ON-LINE METABOLIC PROFILING IN DAIRY COWS

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CHAPTER TITLES ON THE TOP OF EACH PAGE THROUGHOUT THE BOOK ARE VERY PALE

DECLARATION

I declare that this thesis has been written by myself and has not been submitted for another degree from this or any other university. All work has been carried out by myself, with the exception of milk fatty acid analysis which was carried out by Dr Adam Lock, University of Nottingham, and Gerber tests which were carried out by Mrs Dawn Scholey, University of Nottingham. Milk fat, protein and somatic cell count analysis in Chapter 5 were carried out by National Milk Records, Harrogate, Yorkshire, UK. All sources of information have been specifically acknowledged by means of references. The views expressed in this thesis are those of the author and not of the University of Nottingham.

Signed: Lorna J. Massan Date: 7/3/04

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PUBLICATIONS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Acetyl-CoA	Acetyl co-enzyme A
BCS	Body condition score
BHB	Betahydroxybutyrate
BUN	Blood urea nitrogen
CH_4	Methane
CO_2	Carbon dioxide
CO_3^{2-}	Bicarbonate
DEFRA	Department for environment, food and rural affairs
DMI	Dry matter intake
EDTA	Ethylene diamine tretraacetic acid
ERDP	Effective rumen degradable protein
FAs	Fatty acids
FCM	Fat corrected milk
FME	Fermentable metabolisable energy
GC	Gas chromatography
ME(I)	Metabolisable energy (intake)
mM	Millimoles
MPI	Metabolisable protein intake
MUN	Milk urea nitrogen
MY	Milk yield
NADH(NADPH)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NH_3/NH_4^+	Ammonia/Ammonium
NIR	Near infrared
N_2O	Nitrous oxide
PUN	Plasma urea nitrogen
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SBP	Sugar beet pulp
SCC(s)	Somatic cell count(s)
SEM(s)	Standard error of the mean(s)
TCA	Tricarboxylic acid cycle
TMR	Total mixed ration

ABSTRACT

Automatic sampling and analysis of milk composition for on-line metabolic profiling has been proposed as a means of monitoring individual cows to detect and correct problems in nutritional management. Acetone, urea, fat, protein and citrate in milk were investigated independently and jointly as potential indicators for monitoring the dairy cow. A series of experiments and milk analysis were conducted to investigate sources of variation within and between cows, nutritional effects on milk composition and whether dietary changes could be detected through changes in milk composition. Novel methods for on-line acetone and urea analysis were also tested. Significant diurnal variation was found in fat, acetone and urea but not in citrate or protein. Acetone and citrate were the most variable constituents; day-to-day variation was 64.3% of mean acetone and 16.4% of mean citrate. Between cow variation was significant in all milk constituents monitored, in terms of means and normal concentration ranges, highlighting the need for individual cow management. Individual variation could be determined by establishing normal ranges of milk constituents for each cow, so that deviations from normal can be detected. Dietary changes at the group and individual cow levels were not detectable and individual responses to dietary changes were unpredictable. Changing the starch to fibre ratio in the diet had no effect on milk acetone and effects on urea, fat and protein were only significant when dietary changes were extreme. Energy output, in terms of FCM, was more closely correlated with fat:protein ratio (R^2 = 0.3) than with acetone (R^2 = 0.006), so the use of acetone as an indicator of metabolic status is inconclusive. Further research on a larger scale is needed to determine whether on-line monitoring will be feasible for nutritional management and economically justified in practice.

Keywords: on-line monitoring, milk composition, variation, nutritional management

CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

Over the last two decades the structure of the UK dairy industry has changed dramatically. Milk yield per cow has increased significantly, resulting in fewer registered producers and a reduction in cow numbers. From 1995 to 2002 the number of registered milk producers fell from 36 583 to 25 548, cow numbers decreased from 2 601 000 to 2 227 000 and milk yield per cow rose from 5380 to 6320 litres (The Dairy Council, 2002). Herd sizes have increased due to improved milk output and have been accompanied by reduced labour costs and a greater reliance on automation. Increased milk output has been brought about by a combination of improvements in genetics, nutrition, health and management (Mottram 1997). Genetic improvement for high yielding dairy cows has resulted in a greater risk of metabolic or production diseases (Simianer, Solbu and Schaeffer, 1991), particularly in early lactation where metabolic stress is becoming more of a welfare issue and may predispose the cow to other conditions, such as lameness and infertility.

Since the early 1970s, there has been great interest in the development of metabolic profile tests (Payne et al, 1970). These tests may help in the prediction and prevention of metabolic diseases and aid nutritional management by measuring specific metabolites in the blood. However, a more practical and cost-effective way in which to monitor cow and udder health may be to measure milk components on-line during milking, allowing early detection and correction of potential health and nutritional problems. Ensuring optimal nutrition and maximum productivity is difficult when managing large numbers of cows. With rapid advances in the analysis of milk constituents and computer systems for automatic data recording and interpretation, there is great potential for on-line metabolic profiling in dairy cows to optimise nutritional management and animal health.

This review aims to investigate the use of specific milk constituents that could be monitored to determine the metabolic and nutritional status of the cow throughout lactation. Particular attention is given to the measurement of urea, acetone, fat and

protein in milk, factors affecting these, their normal concentrations in milk and the consequences of deviations from normal concentrations in terms of nutrition and productivity. Normal ranges of these constituents are important to define as any deviation from normal may indicate dietary inefficiency and a negative energy balance, predisposing the cow to metabolic disorders such as ketosis. Current sensing technology for on-line milk analysis is also reviewed along with body condition scoring and gaseous emissions as other means by which nutritional status may be assessed.

1.2 METABOLIC PROFILE TESTS

Metabolic profile tests were designed to detect production diseases such as ketosis, milk fever and grass tetany or hypomagnesaemia, by measuring specific components in the blood (Payne et al, 1970). Production diseases arise due to alterations in normal blood chemistry, resulting from an imbalance between the input rate of dietary nutrients and production output. The advantage of the profile test is that many blood constituents can be measured simultaneously. Commonly measured blood metabolites are β-hydroxybutyrate, glucose, urea, specific minerals, non-esterified fatty acids (NEFAs) and albumin.

There are however, several disadvantages of metabolic profiling in blood. Blood sampling is time-consuming, invasive and expensive and several samples should be taken from an individual cow to obtain an accurate picture of health and nutritional status. Although blood tests can provide useful information on metabolic status, metabolites are influenced by seasonal and stage of lactation effects (Rowlands et al, 1979), as well as diurnal (Manston et al, 1981) and individual variation (Gustafsson and Palmquist, 1993). Metabolites are also influenced by feeding practices. Sampling time in relation to feeding is important to consider, with the optimal sampling time varying between metabolites (Herdt, 2000). Different sites of sampling have been used for blood profiles, causing problems in the interpretation of the results. For example, blood samples may be taken from the mammary vein, the jugular vein or the tail vein and differences may exist in blood metabolites at the various sites of sampling (Adams et al, 1978).

Extreme diet changes are needed to produce abnormal concentrations in blood metabolites because levels are regulated by homeostasis (Lee et al, 1978). Therefore the cost of blood metabolic profiles may exceed their value for monitoring individual cows. Metabolic profiles were intended for assessing nutritional efficiency and detecting metabolic problems at the herd or group level (Van Saun, 1997; Whitaker, 2000). A more useful means of monitoring the dairy cow may be through metabolic profiling in milk.

1.3 METABOLIC PROFILING IN MILK

During the last decade there has been great interest in milk profile tests as an indicator of health and production efficiency. The advantages of using milk as a test medium are that milk samples are easy to collect and are taken routinely for laboratory analysis, so no extra cost is involved for sampling and transport. Milk sampling is also less time-consuming and is non-invasive, unlike blood sampling. If milk composition could be monitored on-line with biosensors as the cow is being milked, this would provide real-time data on which quick nutritional management decisions could be made. The advantage of individual cow monitoring over testing bulk samples are that bulk samples may be misleading because they do not take into account factors such as stage of lactation, and they cannot predict dietary efficiency within feeding groups.

Significant deviations from predicted milk yields can be used to detect health problems such as ketosis (Lark, Nielsen and Mottram, 1999). However, there may be a delay in milk yield response to faulty feeding. The theory behind metabolic profiling in milk is that by monitoring changes in milk composition, problems in nutritional management can be detected and corrected before production and health are compromised. As well as identifying potential disturbances in metabolism and reproductive status through progesterone monitoring, milk profiles may allow feeding efficiency to be optimised. However, managing dairy cows through metabolic profiling in milk is in the very early stages of research and is not yet used routinely. Although fat and protein content in milk are already routinely analysed on a monthly basis on commercial dairy farms, additional metabolites such as urea and

acetone would be useful for measuring dietary efficiency and the energy status of the cow respectively. Normal ranges of each milk parameter must be established in individual cows so that when interpreting profiles, values outside the normal range are immediately recognised and corrective action can be taken.

Nutritional management is currently based on feeding the same ration to groups of cows according to lactation stage and productivity. However, diets may not be optimal for all the cows in that group. On-line metabolic profiling would aim to improve nutritional management on an individual cow basis, to ensure that each animal is producing to its full genetic potential. However, this approach would require knowledge of factors affecting milk composition and how feed inputs affect milk composition and yield on a quantitative basis, so that cow responses could be predicted. In this system the cow is effectively being treated empirically, in that output in terms of milk composition is being based on dietary input, with no consideration of the metabolic processes taking place within the individual tissues of the cow.

One disadvantage of on-line monitoring is that the nutritional status of dry cows cannot be assessed. Also, difficulties arise when trying to interpret results of milk composition and relate them to nutritional status of the cow. Both milk composition and energy balance of the cow are affected by dietary energy content, level of energy intake and level of milk production, all of which are interrelated, making it difficult to identify where the deficiency in nutrition lies (Grieve et al, 1986). Although sensors alone may not be able to accurately detect diseases or inadequacies in nutrition, the accuracy of detection can be improved by combining information on diet, milk yield, stage of lactation and parity (Mottram, 1997). This information may be of great value to dairy farmers, nutritionists and veterinarians to achieve optimal nutritional management and ensure cow health and productivity.

1.4 MILK PARAMETERS FOR MONITORING METABOLIC AND NUTRITIONAL STATUS

1.4.1. Ketones

Ketosis or acetonaemia is a multi-factorial disorder of energy metabolism and tends to occur in high yielding dairy cows in early lactation. Ketosis is biochemically characterised by hyperketonaemia, hypoglycaemia and elevated blood NEFAs, and is accompanied by reductions in feed intake, milk yield and liveweight (Baird, 1982). Hyperketonaemia refers to the accumulation of ketone bodies (β-hydroxybutyrate, acetoacetate and acetone) in the blood, milk and urine. Monitoring milk ketones could potentially be used to identify cows that are in negative energy balance and to improve nutritional management.

Ketones arise from the mobilisation of adipose tissue in early lactation. NEFAs produced by the breakdown of adipose tissue undergo β-oxidation to acetyl-CoA (acetyl coenzyme acetyltransferase) and NADH (nicotinamide adenine dinucleotide in the reduced form) in the liver. Under normal conditions acetyl-CoA is either metabolised to acetoacetyl-CoA or oxidised via the Tricarboxylic Acid (TCA) Cycle, which is depended on a sufficient supply of oxaloacetate from its precursor propionate (Lean et al, 1991). Therefore, a deficiency of oxaloacetate will result in limited oxidation of acetyl-CoA and any excess will be metabolised to ketones (Baird et al, 1968). Ketones are also produced in the rumen epithelium. Much of the butyrate produced by rumen fermentation is converted to β-hydroxybutyrate (BHB) in the rumen epithelium and absorbed into the bloodstream (Palmquist et al, 1968). Although low concentrations of ketones can be excreted or used in energy metabolism, high concentrations are toxic (Webster, 1993; Holtenius and Holtenius, 1996). No data are available on what constitutes toxic concentrations of ketone bodies. However, this is likely to vary between individuals depending on their genetic potential and how efficient they are at utilising ketone bodies in energy metabolism.

Most of the research on milk acetone has been carried out in Scandinavian countries (Andersson, 1984; Gustafsson and Emanuelson, 1996; Steen, Østerås and Grønstøl,

1996a; Steen, Østerås and Grønstøl 1996b), due to the higher prevalence of ketosis compared with the UK and it being the second most common cattle disease in Norway (Steen et al, 1996a). Acetone is the most commonly used ketone body for monitoring herd health as it is present in greater quantities than BHB and acetoacetate, and is closely correlated with blood ketones (Table 1.1) (Schultz and Myers, 1959; Anderson and Lundström, 1984a; Reist et al, 2000; Enjalbert et al, 2001). Acetoacetate accounts for only 10% of the total acetone and at room temperature acetoacetate is converted to acetone (Cook, 1999). Therefore, analysis of acetone alone is justified as being the best potential indicator of energy balance in the dairy cow.

Table 1.1. Correlation coefficients among blood and milk concentrations of ketone bodies (Enjalbert et al, 2001)

	Blood			**Milk**		
	Acetone	*Acetoacetate*	BHB	*Acetone*	*Acetoacetate*	BHB
Blood						
Acetone	1.00					
Acetoacetate	0.80	1.00				
BHB	0.53	0.71	1.00			
Milk						
Acetone	0.96	0.77	0.58	1.00		
Acetoacetate	0.74	0.74	0.62	0.75	1.00	
BHB	0.65	0.65	0.66	0.68	0.72	1.00

Measuring ketone concentrations in milk has several advantages over blood testing. Ketones exhibit diurnal variation in the blood, which limits their use as metabolic indicators (Manston et al, 1981; Bines and Morant, 1983). Blood ketones are reported to be highest four to five hours after the start of feeding (Rossow et al, 1991), due to increased metabolism of butyrate in the rumen epithelium (Andersson and Lundström, 1984a). As milk acetone is highly correlated with blood ketone levels then acetone would be expected to show some diurnal variation in relation to feeding. Andersson and Lundström (1984a) found that milk ketones showed similar

diurnal variation to blood ketones, and that all ketone body concentrations peaked three to four hours after commencement of feeding. However significant diurnal variations were only found for milk and plasma BHB. Like urea, these variations could be minimised by milk sampling at the same time each day. In an automatic milking system this may not be possible and more than one sample per day should be taken for analysis.

BHB in milk has also been investigated as a potential metabolic indicator. However, it is present at very low concentrations and is not highly correlated with blood concentrations since it has a role in milk fat synthesis in the mammary gland (Whitaker, 2000). A study by Shultz and Myres (1959) found that increases in blood BHB concentrations were not accompanied by a corresponding increase in BHB in milk. Therefore milk BHB may not be a reliable indicator of energy status. A large amount of variation between individual animals has been reported for BHB concentrations in milk (Hamann and Kromker, 1997). It is possible that milk acetone is also highly variable between cows although Anderson (1984) and Andersson and Lundström (1984b) found no significant effect of cow on milk acetone.

1.4.1.1. Normal ranges of acetone in milk

Various critical levels of acetone in blood and milk have been used to define subclinical ketosis. Typical ranges of milk acetone are between 0 and 2mM (Hansen, 1999) although in cows not suffering from subclinical or clinical ketosis the concentrations are nearer the lower end of the scale (Steen et al, 1996b). Milk acetone has been classified into three categories based on borderline concentrations; ≤0.7mM (normal), 0.7-1.4mM (moderate) and >1.4mM (high) (Gustafsson, 1993). Milk acetone less than 0.7mM has been considered as normal for non-ketotic cows. Concentrations between 0.7 and 1.4mM are indicative of subclinical ketosis and levels over 1.4mM are expected in clinically ketotic cows (Gustafsson and Emanuelson, 1996). However, Andersson (1984) found that the lowest concentration of milk acetone plus acetoacetate for cows showing clinical signs was 1.03mM and that the highest value for cows showing no symptoms was 2.20mM. This indicates that significant variation may exist between cows in their threshold concentrations for symptoms of ketosis.

1.4.1.2. Milk acetone and cow productivity

Elevated milk acetone concentrations are detrimental to productivity, being correlated with subclinical and clinical ketosis, as well as milk yield and reproductive performance (Miettinen, 1994; Gustafsson and Emanuelson, 1996; Cook, 1999; Reist et al, 2000). Gustafsson (1993) found that hyperketonaemic cows with milk acetone concentrations greater than 2mM produced abnormal lactation curves with inverted peaks. Much of the loss in milk yield occurred in the fourth to seventh week of lactation, which is when peak yield is normally reached. Cows with a flattened lactation curve and a low peak in milk yield may not be experiencing considerable metabolic stress in early lactation and this may be considered as a positive event. However, it does indicate a ketosis problem and therefore a potential loss in production (Gustafsson, Andersson and Emanuelson, 1993). Milk yield depression is most likely to be due to reduced glucose supply to the mammary gland since only glucose can be used to synthesise lactose and lactose is the major determinant of milk volume secreted.

Although the majority of cows experiencing subclinical ketosis and hyperketonaemia spontaneously recover, many have reduced milk yields or do not achieve their genetic potential for milk production. Gustafsson and Emanuelson (1996) suggested that milk yields may only be slightly depressed during subclinical ketosis, when milk acetone concentrations are between 0.7 to 1.4mM, with a proportional reduction of 0.1 from weeks two to weeks five to six of lactation. However, no losses in long-term milk yield were evident and responses were inconsistent. With milk acetone concentrations greater than 1.4mM, reductions in yield can be as much as ten to 20% (Gustafsson and Emanuelson, 1996). Miettinen (1994) found that in non-clinically ketotic cows, a two to nine percent reduction in milk yield was evident compared with 26% in clinically ketotic cows and suggested that milk acetone concentrations should not exceed 0.05mM if maximal potential milk yield is to be reached.

A reduction in milk protein content has also been associated with subclinical ketosis (Emanuelson and Andersson, 1986). Since metabolisable energy has a significant effect on milk protein content (Beever, Sutton and Reynolds, 2001), it is reasonable to assume that elevated milk acetone concentrations, indicative of negative energy balance or inadequate dietary energy, will be accompanied by a reduction in milk

protein content. Miettinen and Setälä (1993) found an inverse relationship between milk acetone and milk yield. At three to four weeks postpartum, mean acetone concentration and mean protein contents were 0.43mM and 30.4g/kg respectively. However an increase in mean acetone concentration to 0.66mM was associated with a reduction in milk protein percentage to 2.96%.

Milk analysis for acetone could not only identify cows in negative energy balance or with a dietary energy deficiency, but may also be useful to identify cows with potentially reduced fertility. Hyperketonaemia has been associated with longer calving to conception intervals (Cook et al 2001; Reist et al, 2002), number of inseminations per cow (Andersson, Gustafsson and Emanuelson, 1991) and increased risk of cystic ovaries (Andersson and Emanuelson, 1985). Cows exhibiting acetone concentrations over 1.4mM had on average five days longer from calving to first service and a reduction in milk yield of 190kg over both 100-day and 200-day milk yields, compared with cows with milk acetone levels less than 0.7mM (Gustafsson and Emanuelson, 1996).

1.4.1.3. Factors affecting milk acetone

Although milk acetone may be useful for assessment of metabolic status and energy balance, several factors affect milk acetone concentrations, which are important to consider when interpreting data from individual cows. Milk acetone concentrations are greatly influenced by stage of lactation. Andersson and Emanuelson (1985) reported that milk ketones are commonly high during the second to sixth week of lactation and peak at three to four weeks, which coincides with peak milk yield. Because milk acetone is related to the degree of hyperketonaemia, highest concentrations will be found in early lactation when the cow is in a negative energy balance and the risk of ketosis is highest. According to Duffield et al (1997), the risk of developing subclinical ketosis was four times greater in early lactation than in late lactation.

Most cows are in a negative energy balance for the first eight weeks of lactation, being most severe in weeks two to five (Heuer, Schukken and Dobbelaar, 1999). However, individual variation in negative energy balance between cows can be considerable. De Vries and Veerkamp (2000) found that 17.5 % of cows in their

study did not experience negative energy balance during the first 180 days of lactation. It has been suggested that, if cows are in appropriate body condition at 2.5 and are well fed in early lactation, any adverse effect of negative energy balance tends not to be significant on milk production or fertility (Crooker 1997). However, adequacy of feeding cannot be assessed because no dietary information was presented in this reference.

Parity has also been shown to have an effect on acetone with a greater risk of cows with high acetone concentrations in parity two than in parity one (Gustafsson, 1993). Generally, the risk of ketosis is low in parity one and increases with lactation number (Kauppinen, 1983; Andersson and Emanuelson, 1985). Cows in their first lactation have been shown to have a 60% lower risk of subclinical ketosis than cows in their fourth lactation (Duffield et al, 1997). However, a study by Heuer et al (2000) showed that first lactation cows are in a greater negative energy balance than cows in their second or third lactations, as measured by their milk fat:protein ratio. Cows might be expected to experience a greater negative energy balance in early lactation than heifers, which have lower peak milk yields and more persistent lactation curves (Wood, 1969). On the other hand, heifers have additional requirements for growth and may have lower feed intakes due to their size and the possibility of them being bullied when feeding. These factors, as well as variation between individuals and differences in milk yield, may account for differences between studies.

Genetic improvement in milk yield over the last 20 years has been considerable. Simm (1997) stated that cows born in 1993 will have produced on average 450kg of milk more than those in 1989, based on predicted breeding values. The increase in milk yield in recent years may account for the contradiction in the literature as to whether 1st lactation cows are in a greater negative energy balance than cows in later lactations.

Genetic potential for milk yield may be an influencing factor, with high yielding cows being more likely to experience a greater negative energy balance in early lactation, and therefore with a higher risk of subclinical ketosis. Genetically superior dairy cows may have a higher demand for glucose and a greater metabolic capacity to mobilise body fat (Gravert et al, 1991, cited by Miettinen, 1994), which may result

in higher blood NEFAs and greater production of ketone bodies. Positive relationships between hyperketonaemia and milk yield have been reported (Kauppinen, 1983) and a significant positive partial correlation between the highest individual milk acetone concentration and the highest individual milk yield was found by Andersson and Emanuelson (1985). It should be noted that differences in the relationship between acetone and milk yield between studies may not only be due to differences in genetics and nutrition. Management inputs have a major influence on milk yield and these will vary considerably between herds.

Good nutritional management is important to minimise the risk of ketosis. The risk of ketosis decreases with increasing frequency of concentrate feeding (Andersson et al, 1991). This finding is supported by Gustafsson (1993) who reported that the risk of hyperketonaemia was greater in herds receiving concentrate feeds twice a day than when fed concentrate four times a day. A higher feeding frequency may improve nutrient supply as a result of rumen fermentation being more stable (Gustafsson, 1993). Inferior silage quality with a high level of butyric acid has been linked with clinical ketosis by depressing feed intake (Andersson and Lundström, 1985).

The risk of ketosis may vary according to season. The incidence tends to be higher in winter (Andersson and Emanuelson, 1985) although the incidence of hyperketonaemia is more likely to be related to seasonal changes in nutrition than seasonal effects. Cook (1999) found a greater risk of ketosis in cows grazing late in the grazing season despite access to total mixed ration (TMR) feeding. The risk of ketosis was greater in high producing cows yielding in excess of 8000 litres per lactation. This study suggests that the adequacy of feeding could be determined through the use of milk acetone testing at this time of year, when pasture forage is decreasing in both quantity and quality. Herd factors also significantly influence the incidence of hyperketonaemia (Andersson and Emanuelson, 1985), with the risk being dependent on management and nutritional factors which will vary between herds.

An on-line monitoring system for acetone would be useful as subclinical ketosis usually goes undetected and therefore untreated, yet has detrimental effects on productivity. Analysis of acetone may be a useful and economical means for

detecting hyperketonaemia and ketosis in commercial dairy herds so that corrective action could be taken before milk yield and reproductive performance are compromised. However, individual variation may be substantial and threshold levels to indicate subclinical and clinical ketosis may differ between cows depending on their level of milk production. Therefore it is important that normal ranges for each cow are determined so that hyperketonaemia can be accurately diagnosed.

1.4.2. Urea

Urea is the metabolic end product of protein catabolism in ruminant tissues and by microorganisms in the rumen. It is a small organic molecule commonly found in the blood and other body fluids. Protein digestion within the rumen and protein catabolism in ruminant tissues releases ammonia and absorbed ammonia is detoxified in the liver to urea and can be either absorbed into the bloodstream or utilised by microbes. Excess urea is excreted in the urine and blood urea can also be recycled via the saliva (Moore and Varga, 1996).

Urea is a normal constituent of bovine milk and forms part of the non-protein nitrogen (NPN) fraction. It is measured by its nitrogen content and milk urea and blood urea are commonly referred to as milk urea nitrogen (MUN) and blood urea nitrogen (BUN) respectively. Urea freely diffuses across the mammary tissue from the blood and equilibrates in milk with a lag time of less than one hour (Butler, 1998). Concentrations of urea in blood and milk tend to be similar and a mean difference of 0.8mM has been reported between serum and milk urea (Gustafsson and Palmquist, 1993). Therefore the correlation between blood urea and milk urea is high and monitoring urea in milk is a useful and non-invasive method for assessing protein metabolism and dietary efficiency. Correlations between plasma urea and milk urea have been reported to be as high as 0.98 (Oltner and Wiktorsson, 1983).

In the literature, the terms "milk urea" and "MUN" have been used interchangeably although MUN strictly refers to the nitrogen content of urea only (urea concentration x 0.466), since urea is composed of 46.6% nitrogen. North American research tends to refer to MUN, whereas European and Australasian studies usually refer to milk or blood urea (Westwood, Lean and Kellaway, 1998). However, urea and MUN results

in the literature appear to be comparable and different terms used in this thesis will be due to the terms used in the literature cited. Measurements made throughout the experimental work in this thesis will be reported as urea.

1.4.2.1. Normal ranges of urea in milk

Much of the work on milk urea has been carried out in Sweden and the USA where it has been widely used to assess nutritional status in dairy cows. Despite differences in the breed of cows used in these studies, defined normal concentrations of milk urea are similar. Gustafsson (1993) suggested that optimum urea concentrations in early lactation were 3.5 to 5.5mM and 3.0 to 6.5mM in mid to late lactation and outside these concentrations a dietary adjustment was recommended. Jonker, Kohn and Erdman (1999) developed a model that took into account factors such as protein intake and milk production, to predict target values of MUN when cows were fed according to National Research Council requirements. For a typical lactation, target concentrations were calculated at 10 to 16mg/dl or 3.56 to 5.70mM. However, many of the values in the literature are based on group means. Due to between cow variation, concentrations outside this range may be detected in individual cows. Higher urea concentrations can be expected in summer when cows are grazing due to the high content of rapidly degradable crude protein in grass in relation to its energy content (Carlsson, Bergström and Pehrson, 1995).

1.4.2.2. Interpretation of milk urea profiles

Monitoring urea in milk provides a rapid, non-invasive and inexpensive method of monitoring protein metabolism and energy supply in dairy cows (Table 1.2). Urea is an indicator of protein status, varying according to crude protein intake (Oltner and Wiktorsson, 1983; Payne and Payne, 1987; Carlsson and Pehrson, 1994). Urea concentration also indicates the ratio between protein and energy in the diet and is thought to be influenced more by the balance of protein and energy than by the composition of a feed (Oltner, Emanuelson and Wiktorsson, 1985). However, because milk yield is primarily determined by dietary protein and energy, high milk yields may indirectly affect urea concentrations by a dilution effect (Gustafsson, 1993).

Table 1.2. Interpretation of milk urea concentrations (Gustafsson, 1993)

		Urea Concentration (mM)		
Stage of lactation	*Days*	*Low*	*Moderate*	*High*
Reproductive period	51 - 110	< 3.5 Protein restricted	3.5 - 5.5	> 5.5 Excess protein &/or energy restricted
Mid to late lactation	> 110	< 3.0 Protein restricted	3.0 - 6.6	> 6.5 Excess protein &/or energy restricted

Milk urea may be raised when the diet contains an excess of rumen degradable protein (RDP) and/or if energy is limited for ammonia utilisation by microbes (Pehrson, 1996). An imbalance of RDP and rumen undegradable protein (RUP) will also influence MUN (Baker, Ferguson and Chalupa, 1995; Jonker et al, 1999). In contrast urea should decrease if there is a protein deficiency and/or if the diet is high in energy in relation to protein (Pehrson, 1996). MUN concentrations less than normal may be due to excess carbohydrate in relation to protein, which may inhibit microbial growth due to insufficient production of ammonia within the rumen (Moore and Varga, 1996). Low milk urea indicates that extra RDP would be beneficial if higher milk yields are desired and are not restricted for management reasons. High urea implies that the diet contains too much RDP in relation to fermentable metabolisable energy (FME) and either RDP is in excess or FME is deficient. If FME is deficient, the diet may negatively affect milk yield and/or fertility, whereas excess RDP is wasteful in economic terms. In theory, increasing dietary FME is the best course of action so that spare protein is utilised. If dietary RDP is reduced a reduction in milk yield is likely (Whitaker, Kelly and Eayres, 1995).

1.4.2.3 Urea and reproductive performance

It is important to monitor urea to optimise nutrition and productivity. However, monitoring urea may also be useful to identify cows that may have poorer reproductive performance as a result of feeding excessive protein to maintain high milk production (Elrod and Butler, 1993). Several studies have shown that high

MUN (greater than 6.0 to 6.5mM) is related to reduced fertility (Butler, Calaman and Beam, 1996; Butler, 1998; Mendelez, Donovan and Hernandez, 2000). This effect may be due to feeding crude protein levels of 170 to 190g/kg dry matter or greater (Butler, 1998), which increases ammonia production in the rumen, decreases uterine pH and alters the composition of uterine fluid (Elrod and Butler, 1993). Jordan et al (1983) found that feeding diets with a crude protein content of 23% altered the ionic composition of uterine secretions through changes in magnesium, potassium and phosphorus concentrations. Urea is also spermicidal and depresses sperm motility (Umezaki and Fordney-Settlage, 1975).

Butler et al (1996), reported that when PUN and MUN were greater than 6.8mM, pregnancy rates were reduced by approximately 20% ($P<0.02$). Other studies have reported that fertility is not significantly affected by excess dietary protein or high MUN concentrations (Trevaskis and Fulkerson, 1999; Cottrill et al, 2002). Gustafsson and Carlsson (1993) suggested that optimal fertility could be achieved when milk urea is between 4.5 and 5.0mM. Clearly there are herd differences and differences between studies relating to diet composition in terms of the protein to energy ratio and the proportion of RDP to RUP that may account for various critical levels of urea and their relation to fertility. Differences in management factors between studies may also account for the different relationships reported between milk urea and fertility, such as oestrus detection methods, detection rate and timing of insemination in relation to when oestrus was detected.

1.4.2.4 Factors affecting milk urea

Urea is a very useful analyte to measure in milk since its use as an indicator of nutritional status has been studied in great detail. However, the concentration of urea in milk is affected by several factors which should be taken into account when interpreting urea profiles.

Individual variation between cows in milk urea concentrations can be considerable. Oltner et al (1985) found that concentrations varied by as much as 2 to 3mM between cows on the same diet and feed intake. The authors suggested that urea determined from a single milk sample from an individual cow was not a useful predictor of nutritional status. Since most studies have investigated the use of urea as an indicator

of feeding efficiency at the group or herd level, it is recommended that mean milk urea values from groups of cows should be used to make assumptions on herd feeding efficiency, due to variation between cows (Whitaker 2000; Jenkins et al, 2002b). However, these authors did not say whether making nutritional management decisions for individual cows, based on their milk urea concentration is feasible. This emphasises the importance of establishing normal baseline concentrations of urea for all cows if metabolic profiling and feeding efficiency are to be assessed on an individual cow basis.

Urea shows considerable diurnal variation, which is mainly related to the time of feeding relative to milk sampling. Gustafsson and Palmquist (1993) observed that blood urea increased from approximately 6 to 8mM in one cow after a single daily feed. Urea concentrations in milk tend to peak two to four hours after cows start to feed (Gustafsson, 1993; Gustafsson and Palmquist, 1993). These findings are similar to a study by Carlsson and Bergstrom (1994) who found that milk urea varied significantly throughout the day, with highest values occurring three to five hours after the start of morning feeding, while the lowest concentrations were found between late evening and early morning, which were 60% of maximum values.

However, when investigating the effect of sampling time, Hoff et al (1997) found no consistent differences in MUN between AM and PM samples. Feeding frequency may be an important factor affecting diurnal variation. From reviewing the literature, Eicher, Bouchard and Tremblay (1999) concluded that diurnal patterns of urea are absent in herds with higher feeding frequencies. According to their hypothesis, a more constant dietary input of readily degradable protein is achieved with more frequent concentrate meals. Ammonia production within the rumen is more constant, resulting in a more continuous synthesis of urea. It can be hypothesised that there is an absence of significant diurnal variation in milk urea with *ad libitum* TMR feeding. However, it was not possible to test this theory in the studies reported in this thesis.

Daily variation in urea may also occur. Blood urea has been shown to vary on a daily basis due to changes in the diet, weather, or stress of the sampling procedure (Payne and Payne, 1987). Ideally sampling should be fixed at a specific time after feeding each day. However feeding times are not known in many systems and in automatic

milking systems milking times are not fixed. In this situation, measurements from a few consecutive days should be considered when assessing nutritional status.

Urea concentrations can also vary significantly with season, reflecting the variability in type and quality of dietary nitrogen intake. For example, cows fed on high energy rations in winter show considerably lower concentrations of urea in blood than when on summer grazing where the diet contains highly degradable protein from grass (Moller, Matthew and Wilson, 1993; Carlsson et al, 1995).

Lactation stage is also an influencing factor, with urea concentrations being lower in the first few weeks after calving compared with late lactation. This effect may be due to changes in the dietary protein to energy ratio (Gustafsson, 1993) and partly a result of changes in nutrient demands at various stages of lactation (Bruckental, Oldham and Sutton, 1980). Godden et al (2001) found that concentrations of milk urea were lowest in the first 60 days after calving, increasing to a maximum between days 60 and 150 and decreasing after 150 days. This suggests that if urea is to be used as an indicator of dietary efficiency, corrections should be made according to stage of lactation. However, Schepers and Meijer (1998) found that there was little influence of stage of lactation or parity on milk urea when feeding experiments were controlled for energy and protein balance. Higher urea concentrations in later lactation are more acceptable than in early lactation, since the cow is normally pregnant at this stage and the risk of fertility problems is low (Gustafsson, 1993).

Some studies have shown milk urea to be lower in first parity cows than multiparous cows (Oltner et al, 1985; Gustafsson, 1993; Godden et al, 2001). This could be due to bodyweight gain in first lactation cows, with lean tissue growth being correlated with greater efficiency of amino acid utilisation for protein synthesis and therefore reduced availability of amino acids for gluconeogenesis (Gustafsson, 1993). However Jonker, Kohn and Erdman (1998) found that MUN was higher in first lactation cows than in mature cows. Higher body weight has been correlated with lower milk urea concentrations (Oltner et al, 1985; Gustafsson, 1993) possibly due to a dilution effect where heavier cows have a greater volume for urea distribution compared with cows of a lower liveweight. Therefore the concentration of urea in blood or milk will be lower in a heavier animal if the same amount of urea is formed

in the liver. Oltner et al (1985) found that a 100kg increase in body weight was associated with a 0.6mM decrease in urea concentration. Higher rates of renal clearance of urea are also likely in heavier cows (Swensen and Reece, 1993 cited by Jonker et al, 1998).

The level of milk production may also affect urea concentration. Slightly higher urea concentrations have generally been found in higher yielding cows (Trevaskis and Fulkerson, 1999; Godden et al, 2001), possibly due to increasing dietary crude protein required for high milk production (Kaufmann, 1982 cited by Carlsson et al, 1995; Chalupa, 1984). The factors influencing urea are many and again, this stresses the importance of establishing normal ranges for milk urea per cow if nutritional improvements are to be made on an individual cow basis.

1.4.3. Fat

Fat is the most variable constituent of milk and fat content varies both within breed and between breeds due to differences in genetic potential. Typically milk fat ranges from 38 to 49g/kg (Svennersten-Sjaunja et al, 1997), with milk from Holstein cows having the lowest fat and protein contents and milk from Jersey cows containing the highest amount of fat and protein in breeds found in the UK.

1.4.3.1. Composition of milk fat

Milk fat is composed mainly of triacylglycerols with a wide range of saturated and unsaturated fatty acids (FAs). The rest of the fat is composed of small amounts of phospholipid, mono- and diacylglycerols and steroids. Milk fat contains a wide range of FAs which can be classified into three groups based on their chain length of carbon atoms; short, medium and long chain FAs. There are two main sources of FAs for milk fat synthesis. Short chain FAs (C:4 - C:6) and medium chain FAs (C:8 - C:14) are synthesised *de novo* in the mammary gland from acetate and â-hydroxybutyrate, and the long chain FAs (C16: plus) are taken up by the mammary gland as preformed units in the blood. These may be of dietary origin or from the breakdown of adipose tissue (Moore and Christie, 1979). C16 may be produced within the gland or originate from the blood. Approximately equal quantities of FAs are synthesised *de novo* and from blood lipoproteins. The predominant FAs

(myristic, palmitic and stearic) are long chain FAs contributing 75% of the total FAs in milk. Monunsaturated FAs make up 21%, with oleic acid being the most abundant. The remaining four percent comes from polyunsaturated FAs, which are present mainly as linoleic and linolenic (Mansbridge and Blake, 1997).

In milk it is not possible to identify which specific long-chain FAs originate from adipose tissue or the diet, due to modification of FAs within the rumen and the mammary gland. Triacylglycerols are almost completely hydrolysed by lipoprotein lipase as they pass into the mammary gland (Mansbridge and Blake, 1997). The breakdown products of glycerol and FAs are taken up by the secretory cells and re-esterified to triacylglycerols before being incorporated into milk FAs. In the rumen bacterial lipases hydrolyse triglyceride ester linkages and, once released, the unsaturated FAs undergo saturation by a process called biohydrogenation. However, some FAs escape biohydrogenation and are used in the synthesis of microbial lipids. In addition, some FAs are desaturated in adipose tissue and in the mammary gland so that approximately a 0.1 proportion of the total fatty acid content in milk is unsaturated (Garnsworthy, 1997).

1.4.3.2. Interpretation of milk fat data

Since milk fat is highly responsive to changes in nutrition, it may be useful to assess dietary adequacy. Milk fat content may be used to assess whether there is adequate fibre in the diet, with high milk fat indicating a high fibre diet and low milk fat being typical of high concentrate diets. The dietary component which has the greatest influence on milk fat composition is the amount and composition of dietary fat (Palmquist, Beaulieu and Barbano, 1993). Increasing dietary fat may either increase or decrease milk fat concentration depending on the effect in the rumen (Garnsworthy, 1997), the level of supplementation and the type of dietary fat and whether it is saturated, unsaturated or protected against ruminal biohydrogenation (Thomas and Chamberlain, 1984).

Milk fat could be used to assess energy balance in conjunction with acetone, as high milk fat has been linked with a high incidence of ketosis, with a positive correlation between milk fat and milk acetone concentrations (Simensen et al, 1988; Miettinen and Setälä, 1993)

Fatty acid composition may also give important information about energy balance and whether the cow is mobilising excessive body reserves. Since short and long chain FAs originate from the mammary gland and the blood respectively, a cow's metabolic status will be indicated by the proportions of short and long chain FAs. Consequently, adipose tissue mobilisation will be indicated by an increase in the proportion of long chain and unsaturated FAs in milk fat (Svennersten-Sjaunja et al, 1997). Miettinen and Huhtanen (1989) found a positive correlation between ME (metabolisable energy) balance and the proportion of short and medium chain FAs synthesised within the mammary gland. There was a negative correlation between the proportion of C18 FAs and ME balance and these were also found to be positively correlated with β-hydroxybutyrate, NEFAs and acetoacetate. They further stated that a decreasing ME balance and increases in palmitic, oleic and C18 FAs in milk are related to adipose tissue mobilisation and that information on milk fatty acid composition is a useful indicator of energy status as long as dietary fat content remains constant.

Although changes are observed in early lactation in milk fatty acid composition, which are related to the degree of adipose tissue mobilisation and inhibition of *de novo* fatty acid synthesis, there does not appear to be a specific fatty acid that could be a useful marker of energy balance or nutritional status of the dairy cow. It was hoped that specific FAs could be useful in monitoring the nutritional status of the cow. The direct transfer of dietary fat to milk fat is influenced by three main factors, these being ruminal biohydrogenation, absorption or digestibility, and deposition in adipose tissue (Palmquist et al, 1993). Because of these processes within the rumen and in adipose tissue, it is not possible to estimate the transfer of individual or total FAs from the diet into milk (Wu, Ohajuruka and Palmquist, 1991).

1.4.3.3. Factors affecting milk fat content

There are many factors that influence milk fat such as nutrition, lactation stage, length of milking interval, completeness of milking, age, season and body condition score at calving. These may be important to consider when interpreting daily milk fat measurements.

Stage of lactation has a strong influence on milk fat yield and composition. Fat yield is inversely proportional to milk yield, reaching a minimum at peak yield and gradually increasing towards the end of lactation. The greatest variation in fat content occurs between 60 and 120 days of lactation which may be due to peak milk yield being reached and extensive adipose tissue mobilisation (Lee, 1988). In early lactation the proportion of short chain FAs is low, except for C4, and then increases with maximum proportions of over 90% at eight to ten weeks of lactation (Palmquist et al, 1993). Because cows are in a negative energy balance at the beginning of lactation, FAs are mobilised from adipose tissue and incorporated into the long chain FAs of milk fat. Consequently, short chain fatty acid synthesis is inhibited by the mammary gland due to the high uptake of long chain FAs. Short chain fatty acid synthesis is inhibited to varying degrees, with inhibition increasing from C6 to C12 (Palmquist et al, 1993).

The milking interval influences milk fat content at morning and afternoon milkings. If high yielding cows are milked twice a day at different intervals, the fat content is always higher after the shorter interval due to a lower milk yield. Because fat is expelled from the mammary gland with more difficulty than the rest of the milk, fat content increases during the course of milking, with the first half litre containing not more than 10g fat/kg and the last half litre containing 100 to 150g fat/kg. Residual milk in the udder may contain as much as 200g fat/kg. This is partly due to the gravitational effect and partly due to the fat droplets moving at a slower rate during the milk ejection process as a result of their size and viscosity (Nickerson, 1995). As a result, completeness of milking will also influence milk fat content, with incomplete milking resulting in a lower milk fat content.

Large daily variations in milk fat exist (Lee, 1988; Rook, Fisher and Sutton, 1992). Atwal and Erfle (1990) found gradual changes in milk fat from day-to-day with these patterns being almost cyclical and exhibiting a five to seven day wavelength. The authors suggested that these daily variations were a result of metabolic regulatory processes of lipolysis in adipose tissue.

Parity has an effect on milk composition, with fat content tending to remain relatively constant in the first four lactations and then gradually decreasing with

parity (M^{c}Donald et al, 1995). Seasonal effects on milk composition are also evident (Auldist, Walsh and Thomson, 1998), but these are more likely to be due to dietary changes, such as moving from a pasture-based diet in the summer to a high-energy TMR winter ration, than changes in the weather. The authors found a significant interaction between stage of lactation and season on milk fat yield, with a greater effect of lactation stage in winter than in summer.

The effect of body condition score at calving on milk composition is usually small and insignificant (Table 1.3). Generally, cows that calve at a higher condition score tend to produce milk with a higher fat content and lower protein and lactose contents (Garnsworthy, 1988).

Table 1.3. The effect of body condition score at calving on milk composition (from Land and Leaver, 1981)

	Period of Lactation (weeks)	Condition Score at Calving	Fat (g/kg)	Protein (g/kg)	Lactose (g/kg)
Cows	1-16	2.6	38.6	31.9	49.1
		3.5	39.5	30.6	48.8
Heifers	1-8	2.6	39.8	34.3	49.0
		3.1	41.5	33.6	48.9

1.4.4. Protein

Milk protein content is less variable than fat with the relative daily variation in protein content being estimated at 1.5 to two percent (Svennersten-Sjaunja et al, 1997). The range of protein in bovine milk ranges from 30 to 36g/kg although this varies with several factors (Svennersten-Sjaunja et al, 1997).

1.4.4.1. Composition of milk protein

Milk protein is derived from two sources; proteins may be synthesised in the mammary gland or they may be derived from the blood (DePeters and Ferguson, 1992). There are two distinct types of milk proteins; the caseins and the whey or serum proteins. The caseins are the most abundant milk proteins, accounting for over

80% of the total protein in milk, although stage of lactation affects the relative proportion of whey proteins to casein (Varnam and Sutherland, 1994). The whey proteins are comprised of β-lactoglobulins α-lactalbumins, immunoglobulins and serum albumin (DePeters and Cant, 1992). The major milk proteins (caseins, β-lactoglobulin and α-lactalbumin) are only synthesised within mammary epithelial cells (DePeters and Cant, 1992; DePeters and Ferguson, 1992) and are unlikely to be useful indicators of the cow's metabolic status since they do not reflect proteins present in the blood.

Milk proteins are composed of amino acids derived from the blood. However, the proportion of amino acids present in milk proteins has been found to differ from those absorbed from circulation. Amino acids absorbed from the blood undergo considerable inter-conversion before milk protein is synthesised. For example, the mammary gland absorbs large quantities of ornithine, which does not appear as milk protein. Instead it is used as a precursor for proline, glutamate and aspartate, and within the mammary gland, serine and threonine are phosphorylated before being incorporated into caseins (M^{c}Donald et al, 1995).

The use of casein as a diagnostic tool is limited as it is influenced by lactation stage, parity, season and incidence of mastitis, with higher casein contents in early lactation and reaching a minimum concentration at two months (Ng-Kwai-Hang et al, 1982). Genetic effects also exist with individual cows showing significant variation in their milk casein composition, with the relative proportions of α-, β- and γ-casein showing genetic variation (Varnam and Sutherland, 1994).

Albumin has been considered as a potential indicator in milk for assessing health and nutritional status. It is synthesised by the liver, so albumin concentrations in blood reflect liver health or a poor dietary supply of amino acids. However, significant changes in serum albumin are only apparent with severe long-term underfeeding or significant disease (Whitaker, 2000), so milk albumin would not be a useful indicator of feeding efficiency. Therefore, there does not appear to be a specific milk protein or amino acid that may be useful to monitor protein status in the cow. The total protein content of milk is more likely to be useful to assess nutritional status, since it

is highly influenced by the energy content of the diet (Thomas and Chamberlain, 1984; Beever et al, 2001).

1.4.4.2. Interpretation of milk protein data

Milk protein content is responsive to changes in nutrition although the impact of dietary manipulation is smaller than that seen in milk fat content (Sutton, 1989). The value of measuring milk protein is to evaluate the energy supply to the cow since an energy deficiency would be reflected in low milk protein (Kaufmann, 1976 cited by Svennersten-Sjaunja et al, 1997). Milk protein yield is partly dependent on metabolisable protein supply with inadequate dietary protein resulting in a 1 to 2g/kg decrease in milk protein content (Schingoethe, 1996). Milk protein content, as well as milk yield, was found to be increased by feeding readily fermentable carbohydrates (present in cereal grains, young spring grass, molasses and compound feeds), which stimulate the production of propionic acid in the rumen (Jurjanz et al, 1998). The addition of dietary fat tends to reduce milk protein although fat prills have been found to have a beneficial effect on milk protein (Garnsworthy, 1997).

1.4.4.3. Factors affecting milk protein content

Like milk fat, several non-nutritional factors influence the content of protein in milk. Milk protein varies with stage of lactation, gradually decreasing in early lactation and reaching its lowest concentration at peak milk yield. Protein then slowly rises towards the end of lactation. Seasonal effects are also present, mainly due to temperature extremes with lower milk protein during the warmer months (Ng-Kwai-Hang et al, 1982). Like milk fat, protein content increases with parity, reaching a peak in the third lactation and then declines with age (DePeters and Cant, 1992). Mastitis also influences milk composition with subclinical mastitis increasing some of the whey proteins and γ-casein in the total protein (Urech, Puhan and Schallibaum, 1999). Generally the protein content of milk tends to increase with the onset of mastitis, with a 0.15g/kg increase and a corresponding 0.20g/kg decrease in milk fat content with a two-fold increase in somatic cell count (Hortet and Seegers, 1998). The effects of mastitis tend to be short-term, but without treatment effects on milk composition can overlap into the subsequent lactation (Seegers, Fourichon and Beaudeau, 2003).

1.4.5. Fat:Protein Ratio

As dietary factors usually influence milk fat and protein in opposite directions the ratio of fat to protein in milk may be a more sensitive indicator of energy balance than if they are used separately to monitor the cow (Grieve et al, 1986). In response to a low energy balance, there is an instant increase in the fat:protein ratio. The relationship between fat concentration and energy balance can be explained by adipose tissue mobilisation. An energy deficit in early lactation results in increased mobilisation of body fat and an increase in free FAs and acetyl-CoA in the blood, with more fat being produced by the udder. At the same time an energy deficit reduces the rate at which bacterial protein is synthesised and there is a decrease in supply of amino acids to the mammary gland, thereby reducing milk protein concentration (Steen et al, 1996a). De Vries and Veerkamp (2000) proposed that since changes in protein percentage are only weakly correlated with measures of energy balance, most of the information in the changing fat:protein ratio is derived from the rise in fat concentration with increasing negative energy balance.

Both fat and protein concentration are significantly related to the risk of subclinical ketosis, with the risk increasing over two times with a 10g/kg rise in fat concentration, and the risk being decreased by over 50% with a 10g/kg increase in protein concentration (Duffield et al, 1997). A study by Heuer et al (2000) showed that the fat: protein ratio detected a low energy balance better than cowside tests for β-hydroxybutyrate and acetoacetate. Cows in negative energy balance may more often exhibit an elevation in the milk fat:protein ratio than an increase in ketone bodies in blood or milk. This may be due to the fact that mild cases of ketosis tend to be self-limiting due to a reduction in milk yield.

A fat:protein ratio less than 1.4 is considered to be optimal, with the cow being in positive energy balance, while an energy deficit may be indicated by values higher than 1.4 (Pehrson, 1996). Heuer et al (1999) stated that a fat:protein ratio greater than 1.5 increased the risk of metabolic diseases, with a ratio greater than 1.5 increasing the risk of developing ketosis, cystic ovaries, displaced abomasum, lameness and mastitis. Compared with body condition score or body condition score loss, the fat:protein ratio was a more reliable indicator of disease, milk yield and fertility. Care

must be taken when interpreting results for fat:protein ratio because both of these parameters show considerable variation with stage of lactation and level of milk production.

1.4.6. Fat:Lactose Ratio

Lactose is the main carbohydrate present in milk and is formed from glucose and galactose. Nearly all of the glucose for lactose synthesis is derived from the blood. Most of the galactose originates from blood glucose although small amounts of acetate and glycerol also contribute (M^{c}Donald et al, 1995). Lactose is the principle osmoregulator in milk, controlling the amount of water secreted and hence the quantity of milk secreted. Typical concentrations in milk range from 46 to 48g/kg but generally lactose tends to vary very little due to its osmotic regulatory effect (Svennersten-Sjaunja et al, 1997). Lactose varies in concentration throughout lactation, increasing in early lactation and then declining after peak yield (M^{c}Donald et al, 1995). Other influencing factors include mastitis and age. Nutrition has little effect unless milk yield is reduced by underfeeding, with a slight drop in lactose content being observed (Pehrson, 1996).

The lactose content of milk is fairly constant and would therefore not be a good indicator of nutritional status or cow health. Pehrson (1996) stated that lactose was not a useful indicator of metabolic or nutritional problems since any changes in milk lactose as a result of malnutrition tend to be very small. However, the fat:lactose ratio could be used in a milk profile test as further evidence of subclinical ketosis. A study by Steen et al (1996a) found an association between increasing acetone class and decreased lactose content in milk. Only plasma glucose can be used to synthesise lactose and blood glucose concentrations tend to be low in ketotic cows (Rossow et al, 1991). Consequently milk lactose will be low when acetone is higher than normal. The rate of lactose synthesis is a major determinant of milk volume due to its osmotic properties (Sutton, 1989) and hence milk yield was reduced in cows with elevated acetone.

Energy balance at the individual and herd level were estimated using blood and milk traits in high yielding dairy cows by Reist et al (2002). The most useful trait for

estimating energy balance in milk was the fat:lactose ratio. Surprisingly, acetone was the milk parameter that had the weakest correlation (r = -0.41) while energy balance and fat:lactose ratio, fat and fat:protein ratio had stronger correlations. In individual cows the precision of estimating energy balance was low and was not precise enough for practical application. This is an unexpected result since much work has been carried out on milk acetone as an indicator of energy balance and hyperketonaemia in dairy cows (Emanuelson and Andersson, 1986; Gustafsson and Emanuelson, 1996; Enjalbert et al, 2001).

1.4.7. Citrate

Citrate is a naturally occurring compound in milk and is thought to be involved in maintaining the mineral equilibrium. It occurs in the aqueous phase, mostly in the form of calciocitrate, a fairly soluble complex with Ca^{2+} (Faulkner and Peaker, 1982). The concentration of citrate in milk is typically 7 to 9mM (Davies and White, 1960) and is important in the food processing industry because changes in milk citrate concentration may upset the mineral equilibrium and therefore coagulation of protein (Faulkner and Peaker, 1982). Also the growth of citrate fermenting bacteria in fermented dairy foods may be stimulated by changes in milk citrate content (Brendehaug and Abrahamsen, 1986). Although citrate is thought to originate within the mammary secretory cell and is not derived from citrate present in blood plasma (Faulkner and Peaker 1982), it may be a useful indicator of fatty acid origin, being correlated with *de novo* fatty acid synthesis (Banks et al, 1984a). It is inversely related to milk fat yield (Romo et al, 2000) and is correlated with milk acetone (Baticz et al, 2002), therefore being a potential indicator of energy balance in the dairy cow.

Milk citrate concentrations are affected by many factors. Variation in citrate arises from increased uptake of energy by the udder in lactation and changes in the synthesis of *de novo* FAs in the mammary gland (Kirst, Jacobi and Bauer, 1995). Since dietary changes occur throughout the lactation cycle of the dairy cow, and citrate is responsive to changes in nutrition, it is difficult to assess the effect of stage of lactation on citrate content (Faulkner and Peaker, 1982). Some studies have

reported an increase in citrate in early lactation with a steady decline towards the end of lactation (Konar, Thomas and Rook, 1971; Braunschweig and Puhan, 1999).

As lactation progresses the decrease in citrate may be a result of cytosolic changes in citrate in mammary epithelial cells, due to alterations in the rate of fatty acid synthesis (Faulkner and Peaker, 1982). In early lactation there may be relatively low short- and medium-chain fatty acid synthesis within the mammary gland in relation to total milk fat content, as mobilisation of adipose tissue provides lipids in the plasma for fat synthesis. With advancing lactation, metabolism shifts to utilise substrates entirely from the diet instead of body fat stores. Adipose tissue mobilisation declines and the extent of fatty acid synthesis within the mammary gland increases, reducing the citrate content in milk (Peaker, Faulkner and Blatchford, 1981).

Citrate is affected by short-term changes in nutrition with citrate increasing with high fat diets and decreasing with high concentrate diets (Ormrod, Thomas and Wheelock, 1979; Ormrod, Thomas and Wheelock, 1980). Citrate levels can be highly variable in milk. For example, a 48-hour period of starvation in goats resulted in two-fold increase in milk citrate concentration (Peaker and Faulkner, 1983). Therefore citrate may be useful as an indicator of reduced feed intake during incidences of mastitis or when cows are on heat. Presently there is not enough information on the use of citrate to monitor the fatty acid composition of milk fat. However, citrate is present in measurable amounts and can be easily analysed, making it a potential indicator for monitoring fatty acid origin and metabolic status.

1.5. BIOSENSORS

A biosensor is an analytical device consisting of a biological sensing element that is either closely connected to or integrated within a signal transducer. Basic requirements when developing a biosensor include selective identification of the target biological compound, with the chemical signal produced by biorecognition of the specific analyte being converted to a measurable signal. The signal is usually chemical and related to the concentration of the analyte (Koelsch, Aneshansley and

Butler, 1994). There are various affinity mechanisms that can be used for molecular recognition, the most commonly used being enzymes or antibodies (Powner and Yalcinkaya, 1997). Biosensors can achieve high selectivity and great sensitivity, measuring only the analyte of interest in complex media such as milk. Requirements include high sensitivity and specificity, speed, accuracy, reproducibility of measurements, cheap, easy to calibrate, and be able to withstand field conditions (Braguglia, 1998). Further advances in engineering are required in the field of sensors and biosensors to accurately analyse milk composition on-line.

The field of biosensors is rapidly expanding with applications in the medical, food, environment and agricultural sectors. Much of the research on biosensors in the agricultural industry is being applied to food quality for detecting unwanted microbial activity and biologically active compounds such as pesticides (Richter, 1993). Few sensors have been developed to analyse specific components in milk. Recently, a three-cascaded enzymes biosensor to determine lactose concentration in milk was developed (Eshkenazi et al, 2000). This sensor involves serial reactions of three enzymes; β-galactosidase, glucose oxidase and horseradish peroxidase, which are immobilised onto a glassy carbon electrode. Although lactose can be determined by gas, liquid and high-pressure liquid chromatography, these methods are expensive and time-consuming compared with direct on-line measurement by biosensors (Velasco-Garcia and Mottram, 2003).

Other sensors which have been developed for milk include a screen printed amperometric biosensor which detects D- and L- amino acids to monitor milk ageing effects (Sarkar et al, 1999), and a microbial biosensor for free fatty acids in milk which uses an oxygen electrode based on thick film technology (Schmidt, Standfuss-Gabisch and Bilitewski, 1996). Much research has been carried out on biosensors to determine progesterone concentrations in milk for automatic ovulation prediction and to improve reproductive management (Koelsch et al 1994; Claycomb and Delwiche, 1998; Pemberton, Hart and Mottram, 2001). These sensors are based on immunoassay principles with antibodies as the biorecognition element.

1.5.1. Analytical Methods and Sensor Technology for Milk Analysis

The use of sensors for monitoring milk composition on-line is potentially a rapid and inexpensive way in which to non-invasively monitor the cow. An on-line system with automatic sampling and milk analysis to optimise production performance, has been patented by Larsen et al (2002). At present milk analysis is carried out by laboratory techniques and very few sensors are available for measuring specific milk components. Also, many laboratory techniques are not adaptable to on-line analysis, due to the methodology and complexity of the equipment used.

1.5.1.1. Acetone

There are currently no on-line sensors for measuring milk acetone. Fourier transform infrared spectroscopy combined with multivariate calibration has been investigated as a rapid and inexpensive method for detection of milk acetone since acetone has three main absorptions in the mid-infrared region of the spectrum (Hansen, 1999). Acetone can be easily identified from other milk constituents since its absorptions are isolated from the major fat, protein and lactose peaks. However, the accuracy of this technique was 0.27mM, with an R^2 of 0.81 on test samples and this is equivalent to a relative error of approximately 20%. Therefore Hansen (1999) suggested that this technology would only be useful for screening for ketosis with further analysis by a more accurate method being required to accurately determine acetone concentration.

Milk acetone is most commonly determined by a flow injection analysis (FIA) method as described by Marstorp, Änfalt and Andersson (1983). Although this method has a high accuracy, it can only analyse up to 100 samples/hour and this is a relatively low throughput considering infrared equipment can analyse up to 500 samples per hour for fat, protein, lactose and urea (Hansen, 1999). Cowside tests involving colour strip tests or tests based on sodium nitroprusside have been used to detect ketosis; (Francos, Insler and Dirksen, 1997; Geishauser et al, 1998; Geishauser et al, 2000). However these tests are designed to detect BHB or acetoacetate and some of them have poor sensitivity (Gieshauser et al, 2000).

Gas chromatographic methods have been developed for milk acetone measurement with high accuracy and reproducibility (Winterbach and Apps, 1991; Baticz, Vida and Tömösközi, 2001). Tests involving a colour change include the Vanillin test and the Rothera test. The Vanillin test involves the reaction of acetone with Vanillin to produce the coloured compounds vanillalacetone and divanillalacetone, which can be detected at 415 or 500nm (Hansen, 1999). The Rothera test is a semiquantitative method based on using sodium nitroprusside, which causes a colour change depending on the acetone or acetoacetate concentration in the sample (Nielen et al, 1994). However, these tests are not suited to on-line measurement. Despite a number of laboratory-based methods for acetone detection, the disadvantage that applies to them is that acetone is a highly volatile substance and losses during sample storage and preparation are a potential source of error. Also laboratory methods that are based on chromatography and chemical, enzymatic and fluorimetric techniques tend to be time-consuming and may involve using hazardous chemicals. Therefore there is a need to develop on-line sensing systems that can quickly and accurately predict milk acetone concentration.

A sensor for acetone has recently been developed by Dart Sensors Ltd., Totnes, Devon, UK, to detect acetone in biological fluids such as blood, milk and saliva. The Keto-sensor principle, which measures acetone in the headspace above the sample liquid, could be adapted for on-line measurement of acetone (see Chapter 2).

1.5.1.2. Urea

Many sensors have been developed in the biomedical field for urea due to its use as an indicator of renal health. For example the determination of urea in human urine has been made possible with a sensor based on combining a surface acoustic wave device with urease (Lui et al, 1996). Optical sensors for urea have also been developed by Sansubrino and Mascini (1994) and Kovács et al (2003). The device by Sansubrino and Mascini utilises Brilliant Yellow, a pH dye immobilised on a cellulose acetate membrane, coupled with an Immobilon membrane for urea determination. These sensors utilise the enzyme urease, which hydrolyses urea to ammonium and bicarbonate. However, the majority of these sensors are not designed to handle complex media such as raw milk.

A biosensor to measure milk urea has been developed by Jenkins et al (1999). This device is based on enzymatic hydrolysis of urea to ammonium and bicarbonate and the subsequent measurement of CO_2 partial pressure, which is linearly related to the concentration of urea in the milk sample. Jenkins and Delwiche (2002) tested a similar prototype sensor for on-line analysis during milking and the system had a calibration error of 0.15mM and a standard error of 0.25mM in milk, indicating a high accuracy. The principle of this manometric biosensor has been adapted and an automated biosensor for urea has been developed at Silsoe Research Institute (see Chapter 2).

A variety of laboratory methods exist for measuring milk urea content, such as diagnostic test kits based on diacetyl monoxine or urea hydrolysis to ammonia, which is determined colorimetrically. A flow injection analysis (FIA) method was developed by Andersson, Andersson and Carlström (1986) and involves the breakdown of urea by urease into carbon dioxide and ammonia. Ammonia diffuses through a teflon membrane into a pH indicator solution with the change in colour being determined photometrically. FIA is the reference method in Sweden for calibration with infrared methods. The problem with some of the test kits and FIA is that samples have to be defatted before analysis. The most common and cost-effective method for urea determination is by infrared instruments, due to their accuracy, speed and the fact that simultaneous measures can be made on fat, protein and lactose in monthly milk quality tests. Much research has been carried out on near-infrared (NIR) spectroscopy to monitor milk fat and protein content and this technique is likely to be the preferred technology for on-line urea analysis.

1.5.1.3. Fat and protein

The national milk recording services use a range of laboratory equipment based on infrared spectroscopy to determine milk composition. This is the most widely used technique to analyse milk samples and it is also suited for on-line analysis. Sensors for whole fat and protein content in raw milk are in the very early stages of development. To date, much of the work in monitoring milk fat and protein content has involved NIR spectroscopy (Tsenkova et al, 1999; Schmilovitch et al, 2000; Tsenkova et al, 2000), and this method of analysis seems to be suited to on-line analysis.

The NIR region of the spectrum is defined by wavelengths between 700nm and 2500nm and is situated between the visible and mid infrared region. Modern infrared analysers are single beam instruments, which can measure fat, protein and lactose concentrations in milk by the absorption of infrared light at specific wavelengths. The fat concentration is determined by counting the ester linkages and the measurement of their infrared absorption. The infrared absorption due to nitrogen-hydrogen bonds within the peptide bonds is used to determine the protein content. The absorption of reference wavelengths are also measured to take into account the influence of water absorption (Harding 1995). Infrared methods all have to be calibrated against a reference method. The current laboratory reference method used to determine protein content in milk is the Kjeldahl method, which measures the nitrogen content of milk from which the total protein content can be calculated. The reference method for milk fat determination is the Gerber test where fat is precipitated from the milk sample by addition of sulphuric acid and amyl alcohol. The problem with these reference methods is that they are time-consuming and involve the use of hazardous chemicals.

The short wave region of the infrared spectrum has also been used for quantitative analysis of fat and protein in milk (Sasic and Ozaki, 2001). The short wave region is between 700 to 1100nm. Due to high light transmittance and the availability of good detectors, the short-wave region of the near infrared spectrum may also be useful for on-line milk analysis. There are several advantages of NIR spectroscopy for milk analysis. It is a fast, inexpensive and robust method. Many studies have shown NIR spectroscopy to be very accurate, achieving a close correlation with laboratory methods (Tsenkova, Yordanov and Shinde, 1992; Godden et al, 2000).

1.5.2. Sampling Considerations for On-Line Milk Analysis

Sampling for analysis of milk constituents on-line may involve two different sampling procedures. A representative sample from the whole milking could be taken for analysis. However, the aim of on-line metabolic profiling is to be able to analyse a milk sample during milking and carry out the analysis before the milking system is needed for the next cow. This causes problems in accurate milk analysis because milk composition changes during milking.

1.5.2.1. Acetone

The optimum time to sample for acetone within the milking process has not been determined since there are no data available on within milking variation in acetone content in the literature. The optimal time to sample during milking is important to determine particularly if fat, which increases throughout the milking process, affects the concentration of other milk constituents in the water phase. More research is required in this area before on-line measurements of acetone can be made and accurately interpreted.

1.5.2.2. Urea

Urea does not vary significantly during the milking process. Gustafsson and Palmquist (1993) found that milk samples taken before and after milking were similar in urea content to a sample taken from regular milking. However, Carlsson and Bergström (1994) found significant diurnal variation in urea and suggested that milk with a very high fat content may give misleading results. They suggested that the apparent concentration of urea in whole milk might be affected by the fat content since urea is only dissolved in the water phase of milk. The authors recalculated the urea concentration in the water phase of milk and found that the adjusted urea values were higher than the unadjusted values, although there was no significant difference in diurnal variation. On the other hand, when a blank standard was prepared with 8ml of water and 2ml of cream containing 40% fat (not centrifuged) a urea concentration of 0.4 to 0.5mM was detected. Due to increasing fat content as milking progresses and the displacement of water soluble components, Jenkins et al (2002b) suggested that for an on-line sampling and analysis system the most consistent results would be achieved when sampling occurs early in the milking process, when the fat content is low. Clearly there is conflicting evidence on whether the fat content in milk affects the accurate measurement of urea, and this is likely to depend on the method of analysis used and whether samples are centrifuged to remove the fat before analysis.

Eicher et al (1999) investigated the effect of cow level sampling factors on analysis and interpretation of milk urea. Recommendations for sample collection and analysis involve samples being collected at the same time each day relative to feeding times so as to reduce the influence of the post-feeding surge in urea concentrations on accurate data interpretation. The authors found no significant difference in milk urea

concentrations in samples taken from individual quarters compared with pooled samples, confirming the results of previous studies (Gustafsson and Palmquist, 1993). Sample storage did however have an effect on urea levels with urea being significantly higher in samples that had been refrigerated for a week at 4°C (+0.41 ±0.24mM) and in samples which had been frozen for a month at -20°C (+1.52 ±1.25mM). Analyses should be carried out as soon as possible after sample collection as preservation and storage may bias results (Eicher et al, 1999). This is another reason why on-line sampling and analysis may give more accurate and timely results.

1.5.2.3. Fat and protein

With monthly milk recording schemes for milk fat and protein analysis, a representative sample of whole milk is taken, either as a fractional sample from the milk meter or as a thoroughly mixed milk sample taken after the milk has been agitated with air in the recording jar. Normally a composite 24-hour sample is taken for analysis and the composite sample must be proportional to the volume of milk produced at evening and morning milking. However, there is no recommendation as to the optimum sampling time in an on-line system to obtain a representative sample during the milking process. Ideally sampling should be carried out as early as possible for the analysis to be completed before the milking system is needed for the next cow.

One of the problems with on-line milk analysis may be the non-homogenous nature of milk during milking. Tsenkova et al (2000) investigated the use of NIR spectroscopy to measure fat, protein and lactose in non-homogenised milk samples at 1100 to 2400nm. Samples were collected every 3 litres into milking in one cow during an afternoon milking and milk fat was found to increase significantly while protein remained fairly constant throughout. However, the optimal time throughout milking in which to obtain a representative sample was not discussed.

1.6 ALTERNATIVE MEASURES FOR MONITORING THE DAIRY COW

Currently, farmers monitor the health of their cows by experience and intuition and are not so concerned with data analysis of milk yields and milk composition. Visual inspection and appearance of individuals is key to detecting the first signs of a health problem. Automated monitoring of cows' appearance such as body condition scoring may be useful to improve dairy cow management. As Defra (Department for Environment Food and Rural Affairs) is becoming increasing concerned about, and focusing research on greenhouse gas and nitrogen emissions from dairy cows, nutritional aspects of management may be even more important to consider to improve nitrogen efficiency and reduce methane emissions. These issues are discussed in the following sections.

1.6.1. Body Condition Scoring as a Nutritional Management Tool

Body condition scoring is an important tool to manage the dairy cow and ensure productivity and health. Body condition is related to the degree of body fat and body condition score (BCS) evaluates how much subcutaneous fat is present in a cow. It may be a useful indicator of energy balance as body condition loss is correlated with fat mobilisation (Komaragiri, Casper and Erdman, 1998). Condition scoring is carried out by visual inspection and palpation and gives an indication of body reserves. The UK system measures BCS on a scale from zero to five according to the amount of subcutaneous fat and muscle deposition in the loin region around the lumbar vertebrae and the region between the pelvis and the tailhead (Webster, 1993). A low condition score refers to thin cows and a higher condition score refers to fat cows with target scores for calving being 3.0 to 3.5.

Close attention to condition scores is important for cow health. Significantly more cases of disease are found in fat cows than in thin cows and BCS is particularly important to monitor in early lactation as this is when most body condition loss occurs. Excessive loss of body condition may also compromise fertility (Dechow, Rogers and Clay, 2002). Treacher, Reid and Roberts (1986) examined disease incidence in two groups of Friesian cows fed to achieve condition scores at calving of 2.5 and four. Cows calving at a condition score of four were three times more

likely to develop a health problem and nine diseases were detected overall, including mastitis, ketosis and lameness. Overfat cows tend to have lower dry matter intakes in early lactation and an increased negative energy balance (Garnsworthy and Jones, 1987), thus predisposing them to metabolic disorders. Although overconditioning is undesirable at calving, poor condition should be avoided due to the loss of body condition in early lactation. Cows in poorer body condition in early lactation tend to partition nutrients towards body reserves and have lower milk yields and poorer fertility (Ferguson and Otto, 1989 cited by Chalupa, Galligan and Ferguson, 1996).

1.6.1.1. Automated body condition scoring for integrated management systems

BCS may be a useful parameter to include in an integrated management system for dairy cows. However, despite being a well-established procedure giving consistent results, condition scoring is not routinely carried out on many dairy herds. It is regarded as being too time-consuming and daily records of milk yields and milk composition may be more useful to monitor energy balance and nutritional status more readily and effectively. Body condition scoring is subjective and repeatability between observers may be a potential source of error. For example, Ferguson, Calligan and Thomsen (1994) found that repeatability was approximately 56%, meaning that 56% of the time assessors allocated the same score to the same cow. Furthermore, up to 34% of the time, assessors gave ± 0.25 score to the same cow. However, there was a high correlation for scores between assessors (0.89 to 0.93). BCS has also been criticised because scorers may not be accurate in assessing condition score over time. Therefore changes in condition score over time may be due to inaccuracy by the scorer and not due to actual changes in cow condition (Domecq et al, 1995).

The use of image analysis has been suggested as a potential automated technique in which to assess BCS in dairy cows (Frost et al, 1997), and Coffey, Mottam and M^{c}Farlane (2003) have shown that cow shape characteristics are correlated (0.55 to 0.59) with condition score. The advantage of computer aided condition scoring is that this would allow the collection of large amounts of data, which would be useful for nutritional management. Also an automated system would remove inconsistencies in scoring between individuals by manual assessment and would be less time consuming than weekly condition scoring by the farmer.

However, changes in condition score occur very slowly and it is difficult to formulate rations to achieve optimal BCS at certain stages of lactation due to lack of information on how condition score relates to body composition (Wright and Russel, 1984). Also the subcutaneous fat layer at a given location on a cow is not uniform (Domecq et al, 1995). As condition scoring takes into account visual appraisal as well as manual palpation, image analysis or automated scoring by ultrasound measurement may not be as accurate as manual condition scoring by a trained operator. In addition to these problems, not enough information is known about how cow shape correlates with fat deposits and condition score. There is a need for better understanding of fat deposition and the distribution of fat in different locations in the dairy cow before image analysis and automated condition scoring are used to aid dairy cow management.

1.6.2. Pollutants from Dairy Cows

As well as striving to improve milk production and efficiency, farmers must also consider the effect of gaseous emissions from dairy cows as these are a source of dietary inefficiency. The reduction in emissions from animal agriculture is important due to the contributing effect of greenhouse gases to depletion of the ozone layer and global warming. It is estimated that UK agriculture accounts for 38% of methane emissions, with cows being the principle source, and the Kyoto Protocol has set the target of reducing greenhouse gas emissions in the UK to 12.5% below 1990 levels by 2008 to 2012 (Defra, 2003). Because of recent interest in the effects of agriculture on the environment, optimal feeding strategies may soon have to be taken into account to reduce pollutant emissions from dairy production systems. The main pollutants of interest from cattle are carbon dioxide (CO_2) and methane (CH_4), which arise as end products of rumen fermentation (Tamminga, 1992) and ammonia (NH_3) and nitrous oxide (N_2O). The estimated emissions of pollutants per livestock unit per day are 223g, 6500g and 1.6g for CH_4, CO_2 and N_2O respectively, where one livestock unit equals 500kg liveweight (Jungbluth, Hartung and Brose, 2001).

1.6.2.1 Strategies for reducing emissions

There are several ways in which emissions could be reduced, most of which involve dietary manipulation. The main source of enteric CH_4 is fermentation of forages by

methanogenic bacteria, and the production of CH_4 is influenced by carbohydrate type, level of feed intake, supplementary lipids or ionophores in the diet and changing the rumen microflora (Johnson and Johnson, 1995). Decreasing dietary fibre has the greatest influence on reducing CH_4 formation in the rumen. The addition of lipids and medium chain fatty acids to the diet have also been shown to reduce CH_4 formation in the rumen (Dohme et al, 2001). Various mechanisms of action are responsible for this effect including increased production of propionic acid, biohydrogenation of unsaturated FAs and inhibition of rumen protozoa, which have a major role in CH_4 production in cattle on high concentrate diets (Johnson and Johnson, 1995). Processing of animal feeds may also be useful. If forage is ground or pelleted, CH_4 production may be reduced due to an increased passage rate through the digestive tract (Johnson and Johnson, 1995). Synchronisation of nitrogen and energy availability in the rumen will reduce the output of environmental pollutants, as well as shifting the digestion of starch and protein to the small intestine from the rumen (Tamminga, 1992).

Two to twelve percent of gross energy intake may be lost as CH_4, depending on the composition of the diet (Johnson et al, 1993 cited by Johnson and Johnson, 1995). Feeding higher proportions of fibre in the diet tends to increase CH_4 formation from cell wall fibre degradation. Higher CH_4 production is probably due to a shift in the pattern of fermentation from a propionic to methanogenic fermentation and soluble carbohydrates in the diet tend to be less methanogenic compared with cell wall fibre (Moe and Tyrrell, 1979).

Losses of dietary nitrogen are high with an estimated 75 to 85% of ingested nitrogen being lost in urine and faeces (Charmley, Veira and Aroeira, 1988). Nitrogen excretion from faeces may be reduced by feeding diets with a high content of non-structural carbohydrates and low in protein. This will help to reduce CH_4 production while at the same time maximising milk protein content. However, milk fat content may be compromised (Tamminga, 1992). Strategies to reduce NH_3 emissions should aim to reduce urinary urea excretion and this can be achieved by lowering nitrogen intakes (de Boer et al, 2002). Ammonia can be reduced by maximising the grass content in the diet while keeping rumen degradable protein level to a minimum

(Monteny et al, 2002). Reducing the rate of nitrogen fertilisation on pasture will also significantly reduce NH_3 emissions from grazing dairy cows (Bussink, 1994).

The emission of greenhouse gases may be reduced by the system of manure storage and duration of storage (Kulling et al, 2002). However, strategies to reduce CH_4 production in cattle by nutrition tend to result in a compensatory higher release of CH_4 during manure storage since there may be more organic matter available for fermentation (Kulling et al, 2002). Emissions of NH_3 from dairy cow houses tends to vary according to the housing system and a reduction of up to 50% has been achieved by slurry acidification, flushing floors with diluted formaldehyde or water and nutritional strategies for cubicle housing with slatted floors (Monteny and Erisman, 1998).

Much of the research to date on measuring gaseous emissions from dairy cows involves measuring emissions from animal buildings by air exchange rates or gas concentrations (Monteny and Erisman, 1998). Measuring N_2O from animal buildings has proved to be difficult since emissions tend to be very low and near the detection limit of gas analysers (Jungbluth et al, 2001). Measuring the production of CH_4 or NH_3 from individual animals to assess their dietary efficiency is likely to be very difficult and expensive and optimisation of diets to reduce pollutants from dairy cows would have to be carried out at the group level. Pollution control could potentially be a future objective with on-line monitoring because reducing high urea concentrations in milk would also lead to a reduction in nitrogen excretion to the environment.

1.7 CONCLUSIONS

The project reported was designed to investigate the feasibility of nutritionally managing dairy cows on an individual basis by monitoring changes in milk composition. This was carried out by investigating sources of variation within and between cows in milk composition and by monitoring cow responses to dietary changes. From reviewing the literature, it is clear that several parameters in milk have been identified as being useful indicators of metabolic and nutritional status in

the dairy cow. Much is known about the use of acetone and urea as indicators of energy balance and protein status respectively; however, additional use of fat and protein and perhaps citrate may enhance interpretation of milk composition data and give a more accurate basis on which to make nutritional management decisions. Several factors have been found to influence the concentration of milk constituents and metabolites and therefore much of the previous work in this area has been on evaluating feeding efficiency for a group of cows or at the herd level by assessing bulk tank milk samples. Improving nutritional management on an individual cow basis means that more emphasis must be placed on researching the use of these parameters for individual cow management and whether the sources of variation within and between cows will significantly affect the interpretation of milk composition data.

CHAPTER 2. NOVEL SENSING METHODS FOR ANALYSIS OF ACETONE AND UREA IN MILK

2.1 THE KETO-SENSOR

2.1.1. Introduction

On-line monitoring systems may be a useful tool for dairy cow management, allowing automatic sampling and analysis for milk constituents as the cow is being milked (Larsen et al, 2002). On-line milk analysis will allow frequent, real-time data collection on milk composition and this information could be useful for managing individual cows and optimising nutrition (Mottram et al, 2002). These on-line systems may also be less expensive and less time and labour consuming than current routine analytical methods.

There are currently no on-line sensors for measuring milk acetone. Most of the work in milk acetone has been carried out in Scandinavian countries where analysis is performed by flow injection analysis (Marstorp et al, 1983). A number of sensors based on electrochemical principles have been demonstrated to measure ketones. However, most become fouled by the high concentration of fat in milk. An acetone sensor has been developed by Dart Sensors Ltd. to detect acetone in milk and other body fluids such as blood and saliva (Figure 2.1). The electrochemical device is an adaptation of the widely used Alcosensor for breath alcohol measurement (Intoximeters UK Limited, Totnes, Devon, UK).

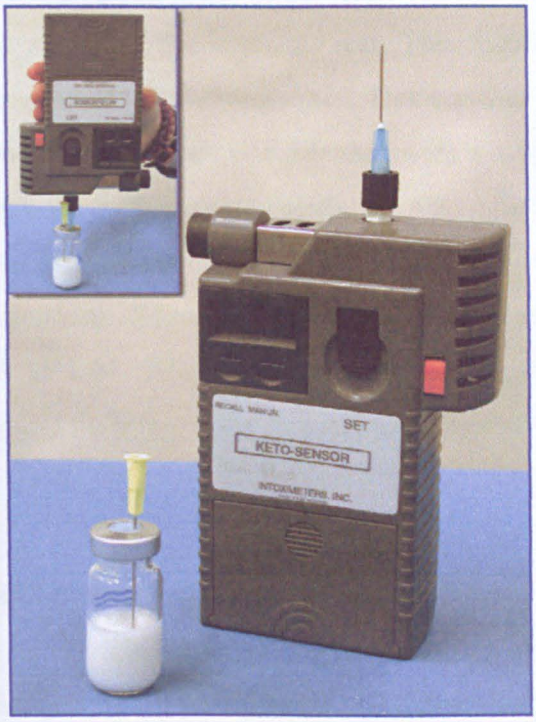

Figure 2.1. The Keto-sensor (inset picture shows the Keto-sensor taking a headspace sample from milk in a sealed vial)

2.1.2. Principle of Operation

The Keto-sensor works on the principle of headspace measurement, sampling vapour above the liquid to be tested. The sensor is composed of a two-electrode fuel cell which is a porous disk covered in a thin layer of platinum black on both sides and saturated with an electrolyte. Pressing the sample button activates the piston-sampling pump and a fixed volume of air is drawn onto the upper surface of the cell. At the sensing electrode, molecules of acetone are electrochemically oxidised, producing water and carbon dioxide. Oxygen is reduced at the counter electrode to complete the reaction. The electric current produced is converted to an acetone-in-liquid concentration which is shown on the digital display in millimoles (mM). The higher the concentration of acetone in the sample, the longer the cycle time. A wait of up to two minutes is required before each test or between a calibration and milk sample to ensure that the baseline has stabilised after a test.

Before a test is carried out, an internal sensor blank test is performed to ensure that the fuel cell has no signal from the previous test. This test must be zero before a calibration or milk sample test can be performed. Detection of acetone occurs within the range of 0.04 to 4mM. The sensor is calibrated with a 1.05mM standard to read 1.00mM. The five percent difference accounts for the milk solids, which occupy space not available to acetone, thereby increasing its effective concentration (King, W. personal communication). Repeatability between samples is between 0.97 and 1.03 but mainly 0.99 to 1.01. All information relating to the Keto-sensor in the current chapter was found on http://www.dart-sensors.com/ketosensor_manual1.htm, accessed 14/02/01.

2.1.3. Calibration and Sample Analysis

With each batch of samples or standards the Keto-sensor should be calibrated with a calibration solution with an acetone concentration of 1.05mM prior to sample analysis. A 5ml-aliquot of acetone standard was pipetted into a 10ml-glass vial and sealed with a rubber septum cap. A five-minute wait was required before analysis to allow acetone to equilibrate between the gas and liquid phases. A vent needle was inserted through the rubber cap of the vial so that the tip was submerged. This needle allows equalisation of pressure between the inside and outside of the vial before analysis, and prevents pressurised liquid being forced up the sample needle. The one-inch needle of the Keto-sensor was passed through the rubber septum to automatically draw off a sample of headspace gas for analysis and the reading was provided on the digital display. The output from the fuel cell was analysed by the microprocessor and the necessary calibration adjustments were made so that the calibration result displayed on the screen reflected the programmed calibration value. Milk samples were prepared in duplicate for analysis.

Temperature control of the samples is very important due to the volatility of acetone, with variation in temperature affecting the vapour pressure of acetone and the accuracy of the method. Higher temperatures will increase the sensitivity of the device although may increase the time taken to determine the acetone concentration in the sample. However, sample temperature is not important if samples are allowed

to equilibrate in a constant temperature and are at the same temperature as the calibration solution. If room temperature is constant, no further control is needed.

Certain decomposition products in milk can be detected by the acetone sensor resulting in an overestimate of acetone in the sample. Therefore milk samples should be analysed immediately after collection. If this is not possible they should be refrigerated for a maximum of two days before analysis or frozen immediately for longer-term storage.

2.1.4. Calibration Results

The Keto-sensor was calibrated with the 1.05mM calibration solution. The accuracy of the Keto-sensor was determined with standards of acetone prepared in water (Figure 2.2) with concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1mM (see Appendix 1).

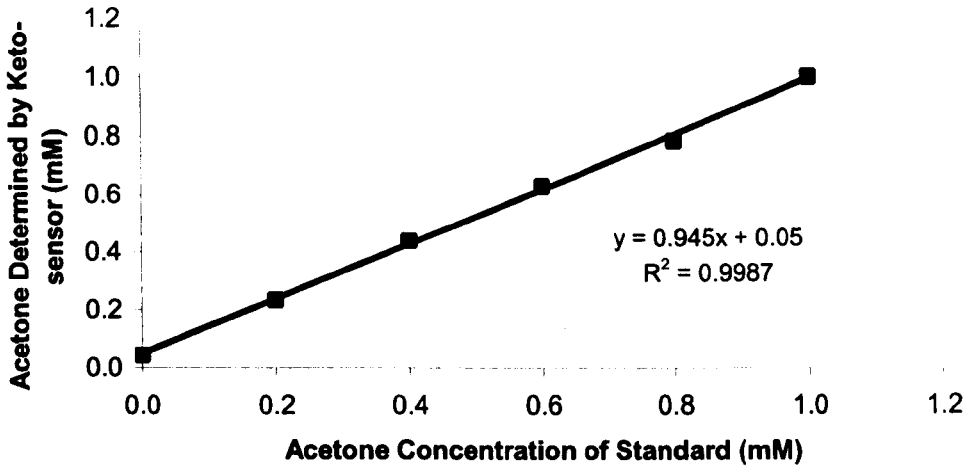

Figure 2.2. Standard curve of acetone standards in water as predicted by the Keto-sensor

Standards were also prepared and analysed in semi-skimmed milk as milk was the test medium. This resulted in a slight decrease in accuracy of the Keto-sensor with an R^2 value of 0.9985 compared with 0.9987 when standards were prepared in water (Figure 2.3). However fewer repeats were carried out with the standards in milk so this may account for the R^2 value being very slightly, but not significantly lower.

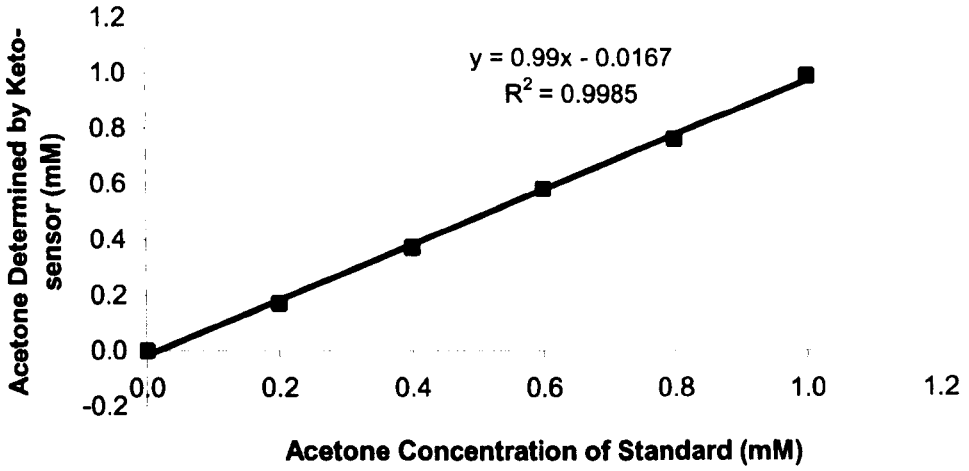

Figure 2.3. Standard curve of acetone standards in milk as predicted by the Keto-sensor

2.1.5. Discussion

The Keto-sensor was accurate and repeatable when tested with standards prepared in both water and milk. Repeatability between duplicates of milk samples tested in experiments was also good (Appendix 1, Table 1c).

Other methods for acetone determination by headspace analysis also exist. Gas chromatography (GC) is an alternative laboratory method which has been used for milk acetone determination (Winterbach and Apps, 1991; Baticz et al, 2001; Heuer et al, 2001a). Acetone determination is well suited to headspace measurement due to its' volatility. With GC analysis a headspace sample is injected onto a chromatographic column through which it is carried by a carrier gas. The compounds in the sample are separated, quantified and identified by a detector. The advantages of this technique are that it is simple and fast. However, it would be difficult to adapt the principle of GC analysis for an on-line analysis system due to the complexity and expense of the difference components used in GC analysers.

The Keto-sensor principle could be adapted for on-line measurement of milk acetone. A temperature control would have to be applied to the system so that the calibration solution and the milk samples were at the same temperature for analysis. Ideally temperature of the calibration solution and the sample should not exceed

34°C, as it may not be possible to achieve the full-scale reading of 4mM. Also, it is recommended that the operating temperature of the Keto-sensor should not vary by more than two to three degrees when performing a series of tests, so as not to affect the calibration and to minimise the effect of temperature on acetone determination.

The sensor within the device is not consumed in use but it deteriorates over time and has to be replaced. Sensor life deterioration can be identified by an increase in analysis time. Although sensors may last at least a year, sensor life may be reduced with heavy use, such as on-line monitoring with daily sampling of cows in the high risk early lactation period. Nevertheless, the investment in an on-line acetone sensor is likely to be outweighed by the benefits of early detection of subclinical ketosis and being able to maintain a high level of milk production.

2.1.6. Conclusions

Preliminary studies showed that using the Keto-sensor is a quick and reliable method to accurately determine milk acetone concentrations. The Keto-sensor is particularly suited to on-line measurements as repeat tests can be performed quickly depending on the acetone concentration of the previous measurement (and taking into account the five minute equilibrium period). This would allow milk sampling and analysis to be carried out before the milking system is needed for the next cow.

2.2 THE UREA PRESSURE SENSOR SYSTEM

2.2.1. Introduction

Milk urea is a useful management tool to monitor nutritional status in dairy cows and is a good indicator of the protein and energy content in the diet. Accurate prediction of urea in milk could result in increased dietary efficiency, reduced nitrogenous waste to the environment and improved reproductive performance (Roseler et al, 1993; Butler et al, 1996).

Laboratory techniques such as flow injection analysis (Andersson et al, 1986) and infrared spectroscopy have been developed to measure milk urea. Many sensors for urea have also been developed in the medical field, due to its importance in monitoring renal health (Ciana and Caputo, 1996; Lui et al, 1996). However, there are no on-farm tests for milk urea measurement. The advantages of an on-line system to measure milk urea are that costs would be reduced for transport of samples and laboratory analysis. Furthermore, on-line analysis could allow more timely application of the information for nutritional management of the herd.

A manometric biosensor to measure urea concentration in milk was developed by Jenkins et al (1999), based on enzymatic hydrolysis of urea and the subsequent measurement of CO_2 partial pressure. A pressure sensor system designed to measure milk urea was developed at Silsoe Research Institute (Ghesquiere, 2000, Appendix 2), based on the biosensor system of Jenkins et al (1999). Requirements of the system were a detection limit in the range 0 to 20mM and good accuracy between 2 to 9mM. Other necessities were that the sensor should be easy to calibrate, have good repeatability, low cost per sample and automatic data transfer.

2.2.2. Principle of Operation

The biosensor to measure urea in milk works by the enzymatic hydrolysis of urea to ammonium (NH_4^+) and bicarbonate (CO_3^{2-}) ions by the enzyme urease. Addition of an acid allows CO_3^{2-} ions to be volatised into CO_2 and the measurement of CO_2 partial pressure is linearly related to the urea concentration in the milk sample.

Reagents housed in reservoirs were pumped through valves to an air-tight chamber where the reaction between urease and the sample took place. The sample and enzyme were incubated together for a fixed time period to allow complete hydrolysis of urea. Citric acid was pumped into the chamber to reduce pH below four and the rise in pressure created was measured by a pressure transducer. A pH 4.1 or lower was required to liberate 99% of CO_2 from the CO_3^{2-} ions (Jenkins et al, 1999).

2.2.3. Automated Sensor Design

The sensor was fully automated and controlled by software developed at Silsoe Research Institute (see Appendix 3 for programme). The reagents in the reservoirs were moved by a 50µl fixed volume pump. Reservoirs containing the reagents required for analysis and the wash procedure were mounted on a common manifold and selected through two-way valves via the positive displacement pump (Figures 2.4 and 2.5).

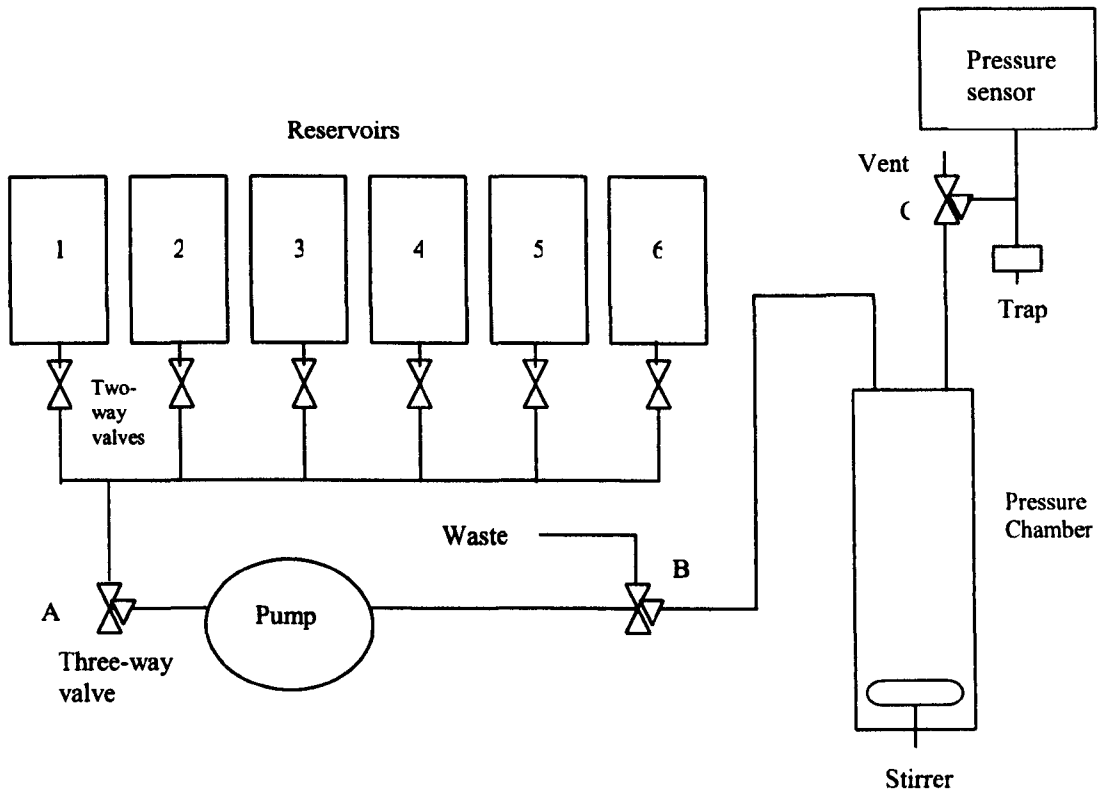

Figure 2.4. Schematic representation of the Urea Pressure Sensor

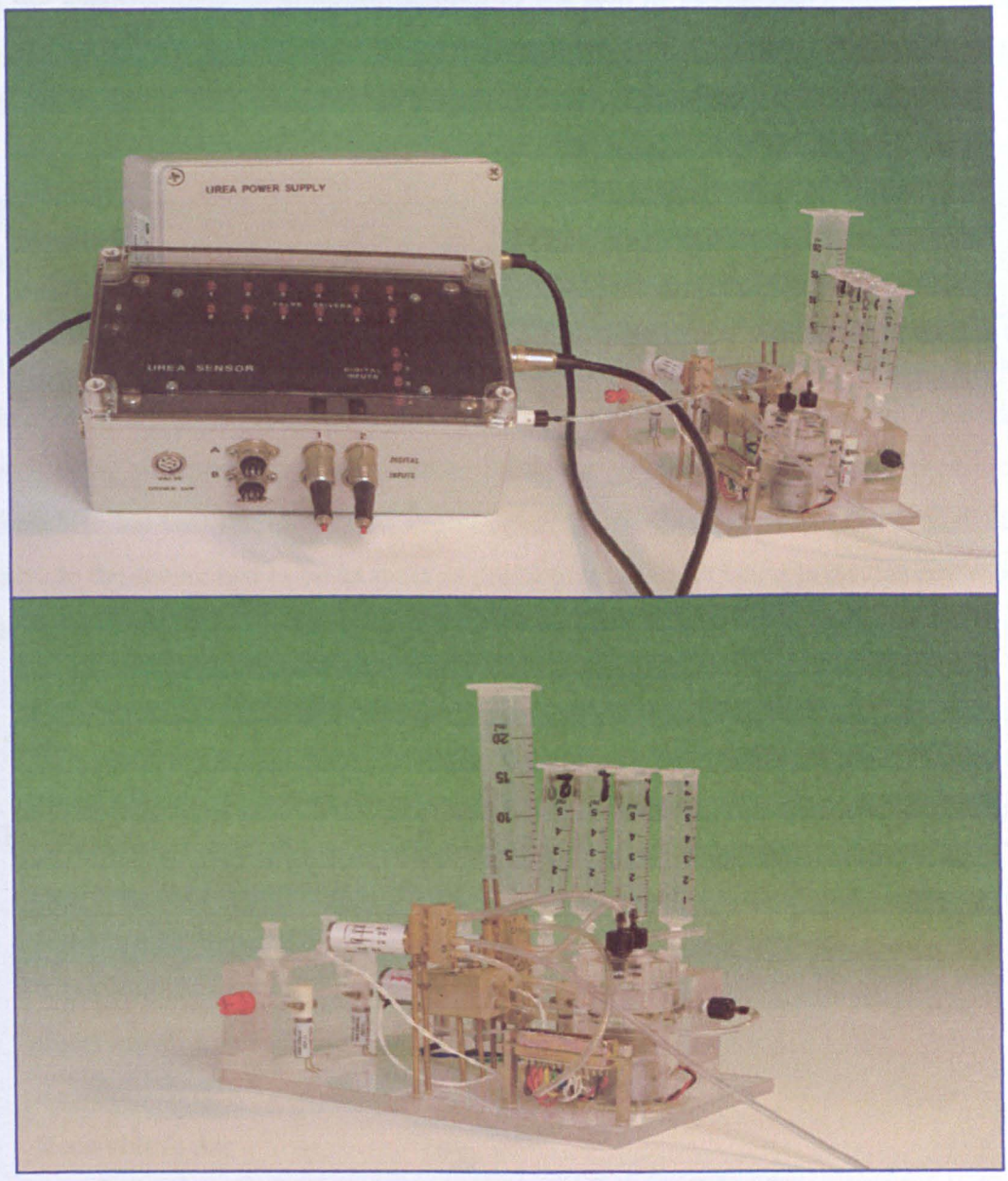

Figure 2.5. The Urea Pressure Sensor

Valves A, B and C were barbed three-way inert solenoid valves. Soft teflon tubing (Lee Products Ltd., Gerrards Cross, Buckinghamshire, UK) with an internal diameter of 1.5mm was used for transporting the liquids from the reservoirs to the chamber and then to waste. The pump and valves all operated at 12 volts and were supplied by Lee Products Ltd. The pressure chamber had a magnetically coupled stirrer which could be operated at various speeds. The stirrer was operated at 40% of maximum speed as above this speed the mixing of reagents was extremely erratic, probably due

to the small volume of liquid in relation to the size of the stirrer. A 142SC series pressure transducer with a pressure detection range of 0-5 psid (G) was used (Farnell Electronics, Leeds, UK).

The design of the system was such that pressure created in the chamber had to pass through tubing to a three-way valve and through another section of tubing before reaching the pressure sensor. It was not possible to have a tube directly connecting the chamber and the pressure sensor. A three-way valve situated between the chamber and the pressure sensor was required to be open to the air when reagents were being pumped into the chamber, so as not to create a build up of pressure. Therefore the two sections of tubing connecting the chamber to the valve and the valve to the sensor had to be as short as possible so that maximum pressures could be recorded from the assay.

2.2.4. Testing of System and Sensor Calibration

The reservoirs were filled as follows:

- Reservoir 1: Urease
- Reservoir 2: Standard/Sample
- Reservoir 3: Citric Acid
- Reservoir 4: Alcohol
- Reservoir 5: Deionised water
- Reservoir 6: Air

The following reagents were required for the assay (chemicals obtained from Sigma-Aldrich, Poole, Dorset, UK):

- Urea standards prepared in water (0, 5, 8, 10 & 20mM)
- Urease in EDTA[1] buffer (0.199g urease and 0.6263g EDTA in 100ml water)
- 1mM Citric Acid (0.096g anhydrous citric acid in 500 ml water)
- 70% Alcohol
- Deionised water
- Tween 20 cleaning solution: 250ml of EDTA buffer (34mM potassium EDTA, pH 7.6) + 0.25% Tween 20 3.13g of (K_2) EDTA salt.

[1] EDTA = Ethylene Diamine Tetraacetic Acid

The volumes and concentrations of reagents used were based on a manual system designed by Ghesquiere (2000), where standards and reagents were syringed into the 5ml glass vial which acted as the pressure chamber, via a three-way stop cock.

Tween 20 cleaning solution at 0.02% was initially used to clean out the system between assays. However the problem with the detergent was that excessive foaming occurred in the tubing and in the chamber, which was difficult to remove. Therefore Tween 20 was not used to clean the system and only 70% alcohol and deionised water were used in the wash procedure.

The system was tested with standards of urea prepared in water. The pressure chamber had a capacity of 2500µl. The first protocol involved pumping 900µl standard and 500µl urease from reservoirs two and one respectively to the pressure chamber. The standard was pumped into the system in two parts, so 450µl of sample, followed by 500µl of urease and another 450µl of sample were pumped into the chamber. This allowed full mixing of the sample with the enzyme. Air from reservoir six was pumped through the system to ensure that any liquid left in the tubing was forced into the chamber. The standard and enzyme were incubated in the chamber for four minutes to allow the urease to breakdown urea to NH_4^+ and CO_3^{2-} ions. A mechanical stirrer was used to mix the liquids. After four minutes the circuit was opened, allowing pressure created between sample and urease to drop before introduction of citric acid. A volume of 1400µl of citric acid was pumped from reservoir three to the chamber. Only approximately 300µl reached the chamber, as there was 1100µl volume of acid remaining in the tubing between the reservoir and the chamber. The system was closed immediately so that the chamber was airtight. Citric acid drove off CO_2 from the CO_3^{2-} and resulted in an increase in pressure. The liquids were stirred continuously for 150 seconds, during which time the increase in pressure was recorded by the pressure transducer. After the data had been collected, the liquids were drained from the chamber and the wash system was initiated. The system was washed out with deionised water and 70% alcohol.

Pressure increases with this system were not detected and when monitoring the accuracy of the pump to determine whether it was dispensing the correct volumes of

reagents from the reservoirs, it was clear that reservoir one was faulty. The pump was guaranteed to operate to within an accuracy of three percent. However the error when pumping liquid from reservoir one was found to be approximately 19% below the required volume and this may have been due to a faulty valve. Therefore the protocol was modified so that standard and enzyme were mixed together in the ratio of 900:500µl and placed in reservoir two as it was found to be more accurate in dispensing the correct volumes than reservoir one. Only 800µl of this mixture was used in the assay with 300µl of citric acid.

Ideally, as small a headspace as possible in the chamber was desired so that higher partial pressures of CO_2 could be recorded. However, the volume of headspace in the chamber was 1400µl despite there only being a maximum of 1100µl of liquid in the chamber at any one time. This was the maximum volume of liquid that could be used in the chamber due to displacement of liquid with the mechanical stirrer when the liquids were being mixed. The displacement caused by the stirrer was difficult to quantify but care had to be taken so that displacement of liquid was not enough to overfill the chamber and force liquid up the opening in the chamber lid and through the tubing that was connected to the pressure transducer.

2.2.4.1. Sensor calibration

The sensor was calibrated with standards of urea prepared in water of concentrations 0, 5, 8, 10 and 20mM. The difference in pressure recorded over the 150 seconds was used to produce the calibration. The assay was carried out at room temperature (approximately 24°C) since the optimum temperature for urease is 25°C. Each standard was run through the pressure sensor system three times and a mean was calculated for each concentration. A graph of mean urea concentration against change in pressure was plotted to produce a calibration curve. The increase in pressure was calculated by the equation ΔP = End P – Start P, where End P refers to the pressure recorded at 150 seconds and Start P refers to the pressure recorded at zero seconds after citric acid addition. ΔP = change in pressure.

2.2.5. Calibration Results

The calibration results show that the system was not accurate or repeatable, as indicated by the high SEM for the higher urea standard concentrations and the variation between repeated measurements for the same standard (Table 2.1). There was also an overlap in pressures obtained between repeated measures for the 8 and 10mM standards.

Table 2.1. Repeated measurements for calibration of the Urea Pressure Sensor

	Run 1	Run 2	Run 3	Mean	SEM
Standard 0mM					
Start	1.1	25.0	0.5		
End	0.6	26.9	-0.2		
ΔP	**0.5**	**1.9**	**0.7**	**1.0**	**0.33**
Standard 5mM					
Start	5.4	2.4	1.9		
End	12.9	7.2	5.9		
ΔP	**7.5**	**4.8**	**4.0**	**5.4**	**1.94**
Standard 8mM					
Start	0.2	25.8	1.6		
End	20.9	42.5	15.0		
ΔP	**20.7**	**16.7**	**13.4**	**16.9**	**7.72**
Standard 10mM					
Start	4.3	2.6	1.6		
End	21.6	25.9	25.7		
ΔP	**17.3**	**23.3**	**24.1**	**21.6**	**5.52**
Standard 20mM					
Start	-1.6	26.7	-2.1		
End	32.1	51.0	26.6		
ΔP	**33.7**	**24.3**	**28.7**	**28.9**	**12.77**

*SEM = standard error of the mean. All pressures are reported in mbar

The increase in pressure with increasing concentrations of standards was not linear. The calibration response was a typical sigmoidal enzyme reaction curve, which was based on mean pressures from Table 2.1 (Figure 2.6).

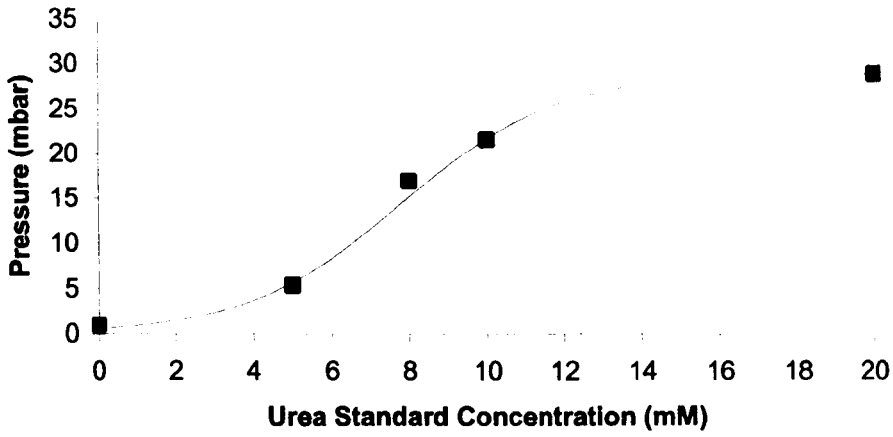

Figure 2.6. Calibration curve for the Urea Pressure Sensor

2.2.6. Discussion

The calibration showed that the system was not accurate or repeatable with the concentrations of standards used and there was considerable variation between repeats for the same standard concentrations (Table 2.1). This would lead to inaccuracy in determining nutritional status of dairy cows with such an on-line system, as only one sample of milk would be taken from each cow on a daily basis and assayed for urea. The system was regularly checked for pressure leaks by pumping air into the chamber and observing whether the pressure remained constant or dropped. Pressure always remained constant so this source of error can be eliminated.

2.2.6.1. Design problems

There were several problems with the automated urea pressure sensor which were mainly related to the design of the system. Firstly, the assay was very time-consuming, taking 22 minutes to complete an assay and wash cycle. If milk samples had been analysed by this system, assay times would have increased due to the extra time required to run a blank. The blank result would have been determined by re-

running the assay, replacing the urease solution with deionised water and skipping the four-minute incubation to measure the effect that other milk constituents may have on the pressure recording. Any response from the blank measurement would be subtracted from the assay response in order to determine the amount of urea in the sample. If the assay time could be reduced to ten minutes or less this system would be useful on-line to monitor milk urea as the assay could be completed and the system washed, ready for the next cow that comes in to be milked. Presently the time required to complete one measurement is too long for integration of the sensor with automatic sampling in an on-line system.

There was a problem with drops of reagents being left in the tubing. Normally air was pumped through the system to ensure that all the standard and enzyme reached the chamber. However, some drops of liquid remained in the tubes so that the programmed volumes did not reach the chamber. This was thought to be due to the pump not being powerful enough to pull the liquids through the tubing into the pressure chamber. Air bubbles were also visible when reagents were pumped from the reservoirs through the system. These air pockets may have increased the resistance to flow through the tubing, contributing to drops of liquid being left in the system. In the case of autoanalysers, this problem is solved by having the tubes full of liquid so that the desired volume of reagent is dispensed. However, with the current design of the urea pressure sensor, having the tubes full of liquid before pumping them to the pressure sensor was not possible since all reagents were pumped to the reaction chamber through the same piece of tubing.

Stirrer displacement resulted in very small volumes of reagents being used in the assay, despite the chamber capacity being 2500µl. As a result, very small pressures were recorded for the standards of low concentration. If higher volumes had been used the sensor may have been more accurate. Initially a chamber with a capacity of 5000µl was designed. However, there were several problems with this design in that it was difficult to determine the optimal volumes and concentrations of reagents and minimise the headspace volume to obtain maximum pressure readings, while taking into account displacement of fluid in the chamber by the stirrer. The optimum concentration of citric acid also had to be determined. Initially the concentration was too strong, resulting in the chamber being acidic after the wash procedure, with

subsequent assay runs not yielding any pressure increases due to suboptimal conditions for the enzyme to work. Also the greater volumes used meant that assay times were longer as the pump could only pump 50µl per stroke and therefore the chamber was modified to half its volume.

Some sections of tubing in the system were not level, having a slight uphill gradient. With small volumes or drops of liquid in the tubes it was very difficult for the pump to force the liquid uphill and this contributed to liquid being left in the tubing despite pumping air through the system. Remaining liquid was pumped through the system with the next run and this may have resulted in increased volumes of liquid in the chamber and thus decreased accuracy of the assay.

Liquid remaining in the tubing between the pump and valve B might have interfered with subsequent assays. This piece of tubing was common to liquids being pumped from the reservoir block to the chamber and liquids going from the chamber to waste. The wash cycle involved pumping several rinses of deionised water and 70% alcohol through the system and dispensing them to waste each time. Therefore, as the wash cycle continued, the risk increased that more liquid would be pumped into the chamber and up the tubing that goes to the pressure sensor. The volumes of wash liquids could have been reduced but this would run the risk of not cleaning the chamber out properly. It was important not to leave chamber acidic since this would denature the enzyme in the subsequent assay. The system could have been programmed to pump greater volumes to waste to ensure that as much wash solution as possible was cleared from the system. However, this would have considerably increased total assay times.

Lastly, the volume of citric acid from reservoir three reaching the chamber may have varied with each run depending on how much standard and enzyme mixture remained in the tubing, despite air being pumped through the system. If insufficient citric acid reached the chamber it is possible that the pH of the reagents was not low enough to liberate all the CO_2 from CO_3^{2-}, resulting in lower pressures being recorded and an underestimate of urea concentration in the standard.

2.2.6.2. Suggestions for further modifications

System parameters have yet to be optimised. There are several factors which may have affected the pressures recorded for the various standards and could be further researched to improve the accuracy of the assay. The optimal volume or concentration of urease in relation to the standard was not determined. Therefore it was not known whether the urease solution used was able to hydrolyse all the urea in the standards. It is possible that with the higher concentration standards not all of the urea was hydrolysed due to there being insufficient enzyme present for all the enzyme binding sites on the urea molecules. Furthermore, it is possible that not all of the urea in the standard was broken down by urease during the incubation period, as the time required for complete hydrolysis of urea with the concentrations used was not known.

The data collection period was 150 seconds. There appeared to be very small increases in pressure towards the end of the data collection period and increases in pressure were greatest in the first 20 seconds after citric acid addition (Figure 2.7). However, it is possible that a longer period of data collection may have resulted in higher pressures being recorded, particularly with higher urea concentrations.

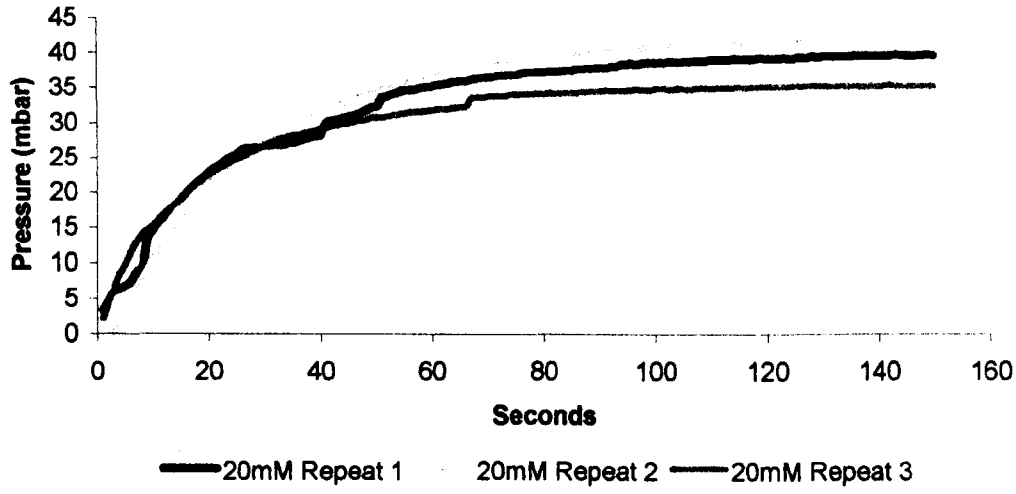

Figure 2.7. Increase in pressure due to CO_2 production after citric acid addition

Sufficient washes with deionised water had to be used so any traces of acid were washed out of the system. Any remaining acid in the chamber or tubing meant that subsequent analysis would not show the desired response as the enzyme urease works best at a neutral pH. However, repeated washes of the system meant that total assay time was increased and a low number of samples could be analysed per day. The length of time of the wash cycle could be reduced by adding a reservoir containing an alkali solution to the system to quickly neutralise the acid remaining in the chamber from the assay. This would reduce the number of water cycles in the wash and also reduce assay time.

A more powerful pump that could handle greater volumes of fluids may be useful to pump more accurate reagent volumes through the tubing to the reaction chamber. This might also help to eliminate drops of liquid remaining in tubing and air bubbles as well as reducing assay time. A variable volume pump would be useful so that more accurate volumes of reagents could be dispensed.

The incubation time for maximum urea hydrolysis has not been determined. The manual system of Ghesquiere (2000) operated on an incubation time of five minutes. However, to reduce assay time the incubation period was reduced to four minutes. It is not known whether this resulted in a significant reduction in response. It is possible that with the shorter incubation time, not all the urea in the standard was hydrolysed and a more accurate response may be achieved when the standard and enzyme has more time to react.

A change in the design of the system may help to improve accuracy. Reagents in reservoirs could be introduced directly into the top of the chamber with a separate entrance for each reagent. Pumping air into the reservoirs, at volumes equal to the required volumes of reagents, would allow filling of the chamber by positive displacement, decreasing the amount of tubing required and therefore inaccuracy due to liquid remaining in tubes. If tubing for each reagent was initially filled with liquid, then more accurate volumes to the pressure chamber could be dispensed, as is the case with autoanalysers.

Improvements to the system design were not possible due to lack of time and the increased costs involved in redesigning the system and installing a variable volume pump. Alternative sensing principles that could be used to monitoring milk urea on-line include mid infrared spectroscopy, which is likely to be more accurate than the automated pressure sensor system. Optical sensors for urea have also been developed (Sansubrino and Mascini, 1994; Kovács et al, 2003) but these are not adaptable for on-line measurement due to the instrumentation required for the analysis.

2.2.7. Conclusions

Calibration of a novel automated sensor for milk urea determination showed that the system was not accurate enough to be used to monitor milk urea. Due to the design problems and inaccuracy associated with the urea pressure sensor system no milk samples were analysed by this method. Several changes to the design of the system would be required for accurate calibration and milk sample analysis before the system could be used on-line. A large reduction in assay time would also be required to complete milk sampling and analysis before the milking station was required for the next cow. Infrared measurement would be a quicker and more accurate method for on-line milk urea determination, rather than further development of the pressure assay.

CHAPTER 3. WITHIN MILKING VARIATION OF ACETONE, UREA, PROGESTERONE, FAT AND PROTEIN AND THE DETERMINATION OF SAMPLING TIME FOR AN ON-LINE SYSTEM

3.1 INTRODUCTION

Sampling for analysis of milk constituents (fat and protein) is a routine procedure on commercial dairy farms. It is assumed that for this procedure a representative sample of the whole milking is taken. The problem with conventional milk sampling and subsequent laboratory analysis is the potential for human error. Sampling errors and transport and storage of milk samples prior to analysis may result in deterioration of the sample if proper storage conditions are not maintained (Ng-Kwai-Hang, Moxley and van de Voort, 1988). Automated on-line sampling and analysis would remove any human error and save on transport and laboratory costs (Pehrson, 1996). It could also provide real-time data on which to base quick nutritional management decisions.

There is no standard procedure for the collection of milk samples in an on-line system. Samplers for milking systems usually take a proportion of milk flowing through the milk line, which is known as a fractional sample and is representative of the whole milking. However, on-line metabolic profiling must aim to take a milk sample as the cow is being milked and analyse that sample for specific constituents, with the result being sent to the herd management database and the system washed before the next cow comes in to be milked. Jenkins and Delwiche (2002) designed a fully automated sampling device for collecting samples during milking. This device allowed milk to drop passively from the bottom of the milk line into the sample reservoir at a fixed time throughout milking. Before milk samples can be taken for analysis in an on-line system, the optimal time for sampling during milking has to be determined. This is particularly important for constituents that show within milking variation and this variation could be due to the way in which the constituents are synthesised and where they originate from, whether this be the blood, mammary gland, or both.

Progesterone was measured in this study due to its use in ovulation prediction for improving insemination times. Progesterone biosensors have been developed by Claycomb and Delwiche (1998) and Pemberton, Hart and Mottram (2001). However, the optimal sampling time during milking has yet to be determined.

The aim of the current experiment was therefore to determine within milking variation of acetone, urea, progesterone, fat and protein and to specify the most appropriate time of sampling during milking to obtain accurate concentrations of these constituents in milk for an on-line monitoring system. The hypothesis was that fat and progesterone would increase during milking, due to progesterone being lipid soluble. Acetone, urea and protein were not expected to vary considerably during milking due to them being present in the aqueous phase of milk (see Section 1.5.2. in Chapter 2).

3.2 MATERIALS AND METHODS

3.2.1. Experimental Design

The trial was conducted at the Royal Veterinary College Holstein Dairy Herd, Boltons Park Farm, Potters Bar, UK in February 2001. Six cows in early lactation (two to three weeks postpartum) were selected for study and milk samples were taken at the afternoon milking. Before the start of milking, a T-piece sampler was inserted into each milk line to allow samples to be collected every four litres. Cow 673 was sampled every five litres due to a higher level of milk production. A 50ml-sample was collected from the long milk tube with the sampler direct into a sample pot, labelled with cow number, date and time within milking that sample was taken. Six samples were collected from each cow throughout milking, including foremilk (sample one) and post-milking samples (sample six), which were composed of strippings from all four quarters. Within milking, four samples were taken (samples two, three, four and five). A whole milk sample from the milk meter for each cow was also collected as a control (sample number seven), to compare the concentration of milk constituents using conventional sampling with sampling at various times throughout milking. This sampling regime was repeated for each cow on four

consecutive days (total 168 samples). A sub-sample of milk was analysed for acetone immediately after sample collection to prevent any loss of acetone during sample transport and storage. Samples were preserved with one Lactab MkIII tablet (Thomson and Capper Ltd., Runcorn, Cheshire, UK) containing 30mg potassium dichromate and refrigerated at 5.5°C until subsequent analysis for urea, progesterone, fat and protein.

3.2.2. Acetone Analysis

Milk samples were analysed in duplicate for acetone with the Keto-sensor by the method discussed in Chapter 2.

3.2.3. Urea Analysis

Urea was determined by the Beckman BUN Analyser 2 (Figure 3.1, Beckman Instruments Limited, High Wycombe, Buckinghamshire, UK). The Beckman Analyser works in conjunction with a Beckman Conductivity Electrode, which measures enzymatic conductivity rate. The sample (a fixed volume) is manually pipetted into a cup containing urease reagent and the electrode detects changes in solution conductivity. The rate of increase in conductivity is directly proportional to the concentration of urea in the sample, which is shown on the digital display in millimoles (mM). When the sample is injected into the cup containing the urease reagent, the following reaction occurs:

$$H_2NCONH_2 + H_2O \rightarrow (NH_4)_2CO_3 \rightarrow NH_4^+ + HCO_3^-$$

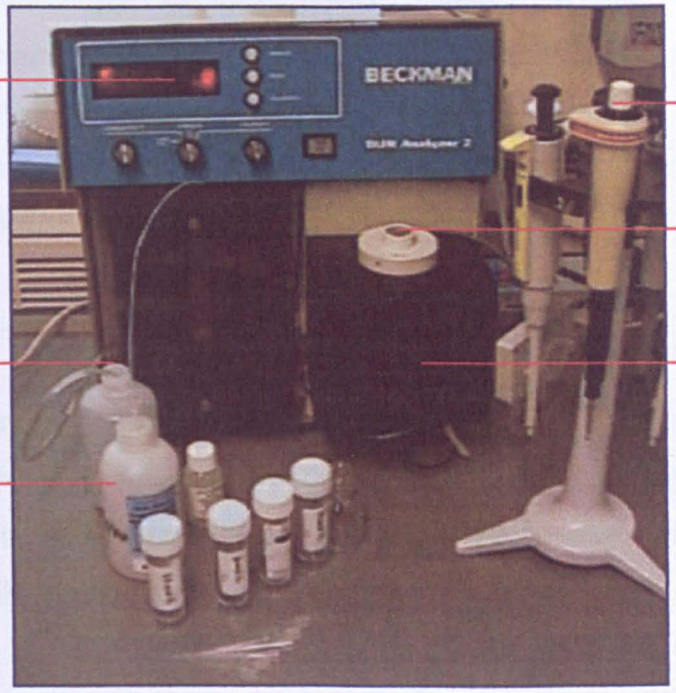

Figure 3.1. The Beckman BUN Analyser 2

The Beckman Analyser is designed to analyse blood samples for urea and this method was adapted for milk. Milk samples and Beckman reagents[1] were warmed to room temperature before the analysis was carried out. The analyser was calibrated prior to sample analysis with a urea standard of 17.8mM. Milk samples were defatted before analysis by centrifugation of a 5ml sample at 3000rpm for ten minutes. The defatted milk was extracted, 10µl pipetted into the Beckman analyser and the reading recorded. Samples were analysed in duplicate (or triplicate if the first two results differed by 0.3mM). The calibration standard was checked every 12 samples. Non-defatted samples were also analysed to investigate whether the fat content in milk had an effect on urea determination. Care was taken to ensure that the samples were thoroughly homogenised and that all the fat was dissolved before analysis.

[1] Beckman reagents were supplied by Beckman Coulter Ltd., High Wycombe, Buckinghamshire, UK and included:

- BUN buffer solution: Tris-(hydroxymethyl) amino methane, 4mmol/l. Ethylene diaminetetraacetic acid, 1mmol/l. Preservatives and stabilisers.

- BUN urease solution: Tris-(hydroxymethyl) amino methane, 4mmol/l. Ethylene diaminetetraacetic acid, 1mmol/l. Urease (plant), 200000 IU/L (37°C). Preservatives and stabilisers.
- Calibration standard: 150mg/dl D-glucose and 50mg/dl urea nitrogen, (107mg/dl urea) in 0.85% sodium chloride. Preservatives and stabilisers.

3.2.4. Progesterone Analysis

Milk progesterone was assayed by the ELISA procedure developed by Ridgeway Science Ltd, Alvington, Gloucestershire, UK. ELISA plates were brought to room temperature and the buffer solution emptied from the wells. A 10µl-aliquot of standard or milk sample was added to each well, followed by 200µl of progesterone-enzyme label. Standards contained 0, 1, 2, 5, 10, 20 or 50ng/ml of progesterone in milk. A wash buffer consisting of 0.05% Tween 20 in wash buffer A[2] was added to blank wells. The plate was agitated and left at room temperature (22°C) for two hours. Wells were emptied and washed in cold water three times. A 200µl-aliquot of ELISA substrate (Sigma 104 phosphatase substrate) was added to each well and left for 10 to 15 minutes. The substrate was composed of Sigma Fast p-Nitrophenyl phosphate tablets (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) which were dissolved in buffer B[3] (140mg/ml). A colour change occurred and the optical density was read by an ELISA plate reader at 405nm after zeroing the machine on the blank well. The concentration of progesterone in milk samples was derived from the standard curve. Four repeats per sample were carried out.

[2] Wash buffer A: Diethanolamine buffer, 100mM. pH 7.0
2.67ml made up to 250ml in distilled H_2O, pH with concentrated HCl (approx. 2ml).
[3] Buffer B: Diethanolamine buffer, 100mM. pH 9.85.
2.67ml made up to 250ml in distilled H_2O, pH with concentrated HCl (approx. 100ml). Add 250mg $MgCl_2$ to give 10mM $MgCl_2$.

3.2.5. Fat and Protein Analysis

Milk analysis for fat and protein was carried out by near infrared (NIR) spectroscopy (NIR Systems 6500, Foss UK Ltd., Warrington, Cheshire, UK). Samples were

warmed to room temperature and mixed so that the fat dissolved and was evenly distributed before analysis. A subset of samples (23) was randomly selected by the computer and analysed for fat and protein content by the Gerber test and nitrogen analysis respectively. The results obtained were used to improve the calibration from the NIR spectroscopy analysis.

3.2.5.1. Gerber test

The Gerber method measures fat content within the range 0 to 80 g/kg with a specially calibrated tube called a butyrometer. A 10ml-aliquot of sulphuric acid (H_2SO_4) was placed in each Gerber tube followed by 10.94ml of milk sample and 1ml of iso-Amyl alcohol. The tubes were continuously inverted for five minutes to dissolve the fat, after which they were incubated in a water bath at 70°C for five minutes. The tubes were centrifuged for five minutes at 1100rpm and replaced in the waterbath for a further three minutes. Samples were allowed to cool for two minutes and the fat level was read off the scale on the butyrometer tube.

3.2.5.2. Nitrogen analysis

Nitrogen analysis was carried out on the same samples used for fat analysis. A 200µl subsample of each milk sample was weighed. Samples were frozen at -80°C overnight and transferred to a freeze drier at -40°C for 24 hours. Samples were prepared in duplicate and analysed for total nitrogen content by a Nitrogen Analyser NA 2000. The nitrogen content of milk samples was converted to protein content by multiplying by 6.38 (M^{c}Donald et al, 1995).

3.2.6. Statistical Analysis

The majority of statistical analyses described in the thesis have used a nested block structure to describe the way in which the experiments were designed and conducted. The symbol "/" means to nest the factor after "/" within the first factor. This corresponds to two layers or strata. For example, in Chapters 4.1, 4.2, 4.3, 5.1 and 5.2, "Day/Cow" expands to Day + Day.Cow and in Chapters 3, 4.2 and 5.3 a three layer experiment was carried out so that a "Day/Stage/Cow" block structure expands to Day + Day.Stage + Day.Stage.Cow. P values for significance testing between

treatments were determined from T distribution tables with the effect of the treatment, or for example, the difference between lactation stages or cows, being significant at $P<0.05$.

Data from the current experiment were analysed by General Analysis of Variance (ANOVA) with specific contrasts in Genstat 4.1. The block structure was "Day/Cow/Sample number" and the treatment structure was "Sample number". The block structure contains the factors describing the experimental design and the treatment structure describes the treatments applied to each of the blocks. The use of contrasts investigated the trend between samples one to five and compared sample seven (the reference sample) with samples one to five in order to identify which sample throughout milking was closest to sample seven in composition. Sample six was not included in the statistical analysis to investigate the linear response because that sample was not collected at a fixed time, whereas samples one to five were collected at regular intervals throughout milking. Sample six was regarded as different from the others and constituted a large source of variation, especially for fat. By eliminating this sample the deviations, or variation left over after the specific contrasts had been compared, were reduced to an acceptable level.

Variation within cows (day-to-day variation) and between cows was investigated by General Linear Regression with Groups for acetone, urea, fat and protein as the response variate and litres of milk as the explanatory variate so that milk composition at each sampling point was taken into consideration in the statistical analysis. Both "Day", "Cow" and "Litres" were used as grouping factors. The model to be fitted was "Day*Cow*Litres" where "*" is an interaction term. Progesterone data were not analysed statistically due to the very low concentrations obtained for most of the cows throughout the sampling period.

3.3 RESULTS

Data on within milking variation in individual cows can be found in Appendix 4.

3.3.1. Acetone

Milk acetone concentrations were very low for all six cows ranging from 0.05mM to 0.19mM, with the mean ± standard error being 0.118 ±0.004mM. There was significant variation in acetone concentration during milking, with a negative linear trend in samples one to five, indicating that acetone decreased throughout milking ($P<0.001$). The reduction in acetone between samples was small with an average decrease of 0.0045mM for each consecutive sample (Table 3.1). When comparing the sample from the milk meter with the average of samples one to five the reference sample was found to be significantly lower ($P<0.001$) as this sample contained the lowest concentration of acetone. Generally, acetone concentration was lowest in the representative sample that was taken from the milk meter and highest in the premilking sample.

Table 3.1. Within milking variation in milk acetone concentration

Sample Number	Sample Description	Acetone (mM)	SEM
1	Premilking sample	0.146	0.005
2	During milking	0.115	0.005
3	During milking	0.127	0.005
4	During milking	0.126	0.004
5	During milking	0.118	0.005
6	Postmilking sample	0.110	0.006
7	Reference sample	0.082	0.004

* SEM = standard error of the mean. $P<0.001$ for linear response in acetone between samples 1 to 5

3.3.2. Urea

3.3.2.1. Defatted samples

Urea concentrations in individual samples ranged from 3.4 to 9.0mM, which is slightly wider than the range of normal urea defined by Plym Forshell (1994), cited

by Whitaker et al, (1995) as 3.5 to 6.0mM. The mean concentration of all the samples was 6.01 ±0.06mM. Urea concentration remained fairly constant throughout milking with little difference between the foremilking sample and the postmilking sample (Table 3.2). There was no significant difference among samples one to five (P=0.067) although there was a very small negative trend in the data with decreasing urea levels throughout milking and an average decrease of 0.036mM between samples. Sample seven was significantly different from the average of samples one to five (P=0.006), having the lowest concentration of urea.

Table 3.2. Within milking variation in milk urea concentration

Sample Number	Sample Description	Urea (mM)	SEM	Urea defatted sample (mM)	SEM
1	Premilking sample	5.43	0.23	6.13	0.27
2	During milking	5.41	0.22	6.08	0.27
3	During milking	5.35	0.23	6.08	0.28
4	During milking	5.21	0.23	5.94	0.28
5	During milking	5.12	0.23	6.01	0.27
6	Postmilking sample	4.74	0.20	5.96	0.28
7	Reference sample	5.08	0.23	5.86	0.26

* SEM = standard error of the mean. P=0.067 for linear response in acetone between defatted samples 1 to 5

3.3.2.2. Non-defatted samples

Samples that had not been defatted prior to urea analysis showed significant within milking variation in samples one to five (P<0.001), with the concentration of urea decreasing throughout milking. The sample from the milk meter also differed significantly from the average of samples one to five (P<0.001), having the lowest concentration of urea. Mean urea concentrations obtained for the defatted samples were slightly higher than the non-defatted samples.

3.3.3. Progesterone

Progesterone concentrations were very low and the majority of samples had progesterone concentrations less than 2ng/ml (SEM = 0.359). Therefore within milking variation for progesterone could not be established. In cow 72, progesterone increased throughout milking but only on days three and four of sampling (Figure 3.2).

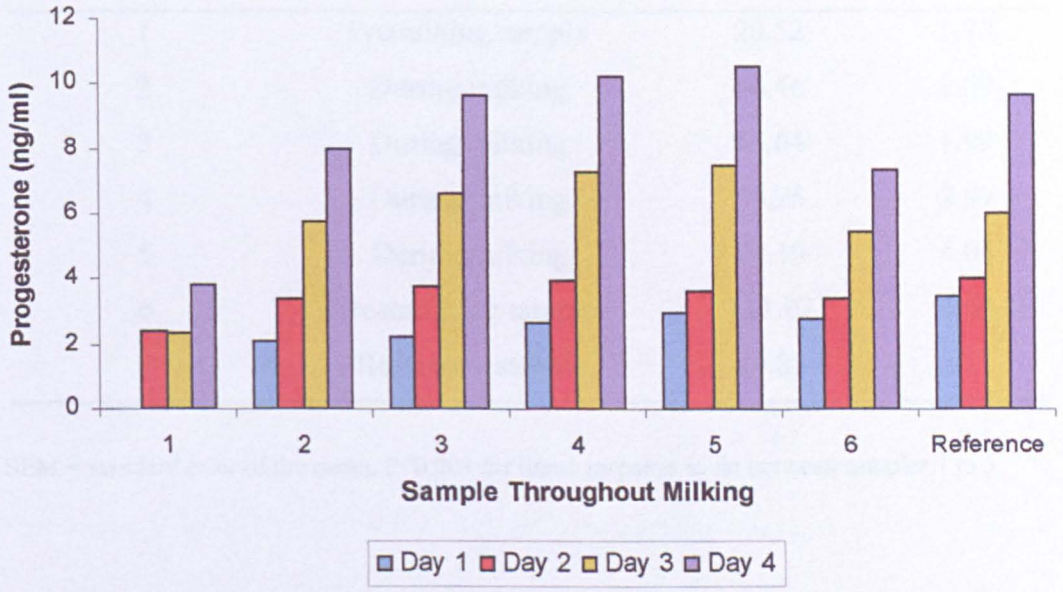

Figure 3.2. Within milking variation in progesterone in cow 72

3.3.4. Fat

The range of fat concentration in the samples was greater than expected (7.0 to 162.9g/kg) with a mean fat content of 56.3 (±2.6g/kg). There was a significant increase in fat concentration throughout milking ($P<0.001$) with an average increase of 1.32g/kg between samples (Table 3.3). There was a greater increase in fat content between sample five collected at 16 litres into milking and the postmilking sample. This is because samples two, three, four and five were collected every 4 litres with sample five being taken at 16 litres into milking. Sample six was the postmilking sample collected after the milking units had been removed and it is possible that these cows were yielding more than 20 litres in the afternoon milking, accounting for

a greater increase in fat content between samples five and six. The reference sample from the milk meter had a fat content which was not significantly different (P=0.255) from the average of samples one to five, indicating that it had a similar fat content to the third sample which was taken at approximately eight litres into milking.

Table 3.3. Within milking variation in milk fat concentration

Sample Number	Sample Description	Fat (g/kg)	SEM
1	Premilking sample	20.52	1.70
2	During milking	34.46	1.69
3	During milking	44.64	1.99
4	During milking	59.25	2.99
5	During milking	74.19	4.04
6	Postmilking sample	111.07	4.69
7	Reference sample	49.83	2.81

* SEM = standard error of the mean. P<0.001 for linear response in fat between samples 1 to 5

3.3.5. Protein

Protein remained fairly constant throughout milking, ranging from 29.1 to 41.2g/kg with an overall mean protein content of 34.3 (±5g/kg). There was no significant trend in protein throughout milking (P=0.605), with an average increase in protein content of only 0.8g/kg between samples (Table 3.4). The sample from the milk meter was significantly different from the average of samples one to five (P=0.033), with a much lower protein content compared with all the other samples.

Table 3.4. Within milking variation in milk protein concentration

Sample Number	Sample Description	Protein (g/kg)	SEM
1	Premilking sample	34.29	0.05
2	During milking	34.22	0.05
3	During milking	34.36	0.05
4	During milking	34.73	0.06
5	During milking	34.45	0.04
6	Postmilking sample	34.90	0.06
7	Reference sample	33.22	0.04

* SEM = standard error of the mean. P=0.605 for linear response in protein between samples 1 to 5

3.3.6. Within and Between Cow Variation in Milk Concentration of Specific Components

There was no significant variation in milk acetone or protein concentration between cows or within cows on a daily basis (Table 3.5). There was a significant between-cow variation in milk urea when averaged over all the days but no day-to-day variation when the urea contents at each sampling point were averaged over all the cows. However, for individual day/cow combinations there were significant differences in urea concentrations at the various sampling points. Being the most variable milk constituent, fat concentration showed significant variation between days and between cows as well as for different day/cow combinations.

Table 3.5. Variation in milk constituents between cows, between days and variation for each cow/day combination taking sample number into account

	Significance of Statistical Model (P value)		
Parameter	*Litres.Day*	*Litres.Cow*	*Litres.Day.Cow*
Acetone	ns	ns	ns
Urea (defatted milk)	0 .002	ns	<0.001
Fat	0.014	<0.001	0.013
Protein	ns	ns	ns

*ns = not significant. Litres.Day = day-to-day variation averaged over all the cows. Litres.Cow = cow-to-cow variation averaged over all the days. Litres.Day.Cow = individual cow/day combinations.

3.4 DISCUSSION

3.4.1. Acetone

There was very little variation in milk acetone concentration between cows and concentrations were very low. This was unexpected since the cows were in early lactation (two to three weeks after calving) and milk ketones are commonly reported to be high during the second to sixth week of lactation (Andersson and Emanuelson, 1985). Low acetone concentrations may be explained by good nutritional management on the farm, with the cows being fed slightly in excess of requirements for milk yield and being on an *ad libitum* high energy diet. Cows were also in good body condition and there was no evidence that cows were overconditioned at calving, which is one of the contributing factors to ketosis in early lactation (Gillund et al, 2001). According to Crooker (1997), cows that are well fed in early lactation and are at the desired condition score will tend to experience little, if any, detrimental effect of negative energy balance and this appears to be the case in these experimental cows.

Low acetone concentrations may be attributed to experimental technique. It is possible that some acetone may have been lost during the preparation of the milk samples prior to analysis, due to the high volatility of acetone. However as samples

were analysed as soon after collection as possible, and care was taken to seal the sample vials quickly, this loss is likely to be minimal. Throughout milking acetone concentration decreased, being highest in the foremilking sample and lowest in the sample from the milk meter. The fractional sample from the milk meter may have had a lower acetone concentration compared to the other samples due to it being held under vacuum for the duration of milking, thereby increasing the loss of acetone from the milk. This would explain why mean acetone concentrations were highest in the foremilk samples, which were obtained by hand and were not under vacuum.

There appeared to be little numerical difference between acetone concentrations in samples taken from the beginning and at the end of milking (despite being statistically significant) and so in this experiment the large variation in fat content throughout milking only had a small effect on acetone concentrations. However, because acetone is highly water soluble and therefore evenly distributed in the water phase of milk, a decrease in acetone concentration throughout milking would be expected due to the higher fat content towards the end of milking.

It is difficult to determine the correct sampling time for an on-line system since the milk meter sample contained the lowest concentration of acetone and was not comparable with any of the samples taken throughout milking (Table 3.1). The concentrations obtained for milk acetone were very low and the difference between each sample would be insignificant in terms of data interpretation for assessing energy status. It can be assumed that at low concentrations, which are not detrimental to health or productivity, the variation within milking would not be critical to determine energy status of the cow. If acetone is high it could be expected that, despite variation within milking, critical levels to ensure health and maintain productivity could still be picked up and the appropriate dietary adjustment made or treatment given.

3.4.2. Urea

There was a small decline in urea concentration throughout milking although the difference between samples was not statistically significant (Table 3.2). Urea has been shown to vary throughout milking and this effect has been attributed to the

higher fat content towards the end of milking displacing water and water-soluble components (Carlsson and Bergström, 1994; Jenkins, Delwiche and Claycomb, 2002a; Jenkins et al 2002b). Therefore Jenkins et al (2002b) suggested that milk sampling should occur early on in milking and after the removal of the foremilk when the fat content is low. This sample should then be low in fat and representative of a composite sample from the cow. However data from this experiment suggest that the urea content of the composite sample was closest to the urea content of samples four and five, which were collected at 12 and 16 litres into milking respectively.

Variation during milking has been previously investigated by Carlsson and Bergström (1994). However, only three samples were taken throughout milking; strippings, when the udder was approximately half empty and from the residual milk in the udder after normal milking. Urea was measured by flow injection analysis and concentrations were found to be highest in the foremilk sample and lowest in the residual milk. However, because urea is not fat soluble, the concentration of urea was adjusted for the water phase of milk only and there was no significant difference in urea throughout milking. This agrees with the results from this study where there was no significant within milking variation in urea for defatted milk samples.

The highest mean concentration of urea was found in the foremilk sample, which contradicts the results of Jenkins et al (2002b) who found that urea was slightly lower in the foremilk sample than the composite sample. The authors hypothesised that foremilk had a slightly different urea content compared with the composite milk sample due to milk in the gland and teat cisterns not being in such close contact with the blood as the milk ejected during full let down. However, as urea readily diffuses from blood, across the mammary tissue and into the milk with a short lag time, this effect is likely to be minimal.

The reference sample from the milk meter was expected to have a urea concentration within the range obtained from samples throughout milking. However, the mean urea concentration in the milk meter sample was lower than the other samples taken throughout milking. This finding cannot be explained. Nevertheless, the mean change in urea content from the premilking to the postmilking sample was only 6.13

to 5.96mM and the differences between the samples were so small that assessment of nutritional status or nutritional management decisions would not be influenced by time of sampling during milking.

Milk samples were also analysed without prior defatting and there was an even greater tendency for urea to decrease throughout milking compared with the defatted samples ($P<0.001$). However, it is thought that milk fat adheres to the conductivity probe in the Beckman analyser and affects the conductivity reading of the reaction mixture, resulting in lower than actual urea concentrations (Mottram, T.T. personal communication). This theory would account for the variation between the defatted samples and the non-defatted samples. Therefore these results were not considered for assessing within milking variation in urea and determining the optimal sampling time.

It can be concluded that time of sampling throughout milking is not a consideration for an on-line system and the sample can be collected at any point. Preferably sampling should occur as close to the beginning of milking as possible so that there is time for the analysis to be carried out before the next cow comes in for milking.

3.4.3. Progesterone

It was expected that the level of progesterone would increase throughout milking because progesterone is lipid-soluble and fat content rises throughout milking. However, milk progesterone concentrations were very low, with five out of the six cows showing concentrations below 4ng/ml and the majority under 2ng/ml. ELISA assays are not very accurate when analysing samples with either very high or very low concentrations of progesterone because the calibration curve is sigmoidal. However, this analytical problem can be ignored as no significant rise in progesterone was observed except in cow 72 (Figure 3.2). Low levels of progesterone can be attributed to the cows having recently calved, and had not yet resumed cyclicity. Data are presented in Appendix 5 on within milking variation in progesterone as determined by ELISA at high and low concentrations.

No trend was observed in the concentration of progesterone throughout milking except in cow 72 where progesterone increased as milking progressed. This trend was only observed on days three and four with progesterone concentrations ranging from 2.37 to 7.48ng/ml and 3.86 to 10.51ng/ml respectively. These results support the hypothesis that progesterone increases throughout milking due to an increase in fat content because progesterone is hydrophobic and lipolytic (Pope and Swindburne, 1980). However, within milking variation needs to be investigated in more detail with more cows that are in mid cycle when progesterone concentrations are high.

A critical factor in accurately determining progesterone concentration is the time of milk sampling. The increase in progesterone with fat content can be considerable, as shown by Hoffman and Hamburger (1973), cited by Pennington, Spahr and Lodge (1981). Based on their data the authors stated that for each percentage increase in milk fat, progesterone concentrations increased by 3ng/ml. Pope et al (1976) measured progesterone in the milk of five pregnant cows and found that progesterone averaged 8.4, 17.5 and 22.5ng/ml for milk samples from foremilk, total yield and strippings respectively.

If the optimal sampling time for progesterone throughout milking is not known then incorrect assumptions about reproductive status may be made, depending on when a sample is taken for analysis. As progesterone rises throughout milking, high concentrations towards the end of milking may be misdiagnosed as cows being pregnant or in mid cycle. Progesterone has been shown to vary from 4.1ng/ml to 12.9ng/ml when samples were collected at 30 and 420 seconds into milking respectively from a cow in mid-cycle (Velasco-Garcia, M. personal communication). It is possible that low concentrations of progesterone, which are found near the beginning of milking, could be interpreted as cows approaching oestrus and thus leading to incorrect timing of insemination. However, progesterone has not been reported as low as <3ng/ml in premilking samples or early on in milking, which is when pro-oestrus starts (Firk et al, 2002).

Mottram, Hart and Pemberton (2000) found that samples taken before 120 seconds into milking could result in an underestimation of progesterone concentration in the milk sample. However, time of sampling may be better expressed on a litres basis

instead of time into milking since cows will vary in their speed of milking. Milk progesterone is lower in milk obtained from the cisternal compartment of the mammary gland than milk from the alveoli (Waldmann et al, 1999), most likely due to the changes in fat content during milking. Because the gland cistern has a capacity of about 100-2000ml (Nickerson, 1995), sampling for progesterone should take place at least 2 litres into milking to obtain alveolar milk and ideally sampling should be carried out as early as possible for the analysis to be completed before the milking system is needed for the next cow.

3.4.4. Fat

Considerable variation in milk fat content was observed in this study with concentrations ranging from 7.0 to 162.9g/kg, which was much greater than expected. Few studies have reported the extent of fat content increase as milking progresses. Carlsson and Bergström (1994) found significant variation in fat content within milking, with means ranging from 32.6 to 101.4g/kg and Tsenkova et al (2000) reported an increase in fat content from just above 10g/kg to almost 80g/kg when sampling every three litres throughout milking.

The increase in fat concentration throughout milking can be explained by the nature of the fat droplets, having a lower specific gravity and a slower rate of removal from the milk ducts during milk ejection due to their size and viscosity (Nickerson, 1995). Therefore, fat may tend to accumulate in the upper regions of the alveoli, larger ducts and cisterns, thereby accounting for the relatively lower fat concentration at the beginning of milking. M^{c}Kusick et al (2002) found significant variation in milk fat percentage in milk from the cisternal compartment of the mammary gland compared with alveolar milk (4.49 and 7.92% respectively) in dairy ewes. However no difference in protein content was observed between cisternal and alveolar milk. This finding suggests that the water soluble components of milk (proteins, lactose, vitamins and minerals) move more freely throughout the duct system in the mammary gland compared with fat globules, thereby accounting for the significant increase in fat content throughout milking, compared with relatively little change in protein content.

The reference sample from the milk meter had a similar fat content to the average of samples one to five throughout milking (Table 3.3). This was to be expected since milk samples for compositional quality analysis are normally taken from the milk meter, which provides a milk sample representative of the whole milking. However, it is difficult to recommend an optimum sampling time that would give an accurate result that was not significantly different from a fractional sample representative of the whole milking. Factors influencing the optimum sampling time may include variation in milk yields between cows and the extent of increase in fat content throughout milking with every few litres. The fat content at the end of milking will also vary considerably between cows due to the length of the milking interval and completeness of milking. Therefore the optimal sampling time is difficult to determine and it is likely that this will vary between cows.

3.4.5. Protein

The protein content in the reference sample from the milk meter was considerably lower than the other six samples taken throughout milking (Table 3.4). A similar effect was observed with urea. In theory the composition of the reference sample should fall within the ranges of milk composition obtained for samples one to five. However, since sampling time did not have a significant effect on milk protein content throughout milking, sampling time in an on-line system is not an important consideration for milk protein determination.

There is little published data on variation in protein content throughout milking, with studies finding protein to be relatively constant (Tsenkova et al, 2000; Velasco-Garcia, M. personal communication (Appendix 7)). Carlsson and Bergström (1994), found a significant increase in protein content throughout milking ($P<0.05$) although the range in protein content was very small (32.3 to 37.1g/kg). Protein was only analysed in a premilking sample, a postmilking sample and a sample taken half-way through milking and it would have been useful to collect more samples throughout milking to obtain a more accurate picture of the change in protein content as milking progressed.

The variation in milk composition throughout milking may be explained by examining the process of milk secretion. Milk removal from the mammary gland occurs by two processes. A passive withdrawal phase removes milk from the cisterns and ducts of the udder and the ejection phase removes the alveolar milk fraction. Passive withdrawal of milk is brought about by a nervous reflex which contracts the smooth muscles lining the ducts and forces milk into the udder cisterns. The milk ejection reflex occurs when the passive withdrawal phase is taking place. The hormone oxytocin is released from the posterior pituitary in response to nerve impulses sent to the brain from the teats due to stimulation from the milkers' hands and attachment of the teat cups. Oxytocin causes a contraction of the myoepithelial cells surrounding the alveoli and small ducts to release milk and force it along the ducts towards the gland cistern. Therefore alveolar milk and that in the narrow ducts draining the alveoli, is secreted after the passive withdrawal phase of milking has taken place. The majority of milk protein is present as small casein micelles and these move freely from the alveolar to the cisternal compartment of the mammary gland, unlike milk fat which is more dependent on the milk ejection reflex for its removal from the alveoli and alveolar compartments. This would account for the little variation in protein content and the significant increase in fat content during milking.

3.4.6. Within and Between Cow Variation

Although many of the data from this experiment seem to agree with the literature, in that urea and fat were variable and protein was more constant between cows and had less variation on a daily basis (Table 3.5), there were only six cows in this study and they were sampled on only four days. To obtain a more accurate picture of day-to-day variation in milk composition, these sources of variation should be investigated with a greater number of cows and over a longer period of time. Variation in acetone was small and acetone concentrations were not significantly affected by interaction between cow and day ($P = 0.262$). However at higher concentrations of acetone and the self-adjustment of lowering milk yields to balance energy requirements, it can be hypothesised that acetone will be more variable on a daily basis and variation between cows will be significant depending on lactation stage and level of milk production.

3.5 CONCLUSIONS

The aim of this experiment was to determine within milking variation of acetone, urea, progesterone, fat and protein and the optimum time for milk sampling in an on-line monitoring system. In summary, the data revealed that there is significant within milking variation in acetone and fat. Previous studies also suggest that the fat content accounts for a significant increase in milk progesterone but this needs to be investigated in greater detail in more cows that have resumed cyclicity postpartum and when the cows are in mid cycle, so that progesterone concentrations are high. These variations throughout milking create problems for determining the best sampling time in an automated system. Samples should be collected at least two litres into milking to obtain alveolar, rather than cisternal milk. Although acetone decreased significantly throughout milking it is thought that since the difference between samples was so small, the optimum sampling time is not critical. Since urea and protein content did not vary during milking a sample could be taken at any point to give an accurate concentration. For fat the milk meter sample was similar to the average of samples one to five, suggesting that around the time when sample three was taken would give a good indication of fat content. However, this would vary with individual cows depending on their level of milk production.

CHAPTER 4. THE USE OF MILK PROFILING TO MONITOR REPONSES TO DIETARY CHANGE AND DETECTION OF NUTRITIONAL STRESS

4.1. EXPERIMENT 1. THE EFFECT OF DIETARY CHANGE ON MILK COMPOSITION

4.1.1. Introduction

Changes to dairy cow diets are a common occurrence throughout the lactation cycle as nutrient requirements change due to changes in milk yield. Cows also experience changes in dietary composition such as moving from a high energy TMR ration to summer grazing and changes in TMR composition, as a result of raw material availability. These dietary changes may affect milk composition to some extent. For example, Jurjanz et al (1998) found that using the same levels of either wheat or potato as the starch source in a TMR significantly affected milk fat yield. For on-line metabolic profiling, the ability to monitor changes in milk composition due to nutrition are important, not only to determine the new level of milk constituents, but to ensure adaptation to the new diet has taken place and that this has not had a detrimental effect on milk quality.

There are no reported data on individual cow responses to dietary changes in terms of milk composition, as most data tend to be analysed at the treatment group level. Milk composition is commonly measured after a period of time, post diet change, without any data on changes in milk composition over the period when cows are adjusting to new diets. Therefore the aims of the current experiment were firstly to investigate the effect of changing the ratio of dietary starch to fibre on milk composition (acetone, urea, fat and protein content) in early lactation. Secondly, to determine whether dietary changes could be detected through changes in milk composition over the period of dietary adjustment in individual cows. The hypothesis was that there would be no significant difference in milk acetone and urea concentrations between treatments since all diets were isoenergetic and isonitrogenous. Dietary manipulation of the ratio of starch to fibre should be

reflected in changes in milk fat and to a lesser extent, protein. High starch diets will reduce milk fat and promote milk protein while the high fibre diets will promote fat and reduce protein content.

4.1.2. Materials and Methods

4.1.2.1. Animals and husbandry

The study was conducted at the University of Nottingham dairy unit. Thirty Holstein-Friesian cows in their second to sixth lactations and calving over the period of July to October 2001 were selected for experiment, which ran until the end of December 2001. Cows were housed in groups of ten in three pens from 20 ± five days and were trained to feed out of individual Calan electronic gates from day 20 onwards (Figure 4.1). Training took place over a period of 20 days during which the cows were fed a standard ration (days 20 to 40). Six cows were allocated to each treatment diet, which was fed from days 41 to 69. The allocation of cows to treatment diets was based on lactation number, weight and condition score at approximately day 30, and milk yield between days ten and 25. Cows were fed a TMR (fixed allocation) so that each group received the same nutrient concentration. Milking was twice daily at approximately 05:00 and 16:30 through an 11/22 Fullwood herringbone parlour. Fresh feed was provided at approximately 16:30 and feed bins were topped up at 07:00.

Figure 4.1. Calan electronic feeding gates

4.1.2.2. Dietary treatments

Diets were formulated to be isoenergetic and isonitrogenous. Grass silage, maize silage and brewers grains were used to formulate the basal diet which was supplemented with concentrates to supply ME (metabolisable energy) and MP (metabolisable protein) requirements for 45 litres of milk production (see Appendix 6 for basal diet). Concentrates were in a meal form and mixed with the basal diet each afternoon to produce a TMR, half of which was fed in the afternoon and half the following morning. Two concentrates were formulated, one with a high starch content and the other with a high fibre content. These concentrates were mixed in the following proportions to achieve the range of five concentrates: 0:6, 2:4, 3:3, 4:2 and 6:0. Diet three was the control diet which was fed for the first 40 days of lactation. This group of cows acted as a control throughout the trial as they received no diet change. No concentrate was offered in the parlour. Details of diet composition (on a fresh weight basis) are shown in Table 4.1.

Table 4.1. Diet composition on a fresh weight basis for cows yielding 45 litres

	Standard Diet (3)	**High Starch Diet (1)**	**High Fibre Diet (5)**
Basal diet (kg/day)	43	43	43
Concentrate (kg/day)	8.90	11.45	6.35
SBP (kg/day)	2.5	0	5
Total (kg/day)	54.4	54.45	54.35
Ratio conc:basal	20.70	26.63	14.77
Ratio SBP:basal	5.81	0	11.63
DMI (kg/day)	23.5	23.5	23.5
MEI (MJ/day)	287	287	287
MPI (g/day)	2432	2423	2440
Starch intake (g/day)	3659	5465	1853

*SBP = sugar beet pulp, DMI = dry matter intake, MEI = metabolisable energy intake and MPI = metabolisable protein intake

4.1.2.3. Milk sampling

Milk samples were collected on days 38, 39 and 40 to determine baseline concentrations of acetone, urea, fat and protein when cows were on the standard diet. Although the cows were transferred onto the treatment diets on day 40, diets were mixed in the afternoon and generally fed after the cows had been milked so milk samples on day 40 still corresponded to the control diets. Daily samples were collected for ten days over the period of dietary adjustment (days 41 to 50). Samples were also collected on days 55, 60, 65 and 69 to determine new levels of milk constituents after dietary adjustment had taken place. AM and PM samples were collected and milk yields were recorded from calving until the end of the trial.

4.1.2.4. Milk analysis

Milk was analysed for acetone, urea, fat and protein by methods discussed in Chapters 2 and 3). It was hoped that urea could be determined by NIR spectroscopy and only a proportion of samples would need to be analysed by the Beckman BUN Analyser 2 for calibration. However, over 300 milk samples were analysed by the Beckman BUN Analyser 2 and the calibration with the NIR method was very poor (R^2=0.201) so all the samples were analysed by the Beckman Analyser. Godden et al (2000) used a mid-infrared method (Fossomatic 4000 Milk Analyser) to measure milk urea and found good accuracy and precision with the mid-infrared method. However, individual cow milk samples showed variation in the analysis and Godden et al (2000) suggested that there were several components in milk that could affect urea measurement by the infrared method. For example, butterfat, total protein, lactose, somatic cell count and citrate also absorb some light at the wavelength that detects urea. This may account for the poor correlation between the Beckman method and the NIR method. It has been suggested that measuring milk urea by NIR spectroscopy is not feasible as the concentrations in milk are too small (0.2 to 0.7g/l) and mid infrared spectroscopy is recommended to measure milk urea (Mathiasen, T. personal communication).

4.1.2.5. Statistical analysis

Two cows were eliminated from the analysis due to them experiencing repeated cases of mastitis throughout the trial period. Other missing values for milk yield, acetone, urea, fat and protein (due to mastitis or missed samples), were replaced by

the means of values around them for the same cow. The mean daily milk composition for each constituent was determined from AM and PM concentrations and milk yields at each milking, and calculated according to the following equation:

((AM milk yield x AM [*X*]) + (PM milk yield x PM [*X*]))/(AM milk yield + PM milk yield) where [*X*] is concentration.

The mean milk composition for all 30 cows was calculated from data collected on days 38, 39 and 40 to determine baseline levels when the cows were on the standard diet (Period one). Milk composition over days 55, 60, 65 and 69 was calculated as a mean per diet (Period three). Differences between the baseline levels and the last four sampling days were investigated by General Analysis of Variance (ANOVA) with diet as the main effect in Genstat 5.0. The block structure used was "Day/Cow" and the treatment structure was "Period/Diet". Statistical significance between the diets was determined from T tables at $P<0.05$. Milk yield and fat:protein ratio data were also analysed in the same way. The distribution of acetone values was skewed so this data set was transformed to a logarithmic scale with base 10, making the data normally distributed before carrying out the statistical analysis. Normality was determined from residual plots of the transformed data. Since all 30 cows were on the standard diet in Period one, the baseline measurements calculated in this period were the same for each diet.

The effect of diet change from days 41 to 50 (Period two) was to be investigated to determine how long it took for dietary adjustment to take place, and whether changes in nutrition could be detected through changes in milk composition. However, from the data it was clear that there was little trend in milk constituents over the period of dietary adjustment and that composition varied from day-to-day. Therefore it was not possible to analyse these data statistically.

Fat corrected milk yield (FCM) at four percent was used to determine energy output of the cows. FCM converts actual milk and fat yields to a milk yield at a specific fat percentage so that volume of milk contains the same energy content to the original milk produced. This was calculated by the following equation:

FCM = (a x milk yield) + (b x fat yield) where a and b are factors for correcting milk and fat yields to different fat percentages. At 4% FCM, a = 0.4 and b = 15

Simple Linear Regression analysis was carried out with milk yield as the response variable and milk acetone as the explanatory variable to investigate the relationship between acetone and milk yield. The relationship between acetone and FCM, and fat:protein and FCM, was also investigated by Simple Linear Regression with acetone and fat:protein as the explanatory variable and FCM as the response variable.

4.1.3. Results

4.1.3.1. Feed intake

All feed intakes are expressed as dry matter (kg/DM). The dry matter contents of diets 1 to 5 were 0.446, 0.447, 0.447, 0.448 and 0.449 respectively. Initial data analysis suggested that the diets had little effect on milk composition. This was probably due to problems with the feeding facilities at the farm. The Calan gates were designed for Friesian cows and were too low for the Holsteins so some cows were able to feed from other feed bins containing different diets. By examining the feed intake data it was possible to identify cows with abnormal intakes. Data were re-analysed excluding seven cows that were identified as stealing. As a result, there were an uneven number of cows per dietary group with only three cows on Diets one and four, six cows on Diets two and three and four cows on Diet five (see Appendix 7 for data on feed intake in individual cows).

The high starch and high fibre diets (one and five respectively) were generally not liked by the cows. For example, cow 112 on Diet five had an average daily intake of 13.8 kg/day and cow 456 on Diet one was only consuming on average 16.1 kg/day. Cows on the control diet were eating approximately 27.0 kg/day. Cow 146 on Diet three had a fairly constant feed intake over the trial period (Figure 4.2). However, cows 456 and 112, which were on Diets one and five respectively, showed a decrease in feed intake over the trial period (Figure 4.2).

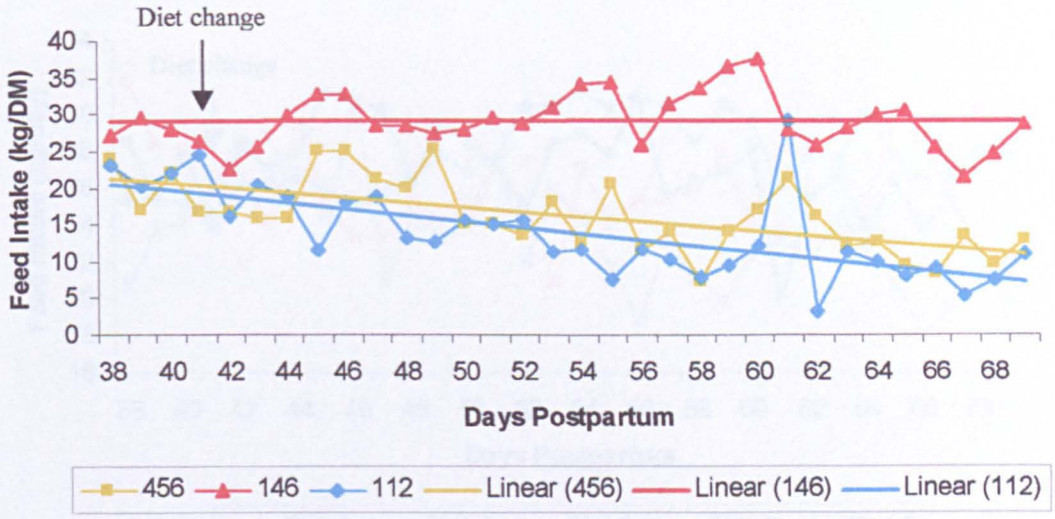

Figure 4.2. Daily feed intakes for three cows on Diets one, three and five

There was a significant effect of diet on feed intake (P=0.045) when intake on days 38, 39 and 40 were compared with intakes recorded on days 55, 60, 65 and 69. The lowest recorded intake was found in cows on Diet four (Table 4.2). Fluctuations in feed intake per day at the group level were considerable and no response to dietary change on day 41 was observed (Figure 4.3).

Table 4.2. Mean feed intake for each group of cows

Diet	Number of Cows	Baseline (mM)	SEM	New (mM)	SEM	P value
1	3	27.2	0.50	26.83	1.81	ns
2	6	27.2	0.50	25.82	1.40	ns
3	6	27.2	0.50	28.57	1.28	ns
4	3	27.2	0.50	21.69	1.81	<0.005
5	4	27.2	0.50	25.46	1.57	ns

*SEM = standard error of the mean, ns = not significant

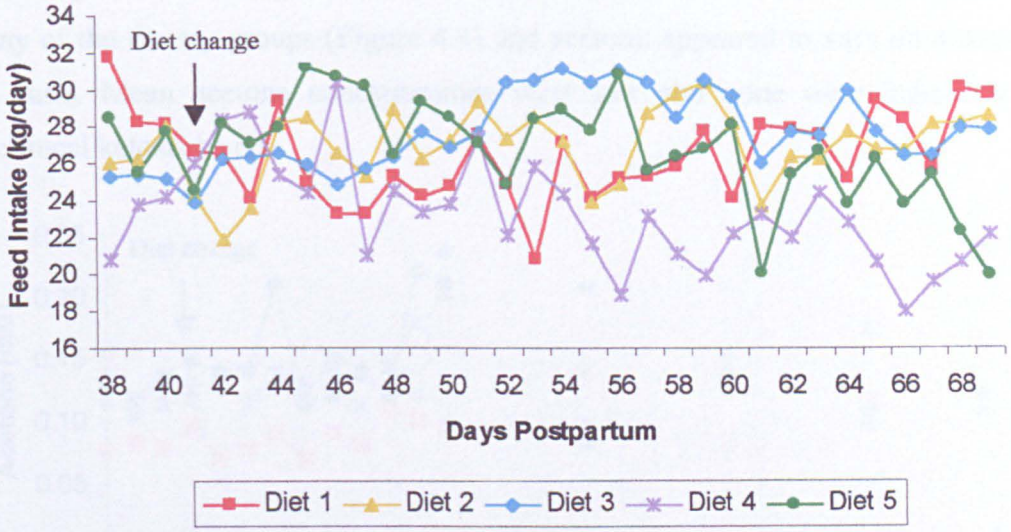

Figure 4.3. Effect of dietary change on feed intake over the trial period

4.1.3.2. Acetone

Mean daily concentrations of milk acetone ranged from 0.04mM to 0.55mM over the trial period, although the majority of measurements were less than 0.4mM. The highest recorded value was 1.02mM in cow 58 which had repeated attacks of mastitis. There was no significant effect of diet on acetone when mean baseline values per diet were compared with new level of acetone calculated in Period three (Table 4.3). The means presented in the table are the back-transformed means from the log base 10 data. SEMs could not be calculated from the log data. All comparisons were made on the log scale. The P value indicates the difference between the control (baseline measurement) and the new level of milk constituent.

Table 4.3. Mean milk acetone concentration for each group of cows

Diet	Baseline (mM)	New (mM)	P value
1	0.107	0.114	ns
2	0.107	0.110	ns
3	0.107	0.110	ns
4	0.107	0.134	ns
5	0.107	0.103	ns

Over the period of dietary adjustment (days 41 to 50) there was no trend in acetone in any of the dietary groups (Figure 4.4) and acetone appeared to vary on a day-to-day basis. Mean acetone concentrations were low and none were indicative of subclinical ketosis levels.

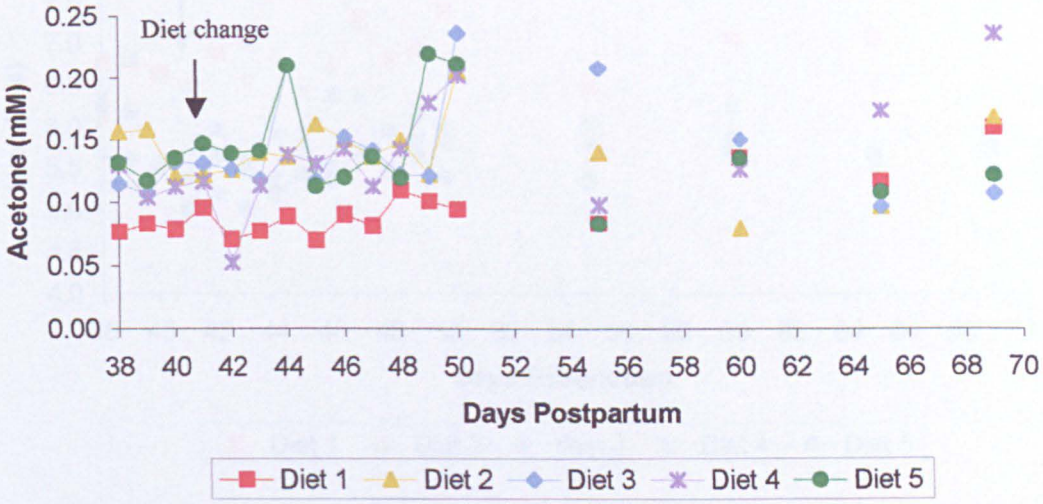

Figure 4.4. Effect of dietary change on mean milk acetone concentrations over the dietary adjustment period

4.1.3.3. Urea

Daily urea concentrations ranged from 3.55mM to 9.41mM, which was slightly outwith the normal physiological range. Dietary change resulted in a decrease in urea for all diets except Diet one, which increased ($P<0.05$) from the baseline value of 6.15mM to 6.70mM (Table 4.4).

Table 4.4. Mean milk urea concentration for each group of cows

Diet	Baseline (mM)	SEM	New (mM)	SEM	P value
1	6.15	0.09	6.70	0.25	<0.05
2	6.15	0.09	5.89	0.19	ns
3	6.15	0.09	5.74	0.17	ns
4	6.15	0.09	5.71	0.25	ns
5	6.15	0.09	5.82	0.21	ns

*SEM = standard error of the mean, ns = not significant

Urea varied on a daily basis and dietary change was not reflected in a change in milk composition over the dietary adjustment period (Figure 4.5). Urea increased from days 41 to 44 with Diet one but then decreased slightly, fluctuating from day-to-day.

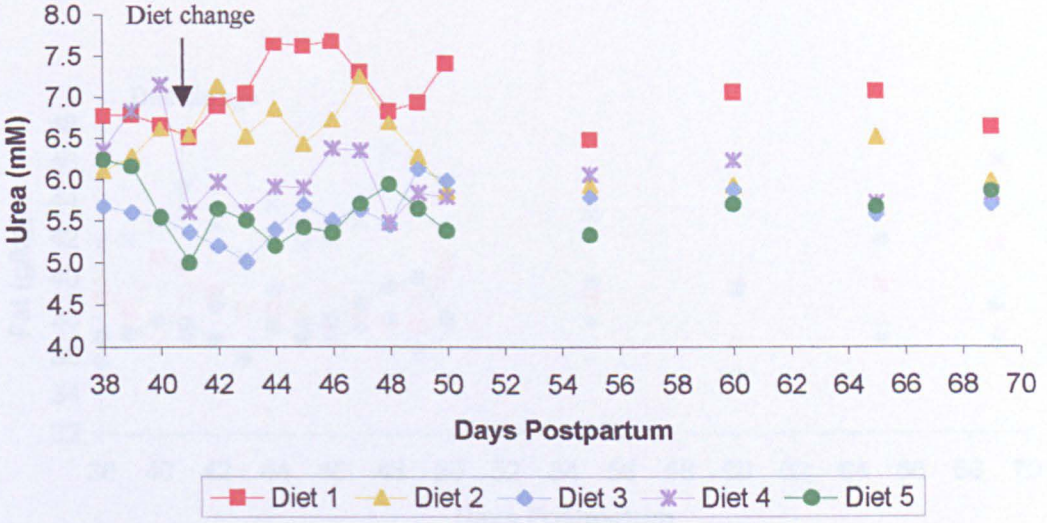

Figure 4.5. Effect of dietary change on mean milk urea concentrations over the dietary adjustment period

4.1.3.4. Fat

Only Diet four had a significant effect on milk fat concentration. Milk fat increased when cows on the control diet were changed to Diet four, which had a higher fibre content (Table 4.5). However Diet five which had the highest fibre content had no significant effect on milk fat.

Table 4.5. Mean milk fat concentration for each group of cows

Diet	Baseline (g/kg)	SEM	New (g/kg)	SEM	P value
1	38.23	0.34	40.06	1.36	ns
2	38.23	0.34	37.71	1.05	ns
3	38.23	0.34	36.52	0.96	ns
4	38.23	0.34	45.21	1.36	<0.001
5	38.23	0.34	39.93	1.18	ns

*SEM = standard error of the mean, ns = not significant

No trend in milk fat was found over the dietary adjustment period (Figure 4.6). Although daily fluctuations were observed, there was a slight increase in milk fat content over the period of dietary adjustment with Diets four and five. Fat content also decreased slowly from days 41 to 46 with Diet one.

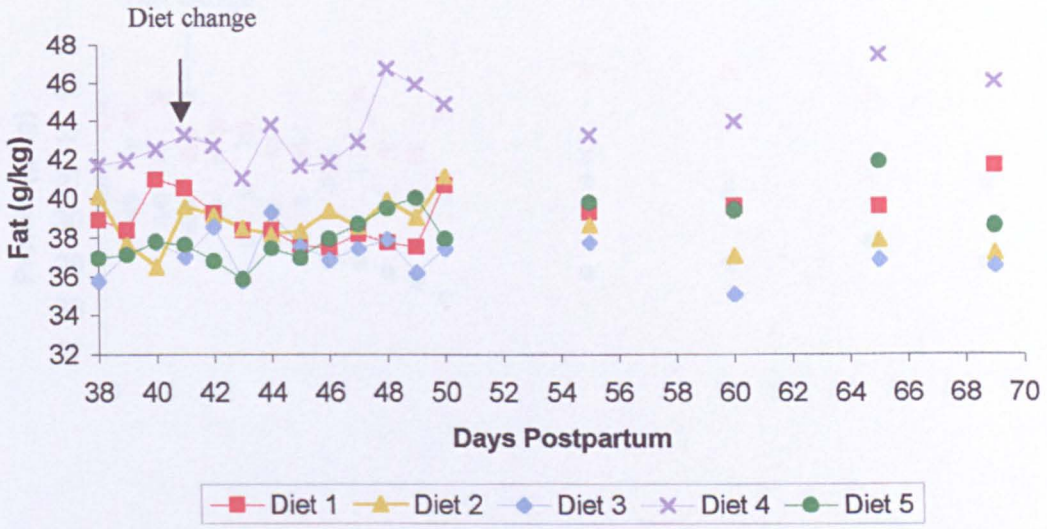

Figure 4.6. Effect of dietary change on mean milk fat concentrations over the dietary adjustment period

4.1.3.5. Protein

Dietary changes significantly influenced milk protein, with an increase ($P<0.001$) in protein content with the high starch diet and a significant decrease ($P<0.001$) with the high fibre diet, compared with the baseline value (Table 4.6).

Table 4.6. Mean milk protein concentration for each group

Diet	Baseline (g/kg)	SEM	New (g/kg)	SEM	P value
1	30.90	0.09	33.25	0.50	<0.001
2	30.90	0.09	30.57	0.15	ns
3	30.90	0.09	30.63	0.35	ns
4	30.90	0.09	31.05	0.50	ns
5	30.90	0.09	29.08	0.43	<0.001

*SEM = standard error of the mean, ns = not significant

During the dietary adjustment period, milk protein concentration declined steadily from days 42 to 50 with Diet five but no increase was observed with Diet one, even though the last four sample days had higher levels of protein compared with baseline values (Figure 4.7).

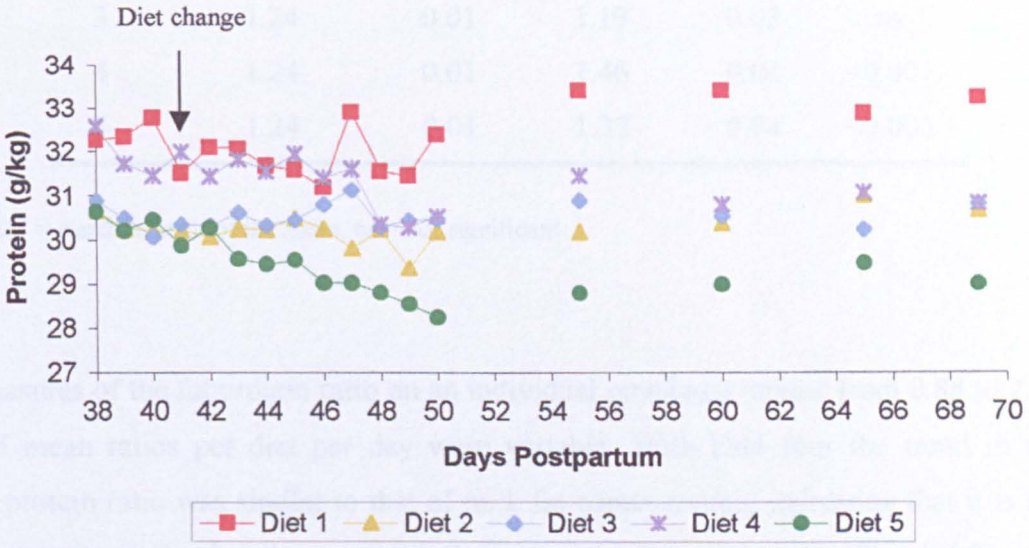

Figure 4.7. Effect of dietary change on mean milk protein concentrations over the dietary adjustment period

4.1.3.6. Fat:protein ratio

The fat:protein ratio was significantly increased by the high fibre diets (Diets four and five), with an increase in fat:protein over the last four sampling days compared with the baseline value. The fat:protein ratio did not change significantly for Diets one, two and three (Table 4.7).

Table 4.7. Mean fat:protein ratios for each group

Diet	Baseline	SEM	New	SEM	P value
1	1.24	0.01	1.20	0.04	ns
2	1.24	0.01	1.24	0.03	ns
3	1.24	0.01	1.19	0.03	ns
4	1.24	0.01	1.46	0.04	<0.001
5	1.24	0.01	1.37	0.04	<0.005

*SEM = standard error of the mean, ns = not significant

Measures of the fat:protein ratio on an individual cow basis ranged from 0.88 to 2.12 and mean ratios per diet per day were variable. With Diet four the trend in the fat:protein ratio was similar to that of milk fat concentration, indicating that it is the fat constituent that has the greatest influence on the fat:protein ratio (Figure 4.8).

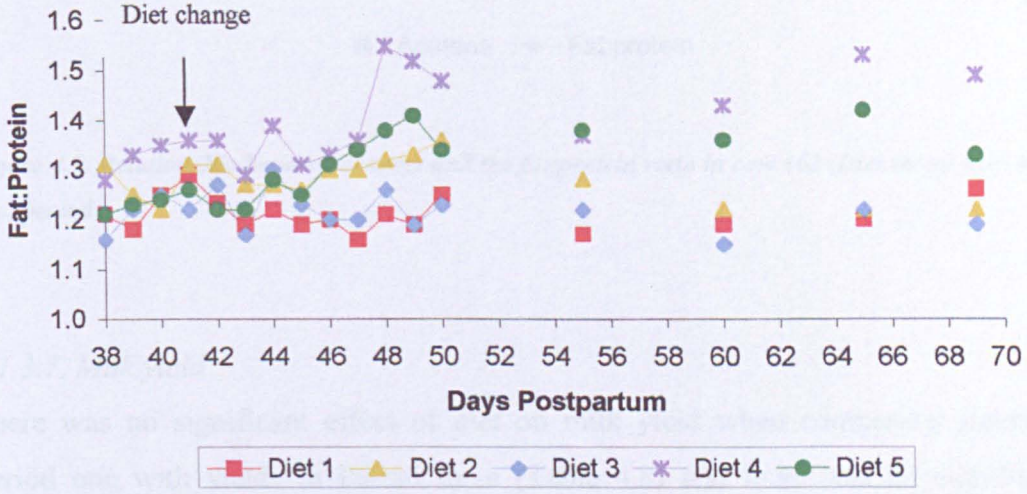

Figure 4.8. Effect of dietary change on the fat: protein ratio over the dietary adjustment period

The fat:protein ratio was also used to assess energy balance and whether this ratio could be related to milk acetone concentrations. Fat:protein ratios were compared with milk acetone data for each cow, as both are putative indicators of energy balance. There were six cows that had a fat:protein ratio greater than 1.4 on eight or

more sampling days out of the 17. When comparing the fat:protein ratios of these cows with their acetone data there appeared to be no relationship (Figure 4.9). Acetone concentrations were classed as high if they were over 0.4mM. In other words a fat:protein ratio indicative of negative energy balance was not always associated with elevated milk acetone concentrations and vice versa; acetone concentrations greater than 0.4mM were not always accompanied by an unfavourable fat:protein ratio greater than 1.4.

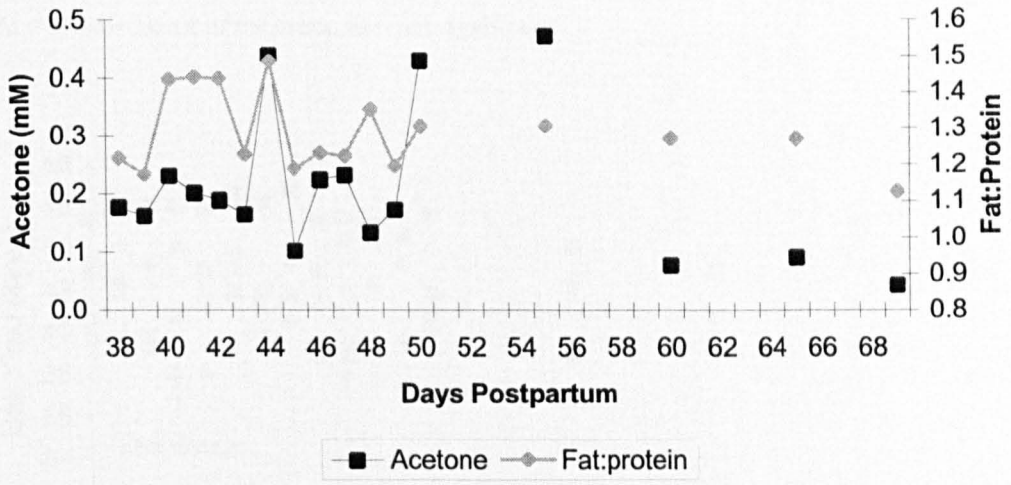

Figure 4.9. Relationship between acetone and the fat:protein ratio in cow 462 (Diet three) over the trial period

4.1.3.7. Milk yield

There was no significant effect of diet on milk yield when comparing yields in Period one with yields in Period three (Table 4.8) and there was no significant change in milk yield when cows received the dietary change on day 41 (Figure 4.10). In this experiment changes in milk yield appeared to be less responsive than changes in milk constituents to dietary adjustments.

Table 4.8. Mean milk yields for each group of cows

Diet	Baseline (litres)	SEM	New (litres)	SEM	P value
1	40.75	0.41	38.85	1.97	ns
2	40.75	0.41	42.63	1.52	ns
3	40.75	0.41	37.80	1.39	ns
4	40.75	0.41	41.30	1.97	ns
5	40.75	0.41	42.61	1.70	ns

*SEM = standard error of the mean, ns = not significant

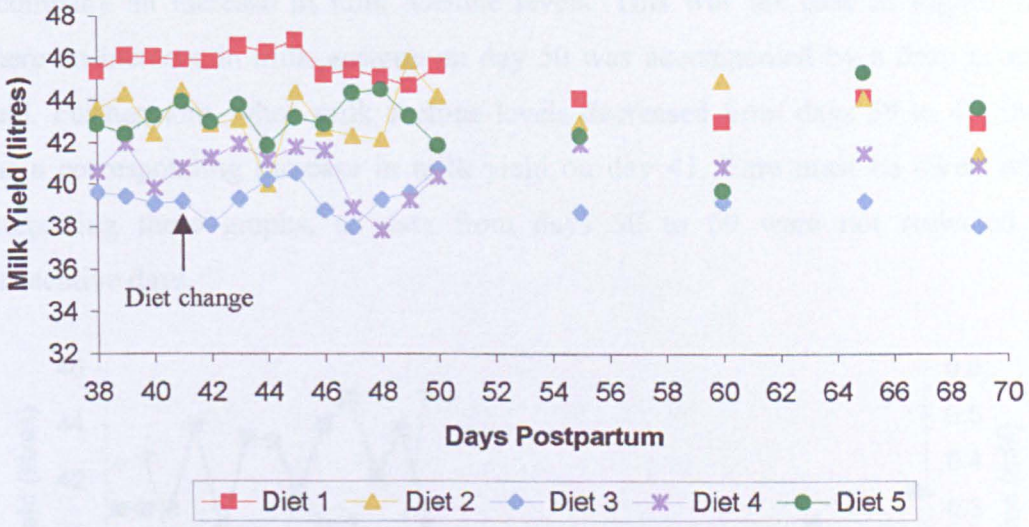

Figure 4.10. Effect of dietary change on mean milk yields over the dietary adjustment period

4.1.3.8 Relationship between milk acetone and milk yield

There was a positive correlation between milk acetone and milk yield with a 1mM increase in acetone being associated with a 4.01 litre increase in milk yield. However this relationship was not statistically significant (P=0.123). Further Linear Regression with Groups was carried out, first with "Diet" as the grouping factor and secondly with "Cow" as the grouping factor. When "Diet" was used as the grouping factor a 1mM increase in acetone was equated to a 4.44 litre increase in milk yield (P=0.08). However, when "Cow" was used as the grouping factor a 1mM increase in acetone was associated with a 1.57 litre decrease in milk yield (P=0.299). Although

different relationships were found between acetone and milk yield with different grouping factors, the relationships were not significant. Therefore no significant relationship was found between milk acetone concentrations and milk yields in this trial.

Acetone concentrations were plotted against milk yields to investigate the relationship between these variates in individual cows. A corresponding drop in milk yield due to high acetone was observed in ten out of 14 cows although it was not possible to determine whether the drop in milk yield was significant for individual cows and whether this response was solely related to an elevation in milk acetone. According to the literature, a reduction in milk yield would be expected to accompany an increase in milk acetone levels. This was the case in Figure 4.11 where an increase in milk acetone on day 50 was accompanied by a drop in milk yield. Furthermore, when milk acetone levels decreased from days 39 to 41, there was a corresponding increase in milk yield on day 41. Care must be taken when interpreting these graphs, as data from days 50 to 69 were not collected on consecutive days.

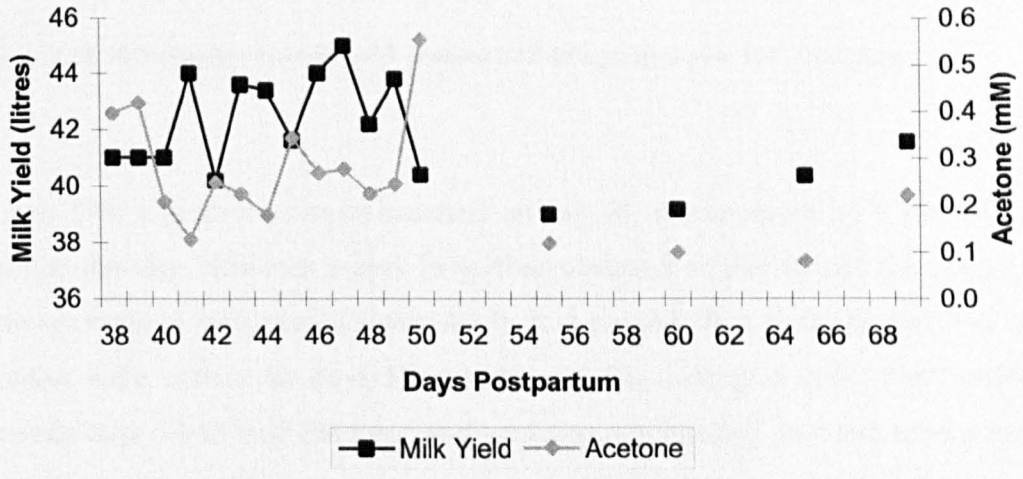

Figure 4.11. Relationship between milk acetone and milk yield in cow 103 (Diet two)

Different patterns of milk yield and acetone were found in different cows. For example cow 177 showed fluctuations in milk yield when acetone concentrations remained fairly constant and higher concentrations of acetone on days 50, 55 and 60

were accompanied by an increase in milk yield on these days (Figure 4.12). However, this is only based on the days that cows were milk sampled and acetone data were not collected on a daily basis from days 50 to 69. Furthermore, it is difficult to separate an effect of milk yield due to increasing levels of acetone from normal day-to-day variation. When an increase in milk acetone was accompanied by a drop in milk yield, the increase in acetone tended to occur approximately one day before milk yield decreased.

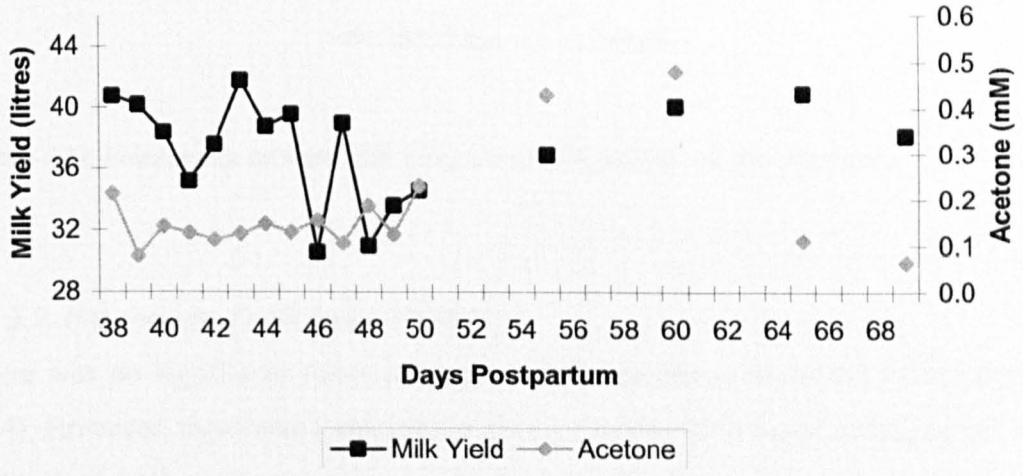

Figure 4.12. Relationship between milk acetone and milk yield in cow 177 (Diet three)

In cow 194, a peak in acetone occurred on day 44, accompanied by a drop in milk yield on this day. However a peak in acetone occurred on day 50 and there was very little response in milk yield (Figure 4.13). It is possible that since the last four milk samples were collect on days 55, 60, 65 and 69, a delayed milk yield response between days 50-55 may have occurred and was not detected. In some cows a raised acetone concentration on one day was not accompanied by a corresponding drop in milk yield.

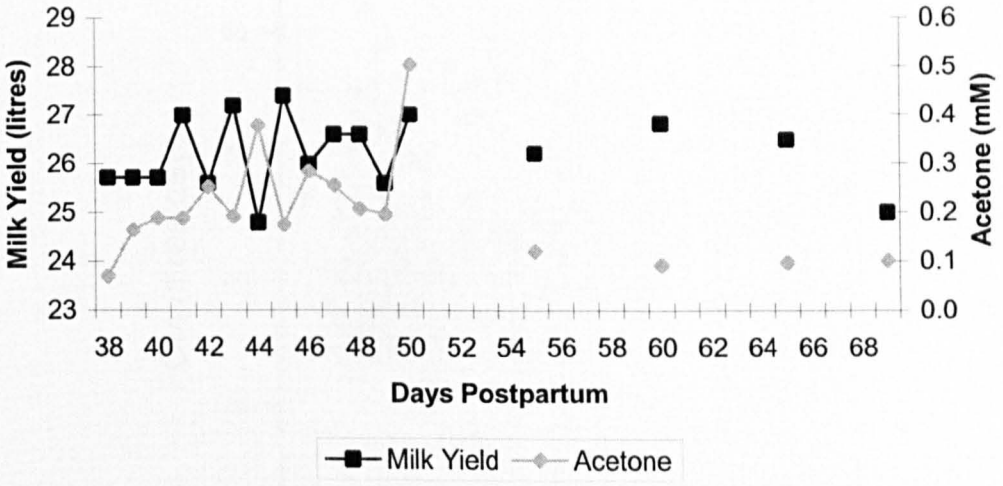

Figure 4.13. Relationship between milk acetone and milk yield in cow 194 (Diet three)

4.1.3.9. Fat corrected milk yield (FCM)

There was no significant relationship (P=0.16) between acetone and FCM (Figure 4.14). However, there was a significant positive relationship between fat:protein and FCM (P<0.001), indicating that an increase in fat:protein is accompanied by an increase in daily FCM (Figures 4.15). A one unit increase in the fat:protein ratio was accompanied by a 23.43 litres/day increase in FCM.

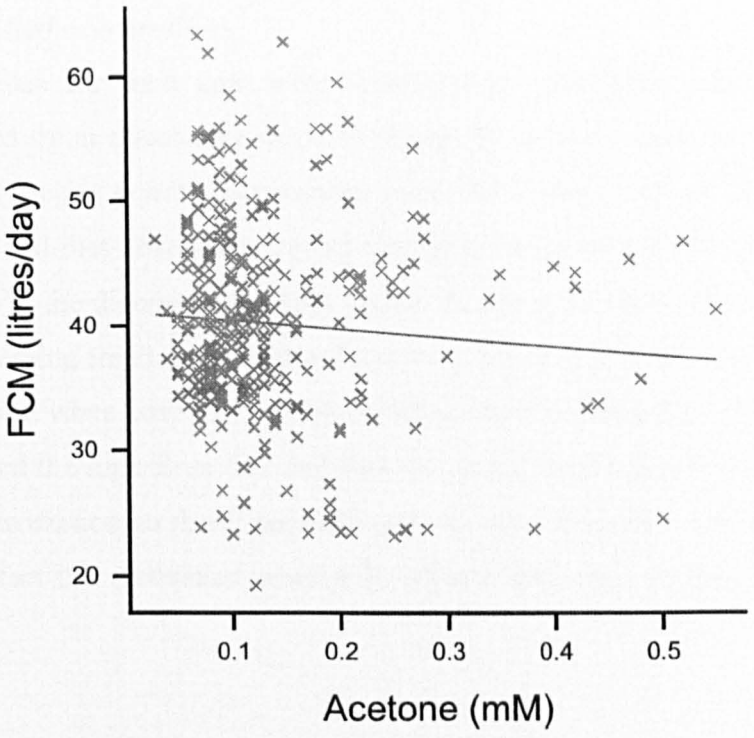

Figure 4.14. Relationship between acetone and FCM. Equation of regression line: Y=-7.07x+41.14. R^2= 0.006

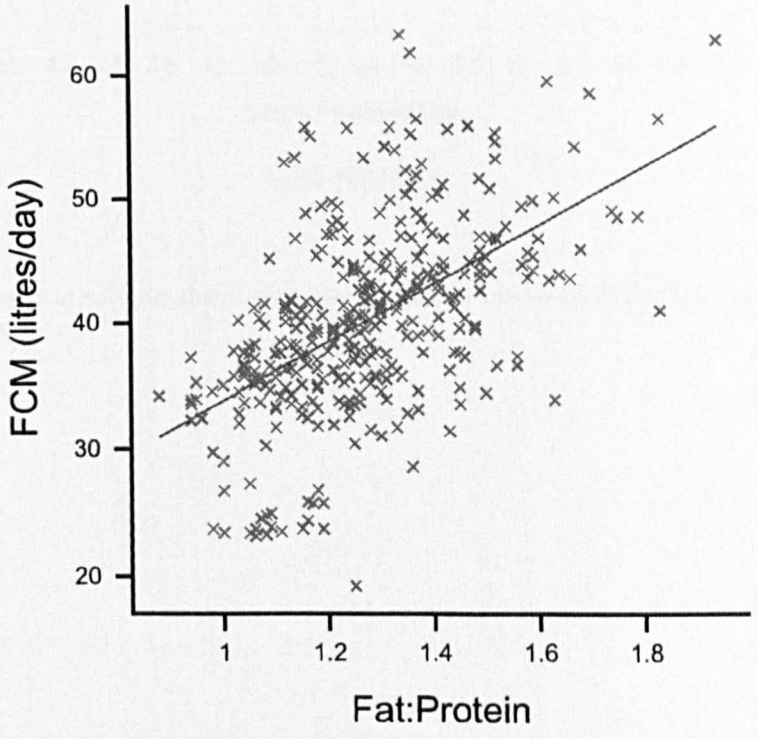

Figure 4.15. Relationship between fat:protein and FCM. Equation of regression line: Y=23.43x+10.36, R^2= 0.30

4.1.3.10. Individual cow profiles

Metabolic profiles for each cow were examined to investigate whether dietary changes resulted in an observable trend or change in acetone, urea, fat and protein concentration. Changes to milk composition were only evident with the extreme diets (one and five) and diet changes were not always obvious by examining individual cow profiles over the dietary adjustment period. Figure 4.16 shows that there was a detectable increasing trend in fat and a decreasing trend in protein over the dietary adjustment period when cow 172 was switched to the high fibre diet. However cow 107 was also fed the high fibre diet and showed only a very slight decrease in milk protein and fat content over the dietary adjustment period (Figure 4.17). This finding highlights the fact that individual cows will respond differently to the same dietary change.

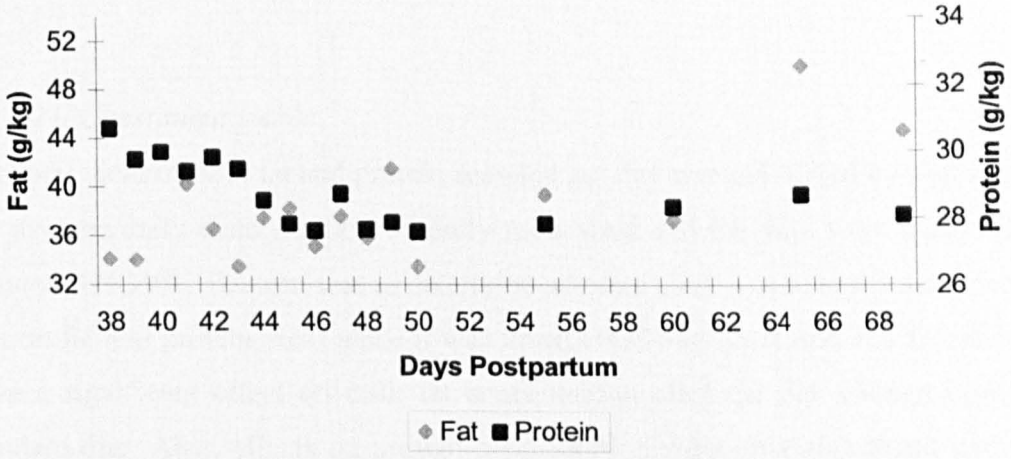

Figure 4.16. Changes in milk fat and protein concentration over the dietary adjustment period in cow 172 (Diet five)

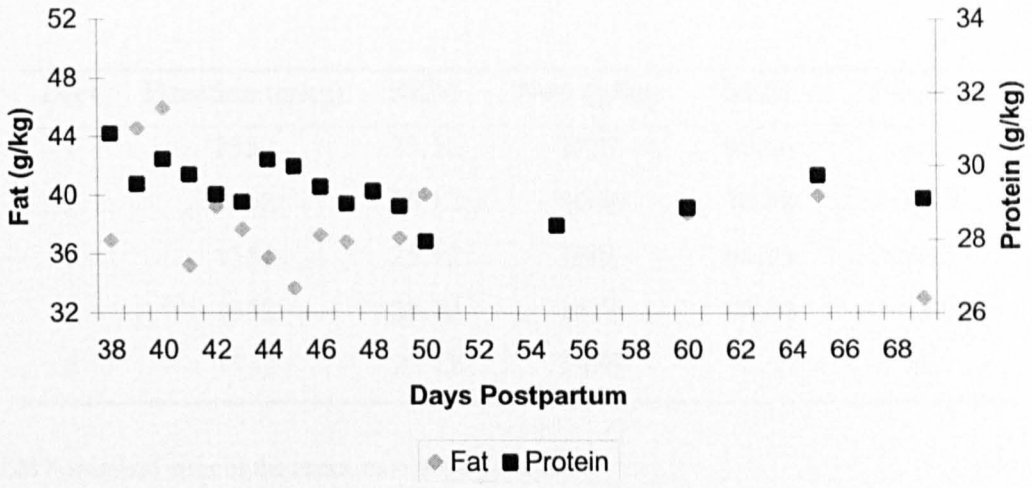

Figure 4.17. Changes in milk fat and protein concentration over the dietary adjustment period in cow 107 (Diet five)

4.1.3.11. Constituent yields

The total yield of milk fat and protein secreted per day was calculated by multiplying the average daily concentration by daily milk yield and the data were analysed by General ANOVA. The aim was to determine whether there was a significant effect of diet on fat and protein yield since it was unexpected that Diets one and five did not have a significant effect on milk fat concentration after the diet change from the standard diet. Also, effects on protein concentration were only significant with the extreme high starch and high fibre diets.

By comparing the total daily yields of fat and protein in the baseline period with the yields measured on the last four sampling days, little difference in total daily yield was achieved with dietary change (Tables 4.9 and 4.10). There was no significant effect of dietary treatment on yield of protein for each of the diets and changes in total fat yield observed were only significant with Diets three and four. Cows on the control diet showed a significant reduction in fat yield when comparing yields in Periods one and three. A significant increase in fat yield was found with Diet four, which was to be expected since milk fat concentration also increased significantly.

Table 4.9. Effect of dietary treatment on mean fat yields

Diet	Baseline (g/kg)	SEM	New (g/kg)	SEM	P value
1	1552	25.12	1532	90.86	ns
2	1552	25.12	1604	70.38	ns
3	1552	25.12	1361	64.25	<0.05
4	1552	25.12	1876	90.86	<0.001
5	1552	25.12	1685	78.69	ns

*SEM = standard error of the mean, ns = not significant

Table 4.10. Effect of dietary treatment on mean protein yields

Diet	Baseline (g/kg)	SEM	New (g/kg)	SEM	P value
1	1256	27.28	1270	61.06	ns
2	1256	27.28	1318	47.30	ns
3	1256	27.28	1131	43.18	ns
4	1256	27.28	1303	61.06	ns
5	1256	27.28	1207	52.88	ns

*SEM = standard error of the mean, ns = not significant

4.1.4. Discussion

This experiment was conducted firstly to determine whether changes in nutrition could be detected over the dietary adjustment period by daily monitoring of milk composition and secondly to investigate the effect of diets ranging in starch to fibre content on milk composition, both at the group and individual cow level. Lastly, acetone and fat:protein data were assessed to determine their use as indicators of energy balance and their relationship with energy output in terms of FCM.

4.1.4.1. Acetone

There was no significant effect of any dietary treatment on milk acetone concentrations (Table 4.3). It was expected that acetone would not vary significantly with dietary change since the diets were isoenergetic. If feed intakes had dropped significantly when switching to the new diets then acetone may have increased in concentration. However the effect of dietary change on feed intake in this study was such that acetone concentrations were not significantly affected. Milk acetone concentrations were generally very low, the majority falling into acetone class one (<0.4mM) as defined by Andersson et al (1991). Milk samples were collected from cows in weeks five to ten of lactation and the highest concentrations of acetone tend to be in weeks two to six, peaking in weeks three to four which is when peak milk yield also occurs (Andersson and Emanuelson, 1985). This would account for the majority of cows generally having low acetone concentrations as the risk period for hyperketonaemia and ketosis had already passed.

Milk acetone appeared to vary considerably across the diets and from day-to-day throughout the trial. Variation in acetone from day-to-day may be accounted for by large individual variation within and between cows and variation in silage quality depending on position in the clamp. Silages with an elevated content of butyric acid, caused by wet conditions, may increase ketones in body fluids as rumen epithelial cells convert butyric acid into ketone bodies (Treacher et al, 1986).

Towards the end of the trial, nine of the cows did not train well on the Calan gates and their milk acetone levels were slightly higher compared with cows that had no problems feeding through the gates. These nine cows relied on people to open their feeding gates and therefore feed intakes were considerably lower in these cows. From approximately day 44, acetone concentrations slowly decreased as these cows became more accustomed to their gates, although they were still slightly higher compared with other cows at the same stage of sampling. Five cows exhibited milk acetone concentrations within the warning class 0.7 to 1.4mM, which has been shown to reduce early lactation milk yield (Gustafsson and Emanuelson, 1996).

4.1.4.2. Monitoring cows via milk acetone concentrations

All cows on the trial exhibited raised acetone levels on at least one day. Acetone concentrations greater than the 0.4mM threshold proposed by Andersson and Emanuelson (1985) as representing hyperketonaemia were observed in 14 out of the 30 cows on trial. Raised levels of acetone were observed in eight cows on day 69. This was most likely due to the cows being blood sampled every two hours from 07:00 to 19:00 as part of another trial, and their time spent feeding was very much reduced. This raises the question whether milk acetone can be used to assess a health or nutritional problem, such as a reduction in feed intake due to mastitis. Cow 58 on Diet two had acetone levels characteristic of subclinical ketosis and the highest acetone measurement was 1.02mM which was most likely due to her experiencing mastitis or four separate occasions between days 35 and 70 postpartum. Her acetone concentrations increased to 1.02mM on days 42 and 0.67mM on day 50. However mastitis occurred on days 38 and 47 at the PM milking which was a few days before acetone peaked so in this case an elevated level of acetone could not be used for early detection of a potential health problem. Also, it was thought that cow 89 might have been under some degree of metabolic stress due to repeated cases of mastitis throughout the trial period. However the range of acetone concentrations observed in this cow were not particularly high, being only slightly greater than 0.4mM (range = 0.09 to 0.46mM).

It is difficult to relate elevated milk acetone levels to reduced feed intake. Feed intakes were extremely variable from day-to-day. The seven cows identified as stealing from other feed bins had relatively low feed intakes. Consequently, some cows had intakes over 80kg/day, most likely due to cows stealing from their feed bins, with higher feed intakes being measured when stealing bouts took place.

Like urea, acetone is thought to vary with time of feeding in relation to milk sampling. As the cows were fed an *ad libitum* TMR, the time of last feeding in relation to milking was not recorded and so it is possible that acetone concentrations may have been raised if milk sampling took place a few hours after the last feed. Blood ketones are reported to be highest four to five hours after the start of feeding (Rossow et al, 1991) and because milk acetone is highly correlated with blood ketone levels then acetone would be expected to show some diurnal variation in relation to

feeding times. However, Gustafsson (1993), Andersson and Lundström (1984a) and Pehrson (1996) stated that milk acetone showed no diurnal variation. Clearly more research is required in this area and any days with raised levels of milk acetone cannot be assumed to be related to the time of milk sampling in relation to feeding.

There was no clear relationship between milk acetone and milk yield in this study despite data in the literature providing evidence for a reduction in milk yield with elevated acetone (Gustafsson and Emanuelson, 1996). Fluctuations in milk yield will occur on a daily basis due to several factors such as uneven milk intervals or changes in the stockmen. Therefore it is difficult to ascertain whether a fall in milk yield is significant and whether it is due to a rise in milk acetone, natural day-to-day variation or a change in management practices.

4.1.4.3. Urea

Urea was significantly increased by the high starch diet (Diet one, Table 4.4). Since diets were isonitrogenous and isoenergetic no differences in milk urea concentration were expected with dietary treatment. It is difficult to relate any changes in urea to varying amounts of starch and fibre in the trial diets as most of the work on urea has been carried out in respect to changing the protein and/or energy content of the diet. Urea is strongly influenced by changes in the protein:energy ratio (Oltner and Wiktorsson, 1983) which remained constant across all the diets.

Time of feeding in relation to sampling is very important to consider as Eicher et al (1999) reported that milk urea reaches maximum concentrations two to four hours after a feed. It is possible that the significant increase in urea with Diet one is due to an effect of time of sampling in relation to feeding if cows were milked soon after their last feed. However as the diets were fed as an *ad libitum* TMR and feeding behaviour was not recorded, assumptions on feeding time in relation to milk sampling cannot be made. High levels of fermentable carbohydrates have been associated with low ruminal concentrations of ammonia due to increased use of ammonia for microbial protein synthesis (Reynolds et al, 1997), which would therefore decrease milk urea concentration. Therefore a reduction in urea would have been expected with Diet one. The significant increase in urea with the high starch diet cannot be explained.

Ideally it would have been useful to conduct a similar experiment with changes to the protein:energy ratio to investigate the effect on acetone and urea. However, resource constraints and costs meant that this was not feasible. However it has been suggested that changes in MUN, in response to changes in experimental conditions, can occur within a few days (Moore and Varga, 1996; Jonker, Kohn and High, 2002). This means that daily measurements of milk urea could be used to monitor dietary changes and could potentially lead to quicker identification of nutritional problems and optimisation of rations if day-to-day variation is not too large.

4.1.4.4. Fat

The results for milk fat content were unexpected in that Diet four resulted in a significant increase in milk fat, yet Diet five, which contained the highest fibre content, had no significant effect (Table 4.5). This may be due to the uneven replication within the diet groups. It was expected that a trend in milk fat across the five diets would be observed, since milk fat is the most easily manipulated component of milk by dietary intervention and dietary fibre has a large influence on milk fat. However it is possible that day-to-day variation may have masked any treatment effect. Also, variation between cows and the fact that data from only three cows on Diets one and four were available for analysis, may have contributed to the relatively little impact of diet change on milk composition.

In theory, the high starch diet should have resulted in a decrease in milk fat content. Production of the volatile fatty acid, propionate, is increased by the starchy concentrate portion of the diet. However there was very little change in milk fat when switching from the control diet to Diets one and two. The most pronounced response to an increase in dietary starch content is a reduction in milk fat (Sutton, 1989). This effect can be attributed to increased relative availability of glucogenic precursors to lipogenic precursors and an increase in propionate production compared with acetate production in the rumen (Reynolds et al, 1997). Due to uneven replication between the dietary groups and only three cows on Diet one it is likely that a more profound effect of the high starch diet would be evident with more cows on this treatment group.

It is possible that the period of dietary adjustment was not long enough to observe changes in milk composition, and that cows take longer to adapt to new diets and respond by changes in milk composition. For example, Engvall (1980) investigated the effect of feeding a diet formulated to reduce milk fat concentration. The author found a lag time between 34 and 65 days from when the diet was first fed until milk fat decreased to below 20g/kg. The decrease in milk fat occurred suddenly, along with a significant alteration to ruminal microflora. However, Griinari et al (1997) stated that changes in milk composition caused by high concentrates and low fibre diets tend to occur within a few days, reducing both milk yield and fat concentration. Clearly there is discrepancy between studies in the responsiveness of milk composition to dietary changes and how quickly these changes appear.

Daily changes to milk composition in response to dietary change on an individual cow basis have not been investigated in great detail. Khorasani and Kennelly (2001) investigated the effect of concentrate to forage ratio and buffer on several production parameters. They suggested that changes in milk composition in response to nutrition are greater in late lactation. It is possible that in the present study, where the cows were in early lactation, the response to dietary change in milk composition could not be observed due to natural variation in milk yield, fat and protein and feed intake over the trial period. Perhaps these variables would be more constant in later lactation and a greater response to dietary change would be observed.

4.1.4.5. Protein

Milk protein content was significantly influenced by the extreme diets, one and five. However, Diets two and four had no effect on protein concentration (Table 4.6). The major influence on milk protein content is energy rather than protein supply and this was constant across all the diets. The increase in milk protein with the high starch diet can be attributed to an increase in propionate production in the rumen, which in turn depresses butyrate and acetate, the latter being the main precursors for milk fat. In contrast, high fibre diets reduce protein at the expense of milk fat due to an increase in acetate production within the rumen.

When protein data were analysed at the group level there was a steady decline in milk protein content over the dietary adjustment period with Diet five (Figure 4.7).

However protein concentrations in milk samples collected on the last four sampling days were slightly higher than concentrations measured in the last few days of Period two. Lee et al (1978) suggested that homeostasis in blood metabolites might be maintained by adjusting milk yield, feed intake, nutrient absorption and intermediary metabolism and excretion and it is possible that the same may be true of milk composition. Elevated concentrations of protein on the last four sample days compared to the dietary adjustment period may be due to homeostasis and the cow maintaining a minimum threshold level for protein content.

In this experiment, Diets one and five were very extreme and these levels of starch and fibre are not likely to be use in commercial practice. Also nutritional changes throughout lactation would not be of the magnitude used in this study. It can therefore be concluded that small dietary changes that occur throughout lactation would not be detectable by monitoring fat and protein on a daily basis in individual cows. However it is possible that significant changes in milk composition would be detectable with changes to the content of raw materials in the TMR depending on their composition, or by moving from indoor feeding to summer grazing.

4.1.4.6. Fat:protein ratio

The fat:protein ratio in each cow was determined to investigate energy status, with a fat:protein ratio over 1.4 being expected for a cow in negative energy balance. Fat:protein data were compared with acetone, which has also been widely researched as an indicator of energy status. Generally, daily peaks in acetone were not accompanied by an increase in the fat:protein ratio, which may have been expected since they are both measures of energy balance in the dairy cow (Figure 4.9). However, it is possible that acetone is a short-term measure of energy balance and can fluctuate from day-to-day, whereas fat:protein is likely to remain more constant and is not so easily influenced. The fat:protein ratio and acetone appear to be fairly crude measures of energy balance in the cow since they do not seem to be related in this study. It would have been useful to calculate the energy balance of each cow and compare the data with the fat:protein ratio and milk acetone concentrations. However, it was not possible to calculate the energy balance per cow in this study due to daily fluctuations in energy intake.

4.1.4.7. FCM

By adjusting milk yields to a standard fat concentration, milk yields can be compared on an equivalent energy basis. This data manipulation removes much of the variability seen when comparing raw milk yield data. Energy output in terms of FCM was expected to decrease with increasing negative energy balance or poorer energy status. Therefore it was unexpected that a positive relationship was be found between fat:protein and FCM. This finding indicates that as the cow approaches a more negative energy balance or poorer energy status, as indicated by a higher fat:protein ratio, energy output is greater in terms of FCM (Figure 4.15). These results confirm the findings of Kauppinen (1983) who found a positive association between milk yield and blood concentrations of acetoacetate and β-hydroxybutyrate, indicating that milk production at peak lactation exceeds dietary energy intake. However, despite Anderson and Emanuelson (1985) detecting a significant positive partial correlation between the highest milk acetone concentration and highest milk yield in an individual, there was a significant negative correlation between milk yield and milk acetone on the same day. In this study there was a stronger relationship between fat:protein and FCM, than acetone and FCM, indicating that fat:protein is more closely correlated with FCM and is therefore a better indicator of energy status (Figures 4.14 and 4.15).

4.1.5. Conclusions

Changes to nutrition were not consistently detected over the period of dietary adjustment for each dietary group of cows or when assessing individual cow profiles. Based on these findings it is likely that dietary changes would not be observed in on-line monitoring of individual cows unless diet change was extreme. This may be due to the fact that dietary treatment did not have a profound effect on milk composition except when dietary change was severe. Individual cow profiles over the trial period showed daily fluctuations, particularly for fat, urea and acetone. It is possible that day-to-day variation in milk constituents is greater than the effect of dietary change, thus masking any response to diet in the adjustment period. Therefore, well-managed dietary changes are not likely to be detected by monitoring daily changes in milk composition on an individual cow basis. Fat:protein ratio appeared to be a better predictor of energy output in the cow in terms of FCM than milk acetone

concentration. Daily monitoring, particularly of milk acetone, may still be useful to detect reduced feed intakes, dietary imbalances or assess silage quality but this requires further research with more cows under study.

4.2. WITHIN AND BETWEEN COW VARIATION IN MILK COMPOSITION

4.2.1. Introduction

Diurnal variation in milk composition may occur due to several factors, such as the length of the milking interval, feeding times, management practices and in response to diurnal variation in blood metabolites (Ward et al, 1995; Nielsen, Ingvartsen and Larson, 2003). For on-line monitoring, it would be useful if once daily samples could be taken for analysis. However it is possible that diurnal and day-to-day variation may contribute to inaccurate data interpretation from a daily sample. For example, milk fat content is known to vary significantly between AM and PM milkings (Rook et al, 1992). There is little information on day-to-day variation in milk constituents in the literature although significant variation can occur between days, particularly in milk fat (Lee, 1988; Atwal and Erfle, 1990; Svennersten-Sjaunja et al, 1997). Day-to-day variation and variation in normal ranges between cows have to be quantified for accurate data interpretation and for detection of milk composition changes outside the normal range. It is important to determine the extent of these variations so that the number of samples required daily, and for how many days can be estimated, to make reliable assumptions on nutritional or metabolic status.

The aim of this experiment was to investigate sources of variation in milk acetone, urea, fat and protein within and between cows. Within cow variation included diurnal and day-to-day variation. It was hypothesised that the concentration of milk constituents would vary between AM and PM samples and on a day-to-day basis, which would be most pronounced for fat. Protein would be less variable but may still show some diurnal variation. Urea and possibly acetone would show diurnal variation, most likely due to the time of feeding in relation to sampling. Some variation on a day-to-day basis should be observed.

4.2.2. Materials and Methods

4.2.2.1. Statistical analysis

Data from the 30 experimental cows in Chapter 4, Experiment 1 were used to investigate within and between cow variation. Diurnal variation in acetone, urea, fat

and protein was investigated per dietary group in all three periods of the trial. Acetone data was not normally distributed and was transformed (log base 10) before analysis. Data were analysed by General ANOVA in Genstat 5.0 and diet and period factors were included in the model. The block structure was "Day/Cow/Time" and the treatment structure was "(Period/Diet)*Time" where the effect of Diet is nested within each Period and "Time" is the interaction term to investigate diurnal variation within each Diet, within each Period. To remove any effect of diet, diurnal variation was also investigated in the control group of cows (on Diet three) from data collected between days 38 and 50 (Block structure = "Day/Cow" and treatment structure = "Time"). In this data set, acetone and protein data were not normally distributed and were transformed in the same way. Therefore, means presented in tables are derived by back-transforming the log data. SEMs could not be calculated for these variables.

Day-to-day variation in each milk constituent was estimated in the control cows and expressed as a standard deviation. This was calculated by taking the square root of the residual mean squares of the day stratum in the ANOVA table (derived from investigated diurnal variation in the control cows) and represented the variability of the measured constituent between days, averaged over all the cows. Day-to-day variation could not be calculated accurately unless complicated statistics were used and assumptions were made about the data. For example, assuming that measurements taken on consecutive days were correlated, so that a high concentration on one day would probably be followed by a high concentration the next day and vice versa. Although the standard deviation is a rough estimate of the day-to-day variability, this estimate also includes variation between the cows and ignores day-to-day correlation. Acetone data from the control cows were transformed and day-to-day variation for acetone was presented as the standard deviation from the raw data since standard deviations cannot be calculated on the log scale.

Day-to-day variation was also estimated on an individual cow basis in the control group. The standard deviation was used to express how milk composition deviated from the mean each day for each cow. Diurnal and day-to-day variation in the fat:protein ratio was not determined since any variation would be taken into consideration when interpreting fat and protein data.

Between cow variation was investigated in the control cows and was analysed by General ANOVA with "Day/Cow" as the block structure and "Cow" as the treatment structure. For each cow a concentration range for each constituent was calculated from the mean ± two standard deviations, which would account for 95% of observations.

4.2.3. Results

4.2.3.1. Diurnal variation

Diurnal variation was investigated by comparing mean AM and PM concentrations in each period of the trial with data from all diets. In the tables, Period one refers to days 38, 39 and 40, Period two refers to samples collected on days 41 to 50 and Period three refers to the last four sample days (55, 60, 65 and 69 postpartum) which determined the new level of milk composition. When data from all diets and periods were analysed there was a significant effect of sampling time on acetone and fat but not on urea and protein concentrations (Table 4.10).

Table 4.10. Diurnal variation in concentrations of milk acetone, urea, fat and protein from data averaged over all five diets and three sampling periods

Parameter	AM	SEM	PM	SEM	P value
Acetone (mM)	0.106	***	0.119	***	<0.001
Urea (mM)	6.08	0.03	6.03	0.03	ns
Fat (g/kg)	36.61	0.27	41.38	0.27	<0.001
Protein (g/kg)	29.89	0.23	30.41	0.23	ns

*** Means presented are derived from back-transforming the log data and SEMs cannot be calculated from log data. With the logged data, mean AM acetone = -0.976 and mean PM acetone = -0.924; SEM = 0.009 (minimum replication). Comparison was made on the log scale. SEM = standard error of the mean, ns = not significant

a) Acetone

There was no significant interaction between time, period and diet (P=0.195) in milk acetone concentrations (Table 4.11). AM samples tended to have slightly lower concentrations of acetone compared to PM samples but these differences were very small and not statistically significant.

Table 4.11. Effect of sampling time on mean milk acetone concentrations according to period and diet

Period	Time	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
1	AM	0.096	0.096	0.096	0.096	0.096
1	PM	0.113	0.113	0.113	0.113	0.113
2	AM	0.080	0.124	0.110	0.117	0.084
2	PM	0.085	0.129	0.135	0.125	0.116
3	AM	0.099	0.106	0.094	0.131	0.080
3	PM	0.123	0.104	0.122	0.112	0.116

b) Urea

There was a significant interaction between time, period and diet (P=0.003) on milk urea concentrations. However, there was no significant difference between AM and PM samples in any period for each of the diets. The significant interaction obtained must be a result of significant differences between diets and between periods in each diet in milk urea concentration. Therefore it can be assumed that there was no pattern of diurnal variation when the experiment was analysed in this way.

Table 4.12. Effect of sampling time on mean milk urea concentrations according to period and diet

Period	Time	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
1	AM	6.16	6.16	6.16	6.16	6.16
1	PM	6.10	6.10	6.10	6.10	6.10
2	AM	7.28	6.71	5.62	5.74	5.37
2	PM	7.09	6.53	5.43	6.03	5.59
3	AM	6.99	6.11	5.83	5.80	5.59
3	PM	6.57	6.02	5.68	6.13	5.69

c) Fat

When the diet and period factors were taken into account in the statistical analysis there was a significant interaction (P=0.002). Generally, milk fat content was found to be lower in AM samples compared to PM samples (Table 4.13) except in Diet 1, Period 2 where milk fat concentrations were similar (38.86g/kg versus 38.33g/kg).

Table 4.13. Effect of sampling time on mean milk fat concentrations according to period and diet

Period	Time	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
1	AM	35.32	35.32	35.32	35.32	35.32
1	PM	41.53	41.53	41.53	41.53	41.53
2	AM	38.86	36.38	35.40	40.75	34.88
2	PM	38.33	42.45	39.50	46.54	41.13
3	AM	39.36	34.76	34.73	42.05	38.30
3	PM	40.87	40.98	37.89	48.58	41.98

d) Protein

There was no significant effect of sampling time on milk protein content when interactions between diet and period were considered (P=0.460). The differences between AM and PM samples for each diet in all three sampling periods were very small and there was no pattern in whether AM samples had a greater protein content than PM samples or vice versa (Table 4.14).

Table 4.14. Effect of sampling time on mean milk protein concentrations according to period and diet

Period	Time	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
1	AM	29.13	29.13	29.13	29.13	29.13
1	PM	29.73	29.73	29.73	29.73	29.73
2	AM	31.22	30.11	29.43	31.38	28.47
2	PM	29.32	30.55	30.66	31.34	29.86
3	AM	33.14	31.05	29.63	31.36	27.50
3	PM	33.27	30.79	30.55	31.84	29.40

4.2.3.2. Diurnal variation in control cows

Diurnal variation was investigated in the control group of cows (Diet 3). As these cows were on a constant diet throughout the sampling period, results from the statistical analysis were thought to be more reliable in determining diurnal variation in milk constituents. Means of constituents in AM and PM samples in the control group of cows are presented in Table 4.15. There was significant diurnal variation which was greatest for fat, then acetone and then urea, as indicated by the P values. Protein content did not vary significantly between AM and PM samples.

Table 4.15. Diurnal variation in milk composition in cows on the control diet

Parameter	AM	SEM	PM	SEM	P value
Acetone (mM)	0.108	***	0.128	***	0.002
Urea (mM)	5.64	0.06	5.45	0.06	0.029
Fat (g/kg)	34.91	0.59	39.79	0.59	< 0.001
Protein (g/kg)	30.85	***	30.56	***	ns

*** With the logged data, mean AM acetone = -0.968 and mean PM acetone = -0.892; SEM = 0.017. Mean AM protein = 1.489 and mean PM protein = 1.485; SEM = 0.004. All comparisons were made on the log scale. SEM = standard error of the mean, ns = not significant

4.2.3.3. Day-to-day variation

Table 4.16 shows the mean and standard deviation associated with each milk constituent when averaged over all the control cows and sampling days. Although the standard deviation for acetone appeared to be very small, it was actually 64.3% of the mean so day-to-day variation in acetone concentrations can be extremely large. The percentage of standard deviations from the mean for urea, fat and protein were 13.0, 6.7, and 2.3% respectively. From these data it can be concluded that day-to-day variation is greatest in acetone and protein is the least variable milk constituent.

Table 4.16. Day-to-day variation in milk composition from data averaged over all the control cows

Parameter	Mean	Standard Deviation
Acetone (mM)	0.14	0.09
Urea (mM)	5.55	0.72
Fat (g/kg)	37.28	2.48
Protein (g/kg)	30.51	0.69

Table 4.17 shows the standard deviations associated with the mean concentration of milk constituents for each cow on the control diet, indicating how much deviation from the mean may be expected with milk sampling and analysis on a daily basis. Again, variation was much greater for fat than for protein with little variation in protein content on a daily basis. Standard deviations for urea ranged from 0.33 to 0.99, indicating that urea varies considerably on a daily basis between cows.

Table 4.17. Day-to-day variation in milk composition on an individual cow basis

Cow	Acetone (mM)	Urea (mM)	Fat (g/kg)	Protein (g/kg)
145	0.016	0.83	2.45	0.78
146	0.013	0.75	2.20	0.46
177	0.043	0.99	1.77	0.56
194	0.110	0.33	1.52	0.62
462	0.102	0.47	3.10	0.45
476	0.028	0.44	3.27	0.86

When data from individual cows were plotted from days 38 to 50, the variation in fat content was greater than the variation in protein, which remained relatively constant between consecutive sampling days (Figure 4.18).

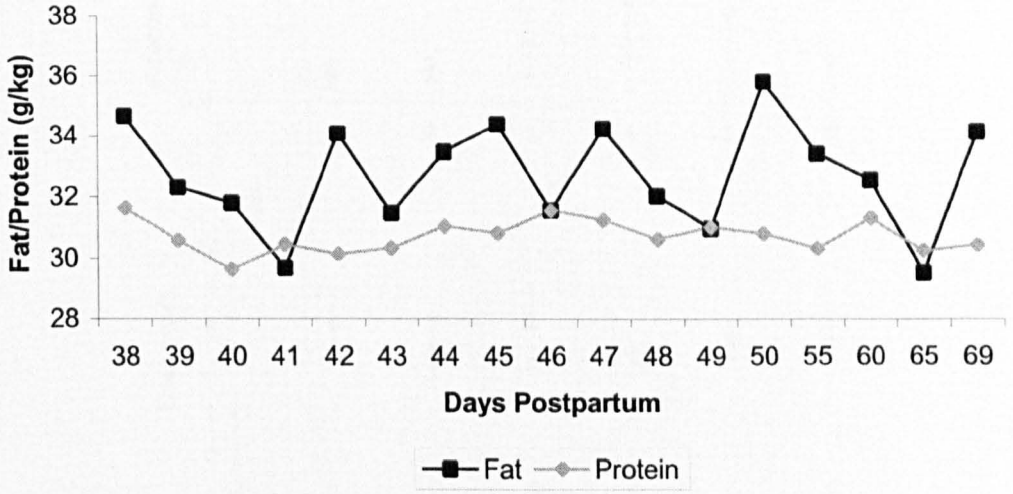

Figure 4.18. Day-to-day variation in fat and protein concentration in cow 177 (Diet three)

4.2.3.4. Between cow variation

There were significant differences between the six control cows in mean concentrations of acetone, urea, fat and protein when data were averaged over all the sampling days ($P<0.001$). These estimated normal ranges, of which 95% of daily measurements should fall into, were very wide and varied in terms of concentration and the extent of the range (Figure 4.19). For example, acetone concentration in cow four ranged from 0.02mM to 0.45mM, whereas in cow six the range was much smaller (0.08mM to 0.11mM).

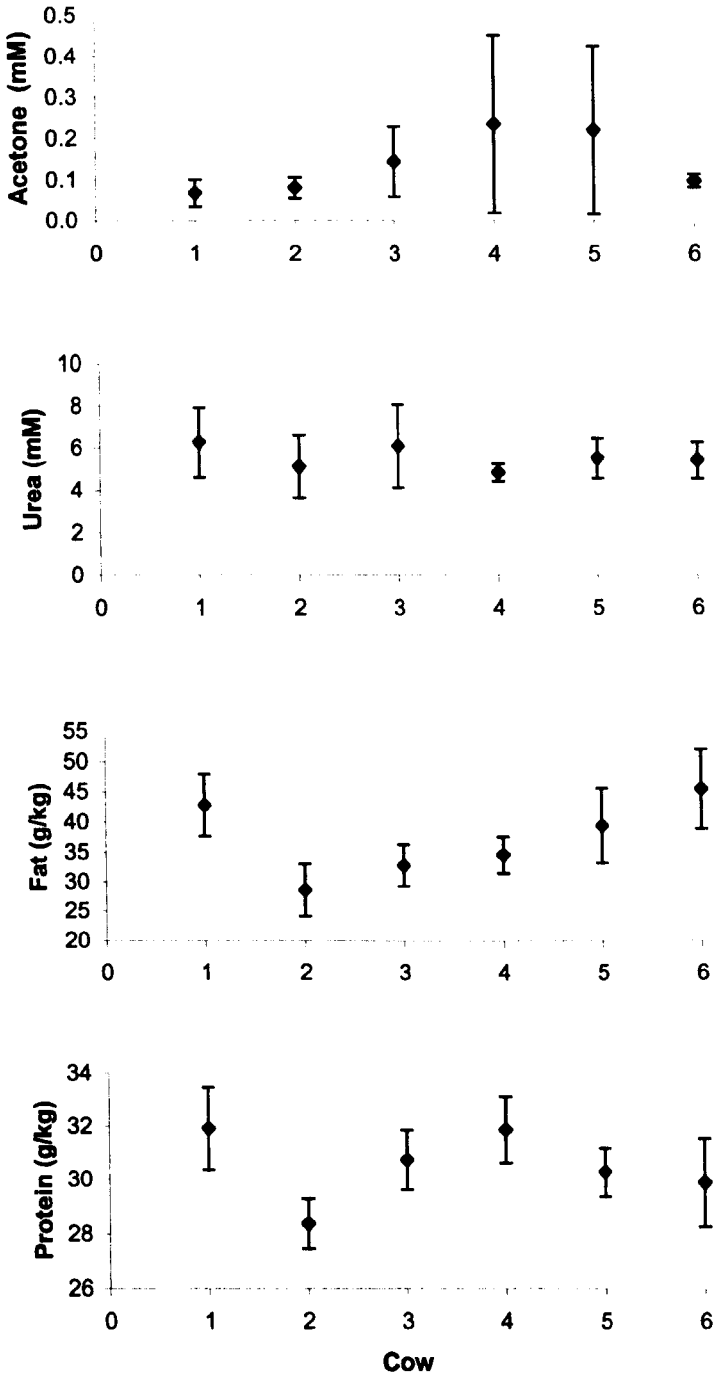

Figure 4.19. Variation between cows in their expected ranges of milk constituents (ranges calculated from mean ± two standard deviations to give a 95% confidence interval)

4.2.4. Discussion

4.2.4.1. Diurnal variation

Significant diurnal variation was found in milk fat concentration from all three analyses; with data averaged over all cows, diets and periods; from data taking account of diet and period factors; and in control cows (Tables 4.10, 4.13 and 4.15). Acetone and urea were significantly influenced in two out of the three analyses and protein did not show any effect of sampling time. Lower concentrations of acetone and fat in the AM milking were probably due to a dilution effect of higher milk yields since the mean AM milk yield was 20.31 litres compared with 17.86 litres at PM milkings ($P<0.001$).

The total yields of milk constituents at each milking in the control group were calculated and the data analysed for diurnal effects (Table 4.18). Both acetone and fat yields were similar at AM and PM samplings, confirming the hypothesis that lower concentrations were observed at AM milkings due to a dilution effect from higher milk yields. The finding of lower acetone and fat concentrations in AM samples and vice versa, provides further evidence for the association between milk fat and acetone with higher levels of acetone being accompanied by a higher milk fat content (Steen et al, 1996a). The significant difference in constituent concentrations and milk yields between AM and PM samples may be partly due to milking intervals being approximately 11.5 and 12.5 hours.

Table 4.18. Diurnal variation in milk constituent yields in control cows

Parameter	AM	SEM	PM	SEM	P value
Acetone (g)	0.124	***	0.129	***	ns
Urea (g)	6.91	0.22	5.77	0.17	<0.001
Fat (g)	707	24	708	26	ns
Protein (g)	623	15	541	13	<0.001

*** With the logged data, mean AM acetone = -0.908 and mean PM acetone = -0.890; SEM = 0.018. Comparison was made on the log scale. SEM = standard error of the mean, ns = not significant

There was a significant effect of sampling time on total protein yield, despite no significant effect on protein concentration. Both yield and concentration of urea varied significantly with time of sampling in the control cows. However, no significant effect of sampling time was seen in milk urea concentration in each sampling period for any of the diets (Table 4.12).

Cows were given fresh feed at approximately 16:30 and feed bins were topped up the following morning at 07:00. Cows were always milked before new feed was given and they tended to eat a lot after milking. It is likely that, because diets were fed as a TMR, dietary energy and protein supply to the rumen were constant throughout the day, with a constant production of urea. As no observations on feeding behaviour were made in this study, diurnal variation cannot be linked to time of feeding before milk sampling. Because cows tended to spend a lot of time feeding after milking, it can be assumed that diurnal variation was due to non-nutritional factors.

4.2.4.2. Day-to-day variation

a) Acetone

The day-to-day variation in acetone concentration was small, ranging from 0.01mM to 0.11mM in cows on the control diet (Table 4.17). However, these standard deviations accounted for 16.0% to 46.8 % of their means respectively. Nevertheless, the extent of this variation is unlikely to affect prediction of energy status in a cow with low concentrations. Only at concentrations above 0.4mM would close attention need to be paid to nutritional management. Daily variation may be important to observe, particularly since cows may have wide ranges of normal acetone concentrations. If cows were sampled on days when acetone was near the 0.4mM threshold, the farmer might think that perhaps corrective action should be taken and rations adjusted to increase energy intake. However, this result may only be due to variation on a day-to-day basis and cows with high acetone concentrations should be sampled and analysed over a few consecutive days to ensure that acetone levels are consistently high or deviating from normal before corrective action is taken.

b) Urea

The standard deviation for individual cows ranged from 0.33mM to 0.99mM, suggesting that day-to-day variation in urea can be considerable. The range of values

that could be expected from individual cows, due to day-to-day variation, was also shown to vary considerably. Again, in more than half the cows the upper limit of the range did not fall into the normal range of urea concentrations, suggesting that rations should perhaps be refined. Therefore these data suggest that nutritional management decisions should be based on a few days results rather than milk composition data from one milking. This applies to all constituents that show considerable day-to-day variation.

c) Fat

Milk fat content was variable on a day-to-day basis with standard deviations for individual cows ranging from 1.52 to 3.27g/kg. Variation between days may be due to several factors, such as the feeding regime, length of milking interval and the completeness of milking, thus making it difficult to interpret daily milk fat measurements. However, in this study the milking units had automatic cluster removal so variation in fat content due to the completeness of milking would not be significant. Based on the daily variation in fat measurements, it would be recommended to base nutritional management decisions on milk fat results averaged from a few days.

There has been little research on the day-to-day variation in milk fat content. Atwal and Erfle (1990) observed a wave like pattern of changes in milk fat in many cows. The change in milk fat was gradual, nearly cyclical and had a wavelength of five to seven days. Dry matter intake was not different between times of high and low milk fat. These alterations in milk fat content were thought to be due to lypolytic metabolic regulation, for example a low milk fat observed in the wavelike cycle may be due to reduced lipolysis to compensate for an energy deficit.

d) Protein

The ranges of milk protein concentration for each cow were fairly narrow, due to the relatively small standard deviation. This indicates that protein content was relatively stable between sampling days in individual cows (Figure 4.18) and differences between protein measurements on a daily basis are not likely to be of concern in an on-line monitoring system.

Based on the results with variation within and between cows in milk composition, it is possible to make assumptions on the accuracy of milk recording schemes and blood metabolic profile tests. Based on the data from this experiment, monthly sampling regimes that base milk composition data on an AM and PM sample could potentially have an error of up to 6.7% for fat and 2.3% for protein. Therefore, due to day-to-day variation in milk composition, these testing schemes may not give an accurate measurement of milk fat or protein content. Monthly samples from the same cow are likely to be significantly different, due to variation between sampling days, as well as a stage of lactation effect.

The sampling procedure for the blood metabolic profile test involves sampling a predefined group of cows in early and mid lactation and in the dry period, with a minimum of seven animals per group (Payne et al, 1970). For each group of cows the mean concentration of each metabolite is calculated and compared to reference values. If variation in blood composition is similar to that of milk composition in terms of diurnal and day-to-day variation, then the metabolic profile test may not be an accurate indicator of nutritional status at the herd level if only one sample per day per group is taken.

4.2.4.3. Between cow variation

There was considerable variation between cows in mean milk composition and normal ranges. The data showed that for acetone, urea, fat and protein there was a significant effect of cow ($P<0.001$) when the data for each cow were averaged over the 13 sampling days. Cows also varied in the extent of their normal concentration ranges, in that some cows had very wide normal ranges of a particular milk constituent and other cows had much smaller ranges for the same constituent (Figure 4.19). These findings highlight the fact that differences exist between individual animals, and milk composition that is normal for one cow may be abnormal for another cow and require an adjustment to nutrition. Therefore, it is important to establish normal ranges of constituents before putting an online monitoring system into practice for nutritional management. Also, these sources of variation highlight the need to manage cows individually instead of feeding at the group level to obtain optimum cow performance.

Due to the extent of the between cow variation it is possible that there were not enough cows in each dietary group to investigate the effect of changing the ratio of starch to fibre in Chapter 4, Experiment 1. If cows vary significantly from each other in milk composition, more cows per group would be required to confirm the significance of diet changes.

4.2.5 Conclusions

There was significant diurnal variation in acetone, urea and fat but not in protein concentration. Variation on a day-to-day basis also exists for all measured milk constituents, therefore nutritional management decisions should not be based on only one measurement. Ideally two samples per day should be collected for those constituents that show diurnal variation and sampling should occur for a few days to obtain a more reliable baseline for accurate assessment of metabolic and nutritional status in the dairy cow. Once estimates of day-to-day variation are known for a particular cow, the sampling frequency can be determined. Obviously if variation between days is large then more samples will be required to obtain a more accurate measure of milk component of interest. Data interpretation should take into account variation between cows in terms of their normal ranges of milk constituents so that individuals can be managed accordingly.

4.3. EXPERIMENT 2. DETECTION OF NUTRITIONAL STRESS (REDUCED FEED INTAKE) BY MONITORING MILK ACETONE

4.3.1 Introduction

Milk acetone, being an indicator of energy balance, may be useful to detect nutritional problems such as insufficient dietary energy or a reduction in feed intake. Many studies have investigated the relationship between milk acetone and energy balance and the use of acetone to detect cows with subclinical and clinical ketosis (Andersson, 1984; Andersson and Lundström, 1984b; Heuer et al, 2001a). Since ketotic cows tend to experience reductions in milk yield and have a reduced appetite (Lean et al, 1991), it could be assumed that elevated acetone concentrations in milk could be used to detect cows with a reduced feed intake and subsequently a health problem.

In the experiment describe in Section 4.1, cows were trained to feed out of individual Calan electronic gates. Feed intakes were observed to drop substantially in the first two days after cows were first allocated to gates, until they learned how to use them. If raised acetone concentrations were observed during this period this would show that they could be useful for early detection and correction of nutritional problems or a reduction in feed intake associated with a health problem such as mastitis or lameness.

The aim of this experiment was to monitor changes in milk composition, in particular milk acetone, in cows under nutritional stress. Nutritional stress included a diet change and a reduction in feed intake as cows went from outdoor grazing to a Calan gate feeding system. It was hypothesised that milk acetone concentrations would increase when the cows switched feeding systems, due to a significant drop in feed intake.

4.3.2 Materials and Methods

4.3.2.1. Experimental design

Seven Holstein-Friesian cows in early lactation were used in this study which was carried out in April/May 2002. From calving to day 19, cows were kept at grass during the day and housed at night, during which time they were fed a high maize silage diet including grass silage and brewers grains (Appendix 6). Concentrates were fed in the parlour according to level of milk production. On day 20, cows were allocated to Calan gates and their diet was changed to a high starch TMR (Diet two in Section 4.1.2.2). Milk samples were collected three days before cows were allocated to gates on days 17, 18 and 19 (Period one) to determine the baseline levels of milk constituents and from days 20 to 27 to monitor any response in milk composition to a reduction in feed intake (Period two). Samples were analysed for acetone, urea, fat and protein. Cows were milked twice daily at 05:00 and 17:00, when AM and PM samples were collected. Milk yields were recorded throughout the experimental period. Feed intakes were measured daily when the cows were using the Calan gates. It was not possible to measure feed intake when the cows were at grass or the first day of using the gates.

4.3.2.2. Statistical analysis

Average daily milk composition was calculated from AM and PM sample data and milk yields. Data were analysed by General ANOVA in Genstat 5.0, comparing the mean values for days 17, 18 and 19 with the mean composition over days 20 to 27. The block structure used was "Day/Cow" and the treatment structure was "Period". Differences in milk parameters before and after the change in feeding system were significant at $P<0.05$. Acetone and fat data were not normally distributed and were transformed to log base 10 before analysis. The relationship between milk acetone and milk yield was investigated by Simple Linear Regression with Groups, with milk yield as the response variable and acetone as the explanatory variable. "Cow" was used as the grouping factor to investigate the relationship between acetone and milk yield in individual cows.

4.3.3. Results

Significant changes were observed in milk fat, protein and urea content and milk yield, when comparing the baseline concentrations with milk composition after changing feeding systems. Surprisingly, milk acetone was not significantly affected (Table 4.19). All measured milk constituents were lower in concentration after changing to the Calan gate feeding system and milk yield increased significantly.

Table 4.19. Effect of transferring cows from a grazing system to individual electronic feeders on milk parameters

Parameter	Baseline	SEM	New	SEM	P value
Acetone (mM)	0.124	***	0.112	***	ns
Urea (mM)	5.28	0.19	4.71	0.12	0.030
Fat (g/kg)	45.19	***	41.30	***	0.030
Protein (g/kg)	31.30	0.28	29.79	0.17	0.001
Milk Yield (l)	40.51	0.92	43.56	0.55	0.019

*** Means for acetone and fat presented in Table 4.19 are back-transformed from the log data. With the logged data, mean baseline acetone = -0.906 and mean new acetone level = -0.950; SEM = 0.03 (minimum replication). Mean baseline fat = 1.655 and mean new fat level = 1.616; SEM = 0.013 (minimum replication). All comparisons were made on the log scale. SEM = standard error of the mean, ns = not significant

4.3.3.1. Feed Intake

Feed intake could not be measured when the cows were at grass. In some cases, feed intakes were not recorded on day 20 as the cows were housed indoors and allocated to Calan gates at approximately 09.30 and feed intakes were recorded daily at 15.30. When intakes were recorded on day 20 they were fairly low since the cows only had access to feed for approximately six hours. There was a gradual increase in feed intake between days 21 and 27 (Figure 4.20), but whether there was a significant reduction in feed intake when the diet and feeding system were changed cannot be determined.

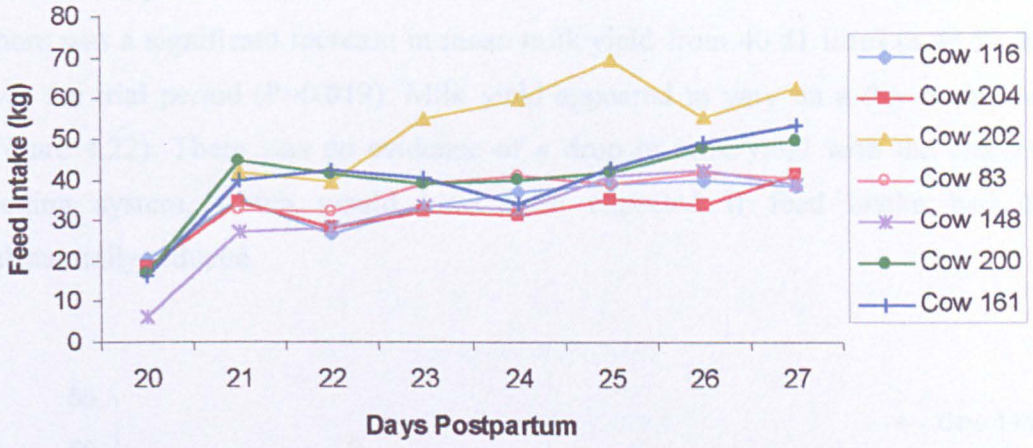

Figure 4.20. Effect of changing feeding systems on feed intake over the trial period

4.3.3.2. Acetone

There was no significant increase in milk acetone with the change of feeding system. Milk acetone concentrations were generally very low except in cow 161 who had acetone concentrations ranging from 0.324mM to 0.348mM on days 18 to 21, before dropping to lower levels ranging from 0.087mM to 0.267mM for the remainder of the trial period (Figure 4.21). Only cow 83 showed a large increase in milk acetone on days 22 to 25, increasing to a maximum of 0.46mM.

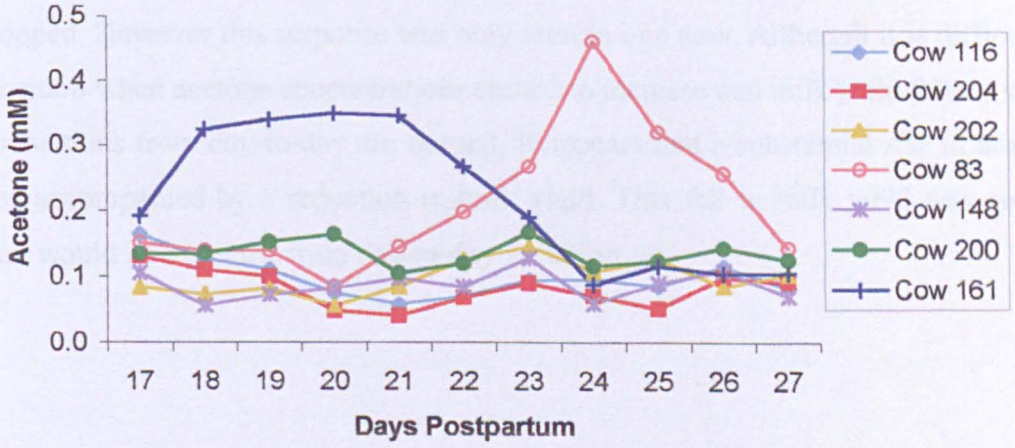

Figure 4.21. Mean milk acetone concentrations over the trial period

4.3.3.3. Milk yield

There was a significant increase in mean milk yield from 40.51 litres to 43.56 litres over the trial period (P=0.019). Milk yield appeared to vary on a day-to-day basis (Figure 4.22). There was no evidence of a drop in milk yield with the change of feeding system, which would have been expected if feed intake had been substantially reduced.

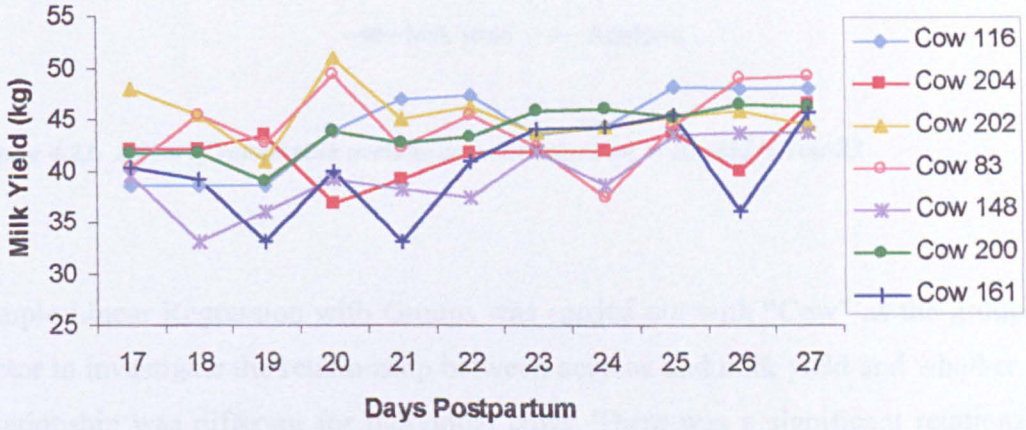

Figure 4.22. Mean milk yields over the trial period

Cow 83 showed a reduction in milk yield with increasing acetone from days 22 to 24 (Figure 4.23). It appears that milk acetone increased two days before milk yield dropped. However this response was only seen in one cow. Although it is difficult to ascertain when acetone concentrations started to increase and milk yield fell, as slight fluctuations from day-to-day are normal, it appears that a substantial rise in acetone was accompanied by a reduction in milk yield. This fall in milk yield was greater than would be expected from day-to-day variation.

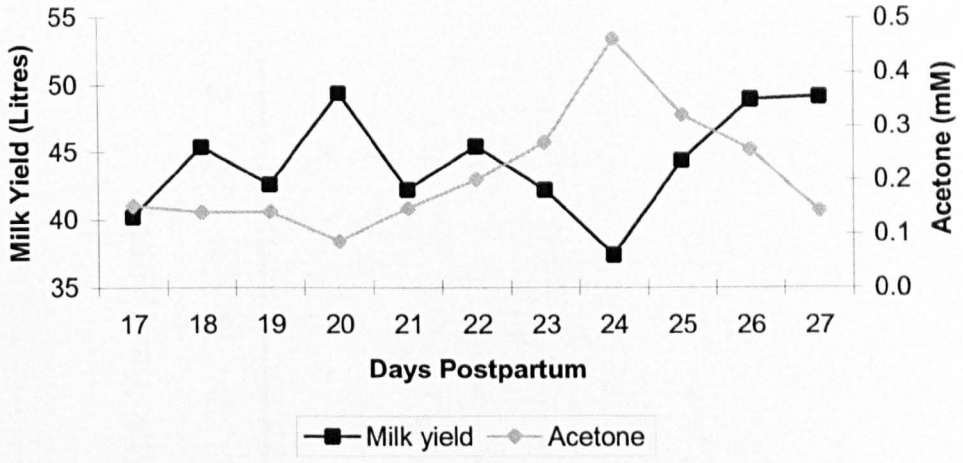

Figure 4.23. Effect of raised milk acetone concentrations on milk yield in cow 83

Simple Linear Regression with Groups was carried out with "Cow" as the grouping factor to investigate the relationship between acetone and milk yield and whether the relationship was different for individual cows. There was a significant relationship between acetone and milk yield when data were averaged over all the cows, with acetone and milk yield being negatively correlated (P=0.023). The decrease in milk yield was estimated to be 12.59 litres per 1mM increase in milk acetone. When cow was used as a grouping factor, a 1mM increase in acetone equated to a 20.74 litre decrease in milk yield (P<0.001).

Five out of the seven cows showed an inverse relationship between milk acetone and milk yield. For cows 148 and 204, as acetone concentration increased, milk yield increased. Data from these cows were removed and the data from the remaining five cows were reanalysed. The relationship between milk acetone and milk yield was more significant (P<0.001), with a 24.56 litre drop in milk yield per 1mM increase in acetone concentration. Accumulated ANOVA indicated that there was no significant difference between the cows (P=0.07) and the relationship between acetone and milk yield could be described by a common regression line (Figure 4.24). Regression analysis was not carried out with fat, urea and protein since these constituents were thought to be influenced by diet change to a greater extent than acetone.

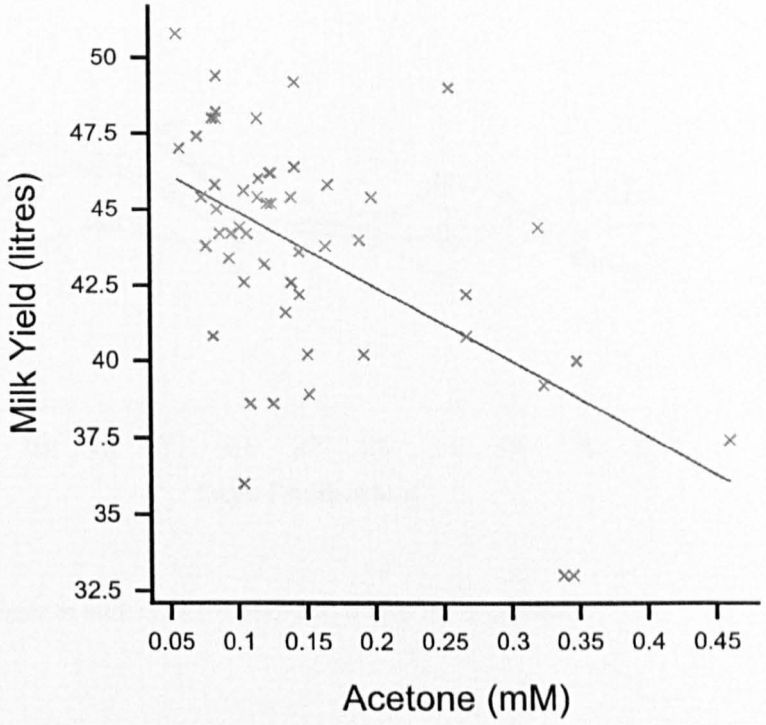

Figure 4.24. Relationship between milk acetone and milk yield in cows 83, 116, 161, 200 and 202, where Y = -24.56x + 47.36. (SEM of the slope = 5.08 and SEM of the intercept = 0.89). R^2 = 0.31

4.3.3.4. Urea

Milk urea significantly decreased from a mean of 5.28mM to 4.71mM over the trial period (P=0.003). There appeared to be some day-to-day variation in urea and there was no obvious trend in urea concentrations with the change in feeding system for individual cows (Figure 4.25). For six out of seven cows, urea concentrations decreased from days 20 to 21 although it cannot be assumed that this effect is solely due to the experimental treatment as day-to-day fluctuation will occur.

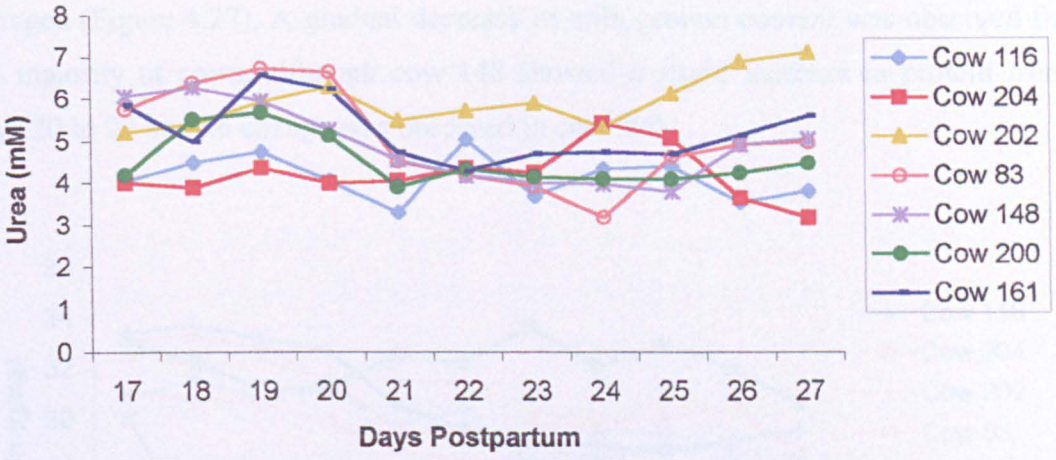

Figure 4.25. Changes in milk urea concentrations over the trial period

4.3.3.5. Fat

Milk fat content was significantly influenced when the feeding system was changed (P=0.03). Milk fat content decreased from 45.19g/kg to 41.30g/kg when the mean of days 17, 18 and 19 were compared with that of days 20 to 27 (Figure 4.26).

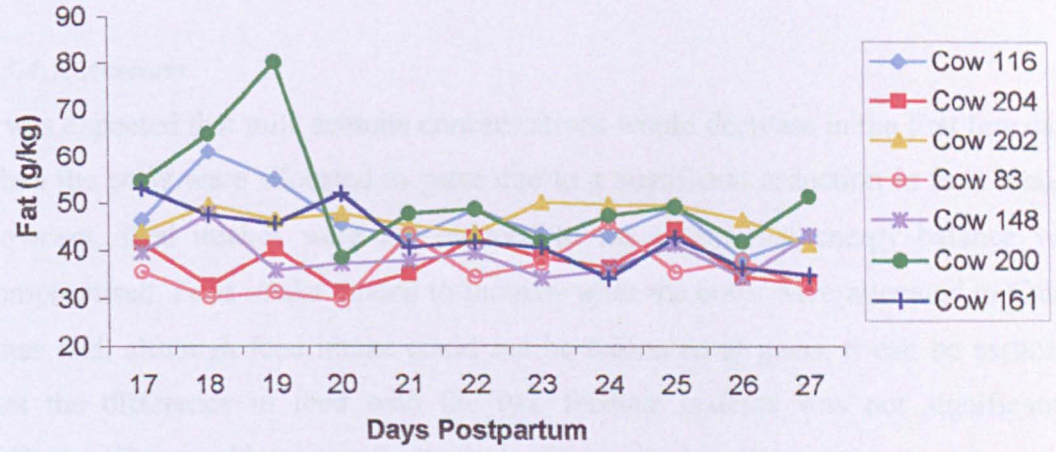

Figure 4.26. Changes in milk fat concentrations over the trial period

4.3.3.6. Protein

A significant decrease in protein content was observed when the feeding system was changed (Figure 4.27). A gradual decrease in milk protein content was observed for the majority of cows, although cow 148 showed a slight increase in protein from days 20 to 27 and no change was observed in cow 200.

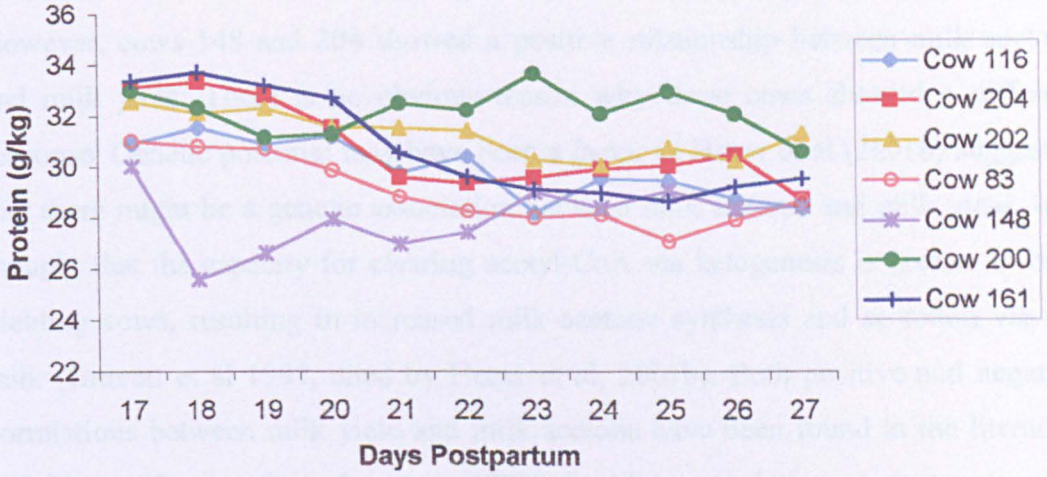

Figure 4.27. Changes in milk protein concentrations over the trial period

4.3.4. Discussion

4.3.4.1. Acetone

It was expected that milk acetone concentrations would decrease in the first few days when the cows were allocated to gates due to a significant reduction in feed intake. However, feed intakes were not reduced to the extent that energy balance was compromised. Feed intake tended to increase after the cows were allocated to Calan gates and, although feed intake could not be measured at grass, it can be assumed that the difference in feed with the two feeding systems was not significantly different. This would account for the lack of response in milk acetone.

There was no obvious reason for cow 161 having raised milk acetone levels compared with the rest of the cows before introduction to the Calan gates. The significant increase in milk acetone for cow 83 could have been caused by reduced

opportunity to feed. Other cows in her pen were blood sampled every two hours from 07.00 until 19.00, which meant that cow 83 was shut away from the feeding gates. However, cow 202 was also under the same experimental conditions and no response in milk acetone was observed (Figure 4.21). Also, feed intake by cow 83 was similar to that of the other cows (Figure 4.20).

Milk acetone concentrations were negatively correlated with milk yield in five cows. However, cows 148 and 204 showed a positive relationship between milk acetone and milk yield. There is no obvious reason why these cows showed a different response. Genetic potential may have been a factor as Heuer et al (2001b) suggested that there might be a genetic association between milk acetone and milk yield. It is thought that the capacity for clearing acetyl-CoA via ketogenesis is greater in high yielding cows, resulting in increased milk acetone synthesis and secretion via the milk (Gravert et al 1991, cited by Heuer et al, 2001b). Both positive and negative correlations between milk yield and milk acetone have been found in the literature and Emanuelson and Andersson, (1986) found a correlation close to zero for hyperketonaemia and milk yield. Miettinen (1994) investigated the relationship between milk acetone and milk yield in individual cows and found a negative correlation between milk yield and milk acetone. Milk yield reductions due to increased acetone were calculated at 2 to 8.5% in non-clinically ketotic cows and a maximum of 26% in cows with clinical ketosis. However, the magnitude of acetone increase was not reported and the response was thought to be partly influenced by sampling month.

The different correlations found in the literature between milk yield and milk acetone or hyperketonaemia may be due to differences in sampling time in relation to the period of ketosis. Concentrations of acetone can vary greatly over time (Heuer et al, 2001b) and cows will vary in response time for acetone levels to increase and for milk yields to decrease (Miettinen 1994). Clearly this relationship needs to be investigated on a larger scale. Because it was not possible to measure feed intake or dietary energy content when the cows were at grass/silage, it cannot be determined whether the response in milk yield and composition to the change in feeding system was due to a change in feed intake, a diet change or due to the cows rapidly approaching peak milk yield.

4.3.4.2. Urea

The reduction in milk urea concentration with the change of feeding system was most likely due to the change in diet rather than the change in feeding system. Milk urea levels tend to be higher when cows are at grass since grass tends to have a high level of rapidly degradable protein. From reviewing the literature, Westwood et al (1998) reported that the crude protein content of ryegrass can reach 30% in spring and autumn. Protein in pasture forage is highly rumen degradable and dairy cows at pasture have been found to have a significantly higher level of milk urea compared with cows fed a TMR (Moller et al, 1993). As there was no information on diet composition before the change in feeding system, it was assumed that the diets contained different protein and energy contents. This would account for the significant effect on milk urea.

4.3.4.3. Fat

Changing the diet and feeding system had a significant effect on milk fat content. This response is more likely to be caused by change in diet than a drop in feed intake. The trial took place in April when grass would have been fairly immature and low in fibre. Because spring grass is also high in sugars, baseline fat levels would be expected to be lower compared with the higher fibre TMR the cows received on the Calan gate system. A large increase in milk fat content was observed in cow 200 (Figure 4.26) from days 17 to 19 (54.73 to 80.12g/kg). This change cannot be explained and is not likely to be accounted for by day-to-day variation alone.

4.3.4.4. Protein

Milk protein content showed a significant decrease when the diet and feeding system were changed (Figure 4.27). Again, this effect is more likely to be due to the diet change, and differences between the energy and crude protein contents of the TMR and grass. Milk yield may have also affected milk composition, as yields were higher after the cows changed feeding system. Increasing milk yields were expected because the cows were in early lactation and had not yet reached peak milk yield. Since all milk constituents decreased in concentration after day 20, dilution by increasing milk yields may be an explanation.

4.3.4.5. Experimental problems

Problems with the experimental facilities affected the results of these experiments. In Section 4.1 the Calan gates were too low for the Holstein cows to operate correctly because they had previously been used with smaller cows. Consequently the cows found it difficult to learn to open the gates as their heads were not in the correct position for the transponders round their necks to make contact with the gates. In preparation for the next nutrition trial, the Calan gates were modified and raised by 40cm so that they were easier for the cows to operate. This modification allowed the cows to learn how to open their gates more quickly, without the reduction in feed intake that was observed in the previous experiment.

The experiments described here had to fit around other trials being carried out at the dairy farm. Improvements could be made by using a greater number of cows and by restricting feed intakes instead of using a change in feeding system to stimulate a reduction in feed intake. Diets before and after the feed restriction should be identical so that any response in milk composition could be attributed solely to the reduction in feed intake.

4.3.5. Conclusions

Milk acetone was not significantly affected by a change in feeding system due to lack of change in feed intake. One out of seven cows showed an increase in milk acetone to 0.46mM with a corresponding reduction in milk yield. This relationship needs to be investigated in a larger scale study, with no diet change and possibly a change from *ad libitum* to restrictive feeding to see whether acetone could be useful to monitor cows on an individual basis. Regression analysis indicated that there was a substantial reduction in milk yield with increasing acetone levels in five out of seven cows. Significant effects on urea, fat, protein and milk yield were most likely due to the diet change and increasing milk yields as cows approached peak yield, since it appears that feed intake was not significantly reduced from the milk acetone concentrations.

CHAPTER 5. MILK CITRATE AS A POTENTIAL METABOLIC INDICATOR

5.1. EXPERIMENT 1. SOURCES OF VARIATION IN MILK CITRATE

5.1.1. Introduction

Citrate is a normal constituent of cows' milk and is involved in cellular energy metabolism, being an intermediate of the Tricarboxylic Acid Cycle or TCA cycle (Figure 5.1). Citrate may be a useful metabolite in milk to assess the energy status of the cow, being correlated with milk acetone (Baticz et al, 2002), milk fat (Romo et al, 2000) and *de novo* fatty acid synthesis (Banks et al, 1984a).

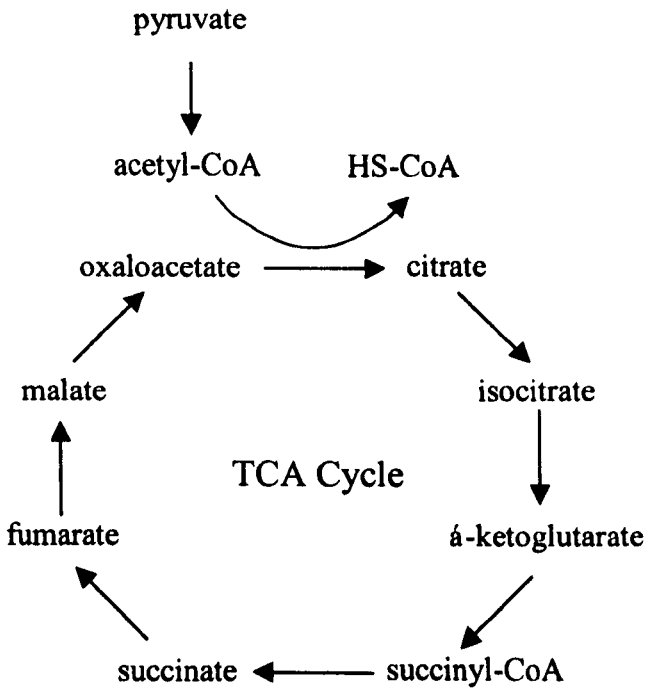

Figure 5.1. The Tricarboxylic Acid Cycle

Citrate concentrations vary throughout lactation, being relatively high in early lactation and then decreasing as lactation progresses. Higher citrate levels are common in healthy, early lactation cows (ten to 20 days in milk) due to the

intensiveness of the TCA cycle at this stage (Baticz et al, 2002) and as a result of changes in the rate of body fat mobilisation, which is in turn related to energy balance. In early lactation adipose tissue is mobilised to support milk production. Fat synthesis in the udder (*de novo*) may be low in relation to the total rate of milk fat secretion, as lipid for milk fat synthesis can be derived from plasma. With advancing lactation, there is less mobilisation of body fat and an increase in the proportion of milk fat produced from *de novo* fatty acid synthesis, thereby reducing the citrate concentration of milk (Peaker et al, 1981).

In the ruminant, citrate is not a significant intermediate for fatty acid synthesis. Nevertheless it may have an indirect role in fat synthesis by providing energy in the form of NADPH, which is required for *de novo* fatty acid synthesis (Faulkner and Peaker, 1982). NADPH is produced by the conversion of isocitrate to 2-oxoglutarate. Isocitrate is an isomer of citrate and these are maintained in equilibrium in the cell (Peaker and Faulkner, 1983). Therefore, as the concentration of isocitrate decreases with increasing synthesis of *de novo* FAs, citrate concentrations will also decrease.

This research focuses on the feasibility of monitoring citrate on a daily basis to assess energy status. If citrate is to be of use for monitoring the dairy cow, several sources of variation need to be established and quantified. Therefore the aims of this study were to investigate diurnal, day-to-day and lactational variation in milk citrate concentration. Secondly, to determine between cow variation in terms of normal ranges of citrate according to lactation stage. It was predicted that citrate concentrations would be greatest in early lactation and would be lower in mid and late lactation cows. Like most other milk constituents, citrate was expected to show some diurnal and day-to-day variation, as well as variation between cows.

5.1.2. Materials and Methods

5.1.2.1. Experimental design

The trial was conducted at the University of Nottingham dairy herd. Twenty-four Holstein-Friesian cows were selected for study according to lactation stage, with eight cows in early, mid and late lactation. Milk sampling for early, mid and late lactation cows took place between days 4 to 29, 103 to 156 and 265 to 306

respectively. The trial took place from August to October 2002. All cows were fed a high maize silage diet (TMR) regardless of lactation stage (Appendix 6). Concentrate feeding took place in the parlour, the amount fed depending on the level of milk production. Milking commenced at 05:00 and 15:30 and milk samples were collected at both AM and PM milkings for ten consecutive days. Milk yields were recorded on sampling days. As the trial cows were amongst the commercial herd and were not milked together as a group, milking intervals were uneven and varied from day-to-day depending on when each cow entered the parlour. Milk samples were frozen until preparation.

5.1.2.2. Milk sample analysis

Milk samples were analysed for citrate by high-performance liquid chromatography (HPLC) and were defatted and deproteinised prior to analysis. Milk samples were thawed at room temperature and defatted by centrifuging a 4ml-aliquot at 4000rpm for ten minutes and removing the fat layer. Deproteinisation was carried out by treatment of a 0.4ml-aliquot of skimmed milk with 3.6ml of three percent cold trichloroacetic acid to precipitate proteins, followed by centrifugation at 17000rpm for ten minutes. A 1.5ml-aliquot of supernatant was placed in an HPLC vial and frozen at -20°C until analysis. The dilution factor of the sample was 1:10.

Sample analysis was carried out by HPLC (model Gynkotek, Jaytee Biosciences Ltd., Whitstable, Kent, UK) with a gradient pump m480G, a Gina 50 autosampler, a UVD340 diode array detector and an Inertsil C8, 5μm column which was 150 x 4.6mm I.D. The eluent or mobile phase used was 98% 0.1M KH_2PO_4 (pH 3.0 by H_3PO_4 + 2% acetonitrile). The flow rate was 1.0ml/min and the ultraviolet detector was set at 218nm. The column temperature was 40°C. Data were analysed by Chromeleon software. A standard curve was produced with a range of standards: 0, 50, 75, 125, 175, 200ppm each day so that the citrate concentrations in each batch of samples were calculated from the standard curve. These standards equated to 0, 2.6, 0.39, 6.5, 9.1 and 10.4mM respectively, once the 1 in 10 dilution factor of the samples was taken into account The mobile phase buffer was used to prepare standards of citric acid and the sample injection size was 50μl.

5.1.2.3. Statistical analysis

Daily citrate concentrations were calculated from the AM and PM data by taking into account both AM and PM milk yields according to the equation in Section 4.1.2.5.

Diurnal and lactational variation were investigated in citrate concentration, citrate yield and milk yield. Data were analysed by General ANOVA in Genstat 6.0. Lactational variation was investigated using "Day/Stage/Cow" as the block structure and "Stage" as the treatment structure. Diurnal variation was investigated with "Day/Stage/Cow/Time" as the block structure and "Stage*Time" as the treatment structure. Significant differences between lactation stages and AM and PM samples within each lactation stage were determined from T tables at $P<0.05$.

Day-to-day variation was expressed as a standard deviation and was calculated from $\sqrt{2.859}$, where 2.859 was the variability of citrate per day averaged over all the cows i.e. the residual mean squares of the day stratum from the ANOVA table derived from investigated stage of lactation variation. However 1.69mM was only an estimate of the day-to-day variation since this could not be accurately calculated for the same reasons as in Section 4.2.2.1.

Between cow variation at each stage of lactation was determined by General ANOVA (block structure = "Day/Stage/Cow" and treatment structure = "Stage*Cow"). An estimated normal range of citrate for each cow was calculated, based on that cow's mean citrate concentration ± two standard deviations. It was expected that when milk sampling on a daily basis, 95% of daily measurements should fall within this range.

5.1.3. Results

5.1.3.1. Diurnal variation

There was no effect of sampling time on milk citrate concentrations when AM and PM samples were compared ($P = 0.873$, SEM = 0.18mM). Furthermore, there was no interaction between time of sampling and stage of lactation ($P = 0.129$). Citrate concentrations were higher in PM samples in early and mid lactation and lower than

AM samples in late lactation although these differences were not statistically significant (Table 5.1).

Table 5.1. Diurnal variation in citrate concentration according to lactation stage

Lactation Stage	AM Citrate (mM)	SEM	PM Citrate (mM)	SEM	P value
Early	10.76	0.12	11.02	0.12	ns
Mid	9.77	0.12	9.79	0.12	ns
Late	10.35	0.12	10.12	0.12	ns

* SEM = standard error of the mean, ns = not significant

Data from individual cows indicated that there was little difference in citrate concentration between AM and PM milk samples at all stages of lactation. There was considerable overlap between citrate concentrations in AM and PM samples with very few outlying measurements (Figure 5.2).

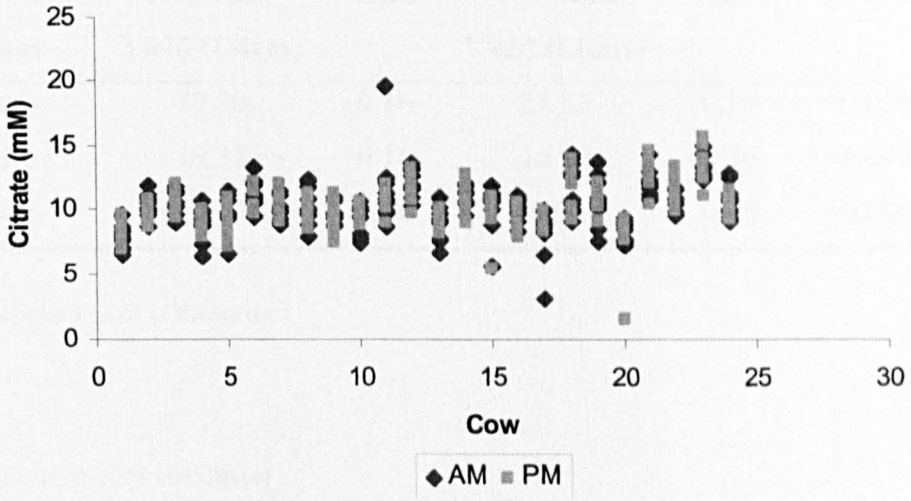

Figure 5.2. Effect of sampling time on milk citrate concentrations in individual cows

Since no effect of sampling time was found in citrate concentration, diurnal variation in total yield of citrate produced at AM and PM milkings was also investigated (Table 5.2). There was a significant effect of sampling time on mean yield of citrate (33.66g citrate produced at AM milking compared with only 26.32g of citrate at PM milking, P<0.001). However, this variation most likely reflects differences in milk yields at each milking, which were significantly higher at AM milking (Table 5.3).

Table 5.2. Diurnal variation in citrate yield according to lactation stage

Lactation Stage	AM Citrate (g/day)	SEM	PM Citrate (g/day)	SEM	P value
Early	39.92	0.42	33.16	0.42	<0.0005
Mid	36.13	0.42	28.31	0.42	<0.0005
Late	24.94	0.42	17.48	0.42	<0.0005

*SEM = standard error of the mean

Table 5.3. Diurnal variation in milk yield according to lactation stage

Lactation Stage	AM Milk Yield (Litres)	SEM	PM Milk Yield (Litres)	SEM	P value
Early	19.30	0.16	15.62	0.16	<0.0005
Mid	19.32	0.16	15.10	0.16	<0.0005
Late	12.47	0.16	8.96	0.16	<0.0005

*SEM = standard error of the mean

5.1.3.2. Day-to-day variation

Day-to-day variation in citrate concentration was considerable and was estimated to be 1.69mM when the data were analysed for all the cows over all the sampling days.

5.1.3.3. Stage of lactation variation

There was a significant effect of lactation stage on milk citrate concentrations, with citrate being highest in early lactation and lowest in mid lactation (P< 0.001). Citrate was higher in late lactation than in mid lactation but not as high as in early lactation (Table 5.4). Since concentrations in late lactation were found to be higher than in mid lactation, and this was an unexpected result, the mean daily yield of citrate according to lactation stage was investigated. There was a significant effect of lactation stage on the daily yield of citrate secreted. Citrate yield decreased from early, to mid and late lactation, most likely reflecting the falling milk yields throughout lactation.

Table 5.4. Effect of lactation stage on citrate concentration, yield and milk yield

	Early	**Mid**	**Late**	**SEM**	**P value**
Citrate (mM)	10.89	9.76	10.26	0.16	<0.001
Citrate Yield (g/day)	73.06	64.39	42.59	0.97	<0.001
Milk Yield (Litres)	34.89	34.43	21.42	0.37	<0.001

*SEM = standard error of the mean

5.1.3.4. Between cow variation

There was a significant effect of cow within each stage of lactation on mean citrate concentrations (P<0.001). Normal ranges of citrate and the size of the range varied between cows (Figure 5.3). Some ranges of citrate concentrations did not overlap, highlighting the difference between individuals in that wide ranges of citrate may be considered as normal in some cows, whereas other cows may have narrow ranges of what would be classed as their normal citrate concentration.

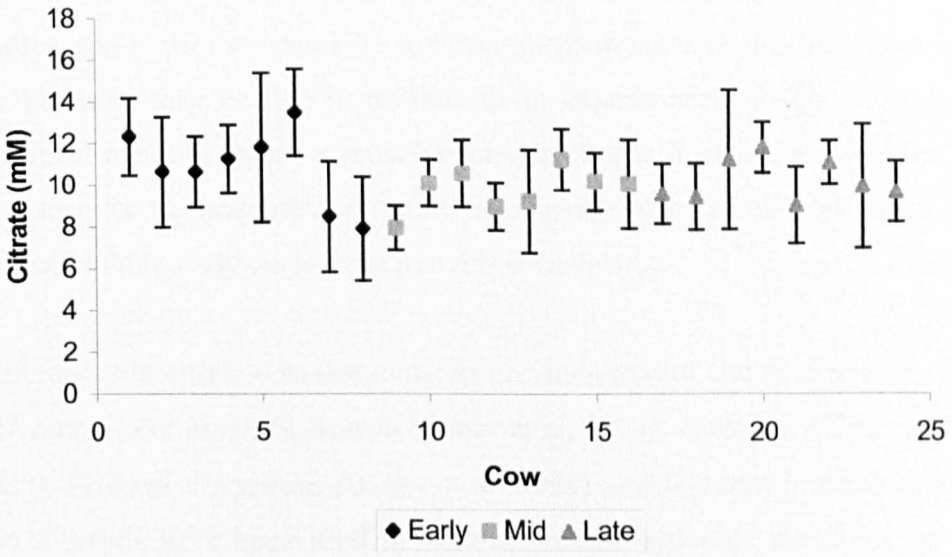

Figure 5.3. Calculated ranges of citrate for each cow based on mean ± two standard deviations

The variation at the stage of lactation level was less than the variation at the individual cow level. The average variability between all the cows at all stages of lactation was 2.60mM. The variation associated between days at each stage of lactation was 1.98mM, which was less than the variation between cows. Therefore variation between the cows is greater than the variation between days for each cow although some cows are more variable than others. For example, in early lactation, variances for individual cows ranged from 0.66 to 3.22mM. Variances for individual cows in mid and late lactation were less than in early lactation confirming that the spread of citrate values is greatest in early lactation when cows are under varying degrees of metabolic stress and are likely to be in a negative energy balance.

5.1.4. Discussion

5.1.4.1. Concentrations of citrate in milk

Typical concentrations of citrate in cows' milk vary greatly in the literature. For example Davies and White (1960) reported citrate to range from 7 to 9mM and a study by Jenness (1988) also found a narrow range of milk citrate from 8.85 to 9.89mM. However, more variable citrate levels have been observed, ranging from 4.69 to 10.41mM (Faulkner and Peaker, 1982). In this experiment, citrate ranged

from 1.61 to 19.63mM with a mean of 10.3mM. Values for citrate from different studies tend to vary substantially and disagreement between these results and those in the literature may be due to differences in experimental design, animals used or analytical methods. Also, it must be considered that figures quoted in the literature tend to refer to mean concentrations in a group of cows and the range of citrate reported in this study came from individual cow values.

Differences in citrate concentrations in previous studies could be attributed to breed differences. For example Jerseys (Konar et al, 1971), Holsteins (Zulak and Keenan, 1983), Friesian x Ayrshire (Banks et al, 1990) and Holstein-Friesian (Baticz et al, 2002) breeds have been used in various studies, although the effect of breed on citrate has not been specifically reported. Variation in results could also be attributed to milk samples being collected from cows at various stages of lactation and differences in diet composition between studies. Therefore based on the literature, the "normal" range of citrate in cows' milk is difficult to define and will vary between individuals, as suggested by the results from this study.

In addition to the above sources of variation, several methods have been used for citrate determination, ranging from HPLC to enzymatic methods, which measure citrate spectrophometrically, fluorimetric methods and capillary electrophoretic determination, all of which will vary in their accuracy of measurement. The method of HPLC was chosen for this experiment due to the large number of samples and minimal sample preparation required before analysis.

5.1.4.2. Diurnal variation

The concentration of citrate in AM samples was not significantly different from PM samples (Table 5.1). This information is useful in that one sample per day could be taken for citrate determination, unlike the conventional pooling of AM and PM samples that is currently employed for milk composition analysis, thereby saving on sampling costs. Although the total citrate yield at each milking was significantly different, this was thought to be related to the difference in milk yield between AM and PM milkings (Tables 5.2 and 5.3). This finding suggests that even though citrate yields and milk yields were significantly different between AM and PM samples, concentrations of citrate in milk tended to be fairly constant throughout the day. This

finding would be of benefit when sampling cows that are milked by automatic milking systems where milking times are not fixed and vary between cows.

Studies in the literature tend to report mean citrate concentrations per cow or by treatment and there has been no investigation into diurnal variation or the effect of sampling time in relation to feeding, which is known to significantly influence urea and possibly acetone. If citrate concentrations were altered shortly after feeding, this would be important to consider if only once daily sampling was used.

5.1.4.3. Day-to-day variation

Large day-to-day variation in milk citrate was observed but this is not surprising considering that citrate is inversely proportional to milk fat (Romo et al, 2000) which can also vary greatly on a day-to-day basis (Atwal and Erfle, 1990; Rook et al, 1992). With considerable day-to-day variation, it is surprising that there was no significant diurnal effect on citrate.

Fluctuations from day-to-day may be a result of metabolic adjustments in response to changes in rations or feed intake. However, in this experiment diet remained constant for all cows at all stages of lactation. Concentrations of citrate have been shown to vary greatly on a short-term basis. For example, in goats that were fasted for 48 hours there was at least a two-fold increase in citrate (Peaker and Faulkner, 1983). Therefore it is possible that citrate could be a useful indicator of a potential health problem that is accompanied by a reduction in feed intake. This theory could be tested by analysing citrate from milk samples taken from cows fed *ad libitum* and then severely restricting their feed intake and monitoring the daily response in milk citrate concentrations.

5.1.4.4. Stage of lactation variation

Milk citrate concentrations can vary widely throughout lactation (Banks et al, 1984b). Therefore, cows sampled in early, mid and late lactation were selected to be within a narrow range of days in milk to reduce this source of variation within each stage. Citrate concentrations were lowest in mid lactation. However, milk yields in early and mid lactation cows were very similar (34.89 litres and 34.42 litres respectively). This is probably due to the cows in early lactation being sampled in the

first two to three weeks of lactation, before peak milk yield. The fact that citrate was significantly different between early and mid lactation provides evidence for an effect of lactation stage on citrate which is independent of milk yield (Table 5.4). These changes are probably related to changes in energy metabolism and in the proportion of FAs synthesised throughout lactation. The daily yield of citrate decreased as lactation progressed, being lowest in late lactation. This finding is most likely due to the significant decrease in milk yield from mid to late lactation.

Although a significant effect of lactation stage was found, the pattern of citrate throughout lactation differed from other reported studies. The majority of studies have reported citrate to be high in early lactation and gradually decreasing as lactation progresses (Konar et al, 1971; Illek et al, 1997). Braunschweig and Puhan (1999) found that citrate in cows milk was significantly lower from days 170 to 215 (9.16mM) compared with 9.94mM between days 30 and 80 of lactation.

Baticz et al (2002) sampled 119 dairy cows randomly from 10 to 90 days after calving. Citrate was greatest in the first four weeks of lactation, although it decreased quickly from approximately 11mM, reaching a minimum concentration about 40 days after calving. The minimum citrate concentration was about 2mM, which was considerably lower than the results reported in this experiment. Citrate increased very gradually until day 90 although levels were only 30% of the basal value. However, the basal value was not defined and measurements were only taken until day 90 of lactation so no data were available on citrate concentrations in late lactation.

As lactation progresses the decrease in citrate may be a result of cytosolic changes in citrate in mammary epithelial cells, due to alterations in the rate of fatty acid synthesis. In early lactation there may be relatively low fatty acid synthesis within the mammary gland in relation to total milk fat content as mobilisation of adipose tissue provides lipids in the plasma for fat synthesis.

It is possible that changes in citrate may also be due to changes in activity of the enzyme ATP-citrate lyase, which cleaves citrate to acetyl-CoA and oxaloacetate. This enzyme is thought to be either absent or present in very low quantities in the

mammary gland, which would account for the relatively high concentrations in early lactation (Bauman and Davies, 1974, cited by Zulak and Keenan, 1983). Concentrations of ATP-citrate lyase have been found to increase in early lactation (Baldwin and Milligan, 1966) and this would account for the decrease in citrate, after peak concentrations. The activity of this enzyme throughout lactation has not been studied and it would be useful to investigate changes in mammary ATP-citrate lyase throughout lactation to determine its influence on citrate concentrations.

In this study citrate concentrations in late lactation were found to be greater than in mid lactation and this was an unexpected result. To rule out the possibility of an analytical error, twenty samples taken from late lactation cows were randomly selected for reanalysis by HPLC to check repeatability of the method and to ensure that the values obtained were still in the range obtained for late lactation cows. The majority of repeated samples had slightly lower concentrations of citrate, with an error rate ranging from 1.4 to 15.4% (Figure 5.4). The mean concentration difference between these sets of samples was 0.9mM. A two sample (paired) T-test was performed between the two sets of measurements and citrate in the samples analysed the second time were on average lower than when first determined (10.42mM compared with 9.80mM, P=0.003). However, the reruns were three months old and it is possible that citrate in the samples may have deteriorated over time despite them being frozen at -20°C between analyses. Also, slight discrepancies are to be expected in the rerun samples due to the way in which citrate concentrations are determined. A standard curve is produced each day with each batch of samples. Therefore the standard curve may be very slightly different from day-to-day and this might account for at least some of the variation between the repeated samples and the original results.

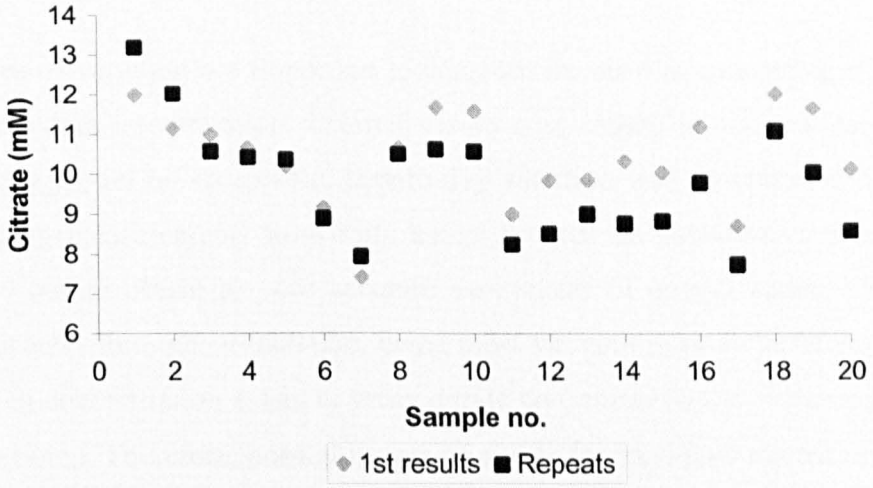

Figure 5.4. Repeated measurements of samples from late lactation to test the validity of the method

Although changes in citrate concentrations throughout lactation can be substantial, it may be difficult to determine how much of that variation is due to a stage of lactation effect since dietary changes are common over the lactation cycle. Seasonal variations have also been reported (Holt and Muir, 1979) but again, these are most likely due to changes in diet such as moving from a gazing system to indoor TMR feeding. In this study it would have been interesting to sample cows after calving and follow them throughout lactation until drying off as this would give a more accurate indication of the effect of lactation stage on milk citrate concentrations in individual cows. However this was not possible due to time constraints.

5.1.4.5. Between cow variation

Between cow variation was statistically significant, indicating that normal ranges of citrate concentrations will vary between cows regardless of lactation stage (Figure 5.3). The extent of the day-to-day variation also varied between cows. Variation within and between cows was greatest in early lactation, probably because cows were under metabolic stress and in a negative energy balance. This variation highlights the importance of establishing normal ranges of citrate for each cow so that deviations from normal can be detected and subsequently corrected via nutrition to improve milk output and/or composition.

5.1.5. Conclusions

Sources of variation are important to consider for on-line monitoring of citrate and accurate data interpretation. Diurnal variation in citrate is minimal so once a day sampling would be acceptable. Day-to-day variation was estimated at 1.69mM and averaging measurements from milk samples taken on consecutive days should be carried out to obtain a more accurate assessment of energy status. Citrate varies significantly throughout lactation, being most variable in early lactation. Significant between cow variation exists in mean citrate concentrations, as well as in the extent of the range. Therefore, normal ranges of citrate for each cow should be established so that deviations from normal can be identified.

5.2. CITRATE, MILK COMPOSITION AND ENERGY OUTPUT

5.2.1. Introduction

The relationship between citrate and certain milk parameters may be useful to determine so that a combination of information could be used to improve the accuracy of assessing the energy status of the dairy cow. As citrate is thought to be correlated with the proportion of *de novo* fatty acid synthesis, citrate concentrations in milk may also indicate the extent of body tissue mobilisation in early lactation (Peaker et al, 1981; Faulkner and Peaker, 1982). Milk citrate has not been correlated with calculated energy balance. However, it would be useful to investigate whether citrate is related to energy output of the cow and milk fatty acid content, to assess its use as an indicator of energy status and for subsequent nutritional management to improve productivity in terms of milk yield and composition.

The aim of this data analysis was to investigate the relationship between citrate and certain milk composition parameters such as fat and protein content, fatty acid composition and somatic cell count (SCC). Secondly, to determine whether citrate could be used as an indicator of energy output of the dairy cow in terms of FCM.

5.2.2. Materials and Methods

Milk samples were collected from the 24 trial cows in Experiment 1 on days one, three, five, seven and nine. A sub-sample was removed and frozen prior to fatty acid analysis (see Appendix 8 for methodology). The remaining sub-samples were preserved with Lactabs and sent to the National Milk Records (NMR, Harrogate, Yorkshire, UK) where they were analysed for fat and protein content and SCC by infrared spectroscopy.

5.2.2.1. Statistical analysis

Data from Chapter 5, Experiment 1 were analysed to investigate the relationship between citrate and measures of milk quality (fat and protein content and SCC) and FCM. Lactational variation in fat concentration, fat yield, protein concentration,

protein yield, SCC and FCM were investigated by General ANOVA with "Day/Stage/Cow" as the block structure and "Stage" as the treatment structure.

Simple Linear Regression with Groups was carried out to investigate the relationship between citrate concentration and fat concentration, fat yield, protein content, protein yield and FCM. Citrate was the explanatory variable and the grouping factor was "Stage" to investigate the relationship between citrate and milk composition parameters at each stage of lactation. Regression analysis was also carried out with SCC as the explanatory variable and citrate as the response variable.

The fatty acid profiles were categorised into <C16 (*de novo* synthesis of FAs with a carbon chain length less than 16), C16 and >C16 (preformed FAs with a carbon chain length greater than 16). The categories of FAs were expressed in mmol/day. *De novo* FAs were also expressed as a ratio to preformed fatty acids (<C16:>C16 in mmol/day) and compared with citrate concentrations. Data were analysed by General ANOVA to determine whether the proportion of *de novo* fatty acid synthesis within the mammary gland varied according to lactation stage. Milk citrate concentrations expressed in mM/l, mmol/day, g/l and g/day were also investigated by ANOVA to determine whether *de novo* fatty acid synthesis could be related to changes in citrate throughout lactation.

Simple Linear Regression with Groups was carried out to investigate the relationship between citrate (mmol/day) and *de novo* fatty acid synthesis (mmol/day) and *de novo* synthesis as a proportion of the total FAs (<C16:total FA in mmol/day). Citrate was the explanatory variable and "Stage" was used as the grouping factor to investigate the relationship between citrate and *de novo* fatty acid synthesis at each stage of lactation.

5.2.3. Results

5.2.3.1. Citrate and milk composition

There was a significant effect of lactation stage in all milk parameters measured (Table 5.5). A similar trend in fat and protein was found throughout lactation in that

concentrations were highest in early lactation and lowest in mid lactation cows (see Appendix 9 for mean data per cow).

Table 5.5. Effect of lactation stage on milk parameters

Parameter	Early	Mid	Late	SEM	P value
Fat content (g/kg)	50.42	40.33	41.37	1.09	<0.001
Fat yield (g/day)	1734.00	1382.00	884.00	32.30	<0.001
Protein content (g/kg)	34.78	32.22	35.16	0.55	<0.001
Protein yield (g/day)	1190.00	1107.00	752.00	7.60	<0.001

* SEM = standard error of the mean

Regression analysis indicated that there was a significant relationship between citrate concentration and fat concentration throughout lactation ($P<0.001$), with a 1mM increase in citrate being equated to a 2.26g/kg increase in milk fat concentration. However, the relationship varied according to lactation stage. In early and mid lactation the relationship between citrate and fat was positive, so as citrate increased, fat also increased. However in late lactation, as citrate increased, fat concentration decreased (Figure 5.5).

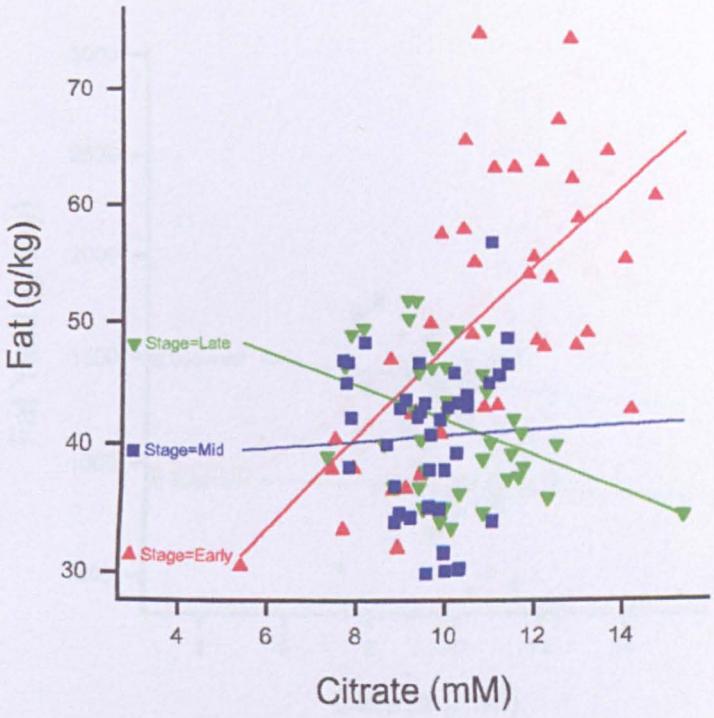

Figure 5.5. Relationship between citrate and fat concentration with lactation stage. Equations for early, mid and late lactation are $Y = 3.52x + 12.35$, $Y = 0.22x + 38.20$ and $Y = -1.41x + 55.74$ respectively. $R^2 = 0.46$

Citrate concentration was also related to the total daily fat yield and these variables were positively correlated with a 1mM increase in citrate being accompanied by a 95.4g/day increase in fat yield ($P<0.001$). However, this relationship only held true in early lactation, with citrate and fat yield being only slightly negatively correlated in mid and late lactation (Figure 5.6).

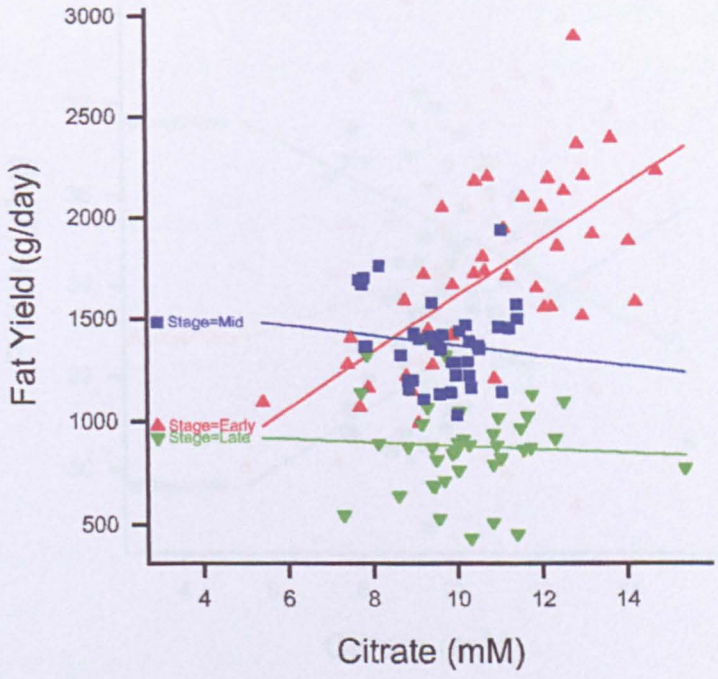

Figure 5.6. Relationship between citrate and fat yield with lactation stage. Equations for early, mid and late lactation are Y = 139.1x + 227, Y = -24x + 1613 and Y = -8.4x + 970 respectively. R^2 = 0.71

A similar trend to citrate and fat content was found between citrate and protein concentration, in that citrate was positively correlated with protein in early and mid lactation but negatively correlated in late lactation (Figure 5.7). The relationship between citrate and protein over the whole lactation was significant (P=0.021) with a 1mM increase in citrate being equal to a 0.32g/kg increase in milk protein content.

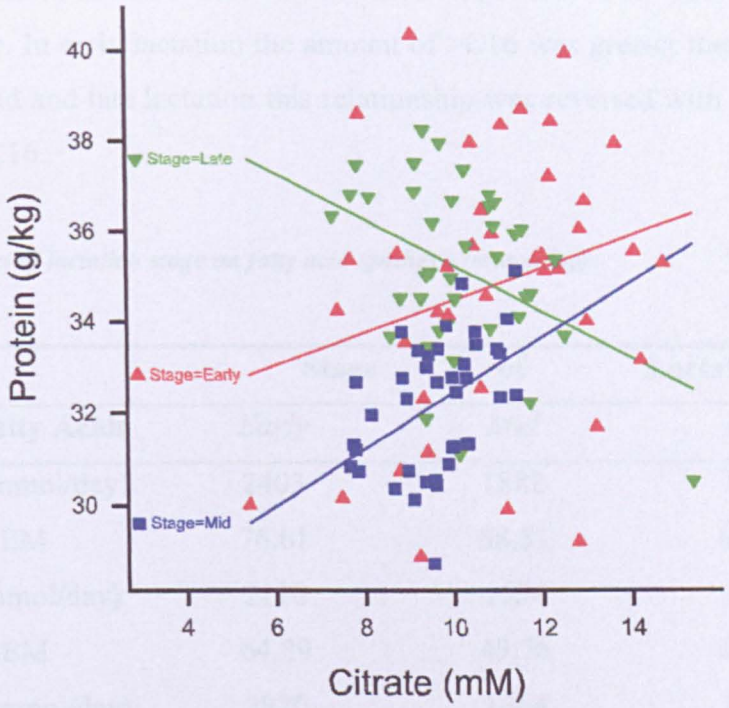

Figure 5.7. Relationship between citrate and protein concentration with lactation stage. Equations for early, mid and late lactation are $Y = 0.36x + 30.86$, $Y = 0.62x + 26.28$ and $Y = -0.51x + 40.36$ respectively. $R^2 = 0.35$

5.2.3.2. Citrate and fatty acid composition

There was a significant effect of lactation stage on citrate concentrations and yields per litre and per day (Table 5.6). Citrate decreased throughout lactation when calculated in g/L and g/day. However, when citrate was calculated in mM/L and mmol/day, the lowest values were found in mid lactation.

Table 5.6. Effect of lactation stage on citrate concentration and yield

	Early	Mid	Late	SEM	P value
Citrate (mM/L)	10.89	9.76	10.26	0.16	<0.001
Citrate (mmol/day)	374.80	219.90	330.60	4.56	<0.001
Citrate (g/L)	73.06	64.39	42.59	0.97	<0.001
Citrate (g/day)	1388.00	1034.00	468.00	35.70	<0.001

* SEM = standard error of the mean

The proportions of *de novo* FAs in relation to C16 and >C16 varied according to lactation stage. In early lactation the amount of >C16 was greater than <C16 (Table 5.7) and in mid and late lactation this relationship was reversed with a greater yield of <C16 to >C16.

Table 5.7. Effect of lactation stage on fatty acid synthesis (mmol/day)

	Stage	**of**	**Lactation**
Milk Fatty Acids	*Early*	*Mid*	*Late*
<C16 (mmol/day)	2403	1882	1178
SEM	76.61	58.51	61.11
C16 (mmol/day)	2110	1654	993
SEM	64.89	49.56	51.77
>C16 (mmol/day)	2820	1664	1100
SEM	98.10	74.93	78.12
P value	<0.001	<0.001	<0.001

* <C16 refers to fatty acids with a carbon chain length less than 16, synthesized within the mammary gland (*de novo*) and >C16 refers to the long chain fatty acids derived from the diet and adipose tissue with a carbon chain length greater than 16. C16 arises from both sources. SEM = standard error of the mean

Figure 5.8 shows the relationship between citrate and the proportion of *de novo* FAs to preformed FAs (<C16:>C16) in mmol/day. There was an inverse relationship between citrate and <C16:>C16 when data from early and mid lactation were compared, confirming the hypothesis that an increase in citrate is accompanied by a decrease in the proportion of *de novo* fatty acid synthesis and vice versa. Citrate concentrations and yields were highest in early lactation when the proportion of *de novo* fatty acids synthesis was the lowest. In mid lactation citrate decreased and the proportion of *de novo* synthesis increased from early lactation. Citrate was significantly lower in late lactation and this decrease was accompanied by only a very small reduction in *de novo* fatty acid synthesis.

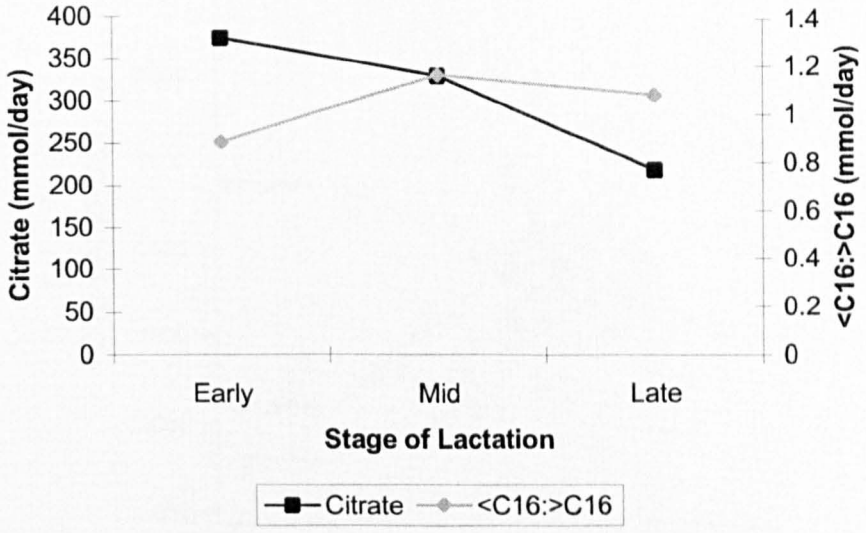

Figure 5.8. Relationship between citrate and de novo fatty acid synthesis

Linear regression analysis revealed that there was a significant relationship between citrate and *de novo* fatty acid synthesis in mmol/day when data from all cows at all stages of lactation were analysed ($P<0.001$). When data at each stage of lactation were considered there was a positive relationship between citrate and *de novo* FAs in early and late lactation, but a negative relationship between these two variables in mid lactation (Figure 5.9).

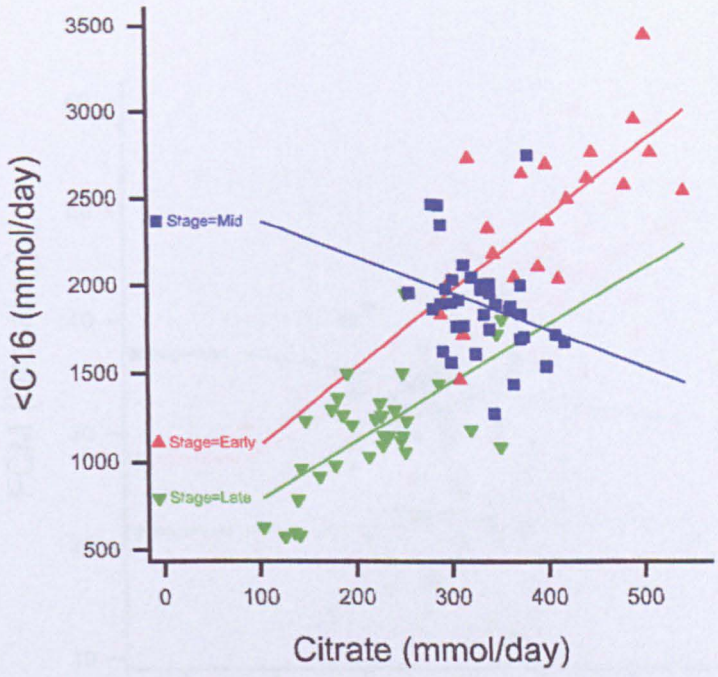

Figure 5.9. Relationship between citrate and de novo fatty acid synthesis with lactation stage. Equations for early, mid and late lactation are $Y = 4.38x + 654$, $Y = -2.11x + 2593$ and $Y = 3.32x + 452$ respectively. $R^2 = 0.78$

When citrate was compared with the proportion of *de novo* fatty acid synthesis in relation to the total amount of FAs (in mmol/day) there was no significant relationship when data from all stages of lactation were analysed (P=0.408).

5.2.3.3. Citrate and energy output

Energy output of the cows was determined by calculating FCM. As expected, FCM was greatest in early lactation and decreased as lactation progressed, being 39.76, 34.49 and 21.84 litres/day in early, mid and late lactation respectively (P<0.001). Regression analysis between citrate and FCM indicated that there was a positive correlation between these two variables throughout lactation, with a 1mM increase in citrate concentration being associated with a 1.54 litres/day increase in FCM (P=0.002). When analysing the data for each stage of lactation the strongest relationship between citrate and FCM was found in early lactation. However, in mid

and late lactation there was no significant relationship between citrate and FCM (Figure 5.10).

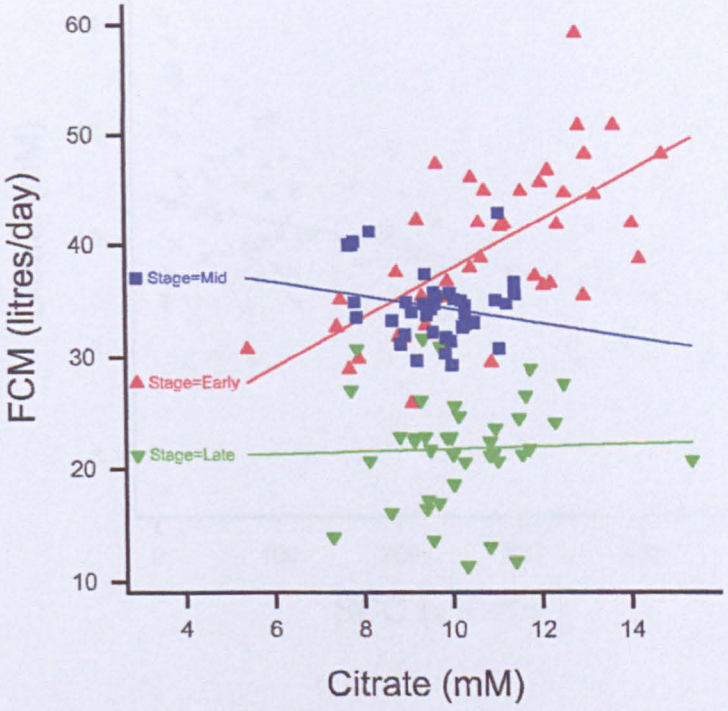

Figure 5.10. Relationship between citrate and FCM with lactation stage. Equations for early, mid and late lactation are Y = 2.216x + 15.76, Y = -0.612x + 40.38 and Y = 0.116x + 20.66 respectively. R^2 = 0.75

5.2.3.4. SCC and citrate

There was a significant effect of lactation stage on SCC with SCC being 27.67 $x10^3$, 139.48$x10^3$ and 143.76$x10^3$ in early, mid and late lactation respectively ($P<0.001$). Regression analysis of SCC against citrate concentration revealed a significant negative relationship ($P=0.002$) when data from all stages of lactation were analysed. SCC was regressed against citrate as it was hypothesised that the increased numbers of SCC may be accompanied by increased cell damage and thus decreased secretion of citrate into milk, due to diffusion into the blood. SCC and citrate concentration were negatively correlated ($P=0.002$) when data from cows at all stages of lactation were analysed. Accumulated ANOVA revealed that there was no significant interaction between citrate and lactation stage ($P=0.945$) and so the relationship between SCC and citrate could be described by a common line (Figure 5.11).

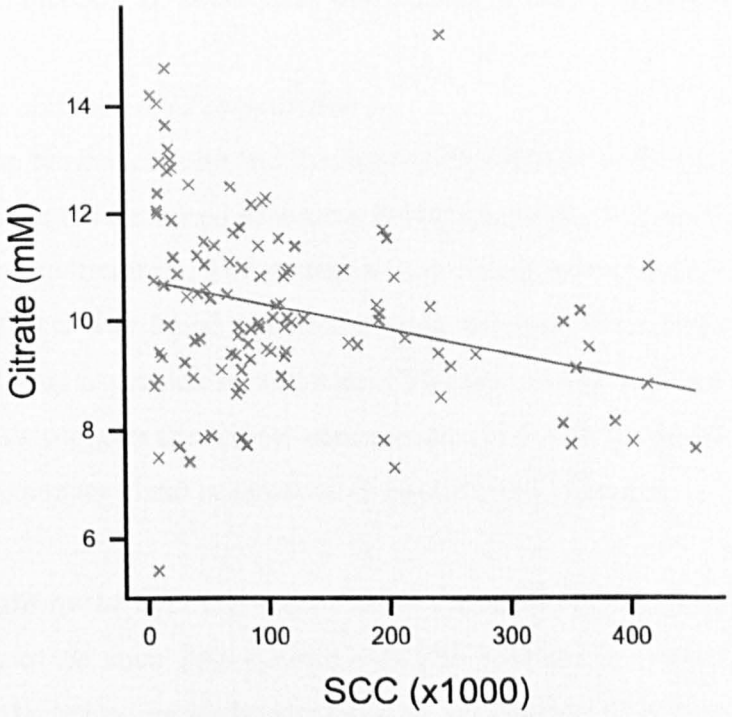

Figure 5.11. Relationship between SCC and citrate. Y=-25x + 418. R^2 = 0.08

5.2.4. Discussion

5.2.4.1. Citrate and milk composition

Significant relationships were found between citrate and fat and protein. However, the nature of these correlations differed according to lactation stage (Figures 5.5, 5.6 and 5.7). Therefore it may be difficult to use citrate in conjunction with milk fat to assess energy status since the nature of the correlation is variable and it is not known at which point throughout lactation the correlation changes from being positive to negative. The change in correlation may be due to change in fat content which decreased from early to mid lactation to a much greater extent than citrate concentration. Fat yield was more than halved between these two periods although citrate yield only decreased from 73.06g to 64.39g. The inversely proportional relationship between citrate yield and fat yield found by Romo et al (2000) in cows

on average 115 days in milk, agreed with the results of this study from mid lactation cows, in that an increase in citrate yield was associated with a reduction in fat yield.

5.2.4.2. Citrate and fatty acid composition

The relationship between citrate and the fatty acid profile of milk was investigated to determine whether citrate varied according to changes in *de novo* fatty acid synthesis, as suggested in the literature. The strongest relationship between citrate and *de novo* fatty acid synthesis was found when data were analysed with citrate and *de novo* fatty acid synthesis expressed in mmol/day. The percentage variance accounted for was 77.2%. This suggests that citrate concentration is a strong determinant of energy status of the mammary gland in terms of *de novo* FAs synthesized.

This study confirms the hypothesis that citrate varies throughout lactation in relation to the amount of *de novo* FAs synthesised. The decrease in citrate yield between early and mid lactation cannot be attributed to a reduction in milk yield since milk yields in early and mid lactation were similar (34.78 and 35.16 litres respectively). There was less *de novo* fatty acid synthesis compared to >C16 (mmol/day) in early lactation (Table 5.7). This was to be expected since in early lactation the proportion of *de novo* synthesis is small compared with the total FAs secreted, due to adipose tissue mobilisation. Later in lactation, body reserves are restored, and more *de novo* FAs are synthesised, reducing the proportion of preformed FAs in the milk fat.

Changes in the concentration of citrate throughout lactation can, to some extent, be related to changes in *de novo* fatty acid synthesis. Citrate and *de novo* fatty acid synthesis appear to be correlated (Figure 5.9). However, the relationship between these two variables tends to change according to lactation stage, and other factors may also affect citrate concentrations, such as energy status, which were not taken into account in this study.

5.2.4.3. Citrate and energy output

Energy output in terms of FCM was positively related to citrate concentration in early lactation (Figure 5.10). High concentrations of citrate were associated with a higher yield of FCM. This information may be useful to improve nutritional management. For example, a low concentration of citrate indicates that FCM may be

improved by increasing the energy content of the diet or providing a higher level of supplemental fat, which would increase milk citrate concentrations (see Section 5.3). The response to this diet change could be monitored in terms of milk citrate concentrations, milk yield and composition.

The only problem with this assumption is that FCM does not give a good representation of a cow's energy status. For example a cow in negative energy balance may produce the same FCM as a cow in a positive energy balance. This highlights the need for monitoring citrate in conjunction with an accurate calculation of energy balance so that citrate concentrations associated with the degree of negative energy balance can be immediately picked up. It must be remembered that citrate is not a reflection of blood citrate and so milk citrate may not accurately reflect metabolic status of the cow since citrate is synthesised within the mammary epithelial cells and does not diffuse into milk from blood. It may however be an indirect indicator of adipose tissue mobilisation and therefore energy status, through changes in *de novo* fatty acid synthesis.

However, citrate alone may not represent a clear picture of an animal's energy status. Milk acetone is another indicator which could be used in conjunction with citrate, which would make assessment of energy status easier and more accurate (Baticz et al, 2002). The authors' hypothesis was that citrate should decrease with increasing ketone body production because oxaloacetate is the key factor for acetone and citrate production via the TCA cycle (Figure 5.12). Increased concentrations of ketone bodies occur when there is insufficient oxaloacetate present to allow all the acetyl-CoA to enter the TCA cycle. This mechanism allows the conversion of acetyl-CoA to various compounds such as ketones, which can then be used as an energy source by other tissues. Consequently, the amount of acetyl-CoA entering the TCA cycle is reduced, thereby reducing the production of citrate which is formed by the condensation of acetyl-CoA with oxaloacetate (Chesworth et al, 1998). Therefore a decrease in citrate would be accompanied by an increase in acetone concentration.

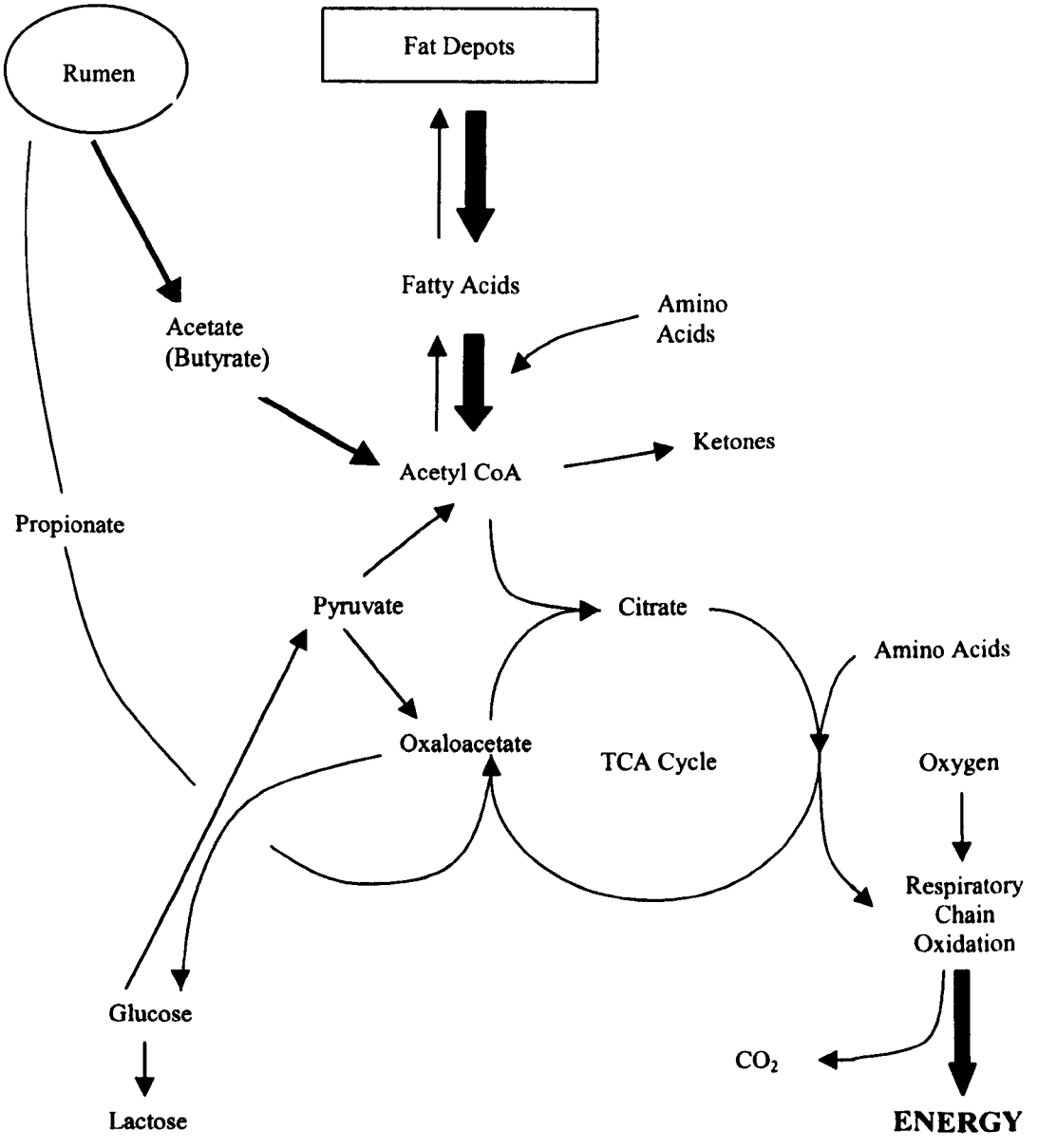

Figure 5.12. The TCA cycle and pathways of energy metabolism in ruminants (adapted from Webster, 1993)

However, Baticz et al (2002) found that there was a significant positive correlation between citrate and acetone in early lactation cows in the high acetone group (cows were allocated to the high acetone group if concentrations exceeded 0.4mM). However in the low acetone group (acetone concentrations less than 0.4mM) the

correlation with citrate was negative and non-significant. Clearly, the relationship between acetone and citrate varies according to lactation stage and may also vary in individual animals depending on their energy status and the extent of negative energy balance in early lactation.

5.2.4.4. SCC and citrate

There was an inverse relationship between SCC and citrate with an increase in SCC being accompanied by lowered citrate concentrations (Figure 5.11). Although the majority of somatic cells are composed of white blood cells or leukocytes, some are epithelial cells from the secretory tissue. An SCC greater than 250,000ml is classed as being abnormal, representing subclinical mastitis (Ozsvari et al, 2003), and may alter the structure of cells in the blood-brain barrier as well as affecting the secretory and synthetic capacity of the alveolar epithelial cells (Sloth et al, 2003). Consequently, as SCC increases, citrate concentrations will tend to decrease due to the diffusion of citrate from milk into the blood (Peaker, 1975; Faulkner and Peaker, 1982).

Milk citrate may therefore be useful in identifying cows which have subclinical mastitis. For example, citrate has been shown to decrease in proportion to the degree of inflammation in cows with subclinical mastitis (Oshima and Fuse, 1981). A milk sample from a mastitic quarter was compared with that from a healthy quarter of the same cow and the degree of mastitis was determined by measuring milk conductivity. The extent of the decrease in citrate concentration was proportional to the degree of mastitis, which was defined by the quarter difference value of Na+Cl concentration in mM/l. The cows sampled varied between 22 and 430 days into lactation and 75% of the cows tested were in late lactation. However, as citrate varies according to lactation stage, the remaining 25% of cows in this study could have influenced the relationship between citrate and the degree of inflammation.

5.2.4.5. Citrate as a metabolic indicator in late lactation

Metabolic indicators are thought to be most beneficial in the early lactation period when producers are aiming to achieve a maximal peak milk yield and the risk of metabolic disorders is high. Monitoring the dairy cow at the end of lactation is also important to avoid overconditioning and to ensure optimal body condition at drying

off and at calving. Citrate may be a useful parameter to monitor the late lactation dairy cow. In late lactation citrate concentrations are reported to be at their lowest, which is due to a relatively higher proportion of *de novo* fatty acids in the milk compared to earlier on in lactation. In later lactation, when cows are putting on body reserves, more milk fat is likely to be derived from *de novo* synthesis as opposed to preformed units in the blood. Therefore increased deposition of body fat is likely to result in relatively lower concentrations of citrate. However, considering the discrepancy between late lactation citrate concentrations in this study and those in the literature, the use of citrate as a body weight gain indicator in late lactation is merely speculation and would have to be researched in great detail.

Ideally it would have been useful to monitor citrate concentrations in cows from mid lactation until drying off and correlate these with measures of body weight and condition score to determine whether citrate could be a potential indicator of body weight gain in late lactation. This may be difficult to assess as growth of the foetus would also result in weight gain and condition scoring would need to be used to determine whether citrate concentrations were reflecting an increase in body weight gain due to foetal growth or increased deposition of body fat. Unfortunately, practical constraints at the dairy farm prevented these measurements from being taken.

5.2.5. Conclusions

Citrate was positively correlated with milk fat and protein concentration, FCM, and *de novo* fatty acid synthesis and negatively correlated with SCC when data from all stages of lactation were analysed. The correlation between citrate and milk composition variables differed according to stage of the lactation cycle. Therefore, it may be difficult to use milk citrate concentrations to improve nutritional management for maximising cow productivity. Further work is required on how citrate changes with calculated energy balances before it can be used as a metabolic indicator to improve individual cow management.

5.3. EXPERIMENT 2. THE EFFECT OF PROTECTED FAT (MEGALAC) ON MILK CITRATE CONCENTRATION

5.3.1. Introduction

Milk citrate is responsive to dietary changes, particularly the fat content of rations (Ormrod et al, 1980 and Ormrod et al, 1979). Megalac is a protected fat supplement, commonly fed to early lactation cows to increase the energy concentration of the diet. The supplement is not altered in the rumen and is a concentrated source of digestible undegradable energy with an ME content of 33.25MJ/kgDM. (Ewing, 2002). Megalac is thought to be of benefit in early lactation rations, due to its energy concentration and aiding the resumption of positive energy balance. Other advantages of feeding Megalac to early lactation cows include increased milk yields and milk fat content (Petit et al, 2001).

Nutritional effects on citrate must be determined if citrate is to be a useful indicator in milk to aid nutritional management. Citrate is positively correlated with FCM (see Section 5.2.3.3.). Therefore feeding Megalac should result in an increase in milk citrate concentration and an increase in energy output in terms of FCM. The aim of this experiment was to determine the effect of supplementary dietary fat (Megalac) on milk citrate concentrations in early lactation and cow performance. It was predicted that supplementary dietary fat would influence milk citrate, with high fat diets increasing citrate, due to an increase in supply of long chain fatty acids which is likely to reduce the proportion of *de novo* fatty acid synthesis.

5.3.2. Materials and Methods

5.3.2.1. Animals and husbandry

Thirty Holstein-Friesian cows from the University of Nottingham dairy herd, calving over the period of April to September 2002 were selected for trial. Cows were housed in three pens of up to ten cows from 20 ± five days of lactation. On day 20 cows were trained to use Calan electronic individual feeding gates, where they remained until day 70. Cows were assigned to six blocks of five animals depending on milk yields between days 30 and 35 of lactation. Within blocks, cows were

allocated to diets at random. Cows were milked at 05:00 and 17:00. Feeding occurred twice daily with fresh feed offered at 16:00 each day and feed bins topped up the following morning at 07:00.

5.3.2.2. Dietary treatments

From calving to day 20 cows were fed a high maize silage TMR (Appendix 6). From day 20 to day 40, cows were fed Diet two from Chapter 4, Experiment 1. Dietary treatments were fed from day 40 until day 70 of lactation. The treatment diets were based on Diet two with the appropriate supplementation of Megalac (Volac Ltd, Royston, Hertfordshire, UK) to supply 0 (Diet one), 0.2 (Diet two), 0.4 (Diet three), 0.6 (Diet four) and 0.8kg (Diet five) of Megalac per cow per day. The Megalac supplement consisted of calcium salts of palm fatty acids and was 80% lipid. Diet one was the control diet as it contained no Megalac.

5.3.2.3. Milk sampling and analysis

Milk samples were collected on days 60, 62, 64, 66 and 68 and analysed for citrate to investigate the effects of feeding different levels of protected fat. Both AM and PM samples were collected and stored at 4°C with a Lactab preservative until sample preparation. Citrate was determined by HPLC according to the procedure and conditions in Section 5.1.2.2. Milk samples were also analysed for fat and protein by National Milk Records (Harrogate, Yorkshire, UK). Milk yields were recorded throughout the trial period.

5.3.2.4. Statistical analysis

Dietary effects on citrate concentration, citrate yield, milk yield, fat concentration, fat yield, protein concentration, protein yield and FCM were investigated by General ANOVA. "Day/Cow" was used as the block structure and "Diet" was used as the treatment structure with differences between diets being significant at $P<0.05$. FCM was calculated at four percent. FCM and fat yield data were not normally distributed and were transformed to log base 10 before analysis. Simple Linear Regression with Groups was carried out with citrate concentration or citrate yield as the explanatory variable and log fat concentration, fat yield, protein concentration, protein yield and log FCM as the response variables. "Diet" was used as the grouping factor to investigate the relationship between variables in the different dietary groups.

5.3.3. Results

There was a significant effect of diet on citrate concentration and yield, fat concentration and yield, milk yield and FCM (Table 5.8). Mean data per cow is presented in Appendix 10.

Table 5.8. Effect of increasing Megalac supplementation on milk parameters. Data for fat yield and FCM are the back-transformed means from the log data

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P value
Citrate (mM)	7.65	7.20	7.56	8.80	8.23	0.37	0.003
Citrate (g/day)	58.4	57.8	57.5	70.3	62.0	3.17	0.003
Fat (g/kg)	32.73	37.299	43.80	38.07	36.70	2.13	0.001
Fat (g/day)	1291.22	1486.62	1694.73	1558.83	1415.14	0.021*	<0.001
Protein (g/kg)	28.86	29.59	29.79	29.24	29.64	0.436	ns
Protein (g/day)	1147	1239	1206	1214	1156	32.2	ns
Milk yield (l)	39.83	41.84	40.35	41.58	39.02	0.88	0.049
FCM (l)	35.33	39.37	41.72	40.08	36.85	0.015*	<0.001

* SEM = standard error of the mean. SEMs on the log scale for fat yield and fat corrected milk yield (FCM) were 0.021 and 0.015 respectively (minimum replication). ns = not significant

Further statistical analysis was carried out by General ANOVA by pooling the results from Diets one, two and three (contrast one) and comparing them with the mean of Diets four and five (contrast two) for both citrate concentration, yield and milk yield (Table 5.9).

Table 5.9. Comparison of contrast one with contrast two on citrate concentration, citrate yield and milk yield

	Contrast 1	Contrast 2	P value
Citrate (mM)	7.45	8.54	<0.001
Citrate (g/day)	56.50	66.40	<0.001
Milk Yield (Litres)	40.75	40.42	ns

*ns = not significant

The interactions between contrast and diet were not significant in citrate concentration and yield, indicating that there was no significant difference between the diets in each contrast. There was a significant difference between contrast one and contrast two for citrate concentration and yield. This suggests that there may be a certain threshold level of Megalac below which there is no significant effect on citrate or milk yield. The difference between the contrasts for milk yield was not significant, indicating that Megalac supplementation had no effect on milk yield.

Simple Linear Regression was carried out to investigate the relationship between citrate concentration and fat yield (P=0.238), log fat concentration (P=0.216), protein concentration (P=0.059), protein yield (P=0.205) and log FCM (P=0.212) and between citrate yield and log fat yield (P=0.766) and log FCM (P=0.171). None of these relationships were significant when data were analysed from all five diets. Therefore citrate was not significantly correlated with milk fat, protein or energy output in terms of FCM.

5.3.4. Discussion

There was little effect of Megalac on milk citrate concentration and yield until higher levels of supplementation (>0.6kg/cow/day) were reached (Table 5.8). The increase in citrate agrees with the literature as citrate tends to increase with high fat diets and vice versa (Ormrod et al, 1980), due to the cows deriving more precursors for milk fat synthesis from preformed FAs in the blood that originate from the diet, compared with *de novo* fatty acid synthesis.

However there is no literature on the effects of Megalac on milk citrate so it is difficult to compare the data from this study with others to know how much supplementary fat would result in a significant change in milk citrate. Citrate concentration was slightly lower with Diet five than Diet four although this difference was not significant. It is possible that a level is reached at which point extra supplementary fat has no effect on milk citrate.

The highest milk yields were found with Diets two and four and there was no linear trend of increasing milk yields across the five diets. Fat concentration and fat yield were greatest with Diet three which only supplied >0.6kg cow/day of Megalac, and cows on this diet also had the greatest energy output in terms of FCM. The lack of a relationship between citrate and fat yield and FCM is most likely due to the fact that citrate concentrations were highest with Diets four and five and fat yield and FCM were highest with Diet three (Table 5.8). These findings were surprising in view of the strong positive relationships previously found between citrate and fat concentration and FCM in early lactation cows (See Sections 5.2.3.1 and 5.2.3.3).

Megalac is normally fed in early lactation as it is thought to be of most benefit when cows are in negative energy balance. The typical daily feeding rate has been suggested as 0.75kg per cow per day (Ewing, 2002) so perhaps it is unlikely to expect Diets two and three, which contained 0.2kg and 0.4kg per cow per day respectively, to have a significant effect on citrate concentrations. However, the greatest yield of fat and FCM was found with Diet three. This finding indicates that citrate may not be as responsive to dietary changes as other milk parameters, such as fat yield and FCM, and that Megalac will begin to influence various parameters at different levels of supplementation.

There are conflicting results in the literature on the advantages of Megalac on dairy cow performance. Holter et al (1992) investigated the effect of supplementary cottonseed and cottonseed plus Megalac in Holstein cows and found that the multiparous cows supplemented with cottonseed and Megalac showed a 2.7kg/day reduction in milk yield and an increase in milk fat percent compared with the control group of cows that were not supplemented. The primiparous cows had higher milk yields and FCM when supplemented with Megalac compared with multiparous cows.

Other studies have also observed no significant increase in milk yield with Megalac supplementation. Lubis et al (1990) found no effect on milk yield and milk composition in 210 cows from feeding 0.54kg Megalac per day for 90 days. The cows in this study were in mid lactation and since Megalac is primarily an early lactation supplement to improve energy balance, it may have a more significant impact on milk production in early lactation.

Much of the research on milk citrate has compared citrate concentrations with the extent of *de novo* fatty acid synthesis, with little or no information on citrate and its relation to energy status in the dairy cow. Supplementary dietary soya oil (both free and protected) has been shown to increase the proportion of long chain FAs and decrease medium chain FAs including C16. The increase in citrate was more marked with the free oil rather than the protected oil (Banks et al, 1990). This might explain why Megalac, which is a protected fat, did not increase citrate significantly with the lower levels of supplementation. This could have been confirmed by looking at the fatty acid composition of the milk. However, milk fatty acid composition was not investigated in this study.

Potential sources of error may have arisen due to uneven replication between the dietary groups, with only data from four cows on Diet one, six cows on Diets two, three and four, and five cows on Diet five. Considering the variability associated between cows and within cows on a day-to-day basis in milk citrate concentrations, it is possible that more cows per dietary group would have been required to obtain significant results with the lower levels of Megalac supplementation.

5.3.5. Conclusions

Citrate was influenced by the fat content of the ration, with higher levels of Megalac in the diet increasing citrate concentrations in milk. However, a clear trend from diets one to five was not observed, with significantly higher citrate concentrations being evident above 0.6kg/cow/day of Megalac supplementation. There was no relationship between citrate and milk yield, fat content or FCM. If citrate is to be useful as a metabolic indicator for nutritional management more research is required on how it

varies with nutrition and its relationship to energy output in terms of milk yield and fat content.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. RESEARCH AIMS AND EXPERIMENTS

The aim of this research was to investigate sources of variation in milk composition that would be important to consider when interpreting data from an on-line monitoring system for individual cow management. Several experiments were carried out to investigate these sources of variation. Firstly, within milking variation in acetone, urea, progesterone, fat and protein were studied in six cows by collecting samples at various points during milking to determine the optimal sampling time during milking to obtain accurate measurements of milk constituents. The optimal sampling time was difficult to determine, especially for fat which was the most variable milk constituent.

Two novel sensing methods to measure acetone and urea in milk were developed and tested to determine their potential for integration into an on-line monitoring system. The Keto-sensor for milk acetone measurement was a quick and accurate method which could easily be adapted for on-line measurements. However, the Urea Pressure Sensor assay was not repeatable and had a long analysis time. Further modifications to the system would be required to improve its accuracy and potential for on-line urea monitoring.

A large experiment involving 30 cows was designed to investigate changing the ratio of starch to fibre in the diet and whether daily monitoring of milk composition could be used to detect dietary changes in individual cows. Only extreme dietary changes were detected at the group level and cow responses differed with the same dietary change. Sources of variation within (diurnal and day-to-day variation) and between cows were studied as well as the use of milk acetone to detect nutritional problems. There was significant diurnal and day-to-day variation in acetone, urea and fat. Protein was the most stable milk constituent and there was significant between cow variation in their normal concentration ranges of all milk constituents. Milk acetone was not reliable for detecting cows with nutritional problems or health incidences such as mastitis.

The potential for citrate to be used as an indicator of energy status was investigated by determining sources of variation, the relationship between citrate and milk composition parameters, and nutritional effects on citrate (supplementary dietary fat). Citrate varied significantly with lactation stage and on a day-to-day basis. No diurnal variation was observed. Megalac significantly increased citrate concentrations but only above a certain level of supplementation.

From these studies it appears that various factors influence the composition of milk, with individual cow responses to diet change being unpredictable, thereby making nutritional management difficult. The factors with the greatest influence on milk composition are likely to be nutrition, day-to-day, lactational variation and health. In the case of milk fat, and to a lesser extent milk protein, genetics also play a part. Environmental factors are difficult to quantify and whether they need to be considered in data interpretation is not known. Whether daily monitoring of milk composition can be used to optimise nutritional management on an individual cow basis has yet to be determined and further research is required in this area.

6.2. SOURCES OF VARIATION

6.2.1. Within Milking Variation

Sources of variation in milk composition can be classed into three categories; within cow variation, between cow variation and external sources of variation, such as nutritional and environmental factors. For on-line milk sampling and analysis, within milking variation is important, particularly for fat content, if fractional samples representative of the whole milking, are not used. The optimal sampling time during milking for fat is difficult to predict due to the extent of variation within milking. However, since analysis by NIR is instantaneous, it would be possible to use fractional samples from the whole milking, which would give a more accurate determination of fat content. For constituents that do not show significant variation throughout milking, or those whose variation is thought to be of little importance such as acetone, urea and protein (Table 6.1), then only one sample per cow, taken at

the optimal sampling time for milk fat, would be adequate for performing a variety of analyses.

Table 6.1 Changes in milk constituents during milking

Parameter	Premilking sample	Postmilking sample	SEM	P value
Acetone (mM)	0.146	0.110	0.09	<0.001
Urea (mM)	6.13	5.96	0.12	ns
Fat (g/kg)	20.52	111.07	0.29	<0.001
Protein (g/kg)	34.29	34.90	0.08	ns

*SEM = standard error of the mean, ns = not significant

6.2.2. Diurnal Variation

Significant diurnal variation exists in acetone, urea and fat, so more than one sample per day would be required for analysis. With robotic milking systems, there is also the problem of milking times varying between the cows. Interpretation of changes in milk constituents that show diurnal variation may not be as accurate compared with conventional milking systems as sampling times can vary from day-to-day. Therefore more than one sample would be required per day, increasing analytical costs and the amount of data for interpretation. However, the aim would be for data interpretation to be managed by computer, presenting the farmer with a range of options of appropriate action to be taken. Friggens and Rasmussen (2001) investigated the use of single milk samples in automatic milking systems and concluded that single daily milk samples produced acceptable estimates of true daily protein, lactose, urea and log SCC content in individual cows. However, estimates of daily fat content were not as accurate and a correction equation was required to improve the accuracy of daily fat determination.

The effect of feeding time in relation to milk sampling may be important. Whether acetone and citrate are significantly influenced by feeding time has yet to be

determined. It could be assumed that because the majority of farms use an *ad libitum* TMR feeding system, then variation throughout the day in response to feeding times is likely to be minimal, due to a continuous supply of protein and energy to the rumen.

6.2.3. Day-to-Day Variation

There was considerable day-to-day variation in all milk constituents which was greatest for acetone (Table 6.2). It could be hypothesised that measurements taken on consecutive days are correlated, in that a high milk fat value on one day would be followed by a similarly high value for milk fat the next day. However fluctuations from day-to-day in all milk constituents appeared to be random. This random variation could be due to a number of factors such as changes in feed intake, ration composition and quality, time of feeding in relation to sampling and length of milking intervals, all of which can vary on a day-to-day basis. The extent of variation from day-to-day varied between cows and this was reflected in their calculated ranges of milk constituents. Therefore nutritional management decisions should not be based on one day's milk composition data alone and data from several days would need to be averaged to determine levels of these constituents.

Table 6.2 Day-to-day variation in milk constituents

Parameter	Mean	Standard Deviation	CV%
Acetone (mM)	0.14	0.09	64.3
Urea (mM)	5.55	0.72	13.0
Citrate (mM)	10.29	1.69	16.4
Fat (g/kg)	37.28	2.48	6.7
Protein (g/kg)	30.51	0.69	2.3

*CV % = coefficient of variation (CV% = 100*standard deviation/mean)

6.2.4. Within Lactation Variation

Milk composition varies according to stage of lactation in conjunction with changes in milk yield and nutrition. With changes in milk composition over the period of a few days or even weeks, it may be difficult to ascertain whether these may be due to dietary changes or due to lactation stage and changes in milk yield. Also diet responses may differ according to lactation stage (Khorasani and Kennelly. 2001).

The use of acetone as an indicator of energy balance is mainly valuable in early lactation, which is the period of negative energy balance and high risk of hyperketonaemia. Little work has been carried out on acetone in mid and late lactation when cows are in positive energy balance and are unlikely to have raised ketone levels in body fluids. Therefore an on-line system for acetone may be appropriate only for cows in early lactation.

Variation in milk constituents due to lactation stage could be accounted for. However this would require building up a database of information on the cow's performance over a whole lactation to establish normal levels of milk constituents and how they vary throughout lactation. It would also have to be shown that individual cows show a consistent pattern of change throughout lactation. This type of management system would therefore take almost a year (i.e. a full lactation) to gather sufficient data on cows on which to base data interpretation and subsequent nutritional management decisions. Because the average herd life of a dairy cow is quite short (the average lactation age has not changed since 1981, at just over three lactations, ADC/NMR, 1998), this may not be a cost effective way in which to manage cows.

6.2.5. Between Cow Variation

Significant between cow variations were found in mean concentrations, normal concentration ranges and also in the extent of the range for acetone, urea, fat, protein and citrate. This finding highlights the need for individual cow monitoring instead of assessing dietary efficiency at the group or herd level which tends to be the current practice, particularly when assessing dietary efficiency based on milk urea concentrations. Due to significant between cow variation, group feeding diets are

very unlikely to be optimal for all cows in a group, even with similar levels of milk production.

Furthermore, it is likely that cows will respond to dietary changes in different ways depending on their homeostatic mechanisms and how tolerant they are to dietary changes. It is also possible that homeostasis may play a part in milk production with genetic potential of the cow determining a minimum target level of milk fat or protein content. The problem of individual variation can be accounted for by establishing mean levels or normal ranges of milk constituents for each cow. If a cow has a particularly wide concentration range of milk constituents then significant deviations from normal in response to dietary changes are less likely to occur than in cows which have a narrow range of milk constituent concentration.

6.3. MONITORING MILK COMPOSITION TO DETECT DIETARY CHANGES

Small dietary changes are not likely to be detected by monitoring daily changes in milk composition because responses in milk protein were observed only when diet change was extreme and milk fat content was significantly influenced by only one of the five treatment diets in Chapter 4, Experiment 1. The response to dietary change is also likely to be different in individual cows and cow responses may also differ according to stage of the lactation cycle. This was evident when investigating the effect of changing the ratio of starch to fibre in the diet and monitoring the response through daily changes in milk composition. It was observed that some cows responded to a diet change through an effect on fat or protein content, whereas other cows did not show a response to the same dietary change. Small dietary changes were not detectable at the group or individual cow level and it is likely that fluctuations in milk composition seen on a daily basis may have masked any treatment effect.

Milk urea has been extensively studied and much is known about the relationship between urea and the energy and protein content of the diet. However much of the work on urea has been carried out at the herd level or in specific groups of cows on the same diet to assess dietary efficiency. This means that on an individual cow basis

problems are not likely to be identified. On-line monitoring of urea may be useful to improve dietary protein and energy efficiency but at the individual level, more factors that affect urea concentrations in milk have to be taken into consideration to correctly interpret the data. Changes in milk urea for individual cows in response to changes in nutrition have not been reported and several authors have stated that milk urea values from individual cows should not be used to assess nutritional status or make dietary adjustments (Oltner et al, 1985; Ropstad et al, 1989; Kohn, 2000). Reasons for this include the large random variation between cows and not having a precise understanding of how to interpret urea measurements from individual cows.

6.4. ACETONE AND CITRATE AS INDICATORS OF METABOLIC AND HEALTH STATUS

6.4.1. Metabolic Status

If we know the sources of variation within and between cows, these may be quantified, making it possible to account for them when interpreting data from individual animals. However, there are some factors that may not be accounted for, such as seasonal influences or stress. Some cows in this study were considered to be under stress, either as a result of low feed intakes with the less palatable high starch or high fibre rations, or due to severe or repeated cases of mastitis. However, increases in acetone were not always observed. Some cows experienced high milk acetone concentrations and were considered to be good milk producers, with high feed intakes and no health problems, while others may have had mastitis or low feed intakes with acetone concentrations lower than the critical level. The use of acetone as an indicator of energy balance or a potential health problem appears to vary depending on the individual cow. Assumptions cannot be made on the basis that cows with mastitis or very low feed intakes are likely to be in negative energy balance and therefore have higher milk acetone concentrations than normal. Clearly, normal acetone levels vary considerably both within and between individuals and cannot be used as a reliable indicator of a potential health problem.

Acetone in conjunction with citrate may be useful to assess metabolic or energy status, being a by-product and an intermediate of the TCA cycle respectively. However the combination of these parameters for assessing energy status, and the correlation between them, was not investigated in this project. Although using two parameters may improve the accuracy of assessment, it would double the number of analyses required and increase costs. Such an increase in cost may not be offset by increased accuracy of data interpretation and the potential benefit derived from a nutritional change or improvement in production. Further research is required to investigate the economics of such a system.

In Chapter 5, citrate was found to be positively correlated with energy output in terms of FCM. However, citrate concentrations and acetone concentrations have not been examined in relation to energy balances of individual cows. Although the purpose of on-line metabolic profiling is to be able to monitor changes in milk parameters that indicate a cow's metabolic or energy status without having to calculate actual energy balance, research into how acetone and citrate vary with calculated energy balances would be useful to determine whether they are significantly correlated and therefore accurate predictors of energy status.

6.4.2. Mastitis

Milk samples from unhealthy mastitic cows were not included in the data analysis because mastitis is known to influence the concentration of milk constituents. Subclinical mastitis has been shown to affect milk urea and citrate concentrations. For example, citrate has been shown to decrease in subclinical mastitic milk, with the decrease being proportional to the extent of mastitis (Oshima and Fuse, 1981). Urea concentrations in milk are also significantly lowered in quarters with symptoms of mastitis, compared with healthy quarters from the same cow (Gutjahr et al, 1997).

One aim of on-line monitoring is to be able to detect potential health problems before they manifest themselves in the clinical state. Therefore, if subclinical mastitis can be detected through abnormal changes in milk composition, early treatment could be used to prevent clinical mastitis and losses in milk yield. Conductivity measurements are routinely used to detect mastitis via electrodes in the milking system, and this

method of detection would be cheaper than the cost of sensors to detect abnormal levels of milk constituents. However, detection of mastitis based on conductivity measures alone are not highly sensitive or specific. For example, Biggadike et al (2002) found that the specificity and sensitivity of conductivity measures of individual quarters to identify mastitis was only 87% and 50% respectively. An on-line system to detect mastitis by monitoring changes in milk composition would be justified, allowing better precision of diagnosis, as long as the milk samples were used primarily for other analysis to aid nutritional management.

6.5. FEASIBILITY OF ON-LINE METABOLIC PROFILING

6.5.1. Predicting Cow Responses

Little work has been carried out to assess how much a specific dietary change will affect milk composition. Tran and Johnson (1991) developed a model to partition food into milk and its main constituents, fat, protein and lactose. The accuracy of prediction of protein and lactose yields with the model was acceptable for farm use. However, the authors found that fat yields consistently peaked every four weeks throughout the study and suggested that any model would not be able to accurately predict milk fat with such fluctuations. The authors attempted to explain the variation in fat yield using dietary fibre. However, this had no effect on the accuracy of fat yield prediction. As protein is likely to be the most stable constituent in milk, it is likely that prediction of urea, acetone or citrate yields will be less accurate and more difficult to control and predict by dietary changes.

In a review of starch digestion in dairy cows, Mills, France and Dijkstra (1999) stated that digestive responses may vary significantly between individuals, even with similar diets and environmental conditions. They suggested that there were several animal factors involved, such as genotype, milk yield, reproductive status, parity, lactation stage, milk composition, body condition and many more which affected digestive responses. Some of these may be accounted for in models, but the effects of stress and environmental factors may be more difficult to take into account since individuals vary in their tolerance to environmental conditions. If these factors do not

significantly affect milk production outwith normal ranges then they can be ignored. However it is not possible to quantify the effect of these factors on milk composition as they will vary in their severity and their effect will depend on an individual's tolerance to changes in environmental conditions.

6.5.2. Technical Aspects

The development of an integrated system of automated milk sampling and analysis depends on advancements in producing robust, accurate and repeatable sensors for milk composition analysis. Many sensors have been tested in the laboratory but not on-line with automatic control. Sensors for fat and protein based on NIR spectroscopy have been tested in the laboratory and this technology could be adapted for on-line monitoring. On-line biosensors for urea and progesterone have also been tested. However no on-line sensors exist for monitoring milk acetone or citrate. There are several reasons for the slow uptake of biosensor technology from the laboratory to the field. Examples include being able to ensure the stability of the sensor's biorecognition elements (enzymes and antibodies), accuracy and reproducibility, and complete integration of the biosensor system (Velasco-Garcia and Mottram, 2003). Automatic sampling with the potential to store samples for analysis may also be required if the analysis time is greater than the time taken to milk one cow. Problems associated with the development of such a sampling system involve the determination of sampling time during milking, obtaining reproducible sample volumes, including an effective wash procedure, and ensuring that all surplus milk samples and reagents are discharged safely.

6.5.3. Economics

If this type of management system could be used to optimise cow performance, monitor health, detect nutritional management problems and to formulate least cost rations, the economics of such a system would have to be analysed in large scale studies to determine where the most profit could be derived from. Since payment schemes are based on milk quality, it is likely that the greatest returns could be made by optimising nutrition to improve the performance of low producing cows and to

increase milk fat and protein content, rather than by monitoring for health incidences, such as ketosis and mastitis.

There is also the issue of labour and how to implement such a system of fine-tuning rations according to milk composition on a regular basis. Labour costs may be increased. Any feeding system unique to individual cows would have to minimise extra labour for interpreting data and making dietary adjustments. The most cost effective means may be to adjust diets by way of in parlour feeding of concentrate with same basal diet or TMR for whole herd or different milking groups based on lactation stage. It is possible that extra expense may be involved if nutritional or veterinary consultation is required to interpret results and modify rations.

6.6. FUTURE WORK

Research into managing cows on an individual basis according to milk composition is in the very early stages and further large-scale trials are required to increase the understanding of individual cow responses to dietary changes and to be able to account for sources of variation in milk composition. There is a need to determine how specific dietary changes will affect milk constituents so that the correct dietary adjustment is made to optimise cow performance. There is a lack of knowledge on individual cow responses to changes in diet and the effects on milk composition. For example, it is not known how much a specific change in dietary energy will influence milk acetone or urea levels. The response to dietary change is likely to differ between cows due to individual variation and genetic potential, as well as dietary composition.

Significant deviations from normal baseline levels of milk constituents must be identified. However, it has not been established how much deviation is required before corrective action should be taken. Also it is difficult to predict what dietary change is required to return metabolite concentrations to normal. Again this is likely to vary according to individual cows. It must be decided whether a deviation is important if it is from the mean milk composition for that cow or from the upper or lower end of the normal range of the constituent being measured.

Further work needs to be carried out in monitoring responses in milk composition to dietary changes, in particular, milk urea, as this is likely to be where the most improvement in least cost ration formulation can be made without compromising cow performance. Also, nitrogen efficiency is likely to assume greater significance in future due to environmental regulations. More work on diurnal variation in response to feeding is required for acetone and citrate, particularly if this type of nutritional management system is used in herds with robotic milking.

There is a lack of knowledge on the use of citrate as an indicator of energy status in conjunction with milk acetone and actual measurements of energy balance. If citrate can be used in early lactation to indicate the extent of body fat mobilisation, being correlated with *de novo* fatty acid synthesis, it is possible that citrate may also be a useful indicator of body weight gain in late lactation. In late lactation, the use of citrate as an indicator of increasing body weight may be useful to prevent overconditioning at drying off and consequently at parturition. Measurements of citrate in conjunction with body condition score may help to assess energy balance. Also, the correct sampling time throughout milking for citrate needs to be determined as no studies have been carried out on within milking variation of citrate. However it is likely that being present in the aqueous phase, citrate will decline slightly but probably not significantly through milking due to the increase in fat content, which is the case for acetone and urea.

Few studies have looked at the effect of nutrition on milk citrate concentrations. If citrate was used as an indicator of energy status, nutritional effects would also need to be quantified so appropriate dietary adjustment could be made in response to deviations from normal citrate concentrations. Also it has to be established whether deviations from normal citrate concentrations are actually indicative of a nutritional problem or whether they are just a reflection of metabolic adjustments from day-to-day. This could perhaps be assessed if milk citrate concentrations were used in conjunction with acetone measurements to detect energy deficiency.

Advances in technology are also required, involving the development of automatic samplers which can store a number of samples for subsequent analysis. Sensors that are robust enough to work in the field and to analyse milk for various components

need to be developed and this system must be integrated with the herd management database so that milk composition data are easily accessible for the farmer or nutritionist. Finally, this kind of nutritional management system needs to be assessed to determine whether it would be economically justified to install such a system.

6.7. CONCLUSIONS

Within milking variation and optimal sampling time are important considerations for an on-line monitoring system, particularly for fat. Variation in constituents present in the aqueous phase of milk are likely to be minimal and can be ignored.

Significant diurnal, day-to-day and lactational variation was found in several milk components. Therefore decisions on nutrition and cow performance should not be based on one daily measurement and sampling should occur for a few days to obtain a more accurate assessment of metabolic and nutritional status. There was significant individual variation in acetone, urea, citrate, fat and protein concentrations in milk. Baseline levels of milk constituents must be determined on an individual basis so that deviations from normal can be detected.

Only extreme dietary changes were detectable by daily monitoring of milk composition. At the individual cow level small dietary changes that are common throughout lactation are not likely to be detected by daily monitoring of milk composition. Responses to dietary changes differ between cows and it is difficult to predict how much a change in a dietary component would be reflected in a known change in a particular milk parameter.

The use of acetone as an indicator of negative energy balance or nutritional stress is inconclusive. Preliminary studies suggest that citrate may be useful to assess energy output, being positively correlated with FCM and being responsive to changes in dietary fat (with levels of supplementation >0.6kg/cow/day), but further work is required in this area.

Because the effects of nutrition on milk composition at the individual cow level may not be detectable and are difficult to quantify, an on-line monitoring system for optimising nutritional management cannot yet be designed. A large amount of information would be required for accurate data interpretation, and optimising productivity and health by fine-tuning rations on an individual basis needs considerable further research.

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APPENDIX 1. CALIBRATION RESULTS OF THE KETO-SENSOR

Table 1a. Repeatability of the Keto-sensor with standards of acetone prepared in water

	Standard (mM)					
	0.0	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Repeat						
1	0.00	0.18	0.36	0.55	0.73	1.00
2	0.00	0.17	0.36	0.54	0.73	0.92
3	0.00	0.17	0.38	0.58	0.80	1.00
4	0.00	0.17	0.38	0.60	0.76	0.98
5	0.00	0.18	0.36	0.60	0.78	1.00
6	0.00	0.18	0.38	0.59	0.76	1.03
7	0.00	0.14	0.39	0.57	0.75	1.00
8	0.00	0.18	0.38	0.59	0.78	1.00
Mean	**0.00**	**0.17**	**0.37**	**0.58**	**0.76**	**0.99**
SEM	0.00000	0.00007	0.00005	0.00018	0.00022	0.00036

*SEM = standard error of the mean

Table 1b. Repeatability of the Keto-sensor with standards of acetone prepared in semi-skimmed milk

Standard (mM)	**1**	**2**	**Mean**	**SEM**
0.0	0.05	0.04	0.045	0.00004
0.2	0.23	0.24	0.235	0.00004
0.4	0.43	0.45	0.440	0.00014
0.6	0.62	0.64	0.630	0.00014
0.8	0.77	0.80	0.785	0.00032
1.0	1.00	1.00	1.000	0.00000

*SEM = standard error of the mean

Table 1c. Keto-sensor repeatability: acetone concentrations (mM) in duplicate samples from two cows in within milking variation experiment (Chapter 3)

	Cow 72			Cow 673		
Day/ Sample	*Duplicate 1*	*Duplicate 2*	***Difference***	*Duplicate 1*	*Duplicate 2*	***Difference***
1/1	-	-	-	0.12	0.12	**0.00**
1/2	0.11	0.13	**0.02**	0.10	0.10	**0.00**
1/3	0.13	0.13	**0.00**	0.10	0.10	**0.00**
1/4	0.14	0.12	**0.02**	0.11	0.10	**0.01**
1/5	0.11	0.12	**0.01**	0.11	0.12	**0.01**
1/6	0.10	0.10	**0.00**	0.10	0.09	**0.01**
1/7	0.05	0.05	**0.00**	0.09	0.09	**0.00**
2/1	0.08	0.07	**0.01**	0.15	0.12	**0.03**
2/2	0.09	0.06	**0.03**	0.11	0.11	**0.00**
2/3	0.10	0.17	**0.07**	0.11	0.13	**0.02**
2/4	0.15	0.13	**0.02**	0.10	0.13	**0.03**
2/5	0.14	0.12	**0.02**	0.14	0.12	**0.02**
2/6	0.08	0.09	**0.01**	0.12	0.12	**0.00**
2/7	0.11	0.11	**0.00**	0.05	0.06	**0.01**
3/1	0.16	0.15	**0.01**	0.16	0.14	**0.02**
3/2	0.11	0.11	**0.00**	0.11	0.12	**0.01**
3/3	0.11	0.13	**0.02**	0.12	0.12	**0.00**
3/4	0.12	0.11	**0.01**	0.13	0.13	**0.00**
3/5	0.11	0.12	**0.01**	0.12	0.13	**0.01**
3/6	0.08	0.09	**0.01**	0.12	0.12	**0.00**
3/7	0.08	0.09	**0.01**	0.10	0.11	**0.01**
4/1	0.17	0.16	**0.01**	0.14	0.15	**0.01**
4/2	0.13	0.13	**0.00**	0.11	0.11	**0.00**
4/3	0.12	0.14	**0.02**	0.11	0.12	**0.01**
4/4	0.14	0.13	**0.01**	0.09	0.10	**0.01**
4/5	0.13	0.15	**0.02**	0.14	0.14	**0.00**
4/6	0.09	0.10	**0.01**	0.12	0.12	**0.00**
4/7	0.07	0.07	**0.00**	0.11	0.11	**0.00**
Mean difference			**0.01**			**0.01**

APPENDIX 2. COMPONENTS OF THE MANUAL UREA PRESSURE SENSOR SYSTEM (*GHESQUIERE, 2000*)

Introduction

The samples and reagents were inserted in the system by a three-way stop cock, then the system was closed and the pressure recorded with a pressure transducer linked to a Multi meter (Fluke and Philips® Scopemeter PM97) that allow computer acquisitions. The enclosed cell was a sealed glass vial (Figure 3a).

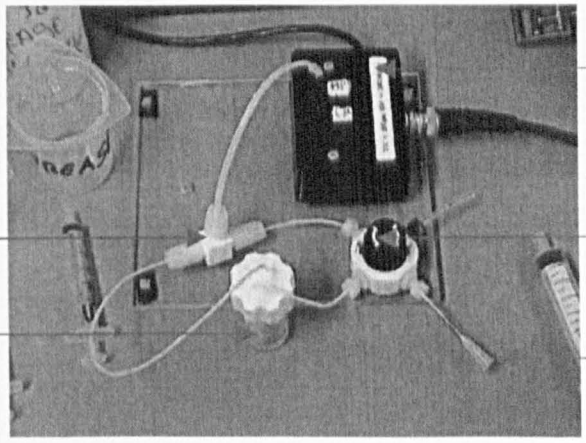

Figure 2a. Apparatus of the manual Urea Pressure Sensor System

Reagents

1. 250ml Citric acid 1 mol/l (48.025g of anhydrous citric acid)
2. 250ml of EDTA buffer (34mM potassium EDTA, ph7.6) + 0.25% Tween 20 (3.13g of K_2 EDTA salt)
3. 100ml of Urease EC 3.5.1.5 (near 200 units/ml) in EDTA buffer (34mM potassium EDTA, pH 7.6) (1.25g of K_2 EDTA salt)

Method

1. Inject 3 ml of the sample or standard.
2. Inject 0.5 ml of urease solution
3. Shake by hand for 5 min with the circuit close to avoid the leaks, an increase of the pressure may be observed due to the reaction.
4. Open the circuit.
5. Insert 1 ml of citric acid solution.
6. Quickly close the circuit.
7. Begin the recording.

8. Shake continuously.
9. Stop after 2 min 30 sec (150 sec)
10. Open the circuit.
11. Drain the solution.
12. Wash the vial with
- 2x 3ml of deionised or tap water
- 2 ml of 70% alcool
- 4 ml of deionised water
- 3 ml of wash solution (EDTA + 0.25% Tween 20)

For milk sample analysis all the stages were rerun, replacing the urease solution by the wash solution and skipping the five-minute incubation, to record the blank. The blank result was subtracted from the assay in order to determine the amount of urea in the sample. No blanks were run for the standards of urea because four different assays showed an insignificant response with difference less than the variation due to the error of measure of liquid volume.

APPENDIX 3. PROGRAMME TO RUN THE AUTOMATED UREA PRESSURE SENSOR

Table 3a. Commands for Urea Pressure Sensor Programme

Command	Command Format	Options	Example
Valve on	Von n	n = 1 to 6	Von 3
		or n = A to C	Von C
Valve off	Voff n	n = 1 to 6	Voff 2
		or n = A to C	Voff A
Stirrer on	StirON n	n = 0 - 100 (% speed)	StirOn 40
Stirrer off	StirOFF	None	Stir OFF
Pump1	Pump1 n	n = amount pump1 to pump in l l	Pump1 500
Delay	Delay n	n = time in seconds	Delay 60
Get data	Get n	n = time in seconds	Get 30

Von A
Voff B
Voff C
Von 2
Pump1 800
Voff 2
StirON 40
Von 6
Pump1 2500
Voff 6
Von C
Delay 240
Von 3
Pump1 1350
Voff 3
Von C
Get 150
Voff C
Voff A
Von B
Pump1 3000
Von A
Voff B
Von 6
Pump1 2500
Voff 6
Voff A
Von B

Pump1 3500
Von A
Voff B
Von 5
Pump1 2000
Voff 5
Voff A
Von B
Pump1 3500
Von A
Voff B
Von 5
Pump1 2000
Voff 5
Voff A
Von B
Pump1 3500
Von A
Voff B
Von 4
Pump1 2000
Voff 4
Voff A
Von B
Pump1 3500
Von A
Voff B
Von 5
Pump1 2000
Voff 5
Voff A
Von B
Pump1 3500
Von A
Voff B
Von 5
Pump1 2000
Voff 5
Voff A
Von B
Pump1 3500
Von A
Voff B
Von 6
Pump1 2500
Voff 6
Voff A
Von B
Pump1 3500
StirOFF

APPENDIX 4. WITHIN MILKING VARIATION IN ACETONE, UREA, PROGESTERONE, FAT AND PROTEIN

Table 4a. Within milking variation in acetone concentration (mM)

	Sample throughout milking						
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	0.145	0.103	0.117	0.124	0.119	0.111	0.064
2	0.124	0.090	0.100	0.100	0.083	0.102	0.068
3	0.157	0.137	0.147	0.136	0.123	0.132	0.068
4	0.170	0.128	0.143	0.144	0.110	0.095	0.088
5	0.132	0.109	0.129	0.130	0.125	0.091	0.099
6	0.138	0.109	0.114	0.111	0.128	0.114	0.090

Table 4b. Within milking variation in urea concentration (mM); (defatted samples)

	Sample throughout milking						
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	6.42	6.08	5.78	5.85	5.91	5.68	6.02
2	7.04	6.90	6.94	6.63	6.71	6.82	6.18
3	5.51	5.36	5.34	5.4	5.57	5.50	5.85
4	4.19	4.26	4.18	4.03	3.88	3.85	3.85
5	7.11	7.00	7.04	7.20	7.22	7.14	6.60
6	7.14	7.24	7.35	7.02	6.86	6.93	6.85

Table 4c. Within milking variation in urea concentration (mM); (non-defatted samples)

	Sample throughout milking						
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	5.46	5.34	5.22	5.18	5.08	4.76	5.13
2	6.18	6.13	6.15	5.90	5.73	5.37	5.55
3	5.18	5.04	4.96	4.79	4.63	4.33	4.75
4	3.88	3.91	3.85	3.68	3.25	3.18	3.30
5	6.25	6.14	6.16	6.03	6.00	5.50	5.75
6	6.29	6.25	6.04	6.03	5.93	5.38	5.91

Table 4d. Within milking variation in progesterone concentration (ng/ml)

			Sample	throughout	milking		
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	0.650	0.422	0.321	0.481	0.528	0.638	0.968
2	0.736	0.691	0.750	0.594	0.733	0.798	1.950
3	0.562	0.320	0.468	0.505	0.603	0.623	1.083
4	0.799	0.540	0.544	0.538	0.678	0.491	1.143
5	2.872	4.822	5.561	6.011	6.159	4.748	5.773
6	0.975	0.967	1.131	0.961	1.042	1.188	1.648

Table 4e. Within milking variation in fat concentration (g/kg)

			Sample	throughout	milking		
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	30.06	47.13	58.30	79.12	82.70	115.85	71.81
2	27.11	40.88	46.95	31.40	73.14	124.12	53.98
3	9.56	25.36	43.34	72.05	99.47	130.32	51.33
4	18.71	27.79	32.40	41.39	79.06	89.29	34.49
5	17.37	30.19	39.57	48.19	52.49	91.77	40.82
6	23.32	35.62	45.59	52.47	60.21	113.88	45.03

Table 4f. Within milking variation in protein concentration (g/kg)

			Sample	throughout	milking		
Cow	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	35.89	34.32	35.59	36.14	32.39	37.78	32.97
2	35.76	36.33	34.25	36.42	36.60	38.12	34.12
3	32.74	33.39	35.92	34.62	36.03	33.07	33.33
4	34.46	34.18	36.08	34.26	35.77	35.17	34.45
5	33.36	34.34	32.27	35.23	33.68	33.34	31.94
6	34.64	33.30	32.34	32.18	33.31	33.02	32.77

APPENDIX 5. WITHIN MILKING VARIATION IN PROGESTERONE (DETERMINED BY ELISA AT HIGH AND LOW CONCENTRATIONS), FAT AND PROTEIN

Thanks to Dr Maria Velasco-Garcia and Dr Toby Mottram of Silsoe Research Institute for supplying this data.

Aim

To investigate within milking variation in progesterone and how it changes in relation to milk fat during milking.

Method

This experiment was carried out at the Royal Veterinary College, Potters Bar, Hatfield, U.K. Three cows were selected for study, one in early lactation, one cycling and one pregnant cow. A T-piece sampler was inserted into the long milk line so that milk samples could be collected at various times into milking. Samples were analysed for fat and protein content at ADAS Laboratories (Woodthorne, Wolverhampton, U.K.) and progesterone concentrations were determined by ELISA (Ridgeway Science).

Results

Table 5a. Within milking variation in progesterone, fat and protein in cow 59 (pregnant)

Milking Time (s)	Progesterone (ng/ml)	Fat (%)	Protein (%)
60	32	1.79	3.35
120	33	2.54	3.43
195	50	3.16	3.37
270	38	4.14	3.28
360	53	4.14	3.27

Table 5b. Within milking variation in progesterone, fat and protein in cow 655 (early lactation)

Milking Time (s)	Progesterone (ng/ml)	Fat (%)	Protein (%)
30	0.34	1.54	3.14
90	0.21	3.42	3.06
180	0.23	3.89	3.05
240	0.24	4.28	3.04
320	0.32	6.93	2.91

Table 5c. Within milking variation in progesterone, fat and protein in cow 46 (mid-cycle)

Milking Time (s)	Progesterone (ng/ml)	Fat (%)	Protein (%)
30	4.1	1.10	3.41
120	9.2	2.12	3.41
180	12.9	3.21	3.36
240	12.1	4.21	3.27
420	8.5	6.78	3.08

APPENDIX 6. COMPOSITION OF BASAL DIET

Table 6a. Diet composition of the high maize silage TMR on a fresh weight basis fed in Experiments 4.1, 4.3, 5.1. and 5.3. FME = fermentable metabolisable energy and CP = crude protein

Ingredient	Amount (kg/cow/day)	FME	CP
Maize Silage	23	10.1	9.0
Grass Silage	13	8.0	12.5
Brewers Grains	8	9.0	25.0
Omega Energy	0.5	7.0	6.6
Palm Kernal	2	10.0	18.0
Hi-Pro Soya	4.5	12.7	55
Wheat	3.94	12.8	13.0
Hi-Phos Minerals	0.3	-	-

APPENDIX 7. FEED INTAKE DATA

Table 7a. Mean feed intake per cow in kg/DM from days 38 to 69

Cow	Diet	Mean Feed Intake (kg/DM)	SEM
118*	1	16.20	0.74
166	1	20.54	0.87
196	1	26.70	0.79
456*	1	16.19	0.87
473*	1	18.32	0.80
45	2	22.67	0.61
58	2	30.39	1.35
103	2	30.29	0.68
135	2	23.05	0.66
184	2	24.90	0.61
483	2	29.78	0.65
89	3	30.64	0.91
145	3	23.68	0.58
146	3	29.13	0.66
177	3	29.00	0.72
194	3	24.01	0.81
462	3	28.49	1.78
476	3	27.78	0.86
7	4	25.73	0.98
73	4	31.71	0.66
76	4	23.46	0.76
150*	4	14.47	1.06
192*	4	10.83	0.65
197*	4	14.54	0.70
413	4	20.40	0.77
107	5	27.57	0.98
112*	5	13.80	1.06
156	5	25.42	0.86
172	5	29.57	0.92
474	5	25.20	1.22

*cows identified as stealing from other feed bins, hence their low recorded feed intakes. SEM = standard error of the mean

Table 7b. Mean feed intakes per diet over the trial period in kg/DM

Day	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
38	31.77	25.99	25.25	20.76	28.46
39	28.25	26.24	25.39	23.78	25.53
40	28.10	28.04	25.17	24.18	27.66
41	26.63	24.19	23.91	26.03	24.49
42	26.55	21.85	26.27	28.37	28.17
43	24.11	23.56	26.36	28.77	27.10
44	29.34	28.19	26.54	25.34	27.95
45	24.92	28.46	25.96	24.38	31.23
46	23.25	26.57	24.82	30.38	30.67
47	23.24	25.31	25.66	20.96	30.18
48	25.22	28.90	26.31	24.57	26.45
49	24.20	26.18	27.68	23.34	29.28
50	24.77	27.20	26.92	23.81	28.47
51	27.47	29.31	27.60	27.44	27.10
52	24.81	27.30	30.33	22.05	24.80
53	20.78	28.44	30.45	25.79	28.40
54	27.05	27.20	31.15	24.36	28.92
55	24.05	23.93	30.36	21.66	27.69
56	25.11	24.80	31.04	18.83	30.81
57	25.25	28.66	30.38	23.16	25.62
58	25.85	29.78	28.49	21.14	26.36
59	27.68	30.41	30.44	19.91	26.71
60	24.14	28.19	29.64	22.19	28.06
61	28.04	23.66	26.06	23.28	20.06
62	27.86	26.36	27.71	21.96	25.38
63	27.50	26.27	27.50	24.47	26.65
64	25.17	27.74	29.96	22.85	23.81
65	29.40	26.73	27.68	20.73	26.19
66	28.32	26.66	26.40	18.00	23.76
67	25.67	28.16	26.48	19.58	25.40
68	30.02	28.11	27.97	20.67	22.24
69	29.73	28.55	27.78	22.19	19.89
Mean	**26.38**	**26.90**	**27.61**	**23.28**	**26.67**
SEM	0.42	0.35	0.36	0.51	0.49

*SEM = standard error of the mean

APPENDIX 8. METHODOLOGY FOR MILK FATTY ACID ANALYSIS

Milk Sample Preparation

Milk samples were centrifuged at 17,800 x g for 30 minutes at 8°C and 300 to 400 mg of fat cake was removed for extraction and methylation. Lipid extraction of milk fat was performed according to Hara and Radin (1978) as described by Chouinard et al (1999) using hexane:isopropanol. Milk fatty acids were trans-esterified with sodium methoxide according to the method of Christie (1982) with modifications. Hexane was added to 40mg of butter oil followed by 40µl of methyl acetate. The mixture was vortexed and 40µl methylation reagent (1.75ml methanol:0.4ml of 5.4 M sodium methylate) was added. The mixture was vortexed and allowed to react for ten minutes, then 60µl of termination reagent (1g oxalic acid/ 30ml diethyl ether) were added. Several grains of anhydrous $CaCl_2$ were added and the solution incubated at room temperature for one hour. The sample was then centrifuged for five minutes at 2400 x *g* at 4°C leaving a clear layer of hexane that contained the fatty acid methyl esters; an aliquot of the hexane was removed and used directly for chromatographic determination.

Conditions of the GC

Fatty acid methyl esters were analysed by gas chromotography (Hewlett Packard GC system 6890+ with a flame ionization detector) equipped with a CP-SIL 88 fused silica capillary column (100m x 0.25mm (i.d.) with 0.2-µm film thickness; Varian, Inc. Walnut Creek, CA). The analysis involved a programmed run with temperature ramps. The oven temperature was initially 70°C for two minutes then ramped to 110°C at 8°C/minute and held for four minutes. The temperature was then ramped again at 5°C/minute to 170°C and held for ten minutes. Finally it was ramped at 4°C/min to 225°C and held for 15 minutes. Injector and detector temperatures were maintained at 250°C. The flow rate for hydrogen carrier gas was 2.1 ml/minute. Each peak was identified and quantified using pure methyl-ester standards (NuChek Prep, Elysian, MN). A butter reference standard (CRM 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was analysed at regular intervals for quality control purposes and was also used to determine recoveries and correction factors for individual fatty acids.

APPENDIX 9. STAGE OF LACTATION TRIAL: MEAN DATA PER COW

Table 9a. Mean milk composition and yield per cow according to lactation stage (* = missing values)

Cow	Stage	Citrate (mM)	Citrate (g)	MY (Litres)	Fat (g/kg)	Protein (g/kg)	FCM (Litres/day)	SCC (x1000)	<C16 (mmol/day)	C16 (mmol/day)	>C16 (mmol/day)
76	Early	12.32	1673.2	34.70	53.37	35.88	40.82	10.82	2469	2200	2178
107	Early	10.63	1293.0	35.26	49.32	36.09	39.29	99.34	2028	1874	2078
188	Early	10.62	1239.7	34.66	59.84	36.69	44.01	57.10	2285	2241	2990
214	Early	11.24	1091.7	30.66	55.33	35.05	36.97	15.94	2037	1742	2469
221	Early	11.80	1964.0	39.11	47.01	31.54	43.19	19.24	*	*	*
227	Early	13.48	2324.0	36.96	65.81	35.71	50.85	21.42	2891	2304	3784
233	Early	8.49	821.0	35.14	37.29	30.87	33.17	9.12	*	*	*
239	Early	7.90	580.1	32.66	35.37	36.43	29.80	40.15	*	*	*
51	Mid	7.93	745.7	36.30	44.74	31.59	39.30	357.7	2211	1875	2042
83	Mid	10.10	1460.9	39.70	31.32	30.98	34.27	112.10	1642	1455	1492
116	Mid	10.56	1065.6	32.70	43.94	32.48	34.49	112.30	1837	1714	1767
148	Mid	8.96	789.7	32.91	41.86	30.50	34.08	73.33	1968	1741	1513
200	Mid	9.22	1014.0	35.50	37.87	33.13	33.15	62.52	1833	1555	1631
202	Mid	11.21	1125.2	31.38	46.89	33.97	35.92	50.93	2118	1971	1759
204	Mid	10.16	1037.2	33.15	38.82	33.08	32.62	1273.00	1788	1578	1474
450	Mid	10.05	1046.3	33.74	37.20	32.00	32.12	379.90	1770	1416	1556
122	Late	9.60	495.4	24.40	34.81	34.49	22.31	104.50	1089	970	1074
143	Late	9.47	228.5	16.70	42.90	33.83	17.09	188.50	912.5	803	880
158	Late	11.27	664.8	24.28	37.36	32.06	23.19	204.00	1272	1077	1052
169	Late	11.85	901.9	26.74	37.81	34.49	26.43	73.14	1539	1079	1310
170	Late	9.08	521.3	26.50	47.31	37.01	29.33	86.86	1536	1465	1452
178	Late	11.12	449.8	20.72	44.02	36.17	21.93	116.10	1224	1025	1151
197	Late	10.00	138.8	12.94	37.55	36.20	12.80	173.10	599	518	698
209	Late	9.72	311.3	19.12	49.20	37.06	21.62	275.40	1274	1086	1151

APPENDIX 10. MEGALAC TRIAL: MEAN DATA PER COW

Table 10a. Mean milk composition and yield per cow in Megalac Trial

Cow	Diet	Citrate (mM)	Citrate (g)	Fat (g/kg)	Protein (g/kg)	MY (Litres)	FCM (Litres/day)
4	1	7.76	63.44	28.23	26.82	42.60	35.09
67	1	6.32	47.44	32.98	30.01	39.06	34.91
146	1	7.52	59.58	34.04	29.68	41.24	37.56
211	1	9.00	63.02	35.68	28.93	36.43	33.94
139	2	6.52	56.01	32.29	29.12	44.72	39.52
140	2	5.16	40.55	55.40	33.45	41.06	50.43
145	2	7.35	52.65	37.77	33.70	37.75	36.04
161	2	7.86	62.95	31.00	29.15	41.39	35.91
200	2	7.94	66.04	35.12	29.39	43.56	40.35
205	2	8.67	71.42	32.12	28.35	42.94	37.98
32	3	7.57	69.91	52.48	33.71	48.07	57.07
84	3	8.47	58.97	38.02	27.64	36.28	35.18
138	3	5.69	42.01	45.44	30.93	38.40	41.54
148	3	5.90	49.25	34.00	27.20	44.00	40.09
166	3	8.97	78.09	39.04	29.59	45.32	44.67
218	3	8.03	56.97	40.98	29.95	36.86	37.39
239	3	8.00	51.38	56.67	29.71	33.50	41.78
25	4	10.51	88.45	36.98	28.12	43.88	42.04
46	4	6.16	54.37	41.22	28.14	45.78	46.66
105	4	7.52	56.08	38.53	30.20	38.85	37.82
211	4	9.41	70.54	33.48	30.04	38.98	35.16
450	4	12.07	100.76	39.87	28.71	43.32	43.32
460	4	7.83	58.09	38.34	30.21	38.68	37.82
57	5	9.95	72.78	34.41	29.11	38.08	33.18
93	5	6.73	49.73	36.55	29.27	37.82	35.86
107	5	8.53	68.38	40.42	29.56	41.72	41.98
233	5	7.46	50.89	38.83	29.70	35.52	34.82
498	5	7.91	63.78	36.30	30.54	41.94	39.59