Rapid measurement of phytosterols in fortified food using gas chromatography with flame ionization detection

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Key words:

Phytosterol; gas chromatography flame ionization detector (GC-FID); plant sterols; rapid analysis; fortified foods.

Chemical compounds studied in this article:

Cholesterol (PubChem CID: 5997); Stigmasterol (PubChem CID: 5280794); Stigmastanol (PubChem CID: 241572); Campesterol (PubChem CID: 173183); Brassicasterol (PubChem CID: 5281327); β–sitosterol (PubChem CID: 222284); 5α-cholestane (PubChem CID: 2723895); 5β -cholestan-3α-ol (PubChem CID: 16219103)

Abstract

A novel method for the measurement of total phytosterols in fortified food was developed and tested using gas chromatography with flame ionization detection. Unlike existing methods, this technique is capable of simultaneously extracting sterols during saponification thus significantly reducing extraction time and cost. The rapid method is suitable for sterol determination in a range of complex fortified foods including milk, cheese, fat spreads, oils and meat. The main enhancements of this new method include accuracy and precision, robustness, cost effectiveness and labour/time efficiencies. To achieve these advantages, quantification and the critical aspects of saponification were investigated and optimized. The final method demonstrated spiked recoveries in multiple matrices at 85-110% with a relative standard deviation of 1.9% and measurement uncertainty value of 10%.

1 Introduction

In the plant world phytosterols are the equivalent to cholesterol in animals and humans. Plant sterols belong to the triterpene family and can be found as free, steryl glycosides (SG), steryl esters (SE), hydroxycinnamic acid ester (HSE) and acylated steryl glycosides (ASG), with the latter four commonly known as phytosterol conjugates (Dutta 2004; Moreau et al. 2002). Phytosterols are believed to be an integral component of plant structural membranes (Dutta 2004; Moreau et al. 2002)with most phytosterols comprised of a 28-29 carbon ring based structure with a hydroxyl group at the Δ-3 position and a double bond at the Δ-5 position (Dutta 2004; Moreau et al. 2002). The structure of a generic sterol molecule is shown in Figure 1 and structures of some of the common phytosterol structures are presented in the Supplementary Material.

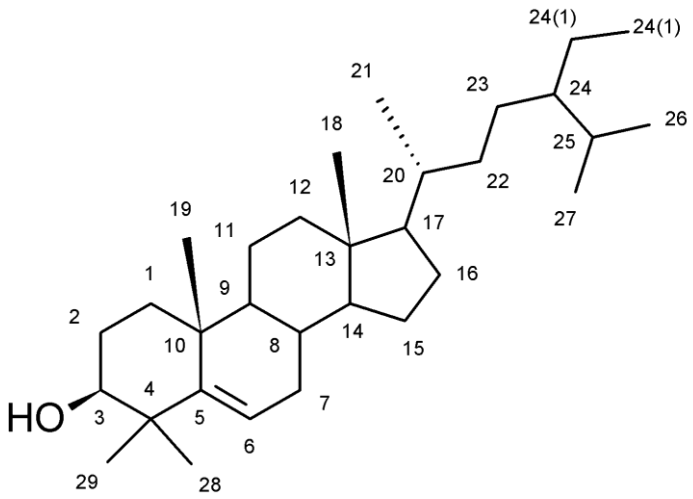

Figure 1. Generic sterol structure.

Food sources naturally rich in plant sterols include a wide range of cereals, fruits, vegetables and plant derived oils (Han et al. 2008; Moreau et al. 2002). In addition to natural foods, some processed foods are fortified with phytosterols, usually with the steryl esters as they are easily incorporated into the fat component of the product. Phytosterols found in these fortified food matrices include β-sitosterol, campesterol, stigmasterol, brassicasterol and stigmastanol (see structures in Supplementary Material). A range of processed food products are commonly fortified with sterols and these include dairy products, fat spreads, chocolates, snack bars and salad dressings.

In the last decade there has been a dramatic increase in public awareness and, consequently, in the consumption of phytosterols due to their demonstrated health benefits. Several reports have shown a direct correlation between phytosterol ingestion and the reduction of low density lipoprotein (LDL) cholesterol (Anon 2005; Katan et al. 2003; Ostlund 2002). The optimal and recommended steryl ester dosage to provide approximately a 10% reduction in LDL cholesterol is 2 g/day (Kritchevsky & Chen 2005). Higher dosages have been shown to offer minimal

additional reduction (Katan et al. 2003; Ostlund 2002). As of 2002, the United States Food and Drug Administration (USFDA) has permitted health claims to be published on any food products containing plant steryl or stanyl esters (Anon 2005; Moreau et al. 2002).

To support the food industry and ensure fortification claims on nutritional labelling are correct, robust analytical techniques for the routine determination of fortified phytosterols in food are required (Chen et al. 2015; Mo et al. 2013; Revathi P, Parimelazhagan T n.d.; Saha et al. 2014; Srigley & Haile 2015). Common analytical procedures for phytosterol determination usually consist of an alkaline saponification mixture and conditions utilising potassium hydroxide or sodium hydroxide at concentrations ranging from 1-6 M (Lagarda, García-Llatas, & Farré, 2006; Liu, Ding, Ruan, Xu, Yang, & Liu, 2007; Moreau, Whitaker, & Hicks, 2002). This is typically followed by an organic solvent extraction with many reported studies showing success using hexane, heptane, toluene, and petroleum ether (Lagarda, García-Llatas, & Farré, 2006; Liu, Ding, Ruan, Xu, Yang, & Liu, 2007; Moreau, Whitaker, & Hicks, 2002). The main benefits of using these organic solvents is their opposing polarity to the aqueous saponifcation mixture, facilitating the extraction of the sterols which are more soluble in organic solvents than water (Dutta 2004). The aqueous phase of the saponification mixture will solubilise the cleaved fatty acid ligands (in salt form) allowing free extraction of the sterols into the organic solvent. This process should eliminate non-targeted compounds that are insoluble in the organic solvent from entering the extract solution (Du & Ahn 2002; Toivo et al. 2000). The selection of the organic solvent will be influenced by several factors including its affinity to the target compounds, low hydrophilicity, availability and safety (Du & Ahn 2002; Toivo et al. 2000).

In theory, organic compounds of similar structure and molecular weight extractable by the saponification/solvent extraction technique may interfere with the sterol quantitation.

Compounds such as tocopherols, tocotrienols, retinol and β-carotene may be expected to interfere, however, in practice the levels of these fat-soluble vitamins are very low when compared to the sterol levels thus rendering any effect to the quantition less than the statistical uncertainty. In addition, the chromatographic method will typically provide sufficient separation from the target sterols so as to negate this anticipated interference (Du & Ahn 2002; Dutta 2004).

Organic solvent extraction is then followed by derivatisation and analysis by gas chromatography with a flame ionisation detector (GC-FID) (Anon 2005; Clement et al. 2010; Lagarda et al. 2006; Moreau et al. 2002). This has been found to be an effective technique for the analysis of most processed foods, in particular those fortified with high levels of steryl esters in the range of 300-8000 mg/100 g. However, the main drawback of these methods is that the extraction procedure is specific for steryl esters and free sterols only. Furthermore, this method is labour intensive and time consuming and therefore ill-suited as a routine procedure. A typical procedure for a batch of 10 samples including a quality assurance (QA) step can take up to 5 hours to complete. In addition, other reported phytosterol analysis techniques include the use of GC-mass spectroscopy (MS) and liquid chromatography coupled with MS, photo diode array or evaporative light scattering detectors (Soupas et al. 2004; Ahmida et al. 2006; Raith et al. 2005; Joseph 2012). A common challenge for sterol analyses is the co-elution of target compounds that have similar column affinity. The FID is non-discriminatory such that compound identification is limited to a referenced retention time. Confirmation can be achieved by either analysing the same extract by a different column stationary phase or by using other techniques such as GC-MS. This method, although less efficient for quantitation, employs the compound retention time in conjunction with the mass-spectrum to characterise the compound. (Skoog et al. 1998).

Previous work on the analysis of phytosterols using GC-FID procedures has predominately used 3 main surrogates for quantification, namely betulin, 5α-cholestane and 5β-cholestan-3α-ol. Despite their structural similarities, some reports have proposed the preferential use of 5β-cholestan-3α-ol. It is suggested that 5β-cholestan-3α-ol which contains a hydroxyl group, is structurally more similar to the target sterols and would therefore provide a better emulation of the process during extraction (Dutta 2004; Katan et al. 2003; Moreau et al. 2002). In this paper, a rapid, accurate and robust method for the measurement of phytosterol esters in a range of fortified food is presented. Emphasis was given to high throughput efficiency while minimising labour and reagent costs to ensure effective implementation in a commercial laboratory.

Terminology

Surrogate standard refers to the use of a similar compound to the target analyte that is added at the beginning of the extraction process. A known amount of surrogate is added to the sample at the beginning of the anlaysis to enable the evaluation of the analyte during extraction (Crosby et al. 1995).

An *internal standard* is a compound (not necessary similar to the target compound) that is added before the instrument analysis for the purpose or instrumentation monitoring (Crosby et al. 1995).

2 Experimental

2.1 Reference standards and reagents

Cholesterol (assay purity 99%), stigmasterol (assay purity 95%), stigmastanol (assay purity 95%), campesterol (assay purity 65%), brassicasterol (assay purity 95%), β-sitosterol (assay purity 97%), 5α-cholestane (assay purity 97%) and 5β-cholestan-3α-ol (assay purity 95%) were

all acquired from Sigma Aldrich (Sydney, Australia). Stock solutions of sterol standards were prepared in cyclohexane at a concentration of 500 mg/L. Further dilutions were made to a concentration of 50 mg/L using the extraction solvent heptane. All stock standards were stored at 4ºC in a spark proof refrigerator and were shown to be stable for 12 months. The quantitation surrogate standards 5α-cholestane and 5β-cholestan-3α-ol were spiked at the beginning of each extraction procedure.

The reagents used in this study were 5 M potassium hydroxide (Sigma Aldrich, Sydney, Australia) with an assay purity of 85% which was prepared in water: absolute ethanol (10:90) (Merck, Melbourne, Australia); N-O-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS) obtained from Grace Davison; n-heptane, pyridine and cyclohexane obtained from Merck (Melbourne, Australia); and 4 M aqueous hydrochloric solution and boiling chips were supplied by BDH (Sydney, Australia). Deionised water was used throughout the experiments and was obtained using a Millipore water purification system (Element A10).

2.2 Samples

Meat homogenate 1546 (certified for cholesterol) was obtained from the National Institute of Standards and Technology (NIST) and was used as a reference material to monitor method recoveries. Vega pure E, a fat paste certified for β-sitosterol, campesterol, stigmasterol, brassicasterol and stigmastanol, was used as the secondary reference material obtained from BASF™ (Melbourne). Matrix recoveries were carried out on fortified fat spread, cheese slice, fortified milk and full cream milk powder obtained from local markets.

2.3 Equipment

The equipment used to perform the experiments included: water bath (Ratekshaking, maximum temperature of 100ºC); 44 mL glass screw-capped vials with teflon septa; 2 mL GC vials and caps; Ratek dry block heater (with GC-vial holding plate); vortex mixer and shaking evaporation manifold with a 44 mL vial holding plate (Thermo Fisher). An Agilent 6890 GC-FID was used and was equipped with a BPX5 column (5% Phenyl Polysilphenylene-siloxane, 25 m x 0.22 mm id x 0.25 μm film thickness) purchased from SGE Analytical (Melbourne, Australia).

2.4 Sample preparation and extraction

A mass of 0.2 g of sample was accurately weighed into a 44 mL glass vial. The sample was saponified with 5 mL of 5 M of KOH with the addition of boiling chips, a surrogate standard and 5 mL of the extracting solvent heptane. This mixture was incubated at 80ºC for 30 minutes in a shaking water bath. Following incubation, the mixture was allowed to cool before 4 mL of deionized water and 7 mL of aqueous HCl was added. It was then vortex mixed for 30 seconds and allowed to settle for a further 1-2 minutes before removing the organic layer for derivatisation. Derivatisation was achieved by transferring a 0.5 mL aliquot to a GC vial, evaporating to dryness, and reconstituting in 300 μL of BSTFA+1%TMCS and 325 μL of pyridine. The vial was crimp-capped, vortex-mixed and then incubated at 80ºC for 20 minutes.

2.5 Gas chromatographic analysis

The derivatised phytosterol extracts were analysed by GC-FID using a 1:30 split ratio injection at 260 °C using hydrogen carrier gas. The initial column temperature was held at 50°C for 0.5 minutes and then increased at a rate of 20°C/min to 320°C and held for a further 10 minutes with a flow of 1.4 mL/min.

2.6 Data analysis

Errors presented in figures are ± 5 percent from the mean value.

3 Results and discussion

Total phytosterol determination in fortified foods was achieved via a process of saponification, solvent extraction, derivatisation/sylation and analysis using GC-FID. The main focus of this research was to develop a method and evaluate its accuracy and efficiency using the recovery of known amounts of sterols from reference material and to confirm nutritional labelling of various fortified foods. Four key parameters were investigated including the selection and quantification of standards, saponification optimization, output improvement and validation.

3.1 Surrogate standard selection

A critical aspect of phytosterol analysis is the process applied for the quantification of the sterols. This can be based on the use of a traditional calibration curve consisting of increasing concentrations of the standard within a linear response range. Alternatively, due to the high cost and limited commercial availability of some reference standards, quantification can be carried out using sterol(s) with simular chemical characteristics to the sterol(s) of interest but is not present in the sample (i.e. similar FID response and chromatographic elution time range). Sterols are routinely analysed using GC-FID due to the broad linear response, robustness, and relatively low cost and operating energy compared to traditional mass spectrometry detectors.

In this study quantitation of individual sterols were achieved by direct comparison from the known concentration from the surrogate standard to that of the sample. This is carried out using relative response factors between phytosterols and this approach has been previously used successfully by other researchers (Clement et al. 2010; Lagarda et al. 2006). The surrogate

standards 5α-cholestane and 5β-cholestan-3α-ol were selected based on published literature (Anon 2005; Clement et al. 2010; Lagarda et al. 2006; Ostlund 2007). These surrogate standards were compared by spiking milk powder to represent dairy matrices and Vega pure E, in order to determine which surrogate standard provided better quantification.

Table 1 shows the quantification of the 5α-cholestane and 5β-cholestan-3α-ol contents in these spiked test matrices. Phytosterol amounts were consistently higher when using 5α-cholestane and lower when using 5β-cholestan-3α-ol in the Vega pure E matrix. Although these differences were not considered to be significant as they were within 10% of the certified or expected values (see Table 1), the results did allude to a possible positive bias in sterol concentrations when 5α-cholestane was replaced by 5β-cholestan-3α-ol. The bias in 5α-cholestane was later confirmed during the validation process and was not attributed to the instrumentation.

Table 1. Quantification of surrogate standards in spiked matrices.

Surrogate	Cholesterol in milk powder (mg/100 mL)	Total sterols in Vega pure E (mg/100 g)
Expected value	13 ± 1.3	59600 ± 5960
5β-cholestan-3α-ol	12.9	53748
5α-cholestane	14.3	56496

The main difference between these surrogates is the absence of a hydroxyl group located on the 3rd carbon in the 5α-cholestane structure (see Figure S1 in Supplementary Material). The absence of the hydroxyl group on the 5α-cholestane renders it unable to entirely reflect the degradation or ligand cleavage of the target sterols during extraction. This was further verified through literature (Lagarda et al. 2006). This would be critical as quantification is based on spiking the surrogate into the sample at the beginning of the extraction. Overall, the relative standard deviation (RSD) for the quantification of 5β-cholestan-3α-ol was determined to be

0.6% in both milk and Vega pure E and that of 5α-cholestane was 1.2% in milk and 5.6% in Vega pure E respectively. Based on these results, the 5β-cholestan-3α-ol was selected as the preferential surrogate due to its consistency and satisfactory recovery that is in accordance with other similar studies (Moreau et al. 2002).

3.2 Establishing and optimising saponification parameters

In fortified foods, the majority of the fortification is achieved by incorporating phytosteryl or stanyl esters into the fat content of the product (Lichtenstein & Deckelbaum 2001). This is an effective method for fortification and provides minimal changes to the taste and appearance of the product. The analysis of these phytosterol esters is therefore imperative to assure an accurate fortification level. By comparison, any naturally occurring sterol conjugates are negligible (<1% of the fortification level) and less than the uncertainty of measurement of the fortification level. In this work, three key aspects of saponification parameters were investigated including alkalinity, incubation temperature and incubation time.

3.2.1 Effect of potassium hydroxide concentration

Various concentrations of potassium hydroxide (KOH) were evaluated to affirm the optimal molarity at which the complete sterols fatty ester hydrolysis is observed. Evaluation of recoveries on Vega pure E and milk powder matrices were compared to certified values or nutritional label. A range of KOH concentrations for saponification have been previously reported ranging from 2 to 6 M (Lagarda et al. 2006; Liu et al. 2007; Moreau et al. 2002). In this study, the results showed acceptable recovery levels from milk and Vega pure E using different KOH mixtures as shown in Figure 2(a). The results demonstrated the KOH concentration used during saponification was not a significant factor in liberating sterols and although 2 M KOH would be ideal for saponification, 5 M was chosen due to the need for

additional alkaline solution during hydrolysis which is commonly employed before saponification to liberate sterol glycosides (mainly from plants matrices) (Laakso 2005; Moreau et al. 2002; Piironen et al. 2002).

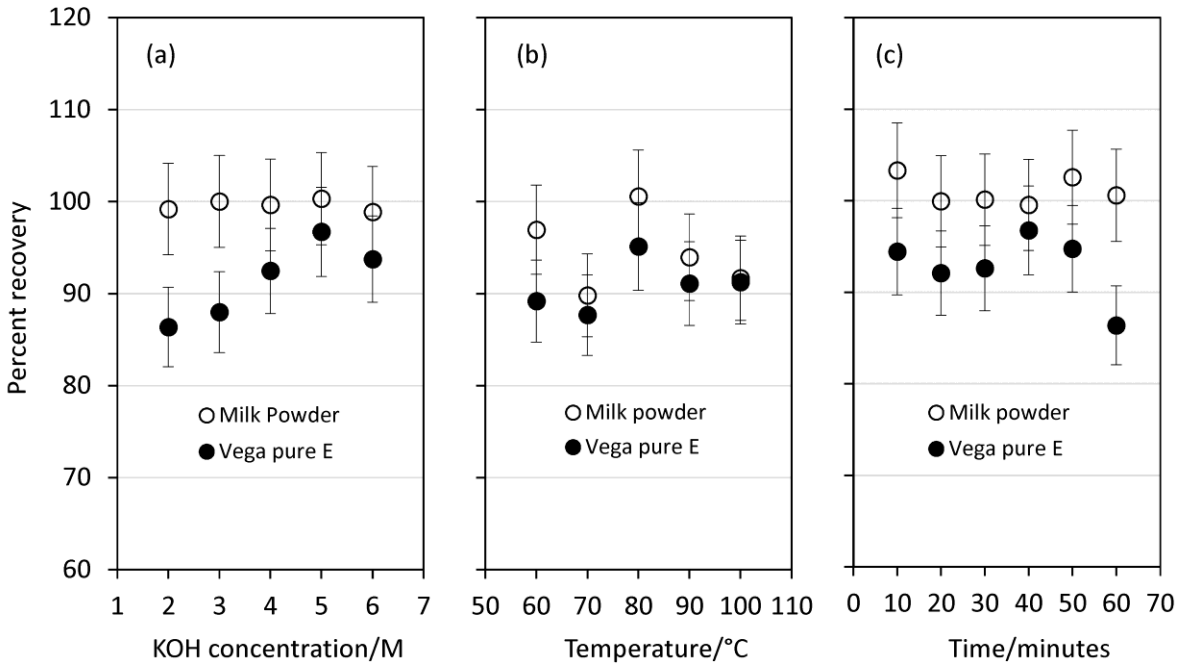

Figure 2. Effect of (a) KOH concentration, (b) incubation temperature, and (c) incubation time during saponification on sterol recovery.

3.2.2 Effect of saponification incubation temperature

Both hot and cold saponification are frequently employed for sterol measurement (Clement et al. 2010; Dutta 2004) with hot saponification employing high temperatures during hydrolysis with incubation times ranging from 10 to 90 minutes. Cold saponification is performed at room temperature for a duration of 8 to 12 hours but this was not investigated as it was not considered to be time efficient. In this work, incubation temperatures ranged from 60 to 100°C, at 10°C increments for a constant time of 60 minutes. Phytosterol recovery from milk powder and Vega pure E ranged from 89-95% at varying incubation temperatures as shown in Figure 2(b). Based

on this recovery data and with consideration of the safety aspects of applying high temperatures to an alcoholic solution, an optimal incubation temperature of 80°C was selected. Recovery determination was based on a comparison between the certified values, nutritional label or known spiked amount (see supplementary material equation 1).

3.2.3 Effect of saponification incubation time

Incubation times ranging from 10-60 minutes at 10 minute increments were tested to determine the minimum period required for the saponification process and the results are shown in Figure 2(c). Complete saponification was observed after an incubation of only 10 minutes for Vega pure E and milk powder, it was noted that the most effective incubation time will vary due to the matrix type. Although prolonged incubation was shown to provide no negative effect on sterol content, an incubation period of 30 minutes was selected to ensure optimum saponification for a variety of matrices. Based on these results, a 50% reduction in incubation time was achieved compared to an existing in-house saponification method performed at 80°C (Dutta 2004; Piironen et al. 2002).

3.2.4 Effect of derivatisation time

For GC analysis, sterol extracts are frequently derivatised using sylating agents such as N-ethyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with trimethylchlorosilane (TMCS) or BSTFA+1%TMCS to render the target analytes thermally stable. Both the BSTFA and MSTFA derivatising reagents form trimethylsilyl esters on the hydroxyl group on the sterols (Brufau et al. 2006; Wu et al. 2008). Derivatisation of the extracts can also reduce potential sterol interaction within the GC inlet or column that may interfere with the analysis.

In this work, BSTFA+1%TCMS was utilised in accordance with the manufacturer's instructions but in order to improve overall efficiencies, the recommended derivatisation incubation periods were investigated. In this work, the maximum sterol recovery was achieved within 10 minutes of incubation time with no changes observed for prolonged incubation (see Figure S2 in Supplementary Material). Based on this result, an incubation time of 20 minutes was selected to ensure thorough derivatisation which equates to a 40 minute reduction compared to the previous in-house method.

3.3 Improving efficiency and output

At this stage, the proposed new method is robust, accurate and suitable for the determination of sterols in fortified food. However, even with improved recoveries and a 70 minute reduction in the total incubation time (30 min from saponification and 40 min from derivatisation), the method is very labour intensive. The majority of the labour arises from the heptane extractions and subsequent evaporation to desired volumes (approximately 1 hour per a batch of 10 samples). To address this, critical parts of the method were studied to determine if processes could be modified to minimize time and labour without compromising sterol recovery. The parameters investigated included: extraction during saponification; extract emulsion reduction techniques; type of saponification solution; and optimum temperatures.

Common practices for sterol measurement include the use of saponification with either sodium hydroxide in methanol or potassium hydroxide in ethanol, the use of hexane, cyclohexane, toluene or heptane for sterol extraction and incubation temperatures ranging from 60-100°C (Dutta 2004; Han et al. 2008; Laakso 2005; Lagarda et al. 2006; Moreau et al. 2002; Piironen et al. 2002). Using a Plackett–Burman experimental design (Tyssedal, 2008), eight parameters were investigated to determine critical aspects of the method including the use of sodium

hydroxide in methanol mixtures, extracting solvent heptane or toluene, incubation temperatures and the use of water, hydrochloric acid or sodium chloride. The experimental design details are outlined in Table S1 of the Supplementary Material (Experiments A-F).

Sodium hydroxide in methanol, toluene and the incubation temperature of 100°C were chosen for comparison as they are commonly used in this type of extraction (Clement et al. 2010; Dulf et al. 2007; Dutta 2004; Lagarda et al. 2006). The addition of hydrochloric acid and saturated sodium chloride after saponification were also investigated in an attempt to reduce emulsification of the extracts.

The results shown in Table 2 demonstrate that the critical parameters in reducing labour and improve time efficiency are the addition of acid after saponification (Experiment D) and the extraction of sterols during incubation (Experiment A-I). Both heptane and toluene extractions were able to demonstrate recoveries from Vega pure E and milk powder ranging from 90-110%. The introduction of the extraction solvent into the saponification mixture eliminated the need to perform multiple manual liquid–liquid extractions after saponification.

It was also shown that the addition of acid after saponification reduced emulsion formation as the acid was able to neutralise the alkaline solution, producing a salt thereby causing the mixture to become ionised. This ionisation of the saponification mixture reduced the potential for emulsification by changing the surface tension between the organic and aqueous layers, creating a hard barrier that is ideal for solvent to solution partition. It has also been suggested that the addition of acid allow for the analysis of fatty acid trimethylsilyl esters by converting the fatty acid to their alcohol conjugates (Clement et al. 2010; Dutta 2004). These aspects were

then applied to the optimised method to determine if this would improve time and labour efficiencies (see Table S1 in Supplementary Material, Experiments G-H).

Table 2. Comparison of cholesterol recovery, total sterol recovery and β-sitosterol from different matrices.

Experiment[#]	Cholesterol recovery from milk powder (%)	Total sterol recovery from Vega pure E (%)	β-sitosterol recovery from Lucerne (mg/100g)
A	89.5	88.9	28.6
B	96.3	86.9	29.1
C	95.7	87.6	30.1
D	66.8	90.0	31.3
E	95.1	102.6	31.2
F	96.8	94.4	31.8
G	79.6	99.1	31.1
H	94.1	99.7	28.6
I	91.7	101.8	30.0

[#]Note: experiments are outlined in Supplementary Material.

The results shown in Table 2 confirm that the adaptation was suitable, however the addition of water was critical after saponification because the water allowed the salt produced from the addition of acid to dissolve into the aqueous phase and provide an ideal organic solvent barrier. However, a weaker acid (4 M) solution was selected for the ionisation of the saponification mixture as this improved laboratory safety. An overall comparison between the existing and the newly developed method was able to reduce the extraction and incubation time by a total of 130 minutes for a typical batch of 10 samples including a QA step. Moreover, the new method was also able to reduce the volume of the extracting solvent by 50%, eliminate the need for extra vials that are used to collect/combine the extracts and extract volume adjustments. Figure 3 shows a comparison of the flow charts of the existing and newly optimised method with considerably fewer steps needed in the new method. Depending on the operator, this has

the potential to impart a total time saving of 60 to 120 minutes which could considerably reduce the costs of analysis. In addition, shown in Figure 4 is a representative chromatogram of the various plant sterols and surrogates which demonstrates an effective separation of the various components.

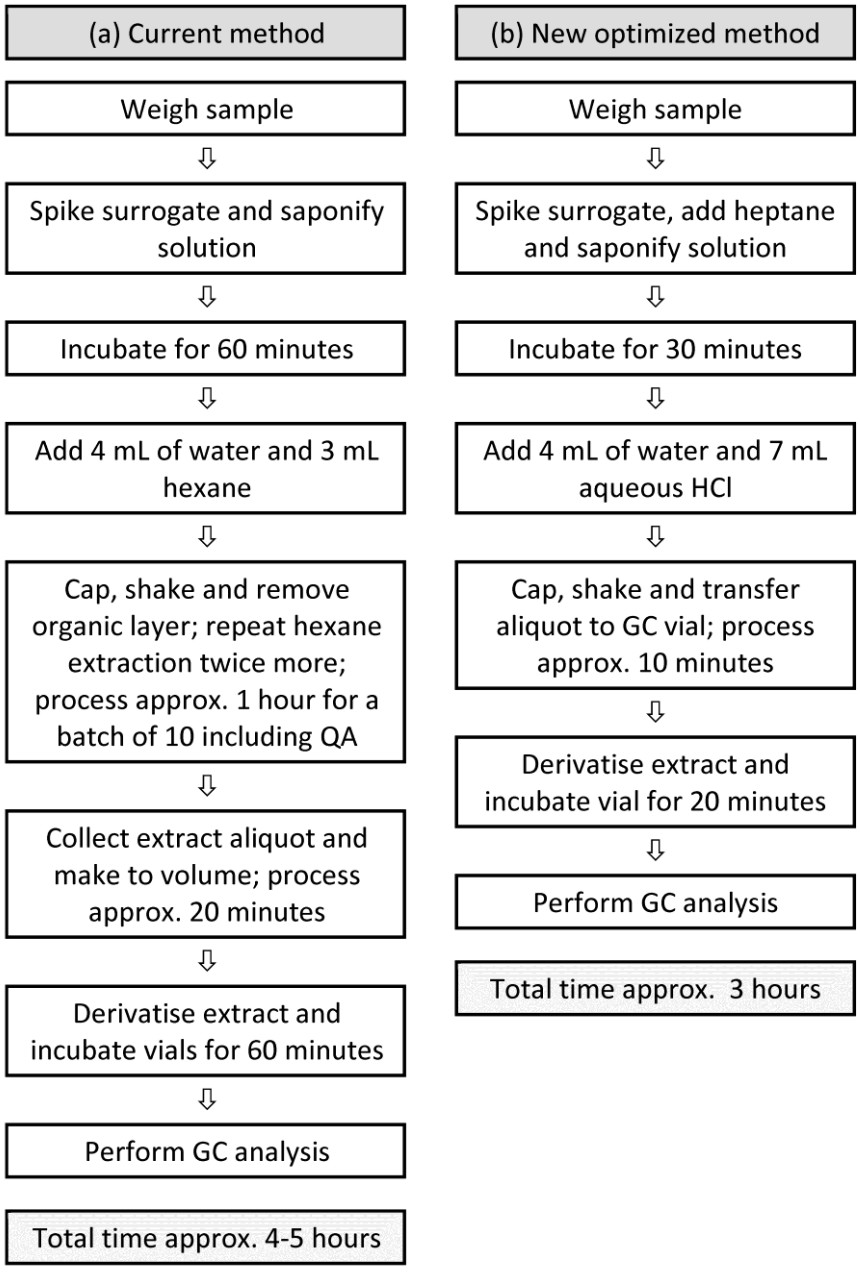

Figure 3. Flow diagrams of (a) existing protocol and (b) optimised method for the extraction of sterols.

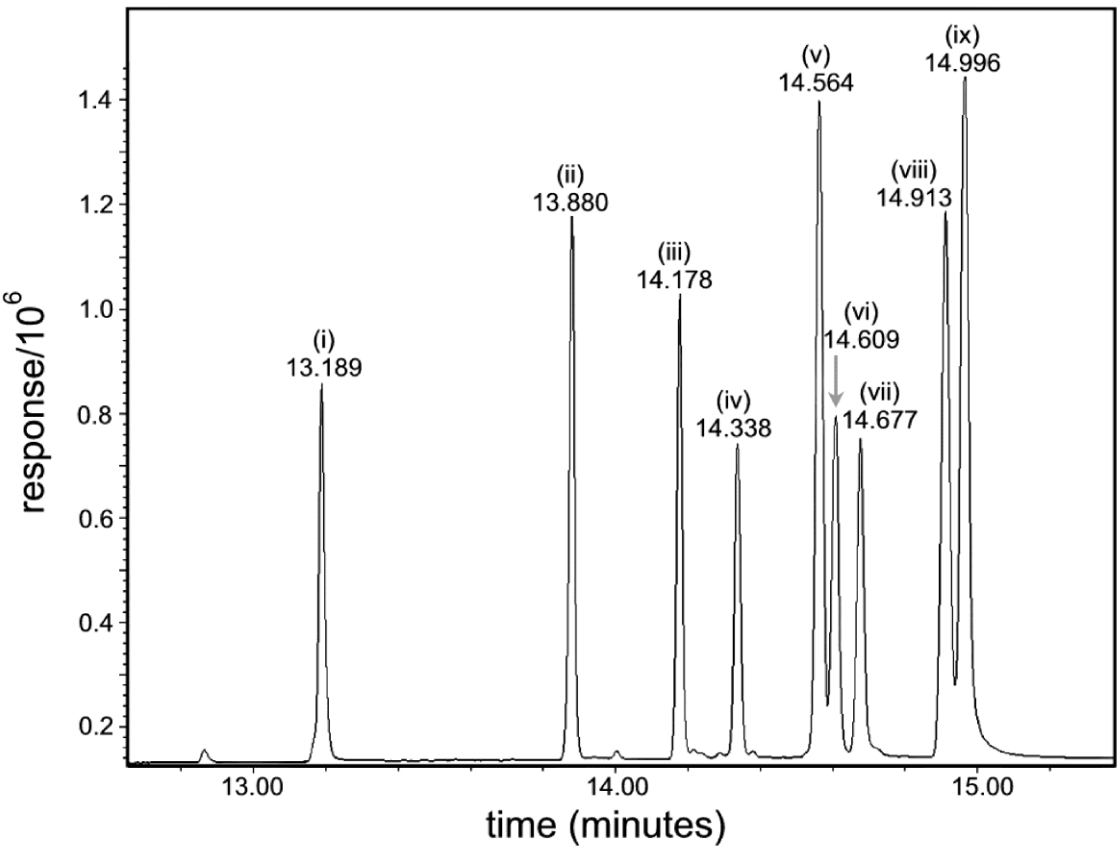

Figure 4. Chromatogram of phytosterols including the surrogate standards and their respective elution order: (i) 5α-cholestane, (ii) 5β-cholestan-3α-ol, (iii) Cholesterol, (iv) Brassicasterol, (v) Campesterol, (vi) Campestanol, (vii) Stigmasterol, (viii) β-sitosterol and (ix) Stigmastanol.

3.4 Method validation and measurement uncertainty

Method validation was performed using certified NIST 1546 meat homogenate (certified for cholesterol only), secondary reference material Vega pure E (certified for plant sterols) and commercial reference materials including: milk, fat spread, and cheese matrices. The surrogate standards 5α-cholestane and 5β-cholestan-3α-ol were both utilised for method validation with both surrogates spiked into the same extracts. The results are shown in Table 3 for Vega pure E and in Table 5 for NIST 1546 and various other fortified food matrices.

393 **Table 3**. Validation using surrogate standards for Vega pure E, NIST 1546 and fortified food
394 matrices.

	Total phytosterol recovery (%)	
	5β -cholestan-3α-ol	5α-cholestane
Vega pure E		
• Total phytosterols	95 ± 0.4	112 ± 1.0
• Brassicasterol	93 ± 0.4	109 ± 1.0
• Campesterol	94 ± 0.4	110 ± 1.0
• Campetanol	101 ± 0.4	119 ± 1.0
• Stigmasterol	118 ± 0.4	139 ± 1.0
• β-sitosterol	94 ± 0.4	110 ± 1.0
• Stigmastanol	109 ± 0.4	128 ± 1.0
Nist 1546 *	89 ± 3.1	108 ± 3.1
Fortified spread	109 ± 0.3	110 ± 0.4
Fortified milk	98 ± 0.8	108 ± 0.6
Soft cheese	91 ± 1.1	106 ± 0.9
Sliced cheese	86 ± 1.9	100 ± 1.9

395 *Note: cholesterol recovery

396

397 The results from the validation demonstrated a satisfactory recovery of sterols ranging from
398 85-110% with a RSD% of 0.3-1.9% when quantitating with the 5β-cholestan-3α-ol surrogate.
399 The method was also able to demonstrate that it is suitable for sterol analysis in a range of
400 commonly fortified foods such as milk, cheese and fat spreads. The major contributing factor
401 to the measurement uncertainty for the method was sterol recovery which was determined to
402 be ±10% with a 95% confidence interval (coverage factor of 2) for total phytosterols. The
403 validation data therefore demonstrated that the method is both precise and applicable for sterol
404 measurement. Quantification using 5α-cholestane, however, demonstrated a clear positive bias
405 with recoveries for all the tested matrices consistently 5-10% greater than the certified or
406 expected value (except for sliced cheese). For this study 5α-cholestane should only be used for
407 quantification if the calculated measurement uncertainty includes a calibrated bias factor or an
408 internal standard for response correction.

4 Conclusions

A new method suitable for plant sterol analysis in fortified food was developed that is capable of providing more rapid analysis with reduced labour and cost. This was achieved by shortening incubation times, eliminating manual extraction, and by reducing solvent use and other consumables. The method enabled the extraction of sterols during saponification and aided in reduction of emulsion formation by the addition of acid during incubation. The results demonstrated that the surrogate 5β-cholestan-3α-ol was well suited for sterol quantification whereas 5α-cholestane provided results with a positive bias. The overall measurement uncertainty for total phytosterols determination for the developed method was ±10%. This method is suitable for the routine analysis of sterols in matrices such as dairy, meat and fat spreads, including oils.

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