The use of analytical techniques for the rapid detection of microbial spoilage and adulteration in milk

A thesis submitted to the University of Manchester for the degree of PhD in Chemistry

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School of Chemistry
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ABSTRACT

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Milk is an important nutritious component of our diet consumed by most humans on a daily basis. Microbiological spoilage affects its safe use and consumption, its organoleptic properties and is a major part of its quality control process. European Union legislation and the Hazard Analysis and the Critical Control Point (HACCP) system in the dairy industry are therefore in place to maintain both the safety and the quality of milk production in the dairy industry.

A main limitation of currently used methods of milk spoilage detection in the dairy industry is the time-consuming and sometimes laborious turnover of results. Attenuated total reflectance (ATR) and high throughput (HT) Fourier transform infrared (FTIR) spectroscopy metabolic fingerprinting techniques were investigated for their speed and accuracy in the enumeration of viable bacteria in fresh pasteurized cows’ milk. Data analysis was performed using principal component - discriminant function analysis (PC-DFA) and partial least squares (PLS) multivariate statistical techniques. Accurate viable microbial loads were rapidly obtained after minimal sample preparation, especially when FTIR was combined with PLS, making it a promising technique for routine use by the dairy industry.

FTIR and Raman spectroscopies in combination with multivariate techniques were also explored as rapid detection and enumeration techniques of S. aureus, a common milk pathogen, and Lactococcus lactis subsp cremoris, a common lactic acid bacterium (LAB) and potential antagonist of S. aureus, in ultra-heat treatment milk. In addition, the potential growth interaction between the two organisms was investigated. FTIR spectroscopy in combination with PLS and kernel PLS (KPLS) appeared to have the greatest potential with good discrimination and enumeration attributes for the two bacterial species even when in co-culture without previous separation. Furthermore, it was shown that the metabolic effect of L. cremoris predominates when in co-culture with S. aureus in milk but with minimal converse growth interaction between the two microorganisms and therefore potential implications in the manufacture of dairy products using LAB.

The widespread and high consumption of milk make it a target for potential financial gain through adulteration with cheaper products reducing quality, breaking labeling and patent laws and potentially leading to dire health consequences. The time-consuming and laborious nature of currently used analytical techniques in milk authentication enabled the study of FTIR spectroscopy and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) as rapid analytical techniques in quantification of milk adulteration, using binary and tertiary fresh whole cows’, goats’ and sheep’s milk mixture samples. Chemometric data analysis was performed using PLS and KPLS multivariate analyses. Overall, results indicated that both techniques have excellent enumeration and detection attributes for use in milk adulteration with good prospects for potential use in the dairy industry.
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DEDICATION

To my husband Giorgos
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PUBLICATIONS


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<td>Dalton (unified atomic mass unit)</td>
</tr>
<tr>
<td>DFA</td>
<td>Discriminant function analysis</td>
</tr>
<tr>
<td>DEFT</td>
<td>Direct epifluorescent filter technique</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTGS</td>
<td>Deuterated triglycerine sulphate</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionisation</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>EM</td>
<td>Electron multiplier</td>
</tr>
<tr>
<td>EMSC</td>
<td>Extended multiplicative scatter correction</td>
</tr>
<tr>
<td>E-nose</td>
<td>Electronic nose</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>E-tongue</td>
<td>Electronic tongue</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>FT-NIR</td>
<td>Fourier transform near infrared</td>
</tr>
<tr>
<td>FT-RS</td>
<td>Fourier transform Raman spectroscopy</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HACCP</td>
<td>Hazard analysis critical control point</td>
</tr>
<tr>
<td>HeNe</td>
<td>Helium neon</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HT</td>
<td>High throughput</td>
</tr>
<tr>
<td>Q^2</td>
<td>Correlation coefficient train set</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Kr</td>
<td>Krypton</td>
</tr>
<tr>
<td>K-PLS</td>
<td>Kernel partial least square</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge</td>
</tr>
<tr>
<td>MA</td>
<td>Mass analyzer</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCP</td>
<td>Microchannel plate</td>
</tr>
<tr>
<td>MCT</td>
<td>Mercury cadmium telluride</td>
</tr>
<tr>
<td>MIR</td>
<td>Mid-infrared</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>n1</td>
<td>Refractive index</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PC-DFA</td>
<td>Principal component – discriminant function analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDO</td>
<td>Protected destination of origin</td>
</tr>
<tr>
<td>PGI</td>
<td>Protected geographical origin</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least square</td>
</tr>
<tr>
<td>PLSR</td>
<td>Partial least square regression</td>
</tr>
<tr>
<td>R²</td>
<td>Correlation coefficient test set</td>
</tr>
<tr>
<td>RBF</td>
<td>Radial base function</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean squared</td>
</tr>
<tr>
<td>RMSEC</td>
<td>Root mean square error calibration</td>
</tr>
<tr>
<td>RMSECV</td>
<td>Root mean square error cross validation</td>
</tr>
<tr>
<td>RMSEP</td>
<td>Root mean square error of prediction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RS</td>
<td>Raman spectroscopy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface enhanced Raman scattering</td>
</tr>
<tr>
<td>SNV</td>
<td>Standard normal variate</td>
</tr>
<tr>
<td>Spp</td>
<td>Species</td>
</tr>
<tr>
<td>Subsp</td>
<td>Subspecies</td>
</tr>
<tr>
<td>ToF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>TVC</td>
<td>Total viable count</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra heat treated</td>
</tr>
<tr>
<td>USB</td>
<td>Universal serial bus</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVRR</td>
<td>Ultraviolet resonance Raman</td>
</tr>
<tr>
<td>ZnSe</td>
<td>Zinc selenium</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 MILK SPOILAGE

The microbial spoilage of food has been a huge problem throughout the history of mankind. This is because food is not only a nutrient source for humans but also for many fungi and bacteria. A wide range of foods, including red (Ellis et al., 2004) and white meat (Lin et al., 2004b), eggs, fish (Tryfinopoulou et al., 2002), milk and dairy products, fruits and vegetables (Frank, 2001) as well as cereals (Brackett, 2001), can be affected by microbial action in a variety of ways.

Animal milk is the principal nutrient source for young mammals and has been a part of the human diet since prehistoric times. Milk for human consumption is most commonly derived from cows, sheep and goats, while milk from other animal species such as water buffalos and camels is also used for the production of some dairy products. The reason behind this widespread use of milk is its highly nutritious nature. Apart from water, it is mainly composed of carbohydrates such as lactose, fat, proteins, non-protein nitrogenous compounds, vitamins and minerals such as calcium. The latter specifically plays an important role in the development and strength of the human skeleton. The broad range of nutrients, which can be utilised as an energy source, and the almost neutral pH of 6.4-6.6, though make milk an excellent growth medium for a variety of microorganisms (Adams and Moss, 2000).

Some of these microorganisms that grow in milk, through the production of metabolites, may cause an unacceptable sensory alteration, such as off flavour or odour or change in texture or appearance, termed as spoilage (Whitfield, 1998, Ellis and Goodacre, 2001, Gram et al., 2002). These microorganisms should be termed as
specific spoilage organisms for milk, as other microorganisms may also grow in milk but without causing any sensory changes. In accordance to this, the type of sensory change causing milk spoilage depends on the microorganism species present in milk, which are sequentially influenced by the raw milk flora, the chemical composition of milk which is affected by the processing and preservation processes, and finally the storage conditions of milk, which reduce the effectiveness of some of the natural growth inhibitors (Frank, 2001, Gram et al., 2002). The commonest spoilage bacteria in milk can be divided into three different groups: the psychrotrophic bacteria, the fermentative non spore-forming bacteria and the spore-forming bacteria. The potential of milk as a good growth medium for bacteria can also sometimes be utilised by some species considered to be pathogenic for humans, such as *Listeria* spp., *Salmonella* spp., *Campylobacter jejuni*, *Staphylococcus aureus* and *Yersina enterocolytica* (Zall, 1990).

The role of milk and dairy product analysis, involving the detection of spoilage and pathogenic organisms through quantitative and qualitative processes, respectively, is of paramount importance for milk product assessment and in the promotion of public health. In an attempt to preserve high quality manufacturing and food production levels at the same time safeguarding the public, European Union legislation (Directive 92/46 EEC) has been introduced and the Hazard Analysis and Critical Control Point (HACCP) system has been implemented in the dairy industry.

*Psychrotrophic bacteria*

The role of psychrotrophic bacteria in milk spoilage only became apparent a few decades ago. As they are the major spoilage factor of milk during refrigeration they
have been causing a major problem to the dairy industry. Psychrotrophic microorganisms that spoil pasteurised and raw milk are capable of growing in milk at temperatures close to 0 °C. They are represented by both Gram-negative and Gram-positive bacteria. Gram-negative bacteria include the families of Pseudomonadsaceae (65-70%) and Neisseriaceae, the genera of Flavobacterium and Alcaligenes and the family of Enterobacteriaceae. Gram-positive bacteria include bacteria from other genera such as Bacillus, Clostridium, Micrococcus, Corynebacterium, Microbacterium and Aerococcus. Gram-negative bacteria can produce heat resistant lipases and proteases which are associated with flavour and quality defects in milk (Sorhaug and Stepaniak, 1997, Frank, 2001). Pseudomonas spp. are the most important psychrotrophic bacteria causing spoilage in pasteurised and raw milk at temperatures below 7 °C. Confirming this, a study by (Deeth et al., 2002) found that at 4 °C all the isolates from skimmed and whole milk were of the Pseudomonas species, with P. fluorescense accounting for approximately half of the isolates.

The organoleptic changes or defects in pasteurised milk due to psychrotrophic bacteria, especially the Pseudomonas species, have been found to be related to the production of bacterial enzymes. When growing in milk many strains are proteolytic and are able to produce extracellular proteases in addition to lipases and phospholipases. Milk defects start to occur once the number of enzymes has reached a high enough value, usually achievable when bacterial counts reach a level of 10^6 colony forming units per ml (cfu/ml) or higher and are stable at high temperatures surviving pasteurisation (72 °C for 15 s) and Ultra Heat Treatment (UHT) (>135 °C for 1-2 s) (Champagne et al., 1994, Shah, 1994, Sorhaug and Stepaniak, 1997, Deeth
et al., 2002). Spoilage of refrigerated milk is most often related to *P. fluorescence*, *P. fragi*, *P. putida* and *P. aeruginosa* bacterial species (Frank, 2001).

The extracellular proteases secreted by microorganisms in milk commonly hydrolyze casein protein (Shah, 1994). These enzymes are heat stable and produced at the late log or stationary phases of bacterial growth resulting in the bitter and putrid defects of proteolysis (Frank, 2001). The action of proteases directly on micellar casein results in casein degradation and release of bitter peptides, with β-casein hydrolysing more rapidly than α-casein, and thus probably being the main culprit for the accumulation of bitter peptides, while the actions of proteases on κ-casein appears to destabilise the casein micelles leading to milk coagulation (Frank, 2001). Further proteolysis of the smaller protein metabolism by-products results in the production of a putrid aroma and flavour (Mabbitt, 1981, Champagne *et al*., 1994, Shah, 1994, Frank, 2001). Major factors which appear to directly affect the production of proteases include temperature and pH; while iron cations appear to inhibit enzymatic action when added to milk (Sorhaug and Stapaniak, 1991, Frank, 2001).

Lipases are lipid hydrolysing enzymes secreted by microorganisms (Shah, 1994). Similar to proteases, in psychrotrophic bacteria lipases are produced during the late log or stationary phase and are often heat stable, unlike the natural milk lipases, causing rancid, and fruity flavours (Shah, 1994, Veld, 1996, Frank, 2001). Microorganism production of lipase enzymes is affected by a variety of factors. These include temperature (Anderson, 1980), the presence of an organic nitrogen source, metal ions, polysaccharides or lecithin (Sorhaug and Stapaniak, 1991, Frank, 2001),
as well as the bacterial strain, triglyceride concentration and growth conditions (Mabbitt, 1981, Frank, 2001).

**Fermentative non spore-forming bacteria**

Fermentative non spore-forming bacteria spoil food by fermenting sugars such as lactose into acids. The increased acidity of milk from a pH of 6.6 to 4.5 promotes casein precipitation (curdling) (Jay et al., 2005). This type of spoilage usually takes place at higher product storage temperatures which promote a faster growth of this type of bacteria compared to psychrotrophic bacteria. In addition, this spoilage occurs when inhibitors present in the product restrict the growth of Gram-negative aerobic organisms (Frank, 2001).

Fermented dairy products are produced using lactic acid bacteria during the manufacturing process, although even these can be spoiled by ‘wild’ lactic acid bacteria. The dairy products most commonly affected by non spore-forming fermentative bacteria are liquid milk, cheese and culture milks (Frank, 2001). Lactic acid bacteria including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Pediococcus* and *Streptococcus*, and coliforms are the major non spore-forming fermentative spoilage microorganisms in milk and dairy products (Sharpe, 1979, Jay et al., 2005).

**Spore-forming bacteria**

Liquid milk products with low acid content can suffer spoilage from spore-forming bacteria. This arises when milk products are heat-treated in substerile temperatures such as aseptically manufactured milk and cream and sweetened and unsweetened
concentrated canned milks (Frank, 2001). The spores of some species or strains appear to be resistant to pasteurisation and UHT. *Bacillus cereus* is one of these, with some strains now appearing to have become psychrotrophic, growing even at temperatures of 4-6 °C (Gilmour and Rowe, 1990, Andersson *et al*., 1995). Spore-forming bacteria may also affect spoilage of hard cheeses with low salt concentrations (Frank, 2001).

Spore-forming bacteria responsible for spoilage of dairy products are usually present in raw milk in very low populations (< 5000 cfu/ml). However, the defects that occur from spore-forming bacteria do not necessarily relate to the population in the original raw product but also to the storage period of the product, as a long storage periods would allow for the overgrowth of these microorganisms causing spoilage. The most common spore-forming bacteria causing dairy product spoilage are *Bacillus* spp. and in particular *B. cereus*, *B. subtilis* and *B. licheniformis* (Mikolojcik and Simon, 1978, Gilmour and Rowe, 1990, Frank, 2001). *Clostridium* spp. also exist in raw milk but in extremely low levels, while in contrast psychrotrophic spore-forming *B. cereus* bacteria are present in a high percentage of raw milk samples (Frank, 2001). The growth of the latter contributes to a milk defect known as sweet curdling, followed by a bitter-flavour once sufficient casein has been degraded (Gilmour and Rowe, 1990, Frank, 2001, Jay *et al*., 2005). Degradation of fat globule membrane with the release of phospholipase C enzyme by the same organism may also result to a ‘bitty’ cream milk defect (Frank, 2001).
1.1.1 Microbial spoilage detection techniques in milk

A number of different techniques have been explored and utilized over the years for the detection and enumeration of microbiological spoilage in milk and dairy products. These include ATP bioluminescence, electrical and microscopy methods, immunoassays, polymerase chain reaction, nucleic acid probing and electronic nose techniques, each with positive and negative aspects in regards to their application and final outcome.

1.1.1.1 ATP Bioluminescence Technique

Adenosine triphosphate (ATP) is a multifunctional molecule present in all living cells, acting as a chemical energy storage unit harvested from free energy liberated during catabolism and later utilized for anabolic processes (Knowles, 1980). Many different organisms possess the ability to produce light through the activity of luciferases, a type of specific enzymes. For this reaction to take place ATP, magnesium ions and a luciferin substrate, acting as a photon emitter, are needed. An ATP molecule is hydrolyzed to form an enzyme-substrate complex which is subsequently oxidized to a higher energy state level. The return of the molecule from its unstable excited state to its stable ground state causes the emission of a photon of light simultaneously liberating the luciferase enzyme (Vasavada, 1993, Adams and Moss, 2000).

Several commercial ATP instruments are available utilizing this enzymatic photon/energy releasing reaction using ATP molecules via luciferin and luciferase. These are able to detect very low levels of emitted light, thus measuring the ATP
ATP levels can be correlated to the number of bacteria present as these are considered to be fairly constant within each type of bacterial cell (Griffiths, 1993). Advantages of these methods include their rapidity and high sensitivity; these methods can detect as low as $10^3$ – $10^4$ bacterial cells when photomultiplier tubes are used (Luo et al., 2009, Hunter and Lim, 2010). However the method only gives good results when the cultures are pure. When applied to food, microbial ATP has to be separated from non-microbial ATP, which is normally present in greater quantities (Griffiths, 1993). This can be achieved in two ways. Mild surfactant and ATPase enzyme can be added to food in order to break up somatic cells and remove ATP, with subsequent filtration of the ATPase and the use of a stronger surfactant to release ATP from the microorganisms. Alternatively, non-microbial ATP can be removed prior to the ATP assay using centrifugation or filtration of liquid food. The latter process though is very difficult to apply in solid food. In addition, different types of microorganisms contain different amounts of ATP and additional techniques need to be applied to remove specific groups of microorganisms and increase sensitivity. As high levels of ATP on a surface are related to poor hygiene, the ATP bioluminescence technique has found a useful application in detecting contamination of equipment and machinery surfaces related with the preparation and processing of food (Adams and Moss, 2000, Luo et al., 2009).

In the dairy industry this method has been successfully used at the industrial level to evaluate raw cows’ milk in regards to its ‘hygienic quality’, within 10 min, discriminating between milk of good and poor quality (Bell et al., 1996). Attempts have also been made for the use of this method in the quantification of
microorganisms in raw milk with limited results (Niza-Ribeiro *et al.*, 2000, Samkutty *et al.*, 2001), while similar results have been obtained for microorganism discrimination (Frundzhyan *et al.*, 1999, Niza-Ribeiro *et al.*, 2000).

ATP bioluminescence has been used for the detection of pathogens in milk with highly sensitive results, originally with the use of bacteriophages (Kricka, 1988) and more recently the use of biofunctional magnetic nanoparticles able to detect up to 20 cfu/ml of *E. coli* within 1 h (Cheng *et al.*, 2009). A more widespread use of the technique in the dairy industry has been the evaluation and monitoring of hygienic conditions of milk tankers (Bell *et al.*, 1994), pasteurization surfaces (Murphy *et al.* 1997), stainless steel milk contact surfaces (Costa *et al.*, 2006) and milking equipment (Vilar *et al.*, 2008). It has also been used to evaluate cleaning performance of various tests (Reinemann and Ruegg, 2000) and validate factory cleaning protocols (Oulahal-Lagsir *et al.*, 2000).

1.1.1.2 Electrical methods

Microbial metabolism in a medium results in the breakdown of complex molecules such as protein, carbohydrates and fats into smaller molecules such as amino acids, the lactate and acetate (Ur and Brown, 1975). As the original molecules are uncharged or minimally charged and their catabolic products are mostly charged, a change in the electrical properties of the medium ensues. The latter can therefore be monitored and measured giving an indication of the bacterial activity and numbers in the medium. Medium capacitance, conductance, and impedance, mostly the latter two, are the electrical properties usually considered (Vasavada, 1993).
Commercially available instruments like the Bactometer (Gnan and Luedecke, 1982, Suhren and Heeschen, 1987) and the Malthus system (Fung, 1994) can measure the impedance and conductivity of the growth medium, respectively. These electrical properties increase along with the concentration of microorganisms growing in that medium. When these increases are plotted against time, the point where a dramatic rise is detected compared to the starting values is the detection time. This is the time required for the microorganisms to grow to a population of approximately $1 \times 10^6$ cfu/ml and can be subsequently used to calculate the initial microorganism counts in the sample (Vasavada, 1993).

These electrical methods have found several applications in dairy microbiology. The Bactometer has been used for detecting specific microorganisms in milk (Kowalik and Ziajka, 2005), milk powder (Neaves et al., 1988) and yoghurt (Pirovano et al., 1995), for monitoring the quality of raw milk (Senyk et al., 1988), estimation of microorganisms in raw and pasteurized milk and cheese (Vasavada, 1993), and for predicting the shelf-life of milk (White, 1993). The Malthus system has been used to detect psychrotrophic microorganisms in raw milk (Easter and Gibson, 1989) as well as detect post-pasteurization contaminants (Visser and De-Groote, 1984).

Despite these wide applications these electrical methods carry some significant limitations for routine application in detecting microbial spoilage in milk. These include the requirement of suitable media that can support the rapid growth of microorganisms with a change in their electrical properties during growth and delays in sample turnover, as for the methods to work there is a pre-requisite of a large
bacterial population \(1 \times 10^6 - 1 \times 10^7\) cfu/ml to be present (Vasavada, 1993, Adams and Moss, 2000).

### 1.1.1.3 Microscopy methods

#### 1.1.1.3.1 Direct Epifluorescent Filter Technique

The Direct Epifluorescent Filter Technique (DEFT) is a method for counting microorganisms by combining membrane filtration and epifluorescent microscopy, and this technique was first used for bacterial enumeration in raw milk in the 1980s (Pettipher et al., 1980). The method involves pre-treatment of the milk sample with an enzyme, filtration of the subsequent surfactant using a polycarbonate membrane filter, staining of the filtered bacteria using fluorescent dyes, and detecting the bacteria by visual inspection using epifluorescent microscopy. Counting of bacteria is either performed manually or with the use of image analysis software (Vasavada, 1993). The use of modified staining techniques, with membrane filter supports and a buffer solution has been shown to increase the sensitivity of the method (Rodriguez and Kroll, 1985).

DEFT with further methodological modifications has been shown to be able to detect *Bacillus* spores in raw milk but with a restricted sensitivity of \(10^5\) spores/ml (Moran et al., 1991). Successful viable bacterial population enumeration in raw milk has also been achieved, with similar restrictions in the sensitivity of the technique at the \(10^4\) cfu/ml (Champagne et al., 1997). Automated (Pettipher et al., 1980, Pettipher et al., 1992) and semi-automated (Hermida et al., 2000) techniques have been investigated with analogous results. In general, this method is associated with many limitations.
including lack of distinction between viable and non-viable bacteria and poor
sensitivity (Vasavada, 1993, Rapposch et al., 2000). Despite the use of image analyser
aids and sophisticated equipment the method does require operator knowledge of
microscopy and lastly it is very laborious and time consuming (Vasavada, 1993).

1.1.1.3.2 Flow Cytometry

Flow cytometry is a technique that measures and analyses various aspects of the
physical and chemical characteristics of cells at the same time, while they flow within
a liquid stream through a point surrounded by various optical/electronic detection
apparatuses, used widely in research since 1975 (Commas-Riu and Rius, 2009). The
main advantage of this technique is its speed and accuracy. Unfortunately though the
majority of instrument designs prohibits the analysis of large sample numbers and
additional processing is required before the technique can be used for bacterial
enumeration (Flint et al., 2006, Commas-Riu and Rius, 2009). The limitations
requiring additional processing occur because some species of bacteria can not be
stained, usually a pre-requirement for flow cytometry, although fluorescence staining
could be employed, while specific stains are required for distinguish viable from non-
viable cells (Rapposch et al., 2000, Flint et al., 2006, Commas-Riu and Rius, 2009).
Furthermore, the growth conditions and origin of the sample directly affects some of
the physical and chemical characteristics of microorganisms, such as their size, shape
and DNA content, introducing variability and loss of accuracy in the final cytometric
analysis (Flint et al., 2006). Additional limitations are associated with the use of flow
cytometry in food microbiology. Milk contents such as protein and lipids may
interfere with the measurements and more elaborate preparation of solid food samples
is required compared to other liquid sample types in order to prepare a homogenous suspension of cells, another pre-requisite of flow cytometry (Flint et al., 2006).

In the 1980s the use of flow cytometry with the Bactoscan method was developed as a routine method for the enumeration of the total number of bacteria in raw milk (Suhren, 1989). This automated technique uses two stains acridine orange or ethidium bromide to stain the nucleic acid of bacteria in raw milk subsequently employing flow cytometry to count the bacterial numbers. The inability of the technique to distinguish between nucleic acid derived from viable or non-viable bacterial cells in pasteurised milk products limits its use in the detection of spoilage in dairy products (Suhren and Walte, 1998, Flint et al., 2006). In addition, the technique underestimates Gram-negative bacterial cultures (Rapposch et al., 2000), some types of mastitis pathogens (Suhren and Reichmuth, 1997), and does not possess sufficient sensitivity; $10^4$ to $10^5$ (Giffel et al., 2001). Towards the end of the twentieth century Gunasekera et al (2000) applied a new technique, fluorescent flow cytometry, to overcome the Bactoscan limitations. This method uses fluorescent stains or a fluorogenic substrate with flow cytometry in order to discriminate and detect viable and non-viable cells in raw milk, within hours with sensitivity of up to $10^4$ bacteria per ml (Gunasekera et al., 2000, Gunasekera et al., 2003b).

Holm and Jespersen (2003) with the use of two different fluorescent stains and flow cytometry have been able to differentiate between Gram-positive and Gram-negative bacteria in bulk tank milk. Flow cytometry has also been used to detect bacteriophage-infected Lactococcus lactis cells, an important bacterium used in the manufacturing of fermented dairy products (Michelsen et al., 2007). In addition,
identification of pathogenic organisms in milk such as *Salmonella typhimurium* (McClelland and Pinder, 1994), *E. coli* 0157:H7 (Seo et al., 1998) and *Listeria monocytogenes* (Masafumi et al., 2009), has been performed employing flow cytometry.

Enumeration of thermophilic bacteria in milk powders has been achieved with flow cytometry within 1h (Flint et al., 2007). Furthermore, Holm et al. (2004) demonstrated that flow cytometry is able to detect the cause of elevated bacterial counts in milk from bulk milk tanks, by quantifying and differentiating bacteria based on three separately assigned groups, each related to a specific cause.

### 1.1.1.4 Immunoassays

Immunological methods are based on the detection of artificially labelled antibodies that bind onto cell surface antigens of specific microorganisms. The microorganisms can be detected in different ways including employing the use of a chromogenic substrate or fluorescent microscopy. Even though various processes and techniques are used to detect the antibody to antigen interaction in immunoassays, the most common method is the enzyme linked immunosorbent assay (ELISA) (Samarajeewa *et al.*, 1991, Adams and Moss, 2000). ELISA involves the mixing of a sample with genus-specific monoclonal antibodies which bind to all their specific antigens forming antibody-antigen complexes. Removal of the remaining sample ensues followed by the addition of a second type of antibody linked to an enzyme which then binds the antibody-antigen complexes that were left behind. The bound antibody-antigen-antibody (thus sandwich ELISA) complexes are subsequently detected using a
chromogenic substrate which changes colour in the presence of these complexes as it is exposed to the activated second antibody’s enzyme (Adams and Moss, 2000).

In assessing milk spoilage employing ELISA *Pseudomonas fluorescens* and psychrotrophic bacteria have been quantified in refrigerated milk with detection threshold for the latter at $10^5$ cfu/ml (Gutierrez *et al.*, 1997a). The enumeration properties of ELISA can be enhanced when combined with immunomagnetic separation techniques for the quantification of *E. coli* to 1 cfu/ml (Reinders *et al.*, 2002). Fluorescent microscopy, flow cytometry or polymerase chain reaction can also be combined with immunology for the identification of specific organisms (Adams and Moss, 2000).

ELISA has also been widely used as a method for detecting animal infection in dairy animals through milk, by the quantification of organisms such as *Salmonella dublin* (Smith *et al.*, 1989), *Mycoplasma bovis* (Heller *et al.*, 1993), *Brucella abortus* (Vanzini et al., 2001) and *Mycobacterium avium* (Winterhoff *et al.*, 2002) and has been tested as a potential dairy herd infection screening tool for *Salmonella typhimurium* (Hoorfar and Wedderkopp, 1995) using milk samples.

### 1.1.1.5 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is used to amplify specific parts of deoxyribonucleic acid (DNA) from the target organism between two known nucleic acid regions. The sample is heated in order to denature and unwind the DNA strand, then organism specific oligonucleotide primers are added to the sample and bind to their complementary DNA sequences on the single DNA templates if the target organism’s
DNA is present. In the presence of the target, the polymerase enzyme synthesises complementary DNA between the primers. The cycle is repeated several times until approximately $10^9 - 10^{12}$ copies of the amplified DNA are produced. Subsequently the target DNA is stained and visualised using UV fluorescence (Vasavada, 1993, Adams and Moss, 2000, Feng, 2001).

The PCR technique is very sensitive and specific and devoid of the requirements for culturing the target organism. This method though has significant requirements as it demands the preparation of appropriate DNA that can be used with the PCR technique and requires a very clean working environment to avoid false positive results (Vasavada, 1993). In the area of food analysis the presence of certain types of PCR inhibitors in various types of food, which can only be circumvent using additional pre-PCR procedures, also make the entire method elaborate and time-consuming for routine use in the food industry (Adams and Moss, 2000).

Nevertheless, PCR remains one of the most important methods for detection of low concentrations of foodborne pathogens, with very significant applications in dairy microbiology. PCR in combination with various DNA isolation techniques can rapidly detect low levels of pathogens such as *Listeria monocytogenes* (1-5 cfu/ml) in fresh milk (O'Grady *et al.*, 2008), *Salmonella spp.* (5 cfu/25ml) (Hein *et al.*, 2006), *Staphylococcus aureus* and *Yersinia enterocolytica* ($10^3-10^4$ cfu/ml) (Ramesh *et al.*, 2002) and *Brucella spp* ($10^2-10^3$ cfu/ml) (Hamdy and Amin, 2002) in raw milk, and *Mycobacterium avium* subsp. *paratuberculosis* in semi-skimmed (5 cfu/ml) (Rodriguez-Lazaro *et al.*, 2005) and raw milk (1 cfu/ml) (Gao *et al.*, 2007). Enterotoxic *Staphylococcus aureus* can also be very reliably identified in skimmed
milk and cheddar cheese with sensitivity of $10^2$ cfu/ml or $10^2$ cfu/20g respectively (Tamarapu et al., 2001). PCR methods have also been successfully used to identify high sensitivity bacteriophages affecting the important fermentative *Lactobacillus casei/paracasei* bacteria (Binetti et al., 2008), to detect *Streptococcus* and *Staphylococcus* bacterial species involved in animal mastitis cross-contamination through milking equipment (Meiri-Bendek et al., 2002, Wu et al., 2008) and probiotic bifidobacteria in raw milk and cheese (Delcenserie et al., 2005).

Quantification of bacteria using the PCR technique in raw milk and cheese samples (Lopez-Enriquez et al., 2007, Lorusso et al., 2007, Rantsiou et al., 2008) and enumeration of specific bacteria from mixed bacterial cultures in artificially contaminated fermented milk (Furet et al., 2002, Grattepanche et al., 2005, Tabasco et al., 2007), has been shown with very good sensitivity. The implementation of PCR in the microbial analysis of pasteurised milk products though has been limited by the technique’s inability to distinct between DNA from viable and non-viable bacteria (Adams and Moss, 2000, Hein et al., 2001, Jung et al., 2005). Recently, real time PCR techniques using a range of methodologies have been developed being able to bypass this problem providing very accurate and sensitive results. They have been able to quantify accurately *L. monocytogenes* (Choi and Hong, 2003) and *M. tuberculosis* (O'Mahoney and Hill, 2004) in artificially contaminated pasteurised whole milk, and *E. coli* 0157:H7 in skimmed milk (Li and Drake, 2001). The main disadvantage behind these techniques though is that they continue to remain laborious and time-consuming, as they take 3-5h for sample analysis.
1.1.1.6 Electronic Nose

Development of electronic noses (e-noses) commenced in the mid-1980s, and has been extensively investigated by research especially towards the end of the previous decade, mainly in the field of food and beverage industry (Hudon et al., 2000, Ellis and Goodacre, 2001). E-nose analyses the input from an array of selective chemical sensors receiving a sample’s odour/volatile compounds, and using an appropriate pattern recognition program recognizes the pattern of odours or fingerprint of specific products (Ellis and Goodacre, 2001, Ampuero and Bosset, 2003). A variety of different sensor technologies are commercially available, including metal oxide sensors, conducting organic polymer sensors, quartz crystal microbalance sensors and quadrupole mass spectrometry (Ampuero and Bosset, 2003).

The e-nose has found many applications in the dairy industry. As already mentioned off-flavours in milk originate mostly from the metabolic and enzymatic activity of bacteria, as well as heat, light (photo-oxidation) and oxidation catalyzed by chemicals (Marsili, 2000, Ampuero and Bosset, 2003). An e-nose system based on solid phase micro-extraction MS with multivariate analysis has been used to classify off-flavours in milk (Marsili, 1999). Metal oxide sensors and principal component analysis (PCA) have been shown to have the ability to classify pasteurized and UHT milk into different age groups (Ampuero and Bosset, 2003) as well as assess milk quality (Jiang et al., 2007). A different e-nose system could also be used to establish the shelf-life of raw milk (Amari et al., 2009). Coated quartz crystal microbalance sensors have shown encouraging results in discriminating E. coli in artificially contaminated and uncontaminated milk samples even though this was not possible for P. fragi, with the authors suggesting lack of fermentative action of the latter organism as a possible
reason for this result (Ali et al., 2003). Differentiation between the bacteria *S. aureus* and *B. cereus* and the yeasts *Candida pseudotropicalis* and *Kluyveromyces lactis* in inoculated milk has been achieved by Magan *et al.* (2001) employing the use of principal component analysis (PCA). Furthermore, Korel and Balaban (2002) have been able to quantify various microbial loads of inoculated sterile milk, with *P. fluorescence* and *Bacillus coagulans*, using e-nose and discriminant function analysis (DFA).

In addition, milk e-nose systems have been tested in other dairy products with good results. In cheese samples, when combined with mass spectrometry, e-nose has been able to differentiate between various bacterial populations (Marilley *et al.*, 2004). The amalgamation of e-nose sensor fusion with an artificial neural network (ANN) has revealed a very promising potential in the monitoring and quality control of yoghurt fermentation (Cimander *et al.*, 2002).
1.2 FOOD AUTHENTICITY

Authenticity of food products is a topic of particular interest to consumers, producers, food processors, retailers and regulators. High value products are especially vulnerable as even small deviations in the constituency of a product, with for example the replacement of a more expensive and scarce ingredient with a cheaper and more abundant one, can prove financially more favourable to the dishonest producer. During the last few decades, the internationalisation of food markets has made this a very lucrative practice (Fuente and Juarez, 2005, Reid et al., 2006, Karoui and Baerdemaeker, 2007). This practice results in a significant loss of product identity with substandard product quality and misleads and exploits consumers through incorrect product labeling and product misrepresentation. In addition, consumer health is placed at risk with potentially avoidable cow milk allergies.

Milk is an expensive raw material, with sheep’s milk being the most expensive followed by goats’ and then cows’ milk, the first two milk species showing seasonal variations in milk production. The widespread use of dairy products, by a broad range of population groups such as children, elderly and pregnant women, makes any successful milk or dairy product adulteration attempt financially very favourable but with a heavier and larger consumer impact. Natural selection through the variability of environmental conditions has led to the development of a variety of animal breeds and species, with distinct milk characteristics (Romero et al., 1996). This milk quality is especially important in the production of different types of cheese, and has implications for cheese yields, quality and characteristics (Summer et al., 2003,
Karoui and Baerdemaeker, 2007). As such, areas with high processing costs can only financially survive if they produce a distinct product of high quality.

Various safe-guards have been developed in order to protect consumer rights and genuine product producers. In an area which is agriculturally as diverse as the European Union (EU), a food safety and traceability regulation EC/178/2002 (Official Journal of EC, 2002) has been implemented. Among its aims is to “pursue a high level of protection of human life and health and the protection of consumers’ interests, including fair practices in food trade…(p.8)” (European Commission, 2002). European regulations on industrial milk processing are strict, only permitting a predefined number of constituent modifications, such as changes in the fat content and the addition of certain minerals, vitamins and milk proteins, despite the potential positive nutritional effect of some additives (Council Regulation (EC) No 2597/97, No 1255/1999, Directive 2001/114/EC) (European Commission, 1997, European Commission, 1999, European Commission, 2001a). Currently, certain dairy products, such as ‘protected destination of origin’ (PDO) and ‘protected geographical indication’ (PGI) products, also require very accurate labeling in regards to their origin and processing and are protected by appellations of origin in an attempt to ensure good product quality for the consumer (Council Regulation (EEC) No 2081/92) (European Economic Community, 1992, European Commission, 2004). Further food labeling legislation is currently under discussion in the EU.

A variety of analytical techniques have been used in the past in order to ensure product authenticity. Unfortunately the advances in dairy chemistry and technology, and the production of newer more specialised milk products, has concurred with the
development of more complicated techniques of product manipulation which are harder to detect (Fuente and Juarez, 2005). Practical techniques of examining product authenticity based on the strict standards and criteria legally required are therefore sometimes unavailable and require constant updating to keep pace with the constantly changing technological advances in the industry. In order to enhance milk product analysis analytical dairy science has therefore employed advanced techniques based on spectroscopy such as near-infrared (NIR), mid-infrared (MIR) and nuclear magnetic resonance (NMR), as well as chromatography, immunoenzymatic assays, PCR, electrophoresis and sensory analyses.

1.2.1 Chromatographic techniques

The chemical process of chromatography involves the dissolution of a mixture into a ‘mobile’ phase such as a liquid or gas, subsequently undergoing differential partitioning and separation of its constituent molecules, including the analyte or molecule under investigation, as it is passed through a ‘stationary’ phase based on the degree of solubility of each component in the stationary phase (Heftmann, 2004). In liquid chromatography (LC) the mobile phase is liquid while in gas chromatography (GC) it is gaseous. These two techniques have been successfully used in food chemistry to identify a great variety of molecules present in food (Nollet, 2000, Nielsen, 2003). High performance liquid chromatography (HPLC), which involves the passing of the mobile phase through the stationary phase at high pressure, detects a range of compounds including proteins, peptides, amino-acids, phenolic compounds and carbohydrates (Reid et al., 2006). GC is used to detect volatile and semi-volatile compounds (Reid et al., 2006). The main drawback for these techniques when applied
to industrial use is that they are time consuming, requiring 30-50 min for data generation and labour intensive, and there is an additional need for data analysis using advanced chemometric techniques (Nollet, 2000, Reid et al., 2006).

1.2.1.1 High pressure liquid chromatography

Various HPLC procedures have been tested for quantification and qualitative analysis of milk. HPLC is a valuable tool for the quantitative analysis of proteins in dairy products. It has been successfully applied in the separation of whey proteins, such as β-lactoglobulin, originating from different milk species, and in the authentication of sheep’s and goats’ cheese adulterated with cow milk up to the 2% level (Urbanke et al., 1992, Ferreira and Cacote, 2003, Chen et al., 2004). The latter study employed HPLC in combination with electrospray ionisation mass spectrometry (Chen et al., 2004). HPLC has also been reported to be able to detect and quantify milk adulteration of goats’ and sheep’s milk with cows’ milk at cow milk levels greater than 5% by separating and quantifying κ-, α- and β-caseins in raw and processed milk (Veloso et al., 2002). By contrast, other studies have failed to identify goats’ milk adulteration in combination with sheep’s or in a triple mixture of goat-cow-sheep’s milk (Noni et al., 1996).

HPLC has also been used for the study of cheese adulteration. Mayer et al. (1997) used HPLC to approximately determine the percentages of goats’, cows’ and sheep’s milk in cheese. More recently, HPLC of para-κ-caseins has been found to be suitable for differentiating between mixtures of goat, sheep and cow milk in cheese at various stages of maturation (Mayer, 2005). Enne et al. (2005) using HPLC of governing fluid (i.e., the liquid in which the cheese is packaged) of water buffalo Mozzarella cheese
were able to detect adulteration with cow milk at the EU 1% limits, with a limiting factor that this technique is only able to be used in cheeses containing adequate governing fluid volumes.

Apart from detecting milk adulteration between different animal milks, HPLC has also been found to be able to detect soybean milk adulteration of milk up to 5% levels (Sharma et al., 2009), and was able to detect soybean proteins in goat, cow and sheep milk (Espeja et al., 2001). HPLC was also able to detect very low levels of bovine whey proteins α-lactalbumin and β-lactoglobulin (1 and 1.3%) in powdered soybean milk (Garcia et al., 1998).

1.2.1.2 Gas chromatography

GC is usually used for detecting adulteration by measuring edible fats (Tsimidou and Boskou, 2003). Using GC and fatty acid identification, it has also been possible to detect buffalo milk adulteration with cows’ milk at levels of up to 5% (Farag et al., 1984). Similarly triglyceride analysis has been able to identify cows’ milk fat in goats’ milk fat (Fontecha et al., 1998), and adulteration of pure cows’ milk fat with other types of fat to levels greater than 3-10% (Ulberth, 1994, Povolo et al., 1999, Kamm et al., 2002, Povolo et al., 2008, Gutierrez et al., 2009). Fontecha et al. (2006b) used GC, to identify the triacylglycerol composition of Carbales blue cheese (cows’ milk or in combination with goats’ milk) and Majorero goats’ cheese. They provided encouraging results for the detection of foreign fats during the early ripening period, provided an adequate number of samples are analysed. Similar results were also reported for the PDO cheeses Mahon (cows’ milk) and Manchego (sheep’s milk) during ripening (Fontecha et al., 2006a).
The characteristic flavour compounds which are present in dairy products, especially the volatile and non-volatile aroma compounds present in milk and cheeses, can also be analysed by GC for qualification and quantification purposes. GC analysis of volatile compounds for the determination of geographical origin of milk (Fernandez et al., 2003) and cheese (Pillonel et al., 2003), in combination with multivariate analysis or mass spectrometry respectively, has also shown good promise.

### 1.2.2 Electrophoresis

#### 1.2.2.1 Isoelectric focusing

Electrophoresis is a separation technique, in which particles usually dissolved in aqueous solution are exposed to a spatially uniform electric field and shift towards their oppositely charged electrode leading to mixture separation particles based on particle/molecule charge to weight ratio (Westermeier, 2005). In isoelectric focusing (IEF) this principle is used to separate a mixture placed in a gel with a pH gradient, and recording protein movement to their individual pH or electrically neutral zones, once an electric current is applied to the gel creating a positive and negative cathode (Westermeier, 2005). This is the European Union’s reference method for detecting sheep’s and goats’ milk adulteration with cows’ milk in fresh milk and cheeses, by measuring cow γ-casein levels to equal or greater to 1% (Addeo et al., 1995, European Union, 1996). This method however appears to be unable to detect sheep’s from goats’ milk adequately and therefore unable to give accurate results in tertiary mixtures containing sheep’s, goats’ and cows’ milk. In an attempt to improve this, IEF has been combined with immunoblotting with anti-bovine polyclonal antibodies.
(Moio et al., 1992, Addeo et al., 1995) and with HPLC (Mayer et al., 1997) but with mildly improved results. A further disadvantage of the technique is that it is lengthy for routine inspection use and requires highly specialised staff and equipment.

1.2.2.2 Polyacrylamide gel electrophoresis

This method has been used to attempt to improve IEF results. Polyacrylamide gel electrophoresis (PAGE) has been shown to be able to detect up to 1% cows’ milk in goats’ milk (Lee et al., 2004), with reduced costs and shorter execution times. Adulteration of cheese with pasteurised cow’s milk or heat-denatured cow’s whey proteins in percentages greater than 1% has been successfully detected using PAGE (Molina et al., 1995a); while electrophoretic analysis of whey proteins using PAGE has been able to predict the percentages of cow’s, sheep’s and goat’s milk in Iberico cheese which contains 25-40% of each milk species (Molina et al., 1995b). Even though this method is an improvement on IEF this technique remains relatively lengthy and labour intensive and unable to visualize small molecules adequately.

1.2.2.3 Capillary electrophoresis

Capillary electrophoresis (CE) analysis with multivariate regression analysis has been shown to be able to quantify the percentage of the different milk species in various mixture combinations from the differences found in the casein fractions of each milk species (Molina et al., 1999). The separation of milk whey proteins using capillary zone electrophoresis (CZE) could also be used to authenticate dairy products (Cartoni et al., 1999) with some restrictions due to genetic variability and protein heat sensitivity. CE analyses have been used in the quantification and qualification of milk
species in cheese using a range of conditions such as acidic isoelectric buffers (Herrero-Martinez et al., 2000) and borate buffer with methyl-silanized capillaries in CZE (Cartoni et al., 1999) respectively. Using CE Recio et al. (2004) were able to detect accurately additions of greater than 2% goat’s and 5% cow’s milk to Halloumi cheese, which is made entirely from sheep’s milk. Similarly, Rodriguez-Nogales and Vazquez (2007) using CE and multiple linear regression analysis were able to predict the percentages of cow’s, sheep’s and goat’s milk present in Panela cheese. The use of uncoated capillaries for the separation and quantification of milk whey proteins using CE (Recio et al., 1995), and identification of goat milk adulteration by cow’s milk to 1% using CZE (Lee et al., 2001) has also been explored with promising results, providing a cheaper and faster alternative to coated capillaries. Speed and simplicity may potentially be achieved by combining CE with mass spectrometry (Muller et al., 2008). In general, CE has been shown to be a more cost-effective and faster technique than PAGE and IEF, with higher resolution results. It does however still require further development for more accurate and sensitive results for use in authenticity checks.

1.2.3 Polymerase Chain Reaction

PCR is a DNA amplification technique involving three steps: DNA denaturation, DNA annealing with primer attachment, and synthesis of multiple new strands by the DNA polymerase enzyme. This DNA amplification of nuclear or mitochondrial DNA fragments is then analysed through a range of molecular biological procedures to identify the original cellular source of the DNA (McPherson and Moller, 2006). As milk from each species contains a number of individual species’ somatic mammary
cells, identification of species-specific DNA sequences in milk should allow for the discrimination of the milk species contained, with the potential for quantification based on the number of cells present. While protein-based detection techniques can be influenced by various dairy product production conditions (Mayer, 2005), it has been demonstrated that PCR is impervious to conditions such as milk heat treatment, drying or freezing (Bottero et al., 1999, Maudet and Taberlet, 2001).

Initial attempts to identify species in milk products using PCR were based on the bovine specific β-casein DNA allowing discrimination of cows’ milk in other types of milk (Plath et al., 1997). More recently, PCR has been used on the mitochondrial cytochrome b (cytb) gene with specific restriction endonuclease enzymes to provide discrimination between cows’, buffalos’ and sheep’s milk (El-Rady and Sayed, 2006). Other primers have also successfully been used to discriminate between cows’, goats’, sheep’s and buffalos’ milk (Maccabiani et al., 2005). Lopez-Calleja et al. using a PCR technique and mitochondrial 12S rRNA gene primers, were able to identify cows’ milk in raw goats’ or sheep’s milk binary mixtures (2004) and goats’, milk in sheep’s milk (2005b), at concentrations as low as 0.1%. Cheng et al. (2006) using a ‘chelex DNA isolation’ technique were able to replicate these results and identified cows’ milk or powder in goats’ milk or powder.

The application of PCR for the identification of animal milk types in cheese has also been very promising. Bottero and colleagues (2003) developed a multiplex PCR approach using a range of primers and managing to identify cows’, sheep’s and goats’ milk in the same cheese product with a maximum sensitivity of 0.5%. Relying on the mitochondrial 12S rRNA gene and using PCR, Lopez-Calleja et al. (2007) managed
to detect up to 1% cows’ milk in both goats’ milk and sheep’s cheese. Identification of cows’ milk in water buffalo milk of mozzarella cheese has also been performed using a PCR technique at a sensitivity level of 0.1% (Lopez-Calleja et al., 2005a), while detection of contamination of sheep’s milk cheese with goats’ milk to 1% levels has also been achieved (Lopez-Calleja et al., 2007). PCR techniques to authenticate feta cheese made exclusively from sheep’s milk have also been described (Branciari et al., 2000, Stefos et al., 2004). Recently, duplex PCR has been used as a faster and more economical alternative in identifying addition of cow’s milk in ‘buffalo’ cheese, (Bottero et al., 2002), cows’ milk in sheep milk cheese (Mafra et al., 2004) and cows’ milk in goats’ milk cheese (Mafra et al., 2007). Many of these techniques have been employed to detect and have verified the wide existence of adulteration of dairy products, indicating the need for more sensitive and specific detection tools such as PCR by the regulating bodies (Maskova and Paulickova, 2006, Mafra et al., 2007, Mininni et al., 2009).

At the moment PCR lacks certain practicality for routine commercial use but with further improvement it could become a very good option. Nonetheless, environmental conditions affecting somatic cell numbers in milk, such as animal mastitis or changes from raw to heat-treated milk, may adversely affect PCR results, even though a few recent techniques have suggested that PCR is unaffected by these conditions (Lopez-Calleja et al., 2005b).
1.2.4 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) is a biochemical technique using specific antibodies to detect specific antigens which have been immobilized, concurrently activating an antibody-linked enzyme which can then be visualized and quantified (Hurley et al., 2004b). The high sensitivity of the antibody-antigen complex makes individual milk species protein differentiation feasible and very useful for the authentication of dairy products. In addition, the technique is relatively fast compared to other techniques and is easy to use without the requirement for highly specialised equipment or staff. For this reason it has been one of the main techniques used in the authenticity of milk species (Moatsou and Aniftakis, 2003, Karoui and Baerdemaeker, 2007, Asensio et al., 2008).

A large number of ELISA techniques have been developed employing a wide variety of species-specific antibodies. These antibodies can be either monoclonal or polyclonal, the latter being more easily to produce but less specific. Monoclonal antibodies have been developed against cow β-casein (Anguita et al., 1997a) and cow IgG₁ and IgG₂ (Hurley et al., 2004a) for the detection of cows’ milk in sheep’s and goats’ milk and against goats’ αₛ₂-casein (Haza et al., 1996, Haza et al., 1997) for detection of goats’ milk in sheep’s milk. Milk polyclonal antibodies such as goats’ IgG derived from sheep (Aranda et al., 1993) have been shown to be able to detect both goats’ and/or cows’ milk adulteration of sheep’s milk or cheese. Polyclonal αₛ₁-casein cow antibodies have been able to detect cows’ milk in sheep’s milk and cheese within a range of 0.125 to 64% and 0.5 to 25%, respectively (Rolland et al., 1993). A polyclonal goats’ αₛ₁-casein antibody has also been developed with good reliability.
(Mimmo and Pagani, 1998). Using an indirect competitive ELISA technique and bovine $\gamma_3$-casein polyclonal antibodies Richter et al. (1997) were able to detect 0.1% cows’ milk adulteration of goats’ or sheep’s milk and cheese to 0.1%.

The development of monoclonal antibodies for the differentiation of milk species in cheese has also been performed. Lopez-Calleja and colleagues (2007), using monoclonal AH4 antibody against bovine $\beta$-casein, were able to identify cow milk in goats’ and sheep’s milk cheese at levels of 1 to 2%. A possible drawback however was that the addition of whey proteins to the sample could make the technique less accurate. Furthermore, a range of other polyclonal antibodies against proteins such as bovine $\beta$-lactoglobulin (Beer et al., 1996) and bovine $\alpha_{51}$-casein (Rolland et al., 1995) have been developed for cows’ milk recognition in sheep’s and goats’ milk cheese or sheep’s milk and cheese, respectively. The recent development of a polyclonal goat anti-bovine IgG antibody for the identification of cows’ milk in sheep’s or buffalos’ milk and goat milk yielded detection limits of 0.001% and 0.01% respectively (Hurley et al., 2006). The same antibody used in soft cheese had detection limits of 0.001% of cows’ milk in goats’ milk cheese and 0.01% in sheep’s and buffalos’ milk cheese (Hurley et al., 2006). Overall ELISA techniques appear at the moment to have great advantages compared to the other qualitative and quantitative techniques as discussed above. The sample analysis time of few hours does however leave space for further improvement of ELISA or the development of other faster and equally sensitive techniques that will be more practical for routine use in the dairy industry.
1.2.5 Spectroscopy

Infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy techniques have been used in the industry for the authentication of food products. These are easy to use, economical and non-destructive techniques. IR spectroscopy in combination with chemometric techniques is routinely used in the industry for quality control of milk and dairy products (Tsimidou and Boskou, 2003).

Mid-infrared reflectance (MIR) spectroscopy identifies molecules based on the absorption of IR light from molecular bonds over the range of 4000-400cm\(^{-1}\), while near-infrared reflectance (NIR) spectroscopy provides more structural information on the vibrations and overtones of bonds in the 14 000 to 4000 cm\(^{-1}\) region of the IR spectra (Banwell and McCash, 1994). NIR has shown good potential for detecting adulteration of cows’ milk with substances such as vegetable oil, urea, NaOH, detergent (Jha and Matsuoka, 2004) and water or whey (Kasemsumran et al., 2007); with the latter providing quantitative analysis limits of 0.244%. MIR and NIR have also shown some fairly good results in identifying the geographical location of production of Emmental cheese (Pillonel et al., 2003, Karoui et al., 2004b, Karoui et al., 2004a) with MIR providing up to 96.7% calibration and validation set accuracies. Despite its ease of use, some of MIR’s informative absorption bands can be obscured in the presence of water, making the analysis of aqueous samples difficult (Karoui and Baerdemaeker, 2007).

NMR spectroscopy has been used to differentiate between cows’ milk (Sacco et al., 2009) and buffalo milk mozzarella cheese (Brescia et al., 2005) from different
geographical locations, as well as in the differentiation between cows’ and buffalos’ milk (Andreotti et al., 2002), with good results. Despite these promising results and the additional information that this technique can provide, especially for heterogeneous samples compared to NIR and MIR, it has been suggested that this approach requires strict optimization of complex very expensive equipment for accurate results (Karoui and Baerdemaeker, 2007).

1.2.6 Sensory analysis

Human response to food involves the five senses: sight, smell, taste, touch and hearing; of which smell and taste appear to be of the most importance with respect to milk (Karoui and Baerdemaeker, 2007). Quantification of food properties such as aroma and flavour can therefore assist the food industry in understanding, measuring and interpreting the human response to particular products aiding in the development of more consumer appealing products. Techniques aimed to identify and quantify these sensory responses or product qualities are called sensory analysis techniques. In the dairy industry these can be used to investigate dairy product quality and authenticity, and they include electronic noses and electronic tongues.

1.2.6.1 Electronic nose

The e-nose system as already described, is a sensor-based system using a number of potentiometric sensor arrays, which creates a unique digital signature of a sample’s set of aromatic compounds, obtaining product information based on its aroma (Reid et al., 2006, Zhang et al., 2008a). A database of these digital patterns of various products
can then be created allowing for the future comparison of investigated samples and their subsequent identification or quality authentication if they are present within the database. E-nose technology has been used to study milk quality and authentication. An example of the studies performed include the use of milk rancidity and its correlation to milk aging (Capone et al., 2001), while Wang and co-workers (Wang et al., 2009) also used e-nose to distinguish six kinds of milk flavours with similar odour profiles. Furthermore, (Yu et al., 2007) successfully used e-nose to identify skim milk samples adulterated with water or reconstituted milk powder and were able to perform age discrimination of the samples for the first 4 days of ageing.

1.2.6.2 Electronic tongue

Electronic tongue (e-tongue) is a similar sensor-based system to the e-nose using the same principles to assess and quantify liquid product taste and flavour, by identifying different solution components using chemical sensors. In milk analysis, E-tongue has provided positive results in bacterial quantification during storage of fresh milk (Winquist et al., 1998). With the use of e-tongue Ciosek et al. (2006) managed to classify milk samples, with a 97% prediction rate, based on their fat content and originating dairy and without performing any sample pre-treatment. More recently, Dias and colleagues (2009) have managed to distinguish goats’ milk adulterated with bovine milk. In this study the authors were able to differentiate between the five basic tastes of sweet, salty, bitter, acid and umami and thus discriminated between goats’, cows’ and goat/cows’ raw skimmed milk with good sensitivity. However, the approach requires further testing and development.
The main disadvantage of these two newly developed techniques is that they can mostly differentiate between individual product characteristics, but are lacking in quantification and differentiation of milk samples adulterated with different types of milk, as these can contain milk types with very similar odours or flavours.

1.2.7 Summary of techniques’ qualities

In general, the chromatographic techniques, HPLC and GC, have been very good in the identification of milk adulteration, also showing promising results in the identification of cheese adulteration. On an industrial level however they are time-consuming, labour intensive and require sophisticated data analysis. Electrophoretic techniques, including the EU’s reference method IEF, despite good detection limits for milk adulteration with cows’ milk and some cost and sample-turnover time improvements achieved with PAGE and CE, are less accurate in detecting adulteration with other milk types; moreover, they remain lengthy and necessitate specialised staff and equipment.

PCR techniques are very accurate in detecting adulteration of milk and cheese, but more on an experimental laboratory basis, lacking practicality for commercialization, PCR also faces some accuracy issues with changes in sample somatic cell numbers depending on certain environmental conditions. ELISA techniques currently appear advantageous over other techniques, with sample turnover times and enhanced practicality for commercial use, being areas for further development.
IR spectroscopic techniques on the other hand, are non-destructive, easily usable and cost-effective, sensitive in the detection of milk adulteration with other liquids or substances. NMR is also promising but there is a very high equipment costs and the method requires optimization. Finally, sensory analysis techniques are new, providing some initial encouraging results but still lacking sufficient accuracy for quantification and identification of adulterated milk samples.
1.3 INFRARED SPECTROSCOPY

Infrared (IR) spectroscopy is a very useful technique for the determination of the structure and identification of different compounds. The greatest advantage of this method is that it can be used for almost any sample in any given state (Stuart, 1997). Because of their simplicity and non-destructive nature, IR analytical techniques have been used in a wide range of applications. Commercially, even though IR spectrometers have been around since the 1940s, it was not until more recently after the introduction of Fourier-transform spectrometers and the advances in computer technology, that spectrum quality and data time turnover has improved greatly. These aspects of IR spectroscopy have made it an important technique for studying and identifying biological molecules rapidly, with minimum sample preparation, giving IR spectroscopy two major advantages over other analytical techniques. Up to now, this technique has been used to distinguish successfully a number of biological systems including proteins, fats and carbohydrates, food and pharmaceutical products as well as cells and tissues from plants and animals (Stuart, 1997).

*Basic principles of IR spectroscopy*

The main principle of IR spectroscopy relies on the irradiation of a molecule with IR radiation and then detection of the energy frequencies absorbed which are highly specific for that molecule (Stuart, 1997). Depending on its shape each molecule can have a number of different fundamental vibrations absorbing energy at specific frequencies, based on the number of atoms contained and their three dimensional translational ability; these can be calculated using established rules (Banwell and McCash, 1994). Molecular vibration may also involve changes in the length of a bond.
or its angle (Banwell and McCash, 1994, Stuart, 1997). The change in the length of a
bond occurs during stretching vibration and bonds may stretch symmetrically (in
phase), or asymmetrically (out of phase). Bond angle changes occur during bending
vibrations (Banwell and McCash, 1994, Stuart, 1997). A molecular dipole moment
change has to occur because of the vibration though in order for the molecule to
absorb infrared radiation. In addition, the intensity of the absorption band increases
proportionally with the change in the dipole moment (Banwell and McCash, 1994,
Stuart, 1997). As each molecule only absorbs specific frequencies of IR energy
corresponding to its fundamental vibrational modes representing its bonds or
functional groups, the resultant IR absorbance spectrum can be used to identify the
molecular contents of an unknown compound and thus the compound itself (Cothup et
Yang and Irudayaraj, 2003).

Most of the useful diagnostic information from the IR spectrum is provided in the
mid-IR range (4000-400 cm\(^{-1}\)). This has two important regions, the group region
(3600 -1200 cm\(^{-1}\)) and the so called fingerprint region (1200 – 600 cm\(^{-1}\)). Group
frequencies are characteristic of molecular groups and are almost independent of the
structure of the whole molecule. The amplitude of the group vibration is only
significant for a small number of molecules. By contrast, the fingerprint region is
derived from the interaction of the vibrational modes and it is highly characteristic of
the molecule under investigation. Using information from both of these highly
specific regions differences in the structure and composition of compounds can be
seen, even when these are very small, enabling the identification of the specific
1.3.1 Development of IR spectrometers

1.3.1.1 Dispersive spectrometers

Dispersive infrared spectrometers were the first type of instrumentation to be used in order to obtain an IR absorbance spectrum. These were used widely from the 1940s onwards, originally using prisms composed of materials such as sodium chloride. During the 1960s cheaper and better quality grating instruments were introduced and the need for prisms declined. The basic components of the dispersive spectrometer are the source, the monochromator and the detector device. Source radiation is dispersed by the monochromator using a dispersive element such as a prism or grating, separating the components of polychromatic radiation based on their wavelength. The dispersed radiation is reflected towards an exit slit which only allows one wavelength element, with or without its multiples, to pass through. This energy then moves through the sample to the detector. The entire sample spectrum can be obtained by adjusting a component within the monochromator and allowing a different wavelength to pass through the exit slit at a time (Stuart, 1997). However, the latter process makes this technique very time-consuming and labour intensive, in addition to its high skill requirements. Furthermore, repeated sample irradiation results in sample overheating and damage. It is not surprising therefore that despite the early advances in spectral interpretation and the accumulation and publication of IR spectra libraries, dispersive IR spectrometry became less popular after the 1950s. The arrival of FT-IR in combination with cheaper and faster computer data processing in the 1980s reignited the interest in analytical IR spectrometry techniques.
1.3.1.2 Fourier transform infrared spectrometry

Fourier transform infrared spectrometry (FT-IR) spectrometers acquire the IR absorbance spectrum by combining the use of an interferometer and the mathematical processes of Fourier transformation. This technique is advantageous compared to others because of its speed (spectra can be obtained within 30s) and requirement for very little sample preparation prior to analysis. Furthermore, it is a simple technique to use, it has high sensitivity, it does not heat the sample and it is inexpensive to operate. For this reason FT-IR spectroscopy has become widely used both in the laboratory and in the industry. More specifically, FT-IR spectroscopy with the use of the interferometer achieves the production of an IR radiation signal across all wavelengths by splitting a signal into two radiation beams of known wavelength, allowing one of the beams to travel a range of different distances and then recombining the two beams into a new signal, allowing for interference and the production of a new wavelength (Stuart, 1997). The IR absorbance of a sample across all the IR frequencies can subsequently be detected. This analogue signal can then be converted into digital and with the use of Fourier transform technique utilizing elaborate mathematical algorithms the sample’s spectrum is calculated from the interferogram (Figure 1)(Stuart, 1997).

1.3.1.2.1 The source

The source of IR is normally an electrically conducting filament varying in type based on the IR range to be studied so as to provide sufficient radiation intensity. For the mid-IR region Nernst filaments, a mixture of zirconium, yttrium and erbium oxides requiring pre-heating to conduct electricity and ‘Globar’ filaments composed of silicon carbide are commonly used (Banwell and McCash, 1994, Stuart, 1997).
**1.3.1.2.2 Michelson interferometer**

The Michelson interferometer is one of the most widespread interferometers used in FT-IR. This consists of three components, a beamsplitter, a moving mirror and a fixed mirror. A parallel beam of radiation is transmitted from the source to the interferometer and originally reaches the beamsplitter. The beamsplitter is a semi-reflecting transparent film that splits the beam into two halves with 50% of radiation each. The amount of radiation in each split beam and the resultant IR spectral region produced depend on the beamsplitter construction material. Potassium bromide or caesium iodide substrates coated with germanium or iron oxide are usually used for the mid-IR region. As the radiation beam is split at the bisecting plane in front of the two aluminized or silver surfaced mirrors, one of the split beams is reflected at 90° to the stationary mirror and the second half is allowed to pass straight towards the moving mirror. In order to ensure accurate distance scanning and more precise measurement, the moving mirror is usually driven by a stepping motor.
measurements, the moving mirror is constantly aligned with the use of a visible helium-neon (HeNe) laser which is focused at the corner of the mirror.

Once the two radiation beams are reflected by the two mirrors, they recombine at the beam splitter and directed as a single transmitted beam, at $90^\circ$ to the source and towards the sample. Since each beam travels a different distance owing to the relative position changes of the moving mirror compared to the stationary mirror, an interference pattern is created where the beams merge. Depending on the length that the second beam has traveled the radiation waves can recombine in phase, interfering constructively with one another, or can recombine out of phase interfering destructively. IR radiation across all wavelengths is thus achieved. Once the transmitted beam is formed it passes through the sample, where some of the energy is absorbed. The remaining portion reaches the detector where the interferogram is recorded and subsequently translated into the sample absorbance spectrum using Fourier transformation (Figure 2) (Banwell and McCash, 1994, Stuart, 1997)
1.3.1.2.3 Detectors

Detectors of IR again vary on the IR range to be studied. Two main types of detector exist, the thermocouple detectors measuring the heating effect of radiation, and the photoconductor detectors measuring the conductivity produced by the resultant IR radiation. Commonly used detectors for the mid-IR region of the first type include the deuterated triglycine sulphate (DTGS) pyroelectric detector normally placed in an alkali halide window which is heat-resistant and of the second type mercury cadmium telluride (MCT) photoconductive detectors requiring cooling to liquid nitrogen.

Figure 2. Michelson Interferometer adopted from Banwell and McCash (1994)
temperatures, but providing higher sensitivity and faster response times (Banwell and McCash, 1994, Stuart, 1997).

1.3.1.2.4 Sampling methods

There are various ways and methods that can be used to examine samples either in the liquid, gaseous or solid form using IR spectroscopy. These include ‘transmission spectroscopy methods’, the most basic and oldest, modern reflectance methods such as attenuated total reflectance (ATR) and other more specialist techniques.

**Attenuated Total Reflectance (ATR)**

ATR FT-IR spectroscopy can be used to study otherwise intractable samples by investigating the chemical composition of their surface utilizing the principles of total internal reflection (Schmitt and Flemming, 1998). In ATR the sample is placed in contact with a crystal possessing a high refractive index and low water solubility, for example diamond, zinc selenide or germanium (Ellis and Goodacre, 2001). Beam radiation is then directed towards the crystal from the side. Total internal reflection then occurs when the incident radiation reaches the boundary between a medium with refractive index \( n_1 \), like the ATR crystal, and a medium with a lower refractive index \( n_2 \), like the sample, at an angle greater than the critical angle \( \theta_c \) (Lentz, 1995). During the total reflection though a fraction of the radiation enters the sample and part of it is absorbed while another part is reflected. The radiation which penetrates the boundary is termed ‘evanescent wave’ (Lentz, 1995). The resultant attenuated reflected radiation is then measured and plotted by the spectrometer. The absorption spectrum created is therefore characteristic of the particular sample (Figure 3) (Stuart, 1997). Furthermore, the area in which radiation may extend beyond the surface of the
crystal is termed ‘evanescent field’, with component vectors in all spatial directions. The intensity of this field decays exponentially as it spreads away from the crystal surface. The depth of penetration \(d_p\), is “the distance within which the intensity decreases to \(1/e\) of the value it had at the crystal-sample boundary (p.64)” (Lentz, 1995). This is given by the equation:

\[
d_p = \frac{\lambda}{2\pi n_1} \sqrt{\sin^2 \theta - \left(\frac{n_2}{n_1}\right)^2},
\]

where \(\lambda\) is the wavelength (in vacuum) and \(\theta\) is the incident angle (Lentz, 1995).

**Figure 3.** Schematic Diagram of ATR adopted from Lentz (1995)

### 1.3.1.2.5 Food Applications

Over the last few years FT-IR spectroscopy has been a very important tool in food analysis including authenticity and adulteration. The former is of most importance especially for the consumer and the food industry. Nutrient determination is time consuming and not appropriate for routine application in the food industries. Vibrational spectroscopy has been used successfully in a variety of areas like the identification of sugar in food, (Kameoka *et al.*, 1998), the identification and discrimination of polysaccharides in food additives (Cerna *et al.*, 2003), the analysis
of modified starches (Dolmatova et al., 1998) and for the rapid determination of total fat in a range of foods (Mossoba et al., 2001).

Investigating meat products Raman and FT-IR spectroscopies have been shown to be able to determine omega-6 and omega-3 fatty acids in pork adipose tissue (Olsen et al., 2008), with the latter technique also being able to discriminate carcasses of suckling lambs according to their rearing system when combined with partial least squares (PLS) and artificial neural network (ANN) analysis (Osorio et al., 2009). FT-IR microspectroscopy has been used to investigate the influence of heating rates and different heating temperatures on protein denaturation in pork (Wu et al., 2007) and beef (Kirchner et al., 2004), as well as to study the influence of ageing and salting on uncooked and cooked pork (Wu et al., 2006).

The quality of oil, an essential constituent of the food processing industry with 80 million tones produced each year (Cooke and Billingham, 1999), has also been investigated. FT-IR can reliably identify adulteration of vegetable (Alexa et al., 2009) and edible oils (Ozen et al., 2003, Vlachos et al., 2006), accurately quantify the moisture in edible oils (Al-Alawi et al., 2005) and the iodine content of palm oil products (Man and Setiowaty, 1999). In addition to these applications, ATR FT-IR in combination with multivariate analysis has also been successfully used in the determination of the origin of honeys (Ruoff et al., 2006, Hennessy et al., 2008).

The combination of FT-IR with multivariate statistical analysis has been used to detect and identify micro-organisms in apple juice (Lin et al., 2005, Al-Qadiri et al., 2006) and similarly ATR spectroscopy has been used successfully for identification
and enumeration of microbial load in poultry and beef (Ellis et al., 2002, Ellis et al., 2004). The latter method calculates microbial spoilage though metabolic fingerprinting which relates the detected metabolites at specific time points to the microorganisms’ metabolic activity. As spoilage in meat and milk is the result of a similar microbiological action, the combination of ATR spectroscopy with appropriate statistical methods can become a useful tool in the rapid enumeration of the total viable bacterial count in milk.

1.3.1.2.6 FT-IR spectroscopy and Milk

In the case of dairy products FT-IR has been used successfully for many applications. FT-IR ATR spectroscopy and chemometrics have been used effectively for the quantification of protein in raw cows’ milk (Etzion et al., 2004), using three different methods, PLS, artificial neural networks and simple band integration; while FT-IR has also been shown to be able to determine the urea concentration in milk (Baumgartner et al., 2003). Furthermore, FT-IR spectroscopy has been employed in the identification of the aerobic heat resistant microflora viable after high heat treatment in extended shelf life milk (Mayr et al., 2004). Diffuse reflectance FT-IR spectroscopy using cluster analysis can identify and differentiate between goat and sheep milk (Pappas et al., 2008), while PLS casein calibration of FT-IR spectrometers can be used rapidly and accurately to determine the casein content of milk (Luginbuhl, 2002), an important industry application as casein is the fraction of milk that precipitates during cheese-making.

The population dynamics of microorganisms on the surface of cheese has been studied using FT-IR (Oberreuter et al., 2003). FT-IR and FT-NIR spectroscopies have
been used to evaluate the shelf-life period of Grespenza cheese (Cattaneo et al., 2005). In this study cheese samples were analyzed at various time intervals during a 20 day period, and spectral data was analyzed using PCA. This was able to detect the reduction in cheese ‘freshness’ and was able to determine the day spoilage reached its critical level during shelf life. In addition, FT-IR ATR may be used for qualitative and semi-quantitative food contamination screening of a large number of ripened and semi-ripened cheeses by detecting cyclopiazonic acid a toxic metabolite produced by contaminating moulds (Monaci et al., 2007).

1.3.1.2.7 Microbiological Applications

FT-IR has become a very useful technique for the discrimination and determination of cultured bacteria (Timmins et al., 1998, Guibet et al., 2003, Winder et al., 2004, Winder and Goodacre, 2004). The identification of molecular structures based on their vibrational spectra can be applied to microorganisms, differentiating between similar bacterial strains rapidly and accurately without the use of any reagents (Maquelin et al., 2002a, Winder and Goodacre, 2004). Naumann and co-workers (1991) suggested this technique as a method of identification of unknown microorganisms. Using this method the infrared spectra of unknown microorganisms can be compared to an existing reference library and can be matched with the most similar spectrum of known species. This technique has already been used successfully for the differentiation of clinically relevant species such as Enterococcus and Streptococcus species (Goodacre et al., 1996) and Yersinia enterocolytica species and subspecies (Kuhm et al., 2009). It has also been employed effectively for the differentiation of Streptomyces isolates (Zhao et al., 2006), a particularly important species for the production of antibiotics, the detection and discrimination of the Genus
Carnobacterium (Lai et al., 2004), the Acinetobacter species (Winder et al., 2004), and different E. coli strains (Gilbert et al., 2009).

ANNs have been combined with FT-IR and Raman microscopy to yield some very encouraging results. Differentiation between Staphylococcus aureus species susceptible and resistant to methicillin has been achieved using the former (Goodacre et al., 1998a), while the latter has been shown to be able to discriminate between clinically significant bacteria causing urinary tract infections (Goodacre et al., 1998b).

FT-IR spectroscopy has been utilized successfully in the identification of cellular components within microorganisms at various stages throughout their lifecycle making it feasible to monitor the dynamic processes taking place within these live cells and identify their status (Helm and Naumann, 1995). Using this principle it has been demonstrated that the degenerative cells of strains such as the solventogenic Clostridium species can be discriminated and quantified from wild type microorganisms based on the different spectra obtained during the normal growth phase of these microorganisms (Schuster et al., 2001). Discrimination between injured and intact Listeria monocytogenes has also been achieved (Lin et al., 2004a). In addition, simple biomarkers can be used to differentiate between cells and spores using FT-IR (Goodacre et al., 2000).
1.4 RAMAN SPECTROSCOPY

Raman spectroscopy (RS) is an old technique which has become very important during recent decades because of the advantages resulting from the use of modern lasers and sensitive detection technology. The application of Raman spectroscopy in biological studies increased significantly in the late 1960s and early 1970s and it can be used for structural determination and distinction between large sample numbers (Maquelin et al., 2002a). Raman spectroscopy and FT-IR spectroscopy complement each other as both techniques produce their own vibrational fingerprint of the same molecules, but differ as FT-IR measures absorption of IR radiation whilst RS measures a change in frequency of the laser light used to probe the sample. RS is also a non-destructive technique and requires only a small amount of sample with minimum sample preparation (Maquelin et al., 2002a). Aqueous solutions can more easily be studied using this technique as water only appears to produce a weak Raman scatter, while conversely it has a very strong infrared absorption spectrum (Ferraro and Nakamoto, 1994).

Raman spectroscopy functions on the principle of Raman scattering (Raman, 1928). Normally, when a photon undergoes a collision with a molecule due to the elastic nature of this collision there is no energy exchange and the photon is re-emitted at the same frequency (Rayleigh scattering). Rayleigh scattering is therefore the dominant effect when radiation of a very narrow frequency is used, such as monochromatic radiation (i.e., a laser) in Raman spectroscopy. Raman scattering or Raman shift is the term used when additional specific discrete frequencies above or below the frequency of the incident beam also become scattered. This occurs because of the inelastic
nature of the photon-molecule collision resulting in an energy exchange during which a molecule gains or loses a minimal amount of energy emitting the radiation at a different frequency. An increase in molecular energy is associated with a lower frequency of scattered radiation in comparison to the incident beam, termed as Stokes radiation, while a loss of molecular energy, only occurring if molecule was previously in an excited state, has a higher frequency and is referred as anti-Stokes radiation (Banwell and McCash, 1994). In general, the majority of an incident beam is Rayleigh scattered resulting in only a very weak Raman effect, with Stokes Raman shift being the more intense and the one usually measured to produce the Raman fingerprint (Figure 4) (Banwell and McCash, 1994, Schrader, 1995, Turrell, 1996).

Figure 4. Vibrational States (Clarke, 2003)
1.4.1 Raman Instrumentation

Raman spectrometers consist of an excitation source, a collecting lens, a sample cell, a monochromator and a detector. The characteristics of a laser having a small diameter and high monochromatic beam and being able to be focused onto very small sample sizes make it an essential part of Raman spectroscopy (Banwell and McCash, 1994). The commonest excitation sources used in Raman spectroscopy are Ar⁺, Kr⁺ and He-Ne or a pulsed laser such as ruby, diode and excimer lasers. The laser beam from the source is focused onto the sample using a microscope or fibre optics. The scattered light is collected by a complex lens system, composed of a collecting and focusing lens, which allow the preliminary focusing of the incoming laser beam. This is then passed into a grating monochromator and subsequently the signal is measured by a sensitive detector device. Finally, it is then amplified and transferred to a computer where the Raman spectrum is plotted (Figure 5) (Banwell and McCash, 1994, Ferraro and Nakamoto, 1994).

The weakness of the Raman signal though creates many problems with its detection and amplification. Several detection techniques have been used over the years including photon counting devices, photodiode array devices and charge-coupled devices (CCDs). The use of the latter, as an optical array detector has been increasing during recent years. Composed of an array of pixels constituted out of silicon-metal-oxide-semiconductor material, the CCD is able to achieve a “low readout noise and high quantum efficiency and sensitivity over a broad range of wavelengths (p.112)” (Ferraro and Nakamoto, 1994). In addition, in an attempt to increase the intensity of the Raman effect, the surface enhanced Raman scattering (SERS) technique has been
developed which utilizes a suitably roughened surface as a substrate in contact with the molecules which enhances the Raman effect (Jarvis et al., 2004).

Figure 5. Schematic diagram of a Raman spectrometer adopted from Banwell and McCash (1994) and Ferraro and Nakamoto (1994)

1.4.2 Microbiological Applications

In the 1970s Raman spectroscopy enjoyed a broad application in biological studies, with UV resonance Raman (UVRR) spectroscopy being the primary Raman technique employed for cellular level study of microorganisms (Nelson and Sperry, 1991, Nelson et al., 1992, Maquelin et al., 2002b). More recent studies have successfully used UVRR spectroscopy for the characterization and differentiation of different microorganisms. For example, this technique not only allows for the discrimination of
**E. coli** strains but also successfully differentiates between pathogenic and benign strains (Yang and Irudayaraj, 2003) discriminates between endospore forming bacteria such as *Bacillus* and *Brevibacillus* at the species levels (Lopez-Diaz and Goodacre, 2004) and is able to classify different strains of lactic acid bacteria from yogurt (Gaus *et al.*, 2006). Furthermore, UVRR in combination with chemometric techniques can discriminate between closely related bacteria (Jarvis and Goodacre, 2004a).

In addition, in 2005 Rosch *et al.* (2005) reported Raman micro-spectroscopy using visible to NIR laser excitation as a fast reliable identification technique for the analysis of single bacteria, and a similar approach has been used as a rapid method for detecting *Acinetobacter* strains for epidemiological purposes (Maquelin *et al.*, 2006), for differentiation and categorization of *L. monocytogenes* strains (Oust *et al.*, 2006) and for single cell analysis as reviewed by Huang and colleagues (Huang *et al.*, 2010). Finally, SERS utilizing substrates such as gold nanoparticles (Premasiri *et al.*, 2005) and colloidal silver (Jarvis and Goodacre, 2004a, Jarvis *et al.*, 2006, Liu *et al.*, 2007, Guicheteau *et al.*, 2008), have been used for bacterial identification, characterization and discrimination.

### 1.4.3 Application in food

Over the last few decades Raman spectroscopy has also found many applications in the field of food science, as a promising analytical tool for quality and authenticity assessments. A major part of the literature focuses on the use of UV resonance Raman spectroscopy in biochemistry for the analysis of proteins and enzymes. In addition,
Raman spectroscopy has been used for the distinction and identification of protein structure and their components in various types of food.

1.4.3.1 Honey, Fruit, Oil, Meat and Fish

Fourier transform Raman spectroscopy (FT-RS) has been used to characterize various types of honey in different states (Oliveira et al., 2002), to determine the level of fructose and glucose in honey (Batsoulis et al., 2005) and has been shown to be able to discriminate between honey from different floral and geographical origins, a potentially simpler and faster technique than currently used techniques (Goodacre et al., 2002). In fruit analysis, RS has been used to characterize and quantify the constituents of certain important fruits (Baranski et al., 2005, Schulz et al., 2005, Baranska et al., 2006, Schulz et al., 2006, Silva et al., 2008), to discriminate olives according to fruit quality (Muik et al., 2004) and to measure the content of carotenoid pigments in fruit and vegetables (Bhosale et al., 2004).

In the oil industry, FT-RS has been shown to be a simple, rapid non-destructive tool for oil analysis (Weng et al., 2003), it is able to authenticate various edible oils, such as virgin olive oil (Lopez-Diez et al., 2003, Baeten et al., 2005), and fats (Yang et al., 2005), with better results compared to FT-IR spectroscopy in extra virgin oil adulteration determination (Yang and Irudayaraj, 2001), but marginally worse than FT-IR when combined with discriminant function analysis (DFA) for the classification of other edible oils and fats (Yang et al., 2005). Similarly, when combined with partial least squares (PLS) RS can accurately estimate the free fatty acid content in olive oil, with a root mean square error of prediction (RMSEP) of only 0.29% and RMSEP of 0.28% directly in olives, making it a potentially useful tool for
on-line quality control (Muik et al., 2003). Near-infrared FT-RS in combination with PLS is able to determine the total unsaturation of fats and oil, a potentially useful tool in industrial quality control limited only by the cost of the Raman spectrometer (Barthus and Poppi, 2001) and has been shown to be accurate in predicting relative oil composition and oil quality (Schulz et al., 2002).

RS has also seen further applications in the meat industry, especially in the assessment of meat quality, displaying good correlation between specific protein structural changes and textural properties of raw pork (Herrero et al., 2008), cooked beef (Beattie et al., 2004b), meat in frozen storage (Herrero, 2008), and has been used in combination with PLS to predict the ‘water-holding capacity’ of meat after slaughter (Pedersen et al., 2003). Combined with PCA it can monitor the transition of meat from edible to inedible, correlating well with microbial load (Schmidt et al., 2009); while other studies using DFA have shown that it can discriminate between various types of pre-packaged meat and poultry muscle types for authentication purposes (Ellis et al., 2005).

Similar to animal meat, RS with PLS has been shown to be a potentially useful technique for the assessment of fish quality, it is able to identify and quantify fat, collagen and pigment content in fish muscle (Marquardt and Wold, 2004). This analytical technique has also been used to study the changes in protein structure of various types of fish during freezing under different conditions (Careche et al., 1999, Ogawa et al., 1999, Sultanbawa and Li-Chan, 2001, Badii and Howell, 2002, Herrero et al., 2005).
1.4.3.2 Milk and Dairy Products

Raman spectroscopy has also found application in the dairy industry. In combination with multivariate data analysis, it has been used to quantify the constituents and assess the nutritional parameters of infant formulae and milk powder (Moros et al., 2007), to quantify different amounts of conjugated linoleic acid in cows’ milk fat (Meurens et al., 2005, Bernuy et al., 2008) and has been found to be an accurate tool for screening milk powder for the inappropriate addition of melamine, a raw ingredient used in plastic manufacture, added in milk powder to increase its nitrogen content and thus its presumed protein content (Okazaki et al., 2009). Furthermore, RS has been used to characterize milk whey protein components (Liang et al., 2006) and FT-RS has been shown to be able to predict accurately the unsaturated fat content of clarified butter potentially for use in quality control (Beattie et al., 2004a).
1.5 MASS SPECTROMETRY

Common analytical techniques, such as the ones previously discussed, work on the principle of detecting the energy released from a molecule returning to its ground energy level after it has been irradiated with electromagnetic radiation which had put the molecule into a higher energy level. In contrast, mass spectrometry (MS), identifies species by selectively detecting ions it creates based on their mass to charge \((m/z)\) ratio, and is thus a destructive technique. In MS, the sample undergoes vaporization and it is ionised by a variety of ionisation methods, often resulting in the gain or loss of electrons when electron ionisation is used. This also results in the fragmentation of the compound, through different fragmentation pathways, into a pattern of ions characteristic for that compound. These ions are then accelerated typically by an electric field which separates ions according to their \(m/z\) ratio. The different ions are detected at the receiver and a mass spectrum is then created, which, in combination with the fragmentation pathways and patterns detected, can be used to identify and quantify the compound under investigation (Figure 6) (Davis and Frewarson, 1987, Siuzdak, 1996).

1.5.1 Sample Introduction

The introduction of a sample into the mass spectrometer has its own specificities as a sample under atmospheric pressure needs to be introduced into the MS vacuum without disturbing the vacuum. This can either take place with the use of a direct insertion probe onto which the sample is introduced into the ionisation region of the
MS using a vacuum lock, or the sample can be infused through a capillary column. The latter technique can be combined with other separation techniques such as GC and LC (Davis and Frearson, 1987, Siuzdak, 1996).

1.5.2 Ionisation Techniques

A number of ionisation techniques exist, having a range of sensitivities and detecting a range of molecular masses, of samples at different phases. One of the first ionisation techniques to be used was the electron ionisation (EI), and was used as the main MS ionisation technique until the 1980s. This requires a gaseous sample that can be obtained by thermal desorption of the sample, this sometimes leads to its decomposition. The gas then interacts with an electron beam, resulting in electron ejection and fragmentation. The main limitation of EI is that it can only be used to analyse compounds with molecular weight below 400 Da and causes fragmentation so sometimes the molecular ion is not detected (Siuzdak, 1996).

Matrix-assisted laser desorption ionisation (MALDI) is a technique which produces ions by applying a laser beam onto a sample which has been embedded into a non-volatile material called a matrix which aids the creation of ions. The matrix contains an excess of small organic molecules in relation to the analyte. The matrix absorbs the incoming radiation, accumulating large amounts of energy via electron excitation of the matrix molecules, leading to desorption and ionisation by proton transfer between the matrix and analyte molecules in the vapour phase over the sample plate (Karas et al., 1987). This technique requires only small sample quantities, can analyse heterogeneous biological samples with very high sensitivity with a very high mass
range of typically up to 300 000 Da for proteins and can therefore be used both for quantitative and qualitative sample analysis (Siuzdak, 1994, Buhimschi et al., 2008).

Electrospray ionisation (ESI), the other commonly used soft ionisation technique, requires a sample in its liquid phase which is then sprayed from a highly electrically charged field to create highly charged droplets evaporated under the subsequent influence of a dry gas, heat or both, creating singly and multiply charged molecules. This technique has a high sensitivity, with a typical mass range of up to 70 000 Da for proteins (Davis and Frearson, 1987, Hoffmann et al., 1996, Siuzdak, 1996, Downard, 2004).

### 1.5.3 Mass Analyzers

The second integral part of the MS, the mass analyzer (MA), is involved in the separation of ions based on their $m/z$ ratio. The original MAs developed in the early 20th century performed this function with the use of magnetic fields. The use of magnetic fields has been replaced in modern MAs to provide higher accuracy and sensitivity over bigger mass ranges, also able to provide some structural information on large biomolecules. The type of MA used can therefore affect the qualities of a particular MS instrument (Siuzdak, 1996).

The quadrupole analyzer, acts as a mass filter, by creating a radio frequency field between four rods carrying direct electricity current, only allowing ions of specific $m/z$ ratio to pass through to the detector. These quadrupole analysers are relatively cheap and are commonly used, especially in conjunction with ESI but poorly
adaptable to MALDI. The ion trap MA works on the same principles as quadrupole analysers and because of its small size and simple design is used to select specific ions for sequential MS which involves further ion fragmentation to obtain structural compound information (Davis and Frearson, 1987, Hoffmann et al., 1996, Johnstone and Rose, 1996, Siuzdak, 1996, Downard, 2004).

Figure 6. Schematic diagram of a time-of-flight (TOF) mass spectrometer adopted from Davis and Frearson (1987) and Siuzdak (1996)

Time-of-flight (TOF) MAs (Figure 6) are simple instruments, accelerating ions with the same amount of kinetic energy towards the detector, which records the time of arrival of the ions, which corresponds to their \( m/z \) ratio. They have no mass limitations, are easily adaptable to MALDI and ESI, but have low resolution. To increase resolution the TOF reflectron MA has been developed, adding a component to reflect the ions back to the detector and thus increasing their time of arrival, but

Fourier-transform ion cyclotron resonance MS (Marshall et al., 1998) and Orbitrap mass analysers (Perry et al., 2008) are newer instruments with high resolution and costs with higher accuracy potential for use with ESI and MALDI (Davis and Frearson, 1987, Siuzdak, 1996).

### 1.5.4 Ion detectors

This is the last part of the MS, which detects ions by converting their kinetic energy into a current which can then be detected and transferred to a computer to be translated into the mass spectrum. A few types of ion detectors exist. The most commonly used are the Electron Multiplier (EM) and the Microchannel Plate (MCP) EM detectors.

In the EM the electrical current is generated by the movement of electrons towards the ions arriving onto the dynode surface of a Faraday cap composed of emitting materials such as BeO, GaP, or CsSb. The emitted electrons are subsequently attracted to neighbouring caps of increasing electrical potential, successively increasing in numbers, with a typical EM current amplification of $10^6$ (Davis and Frearson, 1987, Hoffmann et al., 1996, Siuzdak, 1996, Downard, 2004).
The MCP detector is composed of a plate containing a series of small (10µm) angled channels to the incoming ions. Once the ions enter any of the channels they are deflected along the walls of the channels emitting electrons as they move towards the opposite side of the channel towards the anode. This process results in current amplifications of the order of $10^5$, able to reach gains of the order $10^8$ when several plates are used together (Downard, 2004).

1.5.5 MALDI-MS and Food Analysis

1.5.5.1 Meat, Honey, Fish, Oil and Fruit

In the field of food analysis MALDI-MS has been used for the identification of biological compounds and metabolic pathways, especially protein and peptide characterization and identification, that may be utilized to improve food quality, safety and consistency. MALDI-TOF-MS has been used to study muscle proteomics in post-mortem porcine muscle in relation to meat tenderness (Lametsch et al., 2003, Montowska et al., 2009), in bovine (Jia et al., 2006) and sea bream (Schiavone et al., 2008) muscle during post-mortem storage and in chicken muscle in relation to rapid growth (Doherty et al., 2004). MALDI-MS has also been used to study the effect of various microorganisms during porcine sausage ripening (Basso et al., 2004), in combination with immunomagnetic isolation has been able to detect the *E. coli* 0157:H7 serotype (Ochoa and Harrington, 2005), while its combination with multivariate analytical and chemometric techniques has allowed for the discrimination between the *E. coli* MC1061 serotype and *Yersinia enterocolytica* in beef (Parisi et al., 2008).
Furthermore, MALDI-TOF MS has been employed in the qualitative assessment of oligosaccharides and polysaccharides in bee and acacia honey (Morales et al., 2006, Megherbi et al., 2008), to investigate the proteomic changes during grape berry ripening (Giribaldi et al., 2007, Zhang et al., 2008b) and for the determination and quantification of fatty acid composition in oils (Asbury et al., 1999, Ayorinde et al., 2000, Lay et al., 2006). Importantly, it has been successfully utilized in the authentication of honey (Won et al., 2008, Wang et al., 2009) and fish (Mazzeo et al., 2008), based on protein fingerprinting and characterization, and has been used successfully to distinguish between eight different types of oil based on their triacylglycerol composition (Jakab et al., 2002).

1.5.5.2 Milk

In the area of milk the proteomic analysis properties of MALDI-TOF MS have been used to analyze milk proteins (Dai et al., 1999), the constituents of the milk fat globule membrane of bovine milk (Vanderghem et al., 2008), which possess various health-related properties, and for profiling of milk peptides during the period of thermal processing and subsequently while in storage at 4 °C (Meltretter et al., 2008). MALDI-MS has also been employed in the characterization and fingerprinting of important casein proteins in bovine (Lopes et al., 2007) and goats’ milk (Galliano et al., 2004), as well as phosphopeptides in cows’ milk (Chen et al., 2007, Zhou et al., 2009), in the detection of allergens in cows’ milk (Natale et al., 2004) and in the authentication of raw ewe or water buffalo milk adulterated with cows’ milk (Cozzolino et al., 2001). In combination with LC, MALDI-MS has been used to identify polymorphic variants and low abundance proteins in milk (Ji et al., 2005).
MALDI-TOF MS has been used in other dairy milk products as well. It has been used to detect the changes in milk protein pathways in yogurt caused by the commonly used *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* bacteria, under different conditions (Fedele *et al.*, 1999) and to detect specific casein proteins causing bitterness in cheese (Soeryapranata *et al.*, 2002). Finally, it has been utilized in the authentication of adulterated water buffalo mozzarella and ewe cheese (Cozzolino *et al.*, 2002).
1.6 MULTIVARIATE ANALYSIS

Multivariate analysis or chemometrics is the main statistical method used in qualitative and quantitative analysis of spectroscopic data. These methods attempt to explore the relationship between several related independent variables with the assumption of equal importance at the start of the analysis (Manly, 1994). A range of techniques under this type of analysis are used for the analysis of FT-IR and Raman spectra. The reason behind the use of multivariate analysis is that the vibrational spectra produced are highly complex and multidimensional containing potentially vast amounts of useful data. For example, a spectrum from FT-IR or Raman may contain wavenumbers ranging in numbers from 600 to more than 2000, with each wavenumber representing a separate variable, with the single spectrum containing important quantitative and qualitative biochemical information. The same is true for MS data. However, in contrast to MS, for FT-IR and Raman these data are not independent variables as a peak is sampled many times. Hundreds or thousands of these spectra are collected during a series of experiments with a similar increase in the number of acquired variables. As the visualization of this hyperspace would be very complicated and difficult, multivariate analysis is used in order to simplify the data and reduce the dimensionality (Ellis and Goodacre, 2001, Goodacre, 2003, Goodacre and Kell, 2003). In general, multivariate analysis is separated into two main types, unsupervised and supervised analysis.
1.6.1 Unsupervised techniques

Unsupervised techniques are used when no prior knowledge is required regarding the sample class. These techniques make no assumption regarding the relationships between the samples under investigation and present the obtained data (referred to as X-data) to an algorithm. These algorithms analyse them and attempt to find any interesting features that may represent similarities or dissimilarities between spectra, subsequently providing a visual representation of these patterns (Goodacre, 2003, Goodacre and Kell, 2003). Unsupervised multivariate statistical analysis in this work was achieved using principal component analysis (PCA) and discriminant function analysis (DFA) (this is primarily a supervised technique but can be implemented in an unsupervised fashion) (Figure 7). These techniques can be described as mainly qualitative, as they attempt to distinguish groups or clusters within the data, based on their perceived closeness that may represent a particular characteristic (Ellis and Goodacre, 2001, Everitt et al., 2001). Data analysis using these techniques was performed using the software programme Matlab, version R2007b.

1.6.1.1 Principal component analysis

PCA is a well established analysis technique. It attempts to simplify the collected spectral data by reducing the number of variables into a smaller number of new indices named principal components (PCs) at the same time maintaining the original data variance (Manly, 1994). This is achieved as each PC is a linear combination of a group of variables providing a summary of these data, but is completely uncorrelated to the next PC, and it is represented as a different dimension. The PCs are also
arranged such that the first PC represents the greatest degree of variance among the variables, with subsequent PCs displaying a descending degree of variance. The sum of the variance of all PCs therefore represents all the variation in the original data, with the first five PCs for infrared and Raman data typically representing greater than 95% of this variance (Jolliffe, 1986, Manly, 1994). If the original data are completely uncorrelated between them then PCA cannot work as the number of PCs will be very similar to the number of the original variables. In general, the lower the degrees of variance represented by the PCs, i.e. the lower the original variable variance, the smaller the number of PCs produced, with significant variance between them. PCs can then be plotted and the data can be visualized allowing for better understanding of the original data and allowing further data manipulation (Jolliffe, 1986, Marley et al., 2001).
FT-IR spectrum:
Huge number of qualitative and quantitative information (e.g. 400-1000+ wavenumbers) represented in peak/spectral waveforms

Principal Component Analysis:
Reduces number of variables into new multidimensional indices called PCs, maintaining original sample variance (>95%)

Discrimination Function Analysis:
Primarily a supervised algorithm also able to be used in the second part of ‘unsupervised’ analysis. It minimizes intra group variance and maximises inter group variance using Mahalanobis distances.

Figure 7. Flowchart of the unsupervised multivariate statistical methods used adopted from Goodacre et al. (1998b)

1.6.1.2 Discrimination function analysis

DFA is a supervised algorithm in that a priori knowledge is used to analyze data. However, it can be involved in the second part of ‘unsupervised’ multivariate analysis when this method does not use any knowledge in regards to any ‘biochemical’
relationship between the data and is used to discriminate the data into groups based on
the derived PCs from PCA and the prior knowledge of only spectra replicates (Manly, 1994, Dytham, 1999). Its purpose is to minimize the variance within a group and
maximise the variance between groups. The discrimination approach is mostly based
on Mahalanobis distances (Mahalanobis, 1948, Manly, 1994). This approach takes the
mean vectors of the PCs to represent the true mean vectors of the samples and
calculates the distance of each PC to group centers, allocating each PC to its nearest
group centre. A higher percentage of PCs allocated to their correct group, is an
indication of how good group separation is when employing those variables and the
greater the distance between groups on 2-D or 3-D representation, the more dissimilar
the groups are between them (Manly, 1994).

This method can be validated using different methods, including comparison of
distances. In this, distances between classes must be found to be greater than the
distances within a class. Another way to validate DFA is to test the model. This is
achieved by separating the samples into two sets, a training set used to elaborate the
methods and a test set to validate it (Mariey et al., 2001).

1.6.2 Supervised Techniques

The presence of a ‘gold standard’ data set, in the case of our experimentation the total
viable counts (TVCs) of bacteria and the percentage of milk adulteration, allows for
the use of more powerful techniques for data analysis, namely supervised techniques
(Goodacre, 2003). The aim of supervised learning is to construct the best association
model yielding the smallest difference between inputs, for example the data collected
from spectroscopy, and targets, representing the desired response such as the bacterial TVCs or percentage of milk adulteration, where both inputs and targets are already known (Martens and Naes, 1989). Input and target data form pairs which are conventionally called X-data (inputs) and Y-data (targets). A number of algorithms exist in supervised learning of which some are used for qualitative analysis while others are employed for quantitative analysis (Ellis and Goodacre, 2001, Goodacre, 2003, Goodacre and Kell, 2003). If DFA is used with \textit{a priori} knowledge about biochemical data then it can be used in a supervised way.

1.6.2.1 Partial least squares

Partial least squares (PLS) is a multivariate projection method that uses an algorithm based on linear regression methods, and can be used to analyze the level of metabolites in biological samples and correlate that to microorganism numbers. It can be used to find the primary quantitative relations between two matrices (X and Y) (Martens and Naes, 1989). PLS performs this function by modeling the relationship between dependent variables (X) and independent variables (Y). The optimum model is created by detecting the factors in the input matrix (X) that best describe the input variables’ variance and at the same time provide the greatest correlation to the target variables (Y) (Berrueta \textit{et al.}, 2007). X matrix optimal decomposition occurs by the extraction of these factors in a decreasing relevance order, with Y matrix as a guide. Variations that are irrelevant or be a cause of noise, because of high fluctuation, are therefore underweighted, making PLS less susceptible to errors and maximizing covariance between the X and Y matrices (Martens and Naes, 1989, Cullen and Crouch, 1997, Berrueta \textit{et al.}, 2007).
To obtain the best results from the PLS modeling process three sets of data are usually required, and this was implemented in this thesis. Each set of data contains X and Y data as described above. The first set of data is called the training data and is used to derive the PLS model. The second data set consists of corresponding data matrices and is used to cross validate and calibrate the derived model. The third set of data is obtained from a separate experiment and the input X values are used with the model to obtain the predicted Y values, which are then compared to the actual target Y values.

In addition to PLS, kernel PLS (KPLS) a non-linear extension of the former technique was employed (Shawe-Taylor and Christianini, 2004). This was done in order to explore the possibility that the spectral information obtained may not always be linear and therefore this non-linear regression technique would be able to provide us with additional information and explore the relationships within data even further.
1.7 AIMS AND OBJECTIVES

Responding to the requirements of the analytical dairy industry, this project aims and objectives were focused on the investigation of analytical techniques in combination with multivariate analysis techniques for faster and more accurate results in regards to the microbial spoilage of milk, the quantification and characterisation of certain pathogenic and common milk microorganisms, as well as the detection of milk adulteration.

More specifically, in the first part FT-IR spectroscopy using attenuated total reflectance (ATR), and a high throughput (HT) transmission in combination with supervised learning such as partial least square regression (PLSR), were employed to detect and enumerate viable bacterial cell numbers in the three types (whole, semi-skimmed and skimmed) of pasteurized cows’ milk, with speed and precision.

In the second part, the validity and accuracy of FT-IR and Raman spectroscopies in combination with multivariate analysis were explored, for the identification and quantification of pathogenic and common milk spoilage bacteria such as *Staphylococcus aureus* and *Lactococcus lactis* subsp *cremoris*, inoculated individually or in combination in ultra-heat treatment (UHT) sterilized milk. Furthermore, the growth and metabolic interaction of the two microorganisms in milk was explored.
In both of the previous parts bacterial sample loads derived from the spectroscopic and subsequent multivariate analyses were correlated with bacterial counts obtained from classical microbiological plating methods such as total viable bacterial counts.

The two last parts of the research project explored the potential of FT-IR spectroscopy and MALDI-TOF mass spectrometry, again in combination with multivariate analysis such as PLS and KPLS, as potentially useful techniques for the detection and quantification of adulterated milk. Milk samples tested were from widespread commercially used animal milk types such as cows’ milk and the more expensive goats’ and sheep’s milk.
CHAPTER 2

RAPID AND QUANTITATIVE DETECTION OF THE MICROBIAL SPOILAGE IN MILK USING FOURIER TRANSFORM INFRARED SPECTROSCOPY AND CHEMOMETRICS

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Microbiological safety plays a very significant part in the quality control of milk and dairy products worldwide. Current methods used in the detection and enumeration of spoilage bacteria in pasteurized milk in the dairy industry, although accurate and sensitive, are time-consuming. FT-IR spectroscopy is a metabolic fingerprinting technique that can potentially be used to deliver results with the same accuracy and sensitivity, within minutes after minimal sample preparation.

We tested this hypothesis using attenuated total reflectance (ATR), and high throughput (HT) FT-IR techniques. Three main types of pasteurized milk; whole, semi-skimmed and skimmed, were used and milk was allowed to spoil naturally by incubation at 15°C. Samples for FT-IR were obtained at frequent, fixed time-intervals and pH and total viable counts calculated were also recorded. Multivariate statistical methods, including principal components-discriminant function analysis and partial least squares regression (PLSR), were then used to investigate the relationship between metabolic fingerprints and the total viable counts.

FT-IR ATR data for all milks showed reasonable results for bacterial loads above $10^5$ cfu/ml. By contrast, FT-IR HT provided more accurate results for lower viable bacterial counts down to $10^3$ cfu/ml for whole milk and, $4.10^2$ cfu/ml for semi-skimmed and skimmed milk.

Using FT-IR in combination with PLSR a metabolic fingerprint and quantification of the microbial load of milk samples was accurately and rapidly obtained, with very little sample preparation. The rapid detection and enumeration of bacterial loads in
milk, using metabolic fingerprinting though FT-IR, therefore appears to be a very promising technique for future use in the dairy industry.
2.1 INTRODUCTION

Milk is an important constituent of our diet containing a variety of nutrients, and it is essential for good bone development in infants and children. It is therefore not surprising that a big proportion of the world’s population uses milk on a daily basis. Quality control of milk and milk products is therefore of paramount importance. Outbreaks of foodborne illnesses associated with milk and dairy product consumption have been found in the past to be contaminated with pathogenic microorganisms such as *Listeria* spp., *Salmonella* spp., *Campylobacter jejuni*um and *Yersina enterolytica* (Zall, 1990). Microbial analysis of milk and dairy products therefore has a critical role to play in the quality evaluation of these products, promoting public health safety.

Spoilage is a relative term in dairy products as this is linked to the organoleptic changes occurring within the product making it undesirable to the consumer (Jay, 1996, Whitfield, 1998, Gram *et al.*, 2002). These organoleptic taints, among others, cause defects in appearance, unpleasant odours and flavours, and curdling, characteristics which would make any food unacceptable for human consumption (Whitfield, 1998, Ellis and Goodacre, 2001, Ellis *et al.*, 2002, Gram *et al.*, 2002). The organoleptic changes appear to be the result of microbiological spoilage and the compounds responsible for these changes are the various metabolites produced by the metabolic activity of these microorganisms (Gram *et al.*, 2002). The type of change produced varies according to the species of the microorganisms present in milk, the chemical composition of milk and the physical environment under which it is stored (Frank, 2001).
Milk is an ideal medium for microbial growth as it is mainly composed of water and contains a broad range of nutrients which can be used by microorganisms as an energy source (Frank, 2001). The main components of whole milk are 87.3% water, 4.8% carbohydrates (mainly lactose), 3.7% fat, 3.2% proteins, and 1% non-protein nitrogenous compounds, minerals and vitamins (Frank, 2001). Furthermore, its pH is almost neutral ranging from 6.5 to 6.7 making it an ideal growth environment for bacteria (Adams and Moss, 2000). Temperature is another factor that can influence milk spoilage as the temperature range between 2 to 30 °C is considered to be ideal for spoilage microorganisms’ growth. At the lower limits of this temperature range the growth of one particular bacterial species, the Gram negative *Pseudomonas*, is dramatic. The other major members of the spoilage flora on pasteurised milk include the endospore forming bacteria of the *Bacillus* genera, and other Gram positive rods and cocci such as *Lactobacillus*, *Corynebacterium* and *Lactococcus* species (Chandler and McMeekin, 1985, Griffiths and Phillips, 1988).

The application of the Hazard Analysis and Critical Control Point (HACCP) system in the dairy industry, in order to maintain high quality levels during manufacturing and production of foods for safe consumption, has increased the requirements for rapid and more automated microbiological techniques (Bernard, 2001). In the recent past several methods and instruments have been developed for the identification, detection, enumeration and the characterisation of microorganisms in milk and dairy products (Vasavada, 1993, Fung, 1994, Karwoski, 1996, Adams and Moss, 2000). However, none of these methods have so far been ideal. In the absence of a simultaneously rapid, accurate and sensitive method, the dairy industry currently relies on classical microbiological plate counting techniques and other methods for the detection and
enumeration of spoilage bacteria in pasteurized milk which typically take 1-2 days; but may take longer depending on the growth of the organism. The dairy industry therefore requires a method for the detection and enumeration of bacteria in dairy samples which will be accurate and sensitive, must be able to measure viable bacterial numbers and provide results within 2 to 3 hours so that appropriate measures within HACCP can be made (Ellis et al., 2002, Ellis et al., 2004).

Fourier transform infrared (FT-IR) spectroscopy is a metabolic fingerprinting technique that can potentially be used to reduce this time significantly, by measuring the biochemical fingerprints produced through the metabolic activity of the viable microorganisms in milk and delivering results within minutes after very minimal sample preparation. Its validity has also recently shown in the detection of spoilage in meat (Ellis et al., 2002, Ellis et al., 2004). In FT-IR spectroscopy the sample is irradiated with IR radiation (usually in the mid IR range; 4000-600cm$^{-1}$) resulting in the absorption of specific energy frequencies by a particular molecule within functional groups as it is excited to a higher energy level, usually reaching its first vibrational excited state. As each individual molecule has its own fundamental vibrational modes and only absorbs energy when the frequency of the irradiated infrared energy is the same as the frequency of one of its vibrational modes, a highly specific infrared absorbance spectrum is created (Cothup et al., 1990, Banwell and McCash, 1994, Stuart, 1997, Schmitt and Flemming, 1998, Yang and Irudayaraj, 2003). The main advantage of this technique is that it is very fast as a spectrum can be obtained within a few seconds after minimum sample preparation (Ellis et al., 2002, Ellis et al., 2004). Furthermore, it is a simple technique to use, it has high sensitivity, and it is inexpensive to operate. This technique in combination with appropriate
multivariate statistical methods, including partial least square regression (PLSR), may therefore be an attractive solution for the detection and enumeration of bacteria in dairy samples.

Therefore, the aim of this study was to investigate the ability of FT-IR spectroscopy to quantify the bacterial contamination of the three types (viz., whole, semi-skimmed and skimmed) of pasteurised cow’s milk accurately, sampling using attenuated total reflectance (ATR), and a high throughput (HT) transmission-based technique with PLSR analysis.
2.2 MATERIALS AND METHODS

2.2.1 Sample Preparation

Three cartons of milk with the same use-by-date, each of a different type of milk (whole, semi-skimmed and skimmed milk) were obtained from a national retail outlet. The milk was then separately poured into sterilized flasks 1L and was placed in a rotation incubator at 15°C and 200 revolutions per minute (rpm). Samples were then taken at 8 hourly intervals for 104 hours. Once samples were obtained they were mixed for 1 min and then the organoleptic changes and pH of each type of milk were recorded and the total viable bacterial counts were also determined using classical microbiological plating method (see below). 15 mL of the milk samples were divided to 1 mL volumes and preserved at -80°C. Six of these aliquots were used for ATR FT-IR and HT FT-IR analysis.

2.2.2 Total Viable Counts (TVCs)

TVCs were measured according to the national standard method (Health Protection Agency, 2004). Using peptone saline diluent (containing 1.0 g peptone, 8.5 g sodium chloride in 1L distilled water) serial dilutions were undertaken. Each dilution was mixed for 1 min and 1 mL was inoculated into three Petri dishes. Subsequently, milk plate count agar (containing 2.5 g yeast extract, 5.0 g tryptone, 1.0 g glucose, 1.0 g skimmed milk powder, 15.0 g agar in 1 L distilled water) was added, mixed with the inoculum, and incubated aerobically at 30°C for 72h. The plate colonies were then counted and the total viable count per mL was calculated. The number of colonies per
plate was only taken into account when it was between 30 and 300. The number of viable microorganisms per milliliter of sample was calculated using the equation:

\[ N = \frac{\Sigma c}{(n_1 + 0.1n_2)d} \]

where:

- \( \Sigma c \) is the sum of the colonies counted from all plates (between 30-300 colonies),
- \( n_1 \) is the number of plates counted at the first dilution,
- \( n_2 \) is the number of plates at the second dilution and
- \( d \) is the dilution from which the first counts were obtained (p.8)” (i.e., least dilute) (Health Protection Agency, 2004).

2.2.3 Attenuated Total Reflectance (ATR) FT-IR Spectroscopy

A ZnSe Gateway ATR Horizontal 6 Reflection accessory (Specac Ltd, London) on a Brucker Equinox 55 infrared spectrometer mounted with a DTGS (deuterated triglycine sulfate) detector (Bruker Ltd, Coventry, UK) were utilized for FT-IR analysis. For ATR the evanescent wave allows penetration into the surface above the crystal and this was calculated to be 0.98 µm at 1500 cm\(^{-1}\) (arising from the C=O vibration of Amide I band) and 0.51 µm at 2900 cm\(^{-1}\) (from centre of fatty acids CH\(_x\) stretches) (Stuart, 1997).

Samples were defrosted on ice, one sample at a time, and were then mixed for 1 min. 800µl aliquots were then taken from the sample and positioned directly on the ZnSe crystal in order to acquire the sample’s spectrum. In total 6 replicates were taken from each time-point. Between samples the crystal surface was initially cleaned with
distilled water, then with analytical grade acetone and again with distilled water and
dried with a soft cloth and left for ~5 min to air dry. Prior to making any sample
measurements, reference spectra were acquired from the clean blank crystal. All the
spectra were collected within the wavenumber range of 4000 to 600 cm\(^{-1}\) with a
resolution of 8 cm\(^{-1}\) and the signal-to-noise ratio was improved by co-adding and
averaging 64 scans. In total 252 spectra were collected for every type of milk in the
series of three experiments and the collection time for each spectrum was
approximately 30s (Ellis et al., 2002, Ellis et al., 2004). Spectral acquisition was
achieved using a computer which controlled the spectrometer.

2.2.4 High throughput transmission (HT) FT-IR Spectroscopy
A Bruker Equinox 55 infrared spectrometer equipped with a deuterated triglycine
sulfate (DTGS) detector (Bruker Ltd, Coventry, UK) and employing a motorised
microplate module HTS-XT\(^{TM}\) was used for the FT-IR analysis (Winder et al., 2006).
The previously frozen samples at -80 \(^{\circ}\)C were slowly defrosted in ice and each sample
was mixed with the aid of a rotational mixer for 1 min. 3\(\mu\)L from each sample were
then pipetted onto a ZnSe sample carrier/plate (which can hold 96 samples), and
sample drying was performed in an oven at 50 \(^{\circ}\)C for 30min. In total six replicates
were taken from each sample and were placed randomly onto the ZnSe plates. Again
the wavenumber range collected was 4000 to 600 cm\(^{-1}\), with a resolution of 8 cm\(^{-1}\),
and 64 scans were co-added and averaged. A total of 252 spectra were collected and
each spectrum took 30s to acquire.
2.2.5 Data analysis

2.2.5.1 Pre-processing

For FT-IR, ASCII data recorded using the FT-IR instrument Opus software were collected and introduced into Matlab version 7 (The Mathworks, Inc Matick, MA) analytical software. Employing the Matlab software, the CO$_2$ vibrations visualized in the HT FT-IR measurements were corrected by removing the CO$_2$ peaks at 2403-2272 cm$^{-1}$ and 683-656 cm$^{-1}$ and filling them with a trend, thus diminishing any baseline shift problems. Both HT and ATR FT-IR spectra then were scaled by using extended multiplicative scatter correction (EMSC) (Martens et al., 2003); we also tried the first and second Savitzky-Golay derivatives with 5-point smoothing but this was no better than EMSC. Prior to PCA and PLSR as is normal practice the data were mean centred. At this stage we visually inspected the spectra and checked for outliers using principal components analysis and these were subsequently removed from further analyses. These samples included a single biological replicate from the 24 h time point from the HT spectra collected from whole milk, all the biological replicate from the 104 h time point from the HT spectra collected from skimmed milk and all 3 biological replicates from the 64 h time point from the HT spectra collected from whole; suggesting that this later time point was due to a sampling error.

To investigate the relationship between the FT-IR spectra and the total viable count multivariate statistical methods were used including cluster analysis and partial least squares regression (PLSR). These multivariate analysis methods were performed in PyChem version 3; details of which are available from Jarvis et al. (Jarvis, 2006) and the programme is also available on the web (http://pychem.sf.net/).
2.2.5.2 Cluster analysis

Cluster analysis was performed in two steps as fully described elsewhere (Goodacre et al., 1998b). The first step involved the use of principal component analysis (PCA) (Jolliffe, 1986, Mariey et al., 2001). This statistical process transforms potentially correlated variables into a smaller number of uncorrelated variables which have been named principal components (PCs). PCA identifies data patterns and highlighting differences and similarities and results in a significant reduction in the number of dimensions needed to describe these multivariate data whilst maintaining the majority of the original variance (Goodacre, 2003). In the second step discriminant function analysis (DFA) was used (Manly, 1994). This is a statistical process which employs prior knowledge of which spectra are machine replicates and uses this information to discriminate data based on the PCs. As machine replicates are used this does not bias the cluster analysis in anyway.

Validation of the PC-DFA model was performed for the HT FT-IR data and for the ATR FT-IR data, for every type of milk (whole, semi-skimmed and skimmed) as detailed by Jarvis and Goodacre (2004a). In brief, the data were divided into two subsets, the training set and the test set. The training set consisted of the first two biological replicates (two groups at each time-point), which were used to construct a PC-DFA model. Construction of the PC-DFA model was followed by projection of the test data from the third biological replicate into the PCA space, with the resultant PCs subsequently projected into the DFA space (Jarvis and Goodacre, 2004a). The model was considered valid if the test set data were projected coincident with the training data in the DFA space.
Finally, construction of 95% tolerance regions around the PC-DFA group means was undertaken employing the $\chi^2$ confidence intervals with two degrees of freedom, so that inspection of the tightness of the clusters would become possible (Krzanowski, 2000).

### 2.2.5.3 Partial least squares regression (PLSR)

In order to predict bacterial quantification of TVCs from the FT-IR spectra the multivariate supervised learning method of partial least squares regression (PLSR) was employed (Martens and Naes, 1989) as detailed in Ellis et al (2002). Supervised learning aims in the creation of an association model which can correctly link inputs with targets, where in the calibration phase both input and targets are already known (Goodacre, 2003).

PLSR was calibrated with FT-IR data from the first two spoilage experiments (biological replicates) to predict the known $\log_{10}$ (TVC) values. During calibration these data were divided randomly into training data and cross-validation data, and the number of latent variables used in the model was the point at which the lowest RMS error in the validation data was seen. Once this model was constructed it was challenged with an independent test set data from the third unseen spoilage experiment (biological replicate).
2.3 RESULTS AND DISCUSSION

2.3.1 pH, TVC and organoleptic changes

The pH levels during the 104h of the three spoilage experiments are shown in Figure 8. The initial mean pH for whole milk was 6.72 (Figure 8A). After 104 hours incubation at 15 °C the final mean pH was 7.07. In general, the pH showed mild fluctuation prior to 80h and then increased significantly when the bacterial levels reached $2.10^7$ cfu/ml (Table 1). Similar results were shown for semi-skimmed and skimmed milk were the initial mean pH was 6.72 and 6.74 and then increased to 7.11 and 7.10 respectively (Figures 8B and 8C). It is clear from these results that a change in the sample’s pH would therefore not be an adequate marker of spoilage or residual shelf-life for the different types of milk.
The results of the spoilage experiment for whole milk are shown in Table 1 along with the other types of milk. The initial mean $\log_{10}(\text{TVC})$ was 3.22 which is a usual finding for fresh pasteurised whole milk. After 5 days of incubation at 15°C the final $\log_{10}(\text{TVC})$ increased to 8.08. Examples of the increase in the growth of bacteria in the three types of milk for the three replicate experiments are shown on Figures 9A,
9B and 9C. For semi-skimmed and skimmed milk the initial mean log_{10}(TVC) was between 2.62-2.64 and the final log_{10}(TVC) increased to 7.12 for semi-skimmed and 7.41 for skimmed milk. Both the initial and the final log_{10}(TVC) were lower for the semi-skimmed and skimmed milk than whole milk. This finding is different from previous research which found that the TVC for whole and skimmed milk during the spoilage did not have significant differences when they were stored at 5-8°C (Deeth et al., 2002), and may be a consequence of the incubation temperature for our studying being 15°C.
Figure 9. Plot showing the $\log_{10} (\text{TVC/ml})$ against time for (A) whole, (B) semi-skimmed and (C) skimmed milk samples spoiled at 15°C for 104h. Averages of three replicate measurements for the three biological repeats are shown and error bars show the standard deviations.

In our experiments general organoleptic changes indicative of spoilage became apparent when the number of bacteria reached approximately $10^7$ cfu/ml in all types of milk. Whilst of course these observations are personal and therefore subjective, we found that at this stage the milk started to smell bitter and cheesy. The production of a complex mixture of volatile esters, ketones, aldehydes, fatty acids, ammonia and amines by bacterial metabolism, accounts for these organoleptic changes. The different off-odours are most probably due to protease and lipase enzyme activities (Champagne et al., 1994, Shah, 1994, Sorhaug and Stepaniak, 1997), the production of which has been attributed in the past to the *Pseudomonas* species in milk (Chandler and McMeekin, 1985, Griffiths and Phillips, 1988). It has been shown that the effect of these enzymes becomes apparent once the number of bacteria reaches a level of $10^6$.
cfu/ml or greater, they are resistant to the effects of temperature surviving and remaining stable even after pasteurization (72 °C for 15 sec) and UHT treatment (>135 °C for 1-2 sec) (Champagne et al., 1994, Shah, 1994, Sorhaug and Stepaniak, 1997). Proteases can act directly on micellar casein resulting in its degradation and the release of bitter peptides (Shah, 1994, Frank, 2001), with further proteolysis resulting in the putrid aroma and flavour (Mabbitt, 1981, Champagne et al., 1994, Shah, 1994, Frank, 2001).
<table>
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<th>Time (h)</th>
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<th>Neg SD</th>
<th>Pos SD</th>
<th>Semi-skimmed Mean log_{10} (TVC)</th>
<th>Neg SD</th>
<th>Pos SD</th>
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Pos: positive; Neg: negative; SD: standard deviation
Lipolysis occurs when fatty acids are released from milk triglycerides. More specifically soapy flavours are produced from the release of high molecular weight fatty acids while the oxidization of unsaturated fatty acids to ketones and aldehydes results in a different odour and cardboard-like flavour (Shah, 1994, Veld, 1996, Frank, 2001). Fruity flavours and aroma in pasteurised milk mainly result from the action of \textit{P. fragi} and \textit{P. fluorescence}, esterifying free fatty acids with ethanol, as a result of post-pasteurisation contamination and extend refrigeration times. Ethyl esters such as ethyl acetate, ethyl butanoate and ethyl lexanoate are responsible for the fruity aroma (Whitfield, 1998).

2.3.2 FT-IR ATR Spectroscopy

Representative FT-IR ATR spectra collected from whole milk and the identities of the main absorption bands relating to milk are shown in Figure 10. Using simple visual inspection no obvious quantitative differences were detected between fresh milk at 0h, and spoiled milk at 48h and 104h. Similar spectra were also obtained from semi-skimmed and skimmed milk.

In order to detect any differences between the FT-IR ATR spectra cluster analysis was employed, and the PC-DFA results for the spoilage of the three types of milk from FT-IR ATR are shown in Figure 11. From the whole fat milk PC-DFA results plot it can be seen that the spectra for the first 48h group together in the same region on the bottom left of Figure 11A, as evident from their overlapping 95% tolerance regions; the TVC count during this period was $10^3$ cfu/ml to $9.10^4$ cfu/ml. Spectra of samples incubated after the 48h time point are clearly different from the earlier samples and tended to spread in both axes towards the right hand side in Figure 11A and then
upwards followed by a downward dip. The latter trend appears to occur when the viable bacterial numbers are between $10^6$ cfu/ml and $10^8$ cfu/ml, and for some of these the 95% tolerance regions are seen to overlap sequentially.

**Figure 10.** FT-IR ATR spectra for whole milk at 0h (purple), 48h (blue), and 104h (red)

In semi-skimmed milk similar results in PC-DFA were observed where the spectra for the first 56h appeared in the same region, with total viable counts between $4.10^2$ cfu/ml and $2.10^5$ cfu/ml (Figure 11B). After this time point the spectra again followed a trend correlated to the number of bacteria. For skimmed milk the spectra for the first 56h also appeared clustered together (TVC from $4.10^2$ to $9.10^4$), after which a
A trend relating to the TVC counts from $4.10^5$ to $2.10^7$ cfu/ml was also observed (Figure 11C).
Figure 11. PC-DFA plot of the ATR FT-IR spectra for the 3 repeat experiments of (A) whole, (B) semi-skimmed and (C) skimmed milk. The DFA algorithm used PCs 1-20 (accounting for (A) 98.54%, (B) 98.14% and (C) 96.74% of the total variance) with \textit{a priori} knowledge of machine replicates (i.e., one class per time point, giving 14 classes in total). The different symbols represent the different time-points of spoilage. The $\chi^2$ confidence intervals (two degrees of freedom used) representing the 95% tolerance region are portrayed as circles around the mean.

Since trends were observed in the PC-DFA of all milk types undergoing spoilage, we sought to correlate the known TVC with its representative FT-IR spectra. Therefore supervised learning analysis using PLSR was used to quantify the bacteria in spoilage milk. The FT-IR spectra and the known TVC data were employed for the primary calibration and cross validation of the PLSR algorithm; after calibration these models were tested with FT-IR spectra collected from an independent experiment set (i.e., data that were unseen/new to the model). Preliminary modeling for all milk types was
performed on the FT-IR ATR spectra and it was found that PLSR could not give accurate estimates of TVC for very low bacterial numbers. Therefore depending on the milk type either 48h or 56h to 104 h were used in PLS modeling and this also corresponds to when PC-DFA could not differentiate between the early sampling points.

PLS analysis on FT-IR ATR spectra from whole milk (between 56h to 104h) found that the best model occurred when 5 PLS factors (latent variables) were used. The PLSR result for this model is shown in Figure 12A, where it can be clearly observed that the plot of the predicted TVC versus the known TVC values shows very reasonable prediction (i.e., the estimates lie close to the $y = x$ line shown on this plot). Overall, bacterial enumeration using PLS was achieved at levels higher than $10^6$ cfu/ml (Table 2), and this level is in agreement with the findings of previous research performed for the detection of microbial spoilage in chicken by using ATR FT-IR and PLSR (Ellis et al., 2002). PLS analysis for the FT-IR ATR spectra for the semi-skimmed and skimmed milk are shown in Figures 12B and 12C, respectively.
**Figure 12.** Plot showing the predicted $\log_{10}(\text{TVC})$ from PLS versus the actual $\log_{10}(\text{TVC})$ for (A) whole, (B) semi-skimmed and (C) skimmed milk measured using ATR FT-IR spectroscopy.

**Table 2.** Comparison between HT FT-IR and ATR FT-IR showing the root mean square errors for calibration, validation and test, for each type of milk.
In semi-skimmed and skimmed milk FT-IR ATR spectra from samples between 56h to 104h, and between 48h to 104 h respectively, were analyzed. Table 2 gives the overall performance of the PLS models and shows that PLSR for semi-skimmed milk showed a good predictive value, when the total viable counts above $2.10^5$ cfu/ml could be assessed. By contrast, for skimmed milk reasonable predictions were observed when the total viable counts were above $1.10^4$ cfu/ml which is an order of magnitude lower than those obtained for whole and semi-skimmed milk; remodeling of the whole and semi-skimmed milk including the 48 h time point could not predict $<1.10^4$ cfu/ml.

2.3.3 FT-IR HT Spectroscopy

The same chemometric strategy to that discussed above was also used to analyse the FT-IR spectra obtained from the high throughput screening approach from dried milk. This novel screening approach has not previously been used to investigate food spoilage.

Representative FT-IR HT spectra collected from whole milk with the main absorption bands identified are shown in Figure 13. Qualitative differences between the fresh milk at 0h, and spoiled milk at 48h and 104h were very small; however, further examination of the spectra, especially in the spectral region from 900 to 1600 cm$^{-1}$, revealed certain qualitative differences arising from carbohydrates, proteins and lipids.

Similar spectra were observed for semi-skimmed and skimmed milk with two main differences. The first difference involved the CH$_x$ absorption band related to fatty
acids at 2800 cm\(^{-1}\). Since this involved the absorption of lipids, as expected this was found to be weaker in skimmed milk compared to whole fat milk, as a result of the different fat quantities in the different type of milk. The other major difference appeared on the absorption band related to the C=O group of fatty acids at 1750 cm\(^{-1}\). This absorption was again found to be very strong in whole milk, less strong in semi-skimmed milk and weak in skimmed milk, in keeping with the previous explanation.
Figure 13. FT-IRHT spectra for whole milk at 0h (purple), 48h (blue), and 104h (red)

The PC-DFA results for spoilage of the three types of milk from FT-IR HT are shown in Figure 14. It can be seen in the whole milk results that the spectra for the first 48h appear in the same region on the left of the pane. After that time-point, subsequent time points tend to spread towards the right and upwards. The latter trend occurred when the viable bacterial numbers were between $10^6$ cfu/ml and $10^8$ cfu/ml, and the sample points were more discrete compared to the same analysis on the ATR
accessory (Figure 11) as evident from the 95% tolerance regions not overlapping, suggesting that the FT-IR HT spectra were more information rich. The spectra of semi-skimmed milk during the first 56h appeared within the same region, after which the time-points spread again in a trend that corresponded to the bacterial load. For skimmed milk the trend in PC-DFA space was again very similar to this.
**Figure 14.** PC-DFA plot on HT FT-IR spectra for the 3 repeat experiments of (A) whole, (B) semi-skimmed and (C) skimmed milk. The DFA algorithm used PCs 1-20 (accounting for (A) 99.45%, (B) 99.88% and (C) 98.45% of the total variance) with *a priori* knowledge of machine replicates (i.e., one class per time point, giving 14 classes in total). The different symbols represent the different time-points of spoilage. The $\chi^2$ confidence intervals (two degrees of freedom used) representing the 95% tolerance region are portrayed as circles around the mean.

In contrast to the FT-IR ATR PLSR modeling it was possible to use the full time course for analysis. The PLSR results for whole milk are shown in Figure 15A, where the plot of the predicted TVC versus the known TVC values for whole milk showed good predictive values and gave relatively accurate results even at very low number viable counts ($1.10^3$ cfu/ml). The plots for the other two types of milk are shown in Figures 15B and 15C and results for all three milk types are summarized in Table 2. When semi-skimmed milk was tested, PLSR again gave reasonable
predictions. However, the results from skimmed milk were not quite as good as the results for whole and semi-skimmed milk but were never-the-less still very encouraging.
Figure 15. Plot showing the predicted log_{10}(TVC) from PLS versus the actual log_{10}(TVC) for (A) whole, (B) semi-skimmed and (C) skimmed milk measured using HT FT-IR spectroscopy.

2.3.4 Comparison of the two techniques

The main observed difference between FT-IR HT (Figure 13) and FT-IR ATR (Figure 10) spectra is found at the 2900 cm\(^{-1}\) and 2800 cm\(^{-1}\) region, where CH\(_x\) vibrations involving the peaks related with the acyl chain of fatty acids are only present in the FT-IR HT spectra. Obviously these chemical species will not have disappeared during analysis and this is likely to have occurred because of the FT-IR ATR technique’s spectral sampling method, which is mainly directed to the sample surface providing only a picture of the surface’s chemistry. For ATR the evanescent wave allows penetration into the surface above the crystal and as reported in the Materials
and Methods section for the CH\textsubscript{x} stretched this is \(\sim 0.51 \mu m\). As lipids in milk exist in the form of micellar globules surrounded by a protective membrane which is composed of glycoproteins, lipoproteins and phospholipids, the acyl chains will be internal to these globules with the polar head group exposed to the aqueous environment of the milk, and this may be why the CH\textsubscript{x} stretches are missing from the ATR spectra. By contrast, the FT-IR HT technique employed uses dried milk and is a transmission-based measurement in which infrared light penetrates the whole of the sample and provides a spectrum characteristic of the total components of the milk. Furthermore, there are some additional differences between the two techniques in spectral peak shapes between 1700 and 900 cm\(^{-1}\).

An overall comparison of the PLS modeling between HT FT-IR and ATR FT-IR for the root mean square errors of calibration, validation and test sets, for each type of milk, can be found in Table 2. It can be seen that the accuracy of ATR FT-IR spectroscopy for all whole and semi-skimmed types is better than the HT FT-IR approach, with both having similar predictive ability for skimmed milk. However, HT FT-IR does have significantly lower detection limits compared to ATR FT-IR and generally produces acceptable models with two lower orders of magnitude.
2.4 CONCLUDING REMARKS

FT-IR ATR and HT techniques in combination with multivariate statistical methods, including PC-DFA and PLSR, were able to perform bacterial enumeration and obtain a metabolic fingerprint of milk samples, fast (within 30 seconds) and with precision, requiring very little sample preparation. It appears that metabolic fingerprinting using FT-IR has a very good potential for future use in the dairy industry as a rapid method of viable bacterial detection and enumeration. As such it could therefore be incorporated in the HACCP system increasing consumer safety and lowering product related risks and hazards.
CHAPTER 3

FOURIER TRANSFORM INFRARED AND RAMAN SPECTROSCOPIES FOR THE DETECTION, ENUMERATION AND GROWTH INTERACTION OF *STAPHYLOCOCCUS AUREUS* AND *LACTOCOCCUS LACTIS SUBSP CREMORIS* IN MILK SPOILAGE

In this work guidance on PLS, KPLS and CCA multivariate analysis was provided by Dr Yun Xu.
ABSTRACT

_Staphylococcus aureus_ is one of the main pathogenic microorganisms found in milk and dairy products and has been involved in bacterial foodborne outbreaks in the past. Current enumeration techniques for bacteria are very time-consuming, typically taking 24 h or longer, and bacterial antagonism in the form of lactic acid bacteria (LAB) may inhibit the growth of _S. aureus_. Therefore the aim of this investigation was to establish the accuracy and sensitivity of rapid non-destructive metabolic fingerprinting techniques, such as Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy (RS), in combination with multivariate analysis techniques, for the detection and enumeration of _S. aureus_ in milk, as well as to study the growth interaction between _S. aureus_ and _Lactococcus lactis_ subsp _cremoris_, a common LAB.

The two bacterial species were investigated both in a mono-culture and in a combined inoculated co-culture after inoculation into ultra-heated (UHT) milk during the first 24h of growth at 37 °C. Plating techniques were used in order to obtain reference viable bacteria counts. Principal component discriminant function analysis (PC-DFA), canonical correlation analysis (CCA), partial least square (PLS) and kernel PLS (KPLS) multivariate statistical techniques were employed to analyze the data.

FTIR provided very reasonable quantification results both with PLS and KPLS, the latter providing marginally better predictions, with correlation coefficients in the test set \(Q^2\) and train set \(R^2\) varying from 0.64 to 0.76 and 0.78 to 0.88 for different bacterial sample combinations. RS results were less encouraging with high degrees of error and poor correlation to viable bacterial counts. _S. aureus_ growth was not
inhibited by the presence of the LAB but metabolic fingerprinting of the co-culture indicated that this was closer to that of the LAB.

In conclusion, FTIR spectroscopy in combination with the above multivariate techniques appears to be a promising discrimination and enumeration analytical technique for the two bacterial species. In addition, it has been demonstrated that the *L. cremoris* metabolic effect in milk dominates that of *S. aureus* even though there was no growth antagonism observed.
3.1 INTRODUCTION


With an optimal pH, water activity and nutrients, milk appears to be a very good growth medium for *S. aureus*, being especially favourable to its growth at temperatures between 7 and 48°C (Clark and Nelson, 1961, Schmitt *et al.*, 1990, Adams and Moss, 2000, Qi and Miller, 2000). Pathogenicity of the organism is mainly related to the production of up to 21 different types of staphylococcal enterotoxins (SEs), of most importance types A to E. Unlike the organism itself these SEs appear to be resistant to milk processing, including heat-treatment, storage and preparation (Jarraud *et al.*, 2001, Letertre *et al.*, 2003, SCVPH, 2003, Ono *et al.*, 2008). The toxic strains of *S. aureus* producing SEs are found mainly in the milk of animals suffering from mastitis (Bergdoll, 1989, Gilmour and Harvey, 1990).

The production of SEs is dependent on bacterial numbers, with estimated levels of $10^5$-$10^6$ cfu/mL (colony forming units per mL) at any point during the milk’s life span being sufficient for their production (SCVPH, 2003). Strict regulation therefore exists within the European Union (as well as worldwide) in regards to the levels of *S. aureus*.
present in raw cows’ milk intended for human consumption or products made from raw milk without heat processing, with $2 \times 10^3$ cfu/mL set as the maximum limit allowed in EU. In addition, the identification of enterotoxic strains in milk leads to direct withdrawal of that milk batch from the market (EC 2073/2005 (2005), Council Directive 92/46/EEC (1992)).

The current gold standard for the detection and enumeration of *S. aureus* at industrial level, also used in the ISO 6888-1, is the microbiological plate counting technique using Baird-Parker agar (Baird-Parker, 1962). However, this method is too time-consuming requiring at least 48 h for results, at which point the product may have already been released to the market. Latex agglutination tests available in the market are also time-consuming and also give retrospective answers as they require 20-24 h per sample (Rose *et al.*, 1989, Akkaya and Sancak, 2007).

Polymerase chain reaction (PCR) techniques have also been investigated (Hein *et al.*, 2005, Graber *et al.*, 2007, Ahmadi *et al.*, 2010). These methods are faster than immunological techniques with good detection limits of 100 cfu/g (Tamarapu *et al.*, 2001, Cremonesi *et al.*, 2007) reaching 1 cfu/mL or g with 10 h pre-processing (Chiang *et al.*, 2007). The disadvantages of PCR techniques include the high initial equipment costs and the constant need for highly trained stuff and risk of cross-contamination. Interference and inhibition from foodstuff also increases the requirement for additional processing and costs for purification and DNA isolation. Moreover, as DNA may be present from dead cells PCR does not give an accurate measure of microbial viability. There is clearly the need for more rapid enumeration techniques that give immediate results.
Bacterial antagonism is one of the factors influencing the growth of *S. aureus* in milk and dairy products. It appears that the presence of bacterial competitors can inhibit the growth of *S. aureus* due to changes in the substrate conditions such as reduction in pH, production of volatile compounds, H$_2$O$_2$ or direct competition for nutrients (Genigeorgis, 1989). The presence of high numbers of lactic acid bacteria (LAB) in raw milk or dairy products such as yoghurt and cheese has been shown to inhibit the growth of *S. aureus* and the production of SEs even at *S. aureus* levels of $10^3$ - $10^5$ cfu/mL or g (Lodi et al., 1994, Cleveland et al., 2001, SCVPH, 2003, Ortolani et al., 2010). *Lactococcus lactis* subsp *cremoris* is one of the homofermentative LAB incorporated in starter cultures involved in the production of a number of fermented milk products such as cheeses, buttermilk and sour cream, with a particular effect on the flavour and texture of these products (Johnson and Steele, 2001). The effect of this commonly used LAB on the growth of *S. aureus* in milk has not been investigated in the past.

Fourier transform infrared (FT-IR) spectroscopy is a biochemical fingerprinting technique, which in combination with multivariate statistical techniques has been shown to be a very fast and reasonably accurate method for bacterial detection and enumeration in milk (Nicolaou and Goodacre, 2008). Raman spectroscopy (RS) is also a non-destructive method requiring minimal sample preparation that can be considered to be complementary to FT-IR spectroscopy (Ferraro and Nakamoto, 1994, Goodacre et al., 2002).

The aim of our study was therefore to investigate FT-IR and RS in combination with multivariate analytical techniques for the detection and enumeration of *S. aureus* and
*L. cremoris* in milk. In addition, the use of these techniques will be employed in investigating the growth interaction between *S. aureus* and *L. cremoris* in inoculated milk.
3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

The *S. aureus* strain used was obtained from the American Type Culture Collection (ATCC) (reference strain number 2395), while *L. cremoris* was isolated from raw milk Canestrato Pugliese cheese (Aquilanti *et al.*, 2006). *S. aureus* cultures stored at -80 °C were used. Before these bacteria were used, bacterial cultures were sub-cultured three times in nutrient agar (Oxoid Hampshire, UK), at 30 °C for 18-20 h. *L. cremoris* was also stored at -80 °C and prior to use of the bacteria they were also cultured three times in M17 agar (Oxoid Hampshire, UK), at 30 °C for 18-20 h.

3.2.2 Sample Preparation

Single colonies from *L. cremoris* and *S. aureus* were each inoculated in two different flasks containing tryptone soya broth and incubated at 37°C for 24 h. At this point the optical densities (ODs) for *L. cremoris* and *S. aureus* were measured and correlated with the viable bacterial counts from each strain, by using standard plating methods in tryptone soya agar. This method showed that a concentration of $10^6$ cfu/mL bacterial numbers of *S. aureus* corresponded to an OD of around 2, while for the same bacterial numbers the OD of *L. cremoris* was around 1.2. *S. aureus* and *L. cremoris* were collected containing $10^6$ cfu/ml number of viable bacteria based on the OD values. The samples were centrifuged (5000 g for 5 min), the pellet was washed once with normal saline (0.9% NaCl) and then the pellet was re-suspended and mixed in 2ml of UHT milk using a rotational mixer for 1 min.
The UHT milk used in this experiment was purchased from a national retail outlet in the form of three 500mL cartons of whole UHT milk with the same use-by-date. Three 1L sterile flasks containing 500mL of whole UHT milk were inoculated, the first with *S. aureus*, the second with *L. cremoris* and the third with both *S. aureus* and *L. cremoris* and placed in a rotational incubator at 37°C and 200 revolutions per minute (rpm). Samples were then taken at 0, 60, 120, 150, 180, 210, 240, 300, 360, 420, 480, 720, 960 and 1440 min from all flasks. 1mL samples were obtained at each time point from every flask and mixed in 9mL of physiological saline (0.9%) for 1 min. The viable bacteria from each strain were then determined using a classical microbiological plating method. The latter involved an initial dilution series and lawning of the bacterial suspensions on tryptone soya agar (Oxoid, Hampshire, UK) plates in triplicate with 100 µL of homogeneous sample. The plates were then incubated for 20h at 37 °C and the viable bacteria were recorded as colony forming units (CFU).

Two millilitre samples of milk were also collected at the same time points from each flask, divided to 1mL volumes and preserved at −80 °C. The two of these aliquots were subsequently used for the FT-IR and Raman analyses. The experiment was repeated three times during a 30-day period.

### 3.2.3 FT-IR High Throughput (HT) Spectroscopy

FT-IR analysis and sample preparation were undertaken using the same equipment and protocol as described in Chapter 2, respectively. Sample analysis was also in a randomised order but three replicate samples from each original sample were
analyzed. Identical FT-IR settings and wavenumber range were used as in previously
described experiments with the only difference that the spectral resolution was 4cm\(^{-1}\).
In total 378 spectra were collected.

3.2.4 Raman spectroscopy

Raman analysis was performed using the Renishaw 1000 Raman microscope with a
785nm diode laser delivering typically ~2 mW on the sample (Jarvis and Goodacre,
2004b, Jarvis et al., 2008). The GRAMS WIRE software was employed for
instrument control and data collection. The samples were defrosted in ice, mixed for 1
min and 1 µL from each sample was placed on a stainless steel plate. The plates were
then dried at room temperature for 3 h. Collection of Raman spectra was performed
over the wavenumber shift range of 400-2000 cm\(^{-1}\). Three replicates were analyzed
from each sample, with a total of 378 spectra collected, from the different time points
of bacterial growth in milk. The total time taken to collect each spectrum was 15 min.

3.2.5 Data analysis

3.2.5.1 Pre-processing

ASCII data from FT-IR and Raman were imported into Matlab ver. 7 (The
Mathworks, Inc Matick, MA) and both sets of data were pre-processed using Standard
Normal Variate (SNV) (Barnes et al., 1989, Barnes et al., 1993, Dhanoa et al., 1994).
Multivariate statistical methods such as cluster analysis and supervised regression-
based techniques, PLS and KPLS, were subsequently used to explore the relationship
between the FT-IR and Raman spectra and the number of viable bacteria in milk.
3.2.5.2 Cluster analysis

Cluster analysis was performed as previously described in Chapter 2

3.2.5.3 Exploratory analysis

The exploratory analysis was performed in two steps. Initially principal component analysis (PCA) was performed in order to reduce the large number of variables and dimensionality of the FT-IR and Raman data. The second step involved the use of canonical correlation analysis (CCA). CCA (Hotelling, 1936) is a commonly used method for assessing the correlation between two multivariate matrices or one multivariate matrix and one corresponding vector (e.g. time or VC). CCA seeks a set of linear combinations called canonical variables so that the correlation between the two matrices is maximised. The correlation of the two matrices is expressed as a correlation coefficient in a similar sense of the correlation coefficient (R) between 2 vectors while the significance level of such correlation can be asses by using a $F$-test (Johnson and Wichern, 2007). CCA thus gives us a quick assessment of the correlation (R) between the FT-IR and Raman spectra and bacterial counts in milk and the significance of that probability ($p$ value) before we move to a more robust quantitative analysis.

3.2.5.4 Supervised analysis

In this process the knowledge of both input values, such as the FT-IR spectra, and target/output values, such as those derived from the bacterial counts (as log$_{10}$), are used during calibration. This results in an association model between inputs and
outputs. The linear regression analysis technique partial least squares (PLS) and the non-linear regression method kernel PLS (KPLS) were used in this experiment.

Validation of PLS and KPLS is an important aspect of these analyses because of the great power of supervised analysis methods. In order to perform this validation, for each of the three bacterial combinations (two single and one double), 70% of the total spectra were randomly selected as the training set and the remaining 30% were used as the test set. This selection and test process was repeated 4 times and the average model values were calculated.

3.2.5.5 PLS regression

PLS is a commonly used multivariate regression method (Martens and Naes, 1989), especially in the field of spectroscopic study. This is because PLS is able to handle effectively the problem of multicollinearity, which is always the case in spectroscopic data, while standard multivariate regression will fail due to the rank deficiency problem. PLS can predict either a single predictive variable using a PLS1 model or predict several predictors simultaneously using a PLS2 model. Optimization of the number of PLS components (latent variables) was performed using a $k$-fold cross validation on the training set only.

3.2.5.6 KPLS regression

Kernel PLS (Shawe-Taylor and Christianini, 2004) is a non-linear extension of PLS model which made use of the recent development of the concept of kernel learning. The idea of kernel learning is that by projecting the data into an appropriate higher
dimensional feature space, many non-linear problems (e.g., regression, classification etc) can be solved by using a linear modeling method. The projection is achieved by employing a so called kernel function. In these experiments a radial basis function (RBF) was employed as the kernel function. The optimal combination of the kernel parameter and the number of PLS factors were optimized using a grid search approach coupled with a $k$-fold cross-validation on the training set while $k$ is the number of viable bacterial counts we kept in the training set.
3.3 RESULTS AND DISCUSSION

3.3.1 Viable Counts (VC)

Results from the experiments involving the S. aureus, both when inoculated in isolation in UHT milk at 37°C and when inoculated in combination with L. cremoris, are illustrated in Tables 3 and 4 and are described further below. As can be seen from the growth curves (Figure 16) the initial bacterial inoculation numbers for S. aureus were very similar with a mean log_{10} (VC) of 5.20 and 5.72 for the pure inoculant and the co-culture respectively; the latter being analogous to the initial bacterial inoculation numbers of L. cremoris in the co-culture (mean log_{10} (VC) of 5.67); Table 4). The lag phase of S. aureus growth in both experiments appeared to be from 0 to 120 min, with mean log_{10} (VC) of 5.20 to 5.49 and 5.72 to 5.96 for pure and co-culture inoculations, respectively. The exponential growth phase initiated at 120 min and ended at ~480 min with mean log_{10} (VC) of 5.49 to 8.03 (in pure culture) and 5.96 to 7.81 (in co-culture). The stationary phase commenced thereafter and the final mean log_{10} (VC) were 8.26 and 8.17 after 1440 min (24h) growth for mono-culture and co-culture inoculations, respectively. The bacterial growth curves for L. cremoris both in pure culture and in co-culture are also shown in Figure 16 and show very similar dynamics to those described above for S. aureus. There was certainly no indication that the presence of L. cremoris in the mixture samples interfered in any way with the growth of S. aureus. Although the growth of L. cremoris in the presence of S. aureus did appear to be impeded with a lower final bacterial counts at 24 h of 7.96 (log_{10} (VC)) compared to 9.05 in the pure culture.
Figure 16. Plot illustrating the log$_{10}$ (VC/ml) against time for UHT milk samples inoculated with *S. aureus* and *L. cremoris* either in mono-culture (pure) or in co-culture for 24h. The average values from the three biological replicate are shown for each time-point.
Table 3. Mean log\(_{10}\) (VC) for *S. aureus* growth at 37\(^\circ\)C in UHT milk, both in pure culture and in a co-culture with *L. cremoris*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th><em>S. aureus</em> in mono-culture</th>
<th><em>S. aureus</em> in co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean log(_{10}) (VC)</td>
<td>Negative SD</td>
</tr>
<tr>
<td>0</td>
<td>5.20</td>
<td>0.04</td>
</tr>
<tr>
<td>60</td>
<td>5.71</td>
<td>0.65</td>
</tr>
<tr>
<td>120</td>
<td>5.49</td>
<td>0.16</td>
</tr>
<tr>
<td>150</td>
<td>6.01</td>
<td>0.06</td>
</tr>
<tr>
<td>180</td>
<td>6.68</td>
<td>0.07</td>
</tr>
<tr>
<td>210</td>
<td>7.01</td>
<td>0.07</td>
</tr>
<tr>
<td>240</td>
<td>7.15</td>
<td>0.05</td>
</tr>
<tr>
<td>300</td>
<td>8.18</td>
<td>0.91</td>
</tr>
<tr>
<td>360</td>
<td>7.95</td>
<td>0.13</td>
</tr>
<tr>
<td>420</td>
<td>7.57</td>
<td>0.34</td>
</tr>
<tr>
<td>480</td>
<td>8.03</td>
<td>0.05</td>
</tr>
<tr>
<td>720</td>
<td>8.14</td>
<td>0.13</td>
</tr>
<tr>
<td>960</td>
<td>8.43</td>
<td>0.65</td>
</tr>
<tr>
<td>1440</td>
<td>8.26</td>
<td>0.08</td>
</tr>
</tbody>
</table>
**Table 4.** Mean log$_{10}$ (VC) for *L. cremoris* growth in pure-culture and in a co-culture with *S. aureus*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th><em>L. cremoris in mono-culture</em></th>
<th></th>
<th><em>L. cremoris in co-culture</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean log$_{10}$ (VC)</td>
<td>Negative SD</td>
<td>Positive SD</td>
<td>Mean log$_{10}$ (VC)</td>
</tr>
<tr>
<td>0</td>
<td>5.38</td>
<td>0.06</td>
<td>0.06</td>
<td>5.67</td>
</tr>
<tr>
<td>60</td>
<td>5.76</td>
<td>0.01</td>
<td>0.01</td>
<td>5.88</td>
</tr>
<tr>
<td>120</td>
<td>6.42</td>
<td>0.21</td>
<td>0.14</td>
<td>6.24</td>
</tr>
<tr>
<td>150</td>
<td>7.10</td>
<td>0.07</td>
<td>0.06</td>
<td>6.41</td>
</tr>
<tr>
<td>180</td>
<td>7.20</td>
<td>0.10</td>
<td>0.08</td>
<td>6.74</td>
</tr>
<tr>
<td>210</td>
<td>7.66</td>
<td>0.04</td>
<td>0.04</td>
<td>7.40</td>
</tr>
<tr>
<td>240</td>
<td>7.76</td>
<td>0.11</td>
<td>0.09</td>
<td>7.12</td>
</tr>
<tr>
<td>300</td>
<td>7.92</td>
<td>0.03</td>
<td>0.03</td>
<td>7.55</td>
</tr>
<tr>
<td>360</td>
<td>7.66</td>
<td>0.06</td>
<td>0.05</td>
<td>7.59</td>
</tr>
<tr>
<td>420</td>
<td>7.79</td>
<td>0.09</td>
<td>0.08</td>
<td>7.63</td>
</tr>
<tr>
<td>480</td>
<td>7.74</td>
<td>0.08</td>
<td>0.07</td>
<td>7.66</td>
</tr>
<tr>
<td>720</td>
<td>7.88</td>
<td>0.05</td>
<td>0.04</td>
<td>8.08</td>
</tr>
<tr>
<td>960</td>
<td>8.44</td>
<td>0.57</td>
<td>0.36</td>
<td>7.82</td>
</tr>
<tr>
<td>1440</td>
<td>9.05</td>
<td>0.28</td>
<td>0.17</td>
<td>7.96</td>
</tr>
</tbody>
</table>
3.3.2 FT-IR and Raman data

In order for a molecule’s vibrations to be infrared (IR) active and absorb IR energy they must cause a net change in the dipole moment of the molecule. By contrast it is changes in the polarizability of the molecule due to vibrational motion that make the vibration Raman active (Banwell and McCash, 1994). Therefore the spectra from each technique are expected to be different but complement each other (Goodacre et al., 2002). Thus both methods were investigated for their abilities to generate meaningful metabolic fingerprints from the milk during bacterial growth.

Visual inspection of the spectra obtained using FT-IR HT spectroscopy for the two bacterial species in mono-culture and in co-culture only revealed some differences between spectra. Representative spectra from FT-IR HT spectroscopy at 480 min (6h) are illustrated in Figure 17. Several peaks appear to be of particular interest, namely: the peak at 3300 cm\(^{-1}\), representing the peptide bonds \(\nu(N-H)\) from proteins and \(\nu(O-H)\); the peak at 1100 cm\(^{-1}\), representing the \(\nu(C-O)\) from carbohydrates; and the clear ratio between the Amide I bands at ~1650 cm\(^{-1}\) from \(\nu(C=O)\) with the carbohydrate (1100 cm\(^{-1}\)). Spectral absorption at 3300 and 1100 cm\(^{-1}\) appear to be lower during the growth of \(L. cremoris\) compared to the growth of \(S. aureus\), and the ratio of Amide I to polysaccharide is equivalent in \(S. aureus\) and the co-culture but for \(L. cremoris\) the Amide I:polysaccharide is significantly greater. This represents different metabolic potentials of these organisms with the higher rate of carbohydrate utilization by the fermentative metabolism of \(L. cremoris\). Representative spectra derived from Raman spectroscopy at 480 min (6h) for both bacteria in isolation and from the combined mixture are shown in Figure 18. These Raman spectra appear to be almost identical with no apparent differences on visual inspection. Higher intensity of two peaks can
be seen in the combined mixture spectrum at the 1110 cm\(^{-1}\) \(\nu\)(C-C) mode and the 610 – 630 cm\(^{-1}\) \(\nu\)(C-S) mode, both representing amino acids. The remaining prominent peaks in all spectra appear at the 1230 – 1290 cm\(^{-1}\) amide III mode, 1050 – 920 cm\(^{-1}\) \(\nu\)(C-C) and \(\nu\)(C-N) modes, 810 – 850 and 760 – 790 cm\(^{-1}\) \(\nu\)(C-C) and \(\nu\)(C-S) modes representing various amino acids, 380 – 400 and 280 – 300 cm\(^{-1}\) modes representing lactose (Li-Chan, 1996, Kirk et al., 2007, McGoverin et al., 2010).

**Figure 17.** FT-IR HT spectra at 480 min (6h) for *S. aureus* and *L. cremoris* after inoculated in UHT milk in mono-culture and in co-culture. Spectra are off set so that features can be observed.
Figure 18. Raman spectra at 480 min (6h) for *S. aureus* and *L. cremoris* after inoculated in UHT milk in mono-culture and in co-culture. Spectra are offset so that features can be observed.

### 3.3.3 Trajectory analysis

Cluster analysis using PC-DFA was subsequently performed in an attempt to gain more information regarding potential differences between the milk sustaining bacterial growth from the FT-IR and Raman spectra. PC-DFA results from FT-IR HT data are shown in Figure 19. As an example the results on the *L. cremoris* monocultures are (Figure 19B) described. This trajectory plot indicates that after 480 min there is a clear distinction between time-points in the PC-DFA space. Samples collected from 720-1440 min followed a downwards left trajectory, the mean log_{10}...
(VC) values increase from 7.88 to 9.05, suggesting that whilst there is very little growth the fingerprint of the milk during this time period is changing in a particular manner.

The same general trend is also seen in the PC-DFA space from the Raman data collected from the same bacteria (Figure 20B), with the additional good distinction between time-points at 0 min as well as those after 720 min. However, the spread of these points is much greater in this analysis compared to the PC-DFA FT-IR HT analysis for the same bacteria. This more diffuse clustering of the individual time points suggests that there was a lack of precision in spectral collection. All PC-DFA results from Raman spectroscopy are shown in Figure 20.

Similar findings were obtained for the other mono-culture (S. aureus) as well as for the co-culture for both FT-IR (Figure 19A, 19C) and Raman data (Figure 20A, 20C). Overall these trajectory analyses showed that sample distinction was possible only after the initiation of the stationary phase (after 480 min of growth) for both bacterial species and the co-culture. By contrast, the spectra collected from the lag and exponential phases for all three cultures appeared to be very similar as samples collected from 0-420 min were coincident in PC-DFA space, despite this being when most growth occurred - typically the log_{10}(VC) increased from ~5.2 to ~7.8.
Figure 19. PC-DFA plot of FT-IR HT spectra for (A) *S. aureus* and (B) *L. cremoris* during mono-culture, and (C) both microorganisms in co-culture, in UHT milk. The DFA algorithm used PCs 1–20 (accounting for (A) 99.76%, (B) 99.93% and (C) 99.85% of the total variance) with *a priori* knowledge of machine replicates (i.e. one class per time point, giving 14 classes in total). Each different colour represents a distinct time-point and block arrows indicate the trend of the data.
Figure 20. PC-DFA plot of Raman spectra for (A) *S. aureus* and (B) *L. cremoris* during mono-culture, and (C) both organisms in co-culture, in UHT milk. The DFA algorithm used PCs 1–20 (accounting for (A) 98.33%, (B) 98.41% and (C) 97.98% of the total variance) with a priori knowledge of machine replicates (i.e. one class per time point, giving 14 classes in total). Each different colour represents a distinct time-point and block arrows indicate the trend of the data.

3.3.4 Canonical correlation analysis

In order to investigate these spectral changes further canonical correlation analysis (CCA) was performed on the FT-IR and Raman data obtained for the two microorganisms in mono-culture and in co-culture. Canonical correlation coefficients
(R) of the various plots of canonical variable against VC and time are shown in Table 5 and CCA plots for *S. aureus* in pure and mixed cultures are shown in Figure 21. Visual inspection of these results indicate the presence of a linear pattern in the bacterial concentration levels with the FT-IR and Raman data and that the correlation coefficients appear to be better when calculated against time rather than against VC. The remaining CCA plots are displayed in Figures 22, 23 and 24. The FT-IR data show marginally better results in the mono-cultures, whilst in the co-cultures the same is true for plots against VC, but the Raman data appear to provide significantly better canonical correlation coefficients (R>0.93) against time (Table 5). Although the CCA was better with respect to time, correlations between ~0.73 to ~0.86 were found for all bacteria with respect to VCs. From the microbiological perspective estimates of bacterial numbers rather than growth time would be preferable, therefore these results do suggest that further chemometric analysis should be performed in order to improve bacterial quantification from these vibrational spectroscopic data.

**Table 5.** Canonical correlation coefficient (R) values for FT-IR and Raman data for *S. aureus* and *L. cremoris* mono-cultures and co-cultures against VC and time.

<table>
<thead>
<tr>
<th></th>
<th><strong>FT-IR</strong></th>
<th></th>
<th><strong>Raman</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Canonical correlation coefficient (R)</strong></td>
<td><strong>Canonical correlation coefficient (R)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>log_{10} VC</td>
<td>Time (min)</td>
<td>log_{10} VC</td>
<td>Time (min)</td>
</tr>
<tr>
<td><em>S. aureus</em> mono-culture</td>
<td>0.7528</td>
<td>0.9519</td>
<td>0.8133</td>
<td>0.9431</td>
</tr>
<tr>
<td><em>L. cremoris</em> mono-culture</td>
<td>0.7310</td>
<td>0.9554</td>
<td>0.8208</td>
<td>0.9297</td>
</tr>
<tr>
<td><em>S. aureus</em> in co-culture</td>
<td>0.8635</td>
<td>0.9759</td>
<td>0.7527</td>
<td>0.9484</td>
</tr>
<tr>
<td><em>L. cremoris</em> in co-culture</td>
<td>0.8638</td>
<td>0.9759</td>
<td>0.7928</td>
<td>0.9484</td>
</tr>
</tbody>
</table>
Figure 21. Canonical correlation analysis of FTIR and Raman data against VC and time for *S. aureus* mono-culture
Figure 22. Canonical correlation analysis of FTIR and Raman data against VC and time for *L. cremoris* mono-culture
Figure 23. Canonical correlation analysis of FTIR and Raman data against VC and time for *S. aureus* in co-culture.
Figure 24. Canonical correlation analysis of FTIR and Raman data against VC and time for *L. cremoris* in co-culture
3.3.5 Bacterial quantification

Quantification of each bacterial species was attempted using linear PLS and non-linear KPLS regression as described in the material and methods section. Each model was constructed using 70% of values as the training set and the remaining 30% for test set. Four different models were constructed using different combinations of spectra in the training and test sets and these results were combined in order to obtain the average prediction statistics. Only data collected from the first 480 min of the experiments were used as the bacterial growth curves (Figure 16) demonstrates very little growth after the stationary phase is reached. Figures 25 and 26 illustrate the PLS and KPLS models predicting *S. aureus* and *L. cremoris* numbers from FT-IR data from mono-cultures and co-cultures with each other. Table 6 shows the mean and standard deviation values for each bacterial species in the different growth environments analysed by both PLS and KPLS.

For analysis of the three cultures using FT-IR spectroscopy both PLS and KPLS appeared to have good and similar predictive values for both bacteria (Table 6). In the PLS models the root mean square error of the training set (RMSEC) appeared to vary from log₁₀ 0.25 to 0.52, with RMS errors in the independent set (RMSEP) from log₁₀ 0.38 to 0.59. The correlation coefficients in the test set ($Q^2$) and train set ($R^2$) varied on average from 0.64 to 0.76 and 0.78 to 0.88, respectively. KPLS yielded comparable values with RMSEC from log₁₀ 0.26 to 0.36, and RMSEP from log₁₀ 0.40 to 0.59, the $Q^2$ variation was 0.69 to 0.720 and $R^2$ average values from 0.81 to 0.87. Overall, it appeared that the prediction of *L. cremoris* in the co-culture provided a marginally better predictive model in both PLS and KPLS. By contrast, when the Raman spectra were analysed with PLS and KPLS in exactly the same way as the FT-
iable bacterial load in UHT milk was not satisfactory with large RMSEPs (typically 1.2 log<sub>10</sub>) and RMSECs (typically 0.75 log<sub>10</sub>); therefore, these statistics are not included in Table 6.
Figure 25. Representative plots illustrating the average predicted VC values (log$_{10}$) derived from PLS and KPLS against the average measured VC values (log$_{10}$) measured with FT-IR HT for S. aureus in mono-culture (A) and (B) and in co-culture (C) and (D), respectively. For (A) the $Q^2$ value for the test set was 0.68 and the $R^2$ value for the train set was 0.79, with a RMS error (log$_{10}$) for the calibration of 0.46.
and 0.61 for the prediction. 10 PLS factors were used for this model. For (B) the $Q^2$ value for the test set was 0.69 and the $R^2$ value for the train set was 0.81, with a RMS error ($\log_{10}$) for the calibration of 0.44 and 0.60 for the prediction. 10 PLS factors were used for this model. For (C) the $Q^2$ value for the test set was 0.69 and the $R^2$ value for the train set was 0.84, with a RMS error ($\log_{10}$) for the calibration of 0.35 and 0.47 for the prediction. 3 PLS factors were used for this model. For (D) the $Q^2$ value for the test set was 0.71 and the $R^2$ value for the train set was 0.82 with a RMS error ($\log_{10}$) for the calibration of 0.34 and 0.53 for the prediction. 3 PLS factors were used for this model.
A - PLS

Predicted log$_{10}$ (VC) vs. Known log$_{10}$ (VC)

Data set:
- Test
- Training

B - KPLS

Predicted log$_{10}$ (VC) vs. Known log$_{10}$ (VC)

Data set:
- Test
- Training
Figure 26. Representative plots illustrating the average predicted VC values (log_{10}) derived from PLS and KPLS against the average measured VC values (log_{10}) measured with FT-IR HT for *L. cremoris* in mono-culture (A) and (B) and in co-culture (C) and (D), respectively. For (A) the $Q^2$ value for the test set was 0.68 and the
$R^2$ value for the train set was 0.88, with a RMS error ($\log_{10}$) for the calibration of 0.25 and 0.60 for the prediction. 17 PLS factors were used for this model. For (B) the $Q^2$ value for the test set was 0.61 and the $R^2$ value for the train set was 0.89 with a RMS error ($\log_{10}$) for the calibration of 0.25 and 0.61 for the prediction. 17 PLS factors were used for this model. For (C) the $Q^2$ value for the test set was 0.76 and the $R^2$ value for the train set was 0.83, with a RMS error ($\log_{10}$) for the calibration of 0.26 and 0.43 for the prediction. 10 PLS factors were used for this model. For (D) the $Q^2$ value for the test set was 0.76 and the $R^2$ value for the train set was 0.83, with a RMS error ($\log_{10}$) for the calibration of 0.29 and 0.37 for the prediction. 7 PLS factors were used for this model.
Table 6. Comparison of the partial least squares (PLS) regression and the non linear
Kernel partial least squares (KPLS) results of the FT-IR HT spectra for determining
the number of viable bacteria in UHT milk.

<table>
<thead>
<tr>
<th>S. aureus mono-culture</th>
<th>PLS</th>
<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>RMSECV</td>
<td>0.63</td>
<td>0.08</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.52</td>
<td>0.19</td>
</tr>
<tr>
<td>RMSEP</td>
<td>0.47</td>
<td>0.10</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>0.78</td>
<td>0.14</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.74</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L. cremoris mono-culture</th>
<th>PLS</th>
<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>RMSECV</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.26</td>
<td>0.03</td>
</tr>
<tr>
<td>RMSEP</td>
<td>0.59</td>
<td>0.07</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>0.88</td>
<td>0.02</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.64</td>
<td>0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S. aureus in co-culture</th>
<th>PLS</th>
<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>RMSECV</td>
<td>0.44</td>
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<tr>
<td>RMSEC</td>
<td>0.37</td>
<td>0.02</td>
</tr>
<tr>
<td>RMSEP</td>
<td>0.47</td>
<td>0.06</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
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<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
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<td>0.03</td>
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<table>
<thead>
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<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>RMSECV</td>
<td>0.32</td>
<td>0.01</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>RMSEP</td>
<td>0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>0.87</td>
<td>0.03</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.76</td>
<td>0.04</td>
</tr>
</tbody>
</table>

RMSECV represents the root mean square error for the cross-validation; RMSEC the root mean square error for the calibration; RMSEP the root mean square error for the predictions produced for the independent test set; SD stands for standard deviation; values are based on log$_{10}$
3.3.6 PLS2 projection analysis

In order to investigate whether the metabolic fingerprints from the co-cultured samples were closer to one of the pure cultures, a PLS2 model was first built on the combined data set from the two mono-cultures. In the response matrix $Y$ one column was allocated to the VCs from *L. cremoris* and the other column for the *S. aureus* counts, with the corresponding elements in $Y$ set to zero. That is to say, if the log$_{10}$ (VC) for *S. aureus* was 5.7 then the target response vector in $Y$ would be $[5.7\ 0]$, whilst if the *L. cremoris* was 7.8 this would be $[0\ 7.8]$. In this way the PLS model was ‘forced’ to focus on the *unique* features of each bacterium and actively penalize the common features modeling the cell growth. If the co-culture samples were ‘dominated’ by one type of bacterium then after projection into this model, the PLS scores would be more overlapping with those of the ‘dominating’ bacterium.

In order to test this hypothesis ‘simulated’ co-culture spectra were generated by averaging a series from the two pure bacterial spectra; i.e., each spectrum in the ‘simulated’ co-culture was the mean of the two pure bacterial spectra which had similar VCs at the same time-points. If the previous assumptions in the PLS2 projections were correct then the scores of this simulated co-culture would appear in the middle of the two pure bacterial samples. Figure 27A shows the simulated data projected into PLS2 latent variable space and it can indeed be seen that the simulated data appear in the middle of the valley between the two pure bacterial cultures. Note that the two pure cultures are separated but split in both PLS components 1 and 2 and the direction indicated by the colouring represents the increase in VC.
Figure 27B illustrates the PLS2 projection of the real spectra from the co-cultures and rather than falling in between the two bacteria these data are clearly located closer to the pure *L. cremoris* spectra. This shows that the metabolic fingerprint is dominated by the *L. cremoris* in the co-culture rather than the *S. aureus* phenotype.
Simulated Co-culture samples projected into the PLS model.

Data set:
- L. cremoris
- S. aureus
- Co-culture

Log$_{10}$(Cell Count)
Figure 27. PLS2 models calibrated with pure mono-cultures of *S. aureus* and *L. cremoris* with (A) simulated cultures and (B) co-culture samples projected into the PLS2 model. Colours represent log_{10} (cell counts) and the symbols represent the different cultures or simulated data.
3.4 CONCLUDING REMARKS

In contrast to Raman spectroscopy the metabolic fingerprinting revealed through FTIR-HT spectroscopy was able to give reasonably good estimates of the levels of *S. aureus* and *L. cremoris* in both pure and co-cultures when these bacteria were grown in UHT milk.

Whilst in the literature the growth of LAB in milk has been reported to slow the growth of pathogens including *S. aureus* (Fang *et al.*, 1996, Radovanovic and Katic, 2009), from the growth curve analysis (Figure 16) we certainly did not see any antagonism on the *S. aureus* when grown with *L. cremoris*; if anything the opposite was true in that the growth of *L. cremoris* was slightly lower when cultured with *S. aureus*. The latter may be of particular importance in the dairy industry especially in the manufacture of products using unpasteurized raw milk and LAB, such as cheese and butter.

Interestingly when the phenotype of the co-culture was compared to those from the pure cultures of bacteria in UHT milk using PLS2 (Figure 27), despite the reduction in the growth of *L. cremoris*, this phenotype did appear to dominate the co-culture. This may be due to the fact that this LAB has a higher fermentative metabolism where sugars from the milk are fermented to organic acids which will lower the pH of the milk and cause additional phenotypic changes in the milk substrate, such as curdling of the proteins.
In conclusion, it has been demonstrated that FT-IR spectroscopy when combined with chemometrics is a powerful approach for bacterial enumeration in milk and gives results in a few minutes, rather than in 2 days which is typical of classical methods used in the industry. Moreover, this is the first demonstration that FT-IR can perform enumeration of bacteria in co-cultures without the need for their prior separation.
CHAPTER 4

FOURIER TRANSFORM INFRARED SPECTROSCOPY AND MULTIVARIATE ANALYSIS FOR THE DETECTION AND QUANTIFICATION OF DIFFERENT MILK SPECIES

This work has been published in a peer review journal as:


Dr Yun Xu helped with the PLS and KPLS analyses.
ABSTRACT

The authenticity of milk and milk products is important with extended health, cultural and financial implications. Current analytical methods for the detection of milk adulteration are slow and laborious and therefore impractical for use in routine milk screening by the dairy industry. Fourier transform infrared (FT-IR) spectroscopy is a rapid biochemical fingerprinting technique that could potentially be used to reduce this sample analysis time period significantly.

To test this hypothesis we investigated three types of milk: cows’, goats’ and sheep’s milk. From these, four mixtures were prepared. The first three were binary mixtures of sheep and cow milk, goat and cow milk, or sheep and goat milk; in all mixtures the mixtures contained between 0-100% of each milk in steps of 5%. The fourth combination was a tertiary mixture containing sheep, cow and goat milk also in steps of 5%. FT-IR spectroscopic analysis in combination with multivariate statistical methods, including partial least squares (PLS) regression and non-linear kernel partial least squares (KPLS) regression, were used for multivariate calibration in order to quantify the different levels of adulterated milk. The FT-IR spectra when analysed using PLS showed a reasonably good predictive value for the binary mixtures with an error level of 6.5-8%. These results improved and excellent predictions were achieved (only 4-6% error) when KPLS was employed. When the tertiary mixtures were analysed excellent predictions were also achieved by both PLS and KPLS with errors of 3.4-4.9% and 3.9-6.4% respectively.
These results indicate that FT-IR spectroscopy has excellent potential for future use in the dairy industry as a rapid method of detection and enumeration in milk adulteration.
4.1 INTRODUCTION

Milk quality is of significant importance in the production of all types of cheese especially in regards to their quality and characteristics. Goats’ and sheep’s milk are of higher value than cows' milk and are particularly used for the production of a wide variety of specialty cheeses. This presents the potential for financial gain by unscrupulous producers adulterating either goats’ or sheep’s milk with cows’ milk thus resulting in non-authentic milk products (Maudet and Taberlet, 2001). In addition to the ethical, religious and cultural implications (Shatestein and Ghadirian, 1998), consumers need to be protected from this kind of practice because of potential intolerance and allergic reactions to the cow milk component of these adulterated products (Bischoff, 2006, Venter, 2009, Chafen et al., 2010). Indeed the European Union has legislation in place for the correct display of the constituents of dairy products protecting their authenticity (European Commission, 2001b), while varying legislation on food labelling and authenticity exists among other member countries (Dennis, 1998).

Up to now a number of methods have been investigated both within academic institutes and in industry for their accuracy and practicality in detecting dairy product adulteration. These include several analytical approaches based on immunological, electrophoretic and chromatographic techniques, as well as DNA-based processes such as species-specific polymerase chain reaction (PCR). Antibody-based assays used for the quantification and detections of species-specific milk proteins (antigens) form the basis of immunological methods (Moatsou and Anifantakis, 2003). These have targeted proteins such as cow caseins (whole-, \( \gamma_3 \), \( \beta \)- and as1-), cow \( \beta \)-
lactoglobulin and cow IgG for the detection of cows’ milk adulteration, while goat whey proteins and goat αs2-casein have been used for the detection of goats’ milk adulteration (Chen et al., 2004, Hurley et al., 2004b). Enzyme-linked immunosorbent assays (ELISAs) have been routinely employed for this purpose and are performed using a variety of processes (Levieux and Venien, 1994, Anguita et al., 1996, Beer et al., 1996, Anguita et al., 1997b, Hurley et al., 2006). Even though ELISA techniques require relatively less sample preparation than other techniques they may be considered to be costly, as they rely on the use of expensive antibodies that cannot be reused, and have a limited shelf-life. In addition, the reliance of these methods on specific protein identification and quantification is a potential drawback in the analysis of processed milk as proteolysis and heat denaturation can cause the loss of antibody specific epitopes (Mayer, 2005, Hurley et al., 2006).

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) are the main non-immunological methods employed for the detection of casein or whey proteins in milk but they are slow and laborious for routine use in the dairy industry (Amigo et al., 1992, Levieux and Venien, 1994, Malin et al., 1994, Mayer, 2005, Addeo et al., 2009). Importantly though, IEF has been adopted by the European Commission as the reference method for detecting cow γ-casein, with a detection limit of 1% (vol/vol) cows’ milk (European Commission, 2001b) in other types of milk.

Separation techniques have also been used for the detection of milk adulteration and both high performance liquid chromatography (HPLC) and gas chromatography (GC) have been used, often hyphenated with mass spectrometry (MS), and are based on the detection of characteristic fatty acids and proteins in dairy products (Romero et al.,
1996, Chen et al., 2004, Hurley et al., 2004b, Gutierrez et al., 2009). The main disadvantage of these techniques is that they are also time consuming (the chromatography typically takes 30-60 min) and laborious and the increased requirements for sample handling during preparation can adversely affect the quality of the analysis (Karoui and Baerdemaeker, 2007).

Finally, PCR techniques have been developed over the last decade, aiming to exploit the presence of somatic cells in milk, by detecting genomic DNA from different species. These DNA-based techniques have been used to identify milk adulteration rapidly and with relatively high sensitivity but they are still not very practical for routine industrial use and quantification aspects may be deranged by environmental factors such as mastitis that lead to increase in the numbers of somatic cells in milk or by milk processing factors such as milk heat treatment (Bania et al., 2001, Lopez-Calleja et al., 2005b, Cheng et al., 2006, Maskova and Paulickova, 2006).

In general, there appears to be a useful set of analytical approaches for the detection of milk adulteration; however, all the above techniques have the main disadvantage in that they are slow and laborious and this delay in milk analysis makes these tools of little value for routine screening of milk in the dairy industry. By contrast, Fourier transform infrared (FT-IR) spectroscopy is a very rapid biochemical fingerprinting technique (typically 30 s per sample) that can potentially resolve many of these problems and produce milk analysis results in under a minute after minimal sample preparation (Nicolaou and Goodacre, 2008). Compared to other techniques it is simple to use, with high sensitivity and low operational costs. When combined with appropriate multivariate statistical methods such as partial least squares (PLS)
regression or as we report here kernel PLS, FT-IR spectroscopy may be a more ideal solution for the detection and quantification of the adulteration of milk.

FT-IR in combination with PLS has been used in the past to classify different types of oils (Dahlberg et al., 1997, Ozen and Mauer, 2002) and honey (Hennessy et al., 2008) and detect adulteration of extra virgin oil with palm oil (Rohman and Man, 2010) and hazelnut oil (Ozen and Mauer, 2002) with very good predicting values. In addition, its application in the determination of microbial load in meat such as beef (Ellis et al., 2004) and chicken (Ellis et al., 2002), as well as in cows’ milk (Nicolaou and Goodacre, 2008) has been very promising. Our aim in this study was therefore to investigate whether FT-IR spectroscopy is an accurate and valid technique for the detection and quantification of the adulteration of goats’ or sheep’s milk with cows’ milk in both binary and tertiary mixtures.
4.2 MATERIALS AND METHODS

4.2.1 Sample Preparation

Three types of full-fat fresh pasteurized milk were used in this study: cows’ milk, goats’ milk, and sheep’s milk. The milk samples used were purchased from national retail outlets and analysed immediately. From these, four different milk type combinations were prepared:

1. Sheep milk adulterated with cows’ milk,
2. Goats’ milk adulterated with cows’ milk, and
3. Sheep’s milk adulterated with goats’ milk.
4. A tertiary mixture containing sheep’s, goats’ and cows’ milk was also created.

For each of these combinations, various samples were created, with the primary milk type adulterated with a different type of milk from 0-100% in successive increasing steps of 5%. The concentration levels for the tertiary mixture (4) is shown in the Supporting Information (Table 7).

The different milk combinations were then poured into sterile flasks and placed in a rotational incubator for 15 min to ensure a homogenous mixed sample. 1mL milk samples were then obtained and these were subsequently used for FT-IR analysis.
Table 7. Fourth combination of milk samples containing a tertiary mixture of sheep’s, goats’ and cows’ milk, [%]: percentage concentration

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4.2.2 FT-IR High Throughput (HT) Spectroscopy

The same FT-IR Bruker Equinox 55 infrared spectrometer was used as detailed in Chapters 2 and 3. Identical sample preparation and FT-IR protocol as described in the experiments in Chapter 3 were employed. In total 378 spectra were collected from the different milk combinations.

4.2.3 Data analysis

4.2.3.1 Pre-processing

ASCII data from FT-IR were imported into Matlab ver. 7 (The Mathworks, Inc Matick, MA). The data were pre-processed using Standard Normal Variate (SNV) (Barnes et al., 1989, Barnes et al., 1993, Dhanoa et al., 1994). To perform this correction, each FT-IR spectrum was firstly mean centred and then divided by its standard deviation, a process also called autoscaling (Goodacre et al., 2007). To investigate the relationship between the FT-IR spectra and the concentration of milk, multivariate statistical methods were used including cluster analysis and supervised regression-based techniques.

4.2.3.2 Cluster analysis

The two step cluster analysis was undertaken in an identical fashion to Chapters 2 and 3.
4.2.3.3 Supervised analysis

If the targets are known for some of the data then supervised learning methods can be used where the knowledge of both input (FT-IR spectra) and outputs (% adulteration target values) can be employed in the calibration phase. The aim of supervised learning is to construct a robust valid model that correctly associates inputs to outputs (Martens and Naes, 1989). In this study we used partial least squares (PLS) and kernel PLS, as linear and non-linear regression techniques, respectively.

In order to validate both PLS and kernel PLS for each of the four mixtures a training set and independent hold out set were generated. For the binary mixtures the training set was 0, 10, 20, 30, 40, 50, 60 70, 80, 90, and 100% of one of the milks, and the test set was 5, 15, 25, 35, 45, 55, 65, 75, 85 and 95%. The samples used in the training and test sets for tertiary mixtures, are shown in Table 7.

4.2.3.4 PLS regression

PLS1 was used on the binary milk mixtures (Cow-Sheep, Cow-Goat and Sheep-Goat milk mixtures) while we used PLS2 on the tertiary milk mixture as previously described. The number of PLS components was optimized using $k$-fold cross-validation as previously described in Chapter 3.

4.2.3.5 Kernel PLS

Kernel PLS (Shawe-Taylor and Christianini, 2004) was also employed as a non-linear regression method considering that the response of IR spectra might not always be
linear when measuring different compositions of different milks. Identical parameters and optimization was executed as described in Chapter 3.
4.3 RESULTS AND DISCUSSION

Representative FT-IR spectra collected from pure cows’, goats’ and sheep’s milk are shown in Figure 28. Overall at the qualitative level the spectra look very similar and this is particularly the case for cows’ and goats’ milks. By contrast, some quantitative differences were observed in sheep’s milk. The first difference involved the CH$_2$ absorption band at $ca.$ 2927 cm$^{-1}$, which is related to the acyl chain on fatty acids. As expected the degree of absorption for this band correlates with the fat quantity in each type of milk, with higher fat content resulting in higher IR absorption. As reported in the nutritional information that accompanied these milks, full fat sheep’s milk has a 4.5% fat composition, while cows’ and goats’ milk have 3.1% and 3.7% fat composition respectively, and this was reflected in the CH$_2$ stretch.

The other obvious visible differences between milk types appeared on the absorption bands related to the remaining milk components, protein (at 1654 cm$^{-1}$ and 1544 cm$^{-1}$ for amide I and II respectively) and lactose (at 1159 cm$^{-1}$ and 1076 cm$^{-1}$). These appeared to be in higher quantity in sheep’s milk than in cows’ and goats’ milk, with the former displaying higher IR absorption for these bands. Integration of the bands derived from the spectra for each milk type confirm the above differences in the composition of sheep milk with higher absorption values for the CH$_2$ and C-O absorption bands (Table 8).
**Figure 28.** FT-IR spectra for pure cows’ milk, goats’ and sheep’s milk. These spectra are offset to allow visualization of any difference

**Table 8.** Integrated absorption bands from the three measured spectra for each type of milk; numbers are mean ± standard deviation
4.3.1 Analysis of binary mixtures of milk

Due to the subtle differences highlighted above it was not possible to use simple visual inspection to quantify the level of adulteration of these milk. Thus following spectral collection from the different milk combinations, the relationship between the spectra was investigated using PC-DFA.

Using the combination of goats’ and sheep’s milk as an example (Figure 29A), visual inspection of the results identifies that there is a clear trend in PC-DFA space with respect to mixture levels; although this does not follow a linear trajectory, rather a parabolic one. The samples with a high concentrations of goats’ milk are recovered in the same region on the right of the pane, a decrease in the concentration of sheep’s milk, with a concurrent increase in the concentration of sheep’s milk, appears to cause a spread of the spectra first upwards to the middle of the pane and then latterly towards the bottom left of the pane. Inspection of the PC-DFA loadings matrix (Figure 30) indicates that for the separation of goats' and sheep's milk that there are larger differences in the fatty acid vibrations from CH$_2$ stretches in the region 2800-3000 cm$^{-1}$ (and see Figure 28 for annotation) than for either the protein or polysaccharides regions. For the other two mixtures (cow-sheep and cow-goat) a generally similar parabolic trend in PC-DFA space was also observed (Figures 29B and 29C). It was clear therefore that there was a non-linear trend with respect to milk
concentration and we thus sought to explore the use of linear and non-linear multivariate regression techniques for quantification.
Figure 29. PC-DFA plot on HT FT-IR spectra for the three machine replicates of (A) goats’ milk when added to sheep’s milk, (B) cows’ milk when added to sheep’s milk and (C) cows’ milk when added to goats’ milk. In (A) PCs 1–5 (accounting for 99.15% of the total variance), in (B) PCs 1–13 (accounting for 99.91% of the total variance), in (C) PCs 1–16 (accounting for 99.34% of the total variance), were employed by the DFA algorithm with a priori knowledge of machine replicates. The different numbers show the concentration of goats’ milk in the mixture and the colour scale the level of adulteration. The blue colour indicates low concentration of first milk species in the mixture, the red colour indicates high concentration of the same milk species and purple colour indicates the concentrations where the first milk type and second milk species have very close concentrations. Block arrows indicate the trend of data.
Figure 30. PC-DFA loadings plot from the first vector (discriminant function 1 (DF1)) showing which infrared regions are important: the positive part of DF 1 reflects areas that are increased in goats’ milk and the negative half of the plot those regions that are higher in sheep’s milk.

4.3.1.1 Quantification of binary milk mixtures

As detailed above three different binary milk combinations were produced. For brevity we shall use goat-sheep milk as an example. Samples containing 0 to 100% goats’ milk (in 5% steps) in sheep’s milk were prepared and the 21 mixtures were analyzed in triplicate using FT-IR spectroscopy. As detailed above, the data were pre-processed using SNV and were then split into a training set (0, 10, 20, ..., 90, and 100% goats’ milk) and a test set (5, 15, 25, ..., 85 and 95%) and analyzed by PLS, and KPLS.
During calibration of the PLS model the training data were used and these were sub-sampled using leave-one-out to generate a cross validation set in order to choose the optimum number of PLS factors (latent variables) for calibration. Following this the independent test set was used to challenge the PLS model. The PLSR results for the goats’ and sheep’s milk combinations are shown in Figure 31. In this plot the estimated goats’ milk concentration versus the known goats’ milk concentration values follow the expected $y=x$ and gave relatively accurate results. As detailed in Table 9 the root mean squared (RMS) error for the training data (RMSEC) was 3.73%, the cross validation set selected 3 PLS factors for this model and the RMS error for the cross validation set was 5.57%, the RMS error in the independent test was 8.03% (root mean square error for prediction in the test set; RMSEP). The correlation coefficient $Q^2$ for this model was 0.92.

Root mean square error for predictions and $Q^2$ are two unbiased metrics indicators of the predictive ability of a regression analysis model. In predicting the concentration of unknown samples from a data set not used to construct the model (the test set), RMSEP provides an unbiased estimate of the model’s prediction error, with a small RMSEP value interpreted as an indicator of an accurate model and vice versa. $Q^2$ is an independent metric scale, employed in the same way as the univariate regression analysis squared correlation coefficient $R^2$, in the quantification of the predictive ability of the model, with the $Q^2$ value approaching 1 indicating a more superior model. Thus the predictions from the goat-sheep milk mixture were relatively encouraging at 8.03% and 0.92 for the RMSEP and $Q^2$ respectively.
KPLS is a non-linear extension of PLS and we decided to investigate this algorithm as the PC-DFA had shown a non-linear trajectory. The results for KPLS are also shown in Figure 31 and the associated statistics in Table 9. It can be seen that the greatest improvement of using KPLS over PLS is in the model constructed from the goat-sheep milk mixture with a RMSEP of 3.95% and a $Q^2$ of 0.98. This improvement of KPLS over PLS was also observed for the cow-sheep and cow-goat mixtures (Figure 31 and Table 9) again highlighting the usefulness of employing a non-linear regression algorithm.
Figure 31. Plot showing the predicted concentrations of goats’ milk for the first set of mixtures and cows’ milk predicted concentrations for the second and third mixtures versus the actual concentrations for the three different mixtures of milk using PLS and KPLS. The blue circles represent the training data set and the red circles the test set.
Table 9. Comparison of the partial least squares (PLS) regression, and the non linear Kernel partial least squares (KPLS) of the FT-IR spectra for determining the percentage volume of cows’ milk mixed with goats’ milk and sheep’s milk and goats’ milk mixed with sheep’s milk.

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<td>0.96</td>
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</table>

The RMSECV represents the root mean square error for the cross-validation, the RMSEC the root mean square error for the calibration and the RMSEP the root mean square error for the predictions produced.
4.3.2 Mixtures of the three types of milk

Following a similar strategy to that used for the binary mixtures the first stage in the analysis was to look at the natural relationship between the FT-IR spectra collected from the 63 milk samples, containing various concentrations of sheep’s, goats’ and cows’ milk. The results of the PC-DFA are shown in Figure 32 as a pseudo-3D plot of the first 3 discriminant function, and it is clear that these spectra are spread in three different dimensions. Three colour scales are used for each of the milk species: high concentration of cows’ milk samples are presented with red colour; sheep’s milk is presented with green colour; and a higher proportion of goats’ milk is presented in blue.

It is clear from Figure 32 that when the contribution of one milk type starts to dominate the mixture that a ‘tentacle’ extends from the centre of the milk mixtures and the tips contain that pure milk. Domination of these ‘tentacles’ appears to develop when the concentration of one of the contributing milk types increases above the 55% level. In addition, a clear forth tentacle (yellow colour) develops when the concentration of cows’ and sheep’s milk in the tertiary sample both increase above the 40% contribution level for these milk types. In the middle there also appears to be three smaller tentacles which are coloured dark green, turquoise and purple. These contain samples were the three milk types are in similar concentrations (dark green) and were two milk types are in high concentration; the turquoise is for sheep-goat high concentration milk and purple for cow-goat high concentration milk (a similar plot giving the concentration levels of each milk is shown as Figure 33). In conclusion, the cluster analysis shows that there are clear trends in these data related to the various combinations of milk, but that the ability to quantify the level of milks
in these tertiary mixtures is unlikely from the PC-DFA given the complexity of this space.

Figure 32. PC-DFA plot on HT FT-IR spectra for the three machine replicates of the mixtures from the three types of milk. The DFA algorithm employed PCs 1–20 (accounting for 99.86% of the total variance) with *a priori* knowledge of machine replicates. High concentration of cows’ milk samples are presented with red colour, sheep’s milk is presented with green colour and goat’s milk is presented with blue. In the middle there appears to be samples which contain two out of the three milk types in high concentrations and these appear with different colour from the combination of all three types of milk.
Figure 33. PC-DFA plot on HT FT-IR spectra for the three repeat experiments of the mixtures from the three types of milk. The DFA algorithm used PCs 1–20 (accounting for 99.86% of the total variance) with a priori knowledge of machine replicates. The numbers [c, s, g] refer to the level of cow, sheep and goat milk in the tertiary mixture.
4.3.2.1 Quantification of tertiary milk mixtures

The data were split into training and test set data and then analyzed using linear and non-linear supervised learning techniques; as three milk concentrations were to be predicted we employed three output Y-variables (one for each type of milk) in PLS2 and KPLS2. The results from the PLS2 and KPLS2 models for the training and test data are shown in Figures 34 and 35, and overall showed a good prediction for the three different types of milk. Table 10 shows the summary statistics for PLS2 and KPLS2 using the same training and test set splits and in this case the PLS2 algorithm outperformed KPLS2. In general all milks were predicted with a similar level of accuracy and the RMSEPs were 3.4-4.9% for PLS2 with $Q^2$ correlations of 0.94-0.97.
Figure 34. Plot showing the predicted concentrations of cows’, goats’ and sheep’s milk versus the actual concentrations for the training (A) and test (B) set in the mixtures of the three types of milk by using PLS2.
Figure 35. Plot showing the predicted concentrations of cows’, goats’ and sheep’s milk versus the actual concentrations for the training (A) and test (B) set in the mixtures of the three types of milk by using KPLS2.
Table 10. Comparison of the partial least squares (PLS2) regression, and the non-linear Kernel partial least squares (KPLS2) of the FT-IR spectra for determining the percentage volume of cows’, goats’ and sheep’s milk from mixtures containing the three types of milk together.

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</table>

RMSECV represents the root mean square error for the cross-validation; the RMSEC the root mean square error for the calibration; the RMSEP the root mean square error for the predictions produced.
4.4 CONCLUDING REMARKS

In this study it has been demonstrated that in binary and tertiary mixtures of milk FT-IR spectroscopy in combination with multivariate analysis, such as linear PLS and non linear Kernel PLS, provides an accurate, simple and rapid technique for the quantitative assessment of the adulteration of sheep’s, goats’ and cows’ milk. The typical errors that were found were in the region of 3.5%-8% for all milk species, a level at which a fraudster would unlikely adulterate at because this would not be financially viable. For this reason and the speed of analysis (30s per sample in batches of 96 or 384) provide FT-IR spectroscopy with an excellent potential for use in the food industry in replacing less efficient and more time-consuming techniques for the detection of milk adulteration. For this approach to be employed more broadly within the industry, future studies should also consider geographical as well as seasonal variations in milk production.
CHAPTER 5

MALDI-MS AND MULTIVARIATE ANALYSIS FOR THE DETECTION AND QUANTIFICATION OF DIFFERENT MILK SPECIES

This work has been published in a peer review journal as:


Dr Yun Xu helped with the CCA, PLS and KPLS analyses.
**ABSTRACT**

The extensive consumption of milk and dairy products makes these foodstuffs targets for potential adulteration with financial gains for unscrupulous producers. Such practices must be detected as these can impact negatively on product quality, labelling and even health. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a potentially useful technique, with proven abilities in protein identification and more recently through the use of internal standards for quantification purposes of specific proteins or peptides. In the current work we therefore aim to explore the accuracy and attributes of MALDI-ToF-MS with chemometrics for the detection and quantification of milk adulteration.

Three binary mixtures containing cows’ and goats’, cows’ and sheep’s and goats’ and sheep’s milk and a fourth tertiary mixture containing all types of milk, were prepared and analysed directly using MALDI-ToF-MS. In these mixtures the milk concentrations of each milk varied from 0 to 100% in 5% steps. Multivariate statistical methods including partial least squares (PLS) regression and non-linear kernel PLS (KPLS) regression, were employed for multivariate calibration and final interpretation of the results. The results for PLS and KPLS were encouraging with between 2-13% root mean squared error of prediction on independent data; KPLS slightly outperformed PLS.

Concluding, these findings suggest that MALDI-ToF-MS can be a useful aid and can be routinely employed in the dairy industry for the rapid detection and quantification of adulteration in milk.
5.1 INTRODUCTION

The issue of food safety and authenticity in relation to milk has been described in the previous chapters. Ensuring product authenticity and implementing some of the strict standards and criteria implemented by policymakers across different countries and the EU has been very difficult. Analytical techniques have been employed to perform this hard task, but have been unable to full fill this role effectively, either because they are unable to keep in pace with the constant technological advances and developments concurring at the dairy industry or because of lack of commercial practicality (Karoui and Baerdemaeker, 2007). Advanced techniques such as spectroscopy, near-infrared (NIR), mid-infrared (MIR) and nuclear magnetic resonance (NMR) spectroscopies (Andreotti et al., 2002, Jha and Matsuoka, 2004, Reid et al., 2006, Kasemsumran et al., 2007, Sacco et al., 2009), as well as chromatography (Chen et al., 2004, Gutierrez et al., 2009), immunoenzymatic assays (Anguita et al., 1997a, Hurley et al., 2004a), polymerase chain reaction (Maccabiani et al., 2005, El-Rady and Sayed, 2006), electrophoresis (Addeo et al., 1995, Lee et al., 2004, Recio et al., 2004) and sensory analyses (Yu et al., 2007, Dias et al., 2009) have all been utilized by the analytical dairy science to improve milk product analysis. The main disadvantage of these techniques though, as already described, is that they remain time-consuming and labour intensive, with limited value for routine use in the screening of milk in the dairy industry.
Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a potentially useful technique in the authentication of milk, with proven abilities in protein identification and more recently quantification (Angeletti et al., 1998, Fanton et al., 1998, Ross et al., 2000, Cozzolino et al., 2001, Bucknall et al., 2002, Soeryapranata et al., 2002, Noo et al., 2005). MALDI is an ionisation technique involving the insertion of the sample typically into a UV absorbing matrix composed of a non-volatile material (usually a mild aromatic acid), followed by laser irradiation (typically at 337 nm), absorption, matrix energy desorption and matrix to sample proton transfer resulting in the creation of vaporized ions (Karas et al., 1987). The technique has the advantage of only requiring small sample quantities and can be used for the analysis of heterogeneous biological samples such as milk, as recently demonstrated by Liland and colleagues (2009a, Liland et al., 2009b). In addition, it possess a very high sensitivity and a protein mass range reaching 300 000 Da (Siuzdak, 1996). The aim of our study was therefore to investigate whether the MALDI-TOF-MS technique is able to detect and quantify goats’ and sheep’s milk adulteration with cows’ milk, using the whole spectrum of peaks obtained from the analysis.
5.2 MATERIALS AND METHODS

5.2.1 Sample Preparation

Sample preparation was identical to the one used in the previous chapter utilizing three types of full fresh pasteurized milk: cows’, goats’ and sheep’s milk, tested in four different milk type combinations as follows:

(1) Sheep’s milk adulterated with cows’ milk,
(2) Goats’ milk adulterated with cows’ milk,
(3) Sheep’s milk adulterated with goats’ milk,
(4) A tertiary mixture containing sheep’s, goats’ and cows’ milk

Percentage variation of the different milk types was again the same both for binary and tertiary mixtures (Table 11) as was the mixing protocol. Following this 1 mL milk samples were collected and used for MALDI-ToF-MS analysis.
Table 11. Combinations of milk samples containing a tertiary mixture of sheep’s, goats’ and cows’ milk, [%]: percentage concentration.

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<th>Sheep milk [%]</th>
<th>Goat milk [%]</th>
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<td>Test</td>
</tr>
<tr>
<td>29</td>
<td>30</td>
<td>35</td>
<td>35</td>
<td>Test</td>
<td>61</td>
<td>35</td>
<td>30</td>
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<td>Test</td>
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<td>31</td>
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<td>15</td>
<td>45</td>
<td>Train</td>
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<tr>
<td>32</td>
<td>45</td>
<td>5</td>
<td>50</td>
<td>Test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SID: sample identity

5.2.2 MALDI-ToF-MS

The sample preparation used was based on the method reported by Cozzolino and colleagues (2001). This involved initially taking 100 µL of each homogenized sample.
and diluting these in 1 mL of water containing 0.1% trifloroacetic acid. These samples were then further diluted 1:10 with the same solvent. 5 μL of these samples were then mixed with 5 μL of the matrix solution. For all samples sinapinic acid was used as a matrix having been saturated in a matrix solution composed of 50% acetonitrile and 50% water. From the final mixtures 1μL were then positioned onto a MALDI-MS stainless steel target plate and dried for 2 h at room temperature.

MALDI-ToF-MS analysis was undertaken on a MALDI-TOF mass spectrometer (AXIMA-CRF™ plus; Shimadzu Biotech, Manchester, UK), in conjunction with a nitrogen pulsed UV laser (337nm). A positive ion source in linear ion mode was used for ionisation and separation, and 120 mV of laser power was used. Analysis of every spot was performed using a random raster of 500 profiles, each profile containing data from five laser shots. Each sample was analysed three times and the typical collection times were 4 min per sample.

5.2.3 Data analysis

5.2.3.1 Pre-processing

The mass spectral data were imported into MATLAB (The Math Works, Natick, MA, USA) and processed for analysis. Typically, the data were baseline corrected and normalized. Normalization of each individual spectrum was performed by dividing each individual baseline corrected spectrum with the square root of the sum of squares of the spectrum (Brereton, 2005).
5.2.3.2 Exploratory analysis

The exploratory analysis was performed in two steps (PCA and CCA) as described in Chapters 2 and 3.

5.2.3.3 Quantitative analysis

If there is a strong correlation between the two inputs, i.e. the MALDI-ToF-MS spectra and the adulteration levels, it is then possible to employ a multivariate regression model to predict the adulteration levels using the MALDI-ToF-MS spectra. In this study we used PLS and KPLS, as linear and non-linear regression techniques, respectively.

Whilst supervised methods are very powerful it is possible to over-fit the model, therefore validation of both PLS and kernel PLS was undertaken. We achieved this using an independent test set for each of the mixture types. For each of the binary mixtures the training set contained 0, 10, 20, ..., 90, and 100% of one of the milks, and the test set included 5, 15, 25, ..., 85 and 95%. For the tertiary mixtures, the training and test sets are shown in Table 11.

5.2.3.4 PLS regression

PLS models were generated to predict a single variable and so PLS1 was used for both the three binary mixtures as well as for the tertiary mixture. The number of PLS components was optimized using a $k$-fold cross-validation on the training set only as before, while $k$ is the number of adulteration levels in the training set.
5.2.3.5 Kernel PLS

Kernel PLS was also employed using a radial base function (RBF) as the kernel function, and optimization of the kernel parameter and number of PLS factors was performed as previously described.
5.3 RESULTS AND DISCUSSION

5.3.1 Spectra

MALDI spectra can be used to determine and quantify the protein components of various types of milk, by identifying the different peaks and assigning them to specific proteins based on previously published protein molecular mass data. Individual proteins however may show a variation in their molecular mass and thus the exact position of the peak, this is due to factors such as genetic and non-genetic polymorphisms and milk processing; the latter mainly via thermal denaturation and proteolysis which can affect individual protein structure (Visser et al., 1995, Recio et al., 1997, Moioli et al., 1998, Amigo et al., 2000, Borkova and Snaselova, 2005). Milk samples from different species or from different animal breeds of the same species can therefore display small variations in the molecular mass of the same protein, which will also vary depending on whether the milk is raw or has been processed and how it has been processed.

Figure 36 shows a typical MALDI mass spectrum of fresh full fat pasteurised cow milk, including the original raw data and processed data after baseline correction and normalization that was required before chemometric analyses. Qualitatively the spectra from all three milk species (Figure 37) appear to display similar protein patterns between the different types of milk with small differences in the location of the molecular mass signal of the same proteins; in addition, it is also possible to see some differences in regards to the quantity of certain proteins. A closer inspection of the MALDI mass spectrum of cow milk (Figure 37) and interpretation based on
previously published molecular mass data (Catinella et al., 1996, Angeletti et al., 1998, Fanton et al., 1998, Cozzolino et al., 2001, Soeryapranata et al., 2002), reveals a number of protein related peaks. These include a peak at \( m/z \) 9000 representing the proteoso peptone, seen in all milk types at a similar position, and the peaks at \( m/z \) 15000 and \( m/z \) 18500 relating to \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin, respectively; with the latter displaying a higher peak/content compared to the other milk types. The broad peaks over \( m/z \) 30000, at \( m/z \) 31000 and \( m/z \) 43000, represent dimeric and trimeric species (Catinella et al., 1996).

Inspection of the MALDI spectrum of sheep milk shows the \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin peaks appearing as \( m/z \) 13500 and \( m/z \) 19 000, with an additional peak at \( m/z \) 12 000 representing \( \gamma_2 \)-casein, which is less prominent in the other milk types. Finally, in the MALDI spectrum of pure goats' milk the \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin are located at \( m/z \) 13000 and \( m/z \) 19000 peaks respectively, while a more prominent peak at \( m/z \) 21000 represents \( \gamma_1 \)-casein.

The peaks below \( m/z \) 9000 in all types of milk are representative of species with low molecular mass due to proteolysis of higher mass proteins. The latter appear to be significant and overrepresented in the MALDI spectra obtained in this study compared to spectra from other studies using raw milk and this is most likely due to the effect of milk processing and pasteurization on the various protein species.
Figure 36. Typical MALDI-ToF-MS mass spectra of fresh full fat pasteurised cow samples. The columns are from the initial spectra as they undergo data preprocessing.
Figure 37. Raw MALDI-ToF-MS mass spectra of cow, sheep and goat milk samples. These mass spectra are annotated with protein identifications.
5.3.2 Analysis of binary mixtures of milk

The small differences between the pure spectra became even harder to visualize by eye when mixtures were analysed and so this did not allow direct visual comparison in order to detect the level of adulteration. Therefore the relationship between the spectra collected from the different milk combinations and concentrations was investigated using the exploratory analysis procedures as described in the data analysis section.

The results of from the CCA for the binary milk mixtures analysed using MALDI-ToF-MS are shown in Figure 38. It is very clear from this plot that there is a concentration dependent relationship in the MALDI-ToF-MS data and visual inspection of these results identifies a linear pattern of the mixture levels. The canonical correlation coefficient R for this linear relationship was found to be 0.9953 with a highly significant probability \( p \) value of \( 8.83 \times 10^{-35} \); while similar values (\( R = 0.9893, \ p \ value = 4.07 \times 10^{-30} \)) were found for the cows’ and sheep’s milk mixtures. The goats’ and sheep’s milk mixtures displayed a comparable linear relationship to the other milk mixtures but with a slightly lower value for the correlation coefficient, \( R = 0.9674 \), and a \( p \) value of \( 2.70 \times 10^{-16} \). This suggests that there are very strong correlations between the MALDI-ToF-MS data and their corresponding milk adulteration levels. Linear and non-linear multivariate regression techniques were therefore employed in order to explore these trends even further and to assess whether it was possible to quantify the level of milk adulteration from these mass spectra.
Figure 38. CCA plot on MALDI-ToF-MS spectra of cows’ milk when added to goats’ milk (A) and sheep’s milk (B) and sheep’s milk when added to goat’s milk (C).

(A) PCs 1–15 were used by the CCA algorithm with *a priori* knowledge of concentration of cow milk. The different dots show the concentration of cows’ milk in the mixture in relation to the canonical variable scores, with $R = 0.9953$ and $p$ value $= 8.83 \times 10^{-35}$

(B) PCs 1–15 were used by the CCA algorithm with *a priori* knowledge of concentration of cow milk. The different dots show the concentration of cows’ milk in the mixture in relation to the canonical variable scores, with $R = 0.9893$ and $p$ value $= 4.07 \times 10^{-30}$

(C) PCs 1–15 were used by the CCA algorithm with *a priori* knowledge of concentration of goat milk. The different dots show the concentration of goats’ milk in the mixture in relation to the canonical variable scores, with $R = 0.9674$ and $p$ value $= 2.70 \times 10^{-16}$
5.3.2.1 Quantification of binary milk mixtures

Three different binary milk combinations were created as detailed above. The cow-goat milk binary mixture is subsequently used as an example. Samples containing 0 to 100% cows’ milk (in 5% steps) in goats’ milk underwent preparation and the resulting 21 mixtures produced were analyzed in triplicate using MALDI-ToF-MS. As detailed above, the data were baseline corrected, normalised and then split into a training set (0, 10, 20, ..., 90, and 100% goats’ milk) and a test set (5, 15, 25, ..., 85 and 95%) and analyzed by linear PLS, and non linear KPLS.

During calibration of the PLS model, sub-sampling of the training data took place, leaving one set (i.e. a unique adulteration level; all replicates) out so that a cross validation set could later be generated, this allowed for the selection of the optimum number of PLS factors for calibration. Once this was performed the independent test set was utilized to challenge the derived PLS model. PLS regression results for the cows’ and goats’ milk mixture combinations are shown in Figure 39. The plot of the estimated cows’ milk concentration versus the known cows’ milk concentration values in this figure appeared to show good predictive values and importantly both the training and test sets lie on the expected y=x perfect prediction line. Table 13 shows the detailed results for all the three binary mixtures. For the cows’-goats’ mixture, the root mean squared (RMS) error for the training data (RMSEC) was 0.95%, with 7 PLS factors selected by the cross validation set for this model and the RMS error for the cross validation (RMSECV) set was 5.24%, the RMS error in the independent test was 6.85% (RMSEP). This models’ $Q^2$ value was 0.95, while the $R^2$ for the train set model was 0.99 both very close to the prefect model which would be 1.
Figure 39. Plots showing the predicted levels of milk adulteration estimated from PLS and KPLS models. The rows show the different mixtures analysed with predictions for cow's milk adulteration in goats' (top) and sheep's (middle) milk and goats’ milk adulterated into sheep's milk in the bottom row. The blue circles represent the training data set and the red crosses the test set.
The most dominant features used for PLS modelling for discriminating between two
types of milk can be uncovered by inspecting the highest Variable Importance for
Projection (VIP) plots; these are displayed in Figure 40 and Table 12 for the binary
milk mixtures. Comparison with the pure milk MALDI-MS spectra (Figure 36)
indicates that for the cow-goat binary mixtures the dominant features at 14 100 and 18
020 m/z are clearly present in both the pure cow and goats’ milk spectra and these
peaks represent α-lactalbumin and β-lactoglobulin respectively. Whilst these proteins
are present in both milks the ratio of β-lactoglobulin to α-lactalbumin is different
between cow and goat milk, and this is what is discriminatory. An additional peak at
11 740 m/z, representing γ2-casein, is also selected as a VIP, although it does not
appear as a dominant peak in any of the two pure milk spectra, PLS suggest that
despite its small magnitude this protein is significantly different. In the cow-sheep
binary mixture VIP features at 8600 m/z and 14 100 m/z appear in both the pure cows’
and sheep’s milk spectra representing the proteose peptone and α-lactalbumin proteins
respectively, while features at 11 190 and 11 280 m/z (γ3-casein) and 11 740 m/z (γ2-
casein) are only present in the pure sheep’s milk spectra. In the third binary mixture,
goat-sheep milk, the latter features (γ3-casein and γ2-casein) remain distinctive only in
the pure sheep’s milk spectra with the VIP feature at ~8600 m/z (proteose peptone)
being present in both of the pure milk spectra. The 23 470-700 m/z feature (αS1-
casein) even though appearing as a dominant VIP feature in all three binary mixture
PLS models, does not appear as an intense peak in the pure milk spectra for any of the
three types of milk.
Figure 40. Plots of Variable Importance for the Prediction (VIP) scores used in the PLS modelling for the binary milk mixtures
Table 12. Spectral peaks with the highest Variable Importance for the Prediction (VIP) scores used in the PLS modeling for the binary and tertiary milk mixtures

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Peak m/z</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binary Mixture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow-goat milk</td>
<td>11 750</td>
<td>γ2-casein</td>
</tr>
<tr>
<td></td>
<td>14 100</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td></td>
<td>18 020</td>
<td>β-lactoglobulin</td>
</tr>
<tr>
<td></td>
<td>23 700</td>
<td>αs1-casein</td>
</tr>
<tr>
<td>Cow-sheep milk</td>
<td>8600</td>
<td>Proteose peptone</td>
</tr>
<tr>
<td></td>
<td>11 190, 11 480</td>
<td>γ3-casein</td>
</tr>
<tr>
<td></td>
<td>11 740</td>
<td>γ2-casein</td>
</tr>
<tr>
<td></td>
<td>23 470</td>
<td>αs1-casein</td>
</tr>
<tr>
<td>Goat-sheep milk</td>
<td>8530</td>
<td>Proteose peptone</td>
</tr>
<tr>
<td></td>
<td>11 190, 11 450</td>
<td>γ3-casein</td>
</tr>
<tr>
<td></td>
<td>11 820</td>
<td>γ2-casein</td>
</tr>
<tr>
<td></td>
<td>23 600</td>
<td>αs1-casein</td>
</tr>
<tr>
<td><strong>Tertiary mixture</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow milk</td>
<td>11 470</td>
<td>γ3-casein</td>
</tr>
<tr>
<td></td>
<td>11 730</td>
<td>γ2-casein</td>
</tr>
<tr>
<td></td>
<td>14060</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>Sheep milk</td>
<td>11 190, 11 450</td>
<td>γ3-casein</td>
</tr>
<tr>
<td></td>
<td>14 060</td>
<td>α-lactalbumin</td>
</tr>
<tr>
<td>Goat milk</td>
<td>11 450</td>
<td>γ3-casein</td>
</tr>
<tr>
<td></td>
<td>11 740</td>
<td>γ2-casein</td>
</tr>
<tr>
<td></td>
<td>14 060</td>
<td>α-lactalbumin</td>
</tr>
</tbody>
</table>

In order to examine the results even further we decided to employ the KPLS algorithm, a non-linear extension of PLS. KPLS analysis results are depicted in Figure 39, with the relevant statistical information also shown in Table 13. For our cows’-goats’ binary milk mixture, it is clear that KPLS provides similar results to PLS, with a RMSEP of 6.35% and a $Q^2$ 0.95. The usefulness and improvement of the non-linear KPLS algorithm compared to PLS is more apparent when the results from the other two binary milk mixtures are observed, as shown in Figure 39 and Table 13. KPLS does not allow the generation of loadings or VIP scores so model interpretation in terms of which proteins are important is largely transparent.
Table 13. Comparison of the partial least squares (PLS) regression and the non linear Kernel partial least squares (KPLS) results of the MALDI-ToF-MS spectra for determining the percentage volume of cows’ milk mixed with goats’ milk and sheep’s milk and goats’ milk mixed with sheep’s milk.

<table>
<thead>
<tr>
<th></th>
<th>PLS</th>
<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cow-goat milk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>RMSECV</td>
<td>5.24</td>
<td>4.98</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.95</td>
<td>0.77</td>
</tr>
<tr>
<td>RMSEP</td>
<td>6.85</td>
<td>6.35</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Cow-sheep milk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>RMSECV</td>
<td>7.56</td>
<td>6.16</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.64</td>
<td>0.51</td>
</tr>
<tr>
<td>RMSEP</td>
<td>9.77</td>
<td>8.13</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.89</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Goat-sheep milk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>RMSECV</td>
<td>10.59</td>
<td>10.35</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>RMSEP</td>
<td>12.38</td>
<td>12.87</td>
</tr>
<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.82</td>
<td>0.81</td>
</tr>
</tbody>
</table>

RMSECV represents the root mean square error for the cross-validation; the RMSEC the root mean square error for the calibration; the RMSEP the root mean square error for the predictions produced for the independent test set.

5.3.3 Mixtures of the three types of milk

The MALDI mass spectra collected from the 63 milk samples, containing various concentrations of the three milk types, sheep’s, goats’ and cows’ milk, were analyzed
using a similar strategy to the binary mixture analysis. Initially the natural relationship between the spectra was initially investigated using CCA. Figure 41 presents the CCA results in a pseudo-2D plot constructed from the first 5 PCs. The different concentrations of the three different types of milk are represented in different colours. High concentrations of cows’, sheep’s and goats’ milk in the samples are represented by red, green and blue colour, respectively. Visual inspection of the 2D space indicates a clear pattern of distribution of the different milk mixture concentrations. Domination of a particular type of milk in the mixture at concentrations of greater than 50% appears to create a tentacle towards a specific direction with a pure milk type at the tip. An increasing cows’ milk concentration appears to create a tentacle towards the right of the pane (red), an increase in sheep’s milk concentration extends a tentacle towards the left lower corner of the pane (green), while similarly the increasing goats’ milk concentration forms a tentacle towards the upper left corner of the pane (blue). Furthermore, three additional smaller distinctive tentacles (yellow, purple and turquoise) are observed towards the middle of the pane extending outwards in different directions. Each tentacle appears to lie between two of the bigger tentacles and represents mixtures containing the two associated milk types in concentrations of greater than 40% respectively. For example the small tentacle in turquoise colour extending due west lies between the blue (high goat milk concentration) and the green tentacle (high sheep milk concentration) representing mixtures containing these two types of milk in concentrations of greater than 40%. At the tip of the small tentacles lie the two dominant milk types at 50% concentration each. Even though this analysis of tertiary mixtures using CCA appears to show clear trends regarding the different milk combinations, because these are revealed in their 2D spatial distribution this
would limit accurate quantification and thus PLS and KPLS were also used to quantify the levels of the different milk species in these tertiary mixtures.

**Figure 41.** CCA plot on MALDI-ToF mass spectra for the mixtures from the three types of milk. PCs 1–5 CCA algorithm with *a priori* knowledge of the concentration of the three milks. High concentration of cows’ milk samples are presented with red colour, sheep’s milk is presented with green colour and goat’s milk is presented with blue. In the middle there appears to be samples which contain two out of the three milk types in high concentrations and these appear with different colour from the combination of all three types of milk.
5.3.3.1 Quantification of tertiary milk mixtures

In order to perform quantification of the tertiary mixtures, the data were first divided in two sets, a training and a test set (Table 11), and analyses was undertaken as described above again employing the linear and non-linear supervised learning techniques of PLS and KPLS regression. Although there are three Y-variables to be predicted (one for each milk species), rather than use PLS2 and KPLS2, we chose to use PLS1 and KPLS1 where three models were constructed for each milk. This was because it has been shown that PLS1 generally outperforms PLS2 for quantification of different analytes as there will be different directions in the spectral space that are describing the contributions for the three different milk species; therefore it is better to optimise each of these individually (Goodacre et al., 1994). Figure 42 illustrates the results from the PLS and KPLS models for the training and test data. For both PLS and KPLS models excellent predictions for the three different types of milk was attained. Table 14 shows the summary statistics for both multivariate regression models using the same training and test set splits; in this case the PLS algorithm outperforms KPLS. Rather interestingly these models for the tertiary mixture were better than the models constructed from the binary models (Figure 39 and Table 13), although we can not think of any conceivable reason why this may be the case.

The most dominant spectral peaks used by the PLS modelling for discriminating between the three types of milk with the highest VIP scores for the tertiary milk mixtures are displayed in Figure 43 and Table 12. In all three PLS models for each type of milk the dominant features are largely the same and appear as a peak in one or more of the pure milk spectra. The features at 11190 - 450 m/z ($\gamma_3$-casein) and 11730 - 40 m/z ($\gamma_2$-casein) are only present as spectral peaks in the pure sheep milk
while the 14 060 m/z (α-lactalbumin) VIP score feature is present as a peak in all three types of pure milk samples. Furthermore, the difference found between the number and level of scoring for some features in the tertiary sample mixtures compared to the binary mixtures is most likely due to the effect of the presence of the additional milk type in the mixture.

**Table 14.** Comparison of the partial least squares (PLS) regression, and the non linear Kernel partial least squares (KPLS) results of the MALDI-ToF-MS spectra for determining the percentage volume of cows’, goats’ and sheep’s milk from mixtures containing the three types of milk together.

<table>
<thead>
<tr>
<th></th>
<th>PLS</th>
<th>KPLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cow milk</td>
<td>Sheep milk</td>
</tr>
<tr>
<td>Factors</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>RMSECV</td>
<td>2.58</td>
<td>4.77</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td>RMSEP</td>
<td>2.42</td>
<td>3.29</td>
</tr>
<tr>
<td>Correlation coefficient in the train set (R²)</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Correlation coefficient in the test set (Q²)</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>Factors</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>RMSECV</td>
<td>1.91</td>
<td>2.84</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.50</td>
<td>0.12</td>
</tr>
<tr>
<td>RMSEP</td>
<td>2.02</td>
<td>2.32</td>
</tr>
<tr>
<td>Correlation coefficient in the train set (R²)</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Correlation coefficient in the test set (Q²)</td>
<td>0.99</td>
<td>0.98</td>
</tr>
</tbody>
</table>

RMSECV represents the root mean square error for the cross-validation; the RMSEC the root mean square error for the calibration; RMSEP the root mean square error for the predictions produced for the training set.
Figure 42. Plots showing the predicted concentrations of cows’, goats’ and sheep’s milk versus the actual concentrations for the training and test set in the tertiary mixtures of the three types of milk using (A) PLS and (B) KPLS. The blue colour represent the training data set and the red the test set. The triangles represent the goats’ milk samples, the crosses the sheep’s milk and the stars cows’ milk.
Figure 43. Plots of Variable Importance for the Prediction (VIP) scores used in the PLS modeling for the tertiary milk mixtures
5.4 CONCLUDING REMARKS

In comparison to previous studies demonstrating the qualitative aspects of MALDI-ToF mass spectrometry using selected peaks for milk speciation (Angeletti et al., 1998, Fanton et al., 1998, Ross et al., 2000, Cozzolino et al., 2001, Bucknall et al., 2002, Soeryapranata et al., 2002, Noo et al., 2005), through this study it has been shown that the whole MALDI-ToF mass spectra contains valuable information. However, this can only be revealed when MS is combined with multivariate techniques such as linear PLS and non linear Kernel PLS, and it was shown that it is possible to achieve very accurate predictions of the levels of milk species adulteration. These properties have been demonstrated in analysing binary and tertiary mixtures of fresh pasteurised cows’, sheep’s and goats’ milk using a simple and fast process. Despite the milk processing which may affects protein structure MALDI-ToF-MS was able to achieve high accuracy levels of milk adulteration with typical errors in the region of 2-13%, for cow’s milk a level at which a fraudster would unlikely adulterate at because this would not be financially viable.
CHAPTER 6

CONCLUSIONS
6.1 DISCUSSION

Every year huge quantities of milk are produced and processed for human consumption either directly as milk or as a wide variety of dairy products. According to the latest statistical data from the Food and Agriculture Organization of the United Nations approximately 0.67 trillion tonnes of milk were produced for domestic supply in 2007 alone (Food and Agricultural Organization of the United Nations (FAO), 2010). The yearly individual consumption of milk in western countries such as the UK and USA during the same time period was 241.5 litres per capita and 253.4 litres per capita, respectively, progressively increasing by a few litres per capita every decade.

The widespread and heavy consumption of milk makes this product very valuable. Milk spoilage, attributed to the growth of bacteria in milk, is a very frequent and important problem not only for the milk industry but also for the consumer. Spoilage with its off flavour, odour or change in texture and appearance makes milk unattractive for human consumption (Whitfield, 1998, Gram et al., 2002), while the growth of specific pathogenic organisms can make it unsuitable and dangerous for utilization (Zall, 1990, Mazurek et al., 2004, Gillespie et al., 2010). In an attempt to reduce pathogenicity and prolong shelf-life of fresh milk, various preservation techniques have been adopted throughout the decades, including pasteurization and low temperature storage (Frank, 2001). Despite this milk spoilage appears to occur after few days of processing and storage, although filtration does appear to reduce this (Pafyliaas et al., 1996, Elwell and Barbano, 2006, Goff and Griffiths, 2006, Hoffmann et al., 2006). Ultra heat treatment of milk appears to resolve the issue more effectively
allowing milk to be stored for months but this also results in sensory changes which are unacceptable to a number of consumers (Cais-Sokolinska et al., 2005).

As milk spoilage and pathogenicity appears to be directly related to bacterial growth, a method providing an accurate prediction of the microbial type and/or load in milk would be ideal for evaluating milk quality and promoting public health. As previously discussed, a number of different methods have been investigated in their ability to perform this task (Gutierrez et al., 1997b, Niza-Ribeiro et al., 2000, Samkutty et al., 2001, Reinders et al., 2002, Gunasekera et al., 2003a, Kowalik and Ziajka, 2005, Flint et al., 2007, Jiang et al., 2007, Lopez-Enriquez et al., 2007, O'Grady et al., 2008, Amari et al., 2009). These have however been only partially successful and have been troubled by individual limitations including laborious processing techniques, increased operator demands, use of reagents, sample destruction and delays in sample turnover. Table 15 shows some of the important positive and negative attributes of these methods.

**Table 15.** Attributes of methods involved in the detection and quantification of bacteria in milk

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>• Rapid</td>
<td>• Unable to differentiate between microbial and other background (e.g., eukaryotic) ATP</td>
</tr>
<tr>
<td></td>
<td>• Suitable for checking potentially contaminated surfaces in the industrial setting</td>
<td></td>
</tr>
<tr>
<td>Direct Epifluorescent Filter Technique</td>
<td>• Simple with minimal equipment requirements</td>
<td>• Low repeatability</td>
</tr>
<tr>
<td></td>
<td>• Inexpensive</td>
<td>• Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Difficulty in differentiating between viable and non-viable microorganisms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Food particle debris may interfere with visualisation</td>
</tr>
<tr>
<td>Method</td>
<td>Key Points</td>
<td>Limitations</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| **Electrical**  | • Rapid                                                                     | • Suitable medium required supporting bacterial growth with simultaneous change in electrical charge with growth  
|                 |                                                                             | • Suspending medium has to be very clean  
|                 |                                                                             | • Method only works when bacterial numbers greater than $10^6 – 10^7$ CFU/ml |
| **Flow cytometry** | • Sensitive  
|                 | • Quantitative  
|                 | • Automated                                                                | • Specific stains required for differentiation between viable and non-viable microorganisms  
|                 |                                                                             | • Time consuming 1-3h  
|                 |                                                                             | • Specialized equipment required |
| **PCR**         | • High sensitivity  
|                 | • Specific  
|                 | • Detects low level of pathogenic bacteria                                  | • Target DNA sequence must be known  
|                 |                                                                             | • High rate of false positive results when non-viable microorganisms also present  
|                 |                                                                             | • Time-consuming and elaborate technique because pre-processing food sample preparation requires extraction of various PCR inhibitors |
| **ELISA**       | • High specificity and sensitivity  
|                 | • Reliable                                                                  | • Expensive antibody production  
|                 |                                                                             | • Need for highly specific antibodies and prior knowledge  
|                 |                                                                             | • Labour intensive |
| **Electronic nose** | • Simple  
|                 | • Inexpensive  
|                 | • Rapid for quality control and screening  
|                 | • Minimal sample preparation                                                 | • Sensor problems; e.g. sensor poisoning  
|                 |                                                                             | • High skilled knowledge required for use of chemometrics |

FT-IR spectroscopy is an analytical technique that has been used in the past to investigate the natural spoilage of poultry and beef with very promising results (Ellis et al., 2002, Ellis et al., 2004). The first objective of this work was therefore to investigate the use of ATR and HT FT-IR in combination with multivariate analytical techniques, in achieving rapid (seconds per sample) and accurate viable bacterial quantification in pasteurized milk, devoid of the limitations affecting the other techniques. The most commonly consumed milk species, cow milk, was investigated, and these pasteurized milks included whole, semi-skimmed and skimmed. Bacterial numbers were estimated using TVC plates for each sample; this is the current industry gold standard.

A number of preliminary experiments were performed prior to the start of the main work in order to achieve the best possible quality of results and to standardize the experimental procedure. To obtain high quality spectra various combinations of spectral resolutions and co-adds were tested for the ATR and HT FT-IR. In addition, a technique using ethanol and water to clean the ATR crystal had to be devised in order to avoid interference with the sample analysis. The ideal experimental milk temperature was also explored and set to 15°C, as higher temperatures resulted in very fast spoilage leading to an inability to perform an adequate number of samplings during this short time period. Furthermore, the frequency and duration of sampling was determined after prolonged TVC of milk samples.

In Chapter 1, results after the application of PC-DFA and PLSR analysis indicated that FT-IR ATR can accurately predict bacterial numbers over $10^4$ in skimmed milk, and $2.10^5$ and $10^6$ in semi-skimmed and whole milk, respectively. FT-IR HT using
PLSR appeared to be even more promising allow the prediction of bacterial numbers as low as \(10^3\) for whole milk and \(4.10^2\) in semi-skimmed and skimmed pasteurized milk. The latter uses dried milk samples, involving minimal sample preparation and training requirements and is able to deliver results within 30 seconds. Metabolic fingerprinting through FT-IR spectroscopy therefore appears to be a very promising rapid technique for quantification of bacteria in the industry and could find a role within HACCP protocols.

Following the successful application of FT-IR in detecting natural milk spoilage, the technique’s qualities when combined with multivariate analysis were tested for the identification and quantification of two different bacterial species in milk. This included artificial spoilage either in mono-culture or in co-culture. *Lactococcus cremoris*, a bacterium routinely used in the industry for the production of dairy products such as cheese and butter, and *Staphylococcus aureus*, a pathogenic bacterium and common contaminant of dairy products, were selected for this study and inoculated in UHT cow milk. Raman spectroscopy was also used in these experiments as this is a complementary technique to FT-IR spectroscopy with the potential of providing further sample information. In addition, previously suggested antagonistic effects of LAB inhibiting *S. aureus* were investigated.

Preliminary experiments in Chapter 2 included first assessing the correlation between optical density measurements and bacterial numbers in broth (estimated using plate count methods). This was conducted in order to identify the required numbers of bacteria for the initial inoculation into milk. In addition, a suitable technique for removing the broth and preserving the pellet prior to inoculation of bacteria in milk
was identified, along with the best time-points to collect samples based on bacterial growth curves. The same FT-IR parameters were used as in Chapter 1, while the best parameters for spectrum collection had to be identified for Raman spectroscopy.

The results of the Raman analysis for this work showed poor prognostic value for quantification purposes. By contrast, FT-IR results were more encouraging with the technique showing very good quantification and discrimination qualities; FT-IR was able to identify specific bacterial species within minutes and accurately measure individual species bacterial numbers both in isolation and in co-culture without any need for additional pre-separation of bacterial species. In addition, in contrast to previously reported literature minimal growth inhibition of *S. aureus* on *L. cremoris* was identified but with the latter organism’s phenotype and metabolic effect dominating the co-culture, an effect which could be utilized by the dairy industry in the manufacture of dairy products with LAB, improving the yield of LAB but also being aware of the potential concurrent growth of *S. aureus* in the mixture.

Milk adulteration was the second major focus of this work. As already mentioned enormous quantities of milk are consumed every year. Potential adulteration of expensive types of milk such as sheep’s and goats’ milk with less expensive cows’ milk during milk processing and production of dairy products can therefore be financially very profitable but with negative product identity, cultural, ethical and health implications (Shatestein and Ghadirian, 1998, Maudet and Taberlet, 2001, Chafen *et al.*, 2010). Regulators are relentlessly in a struggle to maintain the upper hand in a constantly developing and specializing analytical dairy industry sometimes in procession of similarly advanced product manipulation techniques (Fuente and
Juarez, 2005). A number of previously described techniques have been investigated in the detection of milk adulteration, but these appear to suffer from inadequacies such as lack of adequate accuracy and sensitivity, are labour intensive and time consuming and may require specialized staff and expensive equipment for routine industrial use (Malin et al., 1994, Anguita et al., 1996, Chen et al., 2004, Hurley et al., 2006, Addeo et al., 2009, Gutierrez et al., 2009). A summary of the main properties of these techniques in relation to milk adulteration are shown in Table 16.

**Table 16.** Summary of properties of techniques used to detect milk adulteration

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS</td>
<td>• Use with any volatile and semi-volatile compound</td>
<td>• Only used with volatile and semi-volatile compounds</td>
</tr>
<tr>
<td></td>
<td>• High cost</td>
<td>• High cost</td>
</tr>
<tr>
<td></td>
<td>• Time-consuming and labour intensive when used with chemometrics</td>
<td>• Time-consuming and labour intensive when used with chemometrics</td>
</tr>
<tr>
<td></td>
<td>• Sample extraction process required</td>
<td>• Sample extraction process required</td>
</tr>
<tr>
<td>Electronic nose/tongue</td>
<td>• Easy to operate</td>
<td>• Sensor problems; e.g., sensor poisoning</td>
</tr>
<tr>
<td></td>
<td>• Rapid for quality control and screening</td>
<td>• Lack of specificity</td>
</tr>
<tr>
<td></td>
<td>• Inexpensive</td>
<td>• High skilled knowledge required for use of chemometrics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Impractical real-time use for multiple sample analysis in the industry</td>
</tr>
<tr>
<td>HPLC</td>
<td>• Separates and identifies any type of molecule present in food sample</td>
<td>• Requires solvent extraction in dairy products</td>
</tr>
<tr>
<td></td>
<td>• Good detection and quantification limits for cows’, goats’ and sheep’s milk</td>
<td>• Time (30-50min) and solvent consuming</td>
</tr>
<tr>
<td></td>
<td>• High repeatability</td>
<td>• Affected by heat treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unable to detect presence of goat milk in tertiary and binary mixtures with cow-sheep and sheep milk, respectively</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Complex data format production (chromatograms)</td>
</tr>
<tr>
<td>Method</td>
<td>Pros</td>
<td>Cons</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• High specificity and sensitivity</td>
<td>• Complex data analysis</td>
<td></td>
</tr>
<tr>
<td>• Reliable</td>
<td>• Affected by somatic cell numbers</td>
<td></td>
</tr>
<tr>
<td>• Unaffected by heat treatment</td>
<td>• Prior specific DNA fragment identification required</td>
<td></td>
</tr>
<tr>
<td><strong>NMR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Rapid</td>
<td>• High cost</td>
<td></td>
</tr>
<tr>
<td>• Sensitive</td>
<td>• Complex equipment optimisation</td>
<td></td>
</tr>
<tr>
<td>• High level of specificity and accuracy</td>
<td>• Time-dependent measurements</td>
<td></td>
</tr>
<tr>
<td>• Easy to use</td>
<td>• High skilled knowledge required for use of chemometrics</td>
<td></td>
</tr>
<tr>
<td>• Can be used in heterogeneous samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Produces high volume of information on sample content</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ELISA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• High detection and quantification levels</td>
<td>• Difficulties in producing the required primary antibody</td>
<td></td>
</tr>
<tr>
<td>• High sensitivity and specificity</td>
<td>• Antibodies are expensive</td>
<td></td>
</tr>
<tr>
<td>• Easy to use</td>
<td>• Labour intensive</td>
<td></td>
</tr>
<tr>
<td>• Reliable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sheep and goat milk adulteration with cows’ milk detected at low levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Permits identification of tertiary samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IEF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Well standardized</td>
<td>• Requires specialized equipment and enzyme treatment</td>
<td></td>
</tr>
<tr>
<td>• EU reference method</td>
<td>• Specially trained staff required</td>
<td></td>
</tr>
<tr>
<td>• Low detection limit</td>
<td>• Time consuming</td>
<td></td>
</tr>
<tr>
<td><strong>PAGE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Inexpensive</td>
<td>• Requires specialized equipment</td>
<td></td>
</tr>
<tr>
<td>• Low level of skills required compared to IEF</td>
<td>• Time consuming</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Labour intensive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Poor visualization of small molecules</td>
<td></td>
</tr>
<tr>
<td><strong>CE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Easy sample preparation</td>
<td>• Low precision</td>
<td></td>
</tr>
<tr>
<td>• Inexpensive</td>
<td>• Not very quantitative</td>
<td></td>
</tr>
<tr>
<td>• High sensitivity</td>
<td>• Results affected by heat treatment</td>
<td></td>
</tr>
<tr>
<td>• Identification of tertiary sample (cow-goat-ewe)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Information combined from De La Fuente and Juarez (2005), Reid et al (2006) and Karoui and Baerdemaeker (2007)
Previous experience with HT FT-IR in quantification indicated that this technique may have some additional advantages to confer such as minimal sample preparation and rapid sample turnover times with potentially similarly accurate results. Sample preparation and FT-IR parameters used for these experiments were the ones identified and employed in Chapter 2.

Results from Chapter 4 after PLS and KPLS multivariate analysis were excellent. The predictive value of the technique for binary mixtures was very good with RMSEPs between 3.95% and 5.84% and $Q^2$ between 0.95 and 0.98 for the binary mixtures using KPLS, providing marginally better results than PLS. Importantly, the analysis of tertiary mixtures using PLS2 showed values of RMSEP between 3.36% and 4.89% and $Q^2$ between 0.94 and 0.97. These results indicate the excellent potential of HT FT-IR in combination with analytical techniques, for routine use in the dairy industry for the detection of milk adulteration.

MALDI-TOF-MS is a technique with potential for industrial use application as it requires minimal sample and time preparation providing rapid results. It is very useful for the identification of proteins and peptides in food (Ross et al., 2000, Soeryapranata et al., 2002) and has been used successfully in the past for the detection of milk adulteration of raw milk (Cozzolino et al., 2001). Previous studies involving the technique have mainly focused on the use of data from specific spectral peaks to perform milk species identification. The presence of fat in the samples and the effects of milk processing such as pasteurization on protein structure though may affect the accuracy and sensitivity of the technique, while full spectrum evaluation may provide additional useful information.
In the fifth chapter of this work MALDI-ToF-MS with multivariate analysis was investigated for its accuracy and sensitivity in detecting the adulteration of full fat, pasteurized milk from three animal species, cows, sheep and goats. The sample preparation method used for this experiment was derived from preliminary experiments and combined with knowledge from previously similar conducted work (Cozzolino et al., 2001). Different sample preparations explored the techniques of sample and matrix placement on the MALDI target plate. Sample-matrix mixing, sandwich or overlay were explored and optimized and the best MALDI-ToF parameters were also identified along with the most useful range for spectrum collection.

Results from the experiments indicated that in binary milk mixtures, MALDI-ToF-MS showed good predictive values both when used with KPLS compared to PLS analysis, with RMSEPs of 6.35%, to 12.87% and $Q^2$ of 0.81 to 0.95 for predicting cow milk in the cows’- goats’ and cows’- sheep’s mixtures and goat milk in the sheep’s - goats’ binary milk mixtures, respectively. The results from the tertiary milk mixture were also very good both with the KPLS and PLS analysis with the former showing RMSEP values from 2.02% to 3.84% and $Q^2$ of 0.97 to 0.99 for predicting individual milk types in the tertiary mixtures. It therefore appears that the application of this technique may be promising in the detection of adulteration of fresh milk.
6.2 CONCLUDING REMARKS

FT-IR spectroscopy provided very good and promising quantitative results both in the detection and enumeration of fresh milk spoilage, bacterial species in co-culture and adulteration of fresh milk. In contrast Raman spectroscopy failed to provide any useful information in regards to the quantification of bacterial species. Finally, MALDI-ToF-MS was able to yield excellent results in the identification of adulteration of fresh milk samples involving cows’, goats’ and sheep’s milk. Table 17 displays the qualities of each individual method in their respective studied area of milk analysis.

Table 17. Qualities of FT-IR, Raman and MALDI-ToF-MS in their studied areas of milk analysis

<table>
<thead>
<tr>
<th>Qualities</th>
<th>FT-IR</th>
<th>Raman</th>
<th>MALDI-ToF-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data analysis</td>
<td>Easy</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Minimum</td>
<td>Minimum</td>
<td>Moderate</td>
</tr>
<tr>
<td>Repeatability</td>
<td>Good</td>
<td>Poor</td>
<td>Average</td>
</tr>
<tr>
<td>Sample size</td>
<td>3µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>Time to acquire spectrum</td>
<td>30 s</td>
<td>15 min</td>
<td>4 min</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>High</td>
<td>High</td>
<td>Fair</td>
</tr>
<tr>
<td>Automatable</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Complex data capture</td>
<td>No</td>
<td>Fair</td>
<td>Fair</td>
</tr>
<tr>
<td>Destructive</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
This thesis shows that the use of analytical techniques such as FT-IR ATR and HT in conjunction with multivariate statistical techniques, was able to provide rapid and accurate detection and enumeration of viable bacterial loads in milk. The metabolic fingerprinting qualities of this technique, and the minimal sample preparation it involves, would make it a very useful technique for industrial scale use. The same analytical principles could be explored further in to investigating microbial spoilage in other types of milk and generally other liquid foodstuffs; this would include further sample preparation techniques needing to be refined. Furthermore, the current commercial availability of portable FT-IR spectrometers such as the Exoscan (A2Technologies, Danbury, Connecticut, USA) and the Mobile-IR (Bruker Optics, Ettlingen, Germany), with universal serial bus (USB) connections for links to a laptop or inbuilt computer for direct statistical analysis, can extend the use of this technique outside the laboratory setting, from analysis of the raw product at the place of production to analysing the final end product on the market shelf.

In the area of milk adulteration FT-IR spectroscopy and MALDI-TOF mass spectrometry combined with PLS and KPLS chemometric techniques appeared to be very promising in the quantification of different types of milk in the same mixture. I believe this work can be expanded further with future experimentation focusing on investigating the accuracy of these techniques in identifying animal milk originating from a variety of geographical locations and animal breeds. Regardless, in its current form and with the current results these techniques appear to be viable methods for use in the milk industry.
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conventional PCR system suggested by the FOOD-PCR project. *Journal of Microbiological Methods*, 66, 538-47.


APPENDIX
Appendix A. Chapter 2 Peer review publication paper

Rapid and quantitative detection of the microbial spoilage in milk using Fourier transform infrared spectroscopy and chemometrics

Nicoletta Nicolaou and Royston Gondacre

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First published as an Advance Article on the web 11th July 2008
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Microbiological safety plays a very significant part in the quality control of milk and dairy products worldwide. Current methods used in the detection and enumeration of spoilage bacteria in pasteurized milk in the dairy industry, although accurate and sensitive, are time-consuming.

FT-IR spectroscopy is a metabolic fingerprinting technique that can potentially be used to deliver results with the same accuracy and sensitivity, within minutes after minimal sample preparation. We tested this hypothesis using attenuated total reflectance (ATR), and high throughput (HT) FT-IR techniques. Three main types of pasteurized milk - whole, semi-skimmed and skimmed - were used and milk was allowed to spoil naturally by incubation at 15°C. Samples for FT-IR were obtained at frequent, fixed time intervals and pH and total viable counts were also recorded.

Multivariate statistical methods, including principal components-discriminant function analysis and partial least squares regression (PLSR), were then used to investigate the relationship between metabolic fingerprints and the total viable counts. FT-IR ATR data for all milks showed reasonable results for bacterial loads above 10⁶ cfu ml⁻¹. By contrast, FT-IR HT provided more accurate results for lower viable bacterial counts down to 10⁴ cfu ml⁻¹ for whole milk and, 4 x 10³ cfu ml⁻¹ for semi-skimmed and skimmed milk. Using FT-IR with PLSR we were able to acquire a metabolic fingerprint rapidly and quantify the microbial load of milk samples accurately, with very little sample preparation. We believe that metabolic fingerprinting using FT-IR has very good potential for future use in the dairy industry as a rapid method of detection and enumeration.

Introduction

Milk is an important constituent of our diet containing a variety of nutrients, and it is essential for good bone development in infants and children. It is therefore not surprising that a big proportion of the world's population uses milk on a daily basis. Quality control of milk and milk products is therefore of paramount importance. Recent outbreaks of foodborne illnesses associated with milk and dairy product consumption have been found to be contaminated with pathogenic microorganisms such as Listeria spp., Salmonella spp., Campylobacter jejuni and Yersinia enterocolitica. Microbial analysis of milk and dairy products therefore has a critical role to play in the quality evaluation of these products, promoting public health safety.

A dairy product is described as spoiled when organoleptic changes within it make it unacceptable to the consumer. These organoleptic traits, among others, cause defects in appearance and unpleasant odours and flavours. These are characteristics which make food unacceptable for human consumption. It is generally accepted that organoleptic changes are a result of microbiological spoilage and that the compounds responsible for these changes are the various metabolites produced by the metabolic activity of the microorganisms. The type of change produced varies according to the species of the microorganisms present in milk, the chemical composition of milk and the physical environment under which it is stored.

Milk is an ideal medium for microbial growth because of its high water content and the large variety of available nutrients which can be used by microorganisms as an energy source. The main components of whole milk are 87.3% water, 4.4% carbohydrates (mainly lactose), 3.7% fat, 3.2% proteins, and 0.1% non-protein nitrogenous compounds, minerals and vitamins. Furthermore, its pH is almost neutral ranging from 6.5 to 6.7 making it an ideal growth environment for bacteria. Temperature also has a part to play as spoilage microorganisms can become active at temperatures between 2 and 30°C. Within this temperature range the growth of one particular bacterial species, the Gram negative Pseudomonas, is dramatic. The other major members of the spoilage flora on pasteurized milk include the endospore-forming bacteria of the Bacillus genera, and other Gram positive rods and cocci such as Lactobacillus, Corynebacterium and Lactococcus species.

The application of the Hazard Analysis and Critical Control Point (HACCP) system in the dairy industry, in order to maintain high quality levels during manufacturing and production of foods for safe consumption, has increased the requirements for rapid and more automated microbiological techniques. In the recent past, several methods and instruments have been developed for the identification, detection, enumeration and the characterisation of microorganisms in milk and dairy products.
products. However, none of these methods have so far been ideal. In the absence of a simultaneously rapid, accurate and sensitive method, the dairy industry currently relies on classical microbiological plate counting techniques and other methods for the detection and enumeration of spoilage bacteria in pasteurized milk which typically take 1-2 days; but may take longer depending on the growth of the organism. The dairy industry therefore requires a method for the detection and enumeration of bacteria in dairy samples which will be accurate and sensitive, must be able to measure viable bacterial numbers and provide results within 2-3 h so that appropriate measures within HACCP can be made.

Fourier transform infrared (FT-IR) spectroscopy is a metabolic fingerprinting technique that can potentially be used to reduce this time significantly, by measuring the biochemical fingerprints produced through the metabolic activity of the viable microorganisms in milk and delivering results within minutes after very minimal sample preparation. Its validity has also recently been shown in the detection of spoilage in meat. FT-IR spectroscopy is based on the principle of detecting the specific frequencies of energy absorbed by a molecule within a functional group once it has been irradiated with IR radiation (usually in the mid IR range, 400-600 cm⁻¹) and has been excited to a higher energy level, usually reaching its first vibrational excited state. As each molecule only absorbs energy when the frequency of the infrared energy directed at the molecule is equal to the frequency of one of the fundamental vibration modes of that molecule, the end result of this absorbance is a highly specific infrared spectrum. The main advantage of this technique is that it is very fast as a spectrum can be obtained within a few seconds after minimum sample preparation. Furthermore, it is a simple technique to use, it has high sensitivity, and it is inexpensive to operate. This technique in combination with appropriate multivariate statistical methods, including partial least square regression (PLSR), may therefore be an attractive solution for the detection and enumeration of bacteria in dairy samples.

Therefore, the aim of this study was to investigate the ability of FT-IR spectroscopy to quantify the bacterial contamination of the three types (vac, whole, semi-skimmed and skimmed) of pasteurized cow’s milk accurately, sampling using attenuated total reflectance (ATR), and a high throughput (HT) transmission-based technique with PLSR analysis.

Materials and methods

Sample preparation

Three cartons of milk, with the same use-by-date, each of a different type of milk (whole, semi-skimmed and skimmed milk) were purchased from a national retail outlet. The milk was then separately poured into sterilized flasks (1 L) and was placed in a rotation incubator at 15 °C and 200 revolutions per minute (rpm). Samples were then taken at eight-hourly intervals for 104 h. Once samples were obtained they were mixed for 1 min and then the organoleptic changes and pH of each type of milk were recorded and the total viable bacterial counts were also determined using a classical microbiological plating method (see below). Fifteen millilitres of the milk samples were divided to 1 ml volumes and preserved at −80 °C. Six of these aliquots were used for ATR FT-IR and HT FT-IR analysis.

Total viable counts (TCVs)

TCVs were measured according to the national standard method. Using peptone saline diluent (containing 1.0 g peptone, 8.5 g sodium chloride in 1 L distilled water) serial dilutions were undertaken. Each dilution was mixed for 1 min and 1 ml was inoculated into three Petri dishes. Subsequently, milk plate count agar (containing 2.5 g yeast extract, 5.0 g tryptone, 1.0 g glucose, 1.0 g skimmed milk powder, 15.0 g agar in 1 L distilled water) was added, mixed with the inoculum, and incubated aerobically at 30 °C for 72 h. The plate colonies were then counted and the total viable count per millilitre was calculated. The number of colonies per plate was only taken into account when it was between 30 and 300. The number of viable microorganisms per millilitre of sample was calculated using the equation:

\[
N = \frac{\sum c}{(n_i + 0.1n_i) d}
\]

were \(\sum c\) is the sum of the colonies counted from all plates (between 30 and 300 colonies), \(n_i\) is the number of plates counted at the first dilution, \(n_{i+1}\) is the number of plates at the second dilution and \(d\) is the dilution from which the first counts were obtained (i.e. least dilute).

Attenuated total reflectance (ATR) FT-IR spectroscopy

FT-IR analysis was undertaken using a ZnSe Gateway ATR Horizontal 6 Reflection accessory (Specac Ltd, London) on a Bruker Equinox 55 infrared spectrometer equipped with a DTGS (deuterated triglycine sulfate) detector (Bruker Ltd, Coventry, UK). For ATR the evanescent wave allows penetration into the surface above the crystal and this was calculated to be 0.98 µm at 1500 cm⁻¹ (arising from the N-H vibration of the Amide II band) and 0.45 µm at 1900 cm⁻¹ (from the centre of fatty acid CH₂ stretches). Samples were defrosted on ice, one sample at a time, and were then mixed for 1 min. Aliquots of 800 µl were then taken from the sample and placed in intimate contact with the ZnSe crystal and the sample’s spectrum was obtained. In total, six replicates were taken from each time point. Between samples the crystal surface was first cleaned with distilled water, then with analytical grade acetone and again with distilled water and dried with a soft cloth and left for approx. 5 min to air dry. Prior to making any sample measurements, reference spectra were acquired from the clean blank crystal. All the spectra were collected within the wavenumber range of 4000-600 cm⁻¹ with a resolution of 8 cm⁻¹ and in order to improve the signal-to-noise ratio 64 scans were co-added and averaged. In total, 252 spectra were collected for every type of milk in the series of three experiments and the collection time for each spectrum was approximately 30 s. Spectral acquisition was achieved using an IBM compatible computer which controlled the spectrometer.
High throughput transmission (HT) FT-IR spectroscopy

FT-IR analysis was undertaken using a ZnSe plate on the same Bruker Equinox 55 infrared spectrometer equipped with a motorised microplate module HTS-XTM utilising a DTGS detector (Bruker Ltd). Samples were defrosted on ice one sample at a time, then mixed for 1 min, and then 5 μl from each sample was placed onto a ZnSe plate (which can hold 96 samples). The ZnSe plates were then oven-dried at 50 °C for 30 min. In total, six replicates were taken from each sample and were placed randomly onto the ZnSe plates. Again the wavenumber range collected was 4000–600 cm⁻¹, with a resolution of 8 cm⁻¹, and 64 scans were co-added and averaged. As also detailed above, a total of 252 spectra were collected and each spectrum took 30 s to acquire.

Data analysis

Pre-processing. For FT-IR, ASCII data were exported from the Opus software used to control the FT-IR instrument and imported into Matlab version 7 (The Mathworks, Inc, Natick, MA). To minimize problems arising from baseline shifts Matlab was used to correct the CO₂ vibrations seen in the HT FT-IR measurements by removing the CO₂ peaks at 2303–2372 and 683–656 cm⁻¹ and filling them with a trend. Both HT and ATR FT-IR spectra then were scaled by using extended multiplicative scatter correction (EMSC)22 we also tried the first and second Savitzky-Golay derivatives with five-point smoothing but this was no better than EMSC. Prior to PCA and PLSR, as is normal practice, the data were mean-centred. At this stage we visually inspected the spectra and checked for outliers using principal components analysis (see below) and these were subsequently removed from further analyses. These samples included a single biological replicate from the 24 h time point from the HT spectra collected from whole milk, all the biological replicates from the 144 h time point from the HT spectra collected from skimmed milk and all the replicates from the 64 h time point from the HT spectra collected from whole, semi-skimmed and skimmed milk; this suggested that this later time point was due to a sampling error, as the sample was collected in the middle of the night.

To investigate the relationship between the FT-IR spectra and the total viable count, multivariate statistical methods were used including cluster analysis and partial least squares regression (PLSR). These multivariate analysis methods were performed in PyChem version 3, details of which are available from Jarvis et al.23 and the programme is also available on the web (http://pychem.sf.net/).

Cluster analysis. Cluster analysis was carried out in two steps. Firstly, principal component analysis (PCA) was used.24 PCA is a well established analysis technique which works by finding the correlation between a set of variables and then creating a new set of uncorrelated variables named principal components (PCs). Subsequently, discriminant function analysis (DFA; also known as canonical variate analysis) was used.25 DFA was programmed to discriminate data based on the first few PCs with the prior knowledge of which spectra are biological replicates.

Validation of the PC-DFA model was performed for the HT FT-IR data and for the ATR FT-IR data, for every type of milk (whole, semi-skimmed and skimmed) as detailed in ref. 27. In brief, the data were divided into two subsets: the training set and the test set. The training set consisted of the first two biological replicates (two groups at each time point), which were used to construct a PC-DFA model. After the PC-DFA model was constructed the test data of the third biological replicate was projected into PCA space and then the projected PCs were projected into DFA space. The model was considered valid if the test set data were projected coincident with the training data.

Finally, in order to inspect the tightness of the clusters 95% tolerance regions were constructed around the PC-DFA group means using the χ² confidence intervals using two degrees of freedom.26

Partial least squares regression (PLSR). For quantitative prediction of TVC from the FT-IR spectra the multivariate supervised learning method of partial least squares regression was used28 as detailed in refs 29, 30 and 31. The aim of supervised learning is to construct a model which correctly associates inputs with targets, where in the calibration phase both input and targets are already known. PLSR was calibrated with FT-IR data from the first two spoilage experiments (biological replicates) to predict the known logs TVC values. During calibration these data were divided randomly into training data and cross-validation data, and the number of latent variables used in the model was the point at which the lowest RMS error in the validation data was seen. Once this model was constructed it was challenged with independent test set data from the third unseen spoilage experiment (biological replicate).

Results and discussion

pH, TVC and organoleptic changes

The pH levels during the 104 h of the three spoilage experiments are shown in Fig. 1. The initial mean pH was 6.72. After 104 h incubation at 15 °C the final mean pH was 7.07. In general, the pH showed mild fluctuation prior to 80 h and then increased significantly as the bacterial levels reached 2 x 10⁹ CFU ml⁻¹ (Table 1). Similar results were shown for semi-skimmed and skimmed milk were the initial mean pH was 6.72 and 6.74 and then increased to 7.11 and 7.10 respectively (data not shown). The results from these experiments suggest that the use of pH as
Table 1: Mean $\log_{10}$ TVC of bacteria acquired from milk spoilage samples from three experiments for the three different types of milk.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Whole Mean $\log_{10}$ TVC</th>
<th>Negative SD</th>
<th>Positive SD</th>
<th>Semi-skimmed Mean $\log_{10}$ TVC</th>
<th>Negative SD</th>
<th>Positive SD</th>
<th>Skimmed Mean $\log_{10}$ TVC</th>
<th>Negative SD</th>
<th>Positive SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.22</td>
<td>0.51</td>
<td>0.23</td>
<td>2.62</td>
<td>0.38</td>
<td>0.29</td>
<td>2.64</td>
<td>1.67</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>3.07</td>
<td>0.55</td>
<td>0.23</td>
<td>2.66</td>
<td>0.21</td>
<td>0.14</td>
<td>2.62</td>
<td>1.38</td>
<td>0.29</td>
</tr>
<tr>
<td>16</td>
<td>3.11</td>
<td>0.65</td>
<td>0.25</td>
<td>2.75</td>
<td>0.28</td>
<td>0.17</td>
<td>2.56</td>
<td>1.16</td>
<td>0.32</td>
</tr>
<tr>
<td>24</td>
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<td>0.32</td>
<td>0.18</td>
<td>2.65</td>
<td>0.28</td>
<td>0.17</td>
<td>2.69</td>
<td>2.29</td>
<td>0.30</td>
</tr>
<tr>
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<td>0.14</td>
<td>2.61</td>
<td>0.63</td>
<td>0.25</td>
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<tr>
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<td>0.44</td>
<td>2.93</td>
<td>1.02</td>
<td>0.28</td>
<td>3.12</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>48</td>
<td>4.99</td>
<td>0.59</td>
<td>0.24</td>
<td>4.07</td>
<td>0.91</td>
<td>0.27</td>
<td>4.21</td>
<td>0.43</td>
<td>0.27</td>
</tr>
<tr>
<td>56</td>
<td>6.11</td>
<td>1.73</td>
<td>0.31</td>
<td>5.47</td>
<td>0.14</td>
<td>0.39</td>
<td>4.96</td>
<td>0.60</td>
<td>0.35</td>
</tr>
<tr>
<td>64</td>
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<td>6.39</td>
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<td>5.99</td>
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<td>0.26</td>
</tr>
<tr>
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<td>7.01</td>
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<td>0.27</td>
<td>6.39</td>
<td>0.44</td>
<td>0.27</td>
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<td>1.46</td>
<td>0.29</td>
<td>7.06</td>
<td>0.80</td>
<td>0.27</td>
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<td>88</td>
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<td>1.17</td>
<td>0.32</td>
<td>7.08</td>
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<td>0.23</td>
<td>7.84</td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td>96</td>
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<td>0.29</td>
<td>0.26</td>
<td>6.89</td>
<td>0.73</td>
<td>0.34</td>
<td>6.92</td>
<td>0.62</td>
<td>0.25</td>
</tr>
<tr>
<td>104</td>
<td>8.08</td>
<td>0.50</td>
<td>0.37</td>
<td>7.12</td>
<td>0.41</td>
<td>0.38</td>
<td>7.41</td>
<td>0.22</td>
<td>0.15</td>
</tr>
</tbody>
</table>

The results of the spoilage experiment for whole milk are shown in Table 1. The initial mean $\log_{10}$ TVC was 3.22 which is a usual finding for fresh pasteurized whole milk. After 104 h of incubation at 15 °C the final $\log_{10}$ TVC increased to 8.08. An example of the increase in the growth of bacteria in the whole milk for the three replicate experiments is shown on Fig. 2. For semi-skimmed and skimmed milk the initial mean $\log_{10}$ TVC was between 2.62 and 2.66 and the final $\log_{10}$ TVC increased to 7.12 for semi-skimmed and 7.41 for skimmed milk. Both the initial and the final $\log_{10}$ TVC were lower for the semi-skimmed and skimmed milk than whole milk. This finding is different from previous research which found that the TVC for whole and skimmed milk during the spoilage did not have significant differences when they were stored at 5–8 °C, and may be a consequence of the incubation temperature for our studies being 15 °C.

**Fig. 2** Plot showing the $\log_{10}$ (total viable count per ml) against time for whole milk samples spoiled at 15 °C for 104 h. Averages of three replicate measurements for the three biological repeats are shown and error bars show the standard deviations.

In our experiments general organoleptic changes indicative of spoilage became apparent when the number of bacteria reached approximately $10^6$ cfu ml$^{-1}$ in all types of milk. Whilst of course these observations are personal and therefore subjective, we found that at this stage the milk started to smell bitter and cheesy. These organoleptic changes result from bacterial metabolism which produces a complex mixture of volatile esters, ketones, aldehydes, fatty acids, ammonia and amines, collectively comprising the off-odours detected. The different off-odours are most probably due to protease and lipase enzyme activities, some well known spoilage effects caused by *Pseudomonas* species, which is one of the most common species in the bacterial population at 15 °C. It has been shown that these enzymes are detectable when the bacterial counts reach a level of $10^6$ cfu ml$^{-1}$ or higher and are stable at high temperatures, surviving pasteurization (72 °C for 15 s). Proteases can act directly on micellar casein resulting in its degradation and the liberation of bitter peptides. When proteolysis is continued the degradation of lower molecular weight products such as ammonia and amines produces the putrid aroma and flavour.

**FT-IR ATR spectroscopy**

Representative FT-IR ATR spectra collected from whole milk and the identities of the main absorption bands relating to milk are shown in Fig. 3. Using simple visual inspection no obvious qualitative differences were observed between fresh milk at 0 h, and spoiled milk at 48 h and 104 h. Similar spectra were also obtained from semi-skimmed and skimmed milk (data not shown).

In order to detect any differences between the FT-IR ATR spectra, cluster analysis was employed, and the PG-DFA results for spoilage of whole-fat milk from FT-IR ATR are shown in Fig. 4. The spectra for the first 48 h group together in the same region on the bottom left of this figure, as evident from their overlapping 95% tolerance regions; the TVC during this period was between $10^4$ and $10^5$ cfu ml$^{-1}$. Spectra of samples incubated after the 48 h time point are clearly different from the earlier samples and tended to spread in both axes towards the right hand side of the figure and then upwards followed by...
FT-IR ATR spectra for whole milk at 0 h (purple), 48 h (blue), and 104 h (red).

FT-IR spectra collected from an independent experiment set (i.e., data that were unseen/new to the model). Preliminary modeling for all milk types was performed on the FT-IR ATR spectra and it was found (data not shown) that PLSR could not give accurate estimates of TVC for very low bacterial numbers. Therefore, depending on the milk type, either 48 or 56 h to 104 h were used in PLS modeling and this also corresponds to when PC-DFA could not differentiate between the early sampling points.

PLS analysis on FT-IR ATR spectra from whole milk (between 56 and 104 h) found that the best model occurred when five PLS factors (latent variables) were used. The PLSR result for this model is shown in Fig. 5, where it can be clearly observed that the plot of the estimated TVC versus the known TVC values shows very reasonable prediction (i.e., the estimates lie close to the $y=x$ line shown on this plot). Overall, PLS gave accurate results at bacterial levels higher than $1 \times 10^6$ cfu ml$^{-1}$ (Table 2), and this level is in agreement with the findings of previous research performed for the detection of microbial spoilage in chicken by using ATR FT-IR and PLSR.

PC-DFA plot of the ATR FT-IR spectra for the three repeat experiments of whole milk. PCs 1-20 (accounting for 96.34% of the total variance) were used by the DFA algorithm with a priori knowledge of machine replicates (i.e., one class per time point, giving 14 classes in total). The different symbols represent the different time points of spoilage. The circles represent the 95% tolerance region constructed around the mean by the $x^2$ confidence intervals using two degrees of freedom.

a downward dip. The latter trend appears to occur when the viable bacterial numbers are between $10^9$ and $10^{10}$ cfu ml$^{-1}$, and for some of these the 95% tolerance regions are seen to overlap sequentially.

In semi-skimmed milk (data not shown), similar results in PC-DFA were observed where the spectra for the first 56 h appeared in the same region, with TVCs between $4 \times 10^6$ and $2 \times 10^7$ cfu ml$^{-1}$. After this time point the spectra again followed a trend correlated to the number of bacteria. For skimmed milk (data not shown) the spectra for the first 56 h also appeared clustered together (TVCs from $4 \times 10^6$ to $9 \times 10^6$ cfu ml$^{-1}$), after which a trend relating to the TVCs from $4 \times 10^6$ to $2 \times 10^6$ cfu ml$^{-1}$ was also observed.

Since trends were observed in the PC-DFA of all milk types undergoing spoilage, we sought to correlate the known TVC with its representative FT-IR spectra. Therefore supervised learning analysis using PLSR was used to quantify the bacteria in spoilage milk. As described above, the PLSR algorithm was first calibrated and cross-validated with FT-IR spectra and the known TVC; after calibration these models were challenged with

Fig. 5 Plot showing the predicted log$_{10}$ TVC from PLS versus the actual log$_{10}$ TVC for whole milk measured using ATR FT-IR spectroscopy. The RMS error (log$_{10}$) in these measurements is 0.25 for the calibration, cross-validation and independent tests.

In semi-skimmed and skimmed milk, FT-IR ATR spectra from samples between 56 and 104 h, and from between 48 and 104 h, respectively, were analyzed. Table 2 gives the overall performance of the PLS models and shows that PLSR for semi-skimmed milk showed a good predictive value, when the total viable counts above $2 \times 10^7$ cfu ml$^{-1}$ could be assessed. By contrast, for skimmed milk reasonable predictions were observed when the total viable counts were above $3 \times 10^5$ cfu ml$^{-1}$, which is an order of magnitude lower than those obtained for whole and semi-skimmed milk; remodeling of the whole-fat and semi-skimmed milk including the 48 h time point could not predict $<1 \times 10^6$ cfu ml$^{-1}$.

FT-IR HT spectroscopy

The same chemometric strategy to that discussed above was also used to analyse the FT-IR spectra obtained from the high throughput screening approach from dried milk. This novel
Table 2  Comparison between HT FT-IR and ATR FT-IR showing the root mean square errors for calibration, validation and test for each type of milk

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>FT-IR technique</th>
<th>TVC prediction range with PLSR</th>
<th>PLS factors</th>
<th>Error calibration (log₁₀)</th>
<th>Error cross-validation (log₁₀)</th>
<th>Error test (log₁₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>HT</td>
<td>10⁹ to 10¹⁰</td>
<td>18</td>
<td>0.35</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>Semi-skimmed</td>
<td>HT</td>
<td>4 × 10⁹ to 10¹⁰</td>
<td>13</td>
<td>0.45</td>
<td>1.20</td>
<td>0.66</td>
</tr>
<tr>
<td>Skimmed</td>
<td>HT</td>
<td>4 × 10⁹ to 2 × 10¹⁰</td>
<td>4</td>
<td>1.10</td>
<td>1.28</td>
<td>0.78</td>
</tr>
<tr>
<td>Whole</td>
<td>ATR</td>
<td>10⁹ to 10¹⁰</td>
<td>5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Semi-skimmed</td>
<td>ATR</td>
<td>2 × 10⁹ to 10¹⁰</td>
<td>4</td>
<td>0.20</td>
<td>0.35</td>
<td>0.41</td>
</tr>
<tr>
<td>Skimmed</td>
<td>ATR</td>
<td>10⁹ to 2 × 10¹⁰</td>
<td>4</td>
<td>0.67</td>
<td>0.82</td>
<td>0.87</td>
</tr>
</tbody>
</table>

screening approach has not previously been used to investigate food spoilage.

Representative FT-IR HT spectra collected from whole milk with the main absorption bands identified are shown in Fig. 6. Minimal qualitative differences were observed between the fresh milk at 0 h, and spoiled milk at 48 and 104 h; however, on close inspection quantitative differences were visible, especially in the spectral region from 900 to 1600 cm⁻¹ which arise from carbohydrates, proteins and lipids.

![FT-IR HT spectra for full milk at 0 h (purple), 48 h (blue), and 104 h (red).](image)

Fig. 6  FT-IR HT spectra for full milk at 0 h (purple), 48 h (blue), and 104 h (red).

Similar spectra were observed for semi-skimmed and skimmed milk (data not shown) with two main differences. The first difference involved the CH₂ absorption band related to fatty acids at 2900 cm⁻¹. Since this involved the absorption of lipids as expected this was found to be weaker in skimmed milk compared to whole-fat milk, as a result of the different fat quantities in the different types of milk. The other major difference appeared on the absorption band related to the C=O group of esters at 1750 cm⁻¹. This absorption was again found to be very strong in whole milk, less strong in semi-skimmed milk and did not appear in skimmed milk. The explanation behind this finding is most likely related to the esterification of fatty acids from lipids, which occurs in higher levels in whole and semi-skimmed milk and in very low levels in skimmed milk.

The PC-DFA results for spoilage of whole milk from FT-IR HT are shown in Fig. 7. It can be seen that the spectra for the first 48 h appear in the same region on the left of the pane. After that time point, subsequent time points tend to spread towards the right and upwards. The latter trend occurred when the viable bacterial numbers were between 10⁹ and 10¹⁰ cfu ml⁻¹, and the sample points were more discrete compared to the same analysis on the ATR accessory (Fig. 4) as evident from the 95% tolerance regions not overlapping, suggesting that the FT-IR HT spectra were more information rich. The spectra of semi-skimmed milk during the first 56 h appeared within the same region, after which the time points spread again in a trend that corresponds to the bacterial load. For skimmed milk the trend in PC-DFA space was again very similar to this.

![PC-DFA plot on HT FT-IR spectra for the three repeat experiments of full milk. PC's 1–20 (accounting for 99.49% of the total variance) were used by the DFA algorithm with a priori knowledge of machine replicates (i.e. one group per time point, giving 10 groups in total). The different symbols represent the different time points of spoilage. The circles represent the 95% tolerance region constructed around the mean by the  ámb-confidence intervals using two degrees of freedom.](image)

Fig. 7  PC-DFA plot on HT FT-IR spectra for the three repeat experiments of full milk. PC's 1–20 (accounting for 99.49% of the total variance) were used by the DFA algorithm with a priori knowledge of machine replicates (i.e. one group per time point, giving 10 groups in total). The different symbols represent the different time points of spoilage. The circles represent the 95% tolerance region constructed around the mean by the  ámb-confidence intervals using two degrees of freedom.

In contrast to the FT-IR ATR PLSR modeling it was possible to use the full time course of the FT-IR HT results for analysis. The PLSR results for whole milk are shown in Fig. 8, where the plot of the estimated TVC were the known TVC values for whole milk showed good predictive values and gave relatively accurate results even at very low number viable counts (1 × 10⁸ cfu ml⁻¹). The results for all three milk types are summarized in Table 2. When semi-skimmed milk was tested, PLSR again gave good predictions. However, the results...
from skimmed milk were not quite as good as the results for whole and semi-skimmed milk but were never-the-less still very encouraging.

Comparison of the two techniques

The most noticeable difference between FT-IR HIT (Fig. 3) and FT-IR ATR (Fig. 6) spectra was the absence of CH₂ vibrations at 2900 and 2860 cm⁻¹ in the FT-IR ATR spectra, the peaks related to the acyl chain of fatty acids. Obviously these chemical species will not have disappeared during analysis and this is likely to have occurred because of the nature of the FT-IR ATR technique, which detects only the surface chemistry of cells or substances. For ATR the evanescent wave allows penetration into the surface above the crystal, and as reported in the Materials and Methods section for the CH₂ stretches this is ca. 0.45 μm. As lipids in milk exist in the form of micellar globules surrounded by a protective membrane which is composed of glycoproteins, lipoproteins and phospholipids, the acyl chains will be internal to these globules with the polar head group exposed to the aqueous environment of the milk, and this may be why the CH₂ stretches are missing from the ATR spectra. By contrast, the FT-IR HIT technique employed uses dried milk and is a transmission-based measurement in which infrared light penetrates the whole of the sample and provides a spectrum characteristic of the total components of the milk. Furthermore, the peak shape of the spectra between 1700 and 900 cm⁻¹ collected with ATR are different from those observed with FT-IR HIT spectrometry.

An overall comparison of the PLS modeling between HT FT-IR and ATR FT-IR for the root mean square errors of calibration, validation and test sets for each type of milk can be found in Table 2. It can be seen that the accuracy of ATR FT-IR spectroscopy for all whole and semi-skimmed types is better than the HT FT-IR approach, with both having similar predictive ability for skimmed milk. However, HT FT-IR does have significantly lower detection limits compared to ATR FT-IR and generally produces acceptable models with two lower orders of magnitude.

Conclusions

The use of FT-IR ATR and HT techniques in combination with multivariate statistical methods, including PCR-DFA and PLSR, was able to acquire a metabolic snapshot and quantify the microbial load of milk samples accurately and rapidly, within 30 s, with little sample preparation. We believe that metabolic fingerprinting using FT-IR has a very good potential for future use in the dairy industry as a rapid method of viable bacterial detection and enumeration. As such it could be incorporated in the HACCP system improving consumer safety and lowering product-related risks and hazards.

Acknowledgements

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Appendix B. Chapter 4 Peer review publication paper

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Fourier transform infrared spectroscopy and multivariate analysis for the detection and quantification of different milk species

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ABSTRACT

The authenticity of milk and milk products is important and has extended health, cultural, and financial implications. Current analytical methods for the detection of milk adulteration are slow, laborious, and therefore impractical for use in routine milk screening by the dairy industry. Fourier transform infrared (FT-IR) spectroscopy is a rapid biochemical fingerprinting technique that could be used to reduce this sample analysis period significantly. To test this hypothesis we investigated 3 types of milk: cow, goat, and sheep milk. From these, 4 mixtures were prepared. The first 3 were binary mixtures of sheep and cow milk, goat and cow milk, or sheep and goat milk in all mixtures the mixtures contained between 0 and 100% of each milk in increments of 5%. The fourth combination was a ternary mixture containing sheep, cow, and goat milk also in increments of 5%. Analysis by FT-IR spectroscopy in combination with multivariate statistical methods, including partial least squares (PLS) regression and nonlinear kernel partial least squares (KPLS) regression, were used for multivariate calibration to quantify the different levels of adulterated milk. The FT-IR spectra showed a reasonably good predictive value for the binary mixtures, with an error level of 6.5 to 8% when analyzed using PLS. The results improved and excellent predictions were achieved (only 4–6% error) when KPLS was employed. Excellent predictions were achieved by both PLS and KPLS with errors of 3.4 to 4.9% and 3.9 to 6.4%, respectively, when the ternary mixtures were analyzed. We believe that these results show that FT-IR spectroscopy has excellent potential for use in the dairy industry as a rapid method of detection and quantification in milk adulteration.

Key words: Fourier transform infrared spectroscopy, milk adulteration, authenticity of milk products, chemometrics

INTRODUCTION

Milk quality is important in the production of all types of cheese, especially in regard to cheese quality and characteristics. Goat and sheep milks are of higher value than cow milk and are used for the production of a variety of specialty cheeses. This presents the potential for financial gain by unscrupulous producers adulterating either goat or sheep milk with cow milk, thus resulting in nonauthentic milk products (Maudet and Taberlet, 2001). In addition to the ethical, religious, and cultural implications (Shatenstein and Ghadirian, 1998), consumers need to be protected from this kind of practice because of potential intolerance and allergic reactions to the cow milk component of those adulterated products (Bischoff, 2006; Venter, 2009; Chafen et al., 2019). Indeed, the European Union has legislation in place for the correct display of the constituents of dairy products protecting their authenticity (European Union, 2001), and various legislation on food labeling and authenticity exists among other member countries (Dennis, 1998).

Until now, several methods have been investigated, both within academic institutions and in industry, for their accuracy and practicability in detecting dairy product adulteration. These include several analytical approaches based on immunological, electrophoretic, and chromatographic techniques, as well as DNA-based processes such as species-specific PCR. Antibody-based assays used for the quantification and detection of species-specific milk proteins (antigens) form the basis of immunological methods. These have targeted proteins such as cow caseins (whole, γ-, β-, and αs1-caseins), cow β-lactoglobulin, and cow IgG for the detection of cow milk adulteration, whereas goat whey proteins and goat αs1-casein have been used for the detection of goat milk adulteration (Hurley et al., 2004). Enzyme-linked immunosorbent assays have been routinely used for this purpose and are performed using a variety of processes including direct, indirect, competitive, and sandwich techniques (Levieux and Venion, 1994; Anguita et al., 1996; Beer et al., 1996; Anguita et al., 1997; Hurley et al., 2006). Even though ELISA techniques require rela-
relatively less sample preparation than other techniques, they are costly, because they rely on the use of expensive antibodies that cannot be reused and have a limited shelf-life. In addition, the reliance of these methods on specific protein identification and quantification is a potential drawback in the analysis of processed milk because proteolysis and heat denaturation can cause the loss of antibody-specific epitopes (Mayer, 2005; Hurley et al., 2006).

Polyacrylamide gel electrophoresis and isoelectric focusing are the main immunological methods employed for the detection of casein or whey proteins in milk but they are slow and laborious for routine use in the dairy industry (Amigo et al., 1992; Levieux and Venien, 1994; Malin et al., 1994; Mayer, 2005; Addeo et al., 2009). Importantly though, isoelectric focusing has been adopted by the European Commission as the reference method for detecting cow s-casein, with a detection limit of 1% (wt/vol) cow milk (European Union, 2001) in other types of milk.

Separation techniques have also been used for the detection of milk adulteration and both HPLC and GC have been used, often with MS, and are based on the detection of characteristic fatty acids and proteins in dairy products (Romey et al., 1996; Chen et al., 2004; Hurley et al., 2004; Gutierrez et al., 2009). The main disadvantage of these techniques is that they are time consuming (chromatography typically takes 30–60 min) and laborious, and the increased requirements for sample handling during preparation can adversely affect the quality of the analysis (Karoui and Boerdenaeker, 2007).

Finally, PCR techniques have been developed over the last decade, aiming to exploit the presence of somatic cells in milk by detecting genomic DNA from different species. These DNA-based techniques have been used to identify milk adulteration rapidly and with relatively high sensitivity. However, they are not very practical for routine industrial use, and quantification aspects may be hindered by environmental factors such as mastitis that lead to increases in the numbers of somatic cells in milk or by milk processing factors such as milk heat treatment (Banis et al., 2001; Lopez-Calleja et al., 2005; Cheng et al., 2006; Maslova and Paulichkova, 2006).

In general, there exists a useful set of analytical approaches for the detection of milk adulteration; however, all the above techniques have the main disadvantage that they are slow and laborious and this delay in milk analysis makes these tools of little value for routine screening of milk in the dairy industry. By contrast, Fourier transform infrared (FT-IR) spectroscopy is a very rapid biochemical fingerprinting technique (typically 30 s or faster per sample) that can potentially resolve many of these problems and produce milk analysis results in under a minute after minimal sample preparation (Nicolaou and Goodacre, 2008). Compared with other techniques it is simple to use, with high sensitivity and low operational costs. When combined with appropriate multivariate statistical methods such as partial least squares (PLS) regression or, as we report here, kernel PLS, FT-IR spectroscopy may be an ideal solution for the detection and quantification of the adulteration of milk.

Fourier transform infrared spectroscopy, in combination with PLS, has been used in the past to classify different types of oils (Dahlberg et al., 1997; Ozen and Mauer, 2002) and honey (Honmassy et al., 2008) and detect adulteration of extra virgin olive oil with palm oil (Rolman and Man, 2010) and hazelnut oil (Ozen and Mauer, 2002) with very good predictive values. In addition, it has been successfully applied in the detection of spoilage in meat such as beef (Ellis et al., 2004) and chicken (Ellis et al., 2002), as well as in cow milk (Nicolaou and Goodacre, 2008). Our aim in this study was therefore to investigate whether FT-IR spectroscopy is an accurate and valid technique for the detection and quantification of the adulteration of goat or sheep milk with cow milk in both binary and tertiary mixtures.

MATERIALS AND METHODS

Sample Preparation

Three types of full-fat, fresh pasteurized milk were used in this study: cow, goat, and sheep milk. The milk samples used were purchased from national retail outlets and analyzed immediately. From these, 4 milk type combinations were prepared: (1) sheep milk adulterated with cow milk; (2) goat milk adulterated with cow milk; (3) sheep milk adulterated with goat milk; and (4) a tertiary mixture containing sheep, goat, and cow milk.

For each of these combinations, various samples were created, with the primary milk type adulterated with a different type of milk from 0 to 100% in successive increasing steps of 5%. The concentration levels for the tertiary mixture (4) are shown in Supplementary Table S1 (http://www.journalofdairyscience.org/).

The different milk combinations were then poured into sterile flasks and placed in a rotational incubator for 15 min to ensure a homogeneous mixed sample. One-mliliter milk samples were then obtained and subsequently used for FT-IR analysis.

FT-IR High-Throughput Spectroscopy

A Bruker Equinox 55 infrared spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector
Supervised methods are very powerful and it is therefore important to validate both PLS and kernel PLS. To do this for each of the 4 mixtures, a training set and independent hold-out set were generated. For the binary mixtures the training set was 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of one of the milks, and the test set was 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95%. The samples used in the training and test sets for tertiary mixtures are shown in the supporting information (Supplementary Table S1; http://www.journalofdairyscience.org/).

**PLS Regression.** Partial least squares is a commonly used multivariate regression method (Martens and Naes, 1989), especially in the field of spectroscopic study. This is because PLS is able to handle effectively the problem of multicollinearity, which is always the case in spectroscopic data, whereas standard multivariate regression will fail because of the rank deficiency problem. Partial least squares can predict either a single predictive variable using a PLS1 model or predict several predictors simultaneously using a PLS2 model.

We used PLS1 on the binary milk mixtures (cow–sheep, cow–goat, and sheep–goat milk mixtures), whereas we used PLS2 on the tertiary milk mixture. The number of PLS components was optimized using a k-fold cross-validation on the training set only.

**Kernel PLS.** We also employed kernel PLS (Shawe-Taylor and Cristianini, 2004) as a nonlinear regression method, considering that the response of IR spectra might not always be linear when measuring different compositions of different milks. Kernel PLS is a nonlinear extension of PLS model that made use of the recent development of the concept of kernel learning. The idea of kernel learning is that by projecting the data into an appropriate higher dimensional feature space, many nonlinear problems (e.g., regression, classification) can be solved by using a linear modeling method. The projection is achieved by employing a so-called kernel function. An introduction to various kernel methods can be found in Shawe-Taylor and Cristianini (2004). In this study, we employed a radial basis function as the kernel function. The optimal combination of the kernel parameter and the number of PLS factors were optimized using a grid search approach coupled with a k-fold cross-validation on the training set; k is the number of concentration levels we kept in the training set.

**RESULTS AND DISCUSSION**

Representative FT-IR spectra collected from pure cow, goat, and sheep milks are shown in Figure 1. Overall, at the qualitative level, the spectra look very similar and
this is particularly the case for cow and goat milks. In contrast, some quantitative differences were observed in sheep milk. The first difference involved the $CH_2$ absorption band at approximately 2927 cm$^{-1}$, which is related to the acyl chain on fatty acids. As expected, the degree of absorption for this band correlates with the fat quantity in each type of milk, with a higher fat content resulting in higher IR absorption. As reported in the nutritional information that accompanied these milks, full-fat sheep milk has a 4.8% fat composition, whereas cow and goat milk have 3.1 and 3.7% fat, respectively, and this was reflected in the $CH_2$ stretch.

The other obvious visible differences between milk types appeared on the absorption bands related to the remaining milk components, protein (at 1654 cm$^{-1}$ and 1544 cm$^{-1}$ for amide I and II, respectively) and lactose (at 1150 cm$^{-1}$ and 1076 cm$^{-1}$). These appeared to be present in higher quantities in sheep milk than in cow and goat milk, with the former displaying higher IR absorption for these bands. Integration of the bands derived from the spectra for each milk type confirm the above differences in the composition of sheep milk with higher absorption values for the $CH_2$ and $C=O$ absorption bands (Table 1).

![Image of Fourier transform infrared spectra for pure cow, goat, and sheep milk. These spectra are used to allow visualization of any differences. Color version available in the online PDF.](image-url)

Journal of Dairy Science Vol. 93 No. 12, 2010
Table 1. Integrated absorption bands from the 3 measured spectra for each type of milk; numbers are mean ± standard deviation

<table>
<thead>
<tr>
<th>Source</th>
<th>CH₃ from fatty acids, 2850–2884 cm⁻¹</th>
<th>C-O from polysaccharides, 1134–1018 cm⁻¹</th>
<th>CH₃/C-O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>98.5 ± 0.09</td>
<td>531.6 ± 7.15</td>
<td>0.182 ± 0.0034</td>
</tr>
<tr>
<td>Sheep</td>
<td>121.7 ± 9.22</td>
<td>614.4 ± 4.67</td>
<td>0.196 ± 0.0141</td>
</tr>
<tr>
<td>Goat</td>
<td>111.2 ± 0.48</td>
<td>521.6 ± 2.30</td>
<td>0.212 ± 0.0005</td>
</tr>
</tbody>
</table>

Analysis of Binary Mixtures of Milk

Because of the subtle differences highlighted above it was not possible to use simple visual inspection to quantify the level of adulteration of these milk. Thus, following spectral collection from the different milk combinations, the relationship between the spectra was investigated using PC-DFA.

Using the combination of goat and sheep milks as an example (Figure 2A), visual inspection of the results identifies a clear trend in PC-DFA space with respect to mixture levels, although this does not follow a linear trajectory, rather a parabolic one. The samples with high concentrations of goat milk are recovered in the same region on the right of the panel; a decrease in the concentration of goat milk, with a concurrent increase in the concentration of sheep milk, appears to cause a spread of the spectra first upward to the middle of the pane and then laterally toward the bottom left of the pane. Inspection of the PC-DFA loadings matrix (Figure 2B) indicates that for the separation of goat and sheep milk, larger differences were observed in the fatty acid vibrations from CH₃ stretches in the region 2900 to 3000 cm⁻¹ (see Figure 1 for annotation) than for either the protein or polysaccharide regions. For the other 2 mixtures (cow sheep and cow goat), a parabolic trend in PC-DFA space was also observed (data not shown). It was clear, therefore, that a nonlinear trend existed with respect to milk concentration and we thus sought to explore the use of linear and nonlinear multivariate regression techniques for quantification.

Quantification of Binary Milk Mixtures

As detailed above, 3 binary milk combinations were produced. For brevity, we shall use goat-sheep milk as an example. Samples containing 0 to 100% goat milk (in 5% increments) in sheep milk were prepared and the 21 mixtures were analyzed in triplicate using FT-IR spectroscopy. As detailed above, the data were preprocessed using SNV and were then split into a training set (0, 10, 20, ..., 90, and 100% goat milk) and a test set (5, 15, 25, ..., 85, and 95%) and analyzed by PLS and KPLS.

During calibration of the PLS model, the training data were used and these were subsampled using leave-one-out to generate a cross validation set to choose the optimum number of PLS factors (latent variables) for calibration. Following this, the independent test set was used to challenge the PLS model. The PLS regression results for the goat and sheep milk combinations are shown in Figure 3. In this plot the estimated goat milk concentration versus the known goat milk concentration values follow the expected y = x and gave relatively accurate results. As detailed in Table 2, the root mean squared (RMS) error for the training data (root mean square error for calibration; RMSEC) was 3.73%, the cross-validation set selected 3 PLS factors for this model, the RMS error for the cross validation set was 5.57%, and the RMS error in the independent test was 8.03% (root mean square error for predictions in the test set, RMSEP). The correlation coefficient for the test set (Q²) for this model was 0.92.

Root mean square error for predictions and Q² are 2 unbiased metrics to assess the predictive ability of a model in regression analysis. Generally, RMSEP gives an unbiased estimation of the error of the prediction when the model is used to predict the concentration of unknown samples from a data set not used to construct the model (the test set). The smaller the RMSEP value, the better the model. The Q² value is an independent metric scale used to quantify the predictive ability of the model with values similar to the commonly used squared correlation coefficient R² in univariate regression analysis. The closer the value of Q² to 1, the better the model. Thus, the predictions from the goat–sheep milk mixture were relatively encouraging at 8.03% and 0.92 for the RMSEP and Q², respectively.

Kernel PLS (KPLS) is a nonlinear extension of PLS and we decided to investigate this algorithm because the PC-DFA had shown a nonlinear trajectory. The results for KPLS are also shown in Figure 3 and the associated statistics are given in Table 2. It can be seen that the greatest improvement of using KPLS over PLS was in the model constructed from the goat–sheep milk mixture, with a RMSEP of 3.95% and a Q² of 0.98. This improvement of KPLS over PLS was also observed for the cow–sheep and cow–goat mixtures (Figure 3 and
Table 2. Comparison of the partial least squares (PLS) regression, and the nonlinear kernel PLS of the Fourier transform infrared spectra for determining the percentage volume of cow, goat, and sheep milk mixtures.

<table>
<thead>
<tr>
<th>Milk mixture</th>
<th>PLS</th>
<th>Kernel PLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat-sheep milk</td>
<td>5.57</td>
<td>3.96</td>
</tr>
<tr>
<td>Cow-sheep milk</td>
<td>6.24</td>
<td>6.24</td>
</tr>
<tr>
<td>Cow-goat milk</td>
<td>5.90</td>
<td>5.75</td>
</tr>
<tr>
<td>RMSECV</td>
<td>6.55</td>
<td>5.84</td>
</tr>
<tr>
<td>RMSEC</td>
<td>7.42</td>
<td>5.62</td>
</tr>
<tr>
<td>RMSEP</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>( Q^2 )</td>
<td>0.94</td>
<td>0.96</td>
</tr>
<tr>
<td>( Q^2 )</td>
<td>0.91</td>
<td>0.96</td>
</tr>
</tbody>
</table>

\( \text{RMSECV} = \text{root mean square error for the cross-validation}; \text{RMSEC} = \text{root mean square error for the calibration}; \text{RMSEP} = \text{root mean square error for the predictions produced}; \text{\( Q^2 \)} = \text{correlation coefficient for the training set}; \text{\( Q^2 \)} = \text{correlation coefficient for the test set.} \)

Table 2), again highlighting the usefulness of employing a nonlinear regression algorithm.

**Mixtures of the 3 Types of Milk**

Following a similar strategy to that used for the binary mixtures, the first stage of the analysis was to look at the natural relationship between the FT-IR spectra collected from the 63 milk samples containing various concentrations of sheep, goat, and cow milk. The results of the PC-DFA are shown in Figure 4 as a pseudo 3-dimensional plot of the first 3 PC discriminant functions (DF), and it is clear that these spectra are spread in 3 different dimensions. Three color scales are used for each of the milk species: high concentrations of cow milk samples are presented in red; sheep milk is presented in green; and a higher proportion of goat milk is presented in blue.

It can be seen from Figure 4 that when the contribution of one milk type starts to dominate the mixture, a “tentacle” extends from the center of the milk mixtures and the tips of the tentacle contain that pure milk. Domination of these tentacles appears to develop when the concentration of one of the contributing milk types increases above the 55% level. In addition, a fourth tentacle (yellow color) develops when the concentration of cow and sheep milk in the tertiary sample both increase above the 40% contribution level for these milk types.

![Figure 2](image)

**Figure 2.** (A) Principal component (PC) - discriminant function analysis (DFA) plot on high-throughput Fourier transform infrared spectra for the 3 repeat experiments of goat milk when added to sheep milk. Principal components 1 to 20 accounting for 99.65% of the total variance were used by the DFA algorithm with a priori knowledge of machine replicates. The different numbers show the concentration of goat milk in the mixture and the color (gray) scale the level of adulteration. The blue (black) color indicates a low concentration of goat milk in the mixture, the red (light gray) color indicates a high concentration of goat milk, and the purple (dark grey) color indicates the concentrations where goat and sheep milk have very close concentrations. (B) Principal component-DFA loadings plot from the first vector (discriminant function 1) showing which infrared regions are important; the positive part of DF 1 reflects areas that are increased in goat milk and the negative half of the plot those regions that are higher in sheep milk. Color version available online PDF.

In the middle 3 smaller tentacles can be observed, which are colored dark green, turquoise, and purple. These contain samples in which the 3 milk types are in similar concentrations (dark green) and in which 2 milk types are in high concentration; the turquoise represents milk
with a high concentration of sheep-goat milk and the purple represents milk with a high concentration of cow-goat milk (a similar plot giving the concentration levels of each milk is shown in Supplementary Figure 1; http://www.journalofdairyscience.org/). In conclusion, the cluster analysis shows that clear trends exist in these data related to the various combinations of milk but that the ability to quantify the level of milks in these tertiary mixtures is unlikely from the PC-DFA, given the complexity of this space.

**Quantification of Tertiary Milk Mixtures**

The data were split into training and test set data and then analyzed using linear and nonlinear supervised learning techniques; as 3 milk concentrations were to be predicted we employed 3 output Y-variables (one for each type of milk) in PLS2 and KPLS2. The results from the PLS2 model for the training and test data are shown in Figure 5 and Figure 6, respectively, and overall showed a good prediction for the 3 different types of
Figure 4. Principal components (PC)-discriminant function analysis (DFA) plot on high-throughput Fourier transform infrared spectra for the 3 repeat experiments of the mixtures from the 3 types of milk. Principal components 1 to 20 (accounting for 96.45% of the total variance) were used by the DFA algorithm with a priori knowledge of machine replicates (i.e., 1 class per time point, giving 63 classes in total). Samples with high concentrations of cow milk are presented in red, those with high concentrations of sheep milk are green, and those with high concentrations of goat milk are blue. In the middle appear to be samples that contain 2 out of the 3 milk types in high concentrations and these samples are a different color from the combination of all 3 types of milk.

milk. Table 3 shows the summary statistics for PLS2 and KLP2 using the same training and test set splits and, in this case, the PLS2 algorithm outperformed KLP2.

In general, all milks were predicted with a similar level of accuracy and the RMSEP values were 3.4 to 4.9% for PLS2 with $R^2$ correlations of 0.94 to 0.97.

Figure 5. Plot showing the predicted concentrations of cow, goat, and sheep milk versus the actual concentrations for the training set in the mixtures of the 3 types of milk by using partial least squares 2. The circles represent the goat milk samples, triangles the sheep milk samples, and the crosses cow milk samples. Color version available in the online PDF.

Figure 6. Plot showing the predicted concentrations of cow, goat, and sheep milk versus the actual concentrations for the test set in the mixtures of the 3 types of milk by using partial least squares 2. The circles represent the goat milk samples, the triangles the sheep milk samples, and the crosses cow milk samples. Color version available in the online PDF.
Table 3. Comparison of the partial least squares 2 (PLS2) regression, and the nonlinear kernel PLS 2 (KPLS2) of the Fourier transform infrared spectra for determining the percentage volume of cow, goat, and sheep milks from mixtures containing the 3 types of milk together.

<table>
<thead>
<tr>
<th>Item</th>
<th>Cow milk</th>
<th>Sheep milk</th>
<th>Goat milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSECV</td>
<td>5.63</td>
<td>3.10</td>
<td>4.96</td>
</tr>
<tr>
<td>RMSEC</td>
<td>5.53</td>
<td>4.94</td>
<td>3.67</td>
</tr>
<tr>
<td>RMSEP</td>
<td>4.89</td>
<td>3.35</td>
<td>4.83</td>
</tr>
<tr>
<td>R²</td>
<td>0.95</td>
<td>0.96</td>
<td>0.98</td>
</tr>
<tr>
<td>Q²</td>
<td>0.94</td>
<td>0.97</td>
<td>0.94</td>
</tr>
<tr>
<td>KPLS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factors</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSECV</td>
<td>6.05</td>
<td>3.97</td>
<td>4.91</td>
</tr>
<tr>
<td>RMSEC</td>
<td>4.20</td>
<td>3.60</td>
<td>2.84</td>
</tr>
<tr>
<td>RMSEP</td>
<td>6.40</td>
<td>5.61</td>
<td>3.89</td>
</tr>
<tr>
<td>R²</td>
<td>0.97</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>Q²</td>
<td>0.92</td>
<td>0.93</td>
<td>0.96</td>
</tr>
</tbody>
</table>

RMSECV = root mean square error for the cross-validation; RMSEC = root mean square error for the calibration; RMSEP = root mean square error for the predictions produced; R² = correlation coefficient for the training set; Q² = correlation coefficient for the test set.

CONCLUSIONS

In this study, we demonstrated that in binary and ternary mixtures of milk, FT-IR spectroscopy in combination with multivariate analysis, such as linear PLS and nonlinear Kernel PLS, provides an accurate, simple, and rapid technique for the quantitative assessment of the adulteration of sheep, goat, and cow milks. The typical errors that were found were in the region of 5 to 8% for all milk species; an unscrupulous producer would unlikely adulterate at such a level because it would not be financially viable. For this reason and the speed of analysis (30 s per sample in batches of 96 or 384), we believe that FT-IR spectroscopy has excellent potential for use in the food industry to replace less efficient and more time-consuming techniques for the detection of milk adulteration. For this approach to be employed within the industry, future studies should consider geographical as well as seasonal variations in milk production.

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Appendix C. Chapter 5 Peer review publication paper

MALDI-MS and multivariate analysis for the detection and quantification of different milk species

Nicoletta Nicolaou • Yun Xu • Royston Goodacre

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Abstract The extensive consumption of milk and dairy products makes these foodstuffs targets for potential adulteration with financial gains for unscrupulous producers. Such practices must be detected as these can impact negatively on product quality, labelling and even health. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a potentially useful technique, with proven abilities in protein identification and more recently through the use of internal standards for quantification purposes of specific proteins or peptides. In the current work, we therefore aim to explore the accuracy and attributes of MALDI-ToF-MS with chemometrics for the detection and quantification of milk adulteration. Three binary mixtures containing cows' and goats' milk, and a fourth ternary mixture containing all types of milk were prepared and analysed directly using MALDI-ToF-MS. In these mixtures, the milk concentrations of each milk varied from 0% to 100% in 5% steps. Multivariate statistical methods including partial least squares (PLS) regression and non-linear Kernel PLS regression were employed for multivariate calibration and final interpretation of the results. The results for PLS and KPLS were encouraging with between 2% and 13% root mean squared error of prediction on independent data; KPLS slightly outperformed PLS. We believe that these results show that MALDI-ToF-MS has excellent potential for future use in the dairy industry as a rapid method of detection and enumeration in milk adulteration.

Keywords Bioanalytical methods • Chemometrics/statistics • Foods/beverages • Mass spectrometry/MALDI-MS

Introduction

In today's era of consumerism and increasing reliability on the food industry to provide food fit for consumption, the issue of food safety and authenticity is becoming progressively more important. The internationalisation of food markets has also made the food industry a very competitive and financially lucrative business. The simple substitution of a scarce ingredient with a more abundant and cheaper ingredient, especially when high value products are involved, can yield huge financial gains. This however may also result in an unwanted and catastrophic chain of events as product quality usually becomes substandard, product identity is lost, incorrect product labelling ensues and consumers are exploited and misled. At the same time, loss of the original high quality and origin-specific product may occur. Furthermore, incorrect product labelling may leave consumers exposed to potential allergens, such as casein protein from cows' milk, becoming detrimental for health.

Milk and dairy products are extensively consumed by large segments of the population during all stages of development and life, including childhood, adolescence, pregnancy and the elderly, due to their high nutritional value and health benefits. In general, however, milk is considered to be an expensive raw material. The current commercial UK price (from national outlets in 2010) for
cows' milk is ~0.70/L, with sheep's over £3.00/L and goats' milk between £1.00/L and £1.34/L, with some seasonal milk production variation. This makes milk and dairy product adulteration, especially of the higher value types of milk, very profitable.

The variation of environmental conditions and the process of natural selection have also lead to the diversity of animal breeds and the production of milk with particular characteristics for that animal type and area [1]. Such milk has subsequently been used by local producers for the production of distinct types of cheeses of recognised quality and characteristics [2]. The production of these products however entails high overall processing costs, and producers can be financially destroyed by the presence of unfair competition, thus there may be a temptation to stretch the more expensive milk with one of lower value.

In an attempt to protect consumers and genuine product producers, policymakers at different countries have developed a number of legislations. The agriculturally diverse European Union has also legislated regulation EC/178/2002, a ‘food safety and traceability regulation’ [3], aiming to protect human life and health, establish consumer rights to food safety and accurate information and protect name misuse and imitation. European regulations on industrial milk processing are strict, only permitting a predefined number of constituent modifications, such as changes in the fat content and the addition of certain minerals, vitamins and milk proteins. Currently, certain dairy products, such as ‘protected destination of origin’ (PDO) and ‘protected geographical indication’ (PGI) goods, also require very accurate labelling in regards to their origin and processing and are protected by appellations of origin [4–6]. Further food labelling legislation is currently under discussion in the EU.

Ensuring product authenticity and implementing some of these strict standards and criteria has been very difficult. Analytical techniques have been employed to perform this hard task but have been unable to fulfill this role effectively, either because they are unable to keep in pace with the constant technological advances and developments occurring at the dairy industry or because of lack of commercial practicality [6]. Advanced techniques such as spectroscopy, near-infrared (NIR), mid-infrared (MIR) and nuclear magnetic resonance (NMR) spectroscopies [7–12], as well as chromatography [13, 14], immunoenzymatic assays [15, 16], polymerase chain reaction [17, 18], electrophoresis [19–21] and sensory analyses [22, 23], have all been utilised by the analytical dairy science to improve milk product analysis. The main disadvantage of these techniques though is that they remain time-consuming and labour intensive, with limited value for routine use in the screening of milk in the dairy industry.

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a potentially useful technique in the authentication of milk, with proven abilities in protein identification and more recently quantification [24–30]. MALDI is an ionisation technique involving the insertion of the sample typically into a UV absorbing matrix composed of a non-volatile material (usually a mild aromatic acid), followed by laser irradiation (typically at 337 nm), absorption, matrix energy desorption, and matrix to sample proton transfer resulting in the creation of vapourised ions. The technique has the advantage of only requiring small sample quantities and can be used for the analysis of heterogeneous biological samples such as milk, as recently demonstrated by Liland and colleagues [31, 32]. In addition, it possesses a very high sensitivity of mass range of up to 300,000 Da for proteins [33]. The aim of our study was therefore to investigate whether the MALDI-ToF-MS technique is able to detect and quantify goats' and sheep's milk adulteration with cows' milk, using the whole spectrum of peaks obtained from the analysis.

Materials and methods

Sample preparation

The fresh full fat pasteurised milk used in this study was purchased from national retail outlets and was analysed immediately. The milk tested included three types of milk: cows', goats' and sheep's milk. Four different milk type combinations were prepared as follows:

1. Sheep's milk adulterated with cows' milk,
2. Goats' milk adulterated with cows' milk,
3. Sheep's milk adulterated with goats' milk,
4. A tertiary mixture containing sheep's, goats' and cows' milk.

Each milk combination included a variation in the percentage of the primary milk adulteration ranging from 0% to 100% in successive increasing steps of 5%. A similar principle was used for the tertiary mixture (concentration levels are shown in Table S1 in the Electronic Supplementary Material). In order to ensure adequate mixing of the different milk types, the different mixtures were placed into sterile flasks and stirred using a rotational incubator for 15 min. Following this, 1 ml milk samples were collected and used for MALDI-ToF-MS analysis.

MALDI-ToF-MS

The sample preparation used was based on the method reported by Cozzolino and colleagues [26]. This involved initially taking 100 μL of each homogenized sample and
diluting these in 1 mL of water containing 0.1% trifluoroacetic acid (Sigma-Aldrich, Dorset, UK). These samples were then further diluted 1:10 with the same solvent. Five microliters of these samples were then mixed with 5 μL of the matrix solution. For all samples, sinapinic acid (Sigma-Aldrich, Dorset, UK) was used as a matrix having been saturated in a matrix solution composed of 50% acetonitrile (Sigma-Aldrich, Dorset, UK) and 50% water. From the final mixtures, 1 μL was then positioned onto a MALDI-MS stainless steel target plate and dried for 2 h at room temperature.

MALDI-ToF-MS analysis was undertaken on a MALDI-ToF mass spectrometer (AXIMA-CFR™plus; Shimadzu Biotech, Manchester, UK), equipped with a nitrogen pulsed UV laser (337 nm), and was operated using a positive ion source in linear ion mode in the positive ion mode. The laser power was set to 120 mV, each spot was analysed using a random raster of 500 profiles and each profile contained data from five laser shots. Each sample was analysed three times, and the typical collection times were 4 min per sample.

Data analysis

Pre-processing

The mass spectral data were imported into MATLAB (The Math Works, Natick, MA, USA) and processed for analysis. Typically, the data were baseline corrected and normalised. Normalization of each individual spectrum was performed by dividing each individual baseline corrected spectrum with the square root of the sum of squares of the spectrum [34].

Exploratory analysis

The exploratory analysis was performed in two steps as described by us elsewhere [12, 35]. Initially, principal component analysis (PCA) was used. PCA is an established procedure for reducing the dimensionality of multivariate MALDI-ToF-MS data whilst preserving most of the variance; this process results in the creation of new variables named principal components PCs which are uncorrelated. This is important because there are a large number of variables in the MALDI-ToF-MS data. The second step involved the use of canonical correlation analysis (CCA). CCA [36] is a commonly used method for assessing the correlation between two multivariate matrices or one multivariate matrix and one corresponding vector (e.g. concentration of adulterant). CCA seeks a set of linear combinations called canonical variables so that the correlation between the two matrices is maximised. The correlation of the two matrices is expressed as a correlation coefficient in a similar sense of the correlation coefficient (R) between two vectors, while the significance level of such correlation can be assessed by using an F test [37]. CCA thus gives us a quick assessment of the correlation (R) between the MALDI-ToF-MS spectra and the adulteration level of the milk and the significance of that probability (p value) before we move to a more robust quantitative analysis. The CCA is performed on the scores from the PCA, and the results shown are based on the number of PCs which yielded the lowest p value.

Quantitative analysis

If there is a strong correlation between the two inputs, i.e. the MALDI-ToF-MS spectra and the adulteration levels, it is then possible to employ a multivariate regression model to predict the adulteration levels using the MALDI-ToF-MS spectra. In this study, we used partial least squares (PLS) and Kernel PLS, as linear and non-linear regression techniques, respectively.

Whilst supervised methods are very powerful, it is possible to over-fit the model; therefore, validation of both PLS and Kernel PLS was undertaken. We achieved this using an independent test set for each of the mixture types. For each of the binary mixtures, the training set contained 0%, 10%, 20%, ..., 90% and 100% of one of the milks, and the test set included 5%, 15%, 25%, ..., 85% and 95%. For the tertiary mixtures, the training and test sets are shown in Table S1. The choice of the training set and the test set is to ensure that both training and test set have a similar coverage of the adulteration levels except that in the test set there was no extrapolation (i.e. there is no pure milk of any kind to be predicted in the test set).

PLS regression

PLS regression is a frequently used multivariate algorithm [38]. It is more useful than standard multivariate regression because PLS is able to deal with multicollinearity in data; this is usual when continuous data are generated (such as those mass spectra shown in Fig. 1). PLS models were generated to predict a single variable, and so, PLS1 was used for both the three binary mixtures as well as for the tertiary mixture. The number of PLS components (latent variables) was optimized using a k-fold cross-validation on the training set only, where k is the number of adulteration levels in the training set.

Kernel PLS

Kernel PLS is a non-linear extension of PLS regression which makes use of Kernel learning concepts [39]. In Kernel learning, one projects the data into an appropriate
higher dimensional feature space, with the result that many non-linear problems can now be solved by using a linear modelling method on this projected feature space [39]. In our KPLS approach, we used a radial base function (RBF) as the Kernel function, and the optimal combination of the Kernel parameter and the number of PLS factors were optimized using a grid search approach again using k-fold cross-validation on the training set as described above.

Results and discussion

Spectra

MALDI spectra can be used to determine and quantify the protein components of various types of milk, by identifying the different peaks and assigning them to specific proteins based on previously published protein molecular mass data. Individual proteins, however, may show a variation in their
molecular mass and thus the exact position of the peak; this is due to factors such as genetic and non-genetic polymorphisms and milk processing, the latter mainly via thermal denaturation and proteolysis which can affect individual protein structure [40-44]. Milk samples from different species or from different animal breeds of the same species can therefore display small variations in the molecular mass of the same protein, which will also vary depending on whether the milk is raw or has been processed and how it has been processed.

Figure S1 (in the Electronic Supplementary Material) shows a typical MALDI mass spectrum of fresh full fat pasteurised cow milk, including the original raw data and processed data after baseline correction and normalization that was required before chemometric analyses. Qualitatively, the spectra from all three milk species (Fig. 1) appear to display similar protein patterns between the different types of milk with small differences in the location of the molecular mass signal of the same proteins; in addition, it is also possible to see some differences in regards to the quantity of certain proteins. A closer inspection of the MALDI mass spectrum of cows’ milk (Fig. 1) and interpretation based on previously published molecular mass data [24, 26, 27, 30, 45] reveal a number of protein-related peaks. These include a peak at m/z 89,000 representing the proteose peptone, seen in all milk types at a similar position, and the peaks at m/z 15,000 and m/z 18,500 relating to α-lactalbumin and β-lactoglobulin, respectively, with the latter displaying a higher peak/contend compared to the other milk types. The broad peaks over m/z 30,000, at m/z 31,000 and m/z 43,000 represent dimeric and trimeric species [45].

Inspection of the MALDI spectrum of sheep’s milk shows the α-lactalbumin and β-lactoglobulin peaks appearing as m/z 13,500 and m/z 19,000, with an additional peak at m/z 12,000 representing γ-casein, which is less prominent in the other milk types. Finally, in the MALDI spectrum of pure goats’ milk, the α-lactalbumin and β-lactoglobulin are located at m/z 13,000 and m/z 19,000 peaks, respectively, while a more prominent peak at m/z 21,000 represents γ-casein.

The peaks below m/z 9,000 in all types of milk are representative of species with low molecular mass due to proteolysis of higher mass proteins. The latter appears to be significant and over-represented in the MALDI spectra obtained in this study compared to spectra from other studies using raw milk, and this is most likely due to the effect of milk processing and pasteurisation on the various protein species.

Analysis of binary mixtures of milk

The small differences between the pure spectra become even harder to visualise by eye when mixtures were analysed, and so, this did not allow direct visual comparison in order to detect the level of adulteration. Therefore, the relationship between the spectra collected from the different milk combinations and concentrations was investigated using the exploratory analysis procedures as described in the “Data analysis” section.

The results from the CCA for mixtures of cows’ and goats’ milk analysed using MALDI-ToF-MS are shown in Fig. 2. It is very clear from this plot that there is a concentration-dependent relationship in the MALDI-ToF-MS data, and visual inspection of these results identifies a linear pattern of the mixture levels. The canonical correlation coefficient R for this linear relationship was found to be 0.9953 with a highly significant probability p value of 8.83 × 10⁻⁵⁵, while similar values (R=0.9893, p value=4.07 × 10⁻⁵⁰) were found for the cows’ and sheep’s milk mixtures (CCA plots not shown). The goats’ and sheep’s milk mixtures displayed a comparable linear relationship to the other milk mixtures but with a slightly lower value for the correlation coefficient, R=0.9674, and a p value of 2.70 × 10⁻⁷³. This suggests that there are very strong correlations between the MALDI-ToF-MS data and their corresponding milk adulteration levels. Linear and non-linear multivariate regression techniques were therefore employed in order to explore these trends even further and to assess whether it was possible to quantify the level of milk adulteration from these mass spectra.

Quantification of binary milk mixtures

Three different binary milk combinations were created as detailed above. The cow–goat milk binary mixture is

![CCA plot on MALDI-ToF mass spectra of cows’ milk when added to goats’ milk. PCs 1–15 were used by the CCA algorithm with a priori knowledge of concentration of cow milk. The different dots show the concentration of cows’ milk in the mixture in relation to the canonical variable scores, with R²=0.9953 and p value=8.83 × 10⁻⁵⁵.](image-url)
Fig. 3 Plots showing the predicted levels of milk adulteration estimated from PLS (left column) and KPLS (right column) models. The rows show the different mixtures analysed with predictions for cow's milk adulteration in goat's (top) and sheep's (middle) milk, and goat's milk adulterated into sheep's milk in the bottom row. The blue circles represent the training data set and the red crosses, the test set.

Subsequently used as an example, samples containing 0% to 100% cows' milk (in 5% steps) in goats' milk underwent preparation, and the resulting 21 mixtures produced were analyzed in triplicate using MALDI-ToF-MS. As detailed above, the data were baseline corrected, normalized and then split into a training set (0%, 10%, 20%, ..., 90% and 100% goats' milk) and a test set (5%, 15%, 25%, ..., 85% and 95%) and analyzed by linear PLS and non-linear KPLS.

During calibration of the PLS model, sub-sampling of the training data took place, leaving one set (i.e. a unique adulteration level; all replicates) out so that a cross-validation set could later be generated; this allowed for the selection of the optimum number of PLS factors for calibration. Once this was performed, the independent test set was utilized to challenge the derived PLS model. PLS regression results for the cows' and goats' milk mixture...
Table 1: Comparison of the partial least squares (PLS) regression and the non-linear Kernel partial least squares (KPLS) results of the MALDI-ToF mass spectra for determining the percentage volume of cows’ milk mixed with goats’ milk and sheep’s milk and goats’ milk mixed with sheep’s milk.

<table>
<thead>
<tr>
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<th>PLS</th>
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<td>RMSEC</td>
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<tr>
<td>RMSEP</td>
<td>6.35</td>
<td>6.35</td>
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<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
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<td>0.99</td>
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<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.95</td>
<td>0.95</td>
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<tr>
<td>Cow—sheep milk</td>
<td></td>
<td></td>
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<tr>
<td>Factors</td>
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<tr>
<td>RMSEP</td>
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<td>8.13</td>
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<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
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<td>0.99</td>
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<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
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<td>0.92</td>
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<tr>
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<td></td>
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<tr>
<td>Factors</td>
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<td>19</td>
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<tr>
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<td>10.35</td>
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<tr>
<td>RMSEC</td>
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<tr>
<td>RMSEP</td>
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<tr>
<td>Correlation coefficient in the train set ($R^2$)</td>
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<td>1.0</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.82</td>
<td>0.83</td>
</tr>
</tbody>
</table>

The RMSECV represents the root mean square error for the cross-validation, the RMSEC represents the root mean square error for the calibration and the RMSEP represents the root mean square error for the predictions produced for the independent test set.

Combinations are shown in Fig. 3. The plot of the estimated cows’ milk concentration versus the known cows’ milk concentration values in this figure appeared to show good predictive values, and importantly, both the training and test sets lie on the expected y=x perfect prediction line. Table 1 shows the detailed results for all the three binary mixtures. For the cow—goat milk mixture, the root mean squared (RMS) error for the training data (RMSEC) was 0.95%, with seven PLS factors selected by the cross-validation set for this model, and the RMS error for the cross-validation (RMSECV) set was 5.24%; the RMS error in the independent test was 6.85% (RMSEP). This model’s $Q^2$ value was 0.95, while the $R^2$ for the train set model was 0.99, both very close to the prefect model, which would be 1.

The most discriminative features used by the PLS regression, which models the adulteration levels of the milks, can be found by inspecting the variable importance for projection (VIP) plots. The most discriminative features generally have higher magnitudes than the non-discriminative ones in the VIP plots. These are displayed in Fig. 4 and Table 3 for the binary milk mixtures. Comparison with the pure milk MALDI-MS spectra (Fig. 1) indicates that for the cow—goat binary mixtures, the dominant features at 14,100 and 18,020m/z are
levels of the milk may not be linear, we decided to employ the KPLS algorithm, a non-linear extension of PLS. The results are depicted in Fig. 3, with the relevant statistical information also shown in Table 1. For our cows’-goats’ binary milk mixture, it is clear that KPLS provides similar results to PLS, with a RMSEP of 6.35% and an $R^2$ of 0.95. The usefulness and improvement of the non-linear KPLS algorithm compared to PLS are more apparent when the results from the other two binary milk mixtures are observed, as shown in Fig. 3 and Table 1. KPLS does not allow the generation of loadings or VIP scores, so model interpretation in terms of which proteins are important is largely hidden.

Mixtures of the three types of milk

The MALDI mass spectra collected from the 63 milk samples, containing various concentrations of the three milk types, sheep’s, goats’ and cows’ milk, were analyzed using a similar strategy to the binary mixture analysis. Initially, the relationship between the spectra and the adulteration levels of the milk was investigated using CCA. Figure 5 presents the significant results in a pseudo-2D plot constructed from the first five PCs. The different concentrations of the three different types of milk are represented in different colours. We used red, green and blue for pure cows’, sheep’s and goats’ milk, respectively. The colours of the mixture samples are represented by mixing the three colours together according to their corresponding relative concentration levels. For example, the colour a mixture sample of cows’, sheep’s and goats’ milk with their relative concentration levels of 70%/20%/10% is a colour with its RGB (red, green and blue) channels having 7:2:1 relative intensities, respectively. Visual inspection of the 2D space indicates a clear pattern of distribution of the different milk mixture concentrations. Dominance of a particular type of milk in the mixture at concentrations of greater than 50% appears to create a tentacle towards a specific direction with a pure milk type at the tip. An increasing cows’ milk concentration appears to create a tentacle towards the right side of the pane (red), an increase in sheep's milk concentration extends a tentacle towards the left lower corner of the pane (green), while similarly, the increasing goats’ milk concentration forms a tentacle towards the upper left corner of the pane (blue). Furthermore, three additional smaller distinctive tentacles (yellow, purple and turquoise) are observed in the middle of the pane extending outwards in different directions. Each tentacle appears to lie between two of the bigger tentacles and represents mixtures containing the two associated milk types in concentrations of greater than 40%, respectively. For example, the small tentacle in turquoise colour extending due west lies between the blue (high goat's milk concentration) and the...
Fig. 6 Plots showing the predicted concentrations of cows', goats' and sheep's milk versus the actual concentrations for the training and test set in the tertiary mixtures of the three types of milk using (a) PLS and (b) KPLS. The blue colour represents the training data set and the red, the test set. The triangles represent the goats' milk samples, the squares, the sheep's milk and the stars, the cows' milk quantifying the levels of the different milk species in these tertiary mixtures.

Quantification of tertiary milk mixtures

In order to perform quantification of the tertiary mixtures, the data were first divided in two sets, a training and a test set (Table S1), and analyses were undertaken as described above again employing the linear and non-linear supervised analyses of cows', goats' and sheep's milk from mixtures containing the three types of milk together.

Table 2 Comparison of the partial least squares (PLS) regression and the non-linear Kernel partial least squares (KPLS) results of the MALDI-ToF mass spectra for determining the percentage volume of Cow milk Sheep milk Goat milk

<table>
<thead>
<tr>
<th></th>
<th>Cow milk</th>
<th>Sheep milk</th>
<th>Goat milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLS Factors</td>
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<td>10</td>
<td>9</td>
</tr>
<tr>
<td>RMSECV</td>
<td>2.58</td>
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<td>4.25</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.19</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>RMSEP</td>
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<td>3.29</td>
<td>3.84</td>
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<td>0.99</td>
</tr>
<tr>
<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.98</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>KPLS Factors</td>
<td>6</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>RMSECV</td>
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<td>RMSEC</td>
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<td>2.02</td>
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<td>Correlation coefficient in the train set ($R^2$)</td>
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<td>Correlation coefficient in the test set ($Q^2$)</td>
<td>0.99</td>
<td>0.98</td>
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</table>

The RMSECV represents the root mean square error for the cross-validation, the RMSEC represents the root mean square error for the calibration and the RMSEP represents the root mean square error for the predictions produced for the training set. The $R^2$ represents the correlation coefficient for the training set, and the $Q^2$ represents the test set.

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Table 3. Spectral peaks with the highest variable importance for the prediction (VIP) scores used in the PLS modelling for the binary and tertiary milk mixtures.

<table>
<thead>
<tr>
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<th>Peak m/z</th>
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<tbody>
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<td>Binary</td>
<td></td>
<td></td>
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<tr>
<td>Cow–goat milk</td>
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<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>14,100</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td></td>
<td>18,020</td>
<td>β-Lactoglobulin</td>
</tr>
<tr>
<td></td>
<td>23,700</td>
<td>α₀-Casein</td>
</tr>
<tr>
<td>Cow–sheep milk</td>
<td>8,690</td>
<td>Peptone proteine</td>
</tr>
<tr>
<td></td>
<td>11,190, 11,480</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>11,740</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>23,470</td>
<td>α₀-Casein</td>
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<td>Goat–sheep milk</td>
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<td>Peptone proteine</td>
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<td></td>
<td>11,190, 11,450</td>
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<td>23,600</td>
<td>α₀-Casein</td>
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<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>11,730</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>14,060</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td>Sheep milk</td>
<td>11,190, 11,450</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>14,060</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td>Goat milk</td>
<td>11,450</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>11,740</td>
<td>γ₂-Casein</td>
</tr>
<tr>
<td></td>
<td>14,060</td>
<td>α-Lactalbumin</td>
</tr>
</tbody>
</table>

Learning techniques of PLS and KPLS regression. Although there are three Y-variables to be predicted (one for each milk species), rather than use PLS2 and KPLS2, we chose to use PLS1 and KPLS1 where three models were constructed for each milk. This was because PLS1 generally outperforms PLS2 for quantification of different analytes, as there will be different directions in the spectral space that are describing the contributions for the three different milk species; therefore, it is better to optimise each of these individually [46]. Figure 6 illustrates the results from the PLS and KPLS models for the training and test data. For both PLS and KPLS models, excellent predictions for the three different types of milk was attained. Table 2 shows the summary statistics for both multivariate regression models using the same training and test set splits; in this case, the PLS algorithm outperforms KPLS. Rather interestingly, these models for the tertiary mixture were better than the models constructed from the binary models (Fig. 3 and Table 1), although we cannot think of any conceivable reason why this may be the case. The most dominant spectral peaks used by the PLS modelling for discriminating between the three types of milk with the highest VIP scores for the tertiary milk.
mixtures are displayed in Fig. 7 and Table 3. In all three PLS models for each type of milk, the dominant features are largely the same and appear as a peak in one or more of the pure milk spectra. The features at 11,190–450 m/z (γ-casein) and 11,730–480 m/z (β-casein) are only present as spectral peaks in the pure sheep milk, while the 14,060 m/z (α-lactalbumin) VIP score feature is present as a peak in all three types of pure milk samples. Furthermore, the difference found between the number and level of scoring for some features in the tertiary sample mixtures compared to the binary mixtures is most likely due to the effect of the presence of the additional milk type in the mixture.

Concluding remarks

In comparison to previous studies demonstrating the qualitative aspects of MALDI-ToF-MS using selected peaks for milk speciation [24–30], through this study, we have shown that the whole MALDI-ToF mass spectra contain valuable information. However, this can only be revealed when MS is combined with multivariate techniques such as linear PLS and non-linear Kernel PLS, and we have shown that it is possible to achieve very accurate predictions of the levels of milk species adulteration. These properties have been demonstrated in analysing binary and tertiary mixtures of fresh pasteurised cows, sheep’s and goats’ milk using a simple and fast process. Despite the milk processing, which may affect protein structure, MALDI-ToF-MS was able to achieve high accuracy levels of milk adulteration with typical errors in the region of 2–10% for cow’s milk, a level at which a fraudster would unlikely adulterate at because this would not be financially viable.

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References