrophobic interactions between the lipophilic backbone of the analytes and the stationary phase provided good peak resolution, even though with retention times as greater as 28 min. Instead, the use of a C_8 stationary phase with acetonitrile–water (86:14, v/v) as the mobile phase resulted in a faster elution while maintaining good resolution. In addition, since a concentration-sensitive detector such as the UV detector was used, in order to achieve greater sensitivity separation was obtained on a narrow-bore column. The enhanced detectability obtained using narrow-bore columns and thus low eluent flow-rates is due to the lowest peak volumes [4σ, $4\sigma = V_0(1+k')/\sqrt{N}$] of the analytes in these columns with respect to standard-bore columns. Less dispersion in the microcolumns causes a favorable higher instantaneous concentration of the solutes in the flow-cell of concentration-sensitive detection systems. In the case of UV detection of phytosterols, a gain in sensitivity is advantageous, since these analytes also present poor molar absorbtivity at the maximum wavelength.

Fig. 2 shows the chromatographic separation of the phytosterols obtained in less than 12 min. The presence of the interferent peak evidenced at 9.8 min and subsequently identified as campesterol by LC–APCI-MS, was due to poor standard purity of sitosterol.

With the aim of applying the HPLC–UV method to the analysis of sitosterol and stigmasterol in soybean oil samples, a sample treatment based on the use of SPE was developed to isolate phytosterols from soybean oil. On the basis of structural similarity between the plant sterols investigated and choles-

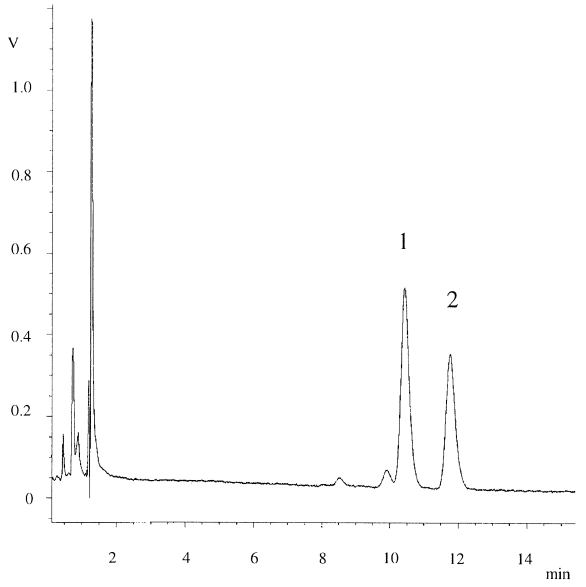


Fig. 2. LC–UV chromatogram of a separation of a standard mixture (100 μg/ml) of phytosterols. For conditions see Experimental section. Peaks identified: 1, stigmasterol; 2, sitosterol.

terol, in this work we devised an SPE sample clean-up method by modifying an SPE procedure developed for isolation of cholesterol oxides [20]. Extractions were carried out on silica solid-phase cartridges using 6-ketocholestanol as the internal standard for extraction recovery determination.

After separation of the saponifiable matter from the unsaponifiable fractions, a three-step SPE procedure was developed. In order to evaluate the influence of naturally occurring substances in vegetable oil on phytosterol recovery, α-tocopherol (vitamin E) at the concentration of 100 μg/ml was considered as a possible interferent and analyzed over the SPE. Elution of vitamin E was quantitatively obtained by maintaining at the same retention time of the analytes on silica gel.

Various hexane–ethyl acetate mixtures at different ratios were evaluated as eluting solvents of phytosterols; recovery of sitosterol and stigmasterol close to 100% was obtained when elution was carried out (Table 1).

Fig. 3 shows the LC–UV chromatogram obtained after phytosterol extraction and purification from a soybean oil sample.

Table 1
Recovery extraction data of phytosterols as a function of the eluting solvent composition

Analyte	Recovery (%)[a]			
	Ethyl acetate (%)			
	5	10	20	40
Sitosterol	n.d.[b]	36±2	55±3	100±2
Stigmasterol	n.d.	30±3	52±2	99±3
6-Ketocholesterol	n.d.	3±2	23±2	94±5

[a] $n=3$.
[b] n.d., Not detected.

3.2. Method validation

A further step of this work was to evaluate method performance. For this purpose, detection limit, quantitation limit, linearity, precision and accuracy were determined.

3.2.1. LOD and LOQ determination

Taking into account the role of phytosterols as micronutrients in foods, in order to develop an analytical method suitable for quantitative determination of trace phytosterols in foodstuff, in this work

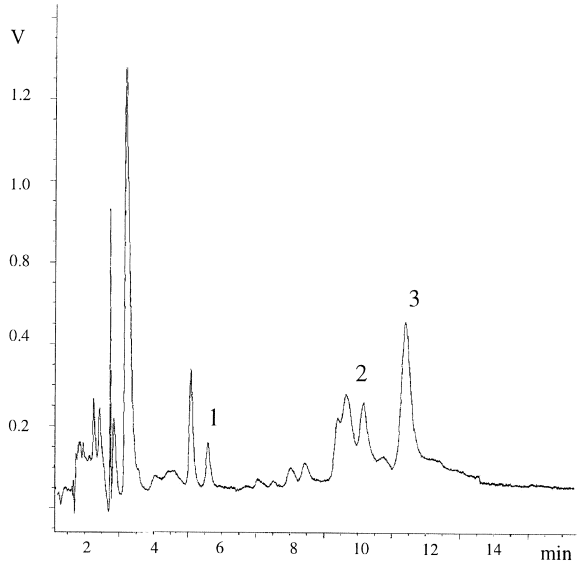


Fig. 3. LC–UV chromatogram of an extract of phytosterols from soybean oil sample. Peaks identified: 1, 6-ketocholesterol (149±2 μg/ml), stigmasterol (610±50 μg/g); 3, sitosterol (1180±40 μg/g).

Table 2
Limit of detection and limit of quantitation of sitosterol and stigmasterol ($n=10$)

Analyte	y_D[a] (mV)	y_Q[b] (mV)	LOD (μg/ml)	LOQ (μg/ml)
Sitosterol	0.070	0.079	0.42	0.52
Stigmasterol	0.071	0.082	0.42	0.54

[a] $y_D = x_b + 2 \cdot 1.83 s_b$.
[b] $y_Q = x_b + 10 s_b$.

we statistically determined the detection limits and the quantitation limits of stigmasterol and sitosterol.

As reported in the Experimental section, the signals corresponding to y_D and y_Q for the analytes were calculated (Table 2). Since a signal value depends on several instrumental parameters (i.e., sampling rate, output range, etc.) and usually cannot be used for direct comparison of different methods, a conversion from the signal to the concentration domain was performed in order to obtain concentration values of detection and quantitation limits.

For the determination of the LOD and the LOQ, a calibration curve was constructed in the 0.8–2.8 μg/ml range close to concentration values expected (data not shown). The first important parameter that we checked was the constant precision (homoscedasticity) of replicate measurements over the concentration range explored. By applying the Bartlett test, the significance values (p) obtained at the 95% confidence level were found to be greater than 0.05 (Table 3), all x_i having equal variance s_i^2. In order to verify if the linear model calculated provided effectively the best fit, a mathematical test of linearity (Mandel test) was performed. The F values found at the 95% confidence level were lower than those reported in the F-tables ($p>0.05$), showing that a quadratic model did not provide a significant better fitting than the linear model. Finally, a lack-of-fit test was run to verify if the error due to model approximation (SS_{lof}) was more significant than the pure error (SS_{PE}). Table 3 shows the p values for all tests performed; these results show that a linear regression model provides a good interpolation of the experimental data and that can be used to convert the limits of detection and quantitation from signal to concentration domain in such a way to calculate them with good precision (Table 2). The method enabled detection of the compounds at a concentration of 0.42 μg/ml and quantitation at concentrations of 0.52 and 0.54 μg/ml for sitosterol and stigmasterol, respectively, making the method useful for trace analysis. These values are comparable with those calculated by using GC–flame ionization detection [6,14].

Table 3
Significance values (p) of the homoscedasticity, Mandel test, lack-of-fit test[a]

Analyte	p[a]		
	Homoscedasticity	Mandel	Lack of fit
Sitosterol	0.217	0.215	0.061
Stigmasterol	0.070	0.099	0.057

[a] Confidence level, 95%.

3.2.2. Linearity

Six equispaced concentration levels in a range of two orders of magnitude were considered starting from a value corresponding to a concentration greater than LOQ of each analyte. Since the Bartlett test evidenced a significant difference among the variance values of replicates at different concentration levels ($p<0.05$), the best fit was obtained using a linear regression model with a $1/s_i^2$ as weighing factor ($r^2>0.999$, $n=24$) (Table 4). From the results of the Mandel test and the lack-of-fit test performed

Table 4
HPLC–UV linearity of sitosterol and stigmasterol[a]

Analyte	Concentration range (μg/ml)[b]	Homoscedasticity p[c]	Mandel p[c]	Lack of fit p[c]	$b_0 \pm s_{b0}$[c]	$b_1 \pm s_{b1}$[c]	r^2 ($n=18$)
Sitosterol	0.7–70	0.001	0.215	0.061	0.022±0.001	0.089±0.001	1.000
Stigmasterol	0.7–70	0.008	0.099	0.057	–	0.102±0.001	0.999

[a] Calibration fitting $y=b_0+b_1x$; y=area values, x=concentration (μg/ml).
[b] Injection volume=1 μl.
[c] ±Significance level 95%.

Table 5
HPLC–UV intra-day repeatability of phytosterols ($n=5$)

Analyte	Concentration level (μg/ml)[a]	RSD (%)
Sitosterol	2	1.16
	100	0.21
Stigmasterol	2	0.78
	100	0.15

[a] Injection volume = 1 μl.

on these data, significance values greater than 0.05 were obtained for all analytes indicating that a linear regression model provides a good interpolation of the experimental data (Table 4).

3.2.3. Method precision

Intra-day repeatability of the HPLC–UV method was evaluated by performing five repetitive analyses of 2 and 100 ng of each sterol, which gave an RSD between 0.15 and 1.16%, showing an excellent precision (Table 5).

The inter-day repeatability was evaluated over 3 different days at the same concentration levels, by calculating RSD and by verifying the homogeneity of variance and applying ANOVA. Significance values greater 0.05 evidenced a non-significant difference in the variances calculated over the 3 days: in addition, ANOVA applied to evaluate data precision provided results in terms of significance value p greater than 0.05, denoting that the mean values were not significantly different over the days considered (Table 6).

3.2.4. Matrix effect

A calculation of the recovery function was performed to test accuracy of the developed method and thus to ascertain the influence of the matrix for both analytes (Table 7), since matrix effects can cause an increase in the imprecision and/or a constant or proportional-systematic deviations of the analytical result from the ''true'' value. The method of standard addition was applied by analyzing unspiked sample and sample spiked to obtain concentrations two and three times higher than that of unspiked soybean oil sample. The slope and the intercept of the recovery functions calculated both for sitosterol and stigmasterol were compared, respectively, with 1 and 0 by means of a t-test (Table 7). Since the t-calculated resulted to be lower than the t-tabulated at the 95% significance level ($\nu=7$), it can be inferred that the calibration curve obtained by spiking oil sample is not significantly different from that obtained using standard solutions. Thus absence of matrix effects allowed us an accurate quantitative determination of phytosterols in soybean oil sample using the external standard method.

Table 7
Recovery function, $y=r_0+r_1x$ ($n=9$)

Analyte	$r_1 \pm s_{r1}$[a]	t-calculated	t-tabulated[a] ($\nu=7$)
Sitosterol	1.08±0.05	1.66	1.90
Stigmasterol	0.97±0.03	1.00	1.90

[a] Significance level 95%.

3.3. LC–APCI-MS analysis of phytosterols

The positive-ion APCI mass spectra of phytosterols are shown in Fig. 4. The spectra are characterized by the protonated molecular ions of the analytes and an abundant signal corresponding to

Table 6
HPLC–UV inter-day reproducibility of phytosterols ($n=15$)

Analyte	Concentration level (μg/ml)[a]	Homoscedasticity p[b]	ANOVA p[b]	RSD (%)
Sitosterol	2	0.92	0.056	1.2
	100	0.35	0.121	0.25
Stigmasterol	2	0.61	0.078	0.86
	100	0.11	0.094	0.13

[a] Injection volume = 1 μl.
[b] Confidence level, 95%.

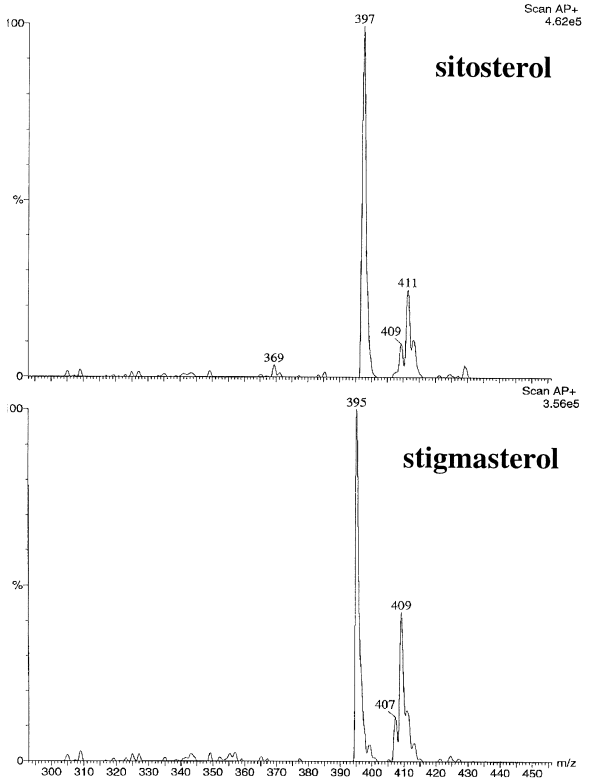


Fig. 4. LC–APCI-MS spectra of: (a) sitosterol; (b) stigmasterol.

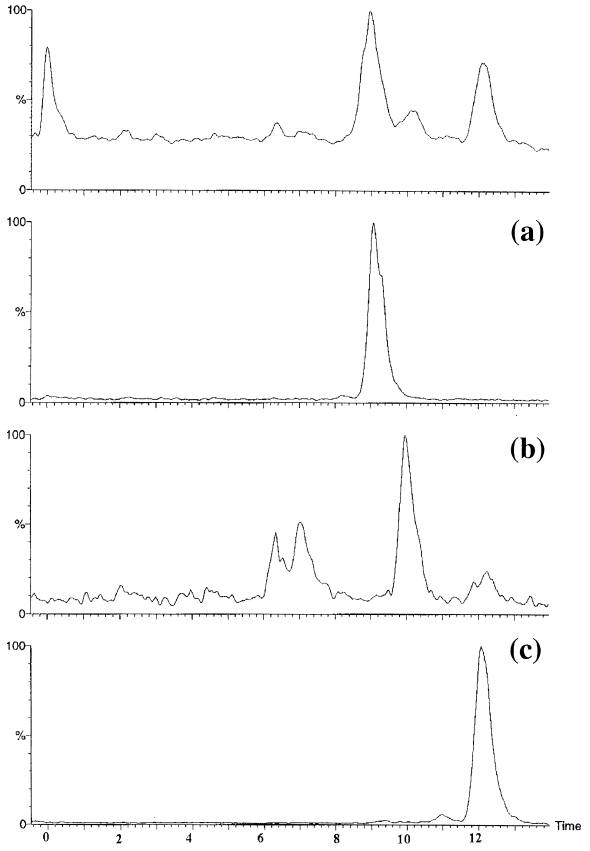


Fig. 5. LC–APCI-MS total ion chromatogram of a soybean oil extract and extracted ion chromatograms of: (a) campesterol; (b) stigmasterol; (c) sitosterol. Time scale in min.

fragment-ion due to the loss of a water molecule. In the sitosterol mass spectrum an interfering signal at m/z 383 was also detected, attributable to the $[M+H-H_20]^+$ ion of campesterol, as impurity in the standard powder of sitosterol.

3.4. LC–UV quantitative assay and LC–MS identification of phytosterols in soybean oil

In the LC–UV trace representing separation of phytosterols in the soybean oil extract (Fig. 3), sitosterol and stigmasterol were detected. To confirm the identification of analytes and to characterize sterol fraction, LC–APCI-MS analysis was carried out on the same sample. In the LC–APCI-MS trace, a peak at 9.15 min was evidenced, which was ascribed to campesterol. Fig. 5 illustrates the total-ion chromatogram together with the extracted ion chromatograms of sitosterol, stigmasterol and campesterol, identified by means of their APCI mass spectra and by comparison with the retention times of compounds.

The quantitative assay of sitosterol and stigmasterol in the sample was carried out by using the LC–UV method developed and a suitable calibration curve obtained by applying the external standard method (data not shown): concentrations determined for sitosterol and stigmasterol were 1180 ± 40 and 610 ± 50 μg/g, respectively. Mean recovery of phytosterol from soybeans was calculated to be in the $95\pm2\%$ range, by spiking samples with 6-keto-cholesterol as reported in the Experimental section. These values are in agreement with those reported for the plant sterols examined in soybean oil [9].

4. Conclusions

In a research program involved in the development of new rapid and sensitive methods for the identification and determination of naturally occurring substances of biological interest in food in this work, the use of a narrow-bore (RP)HPLC–UV system for the analysis of two of the most abundant phytosterols, sitosterol and stigmasterol, was evaluated, and a validation procedure was proposed for their determination. An SPE procedure was developed with particular regard to method accuracy and study of the matrix effect. Finally, HPLC–APCI-MS was proved to be suitable for the characterization of sterol fraction in a complex matrix such as the soybean oil.

Acknowledgements

The work was financially supported by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) Cofin 99 Project.

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