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Gas Chromatographic Determination and Method Validation of Stigmasterol, B-Sitosterol, Campesterol and Brassicasterol Contents of Turkish Cottonseed Oil Samples

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ABSTRACT

Plant sterols are important agricultural products for human health and consequently, for nutrition industries. In the present study, free sterol contents of crude Turkish cottonseed oil samples have been determined in a single analytical run by using a solid phase extraction step prior to the detection with a gas chromatography coupled with a flame ionization detector. Free hydroxyl groups of Stigmasterol, β-Sitosterol, Campesterol and Brassicasterol were derivatized by using *N*-methyl-*N*-trimethylsilyl trifluoroacetamide to enhance their volatility and, sterol content of the samples were, then, separated from their matrix by using a octadecylsilane cartridge. The eluates were injected into the gas chromatographic system and satisfactory recovery ratios were obtained. After having validated the method, it was applied into the analysis for cottonseed oil samples for their free sterol levels.

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Introduction

Plant sterols are important agricultural products for health and nutrition industries. They occur naturally in the diet as minor components of vegetable oils and dietary sterols have received increased attention since they reduce serum cholesterol levels (Plat and Mensink, 2005; Weingartner et al., 2011; Kozlowska et al., 2016). In addition, free sterol levels have been evaluated as factors for discriminating between genuine virgin olive oil and hazelnut-mixed olive oil with a satisfactory statistical certainty (Cert et al., 2000; Aparicio et al., 2000). On the basis of expanding market for vegetable oils, their authenticity has become an important subject from both a commercial and a health perspective (Abidi, 2001). The studies on authentication of vegetable oils by chromatographic analysis are recently reviewed (Garcia, 2013; Aparicio et al., 2013).

Determination of plant sterols in vegetable oils is a difficult task and requires a reliable analytical technique for the extraction, isolation, separation, purification, detection and quantitative data analyses. Traditionally, the analytical method consists of extraction of the total lipid fraction followed by alkaline hydrolysis (saponification), extraction of the non-saponifiable matter, derivatization of sterols followed by gas chromatographic determination of these derivatives using a capillary column (Aparicio, 2000). For routine quality control purposes thin-layer chromatographic (TLC) fractionation of sterols are being laborious and time consuming and it is often replaced with solid phase extraction (SPE) cartridges, which provide faster fractionation in a short time with a small volume of solvent (Toivo et al., 1998; Cercaci et al., 2003). For subsequent characterization and quantification of sterol compounds, the crude isolate can be separated by a wide variety of techniques including gas chromatography (GC) (Lechner et al., 1999; Ham et al. 2000), normal and reverse phase high-performance liquid chromatography (HPLC) with mass spectrometer (MS) detectors (Cañabate-Díaz et al. 2007; Zorrouk, 2009). Multivariate analysis of NMR fingerprint of the unsaponifiable fraction of virgin olive oils is also utilized for authentication purposes (Piironen et al. 2000; Salces et al., 2010).

A recent study propose a rapid determination method for prominent bioactive compounds in canola oil including free sterol contents by coupling the HPLC system with a diode array detection (DAD) and tandem mass spectrometry (MS/MS) (Flakelar et al., 2017). Here, the oil sample was simply diluted in hexane without

derivatisation or saponification prior to the quantification of both free sterols and intact sterol esters. The method was also applied to canola, palm fruit, sunflower and olive oils. However, the cost of such system limits their use in wide applications.

In most of the methods, the sample preparation procedure utilizes a SPE cartridge which constitutes a viable alternative for vegetable oil analyses with support materials serving different purposes. In a study carried out with reversed phase SPE with C_{18} sorbent elution was afforded with chloroform–methanol (95:5) and after evaporation of the solvent, sterol contents were detected by GC-FID as their trimethylsilyl (TMS) derivatives by using betulin as the internal standard (Toivo et al., 1998). In another study, C_{18} sorbent was pre-conditioned by passing acetonitrile and the sterols were eluted with acetonitrile-toluene mixture (Ham et al., 2000).

Alternatively, in a normal-phase mode without saponification, a vegetable oil sample is directly treated with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) in pyridine to give the TMS derivatives of all the oil components that encompass the hydroxyl functionality (Lechner et al., 1999). The derivatized material is then applied to a silica SPE cartridge pre-conditioned with hexane-tert-butyl methyl ether (99:1) followed by elution with the same solvent system prior to the GC analysis. This method provides a simple and reliable procedure for the determination of tocopherols and sterols in a single run.

In this study, it was aimed to determine the free sterol contents namely; stigmasterol, β-sitosterol, campesterol, and brassicasterol of Turkish cotton seed oil samples by following Lechner's method that use SPE cartridges prior to their quantification by GC/FID system. By this means sterol contents of the vegetable oils can be determined in a single analytical run avoiding subsequent time consuming and laborious saponification and TLC steps. The main objective of this study is to validate the method on the basis of estimated parameters applied for Turkish cotton seed oil samples.

Materials and Methods

Stigmasterol, β-sitosterol, brassicasterol, betulin and campesterol (total content >99%) were obtained from Sigma (Germany). Stock solutions containing 1000 µg mL^{-1} of all sterol were prepared in GC-grade pyridine and stored in the dark at 4°C for at least 2 months. Standard solutions were prepared from these solutions and diluted pyridine prior to analysis. N-methyl-N-trimethyl silyltrifluoro acetamide (MSTFA) was purchased from Alltech (USA), HPLC-grade pyridine, n-hexane from Lab-Scan (Ireland), methyl tert-butyl ether from J.T Baker (Holland) and the crude cotton seed oil samples were supplied from a local factory.

Equipment

For sample pretreatment, six milliliters Phenomenex (Madrid, USA) SPE columns packed with 1000 mg silica gel were utilized for SPE together with a vacuum manifold column processor. GC System equipped with AI 3000 auto injector and flame ionization detector (FID) was purchased from Thermo Finnigan (Waltham, USA). The dimensions of ZB-1 (%100 polydimethylsiloxane) column from Phenomenex (Madrid, USA) were 30 m x 0.25 mm x 0.25 µm.

Analytical Procedure

In a vial, 15-25 mg oil sample was weighted and mixed with 40 µL of MSTFA, 50 µL of pyridine and 5 µL of stock betulin solution. The capped vials were placed in a muffle set at 70°C and allowed to react for 20 min. The mixture was loaded onto the SPE cartridge preconditioned with 3 mL of hexane-methyl tertbuthylether (99:1, v/v). The sterol fraction was eluted with 5 mL of hexane-methyl tertbuthylether (99:1, v/v). The eluate was evaporated till dryness in a water bath set at 50°C and the residue was, then, re-dissolved in 0.5 mL of hexane-methyl tertbuthylether (99:1, v/v).

An aliquot of this solution (2 µL) was injected onto the GC-FID system which is programmed as follows: after the sample injection at 70°C the temperature was raised to 230°C at a rate of 15°C /min, then to 250°C at a rate of 5°C/min. The temperature was again raised to 320°C with a rate of 25°C/min and hold for 13 minutes. The injector and detector temperatures were 290°C and 320°C respectively. Nitrogen was used as carrier gas at a flow rate of 1.5 mL/min.

Trimethylsilyl (TMS) derivatives of sterol components were identified by comparing of their retention times to those of pure standard TMS derivatives of sterols. Quantitative analysis was performed by using the area of peaks in the chromatogram based on external calibration method.

Method Validation

The linearity was studied in different concentration ranges and the peak areas were plotted against the nominal concentration levels to construct the calibration curves according to the equation given below;

$$Y = (a \pm ts_a) + (b \pm ts_b)X$$

Where 'Y' is the peak area in mV and 'X' is the concentration in µg/L, 'a' is the intercept and 'b' is the slope, the t value is taken from at the confidence level of 95% for (n-2) degrees of freedom (Miller, 1993).

Limit of detection (LOD) and limit of quantification (LOQ) were expressed as S/N=3 and S/N=10 respectively. The accuracy of the method was tested upon performing recovery studies. Cottonseed oil samples were treated according to the procedure and peak area values were recorded (A). Other aliquots of the sample were spiked with standard sterol solutions at different concentration levels and gone through the same procedure (B). Blank oil sample was passed through the SPE cartridge and then, spiked with standard solution giving a peak area of C. These values were put into the recovery formula given below.

$$\text{Recovery } (\%) = (B - A)/(C - A) \times 100$$

Results and Discussion

Initial studies have been focused on the characteristics of the analysis carried out with blank solutions and sterol standards. Blank chromatogram which was obtained by injecting 2 µL of a mixture containing MSTFA, pyridine and the n-hexane: methyl tertbuthylether (99:1) has displayed a clear baseline in a wide range covering the retention times of sterol derivatives generally located at 18-22 min. The blank solution was spiked with standard sterol compounds along with the betulin as the internal standard prior to the derivatization reaction. Resulting chromatogram is shown in Figure 1 to represent the actual location of the peaks of the sterol compounds in terms of specificity and selectivity. As can be seen from the Figure1, each compound gives a specific and clear Gaussian peak shape with a good baseline resolution.

The linearity was studied in different concentration ranges for all pure sterol standards according to their expected levels in cottonseed oil. Five replicated injections were performed at five different concentration levels and peak area values were plotted against the nominal concentration levels to build the calibration curves. Table 1 summarizes the characteristics of calibration graphs obtained for campesterol, stigmasterol, β-sitosterol and brassicasterol standards. The limit of detection (LOD) and limit of quantification (LOQ) values have revealed that sterol derivatives can be determined in µg/mL level which corresponds to µg/g levels for real oil samples as a separation and preconcentration step is included in the procedure.

The reliability of the method was verified by recovery and repeatability tests. Precision of the method was calculated as both injection (within-day) repeatability and between day reproducibility which was checked on 3 different days at same concentration levels. Results have revealed that the within-day and between-day precision figures are very satisfactory being less than 5% and the method provides a precise analysis of sterol content of cottonseed oil.

The accuracy of the method was tested upon performing recovery studies by using the formula given above and the data for cottonseed oil samples spiked with different concentrations of sterol standards were listed in Table 2.

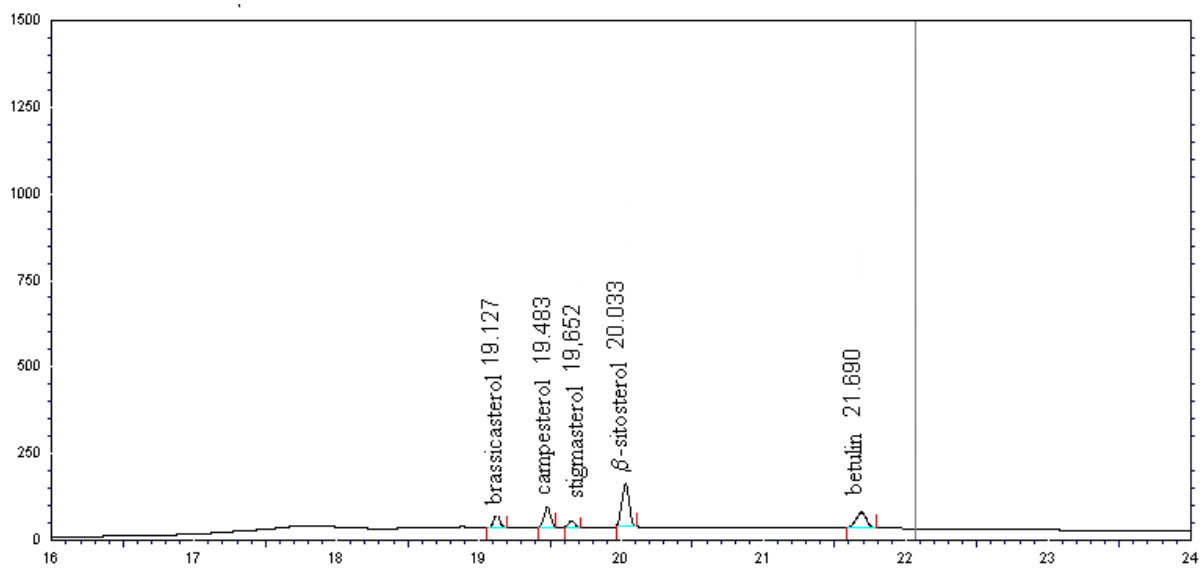

Figure 1 The chromatogram obtained for sterol standards and betulin as internal standard obtained with GC-FID system

Table 1 The data derived from calibration graphs of sterol derivatives obtained by GC-FID system

Analytical characteristic	Brassicasterol	Campesterol	Stigmasterol	β-sitosterol
t_R (min)	19.127	19.483	19.652	20.033
Conc. in the sample (µg/g)	12.5 - 62.5	200 – 500	20 – 150	2000 - 6250
Conc. in the vial (µg/mL)	0.5 - 2.5	8.0 – 20	0.8 – 6.0	80 - 250
Linear equation (peak area /10^5)	y = (18.944±1.21) x –(0.0091±1.96)	y = (18.026±0.73) x + (-75.488±10.28)	y = (20.535±0.46) x –(0.5629±1.48)	y = (21.56±0.64) x + (50.535±97.49)
R^2	0.9869	0.986	0.9999	0.9942
LOD (µg/mL)	1.1	1.05	0.66	0.64
LOQ (µg/mL)	3.63	3.46	2.18	2.12
RSD (%)Within-day	-	(15 µg/mL) 0.88	(1.2 µg/mL) 1.24	(165 µg/mL) 2.35
RSD (%)Between-day	-	(15 µg/mL) 4.37	(1.2 µg/mL)2.48	(165 µg/mL) 3.01

t_R: Retention time, LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation

Table 2 Recovery data for cottonseed oil samples spiked with different concentrations of sterol standards

Type of Sterol	Concentration of spiked standard		Recovery (%)
	In the vial µg/mL)	In the sample (mg/kg)	
Brassicasterol	0.80	20	97.7
Campesterol	10.4	260	90.3
	23.4	585	79.4
Stigmasterol	1.4	35	86.8
	3.0	75	87.8
	6.3	157.5	89.4
β-sitosterol	90	2250	92.4
	85	2125	93.6
	250	6250	86.0

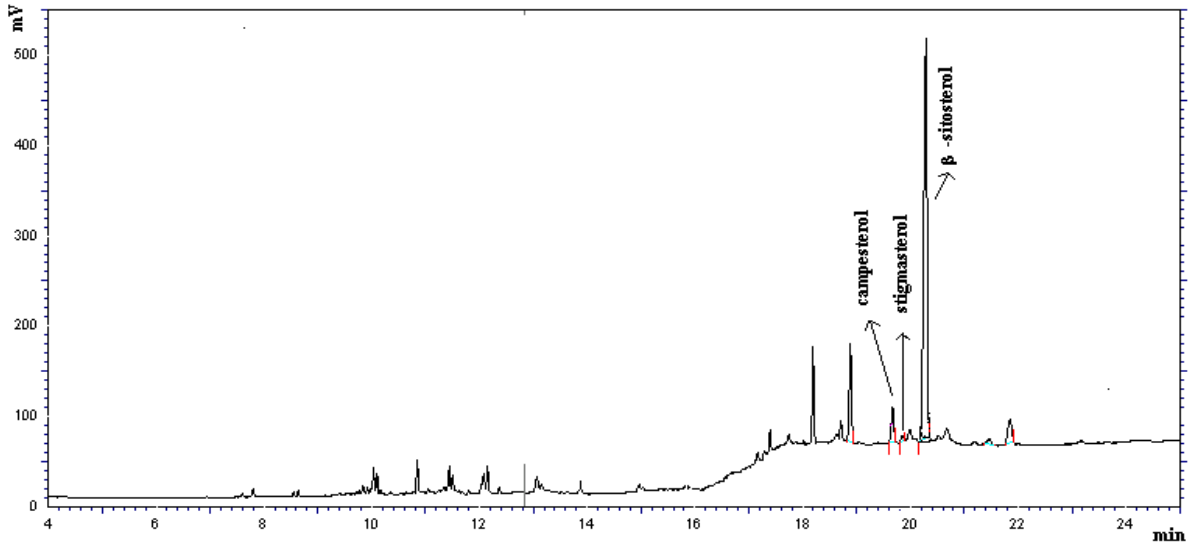

Figure 2 The GC-FID chromatogram obtained for cottonseed oil

Table 3 Mean sterol contents of cotton seed oil samples (n=3)

Type of Sterol	Sterol content (mg/kg)
Campesterol	135±4
Stigmasterol	27±1
β-sitosterol	2411±26
Total	2573

As it is consisted with the figures in the table, the recovery values are very satisfactory probably due to the use of much simpler procedure compared to the conventional methods which include saponification and multiple clean-up steps. Consequently, the possible analyte loss is prevented.

Crude cottonseed oil samples were spiked with internal standard, betulin and analyzed according to the procedure given above and as can be seen from Figure 2, a number of peaks have been obtained with a noisy baseline probably due to the crude oil matrix. The sterol components; campesterol, stigmasterol and β-sitosterol were eluted respectively but, no peak was obtained for brassicasterol derivative. The peak at 21.69 min belongs to betulin and from the relative retention index of the compounds calculated from the chromatograms recorded for vegetable oil samples it may be suggested that peak at 20.6 min belong to Δ^5-Avenastreol.

The peak areas were used in the linear equations obtained with standard solutions of target analytes and sterol contents were given in Table 3.

A number of different limit ranges according to the codex regarding food quality is available for total sterol composition of cottonseed oil samples changing from 2700-6400 µg/g and 3270-3970 µg/g (Verleyen et al., 2002; Codex Alimantarius, 2011). The analyzed result for Turkish cottonseed oil exerted to be 2573 mg/kg as total sterol concentration. This value of crude samples was found to be well below the minimum value of Codex Alimanterius specifications (1999). On the other hand, the phytosterol distribution for the cottonseed oil samples was observed in between esterified and free sterol fraction. The cottonseed oil was reported to contain 18.5% esterified phytosterol (Verleyen et al., 2002) and free sterol content may, therefore, represent the total sterol content of cottonseed oil. Hence, this validated method can be safely used to determine the sterol composition of cottonseed oil.

Conclusion

In this study, the sterol components of crude Turkish cotton seed oil samples were determined with high selectivity and sufficient sensitivity. No peak was obtained for brassicasterol derivative. The reliability of the method was verified by recovery and repeatability tests. The precision of the method was tested upon sequential injections of cotton seed oil sample after pretreatment and RSD values of within and between days were found very satisfactory for a precise analysis.

Recovery values were found very satisfactory probably due to the use of much simpler procedure compared to the conventional methods which include saponification and multiple clean-up steps. Consequently, the possible analyte loss is prevented.

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