Influence of Acid Hydrolysis, Saponification and Sample Clean-up on the Measurement of Phytosterols in Dairy Cattle Feed Using GC–MS and GC with Flame Ionization Detection

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Running title: Phytosterol Content in Dairy Cattle Feed Using GC/MS/FID

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Non-standard Abbreviations:

flame ionization detection (FID); *N,O*-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS); linearity dynamic range (LDR); certified reference materials (CRM); National Institute of Standard and Technology (NIST); secondary reference material (SRM); measurement uncertainty (MU); limit of reporting (LOR).

Keywords: dairy cattle feed, flame ionization detection; phytosterols, sample pretreatment,

Abstract

The fortification of processed foods including dairy products is increasingly commonplace with phytosterols among the many compounds used to improve the nutritional value of food products. It is also increasingly common practice for some dairy cattle feeds to be fortified for their potential to increase phytosterol levels in milk. In this study, a combined, streamlined protocol using acid hydrolysis, saponification and sample clean-up was developed to enable the rapid and reliable. The method was developed with focus on streamlining the overall technique to make it suitable for commercial laboratories, to reduce labor and consumable costs, while maintaining accuracy. A total of twelve different feed types commonly used in the dairy industry were analyzed with the highest and lowest sterol contents found in cotton seed oil and tannin with average phytosterol contents of

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256 and <30 mg per 100 g, respectively. With a limit of reporting of 30 mg/kg for individual sterols and a correlation coefficient >0.99, the method was validated for milk to enable feeding comparison studies with respect to the total phytosterol content in raw milk.

1 Introduction

The Australian dairy industry has a net worth of over 1.5 billion dollars with up to 30% of all production exported internationally, predominately to Asia [1]. With a net share of 6% worldwide, Australian dairy companies are collectively the third largest global dairy industry. To meet production demands, farmers typically invest around 30% of the farming budget towards pasture production which can include planting, harvesting, feeding and storage. In 2014/15, there were approximately 9.7 billion liters of milk produced in Australia [1], but rising costs associated with dairy farming have led to a decline in the total number of productive farms. A growing demands for dairy supplies has required the remaining farms to increase their herd size and, over the past 30 years, numbers have increased from around 85 to the current average of 284 cows per farm [1, 2]. Herd sizes will most likely continue to expand to meet growing demands and this will subsequently increase feed requirements.

Phytosterols are plant based sterols that are the equivalent of cholesterol in animals and with more than 200 forms identified [3], these are vital structural components of plant membranes and other metabolic precursors [4, 5]. There are 5 main forms of phytosterols including free, hydroxycinnamic acid esters, sterol esters, sterol glycosides and acylated sterol glycols, with the latter known as conjugates [3]. The health benefits relating to the consumption of phytosterols are well understood with studies showing a reduction in dietary cholesterol and subsequent lower risk of cardiovascular disease [3, 6, 7]. With the status of “generally recognised as safe” granted by the United States Food and Drug Administration [6, 7], phytosterols are commonly fortified in various food products including milk, cheese, chocolate, pasta and fat spreads, among many others [3, 6, 8]. Worldwide, leading food authorities generally permit producers of products containing phytosterols to advertise health claims such as those related to a reduction in cholesterol or reduced risks of cardiovascular disease [7, 9, 10]. There have been few long-term studies into the effects of high phytosterol consumption, however some short-term studies have demonstrated that a diet high phytosterols can impair vitamin D adsorption and reduce carotenoid levels in plasma by 15–20% [11–13].

There is an ever-increasing interest in enhancing the health benefits of various food products with a major focus on milk and other dairy products [14–16]. Such enhancements are ideally achieved naturally through changes in the feeding of dairy cows to ensure food safety, to minimize human error and subsequent over fortification or consumption. At present, there are a few reported studies investigating the influence of cattle feed on the enhancement of macro- or micro-nutrients in milk. However, these are primarily focused on products that are fortified after milk production

with few related to phytosterol fortification [8, 17, 18]. As a result, there is a growing need for the rapid, streamlined, and accurate measurement of phytosterols and their conjugates in an array of matrixes [19, 20].

Many past studies have used only saponification to determine phytosterol fatty esters as these are the forms commonly found in fortified food products [21–24]. However, due to the significant amount of sterol conjugates, a technique liberating all phytosterol forms including the conjugates is necessary to determine total phytosterol levels [6, 8, 25, 26]. Therefore, acid hydrolysis has been used to liberate sterol glycosides which is then followed by saponification to ensure all sterol conjugates are liberated and are free for extraction [27]. There is an increasing concern with the use of acid hydrolysis, however, since the isomerization of avenasterol and fucosterol has been observed when using this technique. Although this has led to the development of alternative means such as enzymatic treatments to avoid this complication [28–30]. In most food matrixes, avenasterol and fucosterol are generally found in insignificant amounts compared to total phytosterols and therefore the use of acid hydrolysis is generally suitable [6, 26, 31].

Another disadvantage of using acid hydrolysis is the aggressive nature of the reaction that can lead to the increased extraction of non-targeted compounds. Adequate sample clean-up is therefore essential for sterol quantification to improve instrumentation analysis by removing potential interfering compounds. Techniques including TLC have been used in the past and although the method was effective, it was found to be time consuming and lacked the required rigor for routine testing [32, 33]. Other methods based on SPE have been reported as being more efficient and convenient techniques allowing for the purification of sterols before analysis [34, 35].

In our recent study, an optimized saponification method for the determination of total sterols was reported [36]. In the present paper, an extension of this method is reported for the determination of total phytosterols and their conjugates in dairy cattle feed with a view to assessing major cattle feeds commonly used in Australia. A novel extraction technique including acid hydrolysis, sample clean-up and analysis by GC–MS and GC with flame ionization detection (FID) was optimized and a range of feeds were analyzed for total phytosterol content.

2 Materials and methods

2.1 Standards and other chemicals

Sterol standards for quantification (with certified assay purities given in brackets) included: cholesterol (99%), brassicasterol (95%), campesterol (65%), stigmasterol (95%), lanosterol (93%), β -sitosterol (97%), cholesterol (95%), lathosterol (98%), fucosterol (93%), stigmastanol (95%) and demosterol (84%). A surrogate standard, 5 β -cholestan-3 α -ol (98%), was added at the beginning of each extraction to compensate for any degradation or loss from the extraction process, for quality assurance purposes, and as an internal standard during the data interpretation stage. All standards

were purchased from Sigma–Aldrich (Sydney, Australia) or Steraloids (Rhode Island, USA) and were prepared in cyclohexane with a stock concentration of 500 mg L⁻¹.

Solvents including cyclohexane, hexane, toluene, *n*-heptane, ethanol, chloroform, and methanol and hydrochloric acid were purchased from Merck Australia (Melbourne, Australia) with purities greater than 95%. Pyridine was obtained from Sigma–Aldrich (Sydney, Australia). *N,O*-Bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS) was purchased from Grace Davison (Melbourne, Australia). Deionized water was used throughout the experiments and was obtained using a Millipore water purification system (Element A10).

The reagent 5 M potassium hydroxide (assay purity 85% from Sigma–Aldrich) was prepared in a solution of 10:90% v/v water/ethanol. Different hydrochloric acid hydrolysis solutions were used: (i) prepared in 100% water for cattle feed samples (4 M HCl), (ii) 8 M HCl prepared in ethanol for unfortified milk, and (iii) prepared in 50% ethanol in water non-cattle feed samples (4 M HCl). The solvents used for SPE included *n*-heptane, chloroform and a methanol/chloroform mixture (20:80% v/v).

A total of three samples were used to represent the common matrixes studied and to efficiently develop and validate the methods for a large range of matrix types. These representative samples included milk powder with a known cholesterol content of 13 mg/100 mL and Vega pure E with a certified total phytosterols content of 6200 mg/100 g. The milk powder and Vega pure E were used to represent the fatty matrixes and lucerne hay was used for cattle feed samples.

2.2 Sterol quantification

Quantification of the phytosterols was achieved using FID with appropriate reference standards and 5 β -cholestan-3 α -ol as the internal standard to create traditional calibration curves. Confirmation of sterol identification was achieved using MS [37]. The linearity dynamic range (LDR) for the majority of individual phytosterols was found to be 0.1 to 200 mg/L, except for brassicasterol and campestanol where the LDR was 0.1 to 100 mg/L due to the limited of availability of reference standards. The correlation coefficient for all sterols was >0.99. The LDR for cholesterol was determined to be 0.1 to 1000 mg/L with a correlation coefficient >0.99 ($y=0.044x$).

2.3 Consumables and equipment

Incubation of the hydrolysis and saponification sample mixtures were performed in a shaking water bath (WB4D, Ratek, Melbourne, Australia) with a maximum temperature of 100°C. Sample hydrolysis was performed using 44 and 60 mL screwcap vials with Teflon septa. Evaporation of solvents of volumes greater than 1 mL was performed using an evaporating nitrogen manifold (Thermo Fisher, Australia) and derivatization/extract evaporation for volumes less than 1 mL were performed using a heating block with GC holding plate and evaporating manifold (Ratek, Melbourne, Australia).

The SPE cartridges used included a 5 g amino propyl phase cartridge (particle size 40 μm and volume of 20 mL purchased from Agilent Technologies, Melbourne, Australia) and a silica Sep-Pak 690 mg cartridge (particle size 55–105 μm and volume of 2 mL purchased from Waters, Melbourne Australia). The SPE was performed in a vacuum manifold (Sigma–Aldrich, Australia) and all small volumes were accurately measured using piston operated volume aspirator ranging from 10 to 1000 μL , or a positive displacement piston operated volume aspirator.

2.4 GC analysis

Derivatized sample extracts were analyzed using an Agilent 7890 Gas Chromatograph coupled with a 5975 C MS and FID detectors. Chromatographic separation was achieved using a HP-5MS (5-phenyl-methylpolysiloxane 30 m x 0.25 mm, film thickness 0.25 μm) capillary column with a 3 m deactivated silica column (guard column). The GC temperature program for the analysis initially started at 245°C and was ramped to 265°C at 2°C min⁻¹, followed by a ramp to 290°C at 3.5°C min⁻¹ where it was maintained for 8 min. The carrier gas used was helium with a flow 3.1 mL min⁻¹. A split injection mode of 1:5 was used with an injector temperature 310°C. The instrumentation was also program to perform a back-flush after each analysis for 7.5 min at 24.6 psi. The FID temperature was set at 300°C (total detector flow 30 mL min⁻¹) and the MS source at 250°C (scan mode ion 50–500 atomic mass units) with a 1:1 split for each detector.

2.5 Feed sampling and extraction

2.5.1 Feed samples

All feed samples used for this study were provided by the Department of Economic Development, Jobs, Transport and Resources and included lucerne, pasture (rye grass), maize silage, pasture silage, grape marc (dried and ensiled), wheat, canola, tannin, mineral mix, cotton oil and molasses. Replicate samples obtained between 2010 and 2011 from different seasons were also analyzed. All feed samples were stored in the freezer until analysis and were then prepared using a grinding mill (Foss CT 293 CyclotecTM) and a high-powered homogenizer (Robot Coupe Blixer® 3). Total solids were also determined for all cattle feed samples by subtracting the moisture content which was determined gravimetrically by oven drying at 104°C until constant weight was obtained.

2.5.2 Extraction of cattle feed samples

For each sample, 0.5–1.5 g was weighed into a 60 mL screw cap vial containing 2–3 boiling chips. A 5 mL aliquot of heptane, known amounts of 5 β -cholestan-3 α -ol and 10 mL of 4 M aqueous HCl was added to the sample vial which was then capped, mixed and incubated at 80°C for 30 min. The vial was shaken intermittently every 10–15 min during incubation. Following incubation, the vial mixture was cooled to room temperature, then 20 mL of 5 M ethanolic KOH was slowly added to the sample vial to neutralize the acid hydrolysis solution. The vial was then recapped, mixed and incubated at 80°C for 30 min with intermittent shaking every 10–15 min. Following this second incubation, the sample vial was cooled to room temperature before 8 mL of aqueous 4 M

hydrochloric acid and 8 mL of water was added. The vial was recapped, shaken and allowed to settle to form two distinct layers after which the organic layer was transferred to a test tube and the volume reduced to 4 mL using nitrogen gas. Since sterols are non-volatile compounds, they are stable during this evaporation process and are not removed by the nitrogen purge. This step was then followed by sample clean-up using SPE.

2.5.3 Sample clean-up

Before sample clean-up, the amino propyl SPE cartridge was conditioned with 15 mL of heptane. Then 1 mL of sample extract was loaded onto the cartridge and allowed to pass through. Another 15 mL of heptane was passed through the cartridge with this fraction discarded. The sterols were then eluted using 25 mL of 80:20% v/v methanol/chloroform mixture, blown down and then transferred to a GC vial for derivatization.

2.5.4 Extraction of unfortified milk

For each milk sample, 5 mL was weighed into a 60 mL screw cap vial containing 2–3 boiling chips. A 5-mL aliquot of heptane, known amounts of 5 β -cholestan-3 α -ol and 5 mL of 8 M ethanolic HCl was added to the sample vial which was then capped, mixed and incubated at 80°C for 30 min. The vial was shaken intermittently every 10–15 min during incubation. Following incubation, the vial mixture was cooled to room temperature, then 20 mL of 5 M ethanolic KOH was slowly added to the sample vial. The vial was then recapped, mixed and incubated at 80°C for 30 min with intermittent shaking every 10–15 min. Following this second incubation, the sample vial was cooled to room temperature before 4 mL of aqueous was added. The vial was recapped, shaken and allowed to settle to form two distinct layers after which the organic layer was transferred to a test tube and the volume reduced to 1 mL under nitrogen gas to produce the sample extract which was then subjected to derivatization.

2.5.5 Sample derivatization

Sample extracts were evaporated to dryness under nitrogen in the GC vial, after which 300 μ L of BSTFA+1%TCMS and 700 μ L of a 3:4 volume ratio of toluene/pyridine mixture was added. The vial was then capped, shaken and incubated at 80°C for 20 min. For samples with insignificant or no phytosterol glycoside content, i.e. high sterol ester content samples, the extraction method presented in “extraction of unfortified milk” was used with some modifications. In the first incubation step, 5 mL of 5 M ethanolic KOH was added to sample vial rather than aqueous HCl. After incubation, the vial was cooled to room temperature and 4 mL of water was added. The vial was then recapped, shaken and allowed to settle until two distinct layers were observed. The organic layer was collected and reduced to 1 mL using nitrogen. The extract was then transferred to a GC vial and the derivatization process as “sample derivatization” was followed.

2.6 Method validation

Method validation was performed using certified reference materials (CRMs) obtained from National Institute of Standard and Technology (NIST, USA) to obtain samples where the target sterols

were present homogeneously throughout the matrixes. The CRMs used included NIST 3250 *Serenoa repens* seed fruit (certified for β -sitosterol, stigmasterol and campesterol), and meat homogenate NIST 1546 (certified for cholesterol). The secondary reference material (SRM) studied was Vega pure E, a phytosterol fatty ester paste (BASFTM, certified for β -sitosterol, campesterol, stigmasterol, brassicasterol and stigmastanol). In addition, recoveries were performed on milk samples that were purchased from local markets to investigate whether the method was suitable for low level phytosterol measurements.

2.7 Statistical analysis

The measurement uncertainty (MU) determined for this research was based on ISO/IEC Guide 98–3, 2008 using the top-down approach to incorporate validation data including recovery, duplicates, reference standard calibration uncertainty and QC for the final uncertainty estimation. This approach was chosen as it would allow for the precision, accuracy and any systematic bias in the methods [38]. For this study, the recovery and duplicate data included 7 milk matrix spikes, 3 water matrix spikes, 9 NIST 1546 meat homogenate CRM samples, 7 NIST 3250 *Serenoa repens* CRM samples, and 26 Vega pure E SRM samples. Given the high cost of the CRM and SRM materials, it was not possible to perform spike-recovery experiments with hay and silage samples. The uncertainty was determined by the square root of the sum of the RSDs of: the standard preparation, the calibration standard, the recovery, and the duplicates. A coverage factor of 2 was applied to the uncertainty to expand the confidence interval to 95%. In addition, an analysis of variance was performed to determine significances between parameters during the method development with a post-hoc analysis using the Tukey approach.

3 Results and discussion

In our previous study, a method for the determination of total sterols was reported [36] which was optimized based on existing saponification methods [8, 39, 40]. In the present study, further optimization of the method was undertaken with the focus on the acid hydrolysis of samples before GC analysis. The acid hydrolysis method development comprised of an investigation of critical aspects including: hydrolysis solution composition; acid concentration and incubation period; and sample extract clean-up.

3.1 Effect of ethanol content in acid hydrolysis solution

A total of 13 different feed sample matrixes were investigated which included 12 types of animal feed and 1 milk sample. To maximize the extraction of phytosterols from these matrixes, the acid hydrolysis solution composition is a critical factor as it contributes to sample solubility and cell lysis of plant materials. Previous studies have utilized two distinct acid hydrolysis solutions prepared in either water (for food samples) [8, 26] or in ethanol (for plant materials) [41]. Given the broad

range of matrixes in the present study, a hydrolysis solution composition that was suitable for all the matrixes was required. Four solution systems were investigated including acid prepared in: water only; 20% v/v ethanol in water; 50 v/v ethanol in water; and ethanol only. For optimization, milk powder, lucerne and Vega pure E (a reference fatty paste) were selected to represent the dominant sample types, i.e. milk, plant and fatty samples respectively.

Figure 1 shows the quantity of total sterols extracted from the three sample types using the different hydrolysis solution compositions. The data demonstrates that for fatty samples (i.e. Vega pure E), an increase in ethanol content improved sterol recovery whereas higher water contents decreased the solubility for the subsequent saponification reaction. In the case of lucerne, higher ethanol contents in the hydrolysis solution decreased the total sterol recovery with up to 34% lower sterol recovery in ethanol only compared to a water only acid solution. The recovery of total sterols from milk powder was satisfactory at levels up to and including 50% ethanol. It was therefore concluded that for milk and high fat samples, an acid hydrolysis solution prepared using 50% ethanol in water was adequate. For plant-based samples, an aqueous acid hydrolysis solution was the most suitable.

3.2 Effect of acid concentration and incubation time

Acid concentration is critical to efficiently break the glycosidic bonds by hydrolysis to maximize the liberation of sterol glycosides. In this study, HCl concentrations of 4, 5, and 6 M were trialed with results showing that no significant recoveries were gained of the three selected matrixes using higher acid concentration as shown in Table 1. The hydrolysis incubation time was also studied to optimize the time needed to ensure all glycosidic bonds are cleaved. Although the saponification incubation time has been investigated previously [36], the present study further explored the hydrolysis time before the addition to saponification mixture to ensure that the hydrolysis solution did not affect the saponification process. Three incubation time brackets were selected, i.e. 30, 60 and 90 min, and were applied to the hydrolysis of the three representative samples at 4 M. As shown in Table 1, the results demonstrate that complete hydrolysis was obtained with the minimum incubation period of 30 min with no significant increase when incubated for longer times.

It was also observed that doubling the volume of the saponification solution after hydrolysis neutralized the acid with any excess continuing the saponification reaction. Further verification with different feed matrixes was required to ensure the saponification incubation time of 30 min was still applicable from previously optimized conditions [36]. Figure 2 demonstrates that 30 min of saponification incubation time was still applicable and no significant gain was obtained for longer incubation periods. Although grape marc gained *ca.* 7% of total sterol recovery, this was not deemed to be significant to increase the incubation time.

3.3 Effect of acid addition following saponification

The composition of plant matrixes is generally more complex than milk or fat samples and can therefore require additional or modified extraction techniques. In the sterol extraction method, acid and water are added to the sample mixture after saponification with the addition of water facilitating the solubility of salts, glycerine, and fatty acid salts, while leaving the un-saponifiable fraction to be extracted into the organic phase. The addition of acid neutralized the alkaline saponification solution thereby increasing the ionic strength of the aqueous phase to minimize the emulsion between the organic and aqueous layers. This process was very effective for plant samples, however for the milk and fat samples, an emulsion between layers was not observed. In this case, the addition of the acid would potentially result in the conversion of fats to their alcohol conjugate and subsequent solubilization of these compounds into the organic layer thus creating non-targeted interferences.

Experiments were performed to determine whether the addition of acid could be omitted for milk and high fat samples only. For the milk and oil sample extracted with and without the addition of acid, the results showed no significant gain was obtained for the addition of acid. The addition of acid after saponification was therefore omitted for milk, high fat, oil or fat only samples. Conversely, the addition of the acid in plant materials was continued to optimize the extraction. This was demonstrated by performing a recovery with no acid and no SPE clean-up which resulted in a cholesterol level of 11.5 mg/100 mL of milk. No significant gain was achieved with the use of acid and SPE clean-up with only 11.6 mg/100 mL of cholesterol measured suggesting the additional steps could be omitted without detriment to the recovery.

3.4 Optimization of sample clean-up and elution

The use of SPE was further investigated as a clean-up step in this study as sterols can be selectively purified providing the correct phase is used [42, 43]. Several stationary phases are used for this purpose, including cartridges comprised of silica and an aminopropyl phase [6, 26, 44, 45]. For this study, both silica and aminopropyl solid phase cartridges were selected for clean-up investigation. The initial work was performed using reference standards, with both phases able to perform satisfactory recoveries for the Vega pure E ranging from 90–110% for both silica and aminopropyl phase. However, when sample extracts were trialled, no profile change was observed for the silica SPE and it was subsequently confirmed using GC–MS/FID that the silica cartridge was unable to remove non-targeted compounds from the extracts (data not shown). Conversely, the aminopropyl cartridge was able to significantly remove non-targeted compounds while recovering sterols within a satisfactory range (80–120%) for the level of the component measured in accordance with relevant validation guidelines [46].

Shown in Figure 3 (a) and (b) are examples of the lucerne extract chromatograms using the silica and aminopropyl phase in the SPE clean-up, respectively. It is clear that the aminopropyl phase is able to selectively remove non-targeted compounds within the sterol chromatographic range

between 13 and 17 min, whereas the silica is unable to remove non-sterol compounds from the extracts. The aminopropyl SPE clearly demonstrates greater efficiency at removing non-target compounds eluting at 14 min which was identified as the surrogate standard 5 β cholestan-3 α -ol based on the retention time from the reference standard and mass spectral database library. It was observed that for fractions collected by SPE, not all phytosterols were recovered suggesting that the SPE sorbent capacity was too low to retain the target analyte in the extract. A dilution of the lucerne extracts was therefore trialled on the SPE to determine the phase capacity required for methods extracts. Using 320 mg sorbent, a 1 in 5 dilution resulted in recoveries of 102% and 116% of the surrogate (5 β -cholestan-3 α -ol) and β -sitosterol respectively. For a 1 in 2 dilution, recoveries of 23% and 38% were obtained for the surrogate and β -sitosterol respectively suggesting that the 1 in 5 dilution resulted in more satisfactory recoveries.

For the majority of feed matrixes, 1.5 g of sorbent in the SPE stage is typically used effectively. However, in this study 5 g of sorbent was used to safeguard against possible SPE overload capacity issues for unknown cattle feed matrixes. This increase in sorbent material subsequently required the use of additional solvent to discard non-targeted compounds and elute target sterols. As a result of this increase in sorbent, an investigation into appropriate solvent polarity strength was studied to obtain adequate separation between the target and non-target analytes. In this case, the elution of sterols from the grape marc extract was investigated using 45 mL of chloroform and 25 mL of an 80:20% v/v chloroform/methanol mixture. For the chloroform only extraction, 88% and 93% of β -sitosterol and stigmastanol were recovered respectively. For the lower volume mixed solvent, recoveries of 107 and 104% of β -sitosterol and stigmastanol were obtained respectively. The results demonstrate that a reduced volume of the mixed solvent results in a higher recovery than the larger volume of chloroform only which may be due to the greater polarity of the chloroform methanol mixture. It is important to note that during the process of the method development, hazardous and potentially carcinogenic reagents and solvent including toluene, pyridine and chloroform were used sparingly to reduce exposure and to limit the generation toxic waste.

3.5 Method validation

Validation of the total phytosterol and cholesterol extraction methods were performed on two CRMs, one SRM, and additional spiked samples using stigmasterol at the limit of reporting (LOR), 2 $\times$ LOR, and 5 $\times$ LOR where the LOR is 0.02 mg/100 mL for milk and 1 mg/100 mL for other liquid samples. In the case of the spiked samples, the spiking was performed before the hydrolysis process. As shown in Table 2, the RSD for all samples was less than 12% and satisfactory recoveries were obtained for the CRM and SRM samples ranging from 80 to 120% as per the Australian Pesticides and Veterinary Medicines Authority guidelines [46]. Total phytosterol content was calculated based on the summation of the identified sterols (i.e. the sum of campesterol, brassicasterol, campestanol, stigmasterol, stigmastanol and β -sitosterol levels). The average recoveries for the spiked milk samples were *ca.* 64, 69 and 80% for the 1 $\times$, 2 $\times$ and 5 $\times$ LOR samples respectively. This is considered to be satisfactory and the results are as expected for such low-level

determinations. The average process recoveries for spiked samples in water were *ca.* 99, 103, and 96% for the respective LOR spikes and this indicates the lower recovery from the spiked milk samples was due to matrix interference as shown in Table 3. Overall the MU determined for this method for milk at the 1×LOR value of 0.02 mg/100 mL for the individual sterols was ±35% and for the animal feed at the 1×LOR value of 5.0 mg/100 g, the MU determined was ±15% for results falling within the mid-calibration range. In both cases, a 95% confidence interval was used with a coverage factor of 2. The validation data demonstrated that the method is suitable for the analysis of animal feed and milk for both trace and high-level sterol analysis.

3.6 Analysis of cattle feed

Using the validated method, the total sterol levels in a broad range of animal feeds were analyzed. Three separate samples of each feed type were analyzed with the exception of tannin, molasses and cotton seed oil where a single sample from each was analyzed. The average results of the total sterol contents from each sample are presented in Figure 4. The main sterols found in the feed samples were β -sitosterol, stigmasterol, stigmastanol and campesterol. Overall, the highest total sterol content was found in cotton seed oil and the lowest in tannin, although these were among the test feeds comprised of a single sample only. It was also observed that in both the maize and pasture samples that the sterol content was higher in the silage form. Comparison between literature value of total phytosterols was only possible for the wheat and cotton seed oil as, the rest of the animal feed matrixes was not available for direct comparison. The total phytosterols in the wheat sample was comparable to values cited by Ruibal-Mendieta, et al. [45], however for cotton seed oil it was 40–50% lower than the values reported by Gül and Amar [47]. The differences between the latter literature reference and that of the present study for cotton seed oil may be due to differences in cotton varieties, seasonal variations, or the age of the samples. The general SD within each feed matrix over different subsamples was $\leq 25\%$ and given that the subsamples were collected over a course of two years, the results demonstrate that the total sterol content in each matrix was comparable. This suggests that an average result of each matrix can be used to simplify and predict trends associated with feed and sterol content in milk for future studies.

4 Concluding remarks

A method using both acid hydrolysis and saponification was developed in this study to measure total phytosterol levels and was found to be suitable for the analysis of milk and animal feed at trace and naturally occurring levels. An aminopropyl SPE cartridge was found to effectively remove non-targeted analytes from the extract minimizing interference. The method was used to analyse a variety of animal feed types and the highest and lowest total sterol contents were found in cotton seed oil (256 mg/100 g) and tannin (<30 mg/100 g) respectively. Silage samples of pasture and maize had higher sterol content compared to their non-silage counterparts. The average SDs for total sterol levels between subsamples with the same feed matrixes were $\leq 25\%$ suggesting a relativity

insignificant variation between subsamples. It is therefore recommended that average values can be used to compare trends in sterol contents in milk in future animal feed studies.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure Captions

Figure 1. Total sterols extracted from various sample types with different ethanol contents in the acid hydrolysis solution (error bars represent SD between results from the same batch). Notes: (*a) indicates significances within the group for the acid medium with p values <0.5 ; (*) significant differences were found when compared only to the control (water) within the group.

Figure 2. Effect of saponification incubation time on sterol recovery of various feeds. Note: (*) indicates significant differences between data points with p value <0.05 .

Figure 3. Chromatogram of lucerne extract using (a) silica SPE cartridge and (b) aminopropyl SPE cartridge.

Figure 4. Average total sterols within the same matrixes (feed samples marked * were single samples only; error bars represent SD between replicate subsamples). Note the SD within each group was < 25% compared to the overall mean value.

Table 1. Effect of acid concentration and incubation time on sterol recovery

Parameter	Milk powder	Lucerne	Vega pure E
	Cholesterol	β -sitosterol	Total phytosterols
HCl concentration (M)	(mg/100 mL)	(mg/100 g)	(mg/100 g)
4	14	43	57829
5	13	46	57379
6	13	47	54670
Incubation time (min)*			
30	14	38	56721
60	14	31	57511
90	13	32	57275

*Note: 4 M HCl was used to investigate the effect of incubation time.

Table 2. Validation of the extraction of sterols from reference materials

Reference Material	Mean Sterol Recovery $\pm$ RSD (%)		
Sterol	NIST 3250 ^a	NIST 1546 ^b	Vega pure E ^c
Cholesterol		104.3 $\pm$ 5.7	
Brassicasterol			100.6 $\pm$ 7.3
Campesterol	103.0 $\pm$ 5.1		100.0 $\pm$ 7.2
Campestanol			102.8 $\pm$ 10.5
Stigmasterol	98.4 $\pm$ 7.7		102.6 $\pm$ 9.3
β -sitosterol	95.9 $\pm$ 9.2		99.7 $\pm$ 7.2
Stigmastanol			97.6 $\pm$ 11.6
Total phytosterols			98.7 $\pm$ 7.3

Notes: total samples: ^a7 for NIST 3250 (*Serenoa repens*), ^b9 for NIST 1544 (meat homogenate), ^c26 for Vega pure E

Table 3. Recovery of spiked stigmasterol from milk and water samples

Spiked Samples ^a	Mean Sterol Recovery $\pm$ RSD (%)
Milk^b	
1×LOR	64.4 $\pm$ 5.1
2×LOR	69.9 $\pm$ 4.3
5×LOR	80.1 $\pm$ 11.7
Water^c	
1×LOR	98.8 $\pm$ 2.8
2×LOR	102.6 $\pm$ 8.9
5×LOR	95.6 $\pm$ 0.6

Notes: ^atotal samples: 7 for spiked milk, 3 for spiked water; ^b1×LOR = 0.02 mg/100 mL for milk, ^c1×LOR = 1 mg/100 mL for water

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