IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 5, Issue 3 (March 2015), PP. 11-19

Validation and Application of a Gas Chromatographic Method for the Determination of Fatty Acids and Sterols in the Total Diet

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ABSTRACT : *Fatty acids (FAs) and sterols represent the most important fraction of edible oils and fats, which play a significant role in human nutrition and health. A gas chromatographic method for the identification and quantification of FAs and sterols based on the extraction of lipids by accelerated solvent extraction (ASE), saponification of the obtained extract, and direct determination of the isolated FAs and sterols without derivatization, was developed and validated. The proposed method was accurate and precise (mean recovery in the range 80.8% to 98.2%, precision with RSD < 8.6%; LOQ < 0.86 mg/kg; measurement uncertainty < 14.8%). This method was applied for the determination of the most abundant FAs and sterols in the total diet. The obtained results showed that the most abundant FA in the total diet is monounsaturated $C_{18:1}$ with 34.5% of the total FAs. The lowest presence was observed for the short chain FAs: $C_{4:0} - C_{12:0}$ (6.4%). Cholesterol was found to be the most abundant sterol in the diet (57.5%). The total amount of the plant sterols in the diet was found to be 42.5%.*

KEYWORDS: *gas chromatography, fatty acid, sterol.*

I. INTRODUCTION

Edible oils and fats are one of the major components of human diets, comprising as much as 25% of average caloric intake. They play important functional and sensory roles in food products and they act as carriers of fat-soluble vitamins (A, D, E, and K). They also provide an essential linoleic and linolenic acid, responsible for growth [1]. Edible oils and fats are biological mixtures of plant origin consisting of ester mixtures derived from glycerol with chain of fatty acids [2]. Both the physical and the chemical characteristics of oils and fats are greatly influenced by the kind and proportion of the fatty acids (FAs) on the triacylglycerol [3, 4]. FAs can be classified in classes as saturated, mono-unsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. The predominant fatty acids present in the diet are saturated and unsaturated compounds with straight aliphatic chains. An even number of carbon atoms, from 16 to 18, with a single carboxyl group, is the most common [5, 6]. High levels of saturated FAs in the diet are frequently considered to have influence in increasing the concentration of low density lipoproteins (LDL), affecting the ratio of LDL to HDL (high density lipoproteins), promoting clothing and vascular smooth muscle proliferation [7, 8]. Diet with increasing intake of linoleic and linolenic acids increase HDL-cholesterol and decreases LDL-cholesterol, while higher intake of oleic acid decreases LDL-cholesterol, but does not affect HDL cholesterol levels [9].

Therefore, the determination of FAs is an essential requirement in testing food material [10].Gas chromatography (GC) coupled with mass spectrometer (MS) or flame ionization detector (FID) is the most widely used technique for the determination of FAs [11-13]. Commonly, the most methodologies used for determining FAs are lipid extraction followed by conversion of the FAs into corresponding methyl esters (FAMEs) [14, 15]. Such methodologies are usually used for preparing FAMEs from lipids either by basic hydrolysis followed by methylation of the free FAs (FFAs) or by trans-esterification of lipids using acid or base catalyzed as rapid and simple methods [14,16].

The presence of non-lipids at the moment of the derivatization process may interfere with lipids resulting to potential errors and high variable profiles [17].

Beside fatty acids, the most abundant components present in the edible oils and fats are sterols. Cholesterol, sitosterol and stigmasterol are polycyclic steroid compounds with similar chemical structure. Cholesterol is a typical animal sterol, e.g. its content in milk fat is 95–98%, stigmasterol and sitosterol are referred to as phytosterols [18].

The determination of sterols in the total diet is carried out for the following objectives: to measure the total cholesterol content to obtain nutritional information and to detect the presence of vegetable fats [18,19].

The most appropriate and frequently used method for the determination of total sterols content in foods is the direct saponification, followed by the extraction of the unsaponificable residue into the nonpolar solvent, derivatization of the isolated sterols and final gas chromatographic detection [20]. The aim of our study was to develop and validate simple, reliable and accurate GC method for determination of the most abundant FAs and

sterols in the total diet without derivatization of the isolated analytes. The method was applied for determination of FAs and sterols in the total diet (28 samples).

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II. MATERIALS AND METHODS

2.1. Instrumentation

The chromatographic analysis (GC) were performed on a Shimadzu 2010 GC system equipped with flame-ionization detector (FID), and auto injector (AOC- 20i), and the Chrom- Solution software. The sample extraction was performed in a DIONEX Accelerated Solvent extractor, ASE-100 (USA). Stainless steel extraction cells (34 mL) were used for the extraction. Helium (purity 99.999 %) was used as a purge gas. The sample saponification was performed with reflux condenser.

2.2. Reagents and standards

All chemicals and solvent were a special grade for gas chromatography. N-hexane, 96% (V/V) ethanol, absolute ethanol and chloroform were obtained from Merck (Darmstadt, Germany). Anhydrous sodium sulphate, (prepared 3 hours at 650 ^{0}C), was purchased from Sigma-Aldrich/Fluka/Riedel-de-Haen (Zwijndrecht, The Netherlands). The moisture absorbing polymer (MAP) and diatomaceous earth (DE) were purchased from Thermo Fisher Scientific, Waltham, MA (USA). Water was deionized then distilled from glass apparatus. Potassium hydroxide analytical grade was obtained from Merck (Darmstadt, Germany)

The analytical standards: butyric acid ($C_{4:0}$) purity ≥98.5% GC, caproic acid ($C_{6:0}$) purity ≥99.0% GC, caprylic acid ($C_{8:0}$) purity ≥98.5% GC, capric acid ($C_{10:0}$) purity ≥99.5% GC, lauric acid ($C_{12:0}$) purity ≥99.0% GC, myristic acid ($C_{14:0}$) purity ≥98.5% GC, palmitic acid ($C_{16:0}$) purity ≥99.0% GC, palmitoleic acid ($C_{16:1}$) purity ≥98.5% GC, stearic acid ($C_{18:0}$) purity ≥99.0% GC, oleic acid ($C_{18:1}$) purity ≥98.5% GC, linoleic acid ($C_{18:2}$) purity ≥98.5%, linolenic acid ($C_{18:3}$) purity ≥98.5%, cholesterol purity ≥98.5% GC, stigmasterol purity ≥98.5% GC, β- sitosterol purity ≥98.0% GC, campesterol purity ≥98.5% GC and 5-α-cholestane purity ≥98.0% GC were obtained from Sigma-Aldrich/Fluka/Riedel-de-Haen (Zwijndrecht, The Netherlands). Blank samples of dietary non fatty supplements used for fortification were purchased from the local market.

2.3. Preparation of the standard solutions

2.3.1. Preparation of FAs standard solutions

Stock solutions of individual fatty acid (FA) standards were prepared in n – hexane at 1000 mg/L and were stored in a refrigerator at -20 ^{0}C. Standard working solution mixture (i) was prepared by transferring 10 mL of each individual stock standard solution in a 50 mL volumetric flask and diluting with n – hexane to a concentration of 200 mg/L. Stock standard solutions of individual FA and standard working solution mixture (i) was used for the preparation of chromatographic standard solutions (ii) with different FA concentrations: 10 mg/L – 100 mg/L, i.e. 10 mg/L; 25 mg/L; 50 mg/L; 75 mg/L and 100 mg/L. Chromatographic standard solutions were prepared in n-hexane. Stock solutions and standard working solution mixture (i) were used for the fortification of the blank samples.

2.3.2. Preparation of sterols standard solutions

Stock solutions of individual sterols were prepared in chloroform at 1000 mg/L and stored in a refrigerator at -20^{0}C. Standard working solution mixture (i) was prepared by transferring 5 mL of each individual stock standard solution in a 50 mL volumetric flask and diluting with chloroform to a concentration of 100 mg/L. Standard working solution mixture (ii) was prepared by transferring 5 mL of standard working solution mixture (i) to a 50 mL volumetric flask and diluting with chloroform to a concentration of 10 mg/L. Standard working solution mixture (i) was used for the preparation of the chromatographic standard solutions with different stigmasterol and campesterol concentrations: 1 mg/L – 10 mg/L, i.e. 1.0 mg/L; 2.5 mg/L; 5 mg/L; 7.5 mg/L and 10 mg/L. Stock solutions of cholesterol and β sitosterol were used for the preparation of the chromatographic standard solutions with cholesterol and β sitosterol concentrations ranging from 10 mg/L to 100 mg/L, i.e. 10 mg/L; 25 mg/L; 50 mg/L; 75 mg/L and 100 mg/L. Chromatographic standard solutions were prepared in chloroform. Stock solutions and standard working solution mixture (i) and mixture (ii) were used for the fortification of blank samples. Internal standard stock solution of 5-α-cholestane was prepared in chloroform at a 1000 mg/L. Working internal standard solution was prepared by transferring 100 µL of internal standard stock solution (IS) in a 10 mL volumetric flask and diluting with chloroform to a concentration of 10 mg/L. 1 mL of internal standard solution (10 mg/L) was added into the sample prior the extraction.

2.4. Sample preparation

An aliquot of 10 g of homogenized sample was mixed with 2 g of MAP and 2 g of DE. The extraction cell was filled with the homogenized mixture, and placed in the ASE, which worked out under the conditions shown in Table 1.

Table 1. ASE operating conditions

Solvent	n-hexane
Temperature	100 ^{0}C
Pressure	1500 psi
Static time	5 min
Flush volume	60 %
Purge time	140 sec
Static Cycle	1

The obtained extract (15 mL) was evaporated under the stream of nitrogen to dryness. The residue was dissolved in 30 mL solution of KOH in absolute ethanol (0.5 mol/L). The saponification was performed using reflux condenser within 30 min. The solution obtained after the saponification was quantitatively transferred into a 50 mL volumetric flask and filled with absolute ethanol up to the mark. The solution was used for the further analysis.

2.4.1. Extraction of FAs

5 mL of the solution was transferred to a 100 mL separatory funnel. 0.1 mL 25% (V/V) HCl was added together with 25 mL of n-hexane and 25 mL of distilled water. The mixture was vigorously shaken for 5 min and then allowed to stand for 15 min. The water portion was discarded. The hexane layer was collected through the anhydrous sodium sulphate and removed by a rotary evaporator at 40 ^{0}C. The residue was dissolved in n – hexane and adjusted to the volume of 10 mL with the same solvent. The sample solution was used for GC-FID determination.

2.4.2. Extraction of sterols

45 mL of the solution was transferred to a 250 mL separatory funnel together with 100 mL of n-hexane and 50 mL of mixture (96%, V/V) ethanol: distilled water (1:1). The mixture was vigorously shaken for 5 min and then allowed to stand for 15 min. The water portion was discarded. The hexane layer was collected through the anhydrous sodium sulphate and removed by a rotary evaporator at 40 ^{0}C. The residue was dissolved in chloroform and adjusted to the volume of 1 mL with the same solvent. The sample solution was used for GC-FID determination.

2.5. GC determination

Separation of FAs was achieved on a fused silica HP-FFAF capillary column (25 m x 0.32 mm i.d. x 0.52 μm film thickness), supplied by Agilent (USA). Operating conditions were as follows: injector port temperature, 230 ^{0}C; injection volume, 2 μL in split mode (1:10); detector temperature, 260 ^{0}C (make up gas - nitrogen flow 27.5 mL/min); nitrogen as carrier gas at flow rate at 1.5 mL^{0}C /min; oven temperature programme, 180 ^{0}C (1 min) increased with the rate of 2 ^{0}C/min to 200 ^{0}C and held for 19 min. The total run time of the chromatographic analysis was 30 min. The column equilibration time was 3 min.

Sterols separation was achieved on a fused silica HP-FFAF capillary column (25 m x 0.32 mm i.d. x 0.52 μm film thickness), supplied by Agilent (USA). Operating conditions were as follows: injector port temperature, 230 ^{0}C; injection volume, 2 μL in split mode (1:3); detector temperature, 300 ^{0}C (make up gas - nitrogen flow 27.5 mL/min); nitrogen as carrier gas at flow rate at 1.5 mL^{0}C /min; oven temperature was set at 260 ^{0}C and held for 40 min. The column equilibration time was 3 min.

The proposed method was validated in respect to the recovery, linearity, precision expressed as within day repeatability and between day reproducibility of retention time and peak area, stability, limit of detection (LOQ), limit of quantification (LOQ) and measurement uncertainty (U_x).

III. RESULTS AND DISCUSSION

3.1. Method optimization

In order to obtain the best separation for all tested FAs and sterols, a series of preliminary investigations with capillary GC columns with a different polarity of the stationary phase were tested. The optimum separation of components of interest was achieved when capillary column with a strong polarity (Polyethylene glycol-TPA modified) and optimized temperature programmes are used (Fig. 1).

Representative chromatograms for FAs and sterols separation with GC – FID method are shown in Fig. 1 and Fig. 2, respectively.

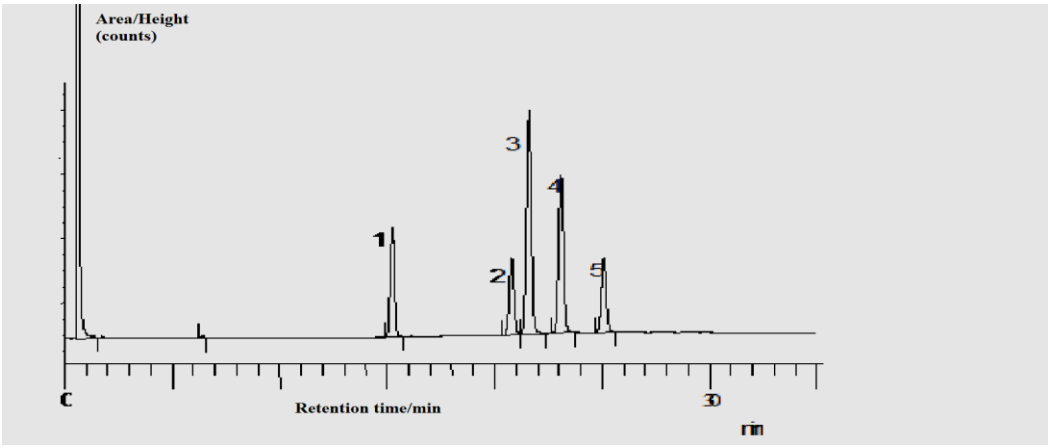

Figure 1. Chromatogram of standard solution of $C_{16:0}$ (1), $C_{18:0}$ (2), $C_{18:1}$ (3), $C_{18:2}$ (4) and $C_{18:3}$ (5), at 10 mg/L

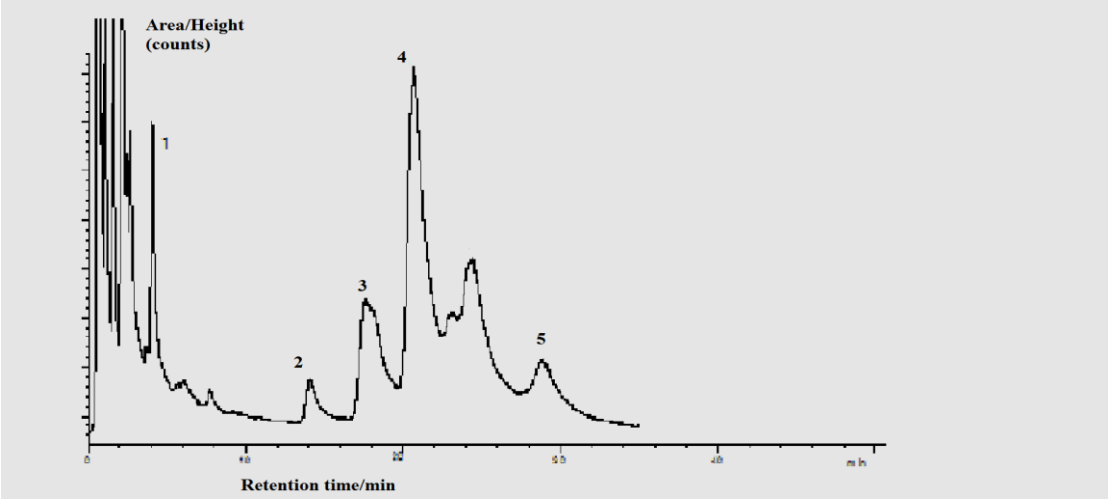

Figure 2. Chromatogram of the sterols extract from total diet sample [1. 5 α-Cholestan (I.S.); 2. Cholesterol; 3. Stigmasterol; 4. Campesterol; 5. β - Sitosterol]

In our research accelerated solvent extraction (ASE) technique was used for samples extraction. The elevated pressure increases the boiling temperature of the solvent and extraction can occur at relatively high temperatures, which leads to faster extractions [21, 22]. The extraction process is therefore significantly faster than traditional methods such as Soxhlet extraction or supercritical fluid extraction [23]. In a study conducted by Omar and Salimon [20] the total time required for extraction of the lipids from the sample with Soxhlet extraction was approximately 3 hours. Soxhlet extraction and SFE are also used as techniques for the isolation of lipids prior to the determination of sterols [24]. In our investigations due to the use of elevated temperature and pressure in the extraction cell the extraction time was approximately 15 min.

Up to our knowledge, no previous data was reported in the literature for direct determination of FAs and sterols by GC. In our study, for the first time we introduce an analytical method for direct determination of free FAs and sterols, avoiding the derivatization step and therefore the possible errors which could be due to this demanding procedure.

3.2. Method Validation

3.2.1. Recovery, Precision and Linearity

The recovery, precision and linearity of all the FAs and sterols was determined using fortified samples of dietary non fatty supplements which were previously tested on the presence of FAs and sterols. In each case, 5 replicates each at 5 levels were fortified into the samples. The samples (10 g) were fortified before the extraction to yield 10 mg/kg; 25 mg/kg; 50 mg/kg; 75 mg/kg and 100 mg/kg, respectively ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, cholesterol and β - Sitosterol); and to yield 1.0 mg/kg; 2.5 mg/kg; 5 mg/kg; 7.5

mg/kg and 10 mg/kg, respectively (stigmasterol and campesterol). Gas tight glass syringes (10 µL, 50 µL and 100 µL) were use for the addition of spiking mixtures.

The recovery, precision and linearity of the tested method for the determination of FAs and sterols are presented in Table 2 and Table 3, respectively.

From the obtained results, it can be noticed that the proposed method is accurate and precise enough for the determination of all the tested FAs and sterols. The obtained values for multiple correlation coefficients, ranged from 0.9904 to 0.9982, indicated that the method has a good linearity for all the tested compounds. High analytical recoveries ranging from 80.8% to 98.2% were obtained for FAs determination, as well as, for sterols determination (82.7% - 91.0%).

Table 2. Statistical data for mean recovery, precision data and linearity of the method for the determination of FAs

FA	Fortification level (mg/kg)	FA found - mean value (mg/kg ± SD)	Recovery (%), n=5	RSD (%)	Regression equation
$C_{4:0}$	10	8.98 ± 0.55	89.8	6.2	y = 0.9912x – 2.2561 R^2 = 0.9987
	25	22.4 ± 1.2	86.2	5.3	
	50	45.6 ± 2.4	91.1	5.2	
	75	71.3 ± 4.0	95.1	5.6	
	100	98.2 ± 4.7	98.2	4.8	
$C_{6:0}$	10	9.12 0.23	91.2	2.5	y = 0.9832x – 1.6415 R^2 = 0.9982
	25	23.5 ± 1.05	94.0	4.5	
	50	44.8 ± 2.5	89.6	5.6	
	75	72.5 ± 3.4	96.7	4.7	
	100	97.5 ± 4.2	97.5	4.3	
$C_{8:0}$	10	8.79 ± 0.61	87.9	6.9	y = 0.969x – 2.7665 R^2 = 0.9954
	25	21.8 ± 1.2	87.2	5.5	
	50	41.5 ± 2.1	83.0	5.1	
	75	70.5 ± 2.3	94.0	3.3	
	100	95.5 ± 4.4	95.5	4.6	
$C_{10:0}$	10	8.65 ± 0.54	86.5	6.2	y = 0.9525x – 3.0378 R^2 = 0.9951
	25	20.5 ± 1.1	82.0	5.4	
	50	40.5 ± 1.9	81.0	4.7	
	75	69.6 ± 2.9	92.8	4.2	
	100	93.2 ± 5.2	93.2	5.6	
$C_{12:0}$	10	8.3 ± 0.40	83.0	4.8	y = 0.9136x – 1.4293 R^2 = 0.9983
	25	21.4 ± 1.4	85.6	6.8	
	50	42.3 ± 2.5	84.6	5.9	
	75	68.9 ± 3.1	91.9	4.5	
	100	89.5 ± 5.9	89.5	6.6	
$C_{14:0}$	10	8.95 ± 0.54	89.5	6.8	y = 0.9089x –1.9915 R^2 = 0.9982
	25	20.5 ± 1.6	82.3	7.8	
	50	41.4 ± 2.8	82.8	6.7	
	75	67.9 ± 3.6	90.5	5.3	
	100	88.6 ± 5.5	88.6	6.2	
$C_{16:0}$	10	8.15 ± 0.6	81.5	7.4	y = 0.9273x –1.8305 R^2 = 0.9987
	25	20.8 ± 1.8	83.2	8.6	
	50	43.2 ± 2.5	86.4	5.8	
	75	69.5 ± 3.6	92.7	5.2	
	100	90.3 ± 5.2	90.3	5.7	
$C_{16:1}$	10	8.8 ± 0.43	88.0	5.5	y = 0.9411x –2.4756 R^2 = 0.9956
	25	22.3 ± 1.3	89.2	5.8	
	50	40.5 ± 2.1	81.6	5.2	
	75	69.4 ± 2.9	92.5	4.2	
	100	92.3 ± 4.8	92.3	5.2	
$C_{18:0}$	10	8.6 ± 0.45	86.0	5.9	y = 0.952x –2.5634 R^2 = 0.9926
	25	23.4 ± 1.6	93.6	6.8	
	50	39.8 ± 2.5	89.6	6.3	
	75	70.5 ± 3.2	94.0	4.5	
	100	93.4 ± 5.1	93.4	5.5	
$C_{18:1}$	10	8.3 ± 0.40	83.0	5.4	y = 0.9464x – 3.3537 R^2 = 0.9906
	25	20.2 ± 1.4	80.8	6.9	
	50	39.6 ± 2.8	89.2	7.1	
	75	72.4 ± 3.0	96.5	4.1	
	100	89.8 ± 4.9	89.8	5.5	
$C_{18:2}$	10	8.1 ± 0.50	81.0	7.0	y = 0.9297x – 3.2634 R^2 = 0.9912
	25	20.5 ± 1.3	82.0	6.3	
	50	38.5 ± 2.1	82.5	5.4	

FA	Fortification level (mg/kg)	FA found - mean value (mg/kg ± SD)	Recovery (%), n=5	RSD (%)	Regression equation
	75	70.5 ± 3.2	94.0	4.5	
	100	88.8 ± 4.7	88.8	5.3	
$C_{18:3}$	10	8.34 ± 0.30	83.4	4.3	y = 0.9192x – 3.5561 R^2 = 0.9904
	25	19.8 ± 1.2	84.3	6.1	
	50	37.5 ± 2.4	85.0	6.4	
	75	69.5 ± 2.9	92.7	4.2	
	100	87.5 ± 3.6	87.5	4.1	

Table 3. Statistical data for mean recovery, precision data and linearity of the method for the determination of sterols

FA	Fortification level (mg/kg)	FA found - mean value (mg/kg ± SD)	Recovery (%), n=5	RSD (%)	Regression equation
Cholesterol	10	8.3 ± 0.42	83.0	6.3	y = 0.8744x – 3.9683 R^2 = 0.9953
	25	18.6 ± 1.6	84.4	8.6	
	50	36.5 ± 2.7	83.0	7.4	
	75	60.5 ± 3.6	82.9	5.9	
	100	85.6 ± 4.5	85.6	5.2	
β - Sitosterol	10	8.93 ± 0.30	89.3	4.3	y = 0.8421x – 2.4683 R^2 = 0.9975
	25	19.2 ± 1.4	86.8	7.3	
	50	37.6 ± 2.4	87.5	6.4	
	75	59.5 ± 3.4	86.3	5.7	
	100	83.4 ± 4.7	83.4	5.6	
Campesterol	1.0	0.863 ± 0.03	86.3	4.3	y = 0.9274x – 0.3024 R^2 = 0.9929
	2.5	2.1 ± 0.15	84.5	7.1	
	5.0	3.9 ± 0.20	85.0	5.1	
	7.5	7.0 ± 0.30	83.7	4.3	
	10	8.9 ± 0.40	89.0	4.5	
Stigmasterol	1.0	0.837± 0.04	83.7	5.5	y = 0.943x – 0.3378 R^2 = 0.9975
	2.5	2.0 ± 0.15	85.4	7.5	
	5.0	4.1 ± 0.22	83.6	5.4	
	7.5	6.9 ± 0.35	92.0	5.1	
	10	9.1 ± 0.30	91.0	3.3	

3.2.2. Repeatability and Reproducibility

The within day repeatability of our method was determined by performing the analysis of 10 blank samples fortified with FAs at 50 mg/g and sterols at 10 mg/kg, respectively. After the isolation of the lipid fraction, saponification of the extracts and extraction of FAs and sterols, obtained extracts were analyzed by GC-FID within the same day under the chromatographic conditions described in the section 2.5. The between day reproducibility of the method was determined by performing the analysis of the extracts obtained from 5 fortified blank sample (50 mg/g of FAs, 10 mg/L of sterols). After the isolation of the lipid fraction, saponification of the extracts and extraction of FAs and sterols, obtained extracts were analyzed by GC-FID within the same day under the chromatographic conditions described in the section 2.5. The calculated RSD values for the retention time (t_R), the peak areas, within day repeatability and between day reproducibility are shown in Table 4 and Table 5, respectively.

The calculated RSD values for within day repeatability in the case of FAs determination for t_R values ranged from 0.112% to 0.190%, whereas for peak areas RSD values ranged from 3.03% to 4.61%, indicating good precision of the t_R, as well as of the peak area, within the same day. Good precision of the t_R, (RSD from 0.165% to 0.196%) and the peak area (RSD from 3.23% to 4.95%) were also observed within different days (between day reproducibility).

The calculated RSD values for within day repeatability in the case of sterols determination for t_R values ranged from 0.182% to 0.192%, whereas for peak areas RSD values ranged from 3.82% to 4.27%, indicating good precision of the t_R, as well as of the peak area, within the same day. Good precision of the t_R, (RSD from 0.196% to 0.205%) and the peak area (RSD from 4.11% to 4.94%) were also observed within different days (between day reproducibility).

Table 4. Statistical data for within day repeatability and between day reproducibility for the determination of FAs

FA	t_R /min	Within day Repeatability (RSD, %); n=10		Between day Reproducibility (RSD, %); n=25	
$C_{4:0}$	4.872	0.112	4.17	0.165	4.11
$C_{6:0}$	6.782	0.162	3.91	0.179	3.23
$C_{8:0}$	8.450	0.190	3.40	0.198	3.78
$C_{10:0}$	9.454	0.110	3.73	0.184	3.94
$C_{12:0}$	12.345	0.121	4.61	0.194	4.97
$C_{14:0}$	14.190	0.103	3.23	0.172	4.56
$C_{16:0}$	15.104	0.105	3.52	0.198	4.46
$C_{16:1}$	21.146	0.163	3.29	0.189	4.95
$C_{18:0}$	21.890	0.142	3.79	0.177	4.65
$C_{18:1}$	22.267	0.164	3.85	0.180	4.90
$C_{18:2}$	23.467	0.161	3.21	0.192	4.34
$C_{18:3}$	25.796	0.176	3.03	0.196	4.95

Table 5. Statistical data for within day repeatability and between day reproducibility for the determination of sterols

FA	t_R /min	Within day Repeatability (RSD, %); n=10		Between day Reproducibility (RSD, %); n=25	
Cholesterol	15.163	0.192	4.27	0.205	4.11
Stigmasterol	18.893	0.182	3.97	0.219	4.23
Campesterol	20.983	0.195	3.82	0.238	4.78
β -Sitosterol	29.124	0.185	3.93	0.196	4.94

3.2.3. Stability

Stock standard solutions and working standard solutions were found to be stable for at least 3 months, respectively, when stored at -20 ^{0}C. Moreover, the stability of a fortified blank sample at a concentration of 50 mg/g kept in the auto injector for 24 hours was assayed, and differences of < 5.0 % were obtained.

3.2.4. Limit of Detection, Limit of Quantification, Measurement Uncertainty

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the formulas LOD = 3.3 · SD/slope and LOQ = 10 · SD/slope [25].

Table 6. Statistical data for LOD, LOQ, U_x for FAs and sterols

Compound	LOD (mg/kg)	LOQ (mg/kg)	U_x (%)
$C_{4:0}$	0.22	0.73	12.5
$C_{6:0}$	0.18	0.59	14.8
$C_{8:0}$	0.16	0.53	11.3
$C_{10:0}$	0.19	0.63	11.9
$C_{12:0}$	0.21	0.69	10.8
$C_{14:0}$	0.15	0.49	9.6
$C_{16:0}$	0.12	0.40	11.6
$C_{16:1}$	0.15	0.49	10.5
$C_{18:0}$	0.16	0.53	8.5
$C_{18:1}$	0.10	0.33	9.5
$C_{18:2}$	0.11	0.36	11.2
$C_{18:3}$	0.12	0.40	9.8
Cholesterol	0.18	0.59	13.7
Stigmasterol	0.25	0.82	13.4
Campesterol	0.22	0.73	11.2
β -Sitosterol	0.26	0.86	12.4

The values for measurement uncertainty (U_x) were calculated according to a Eurachem/CITAC Guide [26]. The obtained values showed in Table 5, ranged from 8.5% to 14.8%.

3.3. Application of the method

In order to demonstrate the applicability of the proposed method for the determination of FAs and sterols in the total diet, the 2 week experiment was conducted. Sampling was done according to the methodology of Thomas et al. [27]. The results of the investigation are presented in Fig. 3 and Fig. 4, respectively.

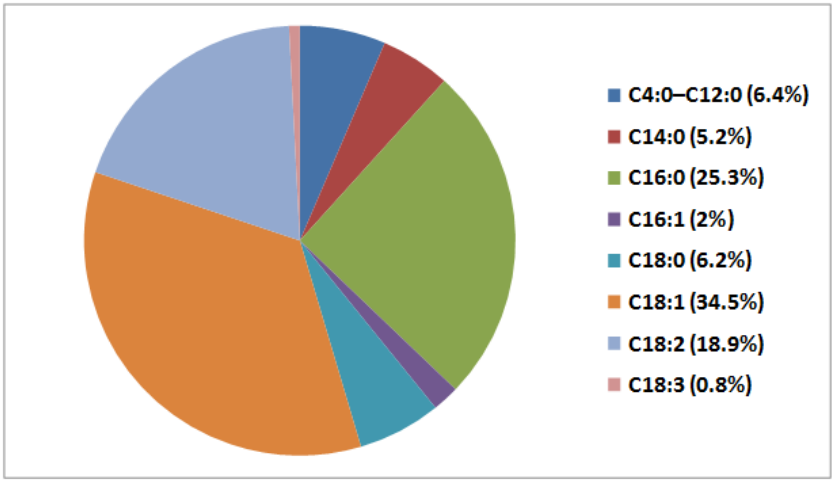

Figure 3. Distribution of the most abundant FAs in the total diet

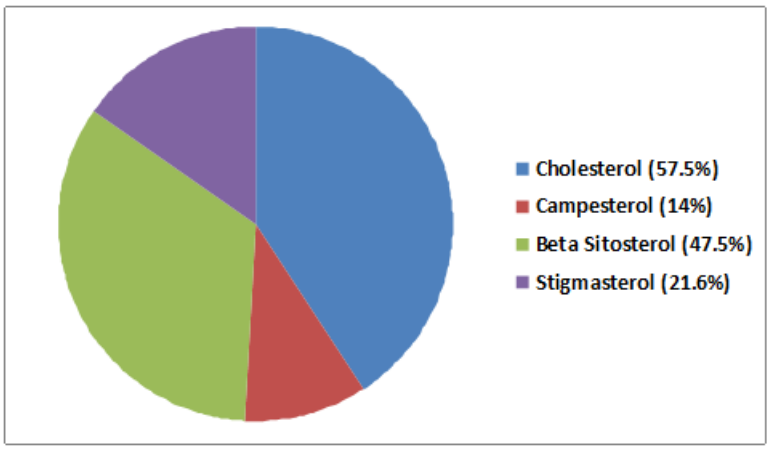

Figure 4. Distribution of the most abundant sterols in the total diet

As can be seen from the obtained results, the most abundant FA in the total diet is monounsaturated $C_{18:1}$ with 34.5% of the total FAs. The lowest presence was observed for the short chain FAs: $C_{4:0} - C_{12:0}$ (6.4%). Cholesterol was found to be the most abundant sterol in the diet (57.5%). The total amount of the plant sterols in the diet was found to be 42.5%.

IV. CONCLUSION

A reliable, accurate and precise GC-FID method, after extraction of the samples with ASE, saponification of the lipid fraction and extraction of the FAs and sterols with n-hexane from the same solution was developed and validated. With the usage of ASE technique, extraction of lipids from the samples was performed within 15 minutes. The proposed extraction procedure of the FAs and sterols enables the single step requiring the same organic solvent. The proposed method, reduce the amount of co- extractible lipids and disables the appearance of the background peaks in the chromatogram, above signal to noise ratio of 3, at the retention times of targeted compounds. FID was chosen for this analysis due to its selectivity for the compounds of interest. Validation parameters obtained for determination of the FAs and sterols in the total diet demonstrate that the developed analytical method meets the method performance acceptability criteria (mean recovery in the range 80.8% to 98.2%, precision with RSD < 8.6%; LOQ < 0.86 mg/kg; measurement uncertainty < 14.8%).

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