Modelling and Predicting the Quality of Cheddar Cheese During Ripening

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Abstract

Cheddar cheese maturation is crucial during cheese manufacture producing distinct flavours and textures. However, it is a slow and expensive process and also not all of the cheese production is capable of reaching the highest quality. Therefore, predictive tools are necessary to allow the optimisation and consistency of supply of mild-mature/vintage Cheddar cheeses, minimising losses.

Six blocks of Cheddar cheese produced on the same day in the same location were graded after a short period of storage by professional cheese graders and the Gilles and Lawrence grading model which is still used in cheese manufacture. After 13 months, these batches were regarded by graders again. Batch to batch variations of cheese ripening developments with different predictive quality were observed up to 450 days from state of water and fat, metabolic profile, aroma profile and texture profile. The batch to batch variation quality markers and ripening evolution markers from instrumental analysis were explored.

In parallel, sensory evaluation combined with the chemo-metrics was studied until 540 days ripening which is about the ripening time for the commercial vintage Cheddar cheese. Sensory profile identified that ‘downgrade’ batch C was found to have bland flavour and lumpy mouthfeel as well as yellower

colour, and whose maturation level was behind schedule as determined sensorically. Whereas 'downgrade' batch E appeared to have strong flavour intensity, dirty aftertaste and texture defects. These suggested that Gilles and Lawrence model is not sufficient to predict the cheese quality,

Time domain water and fat proton Nuclear Magnetic Resonance signals are assigned. Transverse relaxation times (T_2) for water and fat protons decrease and thermodynamic free water percentage increases with cheese ripening up to 450 days. Water and fat state attributes can differentiate between batches of Cheddar cheese after 56 days ripening.

Water soluble metabolites were extracted, identified and analysed using high resolution ^{1}H-^{13}C Nuclear Magnetic Resonance experiments. The analytical methods coupled with chemo-metric analysis offer a profiling of metabolites which showed batch to batch variations and in addition showed a pathway among different ripening time points. Batch C revealed a higher level of serine and β-galactose as well as a lower amount of lactic acid in the aqueous extract. The normalised intensity of citrulline and arginine decreased during maturation.

The aroma profiles of the batches of cheese were studied by solid-phase microextraction gas chromatography-mass spectrometry during ripening. The trajectory of different predictive qualities of Cheddar cheese during ripening was presented. Secondary alcohols, propyl esters and acetic acid can be considered as a defect sign.

Additionally, Protein matrix porosity analysed from microscopy images decreases during ripening with consequential decreases in cohesiveness and springiness.

The correlation between the instrumental analysis and sensory profile was studied. The normalised tyrosine, tyramine, lysine ratio in cheese aqueous extracts; acetoin and branch chain alcohols, and fracturability are highly correlated with a mature Cheddar cheese sensory profile. Conversely, glycerol, β-galactose content, springiness, protein matrix porosity and cohesiveness are associated with a young Cheddar cheese sensory profile. Texture related sensory attributes are correlated with octanoic acid, valeric acid and caproic acid levels. Cohesiveness is the attribute most correlated with sensory attributes among all texture profile analysis parameters.

Finally, a preliminary model was established and the sensory intensity of sweaty flavour, rate of melting, crumbly and onion flavour were well forecasted after 540 days ripening based on the 56 days measurements.

List of Abbreviations

NSLAB	non-starter lactic acid bacteria
LAB	lactic acid bacteria
MFFS	moisture in the fat free substance
S/M	the percentage of salt in moisture
FDM	fat in the water free substance /fat in the dry mass
TD-NMR	time-domain nuclear magnetic resonance spectroscopy
T_2	transverse relaxation times
CPMG	Carr-Purcell-Meiboom-Gill
TGA	thermogravimetric analysis
HSQC	heteronuclear single quantum coherence
TOCSY	total correlation spectroscopy
TPA	texture profile analysis
ANOVA	analysis of variance
MSE	mean squared error
GC-MS	gas chromatography-mass spectrometer
SPME	solid phase microextraction
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
PCA	principal component analysis
CA	correspondence analysis

HSD	honestly significant difference
AHC	agglomerative hierarchical clustering
PLS	partial least squares
DSC	differential scanning calorimetry
RMSE	root mean square error of prediction
IS	internal standard
LRI	linear retention index
PPS	points per second

List of Publications

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Chapter I General Introduction

Cheese has been produced for about thousands of years and is one of the more popular fabricated foods in the human diet. There are more than 1000 varieties of cheese in the world (Fox & McSweeney, 2017b). Cheddar cheese is the most popular type of cheese in the UK cheese market, accounting for more than 50% of total cheese sales (Agriculture and Horticulture Development Board, 2018). Cheddar cheese was firstly manufactured in the town of Cheddar, England. But now it is manufactured all over the world. For example, Cheddar cheese is the most widely consumed cheese in the United States (Serrano, Velazquez, Lopetcharat, Ramirez, & Torres, 2011).

Throughout history cheese has been an essential part of the human diet, both as a dietary staple and a gourmet food. In addition to being delicious, it is a rich source of essential nutrients particularly, proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins and minerals. The high concentration of essential amino acids in cheese contributes to growth and development of the human body. Cheese ripening typically involves the progressive breakdown of casein which increases the digestibility of cheese protein to almost 100% which is more easily absorbed by human body (Walther, Schmid, Sieber, & Wehrmüller, 2008). However, cheese has some health concerns due to high salt and fat contents. However, an online questionnaire study still shows that

76 % of consumers agreed with the statement that Cheddar cheese is a nutritious (Feeney, Regan, Wall, & Gibney, 2015).

Structurally, cheese is a complex gel matrix of milk protein, fats, minerals and other components including water (Dufour, Devaux, Fortier, & Herbert, 2001). Cheese making comprises four key factors: milk composition; key cheese manufacturing processes (including the use of different starter cultures and curd manipulation); water content and storage conditions (temperature and maturation time) (Gan, 2015). These factors lead to diversity in the texture and flavour of cheese.

I.1 Cheddar cheese production

The production of Cheddar cheese which is a rennet-coagulated cheese can be subdivided into two well-defined phases: manufacture and ripening (Figure I-1) (Singh, Drake, & Cadwallader, 2003).

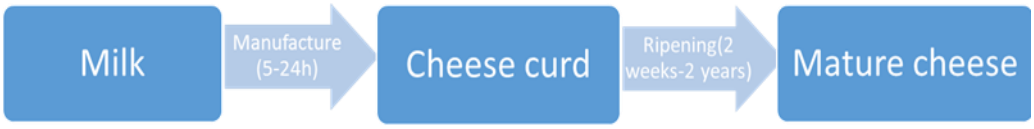


Figure I-1 Outline of Cheddar cheese production

I.1.1 Cheddar Cheese manufacture

The production of Cheddar cheese is illustrated schematically in Figure I-2. Raw milk is the main ingredient of Cheddar cheese which should be free of chemical

taints and antibiotics and high microbiological quality (Fox, Guinee, Cogan, & McSweeney, 2000a). The raw milk needs to be freshly pasteurised at 72 °C for five seconds. Pasteurisation alters the indigenous microbiota and facilitates the manufacture of cheese of more uniform quality (Fox *et al.*, 2017b; Walter & Lochry, 1945). Standardisation of raw milk fat and protein content by modifying cream and milk protein powder is performed in the industry in order to gain consistent cheese quality. The curd manufacture consists of coagulation, cutting, cooking (scalding), stirring and drainage of whey. After the lactic acid starter culture and rennet are added to clot and aggregate all the para caseins, whey protein separates from casein with the help of specific pH and heating. The production of acid at the appropriate rate and time is critical for the manufacture of good quality of cheese curd (Fox *et al.*, 2000a). The coagulum is cut with special cutting tools into small cubes of the desired size (5-10 mm) in order to facilitate expulsion of whey (Tetra Pak, 2015; Walstra, Wouters, & Geurts, 2006a). Rennet-Coagulated cheese curd is quite stable under quiescent conditions but if it is cut or broken, it synereses rapidly, expelling whey (Fox *et al.*, 2017b). The scalding temperature should be around 40 °C in order to produce sufficient acids and aid the expelling of whey from curd (Walstra *et al*., 2006a). Traditionally, Cheddar cheese is made by the milled-curd method. The stirred-curd method is commonly used by large scale commercial manufactures which is presented here (Shakeel ur, Drake, & Farkye, 2008). After cutting and scalding, curds and whey mixture are pumped onto drain table stirring until the pH of curd is 5.9. Salting process is to preserve and modify cheese flavour and consistency. Salt is added in three instalments and thoroughly stirred into the

mass of chipped curd and packed into moulds. The level and time of salting have a major influence on pH changes in cheese (Fox *et al.*, 2000a). The moulds that curds are placed in will be used to press the curds and form the blocks of Cheddar. Such blocks are then vacuum-packed ready for ripening.

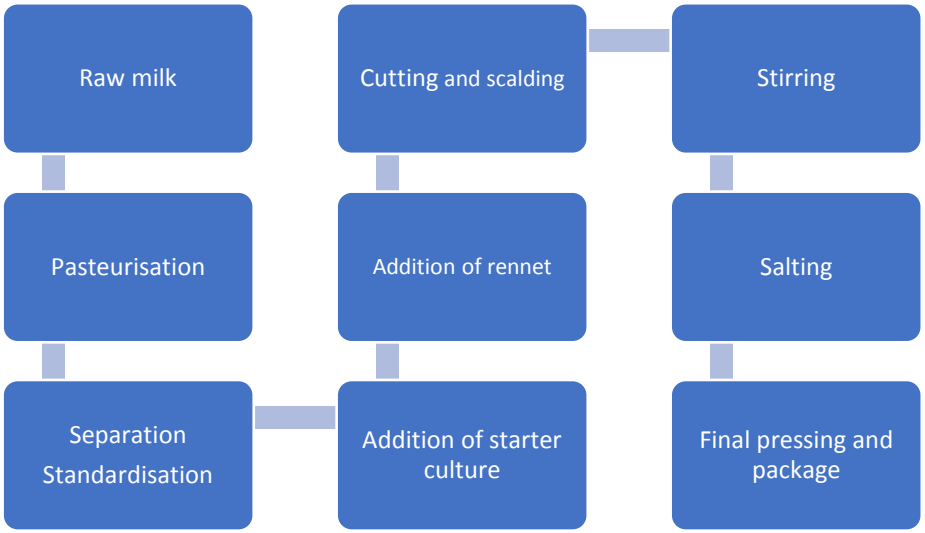


Figure I-2 Process flow in the manufacture of Cheddar cheese curd

I.1.2 Cheddar Cheese Ripening

The final step of Cheddar cheese production is ripening which can take from 3 to 6 months, depending on storage conditions and cheese acidity, whilst the high quality cheeses will improve for up to 2 years (Walstra, Wouters, & Geurts, 2006b). During the ripening phase, the characteristics of flavour and texture of the individual cheese variety develop (Fox *et al.*, 2017b). As ripening of Cheddar cheese takes an extended period and thus incurs considerable inventory and other costs to cheese makers, there has been much interest in accelerating and controlling ripening (McSweeney, 2017). Cheddar cheese ripening usually

involves a microbiome change and a range of biochemical reactions. Ripening changes to the microflora of the cheese, often death and lysis of starter cells, the development of an adventitious nonstarter microflora and in certain cases, the growth of secondary organisms (Fox, Cogan, & Guinee, 2017a). However, in this study the microbiome change during ripening is not the focus of investigation where the biochemical and physical changes of cheese with ageing will form the theme of the experimental enquiry.

I.2 Biochemistry of Cheddar cheese ripening

When compared to other dairy products, cheese is biologically and biochemically dynamic and inherently unstable (Fox *et al.*, 2017b). All of these biochemistry reactions will affect the texture, flavour, appearance quality attributes of cheeses. There are three major biochemical reactions taking place in the ripening process, which are proteolysis, glycolysis and lipolysis, together with the solubilisation of colloidal calcium (Atasoy & Türkoğlu, 2009; Lucey, Johnson, & Horne, 2003). The biochemical changes may be grouped into two levels. The primary level is proteolysis, lipolysis and metabolism of residual lactose. The secondary level is about the metabolism of fatty acids and amino acids (Gan, Yan, Linforth, & Fisk, 2016). All these changes aid in the conversion of fresh curd to mature cheese and markedly influence its rheological and textural functional and flavour characteristics (Fox, Guinee, Cogan, & McSweeney, 2016a).

I.2.1 Proteolysis

I.2.1.1 What is proteolysis?

Proteolysis is the most complex and important primary biochemical event in Cheddar cheese ripening (Lucey *et al.*, 2003). The protein, mainly casein, was gradually degraded under the influence of the rennet enzyme, bacterial enzymes and possibly the enzymes present in the original milk such as plasmin (Dacre, 1953). The further degradation from polypeptides to small peptides and amino acids is affected by the proteinase system of starter and nonstarter bacteria (Lawrence, Creamer, & Gilles, 1987). Catabolism of most of the amino acids appears to be initiated by the action of an aminotransferase which transfers the amino group to an acceptor molecule, usually α-ketoglutarate, thus forming glutamic acid and a new α-keto acid corresponding to the amino acid being degraded (McSweeney, 2007).

I.2.1.2 Why is proteolysis important?

Proteolysis contributes to the development of cheese texture and flavour and perhaps the off flavour of cheese (Upadhyay, McSweeney, Magboul, & Fox, 2004). Cheesy flavour is generated directly by the production of short-chain peptides and further degraded to amino acids. Some of the amino acids are the precursor of volatile compounds through catabolic reactions. Compounds that originate from aromatic amino acids have been shown to impart pungent off-flavours in cheese (Gummalla & Broadbent, 2001). Moreover, amino acid catabolism generates flavour compounds or intermediates. The process

provides energy as ATP for culture and other molecules also essential for cellular survival (Ganesan & Weimer, 2017). Furthermore, peptides and individual amino acids may have a direct impact on cheese flavour, some are bitter or may provide a brothy background flavour to cheese (McSweeney, 2007). Dipeptides are more bitter than the corresponding free amino acids, where proline is a major contributor to bitter taste of a peptide (Lemieux & Simard, 1992). The presence of glycine, alanine, valine, leucine, tyramine, and phenylalanine in peptides also imparts bitterness, while glutamic acid imparts umami taste (Zhao, Schieber, & Gänzle, 2016).

Proteolysis changes the structure and textural properties of cheese. Hydrolysis of a protein matrix and peptide bonds, liberation of carboxylic acid and amino acid which change the water binding capacity, help to develop cheese texture. The reduced number of vertices in the protein network and more compact protein network with thicker protein strands, correlated with stronger proteolysis and softer Cheddar cheese texture, has been reported by Soodam, Ong, Powell, Kentish, and Gras (2017). The most notable change with age, due to proteolytic breakdown of protein matrix, is a decrease in fracture strain and springiness, and an increase creaminess (Gunasekaran & Ak, 2003a).

I.2.1.3 What will affect proteolysis?

The rate of proteolysis is affected by several factors. The gross composition of cheese affects proteolysis. The variations in salt in moisture levels have been shown to have a marked effect on the rate of proteolysis. The cheese with higher salt content had less proteolysis during ageing and increased hardness

and fracturability (Johnson, Kapoor, McMahon, McCoy, & Narasimmon, 2009). Furthermore, the level of autolysis of starter culture or culture adjuncts can affect the abundance and activity of peptidase, which further influence the proteolysis. A Cheddar cheese containing a higher-level autolysis starter system has been found to have an elevated proteolysis level, balanced with musty flavour early in ripening (Hannon, Wilkinson, Delahunty, Wallace, Morrissey, & Beresford, 2003; Poveda, Cabezas, & McSweeney, 2004). The residual level of rennet, the type of coagulant used, also affects the rate of proteolysis, as does the pH and temperature during ripening (Gunasekaran & Ak, 2003b; Lawrence *et al.*, 1987).

I.2.2 Glycolysis

I.2.2.1 What is glycolysis?

98% of the lactose content in the milk used to produce cheese is removed in the whey as lactose or lactate during the manufacturing step and the remaining 2% is fermented by starter bacteria during cheese curd formation or the early stage of ripening (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, Waldron, & Fox, 2004). In Cheddar cheese, most of the lactose has converted to lactic acid (mainly the L-isomer) before salting and moulding (Shakeel-Ur-Rehman *et al.*, 2004). Lactate produced by starter activity is an important starting point for a range of pathways that contribute to the texture and flavour attributes (McSweeney, 2007). From Figure I-3 pathway (1), it can be seen that L-lactate may be racemised to DL-lactate by non-starter lactic acid bacteria (NSLAB) activity after three months of ripening, which may be significant to the

development of the undesirable white specks of calcium lactate crystals (Thomas & Crow, 1983). This may cause the consumer to reject the cheese (Dybing, Wiegand, Brudvig, Huang, & Chandan, 1988). In pathway (2), gas (CO_2 and H_2) production by microorganisms may occur during ripening which is a defect raised by our collaborating industry partner, even though Cheddar cheese is not susceptible to late gas blowing. The late gas blowing and accompanying off-flavour defects are a result of the anaerobic metabolism of lactate by *Clostridium tyrobutyricum* to butyrate, CO_2 and H_2 (Bassi, Puglisi, & Cocconcelli, 2015; McSweeney, Fox, & Ciocia, 2017). H_2 has low solubility in the aqueous phase and so remains mainly as a gas leading to blowing. In pathway (3), the lactate can be metabolised by lactic acid bacteria, depending on strain to acetate, ethanol, formate and CO_2 in cheese containing a high concentration of O_2 (Upadhyay *et al.*, 2004). As in modern Cheddar cheese manufacture, the cheese blocks are vacuum packed for the ripening process, to ensure that the oxidation of L-lactate occurs to a very limited extent (McSweeney *et al.*, 2017).

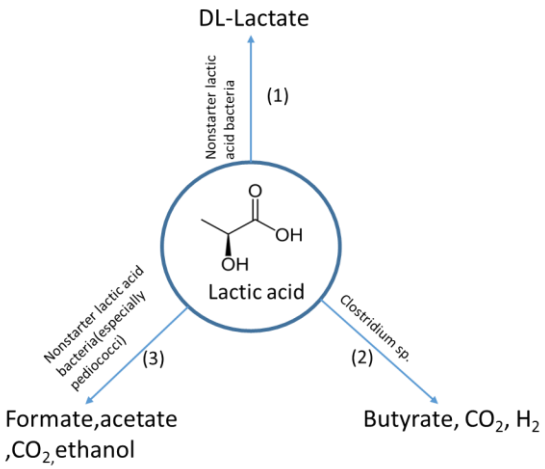


Figure I-3 *Pathway by which lactate is metabolised in cheese during ripening (1) racemisation (2) anaerobic metabolism of lactate to butyrate and H_2 which lead to late gas blowing (3) conversion to formate, ethanol and acetate (McSweeney et al., 2017).*

I.2.2.2 Why is glycolysis important?

The quality of Cheddar cheese is strongly influenced by the failure to ferment residual lactose (McSweeney *et al.*, 2017). If the residual lactose is not fermented by starter instead of heterofermentative non-starter lactic acid bacteria (NSLAB), a defect can be caused related to the acid and CO_2 production, such as slits and bloated packaging.

I.2.2.3 What will affect glycolysis

Fermentation of residual lactose is strongly influenced by the salt in moisture content in curd (O'Connor, 1973). Commercial lactic acid cultures are stimulated by low levels of NaCl but very strongly inhibited at concentrations greater than 2.5 % NaCl (Singh *et al.*, 2003). For example, *Lactococcus*, which is

a common starter culture for Cheddar cheese, is a salt-sensitive bacteria (Legg, Carr, Bennett, & Johnston, 2017; McSweeney *et al.*, 2017). If the starter culture is inhibited, residual lactose will be metabolised by nonstarter lactic acid bacteria (NSLAB) which could lead to the aroma defects.

I.2.3 Lipolysis

Lipolysis in Cheddar cheese during ripening is lower than for Blue or Italian cheese, yet the contribution to cheese specific texture and flavour has received little attention. However, the flavour, texture and physico-chemical properties of cheese are still greatly governed by fat. Also, the state of fat is important for indicating sensory properties of dairy products where it is known that the raw milk Cheddar cheese exhibited a higher concentration of free fatty acids than pasteurised milk cheese (Lopez, Camier, & Gassi, 2007; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993). Indigenous lipase mainly causes significant lipolysis in raw milk cheese, but most indigenous lipase, such as lipoprotein lipase, is a relatively heat-labile enzyme (Atasoy *et al.*, 2009). The principal lipolytic enzymes in Cheddar cheese curd made from pasteurised milk are reported to be lipases and esterase of lactic acid bacteria (LAB) and are most sensitive to salt in moisture content and pH (Atasoy *et al.*, 2009; Collins, McSweeney, & Wilkinson, 2003). Triglycerides are hydrolysed to free fatty acids which may be degraded further to ketone and alcohols. Total free fatty acids concentration and short/long-chain free fatty acid ratio have been related to the type and the amount of lipase used during cheese ripening (Banks, 2011). Especially short-chain fatty acids ($C_{4:0}$-$C_{8:0}$) have lower thresholds and have a

propensity to contribute directly to Cheddar cheese flavour or can act as precursors for important flavour compounds such as ethyl esters, methyl ketones (McSweeney, 2007). Excessive lipolysis is undesirable in Cheddar cheese, where the presence of even a moderate level of free fatty acids would be considered rancid (Fox, McSweeney, Guinee, & Cogan, 2017c). O'Mahony, Sheehan, Delahunty, and McSweeney (2006) and Atasoy *et al.* (2009) have confirmed that the levels of total and individual free fatty acids increased with progressive ripening time.

I.3 Cheddar cheese grading and quality prediction

I.3.1 Cheddar cheese quality affect factor

The characteristic of Cheddar cheese texture and flavour does not depend exclusively on the ageing process. The quality of milk (casein level, protein-to-fat ratio), the bacteriological (the activity of starter cultures and non-starter lactic acid bacteria used), chemical control and manufacture conditions (pasteurisation temperature, cut firmness and the level of salt addition) all lead to batch to batch variation of Cheddar cheese organoleptic attributes (Bintsis & Robinson, 2004; Gómez-Torres, Ávila, Delgado, & Garde, 2016; Guinee, Kilcawley, & Beresford, 2008; Sulejmani, Hayaloglu, & Rafajlovska, 2014; Van Leuven, Van Caelenberg, & Dirinck, 2008). The factors that could affect cheese quality in the same dairy and utilising milk on the same day could be due to the milk storage in different silos, rennet addition, salt addition, and microbial contaminants associated with cheese. As cheese factories become larger,

storage of milk for longer periods becomes necessary, and hence the microbiological quality of milk varies especially if the milk is cold-stored for a long period (Fox *et al.*, 2000a; Fox *et al.*, 2017b). A small variation between different batches and runs of cheese in the same factory is more important to industrial cheese manufacture, but little research about this has been carried out to date.

I.3.2 Cheese grading

Due to the complex nature of Cheddar cheese ripening, the small variations in the milk used and in processing during cheese manufacture could be exacerbated during long periods of maturation. Therefore, it is hard to produce Cheddar cheese of uniform sensory properties and equally hard to predict the Cheddar cheese quality after maturation. The presence of defects in the final products cause financial losses and consequently has a tremendous economic impact on the dairy industry (Engel, Nicklaus, Septier, Salles, & Le Quéré, 2001). Some Cheddar cheese defects are only observed in the late stage of maturation. However, Cheddar cheese maturation can be up to 2 years in order to obtain high-value premium Cheddar cheese, which is a time-consuming and high-cost process. From industry point of view, the flavour and texture qualities of cheese do influence the value of cheese in the marketplace. For quality control, predicting cheese quality as early as possible in the cheese production process is of major interest (Kraggerud, Næs, & Abrahamsen, 2014). Therefore, grading of Cheddar cheese happens during Cheddar cheese maturation which is used to evaluate the potential use and relative value of cheese as it enters

the channels of commerce leading to the consumer (Partridge, 2008). Grading could tell the manufacturer that a cheese in question is suitable for extended ripening for premium Cheddar cheese or must be removed quickly as young, mild cheese. Grading could also attain relatively consistent quality Cheddar cheese samples.

By careful observation of the external appearance and internal body and texture characteristics of cheese and after tasting, an experienced judge can place a given cheese to a quality grade (Partridge, 2008). When specific defects are present in a block of cheese, a lower grade is placed on the cheese and the value of the cheese is reduced due to the downgrade (Smukowski, Ping, Wendorff, & Rao, 2003). From a cheese industry survey, the common defects causing downgrades of cheese when evaluated at 30-60 days of age is body/texture, microbiological and flavour (Smukowski *et al.*, 2003).

Experienced cheese graders agree that Cheddar cheese aged, ranging from a few days to weeks old is more difficult to grade than a more mature product (Partridge, 2008). Even though each dairy has its written criteria for a grader to follow, the grading process is still a subjective evaluation. Muir, Hunter, Banks, and Horne (1995) claimed that sensory assessment early in the maturation period was an unreliable estimate of the ultimate sensory character. However, grading is sensory evaluation in order to predict cheese maturation quality as early as possible, which has been applied in manufacture for a long time. This further suggests that grading cheese requires experienced and professional graders and indicated that sensorial grading is not sufficient for cheese quality

prediction, where a more instrumental analysis should be involved in the grading process in order to aid grading for a more accurate evaluation and early prediction of quality.

I.3.3 Correlation between sensory perception and instrumental analysis

In order to perform better cheese grading, the correlation between sensory perception and instrumental analysis should be studied. Relating sensory perception to instrumental analysis is essential since instrumental measurements would be more cost-effective, provide consistent reproducibility in all manufacturing settings and would be more convenient than sensory tests (Drake & Delahunty, 2017). If a good correlation with sensory perception and instrumental analysis can be demonstrated, the instrumental analysis can be recommended as a routine procedure for Cheddar cheese quality control and classification, possibly then complementary to sensory evaluation. Additionally, instrumental results can help interpret sensorial perception. Various objective chemical and rheological approaches to evaluate cheese quality have been developed. The measurement of total concentration of free amino acids was more effective than HPLC analysis for discrimination between Cheddar cheeses of different maturities, whereas HPLC discriminated more effectively between defective and non-defective cheeses (O'Shea, Uniacke-Lowe, & Fox, 1996). The protein network-related sensory attributes such as surface state, texture length and pastiness are show to be a good prediction from tryptophan fluorescence spectra in soft cheese (Dufour

et al., 2001). Sensory properties of Norvegia cheese have been correlated with spectroscopic methods, but the sensory characteristics at 40 weeks maturity were not very well forecasted from early measurements after eight weeks (Kraggerud *et al.*, 2014). This could be due to the fact that florescence spectroscopy and near infra-red spectroscopy are carried out on the surface of the cheese. However, the concentration of a compound measured by an instrument in cheese is not necessarily a measure of its sensory perception due to the different sensory thresholds and the effects of the food matrix on retention and release (Drake *et al.*, 2017).

I.4 Gilles and Lawrence's model

The quality of the cheese is influenced by the composition, especially moisture content, NaCl concentration, pH, moisture in non-fat substance, fat content (Fox *et al.*, 2017a; Kondyli, Katsiari, Masouras, & Voutsinas, 2002). Compositional factors for premium-quality Cheddar cheese determined by different researchers are listed in Table I-1 (Fox, 1975; Gilles & Lawrence, 1973; Pearce & Gilles, 1979). The Gilles and Lawrence grading scheme model, using simple chemical and physicochemical components that can be used to predict the quality of Cheddar cheese, is still used as an index in the Cheddar cheese grading in the manufacture nowadays (Gilles *et al.*, 1973). The relationship between Cheddar cheese composition and grade quality based on their research is displayed in Figure I-4. Premium grade is predicted when the following parameters are measured: pH: 4.95 to 5.1; salt in moisture (S/M): 4

to 6 %; moisture in solid-not-fat (MSNF): 52 to 56 %; fat in dry matter (FDM): 52 to 55%. The corresponding values for graded quality (1st grade) cheeses are: pH 4.85 to 5.20; 2.5 to 6 %; 50 to 57 % and 50 to 56 %; young cheeses with a composition outside these ranges are considered unlikely to yield good quality matured cheese (Singh *et al.*, 2003).

Table I-1 *Compositional factors for premium quality Cheddar cheese suggested by different researchers.*

pH	Fat in the dry matter, FDM (%)	Moisture in the fat-free substance, MFFS (%)	The percentage of salt in moisture, S/M (%)	Age (weeks)	Reference
4.95-5.10	52-56	52-56	4-6	2	Gilles *et al.* (1973)
<5.4	-	<38	>1.4	10	Fox (1975)
4.95-5.15	-	52-54	4.2-5.2	2	Pearce *et al.* (1979)

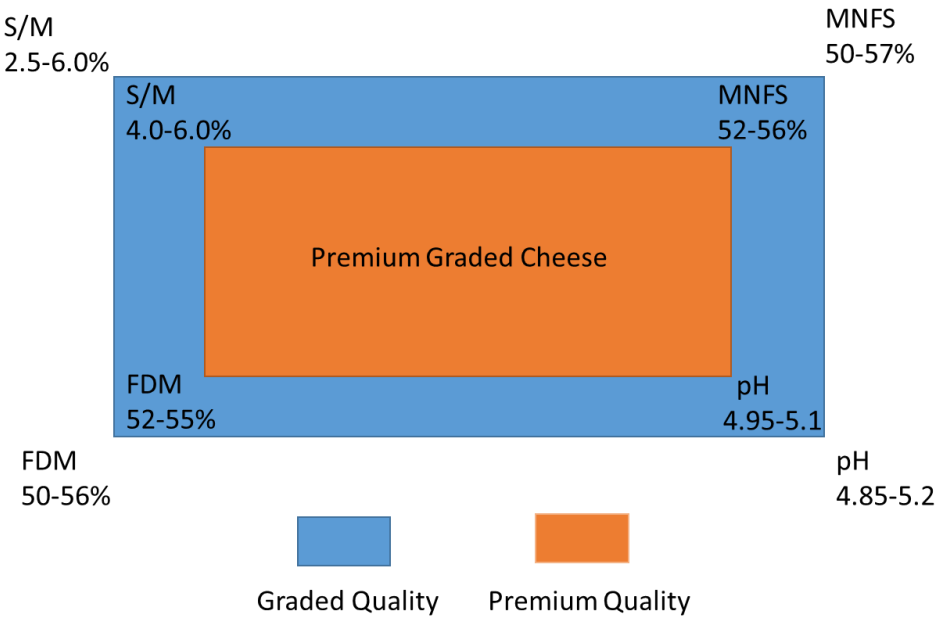


Figure I-4 *Relationship between Cheddar cheese composition and grade quality.*

I.4.1 Moisture in the Fat Free Substance (MFFS)

Moisture in the fat free substance is essentially the relative amounts of moisture and protein in the cheese. A small variability in MFFS can cause major changes to the amount of free moisture associated with the casein network, which can subsequently affect the activity of enzymes and bacteria associated with the network produced during ripening (Lawlor, Delahunty, Wilkinson, & Sheehan, 2001; Soodam, Ong, Powell, Kentish, & Gras, 2015). The water state in the cheese protein matrix is partly responsible for the cheese functional properties (McMahon, Fife, & Oberg, 1999). Lelievre and Gilles (1982), after studying the quality-composition relationship of numerous cheeses manufactured in New Zealand, stated that MFFS is the most important factor affecting cheese quality.

I.4.2 The Percentage of Salt in Moisture (S/M)

Salt is used to control the kinetics of enzymatic reactions, maturation process, microbial growth and flavour of the cheese (Cruz *et al.*, 2011; Santapaola, Maldonado, & Medina, 2013). To be more specific, salt in moisture (S/M) has a marked the effect on the rate and extent of proteolysis (Lawlor *et al.*, 2001). The hydrolysis of β-CN by chymosin is strongly inhibited by 5 % (wt/vol) NaCl and completely inhibited by at 10% (Sulejmani *et al.*, 2014). The high amount of salt content limited the level of proteolysis during maturation. Salt reduction in cheese results in poor flavour development, high levels of bitterness and defects in structure (Guinee, 2004). The premium Cheddar cheese percentage of salt in moisture range is about 4.0-6.0%. All the samples under investigation in the present study, following time are located in that range.

I.4.3 pH

Cheese pH is important for flavour development as it influences the growth of non-starter lactic acid bacteria (NSLAB) in Cheddar cheese (Lawlor *et al.*, 2001). Different pHs will affect the bacteria pathways to produce different metabolites. pH is also critical for cheese texture, as pH changes are directly related to calcium content and protein network formation (Lawlor *et al.*, 2001). Changes in pH induce changes in the amount of calcium-induced cross-linking in the caseins which are the main load-bearing components in cheese (Watkinson *et al.*, 2001). The most apparent effect of pH in hard cheese is brittleness of cheese when pH is less than 5.0 (Gunasekaran *et al.*, 2003b).

Shakeel-Ur-Rehman *et al.* (2004) have also suggested that the pH of cheese is inversely related to the metabolism of lactose during the ripening of Cheddar cheese. Proteins, free amino acids, weak acids, bases and their complexes with metal cations contribute to the pH buffering capacity (Upreti, Bühlmann, & Metzger, 2006a). This indicated that pH is an index for reflecting the cheese biosystem.

I.4.4 Fat in the Water Free Substance (FDM)

Fat in the water free substance is an index comparing the fat and protein ratio in cheese. Fat in cheese not only dissolves lipophilic flavour compounds produced from the hydrolysis of fats and protein but also prevents the casein network of cheese from developing into a tough, rubbery matrix (Lawlor *et al.*, 2001). Low fat Cheese always causes low intensity cheese flavour, due to the lack of precursors from fat, the absence of solvent power of the fat and/or different physical structures of reduced-fat cheese (Kondyli *et al.*, 2002). Indeed, fat reduction increased the instrumental hardness and decreased the cheese meltability and yield, making the microstructure more compact (Madadlou, Khosroshahi, & Mousavi, 2005). From the sensorial point of view, as fat content of cheese is reduced, the cheese develops an undesirable firm, rubbery texture which agrees with instrumental results (Guinee, Auty, & Fenelon, 2000). It appeared that the diminished textural quality in low-fat Cheddar cheese is attributed to changes in the breakdown pattern during chewing, as altered by fat disrupting the cheese network (Rogers, McMahon, Daubert, Berry, & Foegeding, 2010). In contrast, increasing fat content caused

an increase in size of fat globules and a higher percentage of non-spherical fat (Rogers *et al.*, 2010).

I.5 The objective of this study

The aim of this study is to explore a preliminary model using chemical and physiochemical measurements that can be used to predict the sensorial quality of Cheddar cheese and whether a batch of cheese is suitable for extended maturation to yield a high-value mature cheese. Cheddar cheese is a complex and dynamic food. Obviously, it is difficult to have a single perspective to objectively assess the quality of cheese. This study is focused on the water and fat properties, metabolites, aroma, and texture perspectives of Cheddar cheese, linked to and compared with the sensory profiles. The volatile fraction of cheese contributes mainly to its aroma, and the water-soluble fraction is mainly responsible for its taste (O'Shea *et al.*, 1996). Texture and structure are the main features that Cheddar cheese develops during ripening. To date, the knowledge about the relationship between quality and instrumental parameters is still limited.

This study simulates the commercial production and grading looking at different grades of Cheddar cheese production from the same dairy, based on Gilles and Lawrence model. Blocks of cheese are followed during the whole maturation process on a series of individual blocks of cheese using a range of physicochemical and sensorial measurements.

The specific aims of this work were:

(1) To exam the developments in sensory, aroma profile, water and fat state, texture and aqueous metabolites extract during Cheddar cheese ripening,

(2) To study the batch variation of different predictive grading quality with respect to sensory, aroma profile, water and fat state, texture and aqueous metabolites profile in Cheddar cheese,

(3) To determine whether Cheddar cheese sensory profiles can be correlated with specific physiochemical parameters,

(4) To complement the Gilles and Lawrence's quality prediction model,

(5) To find the quality and maturation markers during the ripening process,

(6) To examine whether it is possible to predict the mature sensory profile of cheese from early stage using such objective measurements.

I.6 Thesis structure

This thesis is submitted as a paper format. The work is presented in five papers which make up the core of thesis which is summarized in Figure I-5, thesis structure diagram. Chapter II *Evolution of sensory profiles of different batches Cheddar cheese during ripening* is about the general sensory profile development and evolution of different predictive batches of Cheddar cheese during ripening. From this chapter, the fate of different predictive batches Cheddar cheese up to 540 days ripening was presented. The sensory results are further used in the subsequent chapter to link the instrumental measurements and explore the correlation between the sensory characteristics and

physicochemical properties of these Cheddar cheese samples. Chapter III to chapter VI present the water and fat state, microstructure and texture, aqueous metabolites and aroma profile evolution of different predictive quality Cheddar cheese. The structure from chapter III to chapter VI is similar, in that they all consist of three major parts; change during ripening, batch variation of different predictive Cheddar cheese and the correlation to the sensory profile. Chapter III *The state of water and fat during the maturation of Cheddar Cheese* focuses on the physicochemical state of water and fat in Cheddar cheese measured by time-domain Nuclear Magnetic Resonance spectroscopy (TD-NMR) and Thermogravimetric analysis (TGA). The water and fat protons from transverse relaxation curve in Cheddar cheese has been firstly assigned. In chapter IV *The evolution of aqueous extracts of Cheddar cheese during ripening as a potential model for prediction of quality*, high-resolution NMR spectra of metabolites in aqueous extract of Cheddar cheese during ripening have been identified. The metabolites responsible for the batch variation, ripening and sensory attributes were characterised. Chapter V *Evolution of volatile compounds from different batches Cheddar cheese during ripening* demonstrated the aroma profile evolution trajectory of different predictive quality during ripening. The aroma compounds considered to be off-flavour sign and prediction marker was discussed. Chapter VI *Evolution of texture and microstructure of Cheddar cheese maturation correlated to the sensory profile* demonstrated microstructure and texture profile change among different predictive quality Cheddar cheese. Confocal laser scanning microscopy (CLSM) combined with image analysis was applied to investigate the microstructure

change. Texture profile analysis was performed to observe the development of different predictive quality Cheddar cheese texture during ripening. The final chapter is the summary and concluding remarks. A preliminary 540 days Cheddar cheese sensory profile prediction model-based on instrumental analysis at 56 days is presented and recommendation for Cheddar cheese production is also presented.

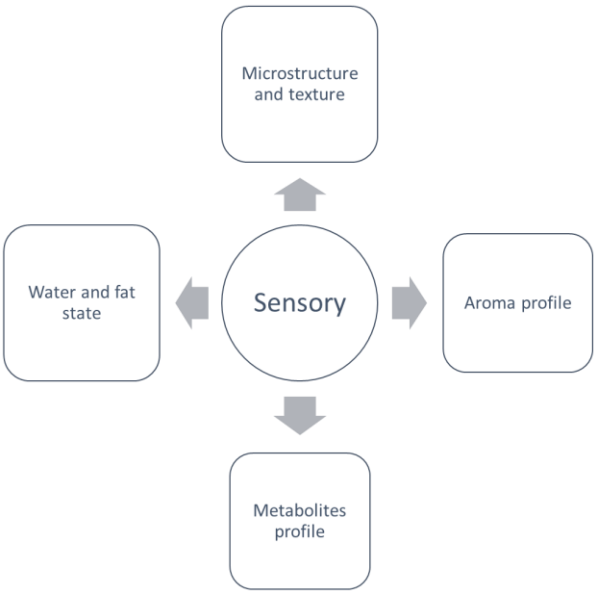


Figure I-5 *Thesis structure diagram with sensory linking the physicochemical characterisation.*

Chapter II Evolution of sensory profiles of different batches Cheddar cheese during ripening

Abstract

Descriptive quantitative analysis and a 'cheese wheel' were developed and used for assessment of the sensory properties of the cheeses during ripening up to 540 days. We have found that cheese predicted to be good quality Cheddar cheese based on the Gilles and Lawrence compositional prediction model show significant sensorial differences after ripening. One has a bland flavour, and lumpy mouthfeel while the other has off-flavour, a bitter, dirty aftertaste and texture defect after ripening. A cheese predicted as premium quality Cheddar cheese, has a more balanced sensory profile. The results show that a quality prediction model based on composition is not sufficient, and that more cheese properties should be considered and involved in future models for the prediction of quality.

II.1 Introduction

Sensory characteristics of Cheddar cheese can be complex and involve different sensory modalities. In practice, it is commonly accepted in the Cheddar cheese industry and market that premium Cheddar cheese is balanced and buttery in flavour (Murray & Delahunty, 2000). The American dairy grading branch provides a general description of AA grade medium-curd to aged Cheddar cheese as having a highly pleasing flavour, firm close smooth cheese body and translucent, uniform colour (Partridge, 2008). White specks, a mouldy appearance and bitter, flat, fruity, fermented, and unclean rancid flavours are all regarded as defects (Partridge, 2008; Zabaleta *et al.*, 2016). In order to understand why Cheddar cheese tastes as it does, a descriptive sensory analysis should be carried out to discriminate the sensory characteristics of a range of cheese but also determine a quantitative description of all sensory differences. O'Mahony *et al.* (2006) used descriptive sensory analysis for assessment of sensory properties of the Cheddar cheese that ripened at different temperatures and -time treatments. They found that Cheddar cheese ripened at high temperatures developed flavour and aroma profiles to an intensity characteristic of typical mature cheese in a relatively shorter time frame. Descriptive sensory analysis was also applied to characterise the seasonal quality control and quality prediction (Kraggerud *et al.*, 2014). The studies implied that sensory characteristics at 40 weeks were not very well forecasted from the early measurement on cheese (eight weeks) based on 153 cheese samples made during all seasons of the year.

The cheese sensory characteristics can generally be described using terms defined within the categories of appearance, flavour and texture (Drake *et al.*, 2017). Appearance characteristics include colour, mould, rind and visual texture (surface cracks). These may create a first impression of the anticipated taste and texture of the cheese. Texture attributes can be assessed either by touch or eating (Fox *et al.*, 2016a). Texture characteristics are the attributes resulting from a combination of physical properties, including size, shape and conformation of constituent structural elements (Jowitt, 1974). Colour perception is determined by how the cheese microstructure scatters or reflects light. The salty perception is governed by the amount of sodium present in saliva which varies as a result of salt content, cheese microstructure affecting salt release, and fat and salt sensory interaction effects (Boisard *et al.*, 2014). The basic taste like saltiness interacts with smell, texture and the trigeminal sensations like pungency (González-Martín *et al.*, 2011).

Additionally, consumers have various Cheddar cheese sensorial preferences, so in order to best satisfy the consumer and accurately convey the cheese sensory characteristic expectations, it is very important to understand the sensory profile journey during cheese maturation in order to begin to provide predictive qualities. In Cheddar cheese manufacturing practice the Gilles and Lawrence cheese quality grading model is based on the elemental cheese composition and is still commonly used in Cheddar cheese manufacture (Gilles *et al.*, 1973). This model is built using the inputs of the percentage of salt in moisture (S/M), moisture in the fat-free-substance (MFFS), fat in the dry matter (FDM) and pH. This prediction model aids the professional cheese grader to

predict the cheese quality at a later date, which is still regarded as an essential index in Cheddar cheese manufacture. However, a test of the model by developing tools by which the mechanism behind cheese ripening and the complexity of the ripening process can be described is required, which links to sensory perception.

Even though the manufacturing processes in Cheddar cheese manufacturing are tightly controlled, we hypothesise that due to variation in material input and artisanal aspects of some parts of the process i.e. at the hand salting stage, that from the same day production within an individual dairy the predictive quality based on the Gilles and Lawrence can be improved due to the significant sensory variations that can be observed during ripening. No such investigation of the sensory profile of Cheddar cheese with the maturation of 540 days has been reported. This study aims to investigate the sensory profile variations among Cheddar cheese with different predictive grades during the ripening process. Based on this aim a quantitative descriptive analysis is employed, which can be further linked to instrumental measurements to understand the chemical and physical components of a product that influence sensory characteristics.

II.2 Materials and Methods

II.2.1 Quantitative descriptive analysis

A modified descriptive sensory analysis was conducted in this study at various ripening time points (56, 90, 180, 270, 360, 450 and 540 days). All data was collected using Compusense (Guelph, ON, Canada).

II.2.1.1 Samples

Six batches of Cheddar cheese were selected based on Gilles and Lawrence quality grading model and professional cheese grading outcomes (Gilles *et al.*, 1973). All cheeses were produced on the same day, and production line, and labelled as batches A, B, C, D, E and F from a commercial Welsh Cheddar producer in the UK. The compositional attribute data for Gilles and Lawrence's model was provided by the industry partner (Table II-1). The grading according to the Gilles and Lawrence model is shown in Figure II-1 which shows a prediction of mature to 'premium quality' for batches B, whereas batches A, C, D, E and F are predicted to develop to 'graded quality'. After 56 days maturation, all the batches were further assessed by an experienced Cheddar cheese quality grader. The grading results from professional cheese grader were that batch C and E were graded as likely to result in a 'downgrade (poor) quality' Cheddar cheese and batch B was graded as likely to mature to 'premium quality', with the other samples requiring further grading. After 13 months of ripening, the grader rechecked all batches and batch B was further confirmed as 'premium quality' and batch E was confirmed as 'downgrade poor

quality' cheese. The rest of the batches (A, C, D, F) were 'graded quality'. The predictive grading and further grading result were hidden from the researcher until all the experiments were finished in order to avoid bias during the experimentation, but the trajectory of quality prediction can also be seen and summarised in Table II-2. From this comparison it can be seen that the current predictive assessment techniques are consistent for batches A, B, D and F but not so for batches C (increase in quality with ripening), E (decrease in quality with ripening).

Table II-1 *Mean of Gilles and Lawrence Cheddar cheese quality predictive grading model composition data on six batches of Cheddar cheese*

Batch / Compositional Factor	A	B	C	D	E	F
Moisture In The Fat Free Substance (MNFS)-%	55	56	53	56	57	57
The Percentage of Salt In Moisture (S/M)-%	5.5	5.4	5.9	5.0	4.5	5.1
Fat in the dry matter (FDM)-%	51	52	50	50	53	53
pH	5.0	5.1	5.1	5.1	5.1	5.1

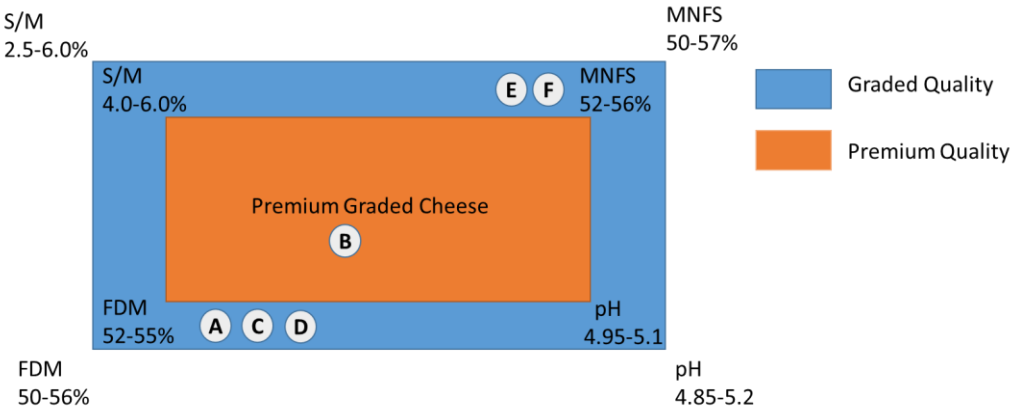


Figure II-1 *Relationship between Cheddar cheese composition and grade quality for six batches of Cheddar cheese. A, B, C, D, E and F were predictively graded as premium and graded quality cheese based on four chemical composition attributes: the percentage of salt in moisture(S/M), moisture in the fat free substance(MNFS), fat in the dry matter(FDM) and pH.*

Table II-2 *Professional grader prediction of quality at different ripening days and Gilles and Lawrence quality prediction result are summarised.*

Batch / Prediction	A	B	C	D	E	F
Professional grader prediction of quality after 56 days	-	Premium	Poor	-	Poor	-
Professional grader prediction of quality after 13 months	Graded	Premium	Graded	Graded	Poor	Graded
Gilles and Lawrence quality prediction	Graded	Premium	Graded	Graded	Graded	Graded

All six blocks were cut into 14 pieces and vacuum packed in bags, further packaged in cardboard and located in the factory warehouse for maturation at a constant controlled temperature of 8 °C. At each time point, one bag of

cheese was removed at random from each block and placed in a 4 °C refrigerator before measurement.

II.2.1.2 Panel

Ten trained sensory panellists (3 males, 7 female) were recruited from Sensory Science Centre panellist database at the University of Nottingham. All trained panellists were invited to attend five training sessions and 14 evaluation sessions at different time points (56, 90, 180, 270, 360, 450, 540 days ripening). Each session lasted approximately 2 hrs.

II.2.1.3 Training sessions

Due to the nature of this study, there is significant sample variation across the time range and different predictive qualities batches Cheddar cheese. There is also a limitation that it is impossible to develop the lexicon based on the same Cheddar cheese samples from different ripening stages at the same time. Thus, different quality variables of Cheddar cheese samples of the various maturities were used for lexicon development, which was suggested by the professional grader in the dairy. The sample-set for lexicon development reflects the time point of sensory evaluation performed. Commercial 'mild', 'medium', 'mature', 'extra mature' and 'vintage' cheese samples from the same cheese factory are roughly ripened for 90 days, 180 days, 270 days, 360 days and 450-540 days, respectively.

Panellists were invited back every three months to evaluate cheese samples during ripening. There is a need to include reference samples before each

evaluation, to enable panellists to refresh their training very quickly on the attribute definition, scale usage and eating protocol. Due to natural variation in commercial cheeses, it is challenging to obtain consistent fresh cheese sample at different time points. Thus, a reference sample system was developed to overcome this limitation.

II.2.1.3.1 Lexicon Development

The first two sessions involved Cheddar cheese attributes generation and identification of critical attributes, selections and familiarisation. The traditional method to generate the sensory attributes is to provide a typical and wide range of samples one by one to panels and obtained descriptors. A new method of lexicon generation was performed in order to generate more attribute descriptors through comparison. There are six comparison groups. Four of them consisted of similar maturity but different graded qualities (three different qualities but similar maturity cheese sample in each comparison group) and two with cheeses having completely different maturities (mild, medium, mature Cheddar) and (mature, extra mature and vintage Cheddar).

In the first session, 18 different maturities and qualities were used for attribute generation. Each panel was asked to generate a series of descriptor attributes from appearance, aroma, taste, flavour and mouthfeel perspectives of all the cheese samples provided. In the sensory field, the term “flavour” refers to the integrated perception of odour, taste, and trigeminal stimuli during mastication and swallowing (Møller, Rattray, Bredie, Høier, & Ardö, 2013). In the first training session, 52 attributes were generated.

In the second training session, based on the attribute list generated in the first session, and the attributes from dairy cheese grading manual and literature (Kraggerud, Skeie, Høy, Røkke, & Abrahamsen, 2008; Partridge, 2008), the discussion was mainly focused on reducing the attributes to a reasonable number through combining similar attributes and selecting the key and unambiguous attributes. During the discussion of each key attribute, the definition and preliminary protocol of consumption were discussed as well. Moreover, samples and reference aqueous solutions representing certain sensory attributes were presented, such as umami samples and open texture samples to help panellist to understand and refine the attribute descriptor. A final descriptor list containing 19 sensory attributes covering appearance, aroma, taste, flavour and mouthfeel properties were developed and are shown in Table II-3. In this session, the palate cleaning method was confirmed, as cheese is a food stuff that has strong flavour and lingering aftertaste. After using cracker, water and apple to cleanse the palate, panellists agreed that apple is the most efficient.

***Table II-3**. Sensory attributes and protocol listed in the assessment order*

Attributes	Definition	Protocol	Scale extremes
Yellow	Yellow colour	Look at the top of the cheese cube	Pale yellow-dark yellow
Pressure firmness	Force perceived by compressing the cheese cube	Gently press the top of the cheese cube with pointer finger	Squishy-firm
Rubbery	Like rubbery, have an elastic texture, capability of resuming original shape after compression with a finger	Gently press the top of the cheese cube	Low-high
Crumbly	Brittle, the degree of the cheese pieces breaks into small fragments after cutting	Cut the whole cheese cube into six pieces	Low-high
Cohesiveness	The degree of reforming the cheese into a small ball	Use the fingertips (one hand only) to form a cheese ball by using one piece of the cheese cube (15 times)	Low-high
Oily	The amount of oil that left on your finger after forming the cheese ball	Look at the fingertips to evaluate the amount of oil left on your fingertips	Dry-oily
Overall odour intensity	The overall odour intensity, the strength of the overall odour	Smell the fingers to evaluate the aroma	Low-high
Dairy odour	The dour of dairy products such as butter or milk	Smell the fingers to evaluate the aroma	Low-high
Rate of melting	How quick the cheese melted in the mouth	Put one piece of cheese in mouth, manipulate the cheese using a tongue without chewing. (Use the timer to aid evaluation)	Slow -fast
Break down	How it feels as the cheese break down, how smooth or lumpy the cheese is	Put one piece of cheese in mouth, manipulate the cheese using a tongue without chewing.	Smooth-lumpy
Attributes	**Definition**	**Protocol**	**Scale extremes**

Salty	The fundamental taste sensation of which NaCl is typical	Put one piece of the cheese in mouth, chew three times then manipulate the cheese using tongue without any additional chewing	Low-high
Umami	The fundamental taste sensation of which certain amino acids, peptides, and nucleotides are typical Taste of MSG, savoury, meaty taste	The same as attribute salty	Low-high
Sour	The fundamental taste sensation of which lactic and citric acids are typical	The same as attribute salty	Low-high
Tangy	Tangy and pungent sensation, like vinegar, appear on the back of the throat	The same as attribute salty	Rounded- tangy/harsh
Overall flavour intensity	The strength of the overall flavour	The same as attribute salty	Low-high
Buttery	The flavour of butter in the mouth	The same as attribute salty	Low-high
Sweaty	Rancid flavour, sweaty, the baby sick flavour in the mouth	The same as attribute salty	Low-high
Astringent aftertaste	Astringent aftertaste	Put one piece of the cheese in mouth, keep chewing the cheese then swallow	Low-high
Linger aftertaste	The remaining intensity that left in mouth ten seconds after swallowing	Put one piece of the cheese in mouth, keep chewing the cheese then swallow. Rate the intensity ten seconds after swallowing.	Low-high

II.2.1.3.2 Attributes scale training

II.2.1.3.3 Sensory reference samples determination

The cheese samples can be evaluated by rating the intensity of each attribute on a scale. Reference samples were presented in every evaluation session allowing panellists to compare the samples with references and facilitating the allocation of attribute intensities on the scale (de Cássia Dos Santos Navarro Da Silva, Minim, Simiqueli, Da Silva Moraes, Gomide, & Minim, 2012). In addition, reference samples aid quick retraining and refreshing the panel memory. As consistent quality reference samples are required for 18 months, two boxes of mild and vintage cheese produced from the same block of 20 kg cheese which have similar sensory properties were frozen in a -20 °C freezer until the day before evaluation in order to keep reference samples consistent. Mild and vintage commercial cheese from the same dairy were set as reference samples as they covered most of the attributes and are at the extreme ends of the scale.

II.2.1.3.4 Standardisation evaluation protocol

Based on the panellists' feedback in the first two sessions, an initial detailed protocol was developed. A rating exercise in subsequent training sessions was conducted to train panellists to understand the attributes in the same way and to obtain the approximate scores for reference samples, and to practice the eating protocol and usage of the scale. The protocol was further amended during subsequent discussion and training. For example, originally the protocol for aftertaste attributes was to manipulate a cheese cube with the tongue until it could be swallowed, after practising and discussing with the panel, the protocol was changed to continue chewing until could the point of swallowing,

which would release more aftertaste than previous protocol. During the rating exercise, some attributes that were not easy to differentiate (milky and buttery aroma) were combined and cut down. A mock evaluation session was held before the actual evaluation session to practice rating, familiarise panellists with the cheese wheel, and emphasise some attribute definitions that could easily be misunderstood.

II.2.1.4 Cheese wheel formation

As the cheese ripening pathway is not fully understood, it could generate some distinct flavour or texture attributes which are beyond the general sensory protocol. Besides, some sensory attributes occur rarely, or only happened in some specific samples. For example, appearance attributes such as surface cracks and calcium crystals only exist in one of the commercial vintage cheese samples. However, these kinds of attributes still need to be captured, which probably only appeared in the latter stage of ripening, as their importance in suggesting the final cheese quality. Therefore, a cheese wheel was developed to capture the outstanding cheese characteristics as supportive evidence of cheese quality, which is a descriptive qualitative analysis with four appearances, twelve aromas, four tastes, thirteen flavours, seven in mouth textures, three aftertastes providing a descriptive vocabulary (Figure II-2). Due to the time and financial limitation, there were no specific training sessions on the attributes, scale and protocol. Panellists were able to check the attributes in the cheese wheel when they found some outstanding characteristics of the

cheese, they were consuming. The cheese wheel sensory profile was characterised by quantifying the frequency of perception of each attribute.

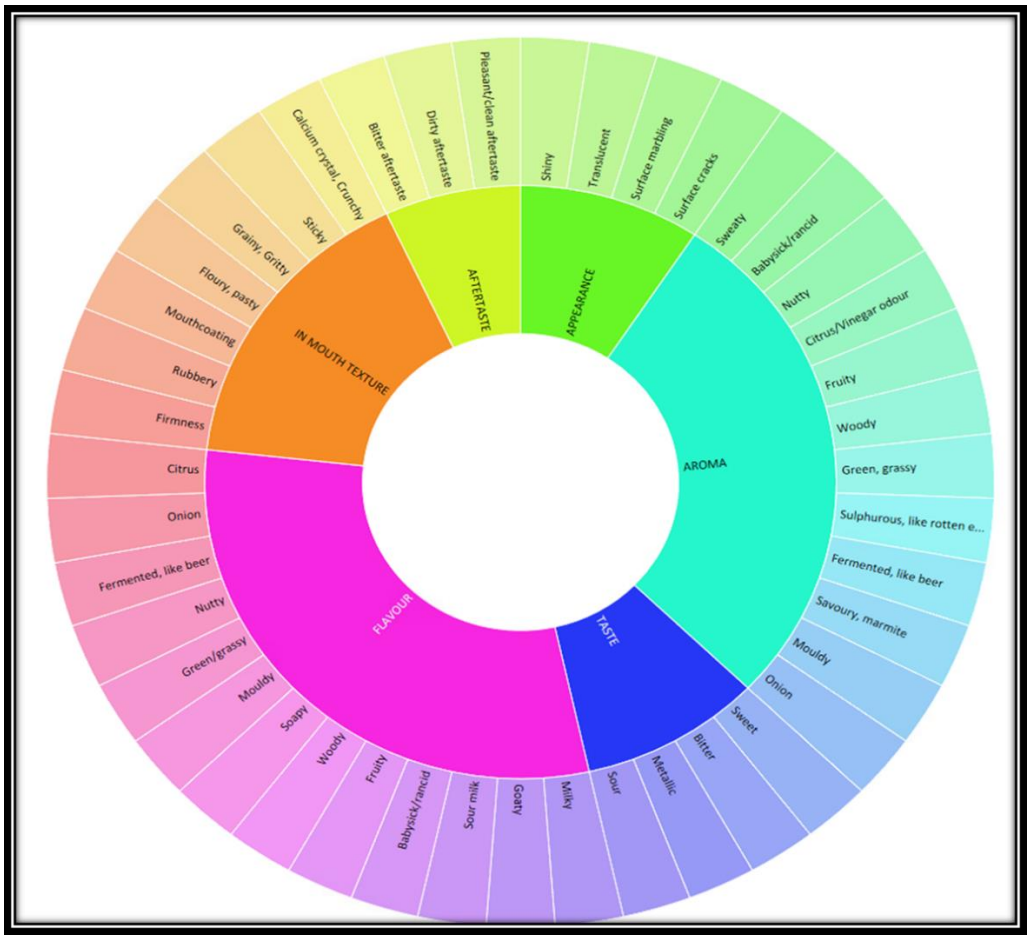


Figure II-2 *Sensory Cheddar cheese wheel consisting of appearance, taste, aroma, flavour, in mouth texture, aftertaste perspectives.*

II.2.1.5 Evaluation sessions

During the measurement sessions, five samples, namely A, B, C, D, E, were evaluated by the panellists at 56, 90, 180, 270, 360, 450 days maturation. All six samples (A-F) were only performed at 540 days maturation. Reference samples were defrosted the night before at 4 °C in the fridge. All the reference and test samples were equilibrated at 18 °C for 5 hrs before the sensory

sessions. The outer layer (5 mm) of each cheese was discarded and each sample cut into 2 cm × 2 cm × 2 cm cubes. Two cubes of each sample were presented to the panellists in a sealed 60 mL plastic pot. One is prepared for the general protocol, the other is for the cheese wheel evaluation. The cheese was coded with randomly selected 3-digit numbers and order of tasting, to account for first-order and carry-over effects (Hannon *et al.*, 2003). Before every evaluation session, a 20-mins introduction along with demonstration was performed to refresh the panel's memory with reference samples. Between two samples, the panellists were instructed to clean their palate with water and apple. The cheese was evaluated in triplicate by each panellist at two-day intervals. In each session, the panel had a ten mins break after they finished the four test samples in that session to avoid sensory fatigue.

II.2.2 Statistical analysis

One-way ANOVA was used to test the ability of discrimination of the descriptive vocabulary attributes between samples at the same time point. Attributes which discriminated significantly ($P<0.05$) between cheese samples were subsequently averaged across replicates and analysed by principal component analysis (PCA). The total number of times for cheese wheel attributes checked by the panel were calculated and further analysed by Correspondence analysis (CA). Only the attributes chosen by panel for more than five times were regarded as a real attribute of the sample rather than by chance. All statistical analysis was performed using XLSTAT (Addinsoft, France).

II.3 Results and discussion

II.3.1 Panel performance

In order to guarantee that the variance obtained in this study is from samples rather than the protocol used, panel performance needed to be assessed before further study. Panel performance was assessed using three criteria (1) Repeatability (to be able to produce a similar score); (2) Discrimination (to be able to discriminate among different qualities of Cheddar cheese); (3) Accuracy (to be able to gain the same samples trend agreement with other panellist) (Ng, 2013). Figure II-3 shows the first time point evaluation session as an example of how to check panel performance before further data analysis.

Figure II-3 (a) indicates P-value for 5 different samples as the function of mean squared error (MSE) (for 3 replicates) for each panellist, and showing the panel discrimination ability, whereas MSE indicates the repeatability of panel performance. Thus, all the panellists' performance point close to origin indicated good discrimination and repeatability. Figure II-3 (b) shows all the panellists followed the same trend for different predictive grades Cheddar cheese samples. There are no interactions between samples and panel which demonstrated the accuracy of panel performance.

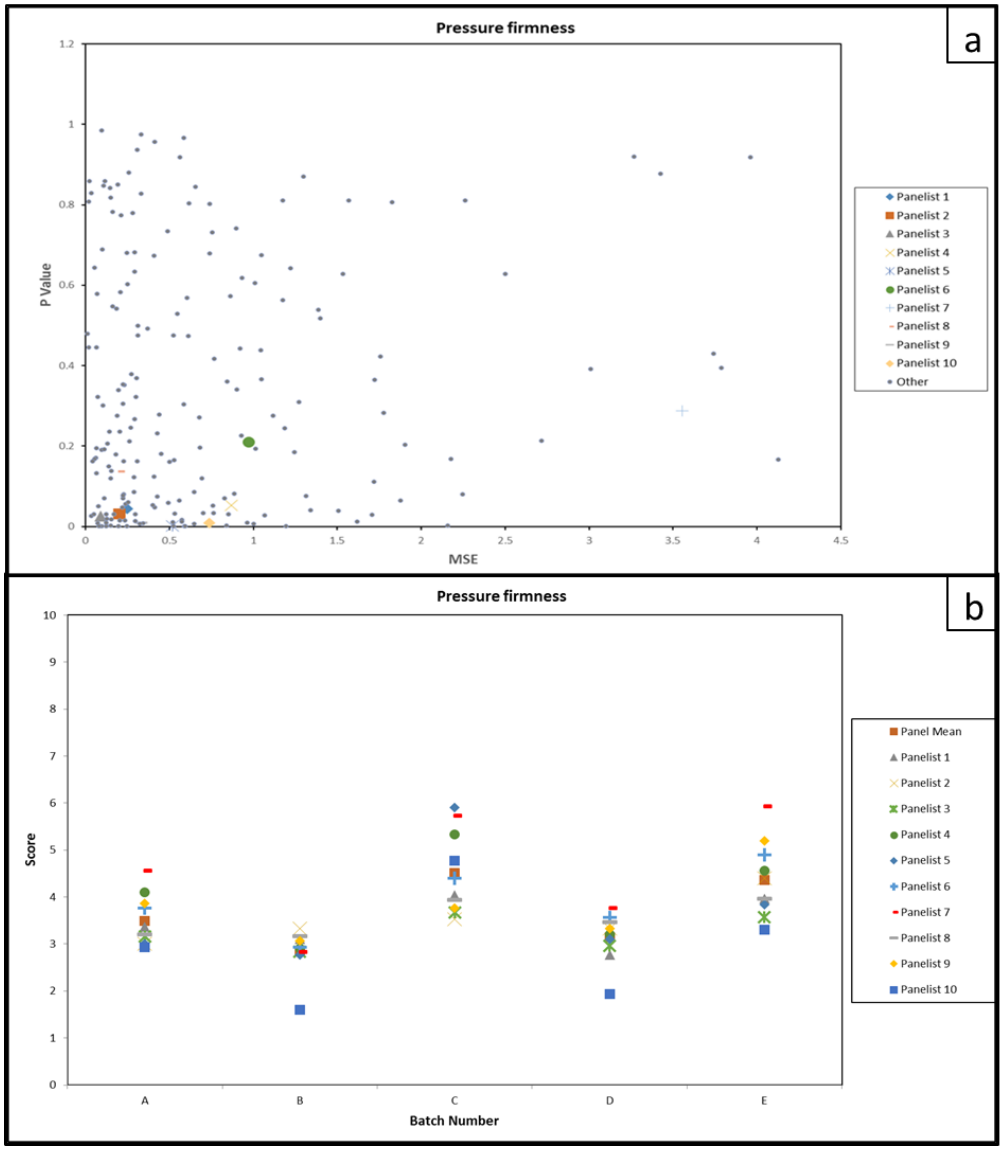


Figure II-3 *(a) P-value from ANOVA and mean squared error (MSE) of each judge for pressure firmness (b) pressure firmness mean score of each judge for all batches of Cheddar cheese*

II.3.2 Sensory properties changes during maturation

II.3.2.1 Appearance

The mean scores, standard deviations, ANOVA test results of all the sensory attributes during ripening are shown in Table II-4. As ripening proceeds, the

‘yellow’ attribute score significantly decreases and levels off after 360 days ripening suggesting that Cheddar cheese changes from dark yellow to pale yellow with ageing. The yellow attribute of cheese has been associated with the melting profile and diffraction of light by the fat fraction of cheese (Madsen, Reinbold, & Clark, 1966). With ageing, fat progressively becomes more solid, which therefore could possibly lead to an increase in the efficiency of light scattering and further decrease the diffraction of light in the fat fraction of cheese (Smith, Vogt, Seymour, Carr, & Codd, 2017). Cheese colour is also affected by the casein fraction, since during the ripening process, casein aggregation causes a dense cluster increasing the ability of casein to reflect white light. Therefore, the cheese becomes paler with age (Johnson, 1999).

Table II-4 *Quantitative sensory descriptive analysis of Cheddar cheese from different ripening times. The sensorial attributes score is presented as mean score with a standard deviation of 150 replicates (10 panellists × 3 replicates × 5 batches). Different lowercase superscripts in the same row indicate significant statistical difference among different ripening time (Tukey's test P<0.05).*

Sensory attributes	56 days	90 days	180 days	270 days	360 days	450 days	540 days
Yellow	4.6 ± 1.1^{b}	5.0 ± 0.9^{a}	3.9 ± 1.4^{c}	3.6 ± 1.1^{c}	3.0 ± 0.5^{d}	2.7 ± 0.7^{d}	2.7 ± 1.0^{d}
Pressure_firmness	3.7 ± 1.2^{d}	3.7 ± 1.0^{d}	5.6 ± 1.3^{c}	5.4 ± 1.2^{c}	5.4 ± 1.3^{c}	6.6 ± 0.9^{b}	7.4 ± 1.0^{a}
Rubbery	6.0 ± 1.5^{a}	6.1 ± 1.4^{a}	3.9 ± 1.7^{b}	4.0 ± 1.5^{b}	4.1 ± 1.6^{b}	2.6 ± 1.0^{c}	2.0 ± 0.9^{d}
Crumbly	2.4 ± 0.9^{c}	2.4 ± 0.8^{c}	3.2 ± 1.1^{b}	3.1 ± 1.1^{b}	3.9 ± 1.2^{a}	4.2 ± 1.4^{a}	4.3 ± 1.7^{a}
Cohesiveness	3.6 ± 1.6^{c}	3.8 ± 1.5^{c}	5.4 ± 1.6^{b}	5.8 ± 1.3^{b}	5.8 ± 1.4^{b}	6.5 ± 1.2^{a}	6.8 ± 1.4^{a}
Oily	6.6 ± 1.3^{a}	6.5 ± 1.4^{a}	5.0 ± 1.5^{b}	4.2 ± 0.9^{c}	4.1 ± 0.9^{c}	3.9 ± 0.8^{c}	3.5 ± 1.0^{d}
Overall_odour_intensity	2.4 ± 1.1^{e}	2.2 ± 0.9^{e}	2.9 ± 0.8^{d}	3.1 ± 0.9^{cd}	3.4 ± 1.0^{c}	4.1 ± 1.2^{b}	4.8 ± 1.5^{a}
Dairy_odour	6.4 ± 1.3^{a}	6.4 ± 1.2^{a}	5.8 ± 1.3^{b}	5.6 ± 1.0^{bc}	5.3 ± 1.1^{c}	4.8 ± 1.2^{d}	4.4 ± 1.3^{e}
Rate_of_melting	5.0 ± 2.0^{cd}	5.5 ± 1.6^{bc}	5.0 ± 1.6^{cd}	5.9 ± 1.2^{b}	6.6 ± 1.0^{a}	5.5 ± 1.4^{b}	5.0 ± 1.6^{d}
Breakdown	4.9 ± 2.0^{a}	4.1 ± 1.9^{b}	3.9 ± 1.2^{bc}	3.7 ± 1.2^{bc}	3.8 ± 1.6^{bc}	3.5 ± 1.0^{c}	2.8 ± 1.1^{d}
Salty	3.7 ± 1.0^{d}	3.9 ± 0.8^{d}	4.4 ± 0.6^{c}	4.6 ± 0.6^{c}	5.0 ± 0.7^{b}	5.2 ± 0.6^{b}	5.5 ± 0.9^{a}
Umami	1.7 ± 0.8^{d}	1.9 ± 0.7^{d}	2.7 ± 0.6^{c}	3.0 ± 0.9^{c}	3.4 ± 0.7^{b}	3.5 ± 1.0^{b}	4.7 ± 1.7^{a}
Sour	3.6 ± 1.0^{e}	3.8 ± 1.0^{e}	4.4 ± 0.6^{d}	4.5 ± 0.6^{cd}	4.8 ± 0.6^{bc}	4.9 ± 0.7^{b}	5.3 ± 1.1^{a}
Tangy	1.9 ± 0.9^{e}	2.1 ± 0.8^{e}	3.3 ± 1.0^{d}	3.7 ± 1.3^{d}	4.4 ± 1.6^{c}	4.9 ± 1.6^{b}	5.8 ± 1.9^{a}
Overall_flavour_intensity	3.7 ± 1.0^{f}	4.1 ± 0.9^{e}	5.1 ± 0.9^{d}	5.3 ± 0.9^{d}	6.0 ± 0.9^{c}	6.4 ± 0.9^{b}	7.2 ± 1.2^{a}
Buttery flavour	6.3 ± 1.4^{a}	6.3 ± 1.0^{a}	5.3 ± 1.2^{b}	5.1 ± 1.2^{b}	4.6 ± 1.1^{c}	4.3 ± 1.1^{c}	3.8 ± 1.2^{d}

Sweaty flavour	1.9 ± 0.7^{d}	1.9 ± 0.6^{d}	2.8 ± 0.9^{c}	3.2 ± 1.1^{c}	3.7 ± 1.3^{b}	4.4 ± 1.3^{a}	4.4 ± 1.8^{a}
Astringent_aftertaste	2.1 ± 0.7^{e}	2.4 ± 0.9^{e}	2.9 ± 0.7^{d}	2.9 ± 0.8^{d}	3.4 ± 0.8^{c}	3.8 ± 0.8^{b}	4.5 ± 1.1^{a}
Linger aftertaste	2.1 ± 0.8^{e}	2.3 ± 0.8^{e}	3.3 ± 0.9^{d}	3.7 ± 1.0^{d}	4.3 ± 1.1^{c}	4.9 ± 1.2^{b}	6.1 ± 1.4^{a}

II.3.2.2 Texture

From Table II-4, it can be seen that, cohesiveness increases gradually with ageing. Cohesiveness from a sensory point of view describes the ability to make a ball by hand. As ageing progresses, proteolysis and coarsening of the casein network results in more deformable cheese (Lucey & Fox, 1993; Rogers, Drake, Daubert, McMahon, Bletsch, & Foegeding, 2009).

During cheese maturation, the score of oily attributes dropped dramatically. Oily in this study was described as the amount of oil on the fingers when the panel forms a cheese ball. This sensory attribute is also associated with the ability of secretion of free oil in cheese during heating and is dictated by the supermolecular structure of fat globules and melting profile of lipid within the globule (Everett & Auty, 2017). The melting profile of Cheddar Cheese of different maturities was conducted in some preliminary research, measured by differential scanning calorimetry (DSC), indicates that the older the cheese, the lower the enthalpy and therefore the oily attribute change during maturation can be linked to lipolysis. Additionally, as cheese ages, smaller fat pools well and embed into the adjacent casein matrix, thus providing a better protective barrier to rupture with less free oil (Everett & Auty, 2008).

II.3.2.3 Flavour, aroma and taste

Buttery flavour and dairy odour decrease during the whole ripening process (Table II-4). The aroma compounds responsible for the buttery and dairy aroma are diacetyl and acetoin. From GC-MS results (seen in chapter V), both chemical compounds decrease during ripening which accounts for the lower scores of

buttery and dairy odours. The decrease of milky and diacetyl flavours was also found during the maturation of American Cheddar cheese (Drake, Yates, & Gerard, 2008).

For all batches of Cheddar cheese, the salty and umami perception increases during maturation. A similar phenomenon has also been observed previously (Drake *et al.*, 2008; Mcsweeney, 1997). The salty perception is governed by the amount of sodium present in saliva, and therefore proteolysis can be linked to more free sodium ions being released (Boisard *et al.*, 2014). On the contrary, Drake *et al.* (2008) proposed that the basic taste salty and sour generally did not change in American Cheddar cheese with ripening. Additionally, umami taste increases during the ripening process, and therefore proteolysis can be assumed to release free amino acids which exhibit the umami taste (Drake *et al.*, 2008).

II.3.3 Batch to batch variation of sensory properties

A bi-plot of the sample scores and sensory attribute loadings for PC1 and PC2 for 56 days and 180 days ripening is shown in Figure II-4. At each ripening time point, the batch variation trend for all cheeses with different predictive qualities is similar. The five batches of cheese can be divided into three distinct groups based on their sensory characteristics with one group comprising batch A, B and D. Here the sensory profile after 56 days and 180 days ripening is presented and discussed as an example. As after 180 days of ripening, the number of significantly different sensory attributes among batches decreases dramatically. This could be due to a lack of distinctive flavour details in general

protocol evaluation. The other batch variation time point PCA plots are presented in Supplementary Figure 2.1.

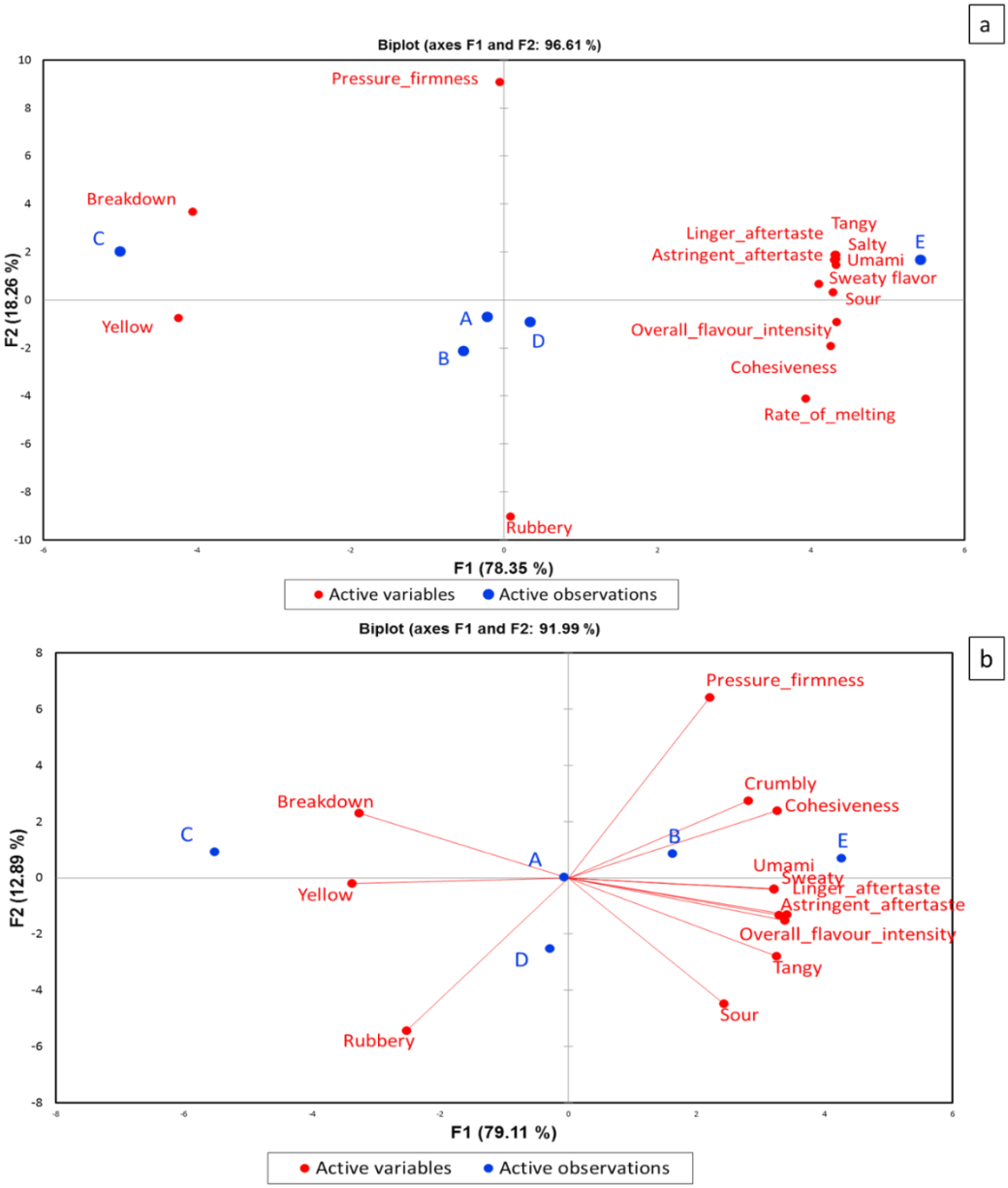


Figure II-4 *The principal components analysis (PCA) biplot on the quantitative sensory profile of 56 days (a) 180 days (b) ripening batches Cheddar cheese. The blue symbols stand for the batch number of different predictive quality Cheddar cheese. The red symbols stand for the different sensory attributes that significantly distinguish batch variations*

‘Premium’ batch B cheese wasn’t significantly separated from ‘graded quality’ batch A and D from the sensory profile. This could be due to a lack of specific details in general protocol and suggested the necessity of ‘cheese wheel’ in this study. But the batch A, B and D group is located in the centre of the PCA plot.

Generally, batch C is significantly lower in most characteristics of the sensory features of mature cheese, whereas batch E is significantly higher in all of those. It appeared that batch E matures much more rapidly than batch C. During the whole ripening process, batch C is significantly higher in yellowness, lower in the break down attribute and overall flavour intensity as well as early stage salty perception. In contrast, batch E is significantly lower in yellowness, higher in break down attributes and overall flavour intensity.

These could be due to the batch C and E having the least and most ‘fat in the water free substance’ and ‘moisture in the fat free substance’, as indicated in the Gilles and Lawrence model. Reduced fat Mozzarella cheese is also seen to be yellower. The reduced fat content can cause a smaller number of colloidal size fat globules able to scatter light (Rudan, Barbano, Joseph Yun, & Kindstedt, 1999). Cheeses high in the break down attribute implied a lumpy mouthfeel. The fat fraction in cheese is to provide lubrication during mastication for the interior to separate (Farkye & Guinee, 2017). Thus, the higher fat content in cheese provides a smoother mouthfeel rather than lumpy. Low fat Cheese always causes low intensity cheese taste and aroma. This is probably because of the lack of precursor from fat or lack of solvent power of the fat, or different physical structure of reduced-fat cheese (Kondyli *et al.*, 2002). The lower

'moisture in the fat free substance' can cause less break down of protein leading to less reformable casein matrix and lumpy mouthfeel as well as a decrease in further flavour development (Calvo, Castillo, Díaz-Barcos, Requena, & Fontecha, 2007; Urbach, 1993). The cheese environment within batch E probably accelerates the autolysis of lactic acid bacteria and promotes the ripening (Hannon, Kilcawley, Wilkinson, Delahunty, & Beresford, 2007).

Surprisingly batch C at the first sensory analysis time point is significant less salty than batch E. However, the percentage of 'salt in moisture' in batch C is the highest among all batches, whereas batch E is the lowest. Therefore, 1.4 % difference in the percentage of 'salt in moisture' still will not play the key role in differentiating the salt perception. An explanation as to why batch E is saltier than batch C is probably due to the lipid-protein ratio. The attributes, 'fat in the water free substance' is concerned with the fat and protein percentage. Batch C protein ratio is higher than batch E, and is the highest one among all the batches. It is stressed that the proportion of bound sodium increased when the protein content increased as the sodium ions were bound by casein (Boisard *et al.*, 2014). The more bound sodium, the lower the salty perception. The difference in the structure of Cheddar cheese therefore appears to have greater influence than a 1.4% salt concentration difference.

In summary, batch C seems to be a bland Cheddar cheese compared to other samples, whereas batch E has a more intense flavour profile. However, this is not necessarily a positive aspect, as the general score from the sensory protocol is not specific enough to tell why batch E is more intense in flavour.

The cheese wheel was applied to demonstrate further whether batch E has a more desirable intense flavour profile or not.

II.3.4 Summary of Cheddar cheese sensorial profile ripening and batch variations interaction via principal component analysis

From the two-way ANOVA results, all the sensory attributes changed significantly during cheese maturation ($p<0.05$). The bi-plot in Figure II-5 shows that the two principal components account for 92.44 % of the experimental variance. Principal component 1 mainly distinguished the Cheddar cheese from different ripening maturity. Younger Cheddar cheese was characterised by the higher score in rubbery, buttery flavour, dairy odour, oily, yellow and breakdown, whereas other attributes are all higher in more mature Cheddar cheese lying in the positive axis. As ripening proceeds, the same age Cheddar cheese are more tightly grouped. As ripening proceeds further, the sensory attributes can significantly ($P<0.05$) discriminate the batch to batch variation reducing from fourteen attributes to five attributes after 540 days ripening. Most of texture related sensory characteristics are still significantly discriminated in the batch variations after 540 days ripening.

Batch E matures noticeably faster than other batches while batch C is slower than other batches. Most of batch E samples clustered into the next maturation time point samples, oppositely batch C sample grouped into the previous maturation time point samples. Only in the last two maturation time points we

observed, namely maturation after 450 and 540 days, all the cheese samples grouped together. This implied that the batch variation could be compensated by long-term ripening from a sensory point of view. Batch C has significantly less aroma and taste than others until 540 days and batch E is still significantly higher in the texture and taste relative sensorial attributes. Not much difference is seen at the late-ripening stage, probably as at such long time ripening, all cheese samples generate distinctive flavour and texture features. Therefore, the cheese wheel has been served as an extra protocol to obtain the standout sensorial characteristics.

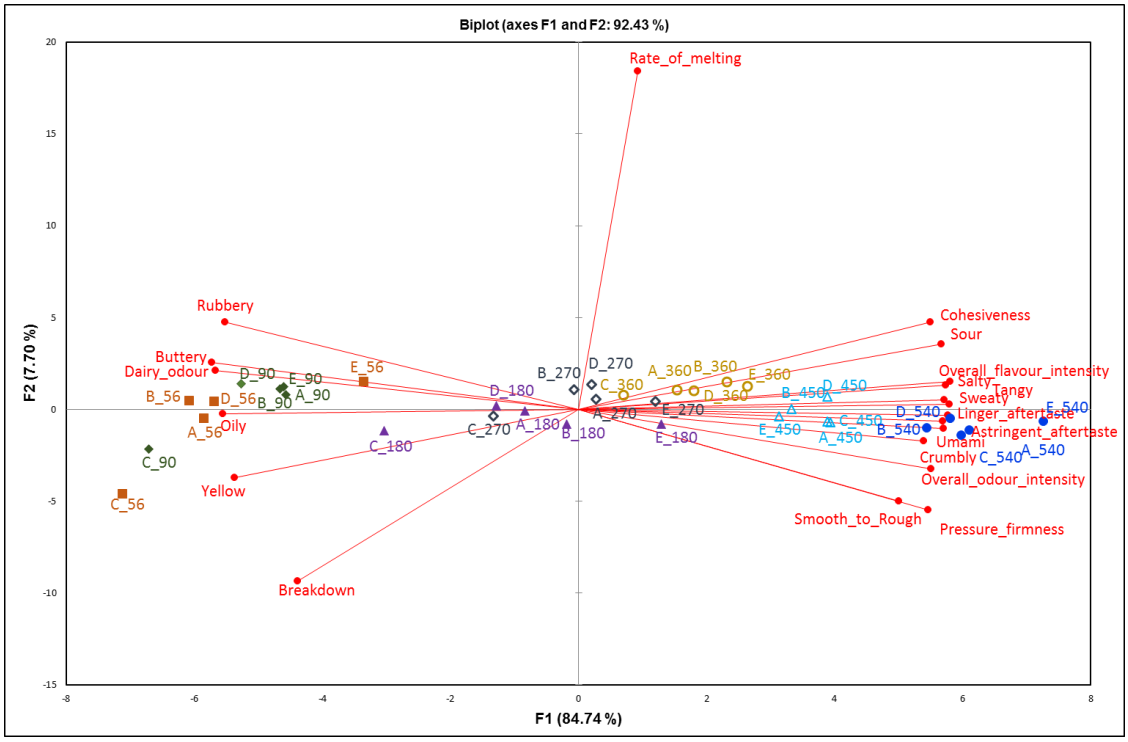


Figure II-5 *PCA bi-plot carried out on sensory attributes by the training panel. All the sample points are labelled as batch number and ripening days. Symbols and colours of the samples indicate different ripening days: 56 days ripening (solid □; orange), 90 days ripening (solid ◊; green), 180 days ripening (solid Δ; purple), 270 days ripening (open ◊; grey), 360 days ripening (open o; yellow), 450 days ripening (open Δ; sky blue), 540 days(●; Navy blue). All the sample points are the mean of three replicates ×10 panel evaluations.*

II.3.5 Cheese wheel profile in late ripening phase batches sample

Commonly Cheddar cheese ripening period is from 3 to 18 months (Banks, Brechany, Christie, Hunter, & Muir, 1992). Due to time limitation, the instrumental analysis was only performed until 15 months. However, the sensory evaluation was continued to the later stages of ripening, to assess the final quality of Cheddar cheese. At the last maturation time point, all six batches of Cheddar cheese were evaluated. As in the early ripening stages, there were not many outstanding characteristics of cheeses chosen by the panel. Cheese wheel data at the late stage of ripening is presented here, to show the batch variation of these batches of Cheddar cheese.

In Figure II-6, most of the outstanding sensorial attributes selected by the panel from cheese wheel are aroma, taste and flavour related attributes rather than textural attributes. A significant flavour deterioration was observed such as dirty and bitter aftertaste in batch E after 540 days ripening. Combining general sensory protocol results and cheese wheel results, batch E Cheddar cheese has a dirty aftertaste in conjunction with bitter and lingering, as well as astringent aftertaste. In literature, poor quality or “old” milk which has been in cold storage for days and used for cheese manufacture is the principal cause of the unclean flavour defect. Proteolytic or lipolytic enzymes, derived from psychrotrophic bacteria may cause undesirable chemical reactions to occur within the cheese, and hence, result in an unclean off-flavour (Partridge, 2008). This only provides a possible reason for the unclean flavour, the further study needs to be performed to confirm this, since while the batches of cheese

studied here were from the same day of production, the origin of the milk from the different on-site storage silos is not known to the authors.

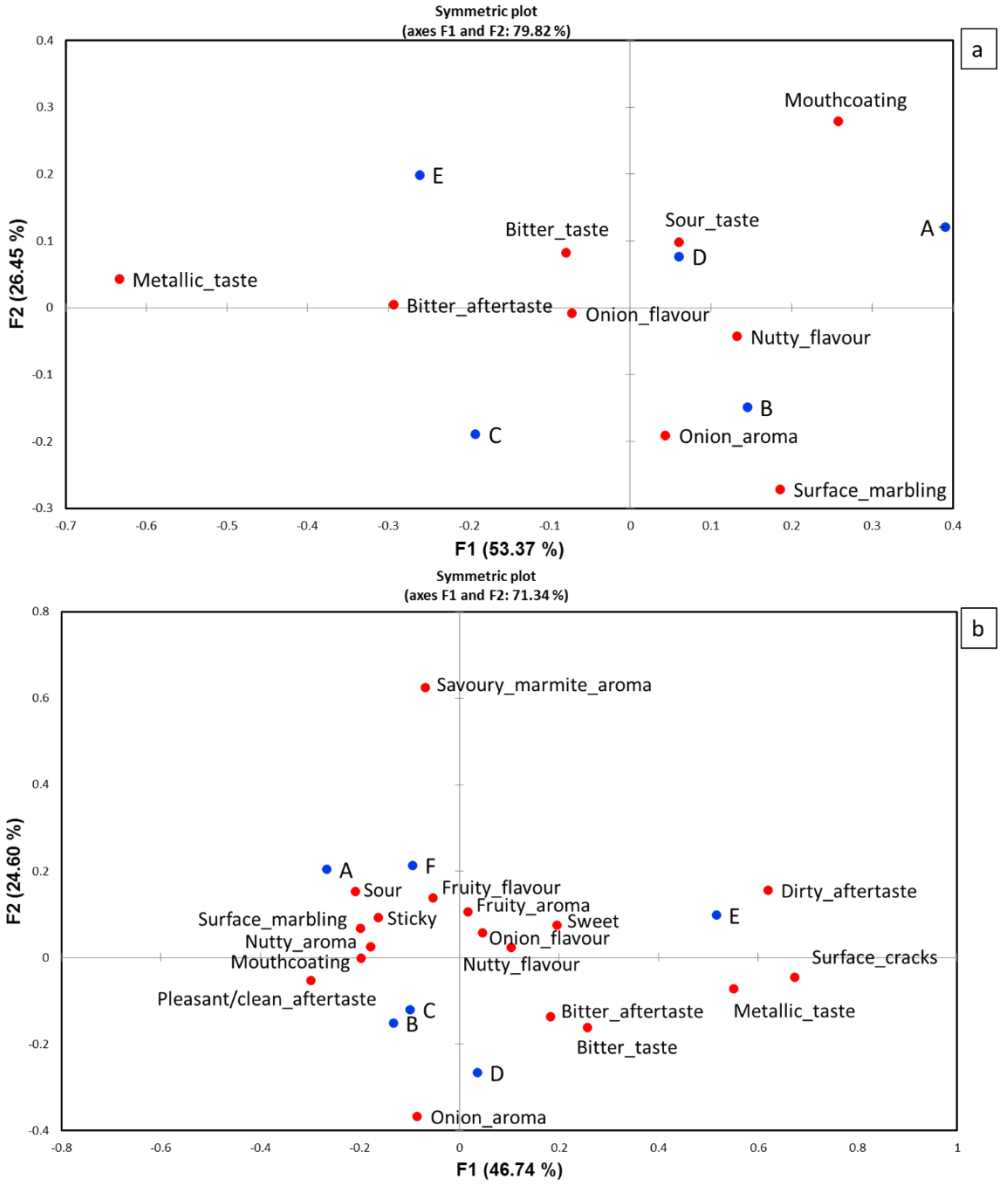


Figure II-6 *The Correspondence Analysis (CA) symmetric plot of cheese wheel sensory profile evaluated at 450 days (a) and 540 days(b) ripening. Blue symbols stand for the batch number, and the red symbols stand for the outstanding sensory attributes.*

The most potent bitter taste and the bitter aftertaste of batch E among all batches cheese are also probably due to the low salt concentration. Even though, the percentage of 'salt in moisture' (S/M) for all grades of cheese match Gilles and Lawrence model premium Cheddar cheese composition range, batch E has the lowest the percentage of salt in moisture, 4.45%. A similar phenomenon was reported that cheese with low salt concentration is very prone to show bitterness, where higher amounts of β-CN(f193-209), β-CN(f176-182) and β-CN(f193-208) were found (Møller *et al.*, 2013). Batch C displays bitter taste and aftertaste at the late stage of ripening. Salt and acidity have been shown to influence the perception of bitterness in previous studies (Engel *et al.*, 2001). As batch C is less sour than other batches beyond the first evaluation ripening point, the panel could sense stronger bitterness. Furthermore, batch C has a lower development of flavour profile, the bitter taste will be more evident without another flavour suppressing cross-modalities.

Only some of flavour characteristics in the Correspondence Analysis (CA) symmetric plot (Figure II-6) can be explained, as the origin of some flavour defects are still unknown due to the complexity of the cheese matrix and the possible interactions between its components during cheese ripening (Engel *et al.*, 2001).

Apparently surface cracks were observed in batch E; this attribute is commonly regarded as a defect in Cheddar cheese manufacture. The cracks or splits in cheese was formatted by the pressure of gases which is mainly CO_2 and H_2 from

the fermentation of lactic acid. This defect is generally accompanied by rancid flavour and unpleasant aroma which is in agreement with our findings that batch E has significant higher sweaty flavour in general protocol and dirty aftertaste in cheese wheel (Garde, Ávila, Gaya, Arias, & Nuñez, 2012).

Combining the general protocol and cheese wheel results, in Figure II-6 batch C is close to the origin of CA symmetric plot at 540 days ripening evaluation point indicating that it has the lowest of all the sensory attributes in the 'cheese wheel'. In Figure II-4, the general protocol also displays that batch C is lower in flavour and aroma during ripening. Batch B is near the origin of CA symmetric plot as well at 540 days ripening, but batch B shows a certain level of flavour and aroma attributes, we can conclude that batch B has a more balanced aroma and flavour profile compared to other batches and batch C just because it is generally lower of flavour and aroma, thus it doesn't have a lot distinctive flavour attributes.

II.4 Conclusions

Cheese predicted as a 'graded quality' based on Gilles and Lawrence quality prediction model still have flavour or texture defects. Therefore, other parameters need to be considered to gain a better prediction of Cheddar cheese quality. Batch C and E both regarded as graded quality in Gilles and Lawrence grading prediction model matured in different rates in terms of sensory profile. Batch C cheese matured slower than other batches throughout the whole ripening process. In contrast, batch E matured relatively ahead of

other batches. After 540 days ripening, batch C matured to a bland flavour profile but yellower and has the lumpy mouth feel whereas batch E matured to be a cheese has a dirty and bitter aftertaste and surface cracks texture defect as well as stronger age cheese flavour. These results indicate that reliance upon the Gilles and Lawrence grading model is not accurate, and we have shown batch to batch variation between cheeses made on the same day in the same production facility, necessitating more robust predictions of cheese quality are required. However, this study investigated only on five batches of Cheddar cheese, more batches of Cheddar cheese need to be observed in order to validate the conclusions.

Chapter III The state of water and fat during the maturation of Cheddar Cheese

Abstract

Cheddar cheese predicted to develop into different quality classes has been evaluated by time domain Nuclear Magnetic Resonance, Thermogravimetric analysis and quantitative sensory analysis. The water and fat proton signals in the transverse relaxation decay curves have been deconvoluted. Proton transverse relaxation values for both the water and fat fractions decrease and the relative %age of the proton peak area, predominantly from the fat increases over a 450-day ripening period. The thermodynamic free water percentage increases during maturation. Water and fat attributes can distinguish between Cheddar cheese batches after 56 days. Cheese batches which have lower transverse relaxation values for the water and fat proton fractions and a higher relative %age of the proton peak area predominantly from fat at 56 days, mature after 270 days to be more yellow, rubbery and smooth, have a less sour and lingering aftertaste and are also harder to form into a cheese ball.

Highlights

- Time domain water and fat proton Nuclear Magnetic Resonance signals are assigned.
- Transverse relaxation times for water and fat protons decrease with cheese ripening.
- Thermodynamic free water percentage increases with cheese ripening up to 450 days.
- Water and fat state attributes can differentiate between batches of Cheddar cheese.
- Batches with different water and fat attributes have distinctive sensory profiles.

Keywords Cheddar cheese; Time domain NMR; TGA; Maturation; Sensory variation

III.1 Introduction

Cheddar cheese ripening is a complicated microbiological and biochemical transformation involving glycolysis, lipolysis and proteolysis (Jiménez-Flores & Yee, 2007). All of these biochemical processes contribute to the overall structure change during maturation. Cheese structure is influenced by factors that include

casein-casein, casein-water and casein-fat interactions; the state of water (bulk or bound to the casein matrix), the state of calcium (ionic or bound to casein matrix); and the extent of proteolysis (Everett *et al.*, 2017). Water migration and binding capacity reflect structural properties such as casein matrix porosity and tortuosity as well as textural properties. They are believed to be an important consequence of ripening and determine many of the sensory and functional characteristics of the cheese (Kuo, Gunasekaran, Johnson, & Chen, 2001; Saldo, Sendra, & Guamis, 2002). There are at least two types of water present in Cheddar cheese distinguished by their extent of association with macromolecules. They are conveniently described as bound and free water. Bound water is the fraction closely associated with the hydrophilic molecules whilst free water is available for biological function (Hickey, Guinee, Hou, & Wilkinson, 2013; Wang, Zheng, Li, Ma, Zhao, & Zhang, 2018). Accurate and detailed definition of these terms depends on the methodology used to measure the free water content. For example, in centrifugation methods, the amount of water removed depends on the conditions used to expel the free water (Saldo *et al.*, 2002). In a hydraulic pressing method, the amount of expressible serum was used to describe water state of the cheese (Guinee *et al.*, 2000). The contribution of lipolysis to maturation in Cheddar cheese is not as significant as in mould-ripened cheese. However, the texture, flavour and physico-chemical properties of cheese are still largely governed by fat. The state of fat is also important in determining the complex sensory properties of Cheddar cheese (Lopez *et al.*, 2007).

Time Domain Nuclear Magnetic Resonance (TD-NMR) analysis is a rapid non-destructive and solvent-free measurement which has been used in many studies

to describe the physical state and distribution of water and fat in materials (Oztop, Bansal, Takhar, McCarthy, & McCarthy, 2014; Rudi, Guthausen, Burk, Reh, & Isengard, 2008; Yang *et al.*, 2016). The transverse relaxation decay time (T_2) is a measurement of relaxation of protons towards an equilibrium state which reflects the molecular dynamics in the neighbourhood of protons but can reflect other phenomena such as chemical exchange and diffusion. It is associated with a particular frequency range and is material specific (Rudi *et al.*, 2008). NMR T_2 relaxometry combined with magnetic resonance imaging (MRI) has been used to quantify spatial water and fat distribution and mobility in methylcellulose coated chicken nuggets (Oztop *et al.*, 2014). A T_2 relaxation analysis has also been used to assess changes in the structure and the mobility of different proton components in Mozzarella cheese during maturation and subsequent heating. A Carr-Purcell-Meiboom-Gill (CPMG) sequence was used where the water and fat proton signals were distinguished by Fourier transformation at specified points in the CPMG decay (Smith *et al.*, 2017). However, the interpretation of such data can often be difficult due to the existence of complex factors in addition to simple measures of mobility, such as proton-proton exchange between water and the hydrophilic polymers present in meat and plant-based products. In this work, TD-NMR was applied to explore the casein-water and casein- fat interactions during maturation and batch variation. In a heterogeneous system such as Cheddar cheese, each proton phase is affected by its surrounding food matrix (Gianferri, D'aiuto, Curini, Delfini, & Brosio, 2007a).

Thermogravimetric analysis is a technique that allows us to determine the loss of mass and thermal response of a material subjected to a controlled heating rate. It

is commonly used in food and pharmaceutics for water content analysis (Ducat, Felsner, da Costa Neto, & Quináia, 2015; Pirayavaraporn, Rades, & Tucker, 2012; Silva, Silva, Andrade, Veloso, & Santos, 2008). Thermogravimetric analysis (TGA) was also used to measure the distribution of free and bound water and the interaction with the polymer in the food matrix (Roozendaal, Abu-hardan, & Frazier, 2012; Saldo *et al.*, 2002). Cheese thermo-physical properties can be measured by TGA. They are influenced by the composition and interaction of the cheese matrix and provide indirect evidence for structural interaction (Schenkel, Samudrala, & Hinrichs, 2013).

Cheddar cheese texture and flavour do not depend exclusively on the ageing process. Grading takes place during Cheddar cheese maturation and is used to inform the manufacturer whether the cheese in question is suitable for extending the ripening period to produce premium Cheddar cheese or must be sold quickly as a young cheese (Partridge, 2008). The Gilles and Lawrence Cheddar cheese predictive grading model, which consists of four chemical composition variables, is still used in Cheddar cheese manufacture to aid the professional Cheddar cheese grader (Gilles *et al.*, 1973). After manufacture, the four chemical composition variables used to grade Cheddar cheese as likely to be premium or lower quality cheese are the percentage of salt in moisture, moisture in the fat-free-substance, fat in the dry matter and pH.

Cheese quality prediction and the control of functional properties require an understanding of the location of the components of cheese in relation to each other and how they interact and change during ripening (Everett *et al.*, 2017). Most of the research reported in the literature to date concerning changes in the state

of water and fat measured by NMR and TGA during maturation, have concentrated on Mozzarella cheese rather than Cheddar cheese, with little research focussing on same batch evolution of structure from early through to late-maturation (Gianferri *et al.*, 2007a; Kuo *et al.*, 2001; Luo, Pan, Guo, & Ren, 2013; Smith *et al.*, 2017). Moreover, there is no work exploring the correlation between the state of water and fat in Cheddar cheese with the sensorial profile and the variation seen between batches.

The objective of this research was to study the change in the biochemical and biophysical state of water and fat during Cheddar cheese maturation over a period from 56 to 450 days, and to examine the potential of water and fat variation in different batches of cheese to predict the differences in sensorial profile.

III.2 Materials and methods

III.2.1 Materials

Batch A, B, C, D, E and F cheese samples used for this chapter are the same block as chapter II. Time-domain ^{1}H NMR relaxometry measurements, thermogravimetric analysis (TGA) and descriptive sensory analysis were carried out at various times during the ripening period, namely 56, 90, 180, 270, 360 and 450 days. At each time point, two bags of cheese were removed at random from each block and placed in a 4 °C refrigerator before measurement. One bag was used for instrumental measurements and the other for descriptive sensory analysis.

A 180 g commercial package of Cheddar cheese from the same factory was delivered at the same time. Half was freeze dried for three days; the other half was vacuum packed and placed in a 4 °C refrigerator before testing. These commercial samples were for identification of proton populations.

III.2.2 Time domain NMR measurement

For low resolution NMR measurements, cheese samples were moulded into cylindrical shaped pieces cut from a central part of a block of Cheddar cheese. To prevent water evaporation and reduce dead space, a glass rod wrapped with polytetrafluoroethylene (PTFE) tape was inserted in the tube over the cheese and finally sealed by parafilm at the top of the tube outside the magnet and NMR coil. All samples were measured in triplicate.

Measurements were performed with a benchtop NMR spectrometer operating at a proton frequency of 21.23 MHz (AMR Ltd, Oxford, UK). The transverse relaxation curves were measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence with a pulse spacing of 2 X tau between the two 180° pulses, where tau = 256 µs and recording at the echo peak value. 1024 data points on the relaxation curve were acquired. The recycle delay (RD) time was chosen as 10 s to avoid saturation of any bulk water fraction present. The relaxation decay curves were fitted to distributed exponential decays based on zeroth order regularisation using software supplier by AMR Ltd, Oxford, UK. A plot of relaxation amplitude against relaxation time was obtained. T_2 values were measured from the peak position and the area under each peak was determined by cumulative integration.

III.2.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analyses were performed on a thermogravimetric analyser TGA/DSC 3+ (Mettler-Toledo GmbH Analytical, Switzerland). The measurements were carried out under nitrogen with a gas flow of 50 cm^3/min. Approximately 15 mg of fresh cheese from the centre of a cheese cube were weighed in 100 µL aluminium crucibles with pierceable lids and heated over the temperature range 25-450 °C, at a scanning rate of 10 °C/min. The analysis was performed in duplicate for each sample. An empty aluminium crucible with a pierceable lid was measured using the same heating program. The blank curve was subtracted from the sample TGA curve in order to minimise the buoyancy effect on the empty crucible during heating. Graphs of the sample weight as a function of temperature and its first derivative (Derivative thermogravimetric (DTGA) with units of %/°C) were constructed and all analysis was carried out using STARe software (Mettler-Toledo GmdH Analytical, Switzerland).

III.2.4 Differential scanning calorimetry (DSC) analysis

Differential scanning calorimetry was performed on DSC823^e (Mettler Toledo, Switzerland). The instrument was calibrated for temperature and enthalpy using the onset transitions and enthalpy values for Indium and cyclohexane. Typically, 10-13 mg of the sample taken from the centre of cheese cubes were placed in aluminium pans and hermetically sealed. The samples were heated from 20 to 60 °C at a heating rate of 5 °C/min.

III.2.5 Descriptive sensory analysis

Quantitative Descriptive Analysis was carried out at the Sensory Science Centre, University of Nottingham (de Cássia Dos Santos Navarro Da Silva *et al.*, 2012). Ten experienced trained panellists (3 males, 7 females) were initially invited to participate in 2-hour training sessions to evaluate the sensory properties of Cheddar cheese. Sensory panels were first presented with a wide range of commercial Cheddar cheeses to generate a list of attributes that would describe the sensory properties perceived. As a result of discussions within the panel and data from the literature, a final descriptor list containing 19 sensory attributes covering appearance, aroma, taste, flavour and mouthfeel properties was developed and is presented in Table II-3 (Drake, McIngvale, Gerard, Cadwallader, & Civille, 2001; Kraggerud *et al.*, 2008; Partridge, 2008). During this long period sensory study, the panel was invited back every 3 months. Reference cheese samples were used in these subsequent sessions to better qualitatively and quantitatively calibrate the panel. Other training sessions were used to train sensory panels on standardising evaluation protocol and quantifying the intensity of sensory attributes using the developed scales.

Reference samples were defrosted the previous night in a 4 °C walk in fridge. All the reference and test sensory samples were equilibrated at 18 °C for 5 h before the sensory sessions. The outer layer (5 mm) of each cheese was discarded and each sample cut into 2 cm×2 cm×2 cm cubes. Two cubes of each samples were presented to the assessors in a sealed 60 mL plastic pot. All samples were

numbered using random 3-digit codes in randomised order. Due to the time limitation, only five batches of sample (A, B, C, D, E) were used for sensory profiling.

Only the sensory analysis data for Cheddar cheese at 270 days are presented, which based on previous studies was considered the most opportune time to reflect mature Cheddar cheese (Hou, Hannon, McSweeney, Beresford, & Guinee, 2014). All data were collected using Compusense Cloud (Compusense, Canada). A more complete set of data will be presented in a future publication.

III.2.6 Statistical analysis

The differences in fat and water state during ripening and batch variations were analysed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. Agglomerative Hierarchical Clustering (AHC) was performed on sensory attributes shown to be significant between batches. For modelling sensory profiles from the water and fat state attributes measured by TD-NMR, Partial Least Squares (PLS) was used. The X data matrix contained water and fat state attributes. The Y matrix contained the results of sensory mean scores for all significantly different cheese attributes. All statistical analysis was performed using XLSTAT (Addinsoft, France).

III.3 Results and discussion

III.3.1 Identification of proton populations and evolution in Cheddar cheese during maturation

In Figure III-1 (a), the distribution of transverse proton relaxation times T_2 as determined by the CPMG pulse sequence in fresh Cheddar cheese (non-freeze-dried Cheddar cheese) at temperatures of 20 and 40 °C is presented. The amplitude of population A labelled in the figure was higher than population B. The transverse magnetisation relaxation curves show multi-exponential behaviour due to the presence of at least 2 components characterised by different T_2 values and amplitudes. T_2 is directly proportional to the molecular mobility and inversely proportional to the level of molecular interactions (Hindmarsh, Smith, Carr, & Watkinson, 2019). For Cheddar cheese, the protons in the protein molecules which are in a solid state will not contribute directly to the NMR signal that was acquired with a tau value of 256 µs. This setting will detect only comparatively mobile protons. However, the proton signal will arise from a weighted average of the intensity and decay times of the water and exchangeable protons on the polymer. The majority of the fat protons being directly connected to the carbon backbone of the lipid fatty acid chains will be non-exchangeable and so will reflect the distinct properties of that phase. The proton signal obtained from Cheddar cheese can therefore be loosely regarded as coming from the water and fat protons. However, the NMR signals from water will interfere with the oil signal in sample at water contents higher than 15% due to the similarity of relaxation times of water and oil

(Todt, Guthausen, Burk, Schmalbein, & Kamlowski, 2006). Thus, the first part of this work had the aim of identifying the water and fat fractions in the relaxation curve.

The transverse relaxation curves of packaged commercial Cheddar cheese were obtained at temperatures of 20 and 40 °C Figure III-1(a) shows that when Cheddar cheese is heated, the relative amount of proton population B increased corresponding to the melting of solid fat. The Differential Scanning Calorimetry (DSC) curve in Figure III-1(b) for the same cheese sample showed the melting of a fraction of the cheese fat between 20-40 °C. This supported the proposal that the proton signal of population B is predominantly from the fat fraction of Cheddar cheese. If the signal in population A were due to a solid component of the fat, we would expect the T_2 value to be in order of microseconds rather than the value of 10 – 20 milliseconds as shown. As heating proceeds, the peak values of the relaxation times of population A and B both increased which probably was related to the effect of heat treatment on the water binding capacity and general mobility in the cheese matrix and mobility of the proton fraction in the fat.

In order to determine the proton population assignment of peak A, Cheddar cheese drying experiments were performed. The same cheese sample after having been freeze dried for three days or room temperature dried for 3 hours were measured at 40 °C. As shown in Figure III-1 (c), a significant decrease in proton population A of room temperature dried and freeze-dried samples compared with non-dried fresh Cheddar was observed. This is associated with the loss of water during the drying process and further supports the allocation of population A to the water protons. The maximum peak value relaxation times obtained for the 3 different treatments of Cheddar cheese are different, with the values for all the dried

systems being less. This is probably due to the generalised decrease in mobility of the sample upon drying affecting all the material phases. A similar phenomenon was observed in dough and microwave expansion of imitation cheese where the relaxation time for the mobile proton population decreased with water decrease (Arimi, Duggan, O'Sullivan, Lyng, & O'Riordan, 2010; Assifaoui, Champion, Chiotelli, & Verel, 2006). Considering Figure III-1 (a) to (c), it is concluded that the protons in population A were primarily protons from the water fraction, whilst protons in population B were predominantly protons from the fat. However, we cannot ignore the possibility that population B contains a significant amount of free serum water protons. Water proton transverse relaxation time constants at 40 °C of about 20 ms and 400 ms were reported in the high field and sampled NMR spectrum of Mozzarella cheese (Smith *et al.*, 2017). As mentioned in the previous text, the relaxation time decay constants of water are similar to that of oil. Using a hydraulic pressing method, Cheddar cheese after 180 days of ripening was found to still have roughly 10 to 15 % expressible serum (Guinee *et al.*, 2000). Previous research has confirmed that lipid in food samples exhibits relaxation times of a few hundred milliseconds. The value of T_2 for proton in solid fat is of the order of tens of μs which cannot be observed on the timescale of this experiment. (Song, 2009; Todt *et al.*, 2006).

Only one main independent compartment of water was detected in Cheddar cheese. Three types of water were detected in Mozzarella di Bufala Campana which were allocated the following descriptions: junction zone water with a T_2 value of 7.3±0.3 ms, entrapped water with T_2=47±3 ms and serum water with

T_2=951±36 ms (Gianferri *et al.*, 2007a). This difference suggests that water distribution is homogenous in Cheddar cheese compared with Mozzarella. This could also be because the size of different water compartments is sufficiently small to allow the complete averaging of the two water pools by diffusive exchange. The water relaxation would then be described by a single mono-exponential curve (Mariette, 2003).

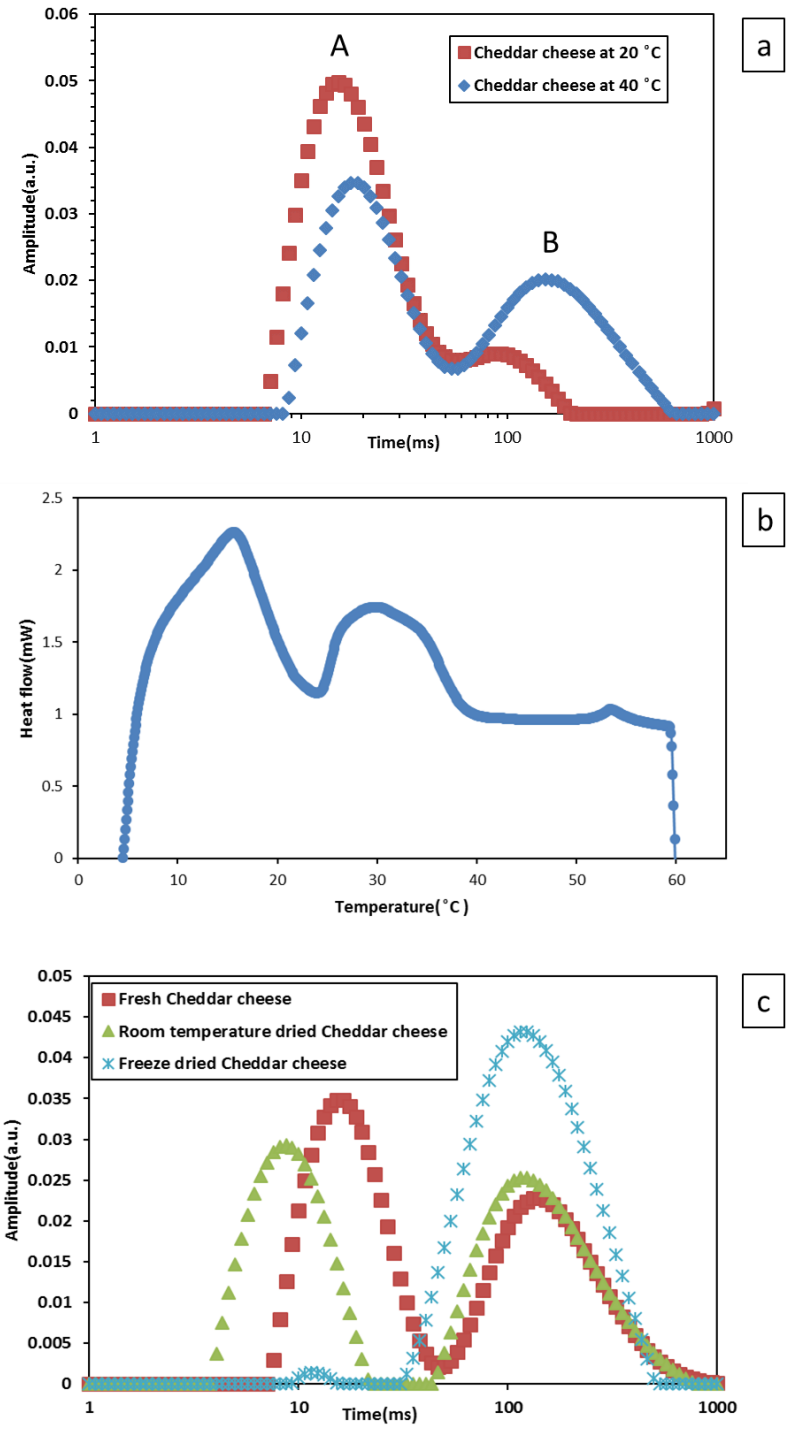


Figure III-1 (a) The distribution of transverse proton relaxation times T_2 as determined by the CPMG pulse sequence in fresh Cheddar cheese (non-freeze-dried Cheddar cheese) upon heating from 20 to 40 °C. The different proton populations are indicated with capital letters A and B. (b) DSC curve showing the melting of fresh Cheddar cheese over the range of 4 °C to 60 °C at a heating rate of

5 °C/min. (c) The distribution of transverse proton relaxation times T_2 as determined by the CPMG pulse sequence in fresh Cheddar cheese, room temperature dried Cheddar cheese and freeze-dried Cheddar cheese all at 40 °C.

As shown in Table III-1, as ripening proceeds from 56 days to 450 days, the T_2 value for both fractions decrease significantly. This indicates that protons from both the water and fat fractions become less mobile. As both fractions are affected this suggests that protons from the water and fat are binding to or immobilised by, for example, the casein rather than any effect on the exchange mechanism which would predominantly affect the water fraction. The decrease in T_2 value for water fractions indicated that increased hydration of casein during ripening may lead to enhanced casein-water interactions. This is in agreement with McMahon's findings. He concluded that serum along with the protein contained therein is absorbed into protein matrix and becomes an integral part of protein matrix by day 21 (McMahon *et al.*, 1999). A similar phenomenon was reported during Mozzarella maturation, the free water surrounding the fat channels gradually being reabsorbed into the protein matrix (Kuo *et al.*, 2001). There are several possible reasons which could explain this phenomenon. Firstly, during the maturation process, as proteolysis progresses, more α-carboxylic acid and α amino groups are produced by the cleavage of peptide bonds producing soluble peptides. They increase the water-binding capacity of casein and restrict proton mobility (Smith *et al.*, 2017; Upadhyay *et al.*, 2004). The second mechanism behind the phenomenon could be the pH change and molecular interaction. During the ripening process, pH increases gradually, when it is higher than casein isoelectric point, the casein molecules have a net negative charge and while hydrophobic

interactions persist, the ionic interactions between the molecules change from attractive to repulsive. Thus, the tight casein aggregates absorb water, partly to solvate the non-neutralized ionic charges. A contributing factor is also the partial exchange of Na^+ for Ca^{2+} in Cheddar cheese further weakening the aggregates (Abdelsalam, Alichanidis, & Zerfiridis, 2012). As a consequence, the casein water binding capacity increases and the T_2 value deceases significantly. A similar phenomenon was reported by Lucey *et al.* (2003) in that young Camembert readily "waters off", but once the pH increases, no watering-off is observed.

There is another similar mechanism taking place in Cheddar cheese. It is widely accepted that the interaction in the Cheddar cheese curd formation after manufacture involves a casein calcium phosphate bridge linkage as shown in Supplementary Figure 3.1. As ripening proceeds, acid levels increase resulting in the solubilisation of the calcium which in turn causes the phosphate ion to be protonated, resulting in a tighter association of the water with the casein. Consequently, the proton mobility in the water fraction decreases as ripening proceeds (Lucey *et al.*, 2003).

One other explanation for the reduction in water mobility during ripening is the decrease in water content. Thermogravimetric Analysis (TGA) data shows that the Cheddar cheese water content decreased from 38.44% to 34.48% from 56 days to 450 days. The proton mobility decreased linearly as the water content decreased (Arimi *et al.*, 2010; Assifaoui *et al.*, 2006). Water within the cheese matrix became less mobile with decreasing water content which suggested that the protons in water were bound more tightly to the cheese matrix or that the exchange mechanism became more effective.

The peak T_2 value of population B decreased during ripening. A similar phenomenon was found by Gianferri *et al.* (2007a), namely that the fat proton T_2 and the serum water proton T_2 value showed a clear and gradual reduction from day 1 to day 14 in Mozzarella. Some of the free serum water is absorbed by the protein matrix during ripening which may contribute to the decrease of T_2 value from population B. This may also have led to an increase of surface contact between fat and protein and reduced mobility (Karami, Reza Ehsani, Ebrahimzadeh Mousavi, Rezaei, & Safari, 2008). The fat also progressively became more solid, reducing both diffusion and T_2 with aging (Smith *et al.*, 2017). Lopez *et al.* (2007) working on Emmental cheese microstructure, suggested that during maturation pockets of serum, which were surrounded by pools of fat, disappeared. The casein matrix then expanded into the fat phase and broke the fat pool into smaller regions causing a possible reduction in mobility of the fat fraction and a decrease in the T_2 proton value. Consequently, there are several possible contributions to the overall decrease in the T_2 value of population B during ripening.

Table III-1 *The distribution of transverse proton relaxation times T_2 from water and fat fractions and proton predominantly from fat fraction peak area proportion at different ripening times in days after Cheddar cheese manufacture.*

Ripening days	Proton from water fraction T_2 (ms)	Proton predominantly from fat fraction T_2 (ms)	Proton predominantly from fat fraction peak area proportion (%)
56	30.27 ± 2.3^{a}	209.33 ± 14.29^{a}	30.43 ± 2.41^{e}
90	28.17 ± 2.71^{b}	196.23 ± 16.63^{b}	31.94 ± 3.33^{d}
180	27.57 ± 1.89^{b}	192.36 ± 15.16^{b}	33.70 ± 3.22^{c}
270	24.85 ± 2.88^{c}	181.62 ± 18.56^{c}	35.64 ± 3.00^{b}
360	24.65 ± 1.91^{c}	175.98 ± 14.33^{c}	36.17 ± 2.89^{b}
450	21.59 ± 1.45^{d}	164.60 ± 11.28^{d}	40.43 ± 2.42^{a}

For each time point the data represents three measurements on the six batches of the cheese and so is an average of 18 samples. Errors present the standard deviation for the eighteen values. Different superscripts in the same column indicate significant statistical difference (Tukey's test $P<0.05$)

III.3.2 Thermal measurements on the state of water in Cheddar cheese during maturation

The thermogravimetric curves describe Cheddar cheese thermal stability which is related to the water holding capacity. Examination of the thermogravimetric and first derivative curves shows at least four different weight loss steps. Water release over the range 25-200 °C is described by points A, B, C in the first derivative curve on Figure III-2 (de Angelis Curtis, Curini, D'Ascenzo, Sagone, Fachin, & Bocca, 1999; Ducat *et al.*, 2015). From a thermal perspective, there are at least two types of water present in Cheddar cheese. The initial shoulder at approximately 92 °C labelled A can be attributed to the water loosely bound to the cheese matrix and is lost at relatively low temperatures, namely free water. Peak B and C can be attributed to water relatively tightly bound to the Cheddar cheese matrix and is lost at a higher temperature (de Angelis Curtis *et al.*, 1999). This is rather

imprecisely defined here as bound water. The state of water as defined by these thermal measurements during maturation, are described by the three attributes; total water content, free water ratio (free water/ total water) and bound water ratio (bound water/total water). Although these three attributes describe primarily the water state, they also provide an indication of the thermodynamic properties of cheese matrix structure.

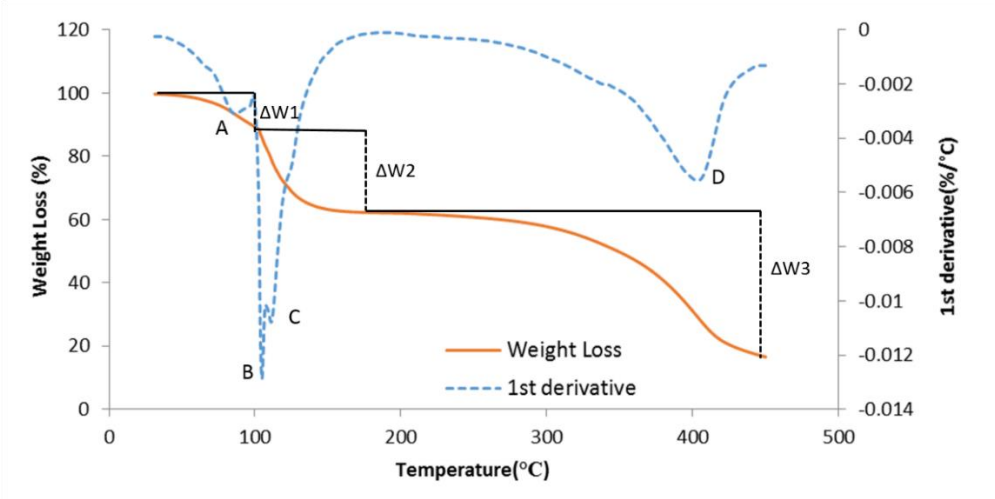


Figure III-2 *Mass loss (solid line) and first derivative (dotted line) TGA curves for a cheese sample heated at a rate of 10°C/min. The water loss peaks in the 1st derivative plot are indicated by the capital letters A, B, C. Three weight loss values (ΔW1- ΔW3) are indicated.*

In Table III-2, during the maturation process, total water content drops from 38.44% at 56 days ripening to 36.68 % at 450 days ripening. Free water and bound water ratio changes more significantly than the overall total water content. As ripening proceeds free water ratio increases from 56 days to 270 days. A dramatic change occurs between 270 days and 450 days where the free water ratio jumps steeply from 28.78 % to 42.65 %.

During heating in the TGA experiment, the first stage of water release is the diffusion of the free water through the cheese matrix which is related to the integrity of cheese matrix (Smith *et al.*, 2017). This is followed by the melting of solid fats and the dissolution of the calcium linkage which holds the casein molecules together reducing the strength of the casein-casein interaction (Lucey *et al.*, 2003). Finally, the protein structure collapses and liquefies. Water in the Cheddar cheese and associated with these components is progressively liberated as the microstructure changes. TGA data therefore reflect the thermal stability of the Cheddar cheese microstructure as well as the water release.

The increase of free water percentage during maturation is most likely due to the loss of integrity of the Cheddar cheese matrix which facilitates the water diffusion and release. The calcium is replaced by hydrogen under acidic conditions which causes casein breakdown resulting in the increase of meltability of Cheddar cheese (Johnson & Lucey, 2006).

Table III-2 *Thermal properties of Cheddar cheese at different stages of maturation. Total water content is calculated by ΔW1+ ΔW2. Free water ratio (Free water /total water) is calculated by ΔW1 divided by the sum of ΔW1 and ΔW2. Bound water ratio (Bound water/ Total water) is calculated by ΔW2 divided by the sum of ΔW1 and ΔW2.*

Ripening Days	Total Water Content (%)	Free water/Total water (%)	Bound water/Total water (%)
56	38.44±1.19^{a}	25.26±1.95^{d}	74.74±1.95^{a}
90	38.31±0.97^{a}	25.64±1.3^{d}	74.36±1.30^{a}
180	37.98±1.58^{a}	29.34±3.01^{c}	70.66±3.01^{b}
270	35.13±1.22^{bc}	28.11±2.80^{c}	71.89±2.80^{b}
360	36.27±1.32^{b}	36.84±6.34^{b}	63.16±6.34^{c}
450	34.68±2.54^{c}	42.65±4.85^{a}	57.35±4.85^{d}

Each time point represents two measurements on the six batches of the cheese and so is a mean of twelve measurements. Errors represent the standard deviation. Different superscripts in the same column indicate significant statistical difference (Tukey's test P<0.05).

This conclusion from TGA appears superficially to conflict with the TD-NMR data. Definitions of free and bound water from the thermal perspective are in a thermodynamic sense. We use the description "thermodynamic" in a loose sense as a way of distinguishing it from NMR definitions. A fraction of the water can be considered to be thermally transient in that it migrates from the "bound" water state to a "free" water state. Therefore, there can be a constant thermodynamic fraction of water which is nevertheless continually exchanging with water in other states. The water is able to move more freely throughout the cheese structure when heated, for cheese of greater maturity, due to the decrease of the tortuosity of cheese matrix during ripening (Smith *et al.*, 2017). The NMR measurements of bound and free water where they are not compromised by exchange phenomena, are fundamentally different from thermal measurements which in the case of TGA is the determination of the temperatures at which various fractions are released.

III.3.3 Batch to batch variation in the state of water and fat

Variations in the state of water and fat have also been detected in different batches of cheese. Thermal measurements on the state of water using TGA did not detect batch to batch variations. However, TD-NMR was capable of distinguishing the behaviour of different batches. Table III-3 shows the distribution of transverse proton relaxation times T_2 from the water and fat fractions and peak area proportion predominantly from the fat fraction at 56 days after Cheddar cheese manufacture for five different batches of Cheddar cheese. At all stages in the ripening process, the same variation trend among batches was observed, however here we present only data for the 56-day time point.

The T_2 value for the water fraction protons for batch C is significantly lower ($P<0.05$) compared with the other batches. The proton peak area %age predominantly from the fat fraction for batch C is significantly higher ($P<0.05$) than all the others. The T_2 value predominantly from the fat fraction protons for batch C is lower than the other batches but at a lower significance level compared with the previous parameters.

Four batches of Cheddar cheese including batch C were predictively graded at an early stage of maturation to produce graded quality cheese according to the Gilles and Lawrence model. Only batch C however was found to be significantly different in terms of the state of fat and water. The lower T_2 value for the water fraction protons for batch C indicated a tighter binding of the water to the casein matrix resulting in lower the water availability in the entrapped water. Bacteria will therefore be more prone to plasmolysis and cell lysis and therefore less biological

activity will be present (Hickey *et al.*, 2013). This could account for the different values measured in the case of batch C.

Table III-3 *Peak values of T_2 for water and fat fractions taken from the distribution of transverse proton relaxation times. Also shown are peak area proportions for five batches of Cheddar cheese. These are predominantly from the fat fraction at 56 days after Cheddar cheese manufacture which occurred on the same day and in the same factory.*

Batch Number	Proton T_2 (ms) for water fraction	Proton predominantly from fat fraction T_2 (ms)	Proton predominantly from fat fraction peak area proportion (%)
A	29.17 ± 1.19^{b}	205.76 ± 8.38^{ab}	31.93 ± 1.13^{b}
B	30.54 ± 0^{ab}	210.6 ± 8.38^{ab}	29.89 ± 0.30^{c}
C	26.56 ± 0^{c}	187.38 ± 0^{b}	34.09 ± 0.55^{a}
D	30.54 ± 0^{ab}	210.6 ± 8.38^{ab}	29.10 ± 0.05^{c}
E	31.27 ± 1.27^{a}	215.79 ± 15.05^{a}	30.63 ± 0.98^{bc}

Data are the average of 3 measurements on different samples. Errors present the standard deviation of the three values. Different superscripts in the same column indicate significant statistical difference (Tukey's test P<0.05)

III.3.4 Batch to batch variation in sensorial profile and correlation with state of water and fat

A significant batch to batch variation was observed in terms of the state of fat and water at 56 days. As Cheddar cheese matured to 56 days has a bland sensorial profile, the same named batches of Cheddar cheese at 270 days ripening were chosen for analysis and the data presented in this section. Cheddar cheese is commonly sold as mature cheese commercially after 270 days ripening and has a more distinctive sensory character. Although the physical measurements at 270

days could have been chosen, the values at 56 days were used in order to create a link to the predictive properties of the grading and the 270 days sensory profile.

Cluster analysis was carried out to understand the variation among the batches of Cheddar cheese based on sensory attributes. Five batches Cheddar cheese can be distributed in two main clusters (Figure III-3a). One cluster contained only batch C; the other cluster comprised batches A, B, D, and E. Batch C sensorial attributes were characterised by a significantly ($P<0.05$) higher yellow colour and break down but relatively lower cohesiveness and sour and lingering aftertaste (data shown in Supplementary Table 3.1).

Partial least squares regression (PLSR) analysis was used to determine the relationship between fat and water state attributes measured by NMR and sensory attributes. A biplot of two latent variables is presented in Figure III-3c and the Pearson correlation coefficient among all the variables is shown in Supplementary Table 3.2. The T_2 value for water and fat fraction protons were highly positively correlated with cohesiveness, sour, lingering aftertaste and negatively correlated with yellow (all Pearson coefficients higher than 0.9). The peak area %age predominantly from the fat fraction protons behaves in the opposite sense compared with these two attributes in terms of sensorial profile.

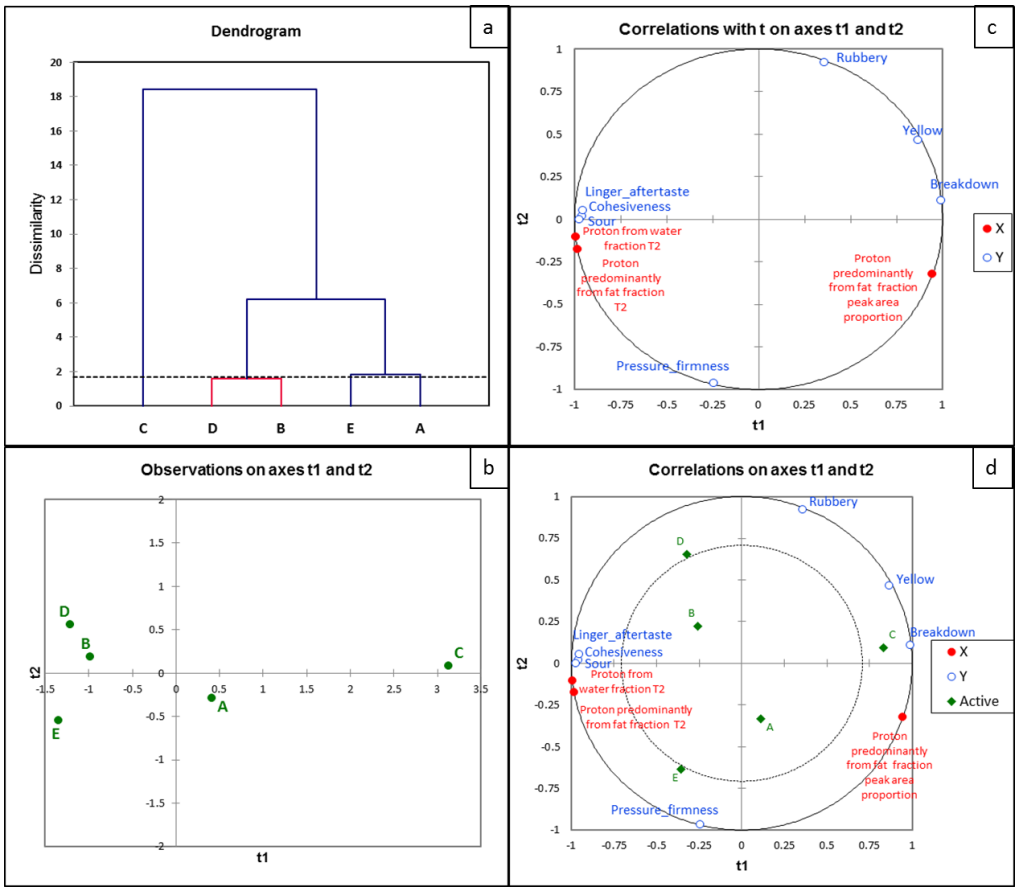


Figure III-3 *(a) Dendrograms representing the five batches of Cheddar cheese. The grouping is based on the 270 days sensory profile. (b) Partial Least Squares observations on axes t1 and t2. The coordinates of the different predictive qualities of Cheddar cheeses in t coordinate space. Batch C is distinct from the other batches (c) Partial Least Squares correlation biplot of sensory attributes evaluated at 270 days and water and fat state attributes measured by TD-NMR for Cheddar cheese of different predictive qualities. (d) Partial Least Squares correlation biplot for cheese batches A, B, C, D, E showing the loadings of sensory descriptors measured at 270 days and water and fat state attributes measured by TD-NMR at 56 days.*

At the same ripening stage, batch C which had a lower T_2 value for protons predominantly from the fat fraction, suggesting that the fat was in a different state, was less prone to dissolve and absorb organic flavour compounds, and so had a less lingering aftertaste due to the influence of flavour release during consumption (Lawlor *et al.*, 2001). Batch C also had a higher colour yellow score which was explained by the diffraction of light in the altered fat fraction of the cheese

(Madsen *et al.*, 1966). Cohesiveness in this sensory evaluation was evaluated by how the cheese formed a ball under finger pressure. The lower cohesiveness of batch C meant that it was harder to make a smooth ball and displayed seams on the ball indicating that the sample exhibited a rubbery and curdy body. The lower proton T_2 value predominantly from the fat fraction in Batch C implied that the fat fraction was more strongly bound to cheese casein matrix resulting in a tighter microstructure of the casein network (Ardo, 1997). This microstructure may result in less susceptibility to deformation and contribute to the rubbery texture of young Cheddar cheese. The sensory profile of batch C is similar to low fat content Cheddar Cheese which has a more intense yellow colour and rubbery body.

III.4 Conclusions

The population with the shorter relaxation time is attributed to water and the longer relaxation component to oil, or oil with a component of modified free serum water. Both transverse relaxation times decrease and the relative peak area predominantly from fat protons and free water increases during Cheddar cheese ripening up to 450 days. The transverse relaxation values for water and fat fraction protons after 56 days ripening were highly positively correlated with cohesiveness, sour and lingering aftertaste and negatively correlated with yellow (related to particle size) sensorial attributes after 270 days ripening. The NMR determined attributes of the fat and water state can be used to monitor Cheddar cheese maturity and predict sensory profile.

Chapter IV The evolution of aqueous extracts of Cheddar cheese during ripening as a potential model for prediction of quality

Abstract

Metabolites in aqueous extracts of Cheddar cheese have been identified by Nuclear Magnetic Resonance. Major differences in metabolic profiles were observed as a function of ripening time among samples graded to attain different quality levels. The ratio of citrulline and arginine to the overall aqueous extract are the most important indices for assessing the ripening; the ratio of both metabolites decreases during ripening. In comparison to the premium batch B cheese, batch C which was predicted to attain a lower quality level, had higher serine and β-galactose as well as lower lactic acid levels and also had a less mature sensorial profile. Tyrosine, tyramine and lysine are highly correlated with mature Cheddar cheese sensory attributes. β-galactose and glycerol are correlated with young Cheddar cheese sensory attributes.

Highlights

- Metabolites of aqueous extracts in cheese during ripening have been identified
- Metabolites responsible for the batch variations in cheese were characterised
- The normalised intensity of citrulline and arginine decreased during maturation
- Tyrosine and lysine are correlated with mature Cheddar cheese sensory attributes
- β-galactose and glycerol are correlated with young cheese sensory attributes

Keywords: Cheddar cheese; NMR; metabolites; Maturation; Sensory evaluation

IV.1 Introduction

Many factors can affect the final quality of Cheddar cheese, such as milk quality, production procedures, and choice of starter culture (Mazzei & Piccolo, 2012; Pisano, Scano, Murgia, Cosentino, & Caboni, 2016). However, some Cheddar cheese defects do not develop, or are only observed, when the cheese is aged. This is due to ripening being a complicated process undergoing many chemical/physical and enzymatic modifications (Consonni & Cagliani, 2008). These biochemical transformations can maximise flavour, taste, and

appearance defects in the samples. The cheese grader must therefore continuously evaluate cheese quality throughout the ageing process. However, Cheddar cheese ripening needs to be performed in a constant temperature and humidly environment, which is a time-consuming and costly process. In Cheddar cheese manufacturing practice, a machine-based predictive grading method is needed to help the manufacturer efficiently manage cheese production and storage. Cheese that has the potential to mature to premium quality needs to be kept longer, whereas other cheese with a lower potential can be sold as low-value young cheese. A grading model using simple initial chemical and physicochemical composition which predicts the quality of Cheddar cheese is still used as an index in Cheddar cheese grading and manufacture. Cheese can be characterised as “premium” or lower quality “graded” cheese. Cheese that fails to meet these levels is referred to as “downgrade” cheese by the professional graders (Gilles *et al.*, 1973).

In order to show the small deviations from normal cheese composition and the resultant quality defects, a metabolomics approach was chosen. Metabolomics produces a fingerprint at a molecular level that accurately represents all aspects of the food product from sensorial taste and flavour to rheological properties (Pisano *et al.*, 2016). All the bio-transformation processes directly or indirectly affect the final metabolome of cheese (Mazzei *et al.*, 2012). In addition, some metabolites formed as a consequence of an undesired fermentation or infection may inhibit bio-reactions (Krause, Bockhardt, & Klostermeyer, 1997). Metabolomics of Cheddar cheese can provide a framework for correlation between the composition and the prediction of

cheese quality during maturation. Water-soluble metabolites in cheese are mainly amino acids, organic acids and carbohydrates. Taste-active compounds contributing to sensations and taste of Cheddar cheese are mostly peptides and free amino acids (Andersen, Ardö, & Bredie, 2010; Lawlor *et al.*, 2001; O'Shea *et al.*, 1996). The acid taste of Swiss cheese for example correlated with the concentration of di-tripeptide and amino acids but not with pH or the concentration of lactic acid (Fox, McSweeney, Cogan, & Guinee, 2016b).

Furthermore, the sensory quality of Parmigiano Reggiano cheese has been suggested to be affected by free amino acids. They contribute indirectly to the cheese flavour by acting as precursors for the production of volatile compounds (Consonni *et al.*, 2008). Free amino acids are hydrolysed from a range of intermediate-sized peptides produced by proteinases and peptidases from the starter lactic acid bacteria. In the cheese matrix, after carbohydrate exhaustion, amino acids are the simplest molecules available for weakly lipolytic bacteria to metabolise to generate adenosine triphosphate (ATP) and so produce compounds that impact flavour (Ganesan *et al.*, 2017).

High resolution nuclear magnetic resonance spectroscopy (NMR) is a method for the structural determination and assignment of major metabolites in cheese (Ruyssen *et al.*, 2013). It is a highly reproducible chemical analysis method, offering in a single experiment, an overview of a wide range of compounds present in the food matrix (Piras *et al.*, 2013). Chemometric methods are commonly used in conjunction with NMR to identify patterns among samples from a large amount of NMR data (Mannina, Sobolev, & Viel,

2012). NMR spectroscopy combined with multivariate chemometric analysis can determine the metabolic profile of intact cheeses such as Mozzarella di Bufala Campana, Parmigiano Reggiano and Emmental cheese and distinguish the geographical origins, ripening and freshness by statistical methods (Consonni *et al.*, 2008; Mazzei *et al.*, 2012; Shintu & Caldarelli, 2005, 2006). NMR allows a thorough analysis of components in the solution extracted from the sample. Unknown and unexpected substances can also be identified (Gianferri, Maioli, Delfini, & Brosio, 2007b).

A substantial body of metabolomic work has been carried out on Parmigiano Reggiano, Mozzarella and Emmental cheese, however, no similar work has been completed on Cheddar cheese. The assignment of ^{1}H spectra of water-soluble cheese extracts in previous studies is not particularly accurate mainly due to the strong overlapping peaks in the water-soluble extracts. To date, there are no studies attempting to predict quality in Cheddar cheese and find correlations between metabolites and sensory variables of cheese. This study investigated the kinetics of ripening in Cheddar cheese batches which were predicted to produce different quality cheeses based on Gilles and Lawrence quality grading model combined with a professional cheese grader grading outcomes. Ripening and sensorial related metabolites markers in cheese aqueous extracts were explored.

IV.2 Materials and methods

IV.2.1 Cheddar Cheese samples

Batch A, B, C, D, E and F cheese samples used for this chapter are the same block as chapter II. Aqueous fractions of cheese were extracted and analysed at various stages during the ripening period namely 56, 90, 180, 270, 360 and 450 days. At each time point, one bag of cheese was removed at random for each batch and placed in a 4 °C refrigerator before measurement. One bag was used for instrumental measurements and the other for descriptive sensory analysis.

IV.2.2 Sample preparation

A chloroform/methanol/water extract for each cheese sample (6 batches at 6 ripening times, n=36) was made in triplicate (36 ×3=108) based on a modified Bligh and Dyer method (Bligh & Dyer, 1959). A 60 mL cold mixed solution was prepared using chloroform and methanol at a volume ratio of 1:2. Cheddar cheese (20 g) was ground in liquid nitrogen with a pestle and mortar and extracted with the cold mixture solution. The suspension was stirred for 2 mins at 4 °C and transferred to a glass tube. The pestle and mortar were rinsed with 20 mL chloroform and the washing solvent combined with the suspension. Distilled water (30 mL) was added to the suspension. After stirring, the suspension was stored in a cold chamber at 4 °C for 40 mins. Phase separation was obtained using a Beckman J2-21 centrifuge with fixed rotor JA-10 at

11700g with the temperature maintained at 4 °C for 30 mins. The supernatant (aqueous phase) was collected. The supernatant was filtered through the filter paper (Whatman, grade1) on a funnel and the aqueous fraction concentrated by vacuum concentration and lyophilisation. The dried sample, containing the hydroalcoholic compounds, was capped and stored at 4 °C (Gianferri *et al.*, 2007b).

Phosphate buffer (ionic strength 25 mM, pH 6.5, in D_2O) containing 0.1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added to weighed samples of water-soluble compounds. The buffer was added by a ratio of 0.028 mL per mg cheese sample. Sample solution (0.6 mL) was then put into an NMR tube.

IV.2.3 High-resolution NMR measurements

NMR experiments were acquired on a Bruker Avance 800 MHz Avance III spectrometer using a 5 mm QCI Cryoprobe. The temperature was set at 25 °C (298.15 K). Proton spectra were acquired using 128 scans of 32K points with a spectral width of 13 ppm. The free induction decays (FID) were multiplied by an exponential weighting function with a line broadening of 0.3 Hz before Fourier transformation.

After phasing and baseline correction, all spectra were referenced to the signal of the added internal standard reference of DSS (0 ppm). All spectra were processed using Bruker Topspin Software. Two-dimensional experiments (1) homo-nuclear ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY) (2) hetero-nuclear

^{1}H-^{13}C (Hetero-nuclear single quantum correlation) HSQC were applied in selected samples to identify spectral components. All two-dimensional experiments were performed at 25 °C (298.15 K) on the same facility as the one-dimensional experiment. HSQC experiments were performed using a spectral width of 11.96 ppm and 200 ppm in F2 and F1 respectively. HSQC spectra were acquired with a time-domain of 1K points (F2) and 256 points (F1) using 8 scans. TOCSY spectra were performed using a spectral width of 15 ppm in both dimensions and 16 scans. Data were compared to the Human Metabolome Database (HMDB: http://www.hmdb.ca/) and literature for further confirmation (Consonni *et al.*, 2008; Piras *et al.*, 2013). The chemical shifts of carbon given by the HSQC spectrum allowed assignment of the spin systems unambiguously by comparison with literature.

IV.2.4 NMR Data Processing

To simplify the statistical analysis, 108 spectra (three replicates per sample) were split into 966 non-overlapping integrated bins of 0.01 ppm width. The spectral range from 4.61 to 4.91 was excluded from integration to avoid interference from residual water. The spectral range from 3.34 to 3.36 was removed from the integration procedure to eliminate variability due to the small amounts of residual methanol. The spectral range due to the DSS internal standard (0.05 to -0.05 ppm) was also excluded. The integrals were normalised to the total area to compensate for the overall concentration differences (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006).

IV.2.5 Sensory analysis

The sensory method and protocol are described in previous work (Chen, MacNaughtan, Jones, Yang, & Foster, 2020). However, a brief outline is given here. All the descriptive sensory analysis data for the six-ripening time points are presented here namely after 56, 90, 180, 270, 360, 450 days ripening. We were only able to use five batches (A, B, C, D, E) of cheese for sensory evaluation at these six ripening time points, but the batch F sensory profile was evaluated with the other five batches after 540 days ripening (data not shown).

IV.2.6 Multivariate analysis

The modulated spectral data matrix (integrals of 6 batch × 6 ripening time point × 3 replicates × 747 bins) was investigated using the Principal Component (PCA) multivariate statistical analysis method embedded in Unscrambler X (CAMO ASA, Trondheim, Norway). All the values of the integrals were mean centre corrected and weighted by dividing by the standard deviation. Some sample replicates were discarded as outliers because they lay outside the ellipse based on the Hotelling' T2 (multivariate t-statistic) corresponding to a 95% confidence limit. A second principal component analysis was performed on having outliers removed. One further PCA was also recalculated using variables whose correlation loading was higher than 70 %.

The quantitative descriptive sensory data for the six ripening time points was analysed firstly by analysis of variance (ANOVA) and all the significantly different sensory attributes were examined by PCA in XLSTAT (Addinsoft,

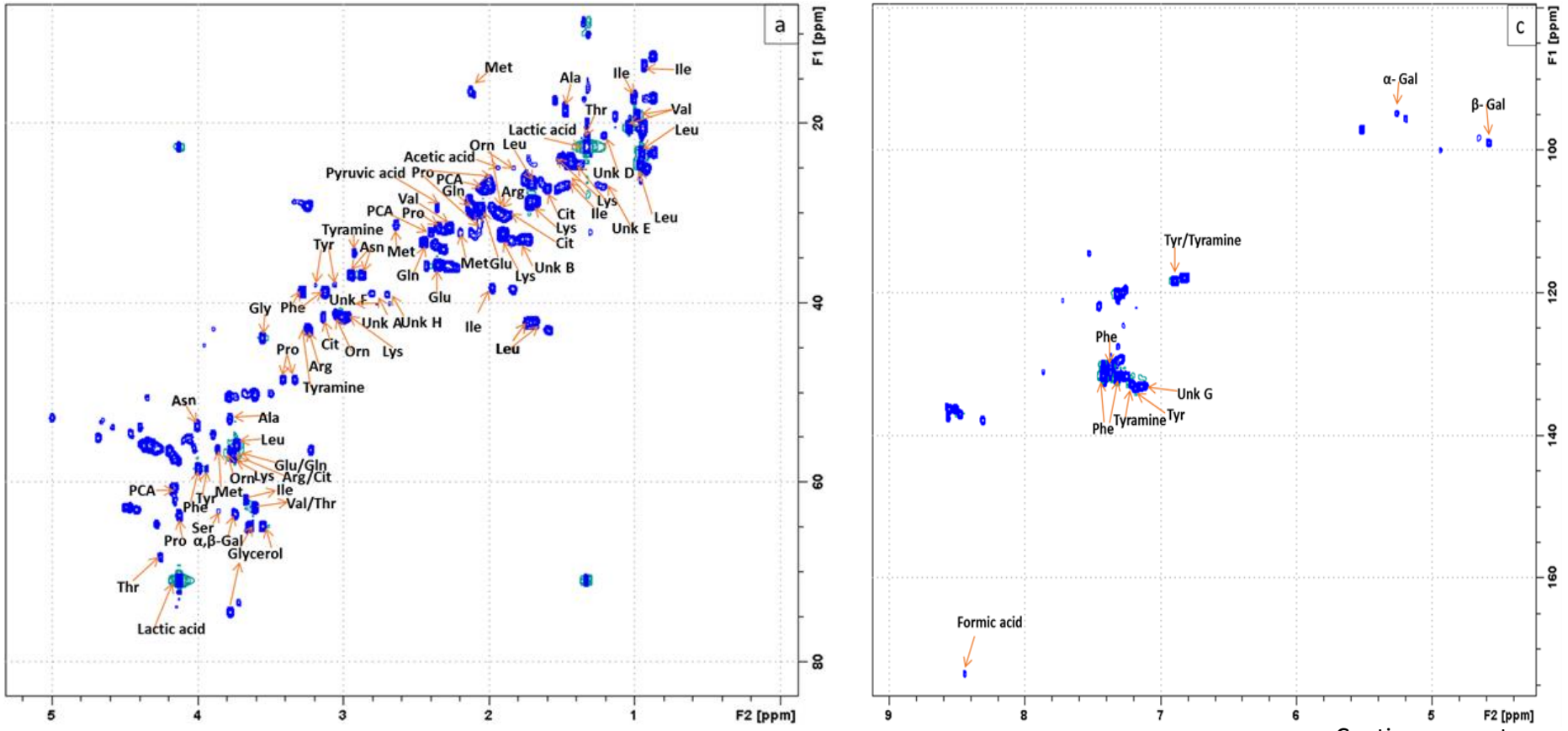
France). For modelling the correlation between the metabolite aqueous extracts with sensory data, Partial Least Squares (PLS) regression was used in Unscrambler X (CAMO ASA, Trondheim, Norway). The X data matrix contained aqueous extract metabolite integral data. The Y matrix contained the results of sensory mean scores for all significantly different cheese attributes. To simplify the PLS analysis, only the four highest associated spectral bins with each sensorial attribute were chosen.

IV.3 Results and discussion

IV.3.1 Metabolite assignments, identification and summary of variance for ripening and batch variation

In Figure IV-1 (a) and (c), two spectral regions of a 2D HSQC spectrum of a water-soluble extract of Cheddar cheese is shown. A representative proton spectrum of one aqueous extract of Cheddar cheese is presented in Figure IV-1 (b) and (d). The ^{1}H NMR spectra of the Cheddar cheese aqueous extracts were assigned from one-dimensional and two-dimensional experiments including HSQC and TOCSY (Figure IV-1). Spectra are dominated by free amino acids in combination with small quantities of organic acids and carbohydrates. In summary, to date, four organic acids, nineteen amino acids, two sugars, one amine and glycerol, have been assigned. Seven resonances involved in maturation have been labelled consistently as unknowns A, B, D, E, F, G, and H. The details of the assignments are shown in Supplementary Table 4.1. As expected, the ^{1}H NMR spectrum reveals the predominance of the lactic acid

resonance signals, but part of the peak is overlapped with a threonine methyl group (Ruyssen *et al.*, 2013).



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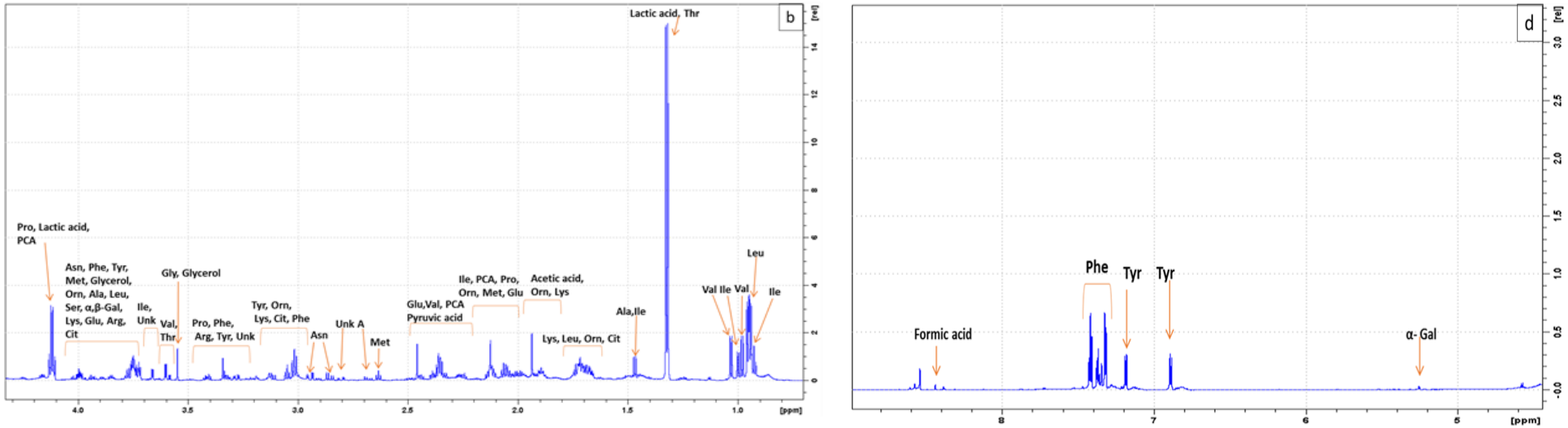


Figure IV-1 *Representative 2D HSQC (a,c) and ^{1}H NMR (b,d) spectra of water-soluble extracts of Cheddar cheese using DSS as a reference showing aliphatic region(a, b) and aromatic (c, d). A range of the most intense peaks are labelled. Some peaks have been omitted for clarity.*

The metabolic trajectories for each batch of cheese are determined by taking the mean position for the ^{1}H spectrum in the PCA score plot (Figure IV-2). The first two principal components of PCA explained 78% of the total variance. From the plot, a separation of samples based on ripening time exists in the direction of the PC1 axis. Batch variation was mainly shown in the PC2 axis direction. The full PC1 and PC2 loading plots for Figure IV-2 are presented in Supplementary Figure 4.1. The PCA plot shows the general trajectories for water-soluble metabolite development during cheese ripening. Cheese ripening trajectories for two batches of cheese are highlighted, namely premium cheese (batch B), and a "downgraded" cheese sample (batch C) (Figure IV-2). The measured data from all six batches are also shown. As ripening proceeds, the cheese sample developed from a positive to negative score along PC1, which is correlated with the changes in metabolites. Batches C and D are initially distinct from the other batches. As ripening progresses batch D gradually merges with the general ripening group, whereas batch C remained on a different trajectory. All the batch C samples had a positive PC2 value, during maturation. Batch B samples in most of the ripening time points were in the lowest score region along the PC2 axis. Batch E, a 'downgraded' sample behaved differently from other batches exhibiting an earlier development at 180 days ripening. The differences between batch E and other samples can be seen along the PC1 axis.

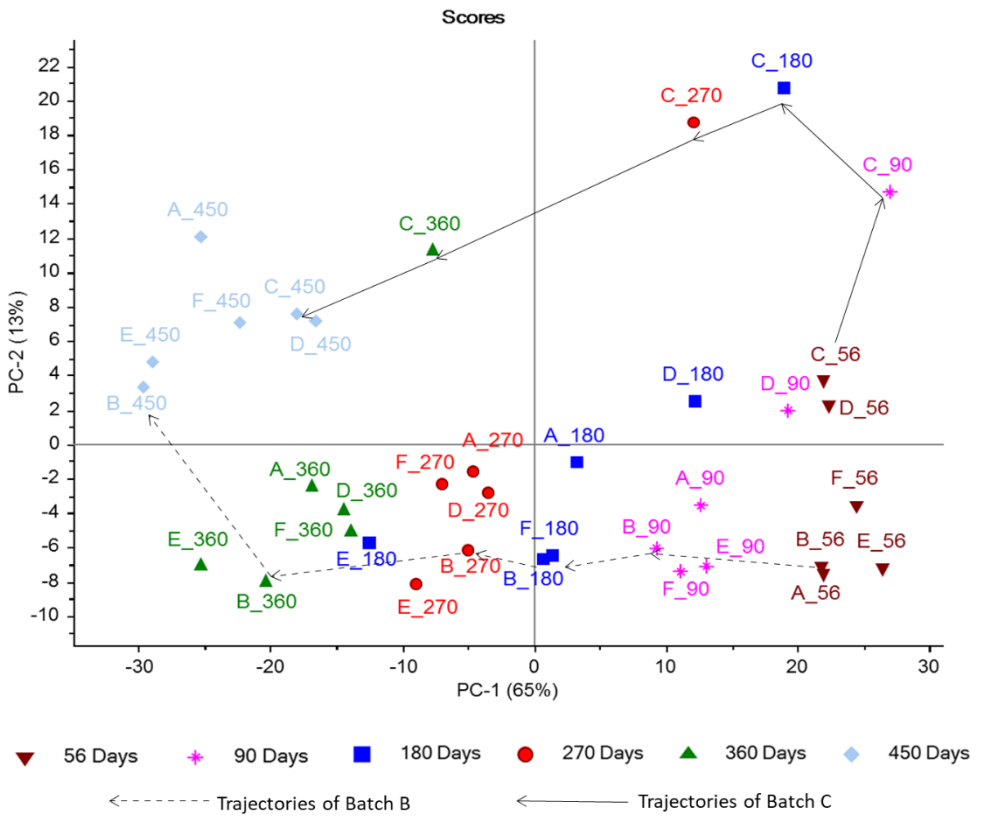
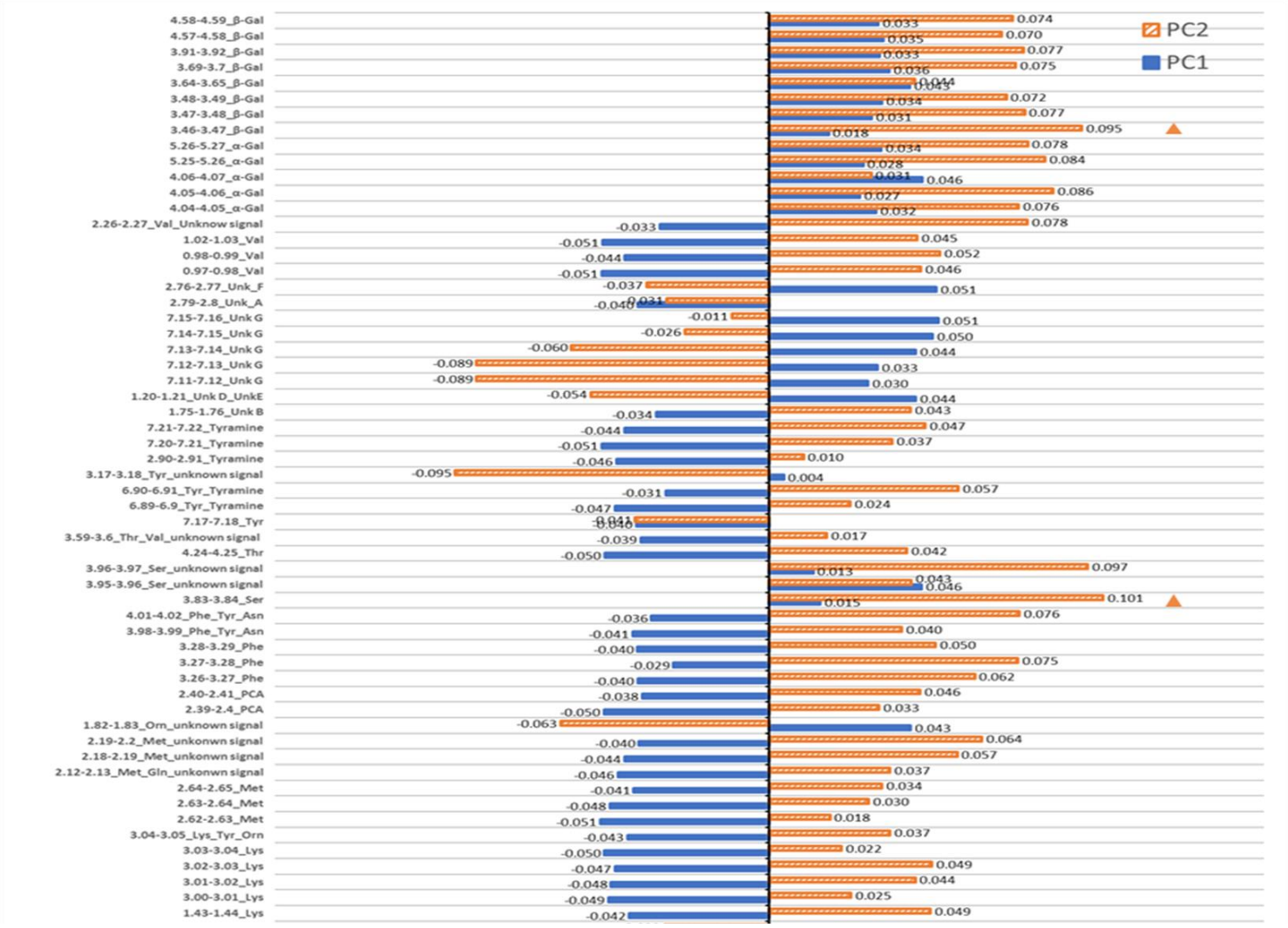


Figure IV-2 *The score plot of the PCA obtained from the mean of each ripening time point for batches A-F. The PCA metabolic trajectory plot maps the average position of the ^{1}H NMR spectra of aqueous extracts for each ripening time. The symbols indicate the batch number and ripening time in days e.g. E-270 is batch E at 270 days.*

In Figure IV-2 at the early stage of ripening, all the batches of cheese were grouped loosely. As ripening proceeded sample C approached the general group of Cheddar cheese samples at a late stage of ripening. A similar phenomenon has been reported in that at an early stage of maturation, the rating of Cheddar cheese flavour and mouth coating character were associated with the composition of cheese and the associations weakened as the cheese matured (Muir *et al.*, 1995). Piras *et al.* (2013) also reported that Fiore Sardo

cheese aqueous extracts that are treated with different adjunct bacterial species became more similar with ripening.

NMR spectra have given a comprehensive picture of the aqueous fraction composition and have enabled the discrimination between samples. The significant loading coefficients for the chemical shift bins from the PCA plot in Figure IV-2 are presented in Figure IV-3. This provides an indication of the extent to which metabolites have changed during ripening and enables discrimination between batches. Not all the bins associated with the same compounds are significant. This is due to overlap and the presence of different components in same bins. The larger the absolute number of coefficient loading, the more important the contribution of the variable to the explanation of the variance. Citrulline (bin from 1.59-1.60 ppm) and arginine (bin from 3.24-3.25ppm) have the most significant contributions to principal component 1 (PC 1). Figure IV-4 (a and b) shows that the normalised intensity of the citrulline (bin from 1.59-1.60 ppm) and arginine (bin from 3.24-3.25ppm) decrease during ripening. Batches A, D and F are grouped together and are compared with individual batches B, C and E in Figure IV-4. Batches A, D and F had similar metabolite development with ageing and the overall batch variance of the group was similar to the other individual batches.



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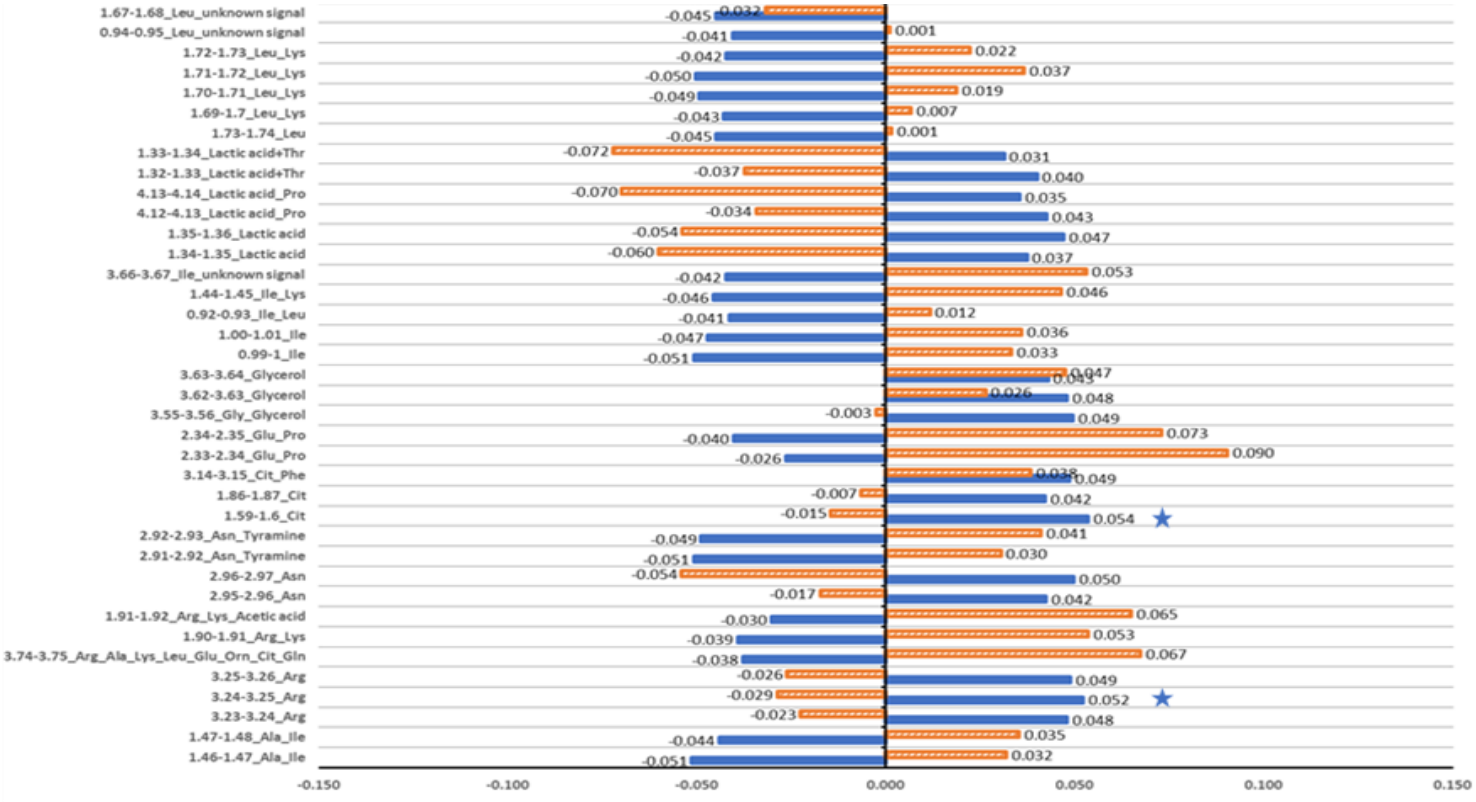


*Key to Compound identification: Ile, Isoleucine; Leu, Leucine; Val, Valine; Thr, Threonine; Lys, Lysine; Ala, Alanine; Cit, Citrulline; Arg, Arginine; Met, Methionine; Glu, Glutamic acid; Pro, Proline; PCA, Pyroglutamic acid; Asn, Asparagine; Tyr, Tyrosine; Phe, Phenylalanine; Gly, Glycine; Ser, Serine; α-Gal, α-Galactose; β-Gal, β- Galactose;

Figure IV-3 *Loading coefficients for the chemical shift intervals from the PCA plot shown on Figure IV-2, comparing different ages and batch variations. The Y-axis is the bin interval with corresponding assignments. The four individual metabolite bins which have the highest loading coefficient in PC1 and PC2 and which discriminate the ripening and batch variation are labelled with star and triangle symbols respectively.*

The chemical shift regions having positive and negative contributions to PC1 are outlined in Supplementary Table 4.3. Arginine, asparagine, citrulline, glycerol, lactic acid, serine, unknown G, α-galactose, β-galactose, glycine and glycerol (bin at 3.55-3.56 ppm), lactic acid and proline (bins at 4.12-4.14 ppm) and lactic acid and threonine (bins at 1.32-1.34 ppm) all have positive contributions to PC1. As ripening proceeds, all the compounds that positively contribute to the PC1 decrease in the Cheddar cheese aqueous extract ratio. Mature Cheddar cheese had a greater content of isoleucine, leucine, lysine, methionine, pyroglutamic acid, phenylalanine, threonine, tyramine, and valine in the Cheddar cheese aqueous extract. Some bins associated with PC1 are not discussed since they are associated with multiple metabolites.

For batch variation, serine (bin at 3.83-3.84 ppm), tyrosine and unknown (bin at 3.17-3.18 ppm) and β-form galactose (bin at 3.46-3.47 ppm) are the most important metabolites. They have the largest contributions to the loadings. Figure IV-4 (c and d) displays the normalised intensity of the serine (bin from 3.83-3.84 ppm) and β-galactose (bin at 3.46-3.47 ppm) among all batches of cheese during ripening. Batch C has a significantly higher amount of both metabolites than the other batches. Batch C was characterized by a smaller amount of lactic acid (bin at 1.34-1.3 6 ppm) and related bins (4.12-4.14 ppm and 1.32-1.34 ppm), asparagine (Asn, bin 2.95-2.97 ppm), arginine (Arg, bin 3.23-3.26 ppm), leucine (Leu, bin at 1.73-1.74 ppm) and tyrosine (Tyr, bin at 3.17-3.18 ppm) and unknowns G, A and F.

Other compounds which are detected in Fiore Sardo cheese by Piras *et al.* (2013) but which have not been observed in these Cheddar cheese spectra are citric acid, succinic acid and glucose. This could be due to the sensitivity limitation of NMR and the abundance of other compounds. Pyruvic acid was identified but was highly overlapped with other metabolites. Formic acid was also observed in aqueous extract spectra but was not significantly discriminating for maturation or predictive quality. There are some compounds which were observed and confirmed but had no distinct non-overlapping bin integrals such as alanine.

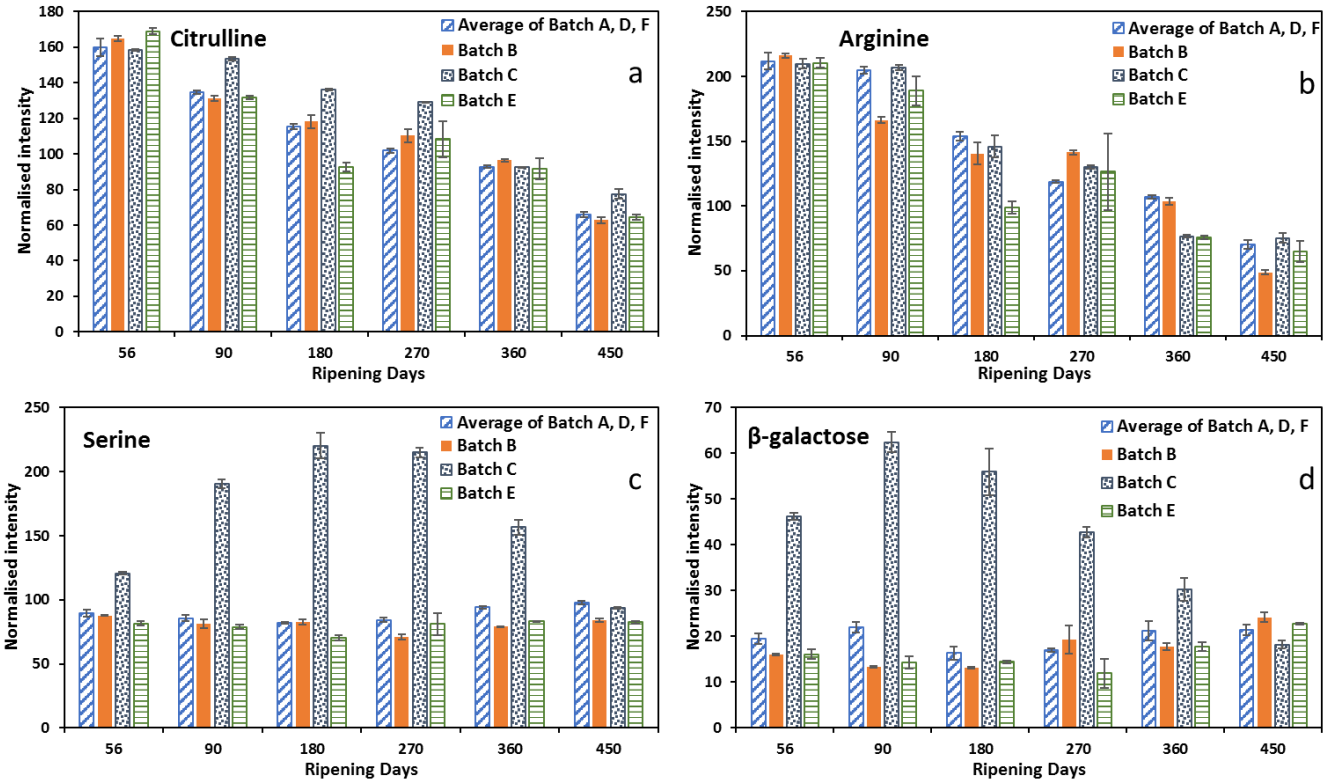


Figure IV-4 *The most significant metabolites that change during the ripening process and the variation in different batches: (a) citrulline bin at 1.59-1.60 ppm (b) arginine bin at 3.24-3.25 ppm (c) serine bin at 3.83-3.84 ppm (d) β-galactose bin at 3.46-3.47 ppm. The displayed values are the mean of the normalised intensity of the metabolite. Error bars are the standard deviation of three replicates.*

IV.3.2 The Evolution of metabolites during maturation

From the literature, the absolute free amino acid content in cheese increases with age (Dacre, 1953). However, in the present work we present the ratio of metabolites in the aqueous extract i.e. the metabolite ratio of all the metabolites in the cheese aqueous extract relative to the overall proton content. As ripening proceeds, the lactic acid (ratio) in the aqueous extract decreases. Previous studies show a decline in lactic acid during ripening (Ganesan *et al.*, 2017; Piras *et al.*, 2013). Lactic acid produced by starter lactic acid bacteria was also consumed by the nonstarter microbiota and by the indigenous cheese microbiota (Eliskases-Lechner, Ginzinger, Rohm, & Tschager, 1999). The reduction in lactic acid ratio in our study is probably due to the decrease of lactic acid but could also be due to the increase in the content of other water-soluble metabolites.

The galactose ratio in the aqueous extract, including α and β forms decreased during ripening. Galactose is a constituent monosaccharide of lactose whereas neither α or β forms of lactose were detected in spectra even at the earliest time of 56 days, which is consistent with the observation in mozzarella and Fiore Sardo cheese (Mazzei *et al.*, 2012; Piras *et al.*, 2013). The rapid decrease of the carbohydrate content during the ripening process is attributed to the metabolism of monosaccharides by homofermentative starter lactic acid bacteria (Piras *et al.*, 2013).

The glycerol ratio of aqueous extracts decreased during maturation. Enzymatic hydrolysis of triglycerides produces fatty acids and glycerol. Glycerol can enter the glycolysis pathway as a carbon source for the growth of lactic acid bacteria (Hatti-Kaul, Chen, Dishisha, & Enshasy, 2018; Mcsweeney & Sousa, 2000). The decrease of glycerol ratio in the aqueous extracts could be due to the extensive depletion of glycerol when used as a carbon source.

Amino acids levels increase during maturation. Increased levels of valine, threonine, phenylalanine, pyroglutamic acid, methionine, lysine, leucine, and isoleucine have been detected. During maturation, it was also observed that some individual amino acid levels reduced, including citrulline, asparagine, tyrosine, arginine and serine. This is consistent with the Parmigiano Reggiano cheese metabolic profile where threonine and methionine are abundant at 18 months and 30 months but tyrosine had a lower content at longer ripening times (Consonni *et al.*, 2008; Shintu *et al.*, 2005).

Citrulline and arginine are the most critical metabolites for monitoring ripening based on the loading plot (Figure IV-3). During ripening the arginine in the total water-soluble extract decreased. This amino acid is closely correlated with maturation, at least in part because starter lactococci are capable of shifting metabolism from sugar to arginine, which is then the first amino acid metabolised for energy (Ganesan *et al.*, 2017). Arginine is hydrolysed to NH_3 and citrulline with the production of ATP (Fox, McSweeney, Guinee, & Cogan, 2000b). Citrulline is an intermediate of the arginine deiminase (ADI) pathway which provides energy in the form of ATP for lactic acid bacteria metabolic

activity. Citrulline is converted from arginine and further metabolised to ornithine (Wenzel *et al.*, 2018). Arginine and citrulline consequently follow the same trend during maturation.

During ripening, the methionine ratio in the aqueous extracts increased. Sulfur fixation may account for the increase in methionine during cheese ageing. The increase in methionine during ageing is accompanied by an increase in the absolute concentration of its precursor amino acid, serine and we observed the serine ratio in most of batches levelled off (Stuart, Chou, & Weimer, 1999). Serine is produced from 3-phosphoglycerate via glycine by lactic acid bacteria (Ganesan *et al.*, 2017; Stuart *et al.*, 1999).

Asparagine is one of the amino acids whose ratio to the overall metabolite content decreases during ripening. Asparagine produces acetic acid and propionic acid via aspartate during the metabolism of lactic acid bacteria and provides oxaloacetate for the production of diacetyl and acetaldehyde (Ganesan, Seefeldt, Koka, Dias, & Weimer, 2004). The increase of acetic acid and propionic acid during the ripening process (unpublished data measured by Gas Chromatography-Mass Spectrometer (GC-MS), data not shown) may account for the decrease of asparagine ratio of aqueous extract.

Glutamic acid and proline levels in the aqueous extracts increased throughout the ripening process. This is an agreement with a previous study whose amino acid content was evaluated by gas-liquid chromatography (Laleye, Simard, Gosselin, Lee, & Giroux, 1987). Proline is a biosynthetic product from glutamic acid and arginine metabolism (Ganesan *et al.*, 2017). Glutamic acid participates

in more than 150 metabolic pathways in lactic acid bacteria (Ganesan *et al.*, 2017). There are therefore no straightforward explanations for the increase in the intensity of glutamic and proline bins.

IV.3.3 Batch to batch variation among different predictive quality Cheddar cheese

Based on the Gilles and Lawrence grading model, batches A, C, D, E and F were predicted to be "graded" quality Cheddar cheese with defects in various characteristics. Aqueous extracts of Cheddar cheese show that batches C and E are different from the other batches. The batch variation among different predictive quality Cheddar cheese from the same dairy can be characterized by NMR analysis of aqueous extracts. The variance is much smaller than cheese from different origins or processing treatments, however these batch and run variations are crucial to grading in the cheese manufacturing industry.

NMR signals are more sensitive to cheese ripening than Cheddar cheese batch variations. For batch variation, principal component 2 (Figure IV-3) loading of bins at 1.32-1.34 ppm and 4.12-4.14 ppm which are both dominated by lactic acid and bin 1.34-1.36 ppm which only contain lactic acid resonances are all much lower in batch C. This suggests that batch C has less lactic acid. Lactic acid is produced by lactic acid bacteria and can be metabolised to CO_2 and water by specific yeast and fungi (Ozturkoglu-Budak *et al.*, 2016). The production of lactic acid from residual lactose depends on the percentage of salt in moisture

S/M, rather than residual lactose (Shakeel Ur, Waldron, & Fox, 2004; Upreti, McKay, & Metzger, 2006b). The percentage of salt in moisture content of batch C is 5.86%, which is 12.8% higher than the average of all batches. It was observed that lactose was not depleted in Cheddar cheese having a high salt concentration which has lower levels lactic acid compared with other cheeses (Guinee *et al.*, 2008; Møller *et al.*, 2013).

All forms of galactose were at lower levels in the aqueous extracts of premium batch B and higher levels in the downgraded batch C. The presence of fermentable carbohydrates may lead to the development of an undesirable secondary flora (McSweeney *et al.*, 2017). The decrease of residual sugar content observed after 15 days of ripening is typical of a secondary fermentation due to the activity of non-starter lactic acid bacteria (Piras *et al.*, 2013). This result is consistent with the lactic acid ratio for batch C, which was lower lactic acid but had a higher carbohydrate level. This indicates that batch C lactose metabolism to lactic acid was not complete and probably had an undesirable secondary flora. Premium Cheddar batch B tend had a higher lactic acid ratio in the aqueous extract rather than carbohydrate. Mazzei *et al.* (2012) found that compared to a good quality Mozzarella cheese whose quality is officially recognised by the Italian Ministry of Agricultural and Forestry Policies (MIPAF), a commercial sample of Mozzarella bought from the local supermarket had a lower content of β-galactose as well as a higher acetic acid content (acetic acid data is shown in chapter V). This is the opposite to the results reported here, which could be due to the different cheese types having different quality criteria.

Most amino acid levels are higher in the predicted low-quality batch C cheese, except for arginine, asparagine, citrulline, and tyrosine. This could be due to the higher salt in moisture content. Higher levels of casein components and free amino acids have previously been observed in Cheddar cheese containing higher sodium chloride levels (Møller *et al.*, 2013).

Predicted low-quality batch C is also lower in arginine than the other samples. It was reported that arginine content was significantly lower in late gassing than in grade one Cheddar cheese (Laleye *et al.*, 1987). Arginine in off flavour Emmental cheese was found to be significantly lower than regular flavour samples and may serve as an indicator of anaerobic contaminating microorganisms (Krause *et al.*, 1997). The intracellular pH regulation and energy production role of arginine may indirectly link to the cheese flavour (Ganesan *et al.*, 2017).

Asparagine is lower in batch C and higher in premium quality sample batch B. Asparagine is related to the sour taste of cheese, as it is a precursor to acetic acid and propionic acid production by lactic acid bacteria (Fox *et al.*, 2000b; Ganesan *et al.*, 2004). Batch C has a significantly lower acetic acid level and is significantly less sour than other samples during maturation which is consistent with the aqueous extract result in that one of the acetic acid pathway precursors is much lower in batch C (sensorial sour attributes data shows in next session and acetic acid data in chapter V). Asparagine in off flavour Emmental cheese is significantly lower than regular flavour samples (Krause *et al.*, 1997).

Serine is highly significant with regard to batch quality variation (Figure IV-4(c)). The serine ratio level of the aqueous extract is higher in batch C predicted low-quality cheese and lower in batch B premium type cheese. The higher the serine content of aqueous extract, the more methionine biosynthesis will occur and therefore more sulphur volatile compounds will be generated. Sulphur volatile compounds are neither desirable nor typical for this type of Cheddar cheese.

IV.4 Sensory profile for batches of Cheddar cheese and correlation with metabolites

The PCA plot on sensory attributes depicted in Figure IV-5(a). PC1 mainly separates sensory attributes from young to mature cheese. As expected, 56 days ripening Cheddar cheese associated with rubbery, buttery, dairy odour, oily, yellow sensorial attributes. Mature Cheddar cheese has higher sour, tangy, umami, astringent and lingering aftertaste as well as being sweaty and crumbly. However, batch C matures noticeably slower than other batches. Most of the batch C sample points are grouped into the previous ripening time point for the other samples. Only at the last ripening time point, namely ripening after 450 days, are all the cheese samples grouped together. Batch C has significantly less overall flavour intensity compared with the others until the 450 days ripening time point. At comparable ripening times batch C is lower in all the mature cheese sensory attributes compared with the other samples. This is probably due to the low lactic acid environment in batch C which causes the lactobacilli

to develop later and generate a lower overall rate of proteolysis (Moynihan *et al.*, 2016). Moreover, lactic acid was recognised as key taste driver in mature Cheddar (Møller *et al.*, 2013) and the overall flavour intensity was associated positively with the extent of protein breakdown (Banks *et al.*, 1992). This is probably the reason for the bland flavour of the batch C sample.

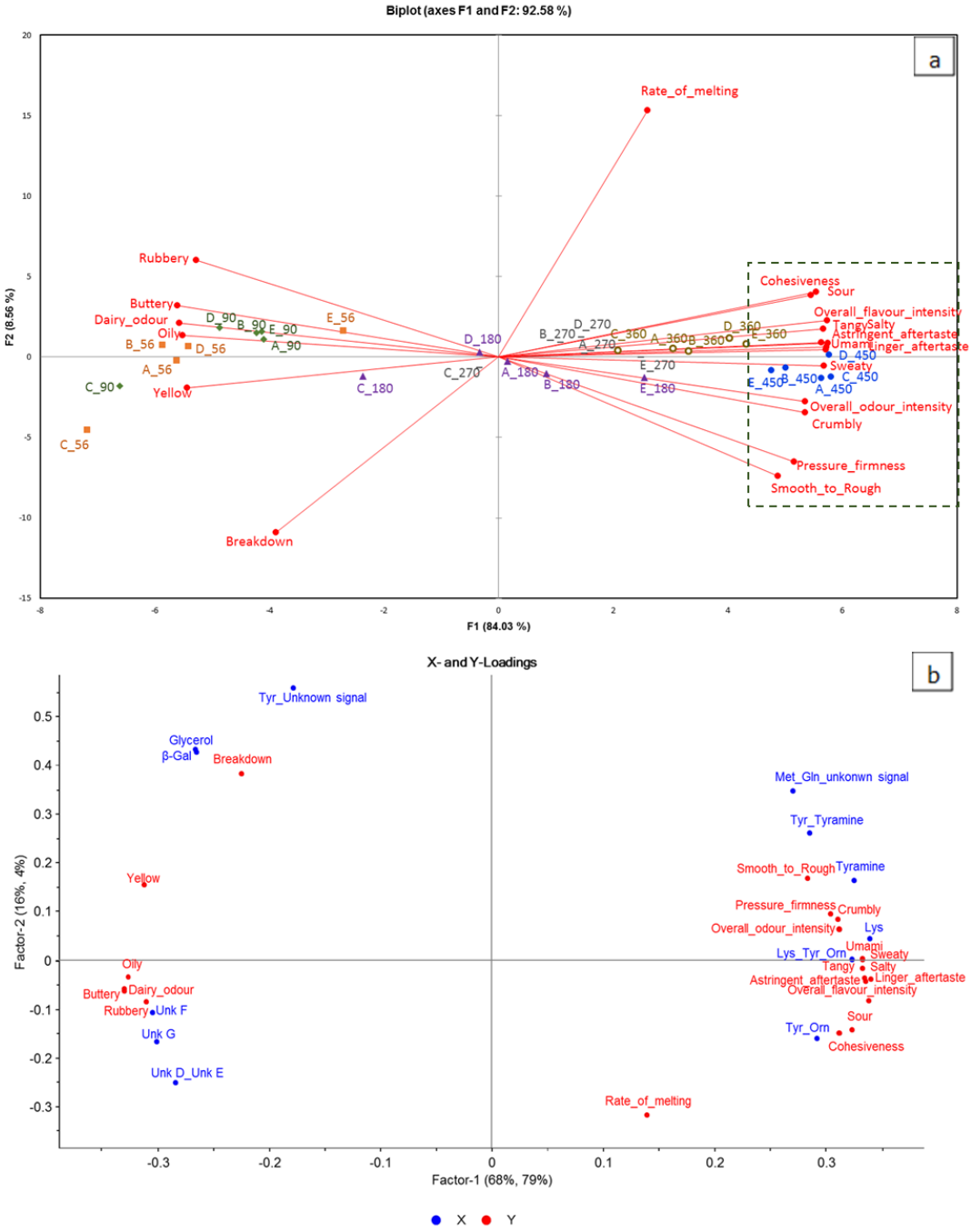


Figure IV-5 *(a) PCA bi-plot carried out on sensory attributes by the trained panel. All the sample points are labelled as batch number and ripening time in days. Symbols and colours of the samples indicate different ripening times: 56 days ripening (□; orange), 90 days ripening (◊; green), 180 days ripening (Δ; purple), 270 days ripening (–; grey), 360 days ripening (o; yellow),450 days (●; blue). All the sample points are the mean of three replicates ×10 panel evaluations. The sensory attributes that correlated with mature Cheddar cheese attributes are in the dashed box (b) Partial Least Squares correlation biplot of sensory attributes evaluated at all ripening days and most relevant metabolites of the Cheddar cheese aqueous extract spectra that explain the most variance of the sensory profile.*

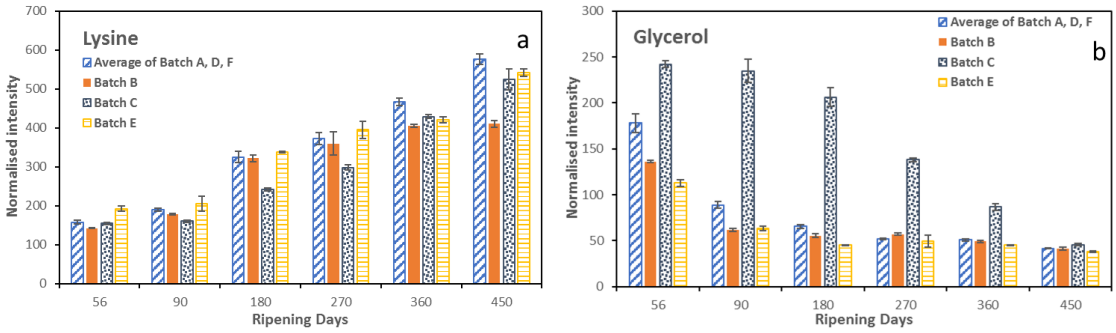


Figure IV-6 *The most important individual metabolites that are correlated with sensory attributes: (a) Lysine bin at 3.03-3.04 ppm (b) Glycerol bin at 3.63-3.64 ppm. The displayed values are the mean of the normalised intensity of the metabolite. Error bars are the standard deviation of three replicates.*

The PLS correlation loading plot shows the correlation between sensory attributes and metabolites. The younger cheese sensorial attributes are associated with β-galactose, glycerol and some unknown compounds (Figure IV-5 (b)). In Figure IV-6, the normalised glycerol and lysine intensity of batch C lags behind the general ripening trend until the later stage of ripening. Tyrosine, tyramine and lysine are highly correlated with more mature sensory attributes. Lysine is catabolized to fatty acids through α-keto acid catabolism which are associated with a mature cheese flavour (Ganesan *et al.*, 2017). Tyrosine is an aromatic amino acid. The amino carbon is transaminated to produce aromatic pyruvate which is further reduced to an aromatic acid, aldehyde or alcohol (Ganesan *et al.*, 2017). All of these flavour compounds are found in mature Cheddar cheese. Tyramine is also an amine derived from tyrosine. Carbon sources such as glycerol and galactose are more likely to be present in less mature Cheddar cheese, as during the course of ripening, they

are depleted. Some metabolites such as serine are highly significant in discriminating between batches but are not highly correlated with sensory attributes because they are not directly or indirectly taste active in the Cheddar cheese.

IV.5 Conclusions

The results have shown that high field NMR spectroscopy can characterise the metabolic profile of Cheddar cheese during maturation and statistically distinguish between Cheddar cheese destined to attain different quality levels. Amino acids and organic acids were the most relevant compounds characterizing the Cheddar cheese aqueous extract samples. The ratio of citrulline and the ratio of arginine in aqueous extracts are the most important index for assessing the ripening of Cheddar cheese. Both of these metabolites in the aqueous extracts decrease during maturation. In comparison to the premium batch B, NMR spectra of batch C revealed a higher level of serine and β-galactose as well as a lower amount of lactic acid in the aqueous extract. This gave batch C a less mature sensorial profile than other batches. Tyrosine, tyramine and lysine are highly correlated with more mature Cheddar cheese sensory attributes whereas β-galactose and glycerol are correlated with young Cheddar cheese sensory attributes. All the metabolites outlined can be used for ripening and sensorial quality control in Cheddar cheese manufacture. The metabolites profile in aqueous extract of Cheddar cheese only significantly discriminate the low-quality batch C to other batches. Due to the complex of

the ripening process, batch E which is another lower quality Cheddar cheese from sensory profile need to be further explored from other physiochemical perspectives.

Chapter V Evolution of volatile compounds from different batches Cheddar cheese during ripening.

Abstract

The volatile profile of six Cheddar cheese samples with different predictive grading qualities based on the Gilles and Lawrence grading model and a professional grader were studied at six stages of ripening (56, 90, 180, 270, 360, 450 days) by solid-phase microextraction gas chromatography-mass spectrometry to characterise aroma compounds and batch variation. Premium Cheddar cheese has higher acetoin and lower branch chain alcohols in the early stages of ripening. Downgrade batch C, which has significantly lower acetic acid and secondary alcohols in the early stages of ripening matures to have higher ethyl esters in the late stages. Another downgrade batch E, which has a higher amount of secondary alcohols in the early stages, matures to be higher in propyl esters in the late stages. Caproic acid, valeric acid and octanoic acid are highly correlated with sensory attributes of Cheddar cheese.

Highlight

- Aroma measurements during ripening of Cheddar cheese complement the Gilles and Lawrence Cheddar cheese quality predictive grading model.
- The trajectory of different predictive qualities of Cheddar cheese during ripening was presented.
- The higher amount of secondary alcohols and propyl esters in the early stage of ripening can be considered as a sign of off-flavour.
- The lower amount of acetic acid and secondary alcohols in the early stage of ripening can be considered as a defect sign.
- Caproic acid, valeric acid and octanoic acid are highly correlated with sensory attributes of Cheddar cheese.

V.1 Introduction

V.1.1 Cheese aroma formation during maturation

Cheese flavour is one of the most important criteria determining consumer choice and acceptance (Delgado, González-Crespo, Cava, García-Parra, & Ramírez, 2010). The unique aroma profile of each cheese is the result of a complex balance of volatile chemical compounds originating from fat, protein and carbohydrate and changes dramatically during ripening (Bintsis *et al.*, 2004; Fox & Wallace, 1997). Ozturkoglu-Budak *et al.* (2016) reported that a longer ripening process could even overrule production site differences. The

changes which occur in the concentration of most volatiles during ripening reflect biochemical changes that are a direct reflection of flavour generation (Urbach, 1993). Cheddar cheese aroma can be generated through several bio-reactions, namely glycolysis, lipolysis and proteolysis. The most important one is amino acid catabolism for Cheddar cheese, which is part of the proteolysis process. The conversion of amino acids to volatile end products takes place in bacteria, in which amino acids are available for physiological maintenance (Ganesan *et al.*, 2017). Lipolysis results in the formation of free fatty acids, which can be the precursors of aroma compounds, such as methyl ketones, alcohols and esters, with low aroma thresholds (Delgado *et al.*, 2010; Van Leuven *et al.*, 2008). Lipolysis is mainly due to mould activity, and much less to lactic acid bacteria (LAB) activity (Kranenburg *et al.*, 2002).

V.1.2 Flavour defects

It is believed that typical cheese flavour results from the blending of a variety of specific individual substances in adequate proportions (Califano & Bevilacqua, 1999). Some extreme higher or lower amounts of flavour compounds can cause flavour defects, which occur during ripening. Urbach (1993) stressed that cheeses from commercial production with flavour defects can be described as soapy, eggy, oniony and fruity, and were found to contain "out of balance" concentrations of fatty acids, hydrogen sulphide, methanethiol and ethyl esters, respectively (Bills, Morgan, Libbey, & Day, 1965; Urbach, 1993). Furthermore, the usual flavour defect of low-fat cheese is burnt/rosy flavour, bitterness, astringency and unclean flavour and a lower

intensity of typical cheese taste and aroma. The phenylethanal was detected as a likely source of rosy off flavour and furanone compounds as well as 1-octen-3-one are likely to be the source of the burnt off-flavour (Drake, Miracle, & McMahon, 2010; Kondyli *et al.*, 2002).

V.1.3 Aroma analysis and correlation with sensory profiles

Most of the techniques for analysing the aroma of cheese are based on mass spectrometry (MS), which is usually combined with a gas chromatography (GC) (Careri, Bianchi, & Corradini, 2002). Solid-phase microextraction (SPME) is the most widely used technique for aroma extraction with only small sample amounts required. The volatile profile of the Spanish soft cheese of the Protected Designation of Origin (PDO) Torta del Casar, made from raw ewes' milk, in four different stages of ripening was studied by the method of SPME–GC–MS by Delgado *et al.* (2010). They found the greater importance of carboxylic acids, with their origin in microbial activity and amino acid degradation than those from lipolysis, are a unique characteristic for aroma of PDO Torta del Casar soft cheese and which could differentiate its aroma from other cheese on the market. GC-MS was also used to investigate the complex mix of volatile compounds in different maturities of commercial Cheddar cheese. 70% success rate in correct prediction of the age of the cheeses based on key headspace volatile profiles has been achieved (Gan *et al.*, 2016). However, GC-MS instrumental analysis of aroma profile cannot mimic human perception, since the aroma compounds' detection threshold and interaction with food matrix need to be taken into account. The same aroma compounds

were detected in full-fat, reduced-fat and low-fat cheese, but the sensory flavour differences were observed, due to the differences in compound concentration as well as differences that influence compound release (Drake *et al.*, 2010).

V.1.4 Gilles and Lawrence's quality prediction model

The quality of the cheese is influenced by the composition, especially moisture content, sodium chloride concentration, pH, fat content (Fox *et al.*, 2017a; Kondyli *et al.*, 2002). A grading scheme model using simple chemical and physicochemical components, namely pH, the percentage of salt in moisture, fat in the water-free substance and moisture in the fat-free substance is still used as indices in Cheddar cheese grading (Gilles *et al.*, 1973). The quality prediction model informs the manufacturer that a cheese in question is suitable for extended ripening for premium Cheddar cheese or must be sold quickly as young cheese.

V.1.5 Aim and hypothesis

So far, no studies have been published on the development of Cheddar cheese aroma profile during ripening of cheeses of different predictive quality, based on Gilles and Lawrence model and professional graders. The investigation of aroma profiles of different predictive quality can improve the quality control and grading prediction of Cheddar cheese during ripening. The main objective of this study is to understand the mechanism behind the formation of aroma compounds during ripening (up to 450 days) of cheeses with different

predictive quality, to confirm or deny and/or complement such predictive capabilities. The variation of volatile compound generation from the different cheese batches is explored, which could be an additional aid for professional cheese grading by using these supplementary attributes at the early stage of ripening. The correlation with sensory attributes and aroma profiles measured by GC-MS is studied in order to characterise the volatile compounds that have the most effect on the sensory perception of a Cheddar cheese matrix. We hypothesise that the batches of Cheddar cheese with different predictive quality would have different volatile profiles. Some predictive poor-quality Cheddar cheese could probably have aroma defects. What is more, aroma defects which occurred in the late ripening stages can be predicted based on the early ripening stage aroma compounds.

V.2 Materials and methods

V.2.1 Cheese samples

Batch A, B, C, D, E and F cheese samples used for this chapter are the same block as chapter II. At each time point, two bags of cheese were removed at random from each block and placed in a 4 °C refrigerator before measurements. One bag was used for instrumental measurements (aroma profile analysis measured by GC-MS) and the other for descriptive sensory analysis.

Vintage commercial Cheddar cheese samples from the same factory were delivered at the same time for preliminary aroma analysis as vintage Cheddar cheese has abundant aroma compounds. They were placed in a 4 °C refrigerator before testing.

A box of medium Cheddar cheese from the same dairy was obtained as a reference to check machine stability in every ripening time point and stored in a -80 °C freezer until the night before measurement and placed in a 4 °C refrigerator before testing (data was presented in Supplementary Figure 5.1).

V.2.2 SPME GC-MS

In most of cheese SPME aroma analysis studies, 25% monosodium phosphate solution is used during incubation using the method of Lee *et al.* (2003) (Delgado *et al.*, 2010; Delgado, González-Crespo, Cava, & Ramírez, 2011a; Lee *et al.*, 2003). However, the optimisation of salt concentration was studied on sodium chloride solution rather than the best salt type monosodium phosphate solution Lee *et al.* (2003). Therefore, it is important to optimise the monosodium phosphate solution concentration in order to apply the method more effectively. Monosodium phosphate solution can not only decrease the solubility of hydrophobic compounds due to the so-called “salting out” effect, but can also solubilise the coagulated milk proteins and loosen the cheese matrix which can further increase sensitivity (Buchholz & Pawliszyn, 1994; Lee *et al.*, 2003). The optimisation was performed by adding sodium phosphate monobasic salt solution to the sample (range from 25 % (w/v) to saturated solution). Finally, a salt solution (70%, w/v) was selected and prepared by

adding 70 g sodium phosphate monobasic (Sigma-Aldrich, Saint Louis, USA) into 100mL ultrapure water and put in roller mixer (Stuart equipment, Staffordshire, UK) for 1h in order to dissolve completely. The external part of the Cheddar cheese was removed. 5g of grated cheese was placed in a 20mL amber glass vial. An internal standard (IS) was prepared by adding 100µL 3-heptanone (Sigma-Aldrich, Saint Louis, USA) into 100mL methanol (Laboratory reagent grade, Fisher Scientific, UK). 5µL of internal standard and 5mL NaH_2PO_4 solution were added into each sample vial. All analytical samples were in a randomised sequence for GC-MS analysis.

Volatile compounds were extracted according to the method of Gan *et al.* (2016). Samples were incubated at 60 °C for 30 min with agitation in order to facilitate analyte extraction and transport from the bulk of the cheese to the fibre. A SPME fibre with 50/30µm divinylbenzene/carboxen on polydimethylsiloxane coating (DVB/CAR/PDMS), purchased from Supelco (Sigma-Aldrich, Saint Louis, USA), was used to extract volatile compounds from the sample headspace (extraction for 15 min then desorption for 1 min). The inlet temperature was set at 250 °C. Helium was the carrier gas with a constant carrier pressure of 18 psi in splitless mode.

A trace 1300 series Gas Chromatograph coupled with the Single-Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used for the analysis of volatile aroma compounds. The separation was carried out on a ZB-WAX Capillary GC Column (length 30m, inner diameter 0.25 mm, and film thickness 1 µm; Phenomenex Inc., Macclesfield, UK). GC oven temperature

was held initially at 40 °C for 2 min, increased by 5 °C/min to 250 °C and held for 2 min. ISQ series mass spectrometer was used with a source temperature of 200 °C, a scanned the mass range of m/z 25-300. The GC to MS transfer line was maintained at 250 °C.

Data from GC-MS were processed with Thermo Scientific™ Xcalibur software. Volatile compounds were identified by comparing their retention times and mass spectra with those of standards provided by Sigma-Aldrich (St. Louis, MO) or their linear retention index (LRI) with those published in databases and the mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library. The relative concentration of each compound was calculated from the GC peak area, by comparison with the peak area of the internal standard (3-heptanone). The equation used to measure the relative concentration of the volatile compound in Cheddar cheese is shown below. Samples were analysed in four replicates in a randomised sequence.

Equation V-I *The equation used to measure the relative concentration of the volatile compound in Cheddar cheese.*

$$The\ relative\ concentration\ of\ volatile\ compound(\mu g/kg) = \frac{Peak\ area\ \ of\ volatile\ compound\ \times Amount\ of\ internal\ standard}{Peak\ area\ of\ internal\ standard \times Cheddar\ cheese\ mass}$$

V.2.3 Sensory analysis

The sensory method and protocol are described in previous work (Chen *et al.*, 2020). However, a brief outline is given here. All the descriptive sensory analysis data for the six-ripening time points of twenty sensory attributes are applied for the correlation. The sensory attributes are from appearance,

aroma, taste, flavour, texture by touch and in mouth texture perspectives of cheese samples. We were only able to perform five batches (A, B, C, D, E) of cheese for sensory evaluation at these six ripening time points.

V.2.4 Statistical analysis

A two-way (Analysis of Variance) ANOVA (factors=ripening days and batch number) was performed on 144 Cheddar cheese samples based on the relative concentration of each volatile compound in order to identify if a significant difference ($p<0.05$) existed for different predictive qualities and ripening days of Cheddar cheese. PCA (Principal Component analysis) was performed on all the samples and volatile compounds by XLSTAT (Addinsoft, France). For aroma category data analysis, each aroma category was normalised to the maximum relative concentration for the given category for all the 144 samples. ANOVA was also performed on normalised volatile concentration category followed by Tukey's honestly significant difference (HSD) post-hoc test. All the ANOVA tests were analysed by IBM® SPSS® Statistics version 25. Partial Least Squares (PLS) regression was used. The X data matrix contained volatile compounds data. The Y matrix contained the results of sensory mean scores for all significantly different cheese attributes. Pearson correlation analysis was performed at the same time by using XLSTAT (Addinsoft, France).

V.3 Results and discussion

V.3.1 Optimisation of SPME-GC-MS analysis

Total peak area from Cheddar cheese samples with 25, 50, 60, 70 % (w/v) of saturated monosodium phosphate solution is shown in Figure V-1. As salt concentration increased from 25% to a saturated solution, the total peak area increased significantly ($p<0.05$). The amount of headspace volatiles in 70% and saturated monosodium phosphate solution treated samples were similar. Saturated monosodium phosphate solution was more time-consuming to prepare. Consequently, 70% monosodium phosphate solution was chosen as the optimum level for the later study.

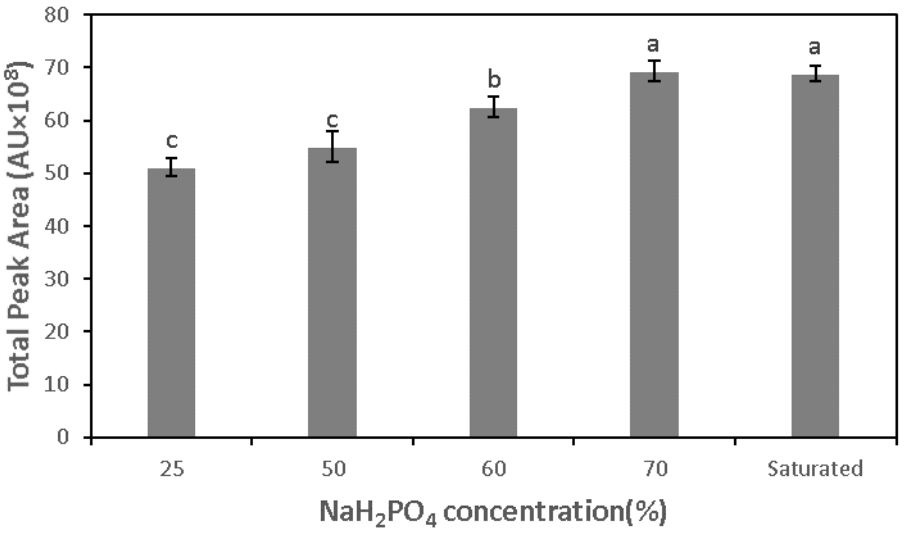


Figure V-1 *Effect of 25, 50, 60, 70 % (w/v) of saturated monosodium phosphate solution on the volatile compounds contained in the headspace of Cheddar cheese trapped by SPME method. Bars with different superscripts are significantly different (p<0.05).*

V.3.2 Determination of aroma compounds of Cheddar cheese batches with different predictive grading qualities during ripening

Twenty-two volatile aroma compounds were identified within the different Cheddar cheese samples and are presented in Table V-1. Volatile compounds included seven alcohols, six esters, three ketones and six organic acids. All the compounds have been previously reported in food materials and especially in cheese (refer to the Table V-1 caption for the relevant literature).

Table V-1 *Log P, molecular weight, Literature odour description for key compounds (include internal standard) with different function groups*

n.	Aroma compounds	Log P	Mol.WT	LRI	LRI-NIST	IM	Odour description [a]	Functional group
1	Ethanol	−0.18	46.07	945	944	MS, L	Alcohol, mild	Alcohol
2	Propyl acetate	1.24	102.13	990	990	MS, L, ST	Pineapple	Ester
3	2-Butanol	0.61	74.12	1037	1036	MS, L	Fruity; sweet apricot	Alcohol
4	1-Propanol	0.25	60.10	1051	1051	MS, L, ST	Alcoholic; fermented fusel; tequila; musty; yeasty	Alcohol
5	Isobutyl alcohol	0.76	74.12	1107	1106	MS, L	fusel; whiskey	Alcohol
6	Propyl butyrate	2.314	130.19	1137	1137	MS, L, ST	Sharp; pungent	Ester
7	1-Butanol	0.880	74.12	1157	1156	MS, L, ST	Fusel; sweet; balsamic; whiskey;	Alcohol
8	IS_3-Heptanone	1.73	114.19	1170	1167	MS, L, ST	Fruit, green, fatty, sweet	Ketone
9	2-Heptanone	1.98	114.19	1199	1198	MS, L	Banana; cinnamon; spicy	Ketone
10	Isopentyl alcohol	1.16	88.15	1220	1217	MS, L, ST	Fruity, alcohol, whiskey	Alcohol
11	Ethyl caproate	2.823	144.21	1249	1248	MS, L, ST	Pineapple; sweet; fruity	Ester
12	Acetoin	-0.36	88.11	1310	1309	MS, L	Sweet; buttery; creamy	Ketone
13	2-Heptanol	2.310	116.20	1331	1332	MS, L	Fresh; lemongrass; herbal; floral	Alcohol
14	Propyl caproate	3.333	158.24	1334	1337	MS, L	Fruity; pineapple; tropical	Ester
15	Lactic acid, ethyl ester	-0.18	118.13	1362	1363	MS, L, ST	Sweet; fruity; acidic	Ester
16	2-Nonanone	3.14	142.24	1408	1405	MS, L, ST	Fruity; soapy; herbaceous	Ketone
17	Ethyl octanoate	3.842	172.27	1450	1451	MS, L, ST	Apricot; banana; floral; pear; wine-like	Ester
18	Acetic acid	0.09	60.05	1478	1476	MS, L, ST	Vinegar; pungent	Organic acid
19	Propanoic acid	0.33	74.08	1567	1565	MS, L	Vinegar; pungent	Organic acid

20	Butyric acid	0.79	88.11	1655	1652	MS, L, ST	Rancid; putrid; sweaty	Organic acid
21	Valeric acid	1.39	102.13	1765	1766	MS, L, ST	Sweaty, rancid, waxy	Organic acid
22	Caproic acid	1.92	116.16	1871	1871	MS, L, ST	Blue cheese; fatty; sour pungent;	Organic acid
23	Octanoic acid	3.05	144.21	2086	2085	MS, L, ST	Oily; goaty, waxy, soapy, musty, rancid, fruity	Organic acid

A Quoted from (Gan *et al.*, 2016; Lecanu, Ducruet, Jouquand, Gratadoux, & Feigenbaum, 2002; The Good Scents Company, 2019)IM=identification method (MS=experimental mass spectra compared to the database; L= literature linear retention index; ST=standard compound, i.e. pure reference compound).

V.3.3 Comparison of all Cheddar cheese batches during ripening via principal component analysis

Principal component analysis (PCA) was performed to assess the acquired GC-MS data for all batches and all ripening time points (Figure V-2). It can be seen that PC1 and PC2 account for 61.57 % and 13.96 % of the variance, respectively. The first principal component (PC1) was mainly associated with maturity. On the right are the mature (later stages of ripening) cheese samples and young (early stage ripening) cheeses to the left. The second principal component (PC2) accounted for 13.96 % of the variance and showed the batch variation among different predictive grading qualities of Cheddar cheese. The general trajectory of aroma compound development during ripening is clearly observed. Before 270 days ripening, all cheese batches group together at the same age. As ripening proceeds further, the same age Cheddar cheese are less tightly grouped, indicating that each batch of Cheddar cheese has developed its distinctive aroma beyond 270 days ripening. In the later stage, at 360 to 450 days ripening, batch B and F group together near the origin of the PCA plot indicating fewer extreme levels of aroma compounds, typical of a well-balanced aroma profile found in the vintage Cheddar cheese control sample. Equally, batch E was furthest away from the other Cheddar cheese samples of the same age.

The batch C sample at 270 days was clearly an extreme sample based on the way it processed through the PCA plot. The bi-plot indicated that the young Cheddar cheese was associated with a higher concentration of acetoin, isopentyl alcohol, isobutyl alcohol and ethanol. Conversely, acids, ketones, esters and straight-chain primary and secondary alcohols were associated with more mature cheeses. Acetic acid and ethanol appeared to be two extreme aroma compounds differentiating Cheddar cheese grading qualities. Globally, batch A and E have a higher amount of propyl esters, ketones, acids and alcohols, whereas batch C and D projected out toward higher levels of branched chain alcohols and ethyl esters and lower levels acetic acid.

In order to evaluate if specific categories of volatiles were significantly dependent on either maturity or different predictive grading qualities, volatile compounds were classified into ten categories: ethyl esters, ethanol, propyl esters, 1-propanol, acetic acid, acids excluding acetic acid, methyl ketones, acetoin, branched-chain alcohols, secondary alcohols, according to chemical properties and development trends.

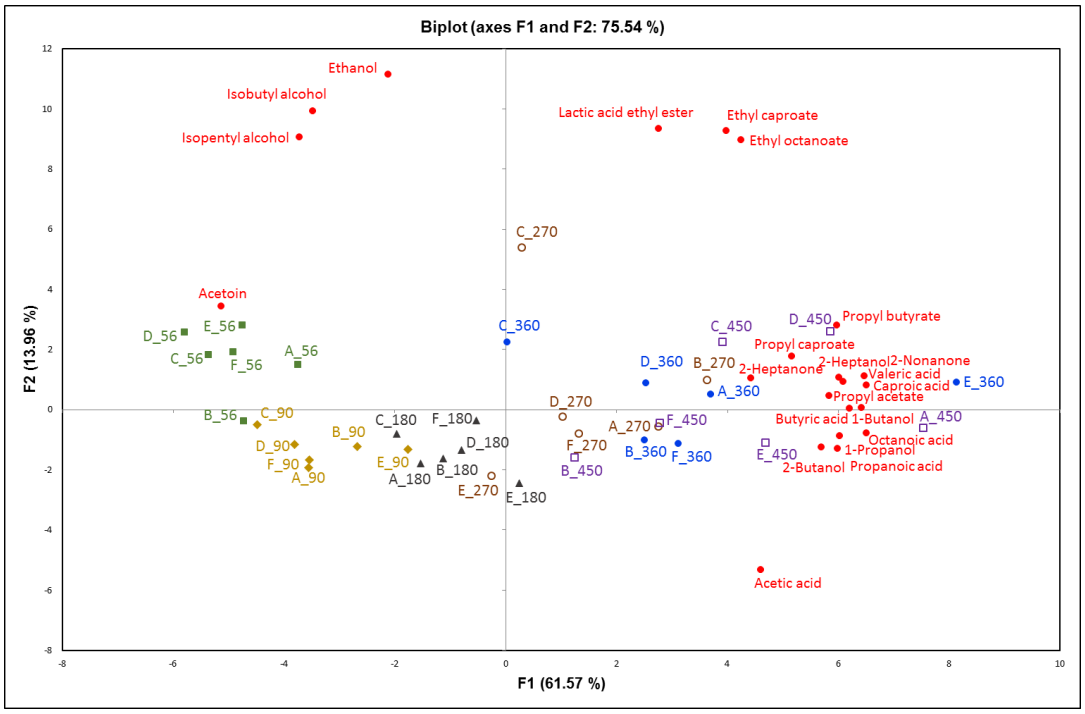


Figure V-2 *Principal components analysis (PCA) bi-plot of the headspace aroma data (average of four replicates) obtained by GC-MS analysis of 36 grated Cheddar cheese samples. Symbols and colours of the samples indicate different ripening times: 56 days ripening (solid square ▪; green), 90 days ripening (diamond ◊; yellow), 180 days ripening (triangle Δ; grey), 270 days ripening (open circle o; brown), 360 days ripening (solid circle ●; blue), 450 day ripening (open square □; purple). The data points were labelled as batch number and ripening days.*

V.3.4 Aroma evolution analysis during Cheddar cheese ripening

V.3.4.1 Esters and related alcohols during maturation

In Figure V-3 (a), the amount of ethanol dramatically decreases to less than one-third level after 56 days ripening then generally levels out through to the end of maturation. The amount of ethanol at 270 days of ripening in batch C seems to be a peculiar subsample, not characteristic of the general trend. Ethyl esters detected in these Cheddar cheeses are ethyl caproate, lactic acid ethyl

ester and ethyl octanoate. In the early phase of ripening, ethyl esters are at relatively low levels and increase three-fold in the later stage of ripening (Figure V-3(b)). 1-propanol is at an extremely low level at very early ripening stage, increases at 180 days to about nine times of the amount in the early stage (Figure V-3(c)). This is maintained throughout to near the final ripening time point. In Figure V-3(d), propyl esters show an exponential increase over the ripening process. Propyl acetate, propyl butyrate and propyl caproate contribute to the development trend of propyl esters. At 56 days of ripening, some of the propyl esters were almost undetectable. The amount of propyl ester at the later stages of ripening is about 40 times that in the early stage. Both ethyl esters and propyl esters increase during Cheddar cheese maturation, but propyl esters increase even greater than ethyl esters. At the early stage of maturation, the ethyl esters are more abundant than the propyl esters, whereas, after 180 days maturation, the propyl esters are the dominant esters in this Cheddar cheese. Equally, ethanol is decreased, whereas 1-propanol is increased on ripening proceeds.

Even though ethanol itself is not a potent odorant, it is a crucial precursor of different compounds. Non-starter lactic acid bacteria can generate ethanol by degrading lactic acid or amino acid metabolism or from acetaldehyde reduction and depleted in esterification (Bintsis *et al.*, 2004; McSweeney *et al.*, 2017). Ethyl esters have been reported to play a vital role in the aroma profile of aged Cheddar cheese (Curioni & Bosset, 2002; Gan *et al.*, 2016). They could be formed from different reactions, one is esterification of ethanol and free fatty acids; the other is alcoholysis of alcohols with acylglycerols or fatty acyl-CoAs

which are derived from metabolism of fatty acids, amino acids and carbohydrates (Liu, Holland, & Crow, 2004; Thierry, Maillard, Richoux, & Lortal, 2006). Ethanol was considered to be the limiting factor of ethyl ester synthesis in Cheddar cheese (Liu, Baker, Bennett, Holland, Norris, & Crow, 2004; McGugan, Blais, Boulet, Giroux, Elliott, & Emmons, 1975). 1-propanol is expected to result from the reduction of corresponding aldehyde, namely propanal. This is produced from volatile fatty acid by a reduction reaction that is catalysed by alcohol and aldehyde dehydrogenases of bacteria (Avsar, Karagul-Yuceer, Drake, Singh, Yoon, & Cadwallader, 2004). Similarly, the increase of propyl esters during cheese ripening was observed in ewes' raw milk in the production of Zamorano cheese (Fernández-García, Carbonell, Gaya, & Nuñez, 2004). Propyl ester formation is similar to that of ethyl esters, through esterification of 1-propanol and fatty acids. The greater increase in propyl esters relative to ethyl esters during ripening could be due to a relatively higher amount of 1-propanol than ethanol at the later stages of ripening (Supplementary Table 5.2), reflecting the metabolic difference between 1-propanol and ethanol.

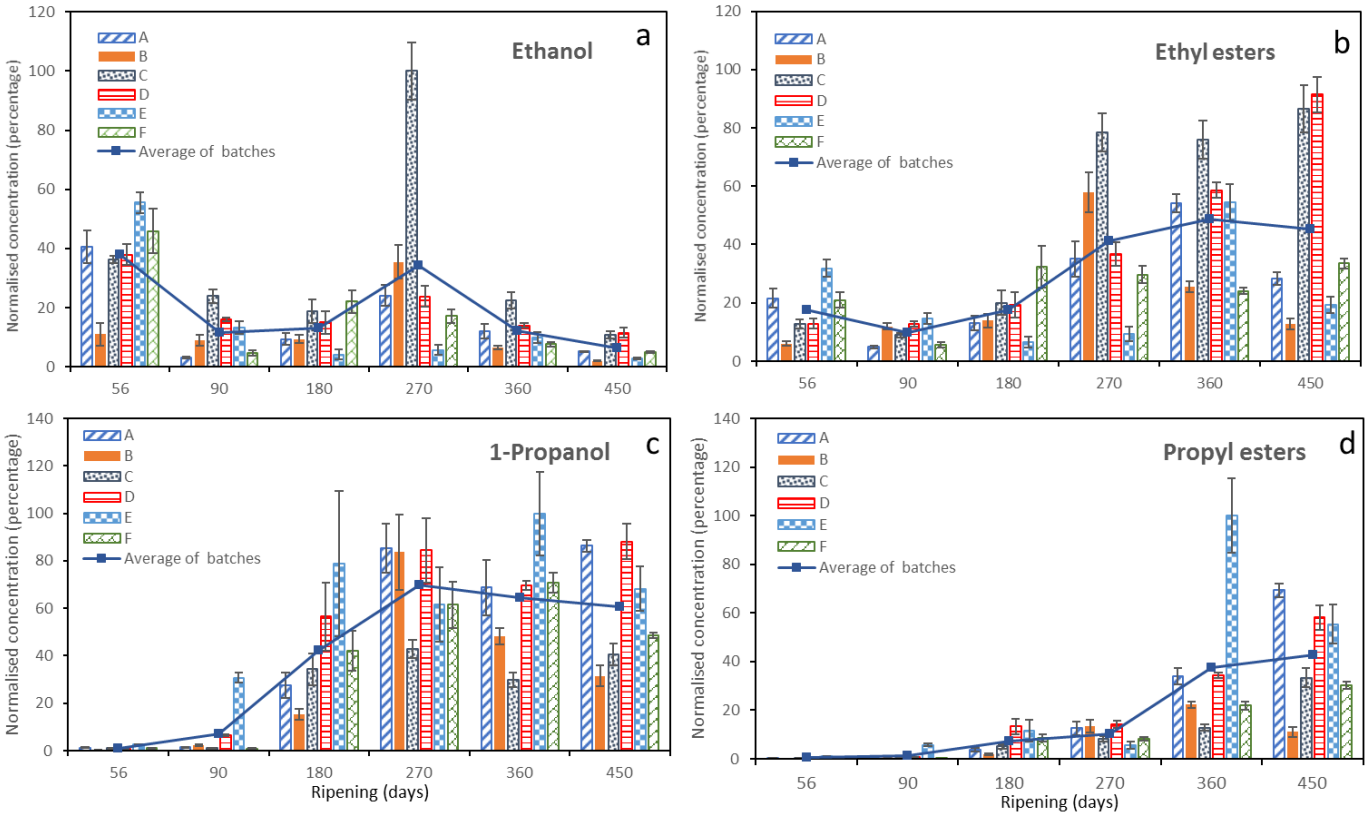


Figure V-3 *Average normalised concentration (calculated as a percentage of maximum concentration for given compounds) of four replicates for four compound classes: (a) ethanol, (b) ethyl esters, (c) 1-propanol and (d) propyl esters in the freshly prepared Cheddar cheese samples. Error bar represents standard deviations. Line charts stand for the average normalised concentration of six batches cheese sample during ripening up to 450 days.*

V.3.4.2 Acids and ketones evolution during maturation

Acids are the most abundant aroma compounds in this Cheddar cheese. Among all the acids, acetic acid is the most abundant acid throughout ripening, and it will be discussed separately. In Figure V-4(a), acetic acid increases to a maximum at 270 days ripening reaching levels about four times that of early stage. It then decreases at the final maturation stage. Other volatile acids (Figure V-4(b)) progressively increase with ripening. Acids, except acetic acid, include propanoic acid, butyric acid, valeric acid, caproic acid and octanoic acid. The amount of other volatile acids at the final stage is about three times that of the early ripening stage.

There are two types of ketones found in Cheddar cheese, the methyl ketones and acetoin. The methyl ketones found in Cheddar cheese are predominantly 2-heptanone and 2-nonanone. As shown in Figure V-4(c), methyl ketones show the least change in aroma concentration during ripening, gradually increasing to about 1.5 times their initial levels. Conversely, in Figure V-4(d) acetoin progressively decreased during ripening, showing an approximately four-fold decrease after 90 days maturation, with the amount in the late ripening time point being about two percent of the initial amount.

Similar trends of acetic acid development during maturation in Spanish raw milk cheese and Turkish Divle cave cheese was observed, but the turning point was 30 days and 120 days for each cheese, respectively (Delgado *et al.*, 2011a; Ozturkoglu-Budak *et al.*, 2016). The different generation pathways of acetic acid and other volatile fatty acids therefore need to be considered. As cheese

ripening proceeds, the acetic acid can be produced from lactose and amino acids (asparagine, glycine, alanine and leucine) (Ganesan *et al.*, 2004; McSweeney *et al.*, 2017). As the lactose depletes completely at an early ripening stage, the increase of acetic acid is mainly associated with the metabolism of amino acids (Ganesan *et al.*, 2017). Straight chain free fatty acids containing four or more carbon atoms (butyric acid, valeric acid, caproic acid and octanoic acid) mainly originate from hydrolysis of milk lipid through the action of esterase and lipase enzymes (Ganesan *et al.*, 2017). Volatile fatty acids also form ketones, esters and lactones in the reduced condition in cheese, which accounts for the decrease of acetic acid in the later stage of ripening (Ganesan *et al.*, 2017).

An increase in the concentration of methyl ketones during ripening was also observed by Bintsis *et al.* (2004); Ozturkoglu-Budak *et al.* (2016) in Turkish Divle Cave cheese and Feta-type cheese, respectively. Methyl ketones are formed from a diversion of the normal β-oxidation pathway. Fatty acids are oxidised to β-keto acids and subsequently decarboxylated to methyl ketones by microorganisms (Bontinis, Mallatou, Pappa, Massouras, & Alichanidis, 2012; Lawlor *et al.*, 2001). They are also intermediate compounds which can be reduced to alcohols (Fernández-García *et al.*, 2004). On the contrary, the decrease of acetoin during maturation was observed by Gan *et al.* (2016). Acetoin was formed by starter bacteria in the adverse growth condition from diacetyl. Starter bacteria in cheese are forced to use an alternative biochemical pathway to survive, which results in the production of acetoin (Upreti *et al.*,

2006b). Starter culture bacteria are also able to reduce acetoin to 2,3-butanediol (Bontinis *et al.*, 2012; Urbach, 1993).

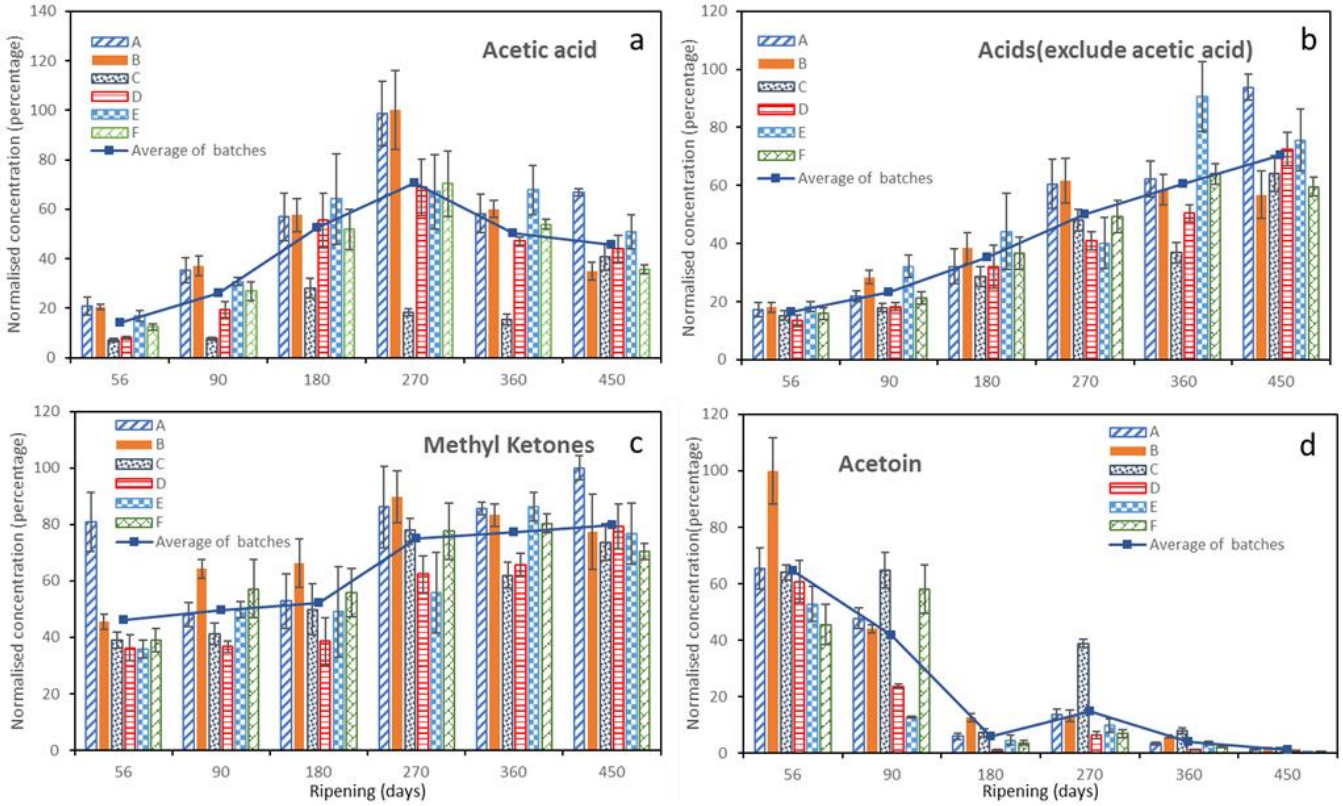


Figure V-4 *Average normalised concentration (calculated as a percentage of maximum concentration for given compounds) of four replicates for four compound classes: (a) acetic acid, (b) acids (exclude acetic acid), (c) methyl ketones, (d) acetoin in the freshly prepared Cheddar cheese samples. Error bar represents standard deviations. Line charts stand for the average normalised concentration of six batches cheese sample during ripening up to 450 days.*

V.3.4.3 Branched-chain alcohols and secondary alcohols evolution during maturation

As presented in Figure V-5(a), it can be observed that branched chain alcohols (isopentyl alcohol and isobutyl alcohol) decrease by about 75 % after 56 days ripening and retain such a low amount until the end of maturation. Secondary alcohols found in Cheddar cheese were 2-butanol and 2-heptanol, where 2-butanol is much more abundant than 2-heptanol being about four hundred times of the amount of 2-heptanol (Supplementary Table 5.1). From Figure V-5(b), a dramatic increase by four times of secondary alcohols was observed after 90 days and then fluctuating increase until the end of maturation.

These branched primary alcohols which have a pleasant aroma of "fresh cheese" occurred in the early stage of cheese aroma evolution (Lecanu *et al.*, 2002). Branched-chain alcohol (isopentyl alcohol and isobutyl alcohol) are attributed to progressive catabolism of branched-chain amino acids (leucine, valine) initiated by an aminotransferase (Gómez-Torres *et al.*, 2016; Van Leuven *et al.*, 2008). The branched chain alcohol will further oxidatively degrade to corresponding branched chain acids (Isovaleric acid and Isobutyric acid), which are attributed to the decrease of branched chain alcohol during ripening (Yvon & Rijnen, 2001). Conversely, secondary alcohols increase during maturation. A different phenomenon was detected in Turkish Divle Cave cheese, 2-butanol increased until 90 days and tended to decrease onwards (Ozturkoglu-Budak *et al.*, 2016). Secondary alcohols are formed from enzymatic reactions (alcohol dehydrogenase) of the corresponding methyl ketones, which

is produced from fatty acid by successive β-oxidation cycles (Molimard & Spinnler, 1996). 2-butanol is exceptional; given it is from methyl ketone 2-butanone as well, which is formed by the action of enzymes from the raw milk microbiota, mainly lactobacilli rather than being due to lipolysis (Keen, Walker, & Peberdy, 1973; Urbach, 1993).

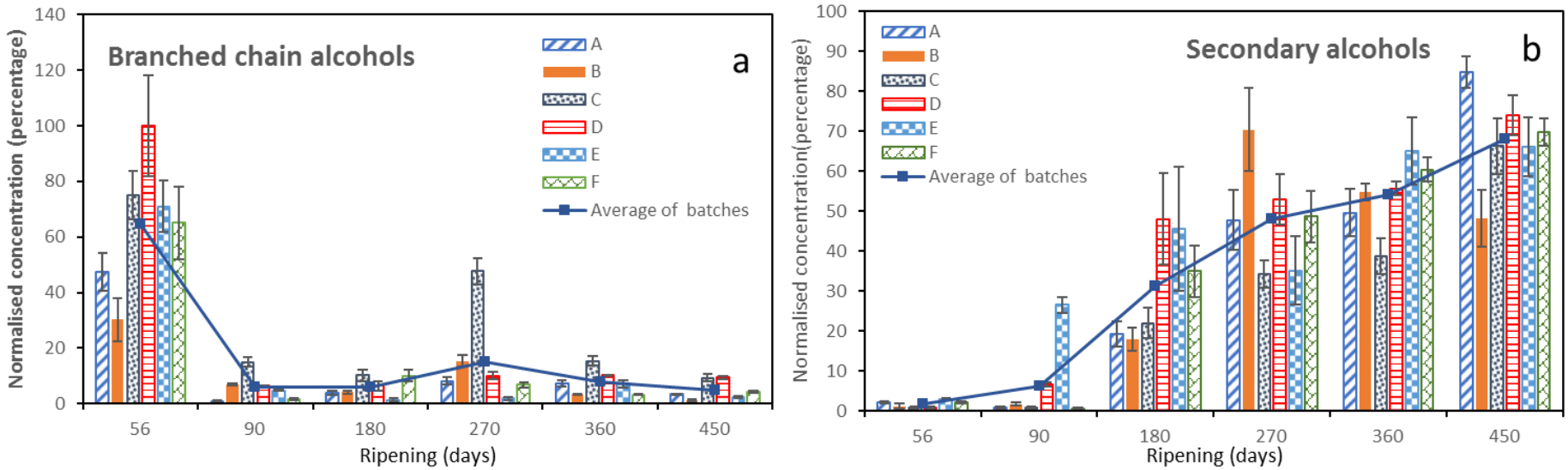


Figure V-5 *Average normalised concentration (calculated as a percentage of maximum concentration for given compounds) of four replicates for two compound classes: (a) branched chain alcohols, (b) secondary alcohols in the freshly prepared Cheddar cheese samples. Error bar represents standard deviations. Line charts stand for the average normalised concentration of six batches cheese sample during ripening up to 450 day*

V.3.5 Batch to batch variation of different predictive qualities Cheddar cheese

Here the discussion of batch variation of different predictive quality Cheddar cheeses is mainly focused on the batch B, C and E, which are extreme samples from Gilles and Lawrence model and professional grader judgements.

'Premium' batch B cheese has a significantly higher concentration of acetoin and lower branched chain alcohol at the earliest phase of ripening but not in the later phase. Acetoin provides a buttery and fresh flavour of curd which is stronger in the early phase of ripening rather than in the later stages.

During the whole ripening process, the amount of acetic acid and secondary alcohols of 'downgrade' batch C are significantly lower than other batches (Supplementary Table 5.3). On the contrary, batch C is markedly higher in acetoin level than other batches in the later stages of ripening. At the midpoint of the ripening process, batch C has significantly higher amounts of ethyl esters and ethanol than other batches.

The acetic acid content of batch C is about 40% of the average amount of other batches Cheddar cheese (Supplementary Table 5.3). Acetic acid is produced from asparagine via aspartate during the metabolism of lactic acid bacteria (Ganesan *et al.*, 2004). The lower level of asparagine in batch C is reported in chapter IV. The lower acetic acid content on batch C could be due to the lower enzyme activity involved in this series of ripening pathways and can be attributed to an initial lower microbial activity.

Ethyl caproate and ethanol in batch C are about 7.8 and 5 times that of 'premium quality' batch B, after 450 days ripening. Ethyl esters could provide fruity notes to the cheese that minimise the strong aroma produced by acids (Delgado *et al.*, 2011a). However, the fruity flavour is regarded as a defect by professional cheese graders (Mcsweeney *et al.*, 2000). An excess of ethyl ester in proportion to other flavour compounds could be responsible for the fruity defect. Bills et al. (1965) demonstrated that ethyl caproate in fruity Cheddar cheese presented at levels from two to ten times greater than standard cheese. They also reported that levels of ethanol was from 6 to16 times greater in the fruity samples. The excessive production of ethanol accentuated esterification of free fatty acids (Bills *et al.*, 1965). Combining the data presented here and previous literature, batch C could have fruity defect after 450 days maturation from an instrumental analysis point of view. However, batch D has the similar amount of ethyl esters and ethanol as batch C after 450 days ripening, but batch D wasn't initially graded as a 'downgrade' cheese, this could be due to the much lower acetic acid content in batch C. This suggests that the ratio of aroma compounds is more important than the absolute amounts.

For the whole ripening process, the total amount of propyl esters of 'downgrade' batch E is significantly higher than other batches. Batch E is significantly higher in secondary alcohol, ethyl esters and propyl esters at the early stage of maturation. Especially at 90 days ripening, the value of secondary alcohols and propyl esters of batch E is nearly twenty-four and fourteen times of premium batch B, respectively. Additionally, propyl acetate and propyl caproate were first found after 90 days ripening in batch E cheese sample,

those in other batches were observed after 180 days ripening. Correspondingly, batch E has higher amounts of 1-propanol than other batches. At the early stage of ripening, secondary alcohol is not desirable in Cheddar cheese which usually can be found in the later stage of maturation. 2-butanol reduced from 2-butanone is associated with off-flavour development, as reported by Keen *et al.* (1973). It is noted that the production of 2-butanone is due to detrimental bacteria which are not necessary for the production of Cheddar flavour (Urbach, 1993). 2-heptanol in musty Cheddar cheese sample is more than 1000 times of this non-musty Cheddar cheese (data not shown) which indicated that 2-heptanol is not a desired aroma for Cheddar cheese. 2-heptanol is formed in cheese via 2-heptanone which can be attributed to the high degree of lipolysis due to the action of mould (Lawlor *et al.*, 2001; Urbach, 1993). Even though a large variation among different predictive quality batch variation in 2-heptanone hasn't been observed in our study, cheese made with reuterin-producing *Lactobacillus reuteri* coupled with glycerol, which can inhibit mould, has much lower levels of 2-heptanone than the control has been reported (Gómez-Torres *et al.*, 2016).

The level of 2-butanol was lower in the highest sensory quality scored for La Serena cheese (Carbonell, Nuñez, & Fernández-García, 2002). Downgrade batch E sample has a higher amount of secondary alcohol in the early stage of ripening which can therefore be regarded as a quality marker for predicting after ripening.

1-propanol exhibited alcoholic and musty note which is not desirable in Cheddar cheese. The difference among batches could indicate that extensive lipolysis probably occurred in batch E cheese sample. Commonly, propyl ester usually occurs in Swiss and brined ripening cheese rather than Cheddar cheese. This could be due to the modified culture used in this cheese in order to add more distinctive aroma. Due to the microbial origin of these esters such as propyl butyrate, their levels should be low at the beginning of ripening (Delgado *et al.*, 2010). Lawlor *et al.* (2001) found that pungent odour is positively correlated with propyl acetate and negatively correlated with ethyl acetate and ethyl butyrate. From our ethyl esters analysis, we can conclude that from an instrumental point of view, batch E should have a more pungent odour and batch C have much less pungent smell. The findings that higher levels of propyl esters and 1-propanol, but no corresponding higher amount of acids in batch E is in agreement that alcohol is the rate limiting factor for esterification process (Fernández-García *et al.*, 2004). At an early phase of ripening the concentration of 1-propanol in batch E is significantly higher than other batches, but not the later due to the depletion of esterification. The level of propyl ester, 1-propanol were lower in the highest sensory quality La Serena cheese reported by Carbonell *et al.* (2002), which is consistent with the observations in this study.

V.3.6 Correlation between Cheddar cheese sensory profile and flavour

Only a small percentage of volatile compounds in food are odour active, therefore it isn't possible to explain all the correlations solely from an aroma perspective.

All the flavour related sensory attributes such as overall odour intensity, dairy odour, salty and umami taste, buttery and sweaty flavour and lingering, and astringent aftertaste are most correlated with 2-heptanol, caproic acid, valeric acid and octanoic acid (Figure V-6 and Supplementary Table 5.4). This could be due to their low aroma threshold and suggests that all these four compounds could be a flavour indicator for this type of Cheddar cheese. Acetoin positively correlated with a buttery flavour and dairy odour. Acetoin has been reported to be a buttery odour note which is consistent with our sensory conclusion. Pressure firmness, crumbly and rubbery sensory attributes are highly correlated with octanoic acid, valeric acid and caproic acid, but not acetic acid or butyric acid. Interestingly, the oily attribute is not only highly correlated with valeric acid and octanoic acid but also 2-butanol. These correlations are not necessarily indicating a causal phenomenon.

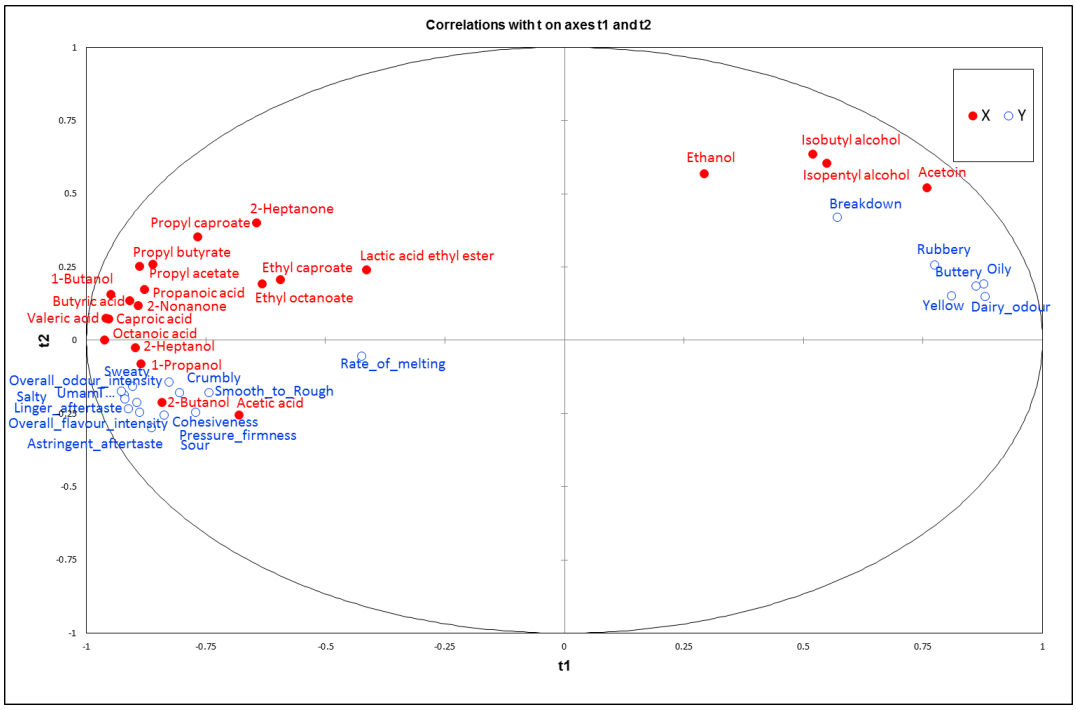


Figure V-6 *Partial Least Squares correlation plot for cheese batch variation A, B, C, D, E showing the correlation of sensory descriptors and aroma profile attributes measured by GC-MS measured six maturation time point days of Cheddar cheese.*

V.4 Conclusions

Broadly speaking, for different predicted quality Cheddar cheese, the maturation trajectory is still similar except for batch C in the late-ripening stage. Premium batch B has significantly higher amounts of acetoin and lower branch chain alcohols and matures to be a cheese having a well-balanced aroma profile, without extreme level of volatiles compounds. Batch C which has significantly lower acetic acid and secondary alcohols in the early stage of ripening but matures to be a cheese that has higher ethyl ester at the later stages of ripening. However, another 'downgrade' cheese, batch E, has higher

secondary alcohols and 1-propanol in the early ripening stages and matures to have higher propyl ester levels. A significantly higher amount of secondary alcohols in the early stage of Cheddar cheese can be regarded as a sign of 'downgrade' cheese after maturation. Cheese with a lower amount of propyl ester are therefore not necessarily a premium cheese but a higher amount of propyl ester should be ranked as a 'downgrade' off-flavour. Cheeses that was predictively graded as 'grade quality', according to Gilles and Lawrence predictive grading model, such as batch C and E can still have aroma defects in the later stage of ripening. Thus, the aroma compounds can potentially be treated as supplementary parameters for quality prediction.

Before applying these aroma attributes to grading, additional validation with more batches would be prudent. As our study was only performed on six batches of Cheddar cheese, future work will be necessary to confirm our conclusion.

Chapter VI Evolution of texture and microstructure of Cheddar cheese maturation correlated to the sensory profile

Abstract

Texture profile analysis coupled with confocal microscopy have been applied to investigate Cheddar cheese texture and structure development during ripening up to 450 days. Additionally, the correlation between sensory profile and the texture and microstructure variables was explored. Protein matrix porosity analysed from microscopy images decreases during ripening with consequential decreases in cohesiveness and springiness. It is found that cohesiveness is the best texture profile analysis parameter to discriminate the different predictive quality Cheddar cheese. Both protein matrix porosity and cohesiveness are highly negatively correlated with tangy, lingering aftertaste and overall flavour intensity.

Highlight

- Protein matrix porosity analysed from confocal micrographs decrease during ripening and is highly correlated with flavour sensory attributes.
- Cohesiveness measured by texture profile analysis is correlated with sensory attributes and is the best attribute to discriminate the cheese batch variation among all the texture profile parameters.
- Cohesiveness and springiness decrease significantly during Cheddar cheese ripening.

VI.1 Introduction

Texture is the primary quality attribute of cheese. The overall appearance and mouthfeel of cheeses are appreciated before their flavour. The textural characteristics are related to the cheese's composition, micro- and macrostructure and molecular properties; more specifically to how the casein matrix gives rise to the rigid texture of cheese (Upadhyay *et al.*, 2004). Cheese texture development after two weeks of ripening changes relatively slowly. The change in rate is determined by the proportion of residual rennet, salt to moisture ratio and storage temperature (Lawrence *et al.*, 1987). Proteolysis was considered to be the key event responsible for the texture changes during ripening (Johnson *et al.*, 2006). O'Mahony, Lucey, and McSweeney (2005) found that the loss of colloidal calcium phosphate (CCP) from the casein is also responsible to the texture change during the early stages of maturation.

Measurements of cheese microstructure can be used to understand and explain what changes in texture indicate and therefore control of cheese properties. The casein matrix, which is the only continuous solid phase in cheese originates from small casein particles held together by various forces (Lucey *et al.*, 2003).

How individual casein molecules or aggregates of many casein molecules interact is vital to cheese physical properties such as texture, body, melt and colour. The early cleavage of α_{s1} casein at Phe_{23}-Phe_{24} by residual chymosin activity has been associated with the decrease in firmness during the ripening process (Lucey *et al.*, 2003). However, an alternative view is that as intact casein content decreases, and the degree of a stable emulsion increases, the hardness and firmness of cheese will increase (Guinee & O'Callaghan, 2013).

Initially, cheese texture is directly measurable only by sensory analysis until a significant correlation between sensory attributes and instrumental parameters are found (Upadhyay *et al.*, 2004). Texture profile analysis (TPA) is a popular double compression test for determining the textural properties of foods which provide insight into how samples behave when chewed. TPA measures the response of cheese to a two-bite deformation and in addition to firmness, fracture stress and strain, also provide a prediction parameter (e.g., cohesiveness, adhesiveness, chewiness) that are important during mastication (Fox *et al.*, 2016a). When compared to sensory evaluation of texture, instrumental methods are easier to perform, standardise, reproduce and require fewer trained people (Pereira, Bennett, & Luckman, 2002). Texture

profile analysis was used to measure the processed cheese texture properties of natural Cheddar cheese in the first 28 days of ripening and lead to the greatest decrease in hardness and flowability in the processed cheese (Brickley, Auty, Piraino, & McSweeney, 2007).

Confocal laser scanning microscopy (CLSM) has been used to assess the microstructure of Cheddar cheese. Soodam *et al.* (2015) used CLSM to observe microstructure changes during ripening from week 1 to week 30 when calcium chloride is added or draining pH altered. The effect of elevated temperature and fat content on the microstructure of Cheddar cheese during ripening has also been examined by CLSM combined with quantitative image analysis. They conclude that an increase in ripening temperature caused thicker protein strands and larger pores in the protein network. An increase in size of fat globules and higher percentage of non-spherical globules were shown in an increasing fat content Cheddar cheese (Rogers *et al.*, 2010; Soodam *et al.*, 2017).

Sensory textual properties of cheese are the result of the temporal pattern of structural breakdown and mixing with saliva during the oral process (Hutchings & Lillford, 1988). This complex dynamic processing is not easy to be described by a series of single attributes. The correlation of texture and microstructure with sensory characteristics can conclude which structural attributes are close to explaining the human sensory response and therefore made relevant for quality control. This correlation can also improve or optimise instrumental

methods to become even more complementary to sensory evaluation (Szczesniak, 1987).

Most microstructure work for Cheddar cheese focuses on the early stage of ripening, up to about 30 weeks, but to date, there is little work investigating ripening until high levels of maturity, and even vintage cheese which usually needs to ripen for 12-15 months. This study investigated texture and structure development during ripening in comparison to the Gilles and Lawrence quality grading model and a professional cheese grader. The correlation between texture, microstructure variables and sensory profile was explored in order find the cheese sensory-related markers and further enable predictive grading of cheese quality.

VI.2 Materials and methods

VI.2.1 Samples

Batch A, B, C, D, E and F cheese samples used for this chapter are the same block as chapter II. Texture and microstructure analyses carried out at various stages during the ripening period namely 56, 90, 180, 270, 360 and 450 days. .At each time point, two bags of cheese were removed at random from each block and placed in a 4 °C refrigerator before measurements. One bag was used for instrumental measurements (texture profile analysis and microstructure analysis) and the other for descriptive sensory analysis.

VI.2.2 The microstructure of Cheddar Cheese

All microscopy work was performed using a Zeiss 710 confocal laser scanning microscope (CLSM) operating in confocal mode. The centre of cheese was cut into 6 mm×6 mm×< 1 mm slices. Seven fluorescent probe combinations in different solvents were trialled prior to the final protocol being determined. 0.01g/L Nile red in 1.2-propanediol was used as a lipid stain and 0.2 g/L fast green in distilled water was used as a protein stain. A thin slice of cheese was immersed in aqueous stain mixture held at 4 °C overnight. Images of representative areas of each sample were taken using ×40 magnification objective with a numerical aperture of 1.3. Nile red was excited at a wavelength of 488 nm and fast green at 633 nm. The emission filter was 550-640 nm for Nile Red and 640-710 nm for fast Green. Zen software was used to acquire digital images of 1024×1024 pixels in size that were average of 8 frames (Ong, Dagastine, Kentish, & Gras, 2010). Four micrographs were obtained from each sample. The micrographs of fast green signal channel were segmented using the similar thresholding process before Gaussian blur filter. Image J program was used to calculate the porosity (fraction of pore area with the respect the total area) of the protein matrix.

VI.2.3 Texture Profile Analysis (TPA)

Texture Profile Analysis (TPA) of cheese was performed using a texture analyser (TA.XT Plus, Stable Micro System Ltd., UK) with 100kg load cell. All the samples are taken from the 4 °C fridge and left to equilibrate at room temperature for

1 hour. Each sample was moulded into a cylinder with a diameter of is 40 mm and height of 20 mm. A 100-mm circular compression plate was lowered at the speed of 1 mm/s onto the sample surface, and the sample was compressed at 2 mm/s with 75% strain using two compression cycles. The same test speed and post-test speed was used. The compression plate is larger than the sample diameter to make sure the sample could be fully contacted and properly compressed so that the TPA tests were largely due to uniaxial compression forces. 75% strain is destructive for old and mature cheese which can imitate the highly destructive process of mastication in the mouth. A 15g trigger force was chosen to avoid triggering in the air and to be sensitive to the true surface of the product. As the old and mature cheese samples are brittle, therefore a data acquisition rate 400 points per second (pps) was used. The height and force were calibrated before experimentation as this is a strain percentage test. Samples were characterized in terms of hardness, springiness, fracturability and cohesiveness. Stable Micro System's software, Exponent was used to consistently quantify the elements of curve. These parameters were defined according to Pons and Fiszman (1996).

VI.2.4 Quantitative descriptive analysis

The details of the sensory protocol have been presented in the previous study and chapter II (Chen *et al.*, 2020). The sensory profiles at six ripening time points (56, 90, 180, 270, 360 and 450 days) were analysed. The sensory analysis was only performed in five batches of Cheddar cheese during this period (A-E).

The sensory protocol includes twenty sensory attributes from appearance, aroma, flavour, taste, texture by touch and in mouth texture perspectives.

VI.2.5 Statistical analysis

Sensory, texture and microstructure image attribute data were analysed firstly by analysis of variance (ANOVA). The means were then compared using variance analysis followed by the Tukey test (significant difference when $p < 0.05$) using IBM SPSS Statistics. For modelling the correlation between the texture profile, microstructure attributes and sensory profile, Partial Least Squares (PLS) regression was used. The X data matrix contained texture profile, microstructure attributes data. The Y matrix contained the results of sensory mean scores for all significantly different cheese attributes. Pearson correlation analysis was performed at the same time by using XLSTAT (Addinsoft, France).

VI.3 Results and discussion

VI.3.1 Confocal microscopy analysis of Cheddar cheese during maturation

A representative image of each ripening time point was presented in Figure VI 1. No obvious serum water was observed after 56 days ripening indicating that most of the water is absorbed into the casein matrix even in young Cheddar cheese. No typical fat globules were witnessed, instead pools of free fat were observed after 56 days ripening. The observation is similar to previous observations that fat globules in the 33 % fat Cheddar cheese appear clumped

and coalesced into non-spherical shapes. The protein matrix appears to coarsen with ageing, and the evolution of the protein matrix porosity obtained from micrographs is statistically analysed in Table VI-1. Protein matrix porosity is seen to decrease during 450 days ripening which indicates that as cheese matures the protein matrix develops to a denser, less open and increasingly interconnected network microstructure. This could be due to the absorbance of serum water that leads to the physical swelling of casein matrix (Karami *et al.*, 2008). It is known that during storage, the number and strength of protein-protein interactions between casein molecules weaken (Lucey *et al.*, 2003). This then indicates that an increase of density of the casein matrix probably is a result of serum protein which is cleaved from peptides during maturation or the cleaved peptide is still attached to the matrix.

A minor lack of structural homogeneity of samples tends to be highly magnified during image analysis. However, we have found that batch variation is small; therefore, microstructure image analysis cannot statistically discriminate small differences and batch to batch variation.

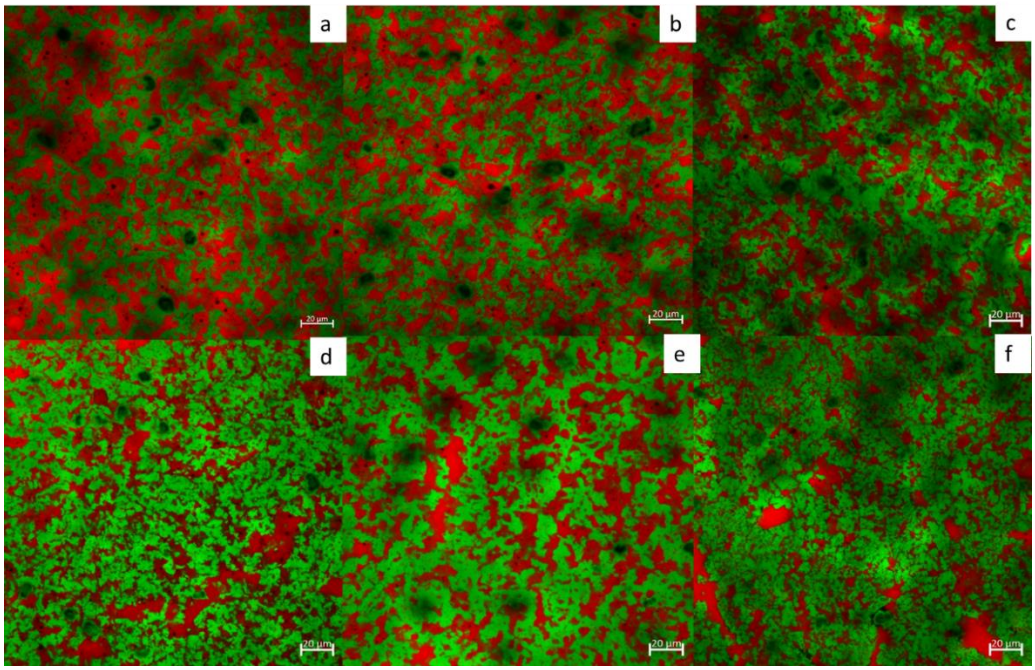


Figure VI-1 *Confocal laser scanning micrographs of batch E Cheddar cheese microstructure during ripening shown as an example. (a), (b), (c), (d), (e), (f) are the Cheddar cheese ripening after 56, 90, 180, 270, 360, 450 days. The scale bars are all 20 µm. The protein matrix in green and fat globules in red. The dark grey shades probably are the crystalline salt, mineral deposits combined with protein matrix.*

Table VI-1 *Protein matrix porosity change during the ripening process. For each time point, the data represent four measurements on the six batches of the cheese and so is an average of 24 samples. Errors present the standard deviation for 24 values. Different superscripts in the same column indicate significant statistical difference (Tukey's test P<0.05).*

Ripening days	Protein matrix porosity (%)
56	56.3 ± 4.07^{a}
90	52.4 ± 4.99^{b}
180	49.8 ± 2.27^{c}
270	49.5 ± 3.53^{c}
360	43.5 ± 2.98^{d}
450	41.4 ± 2.41^{d}

VI.3.2 Texture profile analysis

The microstructure change during maturation was expected to lead to textural differences. Figure VI-2 shows that the hardness, fracturability, springiness and cohesiveness were all significantly affected by the ripening time ($P<0.05$). Generally speaking, all the texture profile attributes do not distinguish the batch to batch variation during ripening very well.

Here chewiness and gumminess are not reported, as cheese is a solid sample and becomes semisolid during sensory mastication. The negative peak in texture profile analysis graph is very small which indicates that adhesiveness is neglectable. In many instances, the samples were stuck to the probe and therefore the parameter adhesiveness is inaccurate and meaningless and therefore is also not reported here.

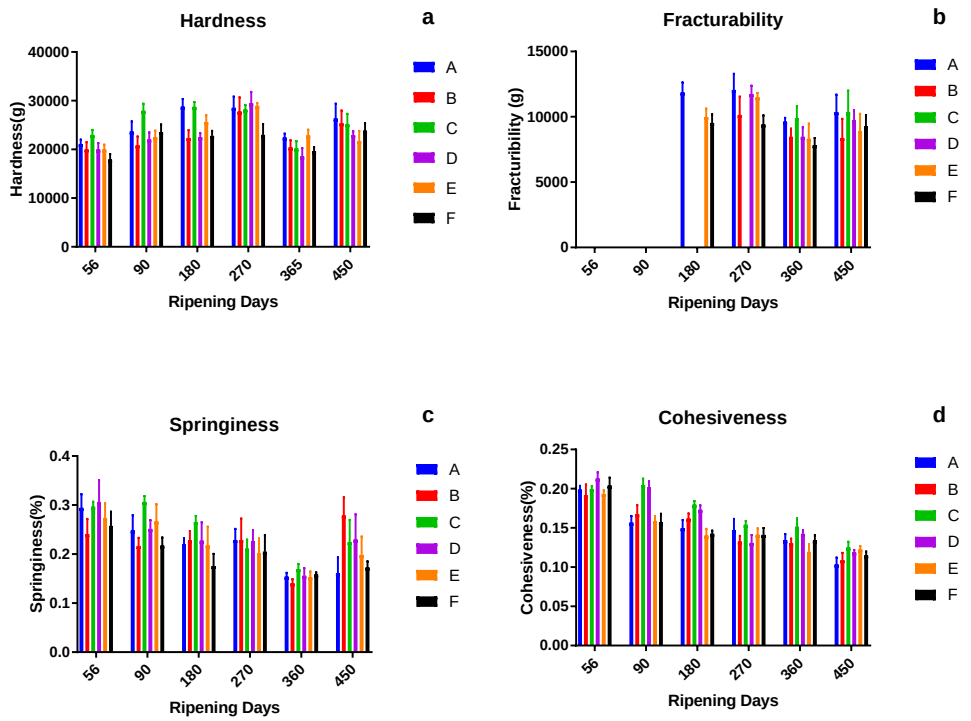


Figure VI-2 *Changes in the hardness (a), fracturability, (b), springiness (c), cohesiveness(d) of different predictive quality Cheddar cheese after ripening at 56,90,180,270,360 and 450 days. The results are expressed as the mean ± standard deviation (n=5).*

VI.3.2.1 Hardness

The cheese hardness was significantly affected by the ripening time ($P<0.05$) (Figure VI-2 and Table VI-2). The hardness of Cheddar cheese increased at the early stage of ripening and reached a peak at 270 days with a subsequent decrease through to the 450 days. Batch C is the hardest batch at the early stage of ripening, but only significantly ($P<0.05$) higher than all batches at 90 days ripening. There are no significant differences in hardness at the last stage of ripening among batches. Similar behaviours have also been reported in the literature (Soodam *et al.*, 2017). The increase of hardness was believed to be a consequence of changes in water binding as a result of proteolysis rather than

solvation of the protein chain (Lawrence *et al.*, 1987). At the late stage of ripening hardness is decreased which is also due to the proteolysis. Brickley *et al.* (2007) suggested that a decrease of hardness is due to the reduced level of intact casein. Another possible reason for the lessening of hardness is the reduction of the concentration of calcium phosphate. As pH increased during ripening, the concentration of calcium phosphate at the surface exceeded its solubility and produced a layer of $Ca_3(PO_4)_2$ which was observed in vintage Cheddar cheese. The reduction of the concentration of calcium phosphate in the interior helps to soften the Cheddar cheese in the late-ripening process (Upadhyay *et al.*, 2004). The hardness of cheese is then described as a compromise between the cheese dehydration and proteolysis (Calvo *et al.*, 2007). However, when comparing Figure VI-1 and VI-2, it is also evident that the microstructure changes, and protein matrix porosity also mirror the changes in measured hardness, certainly up to 270 days (at the peak of measured hardness). A subsequent decrease in hardness with further maturation, can then be attributed to protein matrix properties, as a result of further proteolysis.

Batch C cheese is slightly harder than other batches up to 270 days ripening (Supplementary Table 6.1). The harder textural features could be due to low moisture in the fat free substance which roughly indicating the low moisture to casein ratio. The lower the ratio of moisture to casein, the firmer will be the casein matrix of the cheese (Lawrence *et al.*, 1987). This also possibly due to the lack of acid development which is caused by high salt in moisture phase (Lawlor, Delahunty, Wilkinson, & Sheehan, 2002; Partridge, 2008). Batch C is

shown as a batch of cheese that is lacking acetic acid and lactate (data is shown in chapter IV and V). This could reduce the dissolution of calcium in colloidal calcium phosphate linkages (Johnson *et al.*, 2006). Calcium has a major effect on the cheese texture of hardness which can increase the hardness of matrix (Lawlor *et al.*, 2001). Another possible reason is that batch C has lowest fat in the water free substance, implying that batch C has a higher protein to fat ratio. This then provides extra credence that protein is responsible for the hardness of cheeses, as similar work has found that hardness increase as the mean fat content decrease (Guinee *et al.*, 2000; Madadlou *et al.*, 2005). After 270 days ripening, there is little batch to batch variation in hardness, probably due to bioreactions that compensate for the original composition variation of Cheddar cheese, and that microstructural changes have reached their limit.

VI.3.2.2 Fracturability

None of the batches cheese fracture before 180 days ripening. Only for batches A, E, F does the first fracture occur at 180 days, whilst batch C only shows fracturability at 360 days ripening. Fracture properties have been shown to strongly depend on the size of the largest inhomogeneities or "weak spots" (Luyten & van Vliet, 1996). Whereas the results from batch C also indicate that the composition and subsequent rates of bioreactions in the cheese also have a significant impact. Indeed, a similar phenomenon has shown that high salt cheese has a longer fracture feature compared with low, reduced and normal salt cheese (Møller *et al.*, 2013). As ripening proceeds, Cheddar cheese becomes more brittle associated with the reduction of fracture strain during

the ripening process (Lucey *et al.*, 2003). With ageing, the fat of cheese also becomes easier to melt, where liquid fat acts as a lubricant on fracture surfaces of the casein matrix, and thereby reduces the stress required for fracture surfaces of the casein matrix (Fox *et al.*, 2016a).

VI.3.2.3 Springiness

Springiness is defined as the height of the food between the first and second compression (Texture Technologies Corp). A significant ($P<0.05$) reduction in springiness value from 0.28% to 0.21% indicates that cheese becomes less elastic with increasing maturation time (Figure VI-2 and Table VI-2). A young Cheddar cheese is springy and fairly hard, as described by Hort and Le Grys (2001) and a decrease of springiness is also observed by Delgado, González-Crespo, Cava, and Ramírez (2011b). Springiness can also now be thought of as a result of both microstructure changes during ripening, and the inherent properties of the protein matrix.

VI.3.2.4 Cohesiveness

Cohesiveness is found to decrease significantly ($P<0.05$) during ripening (Figure VI-2 and Table VI-2). Comparing to other textural properties, it changes most during ripening according to the five different significance levels in ANOVA result (Table VI-2). A similar phenomenon has been reported by many other researchers (Delgado *et al.*, 2011b; Soodam, Ong, Powell, Kentish, & Gras, 2014; Soodam *et al.*, 2015, 2017). Cohesiveness is a measure of the extent of deformation that occurs before any rupture occurs. The reduction of the cohesiveness indicates that the internal structure strength is weakened during

maturation. Among all the textural properties of Cheddar cheese, TPA cohesiveness was most related to primary proteolysis with a trend of decreasing with increasing proteolysis (Lane, Fox, Johnston, & McSweeney, 1997). There was a significant correlation between cohesiveness measured from TPA and the number of vertices in the protein network (Soodam *et al.*, 2014). With a reduced vertices number, the casein network expected to be weakened.

There are major two kinds of molecular interaction in a Cheddar cheese casein matrix: one is casein-casein hydrophobic attractive interaction, and the other is electrostatic repulsion (Lucey *et al.*, 2003). As maturation proceeds, the production of acid would result in solubilisation of colloidal calcium phosphate and further increasing electrostatic repulsion. This could be attributed to the decrease of cohesiveness during the maturation (Lawlor *et al.*, 2001). The other mechanism behind that is hydrophobic interactions, which are weakened by the absorption of proteins to solvate the ionic charges (Gunasekaran *et al.*, 2003a).

Batch C has the highest cohesiveness from after 90 days to 450 days ripening which indicated that batch C cheese primary proteolysis is slower than other batches (Lane *et al.*, 1997). This could be due to the higher percentage of salt in moisture in batch C Cheddar cheese. A similar effect of percentage of salt in moisture on intact β-CN has previously been shown in Cheddar cheese (Møller *et al.*, 2013). Another possible mechanism behind this phenomenon is the lower acid development of batch C. Batch C has confirmed as a batch has lower

acetic acid and lactic acid (data is shown in chapter IV and V). This indicated acid development for this batch of cheese is slower which will dissolve less calcium phosphate linkage in casein structure (Johnson *et al.*, 2006).

In summary, the most significant reduction in hardness, springiness, cohesiveness occurred during the first three months, while the most considerable change in fracturability took place after 6-9 months ripening. A gradual decrease in cohesiveness indicates that this textural attribute is more determined by the state of the protein matrix, rather than its porosity.

Table VI-2 *Hardness, fracturability, cohesiveness and springiness values as determined using texture profile analysis observed during ripening up to 450 days. The data presented as mean with a standard deviation of 24 replicates (4 replicates × 6 batches).*

	56 days	90 days	180 days	270 days	360 days	450 days
Hardness	20386 ± 1830^{d}	23434 ± 2709^{c}	24977 ± 3010^{b}	27585 ± 2828^{a}	20685 ± 1958^{d}	23942 ± 2390^{bc}
Fracturability	0 ± 0^{d}	0 ± 0^{d}	5195 ± 5350^{c}	9046 ± 4372^{ab}	8772 ± 1023^{b}	9410 ± 1319^{a}
Springiness	0.28 ± 0.03^{a}	0.25 ± 0.04^{b}	0.22 ± 0.04^{c}	0.22 ± 0.03^{c}	0.16 ± 0.01^{d}	0.21 ± 0.05^{c}
Cohesiveness	0.20 ± 0.01^{a}	0.17 ± 0.02^{b}	0.16 ± 0.02^{c}	0.14 ± 0.01^{d}	0.14 ± 0.01^{d}	0.12 ± 0.01^{e}

VI.3.3 Correlation between Cheddar cheese sensory profile, microstructure and texture profile analysis

Cohesiveness (compression to 75% at 2mm/s) is the attributes most correlated with sensory attributes among all TPA measurement, as the absolute value of Pearson correlation coefficient of cohesiveness with sensory variables is much higher than other TPA measurement parameters (Figure VI-3 and Supplementary Table 6.2). The sensory attributes that cohesiveness is most correlated with are overall flavour intensity, lingering aftertaste and tangy. Cohesiveness measured from TPA is also highly correlated with sensorial cohesiveness, which used to describe how easy to form a ball. Even though the result shows a negative correlation, they indicate a tendency of cheese to remain intact. The cheese is easier to form a ball indicating that the cheese is less resistant to breaking into pieces and reform.

Protein matrix porosity analysed from micrographs is highly correlated with most of sensory attributes; better than the TPA measured springiness, fracturability and hardness. Protein matrix porosity most correlated with flavour taste sensory attributes such as salty, overall flavour intensity, linger aftertaste, while it is negatively correlated with sensorial firmness.

As expected, sensorial crumbly and instrumental fracturability are positively correlated, and sensorial rubbery is positively correlated with springiness measured from texture profile analysis.

Surprisingly, TPA measured hardness (compression to 75% at 2mm/s) is not very well positively correlated the sensorial pressure firmness, which was also observed by Hough *et al.* (1996). The strong correlation between sensory and TPA data, when examining the hardness of cheese, was reported (Półtorak *et al.*, 2015). However, the sensorial hardness evaluated in that study is referred to in mouth hardness rather than in hand texture in this research.

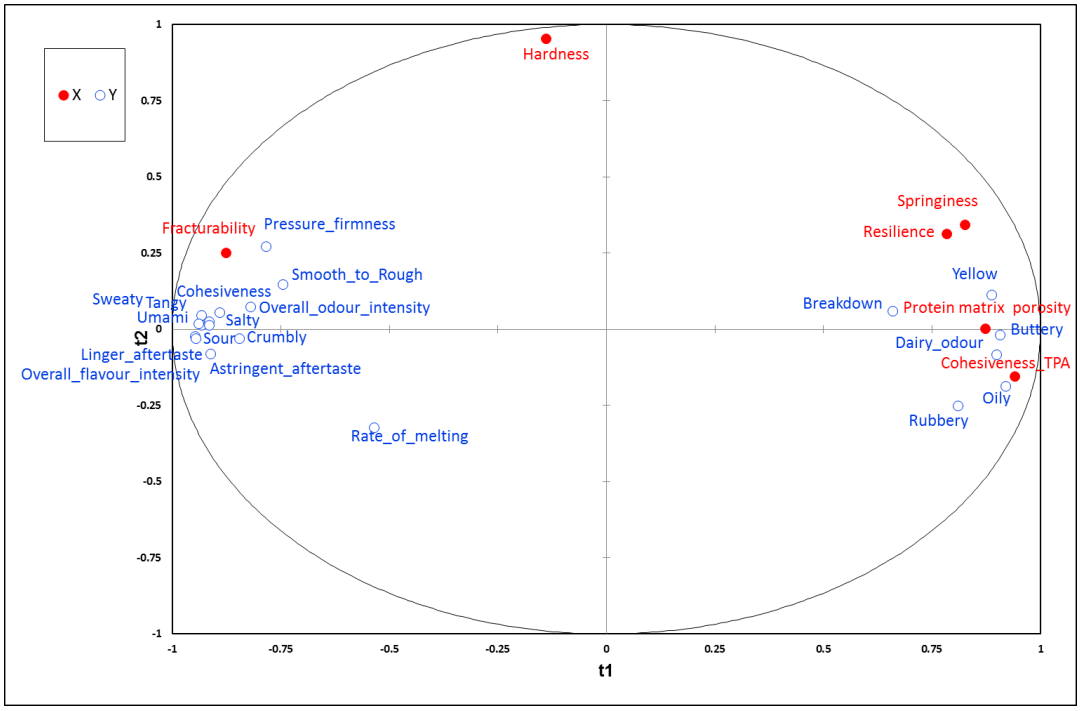


Figure VI-3 *Results of partial least squares regression analysis between the sensory attributes (Y-matrix) and texture profile analysis, microstructure image analysis (X-matrix) of 5 batches Cheddar cheese for 6 ripening time points.*

VI.4 Conclusions

Quantitative analysis of the CLSM images shows an increase of porosity of protein matrix with maturation. Textural properties such as cohesiveness and springiness could be expected to decrease with time. Batch variation among different predictive quality Cheddar cheese can only be observed from

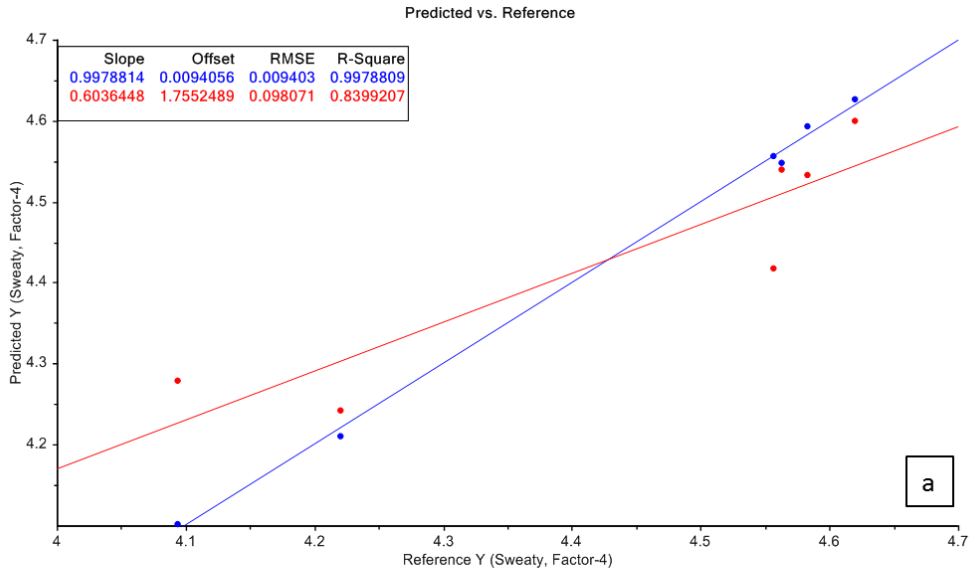
hardness, fracturability and cohesiveness perspectives. In addition, cohesiveness is the best attribute to discriminate cheese variations. Both the porosity of protein matrix and cohesiveness (compression to 75% at 2mm/s) are highly negatively correlated with overall flavour intensity, lingering aftertaste and tangy. Therefore, this work has shown an ability to correlate between structural, textural instrumental parameters and sensory profile which can be applied to evaluate the sensory properties of Cheddar cheese by means of instrumental methods during the maturation process of ripening.

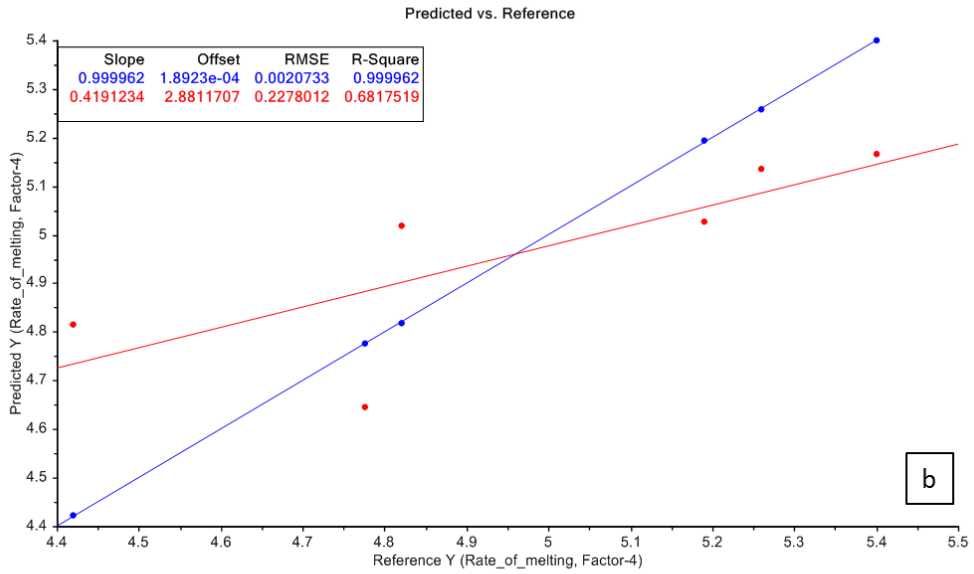
Chapter VII Summary and concluding remarks

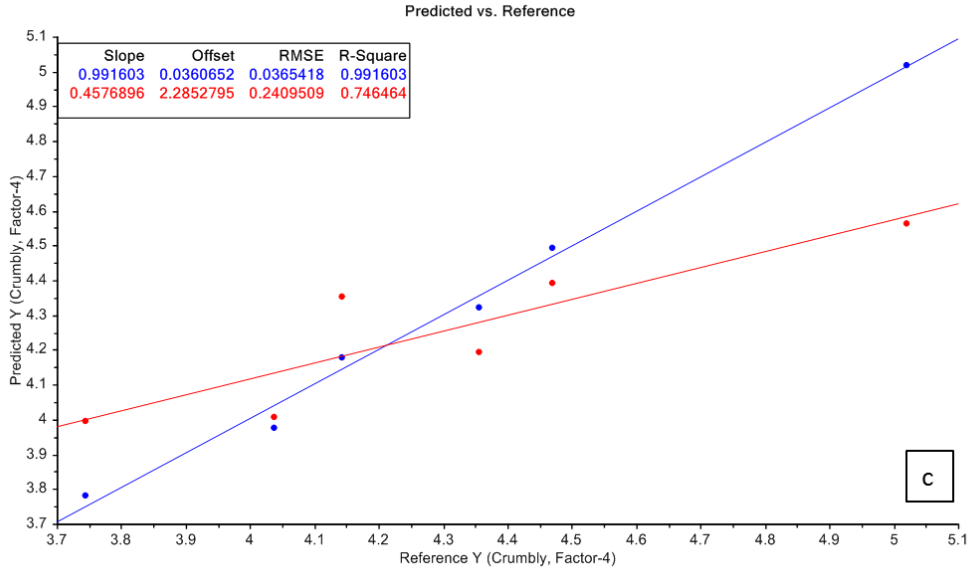
VII.1 Early prediction of sensory profiles

In quality control, predicting product quality as early as possible in the production process is of major interest in Cheddar cheese manufacturing. A broad range of analytical methods have been performed from different perspectives in previous chapters. All the instrumental analysis attributes including all the flavour attributes, metabolites, water and fat attributes, and texture and microstructure parameters measured at 56 days ripening for all batches of Cheddar cheese were used to predict the 540 days Cheddar cheese sensory profile. A preliminary predictive model was determined using Partial Least Squares regression (PLS). Validation of models and choice of the optimal number of components were carried out by cross-validation using Unscrambler (CAMO, Oslo, Norway). Correlation coefficients, R square and root mean square error of prediction (RMSE) were used to evaluate the goodness of fit of models. Only the sensory attributes sweaty, crumbly and onion flavour have a good prediction fit where R square is higher than 65% for both calibration and validation fits after 540 days ripening (Figure VII-1). The sweaty flavour attribute is the best predictor which has lowest RMSE in both calibration and validation curves. Unfortunately, some cheese defect attributes such as surface cracks, unclean aftertaste and bitter flavour can't be well predicted based on

56 days instrumental analysis. If only one measurement can be used to predict the cheese sensory profile after 540 days ripening based on 56 days cheese attributes, it would be cheese water-soluble metabolite profile measured by high-resolution NMR, since the most important loadings of factor 1 and 2 for PLS bi-plot are all from the metabolite profile. The second most important measured attributes are from the aroma profile measured by GC-MS. The sensory characteristics at the end of ripening (after 540 days ripening) were forecasted from early measurements on cheese, which could be useful for prediction of quality development during cheese maturation.







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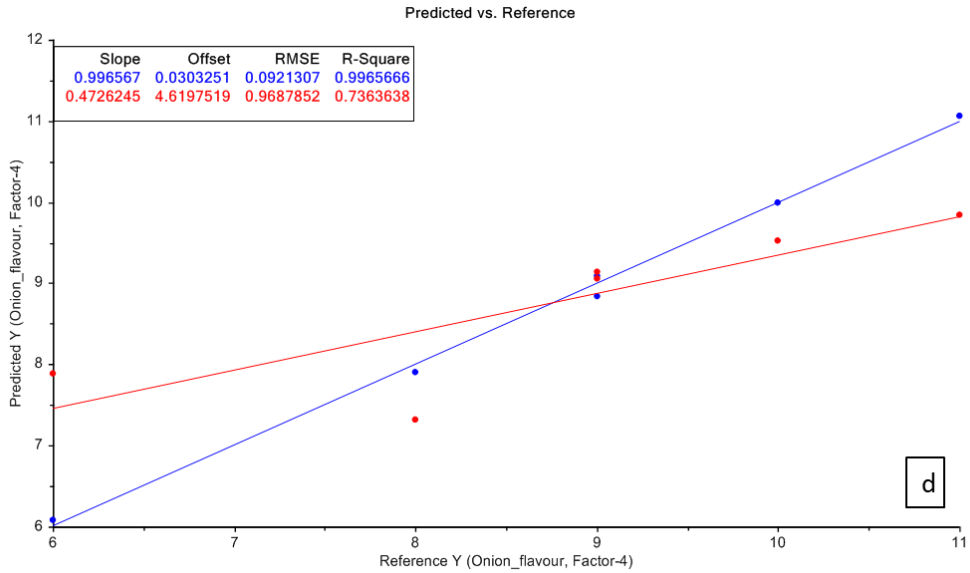

Figure VII-1 The sample models to fit the sensory data after 540 days ripening with 56 days ripening instrumental analysis results were obtained by PLS regression analysis, Only the models obtained for the sensory attributes show a good fit, with R square values for calibration and validation model higher 65%. (a) Sweaty flavour (b) rate of melting (c) crumbly (d) onion flavour fitting models are presented. Red and blue curves are validation and calibration curve respectively.

VII.2 General conclusion

Chapter II concludes that cheese is predicted to be good quality Cheddar cheese based on the compositional prediction model showing significant sensorial differences after ripening. This conclusion agreed with the collaborating dairy industry's concerns that the Gilles and Lawrence model is not sufficient enough to predict the cheese quality. Batch C and E are both predictively graded as 'graded (good) quality' cheese at early stage of ripening. However, batch C has a bland flavour, and lumpy mouthfeel while batch E has off-flavour, a bitter, dirty aftertaste and texture defect after ripening. Thus, the sensory work raised a question concerning which cheese attributes need to be considered in order to complement the composition-based grading model. All the batch to batch variations mainly occur in Cheddar cheese texture, aroma and flavour perspectives, as expected. All the work presented in this PhD thesis, as a series of submitted publications were carried out in parallel due to the nature of this project. Thus, all the perspectives of the Cheddar cheese taken into account for the grading model are based on the preliminary study on commercial cheese samples and a literature review.

In chapter III, the state of water and fat in Cheddar cheese was investigated reflecting indirect structural interactions. Proton transverse relaxation values for both the water and fat fractions decrease and the relative %age of the proton peak area, predominantly from the fat increases over a 450-day ripening period. This implied that casein-water and casein-fat interactions are

enhanced. The thermodynamic free water percentage increases during maturation. 'Downgrade' Cheddar cheese C has significant lower proton transverse relaxation values for water and higher proton predominantly from fat fraction peak area proportion from 56 days ripening until the end of maturation, stressing lower water availability for bacteria in this batch of cheese. Protons predominantly from fat fraction peak area proportion is the best ripening marker compared to the other water and fat state attributes measured in this study, as this parameter has five levels of significant statistical difference during ripening which is much higher than other parameters.

Metabolites in the aqueous extract of Cheddar cheese have been partly identified. Coupled with chemometric analysis, the ripening trajectory was observed. The ratio of citrulline and the ratio of arginine in aqueous extracts are the most important indices for assessing the ripening of Cheddar cheese, both of which decreased with ageing. In comparison to the 'premium' batch B, NMR spectra of 'downgrade' batch C revealed a higher level of serine and β-galactose as well as a lower amount of lactic acid in the aqueous extract. This indicated that batch C has less lactic acid development and could have undesirable secondary flora. A higher serine ratio is related to the sulphur volatile compounds, but such sulphur compounds were not detected in the aroma profile obtained from SPME-GC-MS. This could be due to the aroma extraction method used, not tending to extract sulphur type volatiles. The batch to batch variances in metabolic profile decrease during the ripening process which is well in line with the sensory profile, such that the taste

attributes are not significantly different in the late stage of ripening. However, a rapid maturation was noticed in another 'downgrade' cheese; batch E.

In chapter V a study of aroma profiles of all these batches Cheddar cheese allowed the observation of aroma developments in the different batches during ripening. The different volatile compounds followed different ripening profiles. Among all the volatile compounds, caproic acid is the best ripening marker which increases progressively and significantly during ripening with an abundant amount in cheese samples. Broadly speaking, the aroma profile is the best perspective to distinguish the 'downgrade' batch C and batch E from 'premium' batch B. The aroma profile showed that 'downgrade' cheeses don't have a systematic profile compared to premium cheese. Batch C and batch E aroma profiles are significantly different from each other. Bland tasting batch C has significantly lower acetic acid and secondary alcohols in the early stage ripening yet matures to be the cheese that has higher ethyl ester at later stages of ripening. However, batch E has higher secondary alcohols and 1-propanol in the early ripening stage and matures to higher levels of propyl esters. Whereas, 'premium' batch B has a significantly higher amount of acetoin and lower branched chain alcohols at the early stage of ripening. Even though batch C has a significantly higher amount of ethyl esters, the sensory panel didn't detect strong fruity flavour, from the cheese wheel data. This could be due to the batch C cheese matrix not releasing the volatiles.

Texture profile analysis coupled with confocal microscopy used to analyse structure development during ripening show that cohesiveness and

springiness, as well as protein matrix porosity decrease during ripening. This stressed that the protein matrix is coarsening, and the change of cohesiveness is not due to the porosity of casein matrix instead the state of casein. Hardness increases and reaches a peak at 270 ripening days with a subsequent decrease. Fracturability can discriminate the batch variation but can only be observed from the midpoint of the ripening process so can't be applied for prediction of quality at the earlier stages.

In summary, good ripening markers are cohesiveness measured form texture profile analysis, proton predominantly from fat fraction peak area proportion measured from TD-NMR, the normalised intensity of citrulline and arginine from metabolic profile and caproic acid measure by SPME-GC-MS. Batch to batch quality markers are proton transverse relaxation values for water, the normalised intensity of serine and β-galactose, acetic acid, secondary alcohols, 1-propanol, acetoin and branch chain alcohols. These attributes facilitate the detection of Cheddar cheese quality problems during cheese ripening.

The normalised tyrosine, tyramine, lysine ratio in cheese aqueous extracts measured by NMR, acetoin and branch chain alcohols measured by GC-MS and fracturability measured by texture profile analysis are highly correlated with a mature Cheddar cheese sensory profile. Conversely, glycerol, β-galactose springiness, protein matrix porosity and cohesiveness are associated with young Cheddar cheese. Flavour related sensory attributes are associated with 2-heptanol, caproic acid, valeric acid and octanoic acid, and texture related sensory attributes are correlated with octanoic acid, valeric acid and caproic

acid. Cohesiveness is the attribute most correlated with sensory attributes among all texture profile analysis parameters.

Finally, a preliminary model was established and suggested that measurements after 56 days ripening are possible to well predict the sensory profile after 540 days ripening.

VII.3 Which measurements can observe the batch variation?

Batch to batch variation in the same dairy is small compared to season or regional variation, therefore it is important in the choice of the analytical methods. Table VII-1 summarises the discriminatory ability of measurements and perspectives.

Table VII-1 *The discriminatory ability of measurements for ripening and batch variation has been summarised.'✓' and '✗' indicate that the measurement is and isn't able to discriminate variation respectively.*

Discriminate / Cheese Measurements	Ripening	Batch to batch
Gas chromatography-mass spectrometry (GC-MS) **(Cheese Aroma Profile)**	✓	✓
Texture Analyser **(Texture profile analysis)**	✓	✓
High-resolution NMR **(Water-soluble fraction profile)**	✓	✓
Time Domain NMR **(Proton in water and fat: migration and mobility)**	✓	✓
Thermogravimetric analysis (TGA) **(Thermal stability of cheese microstructure)**	✓	✗
Confocal microscopy **(Cheese microstructure at room temperature)**	✓	✗

VII.4 Recommendation for different types of cheese

Certain cheese batches, which appear to mature more rapidly than others, such as batch E, develop flavour and texture defects after 18 months of maturation, but doesn't show any severe defects at the early and mid-stage of ripening. For this kind of cheese, it should be sold at an early stage of maturation. Slow maturing cheese, such as batch C, does not have specific defects but lack flavour and has a younger cheese texture. If the dairy could keep these for a sufficient time, it may arguably mature into a high-quality product.

VII.5 Future work

Descriptive quantitative analysis was used to establish the sensory profile of each batch of cheese sensory. However, cheese quality is still a personalised term. Cheddar cheese sensory preference mapping can be performed to determine target ranges of intensity and limits for each sensory attribute, so that the industry is enabled in the accurate delivery of cheese characteristics to various consumers.

Water and fat proton signals in the transverse relaxation decay curves have been deconvoluted, but one signal could possibly include the protons from free serum water and fat. The determination and separation of the signal needs to be explored further.

The unknown metabolites in cheese aqueous extracts, especially unknown D, E, G and F which are highly related to sensory profiles and need to be further identified by deconvolution from the 2D spectrum.

The sensory evaluation performed in this study is a general quantitative descriptive analysis rather than texture or flavour profile specific. Some correlation between sensory profile and instrumental analysis attributes need to be validated by further experiments as well as an investigation into the mechanisms behind those.

The preliminary model derived from these studies is only based on six batches of Cheddar cheese produced on the same day in the same production facility. The model needs further validated with more batches of cheese from different

seasons to constitute higher repeatability and reproducibility of the results before routine application in the food industry. From statistical perspectives, it is essential to understand the influence of all the relevant loadings (variables) to the preliminary model which would then simplify the model.

As a limitation of this study, some measurements are destructive. Therefore, rapid and online measurements should be developed in order to optimise the analytical methods. For example, tyrosine ratio of water-soluble fraction of cheese is highly related with mature cheddar cheese sensorial profile. However, only high-resolution NMR is impossible for online application so far, therefore a new analytical method measuring the tyrosine ratio should be developed.

Microorganism activity is important for Cheddar cheese flavour and texture development during ripening. However, this project is only focus on physiochemical measurements. The further investigation of correlation between microorganism activity and physiochemical measurements should be conducted.

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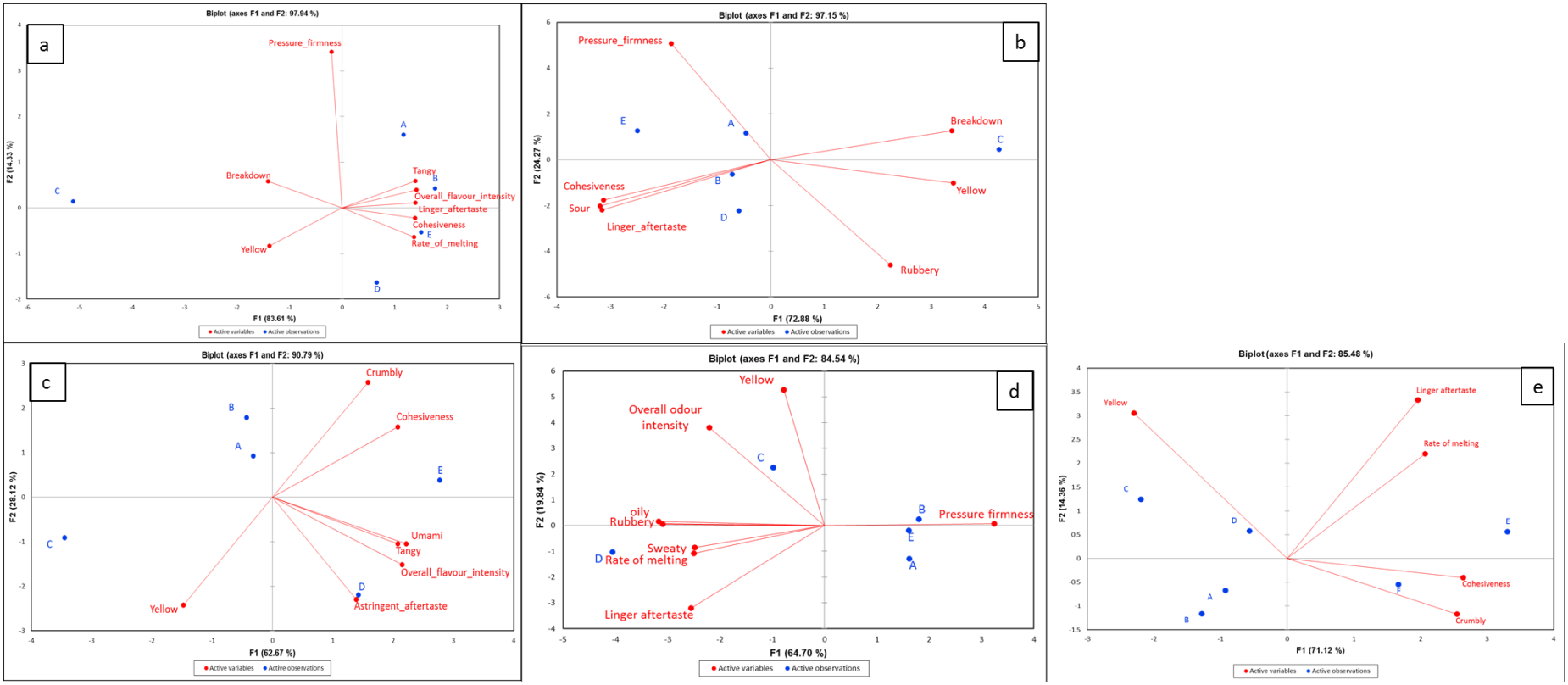
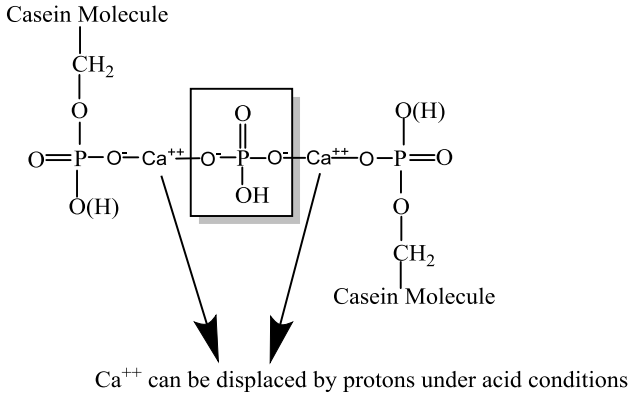
Appendices

Supplementary materials for Chapter II



Supplementary Figure 2.1 *The principal components analysis (PCA) biplot on the quantitative sensory profile of 90 days (a), 270 days, (b) 360 days(c), 450 days (d), 540days (e) ripening batches Cheddar cheese. The blue symbols stand for the batch number of different predictive quality Cheddar cheese. The red symbols stand for the different sensory attributes that significantly (Tuckey's test, P<0.05) distinguish batch variations*

Supplementary materials for Chapter III



Supplementary Figure 3.1 *The linkage involved in the binding of calcium phosphate to the casein molecule (Johnson & Sommer, 2017)*

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Supplementary Table 3.1 *Sensory attributes evaluated at 270 days after maturation that significantly distinguished batches of Cheddar cheese.*

Batch Number	Yellow	Pressure firmness	Rubbery	Cohesiveness	Breakdown	Sour	Linger aftertaste
A	3.43 ± 0.77^{b}	5.67 ± 0.96^{ab}	3.59 ± 1.26^{bc}	5.71 ± 1.21^{ab}	3.68 ± 1.18^{b}	4.48 ± 0.59^{ab}	3.68 ± 0.96^{ab}
B	3.56 ± 0.84^{b}	5.33 ± 0.99^{ab}	4.16 ± 1.37^{abc}	6.16 ± 1.18^{a}	3.37 ± 0.92^{b}	4.56 ± 0.6^{ab}	3.69 ± 1.17^{ab}
C	4.39 ± 1.03^{a}	5.15 ± 1.23^{ab}	4.55 ± 1.42^{ab}	5.05 ± 1.37^{b}	4.74 ± 1.18^{a}	4.25 ± 0.51^{b}	3.16 ± 0.95^{b}
D	3.63 ± 1.06^{b}	4.93 ± 1.36^{b}	4.63 ± 1.61^{a}	5.92 ± 1.28^{ab}	3.33 ± 1.23^{b}	4.68 ± 0.45^{a}	3.95 ± 0.83^{a}
E	3.15 ± 1.08^{b}	5.91 ± 0.97^{a}	3.27 ± 1.03^{c}	5.96 ± 1.39^{ab}	3.15 ± 0.78^{b}	4.68 ± 0.61^{a}	3.83 ± 0.93^{ab}

Data are the average of 30 judgements and errors present the standard deviation of the 30 values. Different superscripts in the same column indicate significant statistical difference (Tukey's test $P<0.05$)

Supplementary Table 3.2 *Pearson correlation coefficient between all water and fat state attributes measured by TD-NMR and sensory attributes that significantly distinguish batch variation.*

Variables	Yellow	Pressure firmness	Rubbery	Cohesiveness	Breakdown	Sour	Linger aftertaste
Proton from water fraction T_2	-0.897	0.337	-0.445	0.951	-0.993	0.970	0.930
Proton predominantly from fat fraction T_2	-0.941	0.410	-0.522	0.944	-0.997	0.957	0.938
Proton predominantly from fat fraction peak area proportion	0.670	0.084	0.042	-0.916	0.904	-0.922	-0.921

Supplementary materials for Chapter IV

Supplementary Table 4.1 *Resonance assignment of Cheddar cheese water soluble solution: ^{1}H and ^{13}C chemical shifts and multiplicity are reported based on TOCSY, HSQC experiments and literature.*

Compound	**Multiplicity**	**^{1}H shift(ppm)**	**^{13}C shift(ppm)**	**Assignment**
Acetic acid	s	1.928	25.26	α-CH_3
Lactic acid	d	1.325	22.62	β-CH_3
	q	4.124	71.11	α-CH
Pyruvic acid	s	2.35	29.46	CH_3
Formic acid	s	8.44	173.48	$HCOO^-$
Valine	d	0.980	19.17	γ'-CH_3
	d	1.033	20.48	γ-CH_3
	m	2.268	31.66	β-CH
	d	3.603	62.91	α-CH
Leucine	d	0.945	23.39	δ'-CH_3
	d	0.956	24.63	δ-CH_3
	unresolved	1.703	26.74	γ-CH
	unresolved	1.684	42.38	β'-CH_2
	unresolved	1.731	42.38	β-CH_2
	unresolved	3.728	55.97	α-CH
Isoleucine	t	0.930	13.60	δ-CH_3
	d	1.001	17.23	γ-CH_3
	m	1.463	26.87	γ-CH_2
	m	1.974	38.44	β-CH

	d	3.664	62.16	α-CH
Alanine	d	1.471	18.61	β-CH_3
	q	3.773	53.07	α-CH
Glutamic acid	m	2.056	29.43	β-CH_2, β'-CH_2
	m	2.345	35.86	γ-CH_2
	dd	3.754	57.05	α-CH
Glutamine	m	2.12	29.37	β-CH_2
	m	2.44	33.32	γ-CH_2
	t	3.75	57.01	α-CH
Methionine	unresolved	2.122	16.52	S-CH_3
	m	2.192	32.25	β-CH_2
	t	2.633	31.40	γ-CH_2
	dd	3.860	56.43	α-CH
Glycine	s	3.551	44.00	α-CH_2
Threonine	unresolved	1.32	21.3	γ-CH_3
	d	3.603	62.82	α-CH
	unresolved	4.252	68.41	β-CH
Lysine	unresolved	1.436	24.16	γ-CH_2
	unresolved	1.500	23.99	γ'-CH_2
	m	1.714	28.84	δ-CH_2
	m	1.898	32.32	β-CH_2
	t	3.010	41.62	ε-CH_2
	t	3.755	57.00	α-CH
Arginine	m	1.906	30.23	β-CH_2
	unresolved	3.237	43.07	δ, δ’ -CH_2
	unresolved	3.755	57.07	α-CH
Asparagine	dd	2.869	36.984	β-CH_2
	dd	2.9427	36.984	β'-CH_2

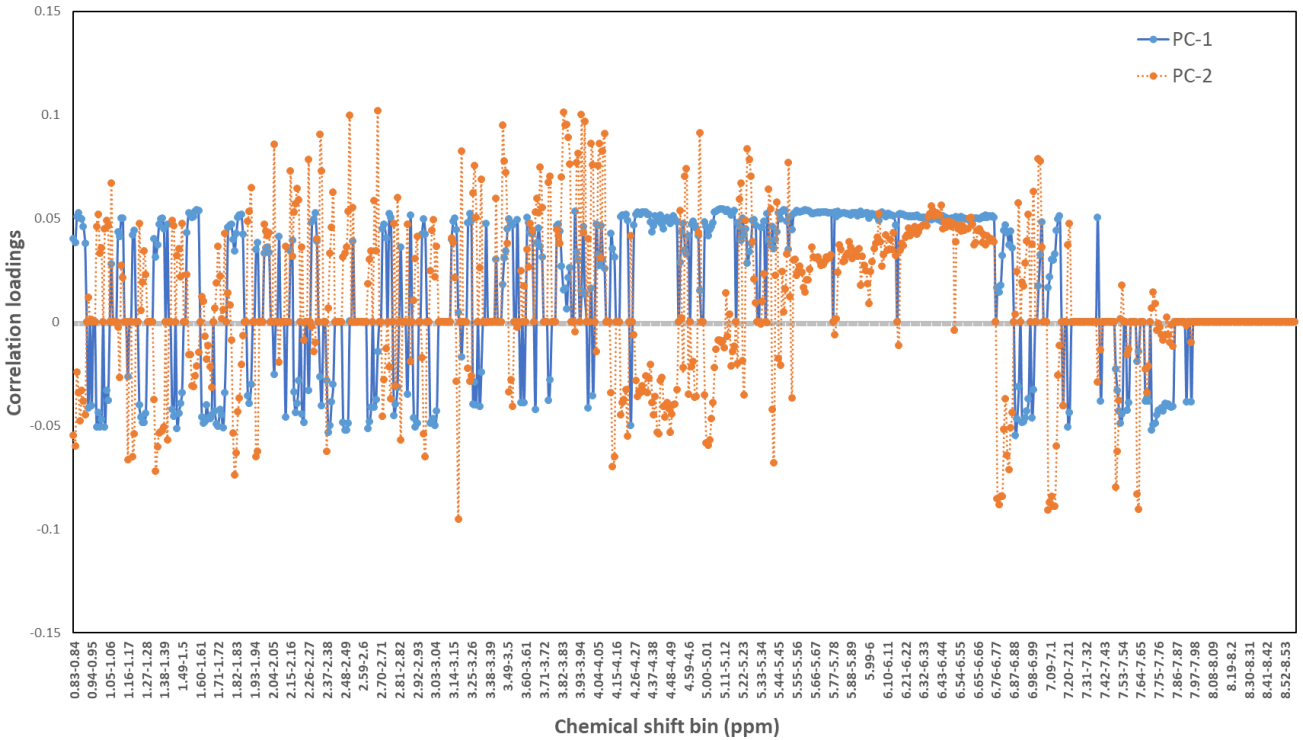
	unresolved	4.002	53.717	α-CH
Proline	m	1.996	26.47	γ-CH_2
	m	2.061	31.16	β-CH
	m	2.342	31.59	β'-CH
	m	3.330	48.54	δ-CH
	m	3.41	48.59	δ'-CH
	m	4.123	63.80	α-CH
Phenylalanine	m	3.122	38.84	β-CH_2
	m	3.279	38.83	β'-CH_2
	dd	3.989	58.50	α-CH
	m	7.310	131.81	$C_{2,6}$ ring
	m	7.417	131.69	$C_{3,5}$ ring
	m	7.367	130.11	C_4H,ring
Pyroglutamic acid	Unresolved	2.021	27.3	β-CH
	Unresolved	2.392	32.18	γ-CH
	Unresolved	2.562	27.87	β'-CH
	dd	4.167	60.82	α-CH
Tyrosine	dd	3.053	38.01	β-CH
	dd	3.188	37.98	β'-CH
	dd	3.935	58.52	α-CH
	d	6.892	118.34	2,6 ring CH
	d	7.183	133.27	3,5 ring CH
Serine	dd	3.839	58.85	α-CH
	dd	3.953	62.63	β, β′ -CH_2
Citrulline	m	1.592	27.22	γ,γ′ -CH_2
	m	1.86	30.26	β',β-CH_2
	q	3.13	41.60	δ,δ′-CH_2
	dd	3.75	57.02	α-CH

Ornithine	m	1.826	24.92	β-CH_2
	m	1.930	30.12	β-CH_2
	t	3.04	41.26	δ-CH_2
	t	3.756	56.99	α-CH
α-galactose	d	3.73	63.59	C6H_2
	m	3.78	70.25	C2H
	m	3.8435	71.759	C3H
	m	3.972	72.165	C4H
	Unresolved	4.050	73.493	C5H
	d	5.256	94.743	C1H
β-galactose	m	3.484	74.29	C2H
	m	3.645	75.11	C3H
	Unresolved	3.699	77.63	C5H
	Unresolved	3.730	63.59	C6H_2
	m	3.917	71.41	C4H
	d	4.577	98.907	C1H
Glycerol	m	3.547	65.01	CH_2
	m	3.640	64.96	CH_2
	Unresolved	3.773	74.55	CH
Tyramine	t	2.92	34.34	β-CH_2
	t	3.25	42.6	α-CH_2
	d	6.90	118.34	2,6 ring CH
	d	7.21	132.75	3,5 ring CH
Unknown A		2.798	38.96	
Unknown H		2.696	39.02	
Unknown B		1.763	32.92	

Unknown D		1.207	21.35	
Unknown E		1.208	27.06	
Unknown F		2.7653	40.119	
Unknown G		7.1388	133.09	

[a] ^{1}H chemical shifts reported with respect to DSS signal(0.00ppm).

[b] Multiplicity definitions: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet.



Supplementary Figure 4.1 *The corresponding loadings plot of principal component 1 and 2 for Figure IV-2 PCA score plot mapping on the average position of ^{1}H NMR spectra of aqueous extracts in Cheddar cheese for each maturation time. Principal component 1(PC1), Principal component 2(PC2)*

. ***Supplementary Table 4.2*** *The assignments of significant corresponding loading (bin integral) of Figure IV-2 PCA plot that has been deconvoluted.*

Chemical shift(ppm)	Compounds	Chemical shift(ppm)	Compounds
0.92-0.93	Ile_Leu	3.02-3.03	Lys
0.94-0.95	Leu_unknown signal	3.03-3.04	Lys
0.97-0.98	Val	3.04-3.05	Lys_Tyr_Orn
0.98-0.99	Val	3.05-3.06	Tyr_Orn
0.99-1	Ile	3.14-3.15	Cit_Phe
1.00-1.01	Ile	3.17-3.18	Tyr_unknown signal
1.02-1.03	Val	3.23-3.24	Arg
1.20-1.21	Unk D_UnkE	3.24-3.25	Arg
1.32-1.33	Lactic acid+Thr	3.25-3.26	Arg
1.33-1.34	Lactic acid+Thr	3.26-3.27	Phe
1.34-1.35	Lactic acid	3.27-3.28	Phe
1.35-1.36	Lactic acid	3.28-3.29	Phe
1.43-1.44	Lys	3.46-3.47	β-Gal
1.44-1.45	Ile_Lys	3.47-3.48	β-Gal
1.46-1.47	Ala_Ile	3.48-3.49	β-Gal
1.47-1.48	Ala_Ile	3.55-3.56	Gly_Glycerol
1.59-1.6	Cit	3.59-3.6	Thr_Val_unknown signal
1.67-1.68	Leu_unknown signal	3.62-3.63	Glycerol
1.69-1.7	Leu_Lys	3.63-3.64	Glycerol
1.70-1.71	Leu_Lys	3.64-3.65	β-Gal
1.71-1.72	Leu_Lys	3.66-3.67	Ile_unknown signal

1.72-1.73	Leu_Lys	3.69-3.7	β-Gal
1.73-1.74	Leu	3.74-3.75	Arg_Ala_Lys_Leu_Glu_Orn_Cit_Gl n
1.75-1.76	Unk B	3.83-3.84	Ser
1.82-1.83	Orn_unknown signal	3.91-3.92	β-Gal
1.86-1.87	Cit	3.95-3.96	Ser_unknown signal
1.90-1.91	Arg_Lys	3.96-3.97	Ser_unknown signal
1.91-1.92	Arg_Lys_Acetic acid	3.98-3.99	Phe_Tyr_Asn
2.10-2.11	Met_Gln_unkonwn signal	4.01-4.02	Phe_Tyr_Asn
2.12-2.13	Met_Gln_unkonwn signal	4.04-4.05	α-Gal
2.18-2.19	Met_unkonwn signal	4.05-4.06	α-Gal
2.19-2.2	Met_unkonwn signal	4.06-4.07	α-Gal
2.26-2.27	Val_Unknow signal	4.12-4.13	Lactic acid_Pro
2.33-2.34	Glu_Pro	4.13-4.14	Lactic acid_Pro
2.34-2.35	Glu_Pro	4.24-4.25	Thr
2.35-2.36	Pro_Pyruvic acid_Glu_unknown signal	4.57-4.58	β-Gal
2.36-2.37	Pro_Pyruvic acid_Glu_unknown signal	4.58-4.59	β-Gal
2.39-2.4	PCA	5.25-5.26	α-Gal
2.40-2.41	PCA	5.26-5.27	α-Gal
2.62-2.63	Met	6.89-6.9	Tyr_Tyramine
2.63-2.64	Met	6.90-6.91	Tyr_Tyramine
2.64-2.65	Met	7.11-7.12	Unk G
2.76-2.77	Unk_F	7.12-7.13	Unk G

2.79-2.8	Unk_A	7.13-7.14	Unk G
2.90-2.91	Tyramine	7.14-7.15	Unk G
2.91-2.92	Asn_Tyramine	7.15-7.16	Unk G
2.92-2.93	Asn_Tyramine	7.17-7.18	Tyr
2.95-2.96	Asn	7.18-7.19	Tyr
2.96-2.97	Asn	7.19-7.2	Tyr
3.00-3.01	Lys	7.20-7.21	Tyramine
3.01-3.02	Lys	7.21-7.22	Tyramine

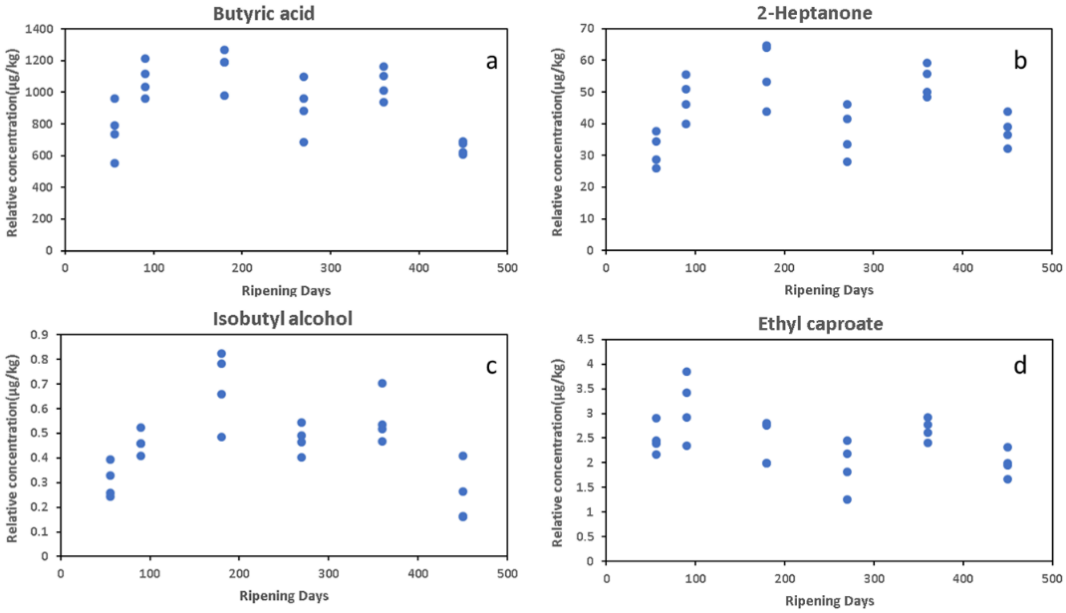
[a] Compounds definition: Ile, Isoleucine; Leu, Leucine; Val, Valine; Thr, Threonine; Lys, Lysine; Ala, Alanine; Cit, Citrulline; Arg, Arginine; Met, Methionine; Glu, Glutamic acid; Pro, Proline; PCA, Pyroglutamic acid; Asn, Asparagine; Tyr, Tyrosine; Phe, Phenylalanine; Gly, Glycine; Ser, Serine; α-Gal, α-Galactose; β-Gal, β-Galactose;

Supplementary Table 4.3 *The chemical shift regions that have assigned have positive or negative contribution to PC1 of Figure IV-2 PCA graph*

Compound Name	Related bin regions that have positive contribution to PC1 (ppm)	Positive or negative contribution to PC1
arginine	3.23-3.26	positive
asparagine	2.95-2.97	positive
citrulline	1.59-1.6; 1.86-1.87	positive
glycerol	3.62-3.64	positive
glycine and glycerol	3.55-3.56	positive
lactic acid and proline	4.12-4.14	positive
lactic acid and threonine	1.32-1.34	positive
lactic acid	1.34-1.36	positive
serine	3.83-3.84	positive
unknown G	7.11-7.16	positive
α-galactose	4.04-4.07; 2.25-2.27	positive
β-galactose	3.47-3.49; 3.64-3.65; 3.69-3.70; 3.91-3.93; 4.57-4.59	positive
isoleucine	1.43-1.44; 3.00-3.04	negative
methionine	2.62-2.65	negative
pyroglutamic acid	2.39-2.41	negative
phenylalanine	3.26-3.29	negative
threonine	4.24-4.25	negative
tyramine	2.90-2.91; 7.20-7.22	negative
valine	0.97-0.99; 1.02-1.03	negative

Supplementary materials for Chapter V

Supplementary Figure 5.1 *The reference Cheese samples are divided into six parts frozen in the -80 °C freezer and tested in every ripening time point in order to check the stability of the machine. Four replicates were run in each time point. (a) Butyric acid; (b) 2-heptanone; (c) Isobutyl alcohol; (d) ethyl caproate are presented as an example.*



Supplementary Table 5.1 *Relative concentration of key aroma compounds mean (µg/kg) of four replicates detected different predictive qualities Cheddar cheese during ripening. Different lowercase superscripts in the same row indicate significant statistical difference among the different predictive qualities' batches Cheese at same time ripening days. Different capital letter superscripts in the same column indicate significant different during maturation for that batch of cheese. (Tukey's test P<0.05).*

Compounds	Ripening days	Batch A	Batch B	Batch C	Batch D	Batch E	Batch F
Ethanol	56	2871.9^{Ab}	777.1^{Bc}	2566.2^{Bb}	2680.2^{Ab}	3928.5^{Aa}	3245.2^{Aab}
	90	224.9^{Dd}	627.2^{BCc}	1690.6^{Ca}	1134.3^{Cb}	929.1^{Bb}	334.5^{Cd}
	180	663.5^{CDcd}	664.0^{Bcd}	1332.7^{CDab}	1057.6^{Cbc}	290.9^{Dd}	1563.3^{Ba}
	270	1707^{Bbc}	2510.5^{Ab}	7079.9^{Aa}	1687.2^{Bbc}	398.6^{CDd}	1216.4^{Bcd}
	360	856.6^{Cb}	453.8^{BCc}	1587.8^{Ca}	988.0^{Cb}	698.5^{BCbc}	543.4^{Cc}
	450	365.1^{CDb}	139.2^{Cd}	767.1^{Da}	815.5^{Ca}	207.1^{Dcd}	347.9^{Cbc}
Ethyl caproate	56	8.1^{Db}	3.1^{Dd}	7.9^{Dbc}	5.8^{Dc}	13.5^{Ba}	9.5^{Bb}
	90	1.5^{Eb}	3.6^{CDb}	3.2^{Db}	2.9^{Db}	6.7^{Ca}	1.6^{Cb}
	180	3.7^{Ebc}	3.8^{CDbc}	6.4^{Db}	6.2^{Db}	2.2^{Dc}	9.6^{Ba}
	270	11.1^{Cc}	20.5^{Ab}	35^{Ca}	12.4^{Cc}	3.0^{CDd}	9.7^{Bc}
	360	27.9^{Ab}	12.8^{Bc}	44.2^{Ba}	30.4^{Bb}	31.5^{Ab}	11.2^{Bc}
	450	17.4^{Bbc}	7.2^{Cd}	56.3^{Aa}	56^{Aa}	11.6^{Bcd}	19.5^{Ab}
Ethyl octanoate	56	1.0^{Db}	0.2^{Dd}	0.6^{Cc}	0.6^{DEc}	1.6^{Ba}	1.2^{Cb}
	90	0.2^{Ec}	0.4^{Db}	0.3^{Cb}	0.4^{Eb}	0.5^{Ca}	0.2^{Dc}
	180	0.7^{DEbc}	0.8^{CDbc}	1.3^{Cb}	1.3^{Db}	0.5^{Cc}	2.1^{Ba}
	270	2.0^{Cc}	3.7^{Ab}	6.8^{Ba}	2.1^{Cc}	0.6^{Cd}	1.8^{Bc}
	360	4.6^{Ab}	2.1^{Bc}	7.1^{Ba}	4.8^{Bb}	5.4^{Ab}	1.8^{Bc}

	450	2.9^{Bbc}	1.1^{Cd}	9.3^{Aa}	9.1^{Aa}	2.0^{Bcd}	3.2^{Ab}
Lactic acid, ethyl ester	56	10.3^{Bb}	2.7^{Cd}	4.6^{Dcd}	5.7^{Bc}	14.1^{Aa}	8.6^{Bb}
	90	2.6^{Dc}	6.5^{Ba}	4.6^{Db}	7.4^{Ba}	6.9^{Ba}	3.0^{Cc}
	180	6.5^{Cbc}	6.9^{Bbc}	9.0^{Cb}	8.6^{Bb}	2.6^{Cc}	15.0^{Aa}
	270	16.6^{Ab}	25.3^{Aa}	26.0^{Aa}	17.0^{Ab}	4.2^{Cc}	13.6^{Ab}
	360	16.5^{Aa}	8.1^{Bc}	18.9^{Ba}	18.3^{Aa}	12.9^{Ab}	8.6^{Bc}
	450	6.0^{CDcd}	3.6^{Ce}	15.5^{Bb}	20.0^{Aa}	4.1^{Cde}	8.2^{Bc}
1-Propanol	56	20.4^{Db}	4.2^{Dd}	17.1^{Cbc}	14.1^{Cc}	42.8^{Ca}	18.9^{Dbc}
	90	23.9^{Dc}	39.0^{Dc}	19.2^{Cc}	110.2^{Cb}	519.4^{BCa}	16.0^{Dc}
	180	467.4^{Cbc}	259.2^{CDc}	580.3^{ABbc}	956.6^{Bab}	1335.4^{Aa}	712.1^{Cbc}
	270	1446.2^{Aa}	1416.4^{Aa}	727.5^{Ab}	1429.2^{Aa}	1043.7^{ABab}	1041.5^{ABab}
	360	1166.2^{Bbc}	814.4^{Bcd}	507.3^{Bd}	1179.4^{ABbc}	1694.3^{Aa}	1199^{Ab}
	450	1461.6^{Aa}	533.2^{BCd}	687.8^{Acd}	1493.8^{Aa}	1156^{ABb}	824.9^{BCc}
Propyl butyrate	56	1.4^{DEb}	0.3^{Cc}	1.0^{Db}	1.2^{Db}	3.6^{Ca}	1.3^{Eb}
	90	0.3^{Ec}	2.7^{Cb}	0.6^{Dc}	2.2^{Db}	9.8^{Ca}	0.2^{Ec}
	180	6.8^{Dbc}	4.0^{Cc}	9.9^{Cbc}	23.3^{Ca}	11.3^{Cb}	20.4^{Ca}
	270	18.4^{Cab}	25.4^{Aa}	21.6^{Bab}	24.0^{Ca}	7.6^{Cc}	14.2^{Dbc}
	360	49.7^{Bb}	28.3^{Ac}	24.4^{Bc}	48.2^{Bb}	115.2^{Aa}	25.6^{Bc}
	450	86^{Aab}	16.1^{Bd}	58.1^{Ac}	89.7^{Aa}	73.1^{Bb}	45.5^{Ac}
Propyl acetate	56	ND^{D}	ND^{C}	ND^{D}	ND^{D}	ND^{C}	ND^{C}
	90	ND^{D}	ND^{C}	ND^{D}	ND^{D}	27.0^{Ca}	ND^{C}
	180	22.1^{Db}	8.4^{Cb}	27.3^{Cb}	83.3^{Ca}	97.3^{Ca}	31.8^{Bb}

	270	92.1^{Ca}	65.8^{Bbc}	24.7^{Cd}	84.4^{Cab}	40.7^{Cd}	47.7^{Bcd}
	360	207.5^{Bb}	148.2^{Abc}	59.4^{Bc}	211.7^{Bb}	511.9^{Aa}	162.8^{Ab}
	450	463.2^{Aa}	54.7^{Bd}	130.3^{Ac}	266.4^{Ab}	319.7^{Bb}	164.0^{Ac}
Propyl caproate	56	ND^{D}	ND^{D}	ND^{C}	ND^{D}	ND^{C}	ND^{E}
	90	ND^{D}	ND^{D}	ND^{C}	ND^{D}	1.3^{Ca}	ND^{E}
	180	0.4^{CDc}	0.2^{Dc}	0.5^{Cbc}	1.3^{CDb}	2.3^{Ca}	0.7^{Dbc}
	270	1.5^{Cabc}	2.0^{Ca}	0.6^{Cd}	1.9^{Cab}	1.0^{Ccd}	1.3^{Cbcd}
	360	7.0^{Bb}	5.1^{Ab}	2.2^{Bb}	7.4^{Bb}	38.0^{Aa}	4.4^{Bb}
	450	16.3^{Aa}	3.2^{Bc}	9.2^{Ab}	16.9^{Aa}	15.3^{Ba}	7.3^{Ab}
Acetic acid	56	1428.9^{Ca}	1398^{Ca}	483.4^{Dd}	556.1^{Ccd}	1154.7^{Cab}	850.8^{Dbc}
	90	2409.3^{Ca}	2525.2^{Ca}	530.4^{Dd}	1316.4^{Cc}	2100.4^{BCab}	1831.8^{CDbc}
	180	3878.7^{Ba}	3930.0^{Ba}	1920.4^{Bb}	3787.5^{ABa}	4372.1^{Aa}	3537.3^{Bab}
	270	6729.6^{Aab}	6816.3^{Aa}	1255.7^{Cd}	4696.5^{Abc}	4572.8^{Ac}	4793.1^{Aabc}
	360	3972.8^{Bab}	4100.1^{Bab}	1050.4^{Cc}	3213.6^{Bb}	4624.7^{Aa}	3679.7^{Bb}
	450	4567.0^{Ba}	2383.9^{Cc}	2779.2^{Abc}	3007.5^{Bbc}	3469.5^{ABb}	2435.3^{Cc}
Propanoic acid	56	2.6^{Dab}	2.0^{Bb}	3.0^{Da}	2.5^{Cab}	3.1^{Ea}	2.2^{Eb}
	90	2.8^{Dcd}	3.9^{Bbc}	1.8^{Dd}	4.6^{Cb}	35.4^{DEa}	1.9^{Ed}
	180	34.5^{Dbc}	16.1^{Bc}	28.4^{Bbc}	66.3^{Bb}	143.4^{BCa}	45.0^{Dbc}
	270	122.8^{CBa}	93.9^{Aab}	17.9^{Cc}	83.3^{Bb}	84.1^{CDb}	68.8^{Cb}
	360	198.7^{Bb}	109.3^{Ac}	27.6^{Bd}	153.7^{Abc}	364.7^{Aa}	196.7^{Ab}
	450	275.0^{Aa}	105.0^{Ac}	53.9^{Ad}	171.9^{Ab}	201.5^{Bb}	116.3^{Bc}
Butyric acid	56	524.3^{Bab}	536.8^{Ca}	511.6^{Cab}	414.8^{Db}	578.8^{Ca}	494.0^{Eab}

	90	656.1^{Bbc}	877.3^{Ba}	566.6^{Ccd}	505.3^{CDd}	815^{BCa}	694.0^{Db}
	180	759.5^{B}a	862.0^{Ba}	698.6^{BCa}	628.4^{Ca}	835.3^{BCa}	845.2^{CDa}
	270	1251.2^{Aa}	1296.0^{Aa}	1165.7^{Aab}	822.7^{Bb}	879.4^{BCb}	1041.0^{ABab}
	360	1163.3^{Ab}	1198.7^{Ab}	796.1^{Bc}	886.0^{ABc}	1435.8^{Aa}	1183.9^{Ab}
	450	1347.1^{Aa}	933^{Bb}	1100.4^{Ab}	1033.3^{Ab}	1135.1^{ABab}	948.5B^{Cb}
Valeric acid	56	4.2^{Ca}	4.5^{Ca}	4.2^{Ea}	3.7^{Da}	4.4^{Ca}	3.7^{Da}
	90	5.2^{Cabc}	7^{BCa}	4.5^{Ebc}	4.4^{Dc}	6.2^{BCab}	4.7^{Dbc}
	180	7.0^{Ca}	8.9^{Ba}	6.4^{Da}	7.0^{Ca}	7.7^{BCa}	7.1^{Ca}
	270	13.5^{Bab}	14.6^{Aa}	12.4^{Babc}	9.9^{Bcd}	8.8^{Bd}	10.5^{Bbcd}
	360	13.2^{Bb}	14.1^{Aab}	9.2^{Cc}	10.6^{Bc}	15.9^{Aa}	12.8^{Ab}
	450	20.8^{Aa}	13.6^{Abc}	16.4^{Ab}	16.4^{Ab}	15.5^{Abc}	12.5^{Ac}
Caproic acid	56	121^{Dab}	123.5^{Ca}	90.6^{Dbc}	85.7^{Dc}	135^{Ca}	115.8^{Dabc}
	90	167.7^{CDc}	225.2^{BCb}	148.7^{Dc}	149.4^{Dc}	289.5^{BCa}	170.7^{Dc}
	180	250.8^{Ca}	310.3^{Ba}	227.6^{Ca}	249.6^{Ca}	315.2^{BCa}	295.1^{Ca}
	270	474.1^{Ba}	517.5^{Aa}	443.2^{Bab}	333.7^{Cb}	321.9^{Bb}	418.8^{Bab}
	360	558.4^{Bb}	571.4^{Ab}	391.9^{Bc}	483.0^{Bbc}	821.1^{Aa}	562.1^{Ab}
	450	930.2^{Aa}	602.0^{Ad}	751.1^{Abcd}	786.3^{Aabc}	813.8^{Aab}	628.5^{Acd}
Octanoic acid	56	15.7^{Dab}	16.8^{Ba}	9.3^{Db}	10.3^{Cab}	14.1^{Dab}	13.6^{Cab}
	90	19.2^{Db}	21.5^{Bb}	11.8^{Dc}	17.1^{Cbc}	32.5^{CDa}	15.5^{Cbc}
	180	36.7^{Ca}	50.0^{Aa}	30.5^{Ca}	37^{Ba}	50.7^{BCa}	45.0^{Ba}
	270	63.7^{Ba}	65.0^{Aa}	45.5^{Bab}	40.0^{Bb}	38.1^{CDb}	57.6^{ABab}
	360	50.7^{BCbc}	48.7^{Abc}	33.7^{BCc}	44.4^{Bbc}	85.9^{Aa}	60.3^{Ab}

	450	97.2^{Aa}	58.6^{Ab}	67.7^{Ab}	78.0^{Aab}	79.4^{ABab}	70.2^{Ab}
2-Heptanone	56	48.4^{Aa}	30.8^{Cb}	27.2^{Cb}	24.9^{BCb}	23.8^{Cb}	24.9^{Cb}
	90	32.7^{Bbc}	42.1^{ABa}	29.1^{Ccd}	23.5^{BCd}	32.1^{BCbcd}	38.1^{ABab}
	180	31.6^{Bab}	38.3^{BCa}	29.6^{Cab}	19.5^{Cb}	27.9^{BCab}	30.8^{BCab}
	270	48.2^{Aa}	47.5^{Aa}	46.5^{Aa}	30.8^{ABb}	31.5^{BCb}	40.5^{ABab}
	360	48.4^{Aa}	45.9^{ABab}	33.2^{BCc}	32.8^{Ac}	48.6^{Aa}	41.9^{Ab}
	450	53.8^{Aa}	41.0^{ABb}	39.6^{ABb}	36.7^{Ab}	39.6^{ABb}	34.9^{ABCb}
2-Nonanone	56	10.4^{Ba}	4.9^{Db}	4.0^{Db}	3.8^{Cb}	4.0^{Cb}	4.6^{Cb}
	90	5.1^{Cbc}	7.3^{CDa}	4.1^{Dc}	4.3^{Cc}	5.8^{BCb}	6.3^{BCab}
	180	6.8^{Ca}	8.9^{BCa}	6.5^{Ca}	6.0^{Ca}	6.7^{BCa}	7.9^{Ba}
	270	12.0^{ABa}	13.2^{Aa}	10.1^{ABab}	9.8^{Bab}	7.7^{Bb}	11.6^{Aa}
	360	11.8^{ABa}	11.8^{ABab}	9.0^{Bc}	10.2^{Bbc}	11.9^{Aa}	12.0^{Aa}
	450	14.5^{Aa}	11.4^{ABb}	10.7^{Ab}	13.1^{Aab}	11.6^{Aab}	11.0^{Ab}
Acetoin	56	738.3^{Ab}	1128.3^{Aa}	722.5^{Ab}	685.7^{Abc}	596.7^{Abc}	513.9^{Bc}
	90	539.5^{Bbc}	498.0^{Bc}	732.1^{Aa}	266.7^{Bd}	143.4^{Bd}	656.3^{Aab}
	180	67.7^{CDbc}	143.1^{Ca}	82.5^{Cb}	11.9^{Cd}	53.0^{CDbc}	42.3^{Ccd}
	270	156.4^{Cb}	149.7^{Cb}	439.3^{Ba}	72.9^{Cc}	111.9^{BCbc}	78.6^{Cc}
	360	39.4^{Dc}	67.5^{Cb}	90.4^{Ca}	16.5^{Cd}	40.7^{CDc}	27.3^{Ccd}
	450	14^{Db}	21^{Ca}	22.6^{Ca}	12.5^{Cbc}	9.2^{Dcd}	7.7^{Cd}
Isobutyl alcohol	56	18.0^{Ab}	16.5^{Ab}	40.4^{Aa}	46.2^{Aa}	26.1^{Ab}	24.7^{Ab}
	90	0.7^{Bd}	2.4^{BCbc}	7.7^{Ca}	3.0^{Bb}	1.8^{Bc}	1.0^{Bd}
	180	1.5^{Bc}	1.8^{BCc}	5.3^{Ca}	2.5^{Bbc}	1.0^{Bc}	3.8^{Bab}

	270	3.4^{Bcd}	6.9^{Bb}	27.8^{Ba}	4.9^{Bbc}	1.0^{Bd}	3.0^{Bcd}
	360	2.9^{Bcd}	1.5^{Ce}	8.8^{Ca}	4.6^{Bb}	3.3^{Bbc}	1.7^{Bde}
	450	2.3^{Bb}	0.9^{Cc}	4.8^{Ca}	5.4^{Ba}	1.6^{Bbc}	2.1^{Bb}
Isopentyl alcohol	56	577.8^{Ac}	257.1^{Ad}	647.6^{Abc}	1034.6^{Aa}	882.7^{Aab}	792.2^{Aabc}
	90	4.8^{Cd}	92.4^{Cb}	138.3^{Ca}	68.0^{Bc}	58.5^{Bc}	13.3^{Bd}
	180	44.9^{BCcd}	44.5^{CDcd}	91.1^{Cab}	77.0^{Bbc}	6.9^{Bd}	123.0^{Ba}
	270	90.6^{Bc}	163.3^{Bb}	365.8^{Ba}	98.4^{Bc}	15.1^{Bd}	75.3^{Bc}
	360	86.6^{Bb}	38.0^{Dc}	122.1^{Ca}	110.8^{Ba}	73.1^{Bb}	28.5^{Bc}
	450	19.1^{BCc}	7.0^{Dd}	84.5^{Ca}	73.4^{Ba}	10.8^{Bcd}	39.7^{Bb}
2-Butanol	56	2.2^{Db}	1.5^{Db}	3.3^{Db}	1.4^{Cb}	45.0^{Ba}	3.6^{Cb}
	90	37.5^{Dc}	102.4^{Dc}	28.9^{Dc}	632.4^{Cb}	2439.2^{Aa}	29.9^{Cc}
	180	2009.8^{Ca}	1748.6^{Ca}	2058.3^{BCa}	3042.3^{Ba}	3243.5^{Aa}	2937.7^{Ba}
	270	4704.5^{Aab}	6164.4^{Aa}	2489.9^{Bd}	4038.7^{Abc}	3074.7^{Acd}	4048.5^{Abc}
	360	3446.7^{Ba}	3258.4^{Ba}	1721.1^{Cb}	2902.9^{Ba}	3510.3^{Aa}	3371.4^{ABa}
	450	4280.6^{ABa}	2057.0^{Cc}	3601.0^{Aab}	3352.1^{ABb}	3069.3^{Ab}	3015.2^{Bb}
2-Heptanol	56	0.7^{Dab}	0.4^{Cbc}	0.3^{Dc}	0.3^{Dc}	0.9^{Ca}	0.7^{Eab}
	90	0.2^{Dbc}	0.3^{Cbc}	0.2^{Dc}	0.5^{Db}	2.3^{Ca}	0.1^{Ec}
	180	1.0^{Dc}	1.3^{Cbc}	1.8^{Dbc}	8.0^{Ca}	6.6^{Ba}	3.8^{Db}
	270	3.3^{Cc}	7.0^{Ba}	4.8^{Cbc}	6.9^{Ca}	3.5^{Cbc}	5.4^{Cab}
	360	7.4^{Bd}	9.7^{Abc}	8.5^{Bcd}	11.0^{Bab}	12.5^{Aa}	11.3^{Bab}
	450	17.1^{Aa}	10.8^{Ad}	12.7^{Acd}	16.0^{Aab}	14.1^{Abc}	15.5^{Aabc}
1-Butanol	56	2.1^{DEa}	1.8^{Ba}	1.7^{Ba}	2.2^{Ca}	2.2^{Ca}	1.8^{Ea}

90	1.2^{Ec}	2.1^{Bb}	1.1^{Bc}	1.8^{Cbc}	2.9^{Ca}	1.7^{Ebc}
180	6.8^{Db}	5.3^{Bb}	4.8^{Bb}	8.9^{Bab}	13.2^{Ba}	7.3^{Db}
270	16.7^{Ca}	19.3^{Aa}	14.0^{Aa}	16.1^{Aa}	12.8^{Ba}	13.0^{Ca}
360	23.1^{Bbc}	18.4^{Ac}	4.4^{Bd}	19.2^{Abc}	33.0^{Aa}	25.9^{Aab}
450	33.5^{Aa}	15.0^{Ad}	16.7^{Acd}	20.0^{Acd}	26.6^{Ab}	21.8^{Bbc}

Supplementary table 5.2 *Relative concentration of key aroma compounds mean (µg/kg) of (four replicates × six batches) cheese samples from different ripening time points. Different lowercase superscripts in the same row indicate significant statistical difference among the different ripening time (Tukey's test P<0.05).*

	56 Days	90 Days	180 Days	270 Days	360 Days	450 Days
Ethanol	2678.198 ^{a}	823.430 ^{c}	928.654 ^{c}	2433.262 ^{b}	854.668 ^{c}	440.332 ^{d}
Ethyl caproate	7.993 ^{d}	3.243 ^{f}	5.310 ^{e}	15.290 ^{c}	26.322 ^{b}	27.994 ^{a}
Ethyl octanoate	0.865 ^{d}	0.329 ^{e}	1.111 ^{d}	2.832 ^{c}	4.297 ^{b}	4.620 ^{a}
Lactic acid ethyl ester	7.690 ^{d}	5.170 ^{e}	8.090 ^{d}	17.124 ^{a}	13.872 ^{b}	9.568 ^{c}
1-Propanol	19.578 ^{d}	121.292 ^{d}	718.467 ^{c}	1184.073 ^{a}	1093.433 ^{ab}	1026.207 ^{b}
Propyl butyrate	1.456 ^{e}	2.631 ^{e}	12.623 ^{d}	18.540 ^{c}	48.590 ^{b}	61.416 ^{a}
Propyl acetate	0.000 ^{c}	4.502 ^{c}	45.015 ^{b}	59.231 ^{b}	216.910 a	233.065 ^{a}
Propyl caproate	0.000 ^{c}	0.220 ^{c}	0.904 ^{bc}	1.411 ^{b}	10.687 ^{a}	11.360 ^{a}
Acetic acid	978.655 ^{e}	1785.567 ^{d}	3571.025 ^{b}	4810.654 ^{a}	3440.210 ^{bc}	3107.071 ^{c}
Propanoic acid	2.581 ^{e}	8.402 ^{e}	55.622 ^{d}	78.470 ^{c}	175.120 ^{a}	153.929 ^{b}
Butyric acid	510.070 ^{c}	685.699 ^{b}	771.496 ^{b}	1076.022 ^{a}	1110.613 a	1082.909 a
Valeric acid	4.115 ^{f}	5.345 ^{e}	7.363 ^{d}	11.630 ^{c}	12.616 ^{b}	15.861 ^{a}
Caproic acid	111.942 ^{f}	191.868 ^{e}	274.763 ^{d}	418.206 ^{c}	564.639 ^{b}	751.962 ^{a}
Octanoic acid	13.304 ^{d}	19.598 ^{d}	41.661 ^{c}	51.672 ^{b}	53.938 ^{b}	75.174 ^{a}
2-Heptanone	29.982 ^{b}	32.907 ^{b}	29.624 ^{b}	40.853 ^{a}	41.798 ^{a}	40.935 ^{a}
2-Nonanone	5.274 ^{d}	5.493 ^{d}	7.134 ^{c}	10.736 ^{b}	11.137 ^{ab}	12.055 ^{a}
Acetoin	730.888 ^{a}	472.673 ^{b}	66.750 ^{d}	168.145 ^{c}	46.969 ^{de}	14.505 ^{e}

Isobutyl alcohol	28.650 [a]	2.765 [c]	2.651 [c]	7.846 [b]	3.818 [c]	2.850 [c]
Isopentyl alcohol	698.657 [a]	62.539 [c]	64.561 [c]	134.746 [b]	76.491 [c]	39.074 [c]
2-Butanol	9.491 [e]	545.041 [d]	2506.694 [c]	4086.765 [a]	3035.135 [b]	3229.176 [b]
2-Heptanol	0.554 [e]	0.609 [e]	3.751 [d]	5.157 [c]	10.090 [b]	14.360 [a]
1-Butanol	1.967 [d]	1.812 [d]	7.705 [c]	15.311 [b]	20.673 [a]	22.244 [a]

Supplementary table 5.3 *Relative concentration of key aroma compounds mean (µg/kg) of (four replicates × six ripening time point) cheese samples from different predictive qualities Cheddar cheese during ripening. Different lowercase superscripts in the same row indicate significant statistical difference among the different predictive qualities' batches Cheese during whole ripening process (Tukey's test P<0.05).*

Aroma Compound	A	B	C	D	E	F
Ethanol	1114.861 ^{c}	861.957 ^{d}	2504.025 ^{a}	1393.811 ^{b}	1075.443 ^{c}	1208.446 ^{bc}
Ethyl caproate	11.609 ^{c}	8.507 ^{d}	25.491 ^{a}	18.943 ^{b}	11.414 ^{c}	10.188 ^{c}
Ethyl octanoate	1.902 ^{c}	1.364 ^{d}	4.244 ^{a}	3.058 ^{b}	1.769 ^{c}	1.716 ^{c}
Lactic acid ethyl ester	9.759 ^{b}	8.856 ^{b}	13.108 ^{a}	12.837 ^{a}	7.466 ^{c}	9.487 ^{b}
1-Propanol	764.280 ^{bc}	511.057 ^{de}	423.195 ^{e}	863.862 ^{ab}	965.260 ^{a}	635.397 ^{cd}
Propyl butyrate	27.108 ^{c}	12.785 ^{e}	19.274 ^{d}	31.429 ^{b}	36.781 ^{a}	17.879 ^{d}
Propyl acetate	130.808 ^{b}	46.180 ^{e}	40.281 ^{e}	107.650 ^{c}	166.101 ^{a}	67.702 ^{d}
Propyl caproate	4.198 ^{b}	1.771 ^{c}	2.088 ^{c}	4.581 ^{b}	9.658 ^{a}	2.286 ^{c}
Acetic acid	3831.054 ^{a}	3525.597 ^{ab}	1336.590 ^{d}	2762.907 ^{c}	3382.368 ^{b}	2854.665 ^{c}
Propanoic acid	106.071 ^{b}	55.045 ^{d}	22.103 ^{e}	80.378 ^{c}	138.698 ^{a}	71.827 ^{c}
Butyric acid	950.260 ^{a}	950.650 ^{a}	806.483 ^{b}	715.085 ^{c}	946.564 ^{a}	867.767 ^{ab}
Valeric acid	10.652 ^{a}	10.452 ^{a}	8.843 ^{bc}	8.665 ^{c}	9.759 ^{ab}	8.559 ^{c}
Caproic acid	417.036 ^{ab}	391.654 ^{bc}	342.187 ^{d}	347.937 ^{d}	449.389 ^{a}	365.176 ^{cd}
Octanoic acid	47.201 ^{a}	43.436 ^{ab}	33.077 ^{c}	37.812 ^{bc}	50.141 ^{a}	43.680 ^{ab}
2-Heptanone	43.848 ^{a}	40.925 ^{a}	34.200 ^{b}	28.029 ^{c}	33.911 ^{b}	35.185 ^{b}
2-Nonanone	10.102 ^{a}	9.560 ^{ab}	7.430 ^{c}	7.875 ^{c}	7.961 ^{c}	8.901 ^{b}

Acetoin	259.203 [b]	334.604 [a]	348.247 [a]	177.702 [d]	159.161 [d]	221.012 [c]
Isobutyl alcohol	4.796 [c]	5.001 [c]	15.823 [a]	11.104 [b]	5.820 [c]	6.037 [c]
Isopentyl alcohol	137.307 [bc]	100.372 [c]	241.563 [a]	243.681 [a]	174.494 [b]	178.650 [b]
2-Butanol	2413.526 [a]	2222.053 [a]	1650.400 [b]	2328.265 [a]	2563.682 [a]	2234.375 [a]
2-Heptanol	4.942 [c]	4.928 [c]	4.696 [c]	7.135 [a]	6.678 [ab]	6.142 [b]
1-Butanol	13.886 [ab]	10.312 [c]	7.115 [d]	11.371 [c]	15.119 [a]	11.909 [bc]

Supplementary Table 5.4 *Pearson correlation coefficient between all aroma compounds attributes measured by GC-MS and sensory attributes of all six ripening time points for all batches of Cheddar cheese.*

Variables	Yellow	Smooth_to_Rough	Pressure_firmness	Rubbery	Crumbly	Cohesiveness	Oily	Overall_odour_intensity	Dairy_odour	Rate_of_melting	Breakdown	Salty	Umami	Sour	Tangy	Overall_flavour_intensity	Buttery	Sweaty	Astringent_aftertaste	Linger_aftertaste
Ethanol	0.35	-0.21	-0.30	0.32	-0.41	-0.31	0.20	-0.31	0.31	-0.09	0.42	-0.27	-0.27	-0.33	-0.34	-0.35	0.26	-0.30	-0.39	-0.35
Propyl acetate	-0.66	0.58	0.57	-0.57	0.70	0.61	-0.62	0.60	-0.69	0.29	-0.38	0.68	0.72	0.64	0.75	0.71	-0.67	0.72	0.73	0.74
2-Butanol	-0.67	0.67	0.67	-0.69	0.62	0.79	-0.85	0.62	-0.73	0.37	-0.56	0.76	0.81	0.76	0.78	0.76	-0.73	0.74	0.70	0.76
1-Propanol	-0.73	0.69	0.66	-0.68	0.67	0.78	-0.83	0.65	-0.74	0.40	-0.55	0.78	0.84	0.77	0.82	0.79	-0.75	0.78	0.76	0.81
Isobutyl alcohol	0.42	-0.32	-0.45	0.43	-0.42	-0.54	0.46	-0.39	0.43	-0.35	0.65	-0.51	-0.51	-0.63	-0.52	-0.58	0.43	-0.46	-0.54	-0.52
Propyl butyrate	-0.67	0.59	0.58	-0.58	0.72	0.63	-0.64	0.71	-0.74	0.33	-0.39	0.75	0.76	0.69	0.79	0.76	-0.72	0.78	0.78	0.79
1-Butanol	-0.79	0.71	0.73	-0.73	0.81	0.79	-0.84	0.73	-0.83	0.37	-0.48	0.81	0.86	0.78	0.87	0.83	-0.81	0.85	0.81	0.85
2-Heptanone	-0.42	0.39	0.43	-0.42	0.46	0.44	-0.51	0.38	-0.50	0.25	-0.20	0.44	0.47	0.41	0.48	0.47	-0.44	0.48	0.39	0.45
Isopentyl alcohol	0.37	-0.42	-0.50	0.47	-0.46	-0.48	0.52	-0.42	0.46	-0.22	0.49	-0.52	-0.52	-0.57	-0.52	-0.56	0.48	-0.49	-0.53	-0.52
Ethyl caproate	-0.46	0.47	0.37	-0.38	0.50	0.42	-0.50	0.67	-0.61	0.38	-0.17	0.67	0.60	0.52	0.60	0.60	-0.61	0.65	0.61	0.61
Acetoin	0.70	-0.67	-0.76	0.76	-0.72	-0.80	0.81	-0.68	0.74	-0.38	0.64	-0.84	-0.84	-0.86	-0.82	-0.84	0.78	-0.78	-0.82	-0.82
2-Heptanol	-0.80	0.77	0.72	-0.74	0.85	0.75	-0.79	0.86	-0.88	0.33	-0.50	0.87	0.87	0.80	0.89	0.88	-0.86	0.91	0.91	0.91
Propyl caproate	-0.56	0.43	0.47	-0.46	0.63	0.50	-0.50	0.56	-0.59	0.28	-0.31	0.60	0.61	0.56	0.64	0.61	-0.55	0.61	0.62	0.63
Lactic acid ethyl ester	-0.24	0.20	0.15	-0.15	0.13	0.33	-0.41	0.31	-0.30	0.48	-0.15	0.45	0.43	0.39	0.37	0.38	-0.36	0.38	0.31	0.37
2-Nonanone	-0.73	0.67	0.69	-0.70	0.72	0.75	-0.81	0.73	-0.80	0.40	-0.48	0.78	0.81	0.75	0.81	0.81	-0.77	0.82	0.76	0.81
Ethyl octanoate	-0.47	0.51	0.42	-0.42	0.52	0.46	-0.55	0.69	-0.63	0.39	-0.20	0.70	0.64	0.56	0.63	0.63	-0.65	0.67	0.63	0.64

Acetic acid	-0.61	0.46	0.57	-0.59	0.46	0.71	-0.69	0.41	-0.54	0.35	-0.61	0.56	0.64	0.65	0.62	0.62	-0.54	0.54	0.54	0.60
Propanoic acid	-0.73	0.61	0.63	-0.63	0.75	0.68	-0.70	0.62	-0.71	0.34	-0.44	0.70	0.76	0.69	0.79	0.74	-0.69	0.74	0.73	0.76
Butyric acid	-0.70	0.63	0.70	-0.69	0.71	0.76	-0.83	0.64	-0.76	0.41	-0.48	0.77	0.80	0.74	0.78	0.78	-0.74	0.75	0.72	0.77
Valeric acid	-0.78	0.76	0.79	-0.79	0.81	0.81	-0.86	0.81	-0.89	0.34	-0.50	0.85	0.88	0.80	0.88	0.87	-0.85	0.89	0.85	0.88
Caproic acid	-0.79	0.74	0.78	-0.78	0.85	0.79	-0.83	0.84	-0.90	0.34	-0.51	0.86	0.87	0.81	0.89	0.88	-0.85	0.89	0.88	0.90
Octanoic acid	-0.80	0.77	0.83	-0.83	0.80	0.84	-0.85	0.79	-0.87	0.28	-0.57	0.84	0.88	0.82	0.88	0.87	-0.83	0.87	0.86	0.88

Supplementary materials for Chapter VI

Supplementary Table 6.1 *Hardness values of different predictive quality Cheddar cheese, namely batch A, B, C, D, E, F was determined using texture profile analysis during ripening up to 450 days. The data presented as mean with a standard deviation of 4 replicates*.

Hardness	Batch A	Batch B	Batch C	Batch D	Batch E	Batch F
56 days	21107 ± 901^{ab}	20333 ± 1076^{b}	22994 ± 1016^{a}	19984 ± 1328^{bc}	19988 ± 988^{bc}	17908 ± 1139^{c}
90 days	23669 ± 2079^{b}	20860 ± 1802^{b}	27943 ± 1450^{a}	22054 ± 1424^{b}	22507 ± 1360^{b}	23574 ± 1552^{b}
180 days	27807 ± 2756^{a}	22337 ± 1618^{c}	28766 ± 975^{a}	22495 ± 843^{bc}	25656 ± 1359^{ab}	22799 ± 1031^{bc}
270 days	28549 ± 2289^{a}	27710 ± 2506^{a}	28231 ± 893^{a}	29475 ± 2331^{a}	28946 ± 605^{a}	22975 ± 2256^{b}
360 days	22456 ± 793^{ab}	20409 ± 1490^{abc}	20171 ± 1532^{bc}	18548 ± 1732^{c}	22894 ± 1144^{a}	19635 ± 862^{c}
450 days	24546 ± 3259^{a}	25398 ± 2589^{a}	25178 ± 2137^{a}	22956 ± 766^{a}	21716 ± 2066^{a}	23858 ± 1499^{a}

Supplementary Table 6.2 *Pearson correlation coefficient between texture profile analysis, microstructure image analysis variable and sensorial attributes*

Sensory attributes	Hardness	Fracturability	Springiness	Cohesiveness	Protein matrix porosity
Yellow	0.031	-0.806	0.685	0.853	0.730
Smooth_to_Rough	0.246	0.656	-0.588	-0.752	-0.664
Pressure_firmness	0.345	0.715	-0.509	-0.830	-0.682
Rubbery	-0.321	-0.758	0.523	0.838	0.716
Crumbly	0.044	0.732	-0.623	-0.834	-0.782
Cohesiveness	0.167	0.764	-0.667	-0.873	-0.770
Oily	-0.322	-0.841	0.712	0.888	0.794
Overall_odour_intensity	0.115	0.740	-0.506	-0.813	-0.785
Dairy_odour	-0.157	-0.815	0.611	0.864	0.845
Rate_of_melting	-0.216	0.386	-0.561	-0.388	-0.425
Breakdown	-0.010	-0.521	0.492	0.642	0.555
Salty	0.138	0.789	-0.725	-0.882	-0.872
Umami	0.184	0.807	-0.743	-0.884	-0.853
Sour	0.153	0.760	-0.711	-0.879	-0.820
Tangy	0.136	0.808	-0.718	-0.905	-0.857
Overall_flavour_intensity	0.094	0.789	-0.735	-0.912	-0.855
Buttery	-0.145	-0.787	0.724	0.842	0.821
Sweaty	0.115	0.770	-0.676	-0.890	-0.838
Astringent_aftertaste	0.018	0.742	-0.705	-0.879	-0.820
Linger_aftertaste	0.076	0.806	-0.722	-0.906	-0.853