MODELLING THE CATABOLITE AND MICROBIOLOGICAL PROFILE OF CHEDDAR CHEESE MANUFACTURED FROM AYRSHIRE MILK

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at the

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Promoter: Prof. P. Venter
Co-promoter: Prof. J.F.R. Lues

BLOEMFONTEIN
May 2010

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, TANIA VENTER, identity number [redacted] and student number 20492006, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree DOCTOR TECHNOLOGIAE: ENVIRONMENTAL HEALTH is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT **DATE**

Acknowledgements

I wish to express my gratitude and appreciation to the following people for their contributions to the successful completion of this study:

Firstly to the **CREATOR OF ALL THINGS**, from whom I received good health and the necessary skills to conduct the research;

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To my co-promoter, **Prof. J.F.R. Lues**, for his advice and assistance throughout this project;

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Summary

Branded dairy products have lately become a global trend. As a result of this, the origin of the milk used in the manufacturing of branded cheeses must be declared by the producer, since it is known that these products are highly adulterated with foreign milk. In South Africa, branded Ayrshire Cheddar cheese has become highly popular due to its unique organoleptic properties and in light of claims that it ripens much faster than cheese made from other milk (not including Ayrshire).

This study was therefore directed to investigate the unique properties of branded Ayrshire Cheddar cheese versus Cheddar cheese manufactured from a mixture of other breeds' milk (not including Ayrshire milk) and to establish a catabolite profile for each cheese type. The outlay of the thesis was constructed into six chapters each with its own outcomes. The first chapter focused on the variations between the two Cheddar cheese batches (produced from Ayrshire and other breeds' milk) with regards to organic acid, selected chemical parameters and starter microbiotia. In the following three chapters mathematical models were developed that would predict organic-; fatty and amino acid fluxtuations respectively in the cheese made from Ayrshire and other milk. In the last chapter two artificial neural networks were designed with the two starter organisms, *Lactococcus lactis* and *Streptococcus thermophilus* as variable indicator respectively.

Thirty-two cheese samples of each batch (pure Ayrshire (4) / mix breed with no Ayrshire (4)) were ripened and samples were analysed under the same conditions on the following days after production: 2, 10, 22, 36, 50, 64, 78, and 92. In the subsequent chapters, the following analysis were done on each day of analysis: organic acid by means of high performance liquid chromatography (HPLC); fatty acids by means of Gas Chromatography Mass Spectometry (GC-MS); amino acids by means of GC-MS; microbial analysis by means of traditional

methods, total DNA extraction and polymerase chain reaction (PCR); and standard chemical analysis for moisture, NaCl and pH.

In the first research chapter, the minimum and maximum (min/max) values, standard deviations and proposed X_{rel} values of organic acids were evaluated in Ayrshire and the mixed-breed Cheddar cheese, and showed that isovaleric acid is the organic acid with the least variation relative to concentration in both cheeses and it was assumed that this organic acid is the most effective indicator of cheese uniformity. Clear differences in organic acids, chemical variables and starter micro-organisms were also evident in the two cheese batches.

Results obtained from the regression models which was defined for each organic -; amino - and fatty acid by means of mathematical equations can be used by the manufacturer to achieve i.e. the selection of cheese for specialist lines, the early exclusion of defective cheeses, and the establishment of brand origin (Ayrshire vs. mixed-breed Cheddar cheeses). The regression graphs also illustrate unique flux patterns in Ayrshire and the mixed-breed in terms of organic -, fatty -, and amino acid content.

In the last chapter, the discrimination between the two batches was respectively done via artificial neural network (ANN) modelling of *Lactococcus lactis* and *Streptococcus thermophilus* as indicator organisms. The ANN consisted of a multilayered network with supervised training arranged into an ordered hierarchy of layers, in which connections were allowed only between nodes in immediately adjacent layers. The construction thereof allowed for two output nodes, connected to an input layer consisting of two nodes to which the inputs were connected. In both cheeses the results from the ANN showed acceptable classification of the cheeses based on the counts of *L. lactis* and *S. thermophilus*.

Contents

Chapter 1

Introduction

1.1. General background

The well-known biochemical pathways, i.e. glycolysis, proteolysis and lipolysis, have been shown to directly influence flavour (*geusi*) and aroma (*evodia*) development in Cheddar cheese (Marilley & Casey, 2004). During the ripening process these pathways mediate the catabolism of sugars, proteins and lipids respectively to yield aromatic and flavour compounds that include organic acids, esters, aldehydes, alcohols, peptides, and aromatic rings. These compounds, when ingested together with casein, lipids, water, salt, and the other compounds that make up Cheddar cheese (Table 1), generate the sharp, tangy flavour that is characteristic of Cheddar cheese. In addition, the bacterial population introduced or autochthonous to the milk prior to cheese formation contributes significantly to the aroma and flavour of the end product, as these organisms facilitate proteolysis and lipolysis (Fox, Sweeney & Lynch, 1998; Lane & Fox, 1996; Peterson & Marshall, 1990). The population composition and metabolic ability of these starter cultures further contribute to the mentioned sensory characteristics of the cheese. The precursors / substrates for the mentioned catabolic pathways of the proteins (i.e. casein and whey proteins), lipids (triglycerides, diglycerides, monoglycerides, phospholipids, carotenoids, sterols and vitamins A, D, E, and K) and sugars (lactose) are all constituents of the raw product, milk (Bylund, 1995), which is fermented into cheese (Fig. 1). The milk composition, in turn, depends on the breed of cow, the physiological state of the animal, and the fodder it consumes (Drackley, Beaulieu & Elliott, 2001; Green & Grandison, 1987).

1.2. South African dairy breeds

The following dairy breeds are popular in South Africa due to the type of milk (high in protein and fat) produced, as well as their adaptability to the South African climate:

1.2.1. Ayrshire

The Ayrshire breed had its origins in the 1800s in the county of Ayr in Scotland. These animals generally produce milk with an average butterfat content

of 4.1%, a protein content of 3.6% and a lactose content of 4.7% (Jensen, 1995). In terms of consistency, the fat globules in Ayrshire milk are much smaller in size compared to the milk of other breeds, and they are widely dispersed throughout the milk. This serves to create greater surface tension, which facilitates enzyme attachment and reactions that subsequently render this milk more digestible by humans than any other type of milk (Ayrshire Breeders' Association of Brandon, Vermont, 1958).

Table 1. Composition of Cheddar cheese

Ingredient	Function of ingredient in cheese-making	Reference
Raw milk		
- Lactose and other sugars	Substrate for starter bacteria in curd formation	Bylund (1995)
- Proteins, of which casein is the main source, and other whey proteins	Catabolic compounds yield flavour compounds, i.e. aromatic rings and peptides	Marilley & Casey (2004)
- Butterfat (*TAGS, MAGS, DAGS, FFAS, sterols, *PL; carotenoids; vitamins A, D, E and K)	Catabolic compounds yield flavour compounds, i.e. secondary alcohols, methyl ketones and esters	Marilley & Casey (2004)
- Water	Medium for reactions to take place	
Additives		
- Rennet	Coagulation of casein	Bylund (1995)
- Calcium chloride	To strengthen the coagulum	Bylund (1995)
- Starter cultures	To produce acid – lowering the pH, assisting syneresis; breaking down protein	Bylund (1995)
- Annatto	Colourant (optional)	Bylund (1995)
- Saltpetre ($NaNO_3$ / KNO_3) (optional)	To counteract butyric-acid bacteria (*Clostridia*) and/or *Coliform* bacteria	Bylund (1995)
- Sodium chloride (salt)	Osmotic effect / Condiment / To retard bacterial processes	Bylund (1995)

*TAGS = Triglycerides; MAGS = Monoglycerides; DAGS = Diglycerides; FFAS = Free fatty acids; PL = Phospholipids

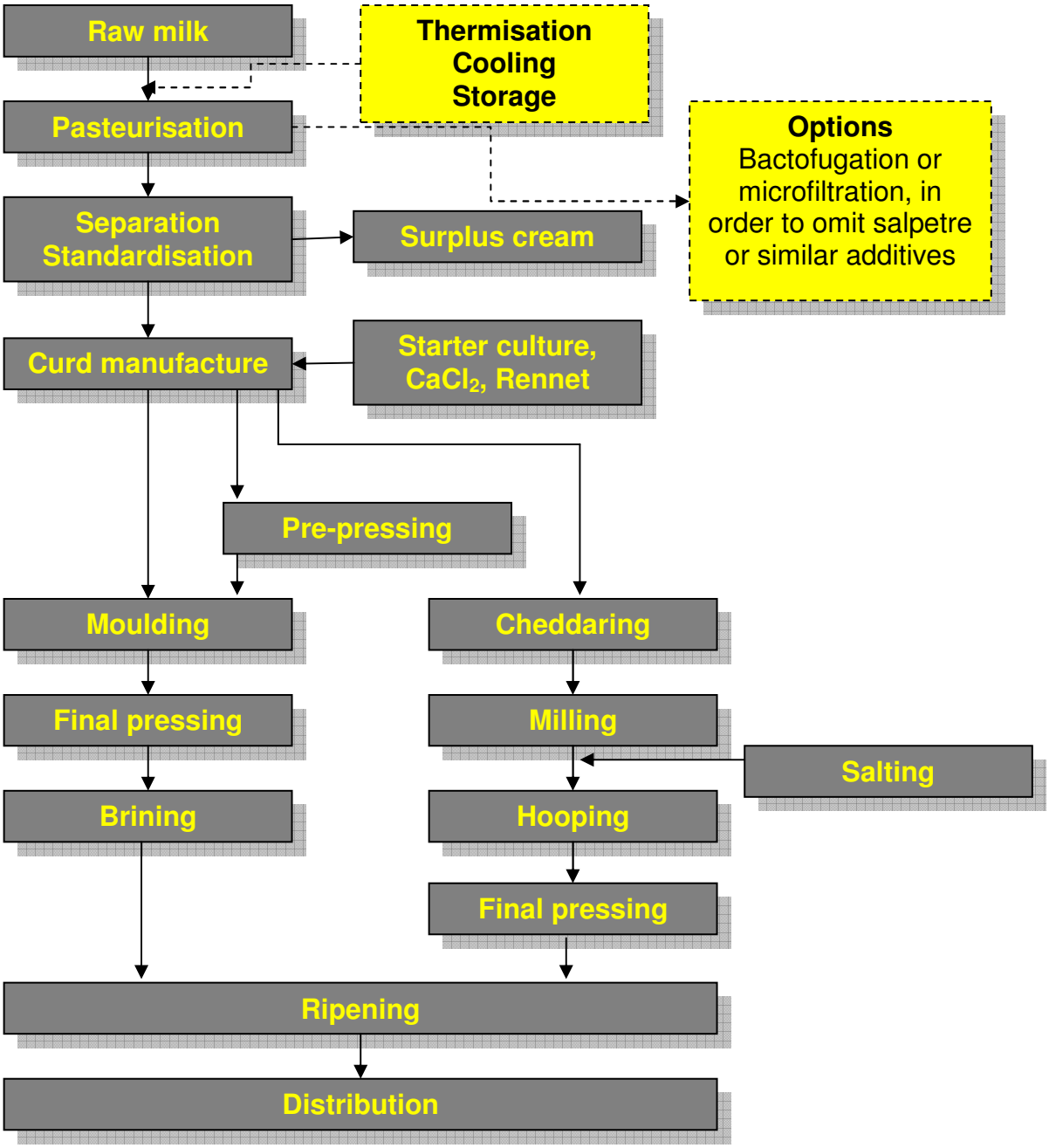


Figure 1. Process flow in the production of hard and semi-hard cheese (adapted from Bylund, 1995).

1.2.2. Brown Swiss

The native home of the Brown Swiss breed is the Alps of Switzerland (Brown Swiss Cattle Breeders' Association of America, 2005). This breed is adaptable to harsh climate conditions (cold and heat), as they were bred under

such conditions. The milk of the Brown Swiss has an average 4% butterfat and 3.5% protein content (Absolute Astronomy, 2005).

1.2.3. Guernsey

Guernsey cattle originated in 1831 on the Isle of Guernsey, which lies in the English Channel off the coast of France (American Guernsey Association, 2005). Guernsey milk has a high percentage of protein (3.59%) and butterfat (4.81%), which enhances its economic value to processors of manufactured dairy products like cheese, butter and ice-cream (WGCF, 2005).

1.2.4. Holstein

The Holstein breed originated in Europe, with the major historical development occurring in what is now known as the Netherlands, and more specifically in the two northern provinces of North Holland and Friesland (Holstein Association, 2005). Compared to Ayrshire milk, the average butterfat content (3.7%) and protein content (3.1%) are lower, but with a higher lactose content (4.9%) (Stallings, 1998).

1.2.5. Jersey

The Jersey breed, which is one of the oldest dairy breeds, originated on the small British island of Jersey in the English Channel off the coast of France (American Jersey Cattle Association, 2004). Jersey milk is very high in fat (5.1%) and protein (3.7%), with an average lactose content of 5.0% (Jensen, 1995).

1.3. Cheddar cheese

Cheese is the generic name for a group of fermented milk-based food products (Fox, 1987) that contains about 30-50% moisture (medium-moisture food product). The water activity of cheese varies from 0.98 to 0.87 and is highly correlated with the total nitrogen and ash content (mainly NaCl) in the cheese (Singh, Drake & Cadwallader, 2003).

1.3.1 Manufacturing process

In general, cheese-making involves a number of steps, which are common to most types of cheese. The main steps in the production of hard/semi-hard cheeses, such as Cheddar cheese, are outlined in Figure 1. In short, the milk intended for the manufacturing of cheese is pre-treated (pasteurised and then cooled to 32°C), followed by the addition of starter bacterial cultures, calcium chloride and sodium nitrate, and is subsequently mixed with rennet enzyme. The rennet causes the milk to coagulate into a solid gel known as the coagulum. The latter is cut into small blocks of a desired size using special cutting tools, in order to facilitate the expulsion of whey (the liquid residue of cheese production, which comprises 80-90% of the total milk volume and contains soluble proteins, lactose, vitamins and minerals). The mechanical treatment of the stirring tools, the heat treatment and the growth of starter bacteria further facilitate whey expulsion. The finished curd is placed into cheese moulds and pressed (Bylund, 1995), followed by a ripening period lasting anywhere from four months to two years, depending on the flavour profile required (Singh *et al.*, 2003).

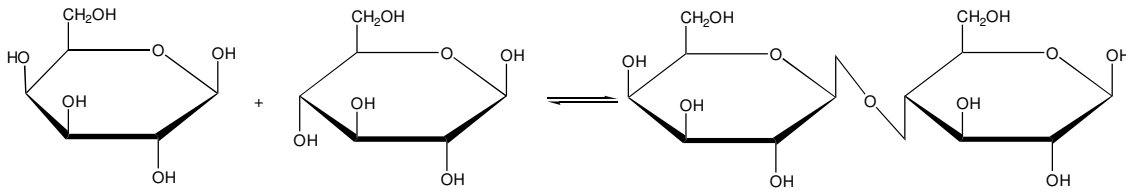
1.3.2 Composition of Cheddar cheese

The main ingredient of cheese is cow's milk, which consists mainly of lipids (hereafter referred to as butterfat), protein (casein), and sugars (lactose and citrate). The primary degradation pathways of the aforementioned milk constituents in cheese curd are referred to as lipolysis (butterfat breakdown), proteolysis (casein breakdown), and glycolysis (lactose breakdown). In the production process of Cheddar cheese, rennet, starter cultures, calcium chloride, sodium nitrate and sodium chloride are added according to the hard- and semi-hard cheese production protocol (Singh *et al.*, 2003). The functions of these ingredients are illustrated in Table 1.

A. Lactose

A.1. Nomenclature

The main sugar present in milk is lactose (Fig. 2). Lactose consists of a glucose molecule and a galactose molecule linked together with a glycosidic bond (Mathews & Van Holde, 1990).



β-D-Galactose β-D-Glucose Lactose

Figure 2. Chemical structure of lactose

A.2. Catabolic pathway of lactose

In cheese-making, the breakdown of lactose occurs through three catabolic pathways (Fig. 3). When lactose is broken down into glucose and galactose, the glucose molecule is further oxidised to pyruvate by the Embden-Meyerhof pathway of glycolysis (Kandler, 1983; McKee & McKee, 1999). Starter cultures that contain galactose-positive starter bacteria and leuconostoc spp. can convert galactose through the Leloir pathway to glucose-6-P (Axelsson, 1993; Kandler, 1983). Lactococci (a bacterial strain present in the starter culture mix for cheese production) in turn has the ability to convert the galactose to glyceraldehyde-3-P through the tagatose pathway (Marilley & Casey, 2004). Subsequently, glyceraldehyde-3-P can be converted to pyruvate, which in turn can be converted to short-chain flavour compounds, i.e. acetaldehyde, diacetyl, acetate, ethanol and acetoin (Fig. 3) (Marilley & Casey, 2004).

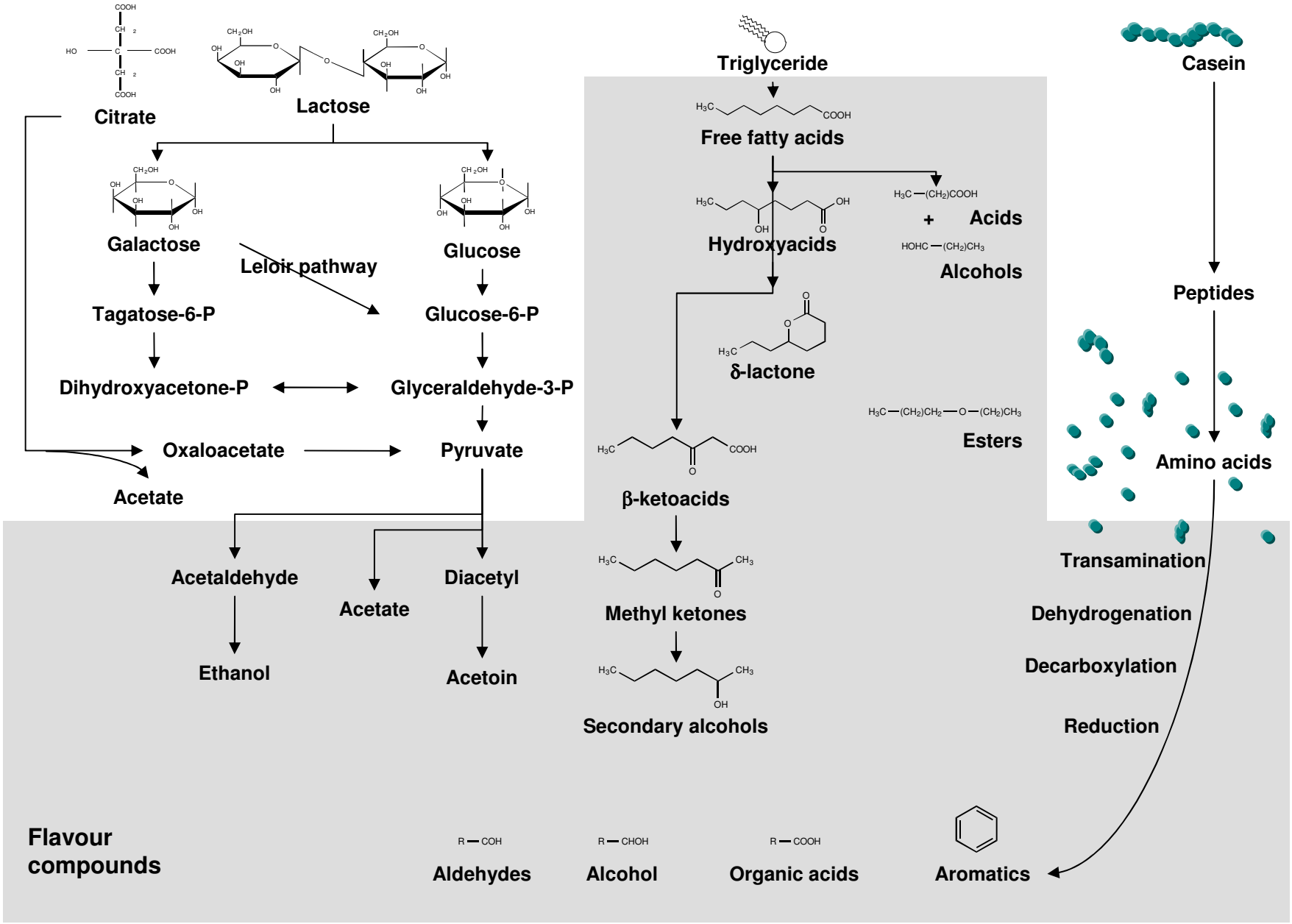


Figure 3. Biochemical pathways leading to the formation of flavour compounds in cheese. The grey surface indicates surface compounds with a flavour note. (Adapted from Marilley & Casey, 2004).

B. Butterfat

B.1. Nomenclature

Triglycerides make up 98% of the butterfat in cheese, with monoglycerides, diglycerides, phospholipids, free fatty acids (FFAs), sterols, carotenoids and vitamins (A, D, E and K) also present, but in smaller quantities (Bylund, 1995). A triglyceride consists of three fatty acids, which are esterified to a glycerol backbone at the SN 1, 2 and 3 positions (Fig. 4). A fatty acid is a carbon chain, which contains a methyl group on the one end and a carboxyl group on the other. They are grouped according to carbon chain length, the number, position and configuration of their double bonds, and by the presence of functional groups along the fatty acid chain (McKee & McKee, 1999).

B.2. Catabolic pathway of fats in cheese

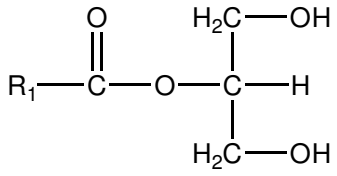
Cheddar cheese contains more than 30.5% fat, which makes lipolytic (enzymatic hydrolysis by lipases and esterases) and oxidative changes likely to occur (Collins, McSweeney & Wilkinson, 2003; Singh *et al.*, 2003). The initial catabolysis of triglycerides is a process called hydrolysis that results in free fatty acids and residual glycerol (Collins *et al.*, 2003). The free fatty acids in turn serve as precursors to three further breakdown processes as illustrated in Figure 3. FFAs, as well as the compounds produced by these pathways (β-oxidation, decarboxylation; esterification or reacting with alcohols), directly impact on the aromatic and flavonoid characteristics of the produced cheese (Marilley & Casey, 2004). β-oxidation and decarboxylation of fatty acids can yield methyl ketones and secondary alcohols (Collins *et al.*, 2003; Marilley & Casey, 2004), which give the characteristic aroma profile to blue-veined cheeses, but only make a limited contribution to the flavour profile of Cheddar cheese (Singh *et al.*, 2003). The reduced product of methyl ketones, namely secondary alcohols, does not, however, contribute to the cheese aroma.

Cheese that contains polyunsaturated and monounsaturated fatty acids can also succumb to auto-oxidation, yielding free radicals and peroxides (Kristensen, Orlien, Mortensen, Brockhoff & Skibsted, 2000). However, oxidation is limited due to the low redox potential in cheese and the presence of natural antioxidants in the cheese, which might prevent or reduce the initiation of the oxidation mechanisms (Singh *et al.*, 2003).

Another possibility for the formation of flavour compounds from fatty acids is by the esterification of hydroxyl fatty acids to form lactones, i.e. δ or γ-lactones. Lactones are cyclic esters that result from the intramolecular esterification of hydroxy acids through the release of water to form a ring (Singh *et al.*, 2003). Lactones possess a strong aroma and contribute to the overall flavour of particularly Cheddar cheese.

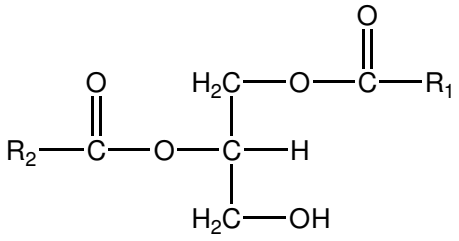
Fatty acids (mainly short - and medium-chained) can also react with alcohol groups to produce esters. The alcohol groups involved may be aliphatic (ethanol), aromatic (phenylethanol) or thiols (methanethiol). These esters play an important role in the flavour and sometimes the off-flavour of Cheddar cheese (Urbach, 1997). According to Singh *et al*. (2003), ester production results from the enzymatic reactions of lactic acid bacteria, but can sometimes also occur from a purely chemical reaction. Some examples of esters in Cheddar cheese are ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and methyl hexanoate (McSweeney, Nursten & Urbach, 1997).

Monoacylglycerol



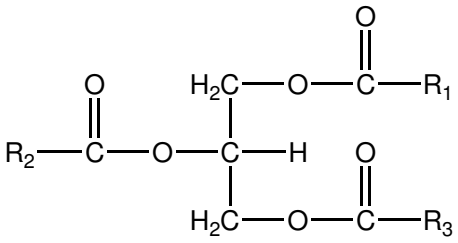
1-Acyl-*sn*-glycerol

Diacylglycerol



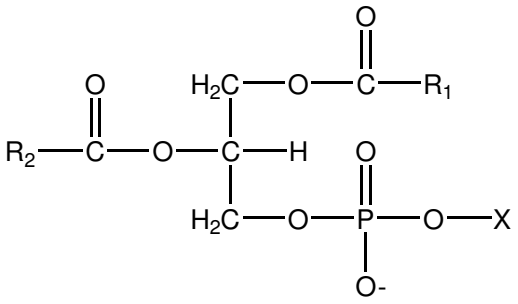
1,2-Diacyl-*sn*-glycerol

Triacylglycerol



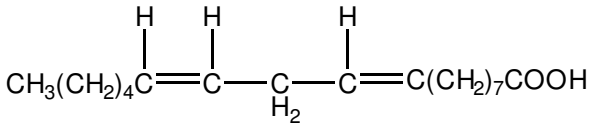
1,2,3-Triacyl-*sn*-glycerol

Phospholipid



Phosphatidic acid

Free fatty acid



Linoleic acid (C18:2)

Figure 4. Fatty acid derivatives mainly present in fats. R_1 CO-, R_2 CO-, R_3 CO- represent fatty acyl groups (Ratledge & Wilkinson, 1988). X = different ligands can be esterified at this point, i.e. hydrogen, choline, serine, etc.

The excessive production of FFA breakdown (hydrolysis and the action of lipases) products during the manufacturing of cheese directly influences the flavour profile of the end product (Akin, Aydemir, Koçak & Yildiz, 2003). According to Bills and Day (1964), the lipolysis of milk triglycerides releases a high concentration of medium- and short-chain fatty acids, which promote the flavour development in cheese. Although lipolysis of fatty acids contributes considerably to the flavour profile of the cheese, it was reported by the latter researchers that intensive lipolysis can cause rancid flavours. Wilkes, Conte, Kim, Holcomb, Sutherland and Miller (2000), on the contrary, showed that short-chain fatty acids only account for 10% of the total fatty acids in milk and contribute less to the off-flavour per mole than the medium- and longer-chain fatty acids. In addition to the mentioned

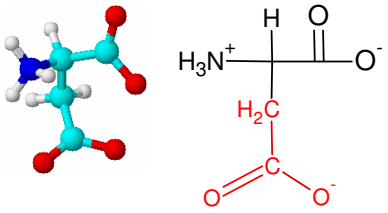
catabolic pathways of lipids in cheese, oxidation of triglycerides could also occur, yielding undesirable flavours. This process is, however, also limited due to the low redox potential of cheese (Marilley & Casey, 2004).

C. Protein

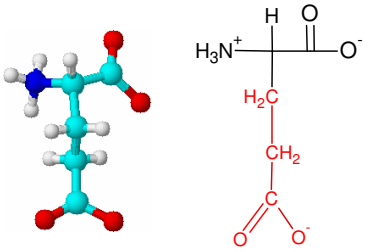
C.1. Nomenclature

Proteins (or polypeptides) are polymers (more than 50 amino acids) of the 20 standard amino acids (Fig. 5). Smaller molecules (fewer than 50 amino acids) are referred to as peptides (McKee & McKee, 1999). The bond between amino acids is called a peptide bond, which is formed when the unshared electron of the α-amino nitrogen atom of one amino acid attacks the α-carboxyl carbon of another in a nucleophilic acyl substitution reaction (Fig. 6(a)). A water molecule is expelled during this reaction (Fig. 6(b)) (McKee & McKee, 1999).

The process of protein breakdown is called proteolyses and it contributes directly to the aroma and flavour of the cheese. Casein is the major protein in cow's milk and constitutes about 80% of the total protein content of milk (Bylund, 1995). The other 20% consists of whey and serum proteins. Caseins can be grouped into α_{S1}-, α_{S2} -, β- and κ-casein (Forde & Fitzgerald, 2000).

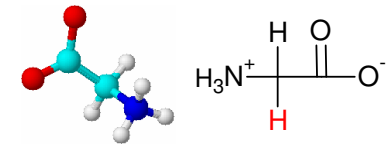


Aspartate

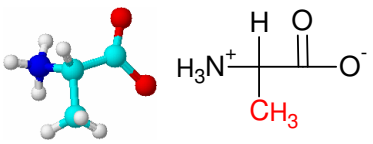


Glutamate

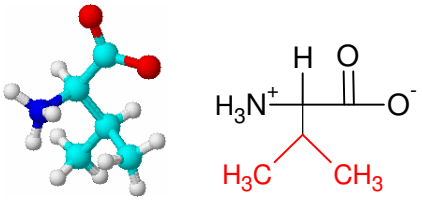
Figure 5(a). Acidic amino acids



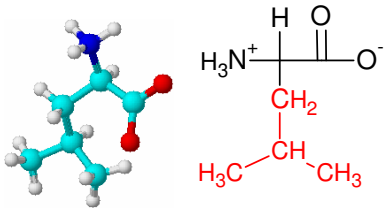
Glycine



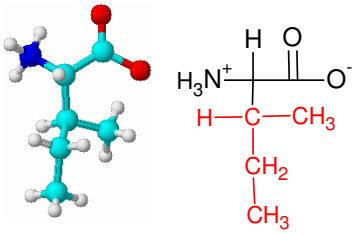
Alanine



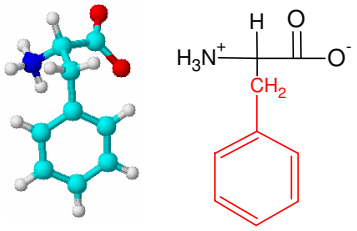
Valine



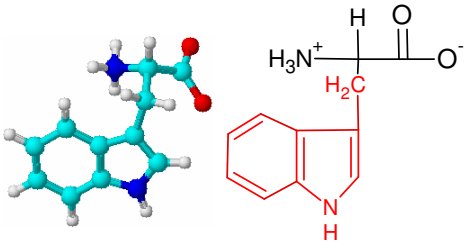
Leucine



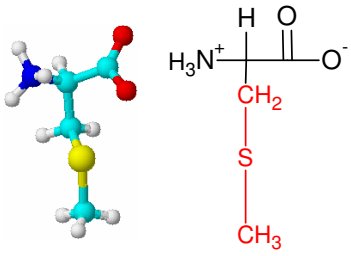
Isoleucine



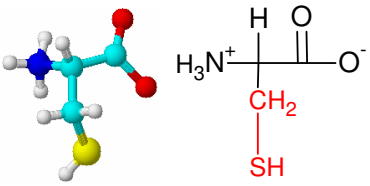
Phenylalanine



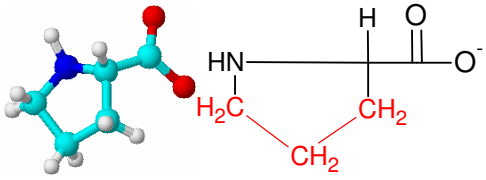
Tryptophan



Methionine

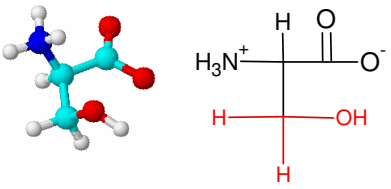


Cysteine

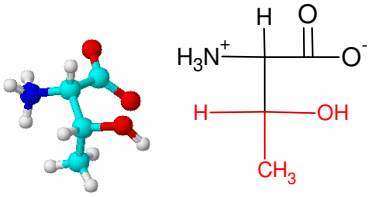


Proline

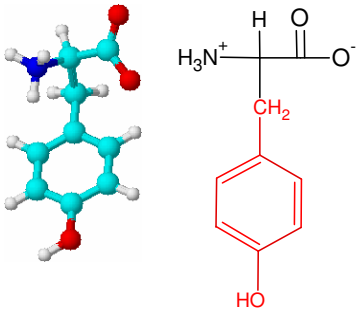
Figure 5 (b). Neutral non-polar amino acids



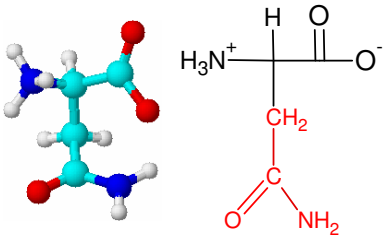
Serine



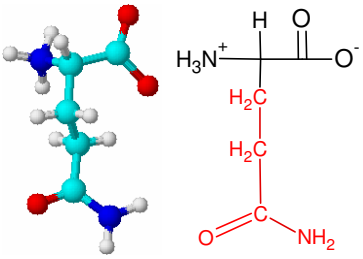
Threonine



Tyrosine

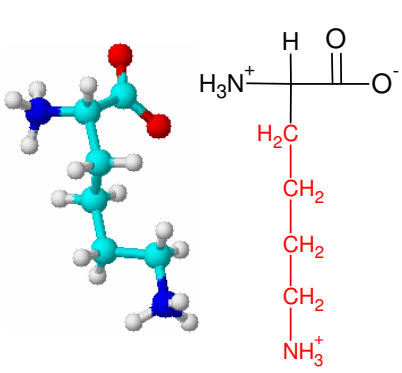


Asparagine

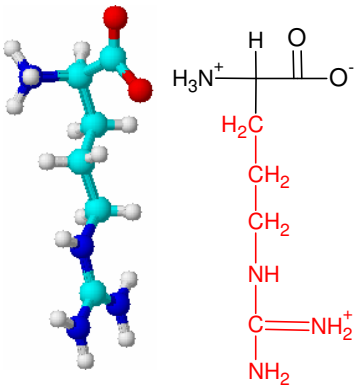


Glutamine

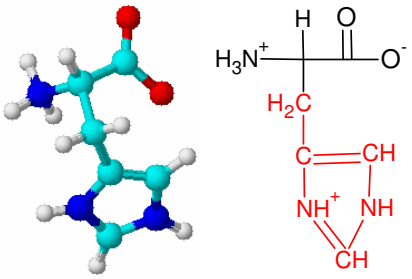
Figure 5(c). Neutral polar amino acids



Lysine



Arginine



Histidine

Figure 5(d). Basic amino acids

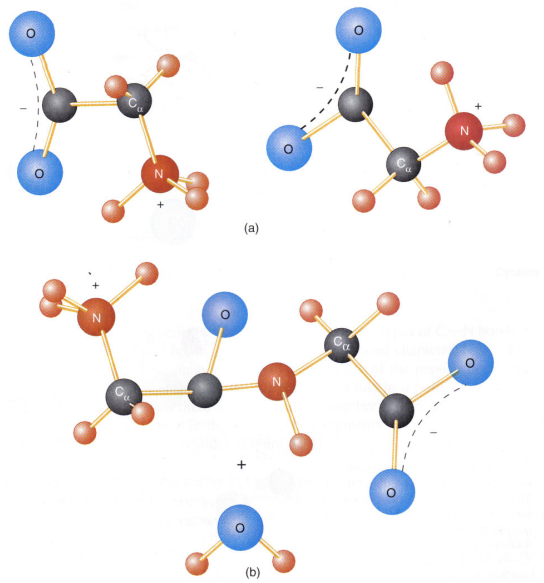


Figure 6. Formation of a protein molecule (dipeptide). (a) The α-carboxyl group of one amino acid reacts with the amino group of another amino acid. (b) A water molecule is lost in the reaction (McKee & McKee, 1999).

C.2. Proteolysis of milk proteins during cheese production

During the cheese-making process, the breakdown of caseins occurs through the combined action of proteolytic enzymes (Singh *et al.*, 2003). The origin of these enzymes is outlined in Table 2.

Table 2. Origin of proteolytic enzymes

Coagulant	**Indigenous milk enzymes**	**Starter and non-starter bacterial enzymes**
(A) Chymosin (genetically engineered)	(C) Plasmin	Cell envelope-associated proteinases (CEP)
(B) Chymosin/pepsin (from calf stomach)	(D) Cathepsin	Peptidases
		(E) Endopeptidases
		(F) Aminopeptidases
		(G) Di- & tripeptidases
		(H) Proline-specific peptidases

The coagulants (A and B) and indigenous milk enzymes (C and D) are active in most ripened cheeses (Singh *et al.*, 2003). The coagulant is responsible for the initial hydrolysis of mainly α_{s1}-caseins (Lane, Fox, Johnston & McSweeney, 1997) and is to a limited extent restricted to β-casein, but not to α_{s2}-casein. At the initial stages of cheese-ripening, the indigenous milk proteinase (C and D) does not play an indispensable part in proteolysis. The production of amino acids and small peptides at this stage is primarily due to the activity of the starter bacterial enzymes (E, F, G and H) (Lane *et al.*, 1997). Of the indigenous milk proteinase, plasmin (C) is to a limited extent responsible for the proteolysis of β-casein in Cheddar, but it is more significantly active in cooked cheeses, i.e. Swiss cheese, where chymosin (A), on the other hand, is completely inactive. The acid protease group (chymosin and pepsin (B)) successfully clots the milk by splitting the κ-casein at the junction

between the para-κ-casein and macropeptide moieties – in bovine milk, that is the bond between the phenylalanine residue 105 and methionine residue 106 (Dalgleish, 1987; Forde & Fitzgerald, 2000). Once sufficient κ-casein has been split, the macropeptide can diffuse off into the serum, as its stabilising influence is now lost, and the micelles then start to coagulate. Chymosin (A) also plays a significant role in the first stages of the maturation process. It breaks down the α_{s1}-casein and this degradation has been shown to directly affect the textural properties of cheese (Forde & Fitzgerald, 2000).

During the manufacturing of Cheddar, the drainage of whey (sineresis) influences the cheese mineral content, the proportion of residual chymosin in the cheese, the final pH and the moisture-to-casein ratio, and is therefore a key stage in the manufacturing process (Walstra, Van Dijk & Geurts, 1987). Chymosin is optimally active at low pH and is responsible for the increased proteolysis in low-pH cheeses (Creamer, Lawrence & Gilles, 1985). Bitterness in the cheese is the result of excessive proteolysis, but can be counteracted with an increase in salt concentration (Guinee & Fox, 1987; Mistry & Kasperson, 1998; Thomas & Pearce, 1981).

C.3. Proteolysis in Cheddar cheese during ripening

The breakdown of casein into small peptides is required for the development of Cheddar cheese flavour (Forde & Fitzgerald, 2000; Lee, Lo & Warthesen, 1996; Singh *et al.*, 2003). During the initial stages of ripening, the residual chymosin hydrolyses α_{s1}-casein at the bond Phe^{23}-Phe^{24} and Phe^{24}-Val^{25}. The hydrolysis of bond Phe^{23}-Phe^{24} results in the formation of a large α_{s1}-CN f24-199 [called α_{s1}-I casein], and small α_{s1}-CN f1-23 peptides (Guinee & Fox, 1987; Singh *et al.*, 2003). According to Lawrence, Creamer and Gilles (1987), the hydrolysis of this single bond of α_{s1}-casein causes the rubbery texture of Cheddar curd to change rapidly into a smoother, more homogeneous product. In certain cases, casein-derived peptides can taste bitter and contribute to off-flavour. Peptides containing a large

volume of hydrophobic amino acids, i.e. leucine, phenylalanine and praline, are well known to cause a bitter taste in cheese (Forde & Fitzgerald, 2000).

On the basis of the cleavage position in the substrate peptide, peptides can be classified into two groups, i.e. endopeptidases and exopeptidases. As the name suggests, endopeptidases cleave susceptible bonds within the peptide chain, while exopeptidases hydrolyse amino acids from the amino- and carboxy-terminal ends of the peptide. The latter prevent extensive protein hydrolysis and subsequent bitterness (Forde & Fitzgerald, 2000).

D. Amino acid metabolism

Amino acids are the building blocks of proteins. They are characterised by the presence of a carboxyl group (COOH) and an amino group (NH_2) attached to the same carbon at the end of the compound (McKee & McKee, 1999). The formation of amino acids and small peptides in Cheddar cheese results from the actions of the micro-organisms applied as starter cultures in the manufacturing of cheese. The major micro-organisms used in the manufacturing of Cheddar cheese are *Lactococcus lactis* spp. *lactis*, *Lactococcus lactis* spp. *Cremoris*, and *Streptococcus thermophilus*. Of these micro-organisms, *Lactococcus lactis* spp. are primarily responsible for the production of small peptides and free amino acids (O'Keeffe, Fox & Daly, 1978).

According to Singh *et al*. (2003), the first degradation step of amino acids by lactococci is transamination, which leads to the formation of α-keto acids (Fig. 7). *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. c*remoris* have been shown to have aromatic aminotransferase enzymes that initiate the degradation of Ile, Leu, Met, Phe, Trp, Tyr and Val – all of which are precursors of cheese flavour compounds (Table 3). It has been reported that α-keto acids correspond to almost every amino acid in Cheddar cheese (Singh *et al*., 2003) and that α-keto-3-methyl butyric acid and α-keto-3-methyl valeric acid have an intense cheese-like odour.

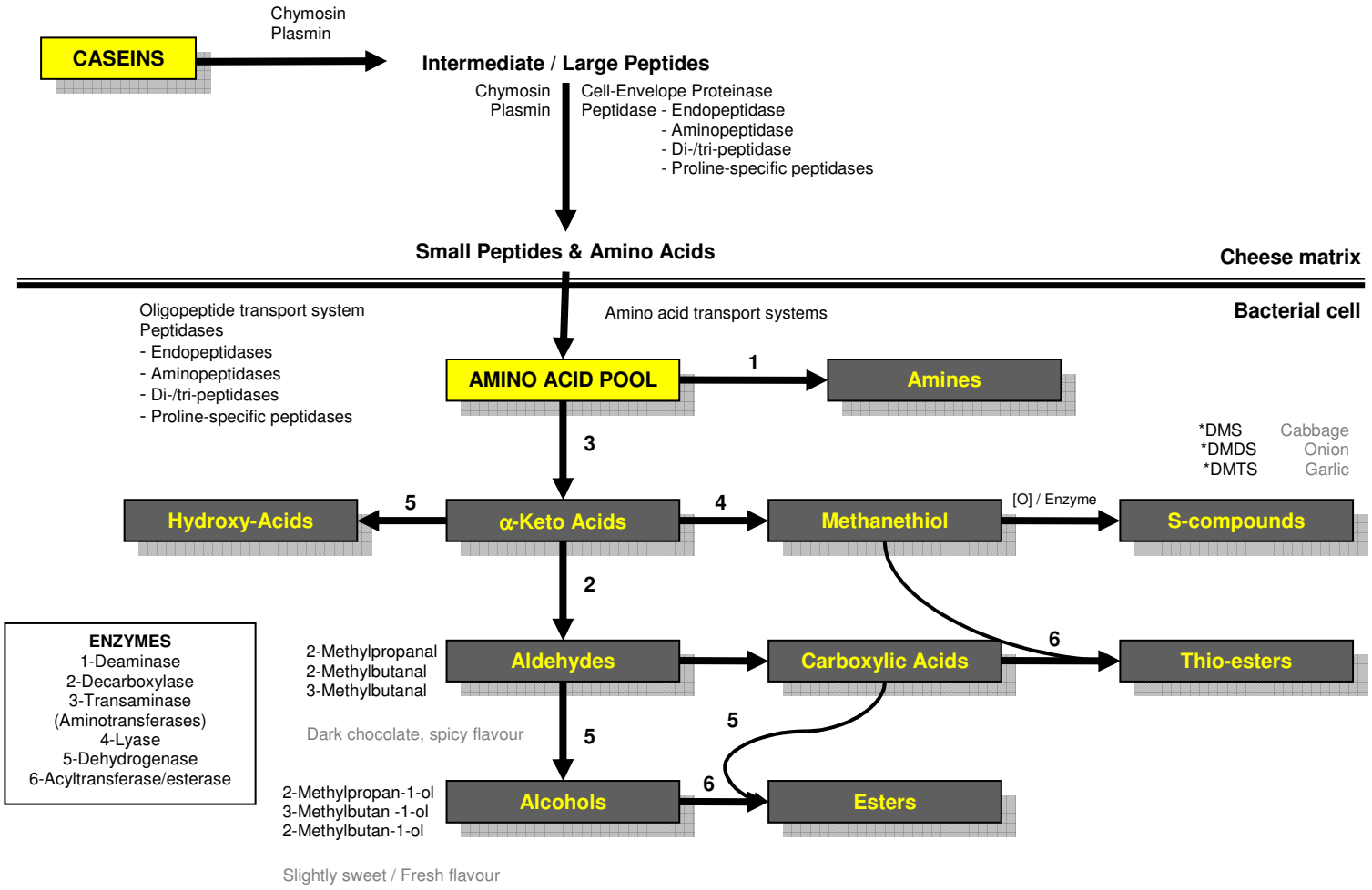


Figure 7. Generation of flavour compounds from milk protein degradation. *DMS – dimethyl sulphide; *DMDS – dimethyl disulphide; *DMTS – dimethyl trisulphide (adapted from Singh *et al.*, 2003).

Table 3. Amino acid catabolites formed by lactic acid bacteria isolated from Cheddar cheese (adapted from Singh *et al.* 2003)

Catabolic product	Precursor	Aroma note
2-Methyl propanoic acid	Valine	rancid butter, sweaty, sweet, apple-like
2-Methyl-1-propanol	Valine	penetrating, alcohol, wine-like
2-Methyl propanal	Valine	malt
3-Methyl butanoic acid	Leucine	cheesy, sweaty, old socks, rancid, faecal, rotten fruit
3-Methyl-1-butanol	Leucine	fruity, alcohol, solvent-like, grainy
3-Methyl butanal	Leucine	dark chocolate, malt
2-Methyl butanoic acid	Isoleucine	fruity, waxy, sweaty-fatty acid
2-Methyl-1-butanol	Isoleucine	—
2-Methyl butanal	Isoleucine	dark chocolate, malt
3-(Methylthio) propanal	Methionine	cooked/boiled potato
3-(Methylthio) propanol	Methionine	cooked/boiled potato
Methanethiol	Methionine / cysteine	cabbage, boiled cabbage, sulphurous
Methyl sulphide	S-containing	cabbage, sulphurous
Dimethyl disulphide	S-containing	onion
Dimethyl trisulphide	S-containing	garlic
Dimethyl tetrasulphide	S-containing	cabbage
Acetophenone	Phenylalanine	almond, musty, glue
Benzaldehyde	Phenylalanine	almond, bitter almond
Phenyl acetaldehyde	Phenylalanine	rosy, violet-like
Phenylethyl alcohol	Phenylalanine	unclean, rose, violet-like, honey
Phenyl acetic acid	Phenylalanine	flowery, rosy, plastic
Phenol	Tyrosine	medicinal
p-OH-phenyl aldehyde	Tyrosine	—
p-OH-phenyl lactate	Tyrosine	—
p-OH-phenyl acetate	Tyrosine	—
p-Cresol	Tyrosine	unclean, medicinal
Indole	Tryptophan	unclean, mothball
Skatole	Tryptophan	unclean, mothball
Benzaldehyde	Tryptophan	almond

Methanethiol is a compound that gives a desirable Cheddar-type sulphur note to cheese and is normally associated with good-quality Cheddar cheese (Gao, Mooberry & Stelle, 1998). This compound is produced from the amino acid methionine (Met), by *Lactococci* via two enzymatic pathways (Singh *et al*., 2003). The first pathway for Met catabolism is via α,γ elimination, where a lyase simultaneously catalyses deamination and demethylation of Met and results in the formation of methanetiol and α-keto butyric acid (Alting, Engels, Van Schalkwijk &

Exterkate, 1995). The second pathway is initiated by transamination of Met to 4-methylthio-2-oxobutyric acid (KMBA). Gao *et al*. (1998) also showed that Met catabolism in lactococci is initiated by aminotransferase and is responsible for the formation of volatile sulphur compounds.

As already stated, amino acid degradation plays a vital role in flavour development in Cheddar cheese. Wallace and Fox (1997) studied the direct addition of amino acids to enhance the amino acid content in Cheddar cheese and the genetic modification of lactococci with increased aminopeptidase N activity. However, the increase in amino acids did not affect the flavour development of the cheese. Yvon, Berthelot and Gripon (1998), on the other hand, hypothesised that the rate-limiting factor in flavour biogenesis is not the release of amino acids, but their subsequent conversion to aroma compounds.

1.3.3. Starter bacteria in cheese-making

The starter bacteria applied in the manufacturing of Cheddar cheese are usually strains *of Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* spp. *lactis* and *Streptococcus thermophilus* (Garde, Babin, Gaya, Nuñez & Medina, 1999; Lane & Fox, 1996; Lick, Keller, Bockelmann & Heller, 1996; Nomura, Kobayashi & Okamoto, 2002). The primary role of starter bacteria is to acidify the milk at an appropriate rate during curd preparation. It also contributes to proteolysis during ripening (Lane & Fox, 1996). Although these lactic acid bacteria (LAB) play a significant role in the flavour of cheese (Giraffa & Rossetti, 2004; Randazzo, Torriani, Akkermans, De Vos & Vaughan, 2002), non-starter lactic acid bacteria (NSLAB) can dominate the cheese microbiota (Fox *et al*., 1998; Peterson & Marshall, 1990; Swearingen, O'Sullivan & Warthesen, 2001). These NSLAB are mainly composed of mesophilic lactobacilli, but pediococci and enterococci have also been found (Hynes, Ogier & Delacroix-Buchet, 2001).

Spores of the *Clostridium* species originating from silage feed could also contaminate the raw milk and cause severe quality problems in cheese, which in turn will have a direct economic impact on the producer. *Clostridium tyrobutyricum* and *C. sporogenes* have the ability to ferment lactic acid, whereas *C. butyricum* produce high concentrations of butyric acid. All these fermented products can

result in late gas blowing in cheese (Le Bourhis, Saunier, Doré, Carlier, Chamba, Popoff & Tholozan, 2005).

1.3.4. Volatiles in Cheddar cheese

The aroma components in Cheddar cheese are generally complex, covering the range of polarity, pH, functional group, solubility, vapour pressure, and volatiles. Beside these complexities, there are certain intrinsic/extrinsic parameters such as oxygen, light, heat and/or pH, and the complexity of the food matrix with which the aroma compounds interact, which must be taken into account when preparing flavour extracts (Singh *et al*., 2003). Most aroma and flavour compounds in cheese are volatile, and procedures for their isolation from cheese have been properly established (Dirinck & De Winne, 1999; Frank, Owen & Patterson, 2004; Jaillais, Bertrand & Auger, 1999).

According to Singh *et al*. (2003), the volatile fraction of cheese contains several sulphur compounds, all of which contribute to the aroma of the cheese, i.e. methanethiol, methional, dimethyl sulphide, dimethyl disulphide, dimethyl trisulphide, carbonyl sulphide and hydrogen sulphide. Besides sulphur-containing compounds, butanoic acid, alkyl-pyrazines, phenolic compounds and benzene derivatives are also present in most Cheddar cheeses and play an essential role in the formation of the basic cheese aroma (Frank *et al*., 2004). In addition, several other volatile compounds have also been identified in Cheddar cheese and are summarised in Table 4 (Singh *et al*., 2003). Table 5 illustrates the relative intensities of these volatile compounds in nine different cheeses, as evaluated by Frank *et al*. (2004).

Table 4. Volatile compounds identified in Cheddar cheese (adapted from Singh *et al*., 2003)

Chemical	Aroma note	Chemical	Aroma note
Acetaldehyde	Sweet, pungent	Indole	Mothball
Acetic acid	Vinegar	Isobutanol	-
Acetone	-	Isohexanal	-
Acetophenone	Almond, musty, glue	Limonene	Citrus
β-Angelicalactone	-	Linalool	Sweet, floral, honey
Benzaldehyde	Almond	Methanethiol	Cabbage, boiled cabbage, sulphurous
Butanal	Pungent	Methyl acetate	-
n-Butanol	Floral, fragrant, fruity, sweet	Methyl propionate	-
2-Butanol	Alcoholic	Methyl hexanoate	Pineapple
2,3-Butanediol	Fruity	2-Methyl butanal	Dark chocolate, malt
2-Butanone	Etheric	3-Methyl butanal	Dark chocolate, malt
2,3-Butanedoine (Diacetyl)	Buttery	2-Methyl-1-butanol	Wine
n-Butyl acetate	Pear	3-Methyl-1-butanol	Fruity, alcohol, solvent-like, grainy
n-Butanoic acid	Sweaty, cheesy, faecal	3-Methyl-2-butanone	Camphor
2-Butyl acetate	-	3-Methylbutanoic acid (Isovaleric acid)	Swiss cheese, waxy, sweaty, old socks, faecal
n-Butyl butyrate	-	3-Methyl-2-pentanone	-
p-Cresol	Unclean, medicinal, cowy, barny	2-Methyl propanal	Malt
(E)-β-Damascenone	Apple sauce	3-(methylthio) propanal (Methional)	Baked/boiled potato
Decanal	Soapy, flowery	Nonanal	Green
n-Decanoic acid	Rancid	(E)-2-Nonenal	Green, fatty
(E,E)-2,3-Decadienal	Mayonnaise, bread, fatty, tallow, fruity	(Z)-2-Nonenal	Green
(E,Z)-2,4-Decadienal	Mayonnaise, bread, fatty, tallow, fruity	(E,Z)-2,6-Nonadienal	Melon, cucumber
Dimethyl sulphide	Cabbage, sulphurous	(E,E)-2,4-Nonadienal	Soapy
Dimethyl disulphide	Onion, sulphurous	2-Nonanone	Green, earthy, blue cheese, fatty, fruity, musty, varnish
Dimethyl trisulphide	Cabbage, garlic, sulphurous	(Z)-1,5-Octadien-3-one	Green, metallic
Dimethyl tatrasulphide	Putrid, cabbage, sulphurous	δ-Octalactone	Fruity, peach, sweet
γ- Decalactone	Coconut	n-Octanoic acid	Body odour, sweaty
δ-Decalactone	Peachy, coconut	Octanal	Green, fatty, soapy, fruity, orange peel
δ-Dodecalactone	Cheese, coconut	2-Octanol	Mushroom, coconut, oil, rancid
6-(Z)-Dodecenyl-γ-lactone	Soapy	2-Octanone	Floral, soapy, ketone, musty
trans-4,5-Epoxy-2-(E)-decenal	Metallic	1-Octen-3-one	Mushroom
Ethanol	Alcohol	2,4-Pentanediol	-
2-Ethyl butanol	-	n-Pentanoic acid	Swiss cheese
Ethyl acetate	Fruity, solvent, sweet	Pentanal	Pungent, almond-like
Ethyl propionate	Fruity	2-Pentanol	Sweet, alcoholic, fruity, nutty
Ethyl butyrate	Bubble gum, fruity	Pentan-2-one	Acetone, sweet, fruity, ketone
Ethyl hexanoate	Fruity	Phenyl acetaldehyde	Rosy
Ethyl octanoate	Fruity	Phenyl acetic acid	Flowery
Furanone, 4,5-dimethyl-3-hydroxy-2(5*H*)-(Sotolon)	Curry, seasoning	2-phenyl ethanol	Rosy
Furanone, 2,5-dimethyl-4-hydroxy-3(2*H*)-(Furaneol)	Sweet, caramel, burnt sugar	α-Pinene	Pine
Furanone, 2-Ethyl-4-hydroxy-5-methyl-3(2H)-(Homofuraneol or Ethyl Furaneol)	Caramel	Propionic acid	Pungent
Geosmin	Earthy, moistened soil	n-Propanol	Pungent
Guaiacol	Smoky, spicy	Propanal	Solvent-like
Heptanal	Fatty, oily, green	Propyl acetate	-
2-Heptanone	Blue cheese, fruity, musty, soapy	n-Propyl butyrate	Pineapple
(Z)-4-Heptenal	Creamy, biscuit	Propenal	-
n-Hexanal	Green	Pyrazine, 2-acetyl	Popcorn
n-Hexanol	Fatty, floral, green	Pyrazine, 2-isobutyl-3-methoxy	Bell pepper-like, green
n-Hexanoic acid	Goat-like	Pyrazine, 2-isopropyl-3-methoxy	Earthy, soil, green, beany
2-Hexanone	Fruity, ketone	Pyrroline, 2-acetyl-1	Roasted
Hexanethiol	Sulphur	Skatole	Unclean, mothball, faecal
2-Hexenal	Almond bitter, green, fatty	Thiazoline, 2-Acetyl-2	Roasted
1-Hexen-3-one	Cooked vegetable, plastic	Thiophen-2-aldehyde	-
Hydrogen sulphide(H_2S)	Rotten eggs, cabbage	2-Tridecanone	-
3-Hydroxy-2-butanone (Acetoin)	Buttery	2-Undecanone	Floral, fruity, green, musty, tallow

Table 5. Relationship between compound and odour / aroma description (modified and adapted from Frank *et al.*, 2004).

Compound name	RI	Odour description
Methanethiol	<900	Fermented cabbage
Dimethyl sulphide	<900	Garlic-rotten
2-Butanone	<900	Sap-acetone
Methyl butanoate	<900	Fruity apple
Diacetyl	923	Buttery-sweet
Ethyl butanoate	954	Fruity-melon, sweet
Dimethyl disulphide	979	Garlic, onion
Methyl butanal	995	Grainy malty
Unidentified	1002	Peppermint/sweet
Unidentified	1007	Rubber-like
Ethyl pentanoate	1016	Melon, fruity, sweet
2-Heptanone	1128	Musty, varnish, sweet
Unidentified	1183	Fatty, sweet, hay
Ethyl hexanoate	1202	Fruity, grape melon
Methylpyrazine	1252	Nutty, grainy
Methylbuty butanoate	1253	Sweet fruity
2-Octanone	1263	Fruity, green
1-Octene-3-one	1299	Fungus, woody
2,5-Dimethyl pyrazine	1317	Nutty, roast grain
2,6-Dimethyl pyrazine	1322	Nutty, roast grain
Dimethyl trisulphide	1358	Sweet, garlic, spicy
Methyl propyl pyrazine	1361	Nutty, savoury
2-Ethyl-5-methyl pyrazine	1381	Peanut, green
2-Nonanone	1390	Floral, fruity, peachy
Trimethyl-pyrazine	1413	Nutty, musty, beans
Sesquiterpene (MW 204)	1424	Dirt, fungus
Methoxy-2-methylbenzene	1439	Raw potato, beans
Methional	1463	Roast potato
Dimethylethyl pyrazine	1480	Savoury broth
Acetic acid	1485	Vinegar sour, sharp
Tetramethyl pyrazine	1499	Raw potato, beans
2-Nonenal	1508	Fatty, cucumber
3,5-Diethyl-2-methyl pyrazine	1516	Savoury, nutty
2,3,5-Trimethyl-6-ethyl pyrazine	1517	Savoury, nutty
Sesquiterpene (MW 204)	1531	Wet wood, fungus
Unidentified	1573	Green exotic
Unidentified	1587	Burnt matches, nutty

Table 5 (*continued*)

Compound name	RI	Odour description
Sesquiterpene (MW 204)	1588	Strong fungus
Unidentified	1632	Garlic, savoury
Butanoic acid	1683	Cheesy, rotten, sharp
Unidentified	1654	Milky, custard
Isovaleric acid	1678	Rotten cheesy
Unidentified	1688	Burnt rubber, plant-like
Dimethyl tetrasulphide	1697	Sulphur, garlic
Naphthalene/unknown	1698	Green, fresh
Methylthio propanol	1702	Garlic sulphur
1,4-Dimethoxybenzene	1716	Woody, earthy, phenol
Allymethyl sulphide	1729	Garlic, savoury
Pentanoic acid (valeric)	1736	Cheesy, meaty, rotten
γ-Hexalactone	1760	Coconut, sweet
Unidentified	1787	Garlic, onion
2-Methoxy phenol	1815	Smoked, phenolic
Hexanoic acid	1817	Sharp, goaty
γ-Octalactone	1846	Coconut, melon sweet
2-Phenylethyl alcohol	1850	Rose, floral
γ-Cyanotoluene	1882	Smoked, chemical
Heptanoic acid	1905	Goaty, cheesy
Diphenyl	1920	Melon, geranium, milky
Furaneol	1951	Caramel, burnt sugar
p-Cresol	1981	Barnyard, phenolic
Unidentified	1990	Honey, floral
Homofuraneol	1984	Caramel, burnt sugar
δ-Decalactone	2036	Coconut, sweet
Ethyl phenol	2047	Disinfectant
Sotolone	2058	Coriander, curry
Unidentified	2086	Wet-dog, cardboard
3-Propyl phenol	2104	Disinfectant
Unidentified	2112	Grass, plant
n-Decanoic acid	2113	Waxy-sweet
δ-Dodecalactone	2187	Fruity-apricots

1.3.5. Sensory studies in respect of Cheddar cheese

The sensory qualities of cheese are as important as the product's appearance when it comes to sales. The dairy industry has therefore developed methodologies such as grading and judging of the product by scoring for overall flavour or texture quality based on an idealised concept and predetermined list of defects (Table 6) (Drake, McIngvale, Gerard, Cadwallader & Ceville, 2001; House & Acree, 2002; Lues, 2000; Singh *et al*., 2003). In Cheddar cheese made with mesophilic cultures, bitterness can be a severe problem. These cultures produce bitter peptides that predominately contain hydrophobic amino acid residues, resulting in a bitter taste (Singh *et al*., 2003). Chymosin has also been implicated in the formation of bitter peptides, but NaCl concentration, which plays a role in the hydrolysis of β-casein by chymosin, can be used as an inhibitory tool to control the bitter flavour in Cheddar cheese (Mistry & Kasperson, 1998). Lee *et al*. (1996) also identified several bitter peptides in Cheddar cheese originating from the N-terminal of α_{s1}-casein. Some scientists suggest that bitterness is related to starter cell numbers, for example fast acid-producing, heat-tolerant strains, while others maintain that there are inherent differences between bitter and non-bitter starter strains with respect to proteinase and peptidase profiles (Lemieux & Simard, 1991). Bitterness also appears to be a problem in low-fat cheese. In normal-fat cheese, bitter peptides, being hydrophobic, probably partition into the fat phase where they are less likely to be perceived as being bitter (Drake & Swanson, 1995).

Unclean-type flavours in Cheddar cheese (also described as subtle "floral" or "rose-like" aftertastes) are mostly found in mature cheeses (Singh *et al*., 2003). This flavour note, according to Dunn and Lindsay (1985), is mostly attributed to compounds like phenyl ethanol and phenyl acetaldehyde, which originate from the hydrophobic amino acid, phenylalanine (Phe), through enzyme-mediated transamination, reduction and decarboxylation reactions.

Table 6. List of cheese flavour defects used when judging/grading cheese flavour (Singh *et al*., 2003)

Flavour defect	Definition
High acid	Excessive acid or sour taste
Bitter	Bitter taste resembling caffeine or quinine
Fruity/fermented	Aroma of fermenting or overripe fruit
Flat	Devoid of flavour
Garlic/onion	Flavour resembling garlic, onions, or leeks
Heated	Not the clean cooked flavour of pasteurised milk but a flavour resembling the odour of old or spoiled milk
Malty	Flavour similar to Grape Nuts cereal
Metallic	A flat metal-like taste and a lingering puckery mouthfeel
Mouldy	Musty, reminiscent of a damp cellar
Rancid	Also called lipase, caused by short-chain fatty acids, flavour described as bitter, soapy, disagreeable
Sulphide	Also called skunky. Similar to water with high sulphur content
Unclean	Dirty aftertaste that fails to clean-up after the cheese is expectorated
Whey taint	Also called sour whey; the dirty sweet acidic taste and odour characteristic of fermented whey
Yeasty	Sour, bread-dough, earthy aroma characteristic of yeast

Dunn and Lindsay (1985) also hypothesised that the presence of branched-chain Strecker-type aldehydes, 2/3-methyl butanal and 2-methyl propanal, contributed to an unclean flavour in Cheddar cheese. On the other hand, Morgan (1970a, 1970b) evaluated the production of 2/3-methyl butanal by lactic acid bacteria in milk and found that it causes a malty flavour, which is considered a defect. More recent work by Avsar, Karagul-Yuceer, Drake, Singh, Yoon and Cadwallader (2004) quantified 2/3-methyl butanal and 2-methyl propanal as the main contributors to nutty flavours in Cheddar cheese.

According to Urbach (1997), esters could also form via the esterification of FFAs and alcohols. Some of the precursor volatile compounds found in Cheddar cheese are ethyl acetate, ethyl butanoate, and ethyl hexanoate. The presence of

low-concentration esters in cheese can contribute to the cheese flavour attributes, but the moment that the ester concentrations increase, fruity flavour defects develop (Morgan, 1970a, 1970b).

Apart from bitter, unclean and fruity flavour defects in Cheddar cheese, meaty-broth odours have also been observed in low-fat Cheddar cheeses. Milo and Reineccius (1997) hypothesised that this odour could be due to a high concentration of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (Furaneol^{TM}), homofuraneol, and methional. This is still to be confirmed by sensory studies, however. Likewise, a mayonnaise/bread-like off-flavour in reduced-fat Cheddar cheese containing soy lecithin was observed by Suriyaphan, Drake and Cadwallader (1999). The chemical agents responsible for these off-flavour notes are (E,E)- and (E,Z)-2,4-decadienal.

1.3.6. Instrumental analysis of Cheddar cheese flavour

1.3.6.1. Enzyme analysis

The production of flavour compounds during the maturation of Cheddar cheese can be measured by quantifying the enzyme responsible for the different metabolic pathways (Marilley & Casey, 2004). Studies comparing enzymatic activities of lactic acid bacteria have shown that enzyme activities allow for apparent differentiation between strains and their ability to produce flavours (Marilley & Casey, 2004). However, this process is time-consuming and it has been shown that some enzyme activities can vary amongst cheese-related strains in genera, some of which are *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Brevibacterium* (Curtin, Angelis, Cipriani, Corbo, McSweeney & Gobbetti, 2001; Gao, Oh, Broadbent, Johnson, Weimer & Steele, 1997).

Peptidase activities play a significant role in amino acid generation and flavour development in Cheddar cheese. Williams, Felipe and Banks (1998) measured peptidase activities with *p*-nitroanilide derivatives that release a chromogen after hydrolysis of the peptide bond, which can easily be spectrometrically measured. A spectrophotometric assay for branched-chain amino

acid transferase was also developed by Cooper, Conway and Hutson (2002), who analysed the aminotransferase activity by means of enzymatic determination of the concentration of keto acids. This was based on the decrease in absorbance at 340 nm due to NADH oxidation in the presence of leucine dehydrogenase and ammonia.

1.3.6.2. Thin-layer chromatography

According to Marilley and Casey (2004), thin-layer chromatography (TLC) can be applied to detect flavour components such as organic acids and sugars. This methodology could, for instance, be applied to evaluate glycolysis, fermentation, and amino acid catabolism. Gao *et al*. (1997) successfully applied this technique to study the production of indole pyruvate, indole acetate, indole-3-aldehyde, and 4-hydroxylbenzaldehyde by lactococci. The advantages of this technique are that it is flexible and it allows for the simultaneous analysis of compounds of a different nature, as well as the parallel analysis of several samples (Marilley & Casey, 2004).

1.3.6.3. Liquid chromatography

Liquid chromatography (LC) can be used to simultaneously monitor the concentrations of organic acids, diacetyl and acetoin (Buffa, Guamis, Saldo & Trujillo, 2004; Lee *et al*., 1996; Lues, 2000; Lues & Bekker, 2002; Lues, Botha & Smit, 1998; Mullin & Emmon s, 1997; Papadakis & Polychroniadou, 2005; Zeppa, Conterno & Gerbi, 2001). High-performance liquid chromatography (HPLC) is well-adapted to the study of glycolysis and fermentative pathways, as well as the first intermediates in the degradation of amino acids. Ion-exchange or reverse-phase chromatography can be used to separate all the components of cheese, i.e. α-ketoisocaproate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, 4-methylthio-2-oxobutyrate, β-phenyl pyrovate, and ρ-hydroxyphenyl pyrovate. The production of these keto acids is related to the aminotransferase activities. Pripp, Shakeel-Ur-Rehman, McSweeney and Fox (1999) conducted a multivariate statistical analysis of peptide profiles and free amino acids to evaluate the effects of single-strain starters on proteolysis by using reverse-phase high-performance liquid chromatography (RP-HPLC). An advantage of HPLC is that several instrumental channels can be used in

parallel. The risk of spoiling the chromatography column is relatively high, however, with complex microbiological samples, which can be a disadvantage of the technique. HPLC is also not often used for flavour analysis, because most flavours are directly amenable to gas chromatography (GC) analysis (Marilley & Casey, 2004).

1.3.6.4. Gas chromatography / Mass spectrometry

Gas chromatography (GC) analysis methods are being increasingly applied in food science and technology due to their exceptional separation and versatility in respect of a wide variety of compounds. Cheese flavour can be characterised by applying GC methods in combination with other analytical methods. These methods include static headspace gas chromatography/mass spectrometry (GC/MS) analysis (Marilley & Casey, 2004; Pérès, Denoyer, Tournayre & Berdague, 2002); purge-and-trap of distillation GC analysis (Wilkes *et al*., 2000); solid-phase micro-extraction-GC analysis (Kataoka, Lord & Pawliszyn, 2000); cryo-trapping-GC analysis (Jaillais *et al*., 1999); and short-path thermal desorption-GC analysis (Wilkes *et al*., 2000). Furthermore, Dirinck and De Winne (1999) characterised and classified cheese by means of GC-MS and simultaneous steam distillation-extraction. Recently, a combination of GC/GC-MS/GC-olfactometry (GC-O) with solid-phase micro-extraction (SPME) has been successfully used to detect cheese aroma compounds (Dirinck & De Winne, 1999; Frank *et al*., 2004; Jaillais *et al*., 1999; Kataoka *et al*., 2000; Marilley & Casey, 2004; Quach, Chen & Stevenson, 1999; Singh *et al*., 2003; Wilkes *et al*., 2000).

1.3.6.5. Electronic nose / GC-olfactometry

GC-O is a technique that is widely used and works with the GC separation of the analyte and the sensitivity of the human nose (Singh *et al*. 2003). According to Wilkes *et al*. (2000) electronic noses are gas-phase sensor arrays combined with pattern recognition into an instrumental package designed to give an objective basis for odour and flavour identification that correlates with human sensory experience.

The chemical sensors that are normally used are metal oxide sensors and polymer sensors.

Electronic noses analyse the sum of volatiles by injecting them together into a mass spectrometer. The technique is not used to analyse individual compounds after chromatographic separation, but according to Marilley and Casey (2004), the instrument mimics the sum of total volatile compounds to an aroma perception. Speed is the main advantage of this technique, and it can also be adapted to the clustering of a large number of bacterial strains. The major drawback of this technique is that it does not identify compounds, nor does it quantify information (Marilley & Casey, 2004), and the instrumental sensors can become saturated and fail to respond, just like the human olfactory sense (Wilkes *et al*., 2000). Avsar *et al*. (2004) characterised the nutty flavour in Cheddar cheese by using GC-O of solvent extracts, as well as GC-O dynamic headspace (GCO-DHS) analysis.

1.4. Food authenticity

Fraud detection in foods often requires the precise characterisation of the product, which involves the use of many diverse analytical tools (Dias, Peres, Veloso, Reis, Vilas-Boas & Machado, 2009; Pillonel, Bütikofer, Schlichtherie-Cerny, Tabacchi & Bosset, 2005; Sacco, Brescia, Buccolieri & Caputi Jambrenghi, 2005; Sacco, Brescia, Sgaramelia, Casiello, Buccolieri, Ogrinc & Sacco, 2009). For instance, the determination of the geographical origin of a foodstuff is a difficult task, especially in the case of cheese, which is microbiologically and biochemically dynamic and undergoes various changes throughout ripening (Luykx & Van Ruth, 2008). Consequently, the data from the selected analytical techniques must be subjected to multivariate analysis, also known as chemometrics.

1.5. Chemometrics

Chemometrics can be defined as the application of mathematical and statistical methods to exploit the chemical information extracted from the data. This

is a powerful tool that is applied in a variety of fields covered by published reviews (Lavine & Workman, 2002; Pillonel *et al*., 2005). Pattern recognition is a specific application of chemometrics that has attracted the attention of chemists involved in the fight against food fraud and which has been comprehensively reviewed (Tzouros & Arvanitoyannis, 2001; Arvanitoyannis & van Houwelingen-Kokaliaroglou, 2003). The techniques that are most commonly used for authentication purposes include principal component analysis (PCA), discriminant analysis (DA), principle component regression (PCR), partial least square (PLS) and artificial neural network (ANN) (Pillonel *et al*., 2005). A decisive point of pattern recognition is the validation of a model. In an over-fitted model, classification into categories may seemingly appear satisfactory, but is in fact not statistically significant. The following simple rule-of-thumb must therefore be applied prior to all classifications: The number of factors $(n - g)/3$, where n is the number of observations and g the number of categories fixed (Defernez & Kemsley, 1997). Furthermore, no classification should be performed without cross-validation. A first possible procedure of cross-validation is to allocate each observation at random to either a "training" set or a "validation" set. The training set is only used to attain a model, which is then applied in a second step to the validation set. In ANN, training sets could contain up to 80% of cases. If the results between training and validation differ to a great extent, the model is over-fitted or insufficiently adapted. An alternative procedure is the "leave-one-out" or "Jacknifed" validation where one observation at a time is omitted from the data set and only the remaining data is used to obtain a model. This is repeated n times, excluding each observation in turn and reintroducing the previously omitted observation. The results from the excluded observations are only then evaluated. Yet again, if normal-set and Jacknifed validation deviate, the model is over-fitted. Both validation procedures seem to deliver similar results (Defernez & Kemsley, 1997; Pillonel *et al*., 2005).

1.5.1 Artificial neural networks

Artificial neural networks (ANNs) can be used as a useful tool for the authentication and classification of foods, depending on their geographical origin. There are several reports on the use of ANNs in chemical data and sensory analysis for the classification of food products, such as wine (Cichelli, Damiani, Murmura, Simonetti, Odoardi & Damiani, 2000; Pérez-Magariño, Ortega-Herasa, González-San José & Boger, 2004), vegetables (Padin, Peña, García, Iglesias, Barro & Herrero, 2001), coffee (Anderson & Smith, 2002), honey (Cordella, Militao, Clement & Cabrol-Bass, 2003) and dairy products (Balestrieri, Damiani & Marini, 2001). ANNs mimic biological neural networks and can be eminent for both their internal architecture and the learning algorithm they use. One of the most common ANNs is the multilayer feedforward network, which is made up of an input layer, one or more hidden layers, and an output layer (Fig. 8).

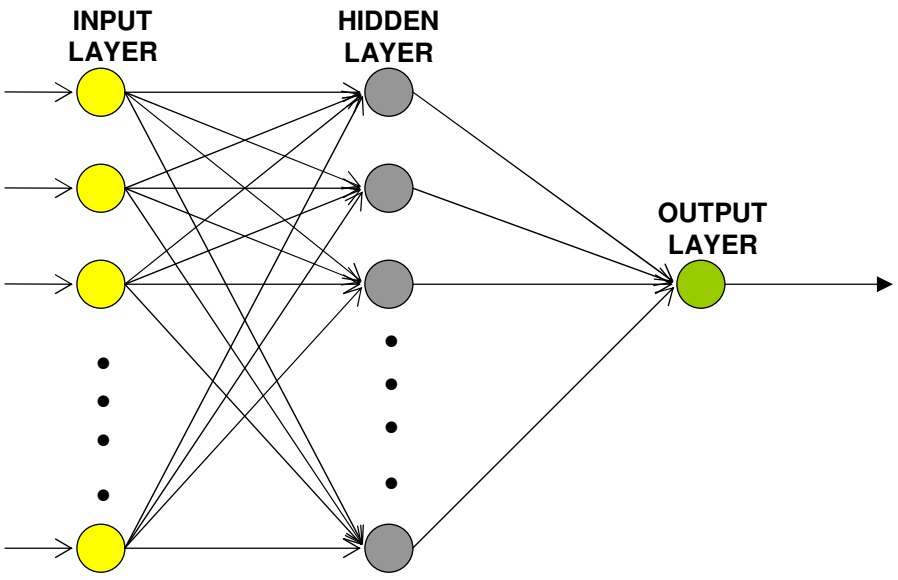


Figure 8. Schematic representation of a multilayer feedforward network (adapted from Barile, Coïsson, Arlorio & Rinaldi, 2006).

This kind of network, made of a single hidden layer with an adequate number of nodes, can approximate any nonlinear function to any required degree of accuracy

(Hornik, Stinchcombe & White, 1989). The proliferation of the signal from node i of one layer to node *j* of the next layer depends on the strength w_{ij} (weight) of the connection. To get the full signal I_j reaching node *j*, the summation over all nodes i, connected to node j, must first be carried out.

$$I_j = \sum_{i=1}^{n} w_{ij} x_i$$

The transfer function must then be applied:

$$f(x) = \frac{1}{1 + e^{-x}}$$

According to Barile *et al*. (2006), there are two types of learning processes: (i) supervised learning or learning with instruction, and (ii) unsupervised learning or learning without instruction. A supervised ANN is presented with both the input and the output and is expected to make the association between them, whereas an unsupervised ANN is presented with the input only and is expected to detect comparisons or relationships in the input where none are pre-specified. The training of a supervised network consists of a series of cycles (epochs) in which the data is presented to the network, which allows for the alteration of weights, initialised at the beginning, to random values uniformly distributed in the interval [-1,1]. According to Ripley (1996), back-propagation is a type of learning strategy based on an algorithm that corrects the weights within each layer in propagation to the error attained from the previous layer. The correction of the weights is given iteratively as follows:

$$\Delta w_{ij}^{l} = \eta \delta_j^{l} \text{out}_i^{l-1} + \mu \Delta w_{ij}^{l} \text{ (previous)}$$

Where l is the index of the layer, δ_j^l is the error of the j th node of the l layer, out is the actual output, μ is the momentum term, and η is the learning rate (Lozano, Novic, Rius & Zupan, 1995).

In the construction of a neural network, attention must be paid particularly to the selection of the architecture, the selection of the learning parameters, and the network validation. During the training, the values for all the parameters involved in

the learning process must be optimised, and the error between the net-predicted output and the correct output must be calculated. The size of the hidden layer and the number of cycles must be appraised by varying their values and checking the accuracy of the resulting prediction. The number of hidden nodes is critical to the design of the network, because if too many hidden nodes are used, the network will over-fit or memorise the training-set data. Conversely, if too few hidden nodes are used, the network will fail to generalise and will become unstable. The optimal number of hidden nodes should be determined iteratively for each dataset and could possibly start with two nodes. They could then be added one at a time until the network has learned the training set. At the end of the training phase, the network should be tested by using samples that were not present in the training set.

1.6. Rationale

One of South Africa's leading retailers aims to supply high-quality, safe and nutritious food products to the general public. Amongst other things, their products originating from milk as raw product are made exclusively from the milk of cattle of the Ayrshire breed. This milk is free from recombinant bovine somatotropin (rBST) and is said to yield secondary products such as yoghurt and cheese with a distinct texture, aroma and taste. In order to protect the brand and assess the quality of their dairy products, this retailer is frequently requested to verify the origin of the milk used in the production of especially cheese by secondary suppliers. However, a method to assess the latter does not exist. The need for such a method was therefore identified and commissioned to the Central University of Technology, Free State, thus yielding the main aim of this study: To model a catabolite profile in addition to an aroma and flavour fingerprint of Ayrshire cheese. This method could in future be applied to profile this supplier's cheese as part of their already thorough quality assurance system.

In order to achieve this aim the study has been structured into six different chapters – every chapter with its own aims:

Chapter 2: The relationship between organic acids, starter microbiota and selected chemical indicators in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 3: Mathematical expressions for organic acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 4: Mathematical indices for fatty acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 5: Mathematical modelling of Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk using amino acid data.

Chapter 6: The discrimination of milk origin in the manufacturing of Cheddar cheese via artificial neural network modelling of *Lactococcus lactis* and *Streptococcus thermophilus.*

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Chapter 2

The relationship between organic acids, starter microbiota and selected chemical indicators in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk

Submitted for publication to Journal of Food Composition and Analysis.

2.1. Title

The relationship between organic acids, starter microbiota and selected chemical indicators in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

2.2. Abstract

This study was directed to investigate the unique properties of branded Ayrshire Cheddar cheese versus Cheddar cheese manufactured from a mixture of other breeds' milk (not including Ayrshire milk). Variations that occur between Ayrshire and non-Ayrshire Cheddar cheese with regard to organic acids, selected chemical parameters and starter microbiota were examined. Thirty-two cheese samples of each batch (Ayrshire (4) / non-Ayrshire (4)) were ripened. Microbial, chemical and organic acid analyses were carried out on the following days after production: 2, 10, 22, 36, 50, 64, 78, and 92. The minimum and maximum (min/max) values, standard deviations and proposed X_{rel} values of organic acids in Ayrshire and non-Ayrshire Cheddar cheese respectively were evaluated. Isovaleric acid happened to be the organic acid with the least variation relative to concentration (X_{rel}) in both cheeses, and it was assumed to be the most effective indicator of cheese uniformity. Chemical parameters such as moisture, salt and pH showed similar values to those reported in the literature. Clear differences in correlation patterns amongst organic acids, chemical variables and starter micro-organisms in Ayrshire and non-Ayrshire Cheddar cheese were evident and this data can be useful to draw disparities between the two types of cheeses for quality control purposes.

2.3. Introduction

Branded Cheddar cheese, i.e. Ayrshire Cheddar cheese (made solely from milk of the Ayrshire breed) is receiving more and more attention due to its distinctive taste and texture properties (SASBA, 2001). Since the unique properties of this cheese had not as yet been investigated at biochemical level, retailers identified the need to determine the disparity between Cheddar cheese made solely from Ayrshire

milk and a cheese made from a mixture of other breeds' milk for quality control purposes.

Variations in the microbiological, biochemical and sensorial parameters of cheese have been ascribed to a variety of factors that can be summarised as milk source and other raw materials, manufacturing protocol, and hygiene status of the manufacturing plant. In general, uniformity is one of the most sought-after attributes in cheese quality, due to its economic implications and the fact that cheese development is influenced by such a vast number of microbiological and biochemical parameters. However, a difference in the uniformity of Cheddar cheese made from Ayrshire and cheese made from a mixture of other breeds' milk is anticipated based on the difference in sensory attributes (according to personal communication with an Ayrshire Cheddar cheese manufacturer).

The applicability of organic acids as classification parameter for different cheese types has been reported (Bevilacqua & Califano, 1992; Bouzas, Kantt, Bodyfelt & Torres, 1991, 1993; Gomis, 1992) and plays an integral part in cheese quality. Quality of cheese is also influenced by a vast number of other factors, i.e. moisture, salt, pH, the starter cultures used, etc. (Axelsson, 1993; Cogan & Daly, 1987; Guinee & Fox, 1987; Høier, Janzen, Hendriksen, Rattray, Brockman & Johansen, 1999; Lawrence & Gilles, 1987; Mistry & Kasperson, 1998; Pastorino, Hansen & McMahon, 2003; Singh, Drake & Cadwallader, 2003;). The moisture and salt content, in particular, is manipulated by cheese-makers to achieve desirable qualities in the cheese (Bouzas *et al*., 1993; Lawrence & Gilles, 1987). The starter culture, on the other hand, also plays a fundamental role in the quality of Cheddar cheese since it produces lactic acid in the coagulation process. In addition, it also plays an important role in proteolysis and lipolysis later during cheese-ripening (Cogan & Daly, 1987; Høier *et al*., 1999).

This study was therefore directed at establishing a maturation pattern based on selected organic acids, starter microbiota and chemical variables in Cheddar cheese made from Ayrshire and a mixture of other breeds' milk (not including Ayrshire milk). Results were aimed at demonstrating the changes that occur in the

organic acid content, the nature and proliferation of the starter micro-organisms, as well as changes in selected chemical parameters in the two different cheeses. The correlation patterns of the selected variables were ultimately utilised to establish typical differences between Cheddar cheese made from pure Ayrshire milk and Cheddar cheese made from a mixture of other breeds' milk.

2.4. Materials and methods

2.4.1. Manufacturing of Cheddar cheese

Two Cheddar cheese batches were manufactured on the same day, under similar conditions in the same factory in order to limit extrinsic and intrinsic variability between cheese batches. Milk source was the only variable between the batches, with one batch being exclusively manufactured from Ayrshire (A) milk and the other from a mixture of other breeds' milk (excluding Ayrshire milk). The Cheddar cheese was manufactured in a closed-vat system with starter cultures consisting of *Streptococcus thermophilus, Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (CHOOZIT RAO24, Danisco). The cheese samples (four samples from each batch) were analysed for moisture, salt (NaCl), organic acid concentration, and microbial population on the following days after production: 1, 8, 22, 36, 50, 64, 78 and 92.

2.4.2. Organic acid extraction

Organic acids were extracted from the cheese by applying the method proposed by Lues (2000). Five grams of Cheddar cheese were ground and added to 25 ml 0.009N H_2SO_4. The mixture was stirred for one hour on a magnetic stirrer, followed by centrifugation (7000 g for 5 minutes). The supernatant was filtered through a 0.2 µm membrane filter (Whatmann) and 10 µl were injected into the HPLC for analysis. Peaks were identified by reference to authentic standards (Sigma-Aldrich, RSA).

2.4.3. Organic acid analysis

Standards of acetic, butyric, citric, formic, isovaleric, malic, oxalic, pyruvic, and *n*-valeric acid were obtained from Sigma-Aldrich RSA and prepared with HPLC-grade water for identification and quantification. Organic acids were separated by HPLC using an automated system (Spectra Physics) equipped with a solvent degasser (SCM 400), quartenary gradient solvent pump (P4000), multi-autosampler (AS1000) fitted with a 50µl loop and a spectral array UV detector (UV3000) set at 210 and 290 nm, and a refractive index detector connected in series. All separations were carried out on a Rezer ROA organic acid hydrogen form (300 x 7.8 mm) ion-exchange chromatography column (Phenomenex, USA), at a constant temperature of 30°C. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1 $ml.min^{-1}$ and detection was done at 210-290 nm scanning with 5 nm intervals. The injection volume was 40 µl with a 40-minute running time.

2.4.4. Chemical analysis

The chemical parameters investigated were sodium chloride (NaCl) content, moisture and pH. Sodium chloride was measured using the Volhard method (Bradley, Arnold, Barbano, Semerad, Smith & Vines, 1993; Helrich, 1990) whereas pH was measured using a calibrated Hanna pH meter. The moisture content of the cheese was analysed using a forced-draft (Labcon, RSA) oven as described by Bradley *et al.* (1993) and Helrich (1990), with some minor adjustments. Three grams of cheese were dried in a pre-weighed dish. Drying parameters were 100°C for a period of 16 hours, after which the sample was cooled in a desiccator for 60 minutes. Moisture content was calculated as the percentage loss of mass.

2.4.5. Microbial analysis

All media and reagents were obtained from Merck and Sigma-Aldrich RSA. One-gram cheese samples (finely ground using sterile equipment) were weighed and blended in 9 ml buffer (Peptone, Merck) after which serial dilutions were

prepared. Dilutions were plated on the following solidified agar media: M17 agar (Merck) for *Lactococci* and specially prepared medium as described by Dave and Shah (1996) for *Streptococcus thermophilus*. Plates were incubated at 30℃ for 48 hours, after which the colonies were enumerated using a colony counter.

2.4.6. Mathematical calculations

Respective minimum/maximum values, as well as standard deviations, were evaluated as a measure of uniformity. Min/max data allows one to determine the upper and lower boundaries of a specific organic acid. The min/max variations were calculated relative to the maximum concentrations of the individual parameters as follows:

$$\frac{(X_{\max} - X_{\min})}{X_{\max}} \times \frac{100}{1} = X_{rel}$$

Where:

$X_{\max}$ = Maximum value recorded

$X_{\min}$ = Minimum value recorded

X_{rel} = Min/max variation of respective parameters as percentiles of their maximum recorded values

2.5. Results and discussion

2.5.1. Indices of uniformity

Figure 1 (A & B) is a visual representation of the respective X_{rel} values for the different organic acids analysed in Ayrshire and non-Ayrshire Cheddar cheese during the ripening period. The uniformity of the cheese flavour was determined by applying X_{rel} (min/max variation of individual parameters as percentiles of their maximum recorded values). In Ayrshire Cheddar cheese (Fig. 1A), isovaleric acid

was found to have the lowest X_{rel} value (0.2%) followed by butyric acid at 0.4% and acetic acid at 0.6%. Formic acid had a higher value (4.04%), whilst citric acid and n-valeric acid both had X_{rel} values of 4.22%. Pyruvic acid (4.76%) was the organic acid with the highest X_{rel} value. The latter two indices of uniformity compare well with the findings of Lues (2000).

In non-Ayrshire Cheddar cheese (Fig. 1B), isovaleric acid was also found to have the lowest X_{rel} value (0.3%) followed by butyric acid at 0.6% and acetic acid at 1.49%. n-Valeric acid had a higher value (2.47%) followed by pyruvic acid and formic acid at 4.22% and 4.95% respectively. The organic acid with the highest X_{rel} value in non-Ayrshire Cheddar cheese was citric acid (7.18%). Considering the fact that all the cheeses analysed were of comparable flavour, those with the least variation relative to their concentration (X_{rel}), were perhaps the most effective indicators of cheese flavour uniformity, whilst the parameters with wide-ranging variations (larger X_{rel}) had little or no relationship with uniformity. When evaluated according to this mechanism, isovaleric, butyric and acetic acids had relatively small X_{rel} values in both Ayrshire and non-Ayrshire Cheddar cheese. These compounds may be deemed possible indicators of uniformity. Nevertheless, formic, n-valeric, pyruvic and citric acids differ in Ayrshire and non-Ayrshire Cheddar cheese in terms of their contribution to cheese uniformity. This probably explains the difference in Cheddar cheese flavour in these two types of Cheddar cheese.

The proposed X_{rel} value should, however, not be used as a stand-alone indicator of cheese uniformity. A number of factors can influence cheese uniformity, amongst them chemical and sensory attributes. However, this model holds great value for cheese quality controllers to apply as a benchmark to indicate the organic acid repertoire of Ayrshire and non-Ayrshire Cheddar cheese respectively for cheese manufactured under similar conditions to those described in this study.

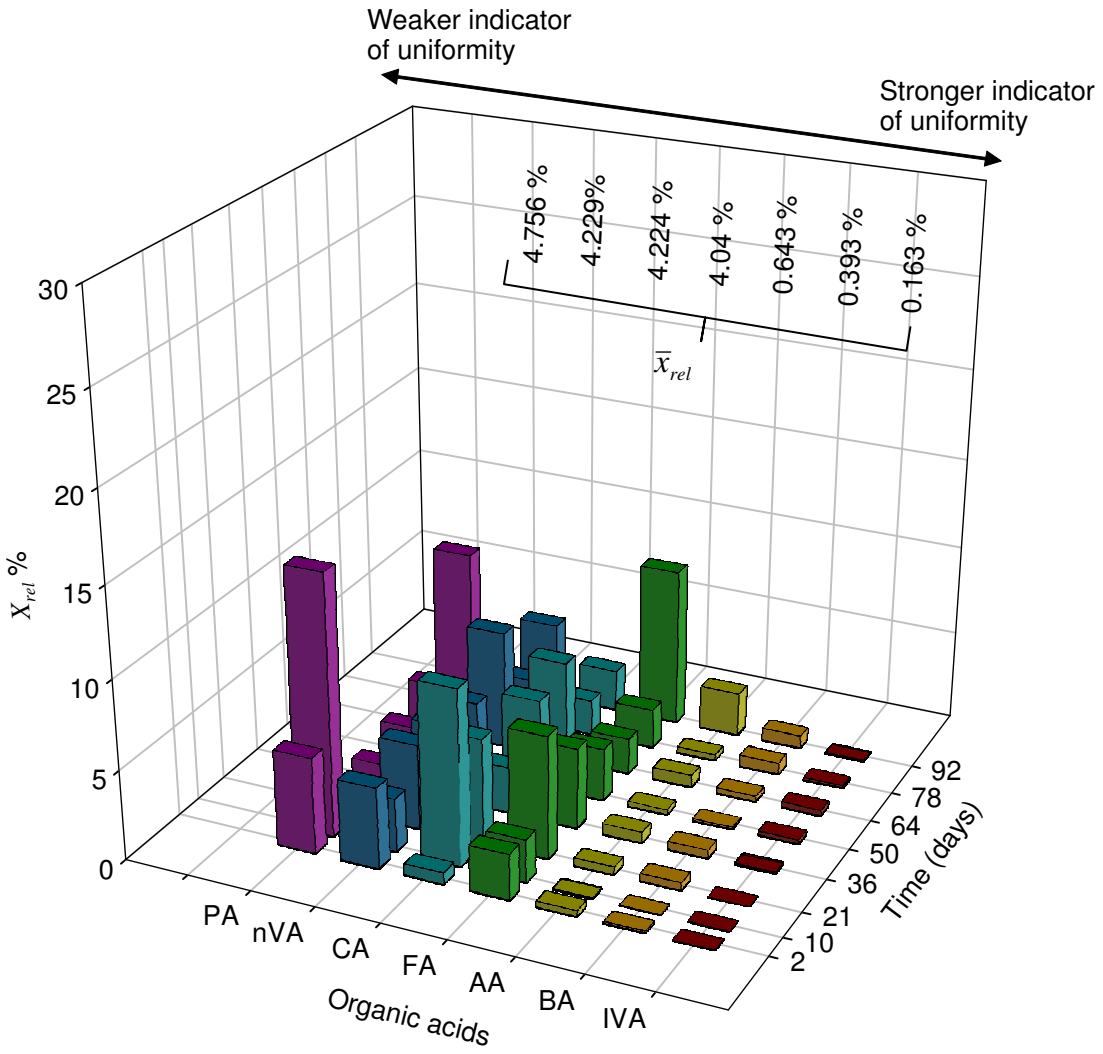

Figure 1A. Variability of selected organic acids in Ayrshire Cheddar cheese (A) as indicated by X_{rel} (min/max variation of respective parameters as percentiles of their maximum recorded values). The parameters with low X_{rel} values are suggested to have a strong relationship with uniformity, whereas the parameters with high X_{rel} values have a weaker relationship with uniformity. IVA = Isovaleric acid; BA = Butyric acid; AA = Acetic acid; FA = Formic acid; CA = Citric acid; nVA = n-valeric acid; PA = Pyruvic acid.

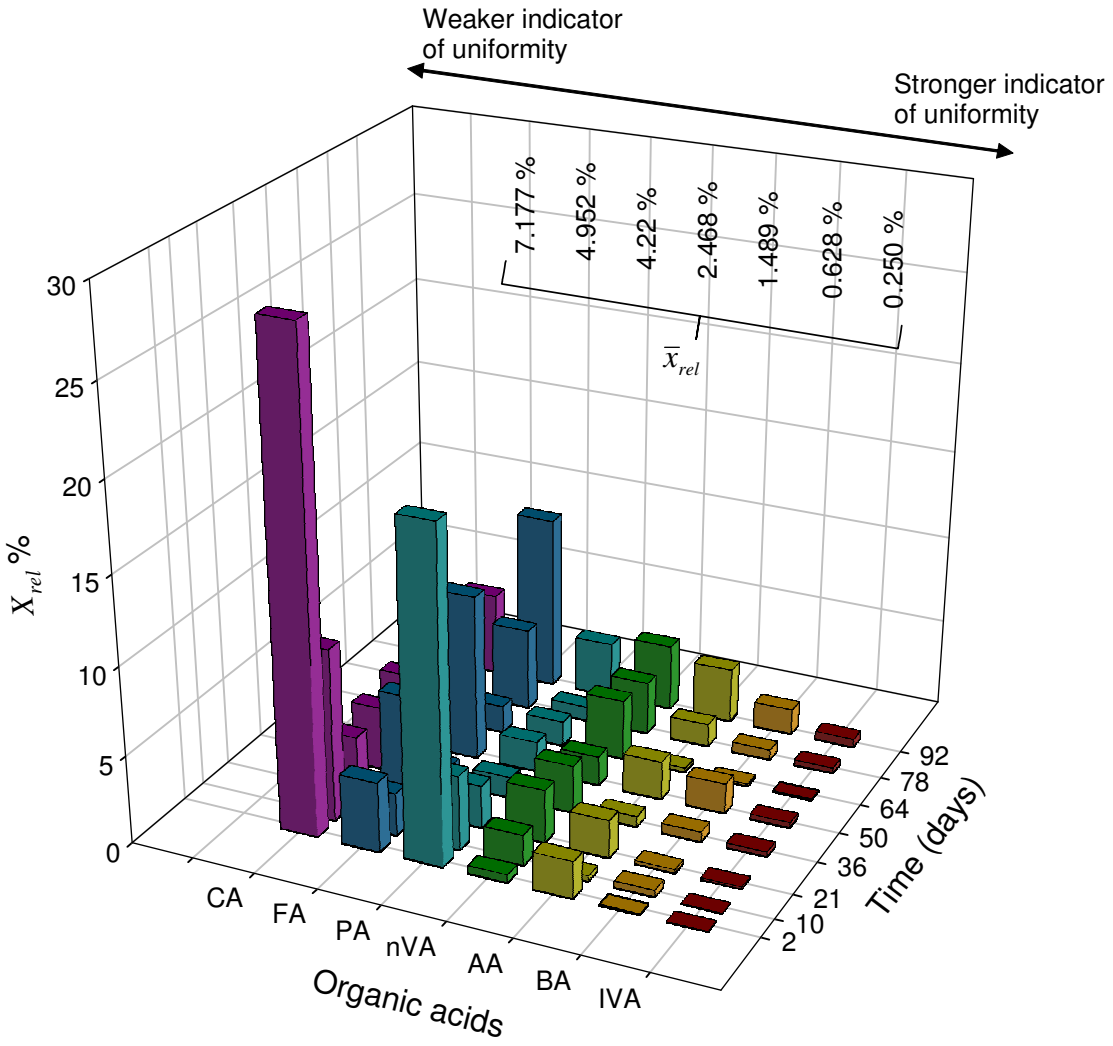

Figure 1B. Variability of selected organic acids in non-Ayrshire Cheddar cheese (B) as indicated by X_{rel} (min/max variation of respective parameters as percentiles of their maximum recorded values). The parameters with low X_{rel} values are suggested to have a strong relationship with uniformity whereas the parameters with high X_{rel} values have a weaker relationship with uniformity. IVA = Isovaleric acid; BA = Butyric acid; AA = Acetic acid; FA = Formic acid; CA = Citric acid; nVA = n-valeric acid; PA = Pyruvic acid.

2.5.2. Chemical variables

Patterns of selected chemical parameters during the maturation of Ayrshire and non-Ayrshire Cheddar cheese are shown in Figure 2 (A–C). The moisture content (Fig. 2A) of Ayrshire (37%) and non-Ayrshire (38.2%) Cheddar cheese remained more or less constant throughout ripening and compares well with the values reported in the literature (Lawrence & Gilles, 1987). The initial moisture percentage was, however, lower in non-Ayrshire than in Ayrshire Cheddar cheese and remained lower throughout ripening.

The pH (Fig. 2B) followed the same trend in both Ayrshire and non-Ayrshire Cheddar cheese, with an initial pH of 5.4 and 5.3 respectively. These values compare well with those in the literature (Guinee & Fox, 1987; Lawrence & Gilles, 1987). An initial downward trend was observed during the first 41 days, followed by an increase up to 78 days, and then a decrease up to 92 days. Surprisingly, the same trend was also observed in citric acid (results not shown), which indicates that the free citric acid is directly proportional to the pH of the cheese. While the same trend was observed throughout ripening for both Ayrshire and non-Ayrshire Cheddar cheese, the pH of Ayrshire Cheddar cheese remained higher throughout.

Similar patterns in NaCl concentration were observed for Ayrshire and non-Ayrshire Cheddar cheese (Fig. 2C). However, the initial concentration of NaCl differed by about 0.5% between the two cheeses. This variation remained present throughout the ripening process. The average NaCl concentration throughout ripening was 1.41% in Ayrshire Cheddar cheese and 1.9% in non-Ayrshire Cheddar cheese. According to Mistry and Kasperson (1998), the intensity of bitterness is lowered as the quantity of salt in the cheese increases, which makes non-Ayrshire Cheddar cheese less bitter in this case. These values are similar to those found in the literature relating to Cheddar cheese (Guinee & Fox, 1987; Lawrence & Gilles, 1987).

Based on the above results for moisture, pH and NaCl, it was noticeable that the analysis of the samples produced figures quite similar to those reported in the

literature (Guinee & Fox, 1987; Huffman & Kristoffersen, 1984; Lawrence & Gilles, 1987; Mistry & Kasperson, 1998).

2.5.3. Bacterial growth during ripening

Figure 3 represents the changes in the starter micro-organisms in Ayrshire and non-Ayrshire Cheddar cheese throughout the ripening period. Three starter organisms were applied in the manufacturing of Cheddar in this study: the thermophilic culture, *Streptococcus thermophilus*, and the mesophilic cultures, *Lactococcus lactis* subsp. *lactis* and *cremoris*.

Streptococcus thermophilus, represented in Figure 3A, is a fast-acidifying culture with an optimal growth temperature of 43°C and is mostly unable to ferment the galactose moiety of lactose (Høier *et al*,, 1999). In Ayrshire Cheddar cheese, an initial increase in *Streptococcus thermophilus* up to day 50 of ripening was observed. This increase is related to the pH of the cheese, as this organism is a fast-acidifying culture, and the moment the organism count increased, the pH decreased. In non-Ayrshire Cheddar cheese, the amount of *Streptococcus thermophilus* remained more or less constant ($\pm 10^5$ cfu.ml^{-1}) throughout the ripening process, except for a sudden decrease at 78 days in one log value, which recovered at 92 days to 10^5 cfu.ml^{-1}.

Lactococcus lactis spp., on the other hand, (Figure 3B), is added for constant acidifying purposes at lower temperatures (30°C) (Høier *et al*,, 1999). In *Lactococcus lactis* spp. the initial numbers during ripening (2 days) were similar for Ayrshire and non-Ayrshire Cheddar cheese (10^6 cfu.ml^{-1}). Counts in Ayrshire Cheddar cheese increased with more than one log for the period of 2 to 22 days, followed by a drastic decrease to approximately 10^6 cfu.ml^{-1}, where it remained more or less constant up to 64 days, followed by a slight increase near the end of ripening (92 days). Concerning non-Ayrshire Cheddar cheese, no dramatic fluctuations in *Lactococcus lactis* spp. transpired, although a slight decrease throughout the entire ripening process was evident.

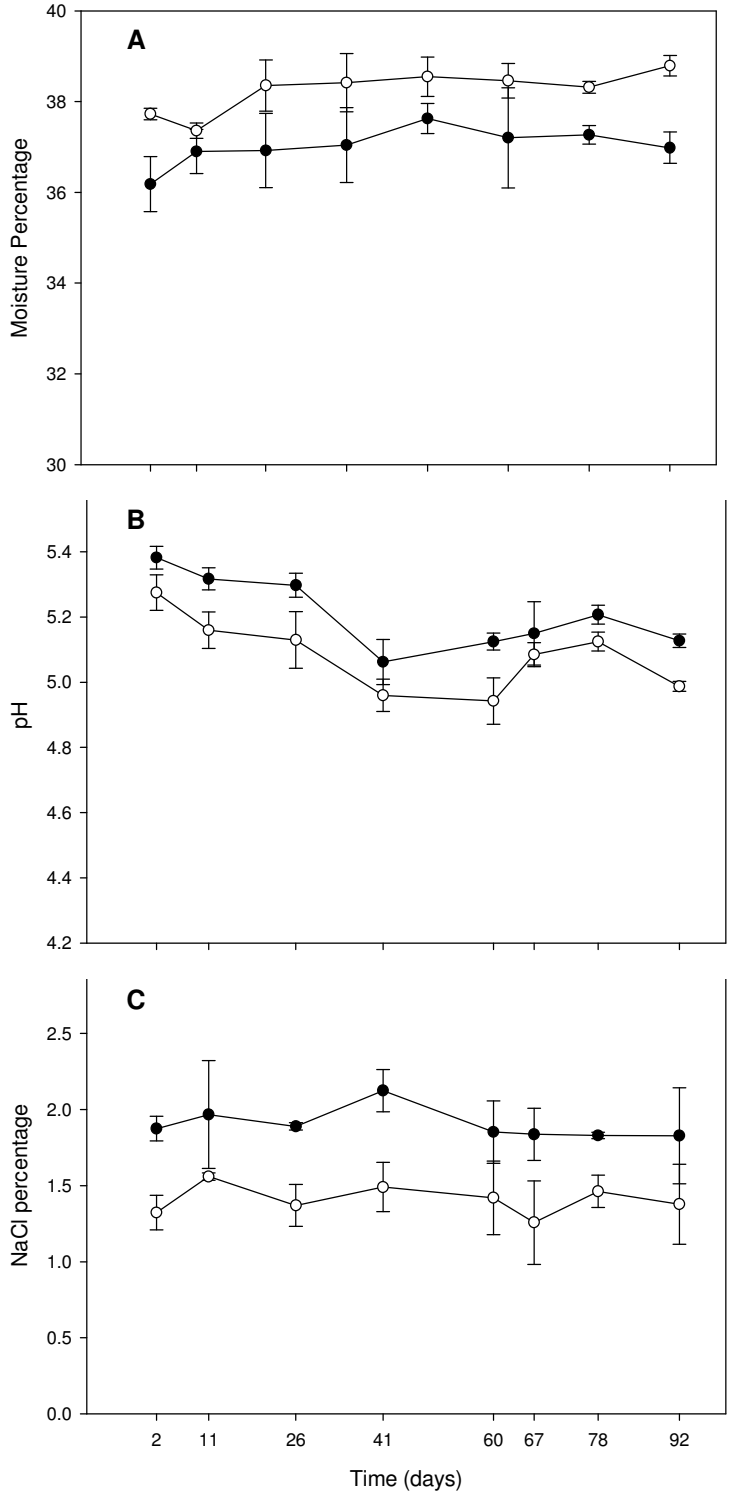

Figure 2. Changes in (a) Moisture; (b) pH and (c) NaCl content in Cheddar cheese made from Ayrshire (—○—) and non-Ayrshire (—●—) milk during ripening.

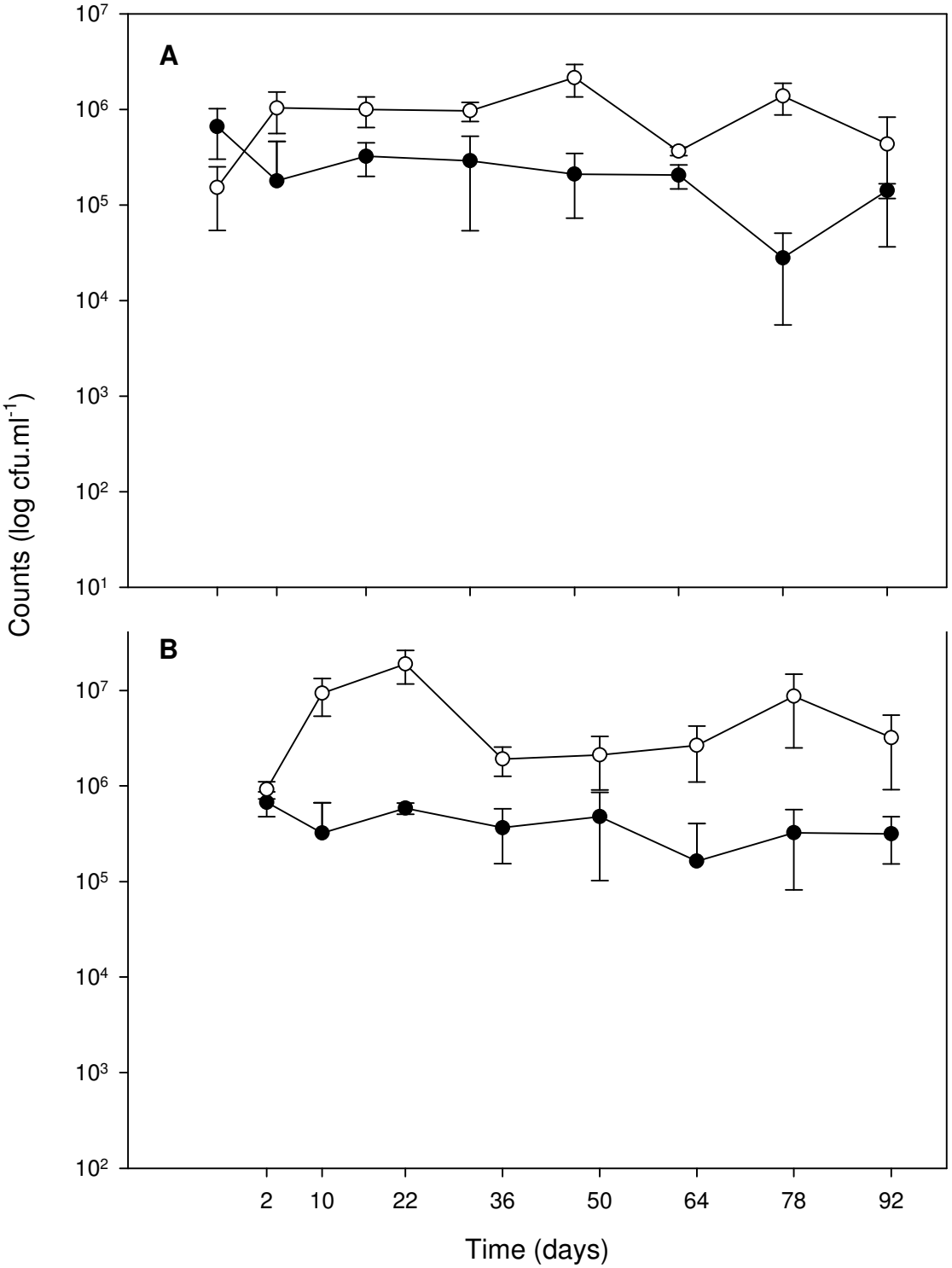

Figure 3. Microbial populations quantified in Ayrshire (—○—) and non-Ayrshire (—●—) Cheddar cheese during ripening: (a) *Streptococcus thermophilus*; (b) *Lactococcus lactis*

2.5.4. Correlations

Table 1 and Table 2 are representations of the correlations between the different organic acids, microbiota (*Lactococcus lactis* and *Streptococcus thermophilus*) and selected chemical components (moisture, pH and NaCl) in Ayrshire and non-Ayrshire Cheddar cheese respectively. Strong correlations were considered as values between ±0.8 and ±1, whereas weaker correlations were deemed values between ±0.4 – ±0.8. No or very weak correlations (-0.4 to +0.4) are indicated as – in the table.

Among these similarities between the two cheese types is the pH that formed positive correlations in both cheeses with citric acid and pyruvic acid, but not with any other organic acid. Another similarity in both Ayrshire and non-Ayrshire Cheddar cheese is the moisture content that formed negative correlations with citric and pyruvic acid, but with no other organic acid. Differences between the two cheese types were the negative correlations between moisture content and the two starter organisms, *Lactococcus lactis* and *Streptococcus thermophilus,* which were only noticeable in non-Ayrshire Cheddar cheese. No correlations between moisture and the starter organisms were detected in Ayrshire Cheddar cheese. The pH, on the other hand, showed positive correlations with all the starter organisms in non-Ayrshire Cheddar cheese, but a negative correlation with only *Streptococcus thermophilus* in Ayrshire Cheddar cheese. No strong correlations were noticeable between the salt content, organic acids and starter organisms in non-Ayrshire Cheddar cheese, although a weak positive correlation between the salt content and *Streptococcus thermophilus* in Ayrshire Cheddar cheese was noticeable.

Table 1. Spearman's correlation between the various micro-organisms, chemical composition and organic acids in Ayrshire Cheddar cheese

	n-Valeric	Oxalic	Citric	Pyruvic	Formic	Acetic	Burytic	Isovaleric	Moisture	pH	Salt	*L. lactis*	*S. therm*
n-Valeric	1												
Oxalic	0.66	1											
Citric	-0.66	-0.59	1										
Pyruvic	-	-0.88*	-	1									
Formic	0.67	0.97*	-0.70	-0.88*	1								
Acetic	0.68	0.97*	-0.66	-0.87*	0.97*	1							
Burytic	0.46	0.88*	-0.59	-0.82*	0.85*	0.85*	1						
Isovaleric	0.57	0.88*	-0.45	-0.80*	0.82*	0.83*	0.93*	1					
Moisture	0.77	0.88*	-0.46	-0.67	0.80*	0.85*	0.79	0.93*	1				
pH	-0.43	-0.69	0.48	0.53	-0.58	-0.71	-0.76	-0.73	-0.74	1			
Salt	-0.74	-	-	-	-	-	-	-	-	-	1		
L. lactis	-0.58	-	0.53	-	-	-	-	-	-	-	-	1	
S. therm	-	-	-	-	-	-	-	-	-	-0.53	0.51	-	1

* Correlation coefficient, significant at tile $p<0.05$ level. Correlations between -0.4 and +0.4 are indicated with a – sign.

Table 2. Spearman's correlation between the various micro-organisms, chemical composition and organic acids in non-Ayrshire Cheddar cheese

	n-Valeric	Oxalic	Citric	Pyruvic	Formic	Acetic	Burytic	Isovaleric	Moisture	pH	Salt	*L. lactis*	*S. therm*
n-Valeric	1												
Oxalic	0.90*	1											
Citric	-	-	1										
Pyruvic	-0.92*	-0.93*	-	1									
Formic	0.60	0.67	-	-0.69	1								
Acetic	0.58	0.69	-	-0.62	0.97*	1							
Burytic	0.79	0.81*	-	-0.84*	0.93*	0.89*	1						
Isovaleric	0.71	0.59	-	-0.81*	0.53	-	0.71	1					
Moisture	0.66	0.63	-0.48	-0.73	0.59	0.46	0.77	0.81*	1				
pH	-0.73	-0.72	0.50	0.74	-0.50	-0.47	-0.75	-0.63	-0.69	1			
Salt	-	-	-	-	-	-	-	-	-	-	1		
L. lactis	-0.48	-0.62	-	0.68	-	-	-	-0.66	-0.51	0.58	-	1	
S. therm	-0.68	-0.75	-	0.83*	-0.61	-0.49	-0.74	-0.88*	-0.77	0.54	0.16	0.76	1

* Correlation coefficient, significant at tile $p<0.05$ level. Correlations between -0.4 and +0.4 are indicated with a – sign.

Similarities between the organic acids in the two types of cheese were that most of the organic acids correlated positively with one another, with the exception of pyruvic and citric acid. In Ayrshire Cheddar cheese, pyruvic acid formed strong negative correlations with all the other organic acids, with n-valeric acid being the exception. Although the same negative correlations between pyruvic and other organic acids were also noticeable in non-Ayrshire Cheddar cheese, none of them were as strongly negative as in Ayrshire Cheddar cheese. This shows the importance of this compound as a key intermediate in the tricarboxylic acid cycle and lipolysis. Citric acid, on the other hand, formed weak negative correlations with most of the organic acids in Ayrshire Cheddar cheese, but no correlations with the organic acids in non-Ayrshire Cheddar cheese. It seems that this pattern is unique to Ayrshire Cheddar cheese and can be used to distinguish between the two types of cheese.

The starter organisms, *Streptococcus thermophilus* and *Lactococcus lactis*, tended to correlate positive with each other in non-Ayrshire Cheddar cheese, but not in Ayrshire Cheddar cheese. Another interesting pattern that was observed in non-Ayrshire Cheddar cheese, but not in Ayrshire Cheddar cheese, was the positive correlations between the starter organisms and pyruvic acid. In Ayrshire Cheddar cheese, no or weak correlations were found between pyruvic acid and the starter organisms. However, a strong correlation between *L. lactis* and citric acid was found only in Ayrshire Cheddar cheese. In general, more negative correlations were observed between the starter organisms and the organic acids in non-Ayrshire Cheddar cheese, whereas in Ayrshire Cheddar cheese, no or weak correlations between the starter organisms and organic acids were evident.

2.6. Conclusions

By applying a concept of the X_{rel} value for cheese uniformity based on the organic acid concentrations, the aim was to identify differences between Cheddar cheese made from Ayrshire milk and that made from non-Ayrshire milk. The organic acids isovaleric, butyric and acetic acid had the least variation relative to their

concentration (X_{rel}) in both cheeses, and it is assumed that they are the most effective indicators of cheese uniformity, whereas those which showed extensive variation (larger X_{rel}) bear little or no relation to cheese uniformity. In Ayrshire Cheddar cheese, those were pyruvic acid, followed by n-valeric acid, whereas in non-Ayrshire Cheddar, those were citric acid and formic acid.

Variations in the correlation of the assessed parameters serve to further discriminate between Ayrshire and non-Ayrshire Cheddar cheese. The fact that citric acid correlates negatively with all the other organic acids in Ayrshire Cheddar cheese, with no correlations evident in non-Ayrshire Cheddar cheese, is one of the key differences between the two cheeses. Another important dissimilarity between the two cheeses is that no to weak relationships were detected between the organic acids and the starter cultures in Ayrshire Cheddar cheese. The opposite was found in non-Ayrshire Cheddar cheese, where correlations (either positive or negative) between almost all the starter organisms and the organic acids were noticeable. Based on the correlation patterns, one could prove that adequate milk separation was maintained and this may be presented during retail audits in order to protect a particular brand.

2.7. Acknowledgements

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Chapter 3

Mathematical expressions for organic acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk

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3.1. Title

Mathematical expressions for organic acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

3.2. Abstract

This study aimed to develop a mathematical model that would predict organic acid fluctuations within Cheddar cheese manufactured from pure Ayrshire milk versus Cheddar cheese produces with milk from a mix of breeds, in light of claims that the former (Ayrshire) ripens faster than the latter (mixed-breed). This mathematical framework can then be applied to the end-product organic acid profile to verify the origin of the raw milk in support of branded products. Thirty-two cheese samples of each batch (pure Ayrshire (4) / mixed with no Ayrshire milk (4)) were ripened. Samples were analysed by means of high-performance liquid chromatography (HPLC) on the following days after production: 2, 10, 22, 36, 50, 64, 78 and 92. Regression models were defined for each of the organic acid patterns by means of mathematical equations. By applying these equations, the manufacturer is able to select cheese for specialist lines and distinguish between Ayrshire and non-Ayrshire Cheddar cheese. The regression graphs illustrate an interesting flux in concentration in four of the eight organic acids analysed, i.e. acetic, formic, butyric and isovaleric acid. This sudden increase in concentration occurred at 50 to 92 days of ripening and was only found in non-Ayrshire Cheddar cheese (never in Ayrshire Cheddar cheese).

3.3. Introduction

In the case of cheese, production mathematical frameworks whereby biochemical control and product regulation can be quantified are becoming ever more popular, as they provide increased control over end-product quality. Profiles of organic acids (OAs) in Cheddar cheese have been applied as a tool to calculate the stage of, and reflect, the microbial metabolic activity during ripening and predict the glycolytic age of the cheese (Bouzas, Kantt, Bodyfelt & Torres, 1993; Buffa, Guamis,

Saldo & Trujillo, 2004; Lues & Bekker, 2002). This reflection of ripening, quantified by OA analysis, is the result of milk fat hydrolysis (lipolysis – free fatty acids such as butyric and acetic acids), normal bovine biochemical metabolism (citric, orotic and uric acid), or bacterial growth (lactic, acetic, pyruvic, propionic and formic acid) (Collins, McSweeney & Wilkinson, 2003; Izco, Tormo & Jiménez-Flores, 2002; Lane & Fox, 1996; Marilley & Casey, 2004). In addition, OAs are the major products of the carbohydrate metabolism of lactic acid bacteria (LAB) and play a significant role in the flavour of cheese (Lane & Fox, 1996; Papadakis & Polychroniadou, 2005). These short-chain acids (OAs) are also important secondary carbon sources for the starter cultures and are intermediates and metabolites of a variety of biochemical processes that occur during cheese-ripening (Bevilacqua & Califano, 1992; Lues, 2000; Marsili, Ostapenko, Simmons & Green, 1981).

Lues and Bekker (2002) investigated the possibilities of predicting the quality of cheese during early maturation by applying linear and non-linear regression modelling for the individual organic acids. Likewise, several papers have provided detailed descriptions of the application of least-square correlation coefficients, two-way multivariate analysis of variance, stepwise regression analysis, stepwise discriminant analysis, and post-hoc multiple comparisons to qualify the most suitable predictors of the glycolytic age of Cheddar cheese (Mohler-Smith & Nakai, 1990; Pham & Nakai, 1983; Bouzas *et al*., 1993). Predictive models of OAs during cheese-ripening were also attained by fitting experimental data for acetic, propionic, butyric and isovaleric acids to exponential functions (Bouzas *et al*., 1993).

In addition to the application of OAs as quality predictors, several studies have evaluated the possibility of manipulating natural aging and subsequently the OA profile of cheese in order to reduce ripening time (Law, 1987). Of course, the latter serves to reduce production costs, leading some manufacturers to opt for milk from selected breeds that boast an OA profile conducive to a reduced ripening period. Consequently, predictive models become more relevant and might even be applicable in the selection of milk type for cheese production.

Cheese manufacturers have also claimed that milk from a single controlled breed (Ayrshire), when applied in the manufacturing of Cheddar cheese, reduces the ripening period when compared to the application of milk from mixed herds (according to personal communication with an Ayrshire cheese manufacturer). Retailers brand this cheese as such and also infer that their product boasts unique organoleptic properties. However, no factual evidence or mathematical models exist to support these claims, thus limiting the information available for a proper biological control analysis.

The aim of this study was therefore to develop a mathematical model that would predict organic acid fluctuations (quality predictors) within Cheddar cheese manufactured from pure Ayrshire milk versus Cheddar cheese produced from milk from a mix of breeds. In addition to predicting product outcome, this study also sought to produce a mathematical framework that could be applied to the end product's OA profile to verify the origin (pure Ayrshire or mixed) of the raw product (milk) in support of branded products. The latter also bears weight where larger producers manufacture cheese for both boutique and bulk retailers. Here, OA profiles and models of the final product could prove that adequate milk separation was maintained and may be presented during retail audits in order to protect a particular brand.

3.4. Materials and methods

3.4.1. Manufacturing of Cheddar cheese

As no single batch of commercially produced Cheddar cheese is identical, care was taken to design experimental procedures that would limit extrinsic / intrinsic variability amongst batches. Therefore, two Cheddar cheese batches were manufactured in parallel, under the same conditions in the same factory / equipment at the same time; the only difference being the milk source. One batch was manufactured exclusively from Ayrshire (A) milk and the parallel batch from a mixture of other breeds' milk (excluding Ayrshire milk) (NA). The Cheddar cheese was manufactured in a closed-vat system with the following standard degree of

variation in chemical parameters of the cheese at time 0 (n=4 / batch): salt = 1.32 ± 0.049 (A), 1.88 ± 0.035 (NA); moisture = 37.73 ± 0.1 (A), 36.18 ± 0.3 (NA); pH = 5.3 ± 0.02 (A), 5.4 ± 0.02 (NA). Subsequently, the organic acid profiles of all eight samples (four from the A batch and four from the NA batch) were monitored for a period of 92 days.

3.4.2. Chemical analysis

The method proposed by Bouzas *et al*. (1991, 1993) was applied to extract organic acids from all cheese samples, followed by quantification using the HPLC method proposed by Lues, Botha and Smit (1998). For the identification and quantification of relevant organic acids the following external standards were used: acetic, butyric, citric, formic, isovaleric, malic, oxalic, pyruvic, and *n*-valeric acids (Sigma-Aldrich, RSA). In this study, organic acids were quantified by means of HPLC using an automated system (Spectra Physics) equipped with a solvent degasser (SCM 400), quaternary gradient solvent pump (P4000), multi-autosampler (AS1000) fitted with a 50µl loop, and a spectral array UV detector (UV3000) set at 210 and 290 nm. All chromatography was carried out on a Rezer ROA organic acid hydrogen form (300 x 7.8 mm) ion-exchange chromatography column (Phenomenex, USA), at a constant temperature of 30°C. A flow rate of 1 $ml.min^{-1}$ was maintained with a 0.1% phosphoric acid mobile phase, run isocratically. The injection volume was 40µl with a 40-minute running time.

3.4.3. Computation

All results used in the computational analysis were the means of quadruplet analysis. Curve fits were performed for the individual organic acids by means of Sigma Plot for Windows version 10.0.

3.5. Results and discussion

3.5.1. Separation, optimisation and validation

Table 1 shows the results obtained from the validation of the HPLC analytical technique for nine different water-soluble organic acids. Three different detectors (UV at 210 and 290 nm, and refractive index) were applied to determine the optimal detector to be used for each of the organic acids analysed. Calibration curves were calculated by analysing 0.01, 0.05, 0.1, 0.5 and 1 M stock solutions. Good linear response was found over a wide range of concentrations for all the organic acids. HPLC detection of organic acids by applying different detectors has previously been reported in the literature (Lues, 2000; Upreti, McKay & Metzger, 2006). This part of the study was done to validate the accuracy of the analytical technique used by these scientists. Not surprisingly, refractive index did not show any remarkable detection properties for organic acids and has been reported in the literature to detect mainly sugars (Lues, 2000; Upreti *et al.*, 2006) and not organic acids. The results show that the optimal detection range of pyruvic acid is at UV 290 nm, while for all the other water-soluble organic acids analysed, optimal detection range is at UV 210 nm.

3.5.2. Organic acid fluctuations and possible explanations

Figure 1 represents the organic acids in Ayrshire and non-Ayrshire Cheddar cheese as detected by HPLC. Citric acid is a compound formed in the tricarboxylic acid and/or glyoxylate cycle by the physiological oxidation of fats, proteins and carbohydrates by starter cultures during cheese-ripening (McKee & McKee, 1999). When this compound is formed by starter bacteria and sometimes yeasts, it is normally excreted as metabolic by-products into the extracellular cheese environment (McKee & McKee, 1999).

Table 1. Retention time (RT) for the different organic acids analysed, their detection and calibration curves

		UV Detection			
Analyte	**RT min**	**210 nm**	**290 nm**	R^2	**Calibration equation**
n-Valeric	6.4	X		0.99	$\frac{\text{Area UV}_{210\text{nm}} + 3.10^7}{2.10^7}$
Oxalic	10.8	X	X	0.99	$\frac{\text{Area UV}_{210\text{nm}} - 1.10^7}{3.10^7}$
Citric	13.3	X		0.96	$\frac{\text{Area UV}_{210\text{nm}} + 1.10^7}{1.10^7}$
Pyruvic	15.4		X	0.95	$\frac{\text{Area UV}_{290\text{nm}} + 1.10^6}{1.10^6}$
Malic	15.8	X		0.99	$\frac{\text{Area UV}_{210\text{nm}} + 5.10^7}{5.10^7}$
Formic	20.9	X		0.95	$\frac{\text{Area UV}_{210\text{nm}} + 7.10^6}{1.10^7}$
Acetic	22.3	X		0.97	$\frac{\text{Area UV}_{210\text{nm}} + 5.10^7}{4.10^7}$
Butyric	33.4	X	X	0.96	$\frac{\text{Area UV}_{210\text{nm}} + 8.10^7}{7.10^7}$
Isovaleric	36.2	X		0.97	$\frac{\text{Area UV}_{210\text{nm}} + 6.10^7}{4.10^7}$

Figure 1 (A) shows the changes in citric acid concentration over the ripening time. Irregular changes in citric acid over the ripening period were detected in both Ayrshire and non-Ayrshire Cheddar cheese. A sharp decrease in citric acid was observed for the first 50 to 64 days after ripening, followed by a dramatic increase in both Ayrshire and non-Ayrshire Cheddar cheese. This decrease is most probably due to the consumption of available carbon sources, lactose, and short-chain fatty acids. A similar pattern in citric acid metabolism in Cheddar cheese was also observed by Akalin, Gönç & Akbast (2002) and Lues and Bekker (2002) in white pickled cheese and Cheddar cheese respectively. In non-Ayrshire Cheddar cheese, the increase after 50 days started earlier than in Ayrshire Cheddar (64 days). The

amount of citric acid produced after 92 days was also higher in non-Ayrshire Cheddar cheese (1.31 M) than in Ayrshire Cheddar cheese (1.28 M), but it did not show any significant difference ($p = 0.17$). The first decrease observed can be explained by the conversion of citrate to pyruvate or acetate by citrate-fermenting micro-organisms (i.e. *Lactococcus*) (Adda, Gripon & Vassal, 1982; Bouzas *et al.*, 1993; Starrenburg & Hugenholtz, 1991). The following increase in citric acid (after 64 days of ripening) can most probably be ascribed to the physiological oxidation of fats and proteins by starter cultures later during cheese-ripening, which forms acetyl-CoA, which in turn can be converted into citric acid (McKee & McKee, 1999) for energy.

Oxalic acid (Fig. 1 (b)) is a relatively strong organic acid, *circa* 10,000 times stronger than acetic acid, due to the joining of two carboxyl groups (McKee & McKee, 1999). The formation of oxalic acid is, however, more closely associated with fungal metabolism (Schlegel, 1992) where oxaloacetate is hydrolysed to oxalic acid by oxaloacetate hydrolase. Since no fungi were incorporated in the production of the cheese for this experiment, its increase in this study is strange and was not expected. Oxalic acid was also the only organic acid studied that could be detected by all the detectors and different wavelengths used (210 nm, 290 nm and RI) (Table 1). An upward pattern was observed for all three detection experiments (210 nm, 290 nm and RI), but the amounts did not correlate well with one another.

According to the literature, the best detectable range for oxalic acid is at 210 nm with a UV detector (Lues & Botha, 1998). In this study, the oxalic acid concentration (Fig. 1 (b)) increased in both Ayrshire and non-Ayrshire Cheddar cheese throughout the ripening period. The initial difference in oxalic acid concentrations can be attributed to either batch differences, or differences in the composition of Ayrshire and non-Ayrshire milk. However, higher concentrations were detected in non-Ayrshire than in Ayrshire Cheddar cheese throughout the ripening process.

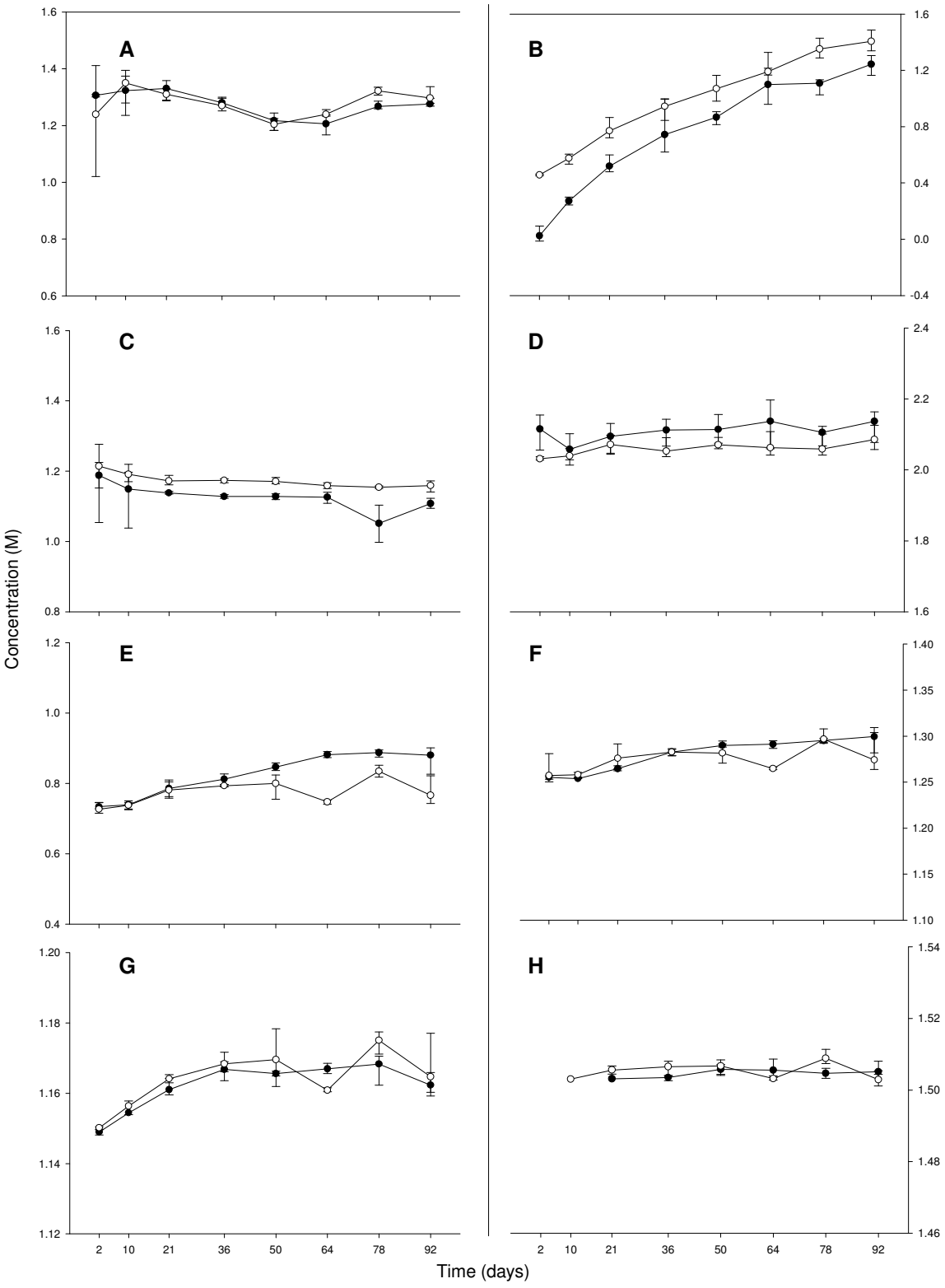

Figure 1. Changes in (a) Citric; (b) Oxalic; (c) Pyruvic; (d) n-Valeric; (e) Formic; (f) Acetic; (g) Butyric and (h) Isovaleric acid during ripening of Ayrshire (—●—) and non-Ayrshire (—○—) Cheddar cheese

As ripening persisted, the oxalic acid concentrations of the two different cheeses grew closer at 92 days. This is most probably where the physiological conditions are optimal for oxalic acid production. This compound increased constantly over time and might thus be a co-product of a different metabolism, i.e. catabolism of fatty acids or proteins that release carboxyl groups, which join together to form oxalic acid.

Pyruvic acid (Fig. 1 (c)) is a key intermediate in lactose metabolism and is readily formed though the glycolytic, tagatose and Leloir pathways (Cogan & Daly, 1987; Upreti, *et al.*, 2006). It also unites several metabolic reactions, i.e. to carbohydrates via gluconeogenesis, to fatty acids or energy through acetyl-CoA, to the amino acid alanine, to ethanol, and to formate (Axelsson, 1993; McKee & McKee, 1999). In this study the pyruvic acid concentration decreased slightly during the ripening process in both Ayrshire and non-Ayrshire Cheddar cheese, by 6.3% and 5.2% respectively over the 92-day ripening period. An initial decrease in pyruvic acid formation was also observed by Akalin *et al.* (2002) during the first and third months of ripening of pickled white Cheddar. The initial concentration of pyruvic acid in non-Ayrshire Cheddar cheese was slightly higher than in Ayrshire Cheddar cheese and remained higher throughout the ripening process. Although a slight decrease was observed, the change in pyruvic acid concentration remained almost constant throughout the ripening period, which shows that this compound is a key intermediate in lactose metabolism (Cogan & Daly, 1987; Upreti, *et al.*, 2006).

Work by Gerson, Hawke and Shorland (1960) indicated that n-valerate might be formed during the addition of acetate to propionate as an intermediate in the synthesis of the higher n-odd numbered acids in the cow's udder. It may seem that the formation of n-valeric acid is thus not microbial related, since it is formed in the udder and remains more or less constant throughout the 92-day ripening period. In Ayrshire Cheddar cheese, the initial concentration of n-valeric acid (Fig. 1 (d)) was found to be higher (2.11 M) than in non-Ayrshire Cheddar cheese (2.03 M). This difference might be ascribed to the different nature and composition of Ayrshire vs. non-Ayrshire milk.

The formation of formic acid (Fig. 1(e)) could be ascribed to the pyruvate-formate lyase system where pyruvate-formate lyase catalyses the reaction of pyruvate and CoA to formate and acetyl-CoA. This system has been shown to be active in several LAB species and becomes active during anaerobic conditions when substrate becomes limiting, resulting in a change from homolactic to heterolactic fermentation (Kandler, 1983; Moat, 1985; Oliveira, Nielson & Förster, 2005; Thomas, Ellwood & Longyear, 1979). The end products formed under this system are lactate, acetate, ethanol and formate (Axelsson, 1993; Derzelle, Botolin, Mistou & Rul, 2005). Formic acid was formed during the first 21 days of ripening in both Ayrshire and non-Ayrshire Cheddar cheese. Similar results were found by Akalin *et al*. (2002) in pickled white cheese and by Califano and Bevilacqua (1999) in mozzarella, where an increase was observed during the second month of ripening, after which it remained constant. This formation might be explained by the presence of *Streptococcus salivarius* subsp. *thermophilus*, which produces formic acid from lactose (Akalin *et al*., 2002). After 21 days, the formic acid concentration in Ayrshire Cheddar cheese increased to a concentration of 0.89 M where it remained constant. In non-Ayrshire Cheddar cheese, a slight decrease was first observed between 36 and 64 days of ripening, after which it increased but to a smaller degree, as was noted in Ayrshire Cheddar cheese at 92 days. Interestingly, the flux pattern was noticed at day 50 to 92 of ripening in non-Ayrshire Cheddar cheese, but not in Ayrshire Cheddar cheese. The same flux pattern was also noted in the acetic, butyric and isovaleric acid concentrations after 50 days of ripening in non-Ayrshire Cheddar cheese.

Acetic acid (Fig. 1 (f)) could be produced from citrate, lactose or amino acids (Izco *et al*., 2002; Upreti *et al*., 2006), which also provides a measurement of heterofermentative metabolism under anaerobic conditions (Kandler, 1983; Moat, 1985; Oliveira *et al*., 2005). Under aerobic conditions, lactate can be metabolised to acetate and CO_2 by stereospecific NAD-independent, flavin-containing lactate dehydrogenases or lactate oxidases (Kandler, 1983). Depending on the carbon source, enzyme capability and pH, acetate can be produced by LAB under aerobic and anaerobic conditions by switching between homolactic to heterolactic

fermentation, using either the tricarboxylic acid, glyoxylate cycle, or pentose phosphate (phosphoketolase) pathway (Moat, 1985; Oliveira *et al*., 2005). In non-Ayrshire Cheddar cheese, a different pattern in acetic acid concentration with fluctuations throughout the ripening period was observed, which emphasises its function as an intermediate in many biochemical pathways. A similar irregular pattern in acetic acid was also reported by Akalin *et al.* (2002) in pickled white cheese. In Ayrshire Cheddar cheese a gradual increase in acetic acid was observed throughout the ripening process, which was also reported by Bouzas *et al*. (1991). The same unstable flux pattern in organic acid concentration was also observed at 50 to 92 days of ripening in non-Ayrshire Cheddar cheese, as was found in formic, butyric and isovaleric acid concentrations.

The formation of free butyric acid (Fig. 1 (g)) during cheese-ripening is mainly due to the hydrolysis of milk fat by lipolytic activity of the starter cultures and secondary microbiota (Akalin *et al*.., 2002; Izco *et al*., 2002; Singh, Drake & Cadwallader, 2003). Lipolysis in milk preferentially releases short- and medium-chain fatty acids, because in milk triglycerides, short-chain fatty acids are esterified predominantly at the sn-3 position. This specificity probably explains the disproportionate concentration of free butyric acid in cheese (Singh *et al*., 2003). Although butyric acid in cheese can be mainly ascribed to the action of esterases and lipases of the starter microbes, Upreti *et al*. (2006) stated that lactose could also be a source of the formation of this acid. Butyric acid increased as ripening progressed in Ayrshire and non-Ayrshire Cheddar cheese respectively. However, the concentration in Ayrshire Cheddar remained somewhat lower than in non-Ayrshire Cheddar cheese. A similar increase in this compound during cheese-ripening has also been observed by other scientists (Akalin *et al*., 2002; Lues & Botha, 1998; Upreti, *et al*., 2006). A flux in concentration was observed in non-Ayrshire Cheddar cheese after 50 days of ripening, which was not observed in Ayrshire Cheddar cheese.

The formation of isovaleric acid (Fig. 1 (h)) can be ascribed to the degradation of branched-chain amino acids, such as leucine (Rijnen, Courtin, Gripon & Yvon, 2000; Yvon, Chambellon, Bolotin & Roudot-Algaron, 2000). It was reported by Yvon

et al. (2000) that the first step in leucine degradation by *Lactococcus lactis* subsp. *cremoris* is a transamination, where this reaction is catalysed by branched-chain or aromatic aminotransferases and requires an α-keto acid as amino group acceptor (Rijnen *et al*., 2000; Thierry, Maillard & Yvon, 2002; Yvon, Berthelot & Gripon, 1998). This step is followed by an enzymatic conversion to isovaleric acid (Yvon *et al*., 2000). In Ayrshire Cheddar cheese, isovaleric acid was only produced after 21 days of ripening, but in non-Ayrshire Cheddar cheese, earlier formation of this compound was observed at 10 days of ripening. This difference in time when it comes to the formation of this compound might be ascribed to the difference in milk composition between Ayrshire and non-Ayrshire milk and consequently leucine degradation afterwards. The isovaleric acid concentration remained more or less constant throughout the ripening period in both Ayrshire and non-Ayrshire Cheddar cheese.

In the case of non-Ayrshire Cheddar cheese, after 50 days of ripening, an interesting fluctuation pattern was observed in the formic, acetic, butyric and isovaleric acids. This fluctuation pattern is unique to non-Ayrshire Cheddar cheese, and more research is required to determine whether this flux pattern could be extrapolated to more than one batch.

3.5.3. Mathematical estimations

The mathematical equations and coefficients describing the changes in eight water-soluble organic acids during maturation of Ayrshire and non-Ayrshire Cheddar cheese are shown in Table 2 and Table 3 respectively. These changes were defined by mathematical equations with R^2 values ranging from 0.92 – 0.99.

All the organic acids (Ayrshire and non-Ayrshire) were best represented by a polynomial equation. The mathematical equations proposed in these tables may be applied to monitor changes in microbial and biochemical parameters influenced directly or indirectly by the organic acids. In addition, some of the organic acids known to contribute directly to the flavour of cheese, such as butyric and isovaleric acids, could be added in quantities calculated by using the mentioned equations to achieve precise quality attributes. In order to save time and money, these equations

can serve as elements of a model for the following: (1) to calculate the age of the cheese using organic acid data as variable; (2) to predict the concentration of a certain organic acid at a certain time during early maturation; and (3) to distinguish between Ayrshire and non-Ayrshire Cheddar cheese. However, some organic acids will be more accurate indicators of maturation than others, and therefore a consolidated formula that relates the information of all the organic acids should give a more accurate estimate of the extent of maturation. Relative to the cheese investigated in this study, the following two formulas (Ayrshire and non-Ayrshire Cheddar cheese) may be proposed as a collective representation of the development of organic acids during maturation:

Ayrshire:

$$y = \frac{-0.0018x^6 - 0.4144x^5 + 0.5491x^4 - 3.0313x^3 + 8.6905x^2 - 11.6583x + 14.7781}{8}$$

Non-Ayrshire:

$$y = \frac{-0.00054x^6 + 0.0135x^5 - 0.1194x^4 + 0.5119x^3 - 1.1131x^2 + 1.3666x + 8.9786}{8}$$

Where:

x = maturation time

y = organic acid concentration

Table 2. Mathematical equations representing the patterns of organic acids during the maturation of Cheddar cheese made from Ayrshire milk

Variable	Estimation summary		
Organic acid	Equation	Coefficients	R^2
Citric	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.0008 **b** 0.018 **c** -0.142 **d** 0.4886 **e** -0.7176 **f** 1.6597	0.9987
Pyruvic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0001 **b** -0.0026 **c** 0.0244 **d** -0.1154 **e** 0.2879 **f** -0.3777 **g** 1.3719	0.9976
n -Valeric	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0002 **b** -0.0059 **c** 0.0631 **d** -0.3403 **e** 0.9712 **f** -1.3561 **g** 2.7733	0.9965
Oxalic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0015 **b** -0.405 **c** 0.4417 **d** -2.4117 **e** 6.8293 **f** -8.9936 **g** 4.2008	0.9945
Formic	$y = ax^3 + bx^2 + cx + d$	**a** -0.0017 **b** 0.0204 **c** -0.0401 **d** 0.7559	0.9940
Acetic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.00004 **b** 0.001 **c** -0.0108 **d** 0.0505 **e** -0.0929 **f** 1.3075	0.9944
Butyric	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.00003 **b** 0.0007 **c** -0.0063 **d** 0.0229 **e** -0.0295 **f** 1.1611	0.9952
Isovaleric	$y = ax^4 + bx^3 + cx^2 + dx + e$	**a** 0.0002 **b** -0.0031 **c** 0.0197 **d** -0.0508 **e** 1.5479	0.999

R^2 = correlation coefficient

Table 3. Mathematical equations representing the patterns of organic acids during the maturation of Cheddar cheese made from non-Ayrshire milk

Variable	Estimation summary		
Organic acid	Equation	Coefficients	R^2
Citric	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.0005 **b** 0.0102 **c** -0.0661 **d** 0.1614 **e** -0.1141 **f** 1.2977	0.9635
Pyruvic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.00007 **b** 0.002 **c** -0.0214 **d** 0.1145 **e** -0.3044 **f** 0.352 **g** 1.0776	0.9987
n -Valeric	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0001 **b** -0.003 **c** 0.0319 **d** -0.1677 **e** 0.452 **f** -0.5612 **g** 2.2806	0.9765
Oxalic	$y = ax^3 + bx^2 + cx + d$	**a** -0.0012 **b** 0.0114 **c** 0.1273 **d** 0.3105	0.9961
Formic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0003 **b** 0.0079 **c** -0.0741 **d** 0.3335 **e** -0.7504 **f** 0.8141 **g** 0.3988	0.941
Acetic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0001 **b** 0.003 **c** -0.0279 **d** 0.1223 **e** -0.2597 **f** 0.2499 **g** 1.1781	0.924
Butyric	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.00007 **b** 0.0018 **c** -0.0176 **d** 0.0848 **e** -0.2083 **f** 0.2506 **g** 1.0387	0.9701
Isovaleric	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0001 **b** 0.0023 **c** -0.0205 **d** 0.0918 **e** -0.2151 **f** 0.248 **g** 1.3966	0.9721

R^2 = correlation coefficient

A less-intricate methodology that may apply to the collective contribution of the organic acids would be to calculate the average age ($\bar{x}$) obtained from solving the individual equations depicted in Table 2 and Table 3.

3.6. Conclusions

The equations represented in this study are applicable to only one South African Cheddar cheese manufacturer, and their applicability to Cheddar cheese made in other manufacturing plants still needs to be established. Nevertheless, their applicability as single equations or as part of a combined mathematical model is promising. Even so, by applying these equations, the manufacturer can see promising outcomes: (1) early exclusion of defective cheeses becomes possible; (2) cheese can be selected for specialist lines; (3) Ayrshire and non-Ayrshire Cheddar cheese can be established at an early stage during ripening. Besides the use of the equations, Ayrshire and non-Ayrshire Cheddar cheese can also be distinguished by simply observing the flux pattern at 50 to 92 days of ripening. In addition to the outcomes of this article, proteolysis and lipolysis mathematical modelling can be added for an entire portrayal of the biochemical events in Ayrshire and non-Ayrshire Cheddar cheese development.

3.7. Acknowledgements

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Chapter 4

Mathematical indices for fatty acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk

Submitted for publication to International Dairy Journal.

4.1. Title

Mathematical indices for fatty acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk

4.2. Abstract

Since Ayrshire Cheddar has been shown to boast unique organoleptic properties compared to other cheeses, this study focused on the differences between Cheddar cheese produced from milk from mixed sources (different bovine breeds) and those produced exclusively from a single source (Ayrshire breed) in terms of its methylated fatty acid content. The aim was also to cast light on regression models for defining the fatty acid patterns by means of mathematical equations. Thirty-two cheese samples of each batch (pure Ayrshire (4) / mixed with no Ayrshire milk (4)) were ripened for 92 days and fatty acid analysis was performed every 14 days on a GC-MS. The regression graphs showed that Ayrshire Cheddar cheese was always accompanied by a peak at day 10 of ripening in all the even-numbered medium-chain saturated fatty acids ($C_{8:0}$; $C_{10:0}$; $C_{12:0}$) and had a higher concentration of unsaturated fatty acids than non-Ayrshire Cheddar cheese. Non-Ayrshire Cheddar cheese, on the other, hand showed a consistently higher concentration of even-numbered medium-chain saturated fatty acids ($C_{7:0}$; $C_{9:0}$; $C_{11:0}$). By applying the mathematical equations from the regression models, specific outcomes – i.e. the selection of cheese for specialist lines, the early exclusion of defective cheeses, and the establishment of brand origin (Ayrshire vs. mixed-breed Cheddar cheeses) – could be achieved.

4.3. Introduction

Cheese quality is mostly determined by its flavour, visual appearance, and rheological properties (Akin, Aydemir, Koçak & Yildiz, 2003). The lipid fraction of cheese has a major effect on the cheese texture and is important for the perception and development of cheese flavour (Singh, Drake & Cadwallader, 2003). Free fatty acids (FFAs), released upon lipolysis and oxidation, contribute directly to cheese

flavour (Partidário, Barbosa & Vilas Boas, 1998) and constitute the principal biochemical transformation during ripening. Lipolysis results from lipolytic enzymes (lipases and esterases), which are hydrolases that cleave the ester bond between a fatty acid and the glycerol backbone of the triacylgleceride (Collins, McSweeney & Wilkinson, 2003), leading to the production of FFAs, diglycerides and monoglycerides, and possibly glycerol (Singh *et al*., 2003). Extensive lipolysis is, however, considered undesirable in cheese varieties such as Cheddar, Dutch and Swiss-type cheeses and can be perceived by customers as rancid (Akin *et al*., 2003; Marilley & Casey, 2004). Lipid oxidation, however, does not occur to a significant extent in cheese, probably due to its low redox potential (-250V) and the presence of natural antioxidants such as vitamin E (Fox & Wallace, 1997; Guinee & Fox, 1987).

Furthermore, the main properties and organoleptic quality of ripened cheeses such as Cheddar are largely due to differences (origin) in the raw material (milk) and key cheese-manufacturing processes (Atasoy & Türkoglu, 2009; Hernández, Barrón, Virto, Pérez-Elortondo, Flanagan, Rozas, Nájera, Albisu, Vicente & De Renobales, 2009; Hickey, Kilcawley, Beresford, Sheehan & Wilkinson, 2006; Pappa, Kandarakis, Anifantakis & Zerfiridis, 2006). In South Africa, a certain leading retailer has stated the intention to supply dairy products made exclusively from the Ayrshire breed, since the retailer believes that this product boasts unique organoleptic properties. It is, however, difficult for this retailer to verify the origin of the milk used in the production of especially cheese by secondary suppliers. A unique catabolite profile of these products, in addition to an aroma and flavour fingerprint, could help the retialer to establish the origin of milk used in the production of their products. This method could be applied in future to profile their cheese as part of their already thorough quality assurance system.

The application of chemometric classification studies to analytical parameters is commonly used to determine the geographical origin or quality of a particular brand of food product (Barile, Coïsson, Arlorio & Rinaldi, 2006; Pillonel, Badertscher, Casey, Meyer, Rossmann, Schlichtherie-Cerny, Tabacchi & Bosset, 2005; Pillonel, Bütikofer, Schlichtherie-Cerny, Tabacchi & Bosset, 2005; Sacco, Brescia, Buccolieri & Caputi Jambrenghi, 2005; Sacco, Brescia, Sgaramella, Casiello, Buccolieri, Ogrinc

& Sacco, 2009). In addition to the application of chemometrics to validate food authenticity, the use of linear regression has also been applied (Bouzas, Kantt, Bodyfelt & Torres, 1993; Buffa, Guamis, Saldo & Trujillo, 2004; Lues & Bekker, 2002). There is a recent trend towards production mathematical frameworks whereby biochemical control and product regulation can be measured, since such frameworks provide better control over the quality of the end product. In the case of cheese, profiles of organic acids have been applied as an instrument to calculate the stage of ripening and also predict the age of the cheese (Bouzas *et al*., 1993; Buffa *et al*., 2004; Lues & Bekker, 2002). Lues and Bekker (2002) investigated the possibility of predicting the quality of cheese during early maturation by applying linear and non-linear regression models to individual organic acids.

The principal aim of this study was to develop a mathematical model that would predict methylated fatty acid fluctuations (quality predictors) within Cheddar cheese produced from the milk of a single breed (Ayrshire) versus cheese produced from a mixture of other breeds' milk (non-Ayrshire). It is anticipated that this mathematical framework will be applicable to the fatty acid profile of the end product so as to verify the origin (mixed or pure Ayrshire breed) of raw milk as a means of supporting a product branded as such.

4.4. Materials and methods

4.4.1. Manufacturing and sampling of Cheddar cheese

Since Cheddar cheese varies amongst batches, care was taken to design an experimental procedure that would limit extrinsic / intrinsic variability between the two batches involved in this study. Two batches of Cheddar cheese were therefore manufactured in parallel, under the same controlled conditions in the same factory at the same time, using the same equipment. The only difference was the milk source used. One batch was manufactured exclusively from Ayrshire (A) milk and the other batch from a mixture of other breeds' milk (excluding Ayrshire milk – NA). The Cheddar cheese was manufactured in a closed-vat system. Subsequently, the methylated fatty acid profiles and total lipid percentages of all eight samples (four

from batch A and four from batch NA) were monitored for a period of 92 days, at 14-day intervals.

4.4.2. Fatty acid extraction

Total lipids were extracted from the Cheddar cheese samples using chloroform/methanol (2:1, by vol.) as described by Folch, Lees and Sloane-Stanley (1957). The lipids were dissolved in diethyl ether and transferred to pre-weighed vials. The samples were dried to a constant weight in a vacuum oven at 50°C over P_2O_5.

4.4.3. Fatty acid analysis

Trans-esterification of cheese lipids was performed through the addition of trimethyl sulphonium hydroxide (TMSOH) according to the method of Butte (1983). The fatty acid methyl ester was analysed and separated on a Finnigan Focus GC equipped with a 30 m × 0.25 mm ZB-1 (Separations, RSA) glass capillary column containing 100% dimethyl polysiloxane (0.25 µm) with helium as carrier gas (constant flow — 3.0 ml min^{-1}) and operated in a split-less mode of injection. The temperature programme can be summarised as follows: 40 to 90 °C at a rate of 8 °C min^{-1} followed by a ramp from 90 to 280 °C at 10 °C min^{-1}. The column was coupled to a Finnigan Focus DSQ mass spectrometer for mass detection of fragments with an *m*/*z* smaller than 1000. Mass analysis was performed at 70 eV with an ion source temperature of 200 °C. Integration of the peaks was performed on the Total Ion (Current) chromatogram using Xcalibur software (Finnigan). All analyses were conducted at least in duplicate.

4.4.4. Computations

The external standards method was used for identification and quantification purposes. All results used in the computational analysis were the means of

quadruplet analysis. Curve fits were for the individual fatty acids by means of Sigma Plot for Windows version 10.

4.5. Results and discussion

4.5.1. Total lipid turnover

Figure 1 shows the total lipid percentage of Ayrshire and non-Ayrshire Cheddar cheese respectively for the 92-day ripening period as quantified by GC-MS. Although non-Ayrshire Cheddar cheese had a slightly higher total lipid percentage (33.8%) than Ayrshire Cheddar cheese (33.2%) throughout the ripening process, the pattern remained the same for both cheeses. The values obtained correlate well with those found in the literature on Cheddar cheese, namely 30.5% or more fat (wet weight) (Renner, 1993). The Cheddar cheese contents and values obtained during this study correlate well with those found in the relevant literature. The lipid fraction is important for the development of typical flavour and texture. It is well known that a higher lipid content leads to a less firm and more elastic body, while products with a low lipid content have the tendency to be harder, more brittle and less smooth (Singh *et al.*, 2003). One possible explanation for the high standard deviation in the lipid percentage throughout ripening could be the variation between the batches.

4.5.2. Methylated fatty acid fluctuations

Figure 2 represents the short-chain methylated fatty acids in Ayrshire and non-Ayrshire Cheddar cheese over time. Irregular changes in both Ayrshire and non-Ayrshire Cheddar cheese were detected over the ripening period. The methylated butanoic acid ($C_{4:0}$) concentration (Fig. 2A) remained more or less constant over time in non-Ayrshire Cheddar cheese (14.4 $mg.g^{-1}$ cheese).

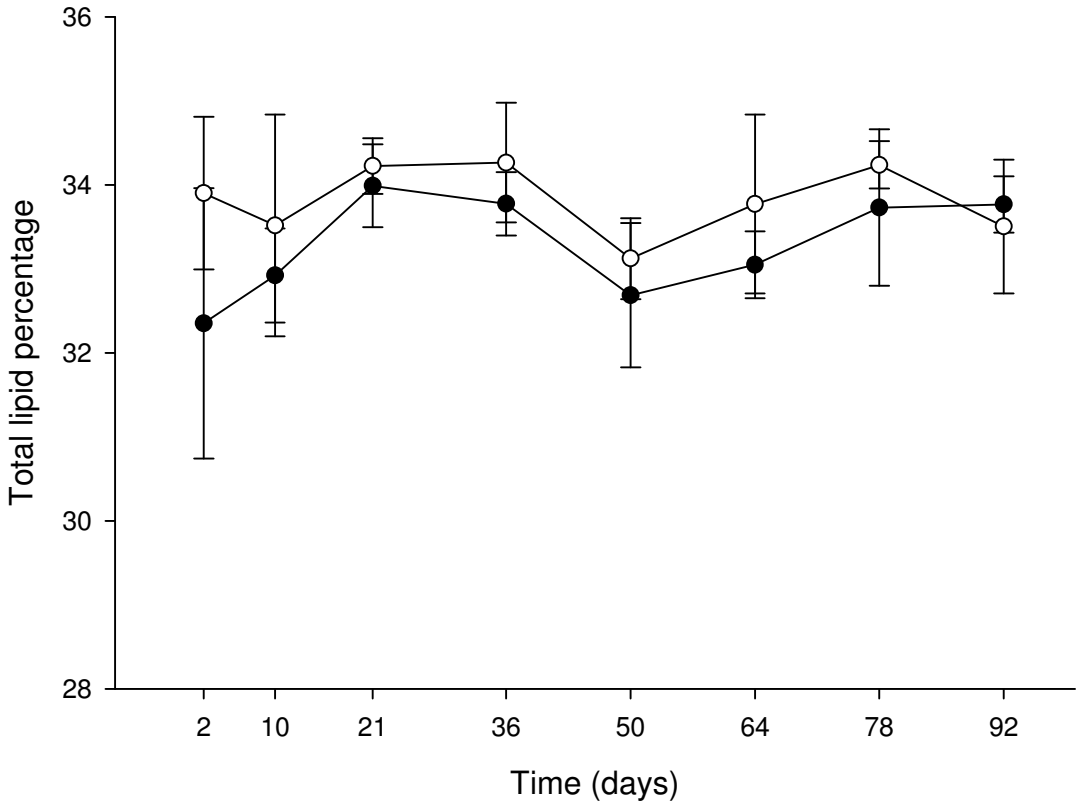

Figure 1. Total lipid content (%) of Cheddar cheese manufactured from Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese respectively

In Ayrshire Cheddar cheese, a sharp increase in methylated $C_{4:0}$ was observed at day 10, after which it decreased again to about the same value as non-Ayrshire Cheddar at that time (13.5 $mg.g^{-1}$). Cheese normally contains a high concentration of free $C_{4:0}$, suggesting that this compound is selectively hydrolysed or synthesised by the cheese microbiota (Singh *et al.*, 2003). Slightly higher concentrations of methylated hexanoic acid ($C_{6:0}$) than butanoic acid ($C_{4:0}$) were reached, with an average of 32.2 and 29.6 $mg.g^{-1}$ respectively in Ayrshire and non-Ayrshire Cheddar cheese. These short-chain fatty acids are known to make a major contribution to the aroma of cheese.

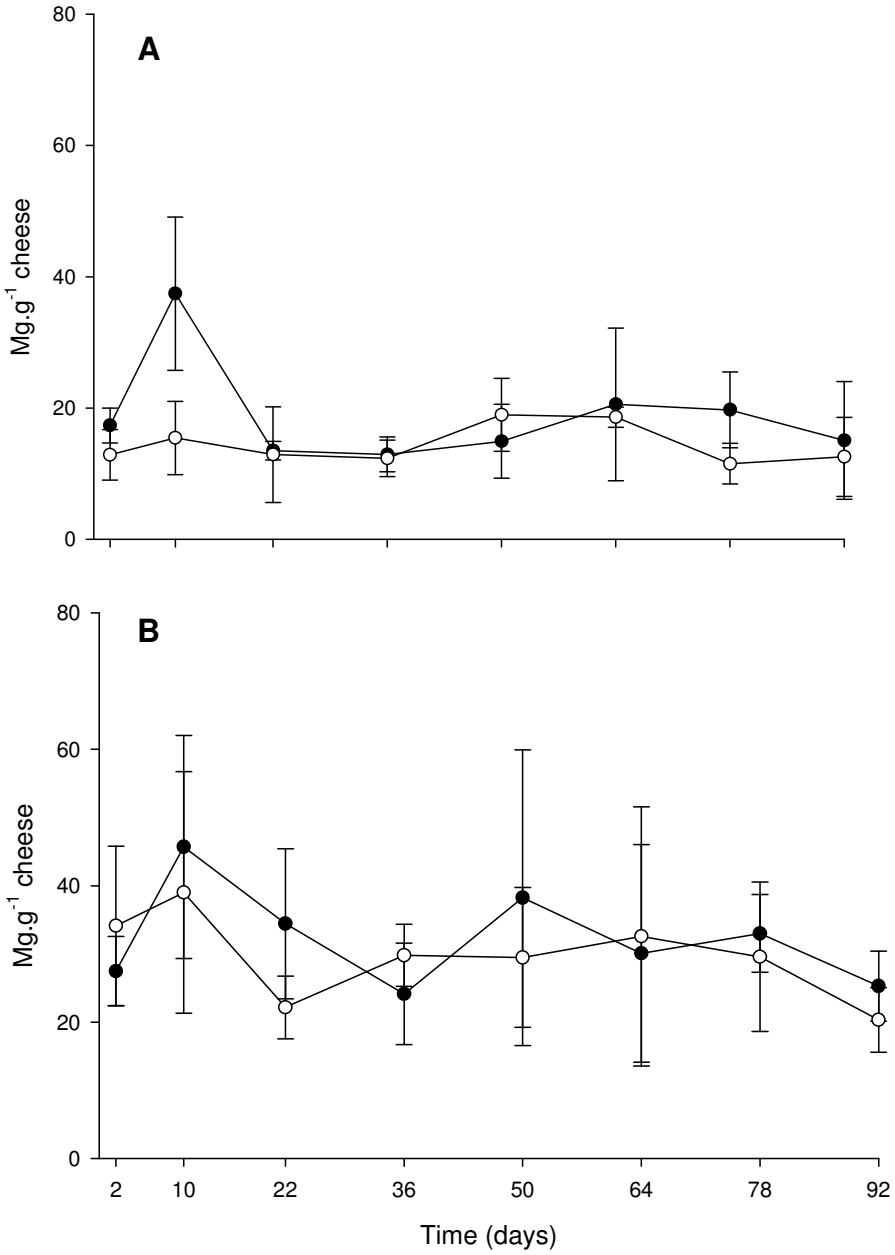

Figure 2. Changes in short-chain fatty acids in Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese respectively. A = Butanoic acid ($C_{4:0}$); B = Hexanoic acid ($C_{6:0}$)

The medium-chain methylated fatty acids present in Ayrshire and non-Ayrshire Cheddar cheese are represented in Figure 3 (A-F). Low concentrations of methylated heptanoic acid ($C_{7:0}$) were detected in both Ayrshire and non-Ayrshire Cheddar cheese (Fig. 3A), with an average concentration of 0.35 and 0.36 $mg.g^{-1}$ in Ayrshire and non-Ayrshire Cheddar cheese throughout the ripening process. A decrease in this methylated fatty acid throughout the ripening process was observed in both cheeses, with the level of this acid in non-Ayrshire Cheddar cheese being almost consistently higher than that in Ayrshire Cheddar cheese. This decrease is probably due to the liberation of free fatty acids from the glycerol backbone of the triglyceride. It has been reported that lipolysis in milk preferentially releases short- and medium-chain fatty acids, because in the case of milk triglycerides, short-chain fatty acids are predominantly esterified at the *sn*-3 position.

Slightly higher concentrations of methylated octanoic acid ($C_{8:0}$) were detected (Fig. 3B). An initial increase in methylated octanoic acid (28.8 $mg.g^{-1}$ cheese) was observed in Ayrshire Cheddar cheese up to day 10 of ripening, after which it decreased again to a concentration similar to the initial concentration (18.8 $mg.g^{-1}$) at 22 days of ripening, remaining more or less constant for the remainder of the ripening period. In non-Ayrshire Cheddar cheese, the initial concentration of methylated $C_{8:0}$ was the same as in Ayrshire Cheddar cheese (19.1 $mg.g^{-1}$), followed by a slight decrease up to 22 days (15.6 $mg.g^{-1}$) and then an increase up to 36 days (20.2 $mg.g^{-1}$). The concentration remained more or less in that region up to 64 days, followed by a decrease up to 92 days (12.9 $mg.g^{-1}$). An interesting pattern was observed in the case of methylated nonanoic acid ($C_{9:0}$) (Fig. 3C), where the concentration in non-Ayrshire Cheddar cheese (0.3 $mg.g^{-1}$) remained higher than in Ayrshire Cheddar cheese (0.4 $mg.g^{-1}$) throughout the entire ripening period. Low concentrations of this fatty acid were detected in both cheeses.

Methylated decanoic acid ($C_{10:0}$) (Fig. 3D) showed a similar trend to that observed for methylated $C_{8:0}$, but at slightly higher concentrations in both cheeses. A sharp initial increase in Ayrshire Cheddar cheese was also observed up to day 10 of ripening (51.4 $mg.g^{-1}$ cheese), after which it decreased up to at 36 days (33.5 $mg.g^{-1}$). There was then a slight increase up to 50 days, followed by a decrease up

to 92 days (30.5 $mg.g^{-1}$). In non-Ayrshire Cheddar cheese, the initial concentration (39.8 $mg.g^{-1}$) decreased slightly up to day 36 of ripening (42.5 $mg.g^{-1}$), after which it remained at 42.5 $mg.g^{-1}$ up to 64 days and decreased towards the end of the ripening period (26.9 $mg.g^{-1}$).

Figure 3E represents the methylated undecanoic acid ($C_{11:0}$) concentrations. In Ayrshire Cheddar cheese, a constant decline in this acid over the ripening period was observed (from 1.1 to 0.34 $mg.g^{-1}$ cheese). The concentration of this acid in non-Ayrshire Cheddar cheese remained higher than in Ayrshire Cheddar cheese throughout the ripening process. An initial decrease up to day 36 (0.7 $mg.g^{-1}$) was observed in non-Ayrshire Cheddar cheese, followed by an increase up to 64 days (0.9 $mg.g^{-1}$) and then another decrease up to 92 days (0.4 $mg.g^{-1}$). Low concentrations of this acid were detected in both cheeses.

The same pattern observed in methylated dodecanoic fatty acid ($C_{12:0}$) was also evident in $C_{8:0}$ and $C_{10:0}$. Characteristic of all three fatty acids was the initial peak in Ayrshire Cheddar cheese at day 10 of ripening. After 10 days, the $C_{12:0}$ concentration decreased to 42.4 $mg.g^{-1}$ and remained in this region up to the end of ripening. In non-Ayrshire Cheddar cheese, the $C_{12:0}$ concentration remained almost constant throughout ripening (43 $mg.g^{-1}$), but with a slight peak at 36 days (58.2 $mg.g^{-1}$).

Interestingly, when evaluating the odd-numbered medium-chain methylated fatty acids ($C_{7:0}$, $C_{9:0}$; $C_{11:0}$), the concentration was always higher in non-Ayrshire Cheddar cheese than in Ayrshire Cheddar cheese. A unique pattern was also established in the even-numbered medium-chain methylated fatty acids ($C_{8:0}$; $C_{10:0}$; $C_{12:0}$) in Ayrshire Cheddar cheese, which reached a peak at day 10 of ripening.

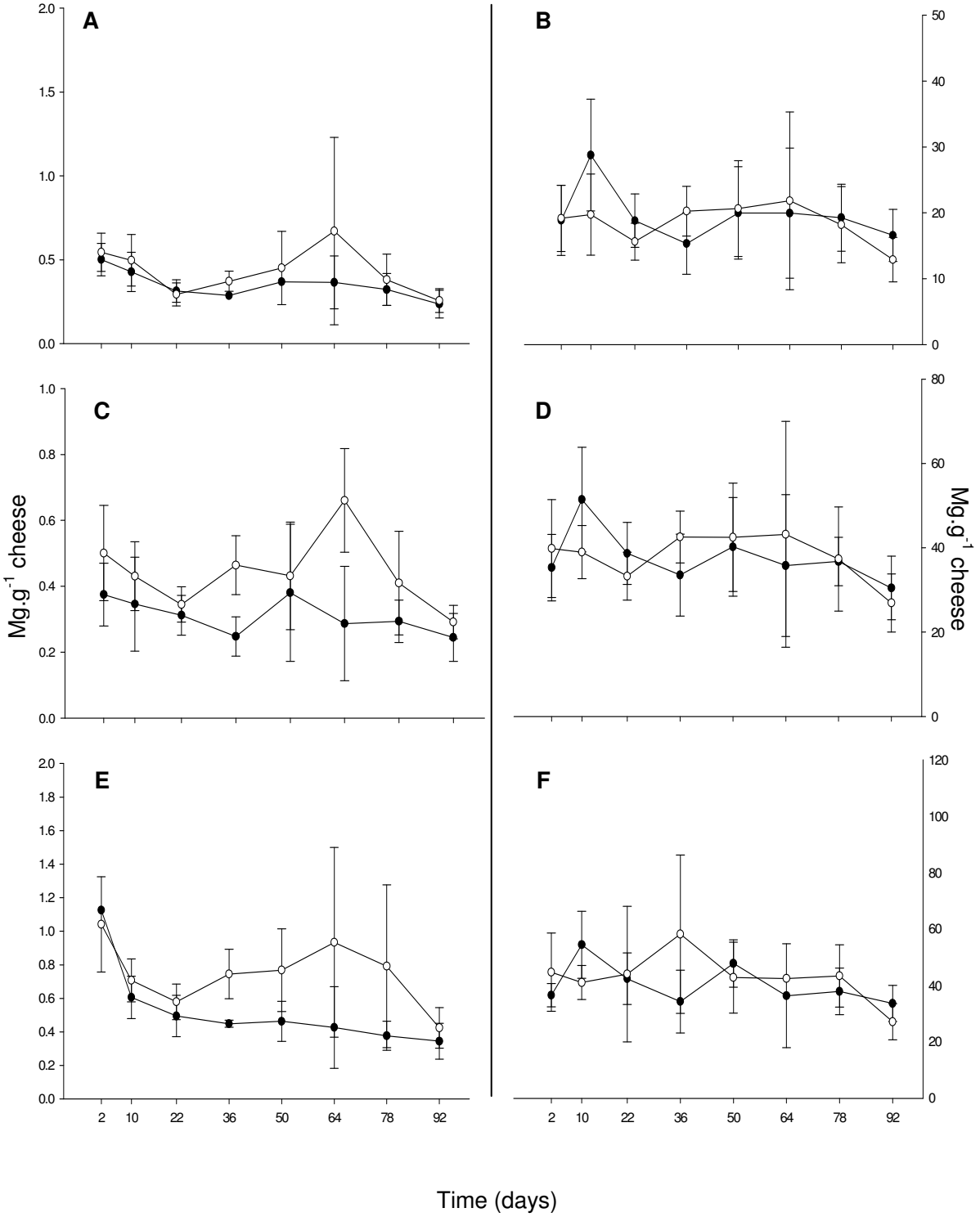

Figure 3. Changes in saturated medium-chain methylated fatty acids in Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese respectively. A = Heptanoic acid ($C_{7:0}$); B = Octanoic acid ($C_{8:0}$); C = Nonanoic acid ($C_{9:0}$); D = Decanoic acid ($C_{10:0}$); E = Undecanoic acid ($C_{11:0}$); F = Dodecanoic acid ($C_{12:0}$).

This peak is characteristic of Ayrshire Cheddar's medium-chain even-numbered fatty acids and is not observed in non-Ayrshire Cheddar cheese. It seems that the liberation of even-numbered medium-chain fatty acids in non-Ayrshire Cheddar cheese is more active than in Ayrshire Cheddar cheese during the initial ripening process, which could be due to more stringent enzymes being present in non-Ayrshire milk than in Ayrshire milk.

Figure 4 (A-F) represents the saturated long-chain methylated fatty acids in Ayrshire and non-Ayrshire Cheddar cheese through ripening. Longer chain fatty acids are predominantly esterified to the *sn*-2 position of the triglyceride (Singh *et al.*, 2003). Small quantities of the methylated tridecanoic fatty acid ($C_{13:0}$) (Fig. 4A) were detected in both Ayrshire and non-Ayrshire Cheddar cheese (average of 1.4 and 1.3 $mg.g^{-1}$ respectively). No specific trend was observed to distinguish Ayrshire from non-Ayrshire Cheddar cheese.

The quantities of methylated pentadecanoic fatty acid ($C_{15:0}$) in Ayrshire and non-Ayrshire Cheddar cheese respectively are shown in Figure 4(B). If compared to the medium-chain odd-numbered fatty acids, a different pattern was observed in the case of $C_{15:0}$, where the concentration in Ayrshire Cheddar cheese was consistently higher than in non-Ayrshire Cheddar cheese. The exact opposite was true for the medium-chain odd-numbered methylated fatty acids.

In contrast to the odd-numbered methylated fatty acids ($C_{7:0}$; $C_{9:0}$; $C_{11:0}$; $C_{13:0}$ and $C_{15:0}$), higher concentrations of even-chain methylated fatty acids in general were found. It has been reported that the most abundant FFAs in milk fat (comprising ~25% and ~27% respectively of the total lipids) are hexadecanoic acid ($C_{16:0}$) and octadecanoic acid ($C_{18:0}$) (Collins *et al.*, 2003). This study also revealed that throughout the ripening process, methylated $C_{16:0}$ (Fig. 4C) was the methylated fatty acid with the highest concentration amongst all the fatty acids, with initial concentrations of 208 and 199 $mg.g^{-1}$ in Ayrshire and non-Ayrshire Cheddar cheese respectively. As with the short-chain even-numbered fatty acids ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$), a

similar peak in $C_{16:0}$ was observed in the first 10 days of ripening of Ayrshire Cheddar cheese.

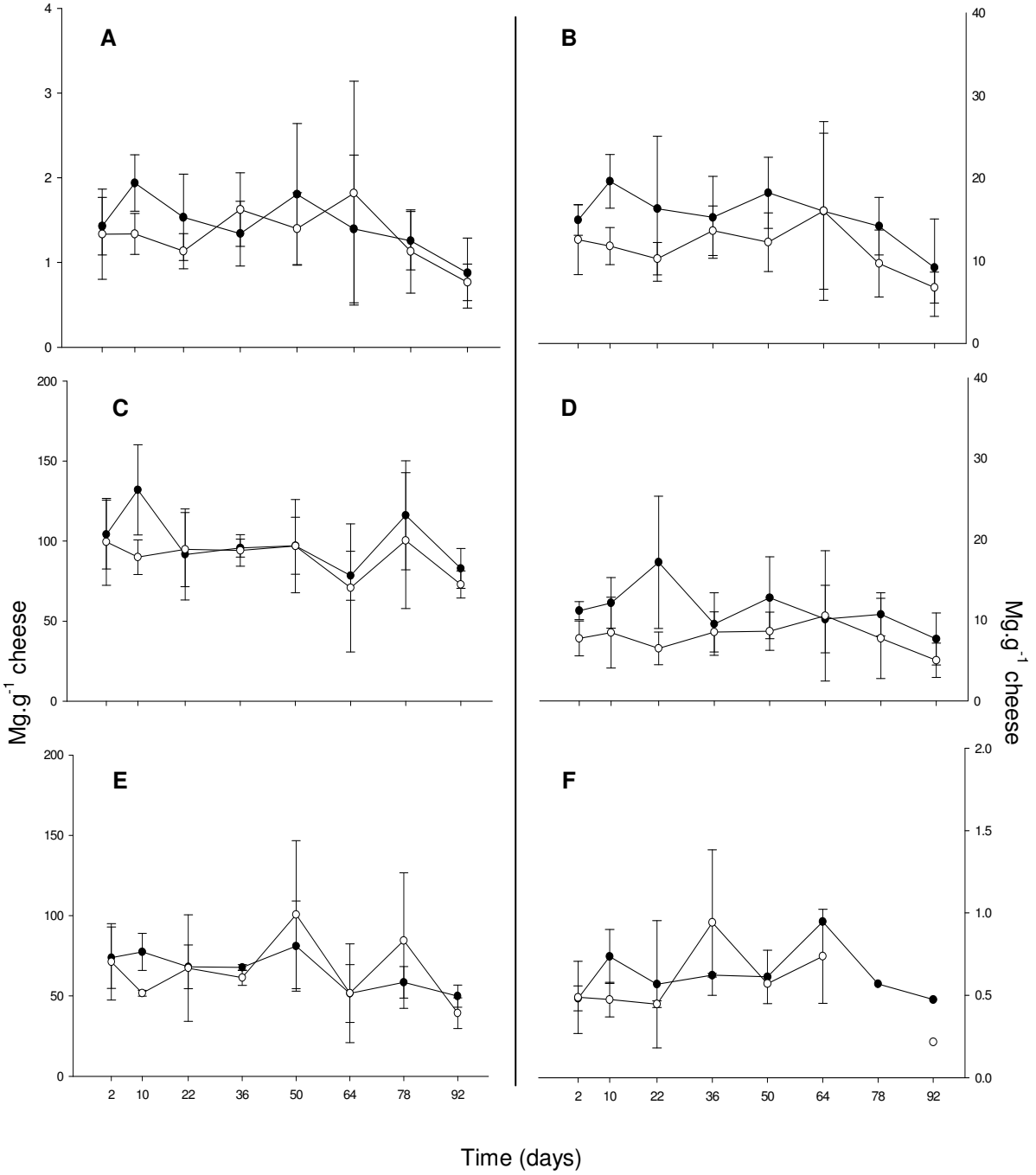

Figure 4. Changes in saturated long-chain methylated fatty acids in Ayrshire (--●--) and non-Ayrshire (--○--) Cheddar cheese respectively. A = Tridecanoic acid ($C_{13:0}$); B = Pentadecanoic acid ($C_{15:0}$); C = Hexadecanoic acid ($C_{16:0}$); D = Heptadecanoic acid ($C_{17:0}$); E = Octadecanoic acid ($C_{18:0}$); F = Nonadecanoic acid ($C_{19:0}$).

Besides the peak, the concentrations in Ayrshire and non-Ayrshire Cheddar cheese remained more or less constant up to day 50 of ripening (±97 mg.g-1). After 50 days there was a sudden decrease (78.3 and 70.8 mg.g-1) up to 64 days and then a sharp increase (116 and 100 mg.g-1) up to 78 days, followed by an immediate decrease (83 and 73 mg.g-1) up to 92 days in Ayrshire and non-Ayrshire Cheddar cheese respectively.

The pattern followed by methylated heptadecanoic acid ($C_{17:0}$) (Fig. 4D) was similar to that of $C_{15:0}$, with concentrations in Ayrshire Cheddar being consistently higher than in non-Ayrshire Cheddar cheese. In non-Ayrshire Cheddar cheese, the movement in concentration was almost identical to that in $C_{15:0}$, but with slightly lower concentrations throughout ripening. Differences between methylated $C_{17:0}$ and $C_{15:0}$ in Ayrshire Cheddar cheese were observed only in the first 22 days, where the initial increase occurred earlier in the former than in the latter.

High concentrations of methylated octadecanoic acid ($C_{18:0}$) (Fig. 4E) were detected in both Ayrshire and non-Ayrshire Cheddar cheese (average 66 $mg.g^{-1}$ in both). Fluctuations in concentration in non-Ayrshire Cheddar cheese were noticeable throughout the ripening process, with sharp peaks at days 50 (100.6 $mg.g^{-1}$) and 78 (84.5 $mg.g^{-1}$). Ayrshire Cheddar followed a steadier downward trend throughout ripening, but with a slight rise in concentration at day 50 (81 $mg.g^{-1}$). Small amounts of methylated nonadecanoic acid ($C_{19:0}$) (Fig. 4F) were detected in Ayrshire and non-Ayrshire Cheddar cheese (average of 0.62 and 0.55 $mg.g^{-1}$ respectively).

Figure 5 (A-D) reflects the long-chain methylated saturated fatty acids in Ayrshire and non-Ayrshire Cheddar cheese. The quantity of methylated eicosanoic acid ($C_{20:0}$) (Fig. 5A) was almost equivalent in both Ayrshire and non-Ayrshire Cheddar cheese throughout ripening, with the initial concentration in Ayrshire Cheddar cheese being slightly higher than in non-Ayrshire Cheddar cheese (up to 36 days). After 36 days, the concentration in Ayrshire and non-Ayrshire Cheddar cheese increased to 2.2 and 2.5 $mg.g^{-1}$ (64 days) respectively, followed by a decrease towards the end of ripening (0.6 and 0.5 $mg.g^{-1}$). Figure 5B shows the

changes in methylated heneicosanoic acid (C21:0) during ripening in Ayrshire and non-Ayrshire Cheddar cheese.

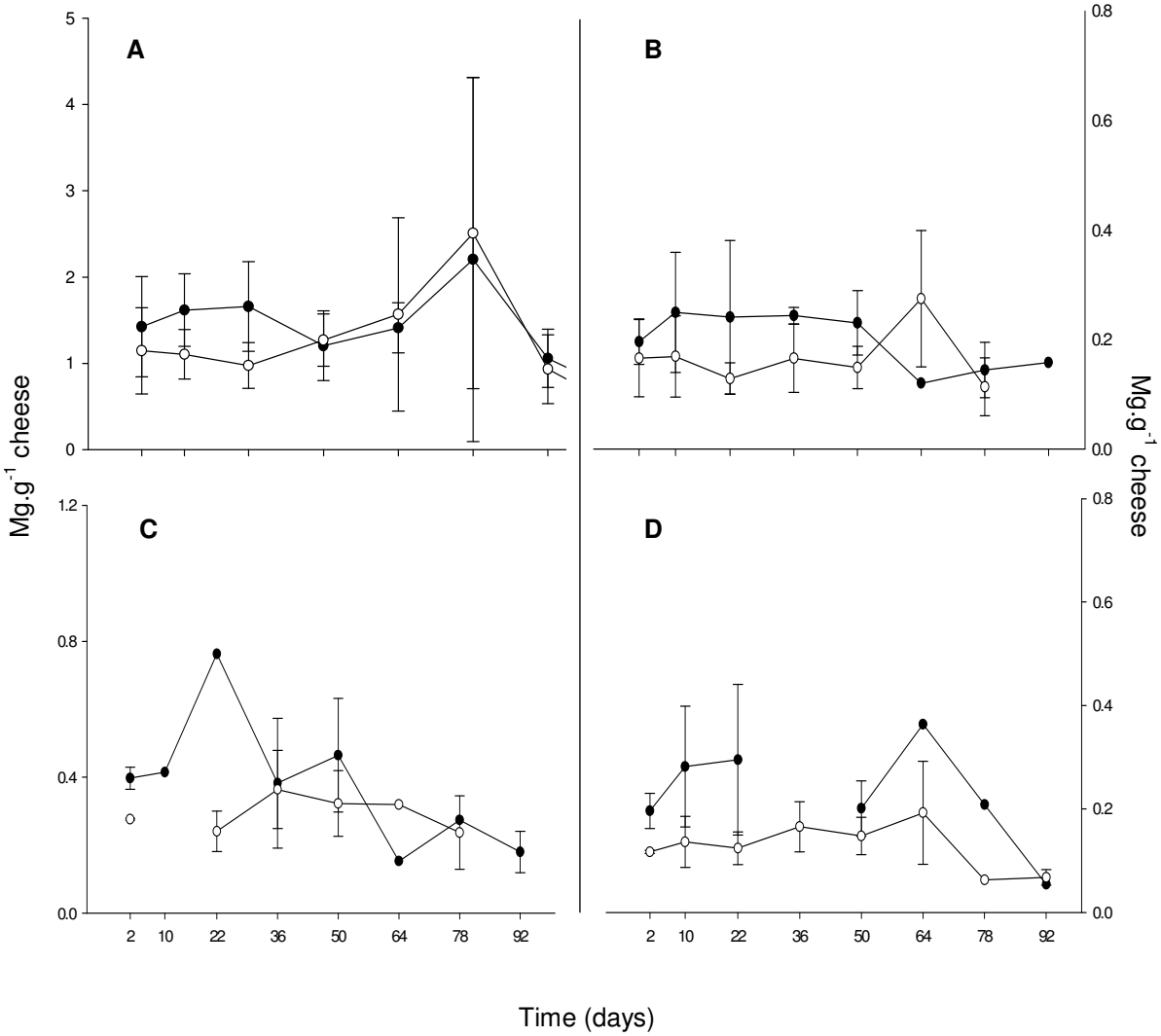

Figure 5. Changes in saturated very-long-chain methylated fatty acids in Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese respectively. A = Eicosanoic acid ($C_{20:0}$); B = Heneicosanoic acid ($C_{21:0}$); C = Docosanoic acid ($C_{22:0}$); D = Tetracosanoic acid ($C_{23:0}$).

Small amounts of this fatty acid were detectable at all times. The initial concentrations were slightly higher in Ayrshire than in non-Ayrshire Cheddar cheese, up to day 64, where an increase was observed in non-Ayrshire Cheddar cheese.

Of all the methylated fatty acids analysed, the concentrations of methylated docosanoic acid ($C_{22:0}$) (Fig. 5C) and methylated tetracosanoic acid ($C_{23:0}$) (Fig. 5D) were the lowest. At some of the time intervals, only one or two of the quadruplet values were detected, which renders the statistical analysis of these two fatty acids impossible. Notable was the constant presence of $C_{23:0}$ in non-Ayrshire Cheddar cheese only. The presence of $C_{22:0}$ in Ayrshire Cheddar cheese was, however, more constant than in non-Ayrshire Cheddar cheese.

Figure 6 (A-F) represents the changes in the unsaturated and methylated fatty acids throughout ripening in Ayrshire and non-Ayrshire Cheddar cheese. In milk fat, unsaturated fatty acids are mainly esterified at the *sn*-1 and *sn*-3 positions of the triglyceride (Balcao & Malcata, 1998). An interesting pattern was observed in tridecanoic 12-methyl ($C_{13:1}$) (Fig. 6A) where the presence of this acid in non-Ayrshire Cheddar cheese was almost undetectable. A constant presence of this acid was, however, perceived in Ayrshire Cheddar cheese throughout the ripening period (average of 1.9 $mg.g^{-1}$).

In methyl Z-11-tetradecanoate (Fig. 6B) a fascinating phenomenon was observed where the concentrations detected in Ayrshire and in non-Ayrshire Cheddar cheese were almost inversely related to each other throughout ripening. The concentrations of this unsaturated fatty acid in Ayrshire Cheddar cheese were almost constantly higher (average 2.7 $mg.g^{-1}$) than in non-Ayrshire Cheddar cheese (average 2 $mg.g^{-1}$). The presence of methyl Z-11-tetradecanoate was limited in both cheeses. High concentrations of methyl tetradecanoate (Fig. 6C) were also detected in Ayrshire and non-Ayrshire Cheddar cheese (average of 84 and 72 $mg.g^{-1}$ respectively). Initially an increase in methyl tetradecanoate was observed in Ayrshire but not in non-Ayrshire Cheddar cheese up to 36 days, with the concentrations in both cheeses remaining more or less equal until the end of the ripening period.

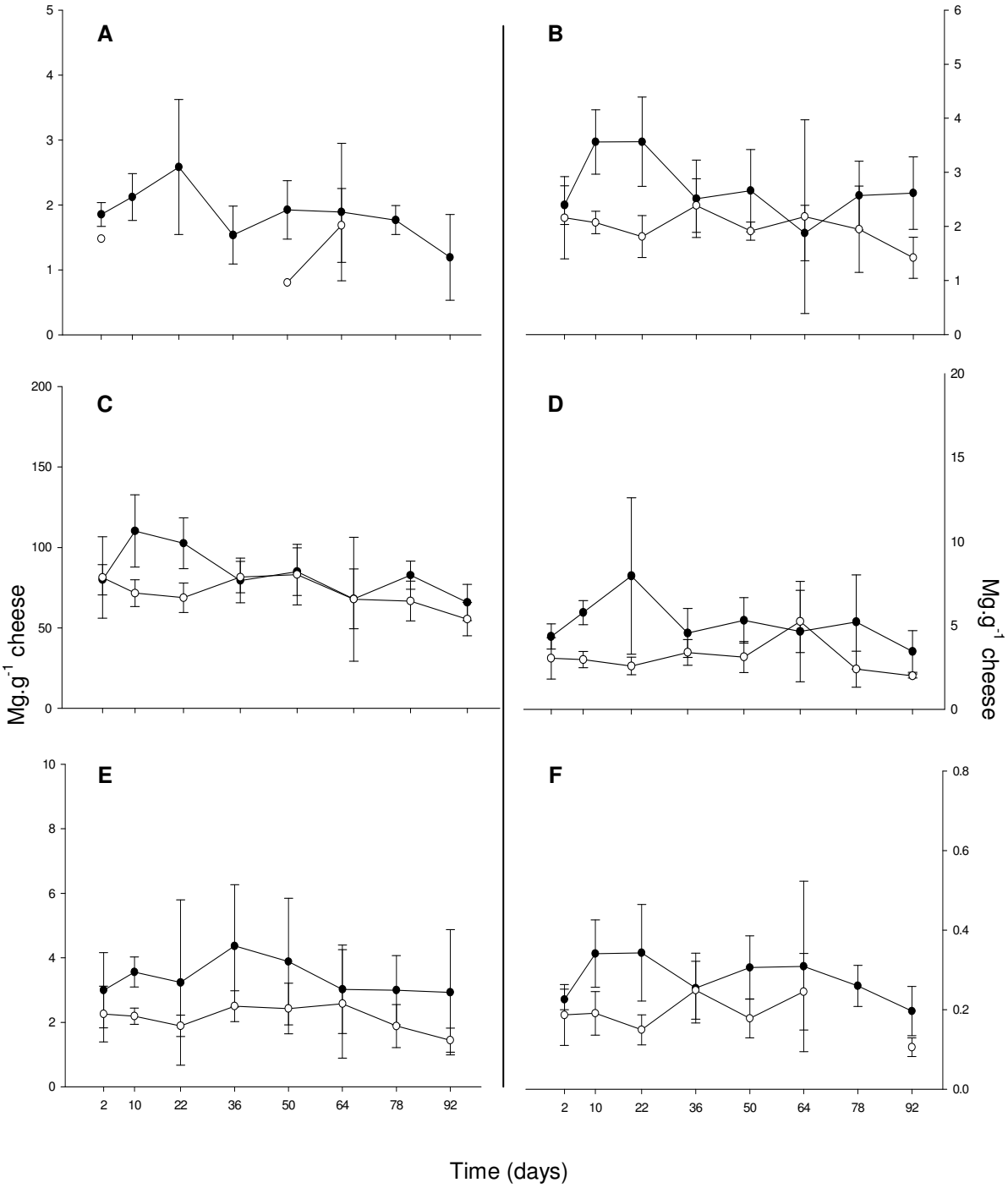

Figure 6. Changes in unsaturated and branched-chain methylated fatty acids in Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese respectively. A = Tridecanoic, 12-methyl ($C_{13:1}$); B = Methyl Z-11-tetradecanoate; C = Methyl tetradecanoate; D = Tetradecanoic acid, 12-methyl ($C_{14:1}$); E = 9-Hexadecanoic acid ($C_{16:1}$); F = 11-Eicosanoic acid ($C_{20:1}$).

Figure 6D represents the changes in tetradecanoic acid, 12-methyl ($C_{14:1}$) throughout ripening. A similar pattern to $C_{13:1}$ in Ayrshire and non-Ayrshire Cheddar cheese was noted, except that the concentrations were lower in $C_{13:1}$ than in $C_{14:1}$. Figures 6E and 6F show the concentrations of 9-hexadecanoic acid ($C_{16:1}$) and 11-eicosanoic acid ($C_{20:1}$) respectively. For both these methylated fatty acids, the Ayrshire Cheddar cheese displayed a consistently higher concentration than the non-Ayrshire Cheddar cheese. A low concentration of $C_{20:1}$ was detected, and at day 78 of ripening in non-Ayrshire Cheddar cheese, this fatty acid was almost depleted.

4.5.3. Mathematical estimations

Table 1 and Table 2 represent the mathematical equations and coefficients that describe the changes in the methylated fatty acid content during the ripening of Ayrshire and non-Ayrshire Cheddar cheese respectively. The alterations were defined by mathematical equations with R^2 values ranging from 0.58 to 0.99. In Ayrshire Cheddar cheese, most of the methylated fatty acids were defined by a six-grade polynomial equation, with the exception of heptanoic acid and methyl tetradecanoic acid, which were best characterised by a five-grade polynomial equation. Undecanoic acid, on the other hand, was best defined by a four-grade polynomial equation in both Ayrshire and non-Ayrshire Cheddar cheese. In the case of non-Ayrshire Cheddar cheese, four of the methylated fatty acids (heptadecanoic, nonadecanoic, heneicosanoic and docosanoic) were best characterised by a five-grade polynomial equation, while the rest were best fitted with a six-grade polynomial equation. These equations can constitute some of the elements of a model to calculate the age of a cheese by using the fatty acid data as variable. Money and time can also be saved by applying these equations to predict the concentration of a particular fatty acid at a certain time during the early maturation process and to possibly distinguish between Ayrshire and non-Ayrshire Cheddar cheese. However, some fatty acids could be more precise indicators of maturation

than others, and therefore a combined formula that relates the information of all the fatty acids would give a more accurate estimate of the extent of maturation.

Table 1. Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Butanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1194 **b** 3.5711 **c** -42.595 **d** 255.75 **e** -797 **f** 1175.8 **g** -560.58	0.9739
Hexanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1001 **b** 2.9691 **c** -35.102 **d** 209.04 **e** -648.5 **f** 961.81 **g** -435.51	0.8017
Heptanoic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0017 **b** -0.0398 **c** 0.3339 **d** -1.203 **e** 1.6668 **f** 0.2428	0.9866
Octanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0449 **b** 1.3989 **c** -17.35 **d** 107.93 **e** -346.82 **f** 524.82 **g** -232.24	0.9859
Nonanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0005 **b** 0.0136 **c** -0.158 **d** 0.9068 **e** -2.6316 **f** 3.4903 **g** -0.8738	0.633
Decanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1027 **b** 3.0007 **c** -34.903 **d** 204.4 **e** -623.94 **f** 911.2 **g** -389.19	0.9591
Undecanoic	$y = ax^4 + bx^3 + cx^2 + dx + e$	**a** 0.0056 **b** -0.1203 **c** 0.9203 **d** -2.9926 **e** 4.4278	0.997

Table 1 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Dodecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1297 **b** 3.7965 **c** -44.043 **d** 256.02 **e** -772.71 **f** 1115.1 **g** 485.24	0.8173
Tridecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0042 **b** 0.125 **c** -1.4581 **d** 8.4911 **e** -25.539 **f** 36.535 **g** -15.302	0.9141
Methyl Z-11-tetradecanoate	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0035 **b** 0.0934 **c** -0.9988 **d** 5.57 **e** -17.316 **f** 27.756 **g** -10.3331	0.8584
Methyl tetradecanoate	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1477 **b** 4.0067 **c** -43.571 **d** 243.13 **e** -731.49 **f** 1095.3 **g** -407.97	0.9035
Pentadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0302 **b** 0.8958 **c** -10.54 **d** 62.102 **e** -189.43 **f** 275.55 **g** -108.73	0.9732
Tetradecanoic, 12-methyl	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0235 **b** -0.6603 **c** 7.20324 **d** -38.387 **e** 102.36 **f** -124.16 **g** 62.24	0.7977
Hexadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.6761 **b** 18.264 **c** -194.51 **d** 1035 **e** -2858 **f** 3780.3 **g** -1572.4	0.9923

Table 1 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
9-Hexadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0151 **b** 0.4196 **c** -4.5785 **d** 24.807 **e** -69.284 **f** 92.661 **g** -39.186	0.9405
Heptadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0538 **b** -1.4929 **c** 16.175 **d** -86.149 **e** 232.07 **f** -289.76 **g** 151.27	0.6558
	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$		0.6558
Octadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.3093 **b** 8.4705 **c** -90.821 **d** 481.36 **e** -1307.1 **f** 1681.6 **g** -626	0.8471
Nonadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0026 **b** -0.0643 **c** 0.5958 **d** -2.5897 **e** 5.3199 **f** -4.4753 **g** 2.1783	0.842
11-Eicosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0007 **b** -0.0169 **c** 0.1615 **d** -0.7186 **e** 1.4614 **f** -1.0543 **g** 0.6169	0.9429
Eicosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0144 **b** -0.3771 **c** 3.8201 **d** -18.942 **e** 47.672 **f** -56.589 **g** 27.252	0.9894
Henecosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.001 **b** 0.0275 **c** -0.289 **d** 1.5 **e** -4.0193 **f** 5.2181 **g** -2.0447	0.9694

Table 1 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Docosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0016 **b** -0.0475 **c** 0.5587 **d** -3.2333 **e** 9.4037 **f** -12.481 **g** 6.5835	0.7489
Tetracosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0038 **b** -0.0989 **c** 0.9997 **d** -4.9063 **e** 12.086 **f** -13.883 **g** 6.1889	0.9234

R^2 = correlation coefficient

Table 2. Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from non-Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Butanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	a 0.0245 **b** -0.4804 **c** 2.9314 d 3.3283 **e** -23.462 **f** 68.58 **g** -18.576	0.9652
Hexanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1279 **b** 3.6411 **c** -41.0021 **d** 230.24 **e** -664.38 **f** 900.18 **g** -360.18	0.9108
Heptanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a -**0.0006 **b** 0.161 **c** -1.6877 **d** 8.7382 **e** -23.145 **f** 28.906 **g** -11.882	0.9152
Octanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0343 **b** 0.9929 **c** -11.304 **d** 63.561 **e** -181.16 **f** 240.07 **g** -73.715	0.9293

Table 2 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from non-Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Nonanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0016 **b** -0.041 **c** 0.4034 **d** -1.9475 **e** 4.873 **f** -6.043 **g** 3.7592	0.7964
Decanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0717 **b** 2.0084 **c** -22.085 **d** 119.54 **e** -326.88 **f** 414.72 **g** -107.48	0.9412
Undecanoic	$y = ax^3 + bx^2 + cx + d$	**a** -0.0368 **b** 0.493 **c** -1.9259 **d** 3.5589	0.9662
Dodecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.1064 **b** 2.593 **c** -23.99 **d** 104.57 **e** -217.41 **f** 196.88 **g** 27.174	0.8682
Tridecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0017 **b** -0.0397 **c** 0.3529 d -1.5058 **e** 3.319 **f** -3.6875 **g** 4.243	0.7835
Methyl Z-11-tetradecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0029 **b** 0.0759 **c** -0.7799 **d** 3.9272 **e** -9.9476 **f** 11.593 **g** -0.5357	0.7068
Methyl tetradecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.2069 **b** 5.549 **c** -57.78 **d** 293.26 **e** -746.1 **f** 874.1 **g** -206.34	0.998
Pentadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0264 **b** -0.6591 **c** 6.3342 **d** -29.942 **e** 73.772 **f** -90.356 **g** 66.054	0.8275

Table 2 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from non-Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Tetradecanoic, 12-methyl	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0223 **b** -0.5722 **c** 5.6935 **d** -27.846 **e** 69.935 **f** -84.427 **g** 43.32	0.7957
Hexadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.3401 **b** 8.6747 **c** -85.738 **d** 414.12 **e** -1012.6 **f** 1161.7 **g** -287.3	0.851
9-Hexadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0015 **b** 0.0487 **c** -0.5976 **d** 3.5067 **e** -10.061 **f** 12.961 **g** -1.3335	0.9058
Heptadecanoic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0433 **b** -1.0078 **c** 8.4683 **d** -31.256 **e** 49.278 **f** -9.998	0.8906
Octadecanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.4295 **b** 11.178 **c** -112.98 **d** 557.55 **e** -1383.1 **f** 1591.7 **g** -522.75	0.5862
Nonadecanoic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.0044 **b** 0.0991 **c** -0.8455 **d** 3.3011 **e** -5.5297 **f** 3.9776	0.6778
11-Eicosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** -0.0023 **b** 0.0556 **c** -0.5154 **d** 2.3727 **e** -5.656 **f** 6.503 **g** -2.3816	0.9891
Eicosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0119 **b** -0.3032 **c** 2.9776 **d** -14.334 **e** 35.4 **f** -42.064 **g** 20.614	0.9421

Table 2 (continued). Mathematical equations representing the patterns of fatty acids during the maturation of Cheddar cheese made from non-Ayrshire milk

Variable	Estimation summary		
Fatty acid	Equation	Coefficient	R^2
Heneicosanoic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.005 **b** 0.0949 **c** -0.6778 **d** 2.2472 **e** -3.3908 **f** 2.0662	0.9174
Docosanoic	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.0075 **b** 0.1665 **c** -1.393 **d** 5.3981 **e** -9.263 **f** 5.6517	0.9993
Tetracosanoic	$y = ax^6 + bx^5 + cx^4 + dx^3 + ex^2 + fx + g$	**a** 0.0007 **b** -0.0162 **c** 0.1554 **d** -0.7294 **e** 1.7499 **f** -1.9861 **g** 1.0617	0.8673

R^2 = correlation coefficient

Relative to the cheese investigated in this study, the following two formulas (Ayrshire and non-Ayrshire Cheddar cheese) may be proposed as a collective representation of the development of fatty acids during maturation:

Ayrshire:

$$y = \frac{-1.584x^6 + 44.296x^5 - 491.438x^4 + 2741.295x^3 - 7983.69x^2 + 11183.412x - 3654.119}{23}$$

Non-Ayrshire:

$$y = \frac{-1.235x^6 + 32.893x^5 - 340.259x^4 + 1733.925x^3 - 4434.669x^2 + 5308.498x - 1420.992}{23}$$

Where:

x = maturation time

y = fatty acid concentration

A less-complicated methodology that may apply to the combined contribution of the fatty acids would be to calculate the average age ($\bar{x}$) obtained from solving the individual equations depicted in Table 1 and Table 2.

4.6. Conclusions

It should be noted that the equations presented in this study are applicable specifically to the manufacturer that produced the cheese in question. Although the applicability thereof to Cheddar cheese produced at other manufacturing plants still needs to be established, their applicability as single equations or as part of a combined mathematical model shows great potential. Even so, by applying these equations, the manufacturer can realise some promising outcomes at an early stage of the cheese-ripening process, i.e.: (1) The selection of cheese for specialist lines; (2) The early exclusion of defective cheeses; and (3) The establishment, soon after ripening, of the origin of the particular Cheddar cheese brand (Ayrshire vs. mixed-breed). In addition to the application of these equations, information could be gathered by simply observing the ripening graphs of the different methylated fatty acids. It would appear that Ayrshire Cheddar cheese is always accompanied by a peak in all the even-numbered medium-chain saturated fatty acids ($C_{8:0}$; $C_{10:0}$; $C_{12:0}$) at day 10 of ripening. It would also appear that Ayrshire Cheddar cheese always has a higher concentration of unsaturated fatty acids than non-Ayrshire Cheddar cheese. During the course of this study, non-Ayrshire Cheddar cheese was revealed to have a consistently higher concentration of saturated medium-chain odd-numbered fatty acids ($C_{7:0}$; $C_{9:0}$; $C_{11:0}$). The stated outcomes of this article could include proteolysis and organic acid mathematical modelling to ensure an accurate overall portrayal of the biochemical events in the development of Ayrshire and non-Ayrshire Cheddar cheese.

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Chapter 5

Mathematical modelling of Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk using amino acid data

Submitted for publication to Journal of Food Composition and Analysis.

5.1. Title

Mathematical modelling of Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk using amino acid data.

5.2. Abstract

Branded dairy products have recently become a global trend. Consequently, the origin of the milk used in the manufacturing of branded cheeses must be declared by the producer, since these products have been known to be adulterated with foreign milk. The aim of this study was to distinguish between Cheddar cheese manufactured from pure Ayrshire milk and that manufactured from the milk of a mixture of breeds in terms of the amino acid content. Samples (four from Ayrshire and four from non-Ayrshire cheese) were analysed for amino acid content on a GC-MS every 14 days for a period of 92 days. Amino acid patterns in Ayrshire and non-Ayrshire Cheddar cheese fluctuated to a great extent, but certain trends were observed in Ayrshire Cheddar cheese, where a peak in concentration was observed at day 36 of ripening in six of the eight amino acids. Regression models for eight amino acids were also determined. These equations will surely come in very handy for the manufacturer, since the specific outcomes of an amino acid can be predicted during early ripening, i.e. in terms of age and the variations between Ayrshire and non-Ayrshire Cheddar cheese.

5.3. Introduction

Proteolysis in cheese is a complex process that begins with the hydrolysis of casein into smaller peptides and eventually single amino acids by proteinases and peptidases of the cheese starter bacteria and non-starter lactic acid bacteria (Coker, Crawford, Johnston, Singh & Creamer, 2005). Amino acids (AAs) could therefore be applied as an instrument to reflect the microbial and enzymatic activity during ripening in Cheddar cheese. Different cheese types mostly reveal special free amino acids at certain stages during ripening that are unique to that specific cheese type. In addition, the milk source, starter cultures and other environmental factors such as

moisture, pH and salt content also play a significant role in the breakdown of proteins, which makes biochemical control difficult to direct (Forde & Fitzgerald, 2000). Recently, the production mathematical frameworks whereby biochemical control can be quantified have grown in popularity, since they offer increased control over end-product quality (Lues & Bekker, 2002).

Branded dairy products have recently become a global trend. Consequently, the origin of the milk used in the manufacturing of branded and origin cheeses must be declared by the producer, since adulteration with foreign milk in these products does tend to occur (Pillonel, Badertscher, Casey, Meyer, Rossmann, Schlichtherle-Cerny, Tabacchi & Bosset, 2005a; Pillonel, Bütikofer, Schlichtherle-Cerny, Tabacchi & Bosset, 2005b; Veloso, Teixeria, Peres, Mendoça & Ferreira, 2004). It sometimes happens that goat's and ewe's milk is adulterated with cow's milk due to seasonal fluctuations in the production of ewe's and goat's milk, as well a higher market prices for these types of milk (Rodriguez-Nogales, 2006). This phenomenon has also been observed in a well-established South African branded cheese, i.e. Ayrshire Cheddar cheese (made exclusively from Ayrshire milk), where the milk of other cow breeds has been added for the production of branded Ayrshire Cheddar cheese due to the low availability and high price of Ayrshire milk (according to personal communication with an Ayrshire milk supplier). The determination of branded cheeses' origin is a complicated task, especially in the case of cheese that is biochemically and microbiologically dynamic and which undergoes various changes during ripening (Pillonel *et al*., 2005b).

Fraud detection and origin determination in foods frequently requires an accurate characterisation of the product, which includes the use of many different analytical techniques. Among these techniques are (1) mass spectrometry, i.e. isotope ration mass spectrometry, inductively coupled plasma mass spectrometry, proton transfer reaction mass spectrometry, and gas chromatography mass spectrometry; (2) spectrometry, i.e. nuclear magnetic resonance spectrometry, infrared spectrometry, fluorescence spectrometry, and atomic spectrometry; (3) separation, i.e. high-performance liquid chromatography, gas chromatography, and capillary electrophoresis; and (4) other techniques, i.e. sensor technology, DNA

technology, and sensory analysis (Bramanti, Sortino, Onor, Beni & Raspi, 2003; Brescia, Omfreda, Buccolieri & Carrino, 2005; Cartoni, Coccioli, Jasionowska & Masci, 1999; Dias, Peres, Veloso, Reis, Vilas-Boas & Machado, 2009; Ferreira & Caçote, 2003; Luykx & Van Ruth, 2008; Mayer, Heidler & Rockenbauer, 1997; Molina, Martín-Ålvarez, & Ramos, 1999a; Molina, Ramos, Alonso & López-Fandiñoet, 1999b; Rodriquez-Nogales, 2006; Sacco, Brescia, Sgaramella, Casiello, Buccolieri, Ogrinc & Sacco, 2008). These techniques are applied to quantify / qualify micromolecules.

Furthermore, Lues and Bekker (2002) investigated the likelihood of calculating the quality of cheese during maturation by applying linear and non-linear regression modelling to the individual organic acids. The articles included as chapters 3 and 4 of this study also describe the mathematical modelling of organic and methylated fatty acids to distinguish between Cheddar cheese made from two different milk sources, i.e. Ayrshire and other breeds' milk. Likewise, several other papers have supplied detailed descriptions of the application of two-way multivariate analysis of variance, least-square correlation coefficients, stepwise regression analysis, stepwise discriminant analysis, and post-hoc multiple comparisons to qualify fraud in the milk of branded and origin cheese (Mohler-Smith & Nakai, 1990; Pham & Nakai, 1983; Bouzas, Kantt, Bodyfelt & Torres, 1993).

The aim of this article was therefore to develop a mathematical model for amino acids that would predict fluctuations within Cheddar cheese manufactured from pure Ayrshire milk versus Cheddar cheese produced from the milk of a mixture of breeds. This study also presents a mathematical framework that could be applied to the end product's amino acid profile to verify the origin (pure Ayrshire or mixed) of the raw product (milk) as support for branded products. This information would be of great value for the manufacturers of branded and origin cheeses, since they will be able to prove that adequate milk separation was maintained, which may be presented during retail audits in order to protect the brands.

5.4. Materials and methods

5.4.1. Manufacturing of Cheddar cheese

Since no batch of commercially produced Cheddar cheese is exactly the same, care was taken to design experimental procedures that would limit extrinsic / intrinsic variability between batches. Therefore two Cheddar cheese batches were manufactured in parallel, under similar conditions in the same factory / using the same equipment at the same time; the only difference being the milk source. One batch was manufactured exclusively from Ayrshire milk and the other batch from a mixture of other breeds' milk (excluding Ayrshire milk) (referred to as non-Ayrshire). The Cheddar cheese was manufactured in a closed vat system. Subsequently, the amino acid profiles of all eight samples (four from the Ayrshire batch and four from the Non-Ayrshire batch) were monitored for a period of 92 days.

5.4.2. Sampling

The cheese samples (four samples selected randomly from the respective batches) were analysed for protein percentage and amino acid concentration on the following days after production: 8; 22; 36; 50; 64; 78 and 92.

5.4.3. Protein analysis

The Kjeldahl method was used to determine the protein content of the cheese. In short, one gram of Cheddar cheese was digested with H_2SO_4, after which K_2SO_4 and copper were added. A distillation step then followed, where ammonia nitrogen was separated from the digestate. The nitrogen was separated by distilling the ammonia and collecting the distillate in a trapping medium. The ammonia bonded to 4% boric acid solution to form ammonium borate. A titration step followed where 0.1 M HCL was used as titrate with indicators bromocresol green and methyl red.

5.4.4. Amino acid analysis

One gram of the Cheddar cheese was ground with a mortar and pestle. Nine millilitres of distilled water were added to the cheese samples to make a cheese paste. The extraction and analysis of free amino acids was done with the EZ:faast amino acid kit manufactured by Phenomenex. The free amino acids were detected on the GC/FID (flame ionization detector) with a Zebron^{TM} ZB-AAA gas chromatography column. Peaks were identified by reference to the standard mixture that was included in the EZ:faast amino acid kit. They were also verified on the GC-MS (Thermo) using the Zebron^{TM} ZB-FFAP (30 m x 0.32 mm x 0.25 µm) column manufactured by Phemomenex.

5.4.5. Computation

All results used in the computational analysis were the means of quadruplet analysis. Curve fits were performed for the individual amino acids by means of Sigma Plot for Windows version 10.0.

5.5. Results and discussion

5.5.1. Protein and amino acid fluctuations

Figure 1 shows the differences in protein percentage in Ayrshire and non-Ayrshire Cheddar cheese, as detected by the Kjeldahl method during ripening. The initial percentage in both Ayrshire and non-Ayrshire cheeses was more or less the same (25.77 and 25.72 Nmoles.g^{-1} respectively). During ripening, the percentage of protein in the non-Ayrshire Cheddar cheese decreased more than in the Ayrshire Cheddar cheese, up to day 60 of ripening. The protein percentage of non-Ayrshire Cheddar cheese was higher than that of Ayrshire Cheddar for only a brief period of time (days 60 – 67), but towards the end of ripening, the percentages remained almost the same in both Ayrshire and non-Ayrshire Cheddar cheese as the initial percentage at the start of ripening. There was, however, no significant difference between Ayrshire and non-Ayrshire Cheddar cheese regarding the total protein percentage ($p = 0.4$).

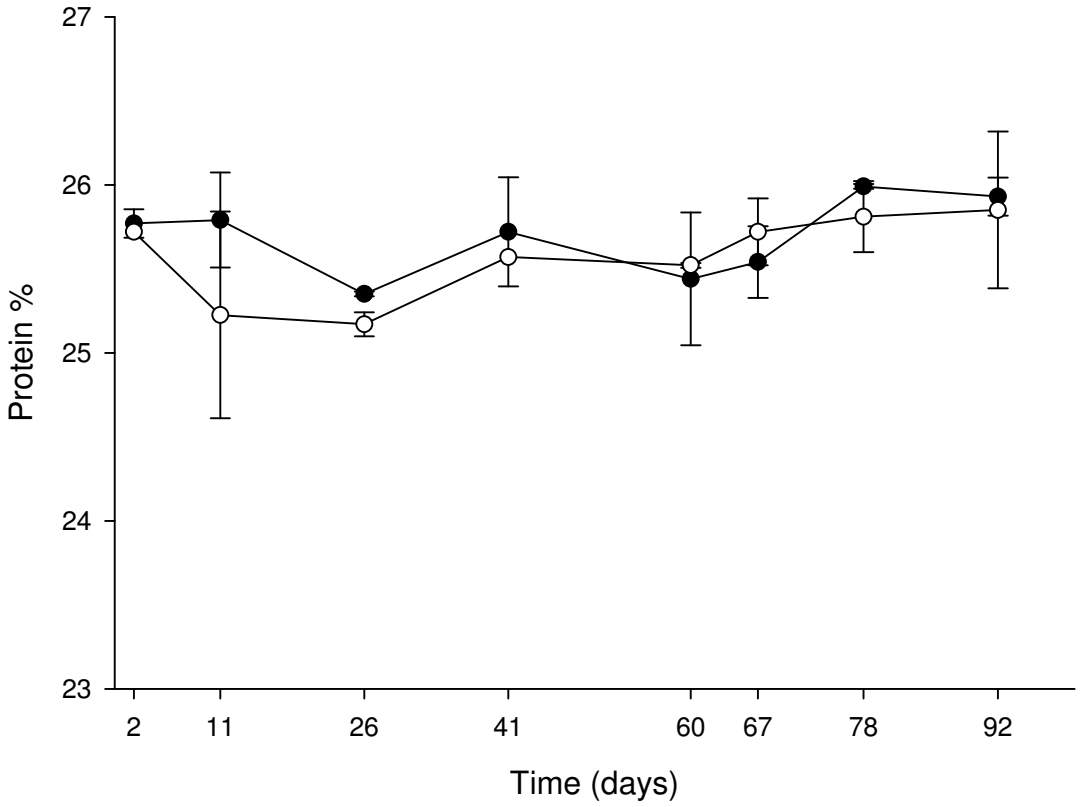

Figure 1. Differences between the respective protein percentages of Cheddar cheese made from Ayrshire (---●---) and non-Ayrshire (--○--) milk during maturation.

The changes in the different amino acid concentrations during ripening in Ayrshire and non-Ayrshire Cheddar cheese, as detected by GC-MS, are shown in Figure 2 (A-H). Only the amino acids that were identified with certainty are shown in Figure 2. This is not surprising, since the amino acid concentrations were in most cases found to increase with the age of the cheese (Bullock & Irvine, 1956). The alanine concentration (Fig. 2A) in non-Ayrshire Cheddar cheese remained more or less constant throughout the ripening process (13.0 Nmoles.g^{-1}), except for a small increase towards the end of ripening (15.0 Nmoles.g^{-1}). However, in Ayrshire Cheddar, a fluctuation in concentration was observed, with a sharp increase at 36 days (17.3 Nmoles.g^{-1}) followed by a sudden decrease at 50 days (10.4 Nmoles.g^{-1}).

Figure 2B represents the glycine concentration in Ayrshire and non-Ayrshire Cheddar cheese. A steady increase in concentration over time was observed in non-Ayrshire Cheddar cheese, with an initial concentration of 20.5 Nmoles.g^{-1}. From day 10 to 78, this concentration remained constant at an average of 26.1 Nmoles.g^{-1}, after which it increased slightly to 31.9 Nmoles.g^{-1} (92 days). The glycine concentration in non-Ayrshire Cheddar cheese was almost consistently higher than in Ayrshire Cheddar cheese throughout the ripening process. Conversely, in Ayrshire Cheddar cheese, the initial glycine concentration (9 Nmoles.g^{-1}) was lower than in non-Ayrshire Cheddar cheese, followed by an increase to almost the same concentration as non-Ayrshire Cheddar cheese at 36 days (28 Nmoles.g^{-1}). After 36 days it remained constant for the remainder of the ripening period at an average of 22.3 Nmoles.g^{-1}. The p-value for glycine throughout ripening showed a statistically significant difference between the two types of cheese ($p \leq 0.05$).

Figure 2C represents the valine concentrations in Ayrshire and non-Ayrshire Cheddar cheese. The initial valine concentration in Ayrhsire Cheddar cheese (26.9 Nmoles.g^{-1}) was slightly higher than in non-Ayrshrie cheese (23 Nmoles.g^{-1}) and remained higher up to day 50 of ripening. The valine concentration followed a downward trend in both cheeses throughout ripening and was depleted at 64 days of ripening in Ayrshire Cheddar cheese, whereas low concentrations were still detected in non-Ayrshire Cheddar cheese towards the end of ripening.

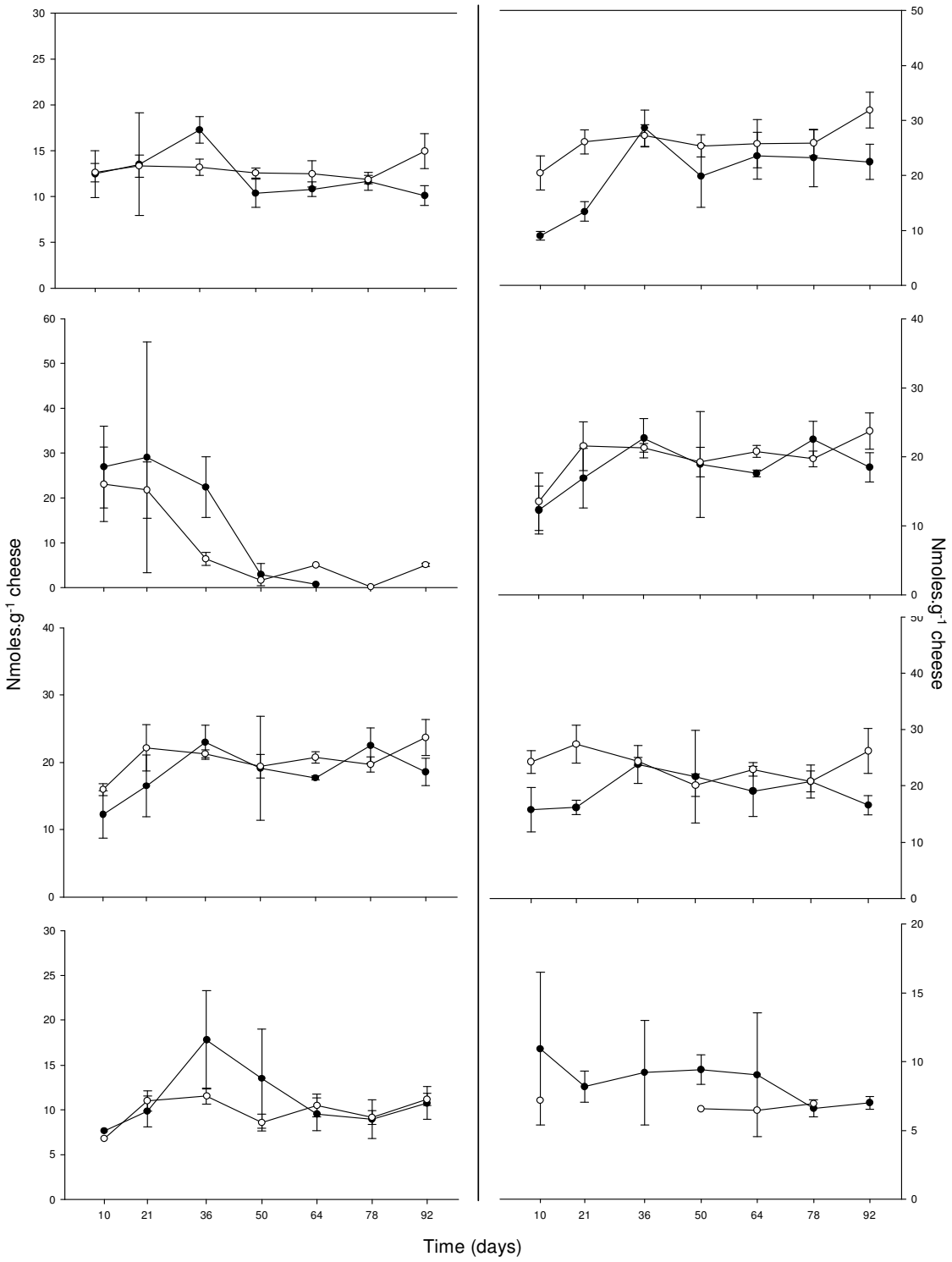

Figure 2. Changes in (a) Alanine; (b) Glycine; (c) Valine; (d) Leucine; (e) Isoleucine; (f) Proline; (g) Asparagine; (h) Phenylalanine during ripening of Ayrshire (---●---) and non-Ayrshire (---○---) Cheddar cheese. Blank spaces = below detectable limits.

The leucine and isoleucine concentrations are represented in Figure 2D and Figure 2E respectively. These two amino acids followed almost indistinguishable patterns throughout ripening and for this reason, will be discussed concurrently. In both amino acids, the initial concentrations in Ayrshire Cheddar cheese were lower than in non-Ayrshire Cheddar cheese. It increased sharply up to day 36, when it exceeded the concentration in non-Ayrshire Cheddar cheese. This was followed by a decline up to day 64 of ripening, with concentrations again lower than in non-Ayrshire Cheddar cheese. An increase up to day 78, followed by a decrease up to day 92, was observed for both leucine and isoleucine in Ayrshire Cheddar cheese. An initial increase in both amino acids in non-Ayrshire Cheddar cheese was observed up to 21 days, where it remained more or less constant until the end of ripening. For both amino acids, no significant differences were observed between the cheeses ($p = 0.4$).

The proline concentration in Ayrshire and non-Ayrshire Cheddar cheese is presented in Figure 2F. The proline concentration in Ayrshire Cheddar cheese (15.8 Nmoles.g^{-1}) was initially lower than in non-Ayrshire cheese (24.2 Nmoles.g^{-1}). A sharp increase in proline concentration was observed at day 36 in Ayrshire Cheddar cheese, which was not noticeable in non-Ayrshire Cheddar cheese. Slight differences in proline concentration were detected between Ayrshire and non-Ayrshire Cheddar cheese from day 36 up to day 78 of ripening. Towards the end of ripening, the proline concentration in non-Ayrshire Cheddar increased to 26.2 Nmoles.g^{-1} whilst the proline concentration in Ayrshire Cheddar cheese decreased to 16.6 Nmoles.g^{-1}. Moreover, the proline concentration for both cheeses was inversely proportional. It has been reported that high proline concentrations in cheese are related to the sweetness of the cheese (Izco & Torre, 2000).

Figure 2G is a representation of the asparagine concentration in Ayrshire and non-Ayrshire Cheddar cheese. The concentration in both cheeses remained almost identical throughout ripening, with the exception of an increase in the concentration thereof in Ayrshire Cheddar cheese at day 36 of ripening (17.8 Nmoles.g^{-1}), followed by a steady downward pattern up to day 64, where it corresponded with the asparagine concentration in non-Ayrshire Cheddar cheese (10 Nmoles.g^{-1}) for the

remainder of the ripening period. High concentrations of asparagine have been associated with sourness in cheese (Molina *et al*., 1999b). A significant difference ($p \leq 0.05$) in the asparagine concentration between Ayrshire and non-Ayrshire Cheddar cheese was detected.

Limited amounts of phenylalanine were detected in both Ayrshire and non-Ayrshire Cheddar cheese (Fig. 2H). The presence of this amino acid was noticeable throughout ripening in Ayrshire Cheddar cheese, but not in non-Ayrshire Cheddar cheese, where only small concentrations were detected at day 10, 50, 64 and 78. It has been reported that the higher the concentration of hydrophobic amino acids such as phenylanaline, the more it contributes to the bitter and astringent sensation of cheese (Molina *et al*., 1999b).

An interesting pattern was noticed in Ayrshire Cheddar for six of the prominent eight amino acids detected. A sharp increase in the concentration of alanine, glycine, leucine, isoleucine, proline and asparagine was observed at day 36 of ripening in Ayrshire Cheddar cheese, which was not observed in non-Ayrshire Cheddar cheese. It may seem that this pattern is unique to the Ayrshire Cheddar cheese profile and might pose certain benefits in the identification of this type of branded cheese. Another interesting difference between Ayrshire and non-Ayrshire Cheddar cheese is the flux pattern that was noticeable in Ayrshire Cheddar cheese, but not in non-Ayrshire Cheddar cheese. In non-Ayrshire Cheddar cheese, the amino acid concentration throughout ripening was generally more consistent for most of the amino acids. A higher standard deviation was also noticeable in Ayrshire Cheddar cheese, which was not observable in non-Ayrshire Cheddar cheese, for seven of the eight amino acids. A specific trend regarding amino acid concentration in Ayrshire Cheddar cheese was also noticeable – the concentrations in Ayrshire Cheddar generally start lower than in non-Ayrshire Cheddar cheese, and also end lower at the end of the ripening process. Besides all these differences between Ayrshire and non-Ayrshire Cheddar cheese, the only significant differences found throughout ripening in the two cheeses were in glycine and asparagine.

5.5.2. Mathematical estimations

Mathematical equations and coefficients describing the changes in amino acids during maturation of Ayrshire and non-Ayrshire Cheddar cheese are presented in Table 1 and Table 2 respectively. These changes were defined by mathematical equations with R^2 values ranging from 0.79 to 0.99. Most of the amino acids in Ayrshire and non-Ayrshire Cheddar cheese were best represented by a five-grade polynomial equation, with the exception of phenylalanine, which was best fitted with a four-grade polynomial equation. The changes in the mathematical models can be used to study the changes in the release of free amino acids by starter culture enzymes during metabolic processes. Moreover, it is known that certain amino acids in Cheddar cheese are extremely important in flavour and taste development, e.g. arginine is related to bitterness, while proline, serine and asparagine are related to sweetness (Izco & Torre, 2000; Pappa & Sotirakoglou, 2008; Sousa, Ardö & McSweeney, 2001). It appears that acidic compounds like asparagine and glutamine could be responsible for the sour taste of cheese, and that high concentrations of phenylalanine result in astringent flavours. Salty tastes are normally associated with the presence of salts, mainly NaCl, although high levels of lysine in cheese might also contribute to the salty taste, since Lys-HCl can be salty (Molina *et al*., 1999b). Umami tastes, on the other hand, originate mostly from sodium salts of glutamic and aspartic acid (Molina *et al*., 1999b). In recognising the fact that selected amino acids contribute to certain flavours, amino acids can be added in quantities calculated by means of the aforementioned equations to achieve specific quality attributes.

To ensure time and cost effectiveness, without the need for repetitive physical analysis, these equations can serve as fundamentals of a model to calculate the age of the cheese by applying amino acid data, or to predict the concentration of a particular amino acid for a certain period of time during early ripening. Furthermore, these equations are useful in determining milk origin in branded cheeses in order to trace adulteration with the milk of other breeds, i.e. whether pure Ayrshire milk or a mixture of other breeds' milk was used in the manufacturing of the cheese.

Even though some amino acids could be more accurate indicators of maturation than others, a combined formula of all the amino acids should give a more accurate estimate of the extent of maturation. Two combined formulas (which are only relevant to the cheese investigated in this study) can therefore be put forward for Ayrshire and non-Ayrshire Cheddar cheese as a representation of the development of amino acids during ripening:

Ayrshire:

$$y = \frac{-1.0821x^5 + 21.3477x^4 - 154.1323x^3 + 492.8373x^2 - 666.027x + 413.906}{8}$$

Non-Ayrshire:

$$y = \frac{0.5447x^5 - 11.323x^4 + 89.5097x^3 - 330.2148x^2 + 547.0872x - 173.2315}{8}$$

Where:

x = maturation time

y = amino acid concentration

With regard to the cumulative contribution of amino acids, it may be easier to calculate the average age $(\bar{x})$ obtained from solving the respective equations depicted in Table 1 and Table 2. As mentioned earlier, these equations are only applicable to the cheese manufactured in this study, and their applicability to similar cheeses manufactured elsewhere is still to be established.

5.6. Conclusions

The application of regression models for eight amino acids as outlined by means of mathematical equations was suitable for the recognition / authentication of

the Cheddar cheeses manufactured from milk originating solely from the Ayrshire breed versus cheese manufactured from milk from a mixture of sources that exclude Ayrshire. By applying these equations, the manufacturer of the branded cheese can realise promising outcomes at an early stage of maturation, i.e. if the cheese conforms to brand specifications. Alternatively, good-quality cheese can be selected for specialist lines (mature Cheddar) and defective cheese can be used in other processes, i.e. processed cheese. This data also shows that a specific trend in Ayrshire Cheddar cheese was established at day 36 of ripening, as indicated by an increase in the concentration of six of the eight amino acids at this time. This phenomenon was, however, not observed in the cheese made from the milk of a mixture of breeds. A quick quality check can be developed, based on increases in amino acids at certain times that occur only in the branded Ayrshire Cheddar cheese. Significant differences between Ayrshire and non-Ayrshire Cheddar cheese were, however, only found in two of the eight amino acids (glycine and asparagine).

Table 1. Mathematical equations representing the patterns of amino acids during the maturation of Cheddar cheese manufactured from Ayrshire milk

Variable	Estimation summary		
Amino acid	Equation	Coefficient	R^2
Alanine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.1145 **b** 2.2197 **c** -15.701 **d** 49.09 **e** -65.42 **f** 42.289	0.79
Glycine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.2111 **b** 4.2584 **c** -31.662 **d** 104.93 **e** -144.61 **f** 76.116	0.84
Valine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.0801 **b** 1.29 **c** -6.2324 **d** 5.8064 **e** 12.565 **f** 13.444	0.98
Leucine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.1738 **b** 3.3631 **c** -23.811 **d** 74.705 **e** -97.716 **f** 55.895	0.99
Isoleucine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.1839 **b** 3.5843 **c** -25.616 **d** 81.4 **e** -108.54 **f** 61.608	0.99
Proline	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.1742 **b** 3.5266 **c** -26.499 **d** 89.88 **e** -131.18 **f** 80.224	0.99
Asparagine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** -0.1445 **b** 3.0101 **c** -23.022 **d** 78.071 **e** -111.2 **f** 60.915	0.97
Phenylalanine	$y = ax^4 + bx^3 + cx^2 + dx + e$	**a** 0.0955 **b** -1.5889 **c** 8.9549 **d** -19.926 **e** 23.415	0.98

R^2 = correlation coefficient

Table 2. Mathematical equations representing the patterns of amino acids during the maturation of Cheddar cheese manufactured from non-Ayrshire milk

Variable	Estimation summary		
Amino acid	Equation	Coefficient	R^2
Alanine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0205 **b** -0.3831 **c** 2.7469 **d** -9.3957 **e** 14.948 **f** 4.6529	0.98
Glycine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0211 **b** -0.4283 **c** 3.5871 **d** -15.378 **e** 32.599 **f** 0.0694	0.99
Valine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.2552 **b** -5.2848 **c** 40.893 **d** -143.76 **e** 215.39 **f** -84.496	0.99
Leucine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0613 **b** -1.2996 **c** 10.62 **d** -41.402 **e** 75.735 **f** -30.252	0.98
Isoleucine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0714 **b** -1.5122 **c** 12.293 **d** -47.433 **e** 85.269 **f** -34.847	0.98
Proline	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0873 **b** -1.8145 **c** 14.331 **d** -52.617 **e** 85.764 **f** -21.625	0.88
Asparagine	$y = ax^5 + bx^4 + cx^3 + dx^2 + ex + f$	**a** 0.0279 **b** -0.6095 **c** 5.1605 **d** -20.916 **e** 39.515 **f** -16.437	0.82
Phenylalanine	$y = ax^4 + bx^3 + cx^2 + dx + e$	**a** 0.009 **b** -0.1218 **c** 0.6869 **d** -2.1328 **e** 9.7032	0.98

R^2 = correlation coefficient

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Chapter 6

The discrimination of milk origin in the manufacturing of Cheddar cheese via artificial neural network modelling of *Lactococcus lactis* and *Streptococcus thermophilus*

Submitted for publication to Food Chemistry.

6.1. Title

The discrimination of milk origin in the manufacturing of Cheddar cheese via artificial neural network modelling of *Lactococcus lactis* and *Streptococcus thermophilus.*

6.2. Abstract

The aim of this study was to design an artificial neural network (ANN) able to distinguish between Cheddar cheese produced from milk sourced from mixed sources (different bovine breeds) and milk produced exclusively from a single source (Ayrshire breed). The two selected variables were the biological indicators, *Lactococcus lactis* and *Streptococcus thermophilus,* which are easily assessable in the standard in-house analytical laboratories of smaller (boutique) cheese-production plants. Thirty-two cheese samples of each batch (pure Ayrshire (4) / mixed with no Ayrshire milk (4)) were ripened for 92 days and microbial analysis was performed every 14 days. *Lactococcus lactis* and *Streptococcus thermophilus* colonies were enumerated and pure cultures were subjected to total DNA extraction and sequence-specific polymerase chain reaction (PCR). A novel ANN was designed and applied, consisting of a multilayered network with supervised training arranged into an ordered hierarchy of layers, which allowed for connections only between nodes in immediately adjacent layers. The construction thereof allowed for two output nodes, connected to an input layer consisting of two nodes to which the inputs were connected. The results from the ANN showed acceptable classification of the cheeses based on the *L. lactis* - (Ayrshire with 96% and mixed with 99% accuracy) and *Streptococcus thermophilus* (Ayrshire with 90% and mixed with 86% accuracy) counts. The study concluded that the neural network that was created, trained and tested for purposes of this study ensured the objective and reliable authentication of the cheese samples, but since growth was tracked over 92 days, endpoint sampling was not possible and a network should therefore be designed to incorporate more than one biological indicator. Retailers that deal in boutique dairy products can now

move towards the continuous application and validation of similar ANN models in their routine quality control and authentication strategies.

6.3. Introduction

The deception of consumers regarding the origin of food products or the raw materials applied in the manufacturing thereof is a regular occurrence (Karoui & De Baerdemaeker, 2007; Pillonel, Badertscher, Casey, Meyer, Rossman, Schlichtherle-Cerny, Tabacchi & Bosset, 2005a; Sacco, Brescia, Buccolieri & Caputi Jambrenghi, 2005; Sacco, Brescia, Sgaramella, Casiello, Buccolieri, Ogrinc & Sacco, 2009). Consequently, manufacturers and retailers rely on the accurate characterisation of products, frequently applying different analytical methods (Brescia, Monfreda, Buccolieri & Carrino, 2005; Luykx & Van Ruth, 2008; Marilley & Casey, 2004; Sacco *et al*., 2005; Sacco *et al*., 2009). However, a product such as cheese poses unique challenges, as its character is influenced by complex biochemical and microbiological interactions that occur during production and ripening. Furthermore, the main properties and organoleptic qualities of ripened cheeses such as Cheddar are largely due to differences (origin) in the raw material (i.e. milk) and key cheese manufacturing processes (Atasoy & Türkoglu, 2009; Hernández, Barrón, Virto, Pérez-Elortondo, Flanagan, Rozas, Nájera, Albisu, Vicente & De Renobales, 2009; Hickey, Kilcawley, Beresford, Sheehan & Wilkinson, 2006; Pappa, Kandarakis, Anifantakis & Zerfiridis, 2006). Therefore the qualification of authenticity requires a combination of selected analytical techniques and compound multivariate analysis, also known as chemometrics.

Several reviews on chemometrics have highlighted its application in the pattern recognition of selected product components in the battle against food adulteration (Luykx & Van Ruth, 2008; Pillonel, Bütikofer, Schlichtherle-Cerny, Tabacchi & Bosset, 2005b; Puerto, Baquero, Rodriguez, Martin & Romero, 2004). From there the definition: The application of mathematical and statistical methods to maximise the chemical/biological information extracted from data (Pillonel *et al*., 2005b). Methods commonly applied include discriminant analysis (DA), principal component analysis/regression (PCA/PCR), partial least square (PLS), and artificial

neural network (ANN) (Barile, Coïson, Arlorio & Rinaldi, 2006; Dias, Peres, Veloso, Reis, Vilas-Boas & Machado, 2009; Pillonel *et al.*, 2005b; Rodriguez-Nogales, 2006; Sacco *et al.*, 2005).

Regardless of the compounds/microbes analysed and the method applied to produce recognition patterns, subsequent model validation is essential and dynamic. Without validation, models applied in food authentication might result in acceptable clustering, but may lack statistical significance. These models are usually overfitted or insufficiently adapted, and suffer from large deviations between the training (model) and validation (evaluation) sets – in many cases a result of compound/microbe concentration fluctuation due to varying production procedures or raw material consistency (Puerto *et al.*, 2004; Rodriguez-Nogales, 2006).

Nevertheless, specific microbes and chemical compounds/parameters are commonly applied in the development of validated recognition patterns (models) of cheese and have successfully been applied in the authentication of age in the case of Cheddar cheese (Lues & Bekker, 2002). Analysis usually includes microbial cultivation followed by rRNA verification, chemical analysis through chromatography, nuclear magnetic resonance (NMR), classical methods, etc., as well as biochemical assays mainly to yield profiles with the potential to solve selected problems in the dairy industry related to the assessment of Cheddar cheese quality (Karoui & De Baerdemaeker, 2007; Luykx & Van Ruth, 2008; Marilley & Casey, 2004; Singh, Drake & Cadwallader, 2003). For example, in cases where the denomination of cheese origin is protected, the origin of the milk used to manufacture this particular cheese is verified (Barile *et al.*, 2006; Luykx & Van Ruth, 2008; Pillonel *et al.*, 2005a; Pillonel *et al.*, 2005b; Sacco *et al.*, 2009).

In addition to the traditional chemometrics approach to authenticating food, several reports on the application of statistical analysis in combination with ANN have appeared in recent years. Here, artificial intelligence (AI) or the neural networks approach is applied as an engineering science whereby intelligent machines are created through the application of intelligent computer programs. The success of ANN relates to the application of AI, which simulates human intelligence

without being confined to approaches that are biologically/humanly observable (Callan, 2003).

ANN is based on collections of nodes or neurons that are connected in a tree pattern to allow communication (Callan, 2003). A single node is a simple processor, which computes by combining the input signals with an activation rule to produce an output signal (Fig. 1) (Callan, 2003). These nodes are interconnected with weighted connections – weight being a multiplying constant for the connection's input. In isolation, these nodes are limited in operation, but interconnections in a multilayered network give them the ability to perform complex tasks such as distinguishing between compound biological systems as they occur in cheese (Barile *et al.*, 2006; Pillonel *et al.*, 2005b).

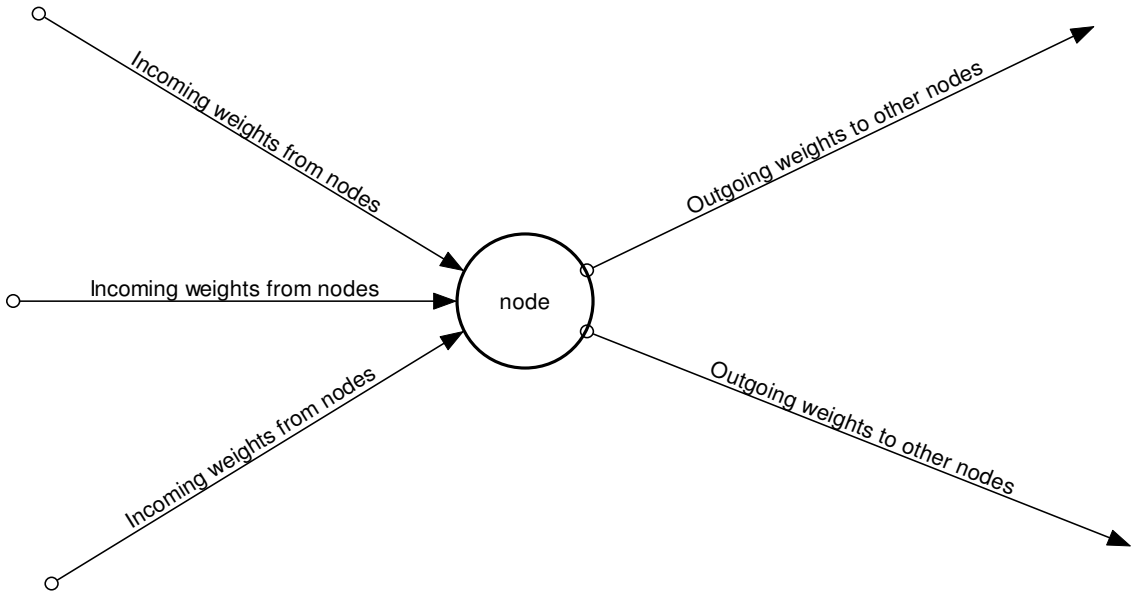

Figure 1. Layout of a single network node

The chemometrics approach to validating food authenticity relies mainly on multivariate statistical analysis (MSA) as an alternative to linear regression. This approach has been presented thoroughly in the literature (Brescia *et al.*, 2005; Sacco *et al.*, 2009). It has also been noted that ANN, in several cases, accomplishes food authentication to a greater and faster extent than the traditional chemometric

approaches (Barile *et al.*, 2006; Pillonel *et al.*, 2005b). Therefore, this particular study deals with the application of two custom-designed ANN's to a selected relevant basic variable (assessable through standard laboratory analysis) to be applied in producing and validating a recognition pattern (model) that could be used to distinguish between Cheddar cheese produced with milk from mixed sources (different bovine breeds) and that produced exclusively from a single source (Ayrshire breed). The selected variable for the two ANN were the biological indicator, *Lactococcus lactis* and *Streptococcus thermophilus* respectively. This paper also sets the stage for retailers dealing in boutique dairy products to move towards the continuous application and validation of models such as in their routine quality control and authentication strategies.

6.4. Materials and methods

6.4.1. Cheese sampling

Sixty-four Cheddar cheese samples were supplied by a local manufacturer specialising in the manufacturing of boutique cheeses. Thirty-two samples originated from whole Cheddar cheese produced from a mixture of bovine breeds' milk (excluding Ayrshire milk), while 32 samples originated from whole Cheddar cheese produced exclusively from Ayrshire milk. Manufacturing of both cheeses was standardised to limit product variability induced by the cheese-making process. After production, the samples were ripened at 10°C for 92 days and samples were collected for analysis at 14-day intervals.

6.4.2. Microbiological analysis

All media and reagents were obtained from Merck (RSA). One gram of cheese sample was finely ground with sterile equipment and blended in 9 ml buffer (Peptone, Merck) after which serial dilutions were prepared. Sterile M17 media were use for the cultivation of *Lactococcus* spp. *Streptococcus thermophilus* isolates were prepared according to the method of Dave and Shah (1996). Dilutions were plated on a solidified agar medium and plates were incubated at 30°C for 48 hours,

after which the colonies were enumerated using a colony counter. Pure cultures of the presumed *L. lactis* and *S. thermophilus* isolates, as well as a reference strain for both, were subjected to total DNA extraction and sequence-specific polymerase chain reaction (PCR). The following primers were used for *L. lactis:* upstream primer *Ll-F* 5'-TGG CTC AGG ACG AAC GCT GGC GGC-3' and downstream primer *Ll-R* 5'-CCT ACT GCT GCC TCC CGT AGG AGT-3' (Ward, Brown & Davey, 1998; Young, Downer & Eardly, 1991). Primers used for *Streptococcus thermophilus* were the following: upstream primer *St-F* 5' CAC TAT GCT CAG AAT ACA 3' and downstream primer *St-R* 5' CGA ACA GCA TTG ATG TTA 3' (Schroeder, Robert, Lenzen, McKay & Mercenier, 1991). DNA extractions on the cells were done with a MagPrep® bacterial genomic DNA kit (Novagen).

6.4.3. Statistical analysis

Every analysis was conducted at least in duplicate, and the values reported are the means. Cheese samples were classified according to the milk source by designing and applying a novel ANN for *L. lactis* and for *S. thermophilus* respectively.

6.4.4. Artificial neural network design

Two multilayered networks, with the supervised training capable of learning a required function, were designed: one for *L.Lactis* and one for *S. thermophilus*. This was accomplished by calculating the error at each net or node for both followed by the adjustment of weights accordingly to produce all the required outputs. This process can be mathematically simulated with the formula of the neuron as follows (Callan, 2003):

$$net_j := \sum_{i=1}^{N} x_{i,j} w_{i,j} \qquad (1)$$

Where:

N is the number of inputs

i is the node number for a specific input

j is the number of the net

x is the input value

w is weights or constants

This is commonly put through a sigmoid function as follows (Callan, 2003):

$$f_j := \frac{1}{1 + \left[e^{\left(-net_j\right)}\right]} \tag{2}$$

Where:

net is the output of the net

j is the number of the net

To calculate the error, the network applies a generalisation of the delta rule by starting at the last layer with (Callan, 2003; Chauvin & Rumelhart, 1995):

$$\delta_j := \left(t_j - o_j\right) \cdot o_j \left(1 - o_j\right) \tag{3}$$

Where:

t is the required output

o is the net output

j is the number of the net

Subsequently, the error at the hidden layers is calculated as follows (Callan, 2003):

$$\delta_j := o_j \left(1 - o_j\right) \cdot \sum_k \delta_k w_{j,k} \tag{4}$$

Where:

o is the net output

j is the number of the net

k is the number of the net from where the error originates

δ_k is the error from the previous layer

l is the number of that specific path

The weight change for each node is then calculated with (Callan, 2003):

$$\Delta w_{i,j} := \eta \cdot \left(x_{i,j} \cdot \delta_j \right) \tag{5}$$

Where:

η is the learning rate

i is the node number for a specific input

j is the number of the net

x is the input value

δ is the error from the each layer

Thereafter the weights are adjusted as follows (Callan, 2003):

$$W_{i,j} := w_{i,j} + \Delta w_{i,j} \tag{6}$$

Where:

Δw is the weight change

w is the old weights

A training data set that simulates a real-world problem was mapped. This training data set consisted of inputs with the corresponding outputs that were fed to the neural network for weight adaptation. It is said to be beneficial to randomise the order of the presentation for each training sample (Callan, 2003; Gurney, 2003).

Finally a test data set, similar to a real-world problem, was given to both the adapted neural networks to verify sufficient generalisation (when the network produces the correct output for the majority of input samples of the test data set). This would imply that the network can produce smooth nonlinear mapping with the ability to interpolate non-exact samples. If the neural network were over-trained, it would be like a memory seeking an output for an input, with interpolation or prediction becoming impossible (Callan, 2003; Gurney, 2003). Therefore, similarly to the traditional chemometrics approaches, accurate validation is fundamental to the success of ANN.

6.5. Results and discussion

The principle of artificial neural network (ANN) modelling has been successfully applied in the field of food authentication. The application of ANN to sensory and chemical data for the classification of selected food products, such as wine (Cichelli, Damiani, Murmura, Simonetti, Odoardi & Damiani, 2000; Pérez-Magariño, Ortega-Herasa, González-San José & Boger, 2004) and honey (Cordella, Militao, Clement & Cabrol-Bass, 2003) has, for example been comprehensively covered in the literature. In addition, there have been reports of the application of ANN to qualify dairy products based on chemical data, although with little reference to the successful application of ANN to biological indicators to authenticate Cheddar cheese manufactured from milk originating from cattle breeds in a South African environment.

Through the application of dedicated software, a functional neural network was designed that was able to effectively associate the proliferation pattern of *Lactococcus lactis* (Table 1) and *Streptococcus thermophilus* (Table 2) in the cheese samples with the corresponding milk origin (pure Ayrshire vs. mixed with no Ayrshire). Training phases were applied to optimise the ANN's through adjustment of the parameters involved in the learning process. This was accomplished by dividing the data into a training set and an evaluation set for both organisms. The

data set would adjust the parameters, and the evaluation set would evaluate whether a realistic prediction could be achieved. The results of the ANN analysis are presented in Table 3 for *L. lactis* and in Table 4 for *S. thermophilus*. The ANN design (Fig. 2) and the related parameters involved in the learning process were selected exclusively on the basis of their ability to recognise and predict. The best results were obtained by sequentially optimising the values of the learning rate and the momentum followed by the values of nodes in the hidden layer and the epoch quantity. Two hidden nodes with *circa* $1x10^4$ epochs yielded the best results for both *L. lactis* (Fig. 3) and *S. thermophilus* (Fig. 4). It was decided that this network design would suffice, as it resulted in root-mean-square errors ranging from 0.2% – 2.76% for the test sets and an overall (including the training sets) root-mean-square error ranging from 0.92% – 1.59% for *L. lactis*. For *S. thermohilus* the root-mean-square errors ranged from 7.82% and 12.56% for the test sets with an overall root-mean-square error ranging between 8.29% and 9.92%.

A multilayered network with supervised training arranged into an ordered hierarchy of layers, only allowing connections between nodes in immediately adjacent layers, was coded for the evaluations (tests). Since there were two outputs (milk origin purely Ayrshire (A) vs. mixed (G)), the decision was made to select two output nodes connected to an input layer consisting of two nodes to which the inputs were connected. The network was designed with two layers of weights, as this kind of network is capable of approximating any continuous functional mapping (Bisop, 2005).

Table 1. Values obtained from the quantification of *Lactococcus lactis* during the ripening of Cheddar cheese manufactured from milk originating from the Ayrshire breed only ($A_{(batch)}$) and from milk originating from a mixture of sources that exclude the Ayrshire breed ($G_{(batch)}$)

Cheese batch	Input values for *L. lactis* (1 Log 7 cfu.g^{-1})							
	Ripening (Days)							
	2	10	22	36	50	64	78	92
A_1	0.085	1.25	2.98	0.1	0.3833	0.461	0.289	0.52
A_2	0.1	1.3	1.405	0.234	0.2	0.308	0.437	0.52
A_3	0.07	0.5183	0.16	0.24	0.135	0.096	1.61	0.11
A_4	0.113	0.67	1.6	0.19	1.125	0.2	1.13	0.135
G_1	0.082	0.084	0.0625	0.0215	0.095	0.0525	0.028	0.037
G_2	0.051	0.011	0.0645	0.0265	0.0483	0.006	0.0165	0.052
G_3	0.05	0.019	0.06	0.068	0.045	0.005	0.06763	0.019
G_4	0.0865	0.15	0.047	0.0305	0.003	0.002	0.01713	0.018

Table 2. Values obtained from the quantification of *Streptococcus thermophilus* during the ripening of Cheddar cheese manufactured from milk originating from the Ayrshire breed only ($A_{(batch)}$) and from milk originating from a mixture of sources that exclude the Ayrshire breed ($G_{(batch)}$)

Cheese batch	Input values for *S. thermophilus* (1 Log 7 cfu.g^{-1})							
	Ripening (Days)							
	2	10	22	36	50	64	78	92
A_1	0.02655	0.14	0.15	0.126	0.325	0.041	0.105	0.0635
A_2	0.020467	0.15	0.07	0.1	0.2	0.03725	0.105	0.0895
A_3	0.007	0.063	0.0805	0.08	0.135	0.032	0.21	0.0128
A_4	0.007	0.062	0.1	0.08	0.2	0.036	0.13	0.00775
G_1	0.036	0.00775	0.040767	0.01445	0.027	0.013	0.00345	0.0171
G_2	0.034	0.06	0.045	0.01505	0.017	0.019	0.0057	0.01505
G_3	0.099	0.00165	0.0235	0.0225	0.036	0.025	0.00105	0.0114
G_4	0.095	0.002	0.02	0.064	0.004	0.025	0.001	0.01315

Table 3. ANN results for *L. lactis* of the eight datasets considered individually ($A_{(batch)}$ – Cheddar cheese manufactured from milk originating from the Ayrshire breed only; $G_{(batch)}$ – Cheddar cheese manufactured from milk originating from a mixture of sources that exclude the Ayrshire breed). $1x10^4$ epochs was maintained with $\eta = 0.5$ throughout the study.

Input (training dataset)	Cheese batch	Input (testing dataset)	Recognition ability (%) (training set)		Recognition ability (%) (test set)	
			A	G	A	G
1_a	A_1		102	-2		
	A_2	A_4	98	2	99.8	0.2
	A_3		100	0.02		
1_b	G_1		0.3	100		
	G_2	G_4	-0.5	100	0.2	99.8
	G_3		-0.1	100		
2_a	A_2		99	0.5		
	A_3	A_1	101	-0.9	103	-3
	A_4		100	-0.04		
2_b	G_2		0.04	100		
	G_3	G_1	0.5	100	1	99
	G_4		-0.3	100		
3_a	A_1		102	-2		
	A_3	A_2	100	0.02	96	4
	A_4		99	1.24		
3_b	G_1		0.5	100		
	G_3	G_2	0.3	100	-0.2	100.2
	G_4		-0.5	101		

Table 4. ANN results for *S. thermophilus* of the eight datasets considered individually ($A_{(batch)}$ – Cheddar cheese manufactured from milk originating from the Ayrshire breed only; $G_{(batch)}$ – Cheddar manufactured from milk originating from a mixture of sources that exclude the Ayrshire breed). $1x10^4$ epochs was maintained with η = 0.5 throughout the study.

Input (training dataset)	Cheese batch	Input (testing dataset)	Recognition ability (%) (training set)		Recognition ability (%) (test set)	
			A	G	A	G
1_a	A_1	A_4	107	-7	90	10
	A_2		96	4		
	A_3		89	11		
1_b	G_1	G_4	8	92	14	86
	G_2		10	90		
	G_3		7	93		
2_a	A_2	A_1	96	4	106	-6
	A_3		90	10		
	A_4		90	10		
2_b	G_2	G_1	16	84	12	88
	G_3		8	91		
	G_4		7	93		
3_a	A_1	A_2	86	14	94	6
	A_3		90	10		
	A_4		107	-7		
3_b	G_1	G_2	5	95	9	91
	G_3		3	97		
	G_4		6	94		

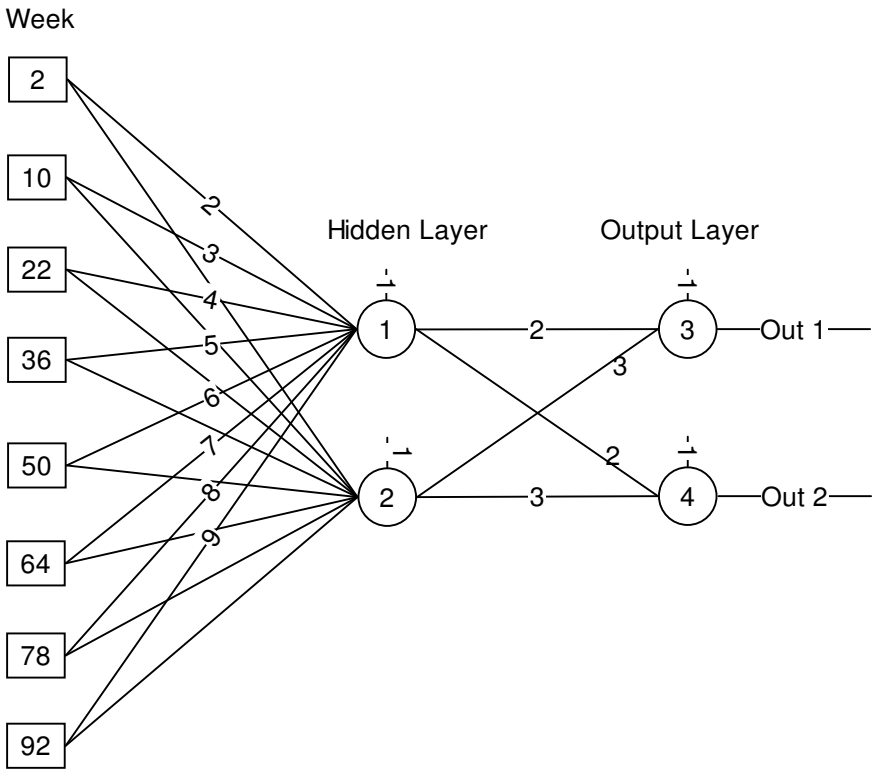

Figure 2. Multilayered network for the five analogue inputs to two nodes to the two outputs, designed to distinguish between the proliferation patterns of *Lactococcus lactis* and *Streptococcus thermophilus* during the ripening (week) of Cheddar cheese manufactured from milk originating from the Ayrshire breed only (Out 1) and from milk originating from a mixture of sources that exclude the Ayrshire breed (Out 2).

The inputs consisted of the corresponding data for *Lactococcus lactis* and *S. thermophilus* of either sample A or sample G as measured at week 2, 10, 22, 36, 50, 64, 78 and 92 of the Cheddar cheese ripening process (Table 1 and 2 respectively for *L. lactis* and *S. thermophilus*). The ANN was trained to yield an Output 1, a percentage of probability for G, and an Output 2, a percentage of probability for A for both organisms. The network was further trained with a training set that consisted of A_1, A_2 and A_3 (batches manufactured) and G_1, G_2 and G_3 (batches manufactured) with their corresponding outputs. As stated, this was randomised. A_4 and G_4 were retained for an evaluation set and after evaluation the network was reset and trained

with a training set that consisted of A_2, A_3 and A_4 and G_2, G_3 and G_4 with their corresponding randomised outputs. In this case, A_1 and G_1 were retained for an evaluation set. After this evaluation, the network was reset and trained again with a training set that now consisted of A_3, A_4 and A_1 and G_3, G_4 and G_1 with their corresponding outputs. This was also randomised. Finally, A_2 and G_2 were retained for an evaluation set (Table 3 & 4 for *L. lactis* and *S. thermophilus* respectively).

The input range was of some concern, as the microbial counts in the cheese were relatively high. Due to the network's sigmoid transfer function, which works best between 0 and 1, a decision was made to divide the input values by $1x10^7$. This resulted in input values < 1. As the sigmoid's optimal resolution for an output is obtained between 0.9 and 0.1, it was trained with the output range of 0.1, indicating a 0% probability, and 0.9, indicating a 100% probability. To ease interpretation, the output was stepped through a function that would give the probability as a percentage. This function is as follows:

$$y = \frac{(x - 0.1)}{0.8} \times 100 \qquad (7)$$

Where:

y is the output probability as a percentage

x is the output of the network in the range of 0.1 for 0% to 0.9 for 100%

To prevent over-training of the network, resulting in the inability to produce smooth non-linear mapping and to interpolate non-exact samples, a selection was made of $1x10^4$ training cycles (epochs) that presented good generalisation at a learning rate of 0.5 (Fig. 3) for *L. lactis*. *S. thermophilus* also showed good generalisation at a learning rate of 0.5 (Fig. 4). It took $1x10^4$ training cycles 0.6 seconds (per biological indicator organism) on a computer with a processing speed of 3.40Ghz and 992Mb ram capability.

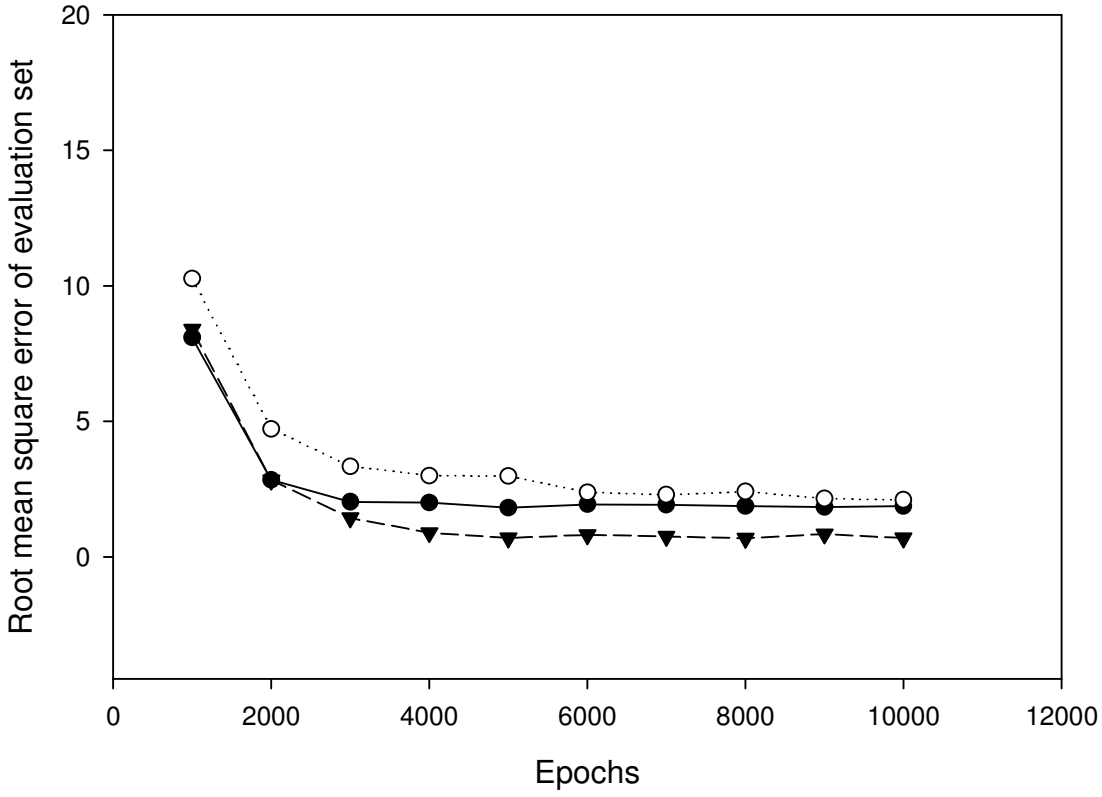

Figure 3. Performance of the training sets with *Lactococcus lactis* (●, ○, ▼ = training set 1, 2, 3). It should be noted that the recognition and prediction ability remained stable after $1x10^4$ epochs. Satisfactory generalisation without overtraining is also noted.

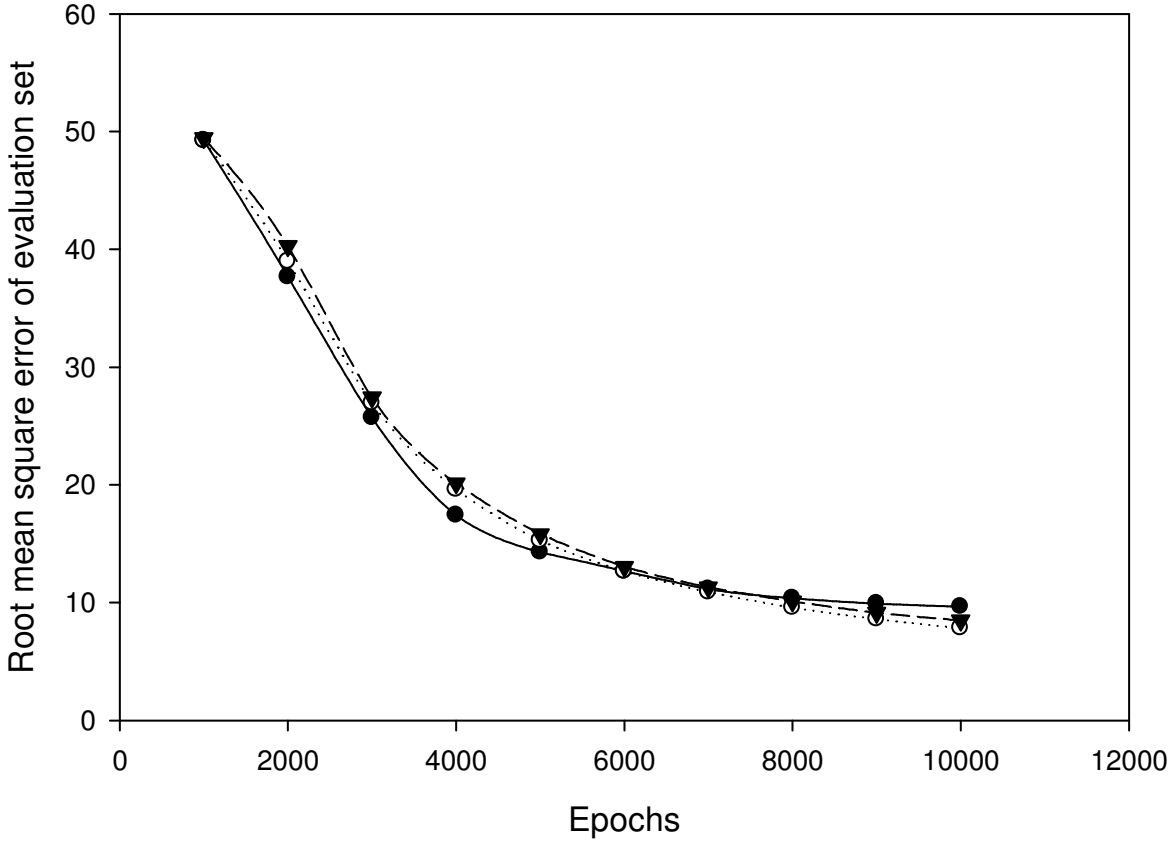

Figure 4. Performance of the training sets with *Sreptococcus thermopilus* (●, ○, ▼ = training set 1, 2, 3). It should be noted that the recognition and prediction ability remained stable after $1x10^4$ epochs. Satisfactory generalisation without overtaining is also noted.

Due to the inherent fluctuation of biological and chemical parameters between cheese batches manufactured, each batch was individually considered as a data set. As indicated in Table 1, for the datasets the prediction ability of the network was assessed by varying the data sets applied for training and tests. The noted number of epochs was sufficient to obtain desirable classification of the cheese samples with no overtraining – also referred to as overfitting or overlearning (Fig. 3 & 4). For the randomised training sets A_1-A_3 and G_1-G_3, the evaluation set consisted of A_4 and G_4. The probability values of the training sets after $1x10^4$ epochs ranged between 98.37% and 101.94% for *L. lactis* and between 89% and 107% for *S. thermophilus*.

Thereafter the ANN was applied to both *L. lactis* and *S. thermophilus* to recognise the test sets. It became evident that both *L. lactis* (A_4 = 99.79%; G_4 = 99.81%) and *S. thermophilus* (A_4 = 90%; G_4 – 86%) can successfully be used to predict the milk origin.

Subsequent to the former evaluation, the ANN was reset and trained again with a training set that comprised A_2-A_4 and G_2-G_4 with their corresponding outputs. A_1 and G_1 were selected as test sets. The resulting probabilities for these training sets ranged from 99.5 – 100.88% for *L. lactis* and from 84 – 96 % for *S. thermophilus*, which was acceptable for both. The resulting evaluation sets for *L. lactis* yielded 102.78% for A_1 and 99.02% for G_1, again with successful prediction similar to the previous set. For *S. thermophilus* it yielded 107% for A_1 and 88% for G_1 which are still successful predictions compared to the previous set. Finally, in order to confirm stability, the ANN was reset again and trained with data sets A_3-A_1 and G_3-G_1 (randomised) with their corresponding outputs. In this case, A_2 and G_2 were applied as the evaluation sets. The prediction results were again within an acceptable range for both organisms, thus confirming good generalisation.

6.6. Conclusions

The ANN approach followed in this study was found to be an effective and promising tool for the recognition and authentication of Cheddar cheese manufactured from milk originating solely from the Ayrshire breed versus milk from a mixture of sources excluding the Ayrshire breed. ANN further provided a satisfactory classification of the cheeses based only on the proliferation pattern of the biological indicators *L. lactis* and *S. thermophilus* during cheese ripening. The suitability of the ANN-based approach was found to result from its high prediction accuracy and the ability to compute the non-linear patterns produced during typical microbiological growth. The neural network created, trained and tested during this study ensured an objective and reliable authentication of the cheese samples. This approach sets the stage for retailers dealing in specialised dairy products, allowing them to move towards the continuous application and validation of ANN models based on

biological markers. However, the ANN presented in this paper might not be quite so suitable for endpoint sampling, as growth was followed over 92 days. Therefore a network designed to incorporate more than one biological indicator in addition to chemical indicators should be considered.

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Chapter 7

Conclusions

7.1. Introduction

The aim of this study was to distinguish between Cheddar cheese produced from milk from mixed sources (different bovine breeds) and that produced exclusively from a single source (Ayrshire breed). To achieve this objective, the chapter outlay was structured into sections that correlate with the methodology of the thesis. In general, all experiments were performed in quadruplet. Two Cheddar cheese batches – one originating from Ayrshire milk only and one from a mix of other breeds' milk – were manufactured according to the procedure for the production of hard and semi-hard cheeses. Subsequently cheese blocks (300 g in weight) were vacuum-packed and left to mature. From these blocks, samples were drawn for analysis on the following days after production: 1, 8, 22, 36, 50, 64, 78 and 92. This study has been structured into six different chapters – every chapter with its own outcomes:

Chapter 2: The relationship between organic acids, starter microbiota and selected chemical indicators in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 3: Mathematical expressions for organic acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 4: Mathematical indices for fatty acids in Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk.

Chapter 5: Mathematical modelling of Cheddar cheese manufactured from Ayrshire and non-Ayrshire milk using amino acid data.

Chapter 6: The discrimination of milk origin in the manufacturing of Cheddar cheese via artificial neural network modelling of *Lactococcus lactis* and *Streptococcus thermophilus.*

7.2. Final concluding remarks

7.2.1. Selection of most stable non-biological component

The evaluations noted in Chapter 2 were directed at assessing the maturation patterns of selected organic acids, starter microbiota and chemical variables in Cheddar cheese made from Ayrshire and a mixture of other breeds' milk. The motivation for this chapter was to assess the stability (least variance) in non-biological parameters (organic acids) by applying a concept of X_{rel} value in cheese uniformity. The organic acid isovaleric acid had the least variation relative to concentration (X_{rel}) in both Ayrshire and non-Ayrshire Cheddar cheese and is therefore the most effective indicator of cheese uniformity, whereas those which showed extensive variation (larger X_{rel}) bear little or no relation to cheese uniformity and are less stable. In Ayrshire Cheddar cheese it was pyruvic acid, whereas in non-Ayrshire Cheddar cheese it was citric acid.

Although isovaleric acid showed the least variance between the two batches, a regression graph (Chapter 3) indicates a fluctuation pattern in the concentration in non-Ayrshire Cheddar cheese, which was not present in Ayrshire Cheddar cheese at 50 to 92 days of ripening.

One of the key differences between Ayrshire and non-Ayrshire Cheddar cheese that came to light in this chapter was the fact that citric acid correlates negatively with all the organic acids in Ayrshire Cheddar cheese, but had no correlations with any of the organic acids in non-Ayrshire Cheddar cheese.

7.2.2. Mathematical models

The results from the experiments performed in Chapters 3, 4 and 5 were designed to define regression models by means of mathematical equations for organic acids, fatty acids and amino acids in Ayrshire and non-Ayrshire Cheddar cheese respectively. By applying these equations, the manufacturer of Ayrshire

Cheddar cheese will now be able to predict specific outcomes of the cheese early during ripening without full maturation of the cheese. Time and money can be saved by applying these equations in order to predict the concentration of certain acids at certain times during the early maturation process, and differences between Ayrshire and non-Ayrshire Cheddar cheese can be verified. Combined formulas, however, gave a more precise indicator of the extent of maturation than single formulas for all the different types of acids (organic, fatty and amino acids) (Table 1).

From the formulas listed in Table 1, one consolidated model for each Ayrshire and non-Ayrshire Cheddar cheese was constructed, taking all the parameters (organic, fatty and amino acids) into account. Since these formulae do not emphasise the characteristic differences between Ayrshire and non-Ayrshire Cheddar cheese in specific fatty, organic and amino acid concentrations, it can therefore not be applied to distinguish between Ayrshire and non-Ayrshire Cheddar cheese.

Ayrshire:

$$y = \frac{-1.582x^6 + 42.8x^5 - 469.541x^4 + 2584.131x^3 - 7482.162x^2 + 10505.727x - 3225.435}{39}$$

Non-Ayrshire:

$$y = \frac{-1.236x^6 + 33.451x^5 - 351.701x^4 + 1823.947x^3 - 4765.997x^2 + 5856.952x - 1585.245}{39}$$

Table 1. Consolidated formulae representing each of the parameters (organic, fatty and amino acids) in Ayrshire and non-Ayrshire Cheddar cheese respectively

Chapter	Cheese type	Parameter	Formula
3	A	OA	$y = \frac{-0.0018x^6 - 0.4144x^5 + 0.5491x^4 - 3.0313x^3 + 8.6905x^2 - 11.6583x + 14.7781}{8}$
3	NA	OA	$y = \frac{-0.00054x^6 + 0.0135x^5 - 0.1194x^4 + 0.5119x^3 - 1.1131x^2 + 1.3666x + 8.9786}{8}$
4	A	FA	$y = \frac{-1.584x^6 + 44.296x^5 - 491.438x^4 + 2741.295x^3 - 7983.69x^2 + 11183.412x - 3654.119}{23}$
4	NA	FA	$y = \frac{-1.235x^6 + 32.893x^5 - 340.259x^4 + 1733.925x^3 - 4434.669x^2 + 5308.498x - 1420.992}{23}$
5	A	AA	$y = \frac{-1.0821x^5 + 21.3477x^4 - 154.1323x^3 + 492.8373x^2 - 666.027x + 413.906}{8}$
5	NA	AA	$y = \frac{0.5447x^5 - 11.323x^4 + 89.5097x^3 - 330.2148x^2 + 547.0872x - 173.2315}{8}$

A = Ayrshire; NA = Non-Ayrshire; OA = Organic acids; FA = Fatty acids; AA = Amino acids

An additional formula for both cheeses therefore needs to be constructed to incorporate only the characteristic differences between the two types of cheese as seen in the regression graphs of each chapter.

The characteristic differences as seen in the regression graphs of Ayrshire and non-Ayrshire Cheddar cheese were the following:

Chapter 3 (organic acids): a fluctuation pattern at 50 to 92 days of ripening was noticeable in acetic, formic, butyric and isovaleric acid, evident in non-Ayrshire Cheddar cheese, but not in Ayrshire Cheddar cheese.

Chapter 4 (fatty acids): Ayrshire Cheddar cheese was always accompanied by a peak at day 10 of ripening in all the even-numbered medium-chain saturated fatty acids ($C_{8:0}$; $C_{10:0}$; $C_{12:0}$). A higher concentration of unsaturated fatty acids was visible in Ayrshire Cheddar cheese than in non-Ayrshire Cheddar cheese. Non-Ayrshire Cheddar cheese had a constantly higher concentration of saturated medium-chain odd-numbered fatty acids ($C_{7:0}$; $C_{9:0}$; $C_{11:0}$).

Chapter 5 (amino acids): In Ayrshire Cheddar, six of the eight amino acids showed an increase in concentration at day 36 of ripening. This phenomenon was not observed in non-Ayrshire Cheddar cheese. Significant differences in the concentrations of glycine and asparagine were noticeable throughout ripening between Ayrshire and non-Ayrshire Cheddar cheese.

Based on the above differences, a formula was constructed that only included all the distinctive differences between the two cheeses as described above. Although there were differences in the concentration of the unsaturated fatty acids and the saturated medium-chain odd-numbered fatty acids, these differences can be seen as batch differences between the two types of cheeses and were therefore not included in the final formula. Only unique peaks at certain times, i.e. the peak in certain organic acids at day 50 to 92 of ripening, which was only present in one of the cheeses, were included in this formula, as represented in Table 2.

Table 2. Major differences between Ayrshire and non Ayrshire Cheddar cheese.

Acids	Equation	Differences	
		Ayrshire	Non-Ayrshire
Formic	$y = -0.0003x^6 + 0.0079x^5 - 0.0741x^4 + 0.3335x^3 - 0.7504x^2 + 0.8141x + 0.3988$	-	Fluctuation pattern at 50 to 92 days of ripening
Acetic	$y = -0.0001x^6 + 0.003x^5 - 0.0279x^4 + 0.1223x^3 - 0.2597x^2 + 0.2499x + 1.1781$		
Butyric	$y = -0.00007x^6 + 0.0018x^5 - 0.0176x^4 + 0.0848x^3 - 0.2083x^2 + 0.2506x + 1.0387$		
Isovaleric	$y = -0.0001x^6 + 0.0023x^5 - 0.0205x^4 + 0.0918x^3 - 0.2151x^2 + 0.248x + 1.3966$		
$C_{8:0}$	$y = -0.0449x^6 + 1.3989x^5 - 17.35x^4 + 107.93x^3 - 346.82x^2 + 524.82x - 232.24$	A peak at day 10 of ripening	-
$C_{10:0}$	$y = -0.1027x^6 + 3.0007x^5 - 34.903x^4 + 204.4x^3 - 623.94x^2 + 911.2x - 389.19$		
$C_{12:0}$	$y = -0.1297x^6 + 3.7965x^5 - 44.043x^4 + 256.02x^3 - 772.71x^2 + 1115.1x + 485.24$		
Alanine	$y = -0.1145x^5 + 2.2197x^4 - 15.701x^3 + 49.09x^2 - 65.42x + 42.289$	A peak at day 36 of ripening	-
Glycine	$y = -0.2111x^5 + 4.2584x^4 - 31.662x^3 + 104.93x^2 - 144.61x + 76.116$		
Leusine	$y = -0.1738x^5 + 3.3631x^4 - 23.811x^3 + 74.705x^2 - 97.716x + 55.895$		
Isoleusine	$y = -0.8139x^5 + 3.5843x^4 - 25.616x^3 + 81.4x^2 - 108.54x + 61.608$		
Proline	$y = -0.1742x^5 + 3.5266x^4 - 26.499x^3 + 89.88x^2 - 131.18x + 80.224$		
Asparagine	$y = -0.1445x^5 + 3.0101x^4 - 23.022x^3 + 78.071x^2 - 111.2x + 60.915$		

From the above table, a single formula for both Ayrshire and non-Ayrshire Cheddar cheese was constructed:

Ayrshire:

$$y = \frac{-0.2773x^6 + 6.5641x^5 - 76.3338x^4 + 422.039x^3 - 1265.394x^2 + 1892.454x + 240.857}{9}$$

Non-Ayrshire:

$$y = \frac{-0.00057x^6 + 0.015x^5 - 0.1401x^4 + 0.6324x^3 - 1.4335x^2 + 1.5626x + 4.0122}{4}$$

7.2.3. Artificial neural network

An artificial neural network (ANN) was designed that incorporated all the parameters (biological and non-biological). However, the ANN based only on one organism at a time proved to be more accurate (results not shown) than those that incorporated non-biological parameters. Chemometrics are more accurate when both biological and non-biological parameters are included in the same network, but in this study the non-biological methods were better defined by traditional methods, i.e. polynomial regression, whereas the biological parameters were best defined by an artificial neural network.

In Chapter 6, novel ANNs were respectively designed and applied, which provided an acceptable classification of the two types of cheeses (Ayrshire and non-Ayrshire cheese) based only on the counts of the different starter cultures used in the manufacturing of the cheese, i.e. *Lactococcus lactis* and *Streptococcus thermophilus*. The two novel ANNs that were designed were based only on the classification of the *Lactococcus lactis* and *Streptococcus thermophilus* counts respectively. Both ANN's showed acceptable classification between Ayrshire and non-Ayrshire Cheddar cheese with 96% accuracy in Ayrshire and 99% accuracy in non-Ayrshire Cheddar cheese for the *L. lactis*. In the ANN with *S. thermophilus* as selected biological indicator, 90% accuracy was showed in Ayrshire Cheddar, whilst

86% in non-Ayrshire Cheddar cheese. Both ANN's presented in this thesis will, however, not be suitable for endpoint sampling, as growth was followed over 92 days. Therefore, a network needs to be developed to incorporate more than one biological indicator in addition to chemical indicators.

7.2.4. Future research

Possible future research could include the following:

- The equations suggested in this study were established for one cheese manufacturer, and their applicability to other processing plants still needs to be investigated.
- A mathematical model for other types of cheese, i.e. gouda, mozzarella, etc., under the same Ayrshire brand also needs to be investigated to verify authenticity thereof.
- Although more than one biological indicator was incorporated with the addition of chemical indicators into neural networks, it was not as accurate as with the single indicators. In future, applied networks should be developed to include at least one biological indicator and one chemical indicator to reduce variation and improve the accuracy of food authentication.