

Review: Automatic on-line analysis of milk constituents (urea, ketones, enzymes and hormones) using biosensors

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Traditional methods of monitoring health changes in animals are based entirely on the human senses. However, in modern dairy production systems humans are rarely present, this is particularly the case with the introduction of robotic milking. In these systems all the functions of milking are automated and cows visit at times of their own choosing. Systems of automatic health monitoring are therefore a priority for research to ensure that the health and reproductive status of the animals can be assessed for management purposes. These systems must be automatic, work in field conditions without technical support and cost a few pence per analysis. The first task is to obtain representative biological samples automatically and non-invasively. As milk is flowing into the milking machine from the cow this can be achieved with ease, except that milk is non-homogeneous with a changing lipid fraction during milking. Lipid soluble components such as progesterone and vitamin A are affected by this change and a model has to be established to determine thresholds at different times during milking. Our main interests in dairy cows are in predicting ovulation, detecting metabolic imbalance and detecting pre-clinical mastitis inflammatory response. Our team is developing a fully automated ovulation prediction system based on the screen printed carbon electrode biosensor for progesterone demonstrated by Pemberton et al. (1998). In recent experiments the automated system was able to detect concentrations of progesterone between 2 and 30 ng/ml in stored milk samples ( $R^2=0.96$ ). The results of field tests are presented showing a good correlation between ELISA and the biosensor ( $R^2=0.91$ ) on samples of fresh milk. The results of the recent field tests show the ability of the biosensor to characterise ovulation cycles of cows and to detect pregnancy. We have identified a major lack of other biological models to detect disease with on-line sensors. Our next objective is to create an integrated system for biological research with sensor systems for urea, ketones, lipids, and enzymes in milk. This will allow the development of diagnostic models based on analysing numerical sensor derived data rather than human visual observations for signs of ill health in dairy cows.

Keywords: biosensor, progesterone, milk, urea, ketones, enzyme

## Introduction

The health and fertility of the dairy cow has until recent years been monitored entirely by visual observation. However, as the availability of skilled labour is reduced automatic systems are needed. The range of conditions feasible for non-invasive monitoring of the lactating dairy cow are described and the sensors that are likely to be effective and the limited experimental data for each system are described.

## The conditions to monitor

### *Fertility*

Esslemont et al. (1985) in numerous publications has shown that significant losses in profit are due to inadequate detection of oestrus. The traditional method of detecting oestrus is by observation for changes in behavioural characteristics. Thus with the reduction in contact with the cows in conjunction with a genetic decline in the fertility of modern cows, losses due to infertility have been rising.

Mottram and Frost (1997) concluded that the needs of the dairy industry was for an automatic sensing system for progesterone that could achieve equivalent detection levels to that achieved by ELISA tests (98% specificity) and that monitoring for progesterone concentrations in the range 3-30 ng/ml of milk would also allow the automatic detection of ovarian malfunction and pregnancy. Milk samples should be analysed as soon as possible during milking so that the on-line analyser can complete the analysis before the milking point is needed for the next cow. However, Pope and Swinburne (1980) showed that as the progesterone is lipid soluble and that lipid concentration changes during milking we can expect that the concentration of progesterone will increase during milking. Due to inaccuracies inherent in the ELISA at low and high concentrations and biological variation, decisions tend to be made on the basis of the concentration of progesterone being above or below a threshold of 10 ng/ml. However, Delwiche et al. (1998) sampling foremilk reported maximum progesterone concentrations below 10 ng/ml. There are thus some biological variables that need further research now that sensors are becoming available.

## Diseases of dairy cattle to monitor routinely

The potential for non-invasive monitoring of the health of dairy cows was reviewed by Mottram (1997). Monitoring systems should only be deployed when a management response to improve health or profit was available, information gathering was inappropriate unless action can be taken with the information.

The most important targets for monitoring disease automatically through the milking system were metabolic conditions and mastitis. Metabolic conditions such as ketosis, can be regarded as a subset of nutritional management.

### *Nutritional status*

The largest single variable cost of an average dairy cow is the feed she consumes either as concentrate (£194 pa) or as grass (£80 pa) (Nix, 2000). The efficient use of the food is of course vital for the health, production and reproductive status of the dairy cow. Nutritional monitoring has largely been conducted by observation of milk yield, body condition score, periodic tests of blood analytes (metabolic profiles) and external nutritional advice.

There are several constituents in milk that may provide useful information on cow health and nutritional status. Milk fat and protein is already routinely monitored at monthly intervals on commercial dairy farms to assess compositional quality for genetic selection but they are also useful metabolic parameters. The potential for improving nutritional management with daily on-line analysis of milk constituents needs the development of new biological models. Existing models are not designed to provide the information needed for routine management. The most useful parameters in milk composition are likely to be acetone, urea, fat and protein as these provide the best indicators of metabolic and nutritional status and are also easily monitored. Other possible analytes in milk that could potentially be used to monitor the dairy cow are  $\beta$ -hydroxybutyrate, citrate, fatty acid composition, casein and purine derivatives.

Acetonaemia (the accumulation of ketone bodies in blood, milk and urine) is characteristic of the metabolic disorder ketosis. Although low concentrations of ketones can be excreted or used in energy metabolism, high concentrations are toxic. Milk acetone is therefore a useful indicator of energy balance, being the most abundant ketone body present in milk and highly correlated to blood ketone levels, compared to  $\beta$ -hydroxybutyrate and acetoacetate (Andersson and Lundstrom, 1984). Milk acetone ranges from 0 to 2mM and is frequently classed into 4 groups, <0.4, 0.41-1.0, 1.01-2.0 and >2.0mM, with the incidence of ketosis increasing with increasing acetone class (Andersson et al., 1991). A range of 0.7 to 1.4mM has been suggested as a suitable warning class where milk yield may be reduced in early lactation. Above this range significant reductions in milk yield and reproductive performance are evident with longer intervals from calving to first service (Gustafsson and Emanuelson, 1996). A high level of acetone in milk indicates that cows are in a negative energy balance and that there is possibly an energy deficit in the diet.

Milk urea is a useful indicator of nutritional status, being highly correlated to blood urea ( $r=0.98$ , Oltner and Wiktorsson, 1983). Monitoring milk urea provides a rapid, non-invasive and inexpensive method of determining protein metabolism and energy supply in dairy cows with the strongest determinant of milk urea being the ratio of protein to energy in the whole diet (Oltner et al., 1985). 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. In contrast, milk urea should decrease if there is a protein deficiency, if the diet is high in energy (Pehrson, 1996) or the ratio of degradable and rumen undegradable protein (RUP) is unbalanced (Jonker et al., 1999). The normal range of urea in milk is 3.0 to 6.5mM (Gustafsson, 1993). Many factors influence milk urea levels including individual variation, season, body weight and stage of lactation, due to changing nutrient requirements throughout lactation. However, the most important factor affecting urea is the time of milk sampling in relation to time of feeding since urea levels tend to increase significantly 2-4 hours after a feed (Gustafsson, 1993). Milk sampling for urea should therefore take place at a specific time after feeding each day. High urea indicates dietary inefficiency and several studies also suggest that high urea is detrimental to fertility. In practical terms 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 FME (fermentable metabolisable energy) 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. Increasing dietary FME is the best course of action so that spare protein is utilised (Whitaker, 2000). If dietary RDP is reduced a drop in milk yield is likely.

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 metabolic status than if used separately to monitor the cow. For example, an energy deficit in early lactation increases mobilisation of body fat and increases free fatty acids in the blood, with more milk 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., 1996). A fat to protein ratio of less than 1.4 is optimal or indicates a positive energy balance. Above this value the cow is in a negative energy balance and over 1.5 there is a risk of developing ketosis due to adipose tissue catabolism and poorer reproductive performance (Heuer et al., 1999). The fat to protein ratio has been suggested as a more sensitive indicator of energy balance than ketone bodies (Heuer et al., 2000) as ketones rarely rise to high concentrations. Mild cases of subclinical ketosis tend to be self-limiting as milk yield only falls to a level at which point nutrient supply and requirement begin to balance out (Webster, 1993). Care must be taken when interpreting results for fat to protein ratio because both of these parameters show considerable variation with stage of lactation and milk yield.

### **Mastitis**

Mastitis or the inflammatory response of the mammary gland is a major source of loss in dairy cows. The detection of clinical mastitis is currently made by a combination of human inspection of animals, by electrodes in the milking system to detect changes in the conductivity of milk (electrical conductivity), and by analysis of data in herd management software to detect changes in milk yield and other factors (Hogeveen & Meijering, 2

to suggest that such systems that attempt to dispense with all human inspection will detect all infectious diseases that are implied by the EU directives 92/46, (Anon, 1992) particularly mastitis, while there is evidence to suggest that it is not possible to detect all types and degrees of mastitis by changes in conductivity even in combination with other data (DeMol et al., 1997). De Mol et al. (2000) reported results of single and combined measures of 29,033 milkings to detect clinical mastitis and concluded that early warning is not reliable with conductivity sensors and software currently on the market.

Enzymes and ions are released as a result of the animal's immune response against infection and changes in cellular membrane chemistry. For example, Kitchen (1981) found N-acetyl  $\beta$ -D-glucosaminidase (NAGase) in concentrations of between 0.021 (normal) and 0.12 (mastitic) units per ml, while Urech et al. (1999) recorded 4-6 U/ml (normal) and ~10U/ml (mastitic), respectively. Mottram et al., (2000) reported that a screen-printed carbon electrode sensor could detect concentrations of NAGase in buffer solution at appropriate concentrations. Similarly Eckersall et al. (in press) reported a retrospective analysis of 29 milk samples from cows with clinical mastitis that indicated that both serum amyloid A and haptoglobin could be used to detect mastitis at concentrations of <.05 to 0.55  $\mu$ g/ml and 0.02 to 2 mg/ml respectively.

The bulk conductivity of milk can be assumed to be the sum of the changes in conductivity induced by changes in ion and enzyme concentrations in milk (Bramley, 1992). The changing electro-chemistry of milk is largely due to different inflammatory responses of the mammary gland but may have other causes.

### **Infectious diseases**

A rapid and non-invasive analytical technique to detect and quantify specific antigens in the body fluids of farm animals would be of great value in the maintenance and improvement of the health and fertility of animals. For example, the cost of the failure to control bovine viral diarrhoea and associated fatal mucosal disease was estimated at £47 million/year to the cattle industry in 1986 (Bennett and Done, 1986). A field test to detect bovine viral diarrhoea virus (BVDV) in saliva or milk would allow the identification of asymptomatic infected cows within a herd and the efficacy of any vaccination regime to be closely monitored and assessed.

### **Sensing Systems**

Sensing systems in the dairy parlour must have a minimal risk of contaminating the milk with reagents or antibodies, thus analysis should be made on samples drawn off line and discharged to waste. The sensor elements should be easy to clean and thus systems that have no contact with the milk or are disposed of after each use seem appropriate.

#### *Progesterone*

Koelsch et al. (1994) used a quartz crystal microbalance, and Claycomb et al. (1995) presented an on-line colour change assay to measure progesterone concentrations in milk but neither of these techniques were suitable for industrial development. The most successful biosensors in terms of sales are those used by diabetics to measure blood glucose. They are based on screen printed carbon electrodes. Pemberton et al. (1998) reported a disposable screen-printed amperometric progesterone biosensor, operated in a competitive immunoassay. The biosensor comprised mAb immobilised on the working area of a screen printed carbon electrode (SPCE). It relied upon a reduction in the binding of alkaline phosphatase-labelled progesterone in the presence of endogenous milk progesterone. The enzyme substrate was naphthyl phosphate. The 1-naphthol generated in the enzymatic reaction was electrochemically oxidised, producing a signal inversely proportional to the concentration of unlabelled progesterone in milk

We have built a system that adopts the technique described by Pemberton et al. (1998) replacing hand processes with electrically operated pumps and valves controlled by software so that we can autom:

in the milking system. The biosensor system can detect concentrations of progesterone in whole fresh milk of between 3 and 20 ng ml<sup>-1</sup> (normal physiological levels), with a good regression ( $R^2 = 0.95$ ). Figure 1 illustrates the calibration graph for progesterone standards in cow's milk (Velasco-Garcia, 2002).

The on-line ovulation prediction system was tested in the field, by taking twice-weekly milk samples from 8 dairy cows for a 6-week period. The biosensor prototype successfully tracked the profile of the ovulation cycles and detected pregnancy in dairy cows. All the data obtained from the biosensor was compared with that obtained using a commercial Enzyme Immunoassay, showing a very good correlation ( $R^2 > 0.9$ ) for the different batch of biosensors fabricated (figure 2) (Velasco-Garcia, 2002).

### *Ketones*

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. A sensor for acetone has recently been developed by Dart Sensors, Totnes, UK ([www.dart-sensors.com](http://www.dart-sensors.com)), to detect acetone in milk and other body fluids such as blood and saliva. The electrochemical device is an adaptation of the widely used Alcosensor for breath alcohol measurement (Intoximeters Inc.). A 1ml sample of headspace is drawn into a sample chamber and the concentration of acetone acts as an electrolyte causing a current to flow. The integrated signal over a number of seconds gives the concentration of acetone. Detection of acetone occurs within the range of 0.04 - 4mM.

This system has been tested to measure ketones in milk. Within milking variation of acetone was investigated by sampling cows in early lactation (weeks 2-3) at various times throughout milking. This was important to determine the optimal sampling time for an on-line monitoring system. A premilking and postmilking sample and 1 sample every 4 litres (depending on total yield) was collected, along with a reference sample from the milk meter (sample 7). This sampling procedure was repeated for 6 cows on 4 consecutive days. Acetone measurements in recently calved cows were very low, ranging from 0.05mM to 0.19mM (figure 3), but with good repeatability between duplicate samples.

The Ketosensor is suited to on-line measurements as repeat tests can be performed every 2 to 3 minutes intervals depending on the acetone concentration of the previous measurement. This allows sampling and analysis to be carried out before the milking system is needed for the next cow. The sensor is calibrated with a 1.05mM standard to read 1.00. The 5% difference accounts for the solids content in milk, which occupy space not available to acetone. Repeatability between samples is between 0.97 and 1.03 but mainly 0.99 to 1.01

### *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 the development of a sensor based on combining a surface acoustic wave device with urease (Lui et al., 1996). These sensors all work with the enzyme urease, which hydrolyses urea. However, the majority of these sensors are not designed to handle complex mediums such as raw milk. An on-line biosensor to automatically measure milk urea nitrogen has been designed by Jenkins et al. (1999) and this system is based on enzymatic hydrolysis of urea and the subsequent measurement of CO<sub>2</sub> partial pressure which is linearly related to the concentration of urea in the milk sample. A test of this system was conducted by the authors (Figure 4).

### *Fat and Protein*

Modern infrared analysers used in analytical laboratories are single beam instruments which can measure fat, protein and lactose concentrations in milk by the absorption of infrared light at specific wavele

wavelengths are also measured to take into account the influence of water absorption. It has been proposed by Tsenkova (1992) that near infrared can be used on line to detect analytes in milk. The fat concentration is determined by counting the ester linkages and the measurement of their infrared absorption. Fat concentration in milk can be determined by 2 different wavelengths: 5.7 $\mu\text{m}$  and 3.5 $\mu\text{m}$ . Protein content is determined at a wavelength of 6.5 $\mu\text{m}$  and the infrared absorption is due to nitrogen-hydrogen bonds within the peptide bonds. This method does not measure all non-protein nitrogen, excluding urea and therefore only measures proteinaceous nitrogen (Harding, 1995)

#### *Ionic Concentration*

The use of an ion-selective electrodes may be an appropriate method to detect early signs of mastitis. Legin et al. (1999) reported the use of an array of 30 chemical sensors ("electronic tongue") to measure changes to simultaneously detect ion changes in an analog of human blood plasma.

#### *Enzymes*

Pemberton et al. (2001) reported laboratory experiments with screen-printed carbon electrodes (SPCEs), having a square working area of 9mm<sup>2</sup> were printed from D14 ink formulation (Gwent Electronic Materials Ltd, Gwent, Wales) onto a PVC base. Electrochemical measurements were made under hydrodynamic conditions using a three-electrode system contained within a modified cylindrical oxygen cell and using an analyte volume of 2ml. The SPCE working electrode, a saturated calomel reference electrode and a platinum counter electrode were connected to a LC-4B amperometric detector (BAS, W Lafayette, IN, USA) and a JJ Instruments chart recorder.

The enzyme substrate 1-naphthyl N-acetyl- $\beta$ -D-glucosaminidase (Glycosynth, Warrington, UK) was dissolved in methanol to 0.12M, then in 0.5M phosphate buffer, to a final concentration of 1mM at a pH of 6.65. A fresh SPCE was taken for each measurement, and having applied an operating potential of +650mV to obtain a steady-state current response in 2ml of the above substrate solution under constant stirring, an aliquot of the enzyme  $\beta$ -N-acetylglucosaminidase (EC 3.2.1.52) from Jack Beans (Sigma-Aldrich Co.Ltd.) was added via a micropipette. The resulting current response was recorded as initial rate for final enzyme concentrations over the range 3.1 to 108 milliunits/ml with a linear response ( $r^2=0.988$ ). Response rates (nA/s) were recorded, logged and plotted against enzyme concentration.

It remains to be seen whether this assay will work in an on-line format to detect mastitis.

#### **Discussion :**

A number of dairy cow health and fertility conditions can be monitored by on-line milk analysis at least in prototype form. The most important of these to the farmer are progesterone for fertility monitoring, markers for mastitis and indicators of metabolic status. Infectious diseases are of less importance to the individual farmer than for the government, responsible as it is for the health of the whole population of animals. Thus the development of sensor systems for detecting antibodies to infectious diseases such as tuberculosis, foot and mouth disease etc. that appear rarely would require a major change of government policy and funding. Zoonotic diseases such as leptospirosis, mycobacterium paratuberculosis, could also be monitored but more as an aid to government policy than because there are large cost savings to be made. Once a sufficient number of farmers are equipped with appropriate on-line analysis devices it will be possible for epidemiological surveys of exposure to disease to be conducted by sending sensors to farms rather than veterinary surgeons.

The specification of an automatic mastitis detection system will be greatly improved if the sources of variability of ion concentration of milk (diet, stage of lactation, breed of cow, type and stage of infection etc) were determined and compared with total conductivity for the same samples. There is a risk that some infections may not show sufficient difference in electrochemical properties to allow them to be classified accurately b

programme of multidisciplinary research to take samples from cows in the process of developing clinical and subclinical mastitis and to analyse them with a view to identifying the constituents most indicative of an infection that will become clinical.

Major biological research is needed to develop models that use routine on-line analysis where appropriate to assess the nutritional performance of the cow. We need to know the most appropriate minimum number of analytes to measure both to know that nutrition is optimal but also that excess waste products are not being produced. Engineering research is needed to develop and integrate on-line sensing systems for urea, ketones, fat and other milk constituents as appropriate. Once these tools are in place it will be possible to propose completely integrated management system for dairy cows that will have a model of how the cow is performing and corrective action needed to supply her with the inputs needed to maximise milk output with minimum detrimental environmental impact.

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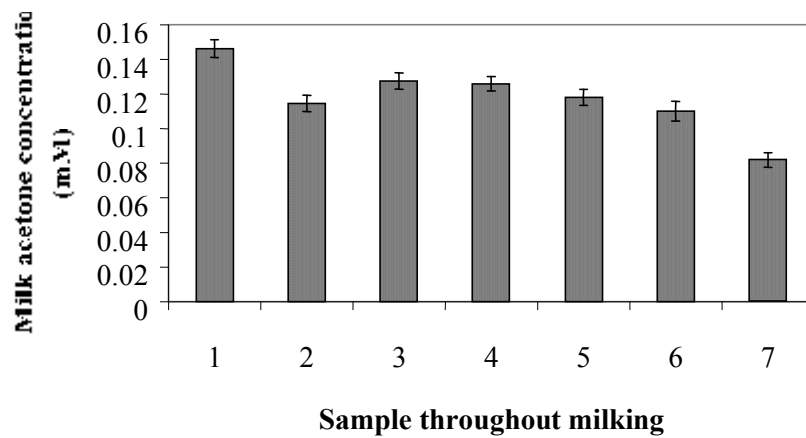


Figure 3. Within milking variation of acetone as determined by the Ketosensor

Figure 4. Tests of standards of urea using a pressure cell system suitable as a low cost milk urea analyser.

