Quantitative Analysis of Phytosterols in Cattle Feed, Milk and Fortified Foods

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This thesis is dedicated to my God, family & friends

“*For God has not given us a spirit of fear and timidity, but of power, love, and self-discipline*.”- 2 Timothy 1:7

“*Anyone who has never made a mistake has never tried anything new*.” - Albert Einstein

ABSTRACT

Over recent decades, research has demonstrated a direct correlation between phytosterol consumption and the lowering of low-density lipoprotein cholesterol. The fortification of phytosterols in processed food products has therefore become increasingly popular and as a result, there is a subsequent need for new and improved techniques for quantification of phytosterols in these products. Natural phytosterol fortification of milk by controlled feeding is also becoming a common farming practice although the efficacy of this approach is relatively unknown. Moreover, there are no known reports regarding the resulting phytosterol content in milk under different animal feeding regimes. This study therefore investigated whether different cattle feeds can influence the profile of phytosterols and cholesterol in the milk produced as an alternative to direct fortification.

A series of five feeding experiments were performed using common feeds used by Australian dairy farmers and selected formulated rumen protected feeds. In order to achieve this main objective, a new reliable and rapid analytical technique was required which could accurately measure total phytosterols (including the conjugates) at naturally occurring levels in cattle feed and the resulting bovine milk. The analytical method development investigated three hydrolysis techniques to liberate the sterols for extraction. This included acid hydrolysis and enzymatic treatments (for glycosidic bonds) and saponification (for fatty acid ester bonds). The method development also included optimisation of a sample clean-up and instrumentation. The final method parameters were selected based on accuracy, time efficiency, labour intensity and the availability of resources.

The optimised analytical method used acid hydrolysis and saponification protocols with simultaneous sterol solvent extraction during the hydrolysis step to avoid the less efficient manual liquid extraction step usually performed at this stage. This was then followed by sample clean-up using an amino propyl phase solid phase extraction for cattle feed samples. All extracts were concentrated to a known volume and derivatised using a silylating reagent to make them thermally stable for analysis. Quantification of sterols was performed using gas chromatography coupled with mass spectrometry and flame ionisation detectors which, allowed for direct quantification and identification of the samples. The total phytosterol determination was based on the sum of the identified plant sterols including brassicasterol, stigmasterol, campesterol, campestanol, *β*-sitosterol and stigmastanol.

A total of twelve different cattle feed types (excluding the rumen protected feed) commonly used in the dairy industry were analysed including lucerne, pasture (rye grass), maize silage, pasture silage, grape marc (dried and wet), wheat, canola, tannin, barley grain, mineral mix, cotton oil and molasses. These feeds were used in various combinations for the controlled feeding trials with the developed analytical method determining that the highest and lowest average phytosterol contents were found in cotton seed oil (256 mg/100 g) and tannin (<35 mg/100 g) respectively. Based on the analytical method developed, the limit of reporting was 35 mg/100 g and 5 mg/100 g for total phytosterols and individual sterols respectively. In addition to these common cattle feeds, a final feeding trial was also conducted with a rumen protected feed with a known high phytosterol content.

The results of the feeding trials showed that statistical significances ($p < 0.05$) were observed for some individual phytosterols and cholesterol in milk under these differing feeding regimes compared to the respective controls. The limit of reporting for the milk was 0.12 mg/100 mL and 0.02 mg/100 mL for total phytosterols and individual sterols respectively. In the case of the phytosterols, where the daily recommended consumption to optimise the health benefits is typically 2 g per day, the levels found in milk were <0.12 mg/100 mL of total phytosterols which is comparatively insignificant. The main phytosterols found in milk included lathosterol, *β*-sitosterol and campesterol, with the average cholesterol content ranging from 12-16.5 mg/100 mL. The cholesterol contents found in the milk samples were within expected values compared to nutritional panels and previous studies. The limited experiment using the rumen protected feed with high phytosterol levels suggested a decreased transfer of cholesterol to the milk by as much as 20% although further work is required to confirm these preliminary results.

Overall, the research suggests that different feeding practices have minimal impact on the quality of milk with regard to the resulting sterol profile. This research has important implications for the dairy industry with the development of reliable, robust and streamlines methods for measuring sterol contents in milk. In addition, it demonstrates that the use of expensive cattle feeds to naturally fortify milk with phytosterols is unfounded and that common, inexpensive feeds result in similar quality milk. Given that cattle feed is the foremost expense for dairy farmers, these findings support the continued use of these more affordable cattle feeds.

PUBLICATIONS ARISING FROM THIS THESIS

Journal Articles

(1) Duong S., Strobel N., Buddhadasa S., Stockham K., Auldist M., Wales W., Orbell J., Cran M., Rapid measurement of phytosterols in fortified food using gas chromatography with flame ionization detection. Food Chemistry, 2016, 211, pp.570–576.

Available from: https://doi.org/10.1016/j.foodchem.2016.05.104

(2) Duong, S. Strobel. N, Buddhadasa. S, Auldist. M, Wales. W, Orbell. J, Cran, M, Quantitative instrumental analysis of phytosterols in fortified foods. *Reference Module in Food Science*, 2017, 7 pp.

Available from: https://doi.org/10.1016/B978-0-08-100596-5.21400-5

(3) Duong, S. Strobel. N, Buddhadasa. S, Stockham. K Auldist. M, Wales. W Orbell. J, Cran, M., Influence of Acid Hydrolysis, Saponification and Sample Clean-up on the Measurement of Phytosterols in Dairy Cattle Feed Using GC/MS and GC/Flame Ionization Detection. *Journal of Separation Science*, 2018, 41(17) 3467-3476.

Available from: https://doi.org/10.1002/jssc.201800484

(4) Duong S., Strobel N., Buddhadasa S., Auldist M., Wales W., Moate P., Cox G., Orbell J., Cran M., Modification of the sterol profile in milk through feeding. *Journal of Dairy Science*, 2019, in press.

Available from: https://doi.org/10.3168/jds.2018-15067

Conference Presentations

Duong S. Influence of animal feed composition on phytosterol content in bovine milk, February 2011. 12th Government Food Analysts Conference, Brisbane Australia, Health and Food Science Precinct.

Duong S., Stockham K., Buddhadasa S., Paimin R., Auldist M., Wales B., 2012. Application and modification of current methods for the measurement of phytosterol content in raw bovine milk. 12th Government Food Analysts Conference. ISBN 978-0-9775968-6-7.

Duong S. Phytosterols - Method development for low level determination, and studies on the relationship between animal feed and milk composition. November 2013. First Collaborative Partnership Symposium, Victoria University, Werribee, Australia.

Duong S. Phytosterols study on the relationship between animal feed and milk compositions. November 2015. Second Collaborative Partnership Symposium, Victoria University, Werribee, Australia.

Duong S. Phytosterols study on the relationship between animal feed and milk compositions. November 2017. Third Collaborative Partnership Symposium, Victoria University, Werribee, Australia.

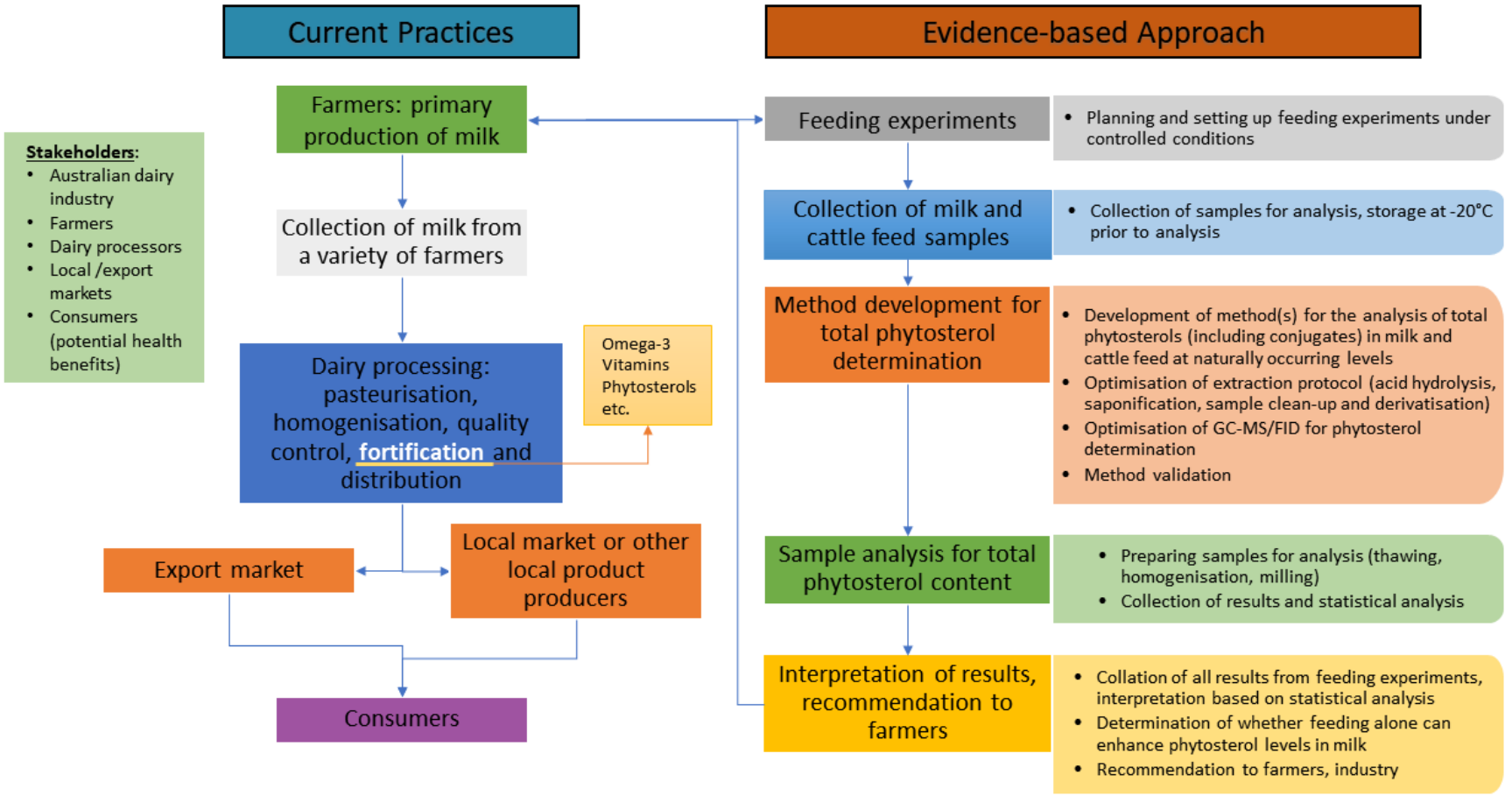
Duong S. A streamlined method for the determination of phytosterols in fortified foods using Gas Chromatography and a Flame Ionisation Detector (GC-FID). August 2017. Food Nutrition and Analytical Chemistry (FNAC) Group Student Symposium, RMIT University, Melbourne, Australia.

Published Interview

Duong S., 2016. Measuring Phytosterols in Fortified Food. LC GC Chromatography online: www.chromatographyonline.com/samantha-duong

RESEARCH SCHEME

Scheme 1: Conceptual framework for phytosterol fortification of milk



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DECLARATION BY AUTHOR

I, Samantha Huynh Duong, declare that the PhD thesis entitled “Quantitative Analysis of Phytosterols in Cattle Feed, Milk and Fortified Foods” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 30/08/2018

NOMENCLATURE

Units	
˚C	Degrees Celsius
µL	Microlitre
µg	Microgram
g	Gram
L	Litre
mg/100g	Milligram per 100 grams
mg/L	Milligram per litre
mg/kg	Milligram per kilogram

Abbreviations	
ADF	Acid detergent fibre
ASG	Acylated steryl glycols
BSTFA + TCMs	N-O-bis-(trimethyl)-trifluoroactatamide + trimethyl silyl
CSO	Cotton seed oil
CSP	Canola/soy protected
DGM	Dry grape marc
DM	Dry Matter
EGM	Ensiled grape marc
FID	Flame ionisation detector
FS	Free sterols
GC	Gas chromatography
GM	Grape marc
HMG-CoA	3-hydroxy-3-methyl-glutaryl CoA
HSE	Hydroxycinnamic acid esters
i.d.	Internal diameter
IPP	Isopentyl diphophate
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LOR	Limit of reporting

MS	Mass spectrometry
MSD	Mass spectrometry detector
MSTFA	n-(ter-butyldimethylsilyl)-N-methyltrifluoroactamide
MU	Measurement uncertainty
MVA	Mevalonic acid
NDF	Neutral detergent fibre
NIST	National Institute of Standards and Technology
NMI	National Measurement Institute
OP	Omega protected
PDA	Photo-diode array
PSE	Pasture supplementation experiment
RM	Rumen protected
SE	Steryl esters
SG	Steryl glycosides
SMT	Sterol methyl transferase
SP	Sterol protected
SPE	Solid phase extraction
TANN	Tannin
TCSO	Tannin & cotton seed oil

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Chapter 1. Introduction

1.1. General Introduction

Plant sterols or phytosterols are the equivalent in the plant world to cholesterol in animals and humans and there are over 200 different types of phytosterols found in plant and marine sources. Similar to cholesterol, they participate in critical metabolic processes with involvement in the cell membrane, signalling and regulatory roles in plants (Moreau *et al.* 2002; Dutta 2004). The predominant types of phytosterols found in plants include *β*-sitosterol, stigmasterol and campesterol but there are several other common types (Moreau *et al.* 2002). Over the past 30 years the importance of dietary phytosterols and subsequent research focusing on them has increased significantly due to the purported health benefits associated with their consumption.

1.2. Phytosterol Structures

Phytosterols are steroid alcohols belonging to the triterpene family of compounds and Figure 1.1 shows a generic structure of a base sterol (Moreau *et al.* 2002; Dutta 2004). Structurally, phytosterols are comprised of four aromatic rings with a hydroxyl group located on the third carbon and an alkyl side chain (ethyl or methyl) on the 24^{th} carbon. Saturated phytosterols, also known as phytostanols, differ from sterols by the lack the double bond on the fifth and sixth carbon in the B-ring or alkyl side chain. Common sterols derived from this structure include campesterol by removing carbon 24^2, and cholesterol by removing carbons 24^1 and 24^2.

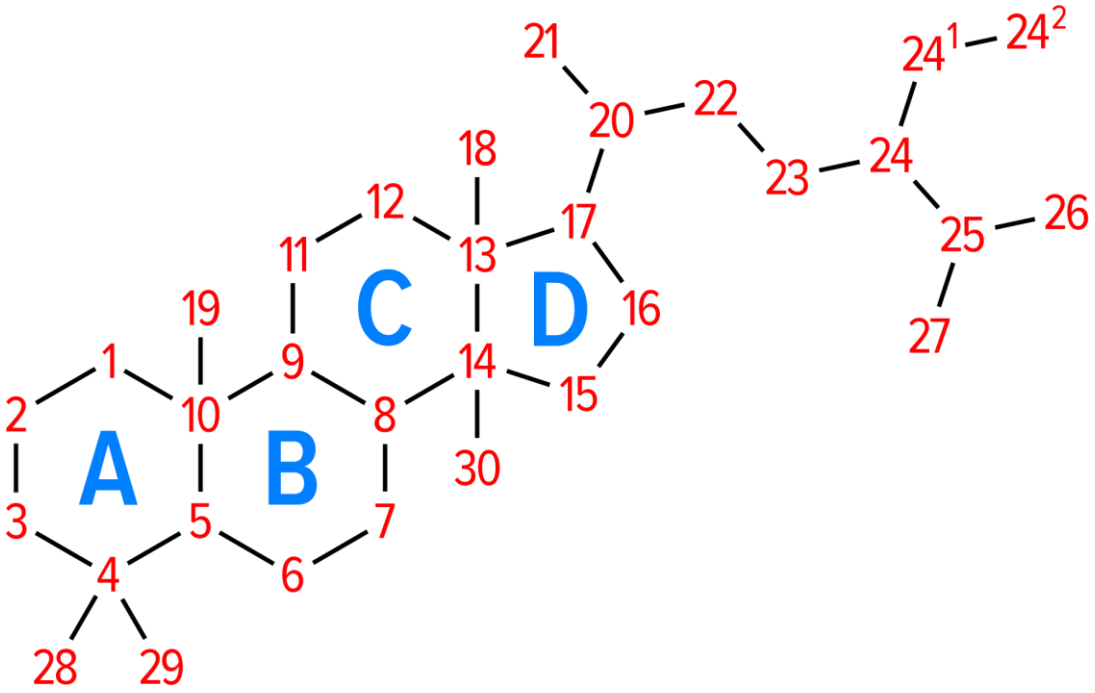

Figure 1.1: Generic structure of sterols (IUPAC-IUB 1989) by Vaccinationist - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=51655849

Phytosterols can be categorised into three groups based on the number of methyl groups on the fourth carbon: 4-dimethyl (two), 4-monomethyl (one) and 4-desmethyl (none) with the first two being intermediate sterols of the 4-desmethyl type. The most common phytosterols are based on 4–desmethyl sterols with either 27 or 29 carbon ring base structures. Phytosterols with a double bond between the fifth and sixth carbon are known as Δ^5 phytosterols, however there are sterols with a double bond between the seventh and eighth carbon and these are known as Δ^7 phytosterols. In addition to the primary structures, phytosterols occur in five main forms depending on the type of moiety bound to them. This includes free sterols (FS), hydroxycinnamic acid esters (HSE), steryl esters (SE),

steryl glycosides (SG) and acylated steryl glycols (ASG). Generically, the last four forms of sterols are known as phytosterol conjugates (Moreau and Hicks 2004). In general, the nomenclature of phytosterols has been confusing and difficult as the standardisation from IUPAC has only been partly implemented with many common names adopted and still used by the scientific community (Moreau *et al.* 2002; Özkan and Hill 2015).

1.3. Phytosterol Synthesis

Phytosterols are synthesised through the production of isopreniod and the post-squalene enzymatic pathway. This process starts with the conversion of sugar to acetate which is then reduced to form mevalonic acid (MVA) by 3-hydroxy-3 methyl-glutaryl-CoA (HMG-CoA) reductase. Phosphorylation of MVA then occurs resulting in the loss of the carboxyl carbon to form Δ^3-isopentyl diphosphate (IPP) (Moreau *et al.* 2002; Behmer and David Nes 2003). Six IPP units then assemble to form squalene (30 carbon units) and a cyclic oxidation process results in the production of either cycloartenol or lanosterol. Sequential interactions between these intermediary structures and sterol methyl transferease (SMT) leads to the formation of different plant sterols. Figure 1.2 summarises the synthesis of sterols through these enzymatic pathways (Behmer and David Nes 2003).

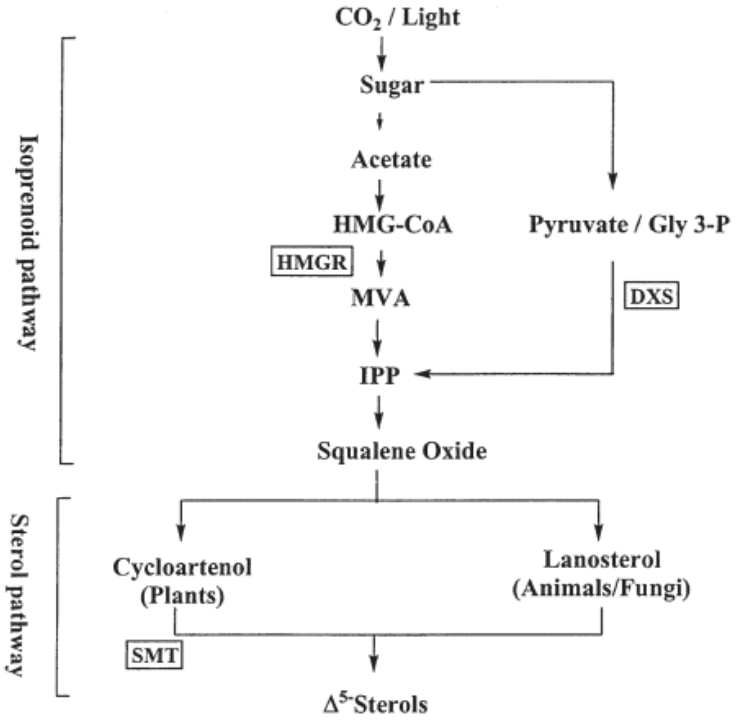

Figure 1.2: Plant sterol synthesis pathway (Behmer and David Nes 2003)

1.4. Phytosterol Fortification

The fortification of phytosterols in food products has become a common practice in western societies and this is a result of the numerous reported benefits and minimal side-effects associated with high sterol consumption. Common foods fortified with phytosterols include dairy products, fat spreads, salad dressings, pasta and chocolate (Moreau *et al.* 2002; Clement *et al.* 2010). The process of phytosterol fortification has been optimised and simplified following the early use of phytosterols or stanol fatty esters first patented by Benecol (Tatu *et al.* 1999). Sterol esters, for example, are pale yellow fatty pastes that can be easily incorporated into processed foods as a fat replacement (Moreau *et al.* 2002; Kritchevsky and Chen 2005). Success have been shown with fortification of

omega fatty acid using cattle feed (Gulati *et al.* 2003; Kitessa *et al.* 2003). Through this research it aims to modify the sterol profile of the milk produced through feed in order to reduce cholesterol and/or increase the level of phytosterols in the milk.

1.5. Phytosterol Quantification

An increase in phytosterol research has resulted from the growth in consumption either from foods that naturally contain or are fortified with phytosterols. As a result, there has been an increased demand for new and modified techniques to quantify phytosterols in order to meet both industry and research needs. Phytosterols are soluble in organic solvents such as chloroform and heptane and can therefore be analysed using either gas chromatography (GC) or liquid chromatography (LC), depending on the application (Clifton *et al.* 2004; Moreau and Hicks 2004; Liu *et al.* 2007; Scholz *et al.* 2015).

When selecting the appropriate analytical method, a number of major factors must be taken into consideration including the application, resources, time constraints, sensitivity and selectivity. In many cases, method modification or variation is required to suit the needs of the application and this can depend on factors such as the availability of instrumentation or reference standards. The food industry is the major facilitator of phytosterol analyses in fortified foods with most analyses performed by commercial laboratories. The extraction methods and analytical techniques must therefore be robust, reliable, cost effective and rapid to meet the demands of industry. Methods must also be appropriate for a

variety of matrices with varying compositions including animal feed, milk and dairy products, through to fortified foods.

1.6. Research Gaps, Significance and Aims

1.6.1. Research Gaps

Past research has been able to demonstrate that some milk quality parameters and milk yields can be manipulated through feed (Auldist *et al.* 2013). Ideally, milk enhancement would be achieved through feeding alone as levels of the target compounds would be at natural occurring levels thereby reducing the risk of over consumption that may subsequently hinder other metabolic processes or human error. A more common practice is the fortification of milk and dairy products with fatty acids, vitamins, minerals and phytosterols to add and further improve the nutritional benefits (Clement *et al.* 2010; Casala *et al.* 2014; Nagarajappa and Battula 2017).

The fortification of processed food products with phytosterols in particular is becoming increasingly popular (Moreau *et al.* 2002; Kritchevsky and Chen 2005; Clement *et al.* 2010). As such, there is a subsequent need for new and improved techniques for quantification of phytosterols in these products. Natural phytosterol fortification of milk by controlled feeding is also becoming a common industrial practice although the efficacy of this approach is relatively unknown. Moreover, there are no known reported studies about the resulting phytosterol content in milk under different animal feeding regimes.

The focus of this thesis is therefore to determine whether there is a relationship between phytosterol content in bovine feed and the milk produced by cows consuming the feed. Through feeding experiments, this research aims to determine whether phytosterol content in milk can be enhanced, which could reduce the need for post-processing fortification of some food products. In addition, there is a pressing need for new and optimised techniques for phytosterol quantification from natural products such as cattle feed, through to milk and foods that are fortified with phytosterols.

1.6.2. Research Significance

The Australian dairy industry is worth over two billion dollars annually and dairy products are an essential commodity for Australian and international consumers (Dairy Australia 2017). In south-eastern Australia, the industry is primarily based around the use of grazed pasture as cheap feed source. The pasture feeding system is an economical and effective way to meet cattle nutritional and production needs. However, over recent years, sustaining dairy outputs to meet the demands of local and international consumers has been difficult for farmers due less predictable rainfall and often unstable weather. This has resulted in a reduction in pasture production and many farmers have been forced to supplement their animal feed to maintain production. On average, 20-30% of the budget of a typical dairy farm is allocated to feed cost, which represents a large financial burden, particularly when the quality of feed can range in cost depending availability and grades (Özkan and Hill 2015; Dairy Australia 2017). Milk prices are governed on milk solids and fat content and, for this reason, there is keen

investment and research into the improving milk quality and production efficacy. In addition, given that feed is one of the more variable costs faced by farmers, the ability to cut these costs while maintaining or improving the quality and yield of milk is highly desirable (Moran 2005; Dairy Australia 2017). Research is therefore critical to ensure the supplemented feed used by the farmer is beneficial or value for money and this is particularly important if the supplemented feed is intended to increase or enhance levels of phytosterols or to decrease cholesterol in milk. This project aims to investigate the effect of cow diet on phytosterol concentration in milk and will additionally add to the scientific knowledge related to quantification of phytosterols down to trace amounts.

1.6.3. Research Aims

The primary aims of this research are to investigate the influence of cow feed composition on the qualitative and quantitative aspects of phytosterol content in raw bovine milk, and to develop new methods for the quantification of phytosterols in a range of matrices.

The specific aims are to:

- Develop and optimise analytical techniques for the extraction and analysis of phytosterols using liquid and gas chromatography
- Validate the developed methods to ensure they are suitable for a range of matrices including milk and cattle feed (pasture, wheat grain, maize grain, pasture silage, canola, cotton seed oil, tannin, grape marc and rumen protected feed)

- Evaluate the total phytosterol content in selected animal feed, milk and fortified foods with validation studies
- Investigate the relationship between various feeding systems and phytosterol profiles and levels in milk

1.7. Thesis Outline

This thesis is divided into the following chapters:

- Chapter 1 presents the general introduction with an overview of phytosterols and their quantitative analysis. The research aims of this work are also presented.
- Chapter 2 encompasses a general overview of the Australian dairy industry and a literature review of different feeding experiments. This chapter also includes a review of the literature pertaining to phytosterols, their health benefits and analytical methods for their quantification.
- Chapter 3 consists of a detailed method and material used for this research including reagents, consumables, sample details, instrumentation set up, extraction protocols, outline of the feeding experiments and the statistical approach used for data interpretation.
- Chapter 4 is a detailed investigation into the method and development process undertaken for this research. This includes the development of phytosterol analysis including extraction (saponification, acid hydrolysis, and sample clean-up), instrumentation optimisation and method validation.

- Chapter 5 presents the results and discussion based on the five-experiment conducted for this thesis research. This includes an examination between feeding groups through comparison of the phytosterol profiles of the milk produced. In addition, this chapter will also examine the financial cost associated with the feeds.
- Chapter 6 presents an overall conclusion of the research and recommendations for future work.
- The references and appendices are given in Chapter 7 and 8 respectively.

Chapter 2. Literature Review

2.1. Dairy in Australia

In Australia, dairy products have been a staple household dietary item since the introduction of the refrigerator into the family home in the mid-1800's. According to the Australian Bureau of Statistics (Australian Bureau of Statistics 2004), annually Australians consume on average 105 L of milk, 13.6 kg of cheese, 7.2 kg of yoghurt and 4 kg of butter per person. Due to this large and increasing demand, dairy and associated industries are among Australia's largest agricultural enterprises (Dairy Australia 2017).

The Australian dairy industry is the third largest exporter worldwide, holding approximately 2% of the world's dairy production and 6% in shares in trade behind New Zealand (38%) and the European Union (32%) (Dairy Australia 2017). With a net worth of more than $4 billion, approximately 40% of milk produced in Australia annually is exported valued at $2.9 billion. At present, the major export markets is Asia, which makes up to 78% of all exports (Dairy Australia 2017).

It is not surprising that the dairy industry is a highly competitive market, with research being an essential part of the industry to ensure the sector continues to grow and remain competitive. Some of the research into the industry includes improving milk production, reducing methane emissions in cattle and improving milk quality (Adin *et al.* 2009; Little *et al.* 2009; Hetti Arachchige *et al.* 2013; Aprianita *et al.* 2017). The fortification of milk and dairy products with a range of supplements to enhance their nutritional profile is also a growing industry

practice. From the wide range of fortifying additives, phytosterols are among the rare compounds that food authorities worldwide allow producers to make health claims associated with reducing cardiovascular disease (Moreau *et al.* 2002; Dutta 2004; Kritchevsky and Chen 2005).

2.1.1. Dairy Farming

The Australian dairy industry has been deregulated since 2000 in order to enable the price to be governed by the free market. As a result, farmers received $1.73 billion in compensation at that time for the restructuring and the number of farms dropped by 40% from 12,500 to 7,500 (Dairy Australia 2015; Dairy Australia 2017). With the decrease in the number of working farms and continuing increase in demands, many farmers increased their herd size to meet production needs. The current average herd size is now 220 compared to 85 three decades ago. At present, there are approximately 1.6 million dairy cows in Australia and herd sizes will most likely continue to increase to meet growing demands on supplies (Dairy Australia 2015; Dairy Australia 2017).

2.1.2. Herd Maintenance

In the simplest terms, “milk production is essentially the conversion of pasture to milk” in herbivores (Moran 2005; Little 2010; Hardie *et al.* 2014). One of the major considerations for farmers is therefore the production or supply of pasture to cattle. For small holder dairy farms, 50-60% of a typical farmer’s expenses is dedicated to the supply of feed to their herds (Moran 2005). Many farmers also plant a perennial ryegrass on their land to harvest as hay and silage and this allows them to conserve feed in low pasture or forage grass periods (Moran 2005; Little 2010; Hardie *et al.* 2014).

Under normal conditions, cows are milked twice a day (early morning and mid-afternoon). Milk production will commence in cows after the birth of a calf and the typical lactation length in south-eastern Australia is 305 days (Moran 2005; Özkan and Hill 2015). The breeding of cows is an immensely important process in dairy farming as this allows for both the production of milk and replacement of the animal (Moran 2005). The process of breeding cows is therefore a very specific and selective process with farmers tending to use semen from bulls with favourable genetic traits such as health, size, milk production, and longevity (Moran 2005; Little 2010).

2.1.3. Milk Production

To optimise milk production, farmers must understand and implement the most efficient methods of converting animal feed to milk. The fundamental step in the process therefore involves the cow's digestive system where the feed is broken down and used by the animal for energy and milk production. A cow's digestive system starts with the mouth, where food is mechanically broken down and combined with saliva, which facilitates chewing and the swallowing of food. Saliva also helps to buffer the pH of ruminal fluids as it contains sodium and potassium (Jacobs and Hargreaves 2002; Moran 2005).

Once the food is swallowed, it travels to the rumen where enzymes produced by the microbial flora naturally occurring in the digestive system start to break it down. Carbohydrates and structural fibres are chemically broken down in a process known as the first fermentation (Jacobs and Hargreaves 2002; Moran 2005). Continuous contraction by the rumen and reticulum mixes and churns the

food and this is then followed by rumination in which the food in the rumen is regurgitated and further mechanically broken down by chewing into small particles. Once the processed food is about 1-2 mm in size, it will travel to the omasum were the fatty acids and minerals are digested. The remaining fat and proteins that are not absorbed in the rumen are absorbed in the small intestine. A second fermentation process occurs in is the large intestine to break down any carbohydrates and starches that have not been metabolised in order to produce volatile fatty acids. Finally, microbes and undigested food are passed as faeces (Elliston and Glyde 2008).

2.1.4. Cattle Feed

Since milk production is essentially the conversion of feed to milk, animal feeds are critical to the quality and quantity of milk produced. The feed supplied to the dairy cow must therefore provide enough energy and nutrition for normal metabolic functions in addition to milk production (Jacobs and Hargreaves 2002; Moran 2005). As discussed by Moran (2005), feed quality is measured through proximate analysis that is broken down into the key aspects outlined in Table 2.1. It is clear from this table that cattle feed is a complex mixture of numerous components that are critical for optimal milk production. Feed compositions that can deliver these essential nutrients are therefore vital to support the dairy industry.

Table 2.1: Major components of dairy cattle feed

Feed Component	Source or Composition
Dry Matter (DM)	• The intake of food consumed by the animal, calculated by the subtraction of the water content in the feed.
Energy The vitality and power of the feed and incorporates different aspects including:	• ***Gross Energy (GE)***, the total energy extracted through feed including faecal energy. • ***Digestible Energy (DE)***, the energy absorbed through the body and used for metabolic functions including gas, heat and urination energy. • ***Metabolised Energy (ME)***, the energy extracted from the feed and utilised by the animal for milk production, body function/maintenance and pregnancy.
Proteins The component of the feed containing nitrogen (up to 16%) which can be further classified as:	• ***Crude protein***, is calculated from the amount of nitrogen in the feed and can be subcategorised into dietary protein and non-protein nitrogen where the latter is produced through microbial or enzymatic reactions. • ***Dietary protein***, includes the subclasses of rumen degradable protein (RDP), which is completely digested in the rumen, and un-degradable dietary protein (UDP) which will pass through to the small intestine where it is digested and absorbed. The UDP also facilitates the slowing down of digestion as it enables an increased time for further microbial breakdown.
Carbohydrates Make up approximately 75% of plant dry matter including fibre but excluding protein. The two main types of carbohydrates are:	• ***Soluble carbohydrates:*** the non-structural carbohydrates that are sourced from cell contents rather than cell walls and include soluble sugars, starches and pectins. • ***Fibre:*** is primarily the structural component of the plant which can be further subcategorised into neutral detergent fibres (NDF), acid detergent fibres (ADF) and lignins. The NDF component, which includes cellulose and hemicellose, is soluble in pH 7 detergents and these are partially digested in the rumen. The ADF component, which is mainly digested in the rumen, is soluble and will dissolve in acidic conditions. Lignins are the more rigid portions of the plant that is the least digestible component of carbohydrates. Crude fibre can be determined by dividing ADF by a factor of 1.15.
Digestibility	• The measurement of the proportion of feed digested by the animal which only includes organic matter. The DM digestibility is calculated as a percentage of feed DM minus faecal DM divided by feed DM.

Adapted from Jacobs and Hargreaves (2002)

2.1.5. Feeding Practices in Australia

Most Australian dairy farms are located in coastal or high rainfall areas and, with the exception of the Northern Territory, dairy is produced in every state. There are two main types of dairy production systems in Australia, namely seasonal and year-round production. Seasonal production is the most common system utilised with two thirds of dairy farms across Australian including Tasmania, Victoria and South Australian implementing this scheme (Australian Bureau of Statistics 2004).This mode of production refers to the calving of cows during the peak pasture availability. In year-round production, as the name suggests, calving is continuous throughout the year allowing for more constant milk production and supply. The bulk of the milk production in this system is used to sustain domestic demands and exportation predominately to Asia (Dairy Australia 2015).

Australian dairy farming practices are among the most efficient and profitable world-wide as a result of the feeding regimes employed. There are five common feeding regimes currently used by farmers and the choice is dependent on the climate, resources (pasture availability), finances, available dairy equipment and preference. The general breakdown of the five different feeding regimes and composition is shown in Table 2.2. The partial mix ration regime shown in this table represents a combination of grain and pasture in prepared bails.

The major feeding system used nationally by farmers is S2, followed by S1, S3, S4 then S5. Farmers in Tasmania, however, predominately use the S1 feeding system (Australian Bureau of Statistics 2004; Elliston and Glyde 2008). Of these feeding systems, S3-S5 require a greater financial investment, however farmers

with larger herd sizes are more inclined to use these systems. The ability to control dietary intake, achieving higher feed conversion efficiencies, reducing the amount of feed wastage, reduction of pasture damage, and variation in pasture availability due to climatic conditions are among the factors influencing farmers decisions on the best feeding regime (Jacobs and Hargreaves 2002; Özkan and Hill 2015).

Table 2.2: Summary of main cattle feeding regimes

Feeding system	Pasture	Forage	Grains[#]	Bail fed concentrate	Partial mixed rations
S1	✓	✓	L	✓	
S2	✓	✓	M/H	✓	
S3	✓		✓	✓	✓
S4	✓				✓
S5					✓

Note: [#]L=low < 1.0 t of grain in dairy, M/H=medium/high > 1.0 t of grain in dairy (Auldist *et al.* 2011).

Feed management is a critical aspect for dairy production as it is the foremost determining factor for milk yield and subsequent financial investment requirements. Management practices are therefore a careful balance between resources including finances and feed availability, an understanding of energy conversion from the feed used by the cattle during lactation, and demand requirements. Optimised feed management can assist in reducing food wastage, promote sustainable pasture production and reduce over spending while maintaining production demands (Jacobs and Hargreaves 2002; Moran 2005; Dairy Australia 2015).

During pregnancy, the cows' appetite will reduce by 50-70% due to the room taken up internally by the calf and in the subsequent lactation period when milk is produced, feed requirements vary significantly. Figure 2.1 shows a schematic representation of the milk production, feed intake and body weight in a Friesian cow during the lactation period (Moran 2005). Peak milk production will occur during the 6-8th week (i.e. the second month) of lactation with a steep drop in body weight due to the animal using its reserves to produce milk. To maintain energy requirements, higher energy feed is required without increasing the volume to compensate for the cow's reducing appetite.

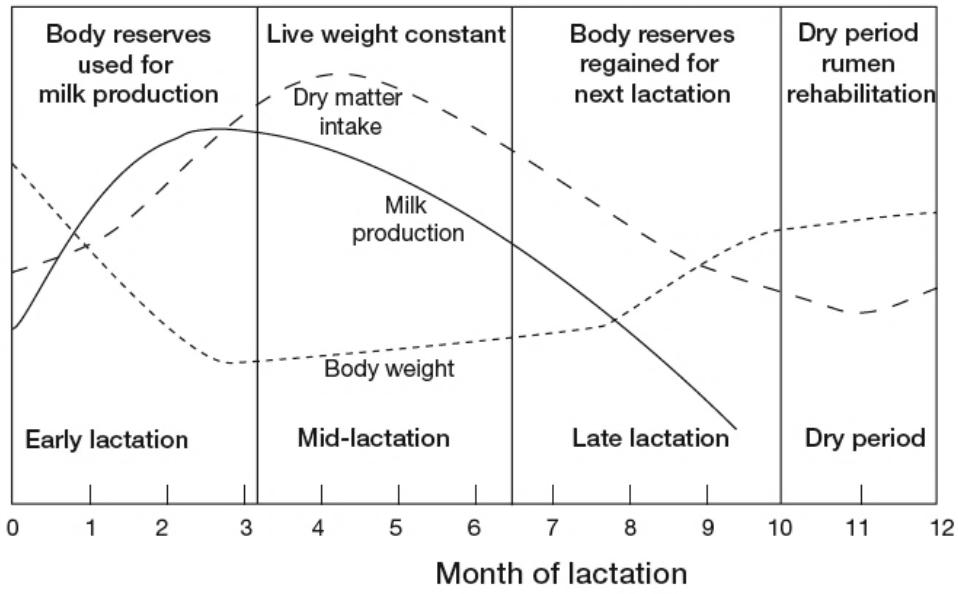

Figure 2.1: Lactation and feed intake chart (Moran 2005)

In early lactation, the cow increases its intake to help maintain body weight but is still limited by physiological capacity. During the mid to late lactation stage, the animal will still require high energy intake even though milk production declines

and in the following dry period Insufficient body reserve maintenance in the animal during this period has shown to reduce the milk production by as much as 220 L of milk in the following season (Moran 2005). Table 2.3 shows the average daily energy requirements to maintain different levels of milk production (Jacobs and Hargreaves 2002).

Table 2.3: Energy requirements for various milk yields

Milk yield (L/day)	ME requirement (MJ/kg DM)	Feed intake (kg DM/day)	
		10 MJ/kg DM	12 MJ/kg DM
20	168	16.8	14.0
30	220	22.0	18.3

Note: DM = dry matter, ME = metabolic energy. Adapted from Jacobs and Hargreaves (2002).

As shown previously in Table 2.2, the Australian dairy industry is heavily reliant upon pasture as an affordable feed source. Over recent decades, changing climates and reduced rainfall in Australia have negativity affected pasture production resulting in farmers supplementing different feeds to maintain production (Hanslow *et al.* 2014; Özkan and Hill 2015). There is therefore a keen interest by the dairy industry to study the effects of feed on dairy production in terms of both milk yield and quality, as well as methane mitigation in order to continually improve dairy practices. Fortification of macro- or micro-nutrients in milk (pre- or post-production) in order to improve human health has also become a common practise. This includes the addition of nutrients such as fat-soluble vitamins, fatty acids and phytosterols. At present, these macro nutrients are general fortified post milk production.

2.2. Milk Quality Research

In Australia, milk fat and protein are the two critical components that govern the value and subsequent pricing of milk with better prices obtained for milk with higher levels of these components in the product (Özkan and Hill 2015). An overview of past research showed there had been feeding research investigating fatty acid content in bovine milk and the effects of feed (Raes *et al.* 2004; Egger *et al.* 2009; Kalač and Samková 2010; Akbaridoust *et al.* 2014; Aprianita *et al.* 2017). However, there are no reported studies that investigate the relationship between phytosterol content in raw, unfortified milk and animal feed.

Phytosterols are plant-based compounds that are an essential component involved with its metabolic function, and structural membrane formation (in plants). Phytosterols come in four forms, one of which is the fatty acid ester conjugate, commonly found in plant oils', it is one of the richest sources of plant sterols. The fatty acid ligand is attached to the sterol by the hydroxyl functional group located on the third carbon. Generally, many metabolic processes and intestinal digestion are heavily influenced by the structural ligands.

For the last 60 years phytosterol studies have extensively demonstrated the health benefits associated with consuming plant sterols as it can reportedly reduce dietary cholesterol (Lichtenstein and Deckelbaum 2001b; Kritchevsky and Chen 2005; Ostlund 2007). Cholesterol has been linked to cardiovascular disease which is one of the health problems currently facing humans. For this reason, phytosterols are a popular health additive commonly fortified in dairy products.

Many reported analytical methods for the analysis of phytosterols are used to measure fortified food or food sources rich in phytosterols (such as nuts or oils) (Dutta 2004; Hyun *et al.* 2005; Laakso 2005; Clement *et al.* 2010; Srigley and Haile 2015). These methods usually include a hydrolysis step to liberate the sterols, followed by an organic extraction, and chemical derivatisation of the sterol extract to make it suitable for gas chromatography flame ionisation detector analysis. The typical sterol levels in these fortified or sterol rich foods ranged from 200-8000 mg/100 g.

This section of the review will focus on the limited studies related to phytosterol in unfortified milk and the relationship between different phytosterols. In nature, phytosterols occur in five forms one of which is the sterol fatty acid. These compounds are mainly found in the fat portion of plant material and on this basis it was hypothesised that phytosterols may respond similarly to that of fatty acid in feed from previous research by Gulati *et al.* (1997); Adin *et al.* (2009) and Meignan *et al.* (2017) among others in terms of modifying milk quality. For this reason, the following section of the review will mainly be focused on feeding studies and their relationship to fatty acid profiles. An in-depth examination is also presented on the analytical methods used for phytosterol research.

2.2.1. Naturally Occurring Sterols in Bovine Milk

It is well known that the predominant sterol found in bovine milk is cholesterol, making up more than 95% of the sterol content, with only minor or trace levels of phytosterols (Wong *et al.* 1988; Jensen 2002). In the last century, a range of sterols in milk were identified by Brewington *et al.* (1970) including lanosterol, *β*-

sitosterol, lathosterol, ergosterol dihydrolanosterol, campesterol and stigmasterol. This was achieved using thin layer chromatography to isolate the sterols fraction, which was later analysed by gas chromatography-mass spectrometry (GC-MS). Parodi (1973), using a similar analytical technique, was also able to confirm the presence of campesterol and *β*-sitosterol in milk fat and butter (Wong *et al.* 1988).

2.2.2. Fatty Acids

Fatty acids (FAs) are chains of hydrocarbons with a carboxyl acid functional group and a saturated or unsaturated aliphatic tail. They are a class of fats/lipids and there are several hundred FAs currently identified (Gunstone 2004; Marchello 2016). Fatty acids including omega FAs are essential for human metabolic processes and they must be obtained from food sources. Although milk is one of the largest consumed commodities in Australia, its consumption is not intended for supplementing omega FAs in the diet. However, it is increasingly common for milk producers to fortify milk with omega FAs to boost its nutritional content (Abu Ghazaleh *et al.* 2003; Dewhurst and Moloney 2013; Dairy Australia 2015; Ben-Ishay *et al.* 2017). As discussed previously, the milk pricing in Australia is reliant on the concentration of milk fat, protein, and as a result there have been numerous dairy cattle feeding experiments to improve the fat content and subsequent composition or FA profile (Gunstone 2004; Marchello 2016).

Fatty Acid Profiles

The breakdown and evaluation of any FA profile usually includes the examination of the main groups of FA including:

- **Saturated fatty acids (SFA)**, where the hydrocarbon chain has no double bonds, i.e. is fully saturated with H atoms. Common SFAs include: butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0) palmitic acid (C16:0) and stearic acid (C18:0) (Gunstone 2004; Marchello 2016).
- **Mono-unsaturated fatty acids (MUFA)**, where the hydrocarbon chain contains a C to C double bond. Common MUFAs include: myristoleic acid (C14:1), palmitoleic acid (C16:1) and oleic acid (C18:1) (Gunstone 2004; Marchello 2016).
- **Poly-unsaturated fatty acids (PUFA)**, where the hydrocarbon chain contains two or more C to C double bonds. Common PUFAs include: linoleic acid (C18:2w6) alpha-linolenic acid (C18:3w3), gamma-linolenic acid (C18:3w6), among many others (Gunstone 2004; Marchello 2016).
- ***Trans* fatty acids (TFA)**, are MUFAs or PUFAs with a *trans* configuration. They occur naturally at low levels, however they are commonly viewed by health and food authorities as unsafe compounds (Li *et al.* 2017). These types of FAs have been linked to coronary disease and are also considered to be carcinogens. They can occur in food when excessive heating is applied to certain PUFAs or MUFAs thereby converting them to the *trans* configuration. (Gunstone 2004; Marchello 2016).

Fatty Acids in Milk

In bovine milk, the predominant FAs are the SFAs which includes myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). In many feeding trial studies, it has been shown that various feeds can affect the FA composition in bovine milk (Adin *et al.* 2009; Kalač and Samková 2010; Akbaridoust *et al.* 2014; Samková *et al.* 2014; Özkan and Hill 2015).The main aims in these feed investigations is to alter the FA profile of the milk by reducing the medium chain SFA and increasing the PUFA (Gulati *et al.* 1999; Laakso 2005; Kalač and Samková 2010; Özkan and Hill 2015). Based on these studies, three main factors that influence FA compounds in milk have been identified: (i) the animal (i.e. breed and lactation cycle); (ii) feed (i.e. types of feed and fat supplements); and (iii) environment (i.e. seasonal variations, feeding regimes, milking practises and herd management) (Kalač and Samková 2010). The FA composition in bovine milk is primarily derived from the feed consumed and the cow's metabolism including the d*e novo* synthesis (DNS) pathway and adipose metabolization (Kalač and Samková 2010). Saturated fatty acids such as the C4:0 to C16 types stem from the DNS whereas the C16:0 and longer chains originate from both the feed intake and the animal's fat reserves (Kalač and Samková 2010). The identification and quantitation of the FA profiles in animal feed based on different grass and feed types, has shown a variation in FA profiles as well as the handling of the feed (i.e. drying and wilting) and grass variety (Kalač and Samková 2010).

Influence of Feed on Fatty Acid Profiles

In a feeding experiment performed by Baldinger *et al.* (2013), comparisons between maize and Italian ryegrass silage feed were used to determine which high-energy forage was superior for milk yield, nitrogen efficacy and overall feed intake. A total of 22 lactating cows were used for this experiment over a period of 15 weeks. Overall the results were able to show cows on the maize diet produced 2.3 kg of milk and 1.5 g/kg of milk protein more than cows fed on Italian ryegrass. Urea levels were also lowered by 57% compared to cows fed the ryegrass diet. The major FA affected by the feed was myristic acid, the level of which increased. In addition, palmitic acid decreased and polyunsaturated FA (PUFA) increased on the maize diet compared to cattle on the ryegrass diet. Overall, the study was able to demonstrate the maize silage feed was able to produce higher milk yield and protein compared to Italian ryegrass as high energy forage and that the feed could also affect the milk FA composition (Baldinger *et al.* 2013).

A study by Samková *et al.* (2014) using Czech Fleckvieh and Holstein cows found the FA profile bovine milk can be altered by feeding the cows fresh lucerne hay as part of their diet. The cows were placed on the feeding experiment in mid-lactation and moderate FA profile changes were observed for the stearic and oleic acid in the Czech Fleckvieh breed, with a greater effect on the Holstein cows. This experiment was able to demonstrate that a change in diet can influence the resulting FA profile and that it was breed specific (Samková *et al.* 2014).

Four different feeding regimes including a control were studied by Hristov *et al.* (2011) to investigate their effects on rumen function, digestibility, milk yield and

FA composition in milk. A total of 8 cows were studied using a Latin square experimental design with the trial including a control feed (consisting of solvent extracted canola meal with high oil content), mechanically extracted canola meal, canola meal consisting of high oleic acid and low PUFA and rapeseed meal consisting of high erucic and low level glucosinolate. An overall comparison of the FA profile from the control feed group showed canola meal feed resulted reduced SFA and enhanced the cis-9 C18:1 and the MUFA. The high oleic acid canola meal diet was able to increase the conjugated linoleic acid (CLA) content and the rapeseed meal increased the milk fat *cis*-13 22:1. The study was able to conclude using feed levels above 12-13% dietary dry matter can alter the FA profile in cows, however, this would decrease the feed intake and subsequent milk production (Hristov *et al.* 2011).

The effects of oilseed supplement were investigated in hay-based feeds with a total of 3 feeding regimes studied by Egger *et al.* (2009). This study consisted of a control (crushed barley and maize), rapeseed oil (crushed barley, crushed maize and ground rapeseed) and linseed oil (crushed barley, crushed maize and extruded linseed) diet using a Latin square experimental design. For both the rapeseed and linseed diets, the SFA were 60.9% and 59.8% respectively which is considerably lower than the control at 66.9%. The PUFA was 3.6% and 4.7% for the rapeseed and linseed treatments respectively compared to the control at 4.1%. Overall, the study demonstrated that the linseed and the control diets could enhance the milk levels of PUFAs (Egger *et al.* 2009). In general, FA milk enhancement through animal feeding has contributed to the understanding of the influence of feed on milk quality. The key to enhancement using normal feed is

to incorporate it into a nutritionally balanced diet since extreme, unbalanced feed arrangements can significantly affect the animal's health, digestibility and milk production yields.

In a review by Steinshamn (2010), experimental data on feeding trials using a variety of grassland legumes such as white clover, red clover and lucerne from literature was investigated. The review was able to demonstrate that in general, a difference in feed resulted in changes to the FA composition of milk when compared to the control. The review showed that cows on the red clover diet produced milk containing higher amounts of PUFA, especially the C18:3n3 type, with the increase postulated to result from the rumen biohydrogenation of PUFA. Since red clover contains lower levels of plant mediated lysis compounds which are essential for rumen microbial hydrogenation, the resulting enhance levels of PUFA are transferred into the milk (Steinshamn 2010).

Feed Modified Fatty Acid Profiles

There is an increasing desire in the food industry to fortify dairy products and other foods by natural means rather than by the use of additives. One way to achieve this is by changing the animal's feed, which can reduce the potential for over-fortification and human error. This can ensure that fortification is maintained at naturally occurring levels, thus reducing the risk of unidentified health problems from over consumption of these macronutrients.

Nevertheless, using feed to enhance FAs or other macronutrients through common cattle feed have resulted in limited success. Greater success has been

obtained by using designed feeds that have been purposely made to withstand the cows' digestive system allowing for the absorbance of these nutrients at the later stage. These types are feed are called "rumen protected feeds" (Ashes *et al.* 1992b).

Ashes *et al.* (1992b) reported that milk produced from cows fed rumen protected canola meal (i.e. canola seeds treated with an inert protein), contained 10% higher fat content compared to the control. Milk obtained from the animals on the controlled feed showed an increase in the MUFA and PUFA by 54% with no effect on milk yield and protein content (Ashes *et al.* 1992b). In another study, the same authors incorporated fish oil using a similar protective inert protein technology in used in their previous study (Ashes *et al.* 1992a). The experiment demonstrated an increase in fatty acids of eicosapentaenoic acid (EPA, 20:5 omega-3) and docosahexaenoic acid (DHA, 22:6 omega-3), from 1% to 13-18% and from 2% to 7-9% respectively in bovine serum. Both EPA and DHA are essential FAs that are associated with brain function in humans and enhancement of these was also observed in the animal's muscle tissue from 1.5% to 14.7% and from 1% to 4.2% respectively. The study was able to show that feed supplement resulted in the successful transfer of both EPA and DHA from the feed to the animal's body (Ashes *et al.* 1992a).

An experiment using two types of rumen protected fish oil was performed by Gulati *et al.* (2003). The research used a DHA enriched and an EPA enriched feed on six Friesian cows for five days, after which the cows returned to their pre-experimental pasture diet. From the study, the researchers were able to observe

and determine the transfer levels of fatty acids into the milk. Animals on the DHA enriched diet was observed to contain 1.25 and 2.19 % (FA profile) of EPA and DHA respectively in the milk, and a transfer percentage of 21.1 and 7.8 % (respectively). While the cows on the EPA enriched diet was observed to contain 1.36 and 0.69% (FA) of EPA and DHA respectively with a transfer percentage of 8.1 and 6.9% respectively. The researchers suggested the lower transfer percentage observed for the EPA enriched cows may have been affected by the lower ruminal protection in the feed. The study was able to demonstrate changes in the fatty acid profile through protected feed, however the protection level of the feed is critical (Gulati *et al.* 2003).

Research conducted by Kitessa and Young (2011) using protected echium oil as an alternative to fish oil was able to enhance PUFA in milk, specifically stearidonic acid (SDA; C18:4 omega−3), *α*-linolenic acid (ALA, 18:3 omega−3), and EPA. Echium oil is a plant-based oil naturally rich in ALA and SDA. From the study, it was indicated EPA and DHA may be enhanced through the use of Echium oil. Echium oil itself does not contain EPA or DHA, however both ALA and SDA share similar metabolic synthesis pathways and may act as precursors for DHA and EPA. Results from the research were able to show an increase of the total omega-3 fatty acid from 553 to 1162 mg/L, ALA from 463 to 877 mg/L, SDA from 38 to 144 mg/L and EPA from 13 to 76 mg/L. The study was able to show echium oil can be used to enhance PUFA in cattle milk, however more research was required to optimise the feed amount and for DHA.

In a review by Gulati *et al.* (2007) the authors summarized many of the experimental studies relating to the alteration of FA profiles using protected rumen feed. Overall, the review surmised that rumen protected feed offers an effective means for altering FA profile in milk, serum and muscle of the animal. The authors suggested the following four key critical aspects for consideration when altering the FA profile in milk and muscle when implementing rumen protected feed:

- Feed used should be rumen protected;
- The amount of protected feed given to the animal should be the minimum effective amount in order to reduce excessive FA enhancement and subsequent over-consumption in the final product;
- Use of the minimum amount of protected rumen feed to reduce undesired effects on the animals' digestive system;
- Vitamin E should be additionally supplemented when using omega protected feed to prevent oxidation of the milk fat.

In general, the main challenges of altering the FA profile of milk include the optimisation of suitable ratios of omega acid enhancement required to achieve optimised health benefits for human consumption (Garnsworthy and Wiseman 2000). A combination of controlled feeding regimes, herd management, metabolic function, lactation cycles and designed supplements can potentially improve the quality of milk quality currently being produced. It is clear that recent studies have demonstrated that using a variety of pasture or rumen protected feed can change the fatty acid profile in the milk (Ashes *et al.* 1992b; Kalač and

Samková 2010; He *et al.* 2012; Aprianita *et al.* 2017). Given the success of some of these studies, it is likely that other nutrients such as phytosterols or vitamins can potentially be enhanced in milk through similar controlled feeding regimes. Since one of the predominant sterol conjugates is a fatty acid ester, it is possible that plant sterols in particular can be influenced through feed.

2.3. Phytosterol Consumption

There are two main reported health benefits associated with phytosterol consumption, namely the lowering of dietary cholesterol and the prevention of tumorous cancers (Kritchevsky *et al.* 1981; Lichtenstein and Deckelbaum 2001b). A considerable number of researches have reported the cholesterol lowering health benefits with the anti-cancer properties relatively new but a rapidly increasing field of research (Kritchevsky *et al.* 1981; Lichtenstein and Deckelbaum 2001b).

2.3.1. Cholesterol Lowering

For more than 60 years, studies have demonstrated a direct correlation between phytosterol consumption and the lowering of low-density lipoprotein (LDL) cholesterol. This type of cholesterol is one of the key factors in cardiovascular disease as it contributes to plaque deposits in the arteries, resulting in damage or loss of cardiac function (Gilani and Anderson 2002; Ostlund 2007; Carr *et al.* 2010). Cardiovascular disease is one of the biggest causes of mortality in developed countries, accounting for approximately 12.8 % of all deaths. It is believed that phytosterols inhibit the dietary uptake of cholesterol and regulate

the synthesis of cholesterol in the body. The reduction of dietary cholesterol *via* phytosterol ingestion is not entirely understood, however it is believed that phytosterols are absorbed mainly in the intestines, preferentially taken in by absorptive micelles, and thereby inhibit cholesterol uptake in the diet. The absorption of phytosterols has also been shown to reduce the stimulus for cholesterol production in the body (Kritchevsky and Chen 2005; Ostlund 2007).

In an early experiments by Pollak (1953), a reduction in plasma cholesterol in rabbits fed with phytosterols was demonstrated. A comparison between rabbits on normal, high cholesterol and sitosterol diets was able to show a cholesterol reduction in the rabbits that were fed the latter diet. This research was later followed by human trials with results also confirming the effectiveness of phytosterols as a cholesterol lowering agent (Pollak 1953). Several years later, Beridge *et al.* (1958) hypothesised that certain components of corn oil were hypo-cholesterolemic agents and this was later confirmed by Ostlund *et al.* (1999), who isolated phytosterols from the corn oil. Subsequently, phytosterol-free and regular corn oils were used in human feeding trials with the results confirming that cholesterol levels in subjects on the phytosterol-free corn oil increased by 38% (Ostlund *et al.* 1999). Feeding trials using phytosterols as cholesterol lowering agents continued until the late 1960s when the interest started to decline due difficulties in the administration of accurate levels of phytosterols to participants. Difficulties in soybean sterol preparation and solubility issues, for example, resulted in uncertainties in administrated dosages, thus leading to questionable outcomes (Kritchevsky *et al.* 1981; Ostlund *et al.* 1999).

In 1989, the development of the steryl fatty esters was patented by the Raisio Group leading to one of the first commercial applications, namely the production of Benecol in 1995 (Kritchevsky and Chen 2005). Studies conducted on the use of steryl esters incorporated into mayonnaise and spreads demonstrated the effectiveness of these compounds in decreasing plasma LDL cholesterol (Gerson *et al.* 1961; Miettinen *et al.* 1995; Ostlund 2002; Thompson and Grundy 2005; Doggrell 2011). In one of the most prominent research reports, Miettinen *et al.* (1995) conducted a study using 153 randomly selected subjects with mild hypocholesterolemia over the course of a year in a double-blind trial. The study, conducted in North Karelia in Finland, showed an average 10.2% reduction in cholesterol plasma compared to 0.1% in the control group. The subjects were administrated with margarine spread containing 1.8 to 2.6 g of sitostanol per day, and the results indicated that a dosage of 1.8 g or greater was most effective (Miettinen *et al.* 1995; Kritchevsky and Chen 2005). In general, collective data from dosage research for steryl and stanol ester consumption showed optimal LDL lowering at approximately 2 g/day with no significant benefits at higher intakes. Research such as that reported by Katan *et al.* (2003) were able to show a plateauing effect in LDL lowering at phytosterol doses above 2.2 g/day (Doggrell 2011; Gylling *et al.* 2014).

2.3.2. Anticancer Properties

The previous examples show that dietary cholesterol inhibition by phytosterol consumption is widely recognised as an effective preventative health practice. It is only in the last decade that new evidence from clinical feeding trials have

reported anti-cancer properties from the ingestion of phytosterols (De Stefani *et al.* 2008; Grattan 2013). For example, the evaluation of epidemiological data has suggested a strong link between the potential protective ability of high phytosterol diets against cancer. Plant-based diets containing significant levels of phytosterols have been shown to significantly reduce the incidence of colon and breast cancer. Comparisons between Asian and western countries demonstrate significant increases in the prevalence of cancer in the west due to the higher animal-based diet. The cancer risk for an Asian person relocating to western countries can also increase due to a reduction in plant-based dietary intake (Awad and Fink 2000; Bradford and Awad 2007; Hu *et al.* 2012).

In a study conducted by McCann *et al.* (2000), 232 endometrial cancer patients within the western New York area were evaluated with regard to diet, reproductive/family/medical history, and general lifestyle including smoking and physical activity. The results suggested that a plant-rich diet reduces the risk of endometrial cancer and a similar experiments conducted in Uruguay also concluded that a high plant diet containing phytosterols could reduce the risk of breast cancer (Mendilaharsu *et al.* 1998). Animal feeding experiments in rodents inoculated (with N-methyl-N-nitrosourea) or otherwise affected by cancer have provided better understanding of the benefits of phytosterols that are not generally well understood. The conclusion drawn from epidemiological studies are further confirmed through these animal trials and the results reiterate the importance of diet in human health (Awad and Fink 2000; Bradford and Awad 2007).

Raicht *et al.* (1980) reported a one-third reduction in incidental colon tumours in rodents fed a controlled diet containing 0.2% *β*-sitosterol. In this research, the rodents were inoculated with *N*-methyl-*N*-nitrosourea (MNU) to initiate cancer development and tumour growth (Raicht *et al.* 1980). Similar results were also demonstrated by Deschner *et al.* (1982) who also used MNU on rats under a controlled feeding regime. The rats were supplemented with a diet containing 0.3-2% plant sterols with the results show a reduction in size and proliferation of tumours. The research was also able to show the sterol was excreted from the test subjects unabsorbed suggesting that phytosterol slowed down the proliferation of epithelial colon cells thus affecting neoplastic transformations (Deschner *et al.* 1982).

More recently, controlled feeding trials using mice have been able to demonstrate the use of phytosterols as a supportive and anti-cancer nutrient (Awad and Fink 2000; Awad *et al.* 2007). Research by Awad *et al.* (2000) involved the use of severe combined immunodeficiency mice fed a control diet supplement of 2% phytosterol or 2% cholesterol with 0.2% cholic acid where the latter is used to assist with the absorption of sterols. After 15 days on the controlled diet, the test subjects were injected with human breast cancer cell (MDA-MB-231) and a total of 8 weeks of tumour growth was designated by tumours detectable within 3 weeks. The results were able to demonstrate mice on the phytosterol diet had 33% smaller tumours and 20% fewer lumps compared to the cholesterol fed subjects. The researchers concluded that diets high in phytosterols are able to retard breast cancer growth (Awad *et al.* 2000).

In similar research, Llaverias *et al.* (2013) conducted feeding trials using breast cancer inherited mouse subjects. The research contained two diets: (i) high fat, high cholesterol and (ii) low fat, low cholesterol diet. With the exception of the control group, these diets were also supplemented with 2% phytosterols. The demonstrated the proliferation and tumour growth at 4, 8 and 13 weeks were reduced in mice fed phytosterols. It is believed that phytosterols promote cell apoptosis in cancer cells or metabolic sterol processes, thus resulting in protective, delaying and inhibiting effects (Llaverias *et al.* 2013).

2.3.3. Excess Consumption

The recommended daily dosage of phytosterol consumption to reduce cardio-vascular diseases is 2 g but this dosage is 4-13 times greater than the normal dietary intake of 150-450 mg/day. At present, there are insufficient long-term studies of high phytosterol diets to determine if there are any adverse, long-term side-effects associated with high levels of phytosterol consumption. Some short-term studies have found a decrease in some fat-soluble vitamins and carotenoids in the blood plasma of subjects are administered with high phytosterol doses. However, this could be easily remedied with the co-administration of increased fat-soluble vitamins or carotenoids during increased phytosterol intake (Clifton *et al.* 2004; Gylling *et al.* 2010).

In a short-term study by Clifton *et al.* (2004), the effects on serum lipid and plasma sterol levels under a high phytosterol diet were investigated. The experiment involved 35 participants over a 12-week trial period where the subjects were on a high plant sterol diet for 6 weeks, with an additional 2-week normalisation

period, and a further continuation of 6 weeks on a high phytosterol diet with increased fruit and vegetable intake. The experiment was able to show high phytosterol diets elevated the phytosterol levels in plasma with a 19-23% decrease in carotenoids. However the experiment also pointed out that the change was potentially not significant compared to normal seasonal changes of up to 70% (Clifton *et al.* 2004).

Research by Goncalves *et al.* (2011) involving *in vivo* and *in vitro* models using the Caco-2 cell line investigated the effects of phytosterols upon vitamin D absorption. The research demonstrated a decrease in vitamin D absorption when the subject was simultaneously administered high levels of phytosterols by showing the uptake of both compounds was *via* the same channels, thus concluding that phytosterols impaired vitamin D absorption. Vitamin D is important for bone development, inflammatory and immunity functions, and cell regulation, however limited sun exposure and reduced fat consumption has caused vitamin D deficiency to be prevalent in western societies. Although long-term high level phytosterol intake can lead to significant side-effects in people with severe vitamin D deficiency, this can be overcome with an increase in vitamin D supplements (Gylling *et al.* 1999).

Research by Noakes *et al.* (2002), 46 human subjects participated in a feeding trial using different types of margarine-based spreads. The trial consisted of ingestion of a control spread containing no phytosterols for 3 weeks, followed by a spread containing 2.3 g of phytosterols for 2 weeks, then 3 weeks of spread containing 2.5 g of stanol esters. The subjects were also advised to consume

more than 5 serving of fruits and vegetables a day and the study was able to demonstrate high carotenoid consumption during high intake of phytosterols can maintain or increase carotenoid levels. This research was able to show that carotenoid lowering effects while on a high phytosterols intake can be easily remedied by increasing carotenoid intake (Noakes *et al.* 2002).

Phytosterolemia, also known as sitosterolemia is a rare autosomal condition that affects the storage and excessive phytosterol absorption and cholesterol metabolism. As a result, patients tend to develop tendon problems such as xanthomas and the development of premature coronary atherosclerosis. Shown in Figure 2.2 is an example of a patient whose hands are presenting xanthomas and although there are less than 50 known cases worldwide, treatment for phytosterolemia includes a controlled low phytosterol diet or the administration of the drug ezetimibe to block sterol absorption (Katan *et al.* 2003; Kritchevsky and Chen 2005; Izar *et al.* 2011).

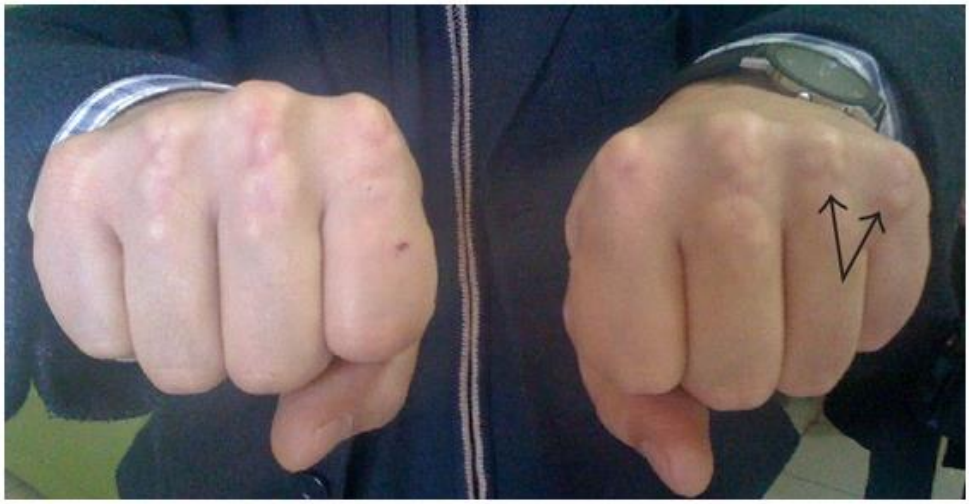

Figure 2.2: Patient with xanthomas located in the hands (Han *et al.* 2015)

2.3.4. Food Safety Authorities

It is well documented that the general consensus among the major world food authorities is that phytosterols are safe and beneficial in aiding the lowering of dietary LDL cholesterol. Food authorities such as United States Food and Drug Administration (US FDA), Health Canada, European Foods Safety Authority (EFSA) and Food Standards Australia New Zealand (FSANZ) have approved phytosterol fortification in foods to the specification outlined by their respective standards. These selling foods fortified with phytosterols have also been permitted to make health claims associated with the benefits of lowering dietary LDL cholesterol and the subsequent health conditions it may influence such as cardiovascular diseases. In general, food authorities recommended a daily consumption ranging from 1.5-2.0 g/day of phytosterol or stanol esters respectively, as these levels have been shown to be effective at lowering dietary LDL cholesterol (Moreau *et al.* 2002; Kritchevsky and Chen 2005).

2.4. Phytosterol Analysis

2.4.1. Overview

Parts of this section have been published in an invited review of phytosterol analysis in: Duong, S. Strobel. N, Buddhadasa. S, Auldist. M, Wales. W, Orbell. J, Cran, M, Quantitative instrumental analysis of phytosterols in fortified foods. *Reference Module in Food Science*, 2017, 7 pp., https://doi.org/10.1016/B978-0-08-100596-5.21400-5.

As discussed previously in Section 1.2, phytosterols can occur naturally as conjugates and in free form. Phytosterols are soluble in organic solvents such as

chloroform and heptane and, as such, can be analysed using either gas chromatography (GC) or liquid chromatography (LC), depending on the application (Moreau *et al.* 2002; Clifton *et al.* 2004; Liu *et al.* 2007)

2.4.2. Liquid Chromatographic Analysis

Liquid chromatography is typically used to analyse or determine groups or individual sterol conjugates. Utilisation of a normal phase is commonly used for lipid groups with a reverse phase used for individual sterol analysis. Liquid chromatographic instrumentation configuration is usually coupled to a photodiode array detector (PDA) or ultraviolet detector (UV), evaporative light scattering detector (ESLD) or Atmospheric pressure chemical ionization mass spectroscopic detector (APCI MSD) for sterol work. Even though LC can be used for total phytosterol determination, GC is the preferred analytical technique for sterol quantification.

The main advantage of using LC for sterol analysis is that the compound can be analysed directly without derivatisation or changes to the analytes (Kesselmeier *et al.* 1985; Moreau *et al.* 2002; Normén *et al.* 2002; Lagarda *et al.* 2006; Sun *et al.* 2017). Several researchers have shown that the use of LC coupled with PDA or MS is able to profile and determine sterol glycosides. These studies were able to identify the main sterol components and show differences in the profiles from a variety of food and plant matrices (Kesselmeier *et al.* 1985; Lagarda *et al.* 2006; Zhang *et al.* 2006; Cañabate-Díaz *et al.* 2007).

Several researchers have shown that the use of LC coupled with detectors mentioned are able to quantitate, profile and determine sterol conjugates (predominately glycosides). These studies were able to identify the main sterol components and show differences in the profiles from a variety of food and plant matrices (Kesselmeier *et al.* 1985; Lagarda *et al.* 2006; Zhang *et al.* 2006; Cañabate-Díaz *et al.* 2007). When MS is used, studies by Rozenberg *et al.* (2003); (Raith *et al.* 2005) and Mo *et al.* (2013) have found that the ionization of the sterol may cause a loss of a water molecule and as a result, the predominant precursor ion fragment will occur as [M+H-H_2O]. Shown in Table 2.4 are some specifications of typical LC columns used for phytosterol studies.

2.4.3. Gas Chromatographic Analysis

Gas chromatography is the preferred instrumentation for total phytosterol determination by many researchers because of its greater ability to resolve structural similar compounds and user friendliness. Typical phytosterol GC analyses use a non-polar column with small amount of phenyl for selectivity with lengths ranging from 15 to 30 m. Oven programs for sterol analysis using GC will commonly use a quick oven temperature ramp up to 200˚C which is then slowed to allow for phytosterol separation. This slower temperature ramp is important since most sterols will elute during the oven temperature program between 240 and 290˚C oven ramp phase. In addition, injector temperature is usually set at 240 to 290˚C to ensure complete or aid in the derivatisation process and volatilisation of the extract. Derivatisation is performed to thermally stabilise the sterol compound by forming a tri-methyl-silyl moiety on the hydroxy functional

group (Du and Ahn 2002; Dutta 2004; Brufau *et al.* 2006; Cunha *et al.* 2006; Lagarda *et al.* 2006; Inchingolo *et al.* 2014). Derivatisation can be achieved using N-O-bis-(trimethylsilyl)-trifluoroacetaminde (BSTFA) or n-(tert-butyldimethylsilul)-N-methy (MSTFA) silylating reagents. In addition, a small amount of pyridine is added in the derivatisation process to neutralise the hydrochloric acid by product created by BSTFA. This addition is essential to prevent any residual hydrochloric acid from degrading the analytical column.

Table 2.4: LC columns used for sterol analyses

Target analyte	Matrix	Column	Detector	Reference
Free and sterol glycosides	Wheat	C18 prevail column	APCI MS	(Rozenberg *et al.* 2003)
Free sterols	Oil	Zorbax XDB-C18 column	APCI MS	(Mo *et al.* 2013)
Free sterols	Cells	Xterra MS C8 reverse phase	APCI MS	(Palmgrén *et al.* 2005)
Free sterols	Medicine	Cosmosil C_8 column	UV detector at 202 nm	(Shah *et al.* 2010)
Free sterols	Capsules	Phenomenex Luna C8 Column	PDA 210 nm	(Nair *et al.* 2006)
Free and steryl ester	Sugar cane	Luna C18 column	UV detector	(Feng *et al.* 2015)
Free and sterol glycosides	Plant matter	Hypersil BDS R18 column	ELSD	(Breinholder *et al.* 2002)
Free sterols	Reference standards	Agilent Poroshell 120 EC-C18	ELSD	(Fu and Joseph 2012)
Sterol conjugate	Pearl barley	LiChrosorb DIOL	UV at 280 nm	(Lampi *et al.* 2004)
Free sterols	Reference standards	Hypersil SiO2	ELSD	(Liu and Ruan 2013)
Steryl ferulates and glycosides	Bran	Supelco PLC-Si	UV detector at 315 nm	(Münger and Nyström 2014)
Sterol conjugates	Foods	LiChrosorb 7 µ DIOL column	ELSD and UV detector	(Moreau and Hicks 2004)

For phytosterol analysis, the GC would typically be coupled to a flame ionisation detector (FID). The advantages of using FID include a large dynamic response, excellent linearity, low cost, robustness, and selectivity to carbon-based compounds which is a critical aspect for quantitation based on equivalence. As FID identification is based on retention times of reference standards, the use of mass spectrometry detector (MSD) has also been popular. The use of the MSD has allowed further confirmation with the use of the mass spectral library to identify sterols without the use of reference standards. Ideally a GC instrumentation set up consisting of FID and MS would be an advantage as this would allow for both quantitation and identification, however identification of plant sterols is generally based on its derivatised form. Shown in Table 2.5 are examples of GC conditions used for different analyses and in each case, sample derivatisation was performed.

Table 2.5 Typical GC conditions used for sterol analysis

Injector temperature	Column type	Oven conditions	Detector	Reference
260˚C	BPX5 (5% phenyl polysil phenylene soloxane) 25 m × 0.22 mm i.d. × 0.25 µm film thickness)	Initial column temp 50˚C (0.5 min) → 20˚C/min to 320˚C (10 min)	FID	(Duong *et al.* 2016)
270˚C	HP-5 (5% phenymethyl siloxane) 30 m × 0.25 mm i.d. × 0.25 µm film thickness	Initial column temp 240 ◦C (10 min) →2˚C/min to 260˚C (30 min)	FID	(Liu *et al.* 2007)
325˚C	DB-5 (5%-Phenyl)-methylpolysiloxane) 30 m × 0.25 mm i.d. × 0.25 µm film thickness	Initial column temp 200˚C–2˚C/min to 300˚C (11.67min)	FID	(Clement *et al.* 2010)
280˚C	Rtx -1701 (14% cyano propylphenyl– 86% dimethylpolysiloxane) 60m × 0.25mm i.d × 0.25-mm film thickness	Initial column temp 280˚C (45 min) →10˚C to 280˚C (3.5min)	FID	(Phillips *et al.* 1999)
300˚C	CP-Sil-13CB (14% phenyl/86% dimethylpolysiloxane) 25 m × 0.25 mm i.d. × 0.25 µm film thickness	Initial column temp 120◦C (0.5 min) → 20˚C to 260→2˚C/min to 300˚C	MS	(Menéndez-Carreño *et al.* 2016)
350˚C	HP-5 (5%phenymethyl siloxane)15 m × 0.25 mm i.d. × 0.22 µm film thickness	Initial column temp 75˚C (1 min) → 40˚C/min to 250˚C 30 min)	FID	(Fernández-Cuesta *et al.* 2012)

2.4.4. Liberation of Sterol Conjugates

Ideally, total sterol analysis should also include all sterol conjugates. To achieve this, many researchers have investigated the use of acid hydrolysis followed by alkaline saponification. The purpose of acid hydrolysis is to liberate steryl glycoside and acylated steryl glycosides. The most direct and simple way to achieve this is to convert all conjugates into a free form using chemical or enzymatic processes to cleave fatty esters or glycosidic bonds. Figure 2.3 shows the different bonds and chemical reaction required to free the various sterols from the ligand moieties (Moreau *et al.* 2002; Dutta 2004; Nystrom *et al.* 2007; Xiang *et al.* 2016).

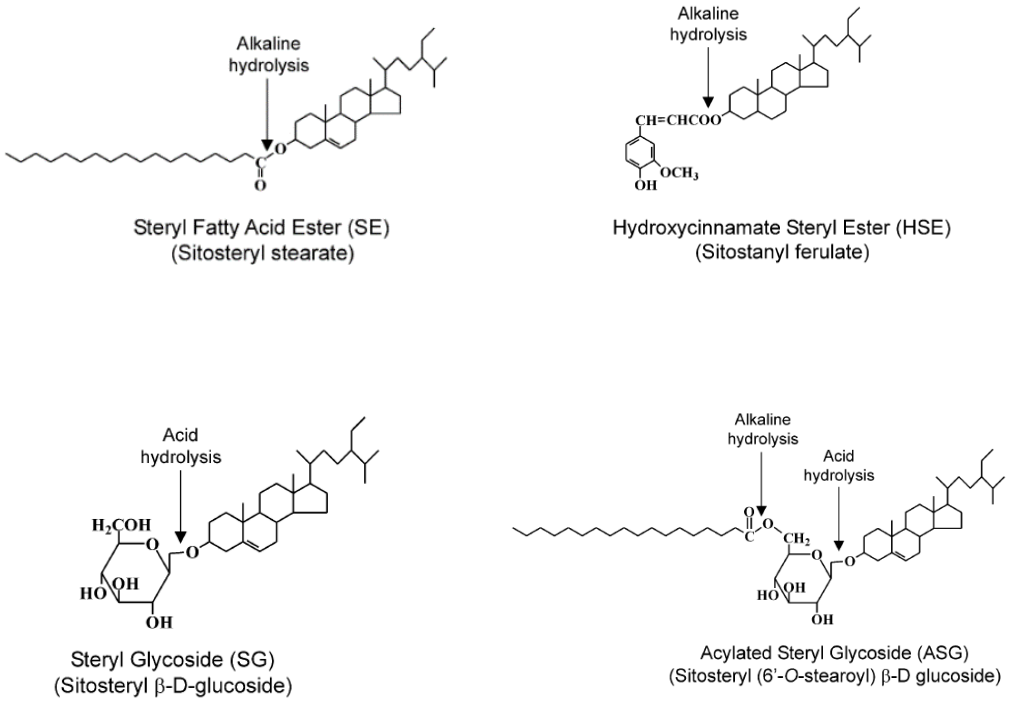

Figure 2.3: Bond cleaving reactions required to free sterols (Moreau *et al.* 2002)

2.4.5. Glyosidic Bonds

In plant sterols, the glyosidic bond between the sterol and the sugar moiety is located in a beta configuration at the 1-4 linkage. These bonds can be cleaved using acid hydrolysis or enzymatic treatment. The process of acid hydrolysis is commonly used as it is economical, robust and efficient, and it is regularly performed using hydrochloric acid for phytosterol analysis. Shown in Figure 2.4 is a generic acid hydrolysis reaction of a glucosidic bond (Moreau *et al.* 2002; Lagarda *et al.* 2006).

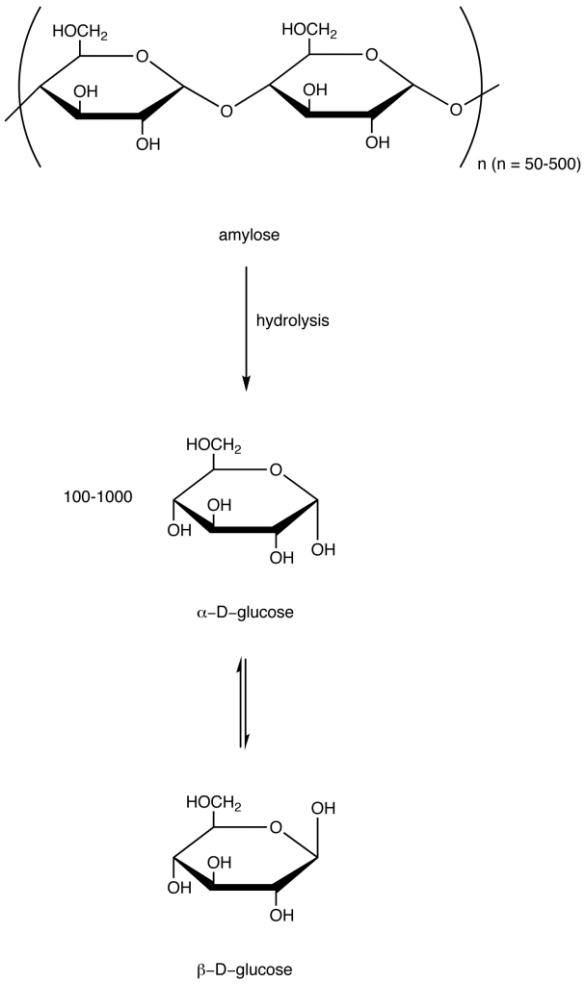

Figure 2.4: Glycosidic linkage bond cleavage using acid (OChempal 2018)

An overview of published literature for the analysis of plant sterols using acid hydrolysis found similarities and common practises used by many co-workers. Toivo *et al.* (2000); Laakso (2005); Liu *et al.* (2007) and Clement *et al.* (2010) were able to successfully use diluted hydrochloric acid for sterol measurement in a variety of foods, wheat, tobacco and oats (Toivo *et al.* 2000; Laakso 2005; Liu *et al.* 2007; Clement *et al.* 2010). It was also noted that acid hydrolysis was always performed prior to saponification, as sterol glycosides are polar compounds and would preferentially remain in the aqueous phase. While sterol esters are non-polar, during the saponification process the aqueous phase is discarded before analysis which results in a potential glycoside loss if saponification was to be performed prior to acid hydrolysis (Dutta 2004; Lagarda *et al.* 2006). Another common practise is the use of aqueous or ethanolic hydrochloric acid ranging from 1-6 M with incubation time and temperature ranging from 60-100 ºC and incubation time of 30-120 minutes is regularly used for this type of work (Moreau *et al.* 2002; Dutta 2004). These published conditions were used as a starting point for this research method development described in this thesis.

The main drawback of acid hydrolysis is the isomerization of ethylidene side chain in sterol with this configuration such as both Δ5- and Δ7-avenasterol and fucosterol. The acid hydrolysis process induces a dehydration upon the carbon 3 site causing degradation of the sterol. Researchers such as Kesselmeier *et al.* (1985); Kamal-Eldin *et al.* (1998) and Toivo *et al.* (2001) reported that levels of Δ5- and Δ7-avenasterol and fucosterol in oats, wheat and coconut oil decreased after acid hydrolysis was performed to the samples.

For this reason, enzymatic treatment using *β*-glucosidase has been explored as an alternative technique for the liberation of glycosides with moderate success (Toivo *et al.* 2001; Nyström 2007). Beta-glucosidase is the main enzyme used for phytosterol work as it is able to actively cleave the 1-4 linkage configuration. The main advantage of using enzyme treatment for sterol analysis is its ability to specifically liberate sterol glycoside/glucosides without negative degradation to sterols. It is also less aggressive and dangerous technique compared to acid hydrolysis as it does not use high temperature and acid. As with any enzymatic treatment, its application must be performed under strict and narrow conditions as it is susceptible to denaturing. Controlled, optimised conditions such as pH, temperature and solution buffer are critical for enzymatic work.

Kesselmeier *et al.* (1985) and Nystrom *et al.* (2007) have successfully used and demonstrate the effectiveness of enzymatic treatment in sterol work in wheat, oats and other cereal grains. In their research, they quantified and profiled total sterols by liberating the conjugates by enzymatic treatment followed by saponification and purification using TLC plates. In addition, Nystrom *et al.* (2007) hydrolysed lipid extracts from the sample using the accelerated solvent extractions (ASE) and not directly on the samples.

The main drawback of using an enzyme treatment is its robustness, as enzymes are susceptible to their physical and chemical environment. Factors such as pH and temperature critical to the enzymatic activity. Utilisation of the enzyme outside its optimal conditions can cause the enzyme proteins to denature, rendering them inefficient or ineffective during the reaction. In general, both Δ5-

and Δ7-avenasterol and fucosterol are not found in many dietary foods nor at significant levels in plants when compared to the other sterols, allowing many researchers to use acid hydrolysis Toivo *et al.* (2001).

2.4.6. Saponification of Fatty Ester and Hydroxycinamate Bonds

Saponification, also known as alkaline hydrolysis, is a process used to break fatty esters and hydroxycinamate bonds. Alkaline hydrolysis is commonly achieved using potassium or sodium hydroxide dissolved in alcohol. The reaction converts fatty acid or triglycerol moiety on the sterol to alcohols and salt (soap), with the concurrent hydrolysis of the fatty acid esters thus freeing the sterols for extraction. Figure 2.5 shows an example of alkaline hydrolysis of a trigyceride. Components of the reaction that are not changed during the reaction are called unsaponifiable and these include the free sterols which are less soluble in water. The salts produced through saponification will solubilise in water and the free sterols can thereby be extracted using organic solvents (Moreau *et al.* 2002). In brief, saponification converts fatty acid or triglycerol moiety on the sterol to alcohols and salt, thereby freeing the sterols (unsaponifiable) for extraction

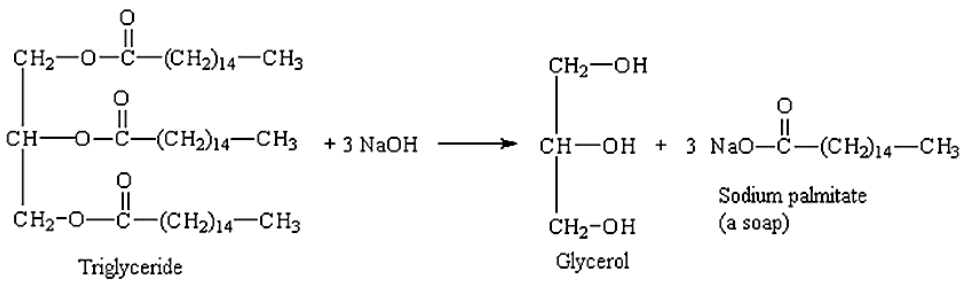

Figure 2.5: Saponification reaction of triglyceride (Wahl and Gallardo-Williams 2011)

2.4.7. Solid Phase Extraction

Sample clean-up using solid phase extraction (SPE) is an effective way to purify and improve sensitivity of the target analysis (Phillips *et al.* 2005; Azadmard-Damirchi and Dutta 2006; Biziuk 2006; Derakhshan-Honarparvar *et al.* 2010). In general, SPE works under the same principle as liquid chromatography except it is intended for more preparative purposes (Sigma-Aldrich 1998; Macherey-Nagel 2012). Similar to liquid chromatography, a solution of the sample is introduced to a solid phase and separation of targeted compound is based on phase interaction and polarity of the eluting solvent/solution. The SPE can be used to bind the target compound, washing the undesired compounds and later eluting the target compound with a specific solvent or vice versa. Shown in Figure 2.6 is an example of basic principle behind SPE which involves a number of steps from conditioning through to elution. Prior to use, the SPE cartridge is usually conditioned to wet the phase bed allowing for even liquid flow in sequential steps.

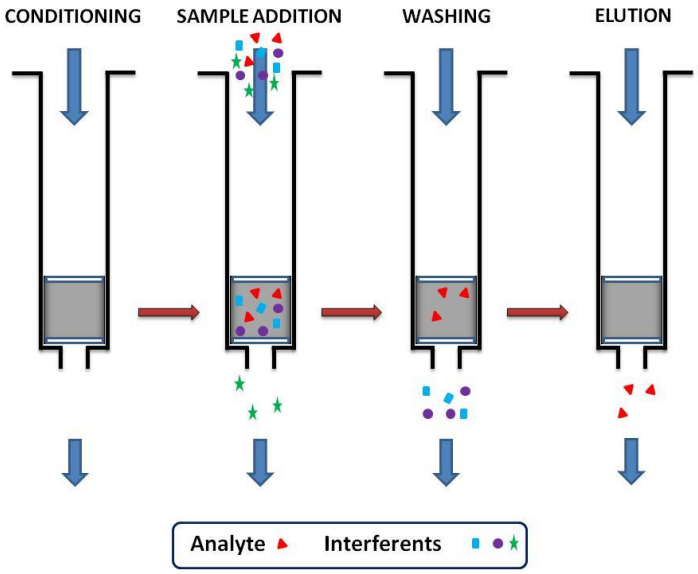

Figure 2.6: General principle behind SPE (Lucci *et al.* 2012)

For phytosterol analysis, traditional thin layer chromatography (TLC) plates were used in the past. However, many modern research techniques have developed and the use of SPE is increasingly popular as it is more user friendly and enables the use of different phases, providing potentially better phytosterol purification. Typically, three phases are used for sterol work including silica, C18 and amino propyl phases, with many researchers reporting success in purifying sterols from oil, plants, food and biological samples after extraction (Phillips *et al.* 1999; Careri *et al.* 2001; Azadmard-Damirchi and Dutta 2006; Derakhshan-Honarparvar *et al.* 2010; Quintin 2010). Shown in Table 2.6 are examples of some of the SPE conditions used by different researchers.

2.4.8. Total Analysis of Sterols

Complex methods including acid hydrolysis, saponification and sample clean-up have been published for total sterol analysis including its conjugates (Laakso 2005; Liu *et al.* 2007; Normén *et al.* 2007; Clement *et al.* 2010). These studies have successfully created elaborate hydrolysis (acidic and alkaline) protocols that can enable the determination of total sterols in a range of cereal, food and plant matrices. These methods use hydrochloric acid ranging from 1-4 M in water or ethanol followed by extraction or direct saponification. The saponification mixture is typically sodium or potassium hydroxide between 1-6 M. The sample is then extracted using organic solvent, derivatised and analysed using GC coupled with FID or MS (Moreau *et al.* 2002; Normén *et al.* 2002; Dutta 2004; Liu *et al.* 2007; Clement *et al.* 2010). The main disadvantage of acid hydrolysis is the isomerization of some phytosterols (Kamal-Eldin *et al.* 1998; Moreau and Hicks

2004), however, the degree of isomerization will vary depending on the hydrolysis conditions, particularly time and temperature. Conversely, Clement *et al.* (2010) used acid hydrolysis for 30 minutes at a temperature of 100˚C for the hydrolysis of beverages and fortified food, however no isomerisation was observed.

Table 2.6: SPE conditions used for phytosterol analysis

Matrices	SPE Phase	SPE conditions	Reference
Food and vegetable oil	C18 SPE	• Condition SPE cartridge with methanol: water • Load sample • Elute sterol with a chloroform: methanol solvent mixture (95:5)	(Abidi 2001)
Rice bran	Silica SPE	• Condition the SPE with hexane • Load sample • Elute sterol with a hexane and diethyl ether solvent mixture (90:10)	(Derakhshan-Honarparvar *et al.* 2010)
Human serum	Aminopropyl SPE	• Condition SPE cartridge with hexane • Load sample • Elute sterol fraction with chloroform: isopropanol (2:1)	(Phillips *et al.* 1999)
Vegetable oil	Silica SPE	• Condition SPE with hexane • Load sample • Wash SPE with diethyl ether solvent mixture (99:1) • Elute 4,4 methyl and 4 mono methyl with hexane diethyl ether solvent mixture (99:2) • Elute 4 methyl sterol with hexane diethyl ether solvent mixture (60:40)	(Azadmard-Damirchi and Dutta 2006)
Soybean oil	Silica SPE	• Condition SPE with hexane • load sample • Wash SPE cartridge with hexane and ethyl acetate (95:5) • Elute sterol with hexane: ethyl acetate solvent mixture	(Careri *et al.* 2001)

There are two main approaches for the quantification of total sterols, namely the use of a traditional calibration curve (consisting of increasing concentrations of the standard within a linear response range) or by equivalence. Due to the high cost and limited commercial availability of some reference standards, quantification by equivalence is regularly carried out using sterols with similar chemical characteristics to the sterol) of interest, but which are not present in the sample (i.e. similar FID response and chromatographic elution time range). Taking advantage of the consistent robust response of the FID, phytosterol quantification based on equivalence has been proven to be accurate and reliable (Dutta 2004; Clement *et al.* 2010; Duong *et al.* 2016)

Overall, a review of the literature indicates that in many cases, method optimisation using published works or existing methods as starting point is critical. It is clear that not all methods are transferable or applicable to specific studies and, as such, method development is important to ensure analytical methods will be suitable for the intended application.

Chapter 3. Analytical Methods and Materials

3.1. Overview

This chapter presents the methods and materials used for this research including standards and reagents, reference materials, sample materials (preparation, extraction protocols, etc.) and analytical instrumentation. Details of the methods used for the feeding trials are given in Chapter 5.

3.2. Chemicals and Equipment

3.2.1. Standards and Reagents

Sterol Reference standards were all acquired from Sigma Aldrich (Sydney, Australia) or Steraliods Inc. (Road Island, USA) this included 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%). The reference standards were then prepared individual stock solution in cyclohexane at a concentration of 500 mg/L. Further dilutions were made using heptane for calibration or spiking standards. All stock standards were stored at 4ºC in a spark proof refrigerator and were shown to be stable for 12 months.

Other standard chemicals including potassium hydroxide and sodium hydroxide were purchased from Sigma Aldrich (NSW, Australia). Solvents including absolute ethanol, n-heptane, hexane, chloroform, methanol, toluene pyridine,

fuming hydrochloric acid and cyclohexane were purchased from Merck Australia. The derivatising agent, N-O-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1%TMCS) was obtained from Grace Davison (Columbia, Maryland, USA). Deionised water was produced onsite using a Millipore system.

The enzymatic reagents used were supplied by Novozyme Corp^{tm} (Sydney, NSW) included Novozym 188 (unit 250 U/g contains *β*-glucosidase), Ultraflo L (unit 45000 U/g contains *β*-glucanases (endo-1,3(4)), Celluclast 1.5L (unit 700 U/g contains Cellulase) and Shearzyme Plus (unit 350 U/g contains cellulase, xylanase (endo-1,4) and activity for *β*-glucanase). For the enzymatic treatment trials, Ultraflo L was diluted by a factor of 1:10 using water with an adjusted pH of 5.0.

3.2.2. Reference Materials

The method optimisation and validation were performed using meat homogenate NIST 1546 (certified for cholesterol) and NIST 3250 *Serenoa repens* seed fruit (certified for *β*-sitosterol, stigmasterol and campesterol) which was obtained from the National Institute of Standards and Technology (NIST, USA). 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™ (Sydney, Australia). Matrix recoveries were carried out on powdered milk, fortified fat spread, cheese slice, fortified milk and full cream milk powder obtained from local markets.

3.2.3. General Equipment and Consumables

The following general equipment and consumables were used: Ratek shaking water bath WB4D (Ratek, Boronia, VIC); dry block heater (with GC-vial holding plate from Ratek, Boronia, VIC); vortex mixer and shaking evaporation manifold with a 44 mL vial holding plate (Thermo Fisher, Scoresby VIC); positive displacement piston operated volume aspirator (POVA); vacuum manifold for SPE set (Sigma, Sydney, NSW); 44 mL glass screw-capped vials with teflon septa; 2 mL GC vials and caps; 10 mL disposable test tubes; silica phase 690 mg SPE sep pak and amino propyl phase SPE 360 mg sep pak (Waters Australia, Rydalmere NSW); and amino propyl phase SPE 0.5 and 5 g (Agilent Technologies, Mulgrave, VIC). Other equipment included: a grinding mill (Foss CT 293 Cyclotec^{TM,} Foss Analytics, Mulgrave VIC); a high-powered homogenizer (Robot Coupe Blixer 3, Robot-Coupe Australia Pty Ltd, Northbridge NSW); and density meter (Anton Paar DMA 4500 M, Anton Paar, North Ryde, NSW).

3.2.4. Gas Chromatography Set-up

An Agilent 7890 GC (Agilent Technologies, Mulgrave, VIC) coupled with a 5975c mass spectrometry detector and a flame ionisation detector (FID) using a HP-5MS capillary column (5%-phenyl-methylpolysiloxane 30m x 0.25 µm x 0.25 µm film thickness) were used to perform the analyses. The optimised GC-MS/FID conditions were as follows:

1) oven program: initial oven temperature was 245ºC held for 0.5 minutes, this was then followed by increasing temperature ramps to 265ºC at 2ºC/min and to 290ºC for 3.5 ºC/min with a hold for 8 minutes. A 7.5-minute

post run program at 240ºC with a back-flush flow at 24.6 psi was then applied for a total run time of 32 minutes.

2) injection conditions: an injection temperature of 310ºC was used for all samples with injection volumes of: 1 µL with a 1:20 split for cholesterol analysis in milk; 2 µL splitless for all phytosterol analysis in milk; and 1 µL with a 1:5 split for animal feed samples.

The following mass spectrometry conditions were applied:

1) MS source temperature set at 230ºC and MS quadrupole at 150ºC, scan parameter from 50-600 amu and a solvent delay at 5 minutes.
2) other columns used for method development include a DB-17MS (50%-phenyl)-methylpolysiloxane 60 m x 0.25 µm x 0.25 µm film thickness capillary column was also used during the optimisation stage.

3.2.5. Animal Feed and Milk Samples

In total 342 samples of animal feed and milk were tested for this research. This included 309 milk and animal feed samples from five different experiments from 2009-2015 (see Chapter 5).

A total of eighteen types of animal feed samples were grouped according to plant type and structure. This was performed to efficiently verify the optimised conditions on as many matrix types as possible. Shown in Table 3.1 is the grouping of the animal feed according to plant species and structure. From each feed class, one feed type was selected to represent the group as highlighted in

yellow. In addition, there were three formulated protected feeds, a sterol protected feed, an omega protected feed and a canola/soy protected feed.

Table 3.1: Animal feed classification

Animal feed classification					
Poaceae (grass)					
Grain/seed	Stems/leaves	Food additive	Pomace	Tree bark	Oils
Wheat	Lucerne	Mineral mix	Grape marc	Tannin	Vege pure E
Barley grain	Maize silage	Palabind molasses powder			Cotton seed oil
Maize grain	Pasture (ryegrass)				
Canola meal	Pit silage				
Dairy concentrate	Pasture silage				
Omega protected feed					
Sterol protected feed					
Canola protected feed					

Subsamples of milk from each cow were collected and combined to make one representative or composite sample from each treatment group. This is common practice for milk research whereby the mixing of many samples is performed to form a representative reflection of a group of samples. This was achieved by taking equal portion from each sample with thorough mixing to ensure it was homogenised in order to normalise the variation within the herd and to reflect normal practises within the dairy production industry. Commercially produced milk is prepared by combining and homogenising milk from different cattle herds

and farms to enable consistent quality control of product distributions (Dairy Australia 2017).

The composition of the sample for this study also allowed for efficient sample management and analysis. For example, if composite samples were not utilised, a total of 192 milk samples would have been collected and analysed for only 1 of the 5 sets of cattle feeding experiments (see Chapter 5). With such large sample numbers, it would have been time consuming and utilised most of the resources available for this research and as a result, would not have allowed for a broader investigation into other feeding regimes.

All milk and cattle feed samples were stored in a freezer at -20˚C until use. Prior to analysis the samples were thawed in a refrigerator, milk samples were than homogenised using a high-powered homogenizer (Robot Coupe Blixer 3) and then cattle feeds were milled as homogenously as possible using grinding mill (Foss CT 293 Cyclotec^{TM}) The specific gravity of each milk sample was also measured using the Anton Paar density meter. In addition, all sterol results are expressed in mg/100 mL for milk samples and in mg/kg for cattle feed samples as per dry matter to account for the moisture content.

It is acknowledged that due to the length of storage of the milk samples, changes to the composition of the milk and milk fat may have occurred (Chang *et al.* 2012; García-Lara *et al.* 2012). However, since all the samples within the experiments were collected, stored and analysed at the same time, any degradation of the milk within the experiments would be similar and therefore comparisons are deemed to be valid. In addition, duplicate samples and comparisons to literature

reports were found to be comparable for both the cattle feed and milk with regards to sterol content (Gorban and Izzeldin 1999; Piironen *et al.* 2002b; Reklewska *et al.* 2002; Ruibal-Mendieta *et al.* 2004; Foods Standards Australia New Zealand 2010).

3.2.6. Moisture Content

The moisture content in the cattle feed was determined gravimetrically using the drying method (O'Kelly and Sivakumar 2014). In brief, a metal crucible with sand and metal paper clip was weighed prior to sample addition. An accurate weight of 1 g of sample was added to the metal crucible containing the sand and metal paper clip. Water was added to the crucible and mixed using the metal paper clip. Sample was then placed in an oven at 100˚C for 24 hours or until a constant a weight was obtained. The sample was then placed in a desiccator to cool to room temperature and was reweighed. The moisture content was determined by the difference before and after drying.

3.3. Extraction Protocols

Two main extraction protocols were developed specifically for milk and for a variety of cattle feeds. In addition, the animal feed protocol also included a SPE clean-up step. All extracts were analysed by GC-FID/MS and quantitation was performed using traditional calibration curves for each identified sterol.

3.3.1. Extraction Protocol for Milk

For the milk samples, 5 mL was transferred into a 44 mL screw cap vial containing 2-3 boiling chips. A 5 mL aliquot of heptane, known amounts of surrogate

standards and 5 mL of 8 M ethanolic HCl was added to the sample vial which was then caped, mixed and incubated at 80˚C for 30 minutes. The vial was shaken intermittently every 10-15 minutes 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 minutes with intermittent shaking every 10-15 minutes. Following this second incubation, the sample vial was cooled to room temperature before 4 mL of deionised water 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 using nitrogen gas. Sample extracts were transferred to a GC vial and evaporated to dryness under nitrogen, after which 300 µL of BSTFA+1%TCMS and 700 µL 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 minutes prior to GC analysis.

3.3.2. Extraction Protocol for Animal Feed

For the animal feed samples, 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 surrogate standards and 10 mL of 4 M aqueous HCl was added to the sample vial, which was then caped, mixed and incubated at 80˚C for 30 minutes. The vial was shaken intermittently every 10-15 minutes 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 minutes with intermittent shaking every 10-

15 minutes. 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. This was then followed by sample clean-up using SPE.

Prior to sample clean-up, the amino propyl solid phase 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 and 80:20% (v/v) methanol: chloroform mixture, blown down and then transferred to a GC vial for derivatisation. Sample extracts were transferred to a GC vial and evaporated to dryness under nitrogen, after which 300 µL of BSTFA+1%TCMS and 700 µL 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 minutes.

3.3.3. Enzymatic Treatment Protocol

A total of four experiments were conducted for the enzymatic studies with all experiments conducted using Novozyme 188 in accordance with the following:

A known volume of *β*-cholestan-3*α*-ol was added to a 44 mL vial. The vial and its contents were then blown down to dryness using nitrogen prior to sample weighing. A 4 mL aliquot of Milli Q water was added to the sample vial and the

pH adjusted to 5 using 0.5 M HCl. The volume of the sample was adjusted to form a 10% suspension using water. The sample was then spiked with 300 µL Novozyme and 20 µL of the diluted Ultraflo L. The sample vial was than capped, mixed and placed in a water bath for 24 hours at 40˚C with magnetic stirring. The samples were then heated to 100˚C for 10 minutes in a water bath to deactivate the enzymes. A 10 mL aliquot of 5 M KOH and 5 mL of heptane was added to the mixture. The sample vial was again capped, mixed and incubated at 80˚C for 30 minutes. After incubation, the samples were allowed to cool to room temperature before 4 mL of water and 7 mL of 6.5 M aqueous HCl was added. The sample was again capped, mixed and centrifuged. A portion of the organic layer was transferred to a GC vial and was blown down to dryness with nitrogen. A volume of 300 µL of BSTFA+1%TCMs and 325 µL of pyridine was added to the GC vial which was then capped, mixed and incubated at 80˚C for 20 minutes. Analysis by GC was then performed as described previously. Details of the trials are presented in Table 3.2.

Table 3.2: Details of enzyme trials

Enzyme:	Novozyme	Ultraflo L	Sherzyme	Celluclast
Trial A	300 µL	20 µL		
Trial B	300 µL		205 µL	
Trial C	300 µL	20 µL		105 µL
Trial D	300 µL	20 µL	205 µL	105 µL

Chapter 4. Method Development and Validation

4.1. Overview

The main focus of this thesis is to investigate the relationship between various dairy cattle feeds and the resulting phytosterol profiles and levels in milk. In order to fulfil this aim, suitable analytical methods are required specifically for milk and the various animal feed matrices. The main driving point of the method development is sensitivity, selectivity, robustness, time and labour efficiency. The method will must also be capable of measuring total phytosterols including conjugates and should be streamlined to efficiently process a large number of samples such as those tested in this study (approximately 350 samples).

This chapter therefore explores a method development process in order to: optimise saponification and acid hydrolysis conditions; explore the potential for enzymatic treatment; investigate SPE clean-up; perform GC-FID/MS optimisation; and perform method validation (i.e. limit of detection, limit of reporting, linearity, robustness, repeatability, recovery and the determination of measurement uncertainty).

The optimisation of the saponification process investigated eight main aspects to attain time, labour, reagent efficiencies, reduced cost (where possible) and sterol liberation. This included the use of quantification standards (i.e. surrogates), the effects of: potassium hydroxide concentration; incubation temperature and time; sample size; extraction solvent; and silylating reagent; and finally, efficiency adaption.

The optimisation of the extraction protocols was performed using GC-FID with confirmation performed when required using GC-MS coupled with FID (see Chapter 3).

The contents of this Chapter and methods from Chapter 3 have been published in two articles:

- Duong S., Strobel N., Buddhadasa S., Stockham K., Auldist M., Wales W., Orbell J., Cran M., Rapid measurement of phytosterols in fortified food using gas chromatography with flame ionization detection. Food Chemistry, 2016, 211, pp.570–576. https://doi.org/10.1016/j.foodchem.2016.05.104

- Duong, S. Strobel. N, Buddhadasa. S, Stockham. K Auldist. M, Wales. W Orbell. J, Cran, M., Influence of Acid Hydrolysis, Saponification and Sample Clean-up on the Measurement of Phytosterols in Dairy Cattle Feed Using GC/MS and GC/Flame Ionization Detection. *Journal of Separation Science*, 2018, 41(17) 3467-3476. https://doi.org/10.1002/jssc.201800484

4.2. Quantification Standards

In this research, quantification of sterols was carried out by using relative response factors between phytosterols. The surrogate standards 5α-cholestane and 5β-cholestan-3α-ol were selected based on their reported use by other

researchers (Lagarda *et al.* 2006; Bradford and Awad 2007; Ostlund 2007; Clement *et al.* 2010) as shown to Figure 4.1. 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.

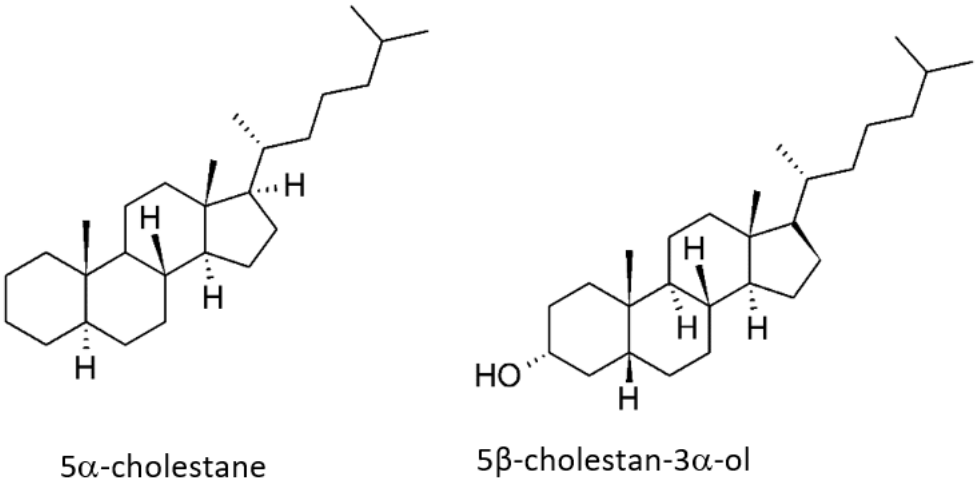

Figure 4.1: Internal standard structures (Steraloids 2018)

Table 4.1 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 4.1), 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.

The main difference between these surrogates is the absence of a hydroxyl group located on the 3rd carbon in the 5α-cholestane structure (Moreau *et al.* 2002). 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 (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).

For the purpose of this research, it was decided that quantitation would be performed on the milk and cattle feed samples using individual reference standards in order to provide greater quality assurance. Moreover, the surrogate 5β-cholestan-3α-ol was used as a response corrector to compensate for any phytosterol loss or degradation during the extraction process.

Table 4.1: Quantification of surrogate standards in spiked samples

Surrogate	Expected value	5β-cholestan-3α-ol	5α-cholestane
Number of samples		7	7
Cholesterol in milk powder (mg/100 mL)	13 ± 1.3	12.9 ± 0.1	14.3 ± 0.2
Total sterols in Vega pure E (mg/100 g)	59600 ± 5960	53748 ± 310	56496 ± 3200

4.3. Optimisation of Saponification

4.3.1. Effect of Potassium Hydroxide Concentration

Various concentrations of potassium hydroxide (KOH) were evaluated to affirm the optimal molarity for steryl ester liberation. Evaluation of recoveries on Vega pure E and milk powder matrices were compared to certified values or those provided on product nutritional labels. A range of KOH concentrations for saponification have been previously reported ranging from 2 to 6 M (Phillips *et al.* 1999; Liu *et al.* 2007; Feng *et al.* 2015). In this study, the results showed acceptable recovery levels from milk and Vega pure E using different KOH mixtures as shown in Figure 4.2. 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 after acid hydrolysis which is commonly employed before saponification to liberate sterol glycosides (mainly from plants matrices) (Moreau *et al.* 2002; Phillips *et al.* 2002; Laakso 2005).

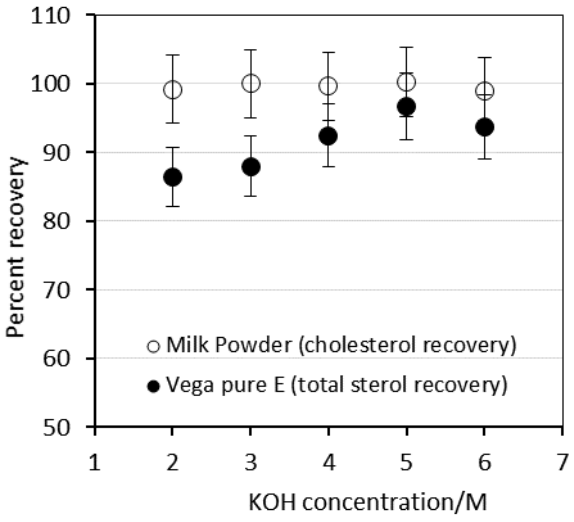

Figure 4.2: Effect of KOH concentration on sterol recovery

4.3.2. Effect of Incubation Temperature

Both hot and cold saponification are frequently employed for sterol measurement with hot saponification employing high temperatures during hydrolysis with incubation times ranging from 10 to 90 minutes (Dutta 2004; Laakso 2005; Liu *et al.* 2007; Clement *et al.* 2010). 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 (Dutta 2004). In this study, 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 4.3. Based on this recovery data and with consideration of the safety aspects of applying high temperatures to solutions containing alcohols, an optimal incubation temperature of 80°C was selected (Piironen *et al.* 2002a; Dutta 2004).

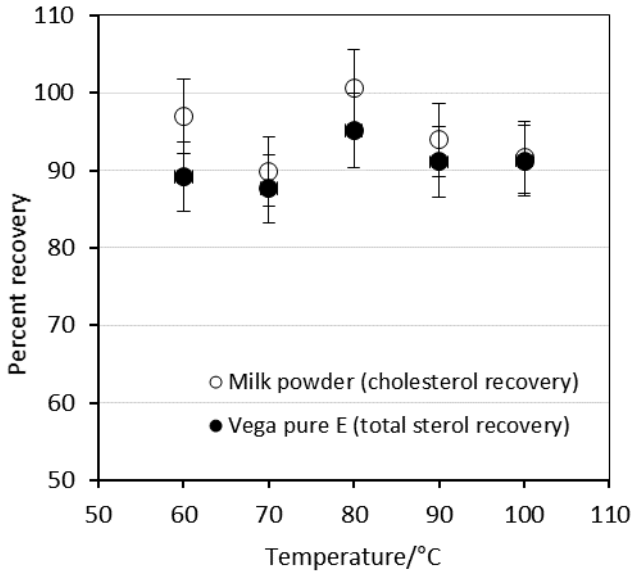

Figure 4.3: Effect of incubation temperature on sterol recovery

4.3.3. Effect of Incubation Time

The most effective saponification incubation time will vary based on the matrix type so it is important to select the minimum incubation period that will suit the majority of matrices. Incubation times ranging from 10-60 minutes at 10-minute increments were evaluated to determine the minimum period required for the saponification process and the results are shown in Figure 4.4. For both the Vega pure E and milk powder samples, complete saponification was observed after an incubation of only 10 minutes. 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 existing saponification methods performed at 80°C (Piironen *et al.* 2002a; Dutta 2004).

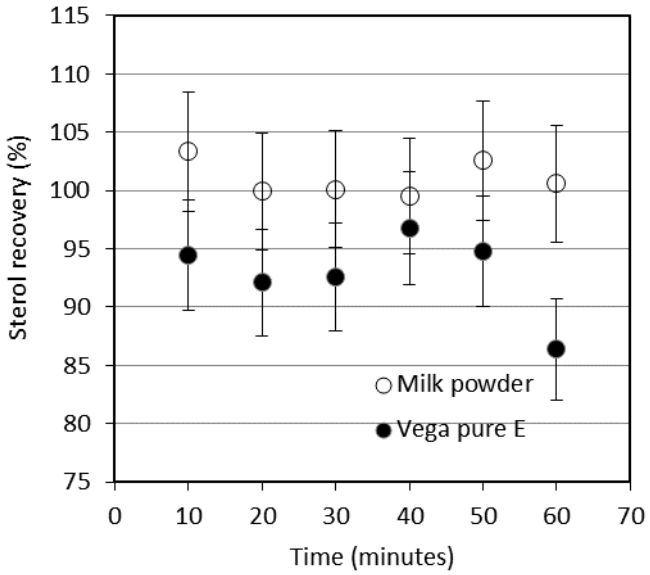

Figure 4.4: Effect of incubation time on sterol recovery

4.3.4. Effect of Sample Size

The amount of sample used in the extraction is a significant consideration when implementing saponification, particularly with regard to the solvent requirements. Insufficient alcohol, for example, can reduce the efficiency and effectiveness of the process (Dutta 2004; Lagarda *et al.* 2006; Clement *et al.* 2010). As mentioned previously the key purpose of saponification is to produce alcohol and salt from the fat portion of the sample thereby liberating the sterol. Ethanol or methanol is a critical factor during the processes as this is the medium in which the saponification will precede in. Ethanol or methanol is used during saponification to disband the lipid to increase surface area resulting in better hydrolysis (Laakso 2005). When insufficient alcohol is implemented during the hydrolysis it can create gel like mixture, making it difficult for extraction. This also indicates that complete hydrolysis may not have occurred as the gel is a cross-linkage between the salt and the fat (Dutta 2004). As a result, this leads to decrease extraction efficiency resulting in poorer recoveries (Laakso 2005). For this research, it was found 0.15 g of fat per 5 mL of alcohol was required during saponification.

4.3.5. Effect of Extraction Solvent

Plant sterols excluding their glycoside conjugates are soluble in a board range of organic solvents. Sterol extraction from published literature has been achieved by many co-workers using such solvents as hexane, heptane, toluene, and petroleum ether only to name a few (Moreau *et al.* 2002; Liu *et al.* 2007; Bedner *et al.* 2008; Clement *et al.* 2010). Heptane, hexane, toluene and petroleum ether was investigated to determine which solvent would provide the highest sterol

recovery, selectivity and liquid partitioning during extraction. These solvents were selected for this investigation based on literature references and their availability in the laboratory.

While maintaining the saponification conditions, sterol extraction from the secondary reference material Vega pure E and milk were performed. Comparisons between the cholesterol in the milk and the total phytosterol in the Vega pure E showed less than 5% difference between the different solvent extracts and insignificant or little changes differences were observed for the sterol profiles in the two matrices. Heptane was selected as the extraction solvent for this research which was selected based on its well-defined liquid-liquid partitioning barrier during extraction and its high boiling point compared to the others solvents. The boiling point of the solvent was taken into consideration as there was the potential to concurrently perform sterol extraction during saponification process.

4.3.6. Effect of Silylating Reagent

For GC analysis, sterol extracts are frequently derivatised using silylating 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 (Supleco 1997; Moreau *et al.* 2002; Thermo Scientific 2008). In this work,

BSTFA+1%TCMS was utilised in accordance with the manufacturer's instructions (Supleco 1997; Thermo Scientific 2008) but in order to improve overall efficiencies, the recommended derivatisation incubation periods and amount were investigated. In this work, the maximum sterol recovery was achieved within 10 minutes of incubation time with no changes observed for prolonged incubation using silylating reagent greater than 100 µL (Figure 4.5). Based on this result, an incubation time of 20 minutes was selected to ensure thorough derivatisation and 300 µL of BSTFA reagent which equates to a 40 minute reduction compared to the previous in-house method. The incubation period of 20 minutes was chosen to give greater robustness to the method and for its application to a potentially broad range of matrices.

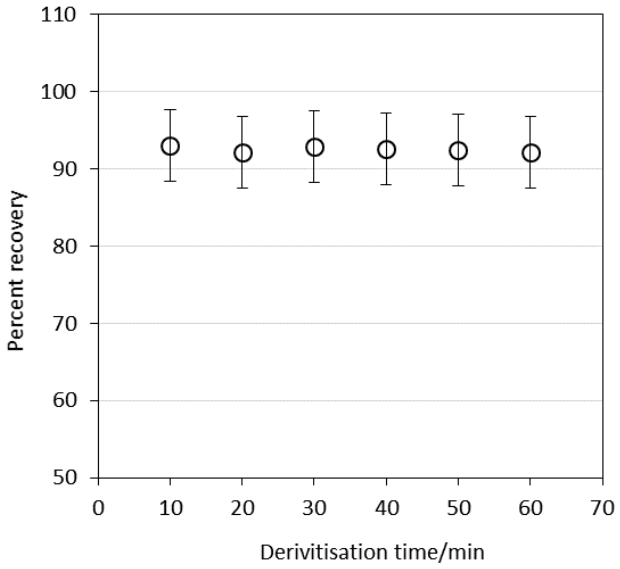

Figure 4.5: Effect of derivatisation time

4.3.7. Efficiency Adaption

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. This is followed by the use of hexane, cyclohexane, toluene or heptane to extract the sterols using incubation temperatures ranging from 60-100°C (Dutta 2004; Lagarda *et al.* 2006; Clement *et al.* 2010). Using a Plackett–Burman experimental design (Ruggeri *et al.* 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 4.2 (Experiments A-F).

Sodium hydroxide in methanol, toluene and an incubation temperature of 100°C were chosen for comparison as they are commonly used in this type of extraction (Dutta 2004; Lagarda *et al.* 2006; Dulf *et al.* 2008; Clement *et al.* 2010). 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 4.3 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.

Table 4.2: Plackett-Burnman experimental design

#	Extraction solvent	Incubation temperature °C	Saponification mixture	Saturated NaCl	Aqueous HCl	Water
A	Toluene	100	2.3 M NaOH in methanol	✓	✓	
B	Toluene	80	2.3 M KOH in methanol	✓	✓	
C	Toluene	100	2.3 M NaOH in methanol	✓	✓	
D	Toluene	100	2.3 M NaOH in methanol	✓	×	
E	Heptane	100	2.3 M NaOH in methanol	✓	✓	
F	Toluene	100	2.3 M NaOH in methanol	×	✓	
G	Heptane	80	5 M ethanolic KOH	×		✓
H	Heptane	80	5 M ethanolic KOH	✓		✓
I	Heptane	80	5 M ethanolic KOH	✓		×

Table 4.3: Recovery of cholesterol, total sterols and *β*-sitosterol from different matrices

#	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

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 (Dutta 2004; Laakso 2005). 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 (Dutta 2004; Clement *et al.* 2010) and this was significant for all cattle feed samples. These aspects were then applied to the optimised method to determine if this would improve time and labour efficiencies (Table 4.2 in Experiments G-H). The results shown in Table 4.3 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 and was shown to be as effective as the 6 M acid used during the experiment.

4.3.8. Saponification of Cattle Feed Matrices

The optimised conditions determined for milk and Vega pure E were applied to the selected cattle feed matrices. To confirm the optimised saponification conditions identified for milk and Vega pure E, variables hydrolysis conditions above and below were applied to the test samples. This included saponification experiments at temperature at 70, 80 and 90˚C at incubation times of 20, 30, 60 and 90 minutes, using 3, 4 and 5 M of potassium hydroxide in ethanol. This experiment was performed on lucerne, grape marc, wheat, tannin and mineral mix which were the selected matrices representing the main groups of cattle feed types. Shown in Table 4.4 are the results for cattle feed verification which presents the overall average, standard deviation, and %RSD. The results show less than 10% RSD from the combined comparison of saponification times and temperature. A further comparison between the molarities also showed <10% variable between the same conditions. This demonstrated that the optimised conditions for milk saponification were also suitable for application to the feed samples.

Table 4.4: Cattle feed verification mg/100 g

		Lucerne			Grape marc			Wheat			Tannin			Mineral Mix		
	Molarity (M)	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5
	Time (min)															
70˚C	20	44.4	44.5	60.5	148.3	145.2	151.3	28.5	27.3	28.9	2.0	2.6	2.6	35.5	34.7	36.2
	30	48.2	48.3	44.9	145.6	147.3	154.9	30.6	32.6	30.9	2.0	2.5	3.0	36.1	37.2	36.6
	40	51.4	50.1	50.4	104.8	151.8	153.1	33.0	32.5	31.6	1.9	2.7	2.7	37.1	37.8	37.1
	60	53.1	53.0	51.0	149.2	150.8	155.5	32.3	33.7	34.5	2.2	2.4	2.8	36.9	37.5	36.7
	90	54.3	53.9	52.1	150.7	148.6	148.2	33.2	33.1	34.3	2.0	2.5	2.7	37.5	37.3	37.8
80˚C	20	48.3	50.2	49.5	151.2	150.0	142.9	30.4	31.3	31.8	2.0	2.5	3.0	43.8	42.0	46.9
	30	50.2	53.5	50.6	150.0	144.4	147.5	34.6	31.7	33.8	1.9	2.7	2.9	43.5	41.9	42.5
	40	54.0	56.2	52.7	148.0	141.1	152.6	32.8	32.9	34.8	1.9	2.3	3.1	44.2	42.5	43.8
	60	55.9	56.6	54.7	152.0	142.1	146.3	36.0	35.3	36.7	1.8	2.5	2.9	47.3	44.4	44.3
	90	58.7	57.4	42.7	153.0	154.7	148.5	36.8	38.5	34.6	1.8	2.2	3.0	48.7	47.9	42.5
90˚C	20	49.1	48.5	49.8	143.1	155.3	153.3	30.8	32.8	35.1	1.8	2.5	3.0	41.4	41.9	41.2
	30	51.7	52.9	50.7	147.2	153.2	158.3	35.8	33.4	34.8	1.8	2.2	2.9	42.1	42.3	41.6
	40	50.0	51.7	51.9	145.5	152.2	155.1	35.7	36.1	34.5	1.7	2.3	2.7	41.2	42.4	41.9
	60	50.4	52.3	52.7	155.2	152.7	151.7	37.3	38.7	35.2	1.8	2.5	3.1	44.2	41.7	42.5
	90	53.7	54.8	54.7	159.6	153.1	150.7	39.5	37.8	36.5	1.8	2.4	2.9	43.2	40.1	42.7
Average		51.6	52.3	51.3	146.9	149.5	151.3	33.8	33.8	33.9	1.9	2.5	2.9	41.5	40.8	40.9
Standard deviation		3.5	3.5	4.1	12.4	4.5	4.1	3.1	3.0	2.1	0.1	0.2	0.2	4.1	3.4	3.3
%RSD		6.9	6.7	8.0	8.4	3.0	2.7	9.0	8.9	6.3	5.9	6.7	5.3	9.9	8.3	8.0

4.4. Enzymatic Treatment

A preliminary enzymatic treatment was investigated to determine if this technique would be suitable for the samples in this research. In this case, method optimisation was not performed and the enzymatic conditions were based on previously reported protocols with small experimental adjustment to suit the available resources and needs of the research. A total of four trials were conducted using the same conditions with varying enzyme reagents. The results from these trials were compared to their acid hydrolysis counter-parts in order to compare the effectiveness of the treatments. The experiments were performed on milk powder, lucerne hay (cattle feed), wheat and Vega pure E (steryl ester). These matrices were chosen as they represented the main sample types used in this overall research. Table 4.5 shows the experimental design used for the enzyme treatments and in each case the same incubation conditions were used (temperature 40°C, pH 5.0, time 24 h) with each enzyme spiked at 60 UI/0.5 g of sample.

Table 4.5: Experimental design for enzyme treatment

	Enzyme			
#	Novozyme 188	Ultraflo L	Celluclast 1.5 L	Shearzyme Plus
A	X	X		
B	X	X		X
C	X	X	X	
D	X	X	X	X

From the trial results shown in Table 4.6, both the milk powder and Vega pure E obtained satisfactory results since the major sterol components are steryl esters. This indicated that the enzymatic treatment did not interfere with the saponification process. A comparison between the *β*-sitosterol content in lucerne was able to show both the hydrolysis results (70 mg/100 g) and the highest value obtained from the enzyme trials (trial A of 63 mg/100 g) were comparable as they were within 10% of each other. This was promising as no optimisation had been performed to gain comparable results. However, the trials also indicated that the other carbohydrate-based enzymes did not compare well to the hydrolysis treatment with poorer sterol recoveries.

Table 4.6: Enzymatic treatment comparison

	Sterol recovery (mg/100 g or mg/100 mL) *					
	A	B	C	D	Hydrolysis	Expected value
Wheat	25	23	27	29	63	-
Lucerne	63	50	51	46	70	-
Milk powder	13	13	14	13	13	13
Vega pure E	57002	58143	48333	56796	62400	62400

*results for wheat and lucerne are of *β*-sitosterol sterol, milk powder of cholesterol and Vega pure E is of total phytosterol.

The results for the wheat were clearly unsatisfactory with the enzyme treatments resulting in 54-74% lower recovery than that of the acid hydrolysis. This is most likely due to sample particulates inhibiting the enzyme treatment whereby the large surface area and the penetration of the reaction in the matrices is limited (Toivo *et al.* 2000). Both lucerne and wheat were milled as finely as possible to facilitate the sterol extraction, however it was observed that the wheat was considerably more hydrophobic and resistant to liquid penetration thereby inhibiting the liberation of the sterol glycosides. Conversely, acid hydrolysis aided

in the destruction of the plant matrices and subsequent sterol extraction (Dutta 2004; Lagarda *et al.* 2006; Clement *et al.* 2010). The addition of other carbohydrate enzymes was expected to similarly aid in the destruction of the sample matrices to free sterol glycosides, however, this was not the case and the poor recoveries led to discontinue enzymatic treatment.

Based on the results of these experiments and a literature search on enzymatic treatments, acid hydrolysis for glycoside liberation was pursued in preference to further enzymatic optimisations. Ideally enzyme treatment would be the preferred technique, as it would prevent the undesirable isomerisation of fucosterol and avensterol (Kamal-Eldin *et al.* 1998; Münger and Nyström 2014). However, it was clear that acid hydrolysis was very efficient and robust compared to enzyme treatment. Although the enzyme treatment technique was not optimised, the long incubation times, the labour required to maintain the narrow pH conditions for each sample, and the expensive nature of the various enzymes deemed this approach unsuitable to pursue further. Clearly acid hydrolysis was more suitable for the high sample output requirements (approximately 350 samples) with limited personal, narrow time-frames and limited economic resources. In addition to these resource limitations, consideration was also given to the possible isomerisation of the Δ5-Avenasterol, Δ7-Avenasterol and fucosterol as a result of acid hydrolysis (Kamal-Eldin *et al.* 1998; Toivo *et al.* 2001). In this case, however, it was concluded that these sterols would be minor components compared to the total phytosterol content and acid hydrolysis was not discounted. It was also problematic to adjust the pH of the raw milk samples without using larger vessels

during extraction and therefore large batch extractions were difficult to manage for enzymatic treatments.

4.5. Acid Hydrolysis Optimisation

To optimise the acid hydrolysis, four main parameters were investigated including acid medium, acid concentration, incubation temperature and incubation time.

4.5.1. Effects of Acid Medium

Previous studies have utilized two distinct acid hydrolysis solutions prepared in either water for food samples (Lagarda *et al.* 2006; Clement *et al.* 2010), or in ethanol for plant materials (Liu *et al.* 2007). Given the broad range of matrices in the present study, a hydrolysis solution composition that was suitable for all the matrices 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 optimisation, milk powder, lucerne and Vega pure E were selected to represent the major sample types, i.e. milk, plant and fatty samples respectively.

Figure 4.6 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 (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 as supported by previous research such as those reported by Piironen *et al.* (2002b); Laakso (2005) and Clement *et al.* (2010).

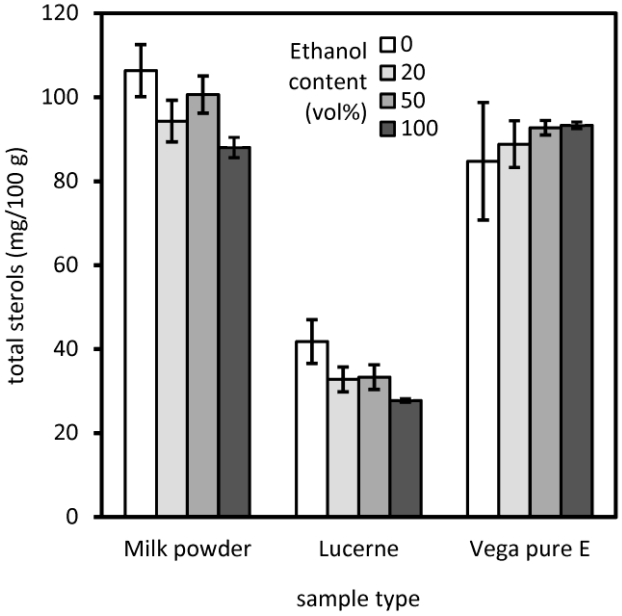

Figure 4.6: Comparison of hydrolysis medium

4.5.2. Effect of Acid Concentration

Acid concentration is critical to efficiently break the glycosidic bonds *via* hydrolysis in order to maximize the liberation of sterol glycosides (Moreau *et al.* 2002; Dutta 2004). In this experiment, HCl concentrations of 4, 5, and 6 M were trialled with results showing that no significant recoveries were gained of the three selected matrices using higher acid concentration as shown in Table 4.7 The hydrolysis incubation time was also studied in order to optimise the time needed

to ensure all glycosidic bonds are cleaved (Moreau *et al.* 2002; Dutta 2004). Although the saponification incubation time has been investigated previously by this research, the present experiment further explored the hydrolysis time prior to the addition to saponification mixture to ensure that the hydrolysis solution did not affect the saponification process. Overall, 4 M HCl solution was selected as the optimal condition.

Table 4.7: Influence of acid molarity on sterol recovery

	Lucerne	**Milk powder**	**Vega pure E**
Acid Concentration	*β*-sitosterol mg/100 g	Cholesterol recovery %	Total sterol recovery %
4 M	43	108	93
5 M	46	100	92
6 M	47	100	88

4.5.3. Hydrolysis Incubation Temperature and Time

Three incubation time brackets were selected, i.e. 30, 60 and 90 minutes, and were applied to the hydrolysis of the three representative samples. As shown in Table 4.8, the results demonstrate that complete hydrolysis was obtained with the minimum incubation period of 30 minutes with no significant increase when incubated for longer times.

Table 4.8: Acid hydrolysis incubation time

	Milk powder	**Lucerne**	**Vega pure E**
Time (min)	Cholesterol recovery %	*β*-sitosterol mg/100 g	Total sterol recovery %
30	108	38	91
60	108	31	92
90	13	32	92

4.6. Combined Saponification and Acid Hydrolysis

The optimised saponification and acid hydrolysis conditions demonstrated that individually, both protocols are suitable for sterol analysis in both milk and animal feed matrices. To confirm the efficacy of the combined hydrolysis protocol, verification of the saponification with the acid hydrolysis was performed in order to verify that the acid hydrolysis did not alter or hinder the subsequent saponification process.

In this trial, three incubation time brackets were selected, i.e. 30, 60 and 90 minutes, and were applied to the hydrolysis of the milk, Vega pure E and lucerne. As shown previously in Figure 4.7, the results demonstrate that complete hydrolysis was obtained with the minimum incubation period of 30 minutes 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 (Skoog *et al.* 1996). Further verification with different feed matrices was required to ensure the saponification incubation time of 30 minutes was still applicable from previously optimised conditions in this study. Figure 4.7 demonstrates that 30 minutes 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.

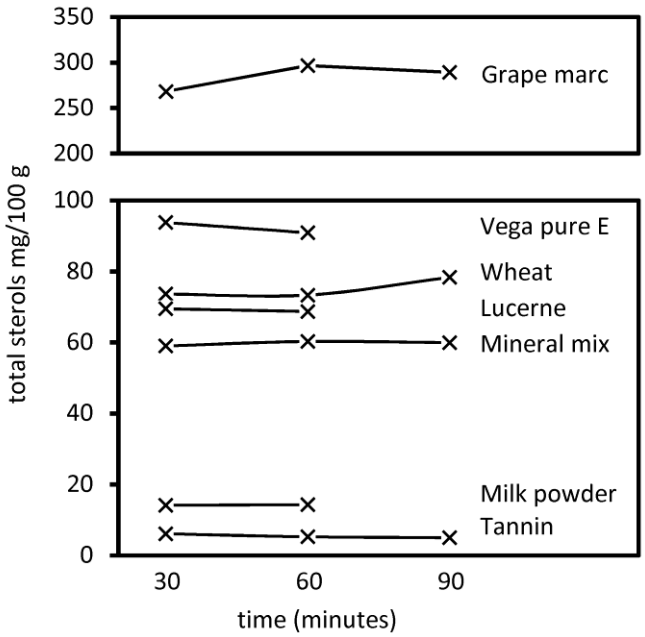

Figure 4.7: Saponification verification for animal feed

4.7. Solid Phase Extraction Clean-up

The initial work was performed using reference standards, with both phases able to perform satisfactorily. Recoveries for the Vega pure E ranging from 90-110% for both silica and aminopropyl SPE cartridges. However, when sample extracts were trialled it was observed that no profile change was observed for the silica SPE and this was later confirmed using GC-MS/FID that the silica 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%). Shown in Figure 4.8 and Figure 4.9 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, whereas the silica is unable to remove non-sterol compounds from the extracts.

It was observed that for fractions collected by SPE, some phytosterols were lost, suggesting that the SPE sorbent capacity was too low for the extract concentration levels. A dilution of the lucerne extracts was therefore trialled on the SPE to determine the capacity required for the extracts. As shown in Table 4.9, a comparison between a 1 in 5 and 1 in 2 dilutions of the extracts with 320 mg sorbent was able to demonstrate significant recovery drops for both the surrogate standard and *β*-sitosterol in the 1 in 2 dilutions.

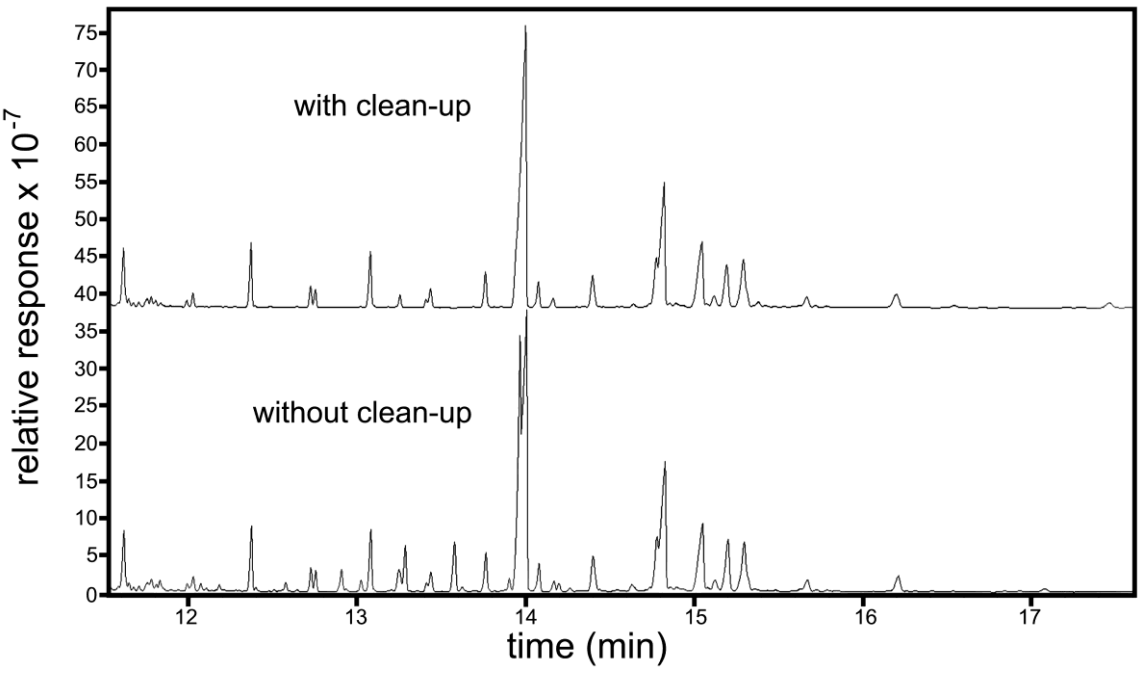

Figure 4.8: GC-FID chromatogram using silica SPE of lucerne extract

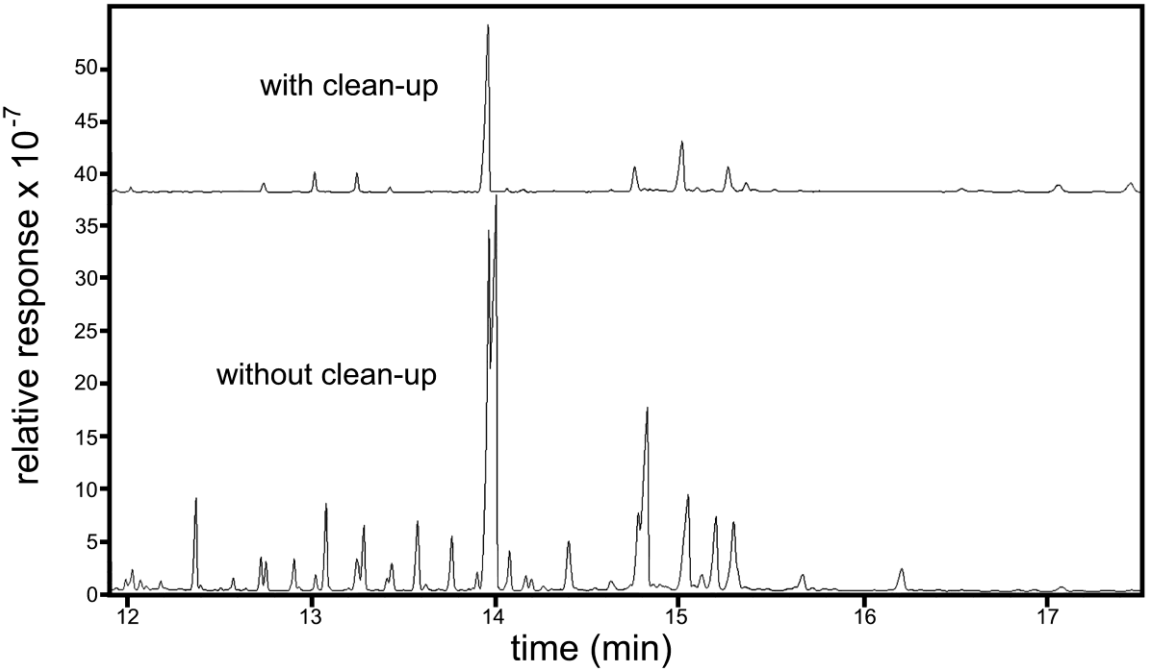

Figure 4.9: GC-FID chromatogram using aminopropyl SPE of lucerne extract

Table 4.9: Effect of extract dilution on SPE loading capacity

	% Recovery	
Dilution factor	Surrogate	*β*-sitosterol
1 in 5	102	116
1 in 2	23	38

For the majority of feed matrices, 1.5 g of sorbent was shown to be sufficient. However, in this study, 5 g of sorbent was used in order to safeguard against possible SPE overload capacity issues for unknown cattle feed matrices. 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, chloroform and a chloroform mixture were investigated with 45 mL of chloroform only required to elute the targeted sterols compared to 25 mL of the 80:20% (v/v) chloroform: methanol mixture. Shown in Table 4.10 is a comparison between these solvent systems for the elution of sterols from the grape marc extract. The results clearly demonstrate that a reduced volume of the mixed solvent results in a higher recovery than the higher volume of chloroform only as a chloroform methanol mixture has a greater polarity index.

Table 4.10: Effect of solvent composition and volume on sterol recovery

	% Recovery	
Eluting solvent	β-sitosterol	stigmastanol
Chloroform only, 45 mL	88	93
Chloroform: methanol (80:20), 25 mL	107	104

4.8. Verification of Different Sample Matrices

The composition of plant matrices is more complex when compared to milk or fat samples. Milk, for example, is primarily comprised of water with proteins, sugars, salts and fat (Foods Standards Australia New Zealand 2010). 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 solvent (Moreau *et al.* 2002). The addition of acid neutralises the alkaline saponification solution thereby increasing the ionic strength of the aqueous phase in order 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 back into their alcohol forms and subsequent solubilisation of these compounds into the organic layer thus creating non-targeted interferences (Skoog *et al.* 1996). 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 in order to optimise the extraction. This was demonstrated by comparing extractions with and without acid and SPE clean up and as shown in Table 4.11, there were no significant differences between treatments.

Table 4.11: Comparison of clean-up treatment on cholesterol recovery

System	Cholesterol recovery, %	Standard deviation
No acid, no clean-up	88.4	8.3
With acid and clean-up	89.0	0.3

*Expected value for the cholesterol content in the milk was 13 mg/100 mL

4.9. Instrumentation Optimisation

4.9.1. GC Separating Conditions

The chromatographic separation conditions reported in the literature were used as a starting point for this phase of the study (Laakso 2005; Brufau *et al.* 2006; Clement *et al.* 2010; Quintin 2010; Fernández-Cuesta *et al.* 2012). There were

two main types of capillary columns commonly used including a 5% Phenyl-95% methylpolysiloxane (HP-5MS) or 50% diphenyl-50% dimethylpolysiloxane (DB17 MS). A mixture of sterol standards was trialled to determine which column was more suitable and from the chromatogram, even with a slow oven rate to allow time for further separation, the DB17 MS column was unable to separate *β*-sitosterol and stigmastanol which are the two main phytosterol components in plant and food. For that reason, chromatographic optimisation was focused on the HP-5MS column.

The optimisation started with a generic low to high oven program ramp with an initial temperature of 50ºC held for 1 minute and ramped to 320ºC at 20ºC/min then held for 10 minutes. This was performed to identify and verify the elution temperature required for the sterols. Figure 4.10 is an example of a chromatogram produced under the above conditions which shows that most of the sterols elute between 14.6-15.5 minutes which related to temperatures above 280ºC. Due to the close elution retention of these sterols, the next step of the optimisation was to slow down the oven ramp leading into 280ºC temperature profile to allow for more separation time. This result also indicated that the starting temperature could also be raised to at least 200ºC.

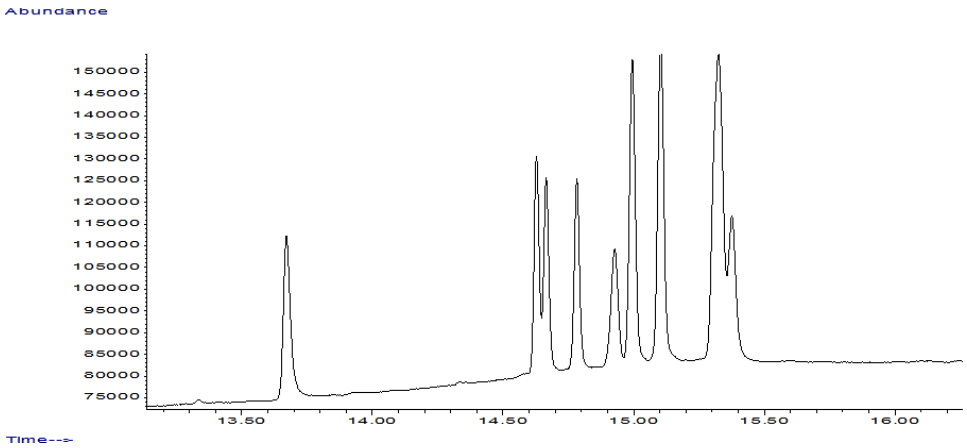

Figure 4.10: GC oven ramp optimisation

Following a series of oven ramp rate adjustments, the final optimisation condition for this study was found to be: initial oven temperature of 245ºC hold for 0.5 minutes, ramp to 265ºC at 2ºC/min then ramp to 290ºC 3.5ºC/ min and hold for 15 minutes. Using these optimised conditions, the separation of the sterol standards mix resulted in successful separation of 10 out of the 11 compounds as shown in Figure 4.11 final GC optimised conditions. Although the optimised GC conditions were unable to separate fucosterol and lanosterol, the final conditions were deemed satisfactory since both lanosterol and fucosterol would rarely occur together in the same matrix since lanosterol is derived from animal sources whereas fucosterol is plant-based (Brewington *et al.* 1970; Moreau *et al.* 2002).

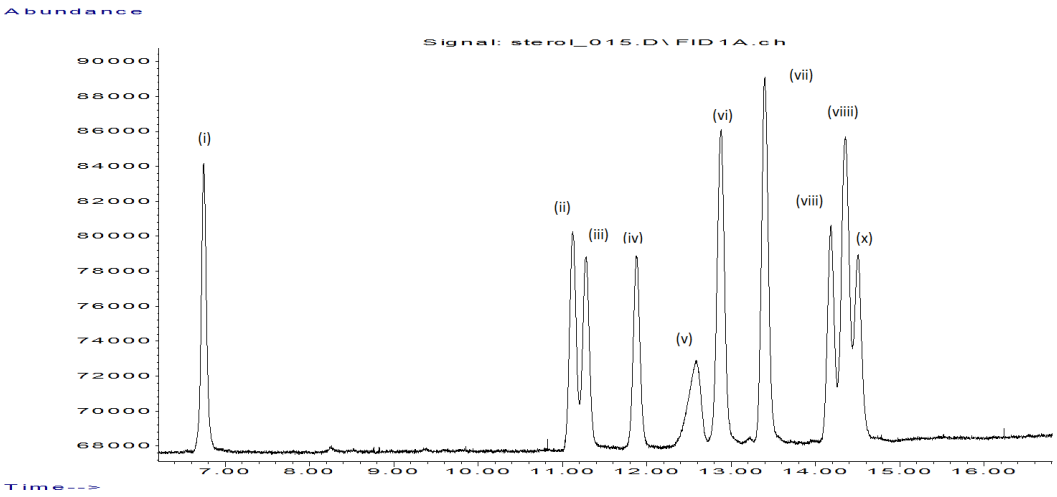

Figure 4.11: Final optimised GC chromatogram
(i) 5β-cholestan-3α-ol**, (ii) cholesterol, (iii) cholestanol, (iv) brassicasterol, (v) lathosterol, (vi) campesterol, (vii) stigmasterol, (viii) fucosterol + lanosterol, (viiii) *β*-sitosterol, and (x) stigmastanol**

4.9.2. Injection Conditions

The injection temperature used during the GC oven program optimisation was found to be suitable for the analysis therefore further optimisation was not required. The injection amount and splitting ratio were trialled on various standards and samples to ensure adequate sensitivity was obtained. From the trials, it was found that for low level phytosterol analysis in milk, a 2 µL splitless injection was required. For cholesterol analysis in concentrated milk extract, a 1 µL injection with a 1:20 split was used and a 1 µL injection with a 1:5 split was required for cattle feed extracts. Overall, the %RSD upon the injection of the standard based on the surrogate is 4.2%, which is considered within the acceptable range as per the standard operating procedure at the National Measurement Institute (2014).

4.10. Method Validation

Method validation was performed on the final extraction protocol using combined acid hydrolysis with saponification and saponification only with the latter method validation performed to determine whether it was suitable for a variety of fortified foods. In addition, method validation was performed to determine the limit of detection (LOD), limit of reporting (LOR), limit of quantitation (LOQ), linearity, repeatability, robustness and the measurement of uncertainty (MU) associated with the methods. Precision and accuracy were also taken into account with calculations of RSD values throughout. The samples used for method validation included CRM NIST meat homogenate 1546, NIST 3250 *Serenoa repens* seed, unfortified milk, fortified milk, fortified fat spread, fortified sliced cheese and fortified soft cheese. The method validation was performed in accordance with the standard operating procedure at the National Measurement Institute (2014) and NATA (2018).

4.10.1. Limits of Detection, Quantitation, and Reporting

In this study, the LOD, LOQ and LOR are defined in accordance with the National Measurement Institute (2014) standard operating procedure. In brief, the LOD is the determination of the lowest concentration of a given standard that could be detected using the instrumentation where the signal-to-noise ratio is greater than 5. The LOQ is the concentration of the standard that can be interpreted and is usually determined by multiplying the LOD by 3. The LOR is determined by taking into consideration the dilution factor, sample size and other extraction processes with reference to the LOD.

The LOD was determined by using diluted standards analysed under the previously optimised GC analysis conditions where the dilution was selected such that 5 times the signal-to-noise ratio was obtained for the standards. The LOD is usually set at 3 times greater than signal-to-noise ratio, however the increase to 5 times provided greater confidence for quantitation purposes (NATA 2018). The LOD was 0.1 mg/L for the flame ionisation detector (FID) and 0.25 mg/L for mass spectrometry (MS) detector. The LOQ was subsequently determined by multiplying the LOD by a factor of 3. The analysis of the milk samples was quantitated using the FID with the LOD at 0.3 mg/L at injection concentration.

The LOR is determined by multiplying the LOQ by a factor of 5 with the consideration of the method dilution factor and sample divisor (sample amount, or % recovery determined experimentally) (NATA 2018). In this research, a LOR of 0.02 mg/100 mL for individual sterols and 0.12 mg/100 mL for total phytosterol in milk was obtained. For cattle feed samples, the LOD of was determined to be 0.25 mg/L with a LOQ of 0.75 mg/L. With consideration of the weight of the feed samples used, dilution factors and recovery data, the LOR was determined to be 5 mg/100 g for individual sterols and 35 mg/100 g for total phytosterols. In addition, due the greater response sensitivity of FID compared to MS for the LOD, it was concluded that the FID would be used for quantification and MS for identification.

4.10.2. Reporting of Total Phytosterols

Total phytosterols were reported as only plant derived sterols and did not include cholesterol, cholestanol, lanosterol and lathosterol as they are mainly animal-

based sterols. For this study, the sum of brassicasterol, campesterol, campestanol, stigmasterol, *β*-sitosterol and stigmastanol was reported as the total phytosterol content. Even though fucosterol levels were measured, the levels were not included in the total phytosterols due to possible isomerisation during the acid hydrolysis.

4.10.3. Linearity

The linearity of analytical methods over the test range is a critical aspect in all analytical techniques. Determination of the linearity range allows for accurate quantitation of targeted compounds within a concentration range that is directly proportional between concentration and instrumentation response (Skoog *et al.* 1998; NATA 2018). A range of linearity of 0.1-200 mg/L was determined for *β*-sitosterol, cholestanol, stigmasterol campesterol, stigmastanol, fucosterol, lanosterol and lathosterol and of 0.1 to 100 mg/L for brassicasterol and campestanol. A broader range for these standards was not tested due to limited reference material although the range obtained was deemed to be sufficient given the sterol contents found in the samples. The linearity range for cholesterol was determined to be 0.1-1000 mg/L for both the FID and MS. Figure 4.12 shows an example of the calibration curve for cholesterol with the calibration curves for other sterols given in Appendix 8.

4.10.4. Ruggedness and Robustness

During the method development and optimisation, the most critical aspects of the method were found to be the use of acid after saponification for animal feed and the hydrolysis medium for the surrogate for quantification. Other changes in the

conditions of extraction and analysis were deemed to be insignificant with slight changes in these conditions demonstrating the method is robust and rugged (NATA 2018). As shown in previously in Table 4.3, the results were able to demonstrate that variables such as differences in incubation temperatures, extraction solvent and saponification mixture solutions were able to provide consistent recoveries for SRM and samples.

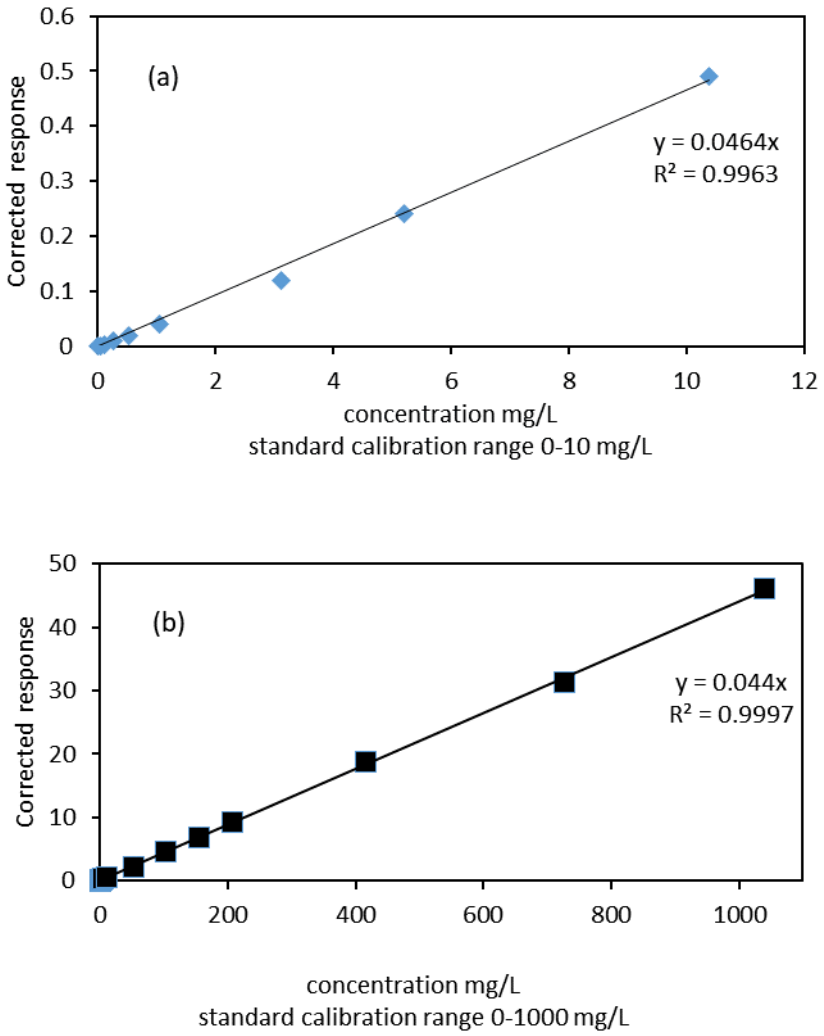

Figure 4.12 Calibration curve of cholesterol using GC/FID for (a) low and (b) high range concentrations

4.10.5. Sterol Recovery

Two main sets of recovery data were collected and the first recovery validation was performed using the saponification portion of the protocol on fortified food samples. These samples were selected since fortified foods contain a negligible amount of sterol glycosides (i.e. less than 1%) this included: a fortified fat spread (expected value of 8000 mg/100g), fortified slice cheese (expected value of 5500 mg/100 g for total sterols), fortified soft cheese (expected value of 5500 mg/100 g for total sterols), fortified milk (expected value of 350 mg/100 g for total sterols), certified meat homogenate NIST1546 (certified for cholesterol at 75 mg/100 g) and Vega pure E (certified for total sterols of 59364 mg/100g).

The second recovery validation data set was performed using both acid hydrolysis and saponification including the SPE step. This second validation was performed on reference material NIST *Serenoa repens* (certified for *β*-sitosterol, campesterol and stigmasterol at 45.4, 4.8 and 11.8 mg/100 g respectively), Vega pure E (certified for total sterols of 59364 mg/100 g) and spiked milk samples (spiked at 1xLOR, 2xLOR and 5xLOR, 0.02, 0.4 and 1 mg/100 mL).

Using the saponification protocol only, the recovery results for the first validation set are shown in Table 4.12 which demonstrates satisfactory recoveries ranging from 86 to 109% for the fortified cheese, fat spread, milk and the reference materials. Recoveries ranging from 80-120% at these levels are deemed satisfactory in accordance with the standard operating procedures at the National Measurement Institute Analytical Branch Victoria. The %RSD obtained for each sample was also small demonstrating good precision of the data.

Table 4.12 Validation of sterol recovery from fortified reference materials

	Total sterol recovery		
	Average recovery %	%RSD	# Replicates
Nist 1546*	89	3.1	7
Vega pure E	95	0.4	7
Fortified milk	109	0.3	3
Fortified fat spread	98	0.8	3
Soft cheese	91	1.1	3
Sliced cheese	86	1.9	3

*Only for cholesterol

The results for the second set of validation data focused on the different reference materials (Vega pure E, *Serenoa repens* and meat homogenate) with recoveries ranging from 95.0 to 104.3%. Again, this was deemed satisfactory with an 11.3% RSD representing the highest RSD as shown in Table 4.13. The recovery from the spiked milk samples shown in Table 4.14 ranged from 64.4 to 80.1% with recovery improving as the milk samples were spiked at higher levels. This was expected as the spiked levels were considered to be with the trace range. It was also concluded that the lower recoveries from the spiked milk were due to the matrix effects which was supported when comparing the recoveries in spiked water. For trace levels, recoveries ranging from 60-110% are deemed acceptable in accordance with the standard operating procedure at the National Measurement Institute (2014).

Table 4.13 Validation of sterol recovery from reference materials

Sterols	CRMs[a]	SRM (Vega pure E) [b]
	Mean % sterol recovery (%RSD)	Mean % sterol recovery (%RSD)
Cholesterol	104.3 ± 5.9[d] (5.7)	
Brassicasterol		100.6 ± 7.4 (7.3)
Campesterol	103.0 ± 5.2[c] (5.1)	100.0 ± 7.2 (7.2)
Campestanol		102.8 ± 10.8 (10.5)
Stigmastanol	98.4 ± 7.6[c] (7.7)	102.6 ± 9.5 (9.3)
β-sitosterol	95.9 ± 8.8[c] (9.2)	99.7 ± 7.1 (7.2)
Stigmastanol		97.6 ± 11.3 (11.6)
Total phytosterols		98.7 ± 7.2 (7.3)

Notes: Total samples: [a]9 for each CRM, [b]26 for SRM,
[c]Nist 3250 *Serenoa repens*, [d]Nist 1546 meat homogenate.

Table 4.14 Milk validation for total sterols

Spiked Sample	Mean % sterol recovery	%RSD
Milk		
1x LOR	64.4 ± 3.3	5.1
2x LOR	69.9 ± 3.0	4.3
5x LOR	80.1 ± 9.4	11.7
Water		
1x LOR	98.8 ± 2.7	2.8
2x LOR	102.6 ± 9.1	8.9
5x LOR	95.6 ± 0.6	0.6

*Note no SPE was performed on spiked milk and water samples

4.10.6. Measurement Uncertainty

The top-down MU approach was used in this research which incorporates validation data including recovery, duplicates, linearity and quality control for the

final uncertainty estimation. This approach was chosen as it would allow for the precision, accuracy and any systematic bias in the methods (NATA 2018). Two sets of MU determinations were performed to cover the broad range of sterol levels in the various samples. The first set was dedicated to the trace level determination of sterol content in milk with a reporting level of <0.02 mg/100 g and for individual sterols, and <0.12 mg/ 100 mL for total phytosterols. The second set was for sterol reporting levels in cattle feed <5.0 mg/100 g for individual sterols and <35 mg/100 g for total phytosterols as shown in Table 4.15.

Table 4.15 Measurement uncertainty

	Measurement Uncertainty %	
Sterol	Milk	Cattle feed
Brassicasterol	25	11
Stigmasterol	35	12
Campesterol	26	10
Campestanol	25	12
Cholesterol	21	13
β-sitosterol	25	12
Stigmastanol	33	14
Total phytosterols	24	11

Overall, the MU determined for the trace level determination at the LOR level of 0.02 mg/100 mL in milk for the individual sterols is ±35% with a 95% confidence interval (coverage factor of 2). The MU determined for the trace level determination at the LOR level of 5.0 mg/100 g in animal feed for the individual sterols is ±15% with a 95% confidence interval (coverage factor of 2, (Samuels

and Witmer 2003; NATA 2018)). For both levels, the highest MU was used to reduce MU underestimation and the analytical factors used for the MU calculation for each sterol are presented in Appendix 8. The validation data demonstrated that the method is suitable for the analysis of animal feed and milk at both trace and normal levels of sterols analyses.

4.11. Summary

The methods developed in this research utilised the used of acid hydrolysis and saponification while simultaneously extracting sterols thereby omitting labour intensive liquid to liquid extraction. Satisfactory recoveries and reduced interference were achieved with the use of an optimised procedure using aminopropyl SPE cartridges for cattle feed samples. Validation of the method was able to demonstrate the developed technique is suitable for trace level analysis of sterols in cattle feed and milk samples. The LOR for individual sterols and total phytosterols in milk was found to be 0.02 and 0.12 mg/100 mL respectively. For cattle feed, the LOR was determined to be 5.0 mg/100 g for individual sterols or 35 mg/100 g for total phytosterols. An overall acceptable MU of 35% was found for sterols at trace level determination and of 15% under normal range determination with an expanded confidence interval of 95%. The research was also able to demonstrate the importance of the surrogate standard as it was able to compensate for any degradation during extraction using 5β-cholestan-3α-ol. Quantitation using individual sterol reference standards and surrogate standards as a response corrector is recommended to add further quality assurance to the analyses.

Chapter 5. Cattle Feeding Experiments

5.1. Overview

This chapter presents the results obtained from various feeding experiments including the phytosterol content in the cattle feed used. A comparison between the different groups and feed rates was made to determine if cattle diet can influence the phytosterol profile in the milk. In addition, this chapter also evaluates the cost effectiveness of the feed in order to determine any potential value to the different feeding regimes. The results of this chapter have been published in the Journal of Dairy Science:

Duong S., Strobel N., Buddhadasa S., Auldist M., Wales W., Moate P., Cox G., Orbell J., Cran M., Modification of the sterol profile in milk through feeding. *Journal of Dairy Science*, 2019, https://doi.org/10.3168/jds.2018-15067.

5.2. General Introduction

Australian dairy farmers commonly support and maintain their cattle based on a diet of in-house pasture grazing which constitutes the bulk of the animals' food intake (Özkan and Hill 2015). Pasture grazing allows farmers to become more financially independent and maintain better control of their animals' diets (Moran 2005; Özkan and Hill 2015). Over recent decades, however, in-house grazing has become difficult due to limited pasture production affected by reduced availability of water. For this reason, supplementation of cattle feed has become more proliferate and is expected to increase in the future to become a major contribution to the diet of dairy cattle. There is a keen interest by the dairy industry

to study effective feeding regimes to obtained better production, improve quality, reduce methane omission from the cattle and keep cost down.

Methane is one of the most predominant contributing greenhouse gases emitted globally. Methane has been directly associated to global warming as it can efficiently trap radiation in the earth's atmosphere thereby increasing the global temperature (US EPA 2018). Methane itself naturally emits from the environment through normal ecosystems process such including wetlands and oceans and termites (US EPA 2018). However, 60% of the total global methane emissions are related to human activities. One of the most significant factors contributing to global methane greenhouse by humans is cattle associated with milk production and livestock (Toprak 2015). Currently, cattle account for approximately 52% of agricultural related methane production in Australia alone (Charmley *et al.* 2016). There is a pressing demand to reduce methane emissions as it has become increasingly evident that unrestricted emissions are negatively impacting human health, livelihoods and the environment.

Many previous *in vitro* studies have been able to demonstrate the administration of certain fats in the diet of cattle and sheep can reduce methane emissions (Czerkawski *et al.* 1966). The application of *in vitro* studies to *in vivo* has been changeling due to environmental factors to allow merging *in vitro* conditions into needs of dairy cattle nutrition. In general, the addition of 1% of supplemented fat into the cows' diet has been shown to reduce methane emissions between 3.8 and 5.6% (Nozière *et al.* 2014). Condensed tannin, another common plant-based additive, has also been shown to reduce methane emission in cattle with the suggestion that tannins reduce methane through indirect fibre digestion or regulation of bacteria in the cows' rumen (Nozière *et al.* 2014).

Research by Bahrami *et al.* (2010) and Mirzaei-Aghsaghali *et al.* (2011) have shown that the use of grape pomace/marc as part of the cattle feed can also reduce methane emissions in dairy cattle. Grape marc is a by-product produced by the wine industry during the production of wine and includes grape skin, seeds, pulp and stems that are removed during wine production. A major cost to the wine industry is the disposal of these by-products which are currently sold for fertiliser or for biogas production via anaerobic digestion.

In addition, fortification of milk nutrients is ideally achieved *via* natural means such as modification of the diet of the cows or biofortification rather than by direct addition of the nutrients to the final product. As biofortification can reduce the possibility of over-fortifying milk thus reducing the risk of potential health problems from over-consumption of the added macronutrients. As discussed in Chapter 2, the literature relating to feeding experiments using cattle feed and protected rumen feed has demonstrated that feed can influence milk quality with regards to FA profile (Ashes *et al.* 1992a; Baldinger *et al.* 2013; Akbaridoust *et al.* 2014). However, at present, no research into the effects of feed with regards to phytosterol content in bovine milk has been published.

5.3. Experimental Overview

A total of five feeding experiments were undertaken with four feeding experiments conducted by researchers from the Department of Economic Development, Jobs, Transport and Resources (DEDJTR, formerly known as the Department of Primary Industries), at the Ellinbank facilities in Victoria, Australia. This included: Pasture Supplementation Experiments I and II (Auldist *et al.* 2013; Auldist *et al.* 2014) under the “milk yield program”; the Tannin and Cotton Seed Oil Experiment (Aprianita *et al.* 2017) and Grape Marc Experiment (Moate *et al.* 2014) under the

"methane mitigation program"; and a Rumen Protected Experiment that was conducted at Naturale Pty Ltd in Ringarooma, Tasmania in 2015. A summary of the feeding programs investigated is shown Figure 5.1.

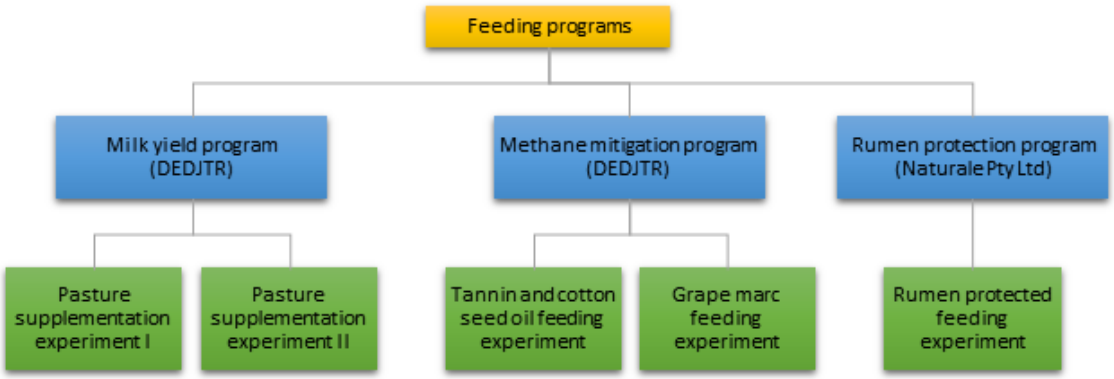

Figure 5.1 Feeding experimental programs

The protocol used for the extraction of sterols from milk are as per section 3.3.1 and from cattle feed as per section 3.3.2 with the optimised GC analysis performed in accordance with the methods described in Chapter 4. All results for milk are expressed in mg/100 mL which accounted for the specific gravity of the samples. All results for the cattle feed are expressed in mg/kg dry matter.

5.4. Statistical Analysis

The statistical approach used for this study was a one-way analysis of variance (ANOVA) that included comparisons between the sterol contents, the feeding type, and feeding rates (feeding rate only applicable to Pasture Supplementation Experiments I and II). This comparison allows for the identification of any differences within the group that could demonstrate the effect of feed upon milk with regards to sterol content. For the Pasture Supplementation Experiments I and II, data points from each feeding group including their feed rate were used

for the statistical analysis with two data points collected for each rate for each feeding regime. To provide greater confidence in the ANOVA, the other feed rates within the same feeding regime were combined for the analysis to give a total of 8 data points. The similar approach was also performed when comparing the different rates between these experiments. Data points from the feeding regime were combined according to feeding rates to increase the number of data points from 2 to 8 in order to provide better statistical confidence. In addition, where differences were found for any of the analyses, a post-hoc (*t*-test) was performed to determine and identify the possible significance. All statistical calculation was achieved using IBM SPSS statistics version 23.

5.5. Phytosterol Content in Cattle Feed

5.5.1. Feed Samples

A total of eighteen different cattle feeds including three formulated protected feed samples were used for this research. The cattle feed includes barley grain, canola, canola protected feed, cotton seed oil, dairy concentrate, grape marc (wet and dried), lucerne, maize grain, maize silage, mineral mix, molasses, omega protected feed, pasture, pasture silage, pit silage, sterol protected feed, tannin and wheat. All milk and cattle feed samples were collected during the period of 2010-2011, except for the protected feed experiment sample and barley grain. The protected feed experiment samples were collected in 2015 and barley grain in 2016. Furthermore, a total of three subsamples of each cattle feed type were collected and analysed, except for tannin, molasses, cotton seed oil and the protected feeds where a single sample was collected.

5.5.2. Phytosterol Content

Analysis of the eighteen types of cattle feed found that the predominant plant sterol found in these feeds was *β*-sitosterol with the total sterol ranging from <350 to 6260 mg/kg. In the feeds, no lanosterol and lathosterol were detected. The results also showed dairy concentrate contained the highest phytosterol (excluding the formulated feeds) and tannin containing less than 350 mg/kg. Shown in Table 5.1 are the phytosterol results of the cattle feed used for this research along with the value of total solids from the feed.

In addition to the cattle feed, three formulated feeds were used for this research, this included the “canola protected feed” (CSP), “omega protected feed” (OP) and “sterol protected feed” (SP). Both the CSP and SP were formulated to contain high phytosterol content. The main difference between these two feeds was the phytosterol source where the SP feed was fortified with plant sterol fatty ester and the CSP feed was fortified with canola and soybean oil instead. The total phytosterol content of the three feeds was able to show that the sterol protected feed contained the highest sterol amount followed by canola and omega. Although unintentional, the total phytosterol content in the omega feed was also considerately high containing significantly more plant sterols compared to the other cattle feeds.

Table 5.1 Phytosterol content in various cattle feed mg/kg

	Brassicasterol	Campesterol	Campestanol	Cholesterol	Cholestanol	Stigmasterol	Stigmastanol	β-sitosterol	Total sterols	Total solid (DM factor) %
Barley grain	<50	133	35	<50	<50	25	86	346	627	89.9
Canola	160	360	<50	<50	<50	<50	<50	740	1300	81.2
CSP feed	210	940	<50	90	<50	80	<50	1250	2590	91.3
CSO	<50	230	<50	<50	<50	<50	<50	2240	2560	100
Dairy concentrate	<50	730	220	<50	<50	380	650	2290	4310	99.6
Grape marc	<50	110	<50	<50	<50	90	80	1500	1820	90.5
Lucerne	<50	<50	60	<50	<50	<50	<50	460	630	86
Maize grain	<50	130	<50	<50	<50	50	130	470	840	86.7
Maize silage	<50	200	<50	<50	<50	190	80	830	1370	35.3
Mineral mix	<50	80	90	<50	<50	<50	<50	130	360	92.3
Molasses	<50	120	60	<50	<50	120	60	280	650	93.9
OP feed	<50	200	<50	<50	<50	140	<50	450	2130	92.9
Pasture	<50	270	<50	<50	<50	70	30	840	1270	22.5
Pasture silage	<50	330	<50	60	<50	150	50	1150	1760	25.6
Pit silage	<50	390	<50	70	<50	140	60	1310	1980	21.1
SP feed	80	2060	<50	130	<50	1740	140	4140	8340	85.7
Tannin	<50	<50	<50	<50	<50	<50	<50	<50	<350	91.8
Wheat grain	<50	100	70	<50	<50	80	90	330	680	88.5

CSP = canola/soybean protected feed, SP = sterol protected feed, OP = omega-3 protected feed, CSO = cotton seed oil

Overall, wheat grain, CSO, and barley grain were the only three matrices where comparisons could be made with other literature at the time of this research. Even though there are reports of phytosterol contents for corn and canola, direct comparison to this research could not be made as they were expressed per oil content and not as the whole grain. Shown in Table 5.2 are the comparisons between the phytosterol levels measured in this study and those reported in the literature. Both wheat and barley grain results were shown to be within stated literature ranges of 500-820 mg/kg and 400-800 mg/kg respectively. Conversely, the phytosterol content of CSO was shown to be approximately 40% lower than the low-end literature value. It is suspected that the age of the oil and cultivar may differ greatly from the literature thus resulting in the significant variation.

Table 5.2: Levels of phytosterols in cattle feed

Matrices	Total phytosterols mg/kg		Reference(s)
	This study	Literature values	
Wheat grain	690	500-820	(Piironen *et al.* 2002b; Ruibal-Mendieta *et al.* 2004)
CSO	2560	4310-5390	(Vlahakis and Hazebroek 2000)
Barley gain	627	400-800	(Piironen *et al.* 2002b)

5.6. Pasture Supplementation Experiments I & II

A milk yield program conducted separately to the present research aimed to determine and understand the effects on feed on milk production (Auldist *et al.* 2013). This work was extended into the present study whereby phytosterol and cholesterol content was concurrently investigated to determine whether milk quality was also influenced under these feeding trials. Two milk yield trials were

selected for the milk quality evaluation that were conducted between 2009 and 2011. Pasture supplementation experiment I was additionally focused assessing the difference in the feeding mode and types supplemented. A total of three lactating seasons were assessed, however only the second and third lactation periods (November 2009 and April 2010) were tested to account for sample distribution and sterol analysis was only performed on milk samples collected during the second and fourth weeks of the experiment. Since the results from experiment I showed significant gains in milk yield at higher feeding rates (Auldist *et al.* 2013), pasture supplementation experiment II also focused on higher feeding rates with the addition of protein in the form of solvent extracted canola. The protein was introduced at the higher end of the feed rate to determine whether an increase in protein would affect milk yield.

5.6.1. Pasture Supplementation Experiment I

In this experiment, three main feeding treatments were investigated to assess differences in the feeding mode and the type of feed supplements. At the start of this experiment, the cows were 227 ± 72.8 (mean ± standard deviation) days in milk (DIM). In this experiment, the feeding regimes included a standard practice control (SPC) treatment (72 cows), and two pasture supplementation experiment (PSE) treatments (72 cows each) where cattle in all groups were initially grazed on ryegrass pasture where the pasture allowance per cow was approximately 14 kg dry matter (DM) per day. In the SPC group, the feed was supplemented twice daily with barley grain fed in the dairy and pasture silage fed in the paddock. Similar to the SPC group, cows in the PSEIa treatment were also supplemented

with barley grain and pasture silage, but these components were fed as a mixed ration on a feed pad. For the third treatment, PSEIb, cows were offered a supplement of barley grain, maize, lucerne hay and maize silage which was also offered as a mixed ration on a feed pad. The cows were then allocated into two replicates within each group set. All cows were fed twice daily and supplements were given in addition to ryegrass pasture. The mode of feeding was different for the SPC diet where the cows were given their supplements in the dairy and in the paddock, whereas the cows on the PSEIa and PSEIb diets were given feed supplements that were mixed in a wagon and placed in a concrete feed pad.

To evaluate the influence of feeding amount, the groups were further divided into four groups of nine cows which were each fed 6, 8, 10, or 12 kg of supplement based on the DM content per day. Shown in Table 5.3 and Table 5.4 are the feeding strategy and the feed composition breakdown for this experiment respectively. Milk samples were collected and combined according to the respective herd feeding regime, i.e. a herd containing nine cows had their milk evenly combined to make a sample. This enabled averaging of the milk sample for the specific feeding regime and rate.

Table 5.3: Pasture supplementation experiment I feeding regime from Auldist *et al.* (2013)

Item	**Control, PSEIa or PSEIb**			
Total number of cows	72			
Replicates	A, B			
Number of cows per replicate	36			
Amount of supplement (kg/day of DM per cow)	6	8	10	12
Number of cows per supplement	9	9	9	9

Table 5.4: Pasture supplementation experiment I feed composition

Feed Type	SPC	PSEIa	PSEIb
Barley grain	75%	75%	25%
Maize grain			30%
Ryegrass silage	25%	25%	
Lucerne hay			25%
Maize silage			20%

% expressed with regards to DM

A summary of the detected sterol levels measured in the milk from the feeding regimes in this experiment is presented in Table 5.5 with the results a combined mean from the different feeding rates for the same treatment. In this experiment, *β*-sitosterol, brassicasterol campestanol and stigmasterol were detected at <0.02 mg/100 mL with total phytosterols detected at <0.12 mg/100 mL. There were differences ($p < 0.05$) in the lathosterol levels for PSEIa and PSEIb with mean lathosterol levels in milk produced under the PSEIa regime 15% higher than in milk produced under the PSEIb regime. Lathosterol was not detected in the feed samples and, in this case, both the SPC and PSEIa shared the same feeding regime with the only difference in the method of offering the supplements to the cows. This change was not observed for the SPC where the cows on this diet were fed barley grain (75%) and ryegrass silage (25%) supplements in the paddock whereas those on the PSEIa were given the same feed but from concrete feed pads. The results suggest that levels of lathosterol in milk may be influenced by the difference in the feed from PSEIa and PSEIb even though lathosterol was not presented in either feed. The main difference in the feed composition is the presence of maize grain and maize silage in the PSEIb which

may have contributed to the lower the lathosterol content in the milk although the mechanism for this is unclear.

Table 5.5: Pasture supplementation experiment I (influence of feed type) sterol contents from milk produced by cows fed the partial mixed rations diet in pasture supplementation experiment

Sterol[#]	Feeding regime*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	SPC	49	14.00	0.37	10.65	18.98
	PSEIa	49	14.44	0.35	10.41	21.21
	PSEIb	51	14.69	0.32	10.95	21.39
	Average		14.38	0.20	10.41	21.39
Lathosterol	SPC	49	0.18	<0.02	0.09	0.30
	PSEIa	49	0.20[a]	<0.02	0.08	0.30
	PSEIb	51	0.17[b]	<0.02	0.08	0.34
	Average		0.18	<0.02	0.08	0.34
Campesterol	SPC	49	0.04	<0.02	0.03	0.12
	PSEIa	49	0.04	<0.02	<0.02	0.07
	PSEIb	51	0.04	<0.02	0.02	0.10
	Average		0.04	<0.02	<0.02	0.12
Lanosterol	SPC	49	0.24	<0.02	0.13	0.55
	PSEIa	49	0.22	<0.02	0.14	0.37
	PSEIb	51	0.21	<0.02	0.02	0.41
	Average		0.22	<0.02	0.02	0.55

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and *β*-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($p < 0.05$): lathosterol p values: a and b = 0.03.*Pasture supplementation experiment (PSE).

The effects of the quantity of feed given to the animals was explored with the results calculated as the mean value of all the same feed rates over the different feeding regimes. As shown in Table 5.6, there were no effects on the sterol

contents in milk as a result of different feeding rates. In the concurrent study, milk yield results were shown to significantly increase at higher rates (Auldist et al., 2013) so the results in the present study suggest that an increase in milk yield or the amount of feed does not appear to influence total phytosterol content with the exception of lathosterol content.

Table 5.6: Pasture supplementation experiment I (influence of feed rate) sterol contents from milk produced by cows fed the partial mixed rations diet in pasture supplementation experiment

Sterol[#]	Feeding rate (kg/day)	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	6	36	14.28	0.44	10.65	21.21
	8	36	14.46	0.38	10.41	18.06
	10	37	14.50	0.46	11.74	21.39
	12	40	14.28	0.32	11.29	18.71
	Average		14.38	0.20	10.41	21.39
Lathosterol	6	36	0.18	<0.02	0.09	0.34
	8	36	0.17	<0.02	0.08	0.30
	10	37	0.18	<0.02	0.10	0.27
	12	40	0.18	<0.02	0.09	0.27
	Average		0.18	<0.02	0.08	0.34
Campesterol	6	36	0.04	<0.02	<0.02	0.07
	8	36	0.04	<0.02	0.02	0.12
	10	37	0.04	<0.02	0.03	0.10
	12	40	0.04	<0.02	0.02	0.06
	Average		0.04	<0.02	<0.02	0.12
Lanosterol	6	36	0.23	<0.02	0.14	0.37
	8	36	0.24	<0.02	0.18	0.55
	10	37	0.21	<0.02	0.02	0.31
	12	40	0.22	<0.02	0.13	0.41
	Average		0.22	<0.02	0.02	0.55

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and *β*-sitosterol were less than the limit of reporting.

5.6.2. Pasture Supplementation Experiment II

This feeding experiment was performed to assess differences in the feeding mode and the type of feed supplements. At the start of this experiment, the cows were 70 ± 15.2 (mean ± standard deviation) DIM. Three main feeding treatments were investigated including a SPC treatment where cattle were fed on ryegrass pasture supplemented with wheat grain in the dairy and pasture silage in the paddock (64 cows). A second feeding treatment based on pasture supplementation (PSEIIa) involved cattle fed on the same ryegrass pasture as the SPC treatment, with the cow's diet supplemented with wheat and corn grain, corn silage and lucerne hay fed as a mixed ration on a feed pad (64 cows). A third dietary treatment (PSEIIb) was investigated that involved cattle grazing ryegrass pasture diet supplemented with the same mixed ration as PSEIIa but in which some of the wheat had been replaced with solvent-extracted canola meal (32 cows).

The pasture allowance for all treatments was approximately 14 kg DM/day for each cow. To evaluate the influence of feeding amount, the SPC and PSEIIa groups were divided into four groups of eight cows (per replicate) which were each fed 8, 10, 12 or 14 kg of supplement based on the DM content per day. Cows on the PSEIIb treatment were divided into two groups eight cows (per replicate) and were each fed 12 and 14 kg of supplement based on the DM content per day. Shown in Table 5.7 and Table 5.8 are the experimental feeding regime and composition respectively.

Table 5.7: Pasture supplementation experiment II feeding regime from: Auldist *et al.* (2014)

	SPC								PSEIIa								PSEIIb			
Total number of cows	64								64								32			
Replicates	A				B				A				B				A			
Cows/replicate	32				32				32				32				16		16	
Cows/treatment	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Amount of feed per cow (kg DM/day)	8	10	12	14	8	10	12	14	8	10	12	14	8	10	12	14	12	14	12	14

Table 5.8: Pasture supplementation experiment II feed composition

Feed Type	SPC	PSEIIa	PSEIIb
Crushed wheat grain	72%	39%	23%
Pasture silage	28%		
Crushed maize grain		20%	20%
Lucerne hay		9%	9%
Maize silage		32%	32%
Canola meal[#]			16%

[#]solvent extracted; % values are with respect to DM content

A summary of the results from this experiment are presented in Table 5.9, which shows a comparison between the mean sterol content of each feeding regime and feeding rate. Similar to the results in the first mixed ration feeding experiment, *β*-sitosterol, brassicasterol campestanol and stigmasterol were detected at <0.02 mg/100 mL with total phytosterols detected at <0.12 mg/100 mL. A comparison between the three feeding regimes of this experiment showed differences for cholesterol, lathosterol, campesterol and lanosterol. A further post-hoc analysis was performed on these individual sterols and the results indicated that the levels in milk were influenced by the differences in feeding regimes. In milk produced under the PSEIIa diet, cholesterol and lanosterol contents were lower when

compared to milk produced under the PSEIIb diet. The main difference between the PSEIIa and PSEIIb is the addition of protein in the form of canola meal (16%) and a lower amount of crushed wheat in the PSEIIb diet (reduced from 39% to 23%). Although the reason for the increased cholesterol and lanosterol in the milk produced by cows fed the higher protein diet are unclear, there is some evidence that lanosterol can reverse protein aggregation (Zhao *et al.* 2015). The results also show that milk produced by cows fed under both PSEIIa and PSEIIb contained the same mean lathosterol content which was lower than the milk from the SPC diet. Moreover, the campesterol level in milk was observed to be highest in the SPC group.

A comparison of total phytosterol content in the feed at the 13.5 kg DM rate was 13200, 12300 and 13500 mg for the control, PSEIIa and PSEIIb respectively with trace levels of cholesterol (Duong *et al.* 2018). This experiment suggests that the addition of protein into the feed in the form of solvent extracted canola meal may increase the levels of cholesterol and lanosterol in the milk produced when compared to the PSEIIa milk. Although the reasons for this are unclear, research by Strzałkowska *et al.* (2010) showed that in a year-long experiment under the same feeding regime, cholesterol content was influenced by the time of year, the stage of lactation, and somatic cell count.

The effects of the quantity of feed given to the cows was also explored with the results calculated as the mean value of all the same feed amounts over the different feeding regimes. In this case, statistical comparisons between the mean feed rates across the different regimes showed differences in the levels of

campesterol as shown in Table 5.10. However, the results overall were considered to be insignificant between the groups.

Table 5.9: Pasture supplementation experiment II (influence of feed type) sterol contents from milk produced by cows fed the partial mixed rations diet in pasture supplementation experiment

Sterol[#]	Feeding regime*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	SPC	25	12.89	0.24	11.12	15.68
	PSEIIa	23	12.24[a]	0.13	11.19	13.07
	PSEIIb	15	13.07[b]	0.19	11.54	14.58
	Average		**12.70**	**0.12**	**11.12**	**15.68**
Lathosterol	SPC	25	0.148[a]	<0.02	0.08	0.28
	PSEIIa	23	0.126[b]	<0.02	0.09	0.17
	PSEIIb	15	0.126[b]	<0.02	0.08	0.16
	Average		**0.135**	**<0.02**	**0.08**	**0.28**
Campesterol	SPC	25	0.050[a]	<0.02	0.03	0.08
	PSEIIa	23	0.040[b]	<0.02	0.03	0.06
	PSEIIb	15	0.043	<0.02	0.03	0.05
	Average		**0.045**	**<0.02**	**0.03**	**0.08**
Lanosterol	SPC	25	0.17	<0.02	0.10	0.35
	PSEIIa	23	0.15[a]	<0.02	0.10	0.20
	PSEIIb	15	0.20[b]	<0.02	0.16	0.25
	Average		**0.17**	**<0.02**	**0.10**	**0.35**

#Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($p < 0.05$): cholesterol *p* values: a and b = 0.02, lathosterol *p* values: a = 0.03 and b = 0.07, campesterol *p* values: a and b = 0.00, lanosterol *p* values: a and b = 0.00. *Pasture supplementation experiment (PSE).

Table 5.10: Pasture supplementation experiment II (influence of feed rate) sterol contents from milk produced by cows fed the partial mixed rations diet in pasture supplementation experiment

Sterol[#]	Feeding rate (kg/day)	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	8	12	12.50	0.26	11.22	13.99
	10	12	13.31	0.34	11.21	15.68
	12	21	12.69	0.18	11.12	14.02
	13.5	18	12.42	0.22	11.19	14.58
	Average		**12.70**	**0.12**	**11.12**	**15.68**
Lathosterol	8	12	0.12	<0.02	0.08	0.22
	10	12	0.15	<0.02	0.10	0.28
	12	21	0.13	<0.02	0.08	0.19
	13.5	18	0.14	<0.02	0.11	0.18
	Average		**0.13**	**<0.02**	**0.08**	**0.28**
Campesterol	8	12	0.05	<0.02	0.04	0.07
	10	12	0.05	<0.02	0.03	0.08
	12	21	0.04	<0.02	0.03	0.07
	13.5	18	0.04	<0.02	0.03	0.06
	Average		**0.04**	**<0.02**	**0.03**	**0.08**
Lanosterol	8	12	0.18[a]	<0.02	0.14	0.30
	10	12	0.18	<0.02	0.14	0.35
	12	21	0.17	<0.02	0.13	0.25
	13.5	18	0.15[b]	<0.02	0.10	0.23
	Average		**0.17**	**<0.02**	**0.10**	**0.35**

#Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($p < 0.05$): campesterol p values: a and b = 0.00.

5.7. Tannin, Fat and Grape Marc Feeding Experiments

Two methane mitigation experiments were performed concurrently with this research, namely the tannin and cotton seed oil (TCSO) feeding experiment and the grape marc (GM) feeding experiment. Methane is a by-product of the dairy

that is produced in the rumen of cattle and *via* manure fermentation and previous studies have investigated whether fat or tannins are able to reduce methane production in cattle (Bahrami *et al.* 2010; Mirzaei-Aghsaghali *et al.* 2011; Moate *et al.* 2014; Aprianita *et al.* 2017). In the parallel study, the use of fat, tannin and combinations were used as feed to determine their potential for methane reduction. Grape marc was used in the second experiment as an alternative source for fat and tannin (Moran 2005). Grape marc is a by-product of the wine industry that is rich in tannins with seeds that also contain fat (Bahrami *et al.* 2010; Moraes *et al.* 2015). It has also shown the potential to reduce methane emissions when used as cattle feed using both the dry and wet (ensiled) forms (Moate *et al.* 2014). For both the TCSO and GM feeding experiments in this study, the phytosterol profile in the milk produced from the animals under these feeding regimes were also investigated to determine if feed can influence sterol profile in the milk.

5.7.1. Tannin and Cotton Seed Oil Feeding Experiment

This experiment was conducted in September 2010 and at the start of this experiment, the cows were 39 ± 13 (mean ± standard deviation) DIM. A total of ten cows were used for the experiment with two cows (donor) placed on the control for the first twelve out of the sixteen weeks of the experiment. These cows were set aside and were only used as a substitute during the experiment if cows one to eight were unable to complete their respective diets. Cows were fed a control diet, or the control diet supplemented with either 800 g/day of tannin (TANN) from black wattle, 800 g/d of cotton seed oil (CSO) or with 400 g/day

each of the TCSO feed. The experimental design was performed using a Latin square scheme for a duration of 16 weeks. Each diet was implemented to the cows for four weeks before moving onto the next respective diet. In addition to their supplemented feed diets, all cows were also offered 6 kg DM of dairy concentrate (crushed wheat, canola meal, mineral mix and molasses powder), and approximately 20 kg DM of alfalfa.

Table 5.11: Tannin and cotton seed oil feeding regime

Cows	Week 1-4	Week 5-8	Week 9-12	Week 13-16
1	CSO	TANN	Control	TCSO
2	Control	CSO	TCSO	TANN
3	TANN	TCSO	CSO	Control
4	TCSO	Control	TANN	CSO
5	TANN	Control	CSO	TCSO
6	CSO	TANN	TCSO	Control
7	TCSO	CSO	Control	TANN
8	Control	TCSO	TANN	CSO
9	Control (donor)			
10	Control (donor)			

Table 5.12: Tannin and cotton seed oil feed composition

Feed type	Control	CSO	TANN	TCSO
Crushed wheat/kg	4.1	4.1	4.1	4.1
Cold-pressed canola/kg	1.5	1.5	1.5	1.5
Mineral mix/kg	0.12	0.12	0.12	0.12
Palabind molasses powder/kg	0.28	0.28	0.28	0.28
Cotton seed oil/kg		0.8		0.8
Tannin/kg			0.4	0.4

In the TCSO feeding experiment, there were no differences in the sterol profiles in the milk produced by the cows given the TANN, CSO or TCSO feeds as shown in Table 5.13. Even though CSO is a naturally rich source of phytosterols, its addition to the cattle diet did not enhance total phytosterol content in the milk in this study. In humans, the consumption of high levels of phytosterols is usually reflected in the plasma levels and a decrease in LDL cholesterol is also observed (Ostlund *et al.* 1999; Kritchevsky and Chen 2005). It is suspected that the same affect may result when cows are fed a diet supplemented in phytosterols that is in excess of their normal consumption. Future work to analyse the blood plasma may aid in the understanding of the cows' metabolism of phytosterols.

Table 5.13: Sterol contents from milk produced by cows fed the TCSO diet

Sterol[#]	Feeding regime	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	Control	7	12.26	0.36	11.33	13.83
	CSO	8	12.60	0.62	10.40	15.93
	TANN	8	12.74	0.88	9.93	17.65
	TCSO	10	12.42	0.35	10.62	13.81
	Average		**12.51**	**0.28**	**9.93**	**17.65**
Lathosterol	Control	7	0.17	0.02	0.11	0.28
	CSO	8	0.17	0.03	0.07	0.36
	TANN	8	0.13	<0.02	0.07	0.22
	TCSO	10	0.15	0.02	0.09	0.27
	Average		**0.15**	**<0.02**	**0.07**	**0.36**
Lanosterol	Control	7	0.21	0.03	0.12	0.30
	CSO	8	0.18	0.03	0.05	0.30
	TANN	8	0.19	0.03	0.12	0.29
	TCSO	10	0.19	0.02	0.07	0.29
	Average		**0.19**	**<0.02**	**0.05**	**0.30**

#Total phytosterols, campestanol, campesterol, stigmasterol, stigmastanol and *β*-sitosterol were less than the limit of reporting. *Cotton seed oil (CSO), tannin (TANN), tannin and cotton seed oil (TCSO).

5.7.2. Grape Marc Feeding Experiment

Grape marc contains various flavonoids, poly-phenols, tannins, with some fat from the seed and is commercially available as either dried GM (DGM) or wet GM (WGM). The main difference between the DGM and WGM is the moisture content and the matrix particulate size. Both DGM and WGM were used in a concurrent study to determine if they have an effect on methane reduction in cattle (Moate *et al.* 2014). This experiment in the present study was conducted in March 2011 and at the start of this experiment, the cows were 203 ± 72.8 (mean ± standard deviation) DIM. A total of thirty-five cows were used and further subdivided into three feeding regimes with sixteen cows given the control feed, ten given the DGM feed, and nine given the WGM feed.

All cows were initially placed on the control diet for three weeks prior to being placed on their respective diets. The daily control diet consisted of 14 kg DM of alfalfa hay and 4.3 kg DM of concentrate mix, the DGM diet consisted of 9 kg DM of alfalfa hay, 4.3 kg DM of concentrate mix and 5 kg DM of DGM, and the WGM diet consisted of 9 kg DM of alfalfa hay, 4.3 kg DM of concentrate mix and 5 kg DM of WGM. In addition to the allocated diets, the cows were also offered alfalfa hay and a dairy concentrate mix (consisting of 93%, 4.7%, and 2.3% (DM) of crushed wheat, dried molasses, and mineral mix respectively). Shown in Table 5.14 and Table 5.15 are the experimental feeding regime and the feed composition respectively for this experiment.

Table 5.14: Grape marc feeding regime

Group	Week 1-3	Week 4-6
Control	Control	Control
DGM	Control	DGM
WGM	Control	WGM

Table 5.15: Grape marc feed composition

Feed type	Control	DGM	WGM
Alfalfa hay kg	14	9	9
Crushed wheat/kg	6	1	1
Molasses/kg	0.2	0.2	0.2
Mineral and lucerne hay/kg	0.1	0.1	0.1
DGM/kg		5	
EGM/kg			5

The GM feeding experiment showed significant differences in the lanosterol levels between the feeding regimes as shown in Table 5.16. It was observed that the mean lanosterol content was highest in the milk from the animals fed on the control diet and lowest in the milk from those feds on the DGM feed, however, this was not reflected in the cholesterol results for the different feed types. Examination of the phytosterol content in the respective diets showed that the control diet contained the lowest amount of phytosterols with 4254 mg compared to the WGM and DGM which contained 9922 mg (Duong *et al.* 2018). The results suggest that high phytosterol levels may affect the endogenous synthesis of lanosterol as this is generally observed in human subjects ingesting a high plant sterol diet (Ostlund *et al.* 1999; Kritchevsky and Chen 2005).

In this case, the WGM and DGM diets were fed at the same weight based on the amount of DM in the feed. This further suggests that the moisture level in the feed may have some influence on the metabolism and digestion of the feed, that may decrease phytosterol absorption and increase endogenous lanosterol levels in the milk which are a metabolic precursor for cholesterol (Jäpelt and Jakobsen 2013).The total amount of phytosterols detected were less than 0.12 mg/100 mL in all cases, which suggests that the feeding of either form of GM to dairy cows did not enhance total phytosterol levels in milk.

Table 5.16: Sterol contents from milk produced by cows fed the GM diet

Sterols[#]	Feeding type	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	Control	25	16.47	0.54	11.99	22.67
	DGM	20	15.43	0.57	11.21	20.97
	WGM	21	16.46	0.59	11.77	23.77
	Total	**66**	**16.15**	**0.33**	**11.21**	**23.77**
Lathosterol	Control	25	0.11	<0.02	0.02	0.21
	DGM	20	0.12	<0.02	0.06	0.23
	WGM	21	0.13	<0.02	0.08	0.21
	Total	**66**	**0.12**	**<0.02**	**0.02**	**0.23**
Campesterol	Control	25	<0.02	<0.02	0.03	0.07
	DGM	20	<0.02	<0.02	0.01	0.07
	WGM	21	<0.02	<0.02	0.03	0.08
	Total	**66**	**<0.02**	**<0.02**	**0.01**	**0.08**
Lanosterol	Control	25	0.26[a]	<0.02	0.12	0.56
	DGM	20	0.18[b]	<0.02	0.06	0.35
	WGM	21	0.22	<0.02	0.12	0.37
	Total	**66**	**0.22**	**<0.02**	**0.06**	**0.56**

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and *β*-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($p < 0.05$): lanosterol p value: a and b = 0.003. *Dried grape marc (DGM), wet grape marc (WGM).

5.8. Rumen Protected Feeding Experiment

The rumen protected (RP) feeding experiment was specifically designed to enhance the phytosterol content in the bovine milk. The feeds were formulated to allow the cattle to adsorb the intended nutrient further down the animal's digestion system hence avoiding degradation during the early stages of digestion. The feed is protected by encapsulating the target compound in a protein and this technique has been successful for the enhancement of omega-3 content in milk through feeding (Ashes *et al.* 1992b; Ashes *et al.* 1997; Gulati *et al.* 1997; Gulati *et al.* 1999). In these examples, a 10% transfer rate of omega-3 fatty acid was achieved. The encapsulation techniques from these studies were adopted for the application of phytosterols for this research.

This experiment commenced in mid-September 2015 for a period of four weeks and the cows were 195 ± 30 (mean ± standard deviation) DIM. In this experiment, seven cows were used with four dietary feeding treatments including control, sterol protected (SP) feed, omega protected (OP) feed containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and canola/soybean protected (CSP) feed. Both the sterol and soybean/canola protected feeds contained high levels of plant sterols with the sterol protected feed consisting of a phytosterol fatty ester paste while the soybean/canola consisting of natural phytosterols originating from the components. All the animals were placed on the control diet on the first two weeks before moving to their respective diets. The daily control diet consisted of 3.6 kg DM of dairy herd concentrate (60% corn and 40% wheat), 3.5 kg DM of maize silage and

approximately 10 kg DM of pasture. The daily protected feeds were the same as the control diet with the addition of 620 g of protected omega feed, 800 g of protected sterol feed, or 1000 g of protected soybean/canola feed. Rumen protection of the feed was achieved using a protein encapsulation process with cinnamaldehyde (a non-toxic food additive) *via* a process similar to that reported by Gulati *et al.* (1999). In brief, the sterol fatty esters, canola oil/soy bean oil or omega EPA and DHA were emulsified before cinnamaldehyde was added to the emulsified lipids to create cross-linkages between the emulsified oil and proteins. The mixture was then dried until it was a free-flowing powder. This type of protection allows the feed to avoid dehydrogenation in the rumen during the digestion process of the cattle (Gulati *et al.* 2000).

Table 5.17 Rumen protected feeding experiment regime

Cow	Week 1	Week 2	Week 3	Week 4
1	Control	Control	OP	OP
2	Control	Control	OP	OP
3	Control	Control	SP	SP
4	Control	Control	SP	SP
5	Control	Control	Control	Control
6	Control	Control	Control	Control
7			CSP	CSP

Similar to the previous experiments, the total phytosterol content found in all milk samples under the rumen protected feeds was <0.12 mg/100 mL. As shown in Table 5.18, significantly higher levels of cholesterol, lanosterol and lathosterol levels in the milk were found in animals fed the OP diet with milk produced from the SP and CSP feed supplements resulting in the lowest cholesterol content. In

humans, the consumption of phytosterols is suggested to reduce dietary cholesterol and regulate metabolic synthesis of cholesterol. The present study suggests that similar to humans, the consumption of high sterol contents by cattle may influence the metabolic synthesis of cholesterol resulting in a reduced cholesterol content expressed in the bovine milk (Lichtenstein and Deckelbaum 2001b; Kritchevsky and Chen 2005; Ostlund 2007). Given that the lathosterol levels in the milk from the SP and the CSP feed experiments were both lower than the control and OP feed, this is in accordance with observations in humans where low levels of lathosterol in blood plasma are consistent with lower cholesterol.

The phytosterol content in the protected portion of the OP, CSP and SP feeds contained total plant sterols of 1319, 2593 and 6673 mg respectively. The results show that levels of cholesterol in the milk produced by cows fed the high plant sterol SP and CSP diets were 22% and 11% lower than the control respectively. In the case of the OP feed, which contained relatively high amounts of protected phytosterol, it also contained 799 mg of cholesterol per day which may have interfered with plant sterol absorption.

In addition, the results for both the phytosterol and cholesterol content for the milk produced were within normal range compared to nutritional panels and previous studies (Gorban and Izzeldin 1999; Piironen *et al.* 2002b; Reklewska *et al.* 2002; Foods Standards Australia New Zealand 2010). Although this experiment was limited in the number of samples, particularly with regard to the OP treatment, the results indicate that cholesterol levels may be influenced by rumen protected

feeding and further experiments would be needed to confirm the results. In all cases, *β*-sitosterol was detected at <0.02 mg/100 mL for this experiment.

Table 5.18: Rumen protected feeding experiment sterol content

Sterol	Feeding types	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol[a]	Control	7	12.96	0.28	11.71	13.73
	OP	1	13.66	N/A	13.66	13.66
	SP	3	10.09	0.21	9.68	10.31
	CSP	1	11.16	N/A	11.16	11.16
	Average		**12.15**	**0.43**	**9.68**	**13.73**
Lathosterol	Control	7	0.07	<0.02	0.06	0.09
	OP	1	0.11	N/A	0.11	0.11
	SP	3	0.06	<0.02	0.05	0.06
	CSP	1	0.05	N/A	0.05	0.05
	Average		**0.07**	**<0.02**	**0.05**	**0.11**
Campesterol	Control	7	0.04	<0.02	0.03	0.04
	OP	1	0.05	N/A	0.05	0.05
	SP	3	0.05	<0.02	0.04	0.06
	CSP	1	0.04	N/A	0.04	0.04
	Average		**0.04**	**<0.02**	**0.03**	**0.06**
Lanosterol	Control	7	0.19	<0.02	0.15	0.23
	OP	1	0.26	N/A	0.26	0.26
	SP	3	0.21	0.02	0.16	0.24
	CSP	1	0.11	N/A	0.11	0.11
	Average		**0.19**	**<0.02**	**0.11**	**0.26**

Total phytosterols, campestanol, stigmasterol, stigmastanol and *β*-sitosterol were less than the limit of reporting; all cholesterol concentrations were significantly different ($p < 0.05$): cholesterol *p* value: a and b = 0.001. N/A = not applicable. *Omega protected (OP), sterol protected (SP), canola soy protected (CSP).

5.9. Cost Efficiencies of Feed

Under suitable climate conditions, pasture production for dairy cattle offers Australian farmers an economical method to feed their animals and maintain production (Moran 2005; Little 2009; Little 2010). However, unpredictable and

extreme weather events over recent decades have limited pasture production forcing farmers to purchase feeds. Effective and economical feeding regimes are therefore vital for the viability of dairy farming.

It is important that any purchased feed enables farmers to meet the animal's nutritional needs while maintaining production demands within the designated financial constraints. The cost of various types of cattle feeds are shown in Table 5.19 with pricing provided by Tasmanian Stockfeed Services (6 George St, Launceston, TAS, 7250) in 2016 except for the three protected feeds which were obtained from Naturale Pty Ltd in 2016.

The results of this research suggest that the range of feeds tested cannot enhance phytosterol content in the bovine milk produced. Even with the more promising preliminary results from the RP feed potentially lowering cholesterol content in the milk (see section 5.7), the costs of the feed would not be financially viable for most farmers (i.e. protected sterol feed $4000/ton). In most cases, the common feeds are an order of magnitude lower in price compared to the protected feeds. Since there is no influence on milk quality with regards to phytosterol content with different feeds, it is more financially sustainable for farmer to continue using the lower priced feeds.

Table 5.19: Cost of cattle feed

Feed type	Cost per ton (AUD)
Dried grape marc	100
Pasture (DM basis)	180
Pasture silage (DM basis)	220
Pit silage (DM basis)	220
Maize silage (DM basis)	250
Barley grain	310
Wheat grain	345
Maize grain	396
Molasses	400
Lucerne	450
Canola	500
Mineral mix	750
Cotton seed oil	2000
Tannin	2500
Protected canola/soybean feed	3000
Protected omega feed	3500
Protected sterol feed	4000

*Price as per 2016

5.10. Summary

Overall, the levels of cholesterol measured in the milk samples from five feeding experiments ranged from 10.3 to 24 mg/100 mL and the majority of the milk samples contained less than 0.12 mg/100 mL of total phytosterols. The major sterols found in the milk were cholesterol, lathosterol and lanosterol, the latter being a precursor sterol for cholesterol (Jäpelt and Jakobsen 2013). Other plant sterols detected in the milk samples included campesterol and *β*-sitosterol, but at minor or trace levels. As shown in Chapter 4, lanosterol and lathosterol were not detected among the range of phytosterols tested (Duong *et al.* 2018). Given that both lanosterol and lathosterol were found in the milk, it suggested that their presence in milk was a result of endogenous synthesis. In general, the rumen

protected feeds containing high phytosterol contents produced milk with cholesterol levels 11-22% lower than the control.

The results of these feeding experiments demonstrate that certain feeds consumed by the cattle can influence individual sterol contents in bovine milk including lanosterol, lathosterol, campesterol and cholesterol, but only to a minor extent. Changes were observed between control groups and diets containing maize silage, maize grain, canola meal, DGM, and high sterol rumen protected feeds. The mode of feed offering was also shown to influence the levels of these sterols. However, given that the safe and beneficial recommended levels of phytosterol consumption by many food authorities is approximately 2000 mg/day (Lichtenstein and Deckelbaum 2001a; Kritchevsky and Chen 2005), the levels determined in this study were less than 0.12 mg/100 mL which is much lower than some fortified levels of 300 mg/100 mL in milk which would require the equivalent consumption of 600 mL of milk to reach the target level (Pollak 1953; Gerson *et al.* 1961; Miettinen *et al.* 1995; Carr *et al.* 2010; Truswell 2010). Of all the feeding experiments, the formulated SP and CSP feeding types resulted in the production of milk with a reduced cholesterol content. However, the phytosterol content in the milk produced under any of the formulated feeding programs was unchanged. In addition, the results also indicate that the phytosterol transfer from feed to the milk was not direct for the rumen protected feed unlike the transfer of omega-3 fatty acid to milk that has previously been reported (Ashes *et al.* 1992a; Gulati *et al.* 1997).

Overall, the results demonstrate that the feeding of diets containing high amounts of phytosterols has an insignificant impact upon the phytosterol content of milk. Thus, the feeding of phytosterol rich feeds to cattle in order to enhance the phytosterol concentrations in milk cannot be recommended. In addition, dairy industries worldwide are generally highly regulated with milk carefully homogenized, pasteurized and fortified to maintain consistent quality control. Thus, any natural fortification achieved on one farm will most likely be diluted during the post-farm processing of milk. It was expected that some dietary treatments would have caused substantial enhancement in the concentrations of phytosterols in milk. These expectations were based on the results of previous studies that reported changes in the fatty acid profile as a result of feeding studies, and given that phytosterol fatty acid esters are a common sterol conjugate found in cattle feed (Gulati *et al.* 2002; Dutta 2004; Nyström 2007; Egger *et al.* 2009; Hristov *et al.* 2011; Samková *et al.* 2014). The rumen protected feeding experiment, however, offered some insight into the potential for producing milk with reduced cholesterol content.

Chapter 6. Conclusions and Recommendations

6.1. Conclusions

The primary aim of this research was to investigate whether cattle feeds can influence phytosterol and sterol content in raw bovine milk. In order to achieve these aims, method development for the analysis of raw milk (phytosterols determination at trace levels) and cattle feed was required. Due to the large number of samples involved, the method developed needed to be suitable for high outputs, streamlined, sensitive, robust and efficient. A total over 300 samples of milk and cattle feed was collected for this research. Therefore, an efficient and optimised method was critical for the success of the research.

Using existing literature methods as a starting point, further development and optimizations were investigated including saponification (for free and sterol fatty esters) as well as enzyme and acid hydrolysis (for sterol glycosides). For this research, it was found that enzymatic treatment was not suitable due to the time efficiency and lack of robustness. The final optimisation condition utilized an aqueous acid hydrolysis treatment for cattle feed and ethanolic hydrolysis for milk and other samples, followed by saponification while simultaneously extracting the sterol with heptane during the digestion stages. Sample clean-up was further applied to cattle feed samples using a 5 g amino propyl phase SPE cartridge followed by sample derivatisation using BSFTA with 1% TCMs. Samples were than analysed using an optimised GC-MS/FID system where the MS detector was used for confirmation and the FID for quantitation.

Excluding the rumen protected feed, analysis of the cattle feed showed that the dairy concentrate feed contained the highest amount of total phytosterols (4310 mg/kg) and tannin contained the lowest total phytosterols (<350 mg/kg). The main phytosterol found in all the cattle feed was *β*-sitosterol, stigmasterol and campesterol. In addition, neither lanosterol or lathosterol was detected in the cattle feed. From the cattle feed varieties analysed, a comparison was made on available cited literature between the reported total phytosterol content with wheat and barley grain within the cited literature range but for cotton seed oil lower than literature values which may be due to the age of the oil and cultivar.

Overall the milk obtained over the course of the 5 feeding experiments was found to contain cholesterol levels between 10.3 to 24 mg/100 mL and < 0.12 mg/100 mL of total phytosterols. No lanosterol and lathosterol was found in the cattle feed, therefore indicating that these sterols were endogenous. The main sterols found in the milk was cholesterol lanosterol, lathosterol campesterol and traces of *β*-sitosterol.

The results for pasture supplementation feeding experiment I was able to show difference between lathosterol when comparing PSEIa and PSEIb feeding regimes with cows on PSEIb producing milk containing 15% lower levels than cows on the PSEIa feed. The variation in feeding rates under these feeding experiments were shown to be insignificant.

In the pasture supplementation experiment II, differences were found in levels of cholesterol, lathosterol, campesterol and lanosterol. Both cholesterol and lanosterol contents were found to be highest in PSEIIb followed by the control

and PSEIIa, whereas lathosterol and campesterol were found to be highest in the control feeding group. An overall comparison between the feeding rates showed differences with respect to campesterol and although this was statistically significant, the value was considerably low.

No significant differences were not observed for the tannin and cotton seed oil experiment, however, the results for the grape marc experiment demonstrated a difference between the dried grape marc and the control with respect to the lanosterol content in the milk which was highest in the control. In this case, the moisture level in the feed was suggested to affect metabolism and digestion leading to influences on endogenous lanosterol synthesis.

Finally, the rumen protected feeding experiment was able to demonstrate a reduction of 11-22% in cholesterol when using CSP and SP respectively compared to the control. This finding is similar to plant sterol consumption in humans where high levels of intake can regulate cholesterol uptake or synthesis thus reducing levels of cholesterol in blood plasma.

In general, the results of these experiments were able to demonstrate that diet had minimal impacts on the sterol profile of milk when compared to the respective control diets. Moreover, the phytosterol levels determined in the milk samples were negligible when considering the 2 g per day recommended by many food authorities. The research was able to indicate phytosterol content in milk cannot be enhanced through the diets trialled in this research and the robustness of milk quality with regards to diet. Supporting and reassuring the industry through studies to confirm milk quality is robust. In addition, at this stage in time for

farmers to continue to use financially cheap feed with regards to phytosterol content in milk. The unexpected results found from the research was the production of lowered cholesterol levels in the phytosterol protected formulated feed. This give rise to further development to the potential of making a feeding type resulting in lower cholesterol content in milk produced.

6.2. Recommendations for Future Work

In this research, the main aim was to optimise the extraction and analysis of phytosterols from a wide range of matrices. This was achieved by selecting the best overall conditions to suit the collective set of samples in order to evaluate samples in a high-throughput laboratory where it is impractical and expensive to change methods, conditions, solvents etc. to suit individual samples.

Future work could focus on optimising conditions for specific matrices and to further investigate some conditions that were excluded. For example, enzymatic treatment was excluded on the basis of time and some initial results that showed poor sterol recoveries. However, it could be further investigated as it has the potential to overcome some of the drawbacks of acid hydrolysis such as isomerization of some sterols and the potential hazards of using strong acids.

Given the overall high costs of analysis of phytosterols in various matrices, future work could investigate reducing the overall sample size. This may lead to a reduction in the amount of SPE sorbents, volumes of solvents, and consumables in order to reduce the cost without compromising the sensitivity of the analysis. This is particularly important since it is becoming common practice to fortify foods

with phytosterols and there is a need for robust quantification for quality and verification. In addition, investigation into the quantification of sterols using triple quadrupole GC/MS instrumentation could be evaluated in order to obtain potentially lower detection limits. In the present study, most of the sterols were undetected using the single quadrupole GG with FID/MS and although this suggests that the levels are inherently low, this may further facilitate sample size reduction.

Future work could be directed towards the understanding of the cows' metabolism of phytosterols. This could be achieved by methods including the analysis of blood plasma from dairy cattle at different times, particularly since the digestion process can take one to three days to complete. Analysis of the digestive enzymes may also assist in further knowledge of the fate of dietary sterols.

Since the rumen protected feeds offered limited evidence of sterol modification in milk, particularly in the case of cholesterol, a larger study group of the formulated feeds should be trialled to confirm these findings. The feeding experiments should also include mode of feeding to determine if the cattle metabolism can also influence the sterol content and profile in the bovine milk. Other studies could be implemented to identify alternative and less expensive methods of producing rumen protected feeds by different modes of encapsulation and/or tailored encapsulation materials.

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Chapter 8. Appendix

8.1. Calibration of Sterol Reference Standards

Calibration curves of the individual plant sterols used in this research.

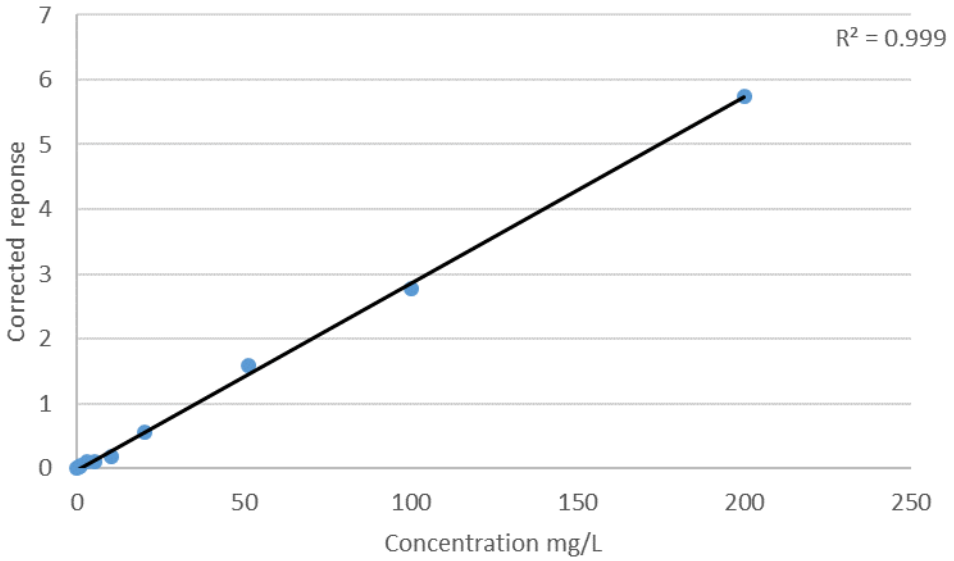

Figure 8.1: Calibration of brassicasterol

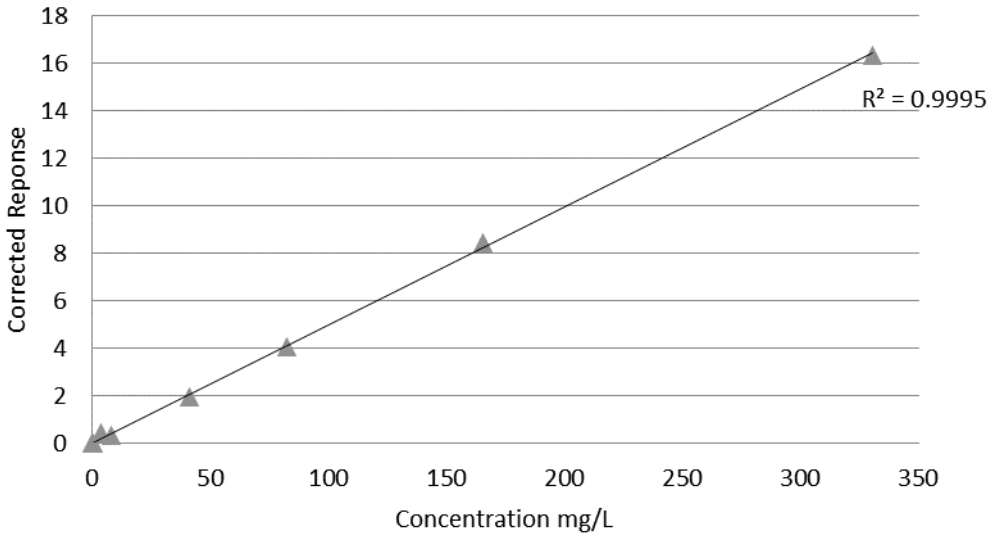

Figure 8.2: Calibration of campesterol

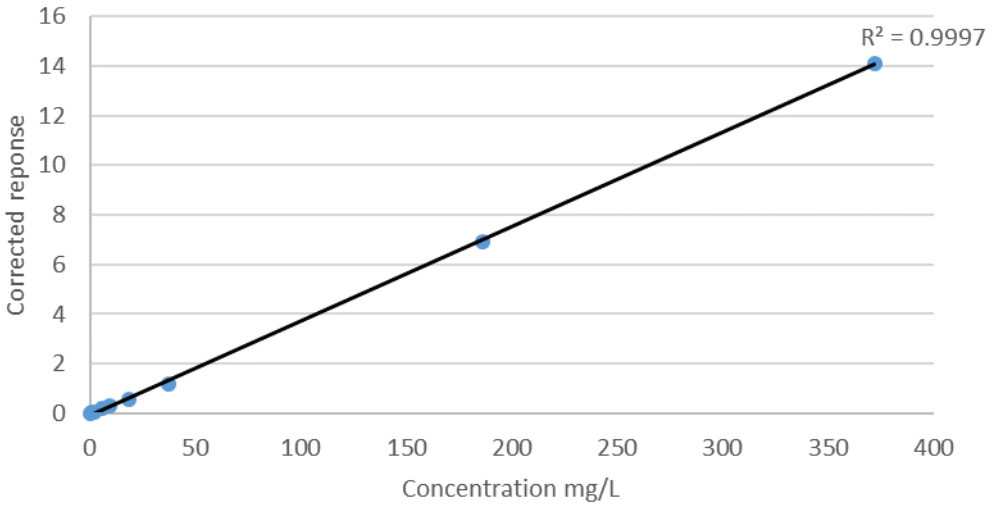

Figure 8.3: Calibration of campestanol

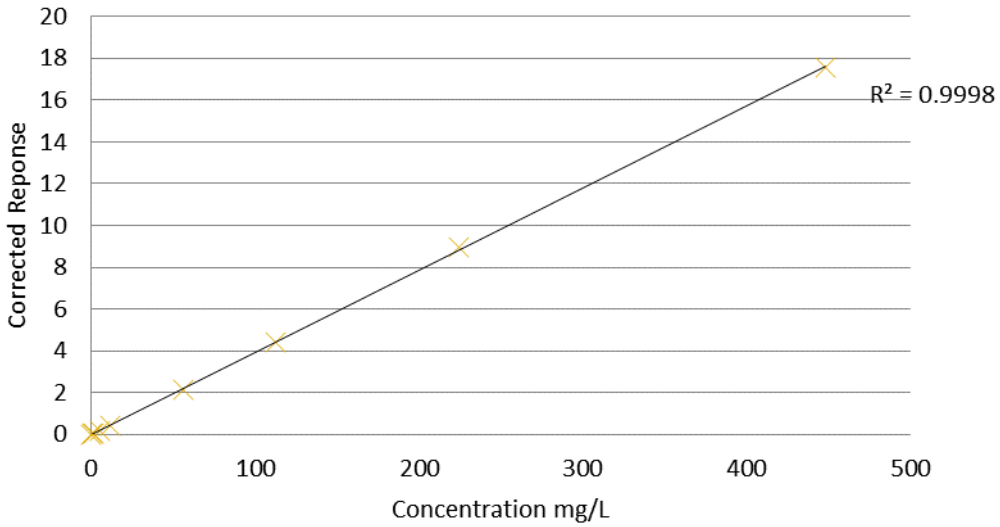

Figure 8.4: Calibration of stigmasterol

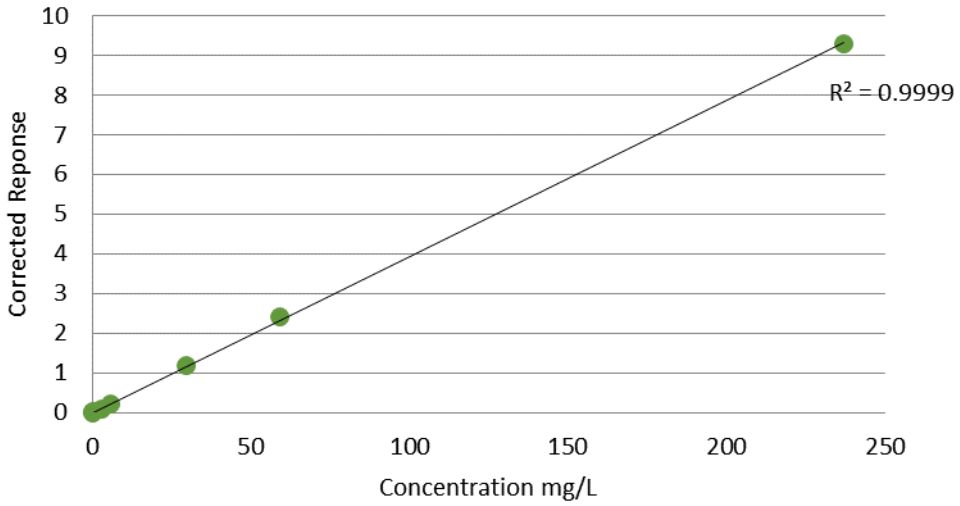

Figure 8.5: Calibration of *β*-sitosterol

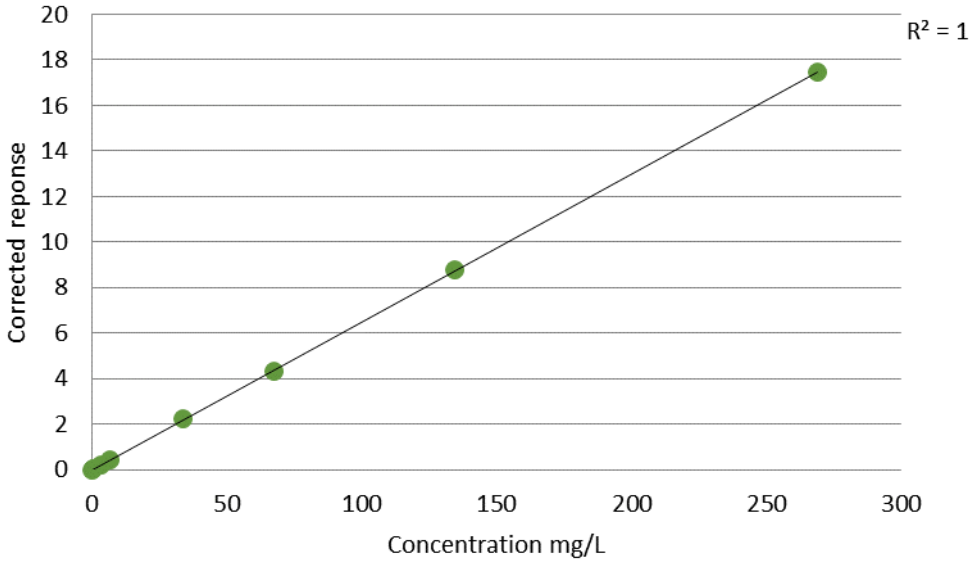

Figure 8.6: Calibration of stigmastanol

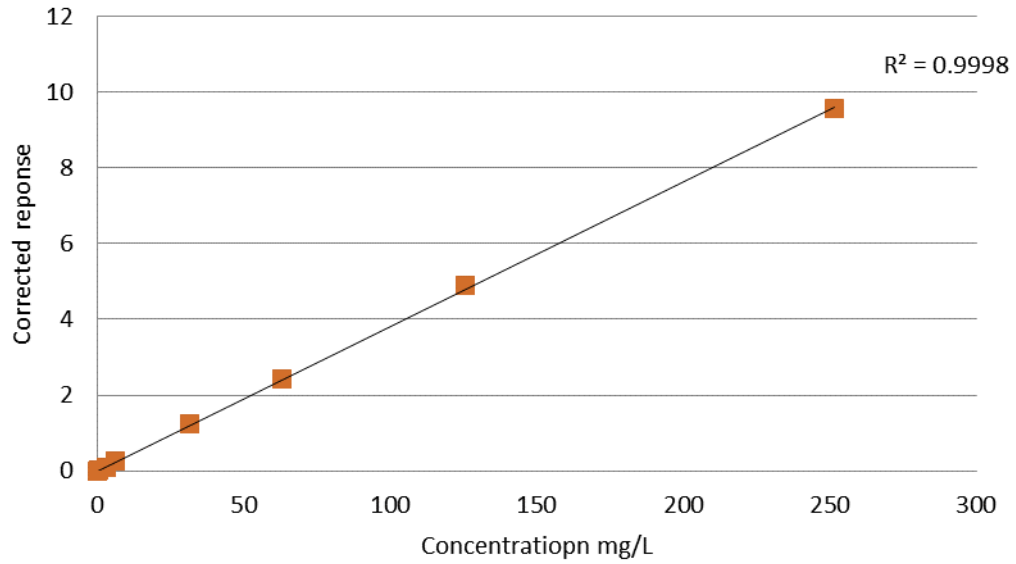

Figure 8.7: Calibration of cholestanol

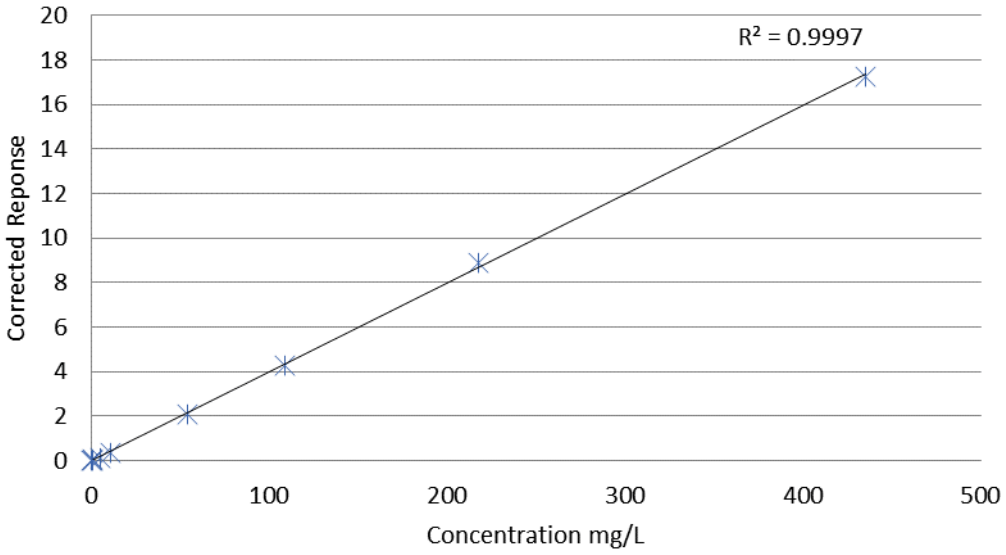

Figure 8.8: Calibration of lanosterol

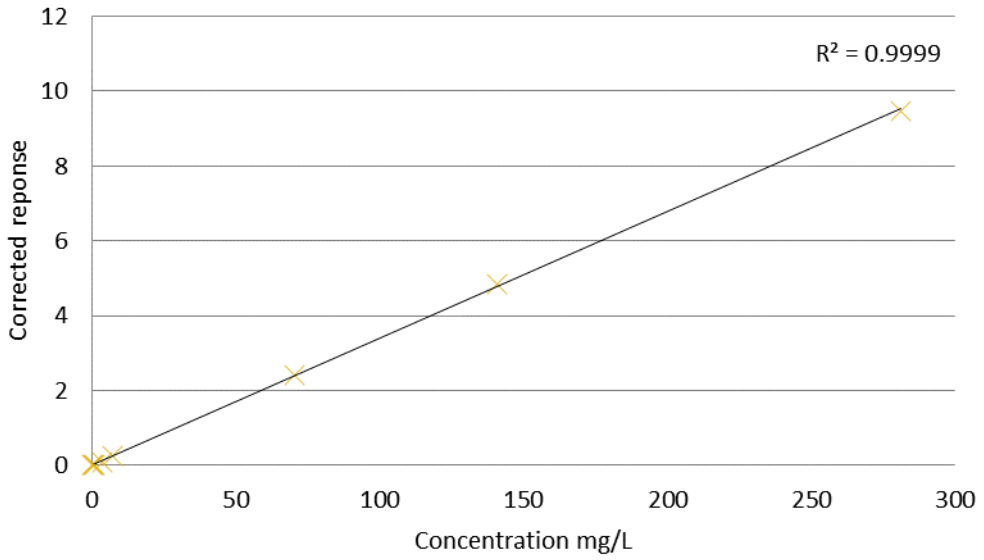

Figure 8.9: Calibration of lathosterol

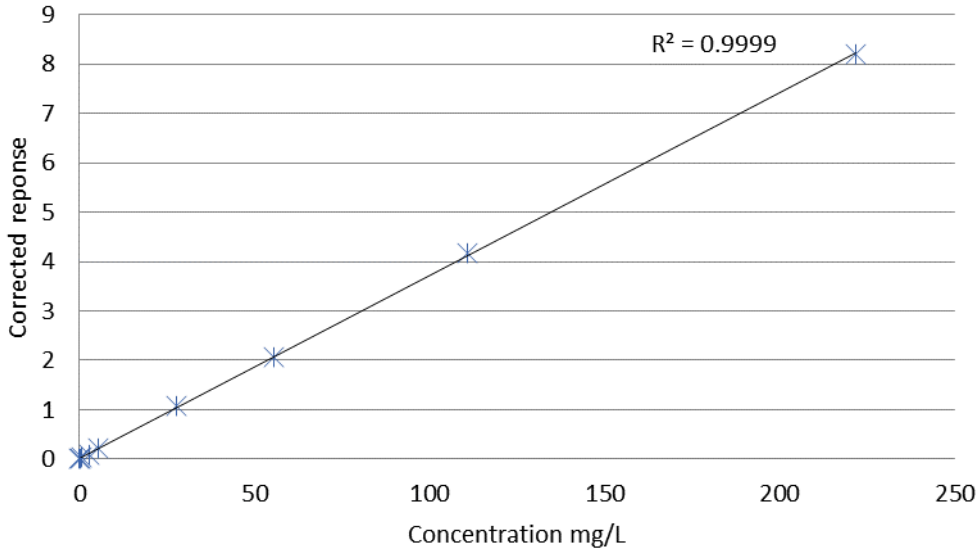

Figure 8.10: Calibration of fucosterol

8.2. Measurement Uncertainty Histograms

Histograms of contributing factors for each individual sterol toward MU:

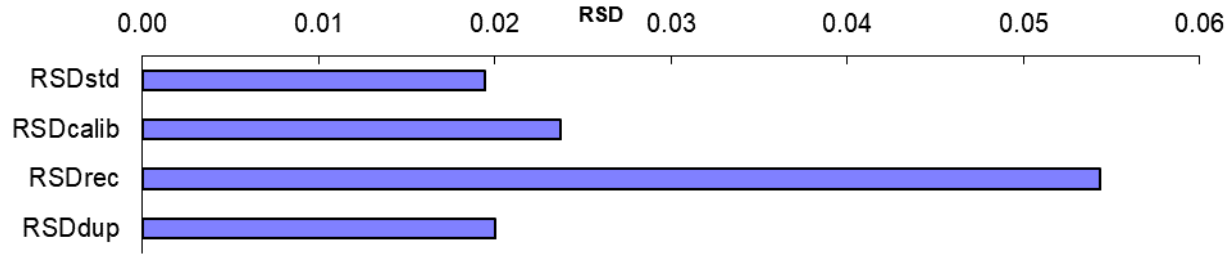

Figure 8.11: Cholesterol contributing factors of MU

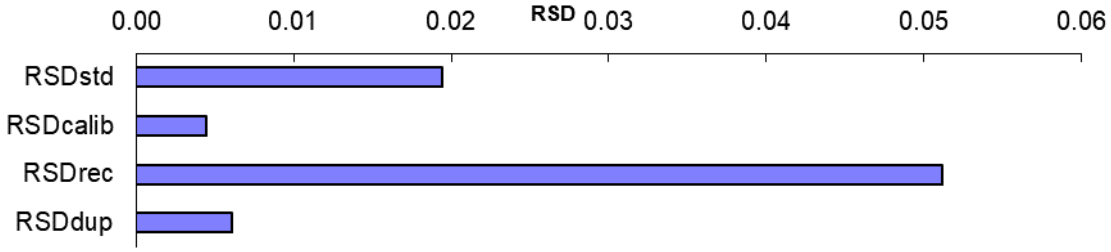

Figure 8.12: Brassicasterol contributing factor of MU

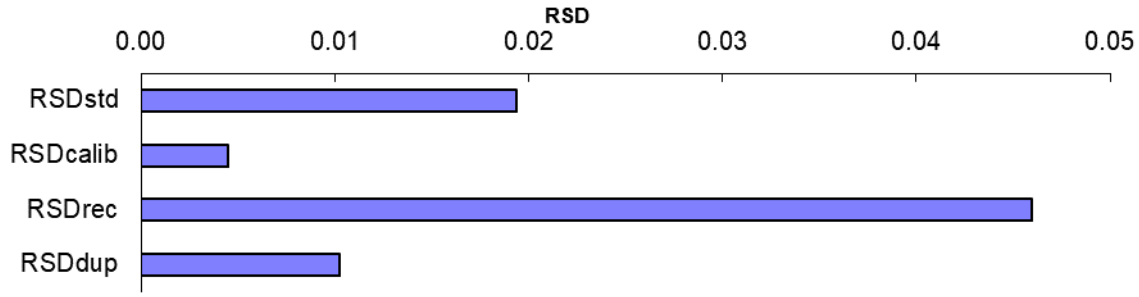

Figure 8.13: Campesterol contributing factors of MU

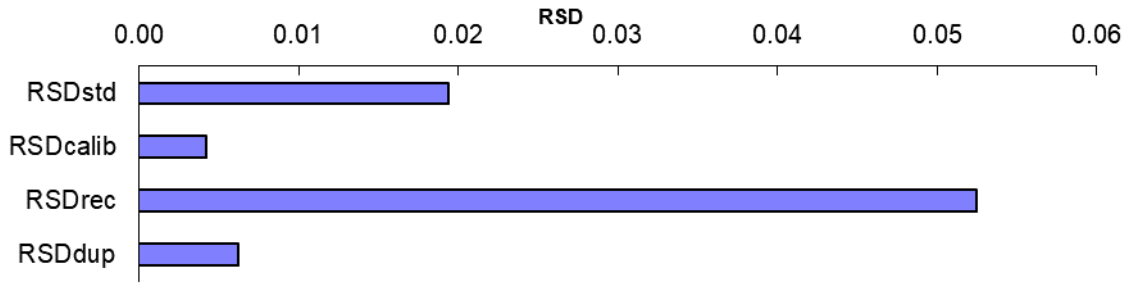

Figure 8.14: Campestanol contributing factors of MU

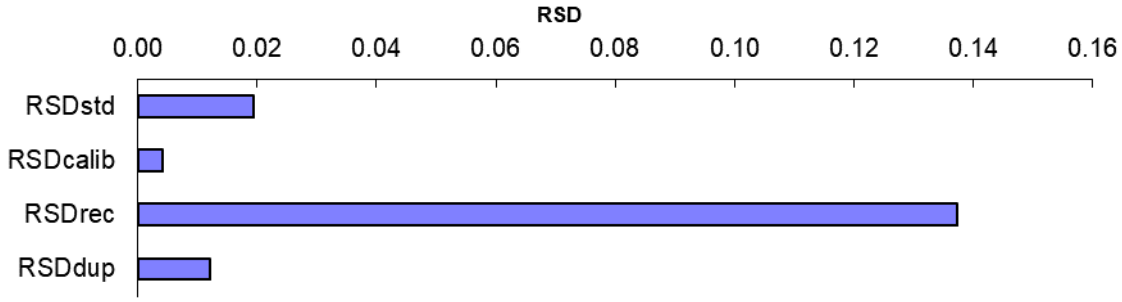

Figure 8.15: Stigmasterol contributing factors of MU

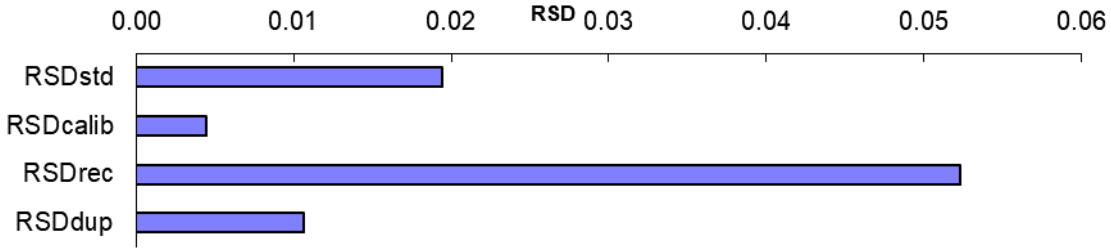

Figure 8.16: *β*-sitosterol contributing factors of MU

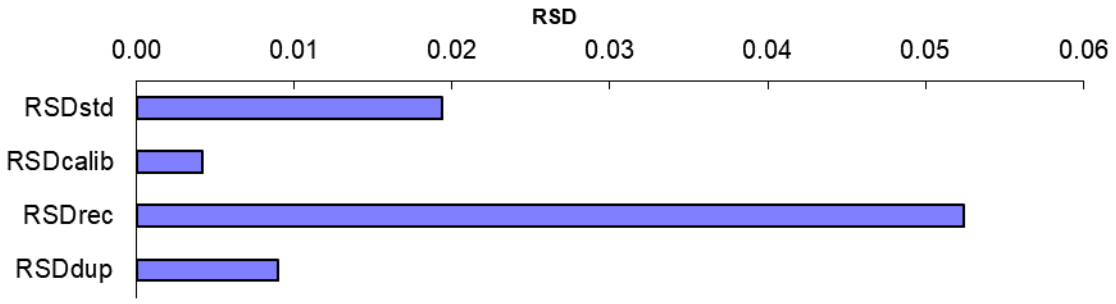

Figure 8.17: Stigmastanol contributing factors of MU

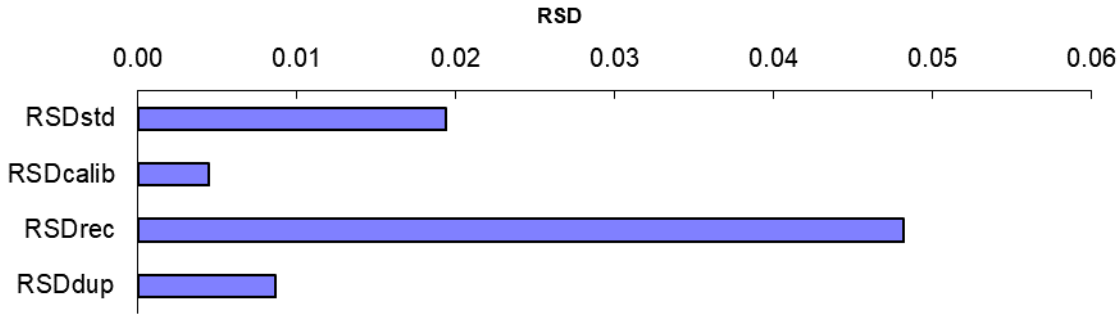

Figure 8.18: Total phytosterols contributing factors of MU

8.3. Pasture Supplementation Experiment I Results

Table 8.1: Results summary for pasture supplementation experiment I

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	β-sitosterol	Total Sterol
			kg		mg/100mL						
V11/009164	SPC	1	10	Nov-09	12.19	<0.02	0.05	0.11	0.23	<0.02	<0.12
V11/009165	PSEIA	2	10	Nov-09	12.87	<0.02	0.04	0.22	0.31	<0.02	<0.12
V11/009166	PSEIIB	3	12	Nov-09	13.13	<0.02	0.04	0.10	0.24	<0.02	<0.12
V11/009167	SPC	1	6	Nov-09	11.68	<0.02	0.04	0.11	0.22	<0.02	<0.12
V11/009168	PSEIIB		10	Nov-09	12.00	<0.02	0.04	0.10	0.25	<0.02	<0.12
V11/009169	PSEIA	2	10	Nov-09	11.74	<0.02	0.03	0.25	0.27	<0.02	<0.12
V11/009170	PSEIIB	3	6	Nov-09	12.40	<0.02	0.03	0.22	0.16	<0.02	<0.12
V11/009171	PSEIA	2	6	Nov-09	13.20	<0.02	0.07	0.26	0.23	<0.02	<0.12
V11/009172	SPC	1	10	Nov-09	13.11	<0.02	0.05	0.27	0.25	<0.02	<0.12
V11/009173	PSEIIB	3	10	Nov-09	13.15	<0.02	0.03	0.18	0.19	<0.02	<0.12
V11/009174	PSEIA	2	12	Nov-09	11.49	<0.02	0.04	0.22	0.22	<0.02	<0.12
V11/009175	PSEIIB	3	6	Nov-09	13.83	<0.02	0.04	0.22	0.34	<0.02	<0.12
V11/009176	PSEIA	2	6	Nov-09	11.74	<0.02	0.04	0.22	0.27	<0.02	<0.12
V11/009177	PSEIA	2	12	Nov-09	13.82	<0.02	0.04	0.27	0.26	<0.02	<0.12
V11/009178	SPC	1	12	Nov-09	12.81	<0.02	0.04	0.12	0.28	<0.02	<0.12
V11/009179	PSEIIB	3	12	Nov-09	12.92	<0.02	0.03	0.11	0.24	<0.02	<0.12
V11/009180	SPC	1	12	Nov-09	12.93	<0.02	0.04	0.11	0.25	<0.02	<0.12
V11/009181	SPC	1	6	Nov-09	11.76	<0.02	0.06	0.09	0.26	<0.02	<0.12
V11/009182	SPC	1	8	Nov-09	11.28	<0.02	0.04	0.10	0.23	<0.02	<0.12
V11/009183	PSEIA	2	8	Nov-09	11.44	<0.02	0.04	0.08	0.29	<0.02	<0.12
V11/009183 dup	PSEIA	2	8	Nov-09	11.77	<0.02	0.03	0.10	0.26	<0.02	<0.12
V11/009183 trip	PSEIA	2	8	Nov-09	11.89	<0.02	0.03	0.09	0.27	<0.02	<0.12

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	β-sitosterol	Total Sterol
V11/009184	SPC	1	8	Nov-09	12.27	<0.02	0.05	0.21	0.31	<0.02	<0.12
V11/009185	PSEIIB	3	8	Nov-09	13.49	<0.02	0.04	0.11	0.24	<0.02	<0.12
V11/009186	PSEIA	2	8	Nov-09	11.13	<0.02	0.03	0.09	0.23	<0.02	<0.12
V11/009187	PSEIIB	3	8	Nov-09	13.00	<0.02	0.04	0.08	0.19	<0.02	<0.12
V11/009212	SPC	1	6	Nov-09	11.35	<0.02	0.03	0.09	0.26	<0.02	<0.12
V11/009213	SPC	1	8	Nov-09	11.14	<0.02	0.04	0.21	0.18	<0.02	<0.12
V11/009215	SPC	1	12	Nov-09	13.22	<0.02	0.04	0.23	0.38	<0.02	<0.12
V11/009216	PSEIA	2	6	Nov-09	12.91	<0.02	0.05	0.21	0.37	<0.02	<0.12
V11/009217	PSEIA	2	8	Nov-09	14.23	<0.02	0.05	0.22	0.26	<0.02	<0.12
V11/009218	PSEIA	2	10	Nov-09	13.34	<0.02	0.04	0.22	0.21	<0.02	<0.12
V11/009219	PSEIA	2	12	Nov-09	14.29	<0.02	0.05	0.23	0.25	<0.02	<0.12
V11/009220	SPC	1	6	Nov-09	10.65	<0.02	0.03	0.21	0.21	<0.02	<0.12
V11/009221	SPC	1	8	Nov-09	12.10	<0.02	0.05	0.21	0.25	<0.02	<0.12
V11/009222	SPC	1	10	Nov-09	13.97	<0.02	0.04	0.21	0.24	<0.02	<0.12
V11/009223	SPC	1	12	Nov-09	13.18	<0.02	0.04	0.19	0.25	<0.02	<0.12
V11/009224	PSEIIB	3	6	Nov-09	10.95	<0.02	0.04	0.19	0.18	<0.02	<0.12
V11/009225	PSEIIB	3	8	Nov-09	14.07	<0.02	0.03	0.17	0.23	<0.02	<0.12
V11/009226	PSEIIB	3	10	Nov-09	12.10	<0.02	0.03	0.15	0.21	<0.02	<0.12
V11/009227	PSEIIB	3	12	Nov-09	12.76	<0.02	0.03	0.26	0.24	<0.02	<0.12
V11/009227 dup	PSEIIB	3	12	Nov-09	11.98	<0.02	0.03	0.22	0.26	<0.02	<0.12
V11/009227 trip	PSEIIB	3	12	Nov-09	13.04	<0.02	0.04	0.23	0.22	<0.02	<0.12
V11/009228	PSEIA	2	6	Nov-09	11.08	<0.02	0.04	0.20	0.20	<0.02	<0.12
V11/009229	PSEIA	2	8	Nov-09	12.86	<0.02	0.04	0.20	0.18	<0.02	<0.12
V11/009230	PSEIA	2	10	Nov-09	12.81	<0.02	0.04	0.21	0.22	<0.02	<0.12
V11/009231	PSEIA	2	12	Nov-09	12.07	<0.02	0.03	0.17	0.18	<0.02	<0.12
V11/009232	PSEIIB	3	6	Nov-09	12.46	<0.02	0.04	0.22	0.22	<0.02	<0.12
V11/009233	PSEIIB	3	8	Nov-09	13.49	<0.02	0.04	0.16	0.18	<0.02	<0.12
V11/009234	PSEIIB	3	10	Nov-09	14.67	<0.02	0.03	0.18	0.20	<0.02	<0.12

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	*β*-sitosterol	Total Sterol
V11/009235	PSEIIB	3	12	Nov-09	11.29	<0.02	0.03	0.17	0.17	<0.02	<0.12
V11/009188	SPC	1	6	Dec-09	11.83	<0.02	0.03	0.18	0.26	<0.02	<0.12
V11/009189	SPC	1	8	Dec-09	12.23	<0.02	0.03	0.23	0.23	<0.02	<0.12
V11/009190	SPC	1	10	Dec-09	12.76	<0.02	0.04	0.21	0.21	<0.02	<0.12
V11/009191	SPC	1	12	Dec-09	13.00	<0.02	0.03	0.23	0.25	<0.02	<0.12
V11/009192	PSEIA	2	6	Dec-09	11.98	<0.02	0.04	0.17	0.21	<0.02	<0.12
V11/009194	PSEIA	2	10	Dec-09	12.86	<0.02	0.04	0.22	0.15	<0.02	<0.12
V11/009195	PSEIA	2	12	Dec-09	11.80	<0.02	0.03	0.19	0.17	<0.02	<0.12
V11/009196	SPC	1	6	Dec-09	11.59	<0.02	0.03	0.18	0.22	<0.02	<0.12
V11/009197	SPC	1	10	Dec-09	12.12	<0.02	0.03	0.18	0.23	<0.02	<0.12
V11/009198	SPC	1	12	Dec-09	11.61	<0.02	0.03	0.17	0.27	<0.02	<0.12
V11/009199	PSEIIB	3	6	Dec-09	11.79	<0.02	0.03	0.19	0.18	<0.02	<0.12
V11/009201	PSEIIB	3	10	Dec-09	12.90	<0.02	0.03	0.20	0.17	<0.02	<0.12
V11/009201	PSEIIB	3	10	Dec-09	12.85	<0.02	0.10	0.14	0.27	<0.02	<0.12
V11/009202	PSEIIB	3	12	Dec-09	11.92	<0.02	0.03	0.26	0.14	<0.02	<0.12
V11/009203	PSEIA	2	6	Dec-09	12.42	<0.02	0.04	0.19	0.19	<0.02	<0.12
V11/009205	PSEIA	2	10	Dec-09	14.32	<0.02	0.05	0.24	0.15	<0.02	<0.12
V11/009206	PSEIA	2	12	Dec-09	13.31	<0.02	0.05	0.19	0.22	<0.02	<0.12
V11/009207	PSEIIB	3	6	Dec-09	13.10	<0.02	0.04	0.10	0.21	<0.02	<0.12
V11/009208	PSEIIB	3	8	Dec-09	14.87	<0.02	0.03	0.10	0.21	<0.02	<0.12
V11/009210	PSEIIB	3	10	Dec-09	14.27	<0.02	0.03	0.12	0.22	<0.02	<0.12
V11/009211	PSEIIB	3	12	Dec-09	11.96	<0.02	0.02	0.09	0.15	<0.02	<0.12
V10/011258	SPC	1	6	21/04/10	14.73	<0.02	0.07	0.12	0.34	<0.02	<0.12
V10/011259	SPC	1	6	21/04/10	15.01	<0.02	0.04	0.12	0.33	<0.02	<0.12
V10/011260	SPC	1	8	21/04/10	16.09	<0.02	0.05	0.29	0.20	<0.02	<0.12
V10/011261	SPC	1	8	21/04/10	16.46	<0.02	0.04	0.23	0.20	<0.02	<0.12
V10/011262	SPC	1	10	21/04/10	14.44	<0.02	0.04	0.10	0.22	<0.02	<0.12
V10/011263	SPC	1	10	21/04/10	14.61	<0.02	0.04	0.10	0.23	<0.02	<0.12

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	*β*-sitosterol	Total Sterol
V10/011264	SPC	1	12	21/04/10	14.83	<0.02	0.04	0.20	0.25	<0.02	<0.12
V10/011265	SPC	1	12	21/04/10	14.70	<0.02	0.04	0.24	0.22	<0.02	<0.12
V10/011266	PSEIA	2	6	21/04/10	16.17	<0.02	0.04	0.25	0.21	<0.02	<0.12
V10/011267	PSEIA	2	6	21/04/10	14.81	<0.02	0.03	0.10	0.20	<0.02	<0.12
V10/011268	PSEIA	2	8	21/04/10	14.74	<0.02	0.04	0.13	0.23	<0.02	<0.12
V10/011269	PSEIA	2	8	21/04/10	14.01	<0.02	0.02	0.13	0.19	<0.02	<0.12
V10/011269	PSEIA	2	8	21/04/10	10.41	<0.02	0.02	0.24	0.21	<0.02	<0.12
V10/011270	PSEIA	2	10	21/04/10	15.60	0.04	0.05	0.15	0.27	0.03	0.12
V10/011271	PSEIA	2	10	21/04/10	17.04	<0.02	0.04	0.18	0.21	<0.02	<0.12
V10/011272	PSEIA	2	12	21/04/10	16.64	<0.02	0.04	0.19	0.24	0.02	<0.12
V10/011273	PSEIA	2	12	21/04/10	17.25	<0.02	0.04	0.17	0.19	<0.02	<0.12
V10/011274	PSEIIB	3	6	21/04/10	15.42	<0.02	0.05	0.14	0.23	0.03	<0.12
V10/011275	PSEIIB	3	6	21/04/10	16.56	<0.02	0.04	0.16	0.16	<0.02	<0.12
V10/011276	PSEIIB	3	8	21/04/10	17.13	<0.02	0.03	0.16	0.19	<0.02	<0.12
V10/011277	PSEIIB	3	8	21/04/10	16.08	<0.02	0.04	0.13	0.23	<0.02	<0.12
V10/011278	PSEIIB	3	10	21/04/10	16.04	<0.02	0.04	0.16	0.22	0.03	<0.12
V10/011279	PSEIIB	3	10	21/04/10	16.72	<0.02	0.04	0.17	0.02	<0.02	<0.12
V10/011280	PSEIIB	3	12	21/04/10	15.04	<0.02	0.04	0.14	0.25	0.02	<0.12
V10/011281	PSEIIB	3	12	21/04/10	15.89	0.03	0.06	0.13	0.41	0.04	0.13
V10/012077	SPC	1	6	4/05/10	15.84	<0.02	0.03	0.21	0.20	<0.02	<0.12
V10/012078	SPC	1	6	4/05/10	16.16	<0.02	0.05	0.16	0.22	<0.02	<0.12
V10/012079	SPC	1	8	4/05/10	16.33	<0.02	0.04	0.17	0.21	0.02	<0.12
V10/012080	SPC	1	8	4/05/10	13.17	<0.02	0.12	0.11	0.55	0.06	0.19
V10/012081	SPC	1	10	4/05/10	15.75	0.02	0.05	0.18	0.30	0.04	<0.12
V10/012082	SPC	1	10	4/05/10	15.74	<0.02	0.03	0.13	0.16	<0.02	<0.12
V10/012083	SPC	1	12	4/05/10	16.00	<0.02	0.04	0.15	0.15	<0.02	<0.12
V10/012084	SPC	1	12	4/05/10	15.97	0.03	0.04	0.18	0.15	<0.02	<0.12
V10/012084	SPC	1	12	4/05/10	14.55	<0.02	0.04	0.17	0.13	<0.02	<0.12

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	*β*-sitosterol	Total Sterol
V10/012085	PSEIA	2	6	4/05/10	17.82	<0.02	0.05	0.19	0.20	<0.02	<0.12
V10/012086	PSEIA	2	6	4/05/10	13.28	<0.02	0.01	0.16	0.21	<0.02	<0.12
V10/012087	PSEIA	2	8	4/05/10	16.01	<0.02	0.04	0.19	0.20	<0.02	<0.12
V10/012088	PSEIA	2	8	4/05/10	16.13	<0.02	0.03	0.20	0.19	<0.02	<0.12
V10/012089	PSEIA	2	10	4/05/10	16.08	0.04	0.05	0.19	0.23	0.02	<0.12
V10/012090	PSEIA	2	10	4/05/10	16.84	0.02	0.04	0.21	0.24	0.02	<0.12
V10/012091	PSEIA	2	12	4/05/10	16.75	<0.02	0.05	0.17	0.15	0.02	<0.12
V10/012092	PSEIA	2	12	4/05/10	14.99	<0.02	0.04	0.17	0.21	<0.02	<0.12
V10/012093	PSEIIB	3	6	4/05/10	16.93	<0.02	0.02	0.16	0.16	<0.02	<0.12
V10/012094	PSEIIB	3	6	4/05/10	16.73	<0.02	0.04	0.15	0.14	0.02	<0.12
V10/012095	PSEIIB	3	8	4/05/10	18.06	<0.02	0.05	0.20	0.20	<0.02	<0.12
V10/012096	PSEIIB	3	8	4/05/10	15.50	0.15	0.04	0.13	0.26	0.03	0.22
V10/012097	PSEIIB	3	10	4/05/10	21.38	<0.02	0.04	0.20	0.21	0.02	<0.12
V10/012098	PSEIIB	3	10	4/05/10	16.12	<0.02	0.04	0.15	0.15	<0.02	<0.12
V10/012099	PSEIIB	3	12	4/05/10	12.29	<0.02	0.02	0.11	0.19	<0.02	<0.12
V10/012100	PSEIIB	3	12	4/05/10	16.41	<0.02	0.06	0.14	0.17	<0.02	<0.12
V10/013063	SPC	1	6	13/05/10	18.98	0.04	0.04	0.26	0.20	<0.02	<0.12
V10/013064	SPC	1	6	13/05/10	16.13	0.02	0.07	0.18	0.32	0.04	0.14
V10/013065	SPC	1	8	13/05/10	17.68	0.05	0.04	0.30	0.19	0.02	<0.12
V10/013066	SPC	1	8	13/05/10	16.54	0.02	0.05	0.17	0.25	0.03	<0.12
V10/013066	SPC	1	8	13/05/10	16.14	0.03	0.05	0.18	0.25	0.04	<0.12
V10/013067	SPC	1	10	13/05/10	16.94	0.02	0.05	0.19	0.22	0.02	<0.12
V10/013068	SPC	1	10	13/05/10	14.71	<0.02	0.04	0.16	0.21	0.02	<0.12
V10/013069	SPC	1	12	13/05/10	16.65	<0.02	0.04	0.16	0.17	<0.02	<0.12
V10/013070	SPC	1	12	13/05/10	18.71	0.03	0.04	0.25	0.16	<0.02	<0.12
V10/013071	PSEIA	2	6	13/05/10	17.87	<0.02	0.05	0.21	0.25	<0.02	<0.12
V10/013072	PSEIA	2	6	13/05/10	21.21	<0.02	0.05	0.25	0.34	0.05	0.12
V10/013073	PSEIA	2	8	13/05/10	16.48	0.03	0.05	0.30	0.32	0.04	0.12

LRN	Feed Type	Feed Type	Rate	Sample Date	Cholesterol	Brassicasterol	Campesterol	Lanthosterol	Lanosterol	*β*-sitosterol	Total Sterol
V10/013074	PSEIA	2	8	13/05/10	16.56	0.04	0.03	0.25	0.22	0.02	<0.12
V10/013075	PSEIA	2	10	13/05/10	18.15	<0.02	0.05	0.23	0.22	0.02	<0.12
V10/013076	PSEIA	2	10	13/05/10	19.09	0.04	0.03	0.21	0.20	<0.02	<0.12
V10/013077	PSEIA	2	12	13/05/10	16.69	0.03	0.05	0.24	0.14	<0.02	<0.12
V10/013078	PSEIA	2	12	13/05/10	15.46	<0.02	0.04	0.17	0.17	0.02	<0.12
V10/013079	PSEIIB	3	6	13/05/10	16.94	<0.02	0.04	0.19	0.22	0.02	<0.12
V10/013080	PSEIIB	3	6	13/05/10	16.65	<0.02	0.04	0.34	0.19	<0.02	<0.12
V10/013081	PSEIIB	3	8	13/05/10	17.85	0.03	0.04	0.23	0.27	0.02	<0.12
V10/013082	PSEIIB	3	8	13/05/10	17.94	<0.02	0.04	0.17	0.26	0.02	<0.12
V10/013083	PSEIIB	3	10	13/05/10	15.89	<0.02	0.04	0.18	0.23	0.03	<0.12
V10/013084	PSEIIB	3	10	13/05/10	17.26	<0.02	0.05	0.23	0.27	0.02	<0.12
V10/013085	PSEIIB	3	12	13/05/10	17.28	<0.02	0.05	0.24	0.31	0.02	<0.12
V10/013086	PSEIIB	3	12	13/05/10	16.37	<0.02	0.04	0.16	0.20	0.03	<0.12
V10/013086	PSEIIB	3	12	13/05/10	16.35	<0.02	0.04	0.15	0.20	<0.02	<0.12

Note: cholestenol, stigmasterol, campestanol, brassicasterol were detected at <0.02 mg/100 mL and total phytosterol <0.12 mg/100 mL.

Table 8.2: Pasture supplementation experiment I ANOVA based on feed type

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	12.258	2	6.129	1.040	.356
	Within Groups	860.352	146	5.893		
	Total	872.610	148			
Brassicasterol	Between Groups	.000	2	.000	.110	.896
	Within Groups	.038	146	.000		
	Total	.038	148			
Lathosterol	Between Groups	.019	2	.009	3.490	.033
	Within Groups	.396	146	.003		
	Total	.415	148			
Campesterol	Between Groups	.000	2	.000	1.194	.306
	Within Groups	.022	146	.000		
	Total	.022	148			
Lanosterol	Between Groups	.013	2	.007	1.880	.156
	Within Groups	.521	146	.004		
	Total	.534	148			
β-sitosterol	Between Groups	.000	2	.000	.287	.751
	Within Groups	.024	146	.000		
	Total	.024	148			
Total Phytosterol	Between Groups	.000	2	.000	.048	.953
	Within Groups	.147	146	.001		
	Total	.147	148			

Table 8.3: Post hoc analysis for lathosterol

Feed type	Feed type	Mean Difference	Std. Error	Sig.
Control	PSEIA	-0.01923	0.010517	0.164
	PSEIB	0.007523	0.010413	0.751
PSEIa	SPC	0.019231	0.010517	0.164
	PSEIB	.0267532*	0.010413	0.03
PSEIb	SPC	-0.00752	0.010413	0.751
	PSEIA	-.0267532*	0.010413	0.03

*Tukey HSD

Table 8.4: Pasture supplementation experiment I ANOVA based on feed rate

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	1.560	3	.520	.087	.967
	Within Groups	871.050	145	6.007		
	Total	872.610	148			
Brassicasterol	Between Groups	.001	3	.000	1.027	.383
	Within Groups	.037	145	.000		
	Total	.038	148			
Lathosterol	Between Groups	.002	3	.001	.213	.888
	Within Groups	.413	145	.003		
	Total	.415	148			
Campesterol	Between Groups	.000	3	.000	.194	.900
	Within Groups	.022	145	.000		
	Total	.022	148			
Lanosterol	Between Groups	.016	3	.005	1.451	.231
	Within Groups	.518	145	.004		
	Total	.534	148			
β-sitosterol	Between Groups	.000	3	.000	.190	.903
	Within Groups	.024	145	.000		
	Total	.024	148			
Total Phytosterol	Between Groups	.001	3	.000	.474	.701
	Within Groups	.146	145	.001		
	Total	.147	148			

8.4. Pasture Supplementation Experiment II Results

Table 8.5: Results summary for pasture supplementation experiment II

LRN	feed type	feed type	Feed rate	sample date	Cholesterol	Brassicasterol	Lanthosterol	Campesterol	Lanosterol	*β*-sitosterol	Total Sterols
V10/031734	SPC	1	13.5	26/10/2010	11.2782	<0.02	0.1491	0.0387	0.1224	<0.02	<0.12
V10/031735	PSEIIA	2	8	26/10/2010	11.7025	<0.02	0.1361	0.0358	0.1472	<0.02	<0.12
V10/031736	PSEIIA	2	8	26/10/2010	11.5013	<0.02	0.1303	0.0391	0.1489	<0.02	<0.12
V10/031737	SPC	1	13.5	26/10/2010	13.3707	<0.02	0.1663	0.0424	0.1478	<0.02	<0.12
V10/031738	PSEIIB	3	13.5	26/10/2010	12.5628	<0.02	0.1428	0.0384	0.1920	0.0202	<0.12
V10/031739	SPC	1	8	26/10/2010	11.4324	0.1214	0.1011	0.0477	0.1700	0.0302	0.1993
V10/031740	PSEIIA	2	12	26/10/2010	11.7009	<0.02	0.1408	0.0332	0.1423	<0.02	<0.12
V10/031741	SPC	1	12	26/10/2010	12.5033	<0.02	0.1295	0.0420	0.1468	<0.02	<0.12
V10/031742	PSEIIA	2	13.5	26/10/2010	12.2254	<0.02	0.1396	0.0287	0.1458	<0.02	<0.12
V10/031743	PSEIIB	3	13.5	26/10/2010	12.9747	<0.02	0.1444	0.0311	0.1598	<0.02	<0.12
V10/031744	SPC	1	12	26/10/2010	11.1184	<0.02	0.1311	0.0439	0.1472	0.0244	<0.12
V10/031745	PSEIIB	3	12	26/10/2010	12.3600	<0.02	0.1259	0.0419	0.2057	0.0236	<0.12
V10/031746	SPC	1	10	26/10/2010	13.2186	<0.02	0.1246	0.0440	0.1618	0.0154	<0.12
V10/031747	SPC	1	8	26/10/2010	11.2175	<0.02	0.2168	0.0745	0.3045	0.0343	0.1269
V10/031748	SPC	1	10	26/10/2010	15.6820	0.0242	0.2834	0.0793	0.3520	0.0338	0.1374
V10/031749	PSEIIA	2	12	26/10/2010	12.5113	<0.02	0.1236	0.0398	0.1363	<0.02	<0.12
V10/031750	PSEIIA	2	13.5	26/10/2010	11.5536	<0.02	0.1239	0.0366	0.1313	<0.02	<0.12
V10/031751	PSEIIA	2	10	26/10/2010	12.7486	<0.02	0.1286	0.0395	0.1486	<0.02	<0.12
V10/031752	PSEIIA	2	10	26/10/2010	11.2126	<0.02	0.1190	0.0348	0.1474	<0.02	<0.12
V10/031753	PSEIIB	3	12	26/10/2010	13.6014	<0.02	0.1150	0.0417	0.2242	<0.02	<0.12
V10/031753	PSEIIB	3	12	26/10/2010	13.3268	<0.02	0.1225	0.0463	0.2275	<0.02	<0.12
V10/031714	SPC	1	13.5	2/11/2010	11.5584	<0.02	0.1394	0.0399	0.1045	<0.02	<0.12

LRN	feed type	feed type	Feed rate	sample date	Cholesterol	Brassicasterol	Lanthosterol	Campesterol	Lanosterol	*β*-sitosterol	Total Sterols
V10/031715	PSEIIA	2	8	2/11/2010	12.4696	<0.02	0.1091	0.0403	0.1370	<0.02	<0.12
V10/031716	PSEIIA	2	8	2/11/2010	13.0681	<0.02	0.1128	0.0458	0.1550	<0.02	<0.12
V10/031717	SPC	1	13.5	2/11/2010	13.1333	<0.02	0.1370	0.0473	0.1575	<0.02	<0.12
V10/031718	PSEIIB	3	13.5	2/11/2010	11.5373	<0.02	0.1100	0.0325	0.1736	<0.02	<0.12
V10/031719	SPC	1	8	2/11/2010	13.9889	<0.02	0.1282	0.0507	0.2020	0.0216	<0.12
V10/031720	PSEIIA	2	12	2/11/2010	11.6165	<0.02	0.1191	0.0338	0.1372	<0.02	<0.12
V10/031721	SPC	1	12	2/11/2010	13.8669	<0.02	0.1335	0.0449	0.1465	<0.02	<0.12
V10/031722	PSEIIA	1	13.5	2/11/2010	12.7187	<0.02	0.1205	0.0309	0.1509	<0.02	<0.12
V10/031723	PSEIIB	3	13.5	2/11/2010	12.9762	<0.02	0.1173	0.0424	0.1807	<0.02	<0.12
V10/031724	SPC	1	12	2/11/2010	12.0302	<0.02	0.1880	0.0428	0.1294	<0.02	<0.12
V10/031725	PSEIIB	3	12	2/11/2010	12.2824	<0.02	0.0980	0.0411	0.1827	<0.02	<0.12
V10/031726	SPC	1	10	2/11/2010	14.1012	<0.02	0.1780	0.0559	0.1968	<0.02	<0.12
V10/031727	SPC	1	8	2/11/2010	13.2690	<0.02	0.0816	0.0563	0.2014	0.0261	<0.12
V10/031728	SPC	1	10	2/11/2010	13.2929	<0.02	0.1560	0.0474	0.1442	<0.02	<0.12
V10/031729	PSEIIA	2	12	2/11/2010	13.0248	<0.02	0.1034	0.0448	0.1312	<0.02	<0.12
V10/031730	PSEIIA	2	13.5	2/11/2010	11.8047	<0.02	0.1437	0.0363	0.1226	<0.02	<0.12
V10/031731	PSEIIA	2	10	2/11/2010	12.7728	<0.02	0.1544	0.0455	0.1593	<0.02	<0.12
V10/031732	PSEIIA	2	10	2/11/2010	12.1363	<0.02	0.1675	0.0506	0.2026	0.0292	<0.12
V10/031733	PSEIIB	3	12	2/11/2010	13.0890	<0.02	0.1331	0.0456	0.1951	<0.02	<0.12
V10/031733	PSEIIB	3	12	2/11/2010	12.6090	<0.02	0.1318	0.0455	0.2178	0.0215	<0.12
V10/031754	SPC	1	13.5	9/11/2010	11.2963	<0.02	0.1806	0.0352	0.1095	<0.02	<0.12
V10/031755	PSEIIA	2	8	9/11/2010	12.1113	<0.02	0.1017	0.0437	0.1383	<0.02	<0.12
V10/031756	PSEIIA	2	8	9/11/2010	12.9841	<0.02	0.0869	0.0567	0.2048	0.0241	<0.12
V10/031757	SPC	1	13.5	9/11/2010	12.8299	<0.02	0.1481	0.0564	0.2052	0.0224	<0.12
V10/031758	PSEIIB	3	13.5	9/11/2010	14.5782	<0.02	0.1577	0.0433	0.2272	<0.02	<0.12
V10/031759	SPC	1	8	9/11/2010	13.0158	<0.02	0.1224	0.0475	0.1766	<0.02	<0.12
V10/031760	PSEIIA	2	12	9/11/2010	11.7048	<0.02	0.1103	0.0395	0.1549	<0.02	<0.12

LRN	feed type	feed type	Feed rate	sample date	Cholesterol	Brassicasterol	Lanthosterol	Campesterol	Lanosterol	*β*-sitosterol	Total Sterols
V10/031761	SPC	1	12	9/11/2010	13.4170	<0.02	0.1506	0.0692	0.1306	<0.02	<0.12
V10/031762	PSEIIA	2	13.5	9/11/2010	12.9209	<0.02	0.1221	0.0304	0.1611	<0.02	<0.12
V10/031763	PSEIIB	3	13.5	9/11/2010	13.0711	<0.02	0.1303	0.0440	0.1899	<0.02	<0.12
V10/031764	SPC	1	12	9/11/2010	11.9054	<0.02	0.1436	0.0415	0.1562	<0.02	<0.12
V10/031765	PSEIIB	3	12	9/11/2010	13.5284	<0.02	0.1488	0.0536	0.2481	0.0273	<0.12
V10/031766	SPC	1	10	9/11/2010	14.3824	<0.02	0.1042	0.0605	0.2431	0.0208	<0.12
V10/031767	SPC	1	8	9/11/2010	13.2218	<0.02	0.1385	0.0459	0.1898	<0.02	<0.12
V10/031768	SPC	1	10	9/11/2010	14.2803	<0.02	0.1435	0.0535	0.1600	<0.02	<0.12
V10/031769	PSEIIA	2	12	9/11/2010	12.6747	<0.02	0.1413	0.0446	0.1284	<0.02	<0.12
V10/031770	PSEIIA	2	13.5	9/11/2010	11.1856	<0.02	0.1269	0.0386	0.1023	<0.02	<0.12
V10/031771	PSEIIA	2	10	9/11/2010	12.9944	<0.02	0.1258	0.0485	0.1470	<0.02	<0.12
V10/031772	PSEIIA	2	10	9/11/2010	12.9426	<0.02	0.1220	0.0441	0.1458	<0.02	<0.12
V10/031773	PSEIIB	3	12	9/11/2010	13.5857	<0.02	0.0807	0.0532	0.1716	<0.02	<0.12
V10/031773	PSEIIB	3	12	9/11/2010	14.0205	<0.02	0.1320	0.0489	0.1765	<0.02	<0.12

Note: cholestenol, stigmasterol, campestanol, brassicasterol were detected at <0.02 mg/100 mL and total phytosterol <0.12 mg/100 mL.

Table 8.6: Pasture supplementation experiment II ANOVA based on feed type

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	7.772	2	3.886	4.625	.014
	Within Groups	50.413	60	.840		
	Total	58.186	62			
Brassicasterol	Between Groups	.000	2	.000	.405	.669
	Within Groups	.018	60	.000		
	Total	.019	62			
Lathosterol	Between Groups	.007	2	.004	4.254	.019
	Within Groups	.052	60	.001		
	Total	.059	62			
Campesterol	Between Groups	.001	2	.001	6.421	.003
	Within Groups	.005	60	.000		
	Total	.006	62			
Lanosterol	Between Groups	.025	2	.012	7.620	.001
	Within Groups	.098	60	.002		
	Total	.122	62			
β-sitosterol	Between Groups	.000	2	.000	4.177	.020
	Within Groups	.002	60	.000		
	Total	.002	62			
Total Phytosterol	Between Groups	.004	2	.002	3.536	.035
	Within Groups	.031	60	.001		
	Total	.035	62			

Table 8.7: Post-hoc pasture supplementation experiment II feed type comparison

Dependent Variable	Feed type	Feed type	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Cholesterol	SPC	PSEIIa	0.64	0.26	0.05	0.01	1.28
		PSEIIb	-0.19	0.3	0.81	-0.91	0.53
	PSEIIa	SPC	-.64*	0.26	0.05	-1.28	-0.01
		PSEIIb	-.83*	0.3	0.02	-1.56	-0.1
	PSEIIb	SPC	0.19	0.3	0.81	-0.53	0.91
		PSEIIa	.83*	0.3	0.02	0.1	1.56
	PSEIIb	SPC	0.19	0.3	1	-0.55	0.93
		PSEIIa	0.83*	0.3	0.03	0.08	1.58
Lathosterol	SPC	PSEIIa	.022*	0.01	0.03	0	0.04
		PSEIIb	0.02	0.01	0.07	0	0.04
	PSEIIa	SPC	-0.022*	0.01	0.03	-0.04	0
		PSEIIb	0	0.01	1	-0.02	0.02
	PSEIIb	SPC	-0.02	0.01	0.07	-0.04	0
		PSEIIa	0	0.01	1	-0.02	0.02
Campesterol	SPC	PSEIIa	.01*	0	0	0	0.02
		PSEIIb	0.01	0	0.09	0	0.01
	PSEIIa	SPC	-.01*	0	0	-0.02	0
		PSEIIb	0	0	0.61	-0.01	0
	PSEIIb	SPC	-0.01	0	0.09	-0.01	0
		PSEIIa	0	0	0.61	0	0.01
Lanosterol	SPC	PSEIIa	0.03	0.01	0.06	0	0.06
		PSEIIb	-0.02	0.01	0.17	-0.06	0.01
	PSEIIa	SPC	-0.035	0.01	0.06	-0.06	0
		PSEIIb	-0.05*	0.01	0	-0.08	-0.02
	PSEIIa	SPC	0.02	0.01	0.17	-0.01	0.06
		PSEIIa	0.05*	0.01	0	0.02	0.08

* The mean difference is significant at the 0.05 level.

Table 8.8: Pasture supplementation experiment II ANOVA based on feed rate

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	6.41	3.00	2.14	2.44	0.07
	Within Groups	51.78	59.00	0.88		
	Total	58.19	62.00			
Brassicasterol	Between Groups	0.00	3.00	0.00	0.97	0.42
	Within Groups	0.02	59.00	0.00		
	Total	0.02	62.00			
Lathosterol	Between Groups	0.01	3.00	0.00	2.22	0.10
	Within Groups	0.05	59.00	0.00		
	Total	0.06	62.00			
Campesterol	Between Groups	0.00	3.00	0.00	5.42	0.00
	Within Groups	0.01	59.00	0.00		
	Total	0.01	62.00			
Lanosterol	Between Groups	0.01	3.00	0.00	1.41	0.25
	Within Groups	0.11	59.00	0.00		
	Total	0.12	62.00			
β-sitosterol	Between Groups	0.00	3.00	0.00	1.33	0.27
	Within Groups	0.00	59.00	0.00		
	Total	0.00	62.00			
Total Phytosterol	Between Groups	0.00	3.00	0.00	2.61	0.06
	Within Groups	0.03	59.00	0.00		
	Total	0.04	62.00			

Table 8.9: Post-hoc analysis for campesterol

Feed rate	Feed rate	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
8	10	-0.00163	0.003589	0.968	-0.01112	0.007856
	12	0.00401	0.003182	0.592	-0.0044	0.012421
	13.5	.0101611*	0.003277	0.015	0.001498	0.018824
10	8	0.001633	0.003589	0.968	-0.00786	0.011123
	12	0.005643	0.003182	0.296	-0.00277	0.014054
	13.5	.0117944*	0.003277	0.004	0.003132	0.020457
12	8	-0.00401	0.003182	0.592	-0.01242	0.004402
	10	-0.00564	0.003182	0.296	-0.01405	0.002769
	13.5	0.006152	0.002824	0.141	-0.00132	0.013618
13.5	8	-.0101611*	0.003277	0.015	-0.01882	-0.0015
	10	-.0117944*	0.003277	0.004	-0.02046	-0.00313
	12	-0.00615	0.002824	0.141	-0.01362	0.001315

*Tukey HSD

8.5. Tannin and Cotton Seed Oil Experiment Results

Table 8.10: Results summary of TCSO feeding experiment

LRN	cow id	feed type	feed type	sample date	cholesterol	Lanthosterol	Campesterol	Lanosterol	*β*-sitosterol
V11/002701	FT 1041	CSO	2	29/09/2010	13.0899	0.1673	0.0266	0.0454	0.0107
V11/002702	FT 1042	Control	1	29/09/2010	11.6297	0.1601	0.0212	0.2569	0.0200
V11/002703	FT 1043	TANN	3	1/10/2010	14.9225	0.1154	0.0284	0.2106	0.0194
V11/002704	FT 1044	TCSO	4	1/10/2010	10.6243	0.1591	0.0206	0.1053	0.0167
V11/002705	FT 1045	TANN	3	6/10/2010	11.7245	0.1752	0.0203	0.2485	0.0147
V11/002706	FT 1046	CSO	2	6/10/2010	10.7138	0.1599	0.0230	0.0963	0.0120
V11/002707	FT 1047	TCSO	4	8/10/2010	11.9345	0.2693	0.0206	0.0674	0.0118
V11/002708	FT 1048	Control	1	8/10/2010	12.9109	0.1866	0.0264	0.2473	0.0183
V11/002709	FT 1049	CSO	2	20/10/2010	11.6334	0.3583	0.0230	0.2508	0.0157
V11/002710	FT 1050	Control	1	20/10/2010	13.8344	0.2833	0.0538	0.2972	0.0406
V11/002711	FT 1051	TCSO	3	22/10/2010	11.6788	0.0790	0.0244	0.1330	0.0146
V11/002712	FT 1052	TCSO	4	22/10/2010	11.4870	0.0922	0.0290	0.2115	0.0201
V11/002713	FT 1053	TCSO	3	27/10/2010	11.7321	0.0912	0.0295	0.2857	0.0128
V11/002714	FT 1054	CSO	2	27/10/2010	12.3603	0.0732	0.0232	0.2259	0.0129
V11/002715	FT 1055	TCSO	4	29/10/2010	13.0745	0.1242	0.0288	0.1471	0.0154
V11/002716	FT 1056	Control	1	29/10/2010	11.3301	0.1507	0.0157	0.1311	0.0125
V11/002717	FT 1065	CSO	2	10/11/2010	13.2278	0.1176	0.0302	0.2989	0.0209
V11/002718	FT 1066	Control	1	10/11/2010	11.4263	0.1150	0.0209	0.1154	0.0107
V11/002719	FT 1067	TCSO	3	12/11/2010	13.3042	0.0703	0.0274	0.1188	0.0142
V11/002720	FT 1068	TCSO	4	12/11/2010	12.6366	0.0897	0.0214	0.2213	0.0144
V11/002720	FT 1068	TCSO	4	12/11/2010	12.5793	0.1329	0.0239	0.2223	0.0107
V11/002721	FT 1069	TCSO	3	17/11/2010	9.9277	0.2181	0.0203	0.1286	0.0105
V11/002722	FT 1070	CSO	2	17/11/2010	10.3977	0.2069	0.0216	0.1453	0.0110

LRN	cow id	feed type	feed type	sample date	cholesterol	Lanthosterol	Campesterol	Lanosterol	*β*-sitosterol
V11/002723	FT 1071	TCSO	4	19/11/2010	13.8080	0.1092	0.0272	0.2300	0.0149
V11/002723	FT 1071	TCSO	4	19/11/2010	13.4967	0.1374	0.0339	0.2938	0.0200
V11/002724	FT 1072	Control	1	19/11/2010	12.8655	0.1146	0.0189	0.2459	0.0137
V11/002726	FT 1082	CSO	2	1/12/2010	15.9265	0.1755	0.0250	0.2574	0.0192
V11/002727	FT 1083	TCSO	3	3/12/2010	17.6515	0.1245	0.0489	0.2910	0.0247
V11/002728	FT 1084	TCSO	4	3/12/2010	11.0316	0.2543	0.0237	0.1250	0.0111
V11/002729	FT 1085	TCSO	3	8/12/2010	10.9894	0.1524	0.0177	0.1301	0.0181
V11/002730	FT 1086	CSO	2	8/12/2010	13.4541	0.1016	0.0259	0.1343	0.0159
V11/002731	FT 1087	TCSO	4	10/12/2010	13.5234	0.1149	0.0259	0.2506	0.0198
V11/002732	FT 1088	Control	1	10/12/2010	11.8121	0.1618	0.0221	0.1452	0.0141

Note: cholestenol, stigmasterol, campestanol, brassicasterol were detected at <0.02 mg/100 mL and total phytosterols <0.12 mg/100 mL.

Table 8.11: TCSO experiment ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	1.018	3	.339	.121	.947
	Within Groups	81.669	29	2.816		
	Total	82.687	32			
Lathosterol	Between Groups	.009	3	.003	.673	.576
	Within Groups	.128	29	.004		
	Total	.137	32			
Campesterol	Between Groups	.000	3	.000	.117	.950
	Within Groups	.002	29	.000		
	Total	.002	32			
Lanosterol	Between Groups	.002	3	.001	.130	.941
	Within Groups	.173	29	.006		
	Total	.175	32			
β-sitosterol	Between Groups	.000	3	.000	.584	.630
	Within Groups	.001	29	.000		
	Total	.001	32			

8.6. Grape Marc Feeding Experiment Results

Table 8.12: Results summary for GM feeding experiment

LRN	cow id	feed type	feed type	sample date	cholesterol	Lanthosterol	Campesterol	Lanosterol	β-sitosterol
V11/015450	GM030	Control	1	16/03/2011	13.6414	0.0748	0.0293	0.2164	<0.02
V11/015451	GM031	EGM	3	16/03/2011	17.4441	0.1422	0.0389	0.2373	<0.02
V11/015452	GM032	DGM	2	16/03/2011	18.1561	0.097	0.0502	0.273	<0.02
V11/015453	GM033	Control	1	16/03/2011	13.2248	0.074	0.0298	0.126	<0.02
V11/015454	GM034	Control	1	16/03/2011	14.8876	0.0819	0.0409	0.2143	<0.02
V11/015455	GM035	EGM	3	16/03/2011	16.3038	0.0939	0.0442	0.2177	0.0208
V11/015456	GM036	Control	1	16/03/2011	13.2678	0.0659	0.0414	0.1209	<0.02
V11/015457	GM037	EGM	3	16/03/2011	14.0504	0.0843	0.0289	0.1332	0.0128
V11/015458	GM038	EGM	3	16/03/2011	11.7674	0.0854	0.0267	0.157	<0.02
V11/015459	GM039	Control	1	16/03/2011	15.497	0.1506	0.0378	0.2878	0.0237
V11/015460	GM040	DGM	2	16/03/2011	12.7609	0.0712	0.0391	0.2254	0.0216
V11/015461	GM041	DGM	2	16/03/2011	13.1276	0.1036	0.0318	0.2231	<0.02
V11/015462	GM042	DGM	2	16/03/2011	16.4694	0.1566	0.0421	0.354	0.0286
V11/015463	GM043	Control	1	16/03/2011	11.9849	0.1011	0.0257	0.1621	<0.02
V11/015464	GM044	DGM	2	16/03/2011	16.0271	0.0553	0.0433	0.1801	0.0297
V11/015465	GM045	EGM	3	16/03/2011	14.2458	0.0823	0.0337	0.2373	0.0215
V11/015466	GM046	DGM	2	16/03/2011	15.5979	0.1047	0.0377	0.2714	<0.02
V11/015467	GM047	EGM	3	16/03/2011	14.5001	0.1671	0.0277	0.2415	<0.02
V11/015468	GM048	Control	1	16/03/2011	16.7927	0.1641	0.0299	0.2228	<0.02
V11/015469	GM049	DGM	2	16/03/2011	12.1173	0.0669	0.0266	0.1514	0.021
V11/015470	GM050	DGM	2	16/03/2011	11.206	0.0759	0.0277	0.1736	0.0127

LRN	cow id	feed type	feed type	sample date	cholesterol	Lanthosterol	Campesterol	Lanosterol	β-sitosterol
V11/015471	GM051	DGM	2	16/03/2011	13.2569	0.115	0.0331	0.2543	0.0215
V11/015472	GM052	Control	1	16/03/2011	18.0341	0.0978	0.0387	0.3616	0.0211
V11/015473	GM053	EGM	3	16/03/2011	14.7926	0.1054	0.0306	0.2807	0.0157
V11/015474	GM054	EGM	3	16/03/2011	15.586	0.1973	0.0379	0.2676	0.0234
V11/015475	GM055	DGM	2	16/03/2011	13.9771	0.1285	0.034	0.3277	0.021
V11/015476	GM056	Control	1	16/03/2011	16.4742	0.1609	0.0343	0.2506	0.0207
V11/015477	GM057	EGM	3	16/03/2011	16.3127	0.0958	0.0444	0.2006	<0.02
V11/015477	GM057	EGM	3	16/03/2011	17.2514	0.0892	0.0463	0.2145	<0.02
V11/015478	GM058	Control	1	16/03/2011	14.4735	0.0602	0.0338	0.3154	0.022
V11/015479	GM059	Control	1	16/03/2011	14.0452	0.0998	0.0396	0.2292	0.0223
V11/015480	GM060	EGM	3	16/03/2011	16.3301	0.121	0.0388	0.3264	0.0233
V11/015481	GM061	Control	1	16/03/2011	13.916	0.0907	0.0273	0.2034	0.015
V11/015482	GM094	Control	1	6/04/2011	15.4053	0.0688	0.0326	0.2976	0.0226
V11/015483	GM095	EGM	3	6/04/2011	22.0016	0.2126	0.0574	0.2905	0.0287
V11/015484	GM096	DGM	2	6/04/2011	17.0118	0.1128	0.0447	0.1187	0.0195
V11/015485	GM097	Control	1	6/04/2011	17.0155	0.0989	0.0343	0.164	0.0105
V11/015486	GM098	Control	1	6/04/2011	17.2027	0.0912	0.0394	0.2552	0.0157
V11/015487	GM099	EGM	3	6/04/2011	16.9665	0.0936	0.0563	0.1742	0.0266
V11/015488	GM100	Control	1	6/04/2011	22.6669	0.0207	0.066	0.2224	0.0248
V11/015489	GM101	EGM	3	6/04/2011	18.5903	0.1051	0.0455	0.1167	0.0189
V11/015490	GM102	EGM	3	6/04/2011	16.2746	0.1451	0.0622	0.1982	0.0171
V11/015491	GM103	Control	1	6/04/2011	20.6205	0.2073	0.0462	0.3254	0.0212
V11/015492	GM104	DGM	2	6/04/2011	15.9499	0.1588	0.0524	0.1743	0.0207
V11/015493	GM105	DGM	2	6/04/2011	15.6888	0.1641	0.0448	0.1198	<0.02
V11/015494	GM106	DGM	2	6/04/2011	13.7224	0.2297	0.0649	0.1118	<0.02
V11/015495	GM107	Control	1	6/04/2011	16.3334	0.1809	0.033	0.2563	0.0209
V11/015496	GM108	DGM	2	6/04/2011	16.906	0.0797	<0.02	0.0864	0.0247

LRN	cow id	feed type	feed type	sample date	cholesterol	Lanthosterol	Campesterol	Lanosterol	β-sitosterol
V11/015497	GM109	EGM	3	6/04/2011	14.6555	0.15	0.0497	0.164	0.0212
V11/015498	GM110	DGM	2	6/04/2011	17.6664	0.1878	0.046	0.1209	0.0131
V11/015499	GM111	EGM	3	6/04/2011	13.5503	0.1502	0.048	0.15	0.0121
V11/015500	GM112	Control	1	6/04/2011	21.4379	0.1995	0.0466	0.3273	0.0226
V11/015501	GM113	DGM	2	6/04/2011	17.2514	0.1518	0.0479	0.0748	<0.02
V11/015502	GM114	DGM	2	6/04/2011	12.1105	0.0844	0.0362	0.0566	<0.02
V11/015503	GM115	DGM	2	6/04/2011	20.9716	0.1725	0.0684	0.1081	0.0215
V11/015504	GM116	Control	1	6/04/2011	19.4337	0.0832	0.0407	0.3328	0.0263
V11/015505	GM117	EGM	3	6/04/2011	16.7523	0.1355	0.0458	0.2744	<0.02
V11/015506	GM118	EGM	3	6/04/2011	17.9346	0.1625	0.0485	0.1877	<0.02
V11/015507	GM119	DGM	2	6/04/2011	18.6378	0.1568	0.0289	0.1423	<0.02
V11/015508	GM120	Control	1	6/04/2011	15.9783	0.1306	0.0254	0.2834	<0.02
V11/015509	GM121	EGM	3	6/04/2011	23.7698	0.1583	0.0824	0.2182	0.0216
V11/015510	GM122	Control	1	6/04/2011	20.0625	0.143	0.0508	0.5564	0.0334
V11/015511	GM123	Control	1	6/04/2011	16.2119	0.1249	0.048	0.2434	<0.02
V11/015512	GM124	EGM	3	6/04/2011	16.531	0.1682	0.0578	0.3696	0.0213
V11/015513	GM125	Control	1	6/04/2011	16.301	0.0981	0.0392	0.275	0.0223
V11/015513	GM125	Control	1	6/04/2011	16.7452	0.116	0.0329	0.2536	<0.02

Note: cholestenol, stigmasterol, campestanol, brassicasterol were detected at <0.02 mg/100 mL and total phytosterols <0.12 mg/100 mL

Table 8.13: GM experiment ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	14.836	2	7.418	1.049	.356
	Within Groups	445.663	63	7.074		
	Total	460.499	65			
Lathosterol	Between Groups	.004	2	.002	1.134	.328
	Within Groups	.123	63	.002		
	Total	.127	65			
Campesterol	Between Groups	.001	2	.000	2.339	.105
	Within Groups	.009	63	.000		
	Total	.010	65			
Lanosterol	Between Groups	.076	2	.038	5.919	.004
	Within Groups	.405	63	.006		
	Total	.481	65			
β-sitosterol	Between Groups	.000	2	.000	.089	.915
	Within Groups	.001	63	.000		
	Total	.001	65			
Total Phytosterol	Between Groups	.001	2	.000	1.462	.239
	Within Groups	.013	63	.000		
	Total	.013	65			

Table 8.14: Post-hoc calculation for lanosterol

Feed type	Feed type	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	DGM	.0827470*	0.0241	0.003	0.025006	0.1405
	WGM	0.0383558	0.0237	0.246	-0.018617	0.0953
DGM	Control	-.0827470*	0.0241	0.003	-0.140488	-0.025
	WGM	-0.0443912	0.0251	0.187	-0.104527	0.0157
WGM	Control	-0.0383558	0.0237	0.246	-0.095328	0.0186
	WGM	0.0444	0.0251	0.187	-0.0157	0.1045

8.7. Rumen Protected Feeding Experiment Results

Table 8.15: Results summary for RP feeding experiment

LRN	Cow ID	feed type	Cholesterol	Lanthosterol	Campesterol	Lanosterol	β-sitosterol	Total Sterols
			mg/100g					
V15/030606	Control (OP) Cow2	1	13.6158	0.0760	0.0444	0.2312	<0.02	<0.12
V15/030607	OP Cow 2	2	13.6642	0.1065	0.0450	0.2580	<0.02	<0.12
V15/030608	Control (Sterol) Cow 3	1	12.4399	0.0682	0.0367	0.1936	<0.02	<0.12
V15/030609	SP Cow 3	3	9.6815	0.0538	0.0364	0.1565	<0.02	<0.12
V15/030610	Control (Sterol) Cow 4	1	11.7130	0.0624	0.0335	0.1826	<0.02	<0.12
V15/030611	PS Cow 4	3	10.3062	0.0627	0.0618	0.2363	0.0218	<0.12
V15/030611	PS Cow 4	3	10.2942	0.0533	0.0602	0.2229	0.0241	<0.12
V15/030612	Control (Cow5)	1	13.0698	0.0852	0.0429	0.1947	0.0232	<0.12
V15/030613	Control (Cow5)	1	13.7288	0.0719	0.0401	0.1942	<0.02	<0.12
V15/030614	Control (Cow6)	1	13.4957	0.0584	0.0396	0.1484	0.0217	<0.12
V15/030615	Control (Cow 6)	1	12.6385	0.0691	0.0373	0.1716	0.0201	<0.12
V15/030616	CSP Cow 7	4	11.1614	0.0471	0.0433	0.1093	<0.02	<0.12

Note: cholestenol, stigmasterol, campestanol, brassicasterol were detected at <0.02 mg/100 mL and total phytosterols <0.12 mg/mL.

Table 8.16: RP experiment ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Cholesterol	Between Groups	20.515	3	6.838	15.611	0.001
	Within Groups	3.504	8	0.438		
	Total	24.019	11			
Lathosterol	Between Groups	0.002	3	0.001	11.946	0.003
	Within Groups	0.001	8	0		
	Total	0.003	11			
Campesterol	Between Groups	0	3	0	2.129	0.175
	Within Groups	0	8	0		
	Total	0.001	11			
Lanosterol	Between Groups	0.012	3	0.004	4.206	0.046
	Within Groups	0.007	8	0.001		
	Total	0.019	11			
β-sitosterol	Between Groups	0	3	0	1.064	0.417
	Within Groups	0	8	0		
	Total	0	11			
Total Phytosterols	Between Groups	0	3	0	1.412	0.309
	Within Groups	0.001	8	0		
	Total	0.001	11			