The analysis of fat-soluble vitamins in dairy cow milk by high performance liquid chromatography-ion trap mass spectrometry

Submitted by

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Abstract

Fat-soluble vitamins A, D, E, and vitamin A precursor β-carotene are essential nutrients for the human body. The analysis of these compounds is difficult due to low levels (ppt - ppm), and physical and chemical similarity to other compounds present in foods. Traditionally, the determinative step uses high performance liquid chromatography (HPLC) coupled to either ultraviolet-visible (A, D, E and β-carotene) or fluorescence (A and E) detection.

The work described in this thesis demonstrates that HPLC coupled with mass spectrometry (MS) is a viable alternative due to its superior selectivity and sensitivity, reducing the need for time-consuming sample preparation, and enabling the analysis of several vitamins in a single analytical method.

High performance liquid chromatography-ion trap mass spectrometry (HPLC-MS^n) and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods were developed to measure the levels of vitamin D_3 in fortified commercial cow's milk (2 μg/100 ml), unfortified commercial and fresh cow's milk (0.01-0.05 μg/100 ml). An additional solid phase extraction step enabled the lower levels (<0.1 μg/100 ml) to be measured. 25-hydroxyvitamin D_3 was also measured, although the extraction procedure was not fully optimised for this compound.

A single HPLC-MS^n method suitable for the analysis of all *trans*-retinol (vitamin A), α-tocopherol (vitamin E) and β-carotene in cow's milk was also developed. This method was used to measure the effects of different supplementary feeding regimes on the fat-soluble vitamin content of milk from pasture-fed dairy cows. Typical levels were all *trans*-retinol, 45 μg/100 ml; α-tocopherol, 150 μg/100 ml; and β-carotene, 12 μg/100 ml.

The results from the feeding experiment showed reductions in the levels of β-carotene and α-tocopherol in milk due to the feeding of Partial Mixed Rations, most likely due to reduced intake of pasture, which was the major source of these compounds in the cows' diet.

Declaration of authenticity

I, Timothy Edward Plozza, declare that the Master by Research thesis entitled "The analysis of fat-soluble vitamins in dairy cow milk by high performance liquid chromatography-ion trap mass spectrometry" is no more than 60,000 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.

Timothy Edward Plozza

14 February 2017

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Finally, I would like to thank my family that grew by three children while I completed this project. They have been a source of much distraction, but that is life ☺

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List of Publications

Publications arising from this thesis are presented below:

Plozza, T., Trenerry, V.C., Caridi, D. (2012). The simultaneous determination of vitamins A, E and β-carotene in bovine milk by high performance liquid chromatography-ion trap mass spectrometry (HPLC-MS^n). *Food Chemistry,* 134(1) 559-563.

Tim Plozza, Craige Trenerry, Domenico Caridi. The determination of vitamins A, E and β-carotene in milk by LC-ion trap MS. 12th Government Food Analysts Conference. 22-24 February 2011, Brisbane (Presentation).

Trenerry, V.C., Plozza, T., Caridi, D., Murphy, S. (2011). The determination of vitamin D_3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemistry,* 125(4) 1314-1319.

Trenerry, V.C., Plozza, T., Caridi, D., Murphy, S. Milk, vitamin D_3 and liquid chromatography-mass spectrometry. 11th Government Food Analysts Conference. 22-24 February 2009, Melbourne (Poster).

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List of Abbreviations and Acronyms

APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure Photo Ionisation
BHT	Butylated hydroxytoluene (2,6-Di-tert-butyl-4-methylphenol)
CID	Collision-Induced Dissociation
%CV	% Coefficient of Variation
DAD	Diode Array Detector
DM	Dry Matter
DPI	Department of Primary Industries, Victoria, Australia (subsequently DEPI and DEDJTR)
DMEQ-TAD	4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl) ethyl]-1,2,4- triazoline-3,5-dione
Em	Emission
ESI	Electrospray Ionisation
Ex	Excitation
Fl	Fluorescence Detector
FTICR	Fourier Transform Ion Cyclotron Resonance Mass Spectrometer
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS^n	Ion Trap Mass Spectrometer
MS/MS	Tandem Mass Spectrometer
m/z	Mass to Charge Ratio
NATA	National Association of Testing Authorities, Australia
PDA	Photodiode Array Detector
PIC	Paired Ion Chromatography
PMR	Partial Mixed Ration
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
PTFE	Polytetrafluoroethylene
RDI	Recommended Dietary Intake

S/N	Signal to Noise ratio
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
TOF	Time of Flight Mass Spectrometer
UHPLC	Ultra-High Performance Liquid Chromatography
UV/Vis	Ultraviolet Visible Detector
2D LC	Two-dimensional Liquid Chromatography
$25(OH)D_2$	25-hydroxyvitamin D_2
$25(OH)D_3$	25-hydroxyvitamin D_3

Chapter 1: Literature review:
Fat-soluble vitamin analysis in dairy cow milk

1.1. Introduction

Fat-soluble vitamins are essential nutrients that are required in sufficient amounts from the diet to perform vital functions within the body, for example vitamin A is an essential component of the retina and has a recommended dietary intake of 900 µg/day in adult males (Ball 1998; National Health and Medical Research Council 2006). Vitamin determinations in food are carried out for a range of reasons, including nutritional labelling, quality assurance of fortified products, nutritional surveys, food composition tables and to assess the effects of environmental and seasonal conditions on levels in food (Ball 1998).

Fat-soluble vitamin analysis is difficult due to the complexity of the compounds, their low levels in foods, and their physical and chemical similarity to other compounds present in foods. Thus, to avoid interferences, the analytical method must extract the vitamins from the sample matrix, separate them from co-extracted compounds and then selectively detect their concentration. This is a tedious, time consuming process, and has traditionally used high performance liquid chromatography (HPLC) to separate the compounds followed by either ultraviolet visible detection (UV/Vis), for vitamins A, D, K and carotenes, or fluorescence detector (Fl) for vitamins A, E, K (Ball 1998; Byrdwell 2009; Byrdwell, DeVries, Exler, Harnly, Holden, Holick*, et al.* 2008; Department of Primary Industries 2007; Heudi, Trisconi and Blake 2004; Hulshof, van Roekel-Jansen, van de Bovenkamp and West 2006; Kurmann and Indyk 1994; Lanina, Toledo, Sampels, Kamal-Eldin and Jastrebova 2007). However, the compounds are usually assayed individually due to their chemical diversity and varying levels within samples.

The relatively recent, rapid advances in HPLC coupled with mass spectrometric detection (HPLC-MS) have seen it emerge as a major breakthrough in analytical science (Willoughby 2002). This technique combines the separating power of HPLC with the extra sensitivity, selectivity and powerful structure elucidation ability of mass spectrometry, potentially allowing for the analysis of all fat-soluble vitamins with one analytical method. Numerous HPLC-MS methods for the analysis of fat-soluble

vitamins in a variety of matrices have been published, including the analysis of vitamins A, D, E, and K in breastmilk (Kamao, Tsugawa, Suhara, Wada, Mori, Murata, *et al.* 2007) and human blood serum (Capote, JimÃ©nez, Granados and De Castro 2007) by HPLC-MS/MS, vitamin D in fortified foods by HPLC-MS/MS (Byrdwell 2009), vitamins A, D, and E in infant formula (Heudi, *et al.* 2004), vitamin D in human blood serum and fat tissue (Blum, Dolnikowski, Seyoum, Harris, Booth, Peterson, *et al.* 2008), and tocopherols in sunflower oil and milk by HPLC-MS (Lanina, *et al.* 2007).

The development of a 'one-method-fits-all' method for the determination of fat-soluble vitamins and β-carotene in milk using HPLC-MS^n or HPLC-MS/MS would reduce the time and increase the cost effectiveness of the analysis of a broad range of fat-soluble vitamins. HPLC-MS^n would also allow for the concurrent acquisition of full-scan MS data, permitting the retrospective analysis of the full-scan data to search for other fat-soluble compounds of biological significance e.g. sterols, which may be of interest at a later date.

1.2. Vitamins

Vitamins are organic compounds in food which are essential for human life, but cannot be synthesised in our bodies (Coultate 2002). Vitamins D and K are the two exceptions to this definition: the body can synthesise its own vitamin D through exposure to ultraviolet B radiation (sunlight), and vitamin K_2 is produced by bacteria in the large intestine. However, in certain circumstances these may be insufficient to meet the body's needs, in which case the vitamins must be sourced from the diet. Australian Recommended Dietary Intakes (RDI) are defined as the average daily dietary intake level that is sufficient to meet the nutrient requirements of 97–98 per cent of healthy individuals in a particular life stage and gender group (National Health and Medical Research Council 2006). The RDI of the vitamins at the various stages of life are summarised in Table 1-1.

Table 1-1: Australian vitamin daily recommended dietary intakes (National Health and Medical Research Council 2006)

	Age (years)															
	Children		Males						Females							
Vitamin	**1-3**	**4-8**	**9-13**	**14-18**	**19-30**	**31-50**	**51-70**	**>70**	**9-13**	**14-18**	**19-30**	**31-50**	**51-70**	**>70**	**Pregnancy**	**Lactation**
A (µg)	300	400	600	900	900	900	900	900	600	700	700	700	700	700	800 700[D]	1100
B_1 (mg)	0.5	0.6	0.9	1.2	1.2	1.2	1.2	1.2	0.9	1.1	1.1	1.1	1.1	1.1	1.4	1.4
B_2 (mg)	0.5	0.6	0.9	1.3	1.3	1.3	1.3	1.6	0.9	1.1	1.1	1.1	1.1	1.3	1.4	1.6
B_3 (mg)	6	8	12	16	16	16	16	16	12	14	14	14	14	14	18	17
B_5[A] (mg)	3.5	4	5	6	6	6	6	6	4	4	4	6	4	4	5	6
B_6 (mg)	0.5	0.6	1.0	1.3	1.3	1.3	1.7	1.7	1.0	1.2	1.3	1.3	1.5	1.5	1.9	2.0
B_7[B] (µg)	8	12	20	30	30	30	30	30	20	25	25	25	25	25	30	35
B_9[C] (µg)	150	200	300	400	400	400	400	400	300	400	400	400	400	400	600	500
B_{12} (µg)	0.9	1.2	1.8	2.4	2.4	2.4	2.4	2.4	1.8	2.4	2.4	2.4	2.4	2.4	2.6	2.8
C (mg)	35	35	40	40	45	45	45	45	40	40	45	45	45	45	60 55[D]	85 80[D]
D (µg)	5	5	5	5	5	5	10	15	5	5	5	5	10	15	5	5
E (mg)	5	6	9	10	10	10	10	10	8	8	7	7	7	7	7 8[D]	11 12[D]
K (µg)	25	35	45	55	70	70	70	70	45	55	60	60	60	60	60	60

[A]Pantothenic acid; [B]Biotin; [C]Folate, [D]Recommended intake for 14-18 year olds.

Vitamins are broadly classified as either water-soluble: B_1 (thiamine), B_2 (riboflavin), B_3 (niacin), B_5 (pantothenic acid), B_6 (pyridoxine, pyridoxal, pyridoxamine), B_{12} (cobalamins), folate, biotin and C (ascorbic acid) or fat-soluble: A (retinol), D_2 (ergocalciferol) and D_3 (cholcalciferol), E (tocopherol), and K (phylloquinone, menaquinone). The B group vitamins have a range of functions including roles in the energy pathways of cells (B_1, B_2 and B_3), metabolism (B_5, B_6, B_{12}, biotin, folate), red blood cell formation, and nervous system maintenance (folate). Vitamin C is required for collagen synthesis, wound healing, immune system function, absorption and utilization of iron, and acts as an antioxidant. Fat-soluble vitamins are required for a range of important biological functions in the body including vision (A), intestinal absorption of calcium and phosphorus and bone mineralisation (D), lipid antioxidant (E), and blood-clotting factor (K) (Ball 1998; Coultate 2002).

1.2.1. Water-soluble vitamins

Water-soluble vitamins travel freely throughout the body and excess amounts are usually excreted by the kidneys. The body needs most of the water-soluble vitamins in frequent small doses. These vitamins are not likely to reach toxic levels, but niacin, B_6, folate, and ascorbic acid have recommended upper consumption limits since prolonged excessive intake can cause adverse effects (National Health and Medical Research Council 2006). A balanced diet usually provides enough of these vitamins, however, vegans in particular may need to use supplements to get enough B_{12}, as specified in Table 1-1 (National Health and Medical Research Council 2006). Common sources of the water-soluble vitamins are shown in Table 1-2.

Table 1-2: Common sources of the water-soluble vitamins (Coultate 2002)

Vitamin	**Sources**
Thiamine	Pork, whole grain breads and cereals, legumes
Riboflavin	Meat, liver, milk, cheese, yeast extract
Niacin	Meat, whole grain breads and cereals, legumes, vegetables
Pantothenic acid	Widespread in foods
Biotin	Widespread in foods
Pyridoxine	Meat, eggs, wheat germ, dairy
Folic acid	Leafy green vegetables, liver
Cobalamin	Meat, liver, eggs, milk
Ascorbic acid	Fruits and vegetables

1.2.2. Fat-soluble vitamins

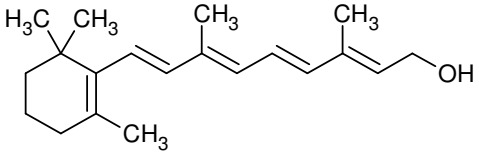
Fat-soluble vitamins are stored in the body's cells and are not excreted as easily as water-soluble vitamins, and subsequently need not be consumed as often as water-soluble vitamins. While adequate amounts, as specified in Table 1-1, are needed, high levels of a fat-soluble vitamin could become toxic, e.g. vitamin A and vitamin D. Common sources of the fat-soluble vitamins are shown in Table 1-3.

Table 1-3: Common dietary sources of the fat-soluble vitamins (Ball 1998; Burild, Lauridsen, Faqir, Sommer and Jakobsen 2016; Magalhaes, Carvalho, Guido and Barros 2007)

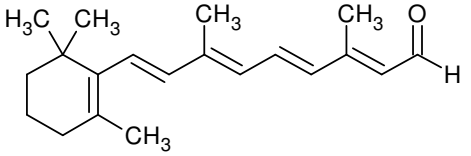
Vitamin	**Sources**
Vitamin A	Meat, eggs, liver, milk, cheese, cream, butter and fortified margarine
β-carotene	Leafy vegetables, apricots, carrots, pumpkin, sweet potatoes
Vitamin D_2	Mushrooms, alfalfa
Vitamin D_3	Eggs, liver, fatty fish, cod liver oil, fortified milk and fortified margarine, pork fat
Vitamin E	Polyunsaturated plant oils (soybean, cottonseed, sunflower), leafy green vegetables, wheat germ, whole grain products, eggs, nuts and seeds
Vitamin K	Leafy green vegetables (cabbage family), liver, cheese. Produced in the intestinal tract by bacteria

1.2.2.1. Vitamin A and carotenes

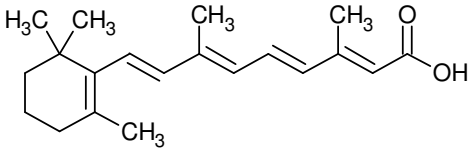
Vitamin A is defined as retinol, however other retinoids also have vitamin A activity and are referred to as vitamers (Ball 1998), the main vitamers being retinal and retinoic acid. The structures are shown in Figure 1-1. Retinoids are found only in animals and originate from the consumption and metabolism of a number of carotenoids, which are referred to as provitamin A (precursors to vitamin A). The most significant of these is β-carotene, which by weight is generally considered to have one sixth the vitamin A activity of retinol when consumed in the human diet. This factor takes into account the efficiency of absorption and the extent to which it is converted to retinol in the body. This is only an approximation as the efficiency of absorption is influenced by a number of factors including the amount of fat in the meal, and its conversion to retinol is inversely related to the amount of β-carotene ingested and the body's vitamin A status (Coultate 2002; Eitenmiller and Landen 1999). Provitamins α- and γ-carotene, β-cryptoxanthin and β-apo-8'-carotenal can also be converted to retinol, but to a much lesser extent than β-carotene due to these compounds containing only one β-ionone ring, whereas β-carotene contains two. The structures of some of the main provitamin A carotenoids are shown in Figure 1-1.



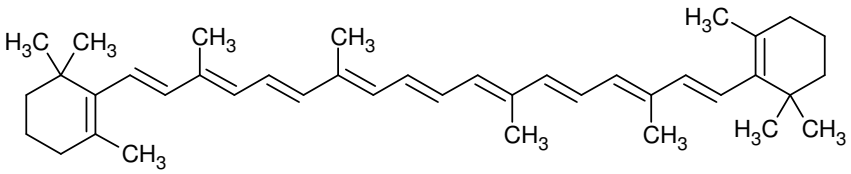
all *trans*-retinol



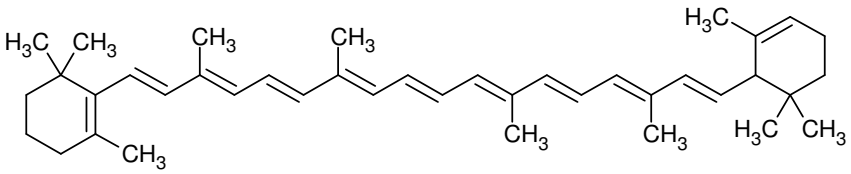
all *trans*-retinal



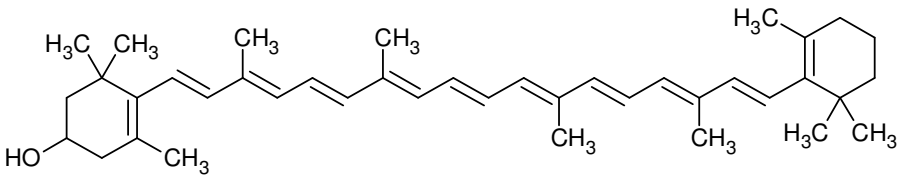
all *trans*-retinoic acid



β-carotene



α-carotene



β-cryptoxanthin

Figure 1-1: The structures of some major retinoids and provitamin A carotenoids (Eitenmiller and Landen 1999)

Vitamin A is an essential compound for vision due to its role in the detection of light in the retina. Other important biological functions in the body include regulation of cell and tissue growth and differentiation. Carotenoids may also act as important antioxidants with a preventative effect for cardiovascular disease, macular degeneration and certain types of cancer (Coultate 2002; Kardinaal, van't Veer, Kok, Ringstad, Gómez-Aracena, Mazaev*, et al.* 1993; Krinsky, Landrum and Bone 2003; Stahl and Sies 2005; Stanner, Hughes, Kelly and Buttriss 2004).

1.2.2.2. Vitamin D

The two major forms of vitamin D are cholcalciferol (D_3) and ergocalciferol (D_2). Both are considered to have the same vitamin D activity. Cholecalciferol is produced in animals (and humans) by the action of ultraviolet light (from sunlight) on 7-dehydrocholesterol in the epidermal cells of the skin and its production is therefore strongly influenced by the amount of sun exposure (Liu, Greenfield, Strobel and Fraser 2013). This reaction is shown in Figure 1-2. Similarly, ergocalciferol is produced by the ultraviolet irradiation of ergosterol, found in plants, fungi and invertebrates. Vitamin $D_{(2 \text{ or } 3)}$ is then converted in the liver to 25-hydroxyvitamin $D_{(2 \text{ or } 3)}$ ($25(OH)D_{(2 \text{ or } 3)}$) which in turn is converted in the kidneys to the physiologically active compound 1,25-dihydroxyvitamin $D_{(2 \text{ or } 3)}$, which promotes the synthesis of the proteins that transport calcium and phosphate ions through cell membranes, thus enabling the uptake of these ions from the intestine and their use for bone growth (Coultate 2002). Vitamin D deficiency in children causes rickets, which is a failure of proper bone development, but adequate levels are important throughout life to maintain bone density and avoid osteoporosis. Toxic levels of vitamin D can result in hypercalcemia, which is caused by excessive consumption of pharmaceutical products, and not from normal diets or sun exposure (Ball 1998).

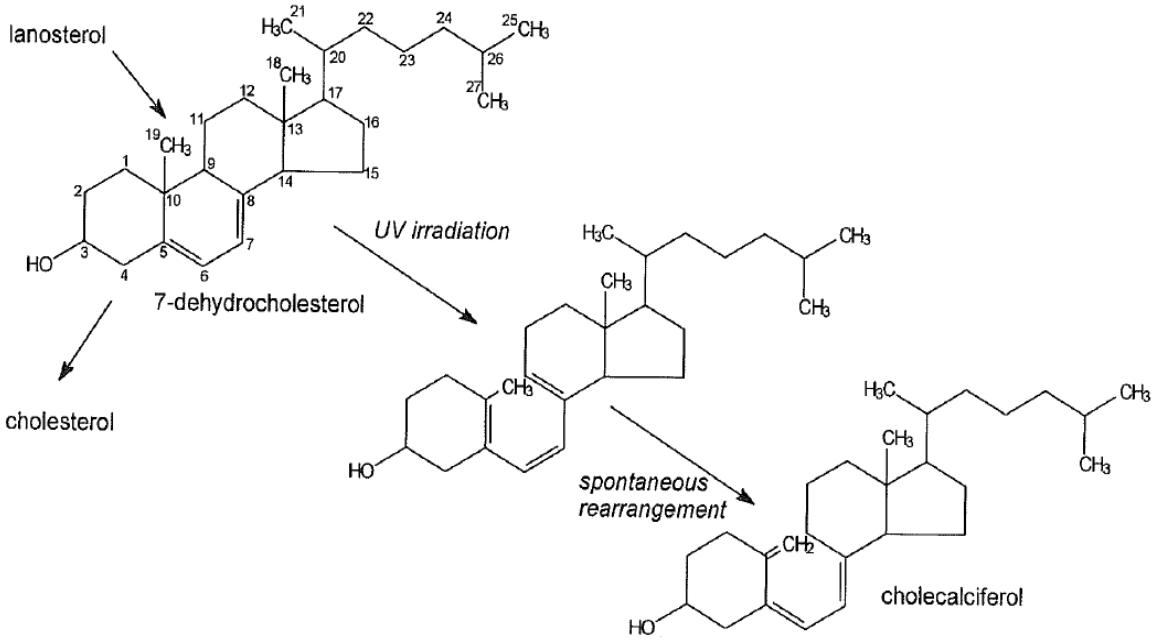


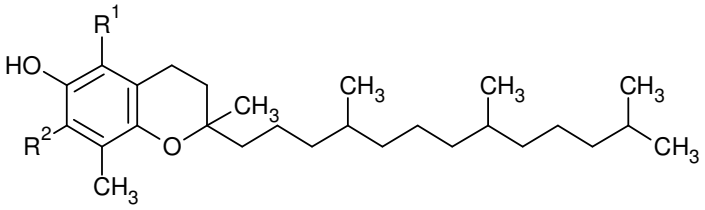
Figure 1-2: The formation of cholecalciferol in skin (Coultate 2002)

1.2.2.3. Vitamin E

The term vitamin E generally refers to α-tocopherol, which is the compound with the highest vitamin E activity. There are three other tocopherols (β, γ and δ), with 27%, 13% and 1% of the activity, respectively, of α-tocopherol. There are also the corresponding tocotrienols, of which only α-tocotrienol has significant vitamin E activity (30%) (Coultate 2002). The structures of the tocopherols and tocotrienols are shown in Figure 1-3.

The role of vitamin E in the body is as a lipid antioxidant, stopping the free-radical chain reactions of lipid peroxidation, and helping to stabilise membrane structures, being particularly prevalent in the membrane lipids of the mitochondria and endoplasmic reticulum of animal cells (Ball 1998; Coultate 2002).

Vitamin E deficiency results in the increased oxidation of cellular membranes, which may lead to disorders of various bodily tissues including muscle, liver, bone marrow and brain (Eitenmiller and Landen 1999). Vitamin E has also been proposed as having a preventative effect for various cancers and cardiovascular disease, however this has not been proven in intervention trials (Kardinaal*, et al.* 1993; Stanner*, et al.* 2004).



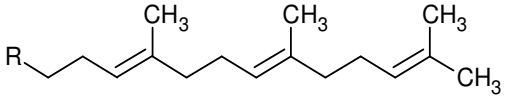
General tocopherol structure

α – R^1 and $R^2 = CH_3$

β – $R^1 = CH_3$, $R^2 = H$

γ – $R^1 = H$, $R^2 = CH_3$

δ – R^1 and $R^2 = H$



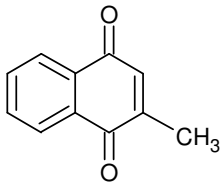
Tocotrienol side chain

Figure 1-3: The structures of the tocopherols and tocotrienols (Coultate 2002; Eitenmiller and Landen 1999)

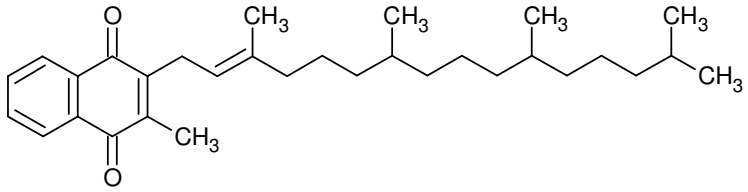
1.2.2.4. Vitamin K

Vitamin K occurs naturally in two forms, phylloquinone (K_1) and menaquinone (K_2), both of which are derivatives of menadione with a side chain at position 3, as shown in Figure 1-4. Vitamin K_1 is produced by plants, most notably green leafy vegetables, whereas vitamin K_2 is produced by bacteria, the richest source in the human diet being the Japanese fermented soybean product natto, but it is also present in smaller amounts in more commonly consumed foods such as cheese and meat. Vitamin K_2 is also produced by bacteria in the large intestine (Weber 2001).

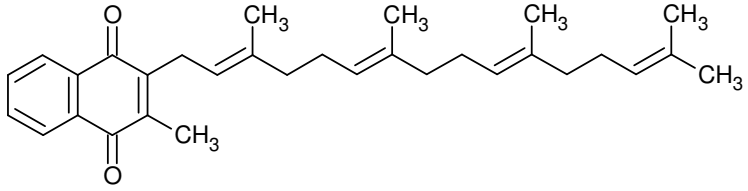
Vitamin K_1 most commonly has a side chain consisting of 4 isoprenoid units, three of which are reduced, vitamin K_2 has a side chain consisting of 4 to 13 isoprenoid units, none of which are reduced (Coultate 2002). Vitamin K was originally recognised as a factor required for blood clotting, but in recent years the role of vitamin K_2 in other processes such as bone mineralisation and the prevention of soft tissue calcification have become apparent (Schurgers, Teunissen, Hamulyák, Knapen, Vik and Vermeer 2007).



Menadione



Phylloquinone



Menaquinone-4

Figure 1-4: The general structures of menadione, phylloquinone and menaquinone (Eitenmiller and Landen 1999)

1.2.3. Vitamin content of dairy cow milk

Dairy cow (*Bos taurus taurus*) milk is a good dietary source of all *trans*-retinol, β-carotene and α-tocopherol (Bergamo, Fedele, Iannibelli and Marzillo 2003; Jensen and Robert 1995), with levels in the order of 49 μg/100g of vitamin A, 18 μg/100g of carotene and 90 μg/100g of vitamin E (Food Standards Australia New Zealand 2014), but contains relatively little vitamin D_3 (<0.2 μg/100 mL) or vitamin K (<1 μg/100 mL) (Gentili, Caretti, Bellante, Ventura, Canepari and Curini 2013; Jakobsen and Saxholt 2009; Jensen and Robert 1995; Mattila, Piironen, Uusi-Rauva and Koivistoinen 1995). Most of the vitamin D_3 is produced by the action of sunlight on the cow's skin and some of the vitamin K is produced in the rumen (Jensen and Robert 1995) with the rest coming from the diet. All *trans*-retinol, β-carotene and α-tocopherol come from the cow's diet, and variations in the levels of these compounds have been attributed to variations in diet (Adler, Dahl, Jensen, Thuen, Gustavsson and Steinshamn 2013;

Bergamo, *et al.* 2003; Hulshof, *et al.* 2006; Marino, Schadt, Carpino, Caccamo, La Terra, Guardiano, *et al.* 2014; Nozière, Grolier, Durand, Ferlay, Pradel and Martin 2006; Slots, Butler, Leifert, Kristensen, Skibsted and Nielsen 2009). β-Carotene is the predominant (>90%) carotenoid found in milk, followed by lutein (1-10%) (Havemose, Weisbjerg, Bredie and Nielsen 2004; Hulshof, *et al.* 2006), although the process of carotenoid absorption in ruminants is not well understood (Nozière, Graulet, Lucas, Martin, Grolier and Doreau 2006). α-Tocopherol is the predominant tocopherol in milk (>94%), followed by γ (<5%) and β (~1%) (Gentili, *et al.* 2013; Lanina, *et al.* 2007).

1.3. Analysis of fat-soluble vitamins in dairy cow milk

1.3.1. Sample extraction

1.3.1.1. Vitamins A, E, D and carotenes

The milk sample is commonly saponified in a boiling mixture of potassium hydroxide, ethanol and water to remove the fat as well as hydrolyse esterified vitamins to the native vitamins (Ball 1998; Byrdwell 2009; Byrdwell, *et al.* 2008; Department of Primary Industries 2007; Heudi, *et al.* 2004; Hulshof, *et al.* 2006; Kurmann and Indyk 1994; Lanina, *et al.* 2007). Cold (room temperature) saponification can also be used but it is more time consuming (Gentili, *et al.* 2013). The vitamins are then extracted from the alkaline mixture with non-polar solvents (e.g. hexane, diethyl ether), and the extract washed with water to remove the soaps (free fatty acids). The organic phase is then concentrated, further purified using solid phase extraction or semi-preparative HPLC if required (D) (Byrdwell, *et al.* 2008; Kurmann and Indyk 1994), and then assayed using HPLC-UV/Vis or HPLC-PDA (A, D, E, carotenes), HPLC-Fl (A, E) or HPLC-MS analysis (A, D, E, carotenes). More recently, Abernethy (2012) described a direct solvent extraction procedure using methanol and isooctane, which avoided the saponification step for vitamin D analysis in fortified milk and milk powder. Vitamin D and its metabolites can also be derivatised with either 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Abernethy 2012; Aronov, Hall, Dettmer, Stephensen and Hammock 2008; Mitamura, Nambu, Tanaka, Kawanishi, Kitahori and Shimada 1999; Shimada and Higashi 2002) or 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4- triazoline-3,5-dione (DMEQ-TAD) (Higashi, Awada and Shimada 2001; Kamao, Tsugawa, Suhara, Wada, *et al.* 2007; Shimada and Higashi 2002) prior to HPLC

analysis to improve the sensitivity, resulting in a lower limit of detection (LOD) and limit of quantification (LOQ).

1.3.1.2. Vitamin K

Vitamin K cannot be assayed by saponification as it is unstable at high pH (pH 13) (Ball 1998; Kamao, Tsugawa, Suhara, Wada*, et al.* 2007), so lipase is used to release vitamin K from the fat prior to solvent extraction, concentration and HPLC analysis. Quantification of vitamin K can be complicated due to the number of related compounds found in milk that possess vitamin K activity, such as phylloquinone and menaquinones 4 - 9 (Ball 1998; Eitenmiller and Landen 1999; Gentili*, et al.* 2013).

1.3.2. Sample analysis

1.3.2.1. High performance liquid chromatography

High performance liquid chromatography is a technique whereby a liquid, referred to as the mobile phase, is pumped at high pressure (up to 400 bar) through a column packed with a stationary phase. When a sample is injected into the mobile phase it travels through the column and the various components of the sample separate according to their partitioning between the mobile and stationary phases, the more the compound resides in the stationary phase, the longer it will take to elute from the column and the longer its retention time. Modification of the properties of the mobile and stationary phases can be used to alter the retention of compounds of interest, and thus a suitable separation of these compounds can be achieved. Recent advances in chromatographic column technology such as small particle size stationary phases (1.7 μm) specifically designed for ultra-high performance liquid chromatography (UHPLC) have the potential to further improve the chromatographic performance by virtue of the greater efficiency of the smaller particles, leading to greater peak capacity and better peak resolution (Rivera and Canela-Garayoa 2012).

Some examples of the analysis of fat-soluble vitamins demonstrating the various HPLC conditions are presented in Table 1-4. A more extensive list of examples is presented in Appendix 1.

Table 1-4: Examples of HPLC conditions used in the analysis of fat-soluble vitamins

Analyte	**Matrix**	**Instrument-conditions**	**Reference**
Retinol, tocopherols, β-carotene	Milk and soy-juice based beverages	HPLC-DAD λ 290 nm tocopherol, λ 440 nm β-carotene, λ 325 nm retinol Luna C18, 250 × 4.6 mm, 5 µm, CH_3OH: tetrahydrofuran:H_2O (67:27:6 v/v/v) 0.8 ml/min	Andrés, Villanueva and Tenorio (2014)
α-tocopherol, β-carotene	Cow milk	HPLC-UV/Vis λ 450 nm β-carotene, HPLC-Fl Ex λ 297 nm, Em λ 340 nm α-tocopherol Zorbax C18, 250 × 4.6 mm, 5 µm, Mobile phase not specified	Marino*, et al.* (2014)
Vitamins D_2 and D_3	SRM 1849a infant formula	Applied Biosystems Sciex 4500 LC-MS/MS, APCI (+) ve ion mode Hypersil aQ, 100 × 2.1 mm, 3 µm (A) CH_3OH:H_2O (75:25) + 0.1% FA, (B) CH_3OH + 0.1% FA, 0 min 70% (B), 0.8-4.5 min 100% (B), 4.51-6 min 70% (B), 0.3 ml/min	Huang, Cadwallader and Heltsley (2014)

1.3.2.1.1. Mobile phases

Typical mobile phases are non-polar solvents (e.g. hexane) for normal phase chromatography and polar mobile phases (e.g. methanol:water or acetonitrile:water) for reversed-phase chromatography. Various additives can also be added to the mobile phase to facilitate separation, such as Paired Ion Chromatography (PIC) reagents (eg alkane sulphonic acids) and buffers to maintain specific pH.

1.3.2.1.2. Stationary phases

The stationary phase generally consists of small particles, with a certain surface chemistry designed to achieve separation of sample components. The most common stationary phases are polar silica (normal phase) and non-polar octadecylsilyl (C18) bonded silica (reversed-phase) particles. The silica can also be modified in a variety of ways to facilitate specific separations, for example intermediate polarity (cyano, diol) and ion exchange (strong cation exchange, strong anion exchange). Examples of some of the most commonly used stationary phases are shown in Table 1-5.

HPLC stationary phases are made of particles with diameters typically between 2.5 and 10 µm, with smaller particle sizes offering greater peak capacity and resolution, but

higher backpressures. Modern 'state of the art' stationary phases can have particle sizes <2 µm, however the high back pressures created by these small particles make them unsuitable for conventional HPLC (Rivera and Canela-Garayoa 2012). The introduction of UHPLC instruments, which can operate at pressures up to 1200 bar, has enabled superior analyte separation and so has become the benchmark for liquid chromatographic separations. Recently, Hampel, York and Allen (2012) reported an ultra-high performance liquid chromatography tandem mass-spectrometry (UHPLC-MS/MS) procedure for the simultaneous analysis of thiamine, riboflavin, flavin dinucleotide, nicotinamide and pyridoxal in human milk using a 50 × 2.1 mm, 1.8 µm HSS T3 column, gradient elution with 10 mM aqueous ammonium formate (A) and acetonitrile (B), a flow rate of 0.3 ml/min and a run time of 4 min. By contrast, (Sakurai, Furukawa, Asoh, KANNO, KOJIMA and YONEKUBO 2005) required three separate HPLC methods and a microbiological assay to determine a similar range of compounds in human breast milk.

Greater peak capacity can also be achieved with two-dimensional liquid chromatography (2D LC) (Quinto Tranchida, Dugo, Dugo and Mondello 2004; Wong and Shalliker 2004). Schadt, Gossl, Seibel and Aebischer (2012) used 2D LC for the on-line clean-up and analysis of sample extracts, reducing the time and effort required to prepare samples for vitamin D analysis.

Table 1-5: Examples of some of the most commonly used HPLC stationary phases (Phenomenex ; Taylor 2015)

Phase	**Ligand**	**Chromatographic mode**	**Applicability**
C18 (alkyl)	$-C_{18}H_{37}$	Reversed phase	Hydrophobic compounds
C18 polar embedded	$-C_{16}H_{33}NO$	Reversed phase	Polar spacer in ligand promotes retention of polar analytes and aids separation based on functional group differences
C8	$-C_8H_{17}$	Reversed phase	Less retention than C18, used to separate very hydrophobic compounds
Cyano (cyanopropyl)	$-(CH_2)_3CN$	Reversed phase	Unique selectivity for polar analytes and solutes with widely differing chemistry
Phenyl (or diphenyl)	$-C_6H_5$ (or $-C_{12}H_9$)	Reversed phase	Aromatic and moderately polar compounds
Pentafluorophenyl	$-C_6F_5$	Reversed phase	Enhanced selectivity for halogenated, polar and isomeric analytes
Amino (aminopropyl)	$-(CH_2)_3NH_2$	Reversed phase	Weak anion exchanger used with pH control to enhance electrostatic retention
Silica	Si-OH	Normal phase	Low polarity compounds soluble in non-polar solvents
Strong cation exchange	$-C_6H_4SO_3^-$	Ion exchange	Positively charged compounds

1.3.2.1.3. Detectors for vitamins analysis

After separation of the compounds in the column, the mobile phase then flows to a suitable detector, with either UV/Vis (A, D, K, carotenes) or Fl (A, E, K) detection being the most common choices for the analysis of fat-soluble vitamins in milk (Ball 1998; Bergamo*, et al.* 2003; Byrdwell*, et al.* 2008; Department of Primary Industries 2007; Hulshof*, et al.* 2006; Kamao, Tsugawa, Suhara and Okano 2007; Kurmann and Indyk 1994). The compounds are usually assayed individually due to their chemical diversity and varying levels within samples.

1.3.2.2. High performance liquid chromatography-mass spectrometry

The relatively recent, rapid advances in HPLC coupled with mass spectrometric detection (HPLC-MS) have seen it emerge as a major breakthrough in analytical science (Willoughby 2002). This technique combines the separating power of liquid chromatography with the extra sensitivity, selectivity and powerful structure elucidation ability of mass spectrometry. The selectivity of mass spectrometry is an advantage over conventional HPLC detectors, particularly when analysing complex sample matrices. Because compounds are separated according to their mass to charge ratio (*m/z*), as long as co-eluting compounds have differing *m/z* values they can be distinguished by the mass spectrometer. The use of collision-induced dissociation (CID) can give further selectivity and specificity to the analysis by fragmenting ions of interest and detecting the resulting product ions (Willoughby 2002).

1.3.2.2.1. Interface and ionisation sources

The HPLC-MS interface must convert dissolved analytes eluting from a HPLC into gas-phase ions at reduced pressure (Willoughby 2002). The processes associated with this conversion are summarised in Figure 1-5.

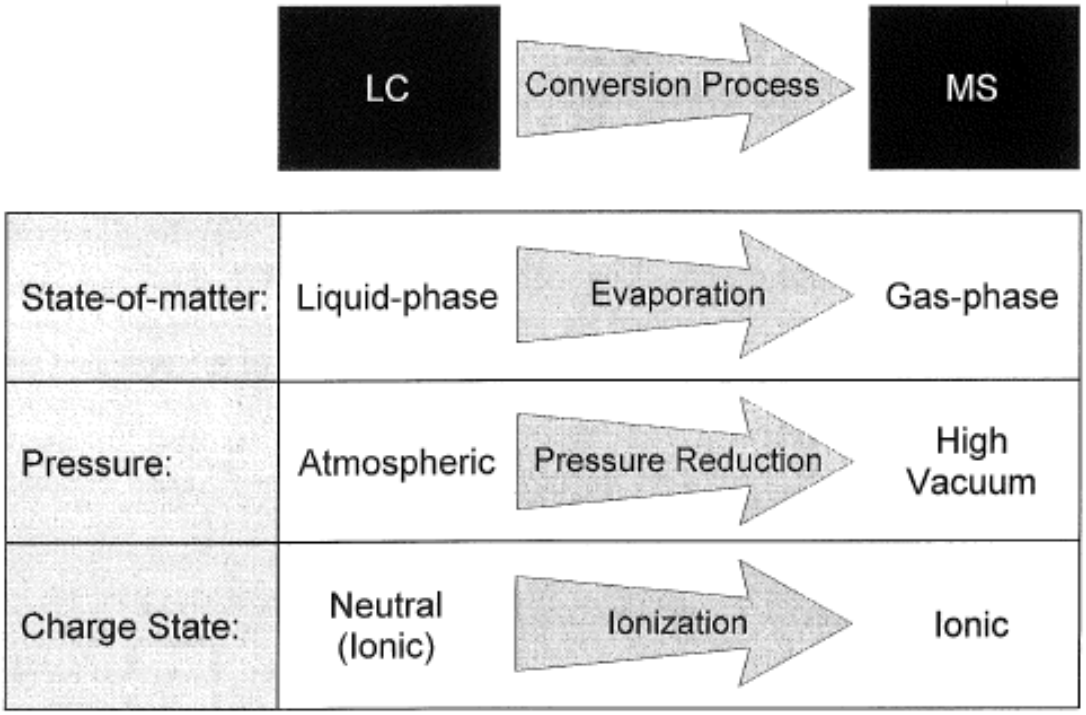


Figure 1-5: Conversion processes required for interfacing liquid chromatography with mass spectrometry (Willoughby 2002)

A number of ionisation and interfacing techniques have been developed, and these include electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo ionisation (APPI), particle beam, continuous flow fast atom bombardment, and thermospray. In practice, the vast majority of HPLC-MS systems used today employ an atmospheric pressure ionisation (API) interface with ESI and APCI ionisation sources (Holčapek, Jirásko and Lísa 2012). These two ionisation sources allow coverage of a wide range of analytes, from polar (ESI) to non-polar (APCI), as shown in Figure 1-6. The overlap of the useful range of the two ionisation techniques is apparent in the use of either for the analysis of fat-soluble vitamins and carotenoids in published methods (Capote*, et al.* 2007; Heudi*, et al.* 2004; Lanina*, et al.* 2007; Su, Rowley and Balazs 2002). Anecdotal evidence has shown that the relative performance characteristics of ionisation sources from different manufacturers vary, and this may also account for the use of either technique. APCI has been shown to have a greater range of linear response for carotenoids compared to ESI, which would be advantageous for the quantitation of these compounds (Rezanka, Olsovska, Sobotka and Sigler 2009).

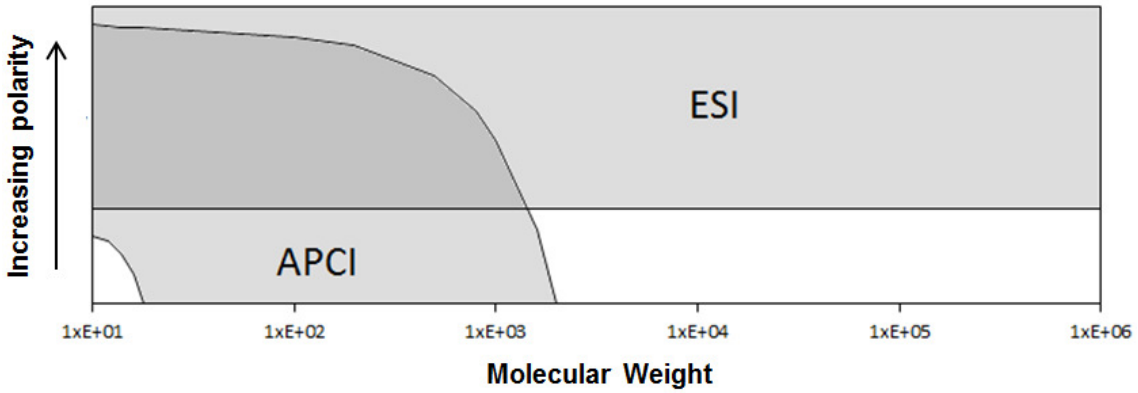


Figure 1-6: The molecular weight and polarity domains of electrospray ionisation and atmospheric pressure chemical ionisation. Adapted from Willoughby 2002.

Electrospray ionisation

Electrospray ionisation is the most widely used ionisation technique for HPLC-MS (Holčapek, *et al.* 2012), due to its simplicity and applicability to a wide range of compounds. The main prerequisite is that the analyte exists in solution as an ion (Willoughby 2002). Willoughby (2002) described it as a 'soft' ionisation technique, as it imparts very little energy to the analyte, and can therefore be used to create molecular ions from relatively unstable compounds such as large biomolecules and pharmaceutical products, which may fragment when ionised by other techniques (Manisali, Chen and Schneider 2006). In principle, the column effluent from the HPLC flows through a hollow needle which is kept at a high electrical potential (approx. 2-5 kV) relative to the entrance to the mass spectrometer. As the liquid exits the tip of the needle, it forms a spray (generally with the assistance of a concentric flow of nitrogen gas) of highly charged droplets. As these droplets evaporate, the charge on each droplet is concentrated, and the repulsion of the like-charged ions leads to the expulsion of solvated ions which quickly desolvate to leave quasi-molecular ions (Willoughby 2002). The ions formed by this process may take on more than one charge if there is more than one ionisable site, and have the form $[M+nH]^{n+}$ when the ion source is operated in the positive ionisation mode or $[M-nH]^{n-}$ in negative ionisation mode. Approximately 1% of the ions produced by this process pass through the sampling orifice and into the vacuum region of the mass spectrometer to the detector, which detects the *m/z* ratio and abundance of the ions.

Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation is also a relatively soft ionisation technique, although not as soft as ESI. The HPLC column effluent is sprayed through a heated (approx. 200-500°C) nebuliser, which evaporates both the solvent and solutes, and into a corona discharge (Rezanka*, et al.* 2009). Electron ionisation of the source gases and mobile phase components by the corona creates reagent gases, which ionise the analytes as they travel towards the sampling orifice, as shown in Figure 1-7. Similar to ESI, approximately 1% of the ions produced are then drawn into the mass spectrometer. Compared to ESI, APCI is generally more rugged, reliable and less susceptible to chemical interferences such as the formation of adducts (Willoughby 2002).

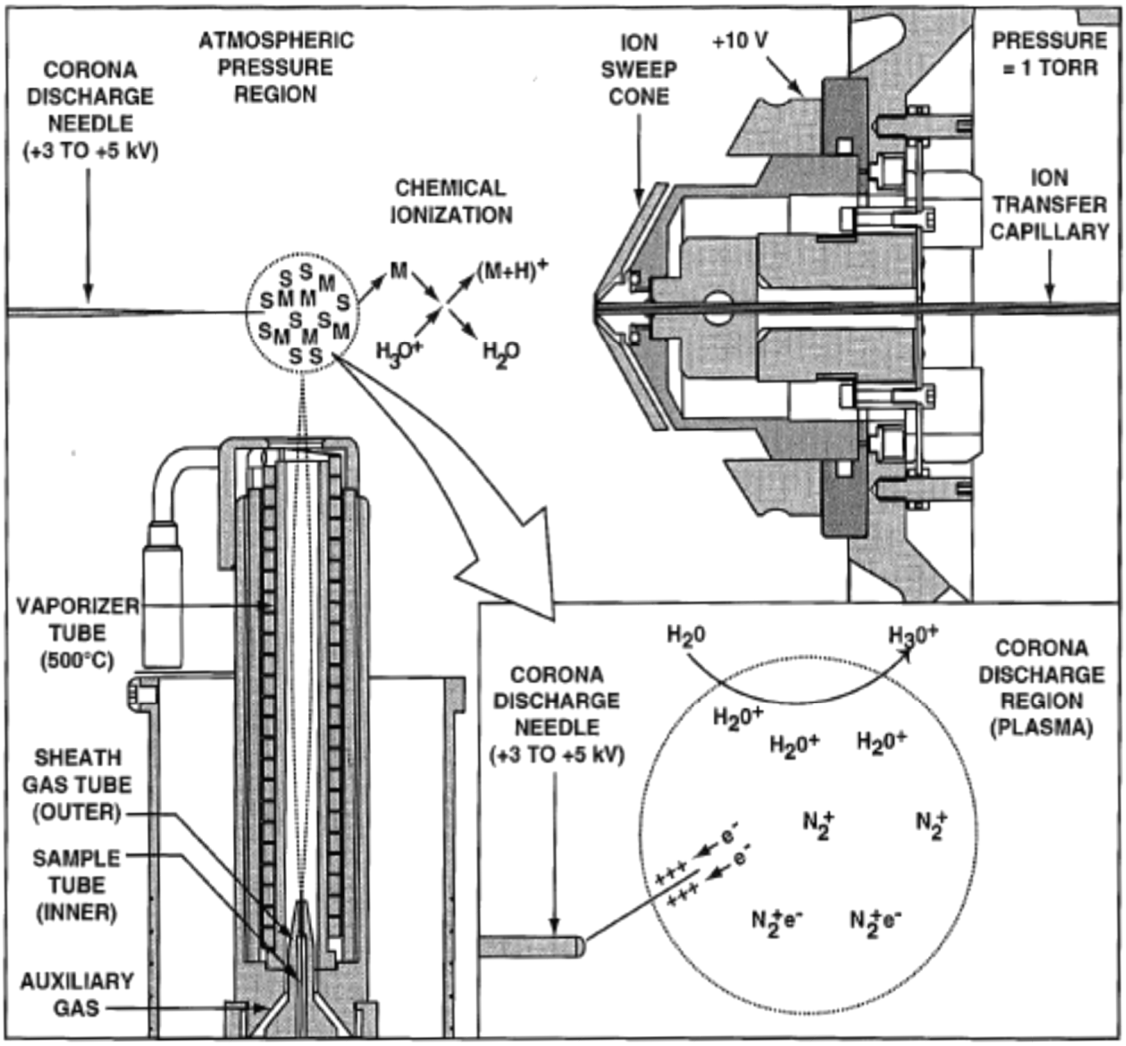


Figure 1-7: Atmospheric pressure chemical ionisation process in the positive ion polarity mode (Thermo Electron Corporation 2003)

1.3.2.2.2. Mass analysers

There are many different types of mass analysers that are typically used for HPLC-MS: low resolution, low mass accuracy instruments such as single quadrupole (MS), triple quadrupole/tandem (MS/MS) and ion trap (MS^n), and high resolution, accurate mass instruments such as time of flight (TOF), Fourier transform ion cyclotron resonance (FTICR) and Orbitrap. There are also a variety of specialised 'combination instruments', such as ion trap-Orbitrap, ion trap-FTICR and ion trap-TOF which are used primarily for research applications.

Single quadrupole

Single quadrupole instruments primarily yield information about the molecular ion, for example a protonated molecule $[M + H]^+$ has a mass/charge ratio one amu higher than the mass of the molecule. Structural information from the formation of product ions is limited to the use of in-source CID.

Triple quadrupole (tandem mass spectrometer)

Triple quadrupole instruments enable isolation and fragmentation of the molecular ion to yield product ions, allowing further structural information. The ability of the triple quadrupole instrument to select a molecular ion, fragment the ion and monitor the product ions is known as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), and makes it the instrument of choice for quantification of targeted analytes at the parts-per-million (ppm) and parts-per-billion (ppb) levels due to its superior sensitivity and selectivity compared to MS, MS^n and TOF instruments (Zhang, Rose and Trenerry 2009).

Ion trap

Ion trap mass spectrometers cover a similar *m/z* range as triple quadrupole mass spectrometers, as shown in Table 1-5, and can also operate in full scan mode at high and low resolution. The major advantage of MS^n instruments is the capability of performing successive fragmentation steps (up to n=10), which provides more structural information than other mass analysers. This information can then be used to assist in the identification of unknown compounds. The sensitivity of traditional three-dimensional ion trap instruments is an order of magnitude less than triple quadrupole mass spectrometers, however, linear ion trap mass spectrometers exhibit sensitivities

approaching that of triple quadrupole mass spectrometers, making them a viable alternative for quantification and metabolomics studies (Rochfort, Trenerry, Imsic, Panozzo and Jones 2008). Figure 1-8 shows internal components of the Thermo LTQ linear ion trap mass spectrometer.

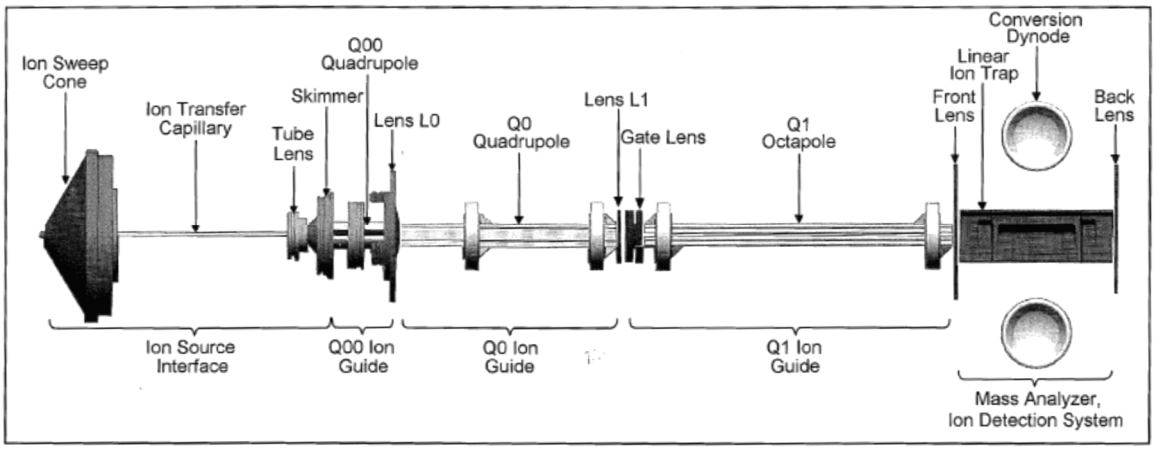


Figure 1-8: Internal components of the linear ion trap mass spectrometer (Thermo Electron Corporation 2003)

Time of flight, Fourier transform ion cyclotron resonance and Orbitrap

Accurate mass instruments (TOF, FTICR, Orbitrap) are very useful for compound identification, as the accurate mass data can be used to deduce the molecular formula of a compound. TOF and FTICR instruments are generally more expensive and not as well suited to targeted analysis as triple quadrupole or ion trap instruments, which have a larger linear range and generally better sensitivity (Holčapek, *et al.* 2012; Willoughby 2002). Typical specifications for different mass analysers are shown in Table 1-5.

Table 1-5: Typical specifications for a range of mass analysers (Hart-Smith and Blanksby 2012)

Mass analyser	**Mass resolving power**	**Mass accuracy**	**Mass range**	**Linear dynamic range**	**Abundance sensitivity**	**Other**
MS MS/MS	100-1000	100 ppm	4000	1×10^{7}	1×10^{4}-1×10^{6}	Low cost, low space requirements
3D MS^n	1000-10 000	50-100 ppm	4000	1×10^{2}-1×10^{3}	1×10^{2}-1×10^{3}	Low cost, low space requirements
Linear MS^n	1000-10 000	50-100 ppm	4000	1×10^{3}-1×10^{4}	1×10^{3}-1×10^{4}	Low cost, low space requirements
TOF	1000-40 000	5-50 ppm	>100 000	1×10^{6}	1×10^{6}	Moderate cost, moderate space requirements
Orbitrap	10 000-150 000	2-5 ppm	6000	1×10^{3}-1×10^{4}	1×10^{4}	Moderate cost, low space requirements
FTICR	10 000-1 000 000	1-5 ppm	>10 000	1×10^{3}-1×10^{4}	1×10^{3}-1×10^{4}	High cost, large space requirements

1.3.2.2.3. Interferences

The compounds present in the sample matrix may affect the ability to obtain accurate identification and quantification with the mass spectrometer in a number of ways, therefore it is generally necessary to obtain a good separation of sample components by HPLC prior to MS analysis. The matrix can cause spectral, chemical or physical interferences (Willoughby 2002). Spectral interference occurs when co-eluting compounds yield ions that overlap with the analyte ions. Chemical interference occurs when other compounds in the sample matrix suppress or enhance the response of the analyte through a chemical process, e.g. the formation of adducts, which alter the apparent mass of the analyte. Adducts are more prevalent with ESI than APCI. Physical interferences may suppress or enhance the response of the analyte through a physical process, e.g. high concentrations of interfering components leading to increased surface tension and reduced desolvation in the API interface, reducing ionisation efficiency. The separation of the analytes by HPLC prior to mass spectral analysis also gives retention time data which can be compared to that of an authentic standard, improving the confidence of the analyte identification in targeted analysis.

1.3.2.2.4. Internal standards

In HPLC-MS analysis, internal standards are often used to correct for the quantitative variations caused by ion suppression or ion enhancement, especially when the target analytes are present in low levels (ppb), e.g. pesticide residues and antibiotic residues in biological samples (Hernández, Sancho and Pozo 2005; Plozza, Trenerry, Zeglinski, Nguyen and Johnstone 2011). Ion suppression is more common in ESI than other ionisation techniques (Stokvis, Rosing and Beijnen 2005). Compounds that exhibit similar chromatographic performance and ionising properties are often chosen as internal standards. For instance, dihydrotachysterol was used as the internal standard for the analysis of vitamins D_2 and D_3 in food (Dimartino 2009) as it is not present in food and has a similar structure to the target analytes. Similarly, vitamin D_2 and 25-hydroxyvitamin D_2 were used as internal standards for the quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in foodstuffs (Bilodeau, Dufresne, Deeks, Clément, Bertrand, Turcotte*, et al.* 2011). Isotopically labelled internal standards, where several atoms in the analyte have been replaced by their stable isotopes, e.g. ^{2}H (deuterium), ^{13}C, ^{15}N or ^{17}O, make ideal internal standards as they share the same physical properties as the target analyte. It is preferable to have at least three stable isotopes incorporated into the structure, so that the molecular ion and key fragment ions are well separated in the mass analyser to prevent 'cross-talk' interference, which occurs when the isotope peaks of the analyte interfere with the signal of the internal standard by virtue of their identical *m/z* ratio(Stokvis*, et al.* 2005). As the abundance of the isotope peaks of a compound become insignificant greater than two mass units higher than the main isotope, an internal standard with *m/z* 3 units or higher than the analyte will avoid this problem. Kalman, Mujahid, Mottier and Heudi (2003) used deuterium labelled tocopherol to quantify α-tocopherol in infant formula, while Abernethy (2012) used 26,26,26,27,27,27-$^{2}H_6$ vitamin D_3 for the quantitative analysis of vitamin D_3 in fortified infant formula, milk and milk powder.

1.3.2.2.5. Mobile phase additives

The choice of mobile phase additives, e.g. acids, bases and buffers is more limited in HPLC-MS compared to other HPLC techniques as any mobile phase additives must be volatile to prevent fouling of the ion source during mobile phase evaporation. Thus, volatile acids (e.g. formic acid), bases (e.g. ammonia) and buffers (e.g. ammonium

formate, ammonium acetate) are preferred over the more traditional additives such as phosphate buffers and alkali salts.

1.4. Analytical method validation

Method validation provides objective evidence that a method is fit-for-purpose, i.e. the particular requirements for a specific intended purpose are fulfilled. The rigour of validation should be sufficient to ensure that test results produced by a method are technically sound and fit for purpose.

The first step in measurement and method validation is specifying what is intended to be measured, both qualitatively describing the entity to be measured and the quantity. A method is then validated against this specification and the client's requirements.

The second step in validation is to determine certain selected performance parameters. A detailed description of each parameter is given in National Association of Testing Authorities, Australia (NATA) Technical Note 17 (2013) and summarised in Table 1-6 below.

Table 1-6: Validation performance parameters (National Association of Testing Authorities 2013)

Characteristics to be evaluated	**Procedure to be followed**
Linearity	Analysis of calibration standards
Sensitivity	Analysis of spiked samples or standards prepared in sample extract solution
Selectivity	Consideration of potential interferences, analysis of samples spiked with possible interferences (method development may have overcome potential issues)
Trueness; bias	Analysis of: • Certified Reference Materials (CRMs) • Other Reference Materials (RMs) • Sample spikes • Comparison with Standard Methods • Results from collaborative studies
Precision; intra-laboratory reproducibility	Replicate analysis of samples, including multiple analysts conducting the analysis: if possible selected to contain analytes at concentrations most relevant to users of test results
Limits of detection (LOD); limits of quantification (LOQ)	Analysis of samples containing low levels of analytes. The determination of LOD and LOQ is normally only required for methods intended to determine analytes at about these concentrations.
Working range	Evaluation of data from bias and possibly LOQ determinations
Ruggedness	Consider those steps of the method, which if varied marginally, would possibly affect the results. Investigate if necessary (1) single variable test and (11) multi variable test
Measurement uncertainty	Calculate a reasonable fit-for-purpose estimate of measurement uncertainty, which is the range where the true measurement could be found. Ensure estimates are aligned with the concentrations most relevant to the users of the results. Utilise other validation data, combined with any other complementary data available, e.g. results from collaborative studies, proficiency tests, round robin tests, in house QC data

1.5. Significance

The development of a single method for the analysis of a large number of fat-soluble vitamins and carotenoids in milk would be a significant advance in the analysis of these compounds since it could potentially reduce the complexity and cost of analysis. The use of mass spectrometric detection could reduce the amount of sample preparation (only 1 extract, not 2 or 3) required and substantially reduce instrument time since one HPLC-MS will replace several HPLC instruments. Achieving good chromatographic performance (acceptable peak shape and separation of compounds from one another and from matrix interferences) and good mass spectrometric performance (good ionisation and fragmentation efficiency with compound specific fragments) without the use of derivatising reagents is preferred in order to keep complexity and cost to a minimum.

HPLC-MS^n has the potential to identify other biologically important compounds in the extract, such as other vitamers and phytosterols, whose levels might change with different feeding regimes.

1.6. Objectives of this study

The aim of this work was to develop a validated method for the determination of a number of fat-soluble vitamins in cow milk by HPLC-MS^n. The method was then used to determine if there are significant differences in the fat-soluble vitamin concentrations of milk sourced from cows fed different feeding regimes.

The specific objectives of this work are:

- Develop and validate a HPLC-MS^n method for the determination of vitamin D_3 in cow milk.
- Develop and validate a HPLC-MS^n method for the simultaneous determination of vitamins A (all *trans*-retinol), E (α-tocopherol), and β-carotene in cow milk.
- Compare these results with those obtained using HPLC with Fluorescence (Fl) and/or Photodiode array (PDA) detection (vitamins A, E and β-carotene) and HPLC-MS/MS (vitamin D_3).
- Determine the levels of these vitamins and β-carotene in milk samples sourced from a dairy cow feeding experiment conducted at the Department of Primary Industries (DPI), Ellinbank, Victoria, Australia.

- Assess whether there is any significant difference in fat-soluble vitamin and β-carotene concentrations in the milk resulting from the different feeding regimes.

Chapter 2: Materials and methods

2.1. Introduction

This chapter provides the details of the materials used for the development and validation of methods for the analysis of fat-soluble vitamins in cow milk, specifically vitamin D_3 and 25-hydroxyvitamin D_3 using HPLC-MS^n and HPLC-MS/MS, and all *trans*-retinol, α-tocopherol and β-carotene using HPLC- MS^n. The methods that were developed are described in Chapters 3 and 4.

Milk samples were sourced from a dairy cow feeding systems experiment. Details of the feeding experiment are provided in this Chapter. The feeding experiment samples were used for (1) validation of the all *trans*-retinol, α-tocopherol and β-carotene analytical method, and (2) to assess the effects of the feeding experiment on the levels of these compounds in the milk (see section 2.8.).

2.2. Materials

The reagents and apparatus used for the analyses in this study are listed in Tables 2-1 to 2-6.

Table 2-1: List of the chemicals and materials used for fat-soluble vitamin extraction and analysis

Name	Specification	Company
Vitamin D_3	≥99%	Sigma Chemical Co (Sydney, Australia)
25-hydroxyvitamin D_3	≥98%	Sigma Chemical Co (Sydney, Australia)
Vitamin D_3-6,19,19-[$^2H_3$]	≥97%	Isosciences, LLC (King of Prussia, PA, USA)
25-hydroxyvitamin D_3-[$^2H_3$]	≥98%	Isosciences, LLC (King of Prussia, PA, USA)
DL-α-tocopherol	≥97%	Sigma Chemical Co (Sydney, Australia)
all *trans*-retinol acetate	100%	USP (Rockville, MD, USA)
β-carotene	30% fluid suspension	Roche (Texas, USA)
Ethanol	100%	CSR Distilleries (Yarraville, Australia)
Ascorbic acid	>99%	Lomb Scientific (now Thermo Fisher Scientific, Melbourne, Australia)
Demineralised water	Milli-Q	Millipore (Melbourne, Australia)
Potassium hydroxide	Pellets	Lomb Scientific (now Thermo Fisher Scientific, Melbourne, Australia)
n-Hexane	GC pesticide grade	Lomb Scientific (now Thermo Fisher Scientific, Melbourne, Australia)

Table 2-1: continued

Name	Specification	Company
2,6-Di-tert-butyl-4-methylphenol (BHT)	≥99%	Sigma Chemical Co (Sydney, Australia)
Methanol	ACS/HPLC grade	Rowe Scientific (Melbourne, Australia)
Acetonitrile	HPLC 190 grade	Lomb Scientific (now Thermo Fisher Scientific, Melbourne, Australia)
Dichloromethane	HPLC & GC grade	Rowe Scientific (Melbourne, Australia)
Ammonium acetate	≥98%	Sigma Chemical Co (Sydney, Australia)
Ammonium formate	≥99%	Sigma Chemical Co (Sydney, Australia)
Ethyl acetate	ACS/HPLC grade	Rowe Scientific (Melbourne, Australia)
Sodium sulphate	Granular, anhydrous	Lomb Scientific (now Thermo Fisher Scientific, Melbourne, Australia)
Solid phase extraction (SPE) cartridges	Sep-Pak silica 500mg	Waters (Milford, MA, USA)
Polytetrafluoroethylene (PTFE) syringe filters	3mm and 13 mm, 0.45µm	Advantec (Japan)

Table 2-2: List of the apparatus used for fat-soluble vitamin extraction

Name	Specification	Company
Conical flask	150 ml	Various
Condenser with water jacket	30 cm	Bartelt Instruments (Melbourne, Australia)
Hotplate stirrer		Industrial Equipment and Control (Melbourne, Australia)
Separating funnel	250 ml	VWR International (Murarrie, Queensland, Australia)
Rotary evaporator	Buchi R-200	InVitro (Melbourne, Australia)

Table 2-3: List of apparatus used for HPLC-MS^n

Name	Model	Company
HPLC	1100 with quaternary solvent delivery, cooled autosampler and column heater	Agilent Technologies (Melbourne, Australia)
Linear Ion Trap Mass Spectrometer	LTQ	Thermo Fisher Scientific (Melbourne, Australia)
APCI source		Thermo Fisher Scientific (Melbourne, Australia)
ESI source		Thermo Fisher Scientific (Melbourne, Australia)
HPLC column	Polaris C18-A, 5µm, 150 × 2.1 mm	Varian (Mulgrave, Australia)
HPLC pre-column	SecurityGuard C18, 4 × 2 mm	Phenomenex (Sydney, Australia)
HPLC-MS software	Xcalibur Version 2.1	Thermo Fisher Scientific (Melbourne, Australia)
UV/Visible spectrophotometer	UV-1601	Shimadzu (Melbourne, Australia)

Table 2-4: List of apparatus used for HPLC-MS/MS

Name	Model	Company
HPLC	2795HT with quaternary solvent delivery, cooled autosampler and column heater	Waters (Milford, MA, USA)
Tandem Mass Spectrometer	Micromass Quattro Micro	Waters (Manchester, UK)
ESI source		Waters (Manchester, UK)
APCI source		Waters (Manchester, UK)
HPLC column	Polaris C18-A, 5 µm, 150 × 2.1 mm	Varian (Mulgrave, Australia)
HPLC pre-column	SecurityGuard C18, 4 × 2 mm	Phenomenex (Sydney, Australia)
HPLC-MS software	Masslynx Version 4.0	Waters (Manchester, UK)

Table 2-5: List of apparatus used for HPLC with both UV/Vis and Fl detection for the analysis of all *trans*-retinol and α-tocopherol

Name	Model	Company
HPLC	2695	Waters (Milford, MA, USA)
HPLC detector 1	996 photodiode array	Waters (Milford, MA, USA)
HPLC detector 2	RF-10A_{XL}	Shimadzu (Melbourne, Australia)
HPLC column	Bondclone C18, 10 µm, 300 × 3.9 mm	Phenomenex (Sydney, Australia)
HPLC pre-column	SecurityGuard C18, 4 × 3 mm	Phenomenex (Sydney, Australia)
HPLC software	Empower version 2	Waters (Milford, MA, USA)

Table 2-6: List of apparatus used for HPLC-UV/Vis analysis of β-carotene

Name	Model	Company
HPLC	2695	Waters (Milford, MA, USA)
HPLC detector	996 photodiode array	Waters (Milford, MA, USA)
HPLC column	SphereClone ODS2 C18 5 μm, 250 × 4.6 mm	Phenomenex (Sydney, Australia)
HPLC pre-column	SecurityGuard C18, 4 × 3 mm	Phenomenex (Sydney, Australia)
HPLC software	Empower version 2	Waters (Milford, MA, USA)

2.3. Analytical methods

Methods for the analysis of vitamin D_3 and for all *trans*-retinol, α-tocopherol and β carotene by HPLC-MS were developed based on a DPI-Werribee Analytical Laboratory 'in-house' HPLC method for the analysis of fat-soluble vitamins in a variety of foods, including dairy products, using photodiode array (PDA), also known as diode array detection (DAD), and Fl detection (Department of Primary Industries 2007). This method was validated according to NATA Technical Note 17 (2013) and accredited by NATA, and therefore was an ideal starting point for the development of an HPLC-MS method as the sample preparation was suitable for extracting a range of vitamins and carotenoids in milk and infant formula. This also enabled a side-by-side comparison of the new HPLC-MS^n method with a fully validated HPLC method for the analysis of all *trans*-retinol, α-tocopherol and β-carotene.

2.4. Standard preparation

2.4.1. Calculation of stock standard concentrations

The concentrations of all stock standards prepared below were checked by UV/Vis, as specified below. The concentration of each stock standard was then calculated using the $E_{1cm}^{1\%}$ (the absorbance of a 1% (m/v) solution, with a 1 cm path length, at the specified wavelength) values obtained from Eitenmiller and Landen (1999).

2.4.2. Preparation of vitamin D_3 and 25-hydroxyvitamin D_3 standards

Stock, intermediate and working standards of vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_3-[$^2H_3$] and 25-hydroxyvitamin D_3-[$^2H_3$] were prepared in methanol.

Stock standard solutions (121 µg/ml vitamin D_3, 68 µg/ml 25-hydroxyvitamin D_3, 53 µg/ml vitamin D_3-[$^2H_3$] and 83 µg/ml 25-hydroxyvitamin D_3-[$^2H_3$]) were shown to be stable for at least 6 months when stored at -20°C.

The intermediate standard solution (0.968 µg/ml vitamin D_3, 0.676 µg/ml 25-hydroxyvitamin D_3) was prepared from stock solutions and was shown to be stable for at least 1 month when stored at -20°C.

The working standard (0.0968 µg/ml vitamin D_3, 0.0676 µg/ml 25-hydroxyvitamin D_3) was prepared daily from the intermediate standards.

The intermediate internal standard solution (0.5 µg/ml vitamin D_3-[$^2H_3$], 0.04 µg/ml 25-hydroxyvitamin D_3-[$^2H_3$]) was prepared from stock solutions and was shown to be stable for at least 2 months when stored at -20°C.

The sample reconstituting solution (0.1 µg/ml vitamin D_3-[$^2H_3$], 0.008 µg/ml 25-hydroxyvitamin D_3-[$^2H_3$]) was prepared weekly from the intermediate standard and stored at -20°C.

2.4.2.1. Analytical standards for the analysis of non-fortified samples

A series of four standards containing between 0.0025 µg/ml and 0.025 µg/ml vitamin D_3, between 0.0017 µg/ml and 0.017 µg/ml 25-hydroxyvitamin D_3, and each containing vitamin D_3-[$^2H_3$] (0.1 µg/ml) and 25-hydroxyvitamin D_3-[$^2H_3$] (0.008 µg/ml) as internal standards, were prepared daily, in methanol, from the working standard and intermediate internal standard solution.

2.4.2.2. Analytical standards for the analysis of fortified samples

A series of six standards containing between 0.01 µg/ml and 0.4 µg/ml were prepared for quantification. Each solution contained vitamin D_3-[$^2H_3$] (0.1 µg/ml) as the internal standard.

2.4.3. Preparation of all *trans*-retinol, α-tocopherol and β-carotene standards

Stock standard solutions of all *trans*-retinyl acetate (82 µg/ml), α-tocopherol (520 µg/ml) and β-carotene (170 µg/ml) were prepared in hexane and the concentrations checked weekly by UV/Vis spectroscopy. Stock standard solutions were stable for at least 3 months when stored at 4°C.

A standard solution of all *trans*-retinol (37 µg/ml) in methanol was prepared weekly by saponification of an aliquot of the all *trans*-retinol acetate stock standard, using the procedure described in section 2.7.1, with the exception that the retinol was extracted using hexane rather than hexane containing 15 mg/l butylated hydroxytoluene (BHT). After removal of the hexane *in vaccuo*, the all *trans*-retinol was dissolved in 4 ml methanol and the concentration checked by UV/Vis spectrometry. The all *trans*-retinol standard solution was then stored at -20°C.

A spiking standard solution containing all *trans*-retinyl acetate (3.3 µg/ml), α-tocopherol (10 µg/ml) and β-carotene (1.7 µg/ml) was prepared daily, in hexane, from the stock standard solutions.

An intermediate standard solution containing α-tocopherol (63 µg/ml) and β-carotene (7 µg/ml) was prepared daily in methanol.

2.4.3.1. Analytical standards

A series of five standards containing approximately 0.05 - 1.2 µg/ml all *trans*-retinol, 0.3 - 6.5 µg/ml α-tocopherol and 0.03 - 0.7 µg/ml β-carotene were prepared daily, in methanol, from the all *trans*-retinol standard and the α-tocopherol and β-carotene intermediate standard.

2.5. Sampling, preparation and storage of milk

2.5.1. Vitamin D_3

Samples of fresh cow milk were sourced from a private dairy farm in Trafalgar, Gippsland, Victoria and DPI Ellinbank research dairy. Commercial cow milk samples were purchased from a retail outlet in Vermont South, Victoria, Australia. All samples were stored at 4°C and assayed within 3 days for the fresh milk samples and before

the recommended use by date for the commercial samples. The homogenised powdered infant formula was kindly supplied by the National Measurement Institute, Port Melbourne, Victoria, Australia. The milk samples were mixed thoroughly by inversion ten times before assaying. The powdered infant formula was mixed with water according to the manufacturer's instructions (1 g with 9 ml water) immediately before analysis. To avoid the destruction of the vitamin by exposure to light, all experiments were conducted under yellow fluorescent lighting which excludes light of less than 500 nm wavelength (Ball 1998), and amber glassware was used where appropriate.

2.5.2. Vitamin A, E and β-carotene

Fresh cow milk was sourced from DPI Ellinbank research dairy, see section 2.8 for details. All samples were stored at 4°C for one day, sub-sampled, and the sub-samples stored at -70°C prior to analysis. The thawed milk samples were mixed thoroughly before assaying. A powdered infant formula sample received from Proficiency Testing Australia was used as a control sample for all *trans*-retinol and α-tocopherol. The infant formula was dissolved in water (1 g with 9 ml water) immediately before analysis. Commercial full cream milk was purchased from a local retail outlet, stored at 4°C and assayed before the recommended use by date. All experiments were conducted under yellow fluorescent lighting and amber glassware used where appropriate.

2.6. Vitamin D

2.6.1. Sample extraction

2.6.1.1. Fortified commercial cow milk and infant formula

A suitable aliquot of the sample (10 ml) was mixed with 0.5 g of ascorbic acid, 40 ml of ethanol and 10 ml of 1:1 (w/v) potassium hydroxide in water and heated at reflux with stirring for 30 minutes. The mixture was cooled in an ice bath and diluted with 50 ml water and 10 ml ethanol. The solution was transferred to a separating funnel and the analytes extracted into 50 ml hexane containing 15 mg/l BHT with gentle mixing. The aqueous phase was separated and extracted twice with 20 ml portions of hexane containing 15 mg/l BHT. The hexane extracts were combined and washed

with water (3 × 100 ml). The hexane solution was transferred to an amber flask and the solvent removed *in vaccuo* at 40°C. The residue was reconstituted with 1.0 ml internal standard solution (vitamin D_3-[$^2H_3$], 0.1 µg/ml) and filtered through a 3 mm 0.45 µm PTFE filter disc before analysis.

2.6.1.2. Non-fortified commercial cow milk and fresh cow milk

The samples were saponified and extracted as described above. The residue remaining after removal of the solvent *in vaccuo* was re-constituted in 5 ml hexane. For vitamin D_3, 2 ml of the hexane solution was loaded onto a 500 mg Silica solid phase extraction (SPE) cartridge. The cartridge was first washed with 3 ml hexane, followed by 3 ml of hexane: ethyl acetate (90:10 v/v) and the vitamin D_3 eluted with 6 ml hexane: ethyl acetate (80:20 v/v). The solvents were removed with a stream of nitrogen, the residue reconstituted with 0.25 ml internal standard solution (vitamin D_3-[$^2H_3$], 0.1 µg/ml) and filtered through a 3 mm 0.45 µm PTFE filter into a tapered vial prior to analysis.

For 25-hydroxyvitamin D_3, a separate 2 ml aliquot of the hexane solution was loaded onto a 500 mg Silica SPE cartridge. The cartridge was washed with 3 ml hexane, 3 ml of hexane: ethyl acetate (90:10 v/v) and the 25-hydroxyvitamin D_3 eluted with 6 ml hexane: ethyl acetate (60:40 v/v). The solvents were removed with a stream of nitrogen, the residue reconstituted with 0.25 ml internal standard solution (25-hydroxyvitamin D_3-[$^2H_3$], 0.008 µg/ml) and filtered through a 3 mm 0.45 µm PTFE filter into a tapered vial prior to analysis.

2.6.2. Analysis

2.6.2.1. High performance liquid chromatography-ion trap mass spectrometry

The analyses were performed with an Agilent series 1100 quaternary solvent delivery system and a cooled autosampler (4°C) connected to a Thermo Electron LTQ linear ion trap mass spectrometer operating in the positive ion Atmospheric Pressure Chemical Ionisation (APCI) mode. The compounds were separated on a 150 × 2.1 mm 5 µm Polaris C18-A column fitted with a C18 guard column and maintained at 30°C. The mobile phase consisted of methanol:water (92:8 v/v) and the flow rate was 0.2 ml/min. The APCI vaporizer temperature was 200°C, the ion current 4.5 µA, the heated capillary was maintained at 200°C and the sheath and auxiliary gases were at

20 and 5 units respectively. Source voltage was 4.4 kV and the capillary voltage set at 16 V. Prior to data acquisition, the system was tuned using a 10 µg/ml vitamin D_3 standard solution. The solution was infused via syringe pump through a T-piece at a rate of 10 µl/min and mixed with HPLC mobile phase (0.2 ml/min) before entering the mass spectrometer. Excalibur V2.1 software was used to process the data.

2.6.2.1.1. Fortified samples

A full scan protocol (3-10 min) with a mass range of *m/z* 150-800 was used for 25-hydroxyvitamin D_3, (*m/z* 401). Three scan events (10-26 min) were used for vitamin D_3 (*m/z* 385.5) and vitamin D_3-[$^2H_3$] (*m/z* 388.5). The first scan event was a full scan from *m/z* 150-800. For the second scan event, *m/z* 385.5 was isolated and fragmented to *m/z* 367.4 and *m/z* 259. For the third scan event, *m/z* 388.5 was isolated and fragmented to *m/z* 370.4. Ion max times of 200 milli sec, isolation widths of 1.0 and normalized collision energies of 20% were used for all SRM transitions.

2.6.2.1.2. Non-fortified samples

Three scan events (3-10 min) were used for 25-hydroxyvitmin D_3 (*m/z* 401) and 25-hydroxyvitamin D_3-[$^2H_3$] (*m/z* 404). The first scan event was a full scan from *m/z* 150-800. For the second scan event, *m/z* 401 was isolated and fragmented to *m/z* 383 and *m/z* 365.2. For the third scan event, *m/z* 404 was isolated and fragmented to *m/z* 386. Ion max times of 200 milli sec, isolation widths of 3.5 and collision energies of 23% were used for all SRM transitions. Three scan events (10-20.9 min) were used for vitamin D_3 (*m/z* 385.5) and vitamin D_3-[$^2H_3$] (*m/z* 388.5). The first scan event was a full scan from *m/z* 150-800. For the second scan event, *m/z* 385.5 was isolated and fragmented to *m/z* 367.4 and *m/z* 259. For the third scan event, *m/z* 388.5 was isolated and fragmented to *m/z* 370.4. Ion max times of 200 milli sec, isolation widths of 2.0 and collision energies of 25% were used for all SRM transitions.

2.6.2.2. High performance liquid chromatography-tandem mass spectrometry

The analyses were performed with a Waters Alliance HT 2795 quaternary solvent delivery system and a cooled autosampler (4°C) interfaced to a Waters Micromass Quattro Micro tandem mass spectrometer operating in the positive ion ESI mode. The compounds were separated on a 150 × 2.1 mm 5 µm Polaris C18 column fitted with a C18 guard column using a mobile phase consisting of methanol:water (92:8 v/v)

containing 5 mM ammonium formate and a flow rate of 0.2 ml/min. The column was maintained at 30°C. Multiple reaction monitoring transitions were: vitamin D_3 *m/z* 385.2 → 107, 159.2, 259.4; vitamin D_3-[$^2H_3$] *m/z* 388.3→110, 162.2, 259.4, 370.4; 25-hydroxyvitamin D_3 *m/z* 401.2 → 105, 159 and 383. The Quattro Micro tandem mass spectrometer capillary voltage was set at 2.6 kV, the cone voltage 23 V and the source and desolvation temperatures set to 100°C and 400°C respectively. The nitrogen gas flow rates were 60 l/h and 500 l/h for the cone gas flow and desolvation gas flow respectively. Argon was used as a collision gas at 3×10^{-3} mbar. MRM functions were divided into three groups (MRM1 25-hydroxyvitamin D_3 0-8 min, MRM2 vitamin D_3 and MRM3 vitamin D_3-[$^2H_3$] 14-24 min) with dwell time of 0.1 sec. Masslynx V4.0 software was used to process the data.

The methodology for vitamin D_3 analysis is summarised in Figure 2-1.

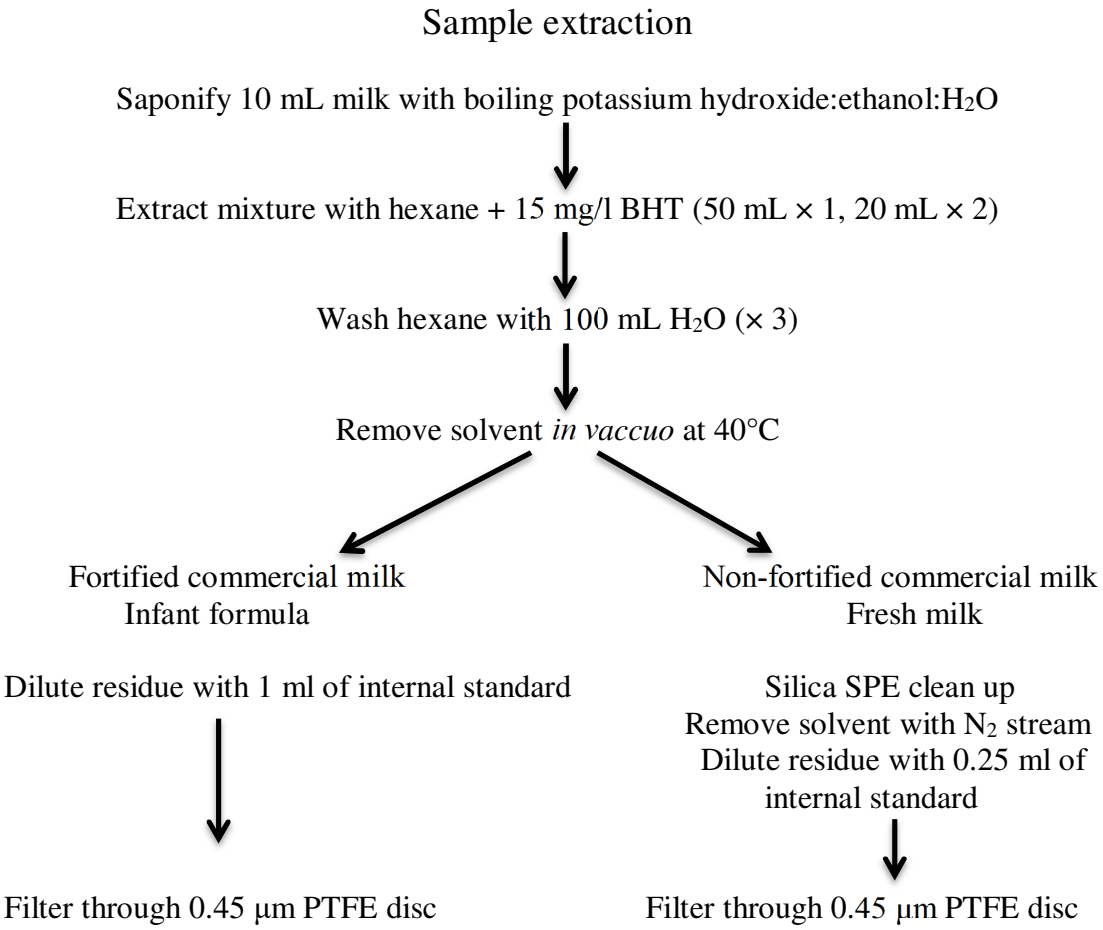


Figure 2-1: Flow diagram of the methodology for the analysis of vitamin D_3 and 25-hydroxyvitamin D_3 in cow milk by HPLC-MS^n and HPLC-MS/MS

2.7. Vitamin A, E and β-carotene

2.7.1. Sample extraction

A 10 ml aliquot of cow milk was mixed with 0.5 g of ascorbic acid, 40 ml of ethanol and 10 ml of 1:1 potassium hydroxide in water and heated at reflux with stirring for 30 minutes. The mixture was cooled in an ice bath and quantitatively transferred to a separating funnel with 50 ml water, 10 ml ethanol and 50 ml hexane containing 15 mg/l BHT. The separating funnel was shaken vigorously for 2 minutes and the phases allowed to separate. The aqueous phase was removed and extracted twice more with 20 ml portions of hexane containing 15 mg/l BHT. The hexane extracts were combined, washed three times with 100 ml of water and then made to 100 ml with hexane. 10 ml of the hexane solution was then transferred to a glass tube and the solvent removed under a flow of nitrogen at room temperature. The residue was reconstituted with 1.0 ml of methanol and filtered through a 13 mm 0.45 µm PTFE filter disc into three separate vials for concurrent analysis by HPLC-MS^n, HPLC-UV/Vis and HPLC-Fl.

2.7.2. Analysis

2.7.2.1. High performance liquid chromatography-ion trap mass spectrometry

The analyses were performed with an Agilent 1100 series quaternary solvent delivery system with a cooled autosampler (4°C) connected to a Thermo LTQ ion trap mass spectrometer operating in the positive ion APCI mode. The compounds were separated with a Polaris 150 × 2.1 mm, 5 µm C18-A column fitted with a C18 guard column and maintained at 30°C. The mobile phase consisted of (A) water and (B) methanol with a flow rate of 0.2 ml/min. The elution profile was: 0 min, 95% B; 5 min, 95% B; 10 min, 100% B (linear gradient); 25 min, 100% B; 26 min, 95% B, and the column equilibrated for 5 min at a flow rate of 0.4 ml/min before the next injection.

The mass spectrometer was tuned by infusing a 10 µg/ml solution of each compound at a rate of 10 µg/100 ml, mixed with 0.2 ml/min HPLC mobile phase via a T-piece before entering the mass spectrometer.

The APCI vaporizer temperature was set at 250°C, the ion current 4.5 mA, the heated capillary was maintained at 200°C and the sheath and auxiliary gases were at 20 and 5 units respectively. Source voltage was set to 4.4 kV and the capillary voltage set at 16 V. A full scan MS^2 protocol was used with the following settings; all *trans*-retinol, parent mass *m/z* 269, normalized collision energy of 30, scan range of *m/z* 70 to 250, quantification ions *m/z* 213 and *m/z* 199, α-tocopherol, parent mass *m/z* 431, normalized collision energy of 20, scan range of *m/z* 130 to 180, quantification ion *m/z* 165 and β-carotene, parent mass *m/z* 537, normalized collision energy of 22, scan range of *m/z* 150 to 490, quantification ion *m/z* 413. Excalibur V2.1 software was used to process the data.

2.7.2.2. HPLC-UV/Vis and HPLC-Fl

2.7.2.2.1. All *trans*-retinol and α-tocopherol

The analyses were performed with a Waters series 2695 quaternary solvent delivery system with a cooled autosampler at 4°C and heated column compartment at 30°C, coupled in series to a Waters 996 PDA detector (all *trans*-retinol λ 320 nm) and a Shimadzu RF-10A_{XL} Fl detector (α -tocopherol Ex 295 nm, Em 330 nm). The compounds were separated on a Bondclone 300 × 3.9 mm, 10 µm C18 column fitted with a C18 guard column. The mobile phase consisted of water:methanol (5:95 v/v), at a flow rate of 1 ml/min.

2.7.2.2.2. β-carotene

The analyses were performed with a Waters series 2695 quaternary solvent delivery system with a cooled autosampler at 4°C and heated column compartment at 30°C, coupled to a Waters 996 PDA (λ 450 nm). The compounds were separated on a Sphereclone 250 × 4.6 mm, 5 µm ODS2 C18 column fitted with a C18 guard column. The mobile phase consisted of acetonitrile:50 mM ammonium acetate in methanol:dichloromethane (75:20:5 v/v) and containing 0.05% v/v triethylamine and 0.1% w/v BHT, at a flow rate of 2 ml/min.

The methodology for the analysis vitamins A, E and β-carotene in milk is summarised in Figure 2-2.

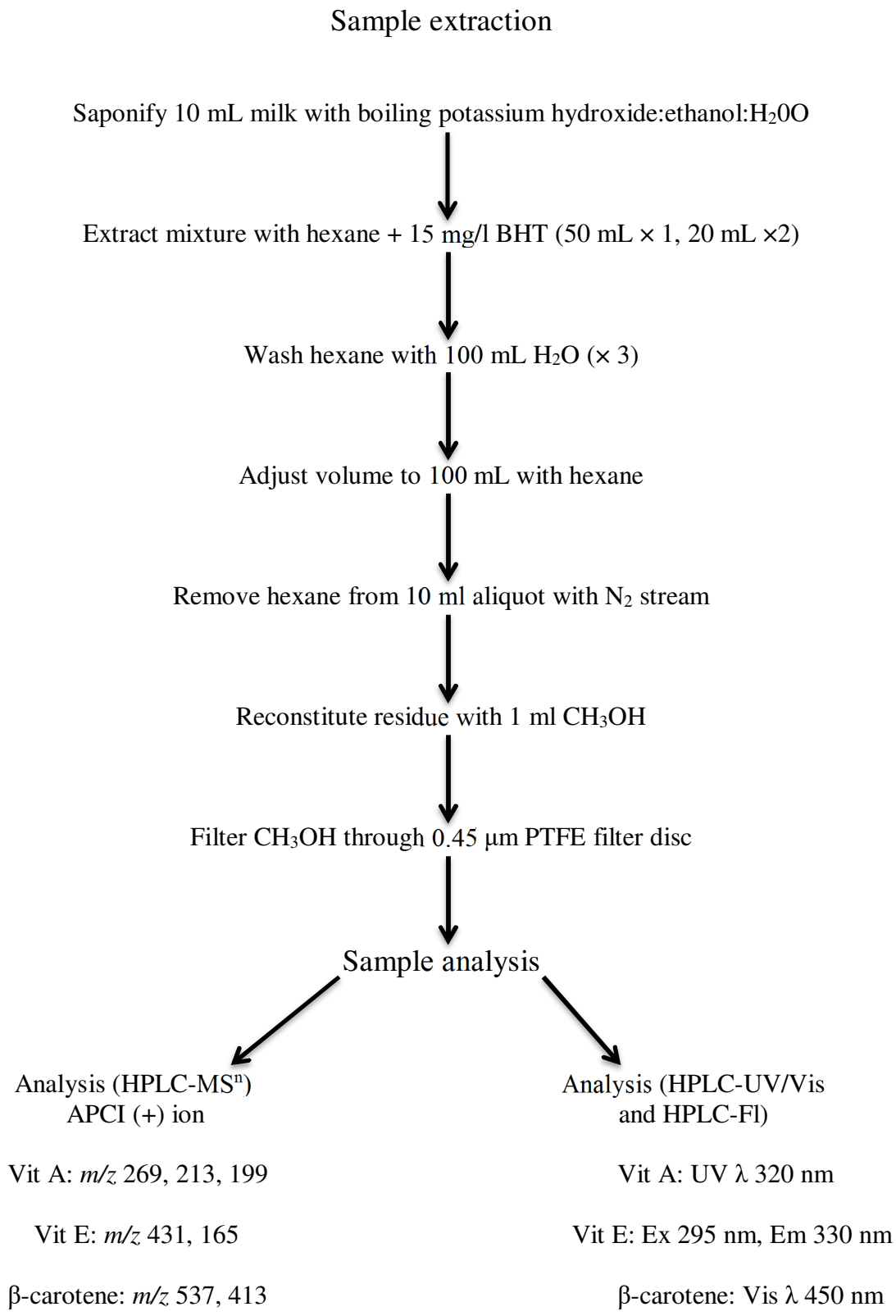


Figure 2-2: Flow diagram of the methodology for the analysis vitamins A, E and β-carotene in cow milk by both HPLC-MS^n and HPLC-UV/Vis and HPLC-Fl

2.8. Dairy cow feeding systems experiment

The milk samples analysed in this study were obtained from a short-term feeding rate experiment that was a component of an experiment primarily designed to examine milk production response curves for dairy cows fed different supplement regimes in addition to the traditional pasture-based diet. The experiment was designed to mimic conditions of low pasture availability and high supplement intake. It was conducted at the DPI Ellinbank research farm, Victoria, Australia (latitude 38°14‘S, longitude 145°56‘E). All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council 2004). Approval to proceed was obtained from the DPI Agricultural Research and Extension Animal Ethics Committee.

2.8.1. Experimental design

The primary study commenced in September 2009 with the selection of 216 cows, which had calved in late winter-early spring and were in at least their second lactation. The cows were allocated into 6 herds of 36 cows, which were balanced for days in milk, age, body weight and prevailing production of milk, milk protein and milk fat according to the method of Baird (1994). Two herds of 36 cows were then randomly allocated to each of three feeding systems and were fed their various rations at a rate of 10 kg DM total supplement/cow per day. The feeding systems are outlined in section 2.8.2. below.

The short-term feeding rate experiment, a component of the primary experiment, commenced in November 2009 with the division of each herd of 36 cows into four groups of nine cows which were randomly assigned to one of four amounts of supplementary feeding (6, 8, 10 or 12 kg DM total supplement/cow per day). This gave a total of 24 treatment groups, as shown in Table 2-7.

Table 2-7: Diagrammatic representation of the experimental design, showing 36 cows for each of 3 replicated dietary treatments (Control, PMR1 and PMR2) in spring 2009 and groups of nine cows offered 4 amounts of supplement (6, 8, 10 or 12 kg DM total supplement/cow per d) (Auldist, Marett, Greenwood, Hannah, Jacobs and Wales 2013)

Feeding system	**Control**								**Partial Mixed Ration 1**								**Partial Mixed Ration 2**							
Number of cows	72								72								72							
Replicate No.	A				B				A				B				A				B			
Number of cows	36				36				36				36				36				36			
	Short term feeding rate experiment																							
Number of cows	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Supplement (kg DM/cow per d)	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12	6	8	10	12

2.8.2. Dietary treatments

Three feeding systems were compared. The Control and Partial Mixed Ration 1 (PMR1) diets had the same formulation, but the Partial Mixed Ration 2 (PMR2) diet was different and contained maize grain, maize silage and lucerne hay. The cows grazed perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens*) based pasture which is traditional in Gippsland, Victoria.

Control: Cows in the control group were supplemented with milled barley grain and pasture silage. The grain:forage ratio of the supplement was 75:25 (DM basis) and is detailed in Table 2-8. Cows fed the Control diet were individually fed their grain twice daily in the milking shed, while pasture silage was provided once per day in the paddock.

PMR1: Cows were also offered the same amounts of supplement as cows fed the Control diet (milled barley grain and pasture silage, as shown in Table 2-8, but the supplements were mixed and chopped in a feed wagon (Richard Keenan and Co. Ltd,

Model K160, Co. Carlow, Ireland) before being presented on a concrete feedpad. In contrast to the cows fed Control diet, cows fed PMR1 were supplemented once a day, after the morning milking.

PMR2: Cows were offered a PMR comprising barley grain (25% of total supplement DM), maize grain (30% of DM), maize silage (20% of DM) and lucerne hay (25% of DM) as shown in Table 2-9. Similar to the cows fed PMR1, cows fed PMR2 were supplemented once a day on a feedpad. The PMR2 diet had the same ratio of grain:forage (including the grain component of the maize silage) as the Control and PMR1 diets, but was formulated to ferment more slowly in the rumen.

Table 2-8: Ration offered to the cows fed Control and PMR1 diets at 4 amounts of supplement

Supplement	**Supplement amount (kg DM/cow per day)**			
	6	**8**	**10**	**12**
Milled barley grain	4.5	6	7.5	9
Pasture silage	1.5	2	2.5	3
Estimated pasture intake	14	14	14	14
Total intake	20	22	24	26

Table 2-9: Ration offered to the cows fed PMR2 diet at 4 amounts of supplement

Supplement	**Supplement amount (kg DM/cow per day)**			
	6	**8**	**10**	**12**
Milled barley grain	1.5	2	2.5	3
Maize grain	1.8	2.4	3	3.6
Lucerne hay	1.5	2	2.5	3
Maize silage	1.2	1.6	2	2.4
Estimated pasture intake	14	14	14	14
Total intake	20	22	24	26

The short-term feeding rate experiment displaced the usual 10 kg supplementary ration of the main feeding systems experiment for a period of 25 days. For all treatments, a pasture allowance of approximately 14 kg DM/cow per day (to ground

level) was available as a single allocation of pasture per day. Cows fed the Control diet had access to pasture immediately after each milking. Cows fed PMR1 and PMR2 diets received their entire supplement after the morning milking, and this was eaten during the day on a feedpad. The pasture component of their diet was only available to the cows between the afternoon milking and the morning milking. All cows had several opportunities each day to access water.

All cows also received a vitamin and mineral supplement (Nutrifeed Hi-Milker; Debenham Australia, Leongatha, Victoria, Australia) containing vitamins A and E. Cows at the highest rate of supplementation (12 kg of DM/cow per day) received this supplement at a rate of 125 g/cow per day, and cows receiving lower amounts of supplement received proportionally less.

2.8.3. Sample collection

Sampling was conducted three times during the experiment, on day 0 at the time of introduction of the different feeding rates, day 9 and after the cows had adapted to the supplement levels (day 23). Cows were milked twice daily at 07:00 and 15:00 hr. Milk samples were collected using in-line milk meters (DeLaval International, Tumba, Sweden) that collected a representative sample from each cow. Cows with clinical mastitis were excluded. Milk samples from the evening milking were kept in the cool room (-4°C) overnight and mixed with the matching milk samples from the next morning milking. The milk samples were pooled for the 9 cows receiving each supplementary feeding rate within each dietary treatment. With two replicates this gave a total of 24 composite samples collected at each sampling time, and 72 samples over the period of the experiment. For each sample, two 1 litre bottles of milk were collected and transported refrigerated to DPI-Werribee. From the first bottle of each sample, a 40 ml subsample was then taken and kept frozen at -80°C until analysis. The second bottle was stored at -20°C for use as a back-up, but was never required.

The various components of the cows' rations were sampled at the time of the final milk sampling.

Pasture was not sampled. It was high quality, short, green perennial ryegrass pasture with a likely high digestibility of approximately 80%, which corresponds to a metabolisable energy of 12 MJ/kg DM and crude protein of approximately 22%.

Fibre content could be quite high given the time of year and the perennial ryegrass content of the pasture.

2.8.4. Sample analysis

Milk samples were analysed for all *trans*-retinol, α-tocopherol and β-carotene according to the method described in Chapter 4 of this thesis.

Milk fat was determined by Hico Australia (Herd Improvement Co-Operative Australia Ltd, Korumburra, Australia) using an infra-red milk analyser (Model 2000, Bentley Instruments, Chaska, MN) (Auldist*, et al.* 2013).

The various components of the cow's rations were analysed for vitamins A, E and β-carotene concentrations using the Werribee Analytical Laboratory 'in-house' HPLC method with UV/Vis and Fl detection.

These concentrations were then used to calculate the daily intake of vitamins in the feed ration of the cows, taking into account the moisture content of the feeds, which was measured by staff at DPI Ellinbank.

2.8.5. Statistical analysis

Milk sample data was subjected to analysis of variance (ANOVA) using Genstat 14th edition.

Chapter 3: Vitamin D analytical method

3.1. Introduction

As detailed in the Materials and Methods chapter, two HPLC-MS methods were developed for the analysis of vitamin D_3. Vitamin D_3 has similar chemical characteristics to the other fat-soluble vitamins (vitamins A and E), therefore the extraction protocol and choice of HPLC conditions were based on a NATA certified DPI 'in-house' HPLC-UV/Vis method for the determination of vitamins A, E and β-carotene in food (Department of Primary Industries 2007). Published HPLC-UV/Vis methods for the analysis of vitamin D_3 incorporate extra clean-up steps, either SPE, preparative HPLC or a combination of both (Ball 1998). HPLC-MS offers at least one order of magnitude sensitivity increase over HPLC-UV/Vis, however, it was anticipated that at least one extra clean-up procedure would be required to provide a cleaner and more concentrated extract for analysis, as instrument sensitivity may not be adequate and matrix interferences may be more of an issue due to the very low levels of vitamin D_3 in milk. For this reason, the determination of vitamin D_3 in milk was developed as a separate method to that used for the other fat-soluble vitamins. However, both methods use the same saponification and extraction protocols, and therefore there is scope for partial integration of the two methods.

Milk sample extracts were assayed on both HPLC-MS^n and HPLC-MS/MS to enable an assessment of the suitability of the linear ion trap mass spectrometer for this work. Vitamin D_3-[$^2H_3$] and 25-hydroxyvitamin D_3-[$^2H_3$] were used as internal standards for the quantification of vitamin D_3 and 25-hydroxyvitamin D_3 respectively.

The development of these methods is described below and has been published as 'The determination of vitamin D_3 in bovine milk by Liquid Chromatography-Mass Spectrometry' in *Food Chemistry* (Trenerry, Plozza, Caridi and Murphy 2011).

3.2. Method development

The HPLC-MS^n and the HPLC-MS/MS methods were developed simultaneously in the following order:

1. The mass spectrometer conditions, API interface and fragmentation patterns, were optimised to give the maximum ion abundance for the ions of interest.

2. The chromatography conditions (choice of HPLC column and mobile phase components) were optimised using the conditions identified in the API optimisation.

3. The sample extraction protocols were developed. The initial work focussed on fortified milk samples, in which the levels of vitamin D_3 were expected to be at least one order of magnitude greater than in fresh milk (fortified milk 0.5-2 µg/100 ml, fresh milk 0.05 µg/100 ml). These conditions were further modified for the analysis of fresh milk.

4. The following method characteristics were evaluated for method validation:

- Linearity
- Precision
- Trueness/bias
- Sensitivity
- Reproducibility
- Limit of Quantification

3.2.1. Mass spectrometer

3.2.1.1. Atmospheric pressure ionisation interface

The same approach was used to optimise the API interfaces for the HPLC-MS^n and HPLC-MS/MS instruments.

Optimisation of the mass spectrometer conditions to give the best peak signal to noise (S/N) ratio was performed whilst infusing a 10 µg/ml solution of each standard (25-hydroxyvitamin D_3, vitamin D_3 and vitamin D_3-[$^2H_3$]) at 10 µl/min into the API interface via a T-piece where it was mixed with HPLC mobile phase (for example, methanol:water 92:8 v/v) at a flow rate of 0.2 ml/min. This allowed optimisation at the flow rate used for the analysis, and also facilitated easy modification of mobile phase components when assessing different solvents or mobile phase modifiers.

The ion trap maximum fill time was set at 200 milli sec for all experiments.

The voltages of the various components of the ion trap mass spectrometer ion optics, which are located after the API interface (ie. inside the mass spectrometer) were optimised using the instrument's auto-tune function.

Two ionisation sources were considered: ESI and APCI.

3.2.1.1.1. Electrospray ionisation

All user-adjustable parameters (positive/negative ionisation mode, spray voltage, sheath, auxiliary and sweep gas flow rates, capillary temperature and probe fore/aft position and insertion depth), were optimised independently, generally starting from the lower end of the available range of adjustment and working incrementally upwards until the value corresponding to maximum sensitivity had been reached. Various mobile phase solvents (methanol, acetonitrile and water) and mobile phase modifiers (ammonium acetate, ammonium formate, formic acid) were also trialled to assess which mobile phase composition gave the best ionisation performance. Only volatile mobile phase modifiers were used for this work as non-volatile compounds deposit in the API causing blockages and loss of signal.

3.2.1.1.2. Atmospheric pressure chemical ionisation

All user-adjustable parameters (positive/negative ionisation mode, discharge current, vaporizer temperature, sheath, auxiliary and sweep gas flow rates, capillary temperature and probe fore/aft position and insertion depth), were optimised independently, as were the choice of mobile phase solvents (methanol, acetonitrile and water) and mobile phase modifiers (ammonium acetate, ammonium formate, formic acid), as described above for ESI.

3.2.1.2. Mass spectrometer fragmentations

The fragmentation conditions for each analyte were optimised while infusing a solution of each standard into the ion source as detailed above. The precursor ion was selected, then a collision energy was applied to cause fragmentation. The collision energy was adjusted to maximise the abundance of the fragment ions, of which the two most abundant ions were chosen for ion trap MS^2 experiments. For the triple quadrupole mass spectrometer, the three or four most abundant fragment ions were selected for MRM experiments.

For the ion trap MS, the isolation width was set at the instrument default value of 1.0 for initial work with fortified milk samples, but for later work with fresh milk samples, the isolation width was optimised for sensitivity according to the instrument manufacturer's instructions.

3.2.2. High performance liquid chromatography column and mobile phase

Three different reversed-phase HPLC columns with differing bonded phase polarities (Phenomenex Luna C18(2) 5 µm, 150 × 2 mm and Varian Polaris C18-A 5 µm, 150 × 2.1 mm) and structure (Varian Pursuit diphenyl 3 µm, 150 × 2 mm) were trialled with a combination of different mobile phases to achieve a suitable separation of the analytes from each other as well as from other compounds present in the sample extract. The smaller particle size of the diphenyl column may have afforded some improvement in peak resolution compared to the two C18 columns, however this was not expected to be significant compared to the selectivity differences between the different phases.

Samples of commercial milk and a fortified milk powder were saponified using hot ethanolic potassium hydroxide and extracted with hexane according to the HPLC-UV/Vis method for the determination of vitamin A, vitamin E and β-carotene (Department of Primary Industries 2007). These extracts, as well as extracts spiked with vitamin D_3, were injected onto each column using isocratic elution with a variety of methanol: water mixtures (75-100% methanol) to determine the ideal column and mobile phase combination. Separation of vitamin D_3 from endogenous interferences proved to be the key criteria, but peak shape, sensitivity and run time were also considered. Once a suitable column was identified, a gradient elution was assessed to see if this gave sharper, taller peaks (and hence better sensitivity), and also to see if vitamin D_3 could be better separated from other endogenous peaks in the extract.

A mobile phase flow rate of 0.2 ml/min was used for all chromatographic work, except where stated otherwise. 0.2 ml/min is commonly used for HPLC-MS as it affords optimum performance of the ion interface. The column dimensions used for this work are the optimum size for this flow rate, as recommended by the column manufacturer.

Column temperature is a parameter which can be manipulated to alter a chromatographic separation, however elevated column temperatures accelerate degradation of the column bonded phase and in this case an adequate separation was achieved at 30ºC. This was high enough above the room temperature for the column

heater to be able to maintain good temperature stability without being affected by fluctuations in the room temperature.

3.2.3. Sample extraction

3.2.3.1. Fortified milk samples

The samples were extracted using the conditions described for the HPLC-UV/Vis analysis of vitamins A, E and β-carotene (Department of Primary Industries 2007). Two modifications were made:

1. The antioxidant BHT was added to the hexane extractant, as preliminary extractions of spiked samples showed unacceptable variation in recoveries, which was found to be alleviated by the addition of BHT at a rate of 15mg/l, as per the method of Dimartino (2007).

2. The complete hexane extract was dried and reconstituted with methanol prior to analysis to maximise the concentration of vitamin D_3 in the final extract.

3.2.3.2. Non-fortified milk samples

A silica solid phase extraction (SPE) method based on the work of Kurmann and Indyk (1994) was developed to provide a cleaner and more concentrated solution for analysis.

All solvents used for silica SPE were dried over sodium sulphate. Water is a very strong solvent for this normal phase system, and even small amounts of water in the solvents could cause inconsistent results.

Aliquots of 25-hydroxyvitamin D_3 and vitamin D_3 standard solutions in methanol were dried and redissolved in 1 ml hexane. The solutions were applied to separate silica SPE cartridges pre-wet with 6 ml of hexane. The vials were washed twice with hexane (1 ml) to quantitatively transfer the analytes to the cartridge. Each cartridge was then eluted with 2 x 1.5 ml aliquots of 90:10 (v/v) hexane:ethyl acetate followed by 4 x 1.5 ml aliquots of 80:20 (v/v) hexane:ethyl acetate for vitamin D_3 elution and 3 x 3 ml aliquots of 60:40 (v/v) hexane:ethyl acetate for 25-hydroxyvitamin D_3 elution. As each solution was applied to the cartridge, the solvent eluting from each cartridge was isolated as a separate fraction, dried under a flow of nitrogen gas, reconstituted

with 1 ml of methanol containing vitamin D_3-[$^2H_3$] and analysed by HPLC-MS, monitoring the most abundant MS^2 fragment for each compound.

The effect of an alternative polar solvent (diethyl ether) and various eluent additives (ammonia, acetic acid, triethylamine and ammonium acetate) on the recovery of the vitamins from the cartridges was also trialled.

3.2.4. Analytical method

The various aspects of the method development were combined to produce a straightforward analytical method suitable for the analysis of fortified commercial milk samples containing greater than 0.1 µg/100 ml of vitamin D_3, and a more complex method for analysis of 25-hydroxyvitamin D_3 and vitamin D_3 in fresh and commercial non-fortified milk samples. The complete method is detailed in section 2.6.

3.2.4.1. Validation

The method was validated according to NATA Technical Note 17 (2013) by simultaneously analysing fortified and non-fortified milk sample extracts, fortified infant formula extracts and spiked extracts by HPLC-MS^n and HPLC-MS/MS. The HPLC-MS/MS was operated in the positive ion ESI mode, and the levels of vitamin D_3 were determined by summing the areas from the MRM transitions (*m/z* 385.2 →107, 159.2, 259.4). For HPLC-MS^n, the levels of vitamin D_3 were calculated from individual MS^1 or MS^2 ions.

The method was further validated through participation in a Proficiency Testing Australia study for the determination of vitamin D_3 in fortified infant formula, in which two identical samples (S1 and S2) were assayed in duplicate (Bunt 2010).

3.3. Results

3.3.1. Mass spectrometer

3.3.1.1. Ion trap mass spectrometer

3.3.1.1.1. Atmospheric pressure ionisation interface

Electrospray ionisation

The optimal ionisation efficiency (signal intensity 1.03×10^5) was achieved in the positive ionisation mode, as shown in Figure 3-1, with a mobile phase composed of methanol containing 0.1 M ammonium acetate. Ammonium acetate was used as the mobile phase modifier as the use of plain methanol or methanol containing other common HPLC additives, such as sodium acetate, resulted in the formation of sodiated adducts, for example $[\text{vitamin } D_3 + Na]^+$ (*m/z* 407), which complicated the mass spectra and made it difficult to identify the molecular ion and associated fragment ions.

The addition of acetonitrile and acidic mobile phase modifiers to the mobile phase had a severe detrimental effect on the ionisation efficiency.

The optimal ionisation efficiency was achieved with the following ESI settings:

Spray voltage	2.8 kV
Sheath gas flow rate	26 arbitrary units
Auxiliary gas flow rate	0 arbitrary units
Sweep gas flow rate	18 arbitrary units
Capillary voltage	16 V
Capillary temperature	200°C

Atmospheric pressure chemical ionisation

The optimal ionisation efficiency (signal intensity 2.57×10^6) was achieved in the positive ionisation mode, as shown in Figure 3-1, with a methanol:water mobile phase containing 0-10% water. The addition of ammonium acetate to the mobile phase had little effect on the ionisation efficiency but produced more chemical noise for *m/z*<300, and was therefore not added.

The addition of acetonitrile and acidic mobile phase modifiers to the mobile phase had a detrimental effect on the ionisation efficiency.

The optimal ionisation efficiency was achieved with the following APCI settings:

Capillary temperature	200°C
Vaporizer temperature	200°C
Sheath gas flow rate	20 arbitrary units
Auxiliary gas flow rate	5 arbitrary units
Sweep gas flow rate	0 arbitrary units
Source current	4.5 µA
Capillary voltage	16 V

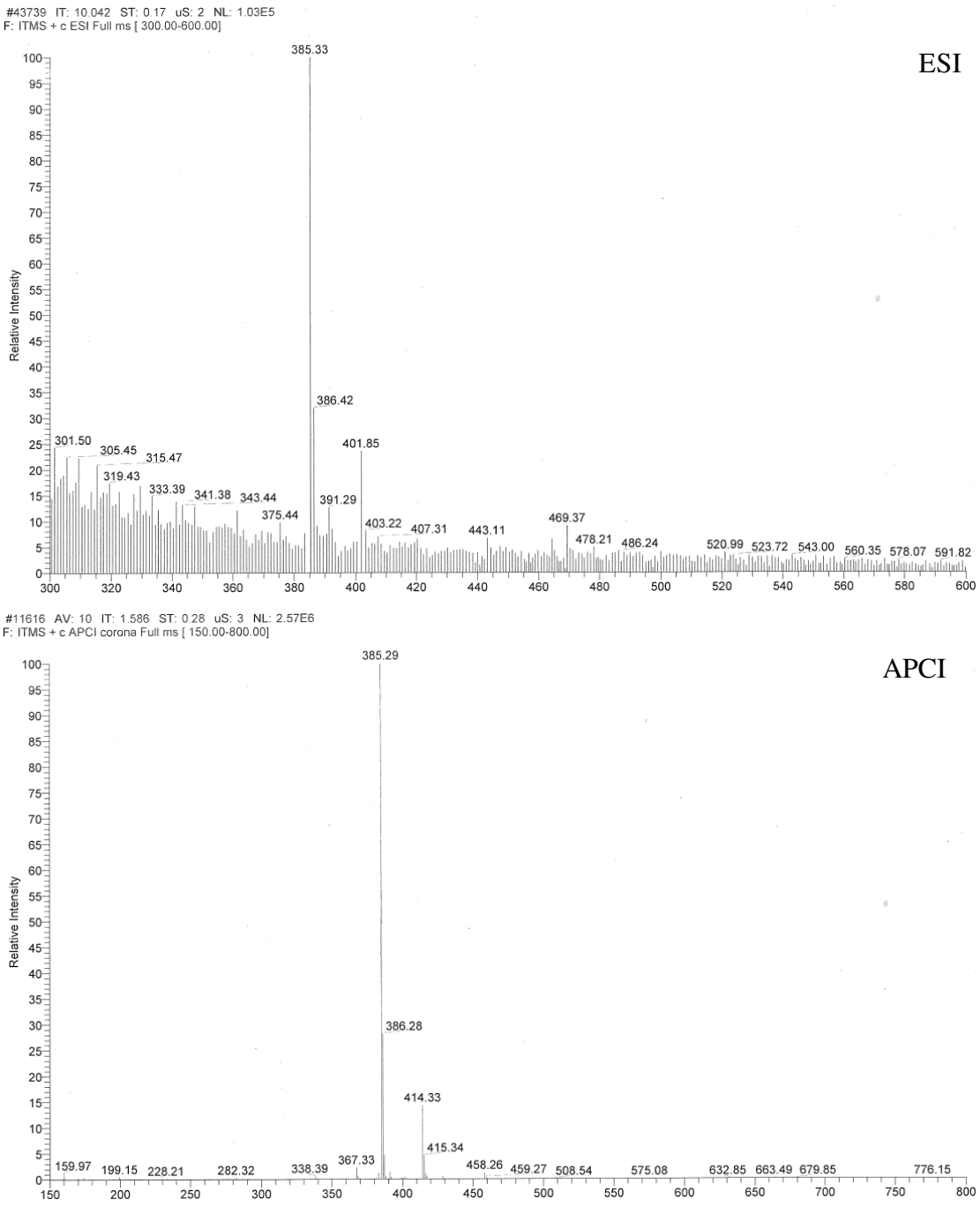


Figure 3-1: Optimised HPLC-MS^n ESI and APCI spectra of 10 µg/ml vitamin D_3 infused at 10 µl/min into 0.2 ml/min mobile phase

3.3.1.1.2. Ion trap mass spectrometer fragmentations

An isolation width of 3.5 and collision energy of 23% (of maximum) was used for 25-hydroxyvitamin D_3 fragmentation, and an isolation width of 2 and collision energy of 25% was used for vitamin D_3 fragmentation. Table 3-1 shows the parent ion and

major MS^2 fragment ions (*m/z*), and Figure 3-2 shows the product ion spectra for vitamin D_3.

Table 3-1: Parent and major fragment ions (*m/z*) for 25-hydroxyvitamin D_3, vitamin D_3 and their corresponding deuterated internal standards for MS^n

Compound	**Parent ion (*m/z*)**	**Major MS^2 fragment ions (*m/z*)**
25-hydroxyvitamin D_3	401	383, 365.2
25-hydroxyvitamin D_3-[$^2H_3$]	404	386
vitamin D_3	385.5	367.4, 259
vitamin D_3-[$^2H_3$]	388.5	370.4

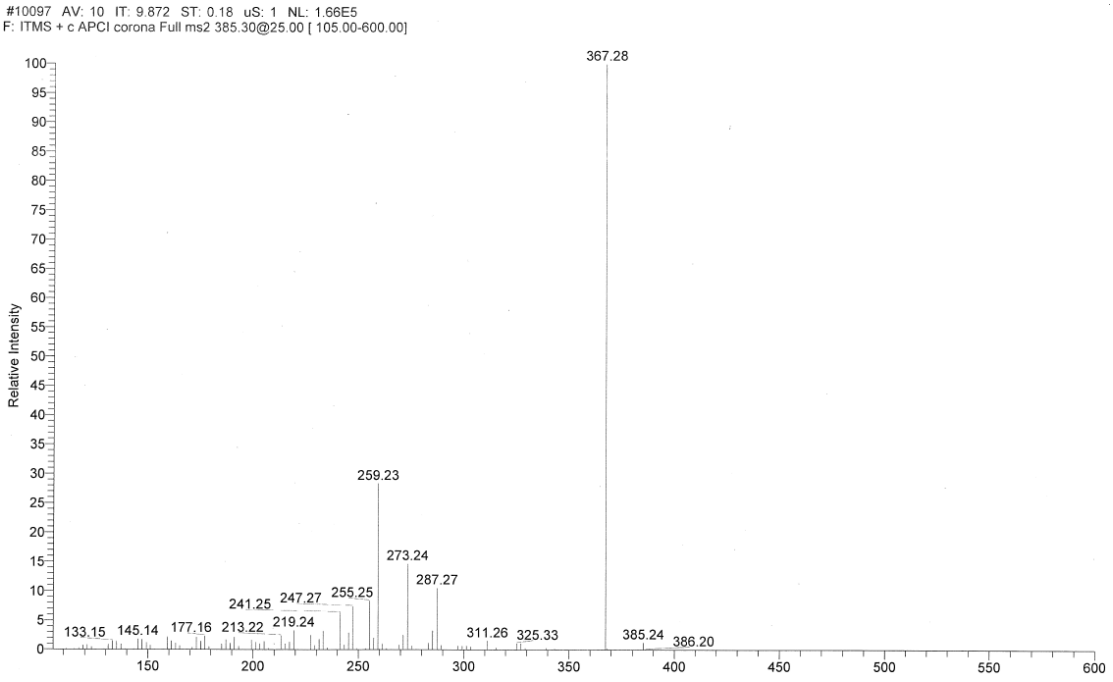


Figure 3-2: HPLC-MS^n product ion spectra for the fragmentation of vitamin D_3

3.3.1.2. Triple quadrupole mass spectrometer

3.3.1.2.1. Atmospheric pressure ionisation interface

Electrospray ionisation

The optimal ionisation efficiency (× 15 gain at full scale whilst infusing 5 µg/ml vitamin D_3 standard solution at 10 µl/min) was achieved with a mobile phase composed of methanol and up to 20% water, with the addition of the most effective mobile phase modifier, 5 mM ammonium formate, increasing ionisation efficiency by a factor of 20.

The use of acetonitrile instead of methanol decreased sensitivity by approximately a factor of two.

The optimal ionisation efficiency was achieved with the following ESI settings:

Capillary voltage	2.6 kV
Cone voltage	23 V
Source temperature	100°C
Desolvation temperature	400°C
Cone gas flow	60 l/h
Desolvation gas flow	500 l/h

Atmospheric pressure chemical ionisation

The optimal ionisation efficiency (× 180 gain at full scale whilst infusing 1 ug/ml vitamin D_3 standard solution at 20 µl/min) was achieved with a mobile phase consisting of 95% methanol, 5% water and 5 mM ammonium formate.

The optimal ionisation efficiency was achieved with the following APCI settings:

Corona current	0.3 µA
Cone voltage	22 V
Source temperature	130°C
Desolvation temperature	550°C
Cone gas flow	50 l/h
Desolvation gas flow	250 l/h

3.3.1.2.2. Tandem mass spectrometry fragmentations

The collision energy was adjusted to maximise the intensity of each fragment, and a dwell time of 0.1 sec was used. The argon collision gas was set at 3×10^{-3} mbar. Table 3-2 shows the parent ion and major MS/MS fragment ions (*m/z*).

Table 3-2: Parent and major fragment ion *m/z* ratios for 25-hydroxyvitamin D_3, vitamin D_3 and their corresponding deuterated internal standards for MS/MS

Compound	**Parent ion (*m/z*)**	**Major MS/MS fragment ions (*m/z*)**
25-hydroxyvitamin D_3	401.2	383, 159, 105
vitamin D_3	385.2	259.4, 159.2, 107
vitamin D_3-[$^2H_3$]	388.3	370.4, 259.4, 162.2, 110

3.3.2. High performance liquid chromatography

3.3.2.1. Phenomenex Luna C18(2)

The best chromatographic performance was achieved using an isocratic mobile phase containing methanol:water (90:10). This enabled separation of 25-hydroxyvitamin D_3 from endogenous interferences, however it was not possible to obtain an interference-free chromatogram for vitamin D_3.

3.3.2.2. Varian Pursuit diphenyl

The best chromatographic performance was achieved using an isocratic mobile phase containing methanol:water (80:20). This column also gave separation of 25-hydroxyvitamin D_3 from endogenous interferences. Vitamin D_3 eluted in-between two other peaks, which was sufficient to allow analysis to a lower limit of 0.05 µg/ml in solution, which is equivalent to 0.5 µg/100 ml in the fortified milk sample.

3.3.2.3. Varian Polaris C18-A

Using an isocratic mobile phase containing methanol:water (92:8), this column gave separation of both 25-hydroxyvitamin D_3 and vitamin D_3 from significant endogenous interferences when analysing fortified milk and infant formula, and unfortified samples at spiking levels of 0.1 µg/100 ml.

Chromatograms of solutions containing 25-hydroxyvitamin D_3-[$^2H_3$] and vitamin D_3-[$^2H_3$] were also free from interfering compounds. These two compounds were not trialled on the other two columns.

Gradient elution was not adopted as this reduced the separation between vitamin D_3 and potentially interfering endogenous peaks.

Monitoring of the full scan MS^1 chromatogram of the HPLC-MS^n showed the elution of a relatively large non-distinct peak in the following chromatogram whenever a milk extract was assayed. This peak co-eluted with 25-hydroxyvitamin D_3, which may have led to interference with the ionisation of the vitamin in the ion source. Increasing the mobile phase flow rate to 0.4 ml/min after the elution of vitamin D_3, reduced the retention time of the large late-eluting peak so that it no longer co-eluted with 25-hydroxyvitamin D_3. This was considered a better option than employing a gradient to elute the peak, which would have necessitated re-equilibration of the column afterwards, and would take significantly longer.

3.3.3. Sample extraction

3.3.3.1. Fortified milk samples

Extraction of the saponified samples using pure hexane gave variations up to 50% in the levels of the vitamins in the fortified milk samples. More consistent data were obtained by adding 15 mg/l BHT to the hexane as per the method of Dimartino (2007).

3.3.3.2. Non-fortified samples

The recoveries of 25-hydroxyvitamin D_3 and vitamin D_3 from silica SPE eluted with various mixtures of hexane and ethyl acetate are shown in Table 3-3.

Table 3-3: Stepwise recoveries of 25-hydroxyvitamin D_3 and vitamin D_3 from silica SPE eluted with various mixtures of hexane and ethyl acetate

Eluent (hexane:ethyl acetate)	**Vol applied (ml)**	**% recovery**	
		25(OH)D_3	**vitamin D_3**
90:10 (wash)	1.5	0	0
	1.5	0	0
80:20 (elution)	1.5	-	7
	1.5	-	71
	1.5	-	20
	1.5	-	1
60:40	3	41	-
	3	54	-
	3	0	-
Total		95	99

Lower recoveries were observed when ethyl acetate was replaced with diethyl ether, or when any of the eluent additives were used.

SPE clean-up also removed a white residue that remained after drying the sample extract with a stream of nitrogen gas, indicating that the SPE was removing some matrix components from the extract.

The HPLC-MS^n chromatograms of vitamin D_3 and vitamin D_3-[$^2H_3$] in a fortified milk sample are displayed in Figure 3-3. The levels of vitamin D_3 were determined from the parent $[M+H]^+$ ion chromatogram (*m/z* 385.5) as well as the two fragment ion (*m/z* 367.4 and *m/z* 259) chromatograms and corrected for any matrix effects using the internal standard (vitamin D_3-[$^2H_3$]).

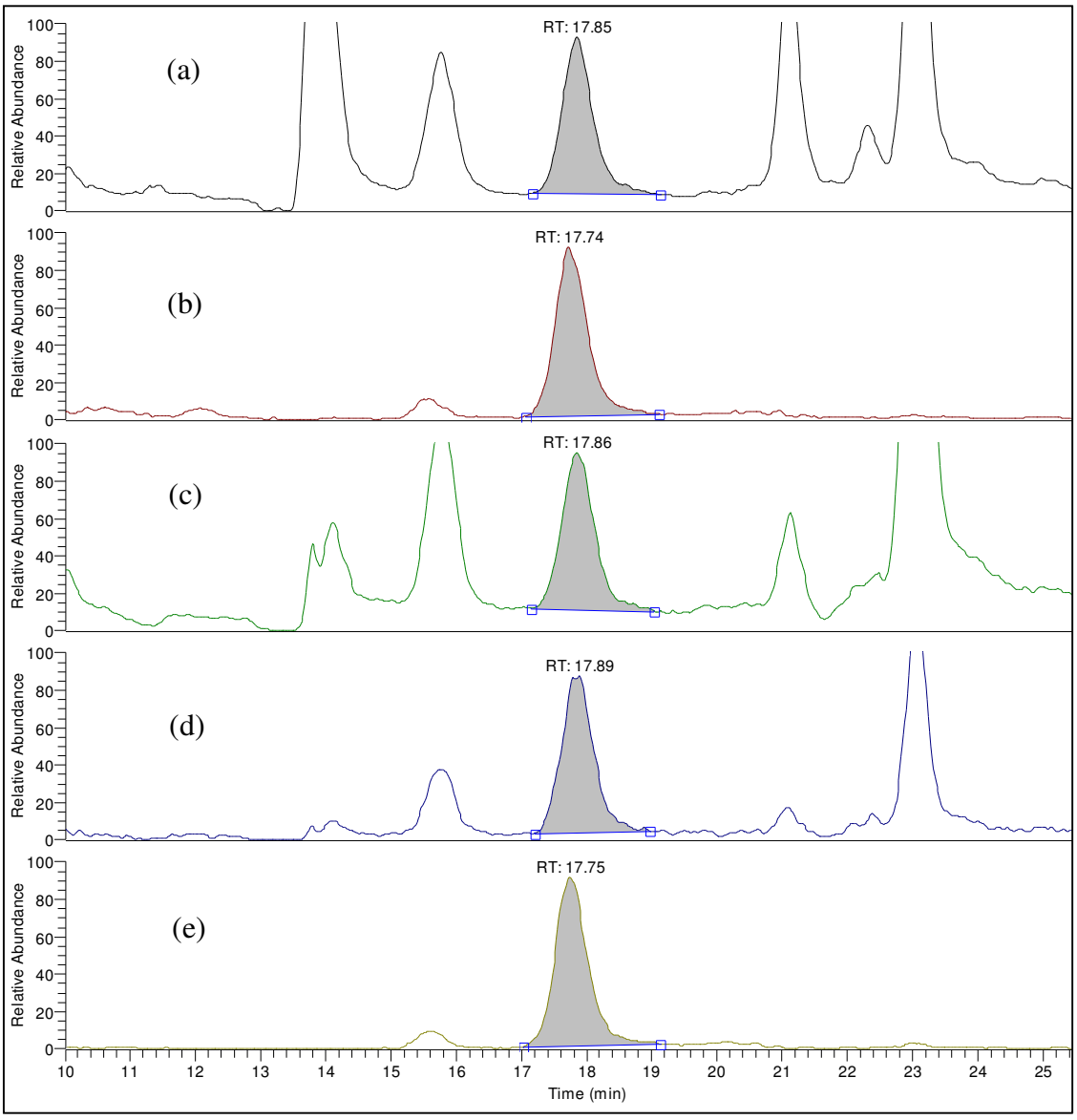


Figure 3-3: HPLC-MS^n chromatograms of a fortified commercial milk sample containing vitamin D_3 at a level of 0.5 µg/100 ml. Vitamin D_3-[$^2H_3$] concentration in the extract was 0.1 µg/ml. The peaks of interest are shaded and are: (a) vitamin D_3 MS^1, *m/z* 385.5, (b) vitamin D_3-[$^2H_3$] MS^1, *m/z* 388.5, (c) vitamin D_3 MS^2, *m/z* 367.4, (d) vitamin D_3 MS^2, *m/z* 259, (e) vitamin D_3-[$^2H_3$] MS^2, *m/z* 370.4.

3.3.4. Method validation

3.3.4.1. Linearity

Linearity was determined at two levels, one for fortified samples and one for non-fortified samples, by injecting a series of standards of various concentrations into the HPLC-MS^n and measuring the peak areas of the corresponding peaks. Each standard solution contained the same concentration of internal standard. The ratio of the peak areas of the standard and internal standard was then used to construct the calibration plots as displayed in Figure 3-4.

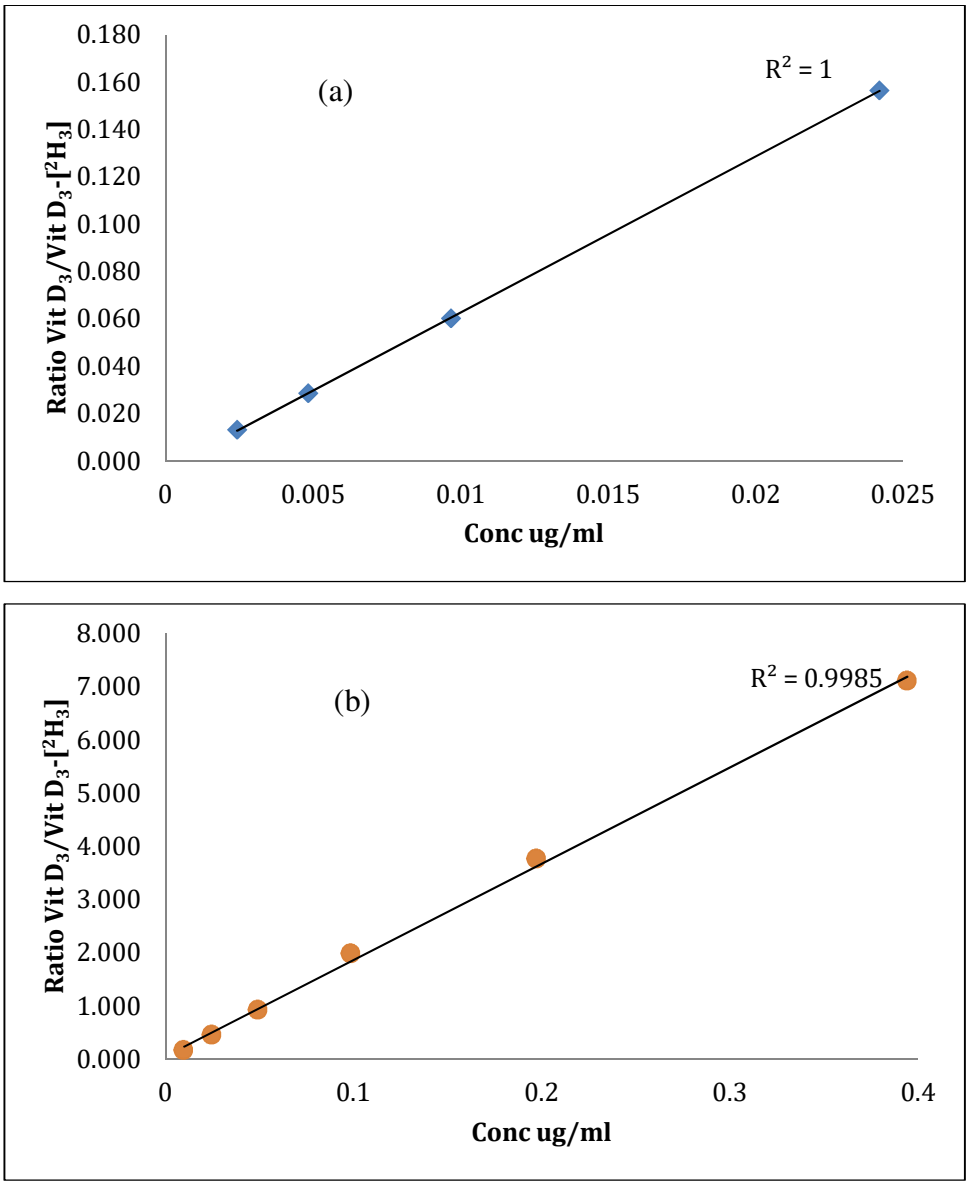


Figure 3-4: HPLC-MS^n calibration plots for vitamin D_3 for (a) non-fortified samples and (b) fortified samples

3.3.4.2. Precision

Method precision was determined by analysing the sample extracts using two different mass analysers (MS^n and MS/MS) and comparing the data with the corresponding label claims, see Table 3-4.

Table 3-4: Vitamin D_3 content of commercially available fortified milk samples (µg/100 ml) and infant formula (µg/100 g)

	HPLC-MS^n			**HPLC-MS/MS**	**Label claim µg/100 ml**
Fat content (label)	$[M+H]^+$ *m/z* 385.5	$[M+H-H_2O]^+$ *m/z* 367.4	$[M+H- C_8H_{14}O]^+$ *m/z* 259	Sum MRM	
0.1 %	0.48	0.51	0.49	0.51	0.5
1.4%	0.55	0.58	0.54	0.55	0.5
0.13%	2.0	1.9	1.9	1.8	2.0
1.4%	1.9	1.8	1.9	1.9	2.0
Infant formula[#]	8.0	8.2	8.4	7.0	7.7

HPLC-MS^n and HPLC-MS/MS data produced from separate samples of infant formula assayed on different days.

3.3.4.3. Trueness or bias

Two fortified infant formulas supplied by Proficiency Testing Australia were used to determine the method trueness/bias. The vitamin D_3 values obtained using the new method were slightly lower than those reported by the Proficiency Study, however, they were within the specified range and deemed satisfactory.

Proficiency test results:

S1, this study 7.8 $\pm$ 0.2 µg/100 g, proficiency study median result 8.7 $\pm$ 2 µg/100 g

S2, this study 7.5 $\pm$ 0.2 µg/100 g, proficiency study median result 9.6 $\pm$ 2.4 µg/100 g n=7) (Bunt 2010).

3.3.4.4. Recovery

Method sensitivity was determined by analysing a series of spiked samples and standards prepared in the sample extract solution.

The average recovery of vitamin D_3 spiked at a level between 0.1-2 µg/100 ml into the samples prior to saponification was 78% (n=19).

Recoveries of vitamin D_3 added prior to saponification ranged from 61% (spiking level 0.01 µg/100 ml) to 86% (spiking level 0.1 µg/100ml) as shown in Table 3-5.

Table 3-5: Vitamin D_3 content (µg/100 ml) and recovery data for milk samples determined by HPLC-MS^n and HPLC-MS/MS

Milk sample	**HPLC-MS/MS**	**HPLC-MS^n**	**% recovery (HPLC–MS^n)**	
Fat content (label)	Sum MRM	$[M+H-C_8H_{14}O]^+$ *m/z* 259	$[M+H-C_8H_{14}O]^+$ *m/z* 259	
			Amount added (µg/100ml)	% rec
0.1 %	<0.02	<0.01	0.01	61
1.0%	<0.02	<0.01	0.02	77
3.8%	0.02	0.02	0.05	72
Fresh cow milk (approx. 4.5% fat)	0.06	0.05	0.1	86

3.3.4.5. Reproducibility

Seven aliquots of a sample of fresh cow milk were assayed to demonstrate method reproducibility (0.02 µg/100 ml, CV = 4.4%).

3.3.4.6. Limit of quantification

The LOQ for vitamin D_3 was based on a S/N ratio of 10:1 and was 0.01 µg/100 g for HPLC-MS^n and 0.02 µg/100 g for HPLC-MS/MS.

25-Hydroxyvitamin D_3 could also be determined by HPLC-MS^n using a separate SPE clean-up of the crude extract. A LOQ of 0.01 µg/100 ml for 25-hydroxyvitamin D_3 in

a sample of fresh cow milk was achieved despite an overall recovery of between 30 and 40%.

25-hydroxyvitamin D_3 was not present at levels greater than LOQ in any of the samples analysed.

3.4. Discussion

For HPLC-MS^n, positive ion APCI was approximately 25 times more sensitive than positive ion ESI, and so APCI was chosen for the ionisation source for the instrument. In contrast, for the triple quadrupole mass spectrometer, ESI was approximately 5 times more sensitive than APCI. This was unexpected, and highlights the difference in relative performance of API sources from different instrument manufacturers such as Thermo Fisher Scientific and Waters.

Despite using compound-specific precursor and fragment ions, the analysis of milk samples still produced numerous interfering endogenous peaks, thus the choice of column and mobile phase was important to achieve chromatographic separation of the analytes from any potential interferences. Of the three columns trialled for this work, the Varian Polaris C18-A column gave the best separation of the analytes from major endogenous interferences, with the optimum elution conditions being an isocratic mobile phase consisting of 92% methanol and 8% water (and containing 5 mM ammonium formate for HPLC-MS/MS).

Initial work using fortified and spiked unfortified milk samples showed that no additional clean-up was required after the initial saponification and extraction when the level of vitamin D_3 in the milk was > 0.1 ug/100 ml. The data presented in Table 3-4 shows that the levels of vitamin D_3 in fortified milks determined by HPLC-MS^n (*m/z* 385.5, 367.4 and 259) and HPLC-MS/MS were nearly identical, and comparable with the levels stated on the label.

When the HPLC-MS^n methodology was applied to a selection of non-fortified commercial milk samples with differing fat contents as well as samples of fresh cow milk, only the *m/z* 259 ion could be used for quantification, as co-eluting compounds interfered with the *m/z* 385.5 and *m/z* 367.4 ions. The quantitative data from the HPLC-MS/MS instrument were also inconsistent. However, silica SPE clean-up of the extract produced a suitable extract for HPLC-MS/MS analysis. The HPLC-MS^n

m/z 259 chromatogram was also cleaner after SPE treatment, however, SPE clean-up failed to completely remove the interfering compounds in the HPLC-MS^n chromatograms for the *m/z* 385.5 and *m/z* 367.4 ions. The levels of vitamin D_3 in the samples determined by HPLC-MS^n (*m/z* 259) and by HPLC-MS/MS after SPE were similar and were also similar to the levels present in milk samples reported in the literature (Jakobsen and Saxholt 2009; Kurmann and Indyk 1994).

After the completion of the experimental part of this project, Huang*, et al.* (2014) reported a systematic error caused by the use of 6,19,19 $^2H_3$ labelled vitamin D_3, which is the same deuterated internal standard as was used for this project. The error relates to the conversion of vitamin D_3 to pre-vitamin D_3, a process which occurs more rapidly at the elevated temperatures used for hot saponification. The positioning of the deuterium atoms in the molecule results in an increased rate of conversion of $^2H_3$-vitamin D_3 to $^2H_3$-pre-vitamin D_3, resulting in a decrease in the peak area of the internal standard relative to the peak area of the analyte, causing a higher calculated result. The work of Huang*, et al.* (2014) involved the addition of the internal standard prior to saponification, whereas for this project, the internal standard was added immediately before mass spectrometric analysis. For this reason, the internal standard would not have converted to the pre-vitamin but the analyte would have, and this may explain the slightly lower result obtained for the proficiency samples.

Silica SPE was chosen for sample clean-up since the retention mechanism was orthogonal, that is, unrelated to the hydrophobic mechanism utilised in the reversed-phase analytical chromatography (Pellett, Lukulay, Mao, Bowen, Reed, Ma*, et al.* 2006). Therefore, it was considered more likely to remove the interfering compounds than a reversed-phase SPE cartridge. Further adjustment of the wash and elution parameters or use of an alternative phase may have achieved a cleaner extract, however time constraints prevented further exploration of the SPE clean-up procedure.

Anecdotal evidence within our laboratory has shown that when analysing antioxidant compounds such as vitamins by HPLC, problems can arise which affect the overall quality of the analytical data, such as non-linear calibrations and unstable peak areas throughout the batch of samples. It is thought that these problems are due to areas of oxidation within the HPLC fluidics which oxidise the analytes, resulting in a loss of analytical signal. To reduce, or 'pacify', these areas of oxidation and minimise their

potential effects on the analysis, six 100µl injections of the highest analytical standard were made at the beginning of each analytical batch of samples run on each of the HPLC systems used for this project. This was then followed by an injection of methanol, prior to injecting the series of calibration standards and samples.

For both HPLC-MS instruments, a number of injections of sample extract were also required to achieve a stable signal, particularly after cleaning the API interface. This effect has been observed previously with nearly all HPLC-MS assays performed in this laboratory, and it is assumed this phenomenon is due to an equilibration of the API interface as it becomes coated in compounds from the sample matrix. Accordingly, six injections of sample extract pacified the HPLC fluidics as well as equilibrating the API interface, ensuring a stable and reproducible signal prior to analysis of the analytical standards and test samples.

The lower recoveries of 25-hydroxyvitamin D_3 in comparison to vitamin D_3 were largely due to the more polar nature of the metabolite. Improved recoveries of the metabolite could have been achieved by extracting the samples with a more polar solvent such as a hexane:ethyl acetate mixture, as detailed by Kamao, Tsugawa, Suhara, Wada*, et al.* (2007), however this was not pursued as the main focus of this work was vitamin D_3.

Limitations of the method include the presence of isobaric interferences in chromatograms, which at present limit the LOQ of the method for the analysis of endogenous levels of vitamin D_3 and 25-hydroxyvitamin D_3 in milk samples. Sample throughput is limited due to the use of liquid-liquid extraction in separating funnels and rotary evaporation of samples. Quicker alternatives may be needed for usage of the method by a commercial laboratory.

Measurement uncertainty would need to be calculated for full validation of the method, however at present there is insufficient data to accurately characterise the method. The time and expense of creating the validation data was not warranted since the method was not used for analysing samples from the feeding study.

3.5. Conclusion

A straight forward, robust method for determining the levels of vitamin D_3 in a range of commercially available fortified and non-fortified cow milk, infant formula and fresh cow milk using HPLC-MS^n has been developed. The use of HPLC-MS^n was validated against an HPLC-MS/MS instrument and found to produce comparable results. Recoveries of vitamin D_3 added to the samples prior to saponification were satisfactory (60-90%). The LOQ were 0.01 µg/100 g and 0.02 µg/100 g for HPLC-MS^n and HPLC-MS/MS respectively. 25-hydroxyvitamin D_3 could also be determined in fresh milk samples using this procedure, although the extraction conditions were not fully optimized for this compound and it gave lower recoveries.

Chapter 4: Vitamins A, E and β-carotene analytical method

4.1. Introduction

Traditionally, fat-soluble vitamins are analysed separately by either HPLC-UV/Vis (vitamin A and carotenes) or HPLC with Fl detection (vitamins A and E). HPLC-MS^n, due to the greater selectivity of the MS analyser, provides an opportunity to assay these compounds in a single run, thus facilitating a faster sample turnaround for large batches of samples that are often collected for research studies. HPLC-MS^n also has the potential to provide mass and structural data on other compounds present in the extracts which can be useful for metabolomics studies.

All *trans*-retinol and α–tocopherol are the major A and E vitamers present in milk (Lanina*, et al.* 2007), and so to reduce complexity, only the levels of these two compounds were measured in this study. A number of positional isomers of retinol and α-tocopherol do exist, however these compounds are not resolved by the reversed-phase chromatographic systems used for this work. β-carotene, on the other hand, exists as a number of isomers that can be separated to some extent by reversed-phase chromatography. The levels of the major isomer, all *trans*-β-carotene, were determined in this work as it is the most abundant carotenoid present in milk (Havemose*, et al.* 2004; Hulshof*, et al.* 2006). The HPLC-MS^n method was based on a fully validated 'in-house' HPLC-UV/Vis and HPLC-Fl method for the analysis of fat-soluble vitamins in foods (Department of Primary Industries 2007). This HPLC-MS^n method was used for the analysis of 72 milk samples collected from the feeding experiment conducted at DPI Ellinbank, Victoria.

In contrast to vitamin D_3, these compounds occur naturally at much higher levels in milk, and therefore no additional clean-up of the sample was expected after the initial saponification and extraction into hexane.

This work has been published as 'The simultaneous determination of vitamins A, E and β-carotene in bovine milk by high performance liquid chromatography-ion trap mass spectrometry (HPLC-MS^n)' in *Food Chemistry* (Plozza, Trenerry and Caridi 2012). The development of the method is described below and used the same protocol as for vitamin D_3.

4.2. Method development

The HPLC-MS^n method was developed in the following order:

1. The mass spectrometer conditions were optimised (API interface and fragmentation patterns).

2. Suitable chromatography conditions were developed.

3. The sample extraction method was developed, taking into account the concentrations of the analytes, the LOQ of the instruments, and the observation that the addition of BHT to the extraction solvent improved the performance of the method for the extraction of vitamin D_3, see section 3.2.3.1.

4. Method validation.

4.2.1. Mass spectrometer

4.2.1.1. Atmospheric pressure ionisation interface

Optimisation was performed while infusing an approximately 10 µg/ml solution of each standard (all *trans*-retinol, α-tocopherol and β-carotene) at 10 µl/min into the API interface via a T-piece where it was mixed with HPLC mobile phase (methanol:water 98:2) at a flow rate of 0.2 µl/min. This mixture was chosen as it gave the best ionisation for vitamin D_3 in the previous study, see section 3.3.1.1.1.

Infusing the standard solutions into the API interface via the T-piece enabled optimisation at the flow rate to be used for the analysis, and also facilitated easy modification of mobile phase components when assessing different solvents or mobile phase modifiers.

4.2.1.1.1. Atmospheric pressure chemical ionisation

All user-adjustable parameters (positive/negative ionisation mode, discharge current, vaporizer temperature, sheath, auxiliary and sweep gas flow rates, capillary temperature and probe fore/aft position and insertion depth) were optimised independently.

4.2.1.1.2. Ion trap fill time

The HPLC-MS^n ion trap fill time was set at the maximum of 200 ms for all experiments.

4.2.1.1.3. Ion optics voltages

The voltages of the various components of the ion optics, which are located after the API interface (i.e. inside the mass spectrometer), were optimised using the instrument's auto-tune function.

4.2.1.2. Ion trap fragmentations

The fragmentation conditions for each analyte were optimised whilst infusing a solution of each standard into the interface. The precursor ion (molecular ion) was selected for each compound and a collision energy applied to cause fragmentation. The collision energy was adjusted to maximise the abundance of the product ions. The two most abundant ions were chosen for the MS^2 protocol used for the analytical method. The isolation width was optimised according to the instrument manufacturer's instructions.

Optimisation of the mass spectrometer showed that retinol could only be ionised in the positive mode, whereas positive or negative ionisation modes gave suitable sensitivity for the analysis of α–tocopherol and all *trans*-β-carotene. A milk extract was analysed eight times by HPLC-MS^n, HPLC-UV/Vis and HPLC-Fl, and the concentrations and %CV for each analyte were calculated to determine the optimum ionisation mode, which product ions gave better results for this analysis, and to compare the data from the MS^n and UV/Vis and Fl detection.

4.2.2. High performance liquid chromatography

Chromatography conditions were developed using the column and mobile phase components identified in the vitamin D_3 study, as these gave a suitable separation of all *trans*-retinol, α-tocopherol and β-carotene and allowed the two methods to be run sequentially if necessary.

4.2.2.1. Column

The Varian Polaris C18-A, 5µm, 150 × 2.1 mm HPLC column used in the vitamin D_3 work was chosen for this analysis.

4.2.2.2. Mobile phase

Isocratic elution using methanol:water (98:2 v/v), and a gradient elution starting at methanol:water (95:5 v/v) and increasing to methanol (100%) were trialled to optimise the separation of the three analytes in standard solutions and a variety of purchased milk sample extracts.

4.2.2.3. Flow rate and column temperature

A mobile phase flow rate of 0.2 ml/min and column temperature of 30 ºC were used for the reasons outlined in 3.2.2.

4.2.3. Sample extraction

The extraction conditions were identical to the 'in-house' HPLC-UV/Vis and HPLC-Fl method, except for the addition of 15 mg/l of the antioxidant BHT to the hexane extractant as described for the extraction of vitamin D_3 from milk. Briefly, 10 ml of milk was mixed with 0.5 g of ascorbic acid, 40 ml of ethanol and 10 ml of 1:1 potassium hydroxide in water and heated at reflux with stirring for 30 minutes. The mixture was cooled in an ice bath, mixed with 50 ml water, 10 ml ethanol and the vitamins quantitatively extracted into hexane containing BHT. The hexane extracts were washed with water and then made to volume (100 ml) with hexane containing BHT. A 10 ml aliquot of the hexane solution was then transferred to a glass tube and the solvent removed under a flow of nitrogen. The residue was reconstituted with 1 ml methanol, sonicated and finally filtered through 0.45 µm PTFE filter disc prior to analysis.

The HPLC–MS^2 chromatogram of a cow milk sample containing all *trans*-retinol (50 µg/100 ml), α-tocopherol (200 µg/100 ml) and β-carotene (12 µg/100 ml) using the gradient conditions described in section 4.3.2.2.1 is shown in Figure 4.1.

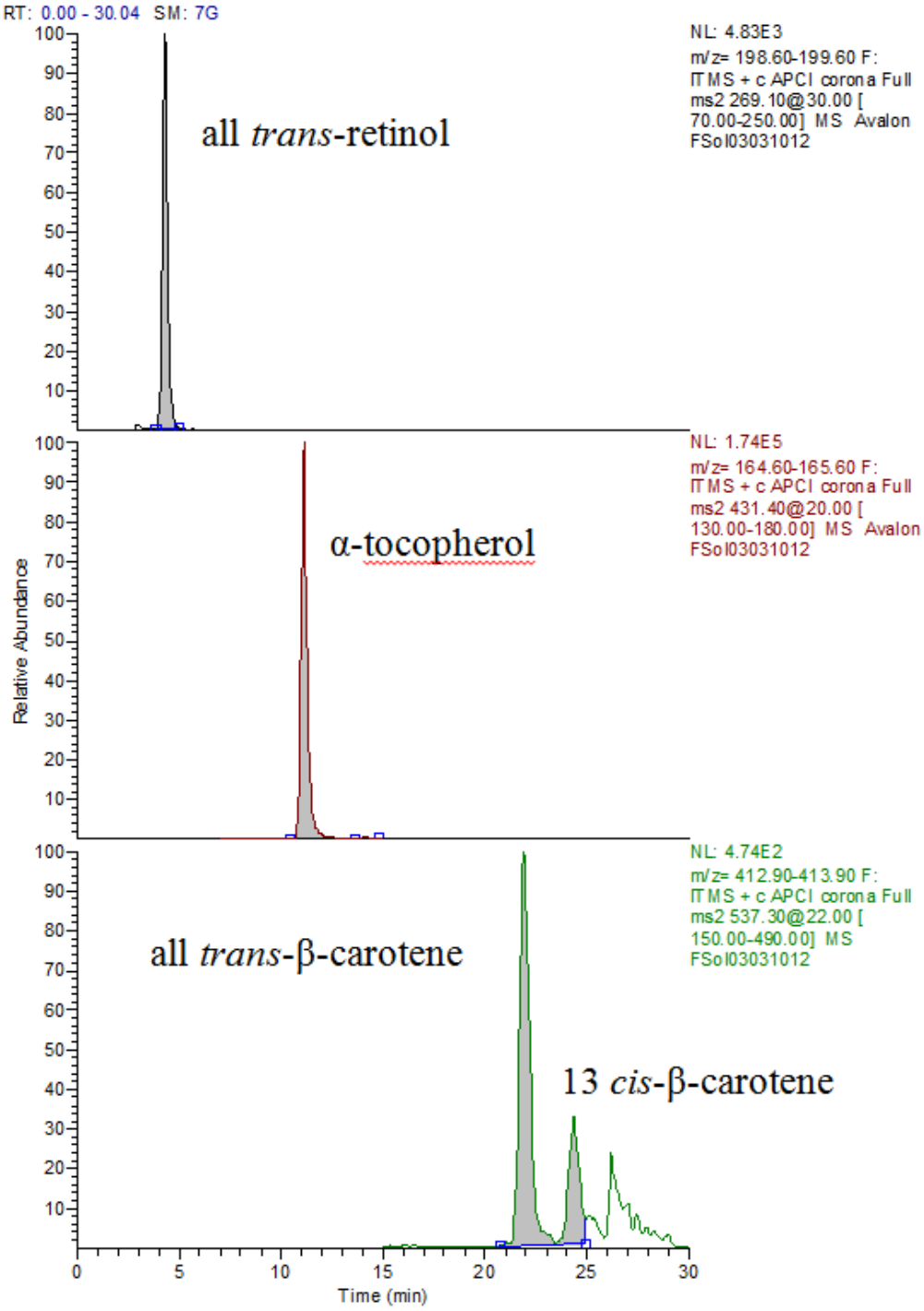


Figure 4-1: HPLC-MS^2 chromatogram of a cow milk sample containing all *trans*-retinol (50 µg/100 ml), α-tocopherol (200 µg/100 ml) and β-carotene (12 µg/100 ml)

4.2.4. Analytical method

4.2.4.1. Validation

The method was validated according to NATA Technical Note 17 (2013) by analysing both unspiked and spiked samples.

Extracts were assayed concurrently on HPLC-MS^n, HPLC-UV/Vis and HPLC-Fl (all *trans*-retinol and α-tocopherol) and HPLC-UV/Vis (β-carotene) so that the performance of the HPLC-MS^n method could be compared with fully validated methods commonly used for this analysis.

A fortified infant formula supplied by Proficiency Testing Australia as part of a proficiency study (Bunt 2010) was used to further validate the method. For quality control purposes, the infant formula reference material was used as an 'in-house' reference material and assayed for all *trans*-retinol and α-tocopherol in every batch of samples.

4.2.4.2. Measurement uncertainty

'In-house' validation data were used to estimate the measurement uncertainty for each analyte at three different levels according to the method of Plozza*, et al.* (2011). The major sources of uncertainty were homogeneity, method recovery and the analytical standard calibration curves. Homogeneity uncertainty was estimated from the relative standard deviation of duplicate analyses run in nine batches of samples. The uncertainty due to the purity of the analytical standards was shown to be insignificant and not included.

4.3. Results

4.3.1. Mass spectrometer

4.3.1.1. Atmospheric pressure chemical ionisation

Methanol and water were used as mobile phase components as these gave the best ionisation efficiency for vitamin D_3 analysis.

The optimal ionisation efficiency was achieved with the following APCI settings:

Capillary temperature	200°C	
Vaporizer temperature	250°C	
Sheath gas flow rate	50 arbitrary units	
Auxiliary gas flow rate	5 arbitrary units	
Sweep gas flow rate	0 arbitrary units	
	Positive polarity	Negative polarity
Source current	5 µA	60 µA
Capillary voltage	12 V	-14 V

4.3.1.2. Ion trap fragmentations

The molecular ion of each analyte was selected and then the fragmentation conditions (collision energy and isolation width) were adjusted to provide the optimum sensitivity. Other settings were left as the default values. The results are shown in Table 4.1, and an example of the spectra obtained for all *trans*-retinol is shown in Figure 4.2.

Table 4-1: Molecular ion, two major fragment ions and optimised collision energy and isolation width for all *trans*-retinol, α-tocopherol and β-carotene

Compound	**Ionisation mode**	**MS^1 Molecular ion (*m/z*)**	**MS^2 Fragment ions (*m/z*)**	**Collision energy (%)**	**Isolation width**
all *trans*-retinol	positive	269.1 $[M+H-H_2O]^+$	213.1, 199.1	30	1.4
α-tocopherol	positive	431.4 $[M+H]^+$	165.1*	20	1.5
	negative	429.4 $[M-H]^-$	414.5, 163.1	24	1.0
β-carotene	positive	537.3 $[M+H]^+$	413, 399	22	2.0
	negative	536.5 $[M+e^-]^-$	444.5, 243.3	27	1.0

* Fragmentation of positively ionised α-tocopherol produced only one fragment ion.

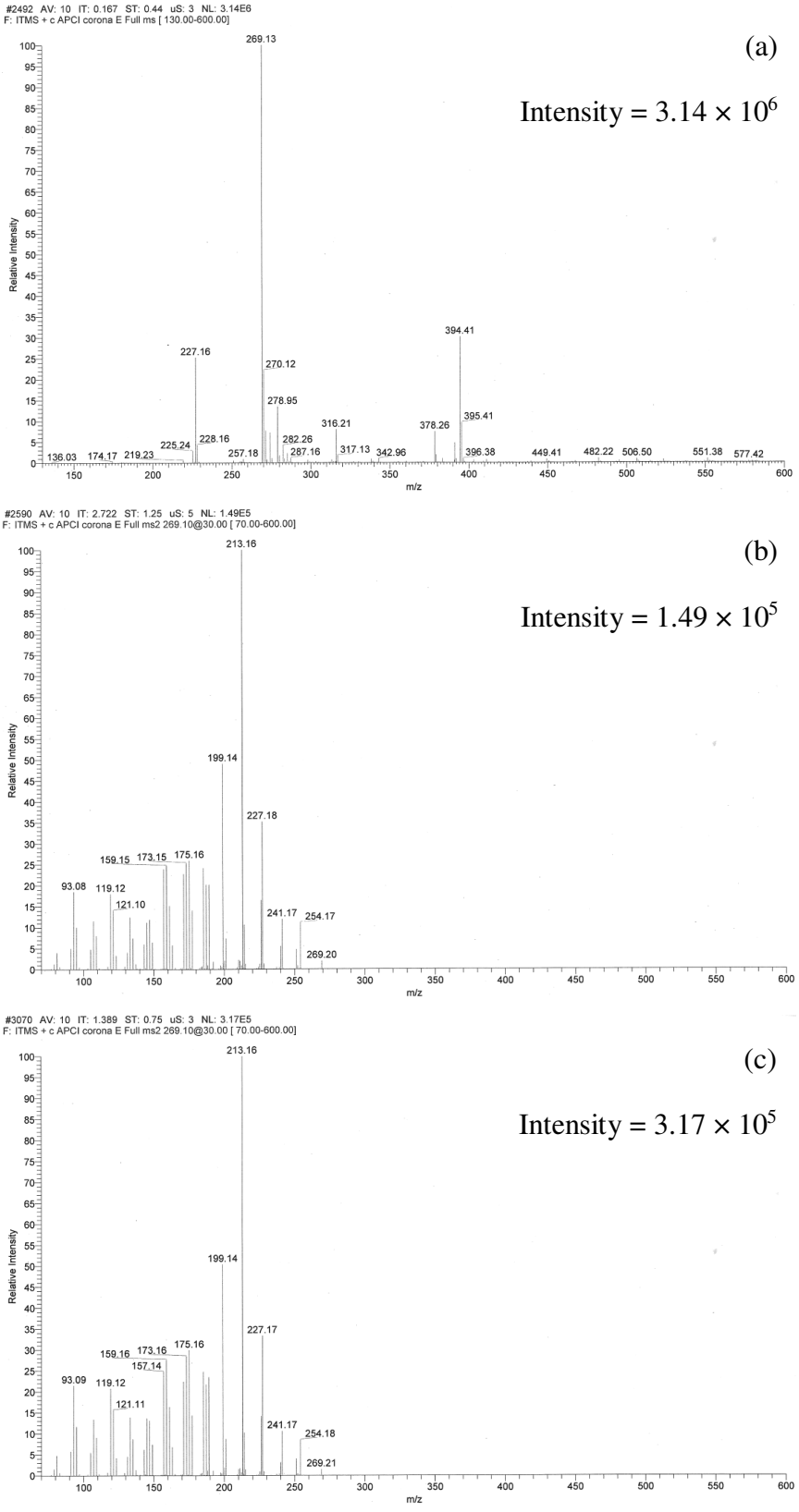


Figure 4-2: Mass spectra of all *trans*-retinol infused into the ion trap MS operating in APCI positive mode, (a) MS^1, (b) MS^2 with isolation width of 1.0, (c) MS^2 with isolation width of 1.4

The parent and the two most intense fragment ions for each compound, along with the %CV of the concentration of each analyte determined from eight successive analyses of a milk extract are shown in Table 4.2.

Table 4-2: HPLC-UV/Vis, HPLC-Fl and HPLC-MS^2 analyte concentrations and coefficient of variation (%) from the repeat analysis of a milk extract (n=8) containing all *trans*-retinol, α-tocopherol and β-carotene

	Concentration µg/100ml				**Coefficient of variation (%)**			
	HPLC-UV/Vis	**HPLC-Fl**	**HPLC-MS^2**		**HPLC-UV/Vis**	**HPLC-Fl**	**HPLC-MS^2**	
			positive	**negative**			**positive**	**negative**
all *trans*-retinol	39.6		*m/z* 213 *m/z* 199 40.9 40.9		1.4		*m/z* 213 *m/z* 199 1.2 2.2	
α-tocopherol		104.5	*m/z* 165* 100.6	*m/z* 163 *m/z* 414 102.3 103.1		1.1	*m/z* 165* 1.2	*m/z* 163 *m/z* 414 3.6 3.6
β-carotene	13.8		*m/z* 413 *m/z* 399 15.0 15.2	*m/z* 243 20.3	2.8		*m/z* 413 *m/z* 399 2.4 4.0	*m/z* 243 22.5

* Fragmentation of positively ionised α-tocopherol produced only one fragment ion.

4.3.2. High performance liquid chromatography

4.3.2.1. Column

The Varian Polaris C18-A, 5 μm, 150 × 2.1 mm HPLC column used in the vitamin D_3 work was chosen for this analysis.

4.3.2.2. Mobile phase

4.3.2.2.1. Isocratic conditions

All *trans*-retinol eluted with a retention time of 3.5 min, α-tocopherol at 7.8 min and β-carotene at 25.8 min using an isocratic mobile phase containing methanol:water (98:2 v/v). A run time of 35-40 min would have been required to ensure complete elution of *cis*-β-carotene isomers and any other late-eluting compounds, although this was not determined experimentally due to time constraints.

4.3.2.2.2. Gradient conditions

A gradient elution, shown in Table 4.3, gave retention times of 4.4 min for all *trans*-retinol, 11.7 min for α-tocopherol and 22 min for β-carotene, with a total run time, including re-equilibration, of 30 min, which was suitable for this work.

Table 4-3: Gradient used for elution of all *trans*-retinol, α-tocopherol and β-carotene using a Varian Polaris C18-A, 5 μm, 150 × 2.1 mm column

Time (min)	**CH_3OH %**	**H_2O %**	**Flow rate (ml/min)**
0	95	5	0.2
5	95	5	0.2
10	100	0	0.2
25	100	0	0.2
26	95	5	0.4

4.3.3. Sample extraction

Samples were extracted as described in the DPI fat-soluble vitamins method (Department of Primary Industries 2007), with some modifications:

1. The effect of the addition of BHT to the hexane extractant was assessed using 'side by side' extractions of aliquots of a purchased milk sample. Extractions were performed in duplicate using hexane containing 15 mg BHT per litre, as was used for the vitamin D_3 extraction. The dried hexane extract was reconstituted with methanol and analysed by HPLC-UV/Vis and HPLC-Fl. The results, shown in Table 4.4, show that the addition of BHT to the extraction solvent resulted in an increase in the concentration of all three analytes.

Table 4-4: Concentrations of all *trans*-retinol, α-tocopherol and β-carotene in a purchased milk sample extracted using hexane or hexane containing 15 mg/l BHT

Analyte (µg/100 ml)	**Hexane**	**Hexane + BHT**
all *trans*-retinol	42.8	46.1
α-tocopherol	198	211
β-carotene	26.8	28.0

2. In the DPI method, two different solvents were used for reconstituting the dried sample extract: methanol for the determination of all *trans*-retinol and α-tocopherol, and a 50/50 (v/v) mixture of ethanol and ethyl acetate containing 30 mg/l BHT for the determination of β-carotene. As a single extract was to be prepared from each milk sample and run on HPLC-UV/Vis, HPLC-Fl and HPLC-MS^n for comparative purposes, a comparison of these two solvents was necessary to decide which gave better recoveries and was more reproducible for the three analytes.

Duplicate aliquots of a hexane extract (not containing BHT) were dried then reconstituted with one of three solvents (methanol, methanol plus 30 mg/l BHT, 50:50 ethanol:ethyl acetate plus 30 mg/l BHT) and analysed by HPLC-UV/Vis and HPLC-Fl. The results are shown in Table 4.5 and show that all three solvents produced very

similar results, and all produced a lower result than the same sample extracted with hexane containing BHT, shown in Table 4.4.

Table 4-5: Comparison of methanol, methanol containing 30 mg/l BHT, and 50:50 (v/v) ethanol:ethyl acetate containing 30 mg/l BHT for the reconstitution of dried hexane extracts

Analyte (µg/100 ml)	**Methanol**	**Methanol:BHT**	**Ethanol:ethyl acetate:BHT**
all *trans*-retinol	42.8	42.4	42.5
α-tocopherol	198	195	206
β-carotene	26.8	25.5	26.9

3. Assessment of the concentrations of the analytes in test samples analysed by HPLC-UV/Vis, HPLC-Fl and HPLC-MS^2 allowed for the determination of an aliquot volume (10ml) of the hexane extract, and reconstitution volume with methanol (1ml) which ensured all analytes for all samples fell within the range of the standards, and were easily detectable on all instruments.

For the fortified infant formula, which was run as a control sample with every batch of samples, 10ml of hexane extract was dried and the extract reconstituted with 2 ml of methanol.

4.3.4. Method validation

4.3.4.1. Linearity

The HPLC-MS^2, HPLC-UV/Vis and HPLC-Fl calibration plots for (a) all *trans*-retinol (λ 320 nm, *m/z* 199), (b) α-tocopherol (Ex 295 nm, Em 330 nm, *m/z* 165) and (c) β-carotene (λ 450 nm, *m/z* 413) are displayed in Figure 4.3. Excellent linearity was achieved for all analytes on all instruments.

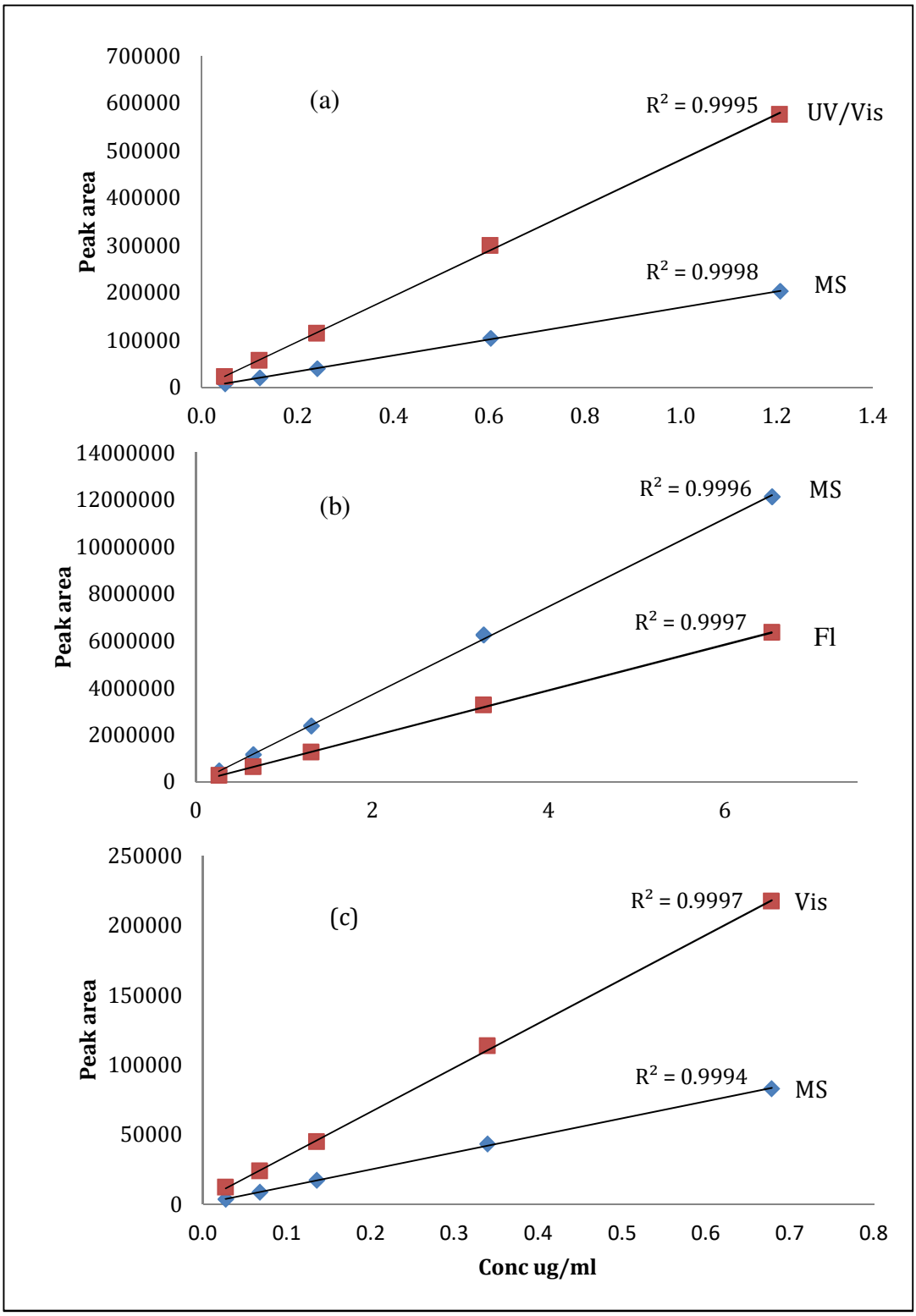


Figure 4-3. HPLC-MS^2, HPLC-UV/Vis and HPLC-Fl calibration plots for (a) all *trans*-retinol, (b) α-tocopherol and (c) β-carotene

4.3.4.2. Vitamin levels in fresh milk samples

The 72 fresh milk samples sourced from the DPI Ellinbank feeding experiment were assayed using HPLC-MS^n and HPLC-UV/Vis and HPLC-Fl detection. The levels of all *trans*-retinol, α-tocopherol and β-carotene determined by HPLC-MS^2 ranged from 24.0-66.3 μg/100 ml, 124-220 μg/100 ml, and 6.7-28.3 μg/100 ml respectively. The complete data set is shown in Appendix 2.

The correlation of results (n=72) obtained from the different instruments is shown in Table 4.6, and has been calculated as the MS^2 result divided by the UV/Vis or Fl result.

Table 4-6: Correlation of results (n=72) for all *trans*-retinol, α-tocopherol and β-carotene obtained from HPLC-MS^n, HPLC-UV/Vis and HPLC-Fl instruments

Analyte	**HPLC-MS^n ÷ (HPLC-UV/Vis or HPLC-Fl)**	**%CV**
all *trans*-retinol	1.09	2.6
α-tocopherol	1.06	3.5
β-carotene	1.10	8

4.3.4.3. Proficiency sample data

The proficiency sample was used as an 'in-house' control sample, and analysed once with each batch of feeding experiment samples analysed (n=9). The range of concentrations determined by HPLC-MS^n and those from the proficiency study (Bunt 2010) are displayed in Table 4.7. The levels of all *trans*-retinol and α-tocopherol determined by HPLC-MS^n were lower than, and within the range of, the results reported in the proficiency study.

The reproducibility of the method, as indicated by the %CV for the analysis of the sample on 9 separate occasions, was very good.

Table 4-7: The ranges of all *trans*-retinol and α-tocopherol determined by HPLC-MS^n for the proficiency sample, compared with the levels reported in the proficiency study

Analyte	**This study**		**Proficiency study**	
	Range (mg/100 g)	**%CV**	**Range (mg/100 g)**	**%CV**
all *trans*-retinol	0.61-0.74	7.1, n=9	0.78-1.2	19.7, n=7
α-tocopherol	9.5-11.8	6.2, n=9	10.5-14.3	8.9, n=6

4.3.4.4. Recovery

Recoveries of spiked milk samples from eight separate analytical batches assayed by HPLC-MS^n are summarised in Table 4.8.

Table 4-8: Spiking level, recovery data and %CV for spiked milk samples analysed in eight separate batches of samples

Analyte	**Spiking level µg/100 ml**	**Recovery % (range and mean)**	**%CV**
all *trans*-retinol	29	91-114; 101	7.6
α-tocopherol	105	91-115; 100	8.3
β-carotene	17	83-112; 93	12.4

4.3.4.5. Instrument repeatability

Instrument repeatability, expressed as the %CV of the peak area, was determined by repetitive injection of standard and sample solutions on HPLC-MS^n, HPLC-UV/Vis and HPLC-Fl. The data are presented in Table 4.9 and show the similar instrument repeatability of HPLC-MS^n in comparison to HPLC-UV/Vis and HPLC-Fl.

Table 4-9: %CV of the peak areas of all *trans*-retinol, α-tocopherol and β-carotene determined from the repetitive injection of standard and sample solutions. Eight repeated injections were used, except where specified.

Analyte	**HPLC-Fl %CV**		**HPLC-UV/Vis %CV**		**HPLC-MS^n %CV**			
	Std	Sample	Std	Sample	Std (n=7)		Sample	
all *trans*-retinol			1.0	0.4	*m/z* 213 1.1	*m/z* 199 1.0	*m/z* 213 0.7	*m/z* 199 0.8
α-tocopherol	0.5	0.4			*m/z* 165 1.6		*m/z* 165 1.0	
β-carotene			1.9	3.4	*m/z* 413 2.3		*m/z* 413 3.1	

4.3.4.6. Method repeatability

Method repeatability was determined by assaying 8 aliquots of a sample of commercial full cream milk. The data are presented in Table 4.10 and show that the method repeatability is very good, and the results produced from HPLC-MS^n are comparable to those obtained from HPLC-UV/Vis and HPLC-Fl. Comparison of the data in Tables 4.9 and 4.10 shows that the variation produced from the analysis of eight aliquots of a milk sample is very similar to that obtained from the repeat injection of a single sample extract, indicating that the instrument is the source of most of the analytical variation.

Table 4-10: %CV of the concentrations of all *trans*-retinol, α-tocopherol and β-carotene determined from the analysis of 8 aliquots of a sample of commercial full cream milk

Analyte	**HPLC-Fl %CV**	**HPLC-UV/Vis %CV**	**HPLC-MS^2 %CV**	
all *trans*-retinol		1.4	*m/z* 213 1.2	*m/z* 199 2.2
α-tocopherol	1.1		*m/z* 165 1.2	
β-carotene		2.8	*m/z* 413 2.4	

4.3.4.7. Limit of quantification

The LOQs for HPLC-MS^n were determined at a S/N ratio of 10:1 from standard solutions of each compound and were equivalent to 0.1 µg/100 ml for all *trans*-retinol and α-tocopherol and 1 µg/100 ml for β-carotene in the actual milk samples.

4.3.4.8. Measurement uncertainty

The expanded measurement uncertainties for all *trans*-retinol, α-tocopherol and β-carotene at levels covering the range found in the milk samples collected from the DPI feeding experiment are shown in Table 4.11. The expanded measurement uncertainties for HPLC-MS^n were comparable with those determined for HPLC-UV/Vis and HPLC-Fl and were suitable for this work. A coverage factor of 2 was used, for 95% confidence.

Table 4-11: The expanded measurement uncertainties for all *trans*-retinol, α-tocopherol and β-carotene at three different levels.

Analyte	**µg/100ml**	**HPLC-MS^n**	**HPLC-UV/Vis**	**HPLC-Fl**
all *trans*-retinol	27	10%	12%	
	45	8%	9%	
	68	8%	7%	
α-tocopherol	110	13%		12%
	150	11%		9%
	205	9%		8%
β-carotene	7	27%	29%	
	12	18%	21%	
	23	14%	19%	

4.3.5. Unknown compound

A large unknown peak was present in the MS^1 total ion chromatogram of the milk samples, and is shown in Figure 4.4 (a). This peak had an intensity approximately two orders of magnitude greater than the MS^1 peak of α-tocopherol at *m/z* 431.4. The mass spectrum of the unknown peak showed the major ion to be *m/z* 369.35, as shown in Figure 4.4 (b).

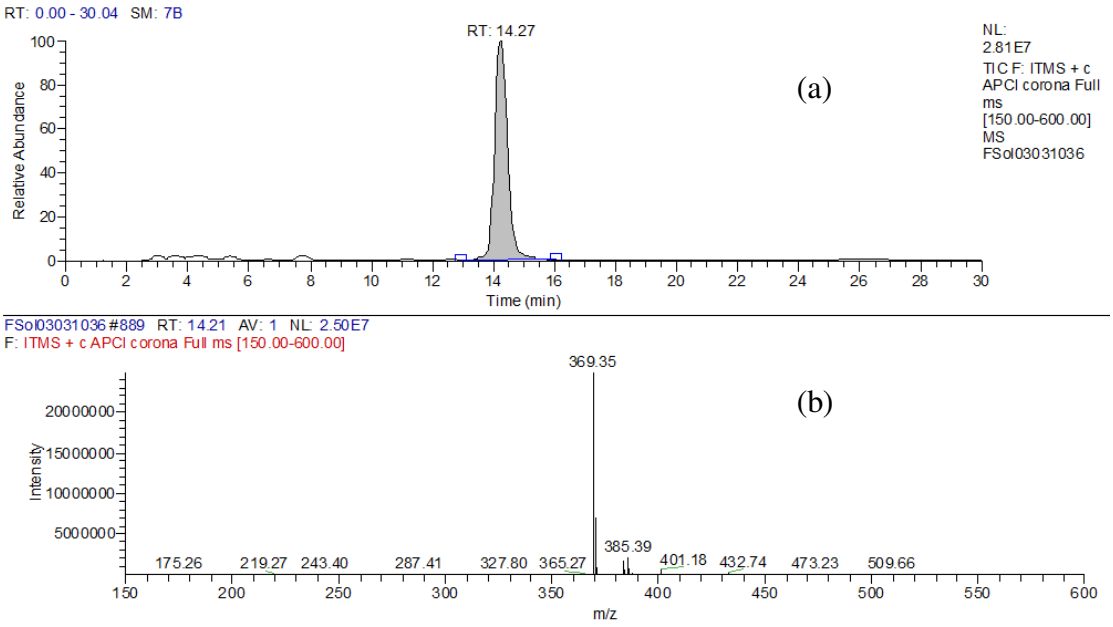


Figure 4-4. Unknown compound in the milk samples (a) MS^1 total ion chromatogram of a milk sample, and (b) MS^1 mass spectrum of the peak at 14.27 min

4.4. Discussion

All *trans*-retinol could only be ionised by positive ionisation mode APCI, whereas both α-tocopherol and β-carotene could be ionised by either positive or negative mode APCI. Although negative ionisation gave larger peak areas for α-tocopherol, positive ionisation gave a similar signal to noise ratio and a lower %CV for the analysis. For β-carotene, positive ionisation gave both increased signal and a much lower %CV for the analysis. For these reasons, positive ionisation was chosen for all analytes in this study. Both MS^1 and MS^2 data were acquired for all samples, however MS^2 was used for calculating the analytical results since it gives greater selectivity than MS^1 (Willoughby 2002), and therefore a lower risk of interference from other species.

The levels determined by HPLC-MS^2 for all *trans*-retinol were measured at both *m/z* 213 and *m/z* 199 and were similar, indicating that either ion could be used for quantification. For β-carotene analysis, although both MS^2 ions gave similar results, the ion with *m/z* 413 gave a lower %CV, and was therefore used for quantification.

The structure of the compound eluting at 24.5 min in Figure 4-1 was assigned as 13 *cis*-β-carotene based on the mass spectral data ($[M+H]^+$ *m/z* 537) and comparison of the UV/Vis spectra with those of Nyambaka and Ryley (1996) as shown in Figure 4-5. The concentration of this compound was estimated to be approximately 20% of the

concentration of all *trans*-β-carotene in the milk samples, based on HPLC-UV/Vis data. However, to minimise the complexity of the analysis, only the all *trans*-β-carotene isomer was quantified for this study.

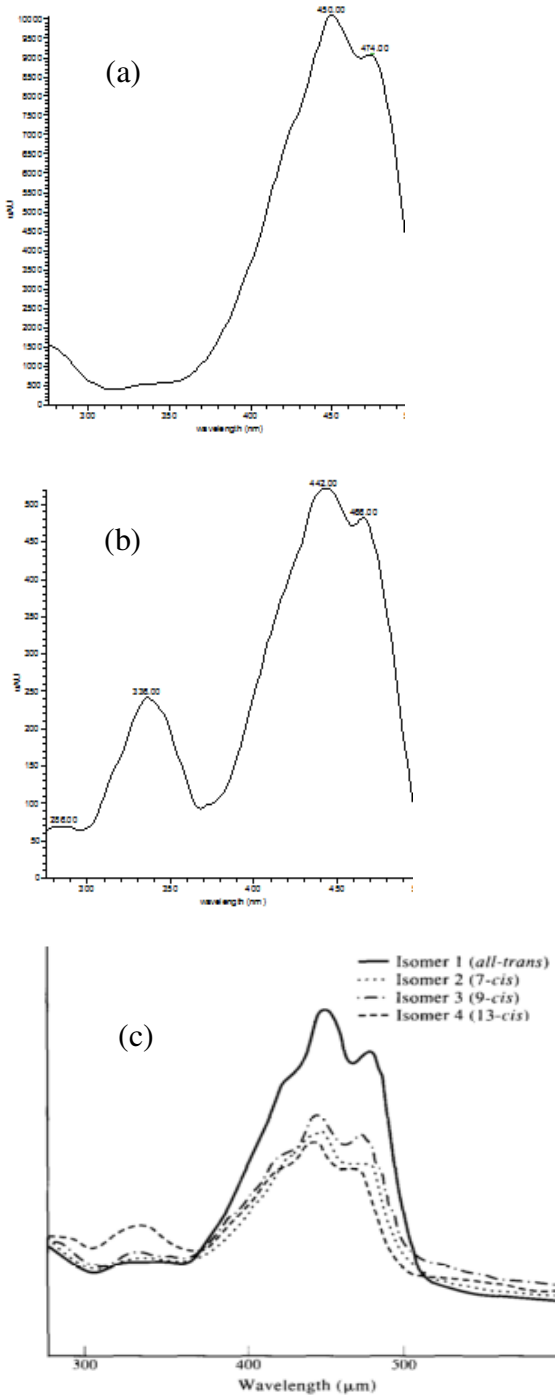


Figure 4-5: UV/Vis spectra of (a) all trans-β-carotene peak, (b) 13 cis-β-carotene peak and (c) all trans, 7 cis, 9 cis and 13 cis-β-carotene isomers

Gradient elution of the mobile phase was preferred over isocratic elution of the mobile phase as the lower concentration of methanol at the commencement of the analytical run (0 min) gave a slightly longer retention time for all *trans*-retinol, potentially giving a better separation from any early eluting compounds that might facilitate suppression or enhancement of the ionisation of the molecular ion. The higher concentration of methanol later in the gradient reduced the retention time of β-carotene and enabled a significantly shorter chromatographic run time for the gradient elution compared to the isocratic elution.

The addition of BHT to the hexane used to extract the analytes from the saponified milk resulted in an increase in the concentration of the analytes. BHT is a synthetic antioxidant and presumably afforded some protection of the analytes from oxidation due to oxygen exposure either during the liquid/liquid extraction step or the drying step of the sample preparation.

An internal standard was not used for this work as the expected levels of all *trans*-retinol, α–tocopherol and all *trans*-β-carotene in milk (40 µg/ml, 200 µg/ml and 30 µg/ml respectively) were sufficiently high enough to make any irregularities in the ion source of the mass spectrometer of minor significance. The HPLC-MS^n instrument repeatability for each compound was similar to those for the traditional HPLC-UV/Vis (all *trans*-retinol and β-carotene) and HPLC-Fl (α-tocopherol) instruments, and excellent correlation was achieved between the levels of the analytes in milk determined by HPLC-MS^n and the levels determined by validated HPLC-UV/Vis and HPLC-Fl methods. This indicates that the extra expense of isotopically labelled internal standards may not be warranted for this analysis.

The levels of all *trans*-retinol and α-tocopherol in the Proficiency Testing Australia sample determined by the HPLC-MS^n method were slightly lower than those obtained from participants in the proficiency study, however the data were useful as a control sample to indicate batch-to-batch method performance. Degradation of the proficiency sample due to aging is possible, but is not expected to have contributed significantly to the lower results, as the sample was only 2-3 months old at the time this analysis was performed, and the results appeared to be stable during the time it was in use.

The large unknown peak in the milk extract chromatogram shown in Figure 4.4 had a MS^1 *m/z* of 369.35, corresponding to cholesterol, which forms the ion $[M+H-H_2O]^+$ when ionised by APCI in the positive ionisation mode (Raith, Brenner, Farwanah, Müller, Eder and Neubert 2005). Milk contains approximately 14 mg/100 g cholesterol (Belitz and Grosch 1999), a fat-soluble compound which is expected to extract from milk along with the fat-soluble vitamins. No further action was taken to positively identify the peak. If this peak was found to be cholesterol, the concentration could be determined from the MS^1 data by comparison to the peak area of a cholesterol standard solution or, for better confidence in the results, a MS^2 protocol for cholesterol could be added to the existing mass spectrometer method and the samples re-analysed along with a series of cholesterol standards.

The main limitation to the usage of this method is the much greater cost of the mass spectrometer compared to UV/Vis and fluorescence detectors, as well as the greater maintenance requirements of the mass spectrometer. Depending on the requirements for the analytical data, these extra expenses may be prohibitive to its adoption as a routine detector for this analysis.

4.5. Conclusion

A straight forward, robust method for determining the levels of all *trans*-retinol, α-tocopherol and β-carotene in cow milk using HPLC-MS^n has been developed. The method was validated using repeatability studies, duplicate analyses, recovery experiments, proficiency study data and comparison with previously validated HPLC-UV/Vis and HPLC-Fl procedures used in our laboratory. The HPLC-MS^n method was faster than using the two separate HPLC methods (UV/Vis and Fl) and could also be used to provide mass data on other compounds present in the extract, which could be useful for retrospective analysis of samples for a new compound of interest or metabolomics studies. The method can also be used in conjunction with the method for determining vitamin D_3 in cow milk by HPLC-MS^n.

Chapter 5: Feeding experiment

5.1. Introduction

The dairy industry is the third largest rural industry in Australia, behind the wheat and beef industries, with a value of $13.5 billion in 2014/15 (Dairy Australia 2016). Gippsland in eastern Victoria is a major dairy region in Australia, producing 1.9 billion litres of milk annually, or 21% of the national milk production (Dairy Australia 2017). The diet of dairy cows in Victoria are based on perennial ryegrass and white clover, with supplementation from grain or pelleted concentrate fed in the dairy shed (Auldist*, et al.* 2013). In times of drought, the lack of pasture is compensated for by increasing supplementary feed (Wales, Marett, Greenwood, Wright, Thornhill, Jacobs*, et al.* 2013). High amounts of grain supplements fed to dairy cows at milking times causes fluctuations in rumen pH and can limit milk production (Wales and Doyle 2003). Researchers at DPI Ellinbank, Victoria, Australia undertook a substantial dairy cow feeding systems experiment to investigate the potential to maximise the quantity and quality of milk by using alternative supplementary rations available throughout the day (Auldist*, et al.* 2013). The effectiveness of the new HPLC-MS^n method for analysis of milk samples will be demonstrated using a series of samples from one of these research programs.

Milk samples were collected from dairy cows in the feeding systems experiment and were analysed for all *trans*-retinol, α-tocopherol and β-carotene using the HPLC-MS^n method detailed in Chapter 4.

In an unrelated feeding experiment carried out at DPI Ellinbank, the newly developed vitamin D method was used to analyse for 25-hydroxyvitamin D_3 and vitamin D_3 in milk collected from cows fed 25-hydroxyvitamin D_3 to investigate the effect on the levels of both 25-hydroxyvitamin D_3 and vitamin D_3 in the milk. The data showed that even when fed 25-hydroxyvitamin D_3 for 10 weeks, the milk contained low levels of both 25-hydroxyvitamin D_3 ($\leq$ 0.05 µg/100 ml) and vitamin D_3 (<0.02 µg/100 ml) (H. Gill, 2009, pers. comm.).

Due to the low levels of vitamin D_3 in the milk samples collected from the 25-hydroxyvitamin D_3 feeding experiment, the samples from the feeding experiment described in Chapter 5 of this thesis were not analysed for vitamin D_3 or it's 25-

hydroxy metabolite, as there was little chance of observing an effect between treatments above the LOQ (0.01 µg/100 ml).

The design of the feeding systems experiment, including treatments and sampling, is outlined in section 2.8 Dairy cow feeding systems experiment.

5.2. Results

This section presents the concentration of each analyte (all *trans*-retinol, α-tocopherol, β-carotene) in the milk at each sampling time and cow daily intake from the diet. The complete set of milk sample results for this experiment are found in Appendix 2.

5.2.1. Milk samples

At the commencement of the short-term feeding rate experiment the milk samples showed a significant difference between all treatments for all *trans*-retinol ($P<0.01$) (Table 5-1) and between Control and PMR2 for β-carotene ($P<0.05$) (Table 5-2). For the samples taken at 9 days, the levels of all *trans*-retinol in milk from the Control and PMR1 treatments had dropped substantially. At day 23 there were no significant differences in all *trans*-retinol levels between treatments.

Table 5-1: All *trans*-retinol concentrations (µg/100 ml) in the milk collected from the short-term feeding rate experiment

		Rate (kg DM supplement/day)				
Sampling time	**Treatment**	**6**	**8**	**10**	**12**	**Average**
0 days	Control	54.5	58.3	59.7	64.4	59.2[a]
	PMR1	52.5	43.9	50.4	51.3	49.5[b]
	PMR2	33.9	42.0	39.0	39.9	38.7[c]
	Average					49.1
9 days	Control	41.6	41.9	40.1	40.9	41.2
	PMR1	35.0	29.2	28.6	28.5	30.3
	PMR2	44.4	32.9	36.2	34.6	37.0
	Average					36.2
23 days	Control	42.3	43.1	43.4	42.2	42.7
	PMR1	44.9	35.6	38.5	35.6	38.6
	PMR2	46.3	49.6	39.7	37.4	43.3
	Average					41.5

For each sampling time, averages with different superscripts differ ($P<0.05$)

Table 5-2: β-carotene concentrations (µg/100 ml) in the milk collected from the short-term feeding rate experiment

Sampling time	Treatment	Rate (kg DM supplement/day) 6	8	10	12	Average
0 days	Control	19.0	15.5	23.6	20.7	19.7[a]
	PMR1	19.6	12.0	18.6	17.7	17.0
	PMR2	15.8	13.4	16.6	16.1	15.5[b]
	Average					17.4
9 days	Control	23.5	23.6	26.8	27.0	25.2
	PMR1	15.1	15.7	16.6	18.0	16.3
	PMR2	12.8	14.9	12.4	10.4	12.6
	Average					18.1
23 days	Control	17.8	12.5	15.8	18.0	16.0[a]
	PMR1	12.4	10.5	10.1	11.9	11.2[b]
	PMR2	10.7	11.4	8.7	7.6	9.6[b]
	Average					12.3

For each sampling time, averages with different superscripts differ (P<0.05)

There was a significant difference (P<0.05) in β-carotene levels between Control and both PMR treatments at 23 days, with higher β-carotene levels in the milk of Control cows compared with PMR1 and PMR2 milk. There was an observed decline in β-carotene levels with increasing feeding rate for PMR2, however this was not statistically significant (Table 5-2).

At each sampling time, there was no significant difference in α-tocopherol levels between treatments (Table 5-3), however a significant (P<0.05) negative feeding rate effect was apparent for PMR2 at 9 days and at 23 days, that is, as the rate of supplement increased the level of α-tocopherol in the milk decreased. PMR1 also showed a negative feeding rate effect, although it was not shown to be statistically significant. There was no apparent effect of feeding rate on α-tocopherol level in the milk for the Control treatment.

Table 5-3: α-tocopherol concentrations (µg/100 ml) in the milk collected from the short-term feeding rate experiment

Sampling time	Treatment	Rate (kg DM supplement/day) 6	8	10	12	Average
0 days	Control	147.3	139.9	156.1	160.5	150.9
	PMR1	148.0	137.2	141.6	149.8	144.2
	PMR2	146.7	157.3	152.6	155.0	152.9
	Average					149.3
9 days	Control	184.6	183.0	198.7	197.7	191.0
	PMR1	163.6	155.5	136.2	151.3	151.6
	PMR2	206.2^{a}	174.6^{b}	167.2^{bc}	138.5^{c}	171.6
	Average					171.4
23 days	Control	186.6	174.0	177.0	179.9	179.4
	PMR1	186.3	166.7	154.1	136.9	161.0
	PMR2	188.2	203.0^{a}	159.8	146.2^{b}	174.3
	Average					171.6

For each sampling time, averages with different superscripts differ ($P<0.05$)
Within rows, means with different superscripts differ ($P<0.05$)

5.2.2. Vitamin content of the dairy cow diets

The vitamin content of the supplementary feed rations for each rate of supplement are provided in Table 5-4.

Table 5-4: Daily intake (mg) of vitamins in each dietary supplement at each feeding rate

	Rate (kg DM supplement/day)			
Control and PMR1	**6**	**8**	**10**	**12**
β-carotene	24	33	41	49
all *trans*-retinol	0.15	0.20	0.25	0.30
α-tocopherol	92	123	153	184
PMR2				
β-carotene	12	16	19	23
all *trans*-retinol	0.15	0.20	0.25	0.30
α-tocopherol	95	126	158	189

Pasture was not sampled, but an estimate of the daily intake of α-tocopherol and β-carotene from pasture was calculated from the levels observed by Adler (2013) of approximately 75 mg/kg DM of α-tocopherol and 45 mg/kg DM of β-carotene. At an estimated intake of 14 kg DM/cow per day, this equates to 1050 mg of α-tocopherol and 630 mg of β-carotene and no retinol as it is metabolised from β-carotene in the animal.

5.3. Discussion

The Control shows that as the rate of supplement was increased there was no obvious change in the levels of all *trans*-retinol, α-tocopherol, or β-carotene despite the presence of both α-tocopherol and β-carotene in the supplement. This is not surprising given the low amounts of these compounds in the supplement compared to the estimated levels of α-tocopherol and β-carotene in the pasture consumed.

The differences in all *trans*-retinol between all treatments at the start of the experiment follow a similar trend to the concentrations of β-carotene. The differences between treatments could be related to the different levels of β-carotene contained in the supplementary feed (Table 5-4) and/or the method of feeding prior to the start of the short term feeding experiment.

Substantial differences between the average results for all *trans*-retinol and β-carotene for samples taken at 9 days and 23 days suggest that the full effect of the dietary treatments for these two compounds had not been reached at 9 days.

β-carotene concentration was significantly higher in the milk of Control cows than both PMR treatments. While the Control had access to pasture all day aside from during milking, the PMR cows only had access to pasture between the evening and morning milking. It is likely that the lack of access to pasture during the day contributed to lower β-carotene in the milk, as pasture was the main source of β-carotene in the diet (section 5.2.2). A similar trend was observed for α-tocopherol however this was not significant. The PMR was designed to be a superior ration to the traditional supplementary feed (Control and PMR1) to increase milk quantity by reducing pH fluctuations in the rumen. This issue of access to pasture was addressed in a subsequent short-term feeding rate experiment in autumn 2010 and found to increase milk production (Auldist*, et al.* 2013).

Feeding large amounts of grain can lead to pasture substitution (Dixon and Stockdale 1999), that is, cows will eat the supplement and not graze as much pasture. It is likely that this occurred with the higher feeding rates of the PMR. All treatments and feeding rates were offered 14 kg pasture DM/head per day, however not all the pasture offered was consumed at the higher feeding rates of PMR (M.J. Auldist, 2014, pers. comm.). Decreased pasture intake would have the effect of decreasing the intake of both α-tocopherol and β-carotene, and a significant negative rate effect was observed for α-tocopherol at 9 days and 23 days. A negative rate effect was also observed for β-carotene at 23 days, but was not statistically significant. A reduction in pasture intake could also have occurred due to the reduced time the PMR cows spent grazing compared to Control cows.

Cows in both PMR 1 and 2, produced lower milk fat concentration at higher feeding rates (Appendix 2). As these vitamins are within the fat component of the milk, a decrease in milk fat concentration could also cause the observed decrease in α-tocopherol levels at higher feeding rates of PMR.

5.4. Conclusion

A reduction in the levels of β-carotene in milk were observed when cows were fed a partial mixed ration instead of the usual practice of feeding grain during milking and silage in the paddock along with the daily allocation of pasture. This was thought to be due to a reduced consumption of pasture, which is the major source of β-carotene.

No difference between treatments was shown for α-tocopherol, however there was a negative correlation between α-tocopherol and feeding rate for the PMR2 treatment. This was due to substitution of pasture with supplement, compounded by a decrease in milk fat concentration at higher feeding rates.

There was no significant difference between treatments for all *trans*-retinol in milk.

Chapter 6: General discussion

6.1. Introduction

Vitamins are organic compounds that cannot be synthesised in sufficient amount within the body, and therefore need to be acquired through the diet. Vitamins D_3 and K_2 are the two exceptions to this definition. The body can synthesise its own vitamin D_3 through exposure to ultraviolet B radiation (sunlight), and vitamin K_2 is produced by bacteria in the large intestine. Vitamins are broadly classified as either water-soluble: B_1 (thiamine), B_2 (riboflavin), B_3 (niacin), B_5 (pantothenic acid), B_6 (pyridoxine, pyridoxal, pyridoxamine), B_{12} (cobalamins), folate, biotin and C (ascorbic acid) or fat- soluble: A (retinol), D_2 (ergocalciferol) and D_3 (cholcalciferol), E (tocopherol), and K (phylloquinone, menaquinone).

Fat-soluble vitamin analysis is difficult due to the complexity of the compounds, their low levels in foods, and their physical and chemical similarity to other compounds present in foods. Traditional HPLC based methods using either UV/Vis (vitamins A, D, K and carotenes) or fluorescence (vitamins A, E, K) detection are time consuming and the vitamins are usually assayed individually due to their chemical diversity and varying levels within samples.

Rapid advances in HPLC-MS have seen it emerge as a major breakthrough in analytical science. This technique combines the separating power of liquid chromatography with the extra sensitivity, selectivity and powerful structure elucidation ability of mass spectrometry. The selectivity of mass spectrometry is an advantage over conventional HPLC detectors, particularly when analysing complex sample matrices. The use of collision-induced dissociation (CID) can give further selectivity and specificity to the analysis by fragmenting ions of interest and detecting the resulting product ions. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the widely used ionisation techniques for HPLC-MS. These two ionisation sources allow coverage of a wide range of analytes, from polar (ESI) to non-polar (APCI).

There are several different types of mass analysers that are typically used for HPLC-MS: single quadrupole (MS), triple quadrupole/tandem (MS/MS), ion trap (MS^n), and accurate mass instruments, such as time of flight (TOF), ion cyclotron resonance

(ICR) and Orbitrap. HPLC-MS^n and HPLC-MS/MS instruments were used for this work.

A good separation of the sample components by HPLC prior to MS analysis, in the majority of instances, is still necessary because of the effects of the sample matrix on the targeted ion(s), either by causing spectral, chemical or physical interferences. The separation of the analyte by HPLC prior to mass spectral analysis also gives retention time data, improving the confidence of the analyte identification in targeted analysis.

The aim of this work was to develop a validated method for the determination of a number of fat-soluble vitamins in cow milk by HPLC-MS^n. The method was then used to determine if there are significant differences in the fat-soluble vitamin concentrations of milk sourced from cows fed different feeding regimes.

The specific objectives of this work are:

- Develop and validate a HPLC-MS^n method for the determination of vitamin D_3 in cow milk.
- Develop and validate a HPLC-MS^n method for the simultaneous determination of vitamins A (all *trans*-retinol), E (α-tocopherol), and β-carotene in cow milk.
- Compare these results with those obtained using HPLC with Fluorescence (Fl) and/or Photodiode array (PDA) detection (vitamins A, E and β-carotene) and HPLC-MS/MS (vitamin D_3).
- Determine the levels of these vitamins and β-carotene in milk samples sourced from a dairy cow feeding systems experiment conducted at the Department of Primary Industries (DPI), Ellinbank, Victoria.
- Assess whether there is any significant difference in fat-soluble vitamin and β-carotene concentrations in the milk resulting from the different feeding regimes.

6.2. Summary of major findings.

6.2.1. Vitamin D_3

A straight forward, robust HPLC-MS^n method for determining the levels of vitamin D_3 in fresh cow's milk, commercially available fortified and non-fortified cow's milk and infant formula has been developed. A HPLC-MS/MS method was developed simultaneously and found to produce comparable results. The LOQ were 0.01 µg/100 ml and 0.02 µg/100 ml for HPLC-MS^n and HPLC-MS/MS respectively. Recoveries of vitamin D_3 added to the samples prior to saponification were satisfactory (60-90%). 25-hydroxyvitamin D_3 could also be determined in fresh milk samples using this procedure, although the extraction conditions were not fully optimized for this compound and so it gave lower recoveries. Isotopically labelled internal standards were used to correct for any quantitative variations caused by ion suppression or ion enhancement in the ion source.

Despite using compound-specific precursor and fragment ions, the analysis of milk samples still produced numerous interfering endogenous peaks, thus the choice of HPLC conditions, such as the choice of HPLC column, mobile phase and flow rate was important to achieve chromatographic separation of the analytes from any potential interferences. A Varian Polaris C18-A column gave the best separation of the analytes from major endogenous interferences, with the optimum elution conditions being an isocratic mobile phase consisting of 92% methanol and 8% water (and 5mM ammonium formate for HPLC-MS/MS).

Initial work using fortified and spiked unfortified milk samples showed that no additional clean-up was required after the initial saponification and extraction when the level of vitamin D_3 in the milk was > 0.1 ug/100 ml. Non-fortified milk samples, which had vitamin D_3 levels below 0.1 ug/100 ml, required a solid-phase extraction step to concentrate the extract and remove compounds which co-eluted with vitamin D_3.

For both HPLC-MS instruments, a number of injections of sample extract were required to achieve a stable signal, particularly after cleaning the API interface. This effect has been observed previously with nearly all HPLC-MS assays performed in

this laboratory, and it is assumed this phenomenon is due to an equilibration of the API interface as it becomes coated in compounds from the sample matrix.

25-hydroxyvitamin D_3 could also be measured in the samples using these methods. The lower recoveries (30-40%) of 25-hydroxyvitamin D_3 in comparison to vitamin D_3 were largely due to the more polar nature of the metabolite. Improved recoveries of the metabolite could have been achieved by extracting the samples with a more polar solvent, however this was not pursued as the main focus of this work was vitamin D_3.

6.2.2. Vitamins A, E and β-carotene

A straight forward, robust HPLC-MS^n method for determining the levels of all *trans*-retinol, α-tocopherol and β-carotene in cow milk was developed. The method was validated using repeatability studies, duplicate analyses, recovery experiments, proficiency study data and comparison with previously validated HPLC-UV/Vis and HPLC-Fl procedures used in our laboratory. In contrast to the HPLC-MS methods for vitamin D_3, an internal standard was not used for this work as the expected levels of all *trans*-retinol, α–tocopherol and all *trans*-β-carotene in milk (40 μg/ml, 200 μg/ml and 30 μg/ml respectively) were sufficiently high enough to compensate for any irregularities in the mass spectrometric detection.

The HPLC-MS^n method was faster than using the two separate HPLC methods (UV/Vis and Fl) and could also be used to provide mass data on other compounds present in the extract, which could be useful for retrospective analysis of samples for a new compound of interest or metabolomics studies. The method can also be used in conjunction with the method for determining vitamin D_3 in cow milk by HPLC-MS^n.

Two other compounds were tentatively identified in the milk extracts. The compound eluting at 24.5 min was assigned as 13 *cis*-β-carotene based on the mass spectral data ($[M+H]^+$ *m/z* 537) and comparison of the UV/Vis spectra with literature spectra. The concentration of this compound was estimated to be approximately 20% of the concentration of all *trans*-β-carotene in the milk samples, based on HPLC-UV/Vis data. The large unknown peak eluting at 14.27 min in the ion chromatogram had a MS^1 *m/z* of 369.35, corresponding to cholesterol, which forms the ion $[M+H-H_2O]^+$ when ionised by APCI in the positive ionisation mode. Cholesterol is a fat-soluble compound which would be expected to be extracted using the conditions described in

this work. It is present in milk at approximately 14 mg/100 g, which is substantially higher than the vitamins assayed in this work.

6.2.3. Feeding systems experiment

Milk samples were collected from a dairy cow feeding systems experiment conducted at DPI Ellinbank. The milk samples analysed in this study were obtained from a short-term feeding rate experiment designed to examine milk production response curves for dairy cows fed different supplement regimes in addition to the traditional pasture-based diet. The experiment was designed to mimic conditions of low pasture availability and high supplement intake. These samples were analysed for all *trans*-retinol, α-tocopherol and β-carotene using the HPLC-MS^n method detailed in Chapter 4.

A reduction in the levels of β-carotene in milk were observed when cows were fed a partial mixed ration instead of the usual practice of feeding grain during milking and silage in the paddock along with the daily allocation of pasture. This was thought to be due to a reduced consumption of pasture, which is the major source of β-carotene.

No difference between treatments was shown for α-tocopherol, however there was a negative correlation between α-tocopherol and feeding rate for the PMR2 treatment. This was due to substitution of pasture with supplement, compounded by a decrease in milk fat concentration at higher feeding rates.

There was no significant difference between treatments for all *trans*-retinol in milk.

6.3. Application and implication of these results

The work presented in this thesis outlines an approach which could be used for the analysis of a range of fat-soluble vitamins in milk using HPLC-MS^n, the advantage of this approach over the traditional approach of using HPLC-UV/Vis or Fl being the ability to analyse a wider range of vitamins with a single sample preparation and analysis, rather than needing to use separate HPLC methods for each vitamin. This has the potential to simplify the process of fat-soluble vitamins analysis in milk, reduce the number of instruments required and save significant amounts of time when analysing the levels of multiple fat-soluble vitamins in milk.

Potentially the most significant time savings come from the analysis of vitamin D_3 in fortified milks, which is a very time-consuming analysis when using HPLC-UV/Vis due to the need for multiple sample clean-up steps to remove chromatographic interferences. In contrast, the use of HPLC-MS^n allows a relatively simple sample preparation as the mass spectrometer is able to detect vitamin D much more selectively, thus avoiding any significant chromatographic interference for this analysis.

Another benefit of using HPLC-MS^n is the potential to identify other biologically important compounds in the extract, such as other fat-soluble vitamers or lipophilic substances such as phytosterols, by examining the appropriate *m/z* values from the full scan MS^1 data, or by setting up MS^2 fragmentation protocols for the extra compounds of interest before analysing samples.

When weighing up the cost/benefit of using the mass spectrometer for this analysis, the significant time savings for the analyst could see a substantial reduction in the cost of analysis, however a laboratory would need to factor in the much higher purchase price of the mass spectrometer (approx. $400,000) compared to conventional HPLC detectors (approx. $20,000) as well as the much higher running costs of the mass spectrometer, which uses approximately 600 l/hr of high purity nitrogen (approx. $5/1000 l), and also has greater servicing requirements.

The extraction method used in this thesis showed good extraction efficiency for a range of fat-soluble vitamins and alteration of the extraction method was beyond the scope of this project. However, in addition to the time efficiencies gained by the use of the mass spectrometer as a HPLC detector for this analysis, it was possible that further time efficiencies could be gained in the sample extraction step by adjustment of the extraction protocol. For example the recent work of Gill, Zhu and Indyk (2016) utilises a single extraction for the analysis of vitamin D_3 and 25-hydroxyvitamin D_3 in cow milk and obtained good recoveries, although this work used stable isotopically labelled standards added at the beginning of the sample preparation process, which would correct for any losses during extraction so the absolute recoveries of these vitamins from the milk is unclear.

6.4. Conclusions

In summary, the findings of this thesis are as follows:

A straight forward, robust HPLC-MS^n method for determining the levels of vitamin D_3 in fresh cow's milk, commercially available fortified and non-fortified cow's milk and infant formula has been developed. The method was validated using a reproducibility study, recovery experiments, participation in a proficiency study and comparison with an HPLC-MS/MS instrument.

A straight forward, robust method for determining the levels of all *trans*-retinol, α-tocopherol and β-carotene in cow's milk using HPLC-MS^n has been developed. The method was validated using repeatability studies, duplicate analyses, recovery experiments, proficiency study data and comparison with previously validated HPLC-UV/Vis and HPLC-Fl procedures.

The HPLC-MS^n instrument repeatability for all *trans*-retinol, α-tocopherol and β-carotene was similar to those for the traditional HPLC-UV/Vis (all *trans*-retinol and β-carotene) and HPLC-Fl (α-tocopherol) instruments. Excellent correlation was achieved between the levels of the analytes in milk determined by HPLC-MS^n and the levels determined by validated HPLC-UV/Vis and HPLC-Fl methods.

The levels of all *trans*-retinol, α-tocopherol and β-carotene were determined in milk samples sourced from a dairy cow feeding systems experiment conducted at the Department of Primary Industries, Ellinbank, Victoria. At the conclusion of the feeding systems experiment, results showed no significant difference in all *trans*-retinol or α-tocopherol levels between treatments, however α-tocopherol levels decreased as the feeding rate of PMR2 increased. A reduction in the levels of β-carotene in milk were observed when cows were fed a partial mixed ration instead of the usual practice of feeding grain during milking and silage in the paddock along with the daily allocation of pasture.

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Appendix 1. Examples of HPLC conditions used in the analysis of fat-soluble vitamins

Analyte	Matrix	Instrument conditions	Reference
α-Tocopherol and α-tocopherolquinone	Cheese	HPLC-UV/Vis λ 290 and λ 265 nm Nova-Pak C18, 390 × 20 mm, CH_3OH:H_2O (95:5) 1 ml/min	Fedele and Bergamo (2001)
α-Tocopherol and α-tocopherolquinone	Human plasma	Applied Biosystems Sciex API 3000 LC-MS/MS, ESI (+) ve ion mode RP8, 150 × 2.1 mm, 3.5 µm; (A) H_2O + 0.1% v/v FA, (B) CH_3CN + 0.1% v/v FA gradient: 0-2 min 25% A, 2.1-15 min 10% A, 16-20 min 0% A, 21-25 min 25% A, 0.3 ml/min	Mottier, Gremaud, Guy and Turesky (2002)
α-Tocopherol	Milk and cereal infant food	Agilent MSD APCI (+) ve ion mode Spherisorb Si, 250 × 4.6 mm, 5 µm; 1% v/v 2-propanol in *n*-hexane, 1.0 ml/min	Kalman, *et al.* (2003)
α, β, γ, δ-Tocopherol	Sunflower oil, milk	Agilent 1000 MSD ESI & APCI (+) ve & (-) ion modes, UV λ 292 nm Fluophase PFP, 200 × 4.6 mm, 5 µm, CH_3OH:H_2O (95:5) 0.5 ml/min	Lanina, *et al.* (2007)
Carotenoids	Tropical fruit	HPLC-DAD-Waters Thermabeam (+) ve ion mode Spherisorb ODS2, 150 × 4.6 mm, 3 µm, CH_3CN + 0.05% v/v triethylamine:CH_3OH:ethyl acetate gradients	Azevedo-Meleiro and Rodriguez-Amaya (2004)
Vitamin A, carotenoids	Cow plasma, milk	HPLC-DAD, 280-600 nm	Nozière, Grolier, *et al.* (2006)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Retinol, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene	Milk and milk products	HPLC-UV/Vis λ 325 nm retinol, λ 450 nm carotenoids Vydac C18, 250 × 3.2 mm, 5 µm, (A) CH_3OH:H_2O: tetrahydrofuran:triethylamine (87.9:10.0:2.0:0.1 % v/v), (B) CH_3OH: tetrahydrofuran:triethylamine (92.4:7.5:0.1 % v/v), 0-0.25 min 100% (A), 0.75-20 min 100% (B) 0.7 ml/min	Hulshof, *et al.* (2006)
Retinol, α-tocopherol, β-carotene	Buffalo and cow milk, mozzarella cheese and dairy products	HPLC-UV/Vis λ 290 nm tocopherol, UV λ 450 nm β-carotene, retinol not specified Nova-Pak C18, 390 × 20 mm; CH_3OH: H_2O (95:5) 1 ml/min	Bergamo, *et al.* (2003)
Retinol, retinyl esters, α-tocopherol, γ-tocopherol, β-carotene	Natural and fortified dairy products	HPLC-UV/Vis λ 326 nm retinoids, λ 294 nm tocopherols, λ 450 nm β-carotene Spheri-5 ODS, 220 × 4.6 mm, 5 µm, CH_3CN:CH_2Cl_2:CH_3OH (70:20:10) 1.3 ml/min	Herrero-Barbudo, Granado-Lorencio, Blanco-Navarro and Olmedilla-Alonso (2005)
Vitamins A and E and carotenoids	Cow plasma and milk	HPLC-DAD 280-600 nm Nucleosil C18, 150 × 4.6 mm, 3 µm coupled with a Vydac TPS4 C18, 250 × 4.6 mm, 5 µm	Calderón, Chauveau-Duriot, Martin, Graulet, Doreau and Nozière (2007)
Vitamins A and E and β-carotene	Cow milk	HPLC-UV/Vis λ 325 nm retinol, λ 291 nm α, δ-tocopherol, λ 450 nm β–carotene Phenomenex C18, 150 × 4.6 mm, 4 µm, CH_3OH:hexane (85:15) 1 ml/min	Ellis, Monteiro, Innocent, Grove-White, Cripps, McLean, *et al.* (2007)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Retinol, tocopherols and carotenoids	Forages, cow plasma and milk	HPLC-UV/Vis λ 325 nm vitamin A, λ 291 nm vitamin E, λ 450 carotenoids (1) Nucleosil C18, 150 × 4.6 mm, 3 µm coupled with a Vydac TPS4 C18, 250 × 4.6 mm, 5 µm, CH_3CN:CH_2Cl_2:0.05 M NH_4Ac:H_2O (70:10:15:5) 2 ml/min (2) Acquity HSS T3, 1.8 µm (A) CH_3CN:CH_2Cl_2:CH_3OH (B) 0.05 M NH_4Ac in H_2O 0-20 min 75% (A), 20-21 min 100% (A), 21-30 min 98% (A), 30-44 min 98% (A), return to 100% (A) 0.4 ml/min	Chauveau-Duriot, Doreau, NoziÃ¨re and Graulet (2010)
Retinol, tocopherols, β-carotene	Milk and soy-juice based beverages	HPLC-DAD λ 290 nm tocopherol, λ 440 nm β-carotene, λ 325 nm retinol Luna C18, 250 × 4.6 mm, 5 µm, CH_3OH:tetrahydrofuran:H_2O (67:27:6 v/v/v) 0.8 ml/min	Andrés, *et al.* (2014)
α-Tocopherol, β-carotene	Cow milk	HPLC-UV/Vis and HPLC-Fl, λ 450 nm β-carotene, Ex λ 297 nm, Em λ 340 nm α-tocopherol Zorbax C18, 250 × 4.6 mm, 5 µm, mobile phase not specified	Marino, *et al.* (2014)
All-*trans*-retinol, α-tocopherol, ergocalciferol (D_2), cholcalciferol (D_3)	Fortified infant formulae	Agilent MSD, APCI (+) ve ion mode Nucleosil Si 100, 250 ×4.6 mm, 5 µm, hexane:dioxan:2-propanol (96.7:3:0.03) 1.45 ml/min	Heudi, *et al.* (2004)
Vitamins A, D_2, D_3, K_1, K_3, α-tocopherol, γ-tocopherol	Human serum	Agilent 6410 LC-MS/MS, ESI (+) ve ion mode Zorbax Eclipse XDB-C18, 4.6mm × 150 mm, 5 µm; (A) 5mM NH_4 formate in acetonitrile: H_2O (90:10 % v/v), (B) 5mM NH_4 formate in methanol: 100% (A) 0-2 min, 100% (B) 7-27 min, 1.0 ml/min	Capote, *et al.* (2007)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Vitamin A, vitamin E, β-carotene, vitamin D, 25(OH)D, vitamin K	Human breast milk	Vitamin D and 25(OH)D derivatisation with DMEQ-TAD Applied Biosystems Sciex API 3000 LC-MS/MS, APCI (+) ve ion mode Capcellpak C18 UG120 (1) Vitamins A, E, β-carotene; (A) CH_3OH:H_2O (90:10 % v/v), (B) CH_3CN, 0-10 min 0% (B), 30 min 90% (B); (2) vitamin D and 25(OH)D; (A) H_2O (B) CH_3CN, 0-5 min 30% (B), 30 min 95% (B)	Kameo *et al* 2007
Tocopherols, carotenoids, ergocalciferol, phylloquinone and menaquinone-4	Plant food	HPLC-DAD-MS/MS (+) ve ion mode. ProntoSIL C30, 250 × 4.6 mm, 3 µm (A) CH_3OH, (B) isopropanol:hexane (1:1 v/v); 100% (A) 0-1 min, 75% (B) 1-15 min, 99.5% (B) 15-15.1 min, 99.5% (B) 15.1-30.5 min. 1ml/min	Gentili and Caretti (2011)
All-*trans*-retinol, tocopherols, carotenoids, cholcalciferol, ergocalciferol, phylloquinone and menaquinone-4	Cow, buffalo, sheep, goat and donkey milk	HPLC-DAD-APCI-MS/MS, λ 450 nm for carotenes, (+) ve ion mode (1) Vitamins A, E, D and K: Supelco C18, 50 × 4.6 mm, 5 µm coupled to a Altima, 250 × 4.6 mm, 5 µm. (A) CH_3OH, (B) isopropanol:hexane (1:1) 0-1 min 100% (A), 15 min 25% (A), 15.1 min 0.5% (A), 22 min 99.5% (A) 1ml/min (2) Carotenoids: ProntoSIL C30, 250 × 4.6 mm, 3 µm (A) CH_3OH, (B) isopropanol:hexane (1:1 v/v) 0-1 min 100% (A), 15 min 25% (A), 15.1 min 0.5% (A), 30 min 99.5% (A) 1ml/min	Gentili, *et al.* (2013)
Vitamin D_3	Fat tissue	Agilent 1100 HPLC-MS APCI (+) ve ion mode ProntoSIL 200-5-C30, 250 × 4.6 mm, 5 µm (A) CH_3OH (B) CH_2Cl_2; 0-10 min 100% (A), 10-25 min 50% (A), 25-35 min 100% (A), 1 ml/min	Blum, *et al.* (2008)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Retinol, retinol acetate, retinol palmitate, tocopherols, β-carotene, lutein, zeaxanthin, cholcalciferol	Infant formula	UHPLC-DAD-APCI-MS/MS λ 285 nm tocopherol and cholcalciferol, λ 450 nm carotenoids, λ 325 nm retinols YMC C_{30} reversed-phase column, 100 × 2.0 mm, 3 μm (A) CH_3OH:H_2O (90:10, v/v) (B) *tert*-butyl methyl ether:CH_3OH (80:20, v/v). 0-8 min 8-40% (B), 8-13 min 40-100% (B), 13-14.5 min 100% (B), 14.5-14.6 min 8% (B), 0.3 ml/min	Nimalaratne, Sun, Wu, Curtis and Schieber (2014)
Vitamin D_3	Fish, fortified foods, shellfish	Preparative: Agilent 1200 HPLC-DAD/ELSD Inertsil Si, 250 × 4.6 mm, 5 μm (A) isopropanol: *tert*-butyl methyl ether:cyclohexane:*n*-heptane (0.5:2.0:48.75:48.75 v/v), (B) isopropanol:*n*-heptane (20:80 v/v); 0-25 min 100% (A), 35-55 min 100% B, 75-85 min 100% (A), 1.3 ml/min Analytical: HPLC-DAD, HPLC-MS/MS APCI (+) ve ion mode Inertsil ODS-2, 250 × 4.6 mm, 5μm, CH_3OH:CH_3CN (20:80) 1.3 ml/min	Byrdwell (2009); Byrdwell, Harnly, Horst, Phillips, Holden, Patterson, *et al.* (2013)
Vitamin D_3 and D_2	Food	HPLC-MS^n	Dimartino (2007)
Vitamin D_3 and 25-hydroxyvitamin D_3	Foodstuff	HPLC-DAD λ 265 nm vitamin D_3 HPLC-APCI/MS/MS 25(OH)D_3 (+) ve ion mode Lichrospher C18, 250 × 3 mm, 5 μm; mobile phase vitamin D_3 methanol:H_2O (98:2 v/v) 0.4 ml/min, 25(OH)D_3 methanol:H_2O (88:12 v/v) with 0.2% v/v acetic acid, 0.42 ml/min	Bilodeau, *et al.* (2011)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Vitamin D_3 and 25-hydroxyvitamin D_3	Meat	HPLC-APCI-IT-MS (+) ve ion mode Prevail Si, 250mm × 4.6mm, 5µm (A) 10% v/v Isopropanol in *n*-heptane, (B) *n*-heptane. 0 min 1% (A), 20 min 25% (A), 28 min 86% (A), 28.01-32 min 100% (A), 32.01-35 min 1% (A)	(Strobel, Buddhadasa, Adorno, Stockham and Greenfield 2013)
Vitamin D_3	Milk based infant formula	Agilent 1200 HPLC-MS/MS ESI (+) ve ion mode Xbridge C18, 150 × 2.1 mm, 3.5 µm (A) 5mM NH_4 formate in H_2O, (B) 5mM NH_4 formate in CH_3OH, 0-20 min 6% (A), 21-65 min 1% (A), 66-70 min 6% (A), 0.2 ml/min	Kwak, Jeong, Lee, Ahn and Park (2014)
Vitamins D_2 and D_3	SRM 1849a infant formula	Applied Biosystems Sciex 4500 LC-MS/MS, APCI (+) ve ion mode Hypersil aQ, 100 × 2.1 mm, 3 µm (A) CH_3OH:H_2O (75:25) + 0.1% v/v FA, (B) CH_3OH + 0.1% v/v FA, 0 min 70% (B), 0.8-4.5 min 100% (B), 4.51-6 min 70% (B), 0.3 ml/min	Huang, *et al.* (2014)
Vitamins D_2, D_3, K_1, K_2 and K_4	Infant formula, infant cereals (D); spinach, lettuce (K)	(1) HPLC-DAD: Agilent 1100 UV λ 245 nm vitamin K and λ 265 nm vitamin D (2) HPLC-MS^n APCI (-) ve ion mode, 8 min for vitamin K; APCI (+) ve ion mode, 8 min-23 min for vitamin D Zorbax C18, 250 × 46 mm, 5 µm (A) CH_3CN:H_2O (70:30 v/v) (B) CH_3CN:isopropanol (60:40 v/v) 0-2 min 100% (A), 2.01 -12 min 100% (B), 13-23 min 100% (A), 1 ml/min	Vinas, Bravo-Bravo, Lopez-Garcia and Hernandez-Cordoba (2013)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Vitamin K	Food	HPLC Fl (1) Ex λ 320 nm, Em λ 430 nm, (2) Ex λ 240 nm, Em λ 430 nm CAPCELL PAK C18, 250 × 4.6 mm with Pt reduction column, (1) MK-4 CH_3OH:H_2O (95:5), (2) PK & MK-7 CH_3OH:C_2H_5OH (95:5), 1 ml/min	Kamao *et al* 2007
25(OH)D_2 and 25(OH)D_3	Human plasma	DMEQ-TAD derivatisation	Higashi*, et al.* (2001)
Vitamin D_2	Fortified toned milk	HPLC-DAD Phenomenex C18, 250 × 4.5 mm, 5 µm, CH_3CN:CH_3OH:$CHCl_3$ (88:8:4), 1 ml/min	Kaushik, Sachdeva, Arora and Wadhwa (2014)
Vitamin D_3	Dietary supplements	HPLC-MS/MS (+) ve ion C18, 100 × 2.1 mm, 2.7 µm, 5mM NH4 formate in CH_3OH + 0.1% v/v FA:5 mM NH_4 formate in H_2O + 0.1% v/v FA (95:5) 0.2 ml/min	Lam, Hung, Wong, Fok and Wong (2014)
Vitamin D metabolites	Human plasma, rat bile	DMEQ-TAD derivatisation PTAD derivatisation	Shimada and Higashi (2002)
Vitamin D metabolites	Human serum	PTAD derivatisation	Aronov*, et al.* (2008)
Vitamin D_3	Feed, food and pharmaceuticals	HPLC-MS/MS	Schadt*, et al.* (2012)
Vitamin D_3 [##]	Fortified infant formula, milk and milk powder	PTAD derivatisation	Abernethy (2012)

Appendix 1. continued

Analyte	Matrix	Instrument conditions	Reference
Vitamin D analogues	Milk	Diels-Alder derivatisation	Gomes, Shaw, Whitfield and Hewavitharana (2015)
Vitamin D_3	Milk	PTAD derivatisation	Gill, *et al.* (2016)

Appendix 2. The complete set of analytical results of the feeding experiment

Sampling Day	Treatment	Rate	Herd	all *trans*-retinol	α-tocopherol	β-carotene	% fat
				µg/100 ml milk			
0	Control	6	A	52.8	150	16.1	4.28
0	Control	6	C	56.3	145	21.8	4.36
0	Control	8	A	56.5	130	14.0	3.89
0	Control	8	C	60.0	150	17.1	4.08
0	Control	10	A	59.1	157	24.3	4.20
0	Control	10	C	60.2	155	23.0	3.94
0	Control	12	A	66.3	155	24.0	3.91
0	Control	12	C	62.5	166	17.4	4.21
0	PMR1	6	B	49.8	144	20.2	4.34
0	PMR1	6	E	55.2	152	18.9	3.89
0	PMR1	8	B	48.9	144	11.2	3.89
0	PMR1	8	E	38.9	130	12.8	3.96
0	PMR1	10	B	47.2	126	16.2	3.96
0	PMR1	10	E	53.6	157	21.0	4.04
0	PMR1	12	B	59.4	167	21.6	4.23
0	PMR1	12	E	43.2	132	13.9	3.79
0	PMR2	6	D	35.1	148	15.1	4.25
0	PMR2	6	F	32.7	145	16.5	4.14
0	PMR2	8	D	41.0	148	13.5	4.35
0	PMR2	8	F	43.0	166	13.4	4.21
0	PMR2	10	D	42.0	164	16.6	4.10
0	PMR2	10	F	36.0	141	16.7	4.01
0	PMR2	12	D	38.3	146	12.4	4.17
0	PMR2	12	F	41.4	164	19.8	3.71
9	Control	6	A	41.9	186	22.6	4.34
9	Control	6	C	41.3	183	24.4	4.20
9	Control	8	A	42.8	166	22.0	4.12
9	Control	8	C	41.0	200	25.2	4.36
9	Control	10	A	41.5	194	25.2	4.25
9	Control	10	C	38.7	204	28.3	4.31
9	Control	12	A	41.1	179	27.5	4.42
9	Control	12	C	40.7	216	26.6	4.09
9	PMR1	6	B	34.0	176	19.2	3.90
9	PMR1	6	E	36.0	152	11.0	4.00
9	PMR1	8	B	33.6	181	18.2	3.73
9	PMR1	8	E	24.7	130	13.2	4.02
9	PMR1	10	B	26.6	147	20.2	3.70
9	PMR1	10	E	30.5	126	13.0	4.07
9	PMR1	12	B	33.0	179	25.2	4.21
9	PMR1	12	E	24.0	124	10.7	3.69
9	PMR2	6	D	45.7	220	15.7	4.18
9	PMR2	6	F	43.1	193	9.9	4.06
9	PMR2	8	D	32.1	176	18.0	4.10
9	PMR2	8	F	33.6	173	11.7	4.31
9	PMR2	10	D	38.0	183	14.8	3.89
9	PMR2	10	F	34.3	152	10.1	4.23
9	PMR2	12	D	33.6	134	11.3	3.95
9	PMR2	12	F	35.7	143	9.5	3.77

Appendix 2. continued

Sampling Day	Treatment	Rate	Herd	all *trans*-retinol	α-tocopherol	β-carotene	% fat
				µg/100 ml milk			
23	Control	6	A	42.9	197	16.8	4.21
23	Control	6	C	41.6	177	18.7	4.14
23	Control	8	A	45.4	163	15.2	4.18
23	Control	8	C	40.7	185	9.7	4.16
23	Control	10	A	39.5	177	16.7	4.44
23	Control	10	C	47.2	177	14.9	4.14
23	Control	12	A	40.1	166	17.9	4.37
23	Control	12	C	44.2	194	18.0	4.37
23	PMR1	6	B	38.5	174	13.2	4.41
23	PMR1	6	E	51.3	198	11.6	4.34
23	PMR1	8	B	31.1	130	8.9	4.28
23	PMR1	8	E	40.1	203	12.1	4.62
23	PMR1	10	B	37.6	149	10.6	3.97
23	PMR1	10	E	39.4	159	9.7	4.21
23	PMR1	12	B	43.3	163	17.1	3.59
23	PMR1	12	E	27.9	111	6.7	3.41
23	PMR2	6	D	42.9	181	10.5	4.27
23	PMR2	6	F	49.7	196	10.9	4.24
23	PMR2	8	D	52.6	211	12.0	4.37
23	PMR2	8	F	46.7	195	10.7	4.33
23	PMR2	10	D	33.1	149	8.1	4.14
23	PMR2	10	F	46.3	170	9.3	4.30
23	PMR2	12	D	34.8	137	6.9	4.16
23	PMR2	12	F	40.0	155	8.4	3.98