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Original Article

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

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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%.

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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

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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,

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