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Original Article

Determination of cholesterol and four phytosterols in foods without derivatization by gas chromatography-tandem mass spectrometry

Yan-Zong Chen [a], Shih-Yao Kao [a], Hao-Cheng Jian [a], Yu-Man Yu [a], Ju-Ying Li [a], Wei-Hsien Wang [b], Chung-Wei Tsai [a,*]

[a] Department of Food Services, SGS Taiwan Ltd., Kaohsiung, Taiwan
[b] Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung, Taiwan

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ABSTRACT

In this study, a method for determination of cholesterol and four phytosterols by gas chromatography coupled with electron impact ionization mode–tandem mass spectrometry without derivatization in general food was developed. The sample was saponified with 7.5% KOH in methanol. After heating on hot plate and reflux for 60 minutes, the saponified portion was extracted with *n*-hexane/petroleum ether (50:50, v/v). The extracts were evaporated with rotary evaporator and then redissolved with tetrahydrofuran. The tetrahydrofuran layer was transferred into an injection vial and analyzed by gas chromatography on a 30 m VF-5 column. Limit of quantification was 2 mg/kg. Recoveries of cholesterol and four phytosterols from general food were between 91% and 100%.

1. Introduction

Sterols are tetracyclic lipid components found in animals, plants, and microorganisms. Several hundred different structures have been identified to date. While cholesterol is the major sterol in animals [1], the most common representatives in the plant kingdom are β-sitosterol [2], campesterol [3], and stigmasterol [3].

Cholesterol is required to build and maintain membranes. Through the interaction with the phospholipid fatty-acid chains, cholesterol increases membrane packing, which reduces membrane fluidity [4]. The structure of the tetracyclic ring of cholesterol contributes to the decreased fluidity of the cell membrane as the molecule is in a *trans* conformation, making all but the side chain of cholesterol rigid and planar [5]. In this structural role, cholesterol reduces the permeability of the plasma membrane to neutral solutes [6], protons (positive hydrogen ions), and sodium ions [7].

Phytosterols are plant compounds that have similar chemical structure and biological functions as cholesterol [8]. Phytosterols contain an extra methyl group, ethyl group, or

* *Corresponding author*. Department of Food Services, SGS Taiwan Ltd., 61 Kai-Fa Road, Nanzih Export Processing Zone, Kaohsiung 81170, Taiwan.
E-mail address: dio.tsai@sgs.com (C-W. Tsai).
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double bond. The suggested daily dietary intake of phytosterols is from 160 mg to 400 mg for different races of humans [9–16]. Phytosterols are known to have hypocholesterolemic properties. Phytosterols analogs are suggested to lower cholesterol absorption and the lower the serum cholesterol level, leading to cardiologic health benefits [1,7].

Cholesterol, corresponding precursors, and phytosterols in human blood have been determined by gas chromatography (GC)–mass spectrometry (MS) [17]. GC-MS is also executed to analyze cholesterol, corresponding precursors and phytosterols in cultured cells [18]. Rocco and Fanali [19] tried to determine phytosterols by nanoliquid chromatography–MS. AOAC published an official method 994.10 as analysis of cholesterol in foods by GC–flame ionization detector after saponification and derivatization with trimethylchlorosilane [20]. However, determination of cholesterol has been treated with derivatization during sample preparation in past reports, and there was no research to show an assay of cholesterol and phytosterols by GC–tandem MS (MS/MS). In this study, we aimed to develop a method of determination of cholesterol and four phytosterols without derivatization by GC-MS/MS in 20 minutes.

2. Methods

2.1. Apparatus

The GC–electron impact-MS/MS (GC-EI-MS/MS) system consisted of a Bruker456-GC system (Bruker, Singapore) connected to a Scion TQ series triple-stage quadrupole mass spectrometer (Bruker, Philadelphia, PA, USA). GC analysis was performed on a VF-5ms (30 m × 0.25 mm, film thickness = 0.25 μm; Agilent Technologies, Amstelveen, The Netherlands) at 280°C. N_2 was applied as carrier gas. Total running time was 20 minutes. The injection volume was 1 μL.

The MS detection system included an electron impact ionization. Its energy was fixed at 70 eV. Temperatures of ion source and transfer line were set at 200°C and 300°C, respectively. Argon was used as the collision-induced dissociation gas at a pressure of 1.5 mTorr.

Heating plates contain heat controls. Rotary evaporator with glass condenser flask between concentration flask and metal shaft were applied. Glassware used included 250-mL Erlenmeyer flasks, 250-mL separatory funnel, volumetric flasks, pipets, 250-mL Rohrig extraction tubes, glass funnels, and graduated cylinders.

2.2. Reagents and solutions

Cholesterol standard (purity > 99%) was purchased from Sigma–Aldrich (St Louis, MO, USA). Brassicasterol (purity > 92.5%), stigmasterol (purity > 89%), and β-sitosterol (purity > 92%) were supplied by ChromaDEX (Irvine, CA, USA). Campesterol (purity > 99%) was purchased from Sigma–Aldrich (Munich, Germany). As an internal standard, 5α-cholestane (purity > 97%) was provided by Sigma–Aldrich (USA). Individual stock standard solutions were prepared at a concentration of 1000 mg/L in tetrahydrofuran (THF; stable for 3 months), apart from 5α-cholestane, which was prepared in *n*-heptane at −25°C. Intermediate single standards solutions of cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol were prepared in THF at a concentration of 10 mg/L and stored in a refrigerator at −25°C (stable for 1 month). Mixtures of all chemicals were freshly made at five different concentrations (2 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L) for the preparation of calibration standards.

THF, *n*-heptane, *n*-hexane, petroleum ether, and methanol were analytical grade and supplied by Merck (Billerica, MA, USA). Potassium hydroxide (KOH), anhydrous sodium sulfate were supplied by Macron (Center Valley, PA, Mexico). Deionized water was obtained using a Millipore purification system (Millipore, Billerica, MA, USA) with a specific resistance of 18.2 MΩ cm. 7.5% KOH in methanol was prepared by adding 75 g KOH in 750 mL methanol. The extraction solvent consisted of *n*-hexane:petroleum ether (50:50, v/v).

2.3. Food samples

Plant oil (containing olive oil and grape seed oil), chicken eggs, milk powder, beverages (milk, tea, and juice), and dietary supplement foods (for elderly people or patients) were collected as testing samples. All were bought from supermarkets and then stored at −20°C. All samples were well homogenized with a blender.

2.4. General procedure

Well-homogenized food samples (1 g pure oil and 5 g general materials) were accurately weighed into 250-mL Erlenmeyer flask and spiked with 0.2 mL 1000 mg/L internal standard into the matrix, then added to a flask containing 50 mL 7.5% KOH in methanol. The flask was placed on a hot plate, a condenser attached, the hot plate turned on with the controller, and the mixture refluxed for 60 ± 10 minutes to ensure complete saponification.

After cooling the solution to room temperature, the saponified test portion was transferred to a Rohrig extraction tube. The saponified test portion was extracted with 50 mL *n*-hexane:petroleum ether (50:50, v/v) three times. The upper layer (organic phase, about 150 mL) was collected into a separatory funnel and the lower layer discarded. The collected organic phase was washed with 40 mL H_2O in a gently rotating separatory funnel. After allowing layers to separate the lower aqueous phase was discarded. The H_2O wash step was repeated at least three times until the layers were neutral (pH = 7). The upper organic phase from the separatory funnel was poured through a glass funnel containing 20 g sodium sulfate in a filter paper into another clean 250-mL Erlenmeyer flask, and the funnel rinsed twice with 5 mL *n*-hexane:petroleum ether (50:50, v/v). All eluates were evaporated to dryness on a rotary evaporator at 40 ± 1°C. The residues were reconstituted with 5 mL THF. The final solution was filtered using a 0.22-μm filter and the sample was transferred into a vial. An 1 μL aliquot was injected onto the GC column.

2.5. Method performance and validation

Validation of this analytical method was performed by assessment of the specificity, linearity, accuracy, precision,

and limit of quantification (LoQ). For evaluating specificity of testing method, six kinds of common food were selected including plant oil, eggs, milk powder, beverages, and dietary supplement foods. We spiked specific concentrations of cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol standards in each kind of testing sample. It was possible to avoid the matrix effects with this procedure.

Linearity was evaluated by fresh preparing of standard solution, at five concentration levels in the interval of 2–100 mg/L for all cholesterol and four phytosterols.

The accuracy was evaluated through participating in proficiency testing from the Food Analysis Performance Assessment Scheme (FAPAS). Certified reference materials such as milk powder for cholesterol testing were also executed during routing tests to maintain reliable quality control. Recovery data were considered acceptable when the accuracy was within ± 10% of the target value. Precision (intra- and interday) was calculated by analysis of samples fortified with each cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol standards at fortification level (20 mg/kg), and the experiments were performed by the same operator in triplicate at the same day and on 12 separate occasions in a month by three different operators. Ion ratios (peak area of confirmation ion pair/peak area of quantitation ion pair × 100%) of the described cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol were 18 ± 2, 24 ± 1, 74 ± 10, 51 ± 2, and 62 ± 5 ($n = 60$).

The LoQ was evaluated with spiking the lowest concentration level of calibration curve as 2 mg/kg in a blank sample. But analyzing cholesterol in eggs and phytosterols in plant oil whenever a real blank are impossible to obtain. Thus, the lowest concentration point on the calibration curve should be accepted as the LoQ. The analyte peak should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% [21].

3. Results and discussion

3.1. *Method development*

The GC-EI-MS/MS method was developed to provide confirmatory data for the analysis of general foods for cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol whose structures are shown in Fig. 1. The MS/MS fragmentation conditions were investigated and collision energies were optimized for each individual compound. In all cases, the tandem mass spectrometer was operated in the electron ionization mode at 70 eV. The retention times and the characteristic fragments of the electron ionization mode mass spectra were determined by multiple reaction monitoring mode. These ions were selected as the precursor ions, the most abundant product ions were selected the most sensitive transition for quantification purposes and a second one for

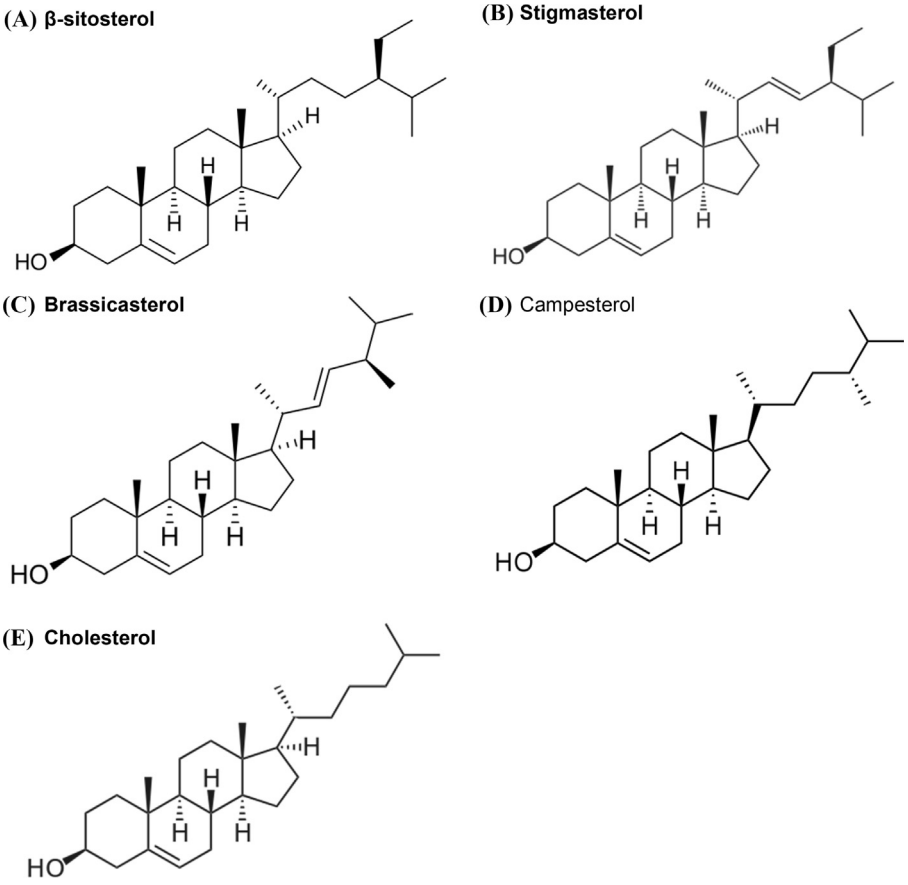

Fig. 1 – Structures of (A) β-sitosterol, (B) stigmasterol, (C) brassicasterol, (D) campesterol, and (E) cholesterol.

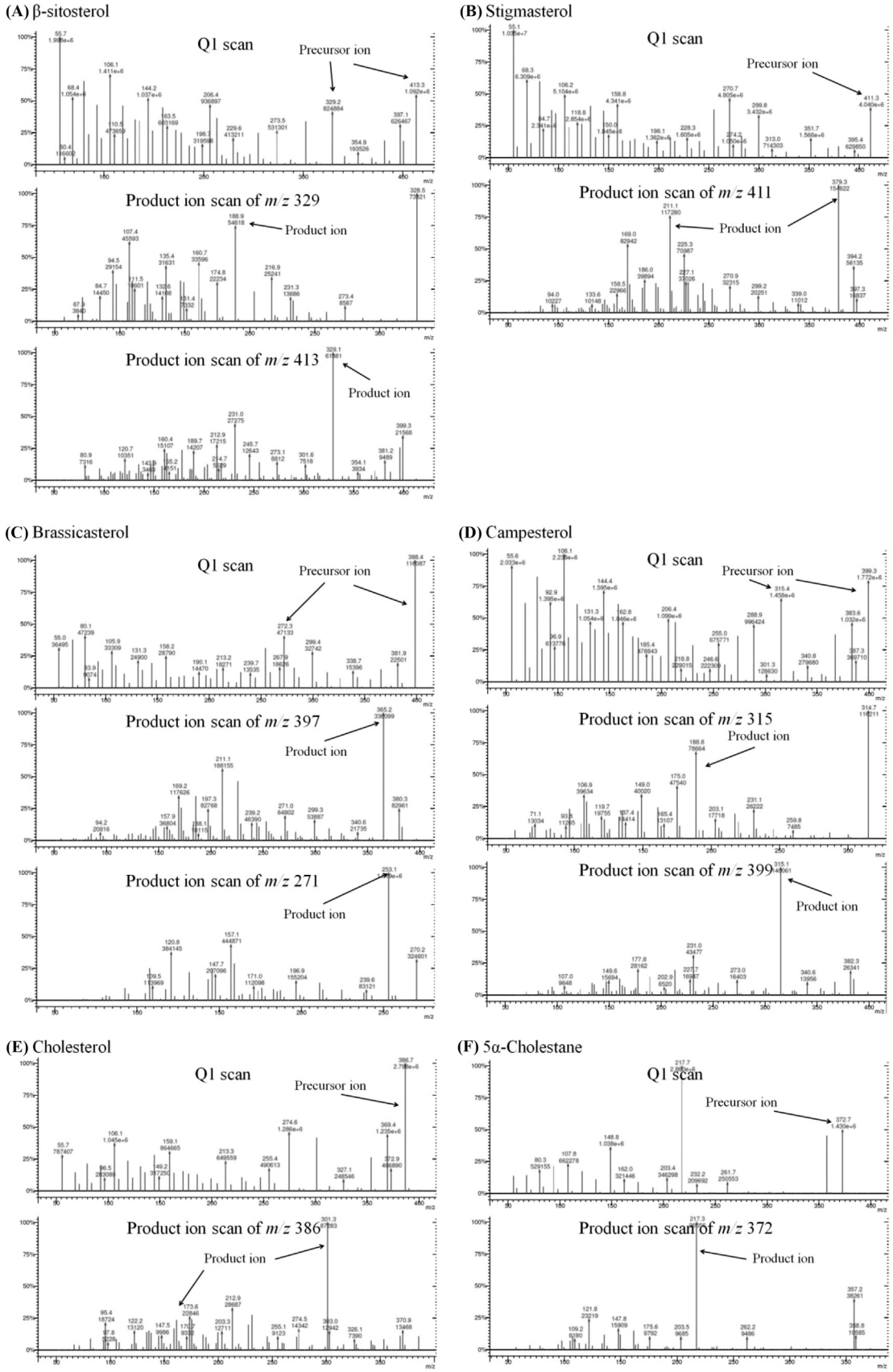

Fig. 2 – Precursor ion (Q1) scan (MS1) and product ion scan (MS2; (A) β-sitosterol, (B) stigmasterol, (C) brassicasterol, (D) campesterol, (E) cholesterol, and (F) 5α-cholestane) spectra.

Table 1 – Transition reactions monitored of cholesterol and four phytosterols by gas chromatography–electron impact–tandem mass spectrometry, retention time, and peak area ratio.

Analyte	Transition reactions (*m/z*)		Retention time (min)	Peak area ratio (%)
	Quantification ion pair	Confirmation ion pair		
β-sitosterol	329 → 189	413 → 329	15.253	51 ± 2
Stigmasterol	411 → 211	411 → 379	13.652	74 ± 10
Brassicasterol	397 → 365	271 → 253	11.475	24 ± 1
Campesterol	315 → 189	399 → 315	12.957	62 ± 5
Cholesterol	386 → 159	386 → 301	10.475	18 ± 2
5α-cholestane[a]	372 → 217	—	6.255	—

[a] Internal standard.

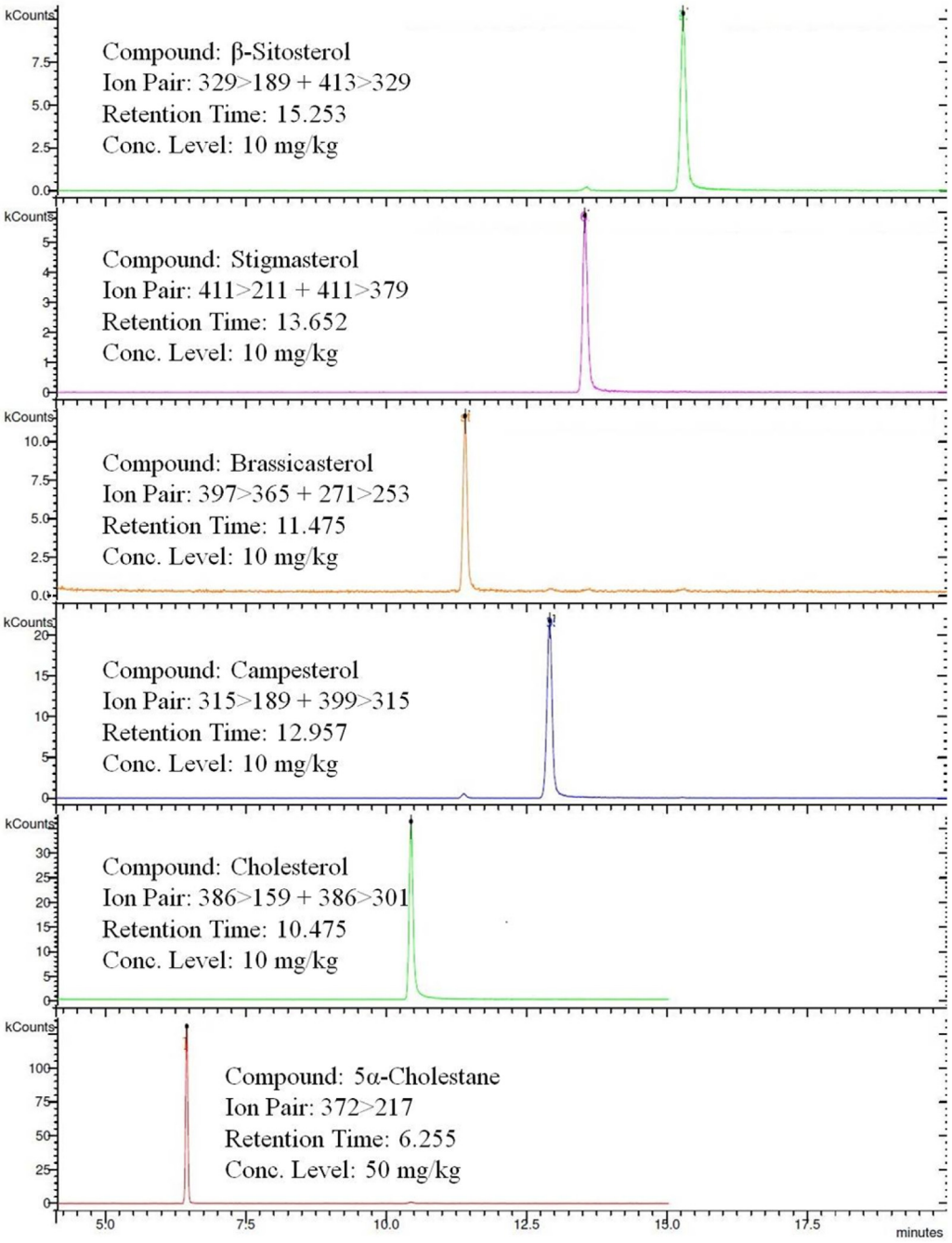

Fig. 3 – Multiple reaction monitoring chromatogram for each of the target analytes in blank matrix (potato starch) extract spiked at 10 mg/kg and 50 mg/kg.

confirmation. It shows MS/MS transitions (Q1 scan and product ion scan) for quantification and confirmation for each of the selected compounds in Fig. 2. For a method to be deemed confirmatory one parent ion and two daughter ions must be monitored (Table 1). This yielded four identification points, which provided a suitable confirmatory method in accordance with 2002/657/EC [22].

GC columns and conditions were studied in order to optimize the chromatographic separation in terms of resolution and overall analysis time. Due to the different properties of compounds under investigation. Helium carrier gas was subsequently found to give the most reliable result, good peak shape, and good resolution on VF-5 ms (30 m × 0.25 mm film thickness = 0.25 μm (Agilent Technologies). Product ion spectra resulting from collision-induced dissociation were examined and suitable ions selected for multiple reaction monitoring schemes (Fig. 3).

Numerous GC methods for the determination of cholesterol and phytosterols, such as cholesterol in serum or brassicasterol, stigmasterol, β-sitosterol, and campesterol in plant oil have been proposed [17,20]. GC-MS has been applied to determine cholesterol and phytosterols [18]. Nanoliquid chromatography has been used to analyze phytosterols in plant oils such as olive oil [19]. Most of them are applied GC–flame ionization detector or GC-MS determine the amount of cholesterol in samples with derivatization procedure during sample preparation [17–20]. Development of determination of cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol in general foods by GC-MS/MS without derivatization is required.

3.2. *Method validation*

The linearity of the chromatographic response was tested using six concentration levels in the range of 2–100 mg/L. The linear regression (r) for all the calibration curves used in this study was ≥ 0.995.

The recoveries of this method were determined using plant oil (n = 20) fortified at 20 mg/kg for four phytosterols and cholesterol. Mean recoveries (interday) of samples of analytes, determined during 1 year (Table 2), were 93%, 94%, 95%, 91%, and 91% for β-sitosterol, stigmasterol, brassicasterol, campesterol, and cholesterol, respectively. The average corrected recovery of cholesterol in different matrix such as beverages, dietary supplement, eggs, and milk powder was 100%, 100%, 95%, and 94%, respectively. The usefulness of a suitable isotope internal standard was demonstrated in the excellent reproducibility and interday and intraday reproducibility is obtained by using the internal standard of cholesterol (Table 2). Although no internal standard (5α-cholestane) is available for β-sitosterol, stigmasterol, brassicasterol, and campesterol, an acceptable repeatability about intraday and interday reproducibility was obtained. The developed method was evaluated by comparison of results when this method was performed and then the results were then passed in FAPAS proficiency tests. The test result of 142.2 mg/100 g of cholesterol in mixed fat spread compared with 133 mg/100 g FAPAS assigned value, report No. 14119, 2013 was 0.8 of z-score. In real sample testing, it was a positive case in terms of residue of cholesterol and phytosterols in milk powder and grape seed oil, respectively. There were no significant matrix effect in this testing method. The chromatogram is shown in Fig. 4.

LoQs were evaluated by spiking the lowest concentration of cholesterol and four phytosterols in blank samples [23,24]. All signal to noise ratios of peaks for analytes after sample pretreatment at lowest spiked concentration level have to be > 10 during triplicate tests. LoQs were 2 mg/kg for four phytosterols and cholesterol.

Based on these acceptable results of method validation, the method that we described in this study could be executed in the analysis of cholesterol and four phytosterols in plant oil, eggs, milk powder, beverages, and dietary supplement foods. Comparison with past method [17–20], we can determine cholesterol and four phytosterols by GC-MS/MS in 20 minutes and get sample pretreatment without derivatization.

4. Conclusion

In this work, we have shown that the combination of GC with MS/MS detection provides reliable simultaneous quantification and confirmation of cholesterol, brassicasterol, stigmasterol, β-sitosterol, and campesterol in plant oil, eggs, milk powder, beverages, and dietary supplement foods. Good

Table 2 – Results for repeatability of interday and intraday reproducibilities of β-sitosterol, stigmasterol, brassicasterol, campesterol, and cholesterol in plant oil, eggs, milk powder, beverages, and dietary supplement foods.

Analyte	Matrix	Fortification concentration level (mg/kg)	Intraday		Interday	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
β-sitosterol		20	97	2	93	6
Stigmasterol	Plant oil	20	97	3	94	8
Brassicasterol	(n = 20)	20	95	4	95	6
Campesterol		20	94	2	91	4
Cholesterol	Beverages (n = 20)	20	99	4	100	6
	Dietary supplement foods (n = 26)	20	98	4	100	8
	Plant oil (n = 20)	20	97	2	91	12
	Eggs (n = 16)	20	98	4	95	9
	Milk powder (n = 14)	20	97	5	94	8

RSD = relative standard deviation.

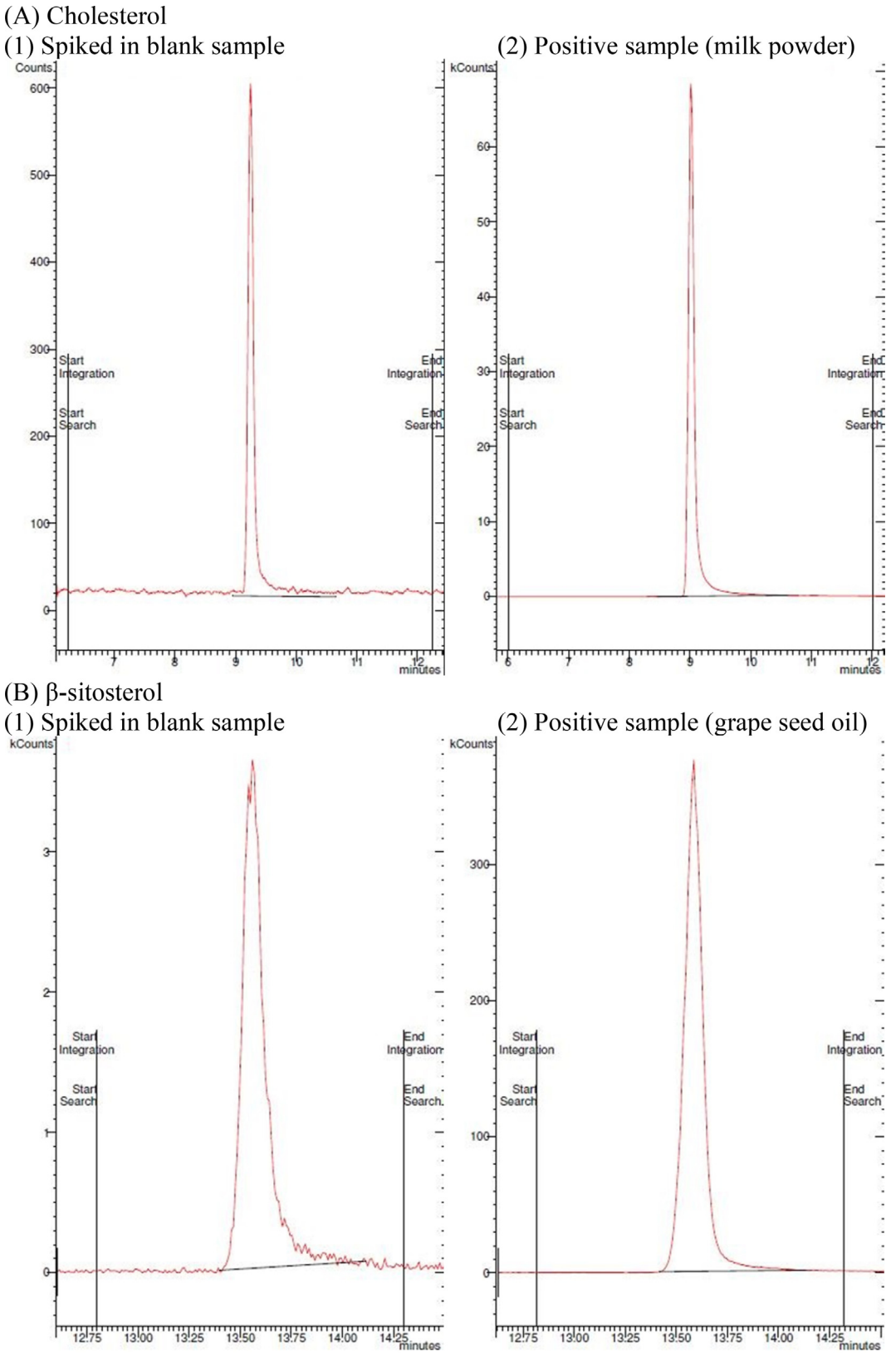

Fig. 4 – Typical chromatograms of spiked fortified concentration at 20 mg/kg for (A) cholesterol, (B) β-sitosterol, (C) stigmasterol, (D), brassicasterol, and (E) campesterol in (1) blank starch sample and (2) positive sample including cholesterol and phytosterols in milk powder and grape seed oil, respectively.

recoveries with excellent relative standard deviations were obtained with multi sample matrix. Simpler sample preparation procedure was executed without derivatization step. Confirmation of the cholesterol and four phytosterols was accomplished using MS/MS, by comparison of peak ratios for two abundant product ions with those of standard samples. The results were satisfactory for the development of a rugged analytical method in this study.

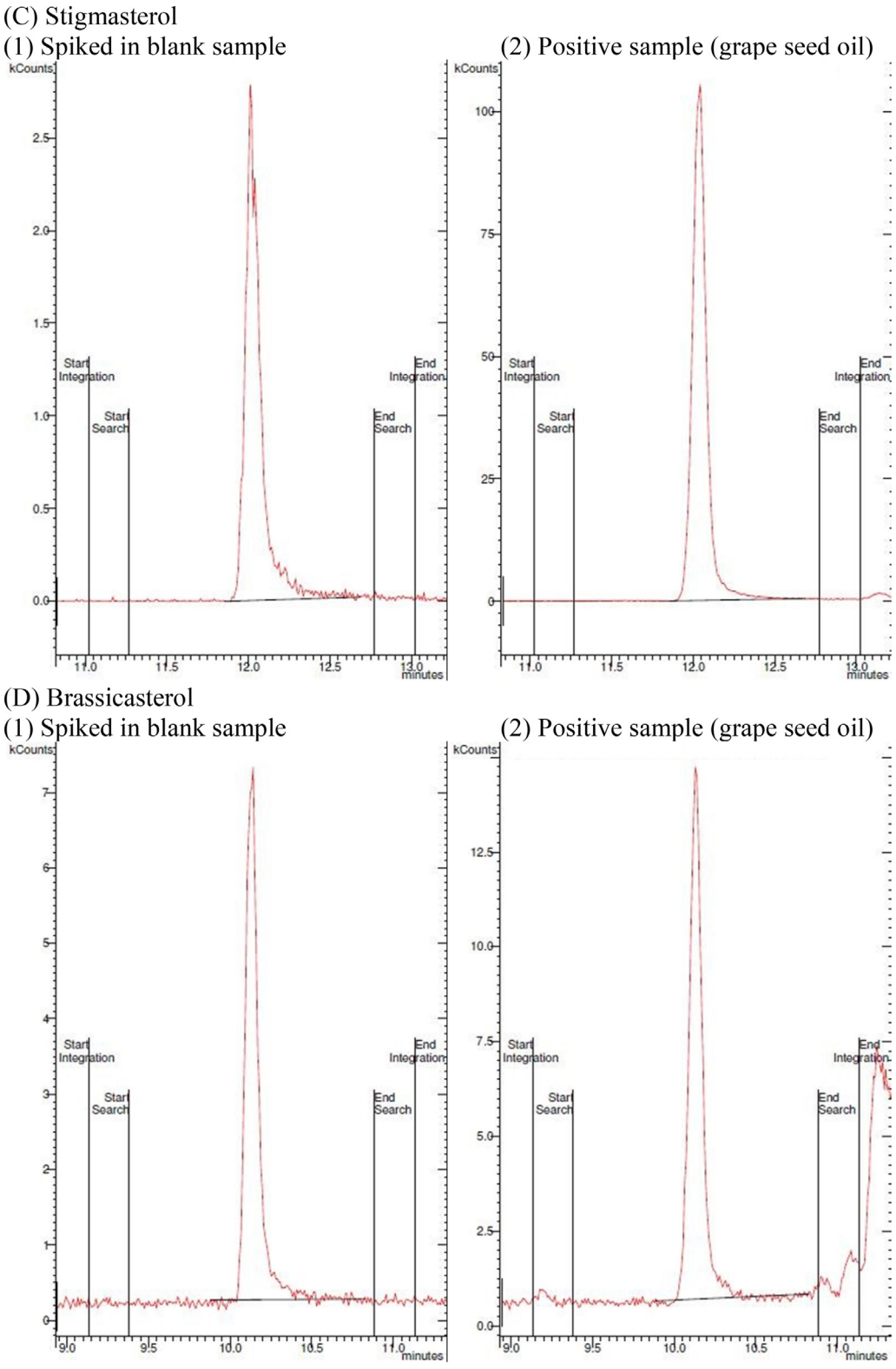

Fig. 4 – (*continued*).

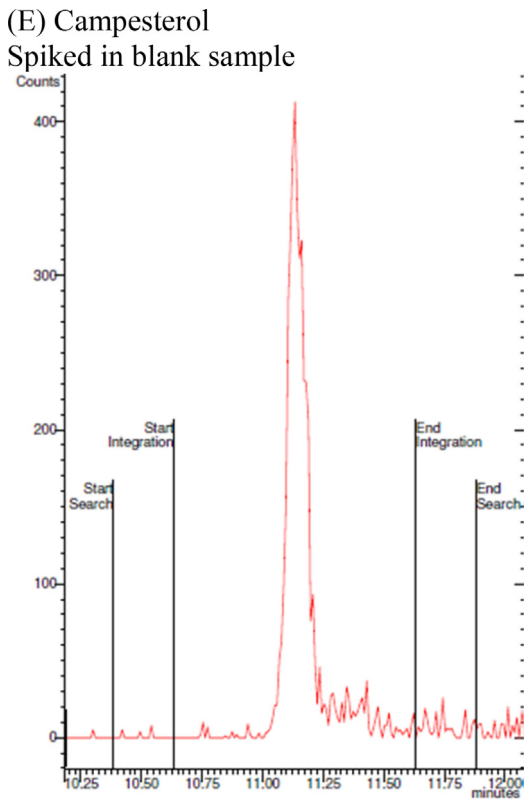

Fig. 4 – (*continued*).

Conflicts of interest

All authors declare no conflicts of interest.

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