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ORIGINAL PAPER

Quantitation of Sterols and Steryl Esters in Fortified Foods and Beverages by GC/FID

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Abstract Direct extraction methods for the quantitation of sterols and steryl esters (SE) in foods, beverages and concentrates as free sterols (trimethylsilane ether derivatives) by gas chromatography with flame ionization detection (GC/FID) are introduced. Theoretical correction factors are used for sterol quantification relative to the internal standard, Epicoprostanol. Conversion of the acylglycerols, when present, into FFA eliminates the time-consuming acylglycerol extraction step and reduces the GC run time since no higher molecular weight acylglycerols need to elute from the column. Accuracy and precision of analytical data are important when formulating food products with sterols or SE to ensure a formulation will meet health claims, while at the same time minimizing costly over formulation. Method accuracy was determined by complete recovery of sterol and SE concentrates as free sterols with subsequent analysis of foods and beverages formulated with sterol and SE concentrates. Triplicate analysis over 5 days demonstrated repeatability for the alkaline saponification and acid extraction with alkaline saponification methods. A relative standard deviation of $<5\%$ demonstrates the repeatability of the methods. Replicate analysis of a common sample by two laboratories and replicate analysis of a common sample by multiple analysts demonstrated reproducibility of the methods.

Keywords Direct extraction · Theoretical correction factors (TCF) · Sterol and steryl esters · Gas chromatography · Flame ionization detection · Data analysis

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Introduction

Phytosterols naturally occur in foods as free sterols, steryl esters (SE), steryl glucosides (SG) and acylated steryl glucosides (ASG). The structure of a common free (alcoholic) sterol is shown in Fig. 1. The double bond is shown at the Δ-5 position; however, it is also common for the double bond to be at the Δ-7 position. The R_1 (side chain) varies between phytosterols [1]. The 3β-hydroxyl group of the sterol is esterified to a fatty acid or a hydroxycinnamic acid in SE, glycosylated with a hexose (usually glucose) in SG, or glycosylated with a 6-fatty acyl hexose in ASG [2]. Phytostanols are fully saturated derivatives of phytosterols with no double bonds either in the ring structure or in the side chain [3].

Methods are introduced for the quantitation of the total free sterols in foods, beverages and concentrates, which includes free sterols and SE converted to free sterols. The alkaline hydrolysis conditions presented herein would only result in partial hydrolysis of ASG to SG and the acid hydrolysis conditions presented herein would only result in partial hydrolysis of SG to free sterol. Because the levels of fortified sterols are considerably higher than that of the SG and ASG, the contribution of SG and ASG to total sterol concentration is negligible. For the determination of total sterols, including glycosidic sterols, acid hydrolysis (6 M HCl for 60 min at 80 °C) can be used [1].

Plant phytosterols have been added to a variety of food products for people wishing to lower their blood low-density lipoprotein (LDL) cholesterol concentration. Phytosterols in the diet efficiently decrease serum cholesterol concentration by reducing the absorption of cholesterol in the digestive tract [4]. The optimal daily dose of sterols or stanols is 2 g/day, which is equivalent to 3.3 g SE per day [4]. On average, 2 g sterols per day results in a 10%

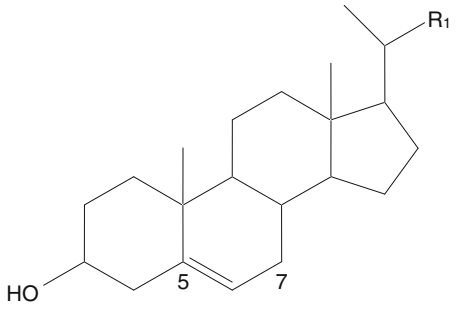

Fig. 1 The structure of a common free sterol is shown with the double bond at the Δ-5 position, however some sterols have the double bond at the Δ-7 position; the R_1 (side chain) varies between phytosterols

reduction in LDL-cholesterol, with higher doses providing little additional effect [4]. In order to formulate a food product to meet the health claims associated with sterol fortification, the sterol concentration must be determined. The health claim associated with sterols in the United States is 0.4 g of sterols per serving (two servings per day) or 0.8 g of sterols per day [5]. The health claim associated with SE in the United States is 0.65 g of SE per serving (two servings per day) or 1.3 g of SE per day. Phytosterols have also been shown to have anti-atherosclerotic, anti-inflammatory and anti-oxidative effects in animals [4]. In Western diets, we consume an average of 250 mg phytosterols and approximately 25 mg of phytostanols (fully saturated derivatives of phytosterols) per day [6].

Methods for the determination of sterols in foods typically include acid hydrolysis and/or extraction of lipids and/or saponification to free sterols, extraction of unsaponifiables and separation/partial purification of sterols, the formation of sterol derivatives and analysis by capillary GC with flame ionization detection (FID) or mass spectrometry (MS) [1–3, 7]. There are no official reference methods for the analysis of phytosterols or phytostanols in sterol-fortified foods and beverages in which the sterol concentration can be as high as 8% and even higher for sterol concentrates [3]. Official reference methods for the analysis of sterols as naturally occurring minor components in food are intended for sterol contents of 1% or less, including the analysis of sterol fractions of fats and oils: ISO 6799 [8], IUPAC methods 2.401 [9] and 2.403 [10], ISO 12228 [11] and AOCS Recommended Practice Ch 6-91 [12]. Codex-Stan 210 [13] refers to ISO 6799 [8] and IUPAC 2.403 [10] methods. AOAC official method 2007.03, "Campesterol, Stigmasterol, and Beta-Sitosterol in Saw Palmetto Raw Materials and Dietary Supplements" is applicable for the determination of sterols at levels >1 mg/100 g [14]. Unofficial methods for the quantitation of sterol fractions using high performance liquid chromatography (HPLC) are also available [2].

Direct extraction methods for the quantitation of total sterols in sterol concentrates, SE concentrates, and foods and beverages formulated with sterol and SE concentrates are introduced herein. Formulated foods and beverages include milk, spoonable yogurt, bread, orange juice, apple juice, margarine spread, coffee creamer and puffed corn cereal. Theoretical correction factors (TCF) are used for sterol quantification [15]. There is no evidence to suggest that the methods presented herein could not be used for the quantitation of stanol or stanol ester fortified foods and beverages.

Experimental Procedures

Materials

Powdered sterol concentrate, emulsified sterol concentrate, SE concentrate and powdered SE concentrate are Cargill's CoroWise™ Naturally Sourced Cholesterol Reducer™ brand plant sterol products (Cargill, Incorporated, Minneapolis, MN) and were obtained from Cargill Health and Nutrition. Soy germ oil, extracted from the soy germ using *n*-hexane, was obtained from the Cargill crush facility at Iowa Falls, IA. Foods and beverages formulated with CoroWise™ plant sterols were submitted by Cargill customers and in most cases are commercially available.

Standard Solutions Preparation

The stock internal standard (IS) solution was prepared by dissolving Epicoprostanol, 5β-cholestan-3α-ol (P/N C-2882, Sigma Chemical Co., St. Louis, MO) in toluene (P/N T291-4, Optima, Fisher Scientific, Inc., Pittsburgh, PA) at a concentration of 5 mg/mL. It is recommended to use Epicoprostanol instead of 5α-cholestan-3β-ol, which has traditionally been used as an IS during sterol and/or cholesterol analysis. Epicoprostanol is similar to sterols in structure and FID response [3]. Epicoprostanol, like most sterols, contains a position-3 hydroxyl group, which can be silylated. 5-α-Cholestane does not contain a hydroxyl group, while betulin, another commonly used IS for sterols determination, contains two hydroxyl groups [3]. Epicoprostanol is also a good choice for IS because it is not present in samples.

For the analysis of standard solutions, 300 μL of each solution was silylated to form trimethyl silane (TMS) ethers by adding 500 μL pyridine (P/N 25104, Pierce Biotechnology Co., Rockford, IL) and 1 mL Bis (Trimethylsilyl) Trifluoroacetamide (BSTFA) + 1% Trimethylchlorosilane (TMCS) (P/N 38834, Pierce Biotechnology Co.).

The stock IS solution was prepared at 5 mg/mL and the designated IS amounts were prepared by serially diluting from the stock IS solution in toluene to achieve a concentration appropriate for the sterol concentration of the samples according to Eq. 1. This equation is a guide to obtain an IS peak area comparable to that of the three major sterols (campesterol, stigmasterol and β-sitosterol)

$$\frac{\text{Amount}_{\text{Sterol}}}{4} \leq \text{Amount}_{\text{IS}} \leq \frac{\text{Amount}_{\text{Sterol}}}{2}. \quad (1)$$

Sample Preparation

The sterols analysis platform is such that the optimum method for each sample matrix is determined by triplicate analysis using 15 min alkaline, 120 min alkaline and 45 min acid/15 min alkaline methods. The method with the highest sterol recovery and lowest variability is further refined as needed by decreasing the saponification time from 120 to 60 to 30 min or decreasing the acid hydrolysis time from 45 to 30 to 15 min with triplicate analysis for each; the highest sterol recovery and lowest variability is used. Repeatability of the optimum method is demonstrated for each sample matrix with triplicate analysis over 5 days. The optimization of the acid concentration used for the acid hydrolysis (3 N in water) is not included in this study.

Acid hydrolysis prior to saponification is required for the complete liberation of steryl esters from certain matrices such as some cereals. The complete hydrolysis of SE is confirmed chromatographically by the absence of residual SE (GC/FID conditions not included). Acid hydrolysis prior to saponification has also been used to improve the variability for certain matrices that appear unsolubilized by the alkaline hydrolysis (e.g. gels). A summary of sample preparation methods is shown in Table 1.

Acid hydrolysis using 6 M HCl for 30 min at 80 °C can isomerize fucosterol, Δ-5-avenasterol, and similar ethylidene side chain sterols to give a mixture of five isomers [16]. However, isomerization was not observed with the acid hydrolysis conditions presented in this article (3 N HCl for up to 45 min), which was demonstrated by identical chromatography of the fucosterol standard (P/N C6685, Steraloids, Inc.) prepared using the 15 min alkaline method and the 45 min acid/15 min alkaline method (data not shown).

Preparation of Foods and Beverages using Alkaline Saponification

A known amount of sample (see Table 1 for sample amounts) was added to a 50-mL reaction flask and to it 5 mL of IS solution and 5 mL 2.3 N NaOH:MeOH (P/N S318-500 and Optima P/N A454-4, respectively, Fisher Scientific, Inc., Pittsburgh, PA) were added. The NaOH/MeOH solution must be stirred before using, since it will settle out with time. Adding water will help to keep NaOH in solution; however, the saponification time is greatly increased. Therefore, no water was added. Evaporating the IS solvent was not necessary as it increased the overall time of sample preparation and saponification time. In this methodology, the organic solvent serves simultaneously as both a carrier for the IS addition and as an extraction solvent. The mixture was boiled on a water bath (P/N 12060-10, Organomation Associates, Inc, Berlin, MA) at 100 °C for 15 min (or longer) while refluxing the solvent. The water in the bath must be boiling to complete the saponification, otherwise, an emulsion will form when the saturated sodium chloride is added. The water level must also be in contact with the glass flask up to the level of the reactants inside the flask to ensure proper heating and reaction completeness (this is specific for the Organomation unit). Seven milliliters 3 N HCl (P/N LC-15330-4, Fisher Scientific, Inc.) was added to mixture after it had cooled to lukewarm temperature. The FA soaps formed during saponification are converted to their acids by the addition of HCl. This allows for chromatography of the FA TMS ethers (although FFA salts could be silylated) and minimizes the formation of emulsions. The organic phase was salted out using approximately 40 mL of saturated $NaCl_{aq}$ solution (reagent grade P/N S640-3, Fisher Scientific, Inc.). A 300 μL aliquot of the organic phase, 500 μL pyridine (P/N 25104, Pierce Biotechnology Co.), 1 mL Bis (Trimethylsilyl) Trifluoroacetamide (BSTFA) + 1% Trimethylchlorosilane (TMCS) (P/N 38834, Pierce Biotechnology Co.) and a small amount of sodium sulfate (reagent grade, P/N S415-1, Fisher Scientific, Inc.) were added to a GC vial. The capped GC vials were shaken briefly before analysis by GC/FID.

Preparation of Foods and Beverages using Acid Hydrolysis Prior to Alkaline Saponification

A known amount of sample (see Table 1 for sample amounts) was added to a 50-mL reaction flask and to it 5 mL of 3 N HCl, then 5 mL of IS solution were added. It is important to add the acid before the internal standard solution because it helps minimize the amount of sample that adheres to the sides of the flask. The mixture was boiled on a water bath at 100 °C for 30 min (or longer) while refluxing the solvent. The organic phase was salted out using a saturated $NaCl_{aq}$ solution after the mixture had cooled to lukewarm temperature. The organic layer was transferred to a clean 50-mL reaction flask and 5 mL 2.3 N NaOH:MeOH was added. As much as possible of the organic layer was transferred without disrupting the solids on the surface. The mixture was returned to the water bath

Table 1 Summary of methods used to obtain total sterols, including free sterols and steryl esters once saponified into free sterols

Sample identification	Sample amount (mg)	Method
Powdered sterol conc.[a]	100	Dilute and shoot
Soy germ oil, emulsified sterol conc. and SE conc.	100	15 min alkaline
Bread with powdered sterol conc.[b] and margarine spread with SE conc.	500	15 min alkaline
Milk or orange juice with emulsified sterol conc.	1,000	15 min alkaline
Spoonable yogurt with powdered SE conc.[c]	1,000	120 min alkaline
Apple juice with SE conc.	1,000	150 min alkaline
Puffed corn cereal with powdered sterol conc.[d]	500	30 min acid, then 15 min alkaline
Powdered SE conc. and coffee creamer with powdered SE conc.	100	45 min acid, then 15 min alkaline

Alkaline preparation using alkaline saponification, *Acid* preparation using acid hydrolysis, *SE* steryl ester, *Conc.* concentrate

[a] Powdered sterol concentrates were ground to a particle size of 0.75 mm or less using a rotor mill (Retsch® Grinder ZM-100, Newton, PA) with a 12-tooth rotor at 18,000 rpm. A 12-tooth rotor is recommended because of the difficulty in cleaning a 24-tooth rotor. Five microliters of IS solution was added to 100 mg of powdered sterols in a 13 × 100-mm test tube. The capped test tube was vortexed to mix. Five hundred microliters pyridine and 1 mL BSTFA + 1% TMCS were added to the test tube. The capped test tube was vortexed to mix. A 300 μL aliquot of the derivatized sample, 1 mL toluene and a small amount of sodium sulfate were added to a GC vial. The capped GC vials were shaken briefly before analysis by GC/FID

[b] One slice of bread was cryoground to a fine powder prior to sampling using a Freezer Mill (Model 6850-115, Spex CertiPrep Inc., Metuchen, NJ). The bread was stored in a moisture-free environment (e.g. desiccator). Additional moisture increased the time necessary to cryogrind the bread to a fine powder. Sterol results could be underestimated due to the added weight of the moisture when sterols results are reported on a percent weight-by-weight basis

[c] Spoonable yogurt containing fruit was homogenized prior to sampling (Polytron® PT3100 bench-top homogenizer, Kinematica, Inc., Bohemia, NY)

[d] The cereal was ground to a fine powder prior to sampling using a rotor mill (Retsch® Grinder ZM-100, Newton, PA) with a 6-tooth rotor and 0.75 mm sieve

at 100 °C for 15 min while refluxing the solvent. Seven milliliters 3 N HCl was added to mixture after it had cooled to lukewarm temperature. The organic phase was salted out using a saturated $NaCl_{aq}$ solution. A 300-μL aliquot of the organic phase, 500 μL pyridine, 1 mL BSTFA + 1% TMCS and a small amount of sodium sulfate were added to a GC vial. The capped GC vials were shaken briefly before analysis by GC/FID.

Gas Chromatographic Analysis

Split Injection GC Conditions

The TMS ethers of the sterols were analyzed by split GC with a non-polar column stationary phase (DB™-5, 30 m × 0.25 mm i.d. × 0.25 μm d_f, Agilent Technologies, Santa Clara, CA) coupled to a FID using a 6850 GC equipped with an autosampler (Agilent Technologies). The temperature program was 200–300 °C at 3 °C/min, hold 11.67 min [17]. Hydrogen was the carrier gas, and inlet pressure was 9 psi in the constant flow mode with a flow rate of 0.8 mL/min. The split ratios were 1:10 or 1:100. The split ratio will depend on the sterol content of the sample being analyzed. In general, the 1:10 split was used for samples requiring IS concentrations of 1 mg/mL or less and the 1:100 split was used for samples requiring IS concentrations greater than 1 mg/mL. Injection volume was 1 μL and the injector and detector temperatures were both 325 °C. A high-pressure microseal septum (P/N 410, Merlin Instrument Company, Half Moon Bay, CA) and Siltek® deactivated split precision liner with deactivated wool (P/N 21022-213.1, Restek, Bellefonte, PA) were used. The detector gas flows were set to 450 mL/min for air, 40 mL/min for hydrogen and 45 mL/min for the nitrogen make up gas.

GC Coupled to Mass Spectrometry

The TMS ethers of the sterols were injected onto an Agilent 6890 GC with a 5873N MS Detector. The samples were separated on a non-polar column stationary phase (DB™-5, 30 m × 0.25 mm i.d. × 0.25 μm d_f, Agilent Technologies) coupled to a MS detector. Sample concentrations were the same as those used for GC/FID. One microliter was injected onto a splitless liner heated at 340 °C. A high-pressure microseal septum (P/N 410, Merlin Instrument Company, Half Moon Bay, CA) was used. The inlet was set a 325 °C and the transfer line was 280 °C. The temperature program was 200–300 °C at 3 °C/min, hold 11.67 min. Helium was the carrier gas at 1.1 mL/min in the constant flow mode. The MS source was set at 230 °C with the quadrupole set at 150 °C. Analysis

were done in electron ionization (EI) mode, voltage of 70 eV, and scanning from 40 to 800 Da.

Sterol Quantitation

Determining Internal Standard Purity

The purity (percent area) of the stock IS solution is accounted for in the final concentrations of the IS by integrating both Epicoprostanol and Coprostan-3-ol (5β-cholest-3β-ol) as one peak. The integration of the IS is shown in Fig. 2 with Coprostan-3-ol being the peak at 27.7 min that elutes in front of Epicoprostanol. The Coprostan-3-ol isomer was identified in the Epicoprostanol standard by matching chromatographic retention time and mass spectra of the peak at 27.7 min in Fig. 2 with a Coprostan-3-ol standard (P/N C7578, Sigma-Aldrich). The mass spectrum of the Coprostan-3-ol peak matched the spectrum of the Coprostan-3-ol standard (data not shown). There exist four different isomers of Epicoprostanol, including itself: Epicoprostanol, Coprostan-3-ol, Dihydrocholesterol (5α-Cholest-3β-ol) and Epicholestanol (5α-Cholest-3α-ol), but only the Coprostanol and Epicoprostanol isomers are present in the Epicoprostanol standard [18]. The percent purity of the Epicoprostanol Stock IS solution is determined by dividing the sum of the Epicoprostanol and Coprostan-3-ol peak areas by the total peak areas. Integration for impurities is from 20 to 32 min for the Stock IS solution. The percentage purity is typically greater than 99%. The concentration of Epicoprostanol in the stock IS solution is determined from chromatographic data as shown in Eq. 2. The purity was determined in triplicate

$$\text{Concentration}_{\text{Epicoprostanol}} = \text{Concentration}_{\text{IS}} \times \text{Purity}_{\text{IS}}. \quad (2)$$

Sterol Quantitation using Theoretical Correction Factors

The total sterols are determined by utilizing theoretical correction factors (TCF) to determine the amount of each sterol relative to the internal standard, Epicoprostanol [15]. TCF are used for sterol quantification as they provide more reproducibility compared to empirical correction factors [15]. In addition, TCF do not require the preparation and analysis time associated with calibration curves nor the need to acquire pure sterol standards that may be difficult to find [15]. Since different sterols have different molecular masses, they can give different responses in a FID. TCF can be used to account for this difference giving very precise results without the need for extensive experimental calibrations [15]. TCF are calculated according to Eq. 3 in which the sum of the molecular weights of all the active carbons is divided by the molecular weight of the entire sterol. Active carbons are defined to be all carbons atoms with the exception of carbonyl carbons, which are not thought to respond in a FID [19]

$$\text{Active carbon mass percent} = \frac{\text{Active carbon molecular weight}}{\text{Total sterol molecular weight}} = \text{TCF}. \quad (3)$$

The TCF of the sterols relative to Epicoprostanol IS are calculated as shown in Eq. 4. The range of TCF for sterols relative to Epicoprostanol IS is from 0.98 to 1 [15]. The amounts of each sterol can be determined from chromatographic data as shown in Eq. 5. The percent of total sterols for a sample can then be determined by summing the amount of each individual sterol in a given sample, dividing by the sample weight and multiplying by 100 [5]. The final SE concentration was determined by

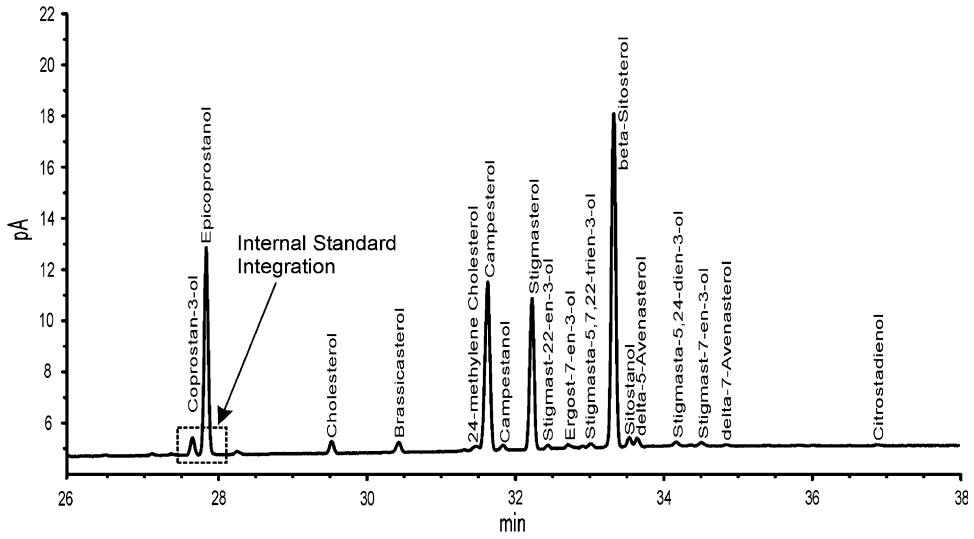

Fig. 2 The gas chromatogram obtained from milk formulated with emulsified sterol concentrate zoomed to show the sterol region of interest

multiplying the total sterols (after extraction and saponification of SE into free sterols) by the conversion factor, 1.634, which converts the free sterols to canola oil based SE. This is based upon the average molecular weight of canola oil fatty acids

$$\frac{\mathrm{TCF_{IS}}}{\mathrm{TCF_{Sterol}}} = \text{Sterol TCF relative to IS}, \quad (4)$$

$$\text{Amount of sterol} = \frac{\mathrm{Area_{Sterol}} \times \mathrm{Amount_{IS}} \times \mathrm{TCF}}{\mathrm{Area_{IS}}}. \quad (5)$$

Results and Discussion

Chromatographic Separation

Free sterols as their TMS derivatives are resolved using the conditions given in the GC analysis section of this article. The gas chromatogram obtained from milk formulated with emulsified sterol concentrate is shown in Fig. 2. The chromatogram was obtained using the conditions given in the Split Injection GC Conditions section with a 1:10 split. Resolution of free stanols as their TMS derivatives can also be obtained using these GC conditions (data not shown).

The entire chromatographic run is 45 min and the sterol peaks elute between 27 and 37 min. Major sterol peaks were identified by comparing the retention times with those of a Plant Sterol Mixture (P/N 1119, Matreya LLC, Pleasant Gap, PA). Minor sterol peaks were identified by MS data (data not shown) [20, 21]. The three major sterols are campesterol, stigmasterol and β-sitosterol. In addition to the three major sterols there can be as many as seventeen other minor sterols in a given sample depending on the source of the sterol material. Hydrocarbons, if present in the sample, will elute from about 2.40 min (C14:0) to 44.55 min (C38:0). Fatty acids as their TMS derivatives will elute from about 2.55 min (C10:0) to 22.60 min (C24:0) for samples containing fat. Squalene and Squalane will elute at about 18.70 and 22.40 min. Tocopherols and tocotrienols as their TMS derivatives, if present in the sample, will elute from about 24.10 to 26.50 min, 28.50 to 29.00 min and 31.40 min.

Accuracy and Repeatability

Sterol Content of Foods and Beverages Formulated with Sterol and Steryl Ester Concentrates

Method accuracy was determined by complete recovery of sterol and SE concentrates as free sterols, with subsequent analysis of foods and beverages formulated with the sterol and SE concentrates. Complete sterol recovery is determined by comparing the recovered sterol content to the

Table 2 Average sterol content, variability and sterol target amounts for sterol concentrates, SE concentrates and foods and beverages formulated with sterol and SE concentrates

Sample identification	*n*	Target sterol content (% w/w)	Average sterol content (% w/w)	SD	% RSD
Soy germ oil (lot a)	178	NA	1.91[a]	0.05	2.6
Soy germ oil (lot b)	147	NA	2.17[a]	0.02	1.2
Emulsified sterol conc.	15	Est. 60	59.41	0.21	0.3
Milk with emulsified sterol conc.	15	0.21	0.21	<0.01	1.3
Powdered SE conc.	32	Est. 33	33.17	0.37	1.1
Coffee creamer with powdered SE conc.	15	Est. 33	33.33	0.51	1.5
Spoonable yogurt with powdered SE conc.	15	NA	0.46	0.01	2.3
Bread with powdered sterol conc.	22	1.40	1.44	0.07	5.1
Powdered sterol conc.	100	NA	94.30[b]	0.94	1.0
Puffed corn cereal with powdered sterol conc.	15	0.90	0.98	0.08	8.3
Orange juice with emulsified sterol conc. a	15	0.15	0.16	<0.01	2.6
Orange juice with emulsified sterol conc. b	15	0.20	0.21	<0.01	0.8
Orange juice with emulsified sterol conc. c	15	0.25	0.26	<0.01	0.7
Orange juice with emulsified sterol conc. d	15	0.35	0.35	<0.01	1.5
SE conc.	19	NA	56.31	0.34	0.6
Apple juice with SE conc.	15	0.29	0.28	<0.01	0.6
Margarine spread with SE conc.	15	13.80	14.39	0.14	1.0

n number, *SD* standard deviation, *RSD* relative standard deviation, *NA* not applicable, *Est.* estimate, *SE* steryl ester, *Conc.* Concentrate

[a] Campesterol, stigmasterol, β-sitosterol, Δ-7-Stigmastenol and Δ-7-avenasterol

[b] Four outlier values have been removed

sterol target amount. Sterol target amounts are based on known formulations. The target sterol content of sterol and SE concentrates will depend on the purity of the concentrate, which can vary slightly between lots. The average sterol content, standard deviation and sterol target amount are shown in Table 2 for sterol concentrates, SE concentrates, and foods and beverages formulated with sterol and SE concentrates. The results in Table 2 are given as total sterols (free sterols plus SE converted to free sterols).

Triplicate analysis over 5 days demonstrated repeatability, or the variability of measurements by one analyst, for the alkaline saponification and acid extraction with alkaline saponification methods. In general, a relative standard deviation (RSD) of <5% demonstrates good repeatability of a method. Replicate analysis of a common sample by two labs or replicate analysis of a common sample by multiple analysts demonstrated reproducibility, or the variability introduced by different analysts, of the methods.

Accuracy and repeatability of the alkaline saponification method is illustrated by complete recovery of the emulsified sterol concentrate determined in triplicate over 5 days. The average sterol content of the emulsified sterol concentrate (59.41% w/w) matches the target sterol content (60% w/w) with 0.3% RSD (Table 2). The accuracy and repeatability of the alkaline saponification method is further illustrated over a range of low concentrations (0.15–0.35% w/w sterols) for four orange juice samples formulated with the emulsified sterol concentrate (Table 2).

Reproducibility of the alkaline saponification method is demonstrated by the analysis of a common sample of fat-free milk formulated with emulsified sterol concentrate at two laboratories. Equivalent results (0.23% w/w and 0.23% w/w) were generated by the two laboratories with 0.4% RSD ($n = 10$) and 1.3% RSD ($n = 10$), respectively. Reproducibility of the alkaline saponification method is also demonstrated by soy germ lots a and b, for which the results generated by at least five analysts had 2.6% RSD and 1.2% RSD, respectively (Table 2).

Accuracy and repeatability of the acid extraction with alkaline saponification method is illustrated by complete recovery of the powdered SE concentrate. The average sterol content of the powdered SE concentrate (33.17% w/w) matches the target sterol content (33% w/w) with 1.1% RSD (Table 2). Accuracy and repeatability of the acid extraction with alkaline saponification method was also demonstrated for coffee creamer formulated with powdered SE concentrate (Table 2). Reproducibility of the acid extraction with alkaline saponification method is demonstrated by the analysis of a common sample of coffee creamer by two analysts. Comparable results (33.33% w/w and 33.39% w/w) were generated by the two analysts with 1.5% RSD ($n = 15$) and 1.1% RSD ($n = 5$), respectively.

Less than 5% RSD of the alkaline saponification method was demonstrated for powdered sterol concentrate, SE concentrate, emulsified sterol concentrate, milk with emulsified sterol concentrate, spoonable yogurt with powdered SE concentrate, orange juice with emulsified sterol concentrate, apple juice with SE concentrate and margarine spread with SE concentrate. Less than 5% RSD of the acid extraction with alkaline saponification method was demonstrated for powdered SE concentrate and coffee creamer with powdered SE concentrate. The methods used for bread with powdered sterol concentrate and puffed corn cereal with powdered sterol concentrate had greater than 5% RSD; however this variability is reasonable for these matrices as they are non-homogeneous.

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