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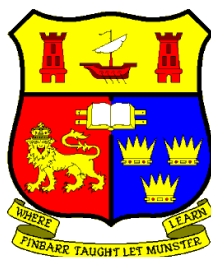
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Factors affecting the quality of low-fat cheeses

Thesis presented by

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for the degree of

Doctor of Philosophy

in

Food Science and Technology

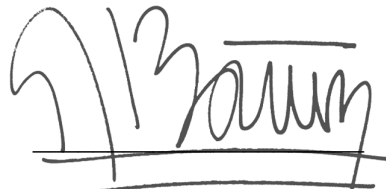
September, 2016

DECLARATION BY THE CANDIDATE

Factors affecting the quality of low-fat cheeses

Rodrigo Ignacio Ibáñez Alfaro

I hereby declare that the work described in this thesis is my own and has not been submitted for another degree, either in University College Cork or elsewhere.



Rodrigo Ignacio Ibáñez Alfaro

September 2016

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SUMMARY

Consumption of cheeses with reduced fat content has experienced significant growth due to health concerns. However, these are associated with defects in appearance, texture and flavour. Strategies developed to improve their properties involve addition of ingredients or modification of cheesemaking protocols. This thesis aimed to evaluate factors affecting the quality of low fat cheeses in terms of (i) optical properties; (ii) use of fat replacers; and (iii) a new approach to improve their properties involving controlling the lactose to casein ratio.

We studied the effect of heating and cooling on the properties of Cheddar cheeses varying in levels of fat. Cheeses with low fat content were translucent and those with high content were opaque. At high temperatures cheeses developed similar levels of opaqueness, independent of the fat content, due to increased hydrophobic interactions between proteins. This was reversed when cheeses were cooled. During ripening, cheeses became more translucent.

Translucency and rheology were studied at high temperatures and different holding times in low-fat cheeses with varying levels of total and insoluble calcium. Increasing temperature and holding times reduced translucency. Excessive heat treatments led to slight increase in translucency. Cheese appearance was affected by heat-induced colloidal calcium phosphate. Increasing of heating time increased cheese stiffness, which was attributed to the formation of heat-induced calcium structures.

The effects of levels of titanium dioxide, annatto and homogenisation pressure on the properties of reduced-fat Cheddar cheese were evaluated. Titanium dioxide led to cheeses with reduced translucency and an opposite trend was observed in those cheeses made with annatto. Homogenisation of cheesemilk only led to an increase in whiteness. During ripening, all treatments were more translucent. CIELAB was highly correlated with Kubelka-Munk analysis, excepting in homogenisation treatment.

Supplementing cheesemilks with three different types of pectins (amidated, high methoxy and low methoxy) were investigated in the properties of reduced-fat cheese. Increased moisture content and reduced insoluble calcium in cheeses made with amidated and low methoxy pectins, led to improved texture and melting. Cheese made with amidated pectin showed increased proteolysis at the first stages of ripening.

Reducing the lactose to casein ratio in low and reduced fat Gouda cheeses by ultrafiltration was proposed as an alternative method to replace whey dilution step to control acid development. Cheeses made with lactose standardization of cheesemilk had higher pH values, lower levels of lactic, residual lactose and residual galactose and insoluble calcium. High variability was found in treatments made with whey dilution. Texture was softer and melting was improved with lactose standardization treatment. Sensory panelist found that cheeses made with lactose standardization were less acidic.

TABLE OF CONTENTS

Declaration by the candidate	i
Acknowledgements	ii
Summary	iv
Literature Review	
Chapter 1 Factors affecting the quality of low-fat cheeses	1
1.1 Introduction	2
1.2 Legal definitions of cheeses with reduced fat content	3
1.3 General properties of low-fat cheeses	5
1.4. Strategies to improve properties of low fat cheeses	21
1.5 Conclusions	35
1.6 References	36
Experimental Section	
Chapter 2 Effect of fat content and temperature on the translucency of Cheddar cheese	52
2.1 Abstract	53
2.2. Introduction	54
2.3 Materials and methods	56
2.4 Results and discussion	63
2.5 Conclusions	83
2.6 Acknowledgments	84
2.7 References	85

Chapter 3	Influence of calcium content on the translucency and rheological properties of low-fat Mozzarella cheese heated at high temperatures for different holding times	90
3.1	Abstract	91
3.2	Introduction	93
3.3	Materials and methods	95
3.4	Results	101
3.5	Discussion	115
3.6	Conclusions	125
3.7	Acknowledgements	126
3.8	References	127
Chapter 4	Effect of titanium dioxide, annatto and homogenisation on the translucency of reduced-fat Cheddar cheese	132
4.1	Abstract	133
4.2	Introduction	134
4.3	Materials and methods	136
4.4	Results	142
4.5	Discussion	159
4.6	Conclusions	167
4.7	Acknowledgements	168
4.8	References	168
Chapter 5	Effect of pectin on composition, microbiology, texture and functionality of reduced-fat Cheddar cheese	172
5.1	Abstract	173
5.2.	Introduction	174
5.3.	Materials and methods	176

5.4. Results	183
5.5. Discussion	200
5.6. Conclusions	208
5.7 Acknowledgements	208
5.8 References	209
Chapter 6 Low and reduced fat Gouda-style cheese: Comparison of lactose standardization of cheese milk and whey dilution techniques	215
6.1 Abstract	216
6.2 Introduction	218
6.3 Materials and methods	221
6.4 Results	231
6.5 Discussion	246
6.6 Conclusions	257
6.7 Acknowledgements	258
6.8 References	259
Chapter 7 Conclusions and recommendations	266
Appendix (Publications)	276
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LIST OF TABLES

Table 1.1 Legal definitions used to claim cheeses with reduced fat content.	Page 4
Table 1.2 Mechanical characteristics used to define texture in food products.	10
Table 2.1 Composition (14 d), percentage of insoluble calcium as a percentage of total cheese Ca and pH values (2 and 180 d) of Cheddar cheeses with various fat levels.	64
Table 2.2 Mean squares, probabilities (in parenthesis) and R^2 values for proteolysis, CIE whiteness (L^*) on black and white backgrounds and contrast ratio based on whiteness during ripening for FF, RF, HF and NF Cheddar cheeses.	67
Table 2.3 Mean squares, probabilities (in parenthesis) and R^2 values for K/S L^* index and CIE L^* whiteness for experimental cheeses.	70
Table 2.4 Pearson correlation (r) and probabilities (in parenthesis) between the ratio of absorbance to scatter (K/S L^*), whiteness (CIE L^*) and contrast ratio (CR L^*) of experimental cheeses at different temperatures during heating and cooling.	77
Table 3.1 Composition, levels of insoluble calcium, pH and whiteness of low-fat directly acidified Mozzarella cheeses incubated in synthetic Cheddar cheese aqueous phase (SCCAP) solutions with low (LCa; 1.39 g L^{-1}), normal (NCA; 2.78 g L^{-1}) and high (HCA; 4.17 g L^{-1}) levels of Ca during 6 h at 22°C .	102
Table 3.2 Mean squares, probabilities (in parenthesis) and R^2 values for whiteness, rheological properties analyzed at two frequencies and insoluble calcium of direct acidified low-fat Mozzarella cheeses during heating.	106
Table 4.1 CIELAB colour of cheesemilks from treatments with different levels of titanium dioxide, annatto and homogenisation.	143
Table 4.2 Fat globule size parameters of cheese-milks homogenized at 0, 10 or 20 MPa.	144
Table 4.3 Kubelka-Munk ratio of absorbance to scatter obtained by CIE whiteness (K/S L^*) and chroma (K/S C^*) of rennet gels and whey obtained from treatments with different levels of titanium dioxide, annatto and homogenisation.	146
Table 4.4 Composition (14 d), pH (d 14 and 180) and proteolysis (d 14 and 180) of reduced-fat Cheddar cheeses with different levels of titanium dioxide, annatto and cheesemilk homogenisation.	148

Table 4.5 Mean squares, probabilities (in parenthesis) and R^2 values for colour and ratio of absorbance to scatter of cheeses treated with titanium dioxide, annatto and homogenisation.	152
Table 4.6 Pearson correlation (r) and probabilities (in parenthesis) between optical properties of reduced-fat Cheddar cheeses estimated by CIELAB and K/S .	160
Table 5.1 Rheological properties of rennet-induced gels made from full-fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH) and low-methoxy pectin (RL) cheesemilks.	184
Table 5.2 Composition (14 d) and proportion of insoluble Ca (2 and 180 d) of full fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH) and low-methoxy pectin (RL) Cheddar cheeses.	185
Table 5.3 Mean squares, probabilities (in parenthesis) and R^2 values for pH, microbiological analysis and proteolysis for experimental cheeses during 180 d of ripening.	187
Table 5.4 Mean squares, probabilities (in parenthesis) and R^2 values for textural analysis, melting properties and release of free oil for experimental cheeses during 180 d of ripening.	196
Table 5.5 Rheological properties of experimental cheeses at 180 d of ripening determined by dynamic small-amplitude oscillatory rheology.	199
Table 5.6 CIELAB colour of experimental cheeses after 14 d of ripening.	200
Table 6.1 Composition of standardized milks used in the manufacture of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses.	222
Table 6.2 Cheesemaking conditions used for cheesemaking protocols of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses.	223
Table 6.3 Definition of the attributes used by trained panelists to evaluate the sensory properties of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses at 12°C using a combination of Spectrum and quantitative descriptive analysis.	228
Table 6.4 Composition of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses manufactured using 30% of whey dilution (WD30), 15% of whey dilution (WD15) and standardization of lactose content of cheesemilk by ultrafiltration (LS) treatments.	233

Table 6.5 Probabilities and R^2 values for pH, lactic acid, insoluble calcium, proteolysis, texture and rheology of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses during 180 d of ripening.	236
Table 6.6 Sensory analysis results of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses manufactured using 30% of whey dilution (WD30), 15% of whey dilution (WD15) and standardization of cheesemilk lactose by ultrafiltration (LS) treatments during 180 d of ripening.	244
Table 6.7 Probabilities and R^2 values for sensorial properties of low-fat (RF) and reduced-fat (RF) Gouda-style cheeses during 180 d of ripening.	245

LIST OF FIGURES

Fig. 1.1 A typical force-time curve obtained by texture profile analysis (TPA). A1 is the area of the first compression; A2 is the area of the second compression; adhesiveness (A3) is the negative force area for the first compression; fracturability (F) is the force at the first significant break in the curve; hardness (H) is the peak force for the first bite; cohesiveness (A2/A1) is the ratio of the positive force area during the second compression to that during the first compression; springiness is the height that the food product recovers during the time that elapses between the end of the first and start of the second bite; gumminess is hardness x cohesiveness and chewiness is gumminess x springiness.	Page 11
Fig. 1.2 Typical profile of the effect of temperature on the storage modulus (G' ; ●), loss modulus (G'' ; ○) and loss tangent (LT; ▲) of cheese obtained by dynamic small amplitude oscillatory rheology. Analysed sample corresponds to 1 mo-old low-fat Gouda cheese disc (50 mm diameter and 3 mm height) heated at 1°C per min, using 0.5% strain at a frequency of 0.08 Hz.	14
Fig. 1.3 Appearance of Cheddar cheeses containing 32 (a), 19 (b) and <1% (c) fat after 30 d of ripening.	18
Fig. 2.1. Changes in proteolysis expressed as pH 4.6 soluble N as a percentage of total N (% pH 4.6 SN/TN) of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses during ripening.	66
Fig. 2.2 Changes in the Kubelka-Munk index determined by L^* values (K/S L^*) during heating and cooling of FF (■), RF (//), HF (□) and NF (=) cheeses at 2 (a) and 180 d of ripening (b).	69
Fig. 2.3 Changes in whiteness (L^* values) during heating and cooling of FF (■), RF (//), HF (□) and NF (=) cheeses at 2 (a) and 180 d of ripening (b).	72
Fig. 2.4 Changes in whiteness measured above black (a) or white (b) backgrounds and contrast ratio (c) of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses during ripening..	74
Fig. 2.5 Changes in the proportion of the temperature soluble N as a percentage of total cheese N (%TSN/TN) during the heating and cooling of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses at 2 (a) and 180 d of ripening (b).	80

Fig. 2.6 Urea-polyacrylamide gel electrophoresis of FF, RF, HF and NF Cheddar cheeses aqueous phase extracts obtained at 180 d of ripening from unheated samples at 4°C (4), samples heat treated at 80°C during 30 min (80) and samples heat treated at 80°C for 30 min and cooled for 4°C during 24 hours (24h). Sodium caseinate was used as standard (S).	81
Fig. 3.1 Visual appearance of normal calcium (NCa) low-fat Mozzarella cheese heated at different combinations of high temperatures and holding times.	103
Fig. 3.2 Changes in whiteness (CIE L* values) of low (LCa; a), normal (NCa; b) and high calcium (HCa; c) low-fat Mozzarella cheeses heated at 60 (■), 70 (●) or 80°C (▽) during 30 min of holding time. Dashed line represents L* value of unheated cheeses.	105
Fig. 3.3 Acid-base buffering curves of unheated (a) NCa low-fat Mozzarella cheese and cheeses subjected to heat treatments of 60 (b), 70 (c) and 80°C (d) for 30 min.	108
Fig. 3.4 Changes in levels of insoluble calcium (INSOL Ca) of low (LCa), normal (NCa) and high calcium (HCa) low-fat Mozzarella cheeses heated at different combinations of temperature and holding times.	109
Fig. 3.5 Changes in storage modulus (G'; ●) and loss tangent (LT; ○) of low calcium (LCa; a – c), normal calcium (NCa; d – f) and high calcium (HCa; g – i) low-fat Mozzarella cheeses heated at 60, 70 and 80°C during 30 min of holding time.	111
Fig. 3.6 Changes in slope (n) obtained from plotting log G' versus log frequency from low calcium (LCa; ■), normal calcium (NCa; ●) and high calcium (HCa; ▼) low-fat Mozzarella cheeses heated at 60°C during 30 min; LCa (■), NCa (●) and HCa (▼) low fat Mozzarella cheeses heated at 70°C during 30 min; and LCa (□), NCa (○) and HCa (▽) low fat Mozzarella cheeses heated at 80°C during 30 min.	112
Fig. 3.7 Score plot (a) and loading plot (b) obtained by principal component analysis (PCA) from whiteness, levels of INSOL Ca and rheological properties of low calcium (LCa; ■ □), normal calcium (NCa; ● ○) and high calcium (HCa; ▼ ▽) low-fat Mozzarella cheeses heated at 60, 70 and 80°C after 0 (black symbols) and 30 min (white symbols) of holding time. Numbers along with symbols in score plot indicate heating temperature (°C).	114
Fig. 4.1 Appearance of reduced-fat Cheddar cheeses manufactured with different levels of titanium dioxide, annatto and homogenisation pressures after 30 d of ripening.	150

Fig. 4.2 Changes in whiteness (CIE L* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa.	151
Fig. 4.3 Changes in chroma (CIE C* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa.	154
Fig. 4.4 Changes in hue angle (CIE h* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa.	155
Fig. 4.5 Changes in the Kubelka-Munk absorbance to scatter ratio determined by whiteness (K/S L*) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa.	157
Fig. 4.6 Changes in the Kubelka-Munk absorbance to scatter ratio determined by chroma (K/S C*) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa.	158
Fig. 5.1 Changes in pH during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) cheeses.	186
Fig. 5.2 Numbers of (a) starter (LAB) and (b) non-starter lactic acid bacteria (NSLAB) during ripening of full fat control (■), reduced-fat control (//), reduced-fat with amidated pectin (■), high-methoxy pectin (=) and low-methoxy pectin (□) Cheddar cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.	189
Fig. 5.3 Changes in (a) pH 4.6 soluble N as a percentage of total N, and (b) free amino acids expressed as g of L-leucine per 100 g of cheese during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses.	191

Fig. 5.4 Urea-Polyacrilamide gel electrophoresis of experimental Cheddar cheeses at 2, 60, 120 and 180 days of ripening of (a) full-fat (FF), reduced-fat (RF) and reduced-fat with amidated pectin (RA) and (b) reduced-fat with high methoxy (RH) and low methoxy pectin (RL) Cheddar cheeses. Sodium caseinate (S) was used as standard for each gel.	192
Fig. 5.5 Changes in (a) accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 2 d and (b) level of intact α_{s1} -CN as a percentage of the level at 2 d for full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses during ripening.	193
Fig. 5.6 Changes in TPA (a) hardness, (b) springiness and (c) cohesiveness values during ripening full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses.	195
Fig. 5.7 Changes in (a) melting determined by the Schreiber test and (b) release of free oil values during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses.	198
Fig. 6.1 Levels of insoluble calcium, expressed as a percentage of total Ca (%INSOL Ca/Total Ca) of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization of cheesemilk by ultrafiltration (▼ ▽) during 180 d of ripening.	235
Fig. 6.2 Levels of proteolysis expressed as pH 4.6-soluble N (%pH 4.6 SN/TN; a) and 12% trichloroacetic acid-soluble N (%12 TCA SN/TN; b) found in of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization of cheesemilk by ultrafiltration (▼ ▽) during 180 d of ripening.	238
Fig. 6.3 Urea-Polyacrylamide gel electrophoresis of low fat (a) and reduced fat (b) Gouda-style cheeses at 1, 30, 90 and 180 d of ripening manufactured with 30% of whey dilution (WD30), 15% of whey dilution (WD15) and lactose standardization (LS). Sodium caseinate (S) was used as standard for each gel.	239
Fig 6.4 Changes in level of intact α_{s1} -CN as a percentage of the level at 1 d (a) and accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 1 d (b) for low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening.	240

- Fig. 6.5 TPA hardness of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening. 241
- Fig. 6.6 Temperature of the crossover point of the storage and loss moduli (or when $LT = 1$) obtained from low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening. 242

CHAPTER 1

LITERATURE REVIEW:

FACTORS AFFECTING THE QUALITY OF LOW-FAT CHEESE

1.1 Introduction

Cheese is a nutritious and versatile food product that contains a high concentration of essential nutrients such as proteins, fat and minerals; however, its composition is greatly influenced by the source and composition of milk and also by manufacturing conditions (O'Brien and O'Connor, 2004; Johnson, 2011). Depending on the variety, cheeses may be a significant source of fat, especially semi-hard or hard varieties. A serving of 50 g of Cheddar cheese (~33-34% fat) provides ~20% of total fat daily intake recommended in a 2,000 kcal diet (O'Brien and O'Connor, 2004). Excessive fat intake has been associated with the development of chronic diseases such as hypertension, obesity, diabetes and cancer. Based on these findings, different health organisations have recommended reductions of fat intake in Western countries (O'Brien and O'Connor, 2004). In the case of cheese, a reduction in its fat content is associated with changes in the proportion of the original components that lead to a modification of cheese properties in terms of texture, flavour, appearance and functionality (Bryant et al., 1995; Rudan and Barbano, 1998; Guinee et al., 2000; Drake et al., 2010; Johnson, 2011). Nevertheless, consumers expect that cheeses with reduced fat content would maintain the same properties as their full-fat counterpart (Childs and Drake, 2009). This is the main reason why the cheese industry has developed several strategies to improve the properties of low fat cheeses (Drake and Swanson, 1995; Mistry, 2001; Banks, 2004; Johnson et al., 2009).

The following sections of this review will describe the main characteristics of low fat cheeses, in terms of legal definitions and their biochemical, microbiological,

textural, functional, and sensorial properties. In addition, we will describe the principal strategies used to improve the properties and acceptability of these products. While remaining of significant industrial importance, much scientific research in low-fat cheese was conducted in the 1990s and early 2000s.

1.2 Legal definitions of cheeses with reduced fat contents

The most common definitions used to classify cheeses with reduced fat content are based in the extent of fat reduction. However, these systems may differ in different parts of the world, based on international or national legislation (Johnson, 2011). Table 1.1 shows different systems used worldwide to classify cheeses based in their fat content. The system proposed by the Codex Alimentarius (FAO, 1978) is based in the fat in dry matter content of cheeses (FDM), ranging from skim cheese ($< 10\%$ FDM) to high fat cheese ($\geq 60\%$ FDM). The United States Food and Drugs Administration (FDA, 2013) classification system is based in the amount of fat contained in a designated serving amount of food (30 g for cheese), which is known as the reference amount customarily consumed (RACC; FDA, 2015). The European Union (EU, 2006) classifies fat levels based on the amount of total fat in 100 g of solid food product (including cheese). In this review, we will refer to low fat cheeses as those products in which the fat content is reduced by at least 25%, unless otherwise specified.

Table 1.1 Legal definitions used to claim cheeses with reduced fat content

Regulation / Claim	Definition
<i>Codex Alimentarius</i> ¹	
High-fat	If the content of fat in dry matter (FDM) is above or equal to 60%.
Full-fat	If the content of FDM is above or equal to 45% and less than 60%.
Medium-fat	If the content of FDM is above or equal to 25% and less than 45%.
Partially skimmed	If the content of FDM is above or equal to 10% and less than 25%.
Skim	If the content of FDM is less than 10%.
<i>United States regulations</i> ²	
Fat-free, free of fat, no fat, zero fat, without fat, negligible source of fat or dietarily insignificant source of fat.	Less than 0.5 g of fat per RACC ³ and per labeled serving size and no added fat or oil ingredient.
Low-fat, low in fat, contains a small amount of fat, low source of fat or little fat.	Maximum 3 g of total fat per 50 g of cheese ⁴ .
Reduced-fat	Minimum 25% reduction of total fat per RACC.
<i>European Union regulations</i> ⁵	
Fat-free	Less than 0.5 g of fat per 100 g.
Low-fat	Less than 3 g per 100 g.
Reduced-fat or light	At least 30% of fat reduction, when compared to similar product.

¹ FAO (1978)

² FDA (2013)

³ RACC of cheese is 30 g (FDA, 2015).

⁴ Based in RACC of cheese.

⁵ EU (2006)

1.3 General properties of low-fat cheeses

Cheese corresponds to a hydrated protein matrix that is interrupted by fat globules (Johnson et al., 2009). In low-fat cheeses, this interruption by fat decreases, leading to a more compact protein matrix (Mistry and Anderson, 1993). A reduction in the fat content of cheese is associated with a shift in the compositional balance of other components which is mainly attributed to an increase in the moisture and protein content, and a decrease in the moisture in the non-fat substance (MNFS; Mistry, 2001). In low-fat cheeses, the percentage of salt in the moisture phase of cheese (S/M) is lower than optimal depending on the fat and moisture contents (Mistry and Kasperson, 1998). Low-fat cheeses have also increased levels of total (Guinee et al., 2000) and insoluble calcium (INSOL Ca; Udayarajan, 2007), mainly attributed to higher levels of protein, as the latter corresponds to the colloidal calcium phosphate fraction that is bound to casein (Lucey and Fox, 1993; Hassan et al., 2004). Solubilization of colloidal calcium phosphate of cheese during ageing leads to an increase in cheese pH (Hassan et al., 2004). Therefore, low-fat cheeses have increased pH values due to high levels of INSOL Ca. Fenelon and Guinee (2000) also reported that increased pH values of low-fat cheeses leads to a reduction in the lactate to protein ratio, altering acidity and buffering capacity. A combination of these differences is responsible for the main changes in the biochemistry, microbiology, texture, functionality and sensory properties of low-fat cheeses. The greater the reduction in fat, the more severe are these effects (Mistry, 2001).

1.3.1 Starter and non-starter lactic acid bacteria

Starter lactic acid bacteria (LAB) play an important role in the final properties of cheese due to their contribution to acid production and proteolysis, affecting directly flavour and texture development (Banks, 2004; Johnson et al., 2009). Cheeses with reduced fat content generally exhibit a decrease in the counts of LAB. Laloy et al. (1996) reported that the fat content of milk directly influenced the growth of LAB in Cheddar cheese before pressing, suggesting that higher LAB counts found in full-fat in comparison to reduced-fat cheese could be attributed to decreased syneresis as fat content increased. Haque et al. (1997) also found that counts of lactococci were lower as fat content of Cheddar cheese was reduced. These observations were in agreement with those of Fenelon et al. (2000) who found that a reduction in the counts of LAB as fat content of cheese decreased was associated with decreased levels of MNFS that may lead to a reduction in the water activity and hence reducing growth of bacteria. In contrast, Tunick et al. (1993) found an increase in the number of LAB when the fat content of Mozzarella cheese was reduced. A possible explanation for these results could be attributed to differences in the manufacturing protocols used in this study, since low-fat cheese was made using a lower cooking temperature (32°C) than full-fat cheese (46°C), which may enhance proliferation and survival of LAB. Nevertheless, the use of LAB suitable for the manufacture of full-fat cheese may not be adequate to obtain a comparable quality in low-fat cheeses, due to the development of excessive acidity and off-flavours, mainly caused by reduction in the S/M content (Johnson, 2011). The growth of LAB in cheese is inhibited when levels of S/M are >2.5% (Irvine and Price, 1961). Mistry and Kasperson (1998) reported that increasing S/M levels from

2.7 to 4.5% in reduced-fat Cheddar cheese led to similar counts of LAB during the first 4 wk of ripening, although cheeses with lower S/M content were more acidic and exhibited a slower rate in the reduction of LAB. LAB suitable for low-fat cheeses should be selected based on reduced acid production and autolytic and proteolytic properties (Banks, 2004).

Non-starter lactic acid bacteria (NSLAB) are a secondary group of bacteria that are found naturally in milk, the dairy environment and surroundings and that develop in cheese during ripening, often contributing desirable flavour notes (Banks, 2004). Similar to starter LAB, NSLAB growth rate decreases as the fat content of cheese is reduced and this has also been related to lower levels of MNFS (Haque et al., 1997; Fenelon et al., 2000). This group of bacteria is also known to use the milk fat globule membrane as a source of carbon (Fox et al., 1998). Therefore, it would be expected that lower fat content of cheese correlates with a reduction in counts of NSLAB.

1.3.2 Proteolysis

Low-fat cheeses exhibit lower breakdown of caseins (CN) when compared to their full-fat counterparts (Fife et al., 1996), which has been mainly attributed to decreased levels of MNFS that leads to a reduction in the retention of chymosin (Tunick et al., 1993a; Rudan et al., 1999; Fenelon and Guinee, 2000). Nevertheless, proteolysis in low-fat cheeses is also influenced by calcium content, pH, salt content and microbial growth. Increased levels of total and insoluble calcium in cheese has been related to

lower levels of proteolysis by residual rennet (Feeney et al., 2002; Joshi et al., 2003a; O'Mahony et al., 2005). High levels of INSOL Ca in low-fat cheeses would make the protein matrix stronger due to increased interaction of proteins mediated by colloidal calcium phosphate nanoclusters, leading to reduced proteolysis (Udayarajan, 2007). Decreasing the fat content of cheese is also associated with changes in the breakdown of individual caseins (Fenelon and Guinee, 2000). Decreased hydrolysis of α_{s1} -CN in cheeses with reduced fat content is attributed to reduced retention of chymosin in the curd due to lower content of MNFS (Fenelon and Guinee, 2000), increased levels of INSOL Ca (O'Mahony et al., 2005; Udayarajan, 2007) and reduced chymosin activity due to an increase in pH (Tam and Whitaker, 1972; Mulvihill and Fox, 1980; Feeney et al., 2002). In contrast, increased pH of low-fat cheeses is associated with higher hydrolysis of β -CN due to enhanced plasmin activity (Fenelon and Guinee, 2000). The optimum pH for plasmin activity occurs at pH 7.5 (McSweeney and Sousa, 2000).

As previously stated, low-fat cheeses also have a reduced S/M content, and proteolysis in cheese is highly influenced by this parameter (Lawrence et al., 1987). A reduction in the S/M content of reduced-fat Cheddar cheese led to an increase in the hydrolysis of α_{s1} -CN (Mistry and Kasperson, 1998). In addition, reduced S/M levels in cheese are also associated with increased hydrolysis of β -CN (Kelly et al., 1996) which may lead to the formation of hydrophobic peptides that contribute to bitterness (Olson and Johnson, 1990; Madsen and Ardö, 2001). Finally, decreased microbial activity in low-fat cheeses would also lead to a reduction in the amount of proteinases and peptidases produced by LAB, affecting secondary proteolysis (Sheehan and Guinee, 2004).

1.3.3 Texture and melting

As described above, as fat content is reduced, the cheese matrix becomes more compact due to decreased interruption by fat globules, an increase in protein and INSOL Ca contents and also a decrease in the MNFS content. These parameters are responsible for the major changes in the textural, rheological and functional properties of low-fat cheeses.

Cheese texture

Cheese texture corresponds to a combination of physical attributes that are perceived by the senses of touch, sight and hearing (Brennan, 1988). A first attempt to review and develop a standard for textural attributes in food products was made by Szczesniak (1963). This author proposed that textural properties of foods could be classified into three main categories: (1) mechanical characteristics, that corresponded to those properties affected by stress; (2) geometrical characteristics, that referred to the arrangements of the constituents of food products and how they effect on senses of touch and pressure (e.g., grainy, crystalline, coarse, etc.); and (3) other properties, which were mainly referred to the composition of food products (e.g., moisture and fat content). The mechanical characteristics (Table 1.2) are considered the most important attributes for texture, since they determine how a food product behaves in the mouth, not only in terms of sensory analysis, but also in rheology. Descriptive sensory methods have been extensively used to evaluate cheese texture to determine differences among

Table 1.2 Mechanical characteristics used to define texture in food products. ¹

Attribute	Definition
Hardness	Force necessary to attain a given deformation.
Cohesiveness	Strength of internal bonds making up the body of the product.
Viscosity	Rate of flow per unit force.
Elasticity	The rate at which a deformed material returns to the original size after deforming force is removed.
Adhesiveness	Work necessary to overcome the attractive forces between the surface of the food product and the surface of the material at which it comes in contact, e.g., tongue, teeth and or palate.
Brittleness (fracturability)	Force at which food product fractures.
Chewiness	Energy required to masticate a solid food to a state ready for swallowing.
Guminess	Energy required to disintegrate a semi-solid food to a state ready for swallowing.

¹ Modified from Szczesniak (1963) and O'Callaghan and Guinee (2004).

cheese varieties and to establish those attributes that are desired by consumers (Drake and Swanson, 1995; Drake et al., 1999; Brown et al., 2003; Yates and Drake, 2007). Nevertheless, the evaluation of cheese texture by sensory analysis has some limitations, since it requires a panel of evaluators that need constant training to obtain reproducible

results, leading to a time-demanding and expensive process (Drake et al., 1999). The use of instrumental methods to evaluate the textural properties of cheese has gained popularity in the food industry, since it is relatively easier to standardize than sensory analysis and it is also more applicable to routine analysis (Fox et al., 2000). Instrumental texture profile analysis (TPA) corresponds to a system of rheological measurements that establishes a relationship with the texture of food products (O’Callaghan and Guinee, 2004). It basically consist of a double compression test aimed to mimic the mastication process performed by molars (Szczesniak, 1963; Bourne, 1978). The parameters obtained by TPA are the same as those described by Szczesniak (1963), which are estimated from a typical two-bite compression (Fig. 1.1).

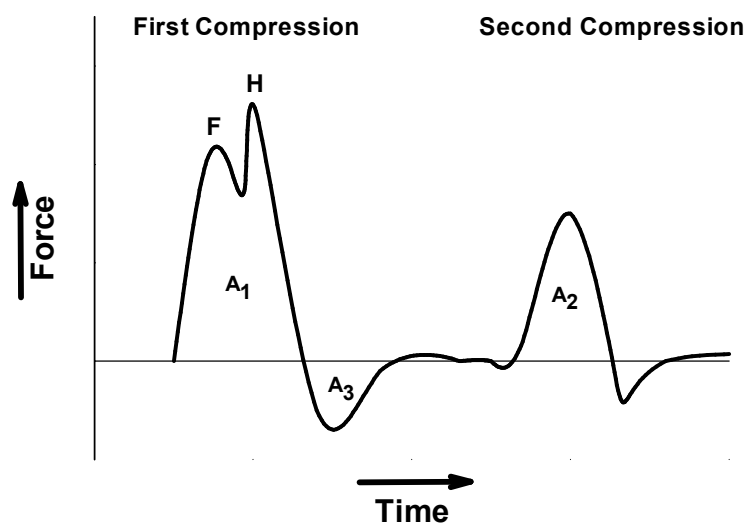


Fig. 1.1 A typical force-time curve obtained by texture profile analysis (TPA). A_1 is the area of the first compression; A_2 is the area of the second compression; adhesiveness (A_3) is the negative force area for the first compression; fracturability (F) is the force at the first significant break in the curve; hardness (H) is the peak force for the first bite; cohesiveness (A_2/A_1) is the ratio of the positive force area during the second compression to that during the first compression; springiness is the height that the food product recovers during the time that elapses between the end of the first and start of the second bite; gumminess is hardness x cohesiveness and chewiness is gumminess x springiness. Modified from Bourne (1978).

Sensory analyses have shown that a decrease in the fat content of different cheese varieties is associated with increased hardness, springiness (rubberiness) and fracturability, decreased cohesiveness and adhesiveness, along with reduced smoothness and increased graininess, when compared to cheeses with higher fat contents (Olson and Johnson, 1990; Bryant et al., 1995; Drake and Swanson, 1996; Johnson et al., 1998; Lobato-Calleros et al., 2001; Banks, 2004; Yates and Drake, 2007; Rogers et al., 2009; Henneberry et al., 2016).

Uniaxial compression tests performed on Cheddar (Fenelon and Guinee, 2000; Guinee et al., 2000; Ozturk et al., 2013), Danbo (Madsen and Ardö, 2001) and Mozzarella (Sheehan and Guinee, 2004; Henneberry et al., 2015) cheeses with decreased fat content recorded higher firmness and fracture strain. Similar to sensory analysis, TPA has shown that low-fat cheeses exhibit increased hardness, chewiness and springiness (Tunick et al., 1991; Bryant et al., 1995; Mistry, 2001; Banks, 2004; Johnson et al., 2009; Cooke et al., 2013; Ozturk et al., 2013; Henneberry et al., 2015; Ibáñez et al., 2016a); however, an inverse relationship has been observed between sensory and instrumental cohesiveness. Bryant et al. (1995) suggested that these differences could be explained based by differences in how these attributes are measured, since sensory analysis uses molars (which have a sharp shape) to masticate samples, whereas TPA uses a flat probe to compress a sample against a bottom plate. Another explanation could be attributed to the temperature of analysis of cheese texture. Despite to the fact that most of the samples are cooled to refrigeration temperature, instrumental texture analysis of cheese is performed at room temperature, whereas in sensory analysis samples are tempered during mastication. Therefore, Abu-Waar et al.

(2013) suggested that temperature of cheese samples during analysis of texture should be controlled to correlate sensory and instrumental measurements.

During ripening, a softening of the cheese matrix mainly occurs due to an increase in proteolysis (Creamer and Olson, 1982) and solubilization of CCP (Lucey et al., 2003; Hassan et al., 2004). As previously stated, since low-fat cheeses exhibited reduced levels of proteolysis and solubilization of CCP, textural changes during ripening are less pronounced when compared to a full-fat cheese (Tunick et al., 1991; Bryant et al., 1995; Fenelon and Guinee, 2000; Johnson et al., 2009).

Cheese melt

From the physical point of view, melting occurs when a substance is transformed from a “solid-like” to a “viscous-like” state. Melt can be defined as the ability of cheese to flow and spread, along of the fusion of individual shreds (Lucey et al., 2003). Changes in the viscoelastic properties of cheese during heating have been extensively studied by dynamic small amplitude oscillatory rheology to measure the elastic or storage modulus (G'), the viscous or loss modulus (G''), the complex modulus (G^*) and loss tangent (i.e., the ratio of viscous to elastic moduli, LT; Steffe, 1996; Guinee et al., 2002; Gunasekaran and Ak, 2003; Tunick, 2011). A typical profile of the viscoelastic properties of cheese obtained during heating is shown in Fig. 1.2. An increase in cheese temperature leads to a reduction of both storage and loss moduli which are associated with a decrease in the number and or strength of bonds in the

cheese matrix (Lucey et al., 2003). Despite the fact that cheese fat is completely melted at $\geq 40^\circ\text{C}$, cheese melting is highly influenced by protein-protein interactions that occur at high temperatures due to a combined effect of increased hydrophobic interactions that are the greatest in the range of $60\text{--}70^\circ\text{C}$ along with increased electrostatic repulsions (Bryant and McClements, 1998). Lucey et al. (2003) suggested that cheese melting occurs when electrostatic repulsions are greater than the combination of all attractive interactions existing in the cheese matrix (i.e., hydrophobic interactions, CCP crosslinks and positive-negative charge bridges).

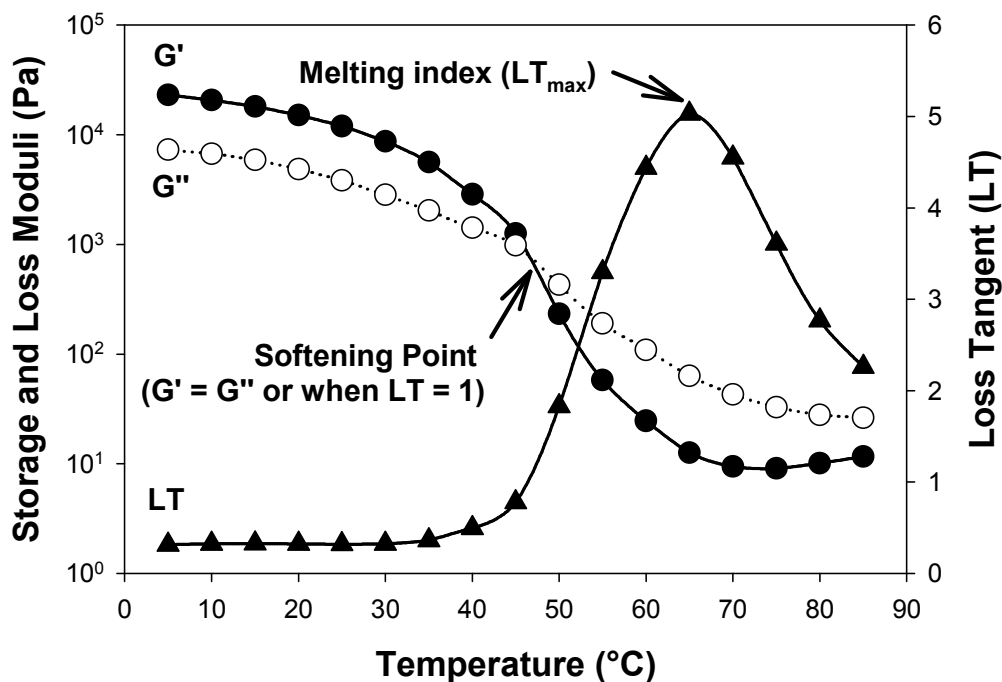


Fig 1.2 Typical profile of the effect of temperature on the storage modulus (G' ; ●), loss modulus (G'' ; ○) and loss tangent (LT; ▲) of cheese obtained by dynamic small amplitude oscillatory rheology. Analysed sample corresponds to 1 mo-old low-fat Gouda cheese disc (50 mm diameter and 3 mm height) heated at 1°C per min, using 0.5% strain at a frequency of 0.08 Hz (Ibáñez et. al, unpublished).

Guinee et al. (2002) reported that a decrease in G' during the heating of cheese is associated with a change of phase from elastic to viscous. Since the LT values (ratio between G'' and G') of cheese exhibits an increase when treated at high temperatures (Fig. 1.2), this indicates that cheese becomes a fluid-like material (Lucey et al., 2003). The temperature where $LT = 1$ (or $G'' = G'$; Fig 1.2) is known as the softening point or cross over point of cheese, since it corresponds to the transition from elastic-like to viscous-like (Gunasekaran and Ak, 2003). At higher temperatures the viscous modulus is higher than the elastic modulus (i.e., $LT > 1$), which is associated with cheese flow. The maximum LT value (LT_{max}) obtained in a heating profile (Fig 1.2) is considered a melting index (Lucey et al., 2003) since has been highly correlated with the melting of cheese obtained by empirical methods (Mounsey and O'Riordan, 1999). A decrease in LT values has been observed after LT_{max} is reached (Fig. 1.2), which may be attributed due to the formation of heat-induced insoluble CCP (Udayarajan et al., 2005).

Meltability of cheese is considerably reduced as fat content is reduced. Ustunol et al. (1995) evaluated the melting properties of Cheddar cheeses with fat content ranging from 34 to 13% by dynamic small amplitude oscillatory rheology and found that cheeses with lower fat content had a more elastic behavior at high temperatures. The authors attributed these differences to lower levels of proteolysis as the fat content of cheese is reduced. Similarly, Udayarajan (2007) observed a reduction of LT_{max} values during the heating of Mozzarella cheese made with reduced fat levels from 26 to 16%, which was mainly attributed due to a combined effect of reduced proteolysis and solubilization of INSOL in cheeses with reduced fat content that maintained increased strength of bonds in the cheese matrix. In addition, this author observed similar trends

when melting properties were evaluated by empirical methods that mainly consisted of evaluating the extent of flow of a cheese sample when subjected to a particular heat treatment (Lucey et al., 2003). Despite of the use of different approaches, empirical methods have also been used to determine melting properties of low-fat cheeses, such as the Schreiber test (Rudan et al., 1999; Sheehan and Guinee, 2004; Ibáñez et al., 2016a), Olson-Price test (Fife et al., 1996; Guinee et al., 2000; Costa et al., 2010; Henneberry et al., 2015) and the UW-Meltprofiler or squeeze flow test (Udayarajan, 2007; Govindasamy-Lucey et al., 2010).

The release of free oil from cheese during heat treatment has a great influence on its melting properties (Rudan et al., 1999). Rudan and Barbano (1998) observed that during the heating of cheese under a pizza baking model, a fat-free Mozzarella cheese had no release of free oil, limited melt and fusion of the shreds, along with skin formation and a burnt appearance, whereas a full-fat low moisture part skim Mozzarella cheese showed extensive release of free oil, melt and fusion of the shreds and no formation of skin. These authors found the importance of free oil release when cheese is heated at high temperatures, since prevents dehydration of the surface of cheese and avoids limited melting. Based on these observations, the authors suggested that applying a thin layer of a hydrophobic coating to cheeses with lower fat content before heating could prevent these defects.

Stretchability is another important functional property related to cheese melt. Lucey et al. (2003) defined stretch as the ability of the casein network to maintain its integrity when a continuous stress is applied to the cheese. In order to form a long thin

stretch, casein molecules have to slide past each other but still have sufficient interaction in order to prevent the strand from breaking (Udayarajan, 2007). During the first stages of ripening, cheeses with reduced fat content exhibit lower stretchability than full-fat cheeses; however, extent of proteolysis and solubilization of INSOL in full fat cheeses become excessive after 28 d of ripening, leading to a considerable reduction in stretch properties (Guinee et al., 2000; Udayarajan, 2007; Henneberry et al., 2015), therefore certain optimum levels of proteolysis and INSOL Ca are necessary to obtain a desired stretch (Lucey and Fox, 1993).

1.3.4 Appearance

The final composition of cheese is one of the most important parameters that determine its appearance. A reduction in the fat content of cheese leads to a reduction in the number of light scattering centres that would contribute with a translucent appearance (Banks, 2004; Johnson et al., 2009). Fig 1.3 shows the appearance of 1 month old Cheddar cheeses differing in fat content from 32 to <1%. Cheese translucency relates to a reduction in the whiteness intensity that is commonly used as an indicator of opacity (Metzger et al., 2000a; Dave et al., 2001; Pastorino et al., 2002; Ibáñez et al., 2016b). Since the concentration of carotenoids is lower, the intensity of yellowness is decreased, as these pigments are found in the fat fraction from bovine milk (Fox et al., 2000). Fife et al. (1996) also reported that low-fat cheeses exhibit higher levels of greenness, due to an increase in the proportion of the aqueous phase of cheese.

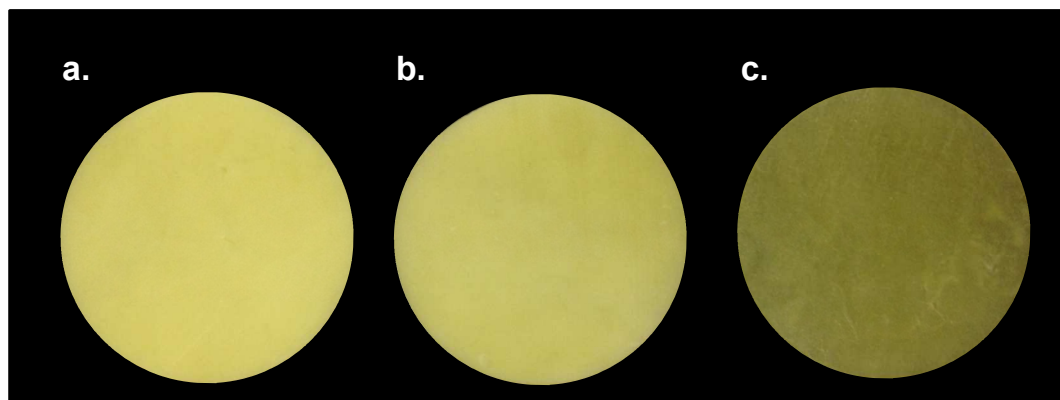


Fig 1.3 Appearance of Cheddar cheeses containing 32 (a), 19 (b) and <1% (c) fat after 30 d of ripening (Ibáñez et al., unpublished).

Factors that affect the appearance of low-fat cheeses

pH plays an important role in the interactions of proteins that may contribute to cheese translucency. Brickley et al. (2008) reported that reducing the pH of directly acidified non-fat Mozzarella cheese from 5.4 to 5.0 decreased cheese translucency. The authors elucidated that a decrease in cheese pH caused a reduction in net charge since caseins approached their isoelectric point leading to aggregation of intact casein in the cheese matrix, increasing light scattering and therefore reducing translucency. Several studies have also shown that cheese ripening has a positive effect increasing translucency independent of the fat content, which could relate to a combined effect of proteolysis and solubilization of colloidal calcium phosphate (Rudan et al., 1998a; Metzger et al., 2000a, 2001a; Dave et al., 2001; Ibáñez et al., 2016b). Reduced levels of S/M found in low-fat cheeses may also contribute to a translucent appearance. Paulson et al. (1998) found, by visual observation, that low-fat Mozzarella cheeses developed a

translucent appearance when the curd was salted, in comparison with an unsalted curd. These authors hypothesized that the addition of salt contributes to reduce hydrophobic interactions of proteins, leading to the absorption of free serum into the matrix, reducing the number of aggregates in the surface and thereby reducing light scattering.

During the heat treatment of cheese, its appearance is modified from translucent to opaque. Rudan and Barbano (1998) first reported changes in the appearance of Mozzarella cheeses varying in fat content when melting properties were evaluated in a pizza baking model heating samples at 232°C for 5 min. Insufficient release of free oil in low-fat cheeses leads to dehydration of the surface and the formation of blisters of brown colour. On the other hand, cheeses with higher fat content, the release of free oil during heating prevented the loss of water and the drying out of the cheese surface, contributing to the formation of a white (opaque) appearance. To prevent loss of moisture in the cheese surface and, therefore, undesirable colour development in low-fat cheeses (browning), the authors suggested the use of a hydrophobic coating to obtain similar results as in full-fat cheeses.

Several changes in appearance take place when cheese is heated to high temperatures. As previously stated, fat globules in cheese scatter light, contributing to opacity. When cheese is heated at ~40°C, fat melting leads to an increase of cheese translucency (Metzger et al., 2000a), whereas, at higher temperatures, cheese appearance is affected by protein-protein interactions with little impact from fat. Pastorino et al. (2002) observed the presence of increased protein aggregates in the microstructure of non-fat Mozzarella cheese heated at 50°C, when studied by electron

microscopy. An increase in the whiteness of cheeses heated at high temperatures could, in part, be explained by the occurrence of hydrophobic interactions that would induce the formation of aggregates, promoting light scattering (Metzger et al., 2000a; Ibáñez et al., 2016b). As previously stated, hydrophobic interactions are greatest in the range of 60-70°C and decrease again thereafter (Bryant and McClements, 1998).

Changes in the proportion of calcium during heating may also contribute to the optical properties of cheese, since the solubility of calcium phosphate is highly temperature-dependent (Fox et al., 2015), which is in agreement with the observations of Rose and Tessier (1959) and Broome and Limsowtin (2002) who found precipitation of calcium phosphate when milk retentates and synthetic buffers matching the aqueous phase of Cheddar cheese were treated at high temperatures. Udayarajan (2007) found that Cheddar cheeses heated at high temperatures ($\geq 70^{\circ}\text{C}$) for 30 min exhibited an increase in the buffering area obtained by acid-base titration, in comparison to unheated sample, suggesting the formation of heat-induced colloidal calcium phosphate. Metzger et al. (2001) observed a linear relationship between levels of water insoluble calcium and the whiteness of directly acidified low-fat Mozzarella cheeses at 60°C, presumably to protein-protein interactions. At high temperatures, milk proteins may also interact via calcium-phosphate linkages (Green, 1971; Dalgleish and Parker, 1980; Dave et al., 2001). Nevertheless, there are no studies that show the influence of these heat-induced structures on the optical properties of cheese.

Current methods to determine cheese appearance

The appearance of cheese has been extensively estimated by measurements of colour based in Hunter or CIELAB scales (Kosikowski and Brown, 1969; Fife et al., 1996; Metzger et al., 2000a, 2001a; Pastorino et al., 2002; Joshi et al., 2003b; Wadhwani and McMahon, 2012; Ibáñez et al., 2016a;b). However, when certain material is translucent (as occurs in a cheese with reduced fat content), its optical properties (i.e., colour) may be affected by interference of light reflection from below the surface, leading to loss of colour (Little, 1964). To avoid this problem, Little (1964) suggested that colour measurements should be performed in thin layers of translucent materials positioned above white and black backgrounds. With these measurements, it is also possible to apply the Kubelka-Munk theory of reflectance (K/S) that corresponds to empirical formulae to estimate the ratio of absorbance to scatter (Judd and Wyszecki, 1975). This method has been used to study the optical properties of translucent materials, including food products such as fruit gels (Calvo and Salvador, 1997), sauces (Little, 1964; Huang et al., 1970), juices (Gullett et al., 1972), tomatoes (Lana et al., 2006), meat, dairy products such as milk and *dulce de leche* (MacDougall, 2002) and even cheese (Ibáñez et al., 2016b).

1.4. Strategies to improve properties of low-fat cheeses

Different types of strategies have been developed to improve the properties of cheeses with reduced fat content in terms of biochemistry, texture, functionality and

appearance that can be achieved by mean of two different approaches: addition of ingredients and or modification/addition of steps during cheese manufacture. In the following subsections, some of those strategies will be summarised.

1.4.1 Addition of ingredients

Starters and adjuncts

As previously stated, low-fat cheeses have reduced S/M content that may lead to excessive development of acidity from lactic acid bacteria. To avoid this problem, the cheese industry has developed and used starters with slow acid production rates in the manufacture of cheeses with reduced fat content to control acid (Johnson et al., 2001, 2009). Isolation of NSLAB from matured cheeses have been used as adjunct cultures in cheeses with reduced fat content to improve their flavour profile (Lee et al., 1992) due to increased secondary proteolysis that relates to flavour development (Ardö, 1993; McSweeney and Sousa, 2000). Fenelon et al. (2002) successfully improved the flavour scores and acceptability of reduced-fat cheeses made with different mixtures of adjunct cultures, which was attributed to higher extent of peptide and free amino acid formation.

The use of exopolysaccharide (EPS)-producing bacteria has also been an alternative to improve the properties of cheeses with reduced fat content. Perry et al. (1997) found that producing low-fat Mozzarella cheese using capsular EPS-producing starters and or adjunct cultures led to cheeses with higher moisture content, since EPS

can reduce whey expulsion. This also resulted in cheeses with increased melting properties, since EPS capsules avoided the fusion of serum channels in the cheese matrix. An important observation from this study is that starter cultures were found to be more efficient than adjuncts in producing EPS. Zisu and Shah (2005) evaluated the use of capsular EPS starter cultures in combination with the addition of whey protein concentrate as a fat replacer and preacidification on the properties of low-fat Mozzarella cheese and found an improvement in yield, texture and melting properties. Costa et al. (2010) evaluated the effects of isogenic (ropy) EPS-producing and non-producing starters on the properties of reduced-fat Cheddar cheese. These authors found that type of starter had similar effect on rennet-induced gelation of cheesemilks. In contrast, cheese made with ropy EPS-producing strain had higher moisture content when compared to non-EPS producing strain that led to increased yield and an interruption of the cheese matrix by EPS that led to a softer texture and increased melting properties (flowability).

Preacidification

Reduction in the contents of total and/or insoluble colloidal calcium phosphate during cheese manufacture are important parameters that affect the properties of full-fat and low-fat cheeses, and which is controlled by the rate and extent of acidification during manufacture (Mistry, 2001; Johnson et al., 2009). The preacidification of cheesemilk prior to cheese manufacture can be an alternative approach to adjust the calcium content of cheese in order to modify its composition, texture and functionality.

In a series of studies, Metzger et al. (2000b, 2001b; a) evaluated the effect of preacidification with acetic acid or citric acid to pH values of 6.0 and 5.8 on the properties of low-fat Mozzarella cheese. The authors found that an increase in the extent of preacidification led to decreased recoveries of nitrogen and fat, especially in those treatments made with citric acid. In terms of composition, increased levels of preacidification led to cheeses with reduced levels of total and water-insoluble calcium, which also had a greater effect in those treatments made citric acid, since it is a calcium-binding agent. Preacidified cheeses with lower levels of total and water insoluble Ca exhibited increased proteolysis, a softer texture when analysed by texture profile analysis and increased melting. In addition, a linear relationship was also found between levels of water-insoluble Ca and CIELAB whiteness.

Coagulants

As previously stated, a reduction in the fat content of cheese leads to a reduction in the MNFS content that may contribute to reduced retention of chymosin in the curd and thus reduced proteolysis that may lead to cheeses with a firmer texture and reduced melting (Fenelon and Guinee, 2000). Increasing levels of coagulants may be an alternative to obtain low-fat cheeses with a softer texture and reduced melting (Madadlou et al., 2005). Similarly, Sheehan et al. (2004) evaluated coagulants with different proteolytic activities (fermentation-produced chymosin, *Rhizomucor miehei* proteinase and *Rhizomucor pusillus* proteinase) on the composition, proteolysis, texture and melting of reduced-fat Mozzarella cheese. The authors obtained cheeses with

similar composition among treatments, but found that cheese made with *R. pusillus* proteinase had higher proteolysis, but similar texture and melting to the cheese made using chymosin. Nevertheless, the development of excessive proteolysis in low-fat cheeses may contribute to undesired off-flavours (Mistry, 2001). Govindasamy-Lucey et al. (2010) compared the use of camel chymosin with calf chymosin in the manufacture of low-fat Cheddar cheese, since camel chymosin has shown reduced bitterness development in full-fat cheeses. The authors obtained low-fat cheeses with similar composition and pH values. Despite the fact that low-fat cheese made with camel chymosin developed reduced bitterness and secondary proteolysis, it had a firmer texture and reduced melting when compared to cheese made with calf chymosin.

Fat replacers

The use of fat replacers has gained popularity, since they can be incorporated into traditional food products, such as in cheese, to give similar functionality as in full-fat counterpart (O'Connor and O'Brien, 2002). Based on their nature, fat replacers can be classified as fat substitutes and fat mimetics.

Fat substitutes are synthetic molecules that may have similar physical and functional properties to conventional fat, even though they do not have nutritional value and may not contribute to the development of flavour (O'Connor and O'Brien, 2002).

Salatrim® (Pfizer Inc.) is a tailored triglyceride ingredient extensively used as a fat substitute since it provides the functionality of fat with only 55% of the calories of natural fat. Rudan et al. (1998b) evaluated the use of Salatrim in the properties of reduced fat Mozzarella cheese. The authors found that cheeses supplemented with this ingredient exhibited higher moisture content, increased proteolysis, harder texture and increased release of free oil during heating, which improved fusion of shreds and colour development when heated.

Sucrose polyesters are non-caloric ingredients synthesized by esterification of fatty acids to the hydroxyl groups of sucrose, and which may be used as fat substitutes. Crites et al. (1997) evaluated the effect of replacing fat with sucrose polyesters at increasing proportions from 10 to 75% in the manufacture of Cheddar cheese. There were no differences in the composition as proportion of fat replacers increased, although the microstructure contained smaller fat globules, probably as homogenisation was required to disperse material when incorporated in cheesemilk.

Fat mimetics are water-soluble fat replacers and are generally derived from protein and or carbohydrates (O'Connor and O'Brien, 2002). McMahon et al. (1996) evaluated the effect of two types of carbohydrates (Stellar™, corn starch and xanthan gum mixture; and Novagel™, microcrystalline cellulose) and protein (Simplese®, whey protein; and Dairy-Lo®, 35% of whey protein concentrate) mimetics on the properties of low-fat Mozzarella cheese at levels recommended by their respective manufacturers. The authors found that the addition of fat replacers increased the moisture content of cheeses, which was related to the water holding capacity of the

ingredients used. This led to improved melting when compared to control samples. In addition, fat mimetics physically interrupted the *para*-casein matrix when observed by electron microscopy, which was influenced by particle size and nature of fat replacer.

Konuklar et al. (2004) evaluated the effect of β -glucan on the properties of low-fat Cheddar cheese and found an increase in the moisture content that resulted in improved texture when compared to control.

Koca and Metin (2004) found an improvement in the texture and melting properties of low-fat Kashar cheese when supplemented with an inulin-based fat replacer. However, cheeses developed off-flavours and became extremely soft with prolonged ripening times (60-90 d).

Cooke et al. (2013) studied the use of gum tragacanth on the properties of reduced-fat Cheddar cheese and found that cheeses with added gum had lower pH values, increased degradation of α_{s1} -CN, reduced hardness and increased melting. However, cheeses made with this fat replacer had lower preference score when compared to control.

Lobato-Calleros et al. (2001) evaluated the use of low-methoxy pectin as fat replacer in low-fat Mexican Manchego cheese and found an increase in the moisture content that led to a softer texture. In addition, the authors found the presence calcium-pectate gels since gelation occurs in the presence of Ca. In a similar study from our group performed on reduced-fat Cheddar cheese (Ibáñez et al., 2016a), we believe that

the addition of low-methoxy and amidated pectin led to the formation of calcium-induced pectate gels that reduced levels of insoluble calcium phosphate, contributing to a softer texture and increased melting. One of the disadvantages of the use of carbohydrate-based fat replacers and its supplementation into cheesemilks, is the interaction with milk proteins that may lead to destabilization of the system and phase separation (Goh et al., 2014).

Buttermilk corresponds to the liquid fraction (by-product) obtained from the destabilization of cream during the manufacture of butter and is mainly composed of all nutrients of milk except fat, together with residual fractions from the milk fat globule membrane. This product has been used as a fat replacer in the manufacture of reduced Cheddar and Mozzarella cheese (Mayes et al., 1994; Mistry et al., 1996; Poduval and Mistry, 1999; Turcot et al., 2002), which has improved retention of moisture in the curd, leading to increased yield. The use of buttermilk leads to reduced cheese hardness. However, it has also been shown to reduce cheese melting, probably due to its emulsifying properties that may lead to reduced release of free oil.

Lecithin is a common emulsifier considered as a pseudo-fat replacer or fat extender since it may improve the rheology of reduced-fat products (Drake et al., 1998). The effect of addition of different types of lecithin at varying levels into cheesemilk was evaluated on the properties of reduced-fat Cheddar cheese by Drake et al. (1998), who found increased moisture content and therefore cheese yield when compared to control and also an increase in cheese whiteness. However, cheeses made with lecithin tended to develop off-flavours when evaluated by sensory analysis.

Colouring agents

Titanium dioxide is a whitening agent extensively used in paintings, textiles and food products (Weir et al., 2012), including cheese (Kosikowski and Brown, 1969). Wadhwani and McMahon (2012) successfully used titanium dioxide to increase whiteness of reduced-fat Cheddar cheeses that also led to an increase in acceptability when evaluated by consumers. Despite to the fact that titanium dioxide is approved as a food ingredient by Codex Alimentarius (FAO, 2015), the United States (FDA, 2005) and European Union legislation (EU, 2008), there are still some concerns as to whether it is safe for the environment and/or human consumption due its small particle sizes (Weir et al., 2012).

Annatto is a natural apocarotenoid pigment obtained from the seeds of a tropical tree (*Bixa orellana* L.), which is extensively used to give red-yellow notes to cheese and dairy spreads, in order to maintain an uniform appearance (Giuliano et al., 2003). Similarly to titanium dioxide, it is considered a safe ingredient (FDA, 2005; EU, 2008). However, it leads to a more translucent appearance when added to reduced-fat cheeses (Wadhwani and McMahon, 2012).

1.4.2 Modification or addition of steps during cheese manufacture

Ultrafiltration of milk

The use of membrane technologies has gained popularity in the cheese industry, since the protein content of cheesemilk can be concentrated, leading to improvements in yields (Johnson and Lucey, 2006). However, lactose content remains constant when milk is concentrated by these techniques, leading to a reduction in the ratio of lactose to casein of concentrated cheesemilks, reducing acid development and increasing buffering capacity (Johnson and Lucey, 2006). This technique could be a good alternative to manufacture low-fat cheeses, since modifying the lactose to casein ratio would allow cheesemakers to control acid development. McGregor and White (1990) found that low-fat Cheddar cheese made from cheesemilk concentrated 5x by ultrafiltration (UF) had higher moisture and protein content than cheese made from control (unconcentrated) cheesemilk. In contrast, the use of low-concentration factor ultrafiltration (LCF-UF; 1.2x – 1.8x) retentates led to a reduction in the moisture content of reduced-fat Minas Frescal cheese that negatively affected the textural properties (Cunha et al., 2006). Govindasamy-Lucey et al. (2004) suggested that some of the processing variables, such as the amount of starters, coagulants and salt levels used during manufacture, have to be modified based on the protein content of concentrated cheesemilks (UF retentate), in order to target desired composition and quality of cheese.

Homogenisation of cheesemilk

Homogenisation of milk has been used in the cheese industry to improve yield, since it reduces loss of fat in the whey, although it has also been associated with a weaker coagulum development that has a direct influence on cheese texture and melting properties, higher extent of proteolysis and development of excessive acidity (Jana and Upadhyay, 1992). Nevertheless different approaches involving homogenisation have been used to improve the properties of low-fat cheeses. Metzger and Mistry (1994, 1995) compared the effect of cream homogenisation alone and its further addition to milk on the properties of reduced-fat Cheddar cheese and found that homogenisation of cream improved yield, improved body and texture and distributed fat globules evenly throughout the cheese matrix, along with reducing their size, when compared to control samples. In a similar study, Rudan et al. (1998a) found that proteolysis was significantly lower in cheeses made with homogenised cream and also found that homogenization of cream and milk decreased the release of free oil, reducing melting. In addition, the authors found that the use of homogenization increased opaqueness of cheese, due to a reduction of the size of fat globules that led to increased light scattering. In a recent study, Deegan et al. (2014) proposed the use of low pressure homogenization to promote a slight increase in proteolysis and lipolysis in order to obtain reduced-fat Emmental cheese product with improved texture and flavour development.

Pasteurization

Guinee et al. (1998) evaluated an increase in the temperature of cheesemilk pasteurization from 72 to 88°C for 15 s and its effect on the properties of reduced-fat Cheddar cheese. The authors found that denaturation of whey proteins increased from 3 to 35%. Higher pasteurization temperatures led to an increase in rennet coagulation times, cheeses with higher moisture content, higher MNFS content, lower content of total protein and total Ca and reduced firmness. In addition, levels of proteolysis and sensory grading were not affected among treatments. In contrast, a similar study by Lo and Bastian (1998) of reduced-fat Havarti cheese, pasteurization of UF cheesemilks was done with temperatures ranging between 72 and 85°C for 17 s and the authors found development of excessive acidity in cheeses made from milk heated at the highest temperature.

Cutting the gel

Increasing moisture content of low-fat cheeses is a common technique that is used to improve their properties and can be easily achieved by cutting the curd into larger pieces (Mistry, 2001; Banks, 2004). Other alternative can be related to cutting the curd at a firmer texture. Johnson et al. (2001) evaluated the effect on the properties of reduced-fat Cheddar cheese of cutting the gel at different levels of firmness, which was achieved by cutting at different rennet coagulation times, ranging from 25 to 65 min.. Cheese manufacture protocol was adjusted to obtain similar pH at different cutting

times. Longer rennet coagulation times led to cheeses with higher moisture content and therefore higher yield. In addition, longer coagulation times led to cheeses with lower pH values, which resulted in a cheese with a softer texture and a smoother body, when compared to those treatments with reduced coagulation times.

Curd washing

Curd washing is a traditional step used in the manufacture of certain cheese varieties that consists of adding cold water into the curd after total drainage of the whey aimed at reducing the amount of lactose and lactic acid in the curd, in order to control cheese acidity (Lee et al., 2005). Chen and Johnson (1996) used curd washing by adding cold water into the curd after whey was completely drained during the manufacture of reduced-fat Cheddar cheese and found that this technique led to cheeses with higher moisture content, reduced residual lactose and lactic acid levels (i.e., controlled acidity) and greater solubilization of colloidal calcium phosphate, which may lead to an improvement in cheese texture and melting.

Cooking and milling

Tunick et al. (1993b) evaluated the effect of two different cooking temperatures (45.9°C and 32.4°C) on the properties of low-fat and full-fat Mozzarella cheese and

found that the lower cooking temperature resulted in higher MNFS content in both fat content levels.

Guinee et al. (1998) evaluated the effect of milling at pH values of 5.35 and 5.75 on the properties of reduced-fat Cheddar cheese and found that an increase of milling pH led to cheeses with increased moisture and MNFS content that would contribute to reduced cheese firmness (Johnson et al., 2009).

Storage temperature

Sheehan et al. (2004) compared the effect of two ripening temperatures (4 and 12°C) for 50 d on the properties of reduced-fat Mozzarella cheese and found that increasing ripening temperature resulted in cheeses with lower pH values, higher levels of primary and secondary proteolysis, lower firmness and higher meltability.

High hydrostatic pressure treatment

The application of high hydrostatic pressure (HPP) in cheese has shown a positive effect on ripening, due to increased lysis of starters and probably modifications to the cheese matrix (Messens et al., 1997; Yokohama et al., 1992). Johnston et al. (2002) evaluated the effect of different levels of HPP (100-500 MPa during 2 h) on the properties of half-fat Cheddar cheese (15% fat content) and found that increasing

pressure levels resulted in a softer texture and increased melting. In contrast, Sheehan et al. (2005) found no major changes in the composition, levels of proteolysis, texture and melting properties of reduced-fat Mozzarella cheese treated by HPP performed at 400 MPa for 5 min compared to untreated (control) cheeses. In a more recent study, Ozturk et al. (2013) evaluated different HPP treatments (50 – 400 MPa for 2.5 – 19.5 min) on the properties of reduced-fat Cheddar cheese and found that pressure levels ≥ 225 MPa led to softer cheeses with increased melting, even though proteolysis was not affected. The authors attributed these differences to a reduction in size of fat droplets as observed by fluorescence microscopy and also due partial changes occurring between protein-protein interactions. The authors also found that increasing pressure levels led to cheeses with higher pH values and reduced counts of LAB and NSLAB.

1.5 Conclusions

Interactions between the biochemistry, microbiology and physical properties of low-fat cheeses can lead to poor consumer acceptability. However, several strategies have been developed by the cheese industry to improve the properties of these products that include the addition of ingredients or modifications to the existing manufacturing protocols. Key factors to improve quality of low-fat cheeses will have to consider the specific cheese variety and the combinations of various strategies to obtain more desirable products.

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CHAPTER 2

EFFECT OF FAT CONTENT AND TEMPERATURE ON THE TRANSLUCENCY OF CHEDDAR CHEESE

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2.1 Abstract

Cheese translucency is influenced by fat content, ripening and temperature; and is traditionally determined by CIELAB whiteness. However, translucency is also affected by thickness of materials. The Kubelka-Munk method (K/S) estimates the ratio of absorbance to scatter from whiteness measurements on thin layers of materials above white and black backgrounds. This study aimed to evaluate the effect of fat content and temperature on Cheddar cheese translucency by K/S and CIELAB methods. Cheddar cheeses varying in fat composition were made and translucency was measured during heating/cooling at 2 and 180 d of ripening. Low fat content in cheese was found to be related to high translucency at $<30^{\circ}\text{C}$. At 40°C , translucency increased due to fat melting. At $>60^{\circ}\text{C}$, a reversible protein aggregation led to a reduction of translucency. Translucency increased as cheeses aged. CIELAB and K/S methods were highly correlated, suggesting that K/S could be a useful method to estimate cheese translucency.

Keywords Cheese translucency, Kubelka-Munk, CIE whiteness, Low-fat cheese.

2.2. Introduction

Colour is considered one of the most important attributes in food appearance; it is associated with certain parameters of quality, such as composition and deterioration and has a big impact on consumers preference (MacDougall, 2002). In cheese, colour is highly influenced by processing conditions, addition of colouring agents, composition, pH, age and temperature (Dave et al., 2001; Joshi et al., 2003; Metzger et al., 2001; Wadhwani and McMahon, 2012). The influence of fat content in cheese not only relates to texture and flavour properties, but also has a big impact on colour and appearance. One of the major appearance defects found in reduced-fat cheeses is associated with an increase in translucency, due to different light scattering properties of fat and protein (Johnson et al., 2009), which are temperature-dependent (Metzger et al., 2000).

The measurement of whiteness has been used extensively to determine the degree of cheese translucency (Dave et al., 2001; Joshi et al., 2003; Metzger et al., 2001, 2000; Rudan and Barbano, 1998; Wadhwani and McMahon, 2012). The level of whiteness is represented on the CIELAB colour scale as the L^* value, which ranges from 0, which represents black, to 100, representing white (i.e., a perfect reflecting diffuser; Hunterlab, 2012). However, in the study of translucent materials, the use of traditional methods of colour measurement may not represent the actual colour of samples as perceived by the human eye, due to reflection of light from below the surface (Gullett et al., 1972) and is greatly influenced by the thickness of samples (Calvo and Salvador, 1997; Little, 1964). Independent of the type of material (translucent or opaque), the reflection of light is highly dependent of the ratio of

absorption to scatter, which is affected by levels of pigmentation, refractive index and light-scattering properties of the material (MacDougall, 2002). The importance of the study of the ratio of absorption to scatter coefficients on translucent materials has to be considered to obtain an adequate concept of colour appearance (Judd and Wyszecki, 1975; MacDougall, 2002).

The Kubelka-Munk index (K/S) is calculated from an empirical formula that determines the ratio of absorbance, K , to scatter, S , based on the measurement of reflectivity, i.e., the reflectance of a certain material at infinite thickness (Judd and Wyszecki, 1975). This analysis has been applied extensively to predict the optical properties of materials used in paint, textile, plastics and other industries (MacDougall, 2002). The application of the Kubelka-Munk theory of reflectance has also been used in different food products such as fruit gels, orange juice, meat products, milk and coffee (Huang et al., 1970; Gullett et al., 1972; Calvo and Salvador, 1997; MacDougall, 2002).

This study aimed to evaluate the optical properties of Cheddar cheeses by means of the Kubelka-Munk analysis; in particular, the effect of fat content, cheese age and heating and cooling were studied. A relationship between this methodology and the measurement of whiteness by the L^* values was also established.

2.3 Materials and methods

2.3.1 Cheese Manufacture

A standard Cheddar cheese manufacturing procedure was conducted at the pilot plant facilities of the School of Food and Nutritional Sciences, University College Cork, Ireland. Experimental cheeses were manufactured on a 50 kg scale, from cheesemilks standardized to fat levels of 3.50%, 2.63%, 1.75% or <0.10% (maintaining casein to fat ratios of 0.70, 1.05, 1.40 and ≥ 24.50 , respectively), in order to obtain four different cheeses: full fat (FF), reduced fat (RF), half fat (HF) and non fat (NF). Each standardised cheesemilk was pasteurised at 73.5°C for 15 s and cooled to 31°C. A concentrated starter culture (R-604Y Cheddar culture starter, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into the vats at a level of 0.03% (w/w) and left to ripen for 30 min under continuous stirring, then 0.09% (v/w) of 1 M CaCl₂ was added and equilibrated for 2 min. Chymosin (Maxiren 180, DSM Food Specialties, Delft, Netherlands) at a strength of 180 International Milk-Clotting Units (IMCU mL⁻¹) was added to each cheesemilk at a level of 0.03% (v/w), diluted 1 to 4 with distilled water to aid dispersion. Once the coagulum developed enough firmness after 45-50 min, curds were cut and cooked from 31 to 39°C during 30 min. The temperature was maintained at 39°C until the pH of curds decreased to 6.2 and the whey was drained from the vats. The curd was cut into blocks and inverted every 15 min until the pH reached 5.4. Curd blocks were milled, salted at a level of 2.5% (w/w) NaCl and equilibrated for 20 min. The salted curd was transferred to 5 kg rectangular moulds and pressed for 14 hours at

2.5 kg cm⁻² at 20°C. The experimental cheeses were vacuum packed and ripened for 6 months at 8°C.

2.3.2 Chemical Analysis

Composition of experimental cheeses was determined after 14 days of ripening. Moisture content was measured by the oven drying method (IDF, 1982), fat by the Gerber method (IIRS, 1955), protein by the macro-Kjeldahl method (IDF, 1986) and salt content by potentiometric determination (Fox, 1963). Total calcium was determined by atomic absorption spectroscopy (IDF, 2007) on the cheesemilk, rennet whey and experimental cheeses. The proportion of insoluble calcium in cheese (INSOL Ca) was determined by the acid-base titration method after 2 and 180 days of ripening as described by Hassan et al. (2004). The pH of experimental cheeses was measured at 20°C on a homogenized mixture of 10 g of cheese and 10 mL of deionized water using a glass pH electrode (Madkor et al., 1987). Proteolysis during ripening was determined by the pH 4.6-soluble N (Kruchoo and Fox, 1982).

2.3.3 Translucency during heating

The degree of translucency of experimental cheeses during heating and cooling was evaluated by the determination of the Kubelka-Munk values and the measurement of CIE *L** values at 2, 14, 30, 60, 120 and 180 days of ripening. Measurements were

performed with a Konica-Minolta colorimeter CR-400 attached to a data processor DP-400 (Konica Minolta Optics Inc., Osaka, Japan). The instrument was set on the CIELAB system based on illuminant D65 and a visual angle of 2°. A white calibration plate CR-A43 (Y: 87.1, x: 0.3186, y: 0.3364) was used to calibrate the instrument before analysis. All the measurements were performed through an 8 mm diameter diaphragm containing a glass light projection tube.

Kubelka-Munk index

The Kubelka-Munk formula relates the coefficient of absorption K and scatter S to the reflectance of a material of infinite thickness R_∞ , as described in Equation (2.1):

$$K/S = (1 - R_\infty)^2 / 2 R_\infty \quad (2.1)$$

where

$$R_\infty = a - b \quad (2.2)$$

$$a = 1 / 2 [R + (R_0 - R + R_g) / R_0 R_g] \quad (2.3)$$

$$b = (a^2 - 1)^{1/2} \quad (2.4)$$

From Equations (2.2) to (2.4), it is possible to establish R_∞ based on the reflectance R of a sample positioned above a white background with a known reflectance R_g and the reflectance of the sample placed on a black background R_0 (Judd and Wyszecki, 1975). Previous studies have shown that K/S values can be expressed on the CIE system (Huang et al., 1970; Gullett et al., 1972; Calvo and Salvador, 1997). In the present

study, the K/S values were based on L^* values ($K/S L^*$) obtained from samples placed above black and white backgrounds (L^*_B and L^*_W , respectively). The contrast ratio (ratio between L^*_B and L^*_W : $CR L^*$), which correspond to an indicator of opacity (Judd and Wyszecki, 1975) was also estimated. A black ceramic tile (Y: 3.8, x: 0.3129, y: 0.3294) was used as a black background and the white calibration tile (previously described) was used as a white background.

Cheese samples were cut into discs (35 mm diameter and 2 mm of height), placed in plastic petri dishes (90 mm internal diameter), coated with a liquid paraffin layer (Fisher Scientific UK Limited, Loughborough, UK) and covered with the petri dish lid to prevent loss of moisture. All the samples were preincubated for 30 min in a cold room at 4°C. Samples were then incubated at 4, 20, 30, 40, 60 and 80°C for 30 min. After treatment, the CIE L^* value was measured in the middle of each cheese disc placed on black and white backgrounds. Five cheese discs were incubated at each temperature and two measurements per cheese disc were made on each background. After measurements, samples were incubated at their respective lower temperature for an additional 30 min and colour was measured again on each background. Petri dishes coated with a layer of liquid paraffin were also preincubated and incubated under the same conditions as experimental cheeses to obtain L^* values on black and white backgrounds and used them in Equation (2.3) as R_0 and R_g , respectively.

CIE L values*

Samples were prepared according the method described by Dave et al. (2001) with some modifications. Cheese samples were cut into cylinders (26 mm of diameter and 28 mm of height), placed into 10 mL Pyrex® beakers ensuring that cheese samples were in contact with the walls and the bottom of the glass. Beakers were sealed with rubber stoppers to prevent loss of moisture during treatment. The preincubation and incubation stages were made under the same conditions as in cheese discs. After heat treatment, the CIE L^* values were measured through the bottom of the beakers. Five cheese cylinders were incubated at each temperature and two colour measurements were performed per cylinder.

2.3.4 Temperature soluble nitrogen (TSN)

Nitrogen solubility in a heat-treated aqueous phase model of experimental cheeses was determined to evaluate the role of protein aggregation on cheese translucency at 2 and 180 d of ripening. Synthetic Cheddar cheese aqueous phase (SCCAP) solutions were prepared for each experimental cheese according the method of Broome and Limsowtin (2002) as described by O'Mahony et al. (2006) with some modifications. The pH of SCCAP was adjusted to the values found for the experimental cheeses and the calcium content was adjusted with calcium chloride to obtain the calcium concentration found in the soluble phase of each experimental cheese at 2 and 180 d of ripening. Fifty grams of grated cheese was mixed with 100 mL of SCCAP at

10,000 revolutions s⁻¹ for 5 min with an Ultra-Turrax® homogeniser (T25 Basic with S25N-18G dispersing element; IKA-Labortechnik, Staufen, Germany). The mixture was stored overnight at 4°C with continuous stirring and centrifuged at 3,000 x g for 30 min at 4°C. The supernatant obtained was filtered at 4°C through glass wool and Whatman 113 filter paper. Aliquots of the filtered supernatants were incubated under the same conditions as of experimental cheeses during heat treatment in a waterbath. After incubation, samples were centrifuged at 15,000 x g for 5 min at 20°C, placed for additional 5 min at the incubation temperature and centrifuged again under the same conditions previously described for additional 5 min. Samples were then filtered through a 0.45 µm filter material (Millex ®; Millipore Corporation; Bedford, MA, USA). The nitrogen content of the filtered sample was determined by the Kjeldahl method to estimate the temperature soluble nitrogen content as a percentage of the total nitrogen found on cheese (%TSN/TN), as described in Equation (2.5):

$$\% TSN / TN = 2 TNS (MAP + MC / 2) / TNC \quad (2.5)$$

where TNS corresponds to the total nitrogen content of the treated sample, MAP is the moisture content of the SCCAP, MC is the moisture content of the cheese sample and TNC is the total nitrogen content of the cheese sample.

To evaluate the reversible effect of cooling on aggregation, samples were heated at 80°C for 30 min, cooled to 4°C for 24 h and the content of %TSN/TN was also estimated. In addition, urea-polyacrylamide gel electrophoresis (Andrews, 1983) was performed on these treated samples to characterize the protein profile of the soluble

aqueous phase model from cheese. Gels were stained with Coomassie blue G250 as described by Blakesley and Boezi (1977).

2.3.5 Experimental design and statistical analyses

Four treatments based on different fat levels (FF, RF, HF and NF) were used in three independent trials, based on a randomized 4 x 3 block design. Analysis of variance (ANOVA) was performed on cheese composition at a significance level of $P < 0.05$. A split-split-plot design (Montgomery, 2013) was used to evaluate the effect of treatment (fat content: FF, RF, HF and NF), ripening time, temperature and their interactions on the translucency of cheese determined by the K/S index, CIE L^* values and TSN. A split-plot design (Montgomery, 2013) was used to evaluate the effects of treatment, ripening and their interactions on proteolysis, L^*_B values, L^*_W values and CR L^* . The ANOVA for both the split-split-plots and the split-plots designs was performed using a general linear model (GLM) procedure. When significant differences ($P < 0.05$) were found, the treatments means were analyzed by the Tukey's multiple comparison test. Pearson correlation coefficients were determined among the translucency values measured with K/S L^* , CR L^* and L^* values at different temperatures during heating and cooling ($P < 0.05$). All analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA).

2.4 Results and discussion

2.4.1 Cheese composition, pH and level of insoluble calcium

Means of three independent trials for composition, percentage of insoluble calcium and pH from experimental cheeses are shown in Table 2.1. As expected, significant differences were found in composition of cheeses as the fat content was reduced ($P < 0.05$), leading to a marked increase in moisture, protein and calcium content ($P < 0.05$). A reduction in the fat content of cheese leads to a shift in the compositional balance of most of the other cheese components (Mistry, 2001). At 2 d of ripening, levels of INSOL Ca ranged about 65%-70%, although HF exhibited a higher percentage than other treatments ($P < 0.05$). A decrease of INSOL Ca was observed at 30 d of ripening ($P < 0.05$; data not shown) and remained constant until 180 d, except for NF cheese that remained constant during ripening ($P > 0.05$). Hassan et al. (2004) reported that the proportion of INSOL Ca in Cheddar cheese decreases during the first 30 d of ripening and remains constant thereafter. On the other hand, Metzger et al., (2001) found no changes in the proportion of water-insoluble calcium and an increase in pH during 90 d of storage of low-fat Mozzarella cheese (6% fat). The pH values at 2 d were slightly different, ranging between 5.2-5.3 ($P < 0.05$). The pH at 180 d remained constant for FF ($P > 0.05$) and increased in RF, HF and NF cheeses ($P < 0.05$). This increase is associated with a reduction of INSOL Ca, causing the release of phosphate ions from colloidal Ca phosphate, leading to their combination with hydrogen ions and hence an increase in pH (Hassan et al., 2004). This increase was also higher in those

cheeses with a lower fat content, given by a higher buffering effect due to a higher protein and calcium content.

Table 2.1 Composition (14 d), percentage of insoluble calcium as a percentage of total cheese Ca and pH values (2 and 180 d) of Cheddar cheeses with various fat levels

Parameter	Treatment				SEM
	FF	RF	HF	NF	
Moisture (%)	37.81 ^c	39.54 ^{cb}	42.34 ^c	47.74 ^a	1.21
Fat (%)	32.44 ^a	23.89 ^b	19.00 ^c	ND	1.98
Protein (%)	24.47 ^d	27.84 ^c	30.46 ^b	41.50 ^a	1.94
Salt (%)	1.69 ^a	1.60 ^a	1.73 ^a	1.82 ^a	0.063
MNFS (%)	55.95 ^a	51.96 ^b	52.25 ^b	-	0.921
FDM (%)	52.17 ^a	39.54 ^b	32.96 ^c	-	2.84
S/M (%)	4.46 ^a	4.05 ^a	4.09 ^a	3.81 ^a	0.158
Total Calcium (mg 100g ⁻¹)	761 ^d	888 ^c	985 ^b	1412 ^a	75.9
INSOL Ca/total Ca					
2d	65.05 ^b	66.38 ^b	71.81 ^a	65.03 ^b	1.15
180 d	52.46 ^b	55.81 ^b	60.68 ^{ab}	64.71 ^a	1.68
pH					
2 d	5.21 ^{ab}	5.17 ^b	5.20 ^b	5.31 ^a	0.019
180 d	5.13 ^c	5.22 ^b	5.24 ^b	5.44 ^a	0.035

Abbreviations are: MNFS, moisture in the non-fat substance; FDM, fat content on a dry basis weight; S/M, salt in the moisture phase of the cheese, ND, not detected by the Gerber method.

Data are means of three replicate trials.

Means within the same row not sharing a common superscript differ ($P < 0.05$).

2.4.2 Proteolysis

Proteolysis expressed as the level of pH 4.6-soluble N of experimental cheeses is shown in Fig. 2.1. It was found that treatment and age of cheese had a significant effect on the level of proteolysis (Table 2.2). During the first stages of ripening, similar levels of pH 4.6-soluble N were found in all treatments. Proteolysis significantly increased during ripening and it was found to be lower in NF after 14 d of ripening and similar in other treatments ($P < 0.05$). This trend was also observed thereafter. At 180 d, no differences were found among cheeses ($P > 0.05$). A reduction in primary proteolysis in cheese with reduced fat content is attributed to a concomitant decrease in the level of moisture-in-non-fat-substances (MNFS; Table 2.1) and hence a reduction in the availability of water for microbial and enzyme activity (Fenelon and Guinee, 2000). In addition, the extent of proteolysis is highly dependent of the moisture and salt content of cheese (Mistry and Kasperson, 1998). In our study, the MNFS content was higher only in FF and no differences in the S/M content were found (Table 2.1), which may be related to the similar levels of proteolysis at the end of ripening.

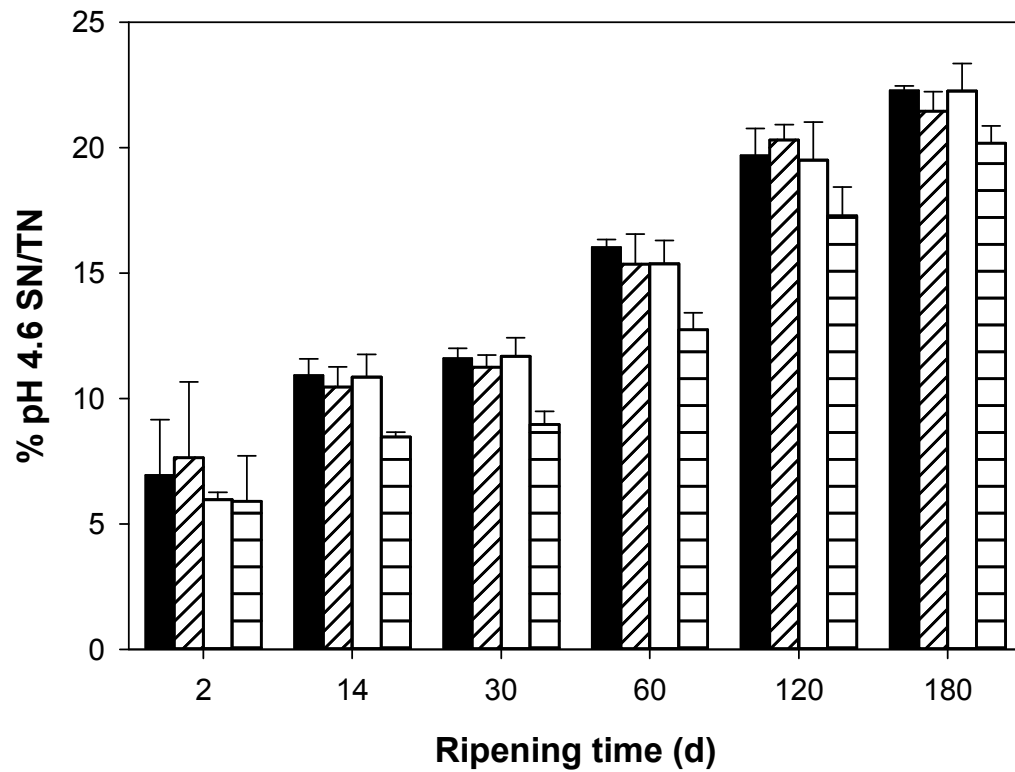


Fig. 2.1. Changes in proteolysis expressed as pH 4.6 soluble N as a percentage of total N (% pH 4.6 SN/TN) of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses during ripening. Values represent mean and standard deviations of three replicate trials.

Table 2.2 Mean squares, probabilities (in parenthesis) and R^2 values for proteolysis, CIE whiteness (L^*) on black and white backgrounds and contrast ratio based on whiteness during ripening for FF, RF, HF and NF Cheddar cheeses.

Factors	df	Proteolysis	L^*_B	L^*_W	CR L^*
Whole Plot					
Day of cheese manufacture (D)	2	2.959 (0.306)	75.410* (0.029)	42.960* (0.031)	0.002* (0.047)
Treatment (T)	3	21.425** (0.008)	2757.420** (<0.001)	851.62** (<0.001)	0.154** (<0.001)
Error (D x T)	6	2.036	11.09	6.600	0.0004
Subplot					
Age (A)	5	392.140** (<0.001)	328.800** (<0.001)	151.670** (<0.001)	0.009** (<0.001)
T x A	15	0.844 (0.694)	14.840* (0.018)	4.090 (0.200)	0.002** (<0.001)
Error	40	1.088	6.440	2.950	0.0003
R^2		0.98	0.98	0.97	0.98

Split-plot design with the 4 treatments (fat content: FF, RF, HF and NF) were analysed as a discontinuous variable and cheese manufacture day was blocked.

Subplot included the effect of age and treatment x age as variables.

Abbreviations are df, degrees of freedom; L^*_B , whiteness determined on a black background at 20°C; L^*_W , whiteness determined on a white background at 20°C; CR L^* , contrast ratio based on L^* values at 20°C.

* $0.01 < P \leq 0.05$; ** $P \leq 0.01$.

2.4.3 Cheese translucency

Kubelka-Munk analysis

Fat content of experimental cheeses had a significant effect ($P < 0.05$) on the $K/S L^*$ values during heating and cooling at different points of ripening (Fig. 2.2; Table 2.3). It was also found that $K/S L^*$ values were affected ($P < 0.05$) by the interaction of treatment x temperature and age x temperature. The K/S index is defined as the ratio of the absorption (K) to the scattering (S) coefficient (Judd and Wyszecki, 1975). At 2 d of ripening $K/S L^*$ values at 4°C decreased as the fat content was reduced ($P < 0.05$; Fig 2.2a). This finding was opposed to the expected, as lower K/S values are related to a higher scattering and hence a lower translucency. However, a reversal of this relation has been previously observed when K/S is expressed in terms of whiteness due to a considerable increase of S and a reduction of K and also to the sensitivity of L^* value on scattering effects (Huang et al., 1970; Gullett et al., 1972). There were no variations in $K/S L^*$ values during heating and cooling for FF, RF and HF cheeses ($P > 0.05$). On the other hand, the $K/S L^*$ values of NF cheese increased as the sample was heated, reaching the same level as other cheeses at 40°C ($P > 0.05$) and remaining constant thereafter. The same trend was observed when heated samples were cooled to their previous temperature stage and held for additional 30 min. We observed no changes in the translucency of FF, RF and HF cheeses, whereas NF exhibited a gradual decrease in $K/S L^*$ as the temperature decreased.

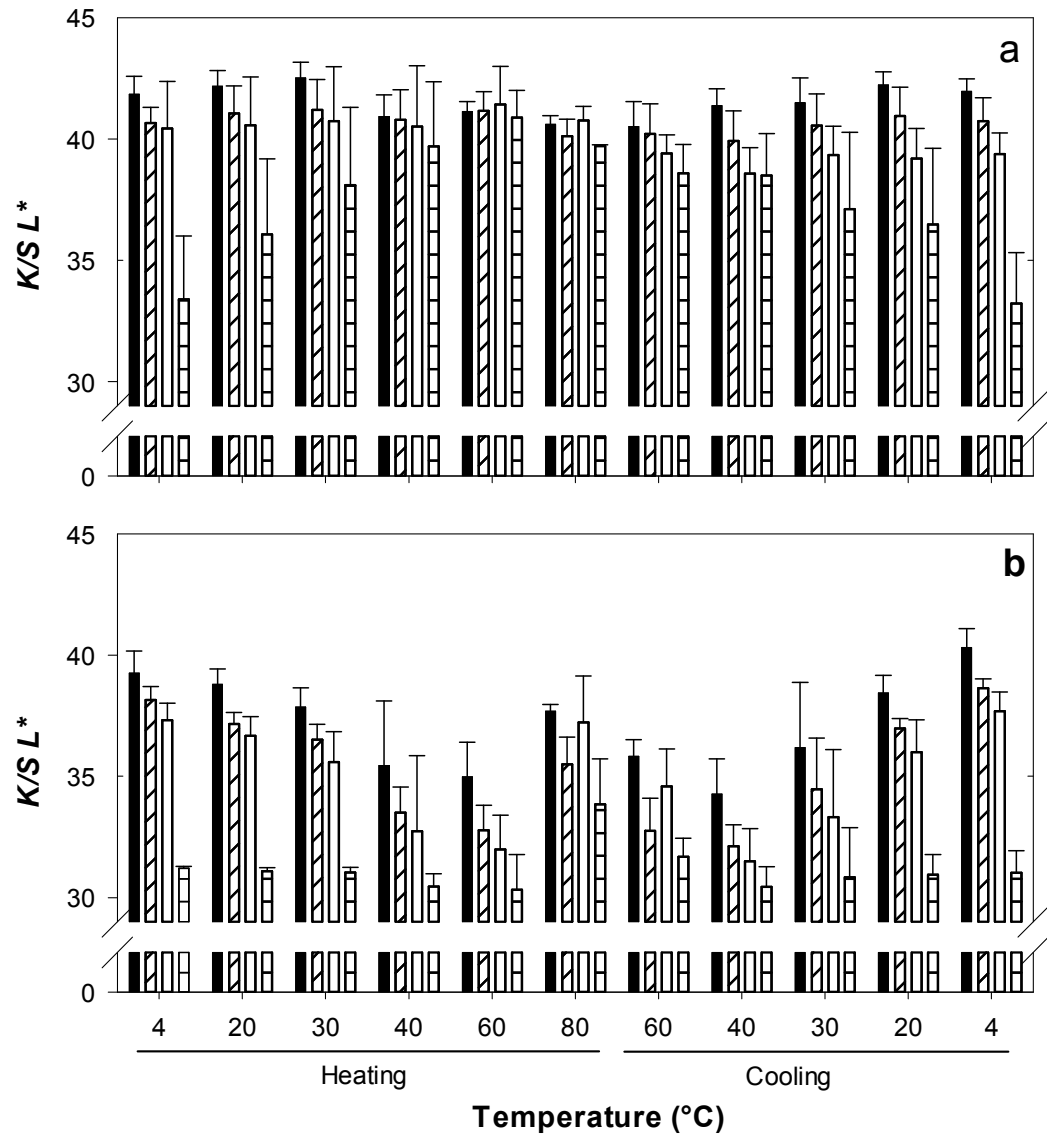


Fig. 2.2 Changes in the Kubelka-Munk index determined by L^* values ($K/S L^*$) during heating and cooling of FF (■), RF (//), HF (□) and NF (≡) cheeses at 2 (a) and 180 d of ripening (b). Values represent mean and standard deviations of three replicate trials.

Table 2.3 Mean squares, probabilities (in parenthesis) and R^2 values for $K/S L^*$ index and CIE L^* whiteness for experimental cheeses.

Factor	df	$K/S L^*$	CIE L^*	df	TSN
Whole-Plot					
Day of cheese manufacture (D)	2	75.107 (0.085)	49.360 (0.630)	2	14,53 (0.636)
Treatment (T)	3	302.330** (<0.001)	3399.64** (0.001)	3	70.260* (0.045)
Whole-Plot Error					
D x T	6	9.042	135.860	6	14.06
Subplot					
Aging (A)	1	1822.922** (0.003)	13554.690** (<0.001)	1	6333.62** (0.003)
T x A	3	10.441 (0.229)	510.590* (0.012)	3	107.810* (0.042)
Subplot Error					
D x A	2	6.310	3.600	2	20.250
D x T x A	6	5.471	55.970	6	20.810
Sub-subplot					
Temperature (H)	10	13.714** (<0.001)	344.640** (<0.001)	6	380.570** (<0.001)
T x H	30	8.544** (<0.001)	167.490** (<0.001)	18	2.570** (0.001)
A x H	10	26.594** (<0.001)	367.800** (<0.001)	6	20.970** (<0.001)
T x A x H	30	0.917 (0.313)	15.970* (0.045)	18	19.160** (<0.001)
Error	160	0.815	10.300	96	1.670
R^2		0.97	0.96		0.99

Split-Split-plot design with the 4 treatments (fat content: FF, RF, HF and NF) were analysed as a discontinuous variable and cheese manufacture day was blocked.

Subplot included the effect of aging of cheese (A) and treatment x age as variables.

Sub-subplot included the effect of temperature of heating and cooling (H), treatment x heat, age x heat and treatment x age x heat as variables.

Degrees of freedom (df) differed for $K/S L^*$, CIE L^* , and TSN, as the temperature points for the analyses were different.

Abbreviations are $K/S L^*$, translucency determined by the Kubelka-Munk analysis; CIE L^* , whiteness; TSN, temperature soluble N as a percentage of total cheese N.

* $0.01 < P \leq 0.05$; ** $P \leq 0.01$

During ripening, the $K/S L^*$ value decreased for all cheeses ($P < 0.05$). As no significant differences were found for $K/S L^*$ values at different temperatures from 14 to 180 d of ripening ($P > 0.05$; data not shown), this study only reports the translucency results obtained after 180 d of ripening (Fig. 2.2b). The $K/S L^*$ values of experimental cheeses at 4°C also decreased as the fat content was reduced ($P < 0.05$). During heating, a decrease in $K/S L^*$ was observed for FF, RF and HF cheeses from 4 to 40°C ($P < 0.05$), remaining constant until 60°C. At 80°C, $K/S L^*$ values increased to the same levels found at 4°C for each respective cheese ($P < 0.05$). On the other hand, $K/S L^*$ values of NF were constant when heated from 4 to 60°C and exhibited an increase only at 80°C. During cooling, a decrease of $K/S L^*$ was observed for FF, RF and HF from 80 to 40°C to increase again to the same levels found before heating. On the other hand, cooling of NF cheese from 80 to 40°C caused higher $K/S L^*$ values than heating.

CIE L^ whiteness*

Similar trends as $K/S L^*$ values were obtained for the measurement of cheese translucency by L^* values (Fig. 2.3). A significant effect on the fat content ($P < 0.05$) was also observed on heating and cooling during ripening (Table 2.3). The L^* values were also affected by the interactions of treatment x age, treatment x temperature, age x temperature and treatment x age x temperature. A decrease in L^* values at 4°C was found after 2 d of ripening as the fat content was reduced (Fig. 2.3a; $P < 0.05$). No differences ($P < 0.05$) in L^* values were found for FF, RF and HF during heating. Only NF exhibited an increase of L^* value from 4 to 40°C, remaining constant at higher

temperatures. The L^* values of FF, RF and HF cheeses showed no differences when samples were cooled down, whereas NF exhibited a decrease.

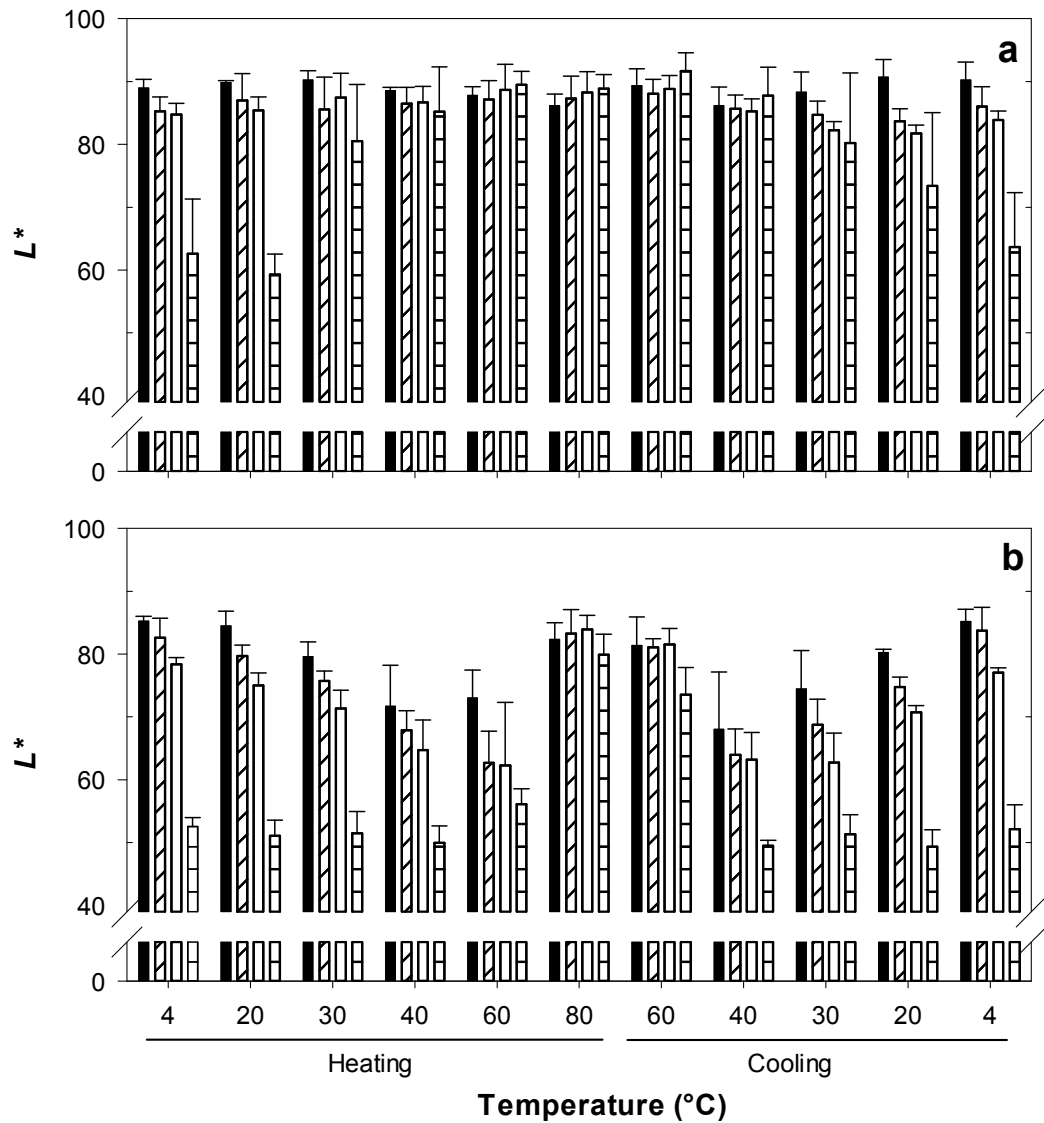


Fig. 2.3 Changes in whiteness (L^* values) during heating and cooling of FF (■), RF (//), HF (□) and NF (≡) cheeses at 2 (a) and 180 d of ripening (b). Values represent mean and standard deviations of three replicate trials.

The L^* values decreased at 180 d of ripening (Fig. 2.3b; $P < 0.05$). FF, RF and HF exhibited a decrease in L^* values from 4 to 40°C, remaining constant at 60°C and increased again at 80°C to the same value as that of FF at 4°C ($P > 0.05$). On the other hand, the L^* values of NF were constant from 4 to 40°C, exhibiting a slight increase at 60°C and at 80°C values reached the same level of whiteness as other cheese samples ($P > 0.05$). We observed higher L^* values in those samples initially heated at 80°C and then cooled to 60°C in all cheeses, when compared to those samples only heated at 60°C ($P < 0.05$).

Contrast ratio

When translucency was calculated by the $K/S L^*$ method, we found differences in the individual measurements of whiteness obtained in cheese discs above black or white backgrounds. Fig. 2.4 shows the CIE L^* values of 2 mm cheese layers measured above black (L^*_B) or white (L^*_W) backgrounds and its contrast ratio ($CR L^*$) at 20°C for different ripening times. For each background, whiteness was found to be higher in those cheeses with a higher fat content (Fig. 2.4a and 2.4b; $P < 0.05$). We also found a decrease in L^* values after 14 days of ripening (Table 2.2). The $CR L^*$ values decreased as the fat content of cheese was reduced and all samples showed a decrease in this parameter with cheese age (Fig. 2.4c; Table 2.2). Judd and Wyszecki (1975) indicated that a contrast ratio of 0.98 is equivalent to a completely opaque material, although it is influenced by the thickness of the sample. Under the conditions studied, only FF, RF and HF were found to be opaque at 2 d of ripening, which corresponds to $K/S L^* > 40$

and CIE L^* values > 85 . Our data agree with the results of Dave et al. (2001) who determined by visual observations that salted and unsalted non-fat Mozzarella cheeses were opaque when L^* values were higher than 85. These authors also indicated that a CIE L^* value of 82 corresponds to the transition point from opaque to translucent. However, visual observations may lead to some mistakes, even under standardized conditions of illumination (Judd and Wyszecki, 1975). In addition, the CR L^* values showed a similar trend to $K/S L^*$ and L^* values during heating and cooling of cheese samples (data not shown).

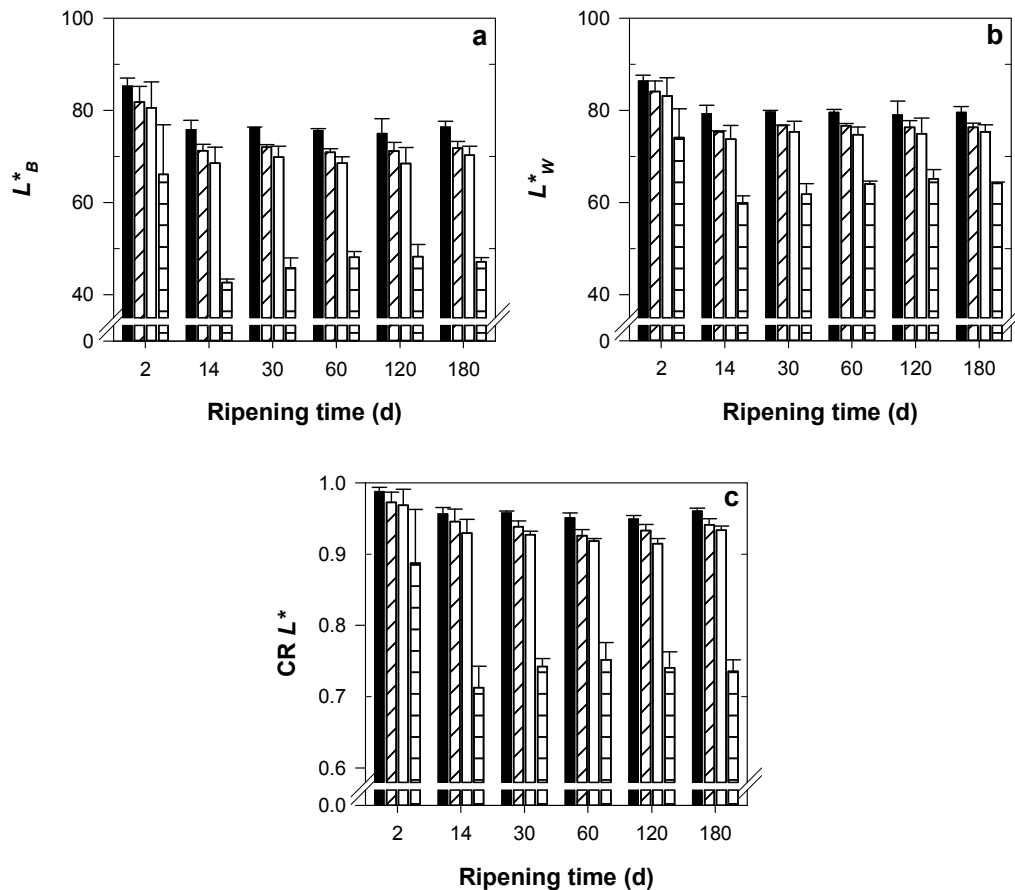


Fig 2.4 Changes in whiteness measured above black (a) or white (b) backgrounds and contrast ratio (c) of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses during ripening. Values represent mean and standard deviations of three replicate trials.

Influence of cheese composition, temperature and ripening

As expected, a lower fat content in cheese is associated with a reduction in the number of light-scattering centers and an increase in the protein and moisture content lead to a translucent appearance (Johnson et al., 2009; Mistry, 2001). The K/S L^* , CR L^* and CIE L^* parameters showed differences in translucency based on the fat content. At 2 d of ripening, none of the methods were able to find differences in the translucency of FF, RF and HF during heating and cooling; however, NF cheese exhibited a decrease of translucency at $\sim 30^\circ\text{C}$. A significant increase in L^* values during heating at 30°C was reported by Dave et al. (2001) for non-fat Mozzarella cheese at 1 d of ripening, probably due to protein-protein interactions. However, cheese fat could also influence the optical properties of FF, RF and HF cheeses. During heating, a high proportion of milk fat melts in the range of $10\text{-}20^\circ\text{C}$ and is completely liquid at about 40°C (MacGibbon and Taylor, 2006), resulting in a reduction of light-scattering and hence an increase in translucency.

At 180 d of ripening, the methods used showed an increase in translucency for all cheese samples. Translucency increased when FF, RF and HF cheeses were heated to 40°C and was reduced again on heating at 80°C , whereas NF exhibited a decrease in translucency at 60°C . A reduction in cheese translucency during heating has been previously related to changes in the serum phase of the cheese. Metzger et al. (2000) found the formation of a white gel when the expressible serum of low-fat and full-fat low moisture part skim Mozzarella cheese was heated from 7 to 49°C , which is associated with a decreased protein solubility as hydrophobic interactions are increased

with temperature. Translucency is also related to changes in cheese structure. A shrinkage of the cheese matrix during heating at high temperatures leads to the expulsion of fat and water (Guinee et al., 2000). Pastorino et al. (2002) found a denser matrix when they observed, by electron microscopy, the microstructure of non-fat Mozzarella cheese heated at 50°C, and found the formation of new serum pockets and protein aggregates of high density, suggesting that protein-protein interactions were promoted by an increase in temperature. We observed a reduction of $K/S L^*$, $CR L^*$ and $CIE L^*$ values as cheese age increased, which might be influenced by an increase in proteolysis (Fig. 2.1) and a reduction of the proportion of INSOL Ca (Table 2.1). An increase in levels of cheese proteolysis with age is related to an increase in translucency and higher heating temperatures to produce a reduction in translucency (Metzger et al., 2001, 2000; Dave et al., 2001). The content of calcium in cheese had no effect on cheese translucency at 5°C; however, a marked reduction was observed at 60°C as the calcium content was increased (Joshi et al., 2003). In addition, a decrease in the proportion of water-insoluble calcium in low-fat Mozzarella cheese during ripening was associated with higher translucency during heating and cooling (Metzger et al., 2001).

An increase in cheese translucency during cooling has been reported previously. Metzger et al. (2000) attributed this phenomenon to an increase in the solubility of light scattering particles formed during heating from the cheese serum phase. At lower temperatures (<40°C), an increase of the cheese whiteness occurs due to solidification of cheese fat.

Table 2.4 Pearson correlation (r) and probabilities (in parenthesis) between the ratio of absorbance to scatter ($K/S L^*$), whiteness (CIE L^*) and contrast ratio (CR L^*) of experimental cheeses at different temperatures during heating and cooling.

Treatment	Temperature (°C)					
	4	20	30	40	60	80
Heating						
$K/S L^* - L^*$	0.990**	0.968**	0.968**	0.964**	0.937**	0.802**
	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
$K/S L^* - CR L^*$	0.970**	0.957**	0.907**	0.873**	0.944**	0.790**
	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
$L^* - CR L^*$	0.989**	0.970**	0.963**	0.927**	0.889**	0.765**
	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
Cooling						
$K/S L^* - L^*$	0.989**	0.985**	0.954**	0.958**	0.922**	-
	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(0.002)	
$K/S L^* - CR L^*$	0.973**	0.932**	0.917**	0.953**	0.914**	-
	(<0.001)	(0.001)	(<0.001)	(<0.001)	(0.001)	
$L^* - CR L^*$	0.984**	0.964**	0.964**	0.897**	0.895**	-
	(<0.001)	(<0.001)	(<0.001)	(0.003)	(0.003)	

*0.01 < $P \leq 0.05$; ** $P \leq 0.01$ *Relationship between Kubelka-Munk, contrast ratio and CIE whiteness*

A high Pearson correlation ($r > 0.900$; $P < 0.05$) was found among the measurement of cheese translucency by $K/S L^*$, CR L^* and L^* whiteness (Table 2.4), suggesting that these methods could be useful to estimate cheese translucency during

heating and cooling. However, a lower correlation was observed ($r \sim 0.800$; $P < 0.05$) when cheeses were heated at 80°C. In a preliminary study of $K/S L^*$ and $CR L^*$ (data not shown), we found loss of moisture when samples were heated at 60 and 80°C, that contributed to a decrease in the diameter of cheese discs. The addition of a layer of liquid paraffin avoided this problem and the $K/S L^*$ and $CR L^*$ values showed no differences when coated samples were compared to control samples (not shown). The hydrophobic surface coating on a cheese pizza baking model was successfully used by Rudan and Barbano (1998) to reduce cheese dehydration and also prevented the formation of blisters.

At elevated temperatures, cheese behaves as a viscous-like material mainly due to a reduction of the CN-CN interactions ($>40^\circ\text{C}$) and melting of cheese fat ($\leq 40^\circ\text{C}$; Lucey et al., 2003). We observed, however, no melting in our cheese samples following any heat treatment. Guinee et al. (2000) reported shrinkage of the cheese matrix when exposed to high temperatures. This phenomenon may present some limitations if shrinkage affects the thickness of cheese samples exposed at high temperatures. Even though the Kubelka-Munk method estimates the ratio of absorbance to scatter based on reflectivity, the thickness of the samples analysed is also an important parameter to consider. Best (1987) established that, in order to obtain accurate, reliable and reproducible results, the ratio of the aperture area of measurement to the thickness of the sample has to be greater than 10. In our study, we obtained a ratio (~ 25) much higher than that recommended, as the aperture diameter of measurement of the colorimeter used was 8 mm (area $\sim 50 \text{ mm}^2$) and the thickness of cheese discs was 2 mm. Another explanation of differences observed at high temperatures could also be the

nature of translucent materials that may reflect light from below the surface (Gullett et al., 1972). To prevent this limitation, Little (1964) concluded that the best conditions to determine the optical properties of translucent materials is measurement of the colour of thin samples positioned above a white background. Calvo and Salvador (1997) found the use of K/S method (based on L^* values) as a better alternative than the measurement of CIE L^* values to estimate the translucency of fruit gels due to a higher correlation with sensorial lightness.

2.4.4 Nitrogen solubility during heating and cooling

We studied the proportion of temperature soluble-N (TSN) of experimental cheeses based on a cheese aqueous extract model to determine the role of temperature on the translucency of cheese. Fat content of cheeses had a significant effect ($P < 0.05$) on the TSN content during heating at different points of ripening (Fig. 2.5; Table 2.3). The TSN was also affected by the interaction of aging x heating and fat content x aging x heating ($P < 0.05$). At 2 d of ripening, levels of TSN were lower in NF cheeses (Fig. 2.5a; $P < 0.05$). These values remained constant between 4 and 40°C ($P > 0.05$). A strong decrease in the levels of TSN was observed in cheeses heated at >40°C, remaining constant at higher temperatures. A reverse effect on nitrogen solubility was observed in samples heated to 80°C and then cooled and stored at 4°C in a waterbath for 24 h, which had the same levels of TSN as untreated samples at 4°C. An increase in TSN levels was observed for all cheeses at 180 d of ripening (Fig 2.5b; $P < 0.05$) and no differences were found among treatments ($P > 0.05$). We also found a decrease in TSN

when samples were heated at $>40^{\circ}\text{C}$, although in a lower intensity than occurred on cheeses at 2 d of ripening. A reversible effect was also observed on heated and cooled samples.

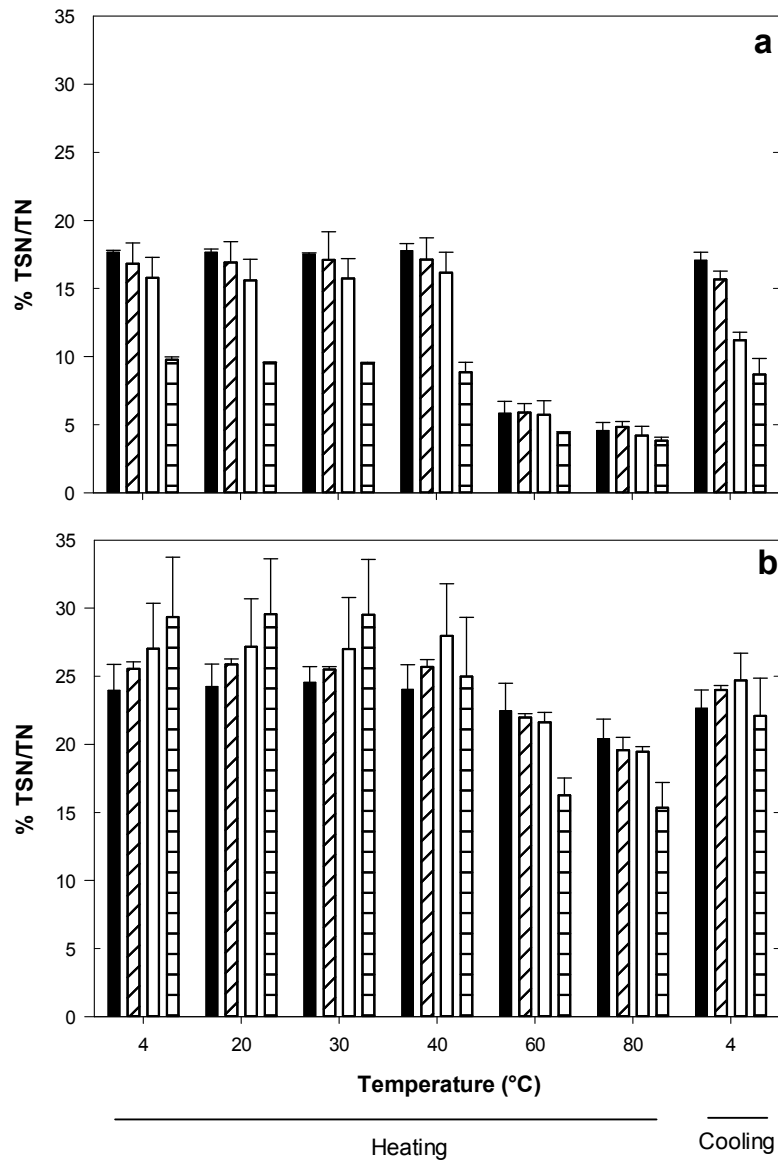


Fig. 2.5 Changes in the proportion of the temperature soluble N as a percentage of total cheese N (%TSN/TN) during the heating and cooling of FF (■), RF (//), HF (□) and NF (=) Cheddar cheeses at 2 (a) and 180 d of ripening (b). Values represent mean and standard deviations of three replicate trials.

An electrophoretic characterization of the soluble phase of cheese samples at 180 d of ripening (Fig. 2.6) showed that untreated samples at 4°C contained mainly intact α_{s1} -CN and β -CN and some of their large hydrolysis products. Differences in band intensity of α_{s1} -CN and β -CN among samples can be related to differences in proteolysis caused by differences in pH (Table 2.1). Post et al. (2012) observed a decrease in the solubility of α_s -CN and β -CN in deionized water and ultrafiltration permeate of milk when pH decreased from 5.5 to 5.0, probably due to the pH approaching these proteins' isoelectric points. The majority of the bands precipitated due to heat treatment of the aqueous phase at 80°C. However, a group of unidentified bands with high electrophoretic mobility remained soluble. Finally, a reverse effect was observed after heat treated samples were stored at 4°C for 24 h.

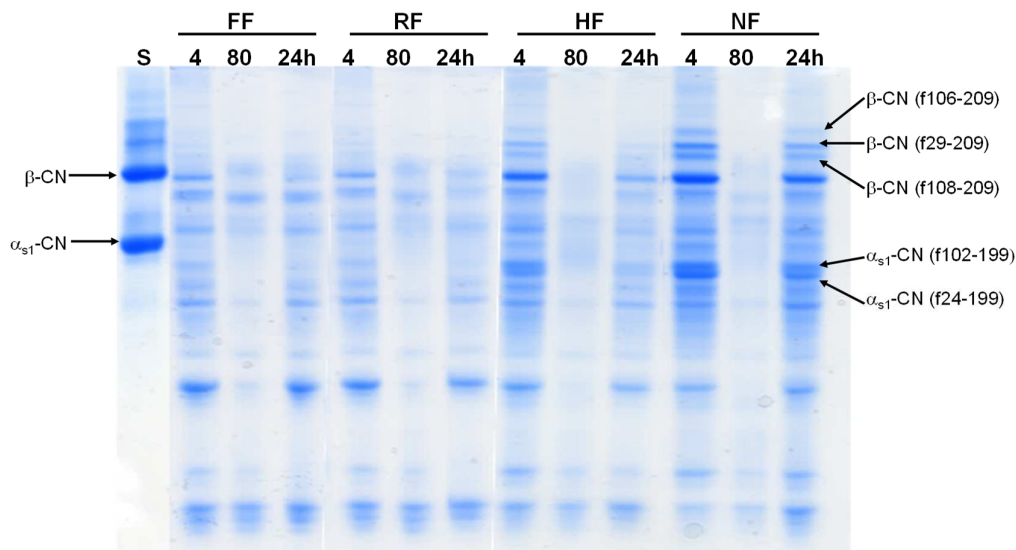


Fig. 2.6 Urea-polyacrylamide gel electrophoresis of FF, RF, HF and NF Cheddar cheeses aqueous phase extracts obtained at 180 d of ripening from unheated samples at 4°C (4), samples heat treated at 80°C during 30 min (80) and samples heat treated at 80°C for 30 min and cooled for 4°C during 24 hours (24h). Sodium caseinate was used as standard (S).

Nevertheless, we observed no changes of translucency during heating of FF, RF and HF at 2 d of ripening, probably due to a counteracting effect of fat melting (Fig. 2.2a and 2.3a); NF exhibited a significant increase in both K/S and L^* values at temperatures higher than 40°C. A decrease in the TSN levels at >40°C was related to the formation of new aggregates that would increase the light scattering and hence reduce the translucency of cheeses. Kim et al. (2011) observed a decrease in the casein solubility when full-fat and reduced-fat Cheddar cheeses were maintained for different holding times at 60°C, due to an increase of the surface hydrophobicity that led to the formation of aggregates. As previously stated, Metzger et al. (2000) suggested that hydrophobic heat-induced aggregates form a white gel at 49°C, leading to a shift in cheese appearance. This gel however, was reversed when sample was cooled for several hours. Based on this observation, Dave et al. (2001) suggested that at low temperatures β -CN migrates from the casein micelle to remain soluble in the serum phase of cheese, due to a higher solubility at cold temperatures (Bingham, 1971), while during heating, β -CN precipitates. From the rheological point of view, hydrophobic interactions during heating might lead to a weakening of the cheese matrix due to a decrease in the contact area among particles and an increase in interactions within particles (Lucey et al., 2003). On the other hand, it has been demonstrated that hydrophobic interactions are greatest at 70°C and that they decrease at higher temperatures (Bryant and McClements, 1998).

In addition, the formation of heat-induced structures might also be related to changes on the proportion of INSOL Ca. Broome and Limsowtin (2002) observed a decrease in the solubility of Ca in SCCAP when heated at >40°C. It has also been

reported that heat treatment promotes the binding of α_{s1} -CN to Ca (Dalglish and Parker, 1980). Udayarajan et al. (2005) elucidated that an increase of cheese stiffness during heating at high temperatures ($>70^{\circ}\text{C}$) is related to the formation of colloidal calcium phosphate that interacts with caseins from the soluble phase, which is believed to be reversible when cheese is cooled. We hypothesized the occurrence of these two phenomena during cheese heating (i.e., hydrophobic interactions and formation of new colloidal calcium phosphate binding to soluble caseins) and its reversible effect during cooling resulted in changes in the proportion of soluble nitrogen (Fig. 2.5) and hence on translucency. This is confirmed by urea-PAGE (Fig. 2.6), where all the soluble fractions heated at 80°C did not contain α_{s1} -CN, β -CN or their degradation products, although became soluble when samples were cooled at 4°C for 24h. Differences in the profiles of TSN obtained at 2 and 180 d can be related to an increase of cheese proteolysis that might lead to a reduction on the proportion of intact α_{s1} -CN and β -CN. These data agree with Guo and Kindstedt (1995) who observed an increase in both the crude protein content and the proportion pH 4.6-soluble nitrogen on the expressible serum of Mozzarella cheese with ageing.

2.5 Conclusions

The results of this study showed that translucency of Cheddar cheese is highly influenced by composition (mainly fat content), temperature and ageing. A lower fat content in cheese resulted in a higher translucency. At low temperatures, cheese fat scatters light, reducing the translucent appearance. During heating, melting of cheese fat

led to an increase of cheese translucency. In addition, a decrease in the proportion of soluble nitrogen, probably caused by increased hydrophobic interaction among the caseins, led to the formation of aggregates that would contribute to a decrease in cheese translucency. After cooling, aggregation was found to be reversible, contributing again to an increase in cheese translucency, whereas solidification of cheesefat counteracts the effect on translucency. It was also believed that changes of translucency at high temperatures were also associated with the formation of new colloidal calcium phosphate; however, further investigation will be required to establish the role of Ca solubility on the optical properties of cheese at high temperatures. Independent of the fat content, cheese age led to an increase in translucency, probably due to an increase in levels of proteolysis and a reduction in the proportion of INSOL Ca. Measurement of cheese translucency by CIE whiteness, the ratio of absorbance to scatter ($K/S L^*$) and the contrast ratio ($CR L^*$) were useful to determine differences and were highly correlated up to 60°C; however, they exhibited a slight reduction in correlation at 80°C, probably caused by shrinkage of cheese matrix when exposed to high temperatures that may influence in thickness of the samples analysed.

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CHAPTER 3

INFLUENCE OF CALCIUM CONTENT ON THE TRANSLUCENCY AND RHEOLOGICAL PROPERTIES OF LOW-FAT MOZZARELLA CHEESE HEATED AT HIGH TEMPERATURES FOR DIFFERENT HOLDING TIMES

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3.1 Abstract

Cheese translucency is a common defect found in low-fat cheeses that is highly influenced by temperature. Heating leads to a reduction of translucency due to increasing cheese whiteness, which has been associated with strengthened hydrophobic interactions between caseins. Another possible cause for increased whiteness is the formation of heat-induced insoluble colloidal calcium phosphate. The impact of holding cheeses at high temperatures for various holding times on appearance is not clear and there is little information on levels of insoluble calcium with increasing cheese temperature. This study aimed to evaluate changes in the whiteness of low-fat Mozzarella cheese differing in levels of total calcium when heated at different combinations of temperatures and holding times. One week old directly acidified, low-fat Mozzarella cheese was incubated with synthetic Cheddar cheese aqueous phase (SCCAP) solutions, differing in levels of Ca (low calcium, LCa, 1.39 g L⁻¹; normal calcium, NCa, 2.78 g L⁻¹; and high calcium, HCa, 4.17 g L⁻¹) to modify the content of total and insoluble calcium in these cheeses. After incubation, cheeses were heated at 60, 70 or 80°C for 30 min. Changes in whiteness were measured with a colorimeter by using the CIE L* scale and levels of insoluble calcium were measured by acid-base titrations. In addition, the rheological properties of cheese samples were evaluated by Fourier transform mechanical spectrometry (FTMS). An increase in calcium levels of cheese, led to increase in cheese whiteness at high temperatures. Holding times also influenced the appearance of cheese. At temperatures $\geq 70^{\circ}\text{C}$, there was a significant increase in levels of insoluble calcium phosphate, which might have contributed to the increased cheese whiteness. Very high temperatures (e.g., 70 or 80°C) and holding

times (e.g., 30 min) led to a slight decrease in cheese whiteness. Rheological measurements showed that increasing levels of total and insoluble Ca (INSOL Ca) led to cheeses with increased stiffness. An increase in heating temperature of cheese samples was related to a softening of cheese matrix due to melting. Increased holding times of cheeses heated at $\geq 70^{\circ}\text{C}$ was associated with an increase in storage modulus values (G') and a reduction in loss tangent values (LT), which was probably attributed to the formation of heat-induced INSOL Ca. These results suggest that thermal behavior of cheese including its appearance and rheological properties are impacted by the levels of insoluble calcium phosphate.

Keywords Cheese whiteness, insoluble calcium, heating temperatures, holding times, rheology.

3.2 Introduction

The appearance of cheese is highly affected by its temperature and composition. A common defect found in reduced-fat cheeses is a translucent appearance due to a reduction of light scattering (Johnson et al., 2009). An increase of the temperature of reduced-fat cheese leads to a change of appearance from translucent to opaque (Rudan and Barbano, 1998; Metzger et al., 2000; Dave et al., 2001; Pastorino et al., 2002; Ibáñez et al., 2016). Cheese translucency is usually estimated by measurements of whiteness based on the CIE L* scale that ranges from 0 (black) to 100 (white; Hunterlab, 2012).

When cheese is heated, its appearance is modified due to changes in the nature of the protein-protein interactions. Metzger et al. (2000) suggested that when full-fat and low-fat Mozzarella cheeses were heated at high temperatures (~50°C), enhanced hydrophobic interactions led to the formation of aggregates of β -casein (CN), from soluble fractions found in the expressible serum phase, leading to an increase in overall cheese whiteness. Pastorino et al. (2002) observed cheese microstructure by electron microscopy and found an increase in protein aggregates when cheese was heated at 50°C. Kim et al. (2011) found that increasing holding times of cheeses treated at high temperatures led to an increase in surface hydrophobicity of proteins. However, there is limited detailed information on the mechanisms by which holding times at high temperatures influence cheese whiteness. Dave et al. (2001) report that, at high temperatures, individual CNs may form of larger complex structures by mean of insoluble calcium phosphate. Metzger et al. (2001) observed that higher levels of total

and water insoluble calcium in non-fat Mozzarella cheese led to an increase of whiteness of cheese when measured at 7 and 71°C. In a similar study, Joshi et al. (2003) observed that increasing the levels of total calcium led to an increase of cheese whiteness when heated to 60°C. In these two studies, the authors attributed these differences to protein-protein interactions occurring in the serum phase of cheese. When milk systems are heated to high temperatures, heat induced colloidal calcium phosphate can be formed (Fox et al., 2015). Udayarajan (2007) found that when Cheddar cheeses were heated at high temperatures, there was an increase in the buffering peak related to insoluble calcium phosphate. The impact of heating conditions on the appearance of cheese and the possible involvement of heat induced insoluble calcium is unclear.

The objective of this study was to investigate changes in the whiteness of low-fat Mozzarella cheese made with different levels of total and insoluble Ca. Cheeses were subjected to various heating temperatures and different holding times and we determined changes in the insoluble calcium phosphate level as well as rheological properties.

3.3 Materials and methods

3.3.1 Manufacture of low-fat cheese base and preparation of samples for storage

Cheese manufacture was performed by licensed cheesemakers at the University of Wisconsin-Madison Babcock Hall dairy plant. Two 9 kg blocks of low-fat directly acidified Mozzarella cheese were made according the protocol described by Brickley et al. (2008). After manufacture, cheeses were vacuum packed and stored at 4°C for 7 d. Cheeses were then cut into discs (50 x 3 mm), packaged in zip-lock bags and stored at -20°C until analysis. Before use, cheese discs were thawed at 4°C for 2 d (Stankey et al., 2011).

3.3.2 Modification of calcium content of cheese samples

The calcium content of the cheese base was adjusted using synthetic Cheddar cheese aqueous phase (SCCAP) solutions (Broome and Limsowtin, 2002). O'Mahony et al. (2006) successfully modified the total and colloidal calcium content of 1 mo old Cheddar cheese by immersing slices of samples into SCCAP made with different levels of Ca. In this study, solutions were prepared according to the procedure described by Stankey et al. (2011) with some modifications, as SCCAP was formulated to reflect the composition of low-fat cheese base. Disodium hydrogen phosphate (6.142 g) was dissolved in 700 mL of deionized water. Then, citric acid-monohydrate (2.3 g), sodium chloride (75.98 g), magnesium sulphate heptahydrate (0.564 g), magnesium chloride

hexahydrate (3.296 g), potassium chloride (3.624 g) and sodium acetate trihydrate (0.6 g) were added in that order. Once salts were solubilized (~15 min) and maintaining continuous stirring, sodium lactate (60% w/w) was added (60.056 g), followed by the addition of 10 mL of manganese chloride tetrahydrate (0.025 g/100 mL). An aliquot of a solution of calcium chloride dehydrate ($34.006 \text{ g } 100 \text{ mL}^{-1}$) was added, varying in the amount to adjust the concentration of Ca of the SCCAP to 1.39, 2.78 or 4.17 g L^{-1} . This was followed by the addition of 10 mL of a solution of zinc chloride ($0.625 \text{ g } 100 \text{ mL}^{-1}$). The pH was then adjusted to 5.4 with 1.0 N HCl and the volume was completed to 1 L using deionized water. As previously stated, SCCAP were made at three different levels of calcium content: low Ca (LCa; 1.38 g L^{-1}), normal Ca (NCa; 2.78 g L^{-1}) and high calcium (HCa; 4.17 g L^{-1}). We studied these levels of Ca as previous studies showed that full-fat Cheddar cheese treated with SCCAP made with NCa did not alter the amount of insoluble calcium phosphate content in full-fat Cheddar (O'Mahony et al., 2006) and non-fat Mozzarella cheeses (Stankey et al., 2011). All reagents used were of analytical grade (Fischer Scientific, Fair Lawn, NJ).

Before incubation, cheese disc samples were equilibrated at room temperature for 1 h. Cheeses were then wrapped in cheesecloth to prevent adhesion with surfaces of Petri dish; placed in the bottom of a plastic Petri dish (70 x 15 mm; Thermo Fisher Scientific Inc., Waltham, MA) and 25 mL of the SCCAP solution were added. Petri dishes were covered during incubation to prevent loss of moisture. Incubation of cheese samples immersed in SCCAP was performed at room temperature ($\sim 22^{\circ}\text{C}$) for 6 h under continuous agitation of the samples at 15 rpm, using a platform rotator (Thermo Fisher Scientific Inc., Waltham, MA). After incubation, cheese samples were placed on

paper tissues (Kimwipes; Kimberly-Clark International, Roswell, GA) to remove excess of moisture and air dried for 30 min (inverted after 15 min). Cheese samples were then placed in plastic bags (Whirl-Pak; Nasco International Inc., Fort Atkinson, WI) and stored at 4°C for 18 h to allow for equilibration. Before analyses, cheese samples were equilibrated at room temperature for at least 30 min. Samples were then analysed for composition, colour and rheological properties.

3.3.3 Cheese composition

The composition of cheese base was analyzed 7 d after manufacture, whereas cheese discs were analyzed before and after incubation in SCCAP for moisture (Marshall, 1992), fat (AOAC International, 2000), protein (AOAC International, 2000), salt (Johnson and Olson, 1985) and total calcium (Park, 2000). The content of total calcium was also measured in cheese milk and rennet whey, with rennet-whey prepared according to Lucey et al. (1993). The proportion of insoluble calcium of cheeses was determined by the acid-base titration method (Hassan et al., 2004). The area of the buffering peak was used as an indicator of the insoluble calcium phosphate content (O'Mahony et al., 2006). The pH of cheeses was measured in a homogenized mixture of 10 g of cheese and 10 mL of deionized water (Ibáñez et al., 2016). All analyses were performed in triplicate.

3.3.4 Cheese whiteness

The whiteness of cheese samples was measured using a HunterLab ColorFlex ® colorimeter (model A60-1010-615, Hunter Associates Laboratory, Inc., Reston, VA). The instrument was set on the CIELAB system based on the illuminant D65 and a visual angle of 10°. Whiteness of cheese was expressed as CIE L* values, ranging from 0 (black) to 100 (white; Hunterlab, 2012). All colour measurements were performed on cheese discs placed in plastic Petri dishes (50 x 15 mm; Thermo Fisher Scientific Inc., Waltham, MA). All heat treatments were performed using the Peltier heating plate of a controlled-stress rheometer (model TEK 150P/UDS, Paar Physica UDS 200, Anton Paar, Ashland, VA). Prior to heating analysis, cheese samples were coated with 0.5 mL of vegetable oil (Ibáñez et al., 2016) and petri dishes were covered with the lid to prevent loss of moisture (Rudan and Barbano, 1998). In preliminary work, the cheese samples did not maintain the desired temperature during heating, probably due to loss of heat. Based on this observation, an expanded polystyrene box was designed to cover the petri dish during heat treatment. This insulation cover was also used while removing samples from the instrument and before colour measurements. Samples were equilibrated on the Peltier plate for 10 min at 20°C and then heated at a rate of 10°C min⁻¹ to 60, 70 or 80°C, allowed to equilibrate for 2 min and held for additional 0, 5, 15 or 30 min at high temperatures. The colour measurements of cheese samples were taken from the bottom of the plastic Petri dish before heat treatment and after each individual heat treatment. At least three replicates were used and five colour measurements were made per sample.

3.3.5 Insoluble calcium content after heating

The proportion of insoluble INSOL Ca phosphate heated of cheese samples was studied to evaluate possible formation of heat induced INSOL Ca phosphate. Samples were prepared according the method described by Udayarajan (2007) with some modifications. A known amount of cheese was weight into a pre-weighted plastic petri dish (50 x 15 mm) and heated to 60, 70 or 80°C with holding times of 0 and 30 min, under the same conditions as previously described for colour measurements (except no addition of vegetable oil) using the Peltier from the rheometer to accurately perform the heating. After incubation, samples were cooled to 25°C for 5 min and reweighed. The weight of the cheese was adjusted to back to the original weight by the addition of deionized water at 25°C (to account for moisture loss during heating). The INSOL Ca phosphate content was estimated on heated samples by the acid-based titration method described by Hassan et al. (2004). All analyses were performed on triplicate.

3.3.6 Rheological measurements

The rheological properties of cheese samples were studied by Fourier transform mechanical spectrometry (Udayarajan et al., 2005) using a controlled stress rheometer (Paar Physica UDS 200, Anton Paar, Ashland, VA). The frequency used ranged from 0.04 to 8 Hz and the total strain was 0.27%, which within the viscoelastic region. Cheese discs (50 x 3 mm) were also used for this study. A serrated parallel plate geometry was used and samples were placed on the bottom plate of the instrument at an

initial temperature of 20°C. A normal force of ~1.8 N was initially applied to the cheese sample and the exposed layers were covered with vegetable oil. When the normal force decreased to ~0.7 N, samples were maintained at 20°C during 10 min and then heated to 60, 70 or 80°C at a rate of 10°C min⁻¹, equilibrated for 2 min and held for additional 30 min. Storage modulus (G'), loss modulus (G'') and loss tangent (G''/G' ; LT) were measured during the heating of cheeses. All analyses were performed in triplicate.

3.3.7 Statistical analyses

One-way analysis of variance (ANOVA) was performed on cheese composition at a significance level of $P < 0.05$. Changes in whiteness, INSOL Ca and rheological properties were analyzed using three-way ANOVA to evaluate differences among Ca treatments (LCa, NCa and HCa), heating temperatures (60, 70 and 80°C), holding times (0, 5, 15 and 30 min) and their interactions. ANOVA was performed using a general linear model. If significant differences were found ($P < 0.05$), treatments means were analyzed by the Tukey's multiple comparison test. In addition, cheese whiteness, rheological measurements and levels of INSOL Ca were analyzed by Principal Component Analysis (PCA) using a covariance matrix. All analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA).

3.4 Results

3.4.1 Cheese composition

The composition of the cheese base was 54.06% moisture, 4.94% fat, 33.96% protein, 2.95% salt, 658 mg of total calcium per 100 g of cheese and pH value 5.57. The incubation of cheeses in SCCAP led to changes in the composition of cheese samples (Table 3.1). Increasing levels of Ca in SCCAP solutions from 1.39 to 4.17 g L⁻¹, LCa and HCa respectively, was associated with a reduction of cheese pH in almost ~0.1 unit between treatments ($P < 0.05$). A reduction in the moisture content, along with an increase in the levels of protein, total Ca and insoluble Ca were observed in cheeses ($P < 0.05$) with an increase in the calcium concentration of the SCCAP.

3.4.2 Whiteness

The whiteness of cheese samples after SCCAP treatment, expressed in L^* values (measured at room temperature) is shown in Table 3.1. Incubation of cheese samples in SCCAP solutions led to a slight decrease in L^* values as the calcium concentration increased. On the other hand, the whiteness of cheese was greatly influenced by heating to high temperatures (Table 3.2). Changes in the whiteness appearance of NCa cheese as both temperature and holding time increased are shown in Fig. 3.1. At room temperature (untreated sample) NCa cheese had a translucent appearance, whereas an increase in temperature and holding time led to a decrease of cheese translucency and

increasing the white appearance. Similar changes in the appearance of LCa and HCa were also observed when treated at high temperatures (results not shown).

Table 3.1 Composition, levels of insoluble calcium, pH and whiteness of low-fat directly acidified Mozzarella cheeses incubated in synthetic Cheddar cheese aqueous phase (SCCAP) solutions with low (LCa; 1.39 g L⁻¹), normal (NCa; 2.78 g L⁻¹) and high (HCa; 4.17 g L⁻¹) levels of Ca during 6 h at 22°C.

Item	Treatment			
	LCa	NCa	HCa	SEM
pH	5.73 ^a	5.65 ^b	5.57 ^c	0.023
Moisture (%)	57.57 ^a	55.69 ^b	54.19 ^c	0.491
Fat (%)	4.33 ^a	4.36 ^a	4.56 ^a	0.056
Protein (%) ¹	29.17 ^c	30.93 ^b	32.02 ^a	0.419
Salt (%)	4.59 ^a	4.59 ^a	4.67 ^a	0.049
MNFS (%) ²	60.17 ^a	58.23 ^b	56.78 ^c	0.492
FDM (%) ³	10.20 ^a	9.84 ^a	9.96 ^a	0.107
S/M (%) ⁴	7.97 ^a	8.24 ^a	8.61 ^a	0.124
Moisture-to-protein ratio	1.97 ^a	1.80 ^b	1.69 ^c	0.041
Total Ca (mg 100 g ⁻¹ cheese)	503 ^c	553 ^b	736 ^a	35.7
Insoluble Ca (mg 100 g ⁻¹ cheese)	395 ^b	432 ^b	538 ^a	23.0
Total Ca (mg 100 g ⁻¹ protein)	1725 ^b	1787 ^b	2299 ^a	92.0
Insoluble Ca (mg 100 g ⁻¹ protein)	1354 ^b	1398 ^a	1680 ^a	58.5
Whiteness (CIE L*) ⁵	63.28 ^a	63.07 ^a	61.05 ^b	0.373

^{a,b,c} Means within the same row not sharing a common superscript differ ($P < 0.05$), as compared by Tukey multiple comparison test.

¹ Total %N x 6.38.

² Moisture in the non-fat substance of the cheese.

³ Fat content on a dry weight basis

⁴ Salt in the moisture phase of the cheese.

⁵ Whiteness measured by CIELAB system

Values represent mean and standard deviation of three replicates.

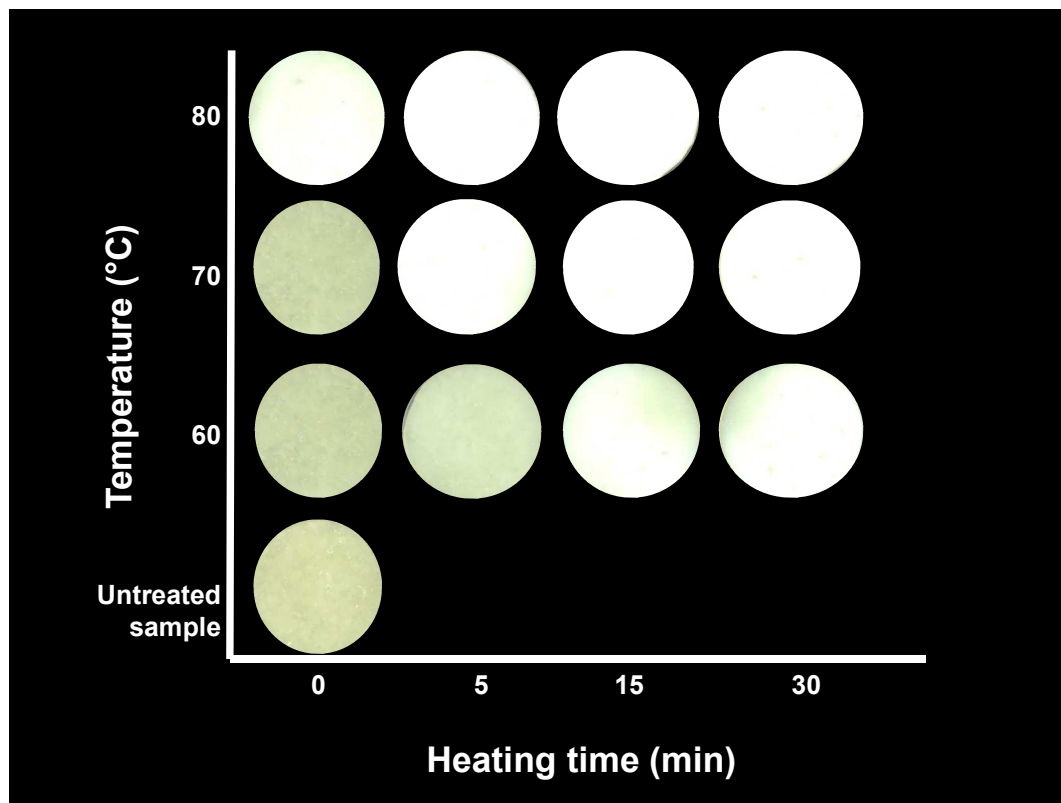


Fig. 3.1 Visual appearance of normal calcium (NCa) low-fat Mozzarella cheese heated at different combinations of high temperatures and holding times.

The whiteness of cheese samples heated at different temperature-time combinations is shown in Fig. 3.2. The L^* values (whiteness) were increased as levels of Ca, temperature and holding time increased. Temperature, holding time and their interaction all had a significant effect on whiteness ($P < 0.05$; Table 3.2). LCa cheese (Fig. 3.2a) heated at 60 and 70°C for 0 min had limited impact on whiteness and the L^* values were similar to those untreated samples ($P > 0.05$). The maximum whiteness of the LCa sample during heating at 60°C was observed at 30 min (~82); the maximum whiteness found at 70°C was observed at 15 min and remained constant thereafter (~88); whereas at 80°C, maximum whiteness was reached after 5 min of holding time (~90) and then whiteness slightly decreased at longer times (e.g. ~85 after 30 min). The whiteness of NCa cheese at 0 min was higher than LCa ($P < 0.05$) at all temperatures (Fig. 3.2b). In addition, a lower holding time was required to achieve a maximum whiteness for NCa or HCa than to LCa cheeses. At 60°C, whiteness from NCa exhibited a maximum at 15 min (~89), remaining constant at longer times, whereas at 70°C the maximum whiteness was reached at 5 min, remaining constant thereafter; and at 80°C, the maximum whiteness was reached at 0 min (~88) and then decreased. HCa cheese (Fig. 3.2c) exhibited higher whiteness values at 0 min than other treatments ($P < 0.05$) when heated to 60 and 70°C. At 60 and 70°C, HCa exhibited a maximum whiteness at 5 min of heating; at 60°C remained constant thereafter, whereas at 70°C, whiteness slowly decreased thereafter (e.g. ~85 at 30 min). On the other hand, HCa cheese heated at 80°C decreased in whiteness from ~88 to ~82 during 30 min of heating.

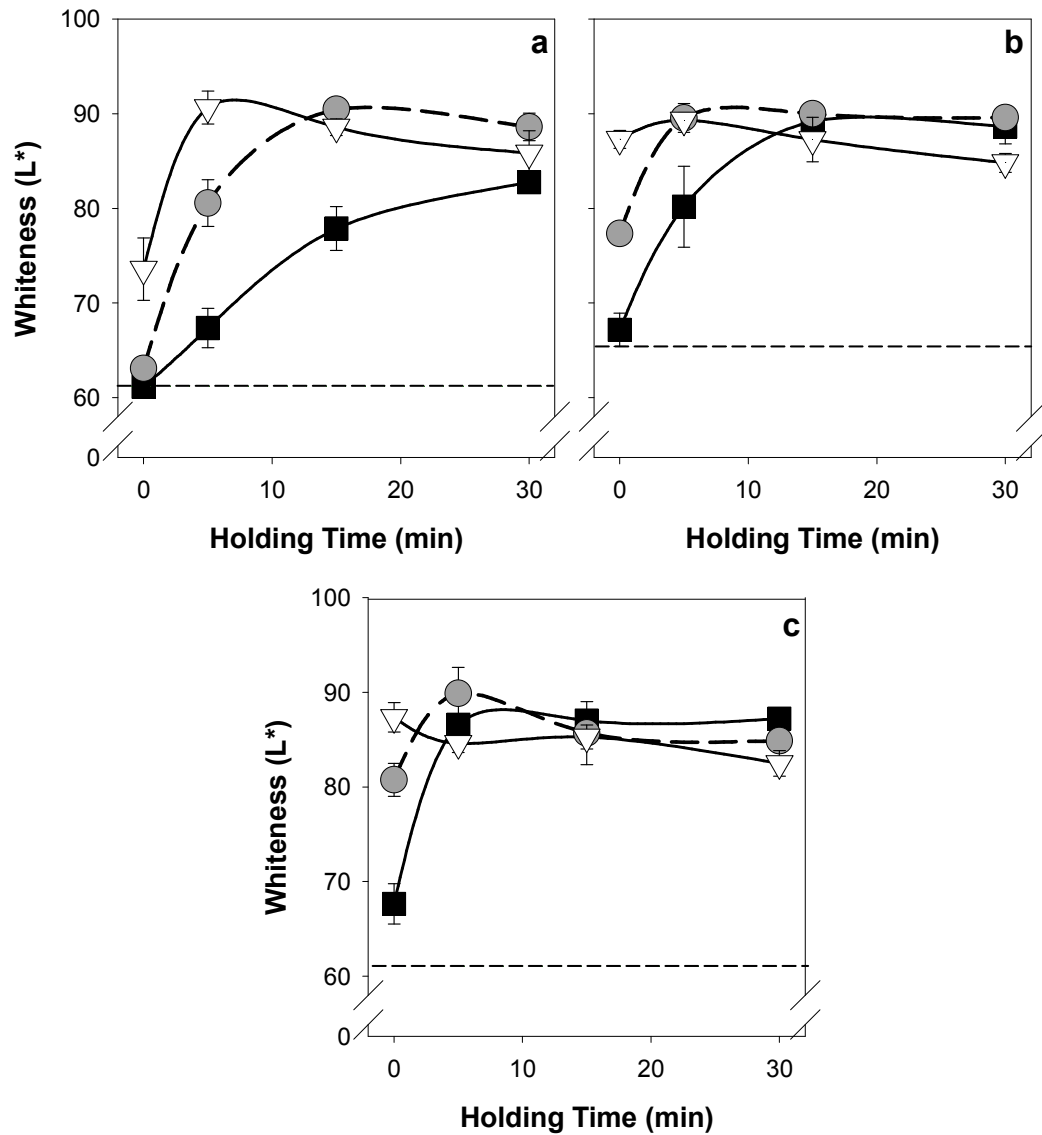


Fig. 3.2 Changes in whiteness (CIE L* values) of low (LCa; a), normal (NCa; b) and high calcium (HCa; c) low-fat Mozzarella cheeses heated at 60 (■), 70 (●) or 80°C (▽) during 30 min of holding time. Dashed line represents L* value of unheated cheeses. Values represent mean and standard deviation of three replicate trials.

Table 3.2 Mean squares, probabilities (in parenthesis) and R² values for whiteness, rheological properties analyzed at two frequencies and insoluble calcium of direct acidified low-fat Mozzarella cheeses during heating.

Factor	df ¹	Whiteness ²	df ¹	INSOL Ca ⁶	df ¹	G' 0.08 Hz ³	LT 0.08 Hz ³	G' 8 Hz ⁴	LT 8 Hz ⁴	df ¹	Slope (n) ⁵
Ca Treatment (C)	2	351.69** (<0.001)	2	337883** (<0.001)	2	1837356** (<0.001)	8.864** (<0.001)	4.78 x 10 ⁸ ** (<0.001)	0.0647** (<0.001)	2	0.05994** (<0.001)
Temperature (T)	2	497.96** (<0.001)	2	183787** (<0.001)	2	188537719** (<0.001)	82.073** (<0.001)	2.65 x 10 ⁸ ** (<0.001)	3.0755** (<0.001)	2	1.69050** (<0.001)
Holding time (H)	3	971.08** (<0.001)	1	242993** (<0.001)	6	40189** (<0.001)	0.3029** (<0.001)	1.08 x 10 ⁷ ** (<0.001)	0.00374** (0.001)	3	0.00593** (<0.001)
C x T	4	72.03** (<0.001)	4	22641** (0.009)	4	902891** (<0.001)	3.0421** (<0.001)	6.41 x 10 ⁷ ** (<0.001)	0.03017** (<0.001)	4	0.00930** (<0.001)
C x H	6	94.40** (<0.001)	2	16657 (0.065)	12	1028 (0.991)	0.0259** (0.002)	959352 (0.513)	0.00032 (0.977)	6	0.00091** (0.004)
T x H	6	171.87** (<0.001)	2	62924** (<0.001)	12	30715** (<0.001)	0.1891** (<0.001)	1.47x 10 ⁶ (0.155)	0.00126 (0.193)	6	0.00402** (<0.001)
C x T x H	12	51.13** (<0.001)	4	3756 (0.621)	24	2259 (0.998)	0.0180** (0.009)	503662 (0.977)	0.0004 (0.990)	12	0.00032 (0.288)
Error	72	3.15	36	5659	126	6419	0.0092	1.02 x 10 ⁶	0.00092	72	0.00026
R ²		0.97		0.97		0.98	0.99	0.93	0.98		0.99

¹ Degrees of freedom differed for whiteness, rheological and levels of insoluble calcium.² Whiteness measured by CIE L* values³ Rheological measurements were performed at a frequency of 0.08 Hz.⁴ Rheological measurements were performed at a frequency of 8 Hz.⁵ Slope of logG' vs log frequency⁶ Levels of insoluble calcium expressed as mg per 100 g of protein.

*0.01 < P ≤ 0.05; ** P ≤ 0.01.

3.4.3 Insoluble calcium

The acid-base titration curves of NCa cheese samples subjected to different heat treatments are shown in Fig. 3.3. Similar types of buffering curves were also obtained from LCa and HCa cheeses (data not shown). Untreated cheese (Fig. 3.3a) and heated samples at 60, 70 and 80°C during 30 min (Figs. 3.3b, 3.3c and 3.3d, respectively) exhibited generally similar shaped peaks in the buffering curves in the vicinity of pH ~4.8, which is related to the solubilization of insoluble calcium phosphate in the cheese (Lucey and Fox, 1993; Hassan et al., 2004). There was an increase in the total buffering area in the vicinity of pH 4.8, as the level of heat treatment increased, which was used to estimate the amount of insoluble calcium (Hassan et al., 2004) for cheese samples (Fig. 3.4). The insoluble calcium content of cheese was significantly affected by Ca level of the cheese, heating temperature and interactions between calcium level x temperature and temperature x holding time ($P < 0.05$; Table 3.2). As expected, an increase in the concentration of total Ca, heating temperature and holding times led to an increase in the levels of INSOL Ca. ($P < 0.05$). LCa heated for 0 min exhibited no increase in levels of INSOL Ca at 60 and 70°C and a slight increase was observed when heated at 80°C. For LCa cheese, after 30 min of heating, INSOL Ca content remained constant at 60°C and a significant increase ($P < 0.05$) occurred during heating at 70 and 80°C. Similar trends were also observed in the NCa and HCa cheeses. Low heat treatments (60°C for 0-30 min and 70°C for 0 min) resulted in cheeses with similar INSOL Ca levels to those samples without heat treatment (Table 3.1).

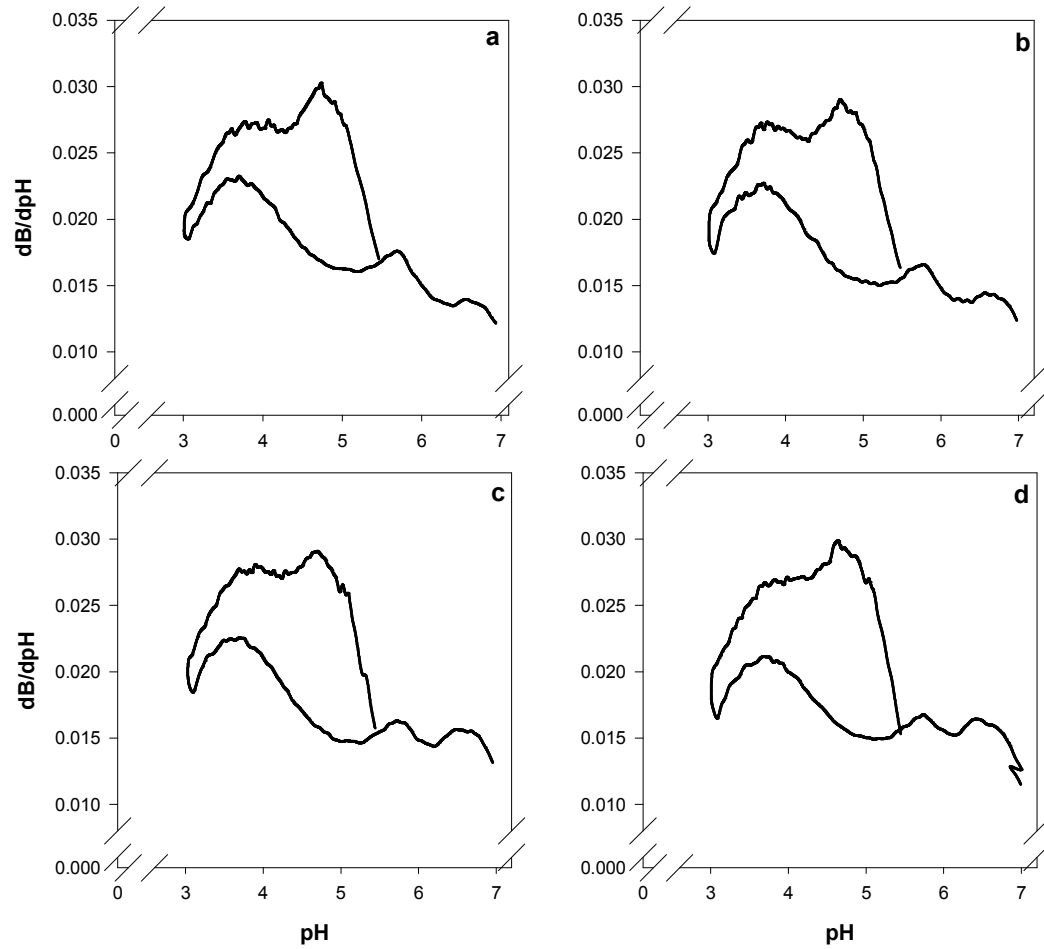


Fig. 3.3 Acid-base buffering curves of unheated (a) NCa low-fat Mozzarella cheese and cheeses subjected to heat treatments of 60 (b), 70 (c) and 80°C (d) for 30 min.

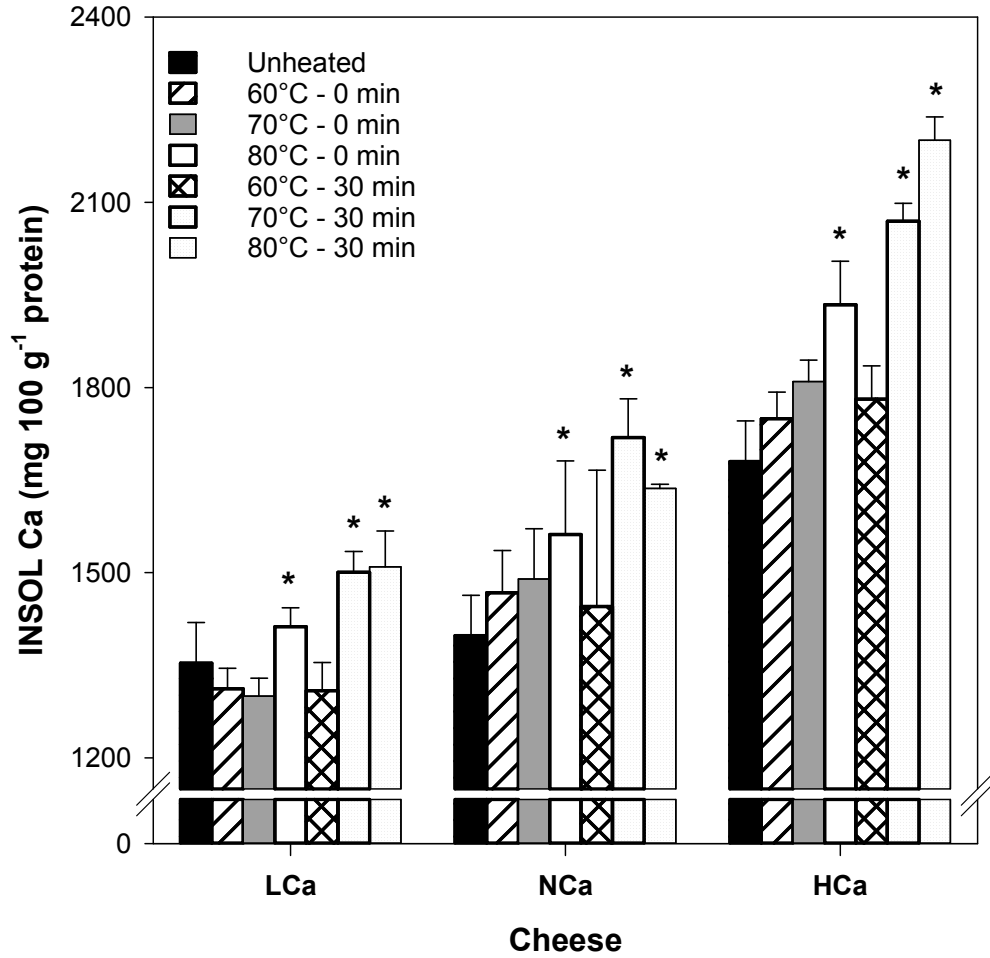


Fig. 3.4 Changes in levels of insoluble calcium (INSOL Ca) of low (LCa), normal (NCa) and high calcium (HCa) low-fat Mozzarella cheeses heated at different combinations of temperature and holding times. Values represent mean and standard deviation of three replicate trials. An asterisk (*) represents significant difference ($P < 0.05$) from unheated cheese.

3.4.4 Rheological properties

The rheological properties of LCa, NCa and HCa cheeses heated at 60, 70 and 80°C for 30 min are shown in Fig. 3.5. In general, there were similar trends at all frequencies measured (data not shown). The storage modulus (G' ; Fig. 3.5) was greatly influenced by the effect of total Ca levels of cheese, temperature, holding times and the interactions between temperature x holding time when evaluated at low and high frequencies (0.08 and 8 Hz, respectively; Table 3.2). An increase in the stiffness (G' values) was found as the levels of total Ca increased in the cheeses, whereas a reduction in G' values was observed when holding temperature was increased from 60 to 80°C. During holding at 60°C, all treatments exhibited a slight decrease in G' values (Figs. 3.5a, 3.5d and 3.5g). In contrast, during heating at 80°C, all treatments exhibited a slight increase in G' values (Figs. 3.5c, 3.5f and 3.5i).

The LT values were significantly affected by levels of total Ca, heating temperature, time as well as their interactions (Fig. 3.5; Table 3.2). An increase in the levels of Ca led to cheeses with lower LT values at all heat treatments. An increase in temperature was associated with an increase of LT values (as expected). During holding at 60°C, the LT values of cheese samples slightly increased up to 5 min and then remained constant (Figs. 3.5a, 3.5d, 3.5g). During holding at 80°C, the LT values from cheese samples heated at 80°C decreased (Figs. 3.5c, 3.5f and 3.5i).

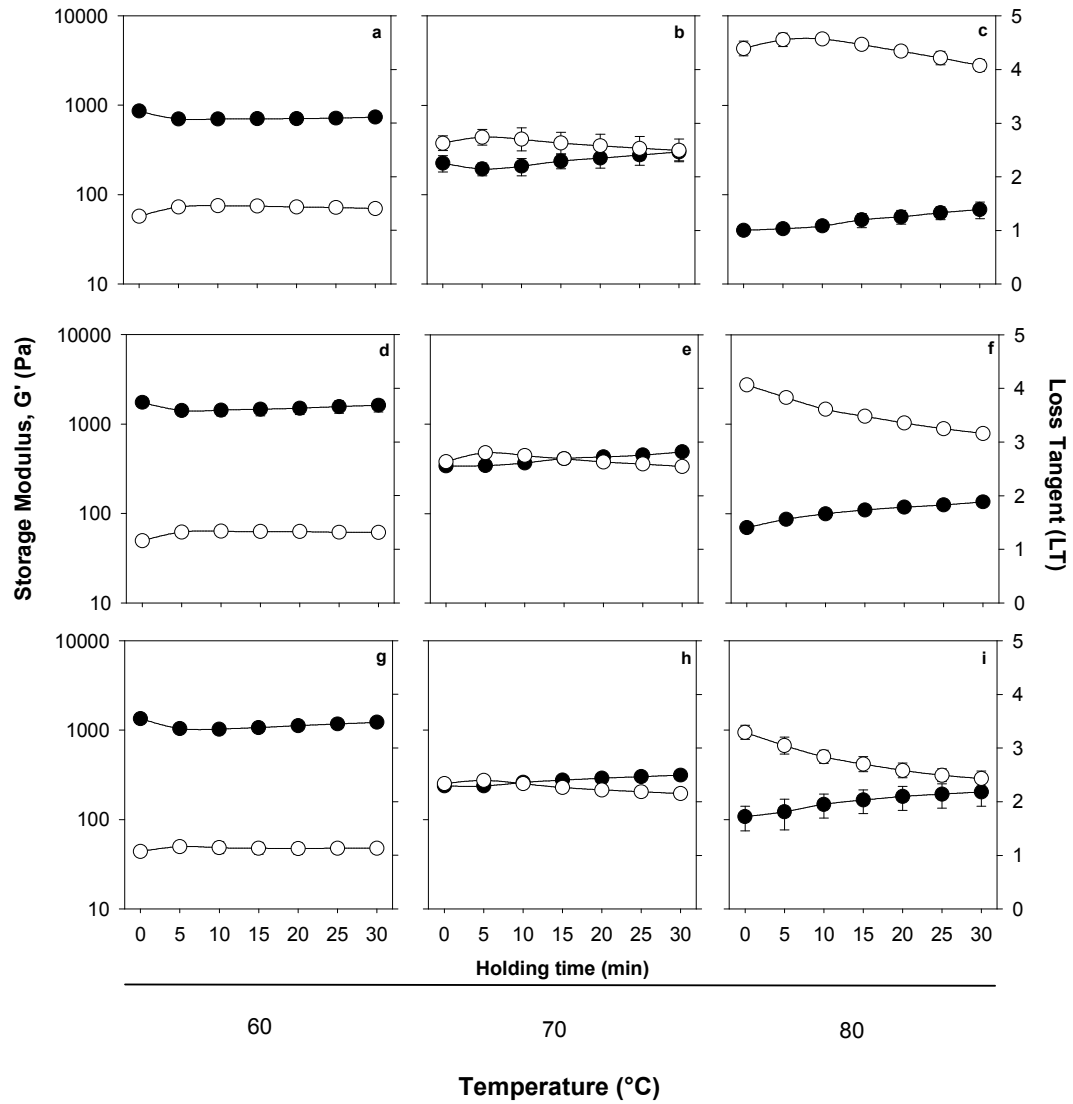


Fig. 3.5 Changes in storage modulus (G' ; ●) and loss tangent (LT; ○) of low calcium (LCa; a – c), normal calcium (NCa; d – f) and high calcium (HCa; g – i) low-fat Mozzarella cheeses heated at 60, 70 and 80°C during 30 min of holding time. Data presented was for 0.08 Hz. Similar trends were observed for other measurement treatments. Values represent mean and standard deviation of three replicate trials.

The frequency dependence of cheese samples treated at different heating temperatures and holding times, was also evaluated by the slope (n) obtained from plotting, on a logarithmic scale, G' versus frequency (Fig. 3.6). An increase in the temperature of cheeses led to an increase in the n values ($P < 0.05$; Table 3.2; Fig 3.6) and an increase in the Ca levels relates to a reduction of these values ($P < 0.05$). Cheeses heated at 60°C and NCa cheese heated at 70°C exhibited a slight increase of n values during the first 5 min of holding time ($P < 0.05$) and remained constant thereafter. In contrast, LCa and HCa cheeses heated at 70°C and all cheeses heated at 80°C exhibited a reduction of n values as holding time increased ($P < 0.05$).

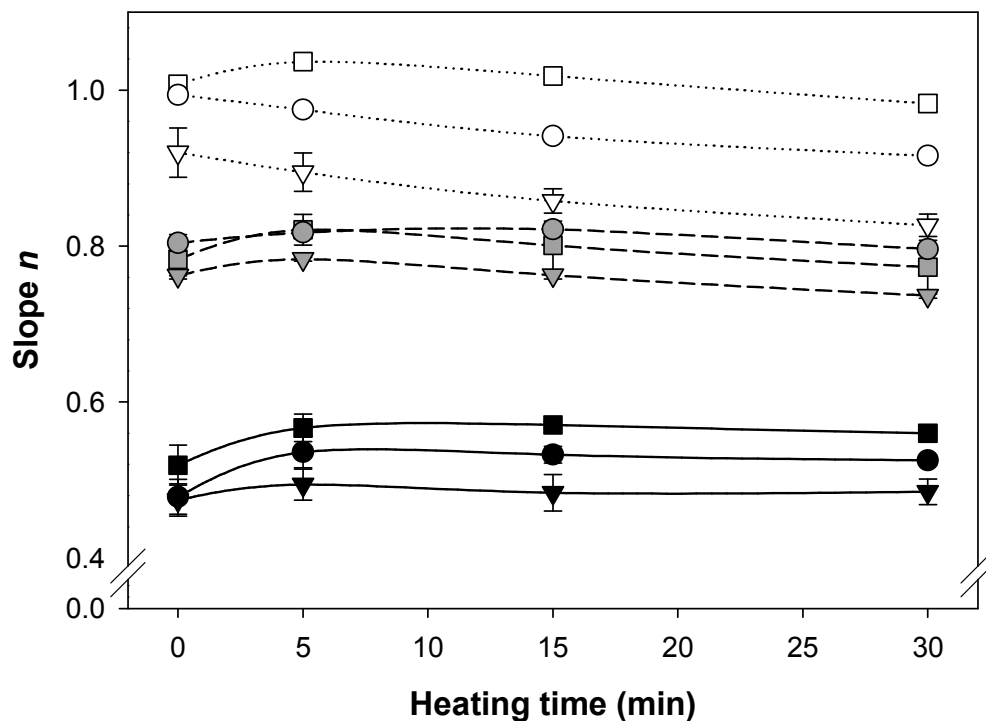


Fig. 3.6 Changes in slope (n) obtained from plotting $\log G'$ versus $\log$ frequency from low calcium (LCa; ■), normal calcium (NCa; ●) and high calcium (HCa; ▼) low-fat Mozzarella cheeses heated at 60°C during 30 min; LCa (■), NCa (●) and HCa (▼) low fat Mozzarella cheeses heated at 70°C during 30 min; and LCa (□), NCa (○) and HCa (▽) low fat Mozzarella cheeses heated at 80°C during 30 min. Values represent mean and standard deviation of three replicate trials.

3.4.5 Principal component analysis

A PCA was performed to cheese samples treated with different levels of Ca, heating temperatures and holding times, in order to obtain a simplified overview of the relationship among the whiteness, levels of INSOL Ca and rheological properties of cheeses (Fig. 3.7). Three components (PC1, PC2 and PC3) were selected that accounted for 96.9% of total variance (59.6%, 23.5% and 13.8%, respectively). The score plot obtained from the three first components (Fig. 3.7a) separated samples among heating temperature, holding times and levels of INSOL Ca. Loading plots (Fig. 3.7b) showed that the PC1 was negatively correlated with G' values (-0.519) and positively correlated with LT and n values (0.523 and 0.599, respectively); the PC2 was positively correlated with L^* values (0.864); and the PC3 was negatively correlated with levels of INSOL Ca (-0.891).

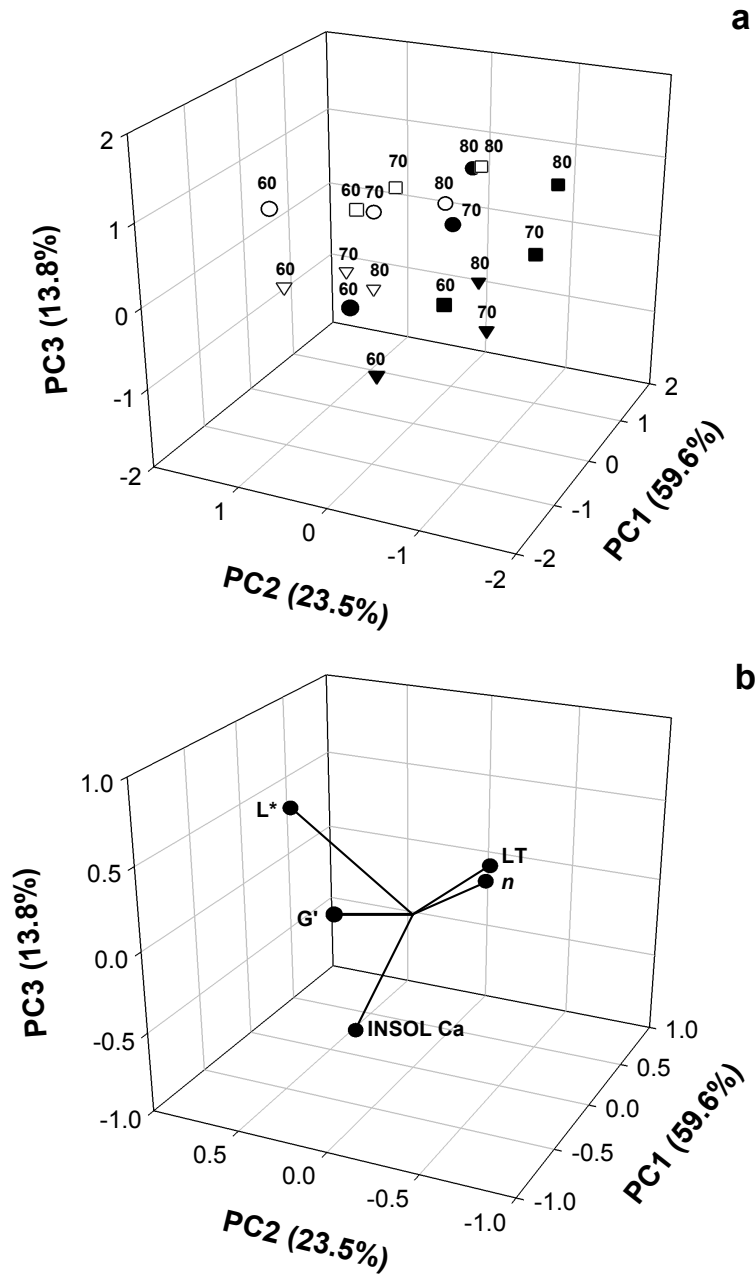


Fig. 3.7 Score plot (a) and loading plot (b) obtained by principal component analysis (PCA) from whiteness, levels of INSOL Ca and rheological properties of low calcium (LCa; ■ □), normal calcium (NCa; ● ○) and high calcium (HCa; ▼ ▽) low-fat Mozzarella cheeses heated at 60, 70 and 80°C after 0 (black symbols) and 30 min (white symbols) of holding time. Numbers along with symbols in score plot indicate heating temperature (°C).

3.5 Discussion

3.5.1 Cheese composition

The incubation of cheese samples in solutions of SCCAP with different concentrations of Ca successfully modified the composition and pH of cheese samples. When cheese base was incubated in SCCAP solutions with LCa and NCa levels, there was a decrease in the total Ca content of cheese, whereas when incubated in HCa solutions, the Ca content increased. However, an increment in Ca content of SCCAP solutions led to an increase of total Ca of cheeses. When results were expressed in mg per 100 g of cheese (Table 3.1), we observed a gradual increase in Ca levels from LCa to HCa. To account for differences in composition between treatments, the Ca content was then normalized to protein content (mg 100 g⁻¹ protein; O'Mahony et al., 2006; Stankey et al., 2011). We also observed an increase in the Ca content; although, there were no differences between LCa and NCa treatments (Table 3.1). This same trend was also observed in levels of INSOL Ca. O'Mahony et al. (2006) successfully used SCCAP solutions with calcium levels ranging between 1.39 and 8.34 g L⁻¹ to decrease or increase the levels of total and insoluble calcium of 1 mo old Cheddar cheese. These authors also found a decrease in the moisture content of cheeses as levels of Ca were higher, which was attributed to a reduction of swelling and hydration of the *para*-casein matrix due to insolubilization of colloidal calcium phosphate. This is in agreement with our study as increasing levels of Ca in SCCAP solutions led to cheeses with reduced moisture content, from 57.57 (LCa) to 54.19% (HCa).

3.5.2 Cheese whiteness

The appearance of cheese is highly affected by its composition. At room temperature, cheese fat acts to scatter light, contributing with an opaque appearance (Johnson et al., 2009). In cheeses made with reduced-fat content, the increase in other components, such as protein and moisture (Fenelon and Guinee, 2000), would also contribute to an increase of cheese translucency (Ibáñez et al., 2016). Brickley et al. (2008) attributed that translucent appearance of directly acidified non-fat Mozzarella cheeses decreased when pH values were reduced from 5.4 to 5.0, due to the approaching of caseins to their isoelectric point, causing increased aggregation of caseins and hence, an increase of whiteness.

The measurement of whiteness based on Hunter or CIELAB colour systems has been extensively used to estimate the degree of cheese translucency in cheese (Metzger et al., 2000; Dave et al., 2001; Metzger et al., 2001; Pastorino et al., 2002; Joshi et al., 2003; Wadhvani and McMahon, 2012; Ibáñez et al., 2016). Dave et al. (2001) evaluated the translucency of non-fat Mozzarella cheese by instrumental and sensory evaluations, and concluded that a cheese had a translucent appearance when $L^* \leq 82$. In our study, we observed that L^* values measured at room temperature ($\sim 25^\circ\text{C}$) ranged from 61 – 63 (Table 3.1), which is associated with a translucent appearance (Dave et al., 2001). This is in agreement with Joshi et al. (2003) who found no differences in the whiteness at 5°C ($L^* \sim 50$) of non-fat Mozzarella cheeses varying in Ca content. These authors also reported an increase in whiteness when cheeses were heated at high temperatures.

During the heat treatment of cheese, its appearance is modified from translucent to opaque, which is reflected in a whiter colour (Figs. 3.1 and 3.2). A first report of how whiteness of cheese changed during heating was observed in a model of pizza baking to evaluate the melting and browning of Mozzarella cheese with fat levels of 21.0, 6.0 and <0.3% (Rudan and Barbano, 1998). Cheeses were subjected to simulated and actual baking treatments (232°C for 5 min). The authors observed that in cheeses with 21% of fat, the release of free oil during baking prevented the loss of water and maintained the temperature at ~80°C, contributing to the formation of a white appearance; whereas in cheeses with lower fat content, the lack of free oil led to a loss of moisture in the surface, an increase of temperature $\geq 100^{\circ}\text{C}$ and hence the formation of a dried surface and or blisters occurred, affecting on melting. Based on these observations, the authors suggested the use of a hydrophobic surface coating and a physical cover to prevent loss of moisture and also the formation of a dried skin and blisters. All of these precautions were taken in consideration to evaluate changes of cheese translucency at high temperatures (i.e., the use of vegetable oil and the covering of samples in Petri dishes) as we used a low-fat cheese as base.

Despite the fact that cheese translucency has been investigated at high temperatures; different methods and approaches have been used by different authors. Metzger et al. (2000, 2001) heated the same cheese samples consecutively for 20 min at different temperatures from 7 to 71°C. A similar approach was used by Dave et al. (2001), but differing in temperature profile, ranging from 10 to 90°C and an equilibration time of 10 min. Pastorino et al. (2002) and Joshi et al. (2003) evaluated cheese samples heated at 10 and 50°C and 5 and 60°C, respectively; however, these

authors did not report the equilibration time. In a recent study, Ibáñez et al. (2016) evaluated individual set of samples heated at only one temperature, from 4 to 80°C for 30 min. An interesting observation from our study is that cheese translucency is not only influenced by heating temperature, but also by holding time (Figs. 3.1 and 3.2).

Several factors take place when cheese is heated to high temperatures that directly influences to its appearance. As previously stated, cheese fat scatters light, contributing to an opaque material. When cheese is heated at ~40°C, fat melting leads to a decrease in light scattering and hence an increase in translucency (Metzger et al., 2000). This effect is also greatly influenced by the fat content of cheese (Ibáñez et al., 2016). In our study, we evaluated colour changes in a low-fat cheese base to avoid the interference of fat melting on the optical properties of cheese.

An increase in cheese whiteness above 40°C is related to protein-protein interactions. Pastorino et al. (2002) observed the presence of protein aggregates in the microstructure of non-fat Mozzarella cheese heated at 50°C, when observed by electron microscopy. Metzger et al. (2000) elucidated that an increase in the whiteness of Mozzarella cheese heated at 49°C is associated with the occurrence of hydrophobic interactions of intact β -CN that remained soluble in the aqueous phase of cheese, leading to the formation of aggregates that would contribute to an increase of light scattering. In addition, these authors observed that when the expressible serum was heated, the formation of a white weak gel occurred that was reversible when cooled. The soluble nitrogen content of Cheddar cheese drastically decreased when Cheddar cheeses were heated at 60°C for 30 min, remaining constant at higher temperatures;

however, solubility increased again when samples were maintained at 4°C during 24 h, suggesting a reversible effect on protein aggregation (Ibáñez et al., 2016). Kim et al. (2011) observed that protein solubility in full-fat and reduced-fat Cheddar cheeses was greatly reduced when heated at increasing holding times. These authors also observed an inverse relationship between protein solubility and surface hydrophobicity. Hydrophobic interactions are known to increase with increments of temperature, reaching a maximum at 70°C and then exhibit a decrease thereafter (Bryant and McClements, 1998). An increase in the whiteness of cheeses heated at high temperatures could, in part, be explained by the occurrence of hydrophobic interactions that would induce the formation of aggregates, promoting light scattering. An increase of whiteness as holding time increase could be then related to the extent of the formation of aggregates.

Differences in the intensity of whiteness among Ca treatments could also be explained by differences in the initial levels of INSOL Ca that might contribute with a different configuration in cheese structure. Metzger et al. (2001) evaluated the whiteness of low-fat Mozzarella cheeses that contained different levels of water insoluble calcium (WIC) and observed that lower levels of WIC were associated with low whiteness values when measured at 60°C and attributed these differences to protein-protein interactions. Differences in whiteness intensity could also be associated with stability of calcium at high temperatures. The solubility of calcium phosphate is highly temperature-dependent and it trends to decrease with increasing temperature (Fox et al., 2015). An early study from Rose and Tessier (1959) found precipitation of calcium phosphate when milk ultrafiltrates and synthetic buffers were treated at high

temperatures, suggesting the formation of new heat-induced structures. These authors also found that a decrease in solubility was more severe in synthetic buffers. This is in agreement with the results of Broome and Limsowtin (2002) who found a permanent precipitation of Ca salts in SCCAP models when heated at temperatures greater than 40°C. In our study, acid-base titration curves obtained from cheeses subjected to different heat treatments (Fig. 3.3) only differed in the magnitude of buffering area, suggesting that heat-induced INSOL Ca may have the same nature as in native form (Udayarajan, 2007). We found that all cheese treatments exhibited an increment in levels of INSOL Ca when heated at $\geq 70^{\circ}\text{C}$ (Fig. 3.4), which may suggest that the formation of new structures may contribute to an increase of cheese whiteness.

We believe that the main driving force involved in colour changes of cheeses heated at 60°C relates to hydrophobic interactions; whereas at higher temperatures, they could be associated with a combined effect of both hydrophobic interactions and formation of new colloidal calcium phosphate. An increase in temperature not only leads to the formation of aggregates of β -CN that could interact with other caseins due to hydrophobic interactions, but also by mean of calcium-phosphate linkages (Green, 1971; Dave et al., 2001). In addition, it has been found that Ca binds to α_{s1} -CN when heated at high temperatures (Dalgleish and Parker, 1980). A decrease in the solubility of α_{s1} -, β -CN and their degradation products was observed when Cheddar cheeses were heated at 80°C for 30 min, and reversed when samples were stored at 4°C for 24 h, which suggests that cheese translucency was influenced by hydrophobic interactions and probably due to Ca-linkages (Ibáñez et al., 2016).

A completely unexpected result was a slight decrease of L^* values of cheeses when treated at the highest combinations of time and temperature (Fig. 3.2). A possible explanation could be associated with the extent of heat treatment and particle size of aggregates. Small aggregates contribute to higher light scattering, whereas an increase in particle size would lead to decreased scattering and hence, a reduction in whiteness. Kalab et al. (1987) observed that under extreme conditions of heat treatments (82°C for 1 – 5 h) larger and denser casein aggregates were formed. Based in this information, we believe that an increase in levels of INSOL Ca, along with temperature and holding times led to larger aggregates, reducing light scattering and hence L^* values.

A decrease in L^* values of cheeses treated at high temperatures has also been associated with the development of browning (Mukherjee and Hutkins, 1994; Ma et al., 2013), which may occur due to heat-induced reaction between free amino groups of proteins and reducing sugars, leading to the formation of high-molecular compounds (Thomas, 1969). Accumulation of reducing sugars in cheese, such as lactose and galactose, along with the occurrence of proteolysis during ripening have a great potential for the formation of browning compounds when exposed at high temperatures (Bley et al., 1985).

A reduction of the thickness of cheese samples at high temperatures may contribute with a decrease of L^* values. In a preliminary study (unpublished data), we found a considerable decrease of the contrast ratio (an indicator of opacity; Ibáñez et al., 2016) when the thickness of full-fat and reduced-fat Cheddar cheeses was reduced from 5 to 2 mm. At macromolecular level, Guinee et al. (2000) observed a shrinkage of the

cheese matrix along with the expulsion of fat and water when heated at high temperatures. This phenomenon could cause a decrease in the thickness of heat-treated cheeses that led to a slight reduction of L^* values.

3.5.3 Rheological properties

A reduction of G' in all cheese treatments as the heating temperature increased was associated with softening of the cheese matrix due to melting (Fig. 3.6). The presence of two driving forces, i.e., hydrophobic interactions and electrostatic repulsions, lead to a weakening of the cheese structure at high temperatures (Lucey et al., 2003). However, an increase of holding time at high temperatures ($\geq 70^\circ\text{C}$) was associated with increased G' values, which is related to the formation of new structures. These observations were not found when cheeses were heated at 60°C , when G' values decreased during the first 5 min of holding time, remaining constant thereafter; suggesting that only hydrophobic interactions took place.

The formation of new heat-induced structures relates to increased levels of INSOL Ca in all treatments, caused by a combined effect of high temperatures and holding times (Fig. 3.4). Udayarajan et al. (2005) found that an increase in the stiffness of cheese heated at $>70^\circ\text{C}$ is related to newly formed colloidal calcium phosphate that could potentially interact with casein-phosphate systems and or crosslink to native casein micelles. We believe that these new structures may also contribute to increased light scattering and directly influence in cheese whiteness at high temperatures. On the

other hand, the development of cheese browning (i.e., a reduction of L^* values) may also be associated with an increase of G' values at high temperatures due to the formation high molecular compounds. Previous studies have shown a negative correlation between melting properties analyzed by empirical methods and browning development of cheese when heated at high temperatures (Hong et al., 1998; Ma et al., 2013). However, further investigation will be required to establish the role of browning development with the rheological properties of cheese at high temperatures.

The LT values are known as an indicator of the mobility of the bonds of the matrix. Increased levels of Ca bound to casein determine a low mobility of the bonds, leading to reduced melting. As expected, an increase of the heating temperature led to an increase in LT values, indicating an increase of the mobility and hence melting of cheese. Nevertheless, an increased holding time at $\geq 70^\circ\text{C}$ led to a reduction LT values (Fig. 3.5), which has been related to the formation of heat-induced calcium phosphate structures (Fig. 3.4), altering the mobility of casein matrix. On the other hand, when cheeses were heated at 60°C , we observed a slight increase in LT values during the first 5 min of heating, remaining constant thereafter. This is in agreement with the report of Kuo et al. (2001), who observed that the extent of melting at 60°C was not affected in 20 min of holding time at early stages of ripening (1 wk) when evaluated Cheddar and Mozzarella cheeses by using an empirical meltability method (UW-Meltprofiler).

The n value is an useful indicator of the type of bonds in materials and is related to their relaxation behavior (Stading and Hermansson, 1990). When n equals zero, the material corresponds to permanent or covalent type gels, which are frequency

independent, whereas when $n > 0$, the material corresponds to a physical gel, where bonds break/reform with time (not permanent; Udayarajan et al., 2005).

In our study, we observed an increase in n values with increasing temperature, suggesting a high frequency dependence with heating temperatures, which also agrees with previous studies that found a considerable increment of frequency dependence when cheeses are heated at $\geq 60^{\circ}\text{C}$ (Udayarajan et al., 2005; O'Mahony et al., 2006). An alteration in the initial levels of INSOL Ca associated with casein would have an effect of n values at high temperatures. This is in agreement with O'Mahony et al. (2006) who found a negative correlation between levels of insoluble colloidal calcium phosphate of Cheddar cheese and the n values obtained at 70°C , suggesting that levels of INSOL Ca determines the mechanical properties of cheese at high temperatures. We also found that the formation of heat-induced INSOL Ca as holding times increased was associated with a decrease in n values, suggesting that the mobility of bonds in the cheese matrix is highly influenced by a combination of high temperatures and holding times.

3.5.4 Principal component analysis

The new variables obtained by PCA showed that heating cheeses at high temperatures with varying holding times have a big impact on the rheology, whiteness and levels of INSOL Ca of experimental cheeses. The PC1 successfully discriminated cheese samples with different levels of INSOL Ca based on their rheological properties,

which were affected by the temperature of heat treatment. Increasing temperature was associated with a decrease in G' and an increase in LT and n values (Figs. 3.5 and 3.6). In addition, PC1 showed how rheological properties changed as holding times were increased. The PC2 separated cheese samples based in whiteness changes during 30 min of holding time (Fig. 3.2). In general, an increase of holding times at high temperature led to whiter cheeses; however, PC2 was also able to discriminate a decrease in whiteness when cheeses were heated to high combination of temperature and holding times (e.g. 80°C during 30 min). The PC3 separated cheeses based on their levels of INSOL Ca (Fig. 3.4), which were highly affected by their initial levels, along with the formation of heat-induced colloidal calcium phosphate at different combinations of temperature-time.

3.6 Conclusions

The results of this study showed that the translucency of low-fat Mozzarella cheeses not only is affected by high temperatures of heating, but also by increased holding times. In addition, an increase in the levels of total and insoluble Ca in cheeses was related to the extent of whiteness appearance when treated at high temperatures. An increase of cheese whiteness occurred when samples were heated at 60°C, even though levels of insoluble calcium were not affected due to heat treatment, suggesting that colour changes could be associated with the formation of aggregates caused by an increase in hydrophobic interactions. The formation of new heat-induced colloidal calcium phosphate when cheeses were heated at $\geq 70^\circ\text{C}$ might have also contributed

with increased cheese whiteness. Despite the fact that new heat-induced insoluble calcium was formed when cheeses were heated at 80°C, whiteness reduced with increasing holding times, probably due to heat-induced modifications of the structure of cheese samples. Rheological measurements performed by FTMS showed similar trends when cheeses were analyzed at different frequencies. A softening of the cheese matrix was observed when cheese samples were heated at high temperatures due to melting. However, when cheeses were heated at >70°C, an increased holding time led to an increase of cheese stiffness, which could be related to the formation of new insoluble Ca that might contribute with cheese structure and hence on appearance.

3.7 Acknowledgements

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CHAPTER 4

EFFECT OF TITANIUM DIOXIDE, ANNATTO AND HOMOGENISATION ON THE TRANSLUCENCY OF REDUCED-FAT CHEDDAR CHEESE

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4.1 Abstract

Colorimetric methods have been used extensively to measure translucency. The Kubelka-Munk index (K/S) estimates the ratio of absorbance to scatter based on the reflectance of a thin layer of sample above black and white backgrounds. A method based on the measurement of CIELAB colour and the application of K/S was proposed to investigate the effect of titanium dioxide, annatto and homogenisation on the translucency of rennet gels, rennet whey and cheese during ripening. Three reduced-fat Cheddar cheeses were manufactured in parallel experiments. For titanium dioxide, levels of 0, 20 or 40 g per 100 kg were added to cheesemilks. For annatto, levels of 0, 8.25 and 16.50 mL per 100 kg were added to cheesemilks. Cheesemilks were homogenised at 0, 10 or 20 MPa at 55°C. CIELAB colour and K/S values were obtained with a colorimeter at 20°C for cheesemilks, rennet gels, whey and cheeses during ripening for six months. Titanium dioxide, annatto and homogenisation significantly modified the optical properties of cheese. Ripening significantly increased translucency for all treatments. A reduction of translucency was observed when titanium dioxide was added. Translucency increased following addition of annatto. Translucency was reduced when milk was homogenised. A high correlation was observed between some K/S and CIELAB values. These results suggest K/S can be considered a useful tool to determine changes of translucency not only in cheese, but also in rennet gels and whey when ingredients such as titanium dioxide or annatto are added or cheesemilks are subjected to homogenisation pretreatments.

Keywords Reduced-fat cheese, cheese translucency, CIELAB, Kubelka-Munk.

4.2. Introduction

The optical properties of cheese are highly influenced by its composition. Fat plays an important role in the appearance of cheese conferring opacity, i.e. whiteness. A common defect observed in cheeses with reduced fat content is an increase in translucency due to a lack of scatter centers (Johnson et al., 2009). The addition of ingredients or new steps during the manufacture of cheese has an impact on its optical properties.

Titanium dioxide has been extensively used as a white pigment in paint, textiles and food products (Weir et al., 2012). Titanium dioxide can be used as a food ingredient (FAO 2015). The United States Food and Drug Administration (FDA) authorizes the use of this ingredient if levels do not exceed 1% (w/w; FDA 2005), whereas the European Union (EU, 2008) establishes its use at *quantum satis* levels. Kosikowski and Brown (1969) and Wadhwani and McMahon (2012) have successfully used titanium dioxide to improve the whiteness of Mozzarella and Cheddar cheese, respectively.

Annatto is a natural carotenoid-based colorant ingredient obtained from the seeds of the tropical tree *Bixa orellana* L. and is extensively used for colouring foods, mainly dairy products such as butter and cheese (Giuliano et al., 2003). The FDA considers that annatto is a safe food ingredient if the amounts used are in accordance with GMP (FDA, 2005). According to European legislation (EU, 2008), levels of annatto in cheese should not exceed 15 mg kg⁻¹.

Homogenisation of milk leads to a reduction in the size of fat globules, affecting its optical properties (Rudan et al., 1998). Despite the fact that homogenisation of milk leads to a series of changes in composition, yield, biochemistry, texture and functionality of cheese, this treatment has been successfully used in a diversity of cheese varieties such as Roquefort, Cottage, Swiss, Mozzarella and Cheddar (Jana and Upadhyay, 1992).

The measurement of colour in cheese is a good indicator of its appearance (Wadhvani and McMahon, 2012). Nowadays, colour is generally measured by the CIELAB scale, which is represented by a three-dimensional plot comprised from L^* , a^* and b^* values. The L^* value represents the level of lightness from 0 (black) to 100 (white); the a^* value indicates greenness or redness, given by negative or positive values, respectively; and the b^* value indicates blueness or yellowness, represented by negative or positive values, respectively (Hunterlab, 2012). The extent of whiteness in cheese is inversely related to the level of translucency and is mainly caused by its fat content (Ibáñez et al., 2016). Little (1964) observed that internal light transmission through translucent materials leads to loss of light which leads to inaccuracy in the measurement of optical properties of food products when subjected to both instrumental and sensory evaluations. To avoid this problem, this author suggested the use of thin layers of materials and the application of Kubelka-Munk reflectance theory (K/S), which is based in the measurement of the reflectance of samples positioned above black and white backgrounds to estimate the ratio of absorbance to scatter (Judd and Wyszecki, 1975). The K/S formula has been extensively used to study the optical properties of translucent materials, including food products such as fruit gels (Calvo and

Salvador, 1997), sauces (Little, 1964; Huang et al., 1970), juices (Gullett et al., 1972) and dairy products such as milk and *dulce de leche* (MacDougall, 2002). A recent study from our group (Ibáñez et al., 2016) successfully used the Kubelka-Munk theory of reflectance based on the CIE whiteness measurements to determine the degree of translucency of Cheddar cheese varying in fat content during heating and cooling at different stages of ripening. Under the conditions studied, we observed that cheese translucency estimated by both *K/S* and CIELAB whiteness methods was highly correlated. Nevertheless, to our knowledge, there is no information if the *K/S* method is suitable to estimate the appearance of cheese when is modified by the addition of certain ingredients or the modification of cheesemaking protocols. This study aims to evaluate the effect of titanium dioxide, annatto and homogenisation on the optical properties of reduced-fat Cheddar cheese at different stages of ripening as measured by CIELAB colour and Kubelka-Munk analyses. The translucency of cheesemilks, rennet gels and whey were also evaluated by these techniques.

4.3. Materials and methods

4.3.1 Cheese manufacture

Reduced-fat Cheddar cheeses were manufactured in triplicate trials as described by Ibáñez et al. (2016), varying in the levels of titanium dioxide, annatto or effects of homogenisation. Experimental cheeses were made on 10 kg cheesemilk scale and standardized to a fat content of 1.75% (w/w), maintaining a casein to fat ratio of 1.4:1.0.

Cheesemilk was batch pasteurized at 63°C for 30 min. After pasteurization, cheesemilk was cooled to 31°C and transferred to individual vats. Titanium dioxide (Sigma-Aldrich, Saint Louis, MO, USA) or single strength annatto (DSM Food Specialties, Delft, Netherlands) were added to cheesemilks at 31°C on their individuals vats at levels of 0, 20 or 40 g per 100 kg of cheesemilk and 0, 8.25 or 16.50 mL per 100 kg of cheesemilk, respectively. The homogenisation step was carried out in cheesemilks prior pasteurization on a two-stage homogeniser (model APV 1000, APV Homogenisers AS, Albertslund, Denmark). Standardized cheesemilks were pre-heated at 55°C and homogenised at 0, 10 or 20 MPa. The ratio of pressure between the first and second stage was 4:1. After manufacture, cheeses were then vacuum packed and ripened for 180 d at 8°C.

4.3.2 Chemical analyses

Composition of experimental cheeses was determined at 14 d of ripening. Moisture content was estimated by the oven-drying method (IDF, 1982), fat by the Gerber method (IIRS, 1955), crude protein (%N x 6.38) by the macro-Kjeldahl method (IDF, 1986) and salt content by potentiometric determination (Fox, 1963). The pH of a mixture of 10 g of cheese and 10 mL of water was measured at 20°C (Ibáñez et al., 2016) and the proteolysis expressed as the pH 4.6-soluble N (Kruchoo and Fox, 1982) were measured at 14 and 180 d of ripening.

4.3.3 Colour measurements

The measurement of colour was performed with a colorimeter (Konika-Minolta CR-400, Konika-Minolta Optics Inc., Osaka, Japan). The instrument was set to the CIELAB system (Hunterlab, 2012) with an illuminant D65 and a visual angle of 2°. Before use, the instrument was calibrated with a CR-A43 calibration plate. The CIE whiteness (L^*), redness/greenness (a^*) and yellowness/blueness (b^*) values were recorded. The metric chroma (C^*) and hue-angle (h^*) were obtained from a^* and b^* values as described in Equations (4.1) and (4.2):

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (4.1)$$

$$h^* = \tan^{-1} (b^*/a^*) \quad (4.2)$$

Colour of milks and experimental cheeses

Colour of cheesemilks was determined using colorimeter accessories (Konika-Minolta Optics Inc., Osaka, Japan): a glass cuvette (model CM-A96; 10 mm thickness) fixed to a specimen holder and attached to a sample holder (model CR-A505). Five measurements were performed per each cheesemilk at 20°C. The measurement of colour of experimental cheeses was performed directly on a fresh cut of the cheese block previously equilibrated at 20°C for 30 min. The L^* , C^* and h^* values were obtained during a ripening period of 180 d. Five random measurements were made in the surface of cheese samples at each timepoint.

Kubelka-Munk measurements

The Kubelka-Munk formula relates the coefficient of absorbance K and scatter S to the reflectance of a material of infinite thickness R_∞ , as shown in Equation (4.3), which is obtained from the reflectance of a material above a black and white backgrounds (Judd and Wyszecki, 1975):

$$K/S = (1 - R_\infty)^2 / 2 R_\infty \quad (4.3)$$

where

$$R_\infty = a - b \quad (4.4)$$

$$a = 1 / 2 [R + (R_0 - R + R_g) / R_0 R_g] \quad (4.5)$$

$$b = (a^2 - 1)^{1/2} \quad (4.6)$$

Note that in Equations (4.4) – (4.6) a and b are constants and do not correspond to CIE a^* and b^* colour values. The reflectance of infinite thickness R_∞ is obtained from the reflectance R and R_0 of a sample positioned above white background with reflectance R_g and black background with reflectance R_0 , respectively. In this study, R_∞ was estimated from individual measurements of CIE L^* and C^* values above black and white backgrounds (Calvo and Salvador, 1997; Ibáñez et al., 2016).

Rennet gels were prepared in triplicate from pasteurised cheesemilks. Aliquots of 200 mL of cheesemilks were supplemented with annatto or titanium dioxide, whereas homogenised cheesemilks were taken before cheese manufacture. Each treatment was supplemented with 132 μ L 1 M CaCl_2 . An aliquot of 11.5 mL was poured to a plastic petri dish (90 mm external diameter) to create a layer of $\sim$ 2 mm height and a volume of 25 μ L of commercial rennet diluted 1 to 100 was then added to the milk, stirred and incubated at 31°C for 50 min. After incubation, samples were equilibrated for additional 15 min at 20°C and colour of the rennet gels placed above black and white backgrounds was measured to calculate K/S values. Two measurements for each background were performed.

The rennet whey obtained from each cheese manufacture treatment was collected and an aliquot of 200 mL was filtered through a cheese cloth. The colour of the filtered whey was measured under the same conditions as cheesemilks, but placing cuvettes against black and white backgrounds in the transmittance specimen holder. At least five measurements were performed for each sample.

Cheese samples were prepared as described by Ibáñez et al. (2016). Cheese discs (35 mm diameter; 2 mm height) were placed on plastic petri dishes, coated with a layer of liquid paraffin and covered with the dish lid to prevent the loss of moisture. Cheese samples were then maintained at 20°C for 30 min. The colour of cheese discs was obtained in the middle of each disc above black and white backgrounds to estimate K/S . Five cheese discs were prepared for each treatment and two colour measurements on each background was made per sample.

4.3.4 Determination of fat globule size in milk

The size distribution of fat globule in homogenised milks was measured with a static laser light-scattering particle size analyser (Mastersizer S, Malvern Ltd., Malvern, Worcestershire, UK) as described by O'Mahony et al. (2005). The parameters reported were volume mean diameter ($D [4, 3]$), surface to volume mean diameter ($D [3, 2]$), specific surface area (SSA) and the average diameter which 10% of all fat volume is contained ($d (v, 0.1)$).

4.3.5 Experimental design and statistical analyses

Three treatments of titanium dioxide (0, 20 and 40 g 100 kg⁻¹ cheesemilk), annatto (0, 8.25 and 16.50 g 100 kg⁻¹ cheesemilk) or cheesemilk homogenisation (0, 10 and 10 MPa) were made in three independent replicate trials, based on a 3 x 3 block design for each treatment. Two-way analysis of variance (ANOVA) was performed on the optical properties of cheesemilks, rennet gels and whey and cheese composition at a significance level of $P < 0.05$. Split-plot designs (Montgomery, 2013) were performed to evaluate the effect of each individual cheesemilk treatment (titanium dioxide, annatto or homogenisation), ripening time and their interactions on the optical properties of cheeses determined by CIELAB and K/S , based on a general linear model (GLM) procedure. When significant differences were found ($P < 0.05$), the means of treatments were analysed by Tukey's multiple comparison test. Pearson correlation coefficients (r) were determined on the optical properties determined by CIELAB and K/S ($P < 0.05$).

All analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA).

4.4. Results

4.4.1 Colour of cheesemilk

As expected, there were no differences ($P > 0.05$) in the fat and protein content of standardised cheesemilks between any treatments (results not shown). The colour of experimental cheesemilks determined by CIELAB is shown in Table 4.1. The addition of titanium dioxide significantly increased the whiteness of cheesemilk, slightly reduced the greenness (increase of negative a^* values) and increased yellowness (b^* values; $P < 0.05$), that lead to a slight increase in C^* values and h^* angle ($P < 0.05$). Cheesemilks supplemented with annatto showed a marked decrease in L^* values and an increase in b^* values as the concentration increased, which also led to an increase in C^* values and a reduction in h^* ($P < 0.05$). An increase in homogenization pressures led to a significant increase in L^* values of cheesemilks, a slight increase in a^* , b^* and C^* values and a slight decrease in h^* values ($P < 0.05$).

Table 4.1 CIELAB colour of cheesemilks from treatments with different levels of titanium dioxide, annatto and homogenisation.

Treatment	L^*	a^*	b^*	C^*	h^* (°)
Titanium dioxide (g/100 kg)					
0	80.61 ^b	-5.03 ^c	7.84 ^c	9.32 ^c	122.71 ^a
20	82.49 ^a	-4.69 ^b	8.40 ^b	9.62 ^b	119.19 ^b
40	83.25 ^a	-4.43 ^a	8.72 ^a	9.78 ^a	116.95 ^c
SEM	0.501	0.089	0.133	0.077	0.839
Annatto (mL/100 kg)					
0	80.50 ^a	-5.34 ^a	7.58 ^c	9.58 ^c	121.33 ^c
8.25	78.93 ^b	-5.34 ^a	12.83 ^b	15.97 ^b	116.59 ^b
16.50	77.90 ^c	-5.26 ^a	17.94 ^a	21.38 ^a	108.26 ^a
SEM	0.388	0.238	1.620	1.710	1.920
Homogenisation (MPa)					
0	81.22 ^c	-5.07 ^c	8.25 ^b	9.69 ^b	121.61 ^a
10	83.84 ^b	-4.25 ^b	9.04 ^a	9.99 ^a	115.21 ^b
20	84.42 ^a	-4.14 ^a	9.07 ^a	9.97 ^a	114.55 ^b
SEM	0.537	0.148	0.139	0.062	1.130

^{a,b,c} Means within the same column for each treatment not sharing a common superscript differ ($P < 0.05$).

Values represent mean and standard error of three replicate trials.

4.4.2 Fat globule size of homogenised cheesemilks

The fat globule size distribution data for homogenised cheesemilks are detailed in Table 4.2. As expected, a significant reduction in the volume mean diameter ($D [4, 3]$) was found as the homogenisation pressure of cheese milk increased ($P < 0.05$), although there were no differences in this parameter between treatments at 10 and 20 MPa ($P > 0.05$). The surface to volume average diameter ($D [3, 2]$) and the mean diameter which 10% of all fat volume is contained ($d (v, 0.1)$) were significantly lower ($P < 0.05$) as the pressure of homogenisation increased. A decrease in the fat globule size caused by an increase in homogenisation pressure, led to a significant ($P < 0.05$) increase in the SSA.

Table 4.2 Fat globule size parameters of cheese-milks homogenized at 0, 10 or 20 MPa.

Item	Treatment (MPa)			SEM
	0	10	20	
$D [4, 3]^1$	2.99 ^a	0.76 ^b	0.55 ^b	0.39
$D [3, 2]^2$	0.93 ^a	0.41 ^b	0.33 ^c	0.09
$d (v, 0.1)^3$	0.31 ^a	0.21 ^b	0.17 ^c	0.02
SSA ⁴	6.47 ^c	14.69 ^b	18.47 ^a	1.77

^{a,b,c} Means within the same row not sharing a common superscript differ ($P < 0.05$).

¹ Volume mean diameter (μm).

² Surface to volume average diameter (μm).

³ 10% of the fat globules have a diameter less than this value (μm).

⁴ Specific surface area of fat globules ($\text{m}^2 \text{g}^{-1}$)

Values represent mean and standard error of three replicate trials.

4.4.3 Kubelka-Munk analysis of rennet gels and whey

The Kubelka-Munk values (K/S) expressed by whiteness (L^*) and chroma (C^*) of rennet gels and whey are shown in Table 4.3. There were no differences in the K/S values of rennet gels as the concentration of titanium dioxide increased ($P > 0.05$). The addition of annatto led to a slight reduction of $K/S L^*$ and a strong increase in $K/S C^*$ ($P < 0.05$), whereas homogenisation only showed a slight decrease in $K/S C^*$ at 10 MPa, remaining constant at higher pressures.

Addition of titanium dioxide to cheesemilk had no effect on the K/S values of whey. A slight, but significant decrease in $K/S C^*$ was found ($P < 0.05$) as the concentration of annatto increased. A marked increase in $K/S L^*$ values of whey was observed ($P < 0.05$) as homogenisation pressure increased and also the $K/S C^*$ values exhibited a slight increase at 10 MPa.

Table 4.3 Kubelka-Munk ratio of absorbance to scatter obtained by CIE whiteness ($K/S L^*$) and chroma ($K/S C^*$) of rennet gels and whey obtained from treatments with different levels of titanium dioxide, annatto and homogenisation.

Treatment	Rennet gels		Rennet whey	
	$K/S L^*$	$K/S C^*$	$K/S L^*$	$K/S C^*$
Titanium dioxide (g/100 kg)				
0	44.48 ^a	6.01 ^a	18.74 ^a	2.87 ^a
20	45.41 ^a	5.60 ^a	18.85 ^a	2.90 ^a
40	46.19 ^a	5.53 ^a	18.72 ^a	2.71 ^b
SEM	0.31	0.08	0.04	0.05
Annatto (mL/100 kg)				
0	44.38 ^a	5.93 ^c	18.45 ^a	2.38 ^c
8.25	43.61 ^{ab}	10.69 ^b	18.40 ^a	3.44 ^b
16.50	42.78 ^b	12.77 ^a	18.09 ^b	4.17 ^a
SEM	0.25	1.03	0.07	0.27
Homogenisation (MPa)				
0	44.35 ^a	6.09 ^a	18.59 ^c	1.78 ^b
10	44.38 ^a	4.74 ^b	19.71 ^b	2.58 ^a
20	44.75 ^a	4.88 ^b	20.31 ^a	3.12 ^a
SEM	0.21	0.24	0.26	0.20

^{a,b,c} Means within the same column for each treatment not sharing a common superscript differ ($P < 0.05$).

Values represent mean and standard error of three replicate trials.

4.4.4 Cheese composition, pH and proteolysis

Cheeses manufactured with different levels of titanium dioxide, annatto and homogenisation had similar compositions ($P > 0.05$; Table 4.4). The pH values at 14 d of ripening were similar between treatments. An increase of pH was observed for all treatments after 180 d of ripening ($P < 0.05$). However, a significant reduction in pH was observed as the extent of homogenisation increased ($P < 0.05$). Similar levels of proteolysis, expressed as pH 4.6 soluble-N as a percentage of total N, between treatments were observed in all treatments at 14 d of ripening, except cheeses made with different levels of annatto that presented slight differences. A significant increase in the levels of pH 4.6 soluble-N was found during ripening and no differences were found ($P > 0.05$) for all treatments at 180 d of ripening.

Table 4.4 Composition (14 d), pH (d 14 and 180) and proteolysis (d 14 and 180) of reduced-fat Cheddar cheeses with different levels of titanium dioxide, annatto and cheesemilk homogenisation.

Treatment	Moisture (%)	Fat (%)	Protein ¹ (%)	Salt (%)	MNFS ² (%)	S/M ³ (%)	pH		pH 4.6 SN/TN ⁴	
							14 d	180 d	14 d	180 d
Titanium dioxide (g 100 kg ⁻¹)										
0	39.79 ^a	22.54 ^a	30.77 ^a	1.61 ^a	51.39 ^a	4.05 ^a	5.22 ^a	5.32 ^a	8.88 ^a	20.31 ^a
20	39.83 ^a	22.33 ^a	30.43 ^a	1.42 ^b	51.29 ^a	3.58 ^a	5.20 ^a	5.30 ^a	9.27 ^a	20.55 ^a
40	39.31 ^a	22.56 ^a	30.87 ^a	1.42 ^b	50.77 ^a	3.60 ^a	5.22 ^a	5.32 ^a	9.07 ^a	21.15 ^a
SEM	0.357	0.246	0.395	0.040	0.609	0.117	0.006	0.001	0.148	0.372
Annatto (mL100 kg ⁻¹)										
0	40.46 ^a	22.67 ^a	30.10 ^a	1.50 ^a	52.19 ^a	3.72 ^a	5.24 ^a	5.33 ^a	9.19 ^b	20.44 ^a
8.25	41.13 ^a	22.78 ^a	29.62 ^a	1.35 ^a	53.26 ^a	3.28 ^a	5.23 ^a	5.30 ^b	9.76 ^a	21.34 ^a
16.50	40.33 ^a	22.55 ^a	30.25 ^a	1.33 ^a	52.21 ^a	3.30 ^a	5.26 ^a	5.33 ^a	9.47 ^{ab}	20.72 ^a
SEM	0.258	0.261	0.236	0.057	0.297	0.154	0.009	0.001	0.190	0.261
Homogenisation (MPa)										
0	40.01 ^a	21.56 ^a	30.86 ^a	1.59 ^a	51.01 ^a	3.98 ^b	5.26 ^a	5.38 ^a	8.90 ^a	19.25 ^a
10	39.44 ^a	22.00 ^a	30.91 ^a	1.66 ^a	50.56 ^a	4.21 ^a	5.27 ^a	5.31 ^{ab}	9.01 ^a	19.36 ^a
20	39.00 ^a	21.78 ^a	31.14 ^a	1.61 ^a	49.86 ^a	4.13 ^{ab}	5.26 ^a	5.28 ^b	8.78 ^a	19.35 ^a
SEM	0.223	0.096	0.215	0.017	0.276	0.045	0.011	0.002	0.260	0.410

^{a,b,c} Means within the same column for each treatment not sharing a common superscript differ ($P < 0.05$).

¹ Total %N x 6.38.

² Moisture in the non-fat substance of the cheese.

³ Salt in the moisture phase of the cheese.

⁴ proteolysis expressed as the percentage of pH 4.6 soluble N

Values represent mean and standard error of three replicate trials.

4.4.5 Cheese colour

The appearance of cheeses treated with different levels of titanium dioxide, annatto and cheesemilk homogenisation at 30 d of ripening are shown in Fig. 4.1, and their optical properties determined by CIE whiteness, chroma and hue angle during ripening are detailed in Figs. 4.2, 4.3 and 4.4, respectively. The addition of titanium dioxide significantly increased the whiteness of reduced-fat cheeses (Fig. 4.2a; Table 4.5). At 2 d of ripening, the L^* values were higher by at least ~8 units in those treatments with titanium dioxide added, when compared to the untreated control cheese. The L^* values exhibited a significant decrease as the ripening time increased; however, cheeses containing titanium dioxide showed a slight decrease with ripening time. Untreated cheese reached their lowest values at 14 d and remained constant thereafter. The addition of annatto significantly reduced the L^* values of experimental cheeses (Fig. 4.2b; Table 4.5). At 2 d of ripening, cheeses containing annatto had reductions of ~4 and ~8 units in L^* values, respectively, when compared to untreated cheese. This trend was also observed during 180 d of ripening. In addition, L^* values reduced with cheese age for all treatments ($P < 0.05$; Table 4.5). All annatto treatments exhibited a decrease of L^* values from d 2 to d 14 and remained constant thereafter. The homogenisation of cheesemilk led to an increase in the L^* values of experimental cheeses (Fig. 4.2c; Table 4.5); however, there were no differences ($P > 0.05$) between 10 and 20 MPa treatments at all timepoints of ripening. As occurred with annatto, a significant decrease of L^* values was reached at 14 d of ripening, remaining constant thereafter.

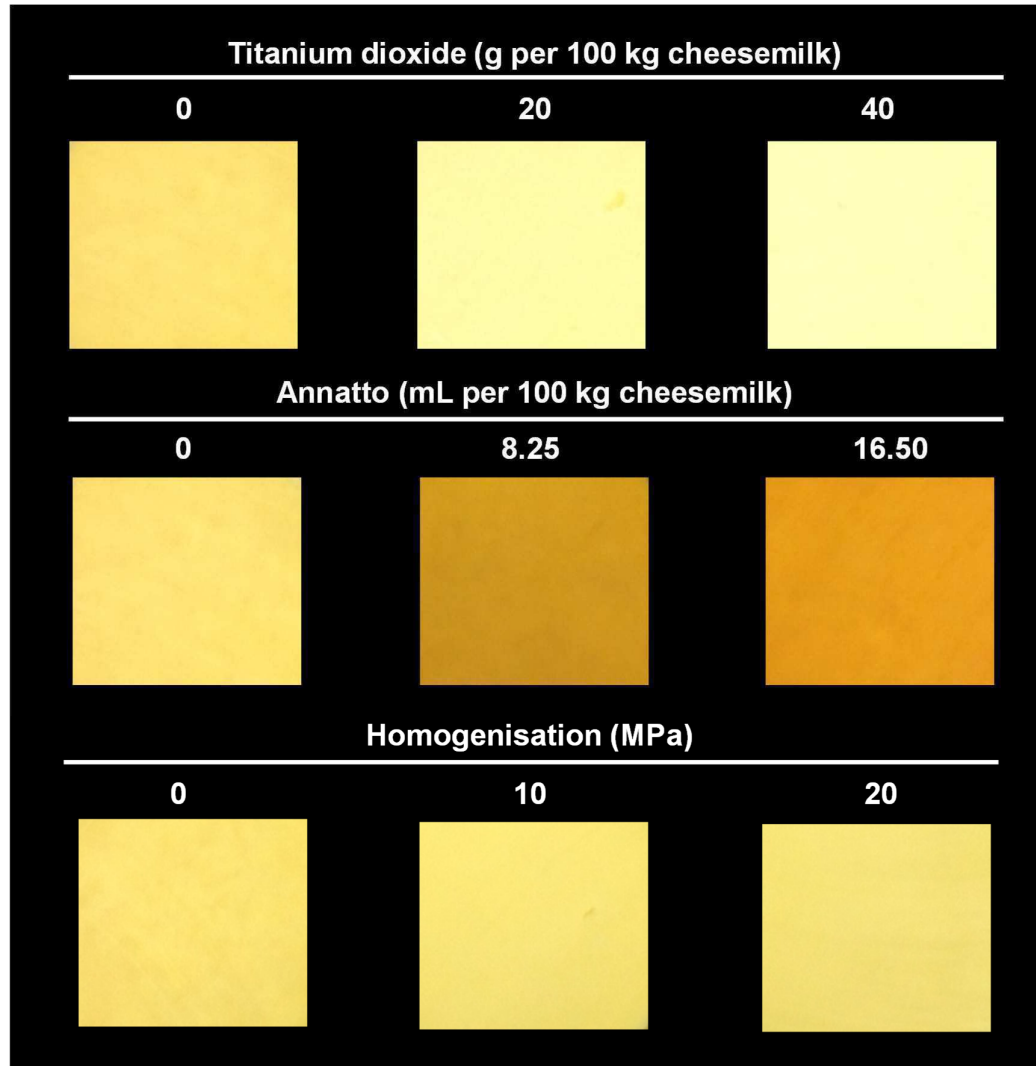


Fig. 4.1 Appearance of reduced-fat Cheddar cheeses manufactured with different levels of titanium dioxide, annatto and homogenisation pressures after 30 d of ripening.

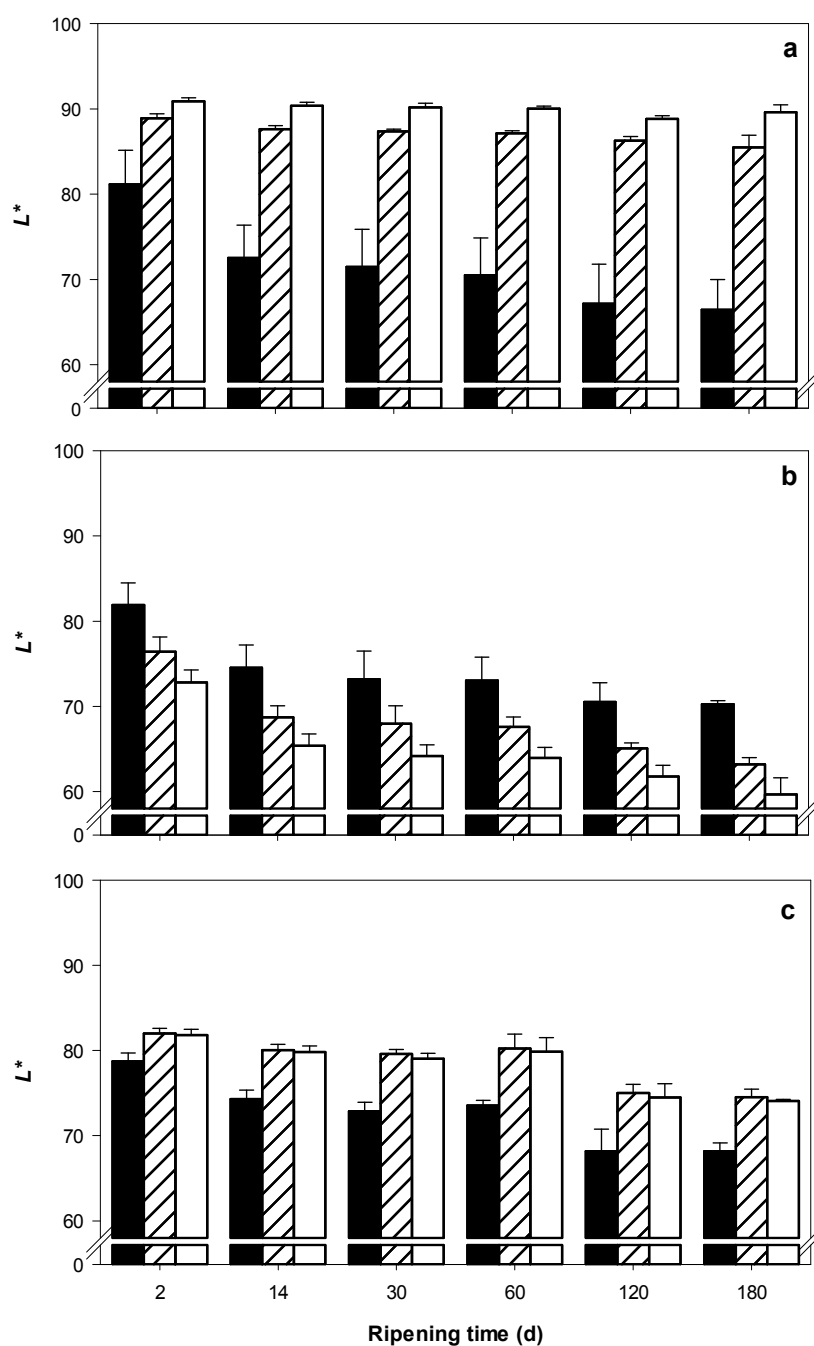


Fig. 4.2 Changes in whiteness (CIE L^* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa. Values represent mean and standard deviations of three replicate trials.

Table 4.5 Mean squares, probabilities (in parenthesis) and R² values for colour and ratio of absorbance to scatter of cheeses treated with titanium dioxide, annatto and homogenisation.

Factors ¹	df ²	L ^{*3}	C ^{*4}	h ^{*5}	K/S L ^{*6}	K/S C ^{*7}
Titanium dioxide						
<i>Whole plot</i>						
Day of cheese manufacture (D)	2	44.120 (0.318)	0.179 (0.937)	0.313 (0.521)	15.110 (0.114)	3.063 (0.096)
Treatment (T)	2	1772.7** (0.001)	245.38** (<0.001)	6.68* (0.012)	402.30** (<0.001)	215.81** (<0.001)
Error (D x T)	4	28.570	2.708	0.406	3.844	0.690
<i>Subplot</i>						
Age (A)	5	49.620** (<0.001)	2.709** (<0.001)	0.394** (<0.001)	8.444** (<0.001)	7.082** (<0.001)
T x A	10	20.000** (<0.001)	0.555 (0.068)	0.112** (<0.001)	5.016** (<0.001)	1.837** (<0.001)
Error	30	0.350	0.276	0.020	0.228	0.187
R ²		0.99	0.98	0.97	0.99	0.99
Annatto						
<i>Whole plot</i>						
Day of cheese manufacture (D)	2	37.320** (<0.001)	2.850 (0.175)	0.520 (0.063)	15.900** (<0.001)	6.303 (0.217)
Treatment (T)	2	396.22 (<0.001)	2662.9** (<0.001)	2825.6** (<0.001)	120.12** (<0.001)	924.99** (<0.001)
Error (D x T)	4	0.369	1.020	0.090	0.098	2.747
<i>Subplot</i>						
Age (A)	5	176.02** (<0.001)	17.520** (<0.001)	4.270** (<0.001)	50.277** (<0.001)	20.693** (<0.001)
T x A	10	0.460 (0.979)	4.140** (<0.001)	0.920** (<0.001)	0.092 (0.958)	1.221 (0.402)
Error	30	1.598	0.380	0.920	0.261	1.122
R ²		0.97	0.99	0.99	0.99	0.98
Homogenisation						
<i>Whole plot</i>						
Day of cheese manufacture (D)	2	0.219 (0.939)	0.032 (0.883)	0.562* (0.016)	0.188 (0.904)	0.006 (0.962)
Treatment (T)	2	198.21** (0.001)	4.780** (0.09)	2.520** (0.001)	35.818** (0.009)	4.859** (0.004)
Error (D x T)	4	3.412	0.02448	0.040	1.822	0.163
<i>Subplot</i>						
Age (A)	5	103.03** (<0.001)	6.662** (<0.001)	0.345** (<0.001)	13.389** (<0.001)	10.503** (<0.001)
T x A	10	1.717 (0.184)	0.7658** (0.005)	0.022 (0.701)	0.356 (0.686)	0.361 (0.096)
Error	30	1.136	0.2255	0.0299	0.485	0.196
R ²		0.97	0.88	0.90	0.92	0.92

¹ Split-plot design with the 3 treatments (within each cheese-milk treatment) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment x age as variables.

² Degrees of freedom

³ Whiteness

⁴ Chroma

⁵ Hue angle

⁶ Kubelka-Munk determined by whiteness

⁷ Kubelka-Munk determined by chroma

*0.01 < P ≤ 0.05; ** P ≤ 0.01

The C^* values of experimental cheeses treated with titanium dioxide decreased as level of addition increased (Fig 4.3a; Table 4.5). A slight increase in C^* values was observed for all titanium dioxide treatments at 14 d of ripening ($P < 0.05$), remaining constant thereafter. Cheeses containing annatto had higher C^* values when compared to control (Fig. 4.3b; Table 4.5). During ripening, all cheeses containing different levels of annatto exhibited a slight increase in C^* at 14 d of ripening ($P < 0.05$) and remained constant thereafter. Homogenisation had no effect on the C^* values of experimental cheeses (Fig. 4.3c; Table 4.5). As occurred with other cheese treatments, a slight increase in C^* values ($P < 0.05$) was observed for all homogenisation treatments at 14 d of ripening, remaining constant thereafter.

The addition of titanium dioxide led to a slight decrease ($\leq 1^\circ$) in the h^* values at 2 d of ripening (Fig. 4.4a; Table 4.5). Cheeses made without titanium dioxide showed no variations in h^* values during ripening ($P > 0.05$), whereas values increased slightly in cheeses containing titanium dioxide during ripening ($P < 0.05$). The h^* values exhibited a marked decrease as the concentration of annatto increased (Fig 4.4b; Table 4.5), which was lower than 90° for cheeses containing annatto, indicating the presence of an orange colour (Fig. 4.1). In addition, all treatments showed a slight decrease of h^* values during ripening ($P < 0.05$). Homogenisation of cheesemilk at 10 MPa led to a slight reduction of h^* values in cheese, when compared to 0 and 20 MPa treatments (Fig. 4.4c; Table 4.5) and, as observed for other treatments, there was a slight decrease as the cheeses aged ($P < 0.05$).

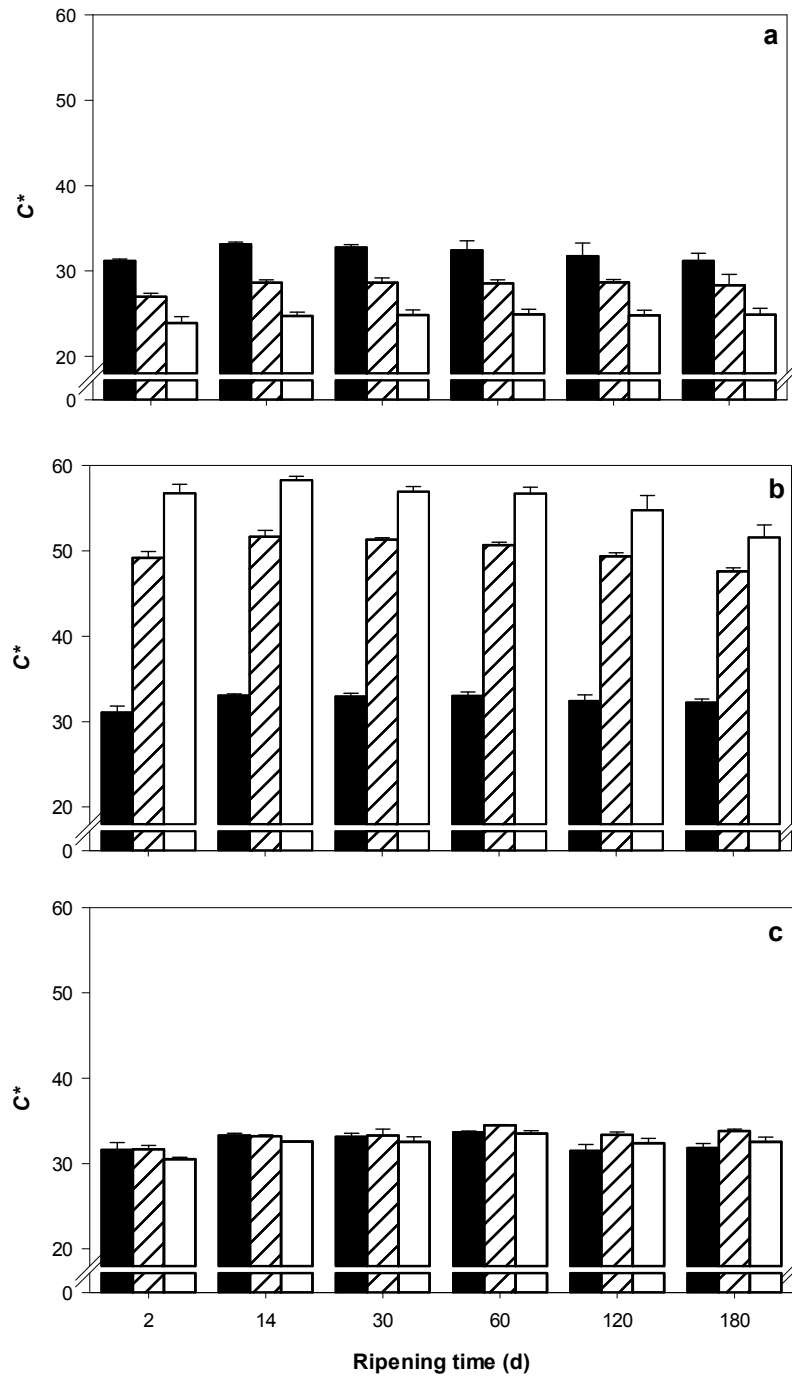


Fig. 4.3 Changes in chroma (CIE C* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (▨) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (▨) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (▨) or 20 (□) MPa. Values represent mean and standard deviations of three replicate trials.

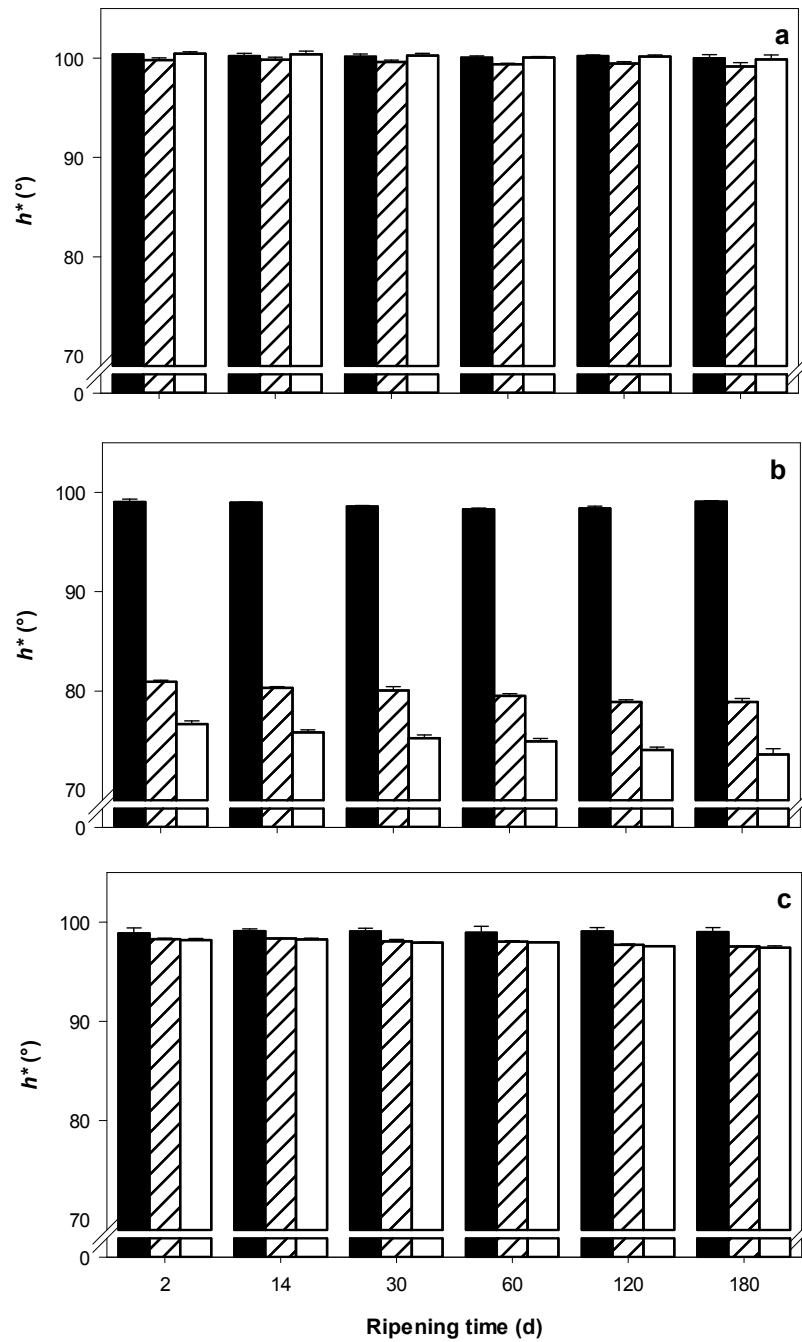


Fig. 4.4 Changes in hue angle (CIE h^* values) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa. Values represent mean and standard deviations of three replicate trials.

4.4.6 Kubelka-Munk analysis of experimental cheeses

The Kubelka-Munk values of experimental cheeses calculated by the measurement of CIE L^* and C^* values, are shown in Fig. 4.5 and 4.6, respectively. Cheeses showed a significant increase of $K/S L^*$ values as the concentration of titanium dioxide added increased (Fig. 4.5a; Table 4.5). During ripening, cheese without titanium dioxide showed a marked decrease in $K/S L^*$ after 14 d of ripening ($P < 0.05$), remaining constant as cheese aged. This trend is also observed in cheeses containing titanium dioxide added, but at a lower decreasing rate. The addition of annatto led to a significant decrease in $K/S L^*$ values in cheese (Fig. 4.5b; Table 4.5). A marked decrease of $K/S L^*$ values was observed at 14 d of ripening ($P < 0.05$), remaining constant beyond for all treatments. The use of homogenisation led to an increase of $K/S L^*$ values (Fig 4.5c; Table 4.5); however, there were no differences between treatments of 10 and 20 MPa ($P > 0.05$). As also observed in other treatments, a marked decrease of $K/S L^*$ values occurred at 14 of ripening ($P < 0.05$) and values remained constant thereafter.

An opposite trend to that found in $K/S L^*$ values was observed for $K/S C^*$ in all treatments (Fig 4.6). The addition of titanium dioxide led to a significant decrease in $K/S C^*$ values (Fig. 4.6a; Table 4.5). The use of annatto significantly increased the $K/S C^*$ values of experimental cheeses (Fig 4.6b; Table 4.5). An increase in the extent of homogenisation led to a slight decrease in $K/S C^*$ values (Fig. 4.6c; Table 4.5). For the different cheese treatments studied, the $K/S C^*$ values were higher at 14 d of ripening ($P < 0.05$) and remained constant beyond.

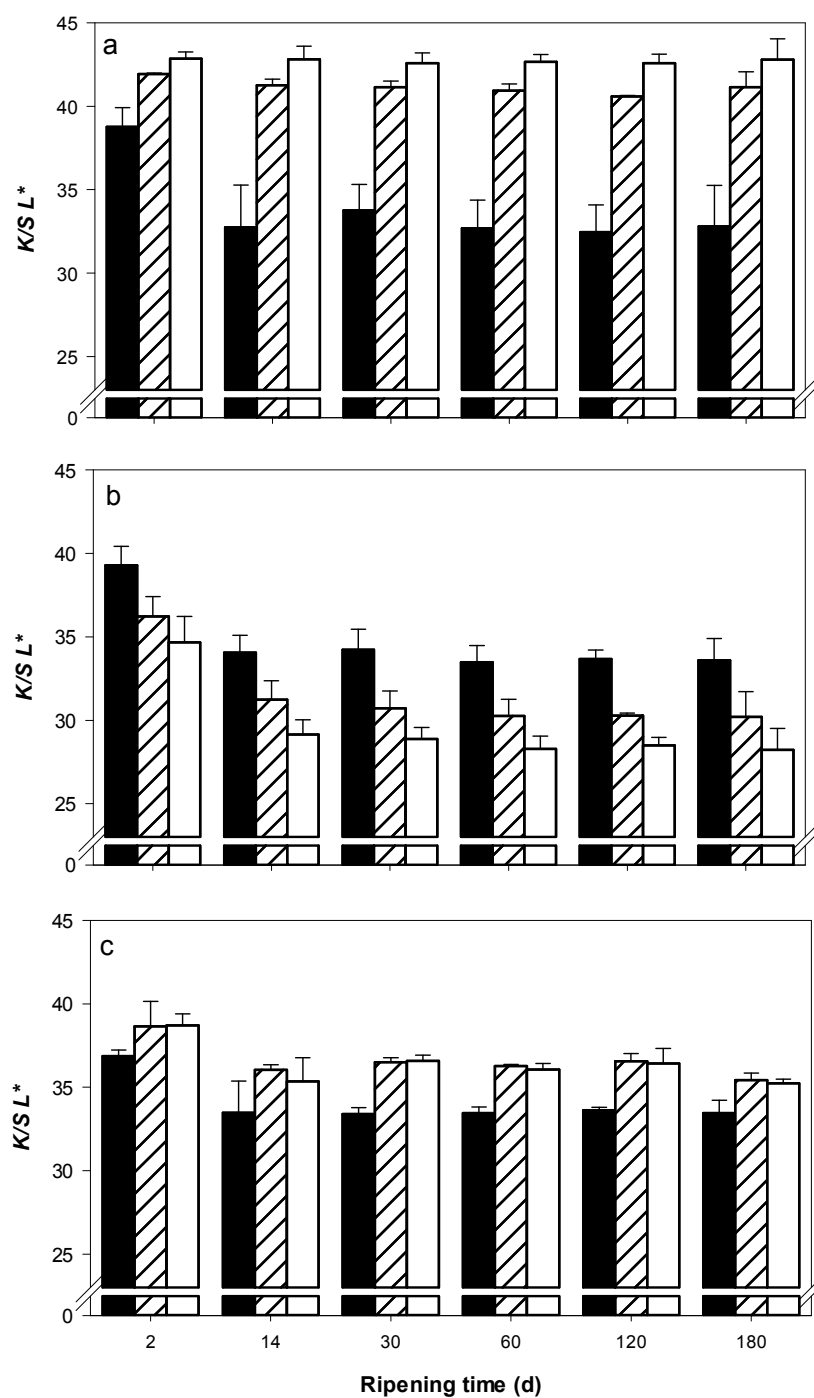


Fig. 4.5 Changes in the Kubelka-Munk absorbance to scatter ratio determined by whiteness ($K/S L^*$) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa. Values represent mean and standard deviations of three replicate trials.

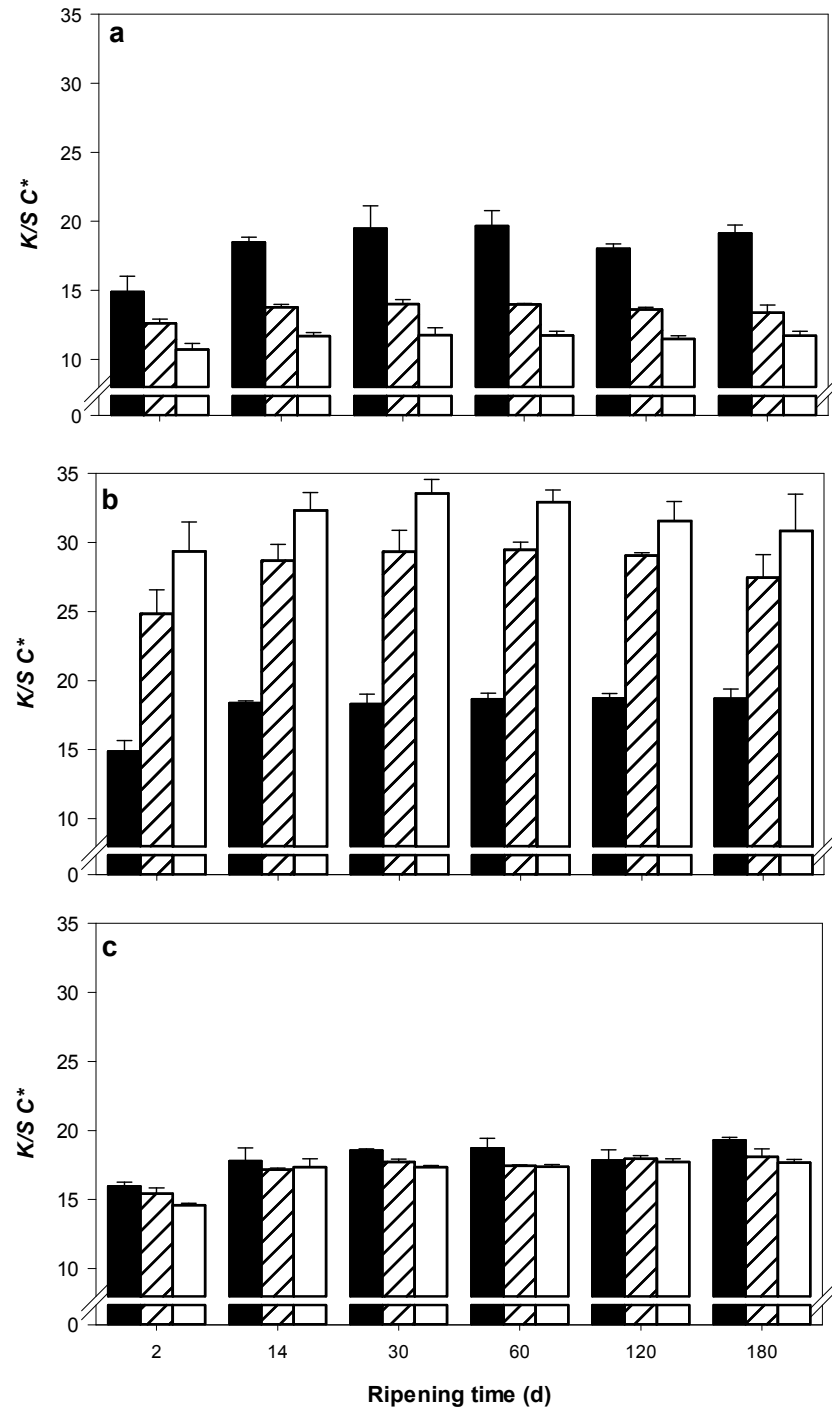


Fig. 4.6 Changes in the Kubelka-Munk absorbance to scatter ratio determined by chroma ($K/S C^*$) during the ripening of reduced-fat Cheddar cheeses manufactured with (a) titanium dioxide at levels of 0 (■), 20 (//) or 40 (□) g per 100 kg of cheesemilk, (b) annatto at levels of 0 (■), 8.25 (//) or 16.50 (□) mL per 100 kg of cheesemilk and (c) homogenisation of cheesemilks at levels of 0 (■), 10 (//) or 20 (□) MPa. Values represent mean and standard deviations of three replicate trials.

4.4.7 Correlation between CIELAB and Kubelka-Munk values

To evaluate the relationship between the optical properties of cheeses analysed by CIELAB and *K/S* methods, Table 4.6 shows Pearson correlations for each individual treatment. The L^* values were negatively correlated with C^* values from titanium dioxide and annatto treatments ($P < 0.05$), whereas there was no correlation with homogenisation treatment ($P > 0.05$). On the other hand, L^* values were positively correlated with *K/S* L^* values and negatively correlated with *K/S* C^* from all treatments ($P < 0.05$). The C^* values were negatively correlated with *K/S* L^* values from titanium dioxide and annatto treatments and there was no correlation with homogenisation treatment ($P > 0.05$), whereas they positively correlated with *K/S* C^* values from all treatments, although there was a lower correlation in cheeses made from homogenised milk ($r \sim -0.55$; $P < 0.05$). The *K/S* L^* values was negatively correlated with *K/S* C^* for all treatments ($P < 0.05$).

4.5. Discussion

A lower fat content in cheesemilk leads to a reduction in the number of light-scattering centers, reducing whiteness and conferring a translucent appearance to cheese (Johnson et al., 2009). Titanium dioxide is an inert nanoparticle extensively used as whitening agent in food industry due to its high refractive index (Weir et al., 2012). These authors have reported a mean particle size of 110 nm in a food-grade titanium dioxide, with a distribution ranged from 30 to 400 nm. Due to these properties, small

concentrations are required to improve optical properties of milk. As expected, the addition of titanium dioxide successfully increased the whiteness of reduced fat cheesemilks. In addition, greenness was reduced and yellowness increased (Table 4.1). These data are in agreement with Phillips and Barbano (1997) who observed the same effect when low-fat milk was supplemented with titanium dioxide at 0.1% (w/v).

Table 4.6 Pearson correlation (r) and probabilities (in parenthesis) between optical properties of reduced-fat Cheddar cheeses estimated by CIELAB and K/S .

Parameters	Titanium dioxide	Annatto	Homogenisation
$L^* - C^*$	-0.873**	-0.643**	0.029
	(<0.001)	(0.004)	(0.910)
$L^* - K/S L^*$	0.989**	0.966**	0.806**
	(<0.001)	(<0.001)	(<0.001)
$L^* - K/S C^*$	-0.962**	-0.783**	-0.737**
	(<0.001)	(<0.001)	(<0.001)
$C^* - K/S L^*$	-0.900**	-0.686**	-0.312
	(<0.001)	(0.002)	(0.208)
$C^* - K/S C^*$	0.942**	0.975**	0.554*
	(<0.001)	(<0.001)	(0.017)
$K/S L^* - K/S C^*$	-0.976**	-0.823**	-0.838**
	(<0.001)	(<0.001)	(<0.001)

*0.01 < $P \leq 0.05$; ** $P \leq 0.01$

Annatto contains apocarotenoid pigments and is primarily used for colouring cheese, conferring a yellow/orange appearance (Giuliano et al., 2003). As expected, its addition to cheesemilk led to a reduction of whiteness and increase of yellowness (Table 4.1).

A reduction in the particle size of the milk fat globules by homogenisation led to an increase in the SSA (Table 4.2), which contributed to increased light scattering and hence an increase in the whiteness of cheesemilk (Table 4.1). These data are in agreement with Rudan et al. (1998) who also observed a significant increase in the Hunter L values of cheese when reduced-fat cheesemilk was homogenised at 18 MPa.

The Kubelka-Munk analysis establishes a relationship between the ratio of absorbance to scatter, by estimating the reflectance of infinite thickness R_{∞} of certain material (Judd and Wyszecki, 1975). Previous studies have also shown the use of tristimulus values and CIELAB to replace the reflectance values in the K/S formulae (Calvo and Salvador, 1997; Little, 1964). However, an opposite relationship has been observed when K/S is expressed in terms of whiteness (L^* values), due to its sensitivity on scattering effects (Gullett et al., 1972; Huang et al., 1970). This is an important observation, as it also agrees with previous work from our group (Ibáñez et al., 2016), where high translucency of cheese (i.e., low opacity or whiteness) was associated with low $K/S L^*$ values and hence lower scattering. Based on this information, for this study we also calculated the Kubelka-Munk values based on CIE chroma ($K/S C^*$). Increased $K/S C^*$ values are associated with increases in absorbance due to an increase of colour.

Despite the fact that there was an increase in the whiteness (reduction of translucency) of cheesemilks treated with titanium dioxide, we found no significant differences in the K/S values obtained from rennet gels, even though the mean values of $K/S L^*$ and $K/S C^*$ increased and decreased, respectively (Table 4.3). The high variability of this data could be then associated with insufficient distribution of titanium dioxide in rennet gels. To ensure a complete distribution of titanium dioxide in cheesemilk and hence in cheese, Kosikowski and Brown (1969) suggested an adequate mechanical agitation of cheesemilk supplemented with titanium dioxide during cheesemaking until the addition of rennet. In our study, colour measurements of rennet gels were performed several times (data not shown), taking precautions given by these authors; however, the same trend in the optical properties was obtained. During cheese manufacture, the majority of titanium dioxide particles could be retained in the curd and hence avoiding its loss in the whey, which might explain the similarity of the optical properties of whey between different levels of added titanium dioxide.

As expected, the addition of annatto caused an increase in the absorbance of rennet gels that led to an increase in $K/S C^*$ and a reduction in $K/S L^*$ values, which is reflected in an increase in yellowness of cheesemilks (Table 4.1). These same trends were observed in rennet whey, suggesting loss of annatto in cheesemaking. In an early study, Barnicoat (1950) estimated that during cheese manufacture approximately 20% of the total annatto added into cheesemilk is lost in the whey.

A reduction in the $K/S C^*$ values of rennet gels as the level of homogenisation increased, is associated with an increase of light scattering due to a reduction in the fat

globule size (Table 4.2). We observed an increase of $K/S L^*$ of rennet whey as the level of homogenisation in cheesemilk increased, probably due to a combined effect of loss of *para*-casein particles caused by a weakening of curd (Jana and Upadhyay, 1992). In addition, an increase in $K/S C^*$ could be associated with a change in the colour of whey probably due to the release of globule fat membrane components caused by homogenisation.

The addition of titanium dioxide and annatto had no effect on the composition, pH and proteolysis of reduced-fat cheeses (Table 4.4), due to the inert nature of these ingredients. Even though we found no differences in composition, homogenisation of milk tends to increase the moisture content of cheese, which is attributed to a reduction of syneresis that leads to increased retention of water (Rudan et al., 1998; Deegan et al., 2014). This effect might also increase salt retention, which also increases S/M content as observed in homogenised treatments. We observed no differences on the pH values at 14 d of ripening. An increase in pH during ripening for all treatments is associated with changes to the buffering capacity of cheese caused by solubilization of colloidal calcium phosphate (Hassan et al., 2004). At 180 d of ripening, we observed lower pH values when homogenisation treatments were at 10 and 20 MPa due to increased rates of acid development during cheese manufacture that might lead to higher acidity (Jana and Upadhyay, 1992). We observed no differences in the levels of pH 4.6-soluble nitrogen at 180 of ripening. Rudan et al. (1998) observed higher levels of pH 4.6-soluble-N of reduced-fat Mozzarella cheese, when milk or cream was homogenised to 18 MPa; however, the authors did not find differences in MNFS and S/M contents.

Similar levels of proteolysis could be then attributed to higher S/M levels as the homogenisation treatment increased.

As expected, the colour of experimental cheeses was also affected by the addition of titanium dioxide, annatto and homogenisation (Figs. 4.1-4.4). The supplementation of cheesemilks with titanium dioxide led to an increase in whiteness and a reduction in chroma caused by a decrease of yellowness. Kosikowski and Brown (1969) observed a considerable improvement in the whiteness of Mozzarella cheese. These authors also found that cheesemilks supplemented with titanium dioxide up to levels of 50 g per 100 kg of cheesemilk maintained uniform cheese whiteness, whereas levels higher than 100 g per 100 kg was associated with a powdery texture and the accumulation of titanium dioxide at the bottom of cheese vats. Nowadays, titanium dioxide is available in form of emulsions (Wadhwani and McMahon, 2012), which facilitates its distribution in cheesemilk. In our study we used titanium dioxide in the form of powder and we observed no sedimentation in cheese vats at the concentration used.

The addition of annatto led to an increased chroma caused by increasing CIE a^* and b^* values (results not shown) that reduced the hue angle, leading to an orange appearance and an increase in cheese translucency. Wadhwani and McMahon (2012) combined the use of titanium dioxide and annatto to evaluate colour and consumer preferences of low-fat Cheddar cheese and observed a synergistic increase in yellowness and whiteness when these colouring ingredients were used simultaneously and found that they could be potentially used to obtain a similar appearance to full-fat

cheese; however, consumers preferred opaque cheeses (whiter appearance), whether they were coloured or not.

A loss of colour in full-fat Cheddar cheese made from homogenised milk was observed by Peters (1956) and was associated with an increase in whiteness. In our study, we observed a significant increase in whiteness even when homogenisation was performed at 10 MPa, which is attributed to a reduction of the size of fat globules (Table 4.2) that led to an increase in light scattering (Rudan et al., 1998). In general, homogenisation of cheesemilk is not only associated with an increase in whiteness of cheese, it also relates to a lower curd tension that leads to a weaker coagulum affecting texture and functionality of cheese, a higher yield due to a reduction of fat loss in whey and also to a higher water retention, increasing the moisture content, higher extent of proteolysis and lower pH (Jana and Upadhyay, 1992). As previously stated, we found a decrease in the pH values of cheeses as homogenisation pressures increased. Brickley et al. (2008) observed a decrease in the translucent appearance of directly acidified non-fat Mozzarella cheese when pH decreased from 5.4 to 5.0, suggesting that changes in the protein arrangements may differ due to approaching isoelectric point of caseins and hence affecting the optical properties of cheese. Despite pH differences between control and homogenized cheeses, which were around 0.1 units, may also have a little impact on the optical properties of cheeses. Rudan et al. (1998) compared the effect of homogenisation of cream and milk at 18 MPa for the manufacture of reduced-fat Mozzarella cheese and observed an increase in whiteness in both treatments when compared to cheese made from unhomogenised milk; however, homogenisation of cream led to lower levels of proteolysis in cheese than homogenisation of milk. Deegan

et al. (2014) found a significant increase in the whiteness of full-fat and reduced-fat Emmental cheese manufactured with cheesemilks homogenised at 10 MPa; in addition, these authors observed that the flavour and taste of reduced-fat cheese made from homogenized milk was improved probably due to a higher extent of lipolysis and a higher S/M content (Jana and Upadhyay, 1992). The extent of lipolysis, which is associated with the release of free fatty acids, was higher as the size of globule fat was reduced (O'Mahony et al., 2005).

A reduction of whiteness in all treatments observed during the first 14 d of ripening might be associated with an increase in the levels of proteolysis and probably to changes in the proportion of insoluble calcium due to solubilization of colloidal calcium phosphate (Ibáñez et al., 2016). This shift in cheese appearance led to an increase of colour intensity as reflected by an increase in chroma values and changes in hue angle values.

Similar trends between on the optical properties of experimental cheeses treated with titanium dioxide, annatto and homogenisation were observed when measured by CIELAB and Kubelka Munk parameters (Figs. 4.2 - 4.5). In general, L^* values were highly correlated with $K/S L^*$ (positively) and $K/S C^*$ (negatively). These data agree with our previous work (Ibáñez et al., 2016) where we found a high correlation between $K/S L^*$ and L^* values when Cheddar cheeses were analysed between 4 and 60°C ($r \geq 0.900$; $P < 0.05$). On the other hand, a lower correlation ($P < 0.05$) and, in some cases, no correlation ($P > 0.05$) was found between C^* values with other parameters, mainly on cheeses made from homogenized cheesemilks. These slight differences could be then

associated with the complexity involved in the optical properties of materials. Little (1964) reported that the measurement of the optical properties of translucent materials in thin layers, as occurred with the measurement of K/S analysis, could be more accurate than in deep layers, as measured directly on the cheese block for CIELAB colour, due to the complicated interactions of transmittance, absorption, reflectance and light loss through internal scattering and trapping.

4.6. Conclusions

Modifications in the optical properties of reduced-fat Cheddar cheeses can be achieved by the addition of ingredients such as titanium dioxide and annatto with no changes in composition. Even though homogenisation modified the optical properties of cheese, it may also affect some chemical properties, such as pH. Titanium dioxide and homogenisation reduced the translucent appearance of reduced fat cheeses by increasing the scattering with a high refractive index ingredient, or reducing the size of fat globule that leads to an increase on the available scattering area. The Kubelka-Munk analysis was able to differentiate translucency not only in cheese, but also in rennet gels and whey. Some of the traditional methods of colour measurements (CIELAB) and Kubelka-Munk were highly correlated, mainly with L^* values; whereas in C^* , correlations were lower. These differences could be associated with the complex phenomena involved in the optical properties of cheese.

4.7 Acknowledgements

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CHAPTER 5

EFFECT OF PECTIN ON COMPOSITION, MICROBIOLOGY, TEXTURE AND FUNCTIONALITY OF REDUCED-FAT CHEDDAR CHEESE

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5.1 Abstract

Hydrocolloids have been extensively studied in low-fat cheeses as a way to improve defects associated with fat reduction, which are often related to texture and functionality (meltability). Pectin is a polysaccharide obtained from plant cells which is commonly used as a stabilizer for acidified dairy beverages. This work aimed to evaluate the effect of three types of commercial pectins on the characteristics of reduced-fat Cheddar cheese during a ripening period of 180 d. Five Cheddar cheeses were made: full fat control (FF), reduced-fat control (RF) and reduced-fat cheeses with amidated (RA), high-methoxy (RH) or low-methoxy (RL) pectin added to milk prior processing at concentrations of 0.175%, 0.100% and 0.075% (w/w) respectively; levels were chosen to avoid phase separation of the casein micelles, due to depletion flocculation. Addition of amidated pectin markedly increased the moisture content of the experimental cheese (~49%), compared to RF (~45%; $P<0.05$). A significant reduction ($P<0.05$) in the proportion of insoluble calcium observed in RA and RL at 180 d (~40% versus ~56% in RF) was probably caused by calcium-induced gelation mechanisms of amidated and low-methoxy pectins. Texture profile analysis showed a softening of cheeses with added pectin (hardness <100 N versus >100 N in RF at 180 d; $P<0.05$). The melting properties of cheeses were significantly improved during ripening, although RF exhibited the lowest values (diameter increase >85% versus <70% at 180 d; $P<0.05$). These results suggest that pectin addition can be used to modify the moisture content, texture and melting properties of reduced-fat Cheddar cheese.

Keywords Pectin, fat replacers, reduced-fat cheese, Cheddar cheese, cheese texture.

5.2. Introduction

In recent years, consumers have shown an increasing interest in the consumption of cheeses with lower fat contents (Childs and Drake, 2009). One of the main properties of reduced fat cheeses is a higher protein to fat ratio which confers a more compact structure leading to a firmer and rubbery texture, lack of flavor, bitterness, development of off-flavours, poorer melting properties and a translucent appearance (Johnson et al., 2009; Mistry, 2001). To compensate for the increased proportion of protein in the matrix, it is possible to increase the moisture content of the cheese by various techniques, such as the modification of processing steps or the addition of ingredients that may increase the water holding capacity (Johnson et al., 2009).

Hydrocolloids have been used to modify the composition, and hence the texture and functionality, of reduced or low-fat cheeses. Mistry (2001) and Johnson et al. (2009) extensively reviewed the manufacture of different types of cheeses supplemented with a variety of carbohydrates-based fat replacers, such as StellarTM, NovagelTM, microcrystalline cellulose, carrageenan, gum arabic, polyanionic gum, starch, β -glucan and gum tragacanth.

Pectin is a class of anionic polysaccharide found in the middle lamella from the cell wall of higher plants and is responsible for the firmness and structure of plant tissues, acting as a hydrating agent and providing a cementing material for the cellulose network (Thakur et al., 1997). Although pectin is found in most plant tissues, the main commercial sources of pectins are extracted from apple pomaces and citrus peels. The

structure consists of a homopolymer of α -(1 $\rightarrow$ 4)-D-galactopyranosyluronic acid with repeating α -(1 $\rightarrow$ 2)-L-rhamnosyl- α -(1 $\rightarrow$ 4)-D-galacturonosyl sections that may contain branched sections with neutral side chains. The carboxylic groups from the galacturonans contain varying degrees of methyl esterification (DM). Pectins with more than 50% DM are classified as high-methoxy pectin (HMP), whereas those ones with lower than 50% DM are known as low-methoxy pectin (LMP; Thakur et al., 1997). In addition, a modified LMP may contain degrees of amidation (DA) on its carboxyl groups, and is referred as amidated pectin (AMP). One of the main characteristics of pectin is its ability to form gels. In general, HMP gels under acidic conditions ($\text{pH} \leq 3$) and the presence of high concentration of sugars ($\geq 55\%$), where the driving forces of gelation are hydrogen bonding and hydrophobic interactions, whereas LMP gels in the presence of Ca^{+2} , based on an egg-box model (Thakur et al., 1997). The main difference between LMP and AMP is that the latter gels at low concentrations of Ca^{+2} (Matia-Merino et al., 2004).

The main application of pectin in dairy industry is to stabilize caseins in acidified milks to avoid syneresis (Marozienne and de Kruif 2000; Harte et al. 2007). Some other applications have focused the use of pectin in rennet gels (Acero Lopez et al., 2009; Fagan et al., 2006; Tuinier et al., 2002). However, the concentration of pectin in milk plays an important role in stability, due to interactions between caseins and polysaccharides. Above a certain concentration of pectin, depletion interactions may cause destabilization of milk and hence a phase separation (Marozienne and de Kruif 2000; Tuinier et al. 2002; Acero Lopez et al. 2009). Lobato-Calleros et al. (2001) successfully used LMP in milk at a concentration of 0.2% (w/w) to increase moisture

content and improve the texture of low-fat Mexican Manchego cheese, probably due to the formation of calcium pectate gels that may lead to an interruption of the compact *para*-casein matrix. The authors did not report the presence of phase separation in milk due to the interaction of pectins and caseins. However, the proportion of calcium associated with caseins plays an important role determining the texture and melting properties of cheese and it is highly influenced by pH and ageing (Hassan et al. 2004, Lucey et al. 2003). As the gelation of pectin has an impact on the calcium equilibrium of milk (Harte et al. 2007), cheeses made with added pectin could potentially reduce the proportion of insoluble Ca of cheese, leading to a weakening of the *para*-casein matrix and hence modify its textural and melting properties. This study aimed to evaluate the effect of different types of pectins on the composition, proportion of insoluble Ca and rheological properties of reduced-fat Cheddar cheese during ripening. Pectins were added into milks at concentrations below this critical point to avoid destabilization.

5.3. Materials and methods

5.3.1 Pectin solutions

One day prior cheese manufacture, solutions of amidated (AMP; 34% DM and 15% DA) high-methoxy (HMP; 67% DM) and low-methoxy (LMP; 39% DM) pectins (Herbstreith & Fox KG, Neuenbürg, Germany) were prepared at levels of 4.55 % (w/v), 2.60 (w/v) and 1.95% (w/v), respectively. Pectins were mixed with 1.5 L of deionized water at 65°C for two hours using an overhead stirrer to ensure complete solubilization.

Pectin solutions were then cooled to 25°C, adjusted to 2 L, stored overnight at 4°C and heated again at 65°C using a waterbath before use.

5.3.2 Cheese manufacture

Cheddar-type cheese was manufactured in the pilot plant facilities of the School of Food and Nutritional Sciences, University College Cork, Ireland. Experimental cheeses were manufactured on a 50 kg scale based on cheesemilks standardized to a casein to fat ratio of 0.7 (full-fat: 35 g of fat per L of cheesemilk) or 1.4 (reduced-fat: 17.5 g of fat per L of cheesemilk) to obtain five different vats: full-fat Control (FF), reduced-fat Control (RF), reduced-fat with AMP (RA), reduced-fat with HMP (RH) and reduced-fat with LMP (RL). Each cheesemilk was batch pasteurized at 63°C for 30 min. During this step and when cheesemilks reached 63°C, 2 L of each pectin solution at 65°C was added to their corresponding vat. In order to maintain the same volume in all the treatments, 2 L of deionized water at 65°C was added to control vats (FF and RF). The final concentration of AMP, HMP and LMP in cheese milks were 0.175, 0.100, 0.075% (w/w), respectively. These levels were selected based on preliminary work performed on 10% (w/w) reconstituted low heat skim milk and RF cheese milks, to maximize addition of pectin while preventing depletion interaction (Marozienne and de Kruif 2000). Once pasteurization was finished, milks were cooled to 31°C. A Cheddar cheese starter culture (R-604Y, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added to a level of 0.03% (w/w) and left to ripen for 30 min with continuous stirring. Cheesemilks were supplemented with 0.09% (v/w) of 1 mol • L⁻¹ CaCl₂ and

equilibrated for additional 5 min. Chymosin (Maxiren 180, 180 IMCU • mL⁻¹, DSM Food Specialties, Delft, Netherlands) was added to each vat at a level of 0.03% (v/w) diluted 1 in 4 with distilled water to aid water dispersion. Once the curd developed enough firmness after 45-50 min, the coagulum were cut and cooked from 31 to 39°C in a period of 30 min and held to that temperature until the pH dropped to 6.2 and the whey was drained from the vats. The curd was then cut into blocks and inverted every 15 min until the pH decreased to 5.4. Curd blocks were milled, salted at a level of 2.5% (w/w) NaCl and equilibrated for 20 min. The salted curds were transferred to 5 kg rectangular molds and pressed during 14 h at a pressure of 2.5 kg • cm⁻². Experimental cheeses were vacuum sealed and ripened for 6 months at a temperature of 8°C.

5.3.3 Rennet coagulation properties

The rheological properties of cheesemilks during rennet coagulation were studied with a dynamic small amplitude oscillatory rheometer equipped with a Peltier concentric cylinder system and a conical rotor (28 mm diameter and 42 mm length; model AR-G2; TA Instruments, Waters LLC, Leatherhead, Surrey, UK) at a frequency of 1 Hz and 0.1% strain. Chymosin was added to cheesemilks previously heated at 31°C at a level of 0.03 % (w/v) and stirred for 1 min. Two additional min elapsed between the mixing of rennet in milks and the starting of oscillation. The storage modulus (G') and the loss modulus (G'') were measured during a gelation time of 45 min. The loss tangent (LT; G''/G') was also estimated. Gelation time (GT), was defined as the time required for rennet gels to reach $G' \geq 1$ Pa. Each treatment was analysed in triplicate.

5.3.4 Compositional analysis

Composition of cheeses was determined at 14 d of ripening. Moisture content was determined by the drying-oven method (IDF, 1982), fat by the Gerber method (IIRS, 1955), protein (%N x 6.38) by the macro-Kjeldahl methodology (IDF, 1986), salt by potentiometric titration with AgNO₃ (Fox, 1963) and calcium by atomic absorption spectroscopy (IDF, 2007). The proportion of insoluble calcium (INSOL Ca) was estimated by the cheese juice extraction method at 2 and 180 d of ripening as described by Hassan et al. (2004). The pH was measured at 2, 30, 60, 120 and 180 d of ripening on a homogenized mixture of 10 g of cheese and 10 mL of water at 20°C (Madkor et al., 1987). All analyses were performed in triplicate.

5.3.5 Microbiological analysis

Samples were prepared as described by Fenelon et al. (2000). Starter lactic acid bacteria (LAB) were enumerated on LM 17 agar (Terzaghi and Sandine, 1975) using aerobic incubation at 30°C for 3 d and non-starter lactic bacteria (NSLAB) were counted on Rogosa agar (Rogosa and Mitchell, 1951) incubated anaerobically at 30°C for 5 d. Enumeration of LAB and NSLAB were performed in duplicate at 2, 14, 30, 60, 90, 120 and 180 d of ripening.

5.3.6 Proteolysis

Proteolysis was assessed by the pH 4.6 soluble-N (pH 4.6 SN/TN) method (Kruchoo and Fox, 1982) and the level of total free amino acids (FAA) by the trinitrobenzenesulphonic acid (TNBS) method (Kruchoo et al., 1983) at 2, 60, 120 and 180 d of ripening. Urea-polyacrylamide gel electrophoresis (urea-PAGE) was performed directly on cheese samples as described by Andrews (1983) to monitor the breakdown of α_s - and β - casein (CN) during ripening. Gels were stained with Coomassie blue G250 as described by Blakesley and Boezi (1977). Densitometric analysis of scanned gels was performed with an image processing software (ImageJ 1.48v, National Institutes of Health, Bethesda, MD, USA). All treatments were analysed in triplicate.

5.3.7 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 14, 30, 60, 120 and 180 d of ripening. Cheese samples were cut into cylinders (20 mm diameter, 20mm height) and stored overnight at 8°C. Cheese cylinders were compressed to 75% of strain in two consecutive cycles at a rate of $1 \text{ mm} \cdot \text{s}^{-1}$. Hardness, springiness and cohesiveness were estimated as previously described by Bourne (1978). Five cheese cylinders were analysed per treatment.

5.3.8 Meltability and release of free oil

Melting properties of cheeses during ripening was performed by the Schreiber meltability test as described by Altan et al. (2005), by heating cheese discs at 232°C for 5 min. Meltability was calculated as the percentage of increase in diameter of unmelted samples. The release of free oil of experimental cheeses at 60°C during ripening was determined by the modified Gerber method described by Kindstedt and Fox (1991). Results were expressed as the amount of free oil, as a percentage of the total cheese fat, released at 60°C. Analyses were performed in triplicate at 7, 30, 90 and 180 d of ripening.

5.3.9 Dynamic small amplitude oscillatory rheology

The rheological properties of cheese samples were determined using a controlled stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) at 180 d of ripening. Serrated parallel plate geometry was used and cheese discs (40 mm diameter, 2 mm height) were placed on the bottom plate at an initial temperature of 20°C. A normal force of ~1.8 N was initially applied to the cheese disc. Liquid paraffin was used to cover the exposed layers of the sample to prevent loss of moisture. When the normal force decreased to ~0.7 N, sample was heated to 80°C at a heating rate of 2°C/min. Analyses were performed using a total strain of 1% and a frequency of 1 Hz, which were found to be within the linear viscoelastic region (this was confirmed by performing strain sweep and frequency sweep tests). Storage modulus (G'), loss

modulus (G'') and loss tangent (LT) were measured during heating. The maximum LT ($LT_{\max}$), which is an indicator of melting, was also recorded. Each treatment was analysed in triplicate.

5.3.10 Colour analysis

Colour of experimental cheeses was performed with a Konika-Minolta colorimeter CR-400 (Konika-Minolta Optics Inc., Osaka, Japan) at 2, 14, 30, 60, 120 and 180 d of ripening. The instrument was set on the CIELAB system based on illuminant D65 and a visual angle of 2°. Five random measurements were performed directly on a fresh cut of cheese block at 20°C.

5.3.11 Experimental design and statistical analysis

Five treatments (fat content and pectin type: FF, RF, RA, RH and RL cheeses) were manufactured in three independent trials, based on a 5 x 3 randomized block design. Analysis of variance (ANOVA) was performed on cheese composition, INSOL Ca, colour and rheological properties at a significance level of $P < 0.05$. A split-plot design (Montgomery, 2013) was used to evaluate the effects of treatment, ripening time and their interactions on pH, microbiological analysis, proteolysis, texture, melting and free oil release. The ANOVA for the split-plot design was carried out using a general linear model (GLM) procedure. If significant differences were found ($P < 0.05$), the

treatments means were analyzed by the Tukey multiple comparison test. All analyses were performed using Minitab 16® (Minitab Inc., State College, PA, USA).

5.4. Results

5.4.1 Rennet coagulation properties

The rheological properties of rennet-induced gels are shown in Table 5.1. The addition of pectins had an effect on the gelation time (GT) and the stiffness (G') of rennet curds. Cheesemilks containing pectin had a reduction of ~4 min in GT compared to controls ($P < 0.05$). During the first 15 min of renneting, cheesemilks containing pectin exhibited similar increases in G' as the time increased. Beyond this time, RA increased at a slower rate than other milks (data not shown). At 45 min, the highest G' values were found for RH and RL, followed by FF and RF and finally on RA milk ($P < 0.05$). Even though RA gel exhibited a lower LT at 45 min when compared to other treatments ($P < 0.05$), differences were lower than 0.003 units.

Table 5.1 Rheological properties of rennet-induced gels made from full-fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH) and low-methoxy pectin (RL) cheesemilks.

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
Gelation time (min)	15.03 ^a	14.99 ^a	10.90 ^b	10.86 ^b	11.30 ^b	0.527
Storage modulus (G') at 45 min (Pa)	32.87 ^b	34.17 ^b	26.88 ^c	52.06 ^a	51.70 ^a	2.790
Loss tangent (LT) at 45 min	0.244 ^a	0.246 ^a	0.238 ^b	0.244 ^a	0.242 ^a	0.001

Gelation time corresponds to the point when gels had a storage modulus (G') of ≥ 1 .

G' and LT were measured for 45 min after rennet addition.

Data are means of three replicate trials.

Means within the same row not sharing a common superscript differ ($P < 0.05$).

5.4.2 Cheese composition, levels of insoluble calcium and pH

The composition and percentage of INSOL Ca of cheeses are shown in Table 5.2. As expected, a significant decrease in fat content was found between full-fat and reduced-fat cheeses ($P < 0.05$). Levels of salt of experimental cheeses ranged around 1.95- 2.15%, however a higher content of salt in the moisture phase of cheese (S/M) was found only FF ($P < 0.05$) and no differences were found between reduced-fat cheeses. The addition of pectin led to an increase in the moisture content of RA, when compared to RF. No changes ($P > 0.05$) in the total Ca content was found between treatments when results were expressed per 100 g of protein. Similar levels of INSOL Ca were observed at 2 d of ripening for all treatments, which was around 60-70% ($P > 0.05$). At 180 d, the INSOL Ca significantly decreased for all reduced-fat treatments (P

< 0.05); RA and RL cheeses had a lower proportion of INSOL Ca when compared to RF ($P < 0.05$) and RH exhibited similar levels of INSOL Ca to RF, RA and RL cheeses.

Table 5.2 Composition (14 d) and proportion of insoluble Ca (2 and 180 d) of full fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH) and low-methoxy pectin (RL) Cheddar cheeses.

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
Moisture (%)	38.95 ^c	44.47 ^b	49.11 ^a	45.86 ^b	46.25 ^{ab}	0.924
Fat (%)	31.33 ^a	18.78 ^b	15.00 ^c	17.66 ^b	17.77 ^b	1.550
Protein (%)	23.52 ^b	29.40 ^a	29.08 ^a	28.28 ^a	29.13 ^a	0.640
Salt (%)	2.12 ^a	2.03 ^{bc}	2.14 ^a	1.96 ^c	2.09 ^{ab}	0.034
MNFS (%)	56.72 ^a	54.76 ^a	57.78 ^a	56.47 ^a	55.50 ^a	0.415
FDM (%)	51.32 ^a	33.81 ^b	29.48 ^c	33.72 ^{bc}	32.04 ^{bc}	2.110
S/M (%)	5.46 ^a	4.56 ^b	4.37 ^b	4.36 ^b	4.43 ^b	0.132
a_w	0.964 ^b	0.968 ^a	0.969 ^a	0.970 ^a	0.969 ^a	0.0006
Total Calcium (mg 100g ⁻¹ protein)	2820 ^a	2869 ^a	2731 ^a	2832 ^a	2853 ^a	34.8
% INSOL Ca/total Ca						
2 d	69.89 ^a	65.85 ^a	61.83 ^a	60.82 ^a	58.65 ^a	1.38
180 d	57.33 ^a	55.89 ^{ab}	39.51 ^c	44.03 ^{bc}	42.35 ^c	2.30

Abbreviations are: MNFS, moisture in the non-fat substance; FDM, fat content on a dry weight basis; S/M, salt in the moisture phase of the cheese; a_w , water activity.

Data are means of three replicate trials.

Means within the same row not sharing a common lowercase superscript differ ($P < 0.05$).

The pH values of experimental cheeses during ripening are shown in Fig 6.1. No significant differences ($P > 0.05$) were observed in the pH values of experimental treatments at 2 d of ripening. The pH of FF cheese showed no variations during 180 d of ripening ($P > 0.05$). An increase in the pH values was observed for other treatments after 60 d of ripening ($P < 0.05$). At 180 d, similar pH values were similar between RF, RH and RL cheeses and they were higher than those found in FF and RA ($P < 0.05$).

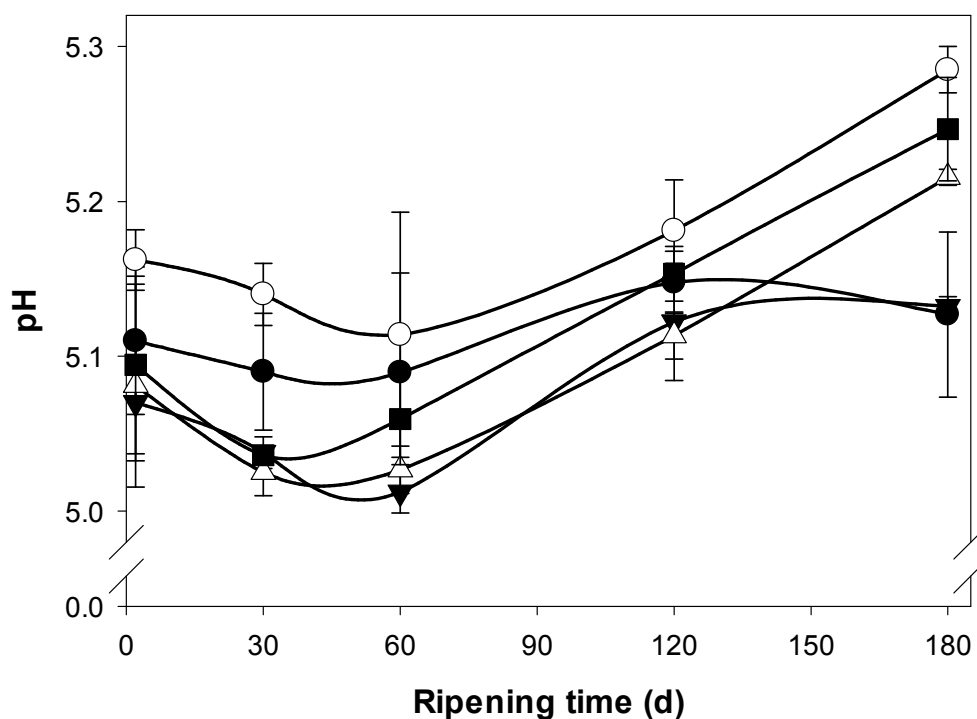


Fig. 5.1 Changes in pH during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.

Table 5.3 Mean squares, probabilities (in parenthesis) and R^2 values for pH, microbiological analysis and proteolysis for experimental cheeses during 180 d of ripening.

Factor	df	pH	df	LAB	NSLAB	df	pH 4.6 SN/TN	FAA	β -CN (f1- 189/192)	α_{s1} -CN
Whole Plot										
Trial (T)	2	0.0039 (0.284)	2	0.6884 (0.161)	7.186* (0.048)	2	0.938 (0.690)	48346 (0.078)	179.78** (<0.001)	273.50** (<0.001)
Treatment (t)	4	0.0222** (0.006)	4	0.0699 (0.911)	7.631* (0.028)	4	10.321* (0.039)	73015* (0.021)	120.93** (<0.001)	218.40** (0.004)
Error (T x t)	8	0.0026	8	0.2972	1.575	8	2.418	13549	6.32	23.60
Subplot										
Age (A)	4	0.0518** (<0.001)	6	25.4905** (<0.001)	135.257** (<0.001)	3	418.05** (<0.001)	1935034** (<0.001)	2061.11** (<0.001)	17168.3** (<0.001)
A x t	1	0.0028** (0.003)	24	0.1993 (0.489)	2.431 (0.078)	12	0.959 (0.441)	5850 (0.122)	17.15* (0.042)	33.70** (0.007)
Error	4	0.0009	60	0.2008	1.537	30	0.922	3482	7.89	11.30
	0									
R^2		0.91		0.93	0.91		0.98	0.98	0.97	0.99

Split-plot design with the five treatments (FF, RF, RA, RH and RL) were analysed as a discontinuous variable and trial was blocked.

Subplot included the effect of aging of cheese (A) and the interaction age x treatment (A x t) as variables.

Degrees of freedom (df) differed for pH, microbiology and proteolysis measurements as the time points for the analyses were different.

Abbreviations are LAB, number of starter lactic acid bacteria; NSLAB, number of non-starter lactic acid bacteria; pH 4.6 SN/TN, pH 4.6 soluble N as a percentage of total N; FAA, free amino acids expressed as mg of L-leucine per 100 g of cheese; β -CN (f1-189/192), accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 2 d; α_{s1} -CN, level of intact α_{s1} -CN as a percentage of the level at 2 d.

* $0.01 < P \leq 0.05$; ** $P \leq 0.01$.

5.4.3 Starter and non-starter lactic acid bacteria

The number of starter LAB exhibited a significant decrease of 2 – 4 log units for all cheeses over the ripening period of 180 d (Fig. 5.2a; Table 5.3). However, no significant differences were found between treatments (Table 5.3). The counts of NSLAB increased for all cheeses during ripening (Fig. 5.2b; Table 5.3). NSLAB in FF cheese increased and reached a maximum at 14 d ($P < 0.05$; Table 5.3), remaining constant beyond, whereas the number of NSLAB in RF and cheeses containing pectin also exhibited an increased during ripening, but at a slower rate of growth, where RF, RA and RL reached a maximum at 60 d and RH at 30 d ($P < 0.05$), remaining constant thereafter. The number of NSLAB were similar ($P > 0.05$) between treatments at 180 d of ripening.

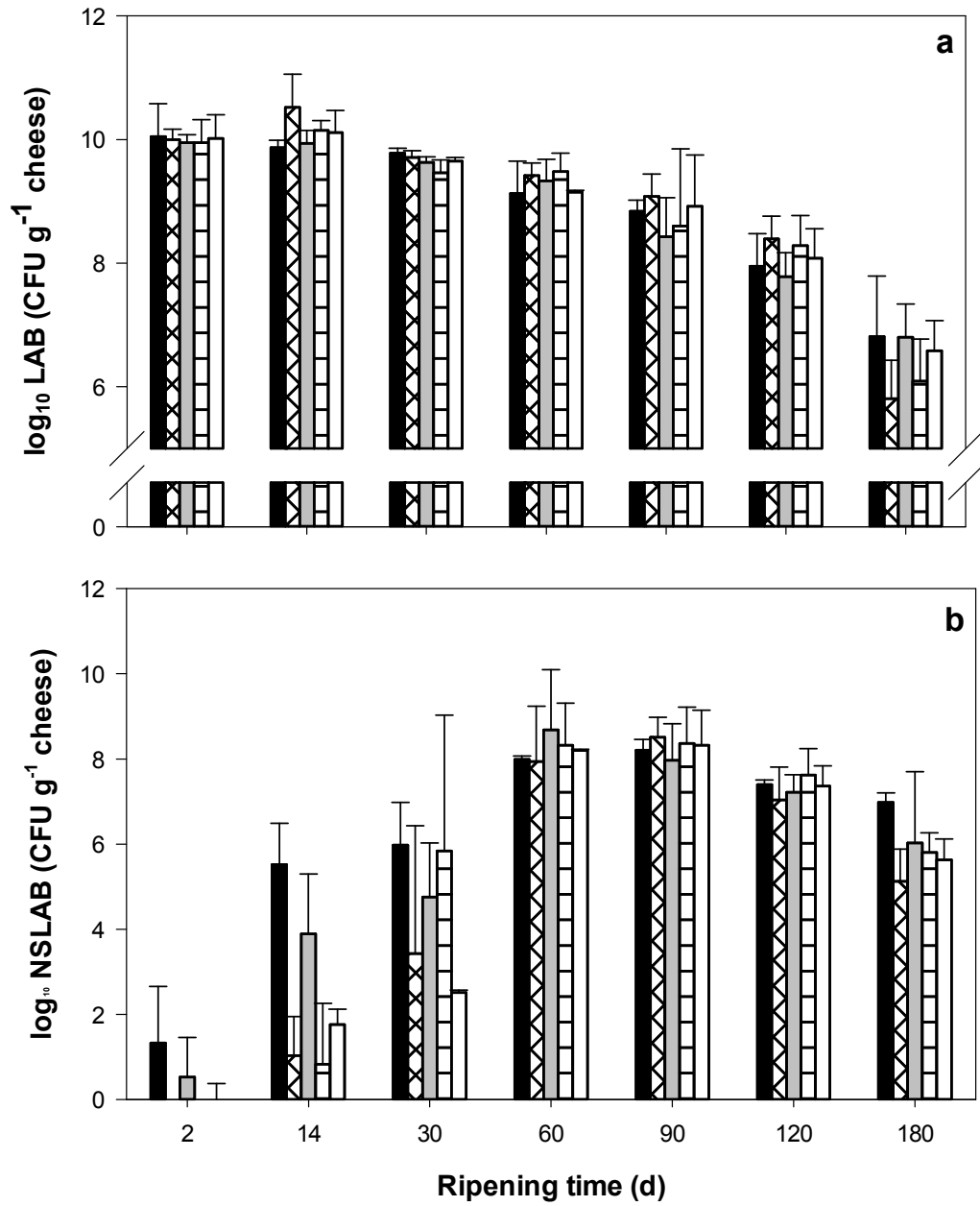


Fig. 5.2 Numbers of (a) starter (LAB) and (b) non-starter lactic acid bacteria (NSLAB) during ripening of full fat control (■), reduced-fat control (//), reduced-fat with amidated pectin (■), high-methoxy pectin (=) and low-methoxy pectin (□) Cheddar cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.

5.4.4 Proteolysis

The primary proteolysis expressed as the level of pH 4.6-soluble N of cheeses during ripening is shown in Fig. 5.3a, whereas the secondary proteolysis expressed as the concentration of total FAA is shown in Fig. 5.3b. A significant effect on treatment and cheese age was observed for both parameters (Table 5.3). A lower level of pH 4.6 SN/TN was observed for FF cheese at 2 d of ripening. Proteolysis significantly increased during ripening for all cheeses ($P < 0.05$). RA exhibited an increase of pH 4.6 SN/TN at a higher rate than other treatments ($P < 0.05$). However, no differences between treatments were observed at 180 d. A significant increase in levels of FAA was observed during ripening for all the treatments ($P < 0.05$). In addition, FF cheese contained a lower amount of FAA than reduced-fat cheeses ($P < 0.05$).

Urea-PAGE electrophoretograms of cheese samples during ripening are shown in Fig. 5.4. Treatments and cheese age had an effect on the breakdown of β - and α_{s1} -CN. We observed a significant increase in levels of β -CN (f1-189/192) during ripening for all treatments (Fig. 5.5a; Table 5.3). FF cheese had significantly lower accumulation of β -CN (f1-189/192) than RF and all cheeses containing pectin ($P < 0.05$). RA exhibited higher amounts of β -CN (f1-189/192) than other treatments at 60d ($P < 0.05$) and were similar to RF cheeses after 120 d ($P > 0.05$). Degradation of α_{s1} -CN significantly ($P < 0.05$) increased during ripening for all treatments (Fig. 5.5b; Table 5.3). RA and RL cheeses showed a higher proportion of intact α_{s1} -CN after 120 d ($P < 0.05$), when compared to FF and RF, whereas there were no differences ($P > 0.05$) between treatments after 180 d of ripening.

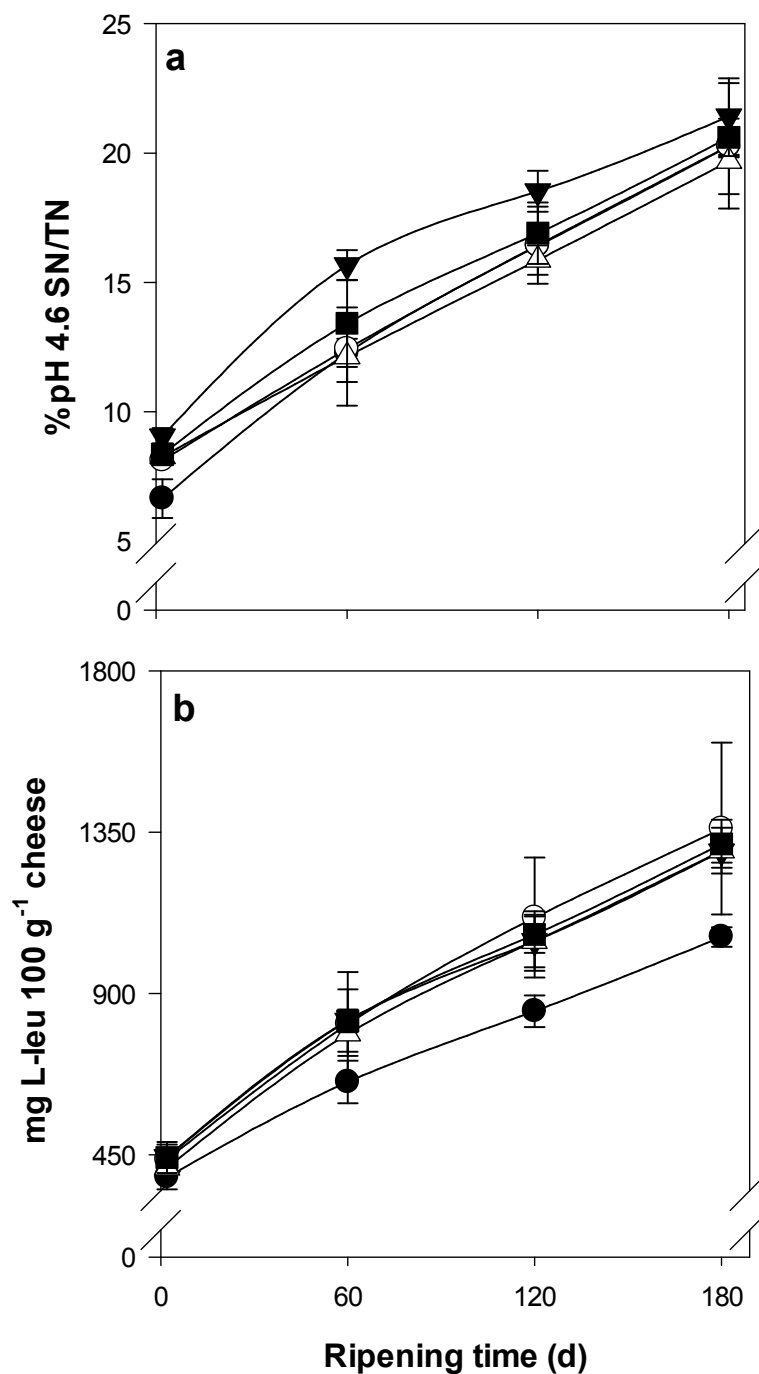


Fig. 5.3 Changes in (a) pH 4.6 soluble N as a percentage of total N, and (b) free amino acids expressed as g of L-leucine per 100 g of cheese during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.

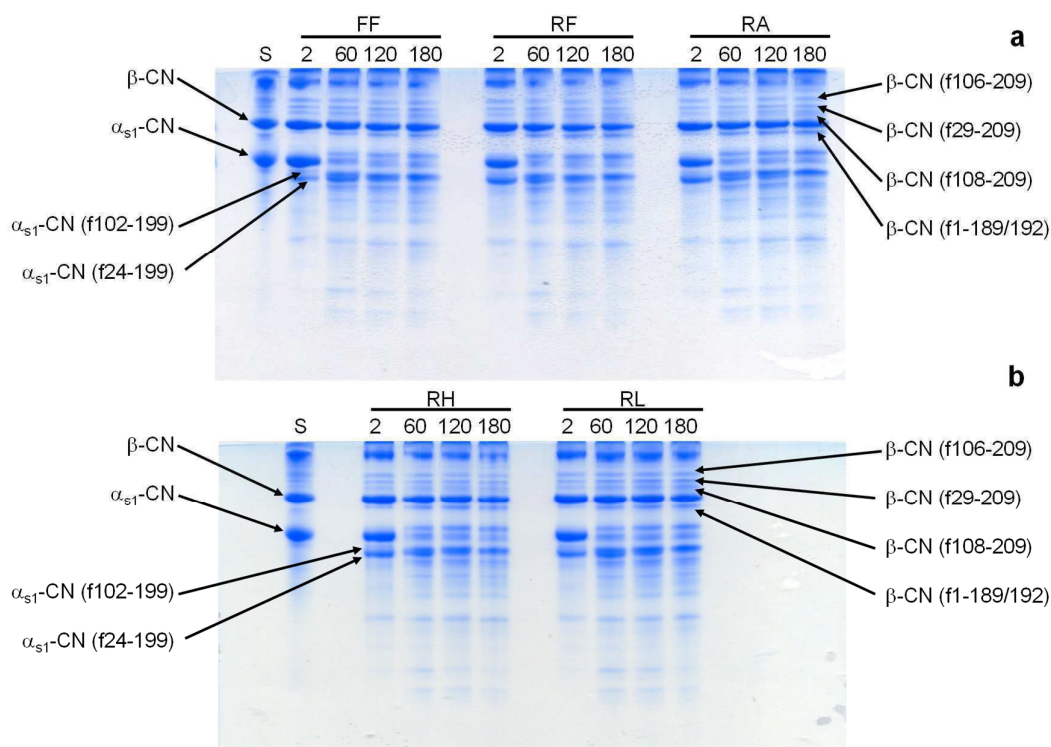


Fig. 5.4 Urea-Polyacrilamide gel electrophoresis of experimental Cheddar cheeses at 2, 60, 120 and 180 days of ripening of (a) full-fat (FF), reduced-fat (RF) and reduced-fat with amidated pectin (RA) and (b) reduced-fat with high methoxy (RH) and low methoxy pectin (RL) Cheddar cheeses. Sodium caseinate (S) was used as standard for each gel.

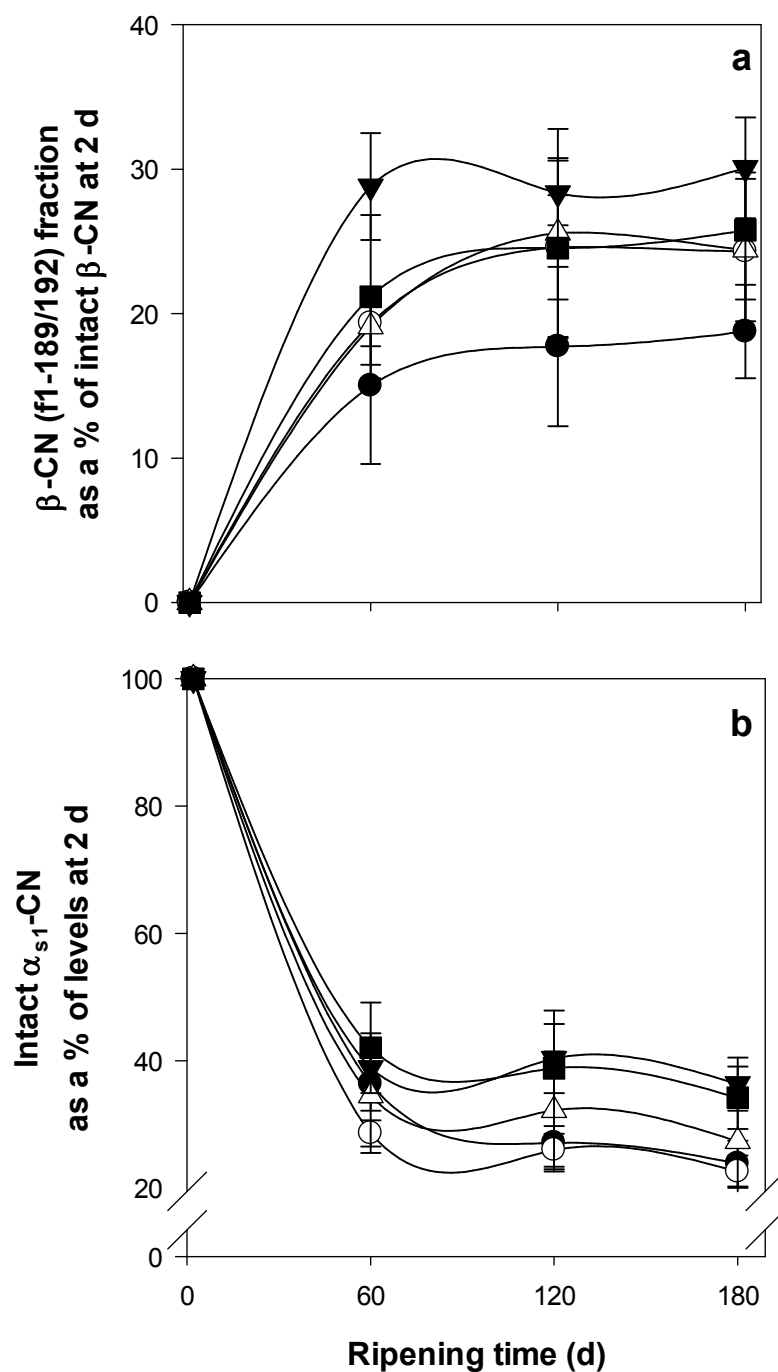


Fig. 5.5 Changes in (a) accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 2 d and (b) level of intact α_{s1} -CN as a percentage of the level at 2 d for full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses during ripening. Values are means of 3 replicates. Error bars indicate standard deviation.

5.4.5 Texture profile analysis

The TPA properties of experimental cheeses are shown in Fig. 5.6. A significant decrease in hardness values was observed during ripening for all cheeses (Fig. 5.6a; Table 5.4). A reduction in the fat content of cheese led to an increase in hardness of RF, when compared to FF ($P < 0.05$). The hardness of cheeses containing pectin was lower than that of the RF cheese ($P < 0.05$).

The TPA springiness of experimental cheeses decreased during ripening for all treatments (Fig. 5.6b; Table 5.4). No differences between treatments were observed at 14 d of ripening ($P > 0.05$). RF exhibited a higher springiness than FF after 30 d ($P < 0.05$). Cheeses containing pectin had similar values than FF until 60 d ($P > 0.05$) and then became higher beyond ($P < 0.05$). However at 180 d, cheeses containing pectin exhibited similar values than RF ($P > 0.05$).

No differences were observed between treatments in TPA cohesiveness ($P > 0.05$). Cheese cohesiveness showed a decrease during ripening (Fig. 5.6c; Table 5.4).

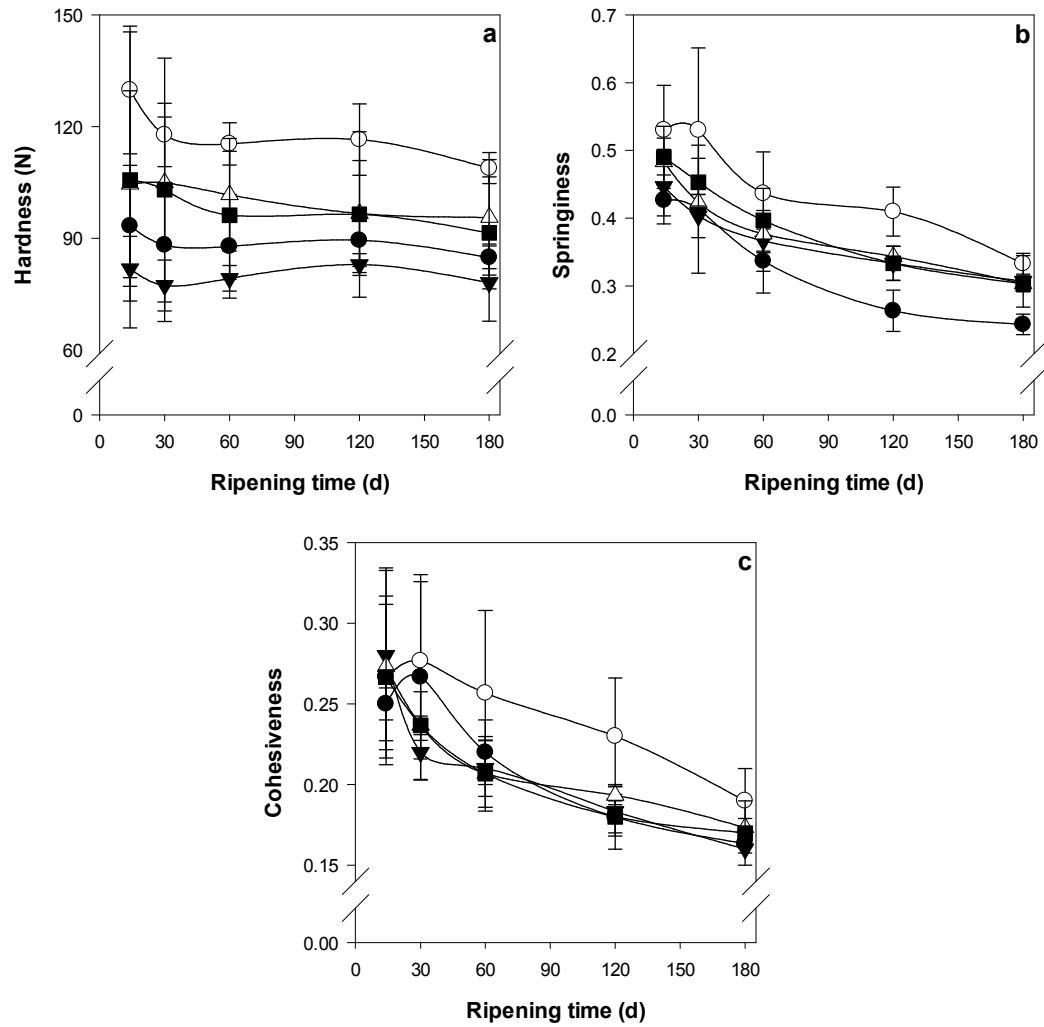


Fig. 5.6 Changes in TPA (a) hardness, (b) springiness and (c) cohesiveness values during ripening full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.

Table 5.4 Mean squares, probabilities (in parenthesis) and R² values for textural analysis, melting properties and release of free oil for experimental cheeses during 180 d of ripening.

Factor	df	Hardness	Springiness	Cohesiveness	df	Melting	Free oil
Whole Plot							
Trial (T)	2	4993.6*	0.007109	0.0042893	2	44.27	7.44
		(0.024)	(0.319)	(0.223)		(0.725)	(0.448)
Treatment (t)	4	3949.4*	0.024635*	0.0028280	4	548.21*	1859.30**
		(0.028)	(0.032)	(0.380)		(0.041)	(<0.001)
Error (T x t)	8	810.9	0.005378	0.0023560	8	131.97	8.37
Subplot							
Age (A)	4	654.2**	0.081001**	0.0227513**	3	4656.97**	98.30**
		(<0.001)	(<0.001)	(<0.001)		(<0.001)	(<0.001)
A x t	16	68.8	0.000905	0.0005780	12	101.61**	12.05*
		(0.778)	(0.764)	(0.508)		(0.005)	(0.017)
Error	40	94.4	0.001269	0.0005977	30	31.46	4.65
R ²		0.89	0.91	0.85		0.95	0.98

Split-plot design with the five treatments (FF, RF, RA, RH and RL) were analysed as a discontinuous variable and trial was blocked.

Subplot included the effect of aging of cheese (A) and the interaction age x treatment (A x t) as variables.

Degrees of freedom (df) differed for texture and functional properties as the time points for the analyses were different.

* $0.01 < P \leq 0.05$; ** $P \leq 0.01$.

5.4.6 Melt analysis and release of free oil

The melting determined by the Schreiber analysis of experimental cheeses is detailed in Fig. 5.7a. A significant increase in melting was observed for all treatments during cheese age ($P < 0.05$; Table 5.4). At 7 d of ripening, a higher meltability was observed in FF, when compared to reduced-fat cheeses ($P < 0.05$) and similar levels of melting were observed between RF and pectin-containing cheeses. At 90 d of ripening, RA showed similar meltability than FF. At 180 d of ripening, cheeses containing pectin exhibited a higher meltability than RF ($P < 0.05$).

The release of free oil was higher in FF than all other treatments ($P < 0.05$) and only FF and RH exhibited an increase during ripening ($P < 0.05$). There were no differences in the release of free oil between RF and cheeses containing pectin.

5.4.7 Dynamic small amplitude oscillatory rheology

The G' values of experimental cheeses showed a decrease during heating (Table 5.5). At 20°C, RF exhibited higher G' than FF and pectin-containing cheeses ($P < 0.05$). At 70°C, no significant differences were found in G' values between FF and RF. RL exhibited lower G' than RA and RH ($P < 0.05$). RF exhibited lower $LT_{\max}$ than FF. RL cheese had similar $LT_{\max}$ than FF cheese, whereas RA and RH exhibited similar $LT_{\max}$ than RF. There were no differences in the temperature of $LT_{\max}$ between FF, RF and RL; however, RA and RH exhibited $LT_{\max}$ at a lower temperature ($P < 0.05$).

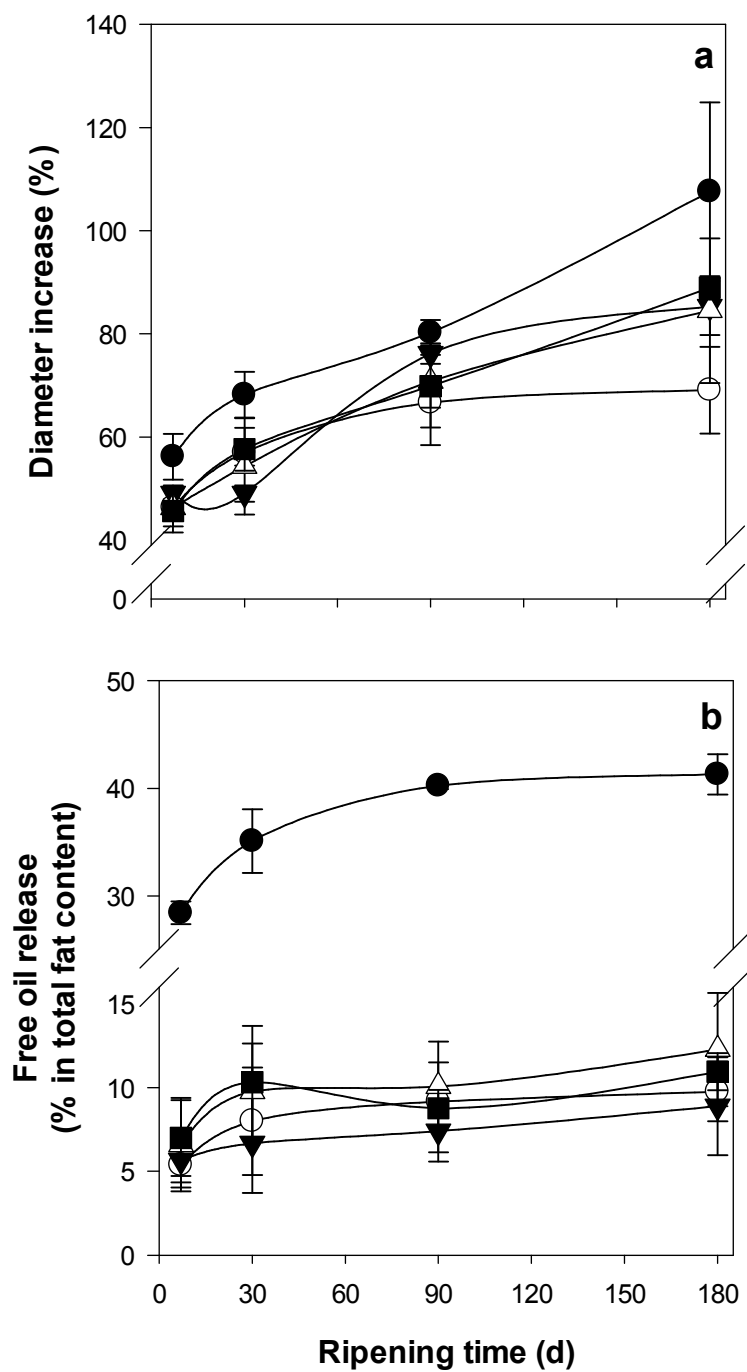


Fig. 5.7 Changes in (a) melting determined by the Schreiber test and (b) release of free oil values during ripening of full fat control (●), reduced-fat control (○), reduced-fat with amidated pectin (▼), high-methoxy pectin (Δ) and low-methoxy pectin (■) Cheddar cheeses. Values are means of 3 replicates. Error bars indicate standard deviation.

Table 5.5 Rheological properties of experimental cheeses at 180 d of ripening determined by dynamic small-amplitude oscillatory rheology.

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
G' at 20°C (Pa)	37159.1 ^{bc}	43236.0 ^a	38466.7 ^b	34079.8 ^c	39394.1 ^b	873.0
G' at 70°C (Pa)	336.1 ^{bc}	538.5 ^{abc}	652.5 ^a	591.6 ^{ab}	290.2 ^c	43.1
LT _{max}	1.10 ^a	0.82 ^b	0.77 ^{bc}	0.73 ^c	1.17 ^a	0.05
Temperature at LT _{max} (°C)	67.18 ^a	65.89 ^a	62.42 ^b	62.89 ^b	66.12 ^a	0.52

G' corresponds to storage modulus.

LT_{max} corresponds to loss tangent maximum.

Data are means of three replicate trials. Means within the same row not sharing a common superscript differ ($P < 0.05$).

5.4.8 Colour

The colour of experimental cheeses at 14 d of ripening is detailed in Table 5.6. As expected, FF exhibited higher whiteness (L^* values) than reduced-fat treatments. At 2 d of ripening (data not shown) L^* values were ~88 for FF and ~85 for reduced-fat cheeses. At 14 d, a decrease in whiteness was observed for all treatments ($P < 0.05$) and this difference remained at longer ripening times. No differences were observed in L^* values of cheeses containing pectin, when compared to RF ($P > 0.05$). The greenness of RA and RL cheese was significantly lower (higher a^* values) than RF ($P < 0.05$) and similar to FF. The addition of pectin had no effect on cheese yellowness (b^* values; $P > 0.05$).

Table 5.6 CIELAB colour of experimental cheeses after 14 d of ripening.

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
L*	83.45 ^a	78.34 ^b	78.57 ^b	79.71 ^b	78.80 ^b	0.564
a*	-3.92 ^{ab}	-4.38 ^b	-3.40 ^a	-3.92 ^{ab}	-3.38 ^a	0.111
b*	36.91 ^a	30.88 ^{ab}	27.71 ^b	27.99 ^b	29.13 ^b	1.06

L*, whiteness; a*, greenness or redness; b*, yellowness or blueness.

Data are means of three replicate trials.

Means within the same row not sharing a common superscript differ ($P < 0.05$).

5.5. Discussion

A reduction on the GT during renneting of cheesemilks supplemented with pectin is associated with an increase in the viscosity of the aqueous phase of milk (Fagan et al., 2006). The concentration of pectin has a strong influence on the microstructure and hence the rheological properties of rennet-induced gels. The results obtained from RH and RL are in agreement with those of Tan et al. (2007), who observed an increase in the stiffness of rennet gels and a reduction in syneresis as the concentration of HMP increased from 0 to 0.1% due to a more compact microstructure when observed by confocal laser microscopy. At higher concentrations of pectins (0.12 – 0.15%), a more open network of depleted caseins led to the formation of a weaker gel (Acero Lopez et al., 2009), as was observed in RA rennet gel.

A reduction in the fat content of cheese leads to a shift in the compositional balance such as in moisture and protein content (Mistry, 2001). The addition of pectin only led to an increase in the moisture content of RA cheese; however, moisture content of RL cheese is similar to both RF and RA cheeses. The capacity of AMP and LMP to form gels could increase the moisture content of experimental cheeses. However, amidation of LMP improves the gel-forming ability of AMP, leading to a reduction of syneresis (Thakur et al., 1997) which could increase the moisture content of RA cheese. Another explanation for the increase in moisture content could be associated with the amount of pectin added to cheesemilks, as HMP and LMP were added at $\leq 0.1\%$ (w/w), comparing with AMP, which was added at 0.175% (w/w). Lobato-Calleros et al. (2001) attributed an increase in the moisture content of low-fat Manchego cheese made from cheesemilks supplemented with LMP at levels of 0.2% (w/v) to the water binding capacity of pectin due to the formation of gels in the presence of calcium. Similar levels of total Ca probably resulted from the pH values at critical points during cheese manufacture being similar between treatments.

The proportion of INSOL Ca in Cheddar cheese is reduced during the first 30 d of ripening and remains constant beyond (Hassan et al., 2004). Differences in the proportion of INSOL Ca between RF with RA and RL cheeses could be attributed to gelation mechanisms of AMP and LMP. Lobato-Calleros et al. (2001) found the presence of calcium pectate particles when they observed the microstructure of low-fat Mexican Manchego cheese supplemented with LMP. Harte et al. (2007) proposed a gelation model of LMP during the acidification of milk that could also be applied to cheese. As pH decreases during acidification, solubilization of Ca from the colloidal

calcium phosphate interacts with LMP, promoting cross linking of chains and hence the formation of a gel structure. This phenomenon probably occurred during cheese manufacture due to acidification of RA and RL cheesemilks caused by action of starter LAB.

An increase in cheese pH during ripening is attributed to the buffering capacity of cheese due to a reduction in the levels of INSOL Ca (Hassan et al. 2004; Table 5.2). In addition, pH plays an important role in the interaction between pectins and casein micelles. As previously stated, pectin remains dispersed in the serum phase of milk at neutral pH and it adsorbs onto casein micelles at pH 5.3. If the concentration of pectin is not enough to cover casein micelles, then pectin interacts with casein micelles by mean of bridging flocculation; whereas, if casein micelles are fully covered by pectins, then they are sterically stabilized. However, a further increase in the pectin concentration leads to depletion interaction (Marozienne and de Kruif 2000). In addition, below pH 5.0 pectin and casein micelles are associated by mean of electrosorption (Tuinier et al., 2002). These authors also indicated that renneting had no influence on the interaction of pectins and κ -casein depleted micelles. As the pH of pectin-added cheeses was close to 5.3, pectin may interact with the protein matrix by adsorption mechanisms, interrupting the compact protein structure, which will influence water retention (i.e., higher moisture content, as occurred with RA cheese) and also cheese functionality. The use of carbohydrate-based fat replacers has shown a more open structure in low-fat cheeses, which might be caused by the presence of amorphous carbohydrate particles that interrupts the compact protein matrix (McMahon et al., 1996; Lobato-Calleros et al., 2001), altering the composition, texture and functionality (Mistry, 2001).

Similarities in the counts of LAB between treatments could be explained due to similar levels of MNFS (Table 5.2). Fenelon et al. (2000) found a reduction in the counts of starter LAB in Cheddar cheeses with lower fat content, which was attributed to a decrease in the MNFS as fat content was reduced. As NSLAB likely use the milk fat globule membrane as a source of carbon for their growth (Fox et al., 1998) it would have been expected that propagation of NSLAB would be lower in reduced-fat cheeses.

Similar levels of primary proteolysis between FF and reduced-fat cheeses can be related to their similar levels of MNFS (Table 5.2). A significant increment in the levels of pH 4.6 SN/TN as the fat content of cheese was increased has been associated with a concomitant increment of MNFS that may lead to a higher retention of chymosin (Fenelon et al., 2000; Guinee et al., 2000). An increase in the levels of FAA has been previously reported for low-fat cheese (Fenelon et al., 2000), which is attributed to a concomitant increase in the protein content as the fat content is reduced (Guinee et al., 2000).

The hydrophobic peptide β -CN (f193-209) formed by cleavage at Leu₁₉₂-Tyr₁₉₃ which also forms β -CN (f1-189/192), contributes bitterness to cheese (Visser et al., 1983). The concentration of salt to moisture content (S/M) has a direct influence on the residual activity of chymosin. We observed lower levels of S/M in reduced-fat cheeses, which might increase the accumulation of β -CN (f1-189/192) due to an enhancement of the residual activity (Fenelon and Guinee, 2000). As previously stated, higher accumulation of β -CN (f1-189/192) in RA cheese can also be influenced by its lower pH. Fenelon and Guinee (2000) found a lower degradation of α_{s1} -CN as the fat content

of cheese is reduced, due to a decrease of residual activity of chymosin. In our study, similar levels of intact α_{s1} -CN between treatments could be associated with similar levels in the MNFS (Table 5.2), which influences in the residual activity of chymosin.

The softening of cheese during ripening is partly associated to the extent of proteolysis, mainly caused by the degradation of α_{s1} -casein (Fig. 5.5b; Creamer and Olson 1982). In addition, the reduction of the INSOL Ca levels during ripening (Table 5.2), which corresponds to the release of colloidal calcium phosphate (CCP) to the serum phase, has a strong influence on cheese hardness, causing a weakening of the protein matrix (Lucey et al., 2003). An increase in TPA hardness as the fat content is reduced is attributed to a concomitant increase in the protein content (Fenelon and Guinee, 2000) and also to a decrease in the interruption of the cheese matrix by fat globules, leading to a more compact structure (Johnson et al., 2009; Bryant et al., 1995). As previously stated, the addition of pectin may cause an interruption of the cheese matrix, probably due to protein-polysaccharide interactions (Marozienne and de Kruif 2000; Tuinier et al. 2002) and/or the formation of calcium pectate gels (Lobato-Calleros et al., 2001), conferring a less compact structure and hence reduce hardness. A similar effect has been reported by McMahon et al (1996), who observed the presence of amorphous carbohydrate-based fat replacers immersed in the cheese matrix. In addition, lower levels of INSOL Ca, when compared to RF, was found in RA and RL cheeses (Table 5.2), probably due to the formation of calcium-pectate gels (Lobato-Calleros et al., 2001; Harte et al., 2007), which might contribute to a softer texture. The addition of AMP was more efficient in reducing hardness, leading to similar values to FF, probably caused by higher concentrations of pectin added than in the other treatments.

An increase in springiness as the fat content of is reduced was also observed by Lobato-Calleros et al. (2001). These authors also observed a reduction in the springiness of reduced-fat Manchego cheese with LMP added when compared to control which was attributed to an interruption of the protein matrix by pectin particles. The same effect in the protein matrix might have caused a reduction of springiness in RA, RH and RL cheeses, when compared to RF.

Bryant et al. (1995) observed an increase in the cohesiveness of Cheddar cheese as the fat content was lower than 21%. On the other hand, Tunick et al. (1991) found that higher moisture content in Mozzarella cheese is associated to higher cohesiveness values. In our study, we found no differences in the cohesiveness of experimental cheeses, which could be explained by higher moisture content of reduced-fat cheeses, when compared to FF (Table 5.2) and also with the presence of fat replacers, i.e., pectin, that would reduce casein-casein interactions, leading to a loosening of the protein matrix (Lobato-Calleros et al. 2001).

An increase of melting during ripening for all cheeses is associated with the extent of proteolysis (Fig. 5.3) and changes in levels of INSOL Ca (Table 5.2; Lucey et al. 2003). A lower melting in reduced-fat cheeses is associated with a higher protein content, which leads to a more compact structure (Guinee et al., 2000). In addition, levels of free oil release might also be influenced by the melting properties (Fig. 5.7b). Rudan and Barbano (1998) observed the formation of a thick skin and/or blisters during the baking of low-fat Mozzarella cheese that prevented its softening and hence flow, due to dehydration of the surface caused by a lack of free oil release, which may act as a

hydrophobic surface coating. To avoid this problem, the authors found that covering the surface of cheese with a small amount of a hydrophobic surface coating considerably improved its melting properties. As previously stated, a more open structure due to the addition of pectin may eventually increase melting (McMahon et al., 1996). A higher meltability found in cheeses containing pectin could also be attributed to lower levels of INSOL Ca at 180 d of ripening, when compared to control (Table 5.2), due to a lower amount of CCP crosslinks, which leads to a reduction in the attractive interactions of the cheese matrix (Lucey et al., 2003).

A decrease in storage modulus (G') during heating of cheese indicates a weakening of the casein matrix structure (Lucey et al., 2003). A higher G' value in RF at 20°C is probably associated to higher protein content. Lower G' values found in cheeses containing pectin could be associated with a more open structure (McMahon et al., 1996; Lobato-Calleros et al., 2001) and lower levels of INSOL Ca (Table 5.2). During the heating of cheese at high temperatures ($>$ temperature at LT_{max}), Udayarajan et al. (2005) observed an increase in G' , probably caused by a heat-induced formation of INSOL Ca that may interact with caseins to form new structures. This same phenomenon might have caused increases in G' values in RA and RH cheeses at 70°C, due to a lower temperature at LT_{max} . The LT values at temperatures $<40^{\circ}\text{C}$ (results not shown) were constant for all treatments (0.3-0.4), indicating the presence of a solid-like matrix. At higher temperatures, the LT values increased to a maximum (LT_{max}) reached at 60-70°C (Table 5.5) and then decreased at higher temperatures (results not shown). Higher LT values relates to a higher melting (Lucey et al., 2003). As expected, RF had a lower LT_{max} than FF. Only RL exhibited a similar LT_{max} than FF cheese, whereas RA

and RH had similar values to RF. A lower temperature at LT_{max} found in RA and RH cheeses could be associated with a combined effect in the levels of INSOL Ca and the extent of proteolysis. However, Udayarajan et al. (2005) found that LT_{max} is highly frequency dependent and hence these authors suggest caution in using this parameter as melting index.

We observed differences in the results obtained between the Schreiber melting test and dynamic small amplitude rheology. Cooke et al. (2013) studied the melting properties of full-fat and reduced-fat Cheddar cheese made from milk supplemented with gum tragacanth and attributed these differences to a higher extent of fat liquefaction in the Schreiber test due to the high temperatures of exposure of cheese samples. In addition, these authors suggested the presence of interactions between casein and gum tragacanth that inhibited cheese melting at the temperatures of dynamic small amplitude rheology. This type of interactions may also inhibit cheese melting in pectin-containing cheeses.

In unmelted cheese, fat acts to scatter light and a reduction in fat content leads to a translucent appearance, which is reflected by a reduction in whiteness (Johnson et al., 2009). A decrease of cheese whiteness during ripening could be attributed to an increase in proteolysis (Fig. 5.3) and also a decrease in INSOL Ca levels (Table 5.2). The addition of pectin had no effect on cheese whiteness; however greenness of RA and RL cheeses was reduced (Table 5.6). This finding was not expected as we assumed that pectin would act as a light scatter center and hence increase whiteness.

5.6. Conclusions

Depending the type of pectin, its addition to milks for the manufacture of reduced-fat Cheddar cheese had to be below certain concentration in order to prevent phase separation due to depletion flocculation. Amidated Pectin had an impact on pH and composition of experimental cheeses, by increasing the moisture content due to its water holding capacity. The levels of INSOL Ca were modified by the use of AMP and LMP, probably due to the formation of calcium-pectate structures that influenced on texture and meltability. The addition of pectin had no effect on the number of LAB and NSLAB during ripening, when compared to reduced-fat control. The TPA hardness was significantly reduced in cheeses supplemented with pectins, especially RA. Melting properties of reduced-fat cheeses were modified when analyzed by Schreiber melting test and dynamic small amplitude rheology. Pectin had no effect in the whiteness of cheeses, when compared to RF. These results suggest that the use of pectin might be a useful strategy to modify the composition, texture and functionality of reduced-fat Cheddar cheeses.

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CHAPTER 6

LOW AND REDUCED FAT GOUDA-STYLE CHEESE: COMPARISON OF LACTOSE STANDARDIZATION OF CHEESE MILK AND WHEY DILUTION TECHNIQUES

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6.1 Abstract

Control of acidity is critical in Gouda cheese, especially in reduced fat versions as high acidity is associated with poor flavour and textural attributes. We investigated an alternative method to control acidity in Gouda-style cheese, which involved standardization of the lactose content of cheese milk. In traditional Gouda cheese manufacture, a key technique to control acidity is whey dilution (WD), i.e. partial removal of whey and its replacement with water. Standardization of the lactose content of cheesemilk could be a simpler and more efficient technique to control cheese acidity. This study aimed to compare the effect of WD during cheese manufacture, with the alternative approach of adjustment of the lactose content of milk using low-concentration-factor ultrafiltration (LCF-UF) on the composition, texture, functionality and sensory properties of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses during 180 d of ripening. A milled curd direct-salted cheese manufacture was used, and WD levels were varied from 0 to 30%. The RF and LF milks used for WD at levels of 30 and 15% (WD30 and WD15, respectively) had a lactose-to-casein (L:CN) ratio of ~1.8, which is the typical ratio found in milk, whereas treatments with no WD (lactose standardized, LS) were made from LCF-UF standardized milks and had a L:CN ratio of ~1.1. Similar trends between treatments were observed in both RF and LF cheeses. Cheeses made with LS exhibited lower residual lactose and lactic acid contents than WD30 and WD15, leading to higher pH values ($P < 0.05$). LS cheeses were softer when evaluated by texture profile analysis. Dynamic small amplitude oscillatory rheology indicated that the use of LS led to cheeses with a lower melting point than the WD treatments ($P < 0.05$). LS cheeses also had a lower proportion of insoluble Ca caused by

the addition of water required to achieve the lower L:CN ratio in milks. Sensory analysis also indicated that LS cheeses had lower acidity and softer texture. These results suggest that standardization of the L:CN ratio of cheesemilk could be a useful alternative to WD in Gouda-Style cheeses in order to reduce acidity, improve texture and functional properties.

Keywords Low-concentration-factor ultrafiltration; lactose-to casein ratio; cheese acidity; lactic acid; cheese texture; cheese rheology; low-fat cheese.

6.2 Introduction

Reduced fat cheeses can be challenging to manufacture, since consumers expect the same properties as full-fat counterparts (Childs and Drake, 2009). A reduction in the fat content of cheese can alter starter activity, flavour development, texture, melting and appearance; unless corrective action is taken (Bryant et al., 1995; Fenelon and Guinee, 2000; Johnson et al., 2009). One of the most common defects of reduced-fat cheeses is the development of excessive acidity due to lower levels of salt in the moisture phase of cheese (S/M; Johnson et al., 2009).

Whey dilution (WD) is a common step traditionally used during the manufacture of Dutch style cheeses, like Gouda or Edam (Van den Berg et al., 2004), although it has also been used in other cheese varieties, such as Colby (Lee et al., 2011) and sometimes Cheddar-types (Shakeel-Ur-Rehman et al., 2004; Upreti and Metzger, 2006; Hou et al., 2014). During cheesemaking, some of the whey is drained and replaced with warm water, improving syneresis and reducing some of the lactose, which helps to control the production of lactic acid and hence the final cheese pH (Van den Berg et al., 2004). A combination of the amount of water used, the temperature of water and the holding time of curd immersed in diluted whey are critical factors that determine the final properties of cheese made with WD (Lolkema, 1991; Van den Berg et al., 2004; Lee et al., 2011; Hou et al., 2014). Lolkema (1991) observed that increasing the level of WD in the manufacture of Gouda cheese, led to a curd with lower levels of lactose and cheeses with higher moisture and pH values. Therefore, this author

suggested that the percentage of WD used during cheesemaking has to be based on the composition of milk, as the lactose content changes during lactation and seasonality.

Several studies have investigated the effect of WD on cheese properties. Shakeel-Ur-Rehman et al. (2004) used 25% of WD (as a percentage of the total volume of curd and whey) to reduce levels of lactose to compare its effect with Cheddar-style cheeses made from milks containing regular (4.2%) and high (8.4%) lactose content. The authors found that modifying the lactose content of cheese had little or no effect on the gross and chemical composition, primary proteolysis or the numbers of starter and non-starter lactic acid bacteria (LAB). Cheese made from high lactose milk exhibited lower pH values and sensory analysis indicated that it was more acidic. In contrast, cheese made with WD (low lactose) had lower acidity and was less firm. In a recent study, Hou et al. (2014) increased levels of WD from 0 to 33% (as a percentage of volume of cheesemilk) with 38°C water in the manufacture of Cheddar cheese to reduce the levels of lactose and lactic acid. An increase in the levels of WD led to cheeses with similar composition and primary proteolysis, but higher pH values and a firmer texture. Sensory analysis also showed that increasing levels of WD led to cheeses with lower acidity and the development of different flavour profiles (Hou et al., 2014). Lee et al. (2010, 2011) evaluated the effect of different approaches of curd washing (i.e., addition of cold water into curds after total drain of the whey; CW) in the manufacture of Colby cheese and found that, as same as found in WD, the use of CW led to decreased amounts of residual lactose and lactic acid in cheese. In addition, the authors found that the use of CW led to solubilization of insoluble colloidal calcium phosphate (CCP). CW has also been used in the manufacture of reduced-fat Cheddar cheese and leads to

increased moisture content, reduced amount of residual lactose and production of lactic acid and solubilization of CCP (Chen and Johnson, 1996). The textural and rheological properties of cheese are greatly influenced by the proportion of calcium bound to casein, which also impacts the buffering capacity (Lucey et al., 2003; Hassan et al., 2004).

Ultrafiltration (UF) has gained popularity in the cheese industry. With this technique, milk is separated into two different fractions: retentate, which is an enriched fraction of concentrated protein and fat; and permeate, which corresponds to a serum fraction containing water, lactose and minerals (Johnson and Lucey, 2006). The use of low-concentration-factor UF (LCF-UF) has been successfully used for milk protein standardization in the manufacture of Parmesan (Govindasamy-Lucey et al., 2004), pizza-style (Govindasamy-Lucey et al., 2005) and Swiss (Govindasamy-Lucey et al., 2011) cheeses. These studies have shown that cheeses of similar quality and composition can be obtained if certain process variables (e.g., coagulation conditions, amount of starters, rennet and salt) were modified to account of the increased protein content of the cheesemilk. However, when increasing the protein level with LCF-UF, the lactose content remains constant, which alters the lactose to casein ratio (L:CN) and thereby influence on the texture and acidity of cheese. Johnson and Lucey (2006) pointed out that the use of diafiltration (i.e., the addition of water to the retentate followed again by ultrafiltration) could be an useful tool to control the lactose content of UF retentates. Therefore, we hypothesized that we could replace the WD step in Gouda-type cheeses with LCF-UF by reducing lactose levels with water addition, if needed. This study compared the use of two levels of WD (30 and 15%) during cheesemaking

with the standardization of the L:CN of cheese milk by LCF-UF on the composition, texture, rheology and sensory properties of low-fat (LF) and reduced-fat (RF) Gouda cheeses during 180 d of ripening.

6.3 Materials and methods

6.3.1 Ultrafiltration of milk

Raw skim milk was obtained from the University of Wisconsin-Madison dairy plant. Two days before cheese manufacture, skim milk was subjected to LCF-UF similar to described by Govindasamy-Lucey et al. (2005), until levels of total solids (TS) were approximately 13.5% (w/w). Processing was performed at $\leq 7^{\circ}\text{C}$ and milk was recirculated through a UF unit (modified APV North America Inc., Tonawanda, NY) fitted with 4 spiral-wound polyethersulphone membranes (model ST3B4338, Synder Filtration, Vacaville, CA) with a molecular cut-off 10 kDa and a total area of 32.8 m². The final retentate and permeate were stored overnight at 4°C and analyzed for composition.

6.3.2 Standardization of cheesemilks and cheese manufacture

Four independent trials of milled-curd, direct-salted (not brined) Gouda-style cheeses were made at the University of Wisconsin-Madison dairy processing plant.

Each cheesemaking trial used six 272-kg vats of cheesemilk to manufacture cheeses at two fat levels: LF and RF. For each fat level, two cheesemilks were standardized using raw skim milk (9.4 ± 0.2 TS; $2.5 \pm 0.1\%$ CN; $0.4 \pm 0.1\%$ fat; $4.3 \pm 0.1\%$ lactose) and sweet cream ($37.7\% \pm 6.2\%$ TS; $1.6 \pm 0.2\%$ CN; $31.4 \pm 6.7\%$ fat; $3.00 \pm 0.3\%$ lactose) to obtain cheesemilks at a lactose-to-casein ratio (L:CN) of ~ 1.8 (normal lactose); whereas the third vat was standardized by blending UF retentate ($13.5 \pm 0.1\%$ TS; $5.6 \pm 0.2\%$ CN; $0.2 \pm 0.02\%$ fat; $4.3 \pm 0.2\%$ lactose), permeate ($5.4 \pm 0.1\%$ TS; 4.1 ± 0.1 lactose), sweet cream and high quality potable water to adjust the L:CN ratio to ~ 1.2 (reduced lactose). All milks had the same CN:fat ratio. The mean composition of cheesemilks from each vat is shown in Table 6.1. Before manufacture, cheesemilks were pasteurized at 73°C for 19 s and cooled to 31°C .

Table 6.1 Composition of standardized milks used in the manufacture of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses.

	Low-fat (LF)			Reduced-fat (RF)			SEM
	WD30 ¹	WD15 ²	LS ³	WD30	WD15	LS	
Total solids (%)	9.12 ^d	9.36 ^c	7.15 ^f	9.80 ^b	10.06 ^a	8.11 ^e	0.211
CN (%)	2.42 ^a	2.44 ^a	2.43 ^a	2.35 ^a	2.43 ^a	2.42 ^a	0.011
Lactose (%)	4.08 ^b	4.20 ^{ab}	2.66 ^d	4.18 ^{ab}	4.25 ^a	2.93 ^c	0.138
L:CN ratio	1.69 ^a	1.72 ^a	1.10 ^c	1.78 ^a	1.75 ^a	1.21 ^b	0.059
CN:fat ratio	5.34 ^a	5.35 ^a	5.34 ^a	1.96 ^b	1.94 ^b	2.02 ^b	0.357
Total Ca (mg/100 g CN)	4556 ^a	4478 ^{ab}	4003 ^{cd}	4351 ^{abc}	4185 ^{bcd}	3973 ^d	62.5
INSOL Ca (mg/100 g CN) ⁴	3219 ^a	3146 ^a	2997 ^a	3017 ^a	2932 ^a	3004 ^a	48.1

^{a,b,c} Means within the same row for each fat level not sharing a common superscript differ ($P < 0.05$), as compared by Tukey multiple comparison test.

¹ Whey dilution at level of 30%.

² Whey dilution at level of 15%.

³ Lactose standardization by LCF-UF.

⁴ Proportion of insoluble colloidal calcium phosphate.

The cheesemaking protocol for the manufacture of LF and RF treatments was slightly modified (Table 6.2), in order to obtain similar firmness of rennet gels at cutting and similar moisture contents.

Table 6.2 Cheesemaking conditions used for cheesemaking protocols of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses

Item	Treatments	
	Low-Fat	Reduced-Fat
pH of cheesemilks after preacidification with 25% (wt/wt) lactic acid	6.38±0.02	6.45±0.02
Starter cultures added (g per 100 kg of cheesemilk)		
CHN-19	39.7	36.4
LN-32	3.7	3.3
Amount of 33% CaCl ₂ added (mL per 100 kg of cheesemilk)	35.7	32.7
Amount of double strength coagulant added (mL per 100 kg of cheesemilk)	8.8	8.1
pH at cutting the gel	6.23±0.03	6.26±0.04
Size of wire knives for cutting rennet gels (cm)		
Vertical	1.27	1.27
Horizontal	1.91	1.27
Amount of NaCl added during salting (g per 100 kg cheesemilk)	285	262

Cheesemilks were preacidified with 25% (w/w) lactic acid to reduce pH (Table 6.2) and inoculated with direct-vat-set mesophillic cultures comprising *Lactococcus lactis* ssp. *cremoris*, *Leuconostoc* sp., *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* blend (CHN-19) along with *Lactobacillus helveticus* (LN-32; Chr. Hansen, Milwaukee, WI), added at a ratio of 11:1, respectively

(Table 6.2) and left to ripen for 30 min with continuous stirring. Cheesemilks were supplemented with 33% (wt/wt) CaCl_2 solution and equilibrated for additional 10 min. Double strength coagulant (600 IMCU mL^{-1} , CHY-MAX® Extra, Chr. Hansen, Milwaukee, WI) was then added to each vat at the levels detailed in Table 6.2. The coagula were cut on similar firmness (~50 min) by an experienced licensed Wisconsin cheesemaker. The coagulum was cut with vertical and cross wire cut knives, according to the sizes given in Table 6.2. After 10 min of gentle agitation, whey was partially removed from vats at levels of 30 (WD30) and 15% (WD15) based on the total volume of curd and whey and replaced to their original volume with 34.4°C high quality tap water. The LCF-UF standardized treatments did not have any WD. Curds were then cooked and stirred at 36.7°C until the pH of curds decreased to ~6.05, when whey was drained from the vats. The curds were then cut into slabs, stacked 3 high, inverted every 15 min and milled when pH decreased to ~5.5. Milled curds were then salted with NaCl to their appropriate levels (Table 6.2) over a 15 min period. The cheeses were hooped and pressed for 3 h at a pressure of 2.81 kg cm^{-2} and maintained at room temperature overnight. Cheeses were vacuum packaged, initially stored at 10°C for the first 21 d and then stored at 5°C for the rest of the 180 d of ripening.

6.3.3 Compositional analyses

The composition of raw skim milk, sweet cream, UF retentate, UF permeate and cheesemilks were analyzed to determine the levels of TS (Green and Park, 1980), total protein (Kjeldahl method; AOAC International, 2000), CN (AOAC International,

2000), NPN (AOAC International, 2000), fat (Mojonnier method; AOAC International, 2000), lactose (HPIC; Møller et al., 2012) and total calcium (inductively coupled argon plasma emission spectroscopy). The proportion of insoluble calcium (INSOL Ca) of cheesemilks was measured by analyzing the calcium content of rennet whey to estimate the content of soluble Ca (Lucey et al., 1993; Hassan et al., 2004). The composition of experimental cheeses was determined after 14 d of ripening for moisture (Marshall, 1992), fat (AOAC International, 2000), total protein (AOAC International, 2000), salt by the chloride electrode method (Johnson and Olson, 1985) and total Ca (Park, 2000). The pH of cheeses was measured at 1, 7, 30, 90 and 180 d of ripening by inserting a spear tip pH probe into a cheese block previously equilibrated at 20°C for 45 min. The content of residual lactose, residual galactose and lactic acid in cheese were measured by HPIC (Dionex ICS-5000 RFIC-EG™ Dual System, Thermo Fisher Scientific Inc., Waltham, MA.; Møller et al., 2012) at 1, 7, 30, 90 and 180 d of ripening. Cheese extracts used for chromatographic analyses were prepared according to the method described by Zeppa et al. (2001). The proportion of INSOL Ca in cheese was determined by the acid-base titration method (Hassan et al., 2004) at 1, 14, 30, 90 and 180 d of ripening. All analyses were performed in duplicate.

6.3.4 Proteolysis

Proteolysis was assessed by pH4.6-soluble nitrogen (pH 4.6 SN/TN; Kruchoo and Fox, 1982) and 12% trichloroacetic acid-soluble nitrogen (12% TCA SN/TN; AOAC International, 2000) methods at 1, 14, 30, 90 and 180 d of ripening. Urea-

polyacrylamide gel electrophoresis was performed directly on cheese samples to monitor the breakdown of α_{s1} - and β -CN during ripening as described by Ibáñez et al. (2016). Densitometric analysis of scanned gels was performed with an image processing software (GelAnalyzer 2010 1.6, Lazarsoftware, Debrecen, Hungary). All treatments were analyzed in duplicate.

6.3.5 Textural and rheological measurements

Texture profile analysis (TPA) was performed using a Texture Analyzer TA-XT2 (Stable Micro Systems, Godalming, Surrey, UK) at 14, 30, 60, 90 and 180 d of ripening as described by Ibáñez et al. (2016) with some modifications. Cheese samples were cut into cylinders (16 mm diameter, 17.5 mm height) and stored overnight at 4°C. Analysis was performed by compressing a cheese sample to 30% of strain in two consecutive cycles at a rate of 0.8 mm s⁻¹. At least five cheese cylinders were analyzed per treatment.

The rheological properties of cheeses were studied by dynamic small amplitude oscillatory rheology (Govindasamy-Lucey et al., 2005) using a controlled stress rheometer (Anton Paar MCR 302, Anton Paar Inc., Ashland, VA) at 14, 30, 60, 90 and 180 d of ripening. Cheese samples were cut into discs (50 mm diameter, 3 mm height), placed in sealed bags and stored at 4°C for at least 8 h before analysis. A serrated parallel geometry was used and samples were placed on the bottom plate of the instrument at an initial temperature of 5°C. A normal force of ~1.8 N was initially

applied to the cheese sample and the exposed layers were covered with vegetable oil to prevent drying out. When the normal force decreased (relaxed) to ~ 0.7 N, cheese was heated to 85°C at a rate of $1^{\circ}\text{C min}^{-1}$, using a frequency of 0.08 Hz and a total strain of 0.5%. Storage modulus (G'), loss modulus (G'') and loss tangent (G''/G' ; LT) were measured during cheese heating. The temperature at the crossover point (i.e., where $G'' = G'$ or $\text{LT} = 1$) was also calculated, as this corresponds to the transition from solid to liquid-like system. All analyses were performed on duplicate

6.3.6 Descriptive sensory analysis

The texture and flavour attributes of experimental cheeses were studied by a combination of the sensory Spectrum technique and quantitative sensory analysis (Meilgaard et al., 1999). At least 12 panelists (20 h training) evaluated samples in duplicate at 30, 60, 90 and 180 d of ripening. Each sample was identified with a random 3-digit code. Cheese cubes ($2 \times 2 \times 2$ cm) were evaluated at 12°C using a numerical scale, ranging from 0 to 15. Attributes described (Table 6.3) were texture (hand firmness, cohesiveness, chewiness and particle size), basic taste and flavors (sweet, salt, acid, bitter, milkfat, brothy, sour and cardboard) and chemical feeling (burn and astringent).

Table 6.3 Definition of the attributes used by trained panelists to evaluate the sensory properties of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses at 12°C using a combination of Spectrum and quantitative descriptive analysis.

Attribute	Definition and evaluation procedure	References used, preparation instructions and anchor points (0-15)
Hand firmness	Force required to compress the cheese between finger and thumb.	Green-coloured Thera-Putty (#5075; Sammon Preston) = 4.5
	Place the cheese cube between thumb and fore finger. Compress cheese cube to approximately 30% its original size; do not fracture.	Green-coloured Thera-Putty (#5077; Sammon Preston) = 7.0 Flesh-coloured Thera-Putty (Graham-Field, Inc.) = 9.5 Grey eraser (Primacolor Kneaded Rubber) = 12.0 White eraser (School Select White) = 15.0
Cohesiveness	Degree to which sample holds together in mass.	Carrots (Meltalfe's Sentry Foods) = 1.0
	Put cheese sample in molars and chew 7 times. Gather to the middle of mouth and evaluate cohesiveness of mass.	Mushrooms (Metcalf's Sentry Foods) = 3.0 Tostitos chips (Frito-Lay Brand) = 7.0 Dried apricots (Metcalf's Sentry Foods) = 11.0 White bread (Wonder Brand) = 14.0
Chewiness	The total amount of energy required to masticate the sample to a state pending swallowing.	Philadelphia full-fat cream cheese (Kraft Foods) = 1.0 Beef frankfurters (Hebrew National Brand) = 4.0
	Place cheese cube between molars, chew cheese cube at an even rate, both sides of mouth may be used. Measure total energy required.	Gum Drops (Dots Brand) = 9.0 Beef Jerky (Jack Links Brand) = 13.5

Continued

Table 6.3 (Continued)

Attribute	Definition and evaluation procedure	References used, preparation instructions and anchor points (0-15)
	Chewiness is a product of cohesiveness, hardness and springiness. The longer time required to chew, the chewier the sample.	
Particle size	Size of the particles in the chewed mass. Chew 12-15 times and evaluate.	Philadelphia full-fat cream cheese (Kraft Foods) = 0.0 Parmesan Cheese (BelGioioso Cheese) = 5.0 Juustoleipa Cheese (Babcock Dairy Hall, UW-Madison) = 10.0 Medium Cheddar (Kraft Foods) = 15.0
Sweet	Basic taste sensation elicited by sweet compounds.	None to pronounced.
Salt	Basic taste sensation elicited by salt.	None to pronounced.
Acid	Basic taste sensation elicited by acids.	None to pronounced.
Bitter	Basic taste sensation elicited by bitter compounds	None to pronounced.
Milkfat	Aromatics and flavor commonly associated with milk or fresh cream.	None to pronounced.
Brothy	Aromatics associated with boiled meat or vegetable soup stock.	None to pronounced.

Continued

Table 6.3 (Continued)

Attribute	Definition and evaluation procedure	References used, preparation instructions and anchor points (0-15)
Sour	Aromatics and flavours commonly associated with acid compounds.	None to pronounced.
Cardboard	Aroma associated with wet cardboard.	None to pronounced.
Burn	Chemical feeling factor associated with high concentrations of irritants to the mucous membranes of the oral cavity	None to pronounced.
Astringent	Harsh, drying, puckering sensation on the surfaces of the mouth.	None to pronounced.
Attributes were evaluated using Spectrum and quantitative descriptive analysis (Meilgaard et al., 1999).		

6.3.7 Experimental design and statistical analyses

Six treatments were based on the two milk fat levels and three lactose modification techniques, namely low-fat cheese, normal lactose, 30% of whey dilution (LF-WD30); low-fat cheese, normal lactose, 15% of whey dilution (LF-WD15); low-fat cheese, lactose standardized by UF (LF-LS); reduced-fat cheese, normal lactose, 30% of whey dilution (RF-WD30); reduced-fat cheese, normal lactose, 15% of whey dilution (RF-WD15) and reduced-fat cheese, lactose standardized by UF (RF-LS). These treatments were manufactured in four independent trials, based on a 6 x 4 randomized block design. Analysis of variance (ANOVA) was performed on cheese composition at a significance level of $P < 0.05$. A split-plot design (Montgomery, 2013) was used to evaluate the effect of treatment, ripening time and their interactions on pH, levels of lactic acid, proteolysis, INSOL Ca, texture, rheology and sensory properties. When significant differences ($P < 0.05$) were found, the treatment means were analyzed by Tukey's multiple comparison test. All analyses were performed using Minitab® 16 (Minitab Inc., State College, PA).

6.4 Results

6.4.1 Cheese milk composition

The composition of standardized cheese milks is shown in Table 6.1. LF cheesemilks had lower levels of TS when compared to RF treatments ($P < 0.05$), due to

their lower fat contents. The lactose adjustment by UF significantly ($P < 0.05$) reduced the lactose content, leading to decreased L:CN from 1.7-1.8 to 1.1-1.2, when compared to milks without lactose standardization (WD30 or WD15; $P < 0.05$). In addition, LS treatments exhibited lower levels of total Ca, when compared to WD treatments ($P < 0.05$).

6.4.2 Cheese composition

The composition of LF and RF Gouda-style cheeses is shown in Table 6.4. As expected, there was a significant decrease in the fat content between RF and LF cheeses that also led to a corresponding increase in protein content ($P < 0.05$). In general, the moisture content of all treatments was close to 50% and was higher in LF cheeses ($P < 0.05$). Despite the fact that the moisture content between LF cheeses was similar, the content of RF-LS cheese was slightly higher when compared to RF-WD15 ($P < 0.05$). This trend was also found in the MNFS content of RF cheeses. LF cheeses had similar MNFS among treatments and they were lower when compared to RF cheeses ($P < 0.05$). Levels of salt in LF cheeses were higher than RF cheeses; however they exhibited similar salt in the moisture phase (S/M; $P > 0.05$). There were no differences in the content of total Ca in LF and RF cheeses when values were expressed on protein content ($P > 0.05$).

Table 6.4 Composition of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses manufactured using 30% of whey dilution (WD30), 15% of whey dilution (WD15) and standardization of lactose content of cheesemilk by ultrafiltration (LS) treatments.

Item	Low-Fat			Reduced-Fat			SEM
	WD30	WD15	LS	WD30	WD15	LS	
Moisture ¹ (%)	51.94 ^{ab}	51.32 ^{ab}	52.16 ^a	49.41 ^{cd}	48.90 ^d	50.75 ^{bc}	0.309
Fat ¹ (%)	6.01 ^c	6.08 ^c	6.04 ^c	14.51 ^{ab}	14.79 ^a	13.89 ^b	0.189
Protein ^{1,2} (%)	35.44 ^a	35.81 ^a	35.38 ^a	30.55 ^b	30.59 ^b	30.19 ^b	0.155
Salt ¹ (%)	1.69 ^a	1.57 ^{ab}	1.63 ^{ab}	1.42 ^b	1.39 ^b	1.37 ^b	0.033
MNFS ^{1,3} (%)	55.25 ^c	54.64 ^c	55.52 ^c	57.80 ^{ab}	57.38 ^b	58.93 ^a	0.257
FDM ^{1,4} (%)	12.49 ^b	12.49 ^b	12.64 ^b	28.69 ^a	28.95 ^a	28.18 ^a	0.248
S/M ^{1,5} (%)	3.26 ^a	3.05 ^a	3.12 ^a	2.87 ^a	2.84 ^a	2.70 ^a	0.074
Total Ca ¹ (mg/100 g protein)	2718 ^a	2728 ^a	2500 ^a	2701 ^a	2587 ^a	2598 ^a	37.2
Residual lactose at 1 d (mg/100 g)	4.00 ^b	5.00 ^b	0.38 ^c	5.95 ^b	12.92 ^a	0.21 ^c	1.62
Residual galactose at 1 d (mg/100 g)	7.22 ^d	10.04 ^c	1.80 ^e	13.37 ^b	23.32 ^c	0.90 ^e	1.30
Lactic acid (%)							
1 d	1.70 ^{ab,B}	1.78 ^{ab,B}	1.26 ^{c,A}	1.63 ^{b,B}	1.85 ^{a,B}	1.44 ^{c,A}	0.047
7 d	1.85 ^{ab,AB}	2.04 ^{a,A}	1.33 ^{c,A}	1.81 ^{b,A}	1.98 ^{ab,AB}	1.46 ^{c,A}	0.059
30 d	1.97 ^{ab,A}	2.16 ^{a,A}	1.41 ^{c,A}	1.85 ^{b,A}	2.07 ^{a,A}	1.56 ^{c,A}	0.059
90 d	1.92 ^{ab,A}	2.04 ^{a,A}	1.36 ^{d,A}	1.80 ^{b,A}	2.00 ^{a,AB}	1.54 ^{c,A}	0.054
180 d	1.93 ^{ab,A}	2.07 ^{a,A}	1.38 ^{c,A}	1.84 ^{b,A}	1.99 ^{ab,AB}	1.47 ^{c,A}	0.059
pH							
1 d	5.04 ^{c,D}	4.99 ^{cd,D}	5.21 ^{a,D}	5.01 ^{c,D}	4.94 ^{d,C}	5.11 ^{b,C}	0.019
7 d	5.11 ^{c,C}	5.07 ^{c,C}	5.31 ^{a,C}	5.09 ^{c,C}	5.01 ^{d,B}	5.21 ^{b,B}	0.022
30 d	5.19 ^{c,B}	5.14 ^{c,B}	5.40 ^{a,B}	5.16 ^{c,B}	5.08 ^{d,A}	5.33 ^{b,A}	0.025
90 d	5.24 ^{c,AB}	5.18 ^{de,AB}	5.49 ^{a,A}	5.21 ^{cd,AB}	5.13 ^{c,A}	5.38 ^{b,A}	0.027
180 d	5.27 ^{c,A}	5.22 ^{c,A}	5.49 ^{a,A}	5.22 ^{c,A}	5.12 ^{d,A}	5.38 ^{b,A}	0.028

^{a,b,c} Means within the same row not sharing a common superscript differ ($P < 0.05$), as compared by Tukey's multiple comparison test.

^{A,B,C} Means within the same column (for a particular item) not sharing a common uppercase superscript differ ($P < 0.05$; comparing the effect of ripening at a single treatment).

¹ Composition measured at 14 d of ripening

² Total %N x 6.38.

³ Moisture in the non-fat substance of the cheese.

⁴ Fat content on a dry weight basis

⁵ Salt in the moisture phase of the cheese.

Values represent mean and standard error of four replicate trials.

The adjustment of lactose in cheesemilk by UF led to LF and RF cheeses with lower amounts of residual lactose at 1 d of ripening when compared to cheeses made with WD ($P < 0.05$; Table 6.4). The RF-WD15 cheese had significantly higher amounts of residual lactose than other WD treatments ($P < 0.05$). After 7 d of ripening, residual lactose was not detected in any treatment (results not shown). Similarly, lower levels of galactose at 1 d were observed in LS cheeses when compared to WD cheeses ($P < 0.05$; Table 6.4). The WD15 treatment resulted in higher levels of galactose than WD30 in both LF and RF cheeses ($P < 0.05$). After 30 d of ripening, residual galactose was not detected in any treatment (results not shown). A significant effect on treatment and cheese age was observed for the levels of lactic acid (Table 6.5). Cheeses made with LS cheesemilks had lower levels of lactic acid at all ripening periods, when compared to cheeses made with WD treatments ($P < 0.05$; Table 6.4). The cheeses made with WD exhibited high variability in their levels of lactic acid. For the WD cheeses, the levels of lactic acid exhibited a slight increase after 7 d of ripening and then remained relatively constant for the rest of ripening (Table 6.4).

The pH values of experimental cheeses exhibited a significant effect of treatment, cheese age and the interaction of treatment x cheese age ($P < 0.05$; Table 6.5). At 1 d of ripening, the pH values from LS cheeses were higher than those of WD cheeses (Table 6.4). The pH values of cheeses obtained from WD15 treatments tended to be lower than WD30 treatments. An increase in the pH values was found for all treatments during ripening ($P < 0.05$), but followed the same trend as at d 1.

The proportion of INSOL Ca of cheeses during ripening is shown in Fig. 6.1. Treatment, cheese age and their interaction had a significant effect on the levels of INSOL Ca (Table 6.5). At 1 d of ripening, RF-WD30 and RF-WD15 cheeses exhibited the highest levels of INSOL Ca (>90%) and followed then by LF-WD30 and LF-WD15 (~85%) treatments. Cheeses made with LS had the lowest levels in INSOL Ca compared to cheeses made with WD ($P < 0.05$) and this trend was also observed during ripening. All cheeses exhibited a significant reduction in the amount of INSOL Ca during ripening ($P < 0.05$) and all cheeses made with WD treatments had similar proportion of INSOL Ca after 90 d of ripening.

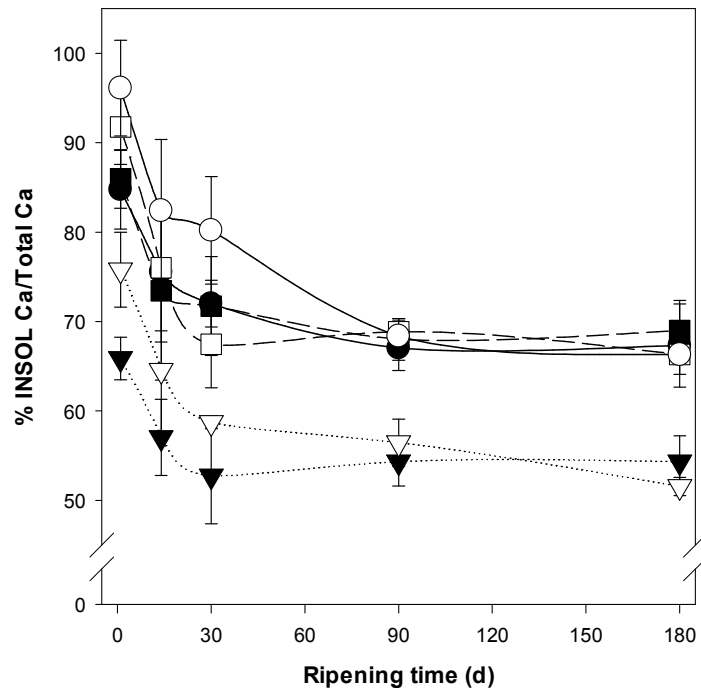


Fig. 6.1 Levels of insoluble calcium, expressed as a percentage of total Ca (%INSOL Ca/Total Ca) of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization of cheesemilk by ultrafiltration (▼ ▽) during 180 d of ripening. Values represent mean and standard deviation of four replicate trials.

Table 6.5 Probabilities and R² values for pH, lactic acid, insoluble calcium, proteolysis, texture and rheology of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses during 180 d of ripening.

Factors ¹	df ²	pH	Lactic Acid	INSOL Ca ³	pH 4.6 SN/TN ⁴	12%TCA SN/TN ⁵	TPA Hardness ⁶	LT=1 ⁷	df ²	α_{s1} -CN ⁸	β -CN f1-192 ⁹
<i>Whole plot</i>											
Day of cheese manufacture (D)	3	0.391	0.630	0.050	0.013	0.012	0.002	<0.001	3	0.005	0.049
Treatment (T)	5	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	5	0.052	0.010
Error (D x T)	15								15		
<i>Subplot</i>											
Age (A)	4	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	3	<0.001	<0.001
A x T	20	0.004	0.575	0.010	<0.001	0.041	<0.001	0.009	15	0.232	0.003
Error	72								54		
R ²		0.98	0.94	0.92	0.99	0.99	0.95	0.92		0.99	0.96

¹ Split-plot design with the six treatments (LF and RF cheeses made with 30% of whey dilution, 15% of whey dilution and cheesemilk lactose standardization by ultrafiltration) were analyzed as a discontinuous variable and day of cheese manufacture (D) was blocked.

Subplot included the effect of aging of cheese (A) and the interaction age x treatment (A x T) as variables.

² Degrees of freedom differed for variable measurements, as the time points for the analyses were different.

³ Percentage of insoluble calcium, as a percentage of total calcium in cheese.

⁴ pH 4.6-soluble nitrogen as a percentage of total nitrogen.

⁵ Trichloroacetic acid-soluble nitrogen as a percentage of total nitrogen.

⁶ TPA hardness was measured by texture analyser.

⁷ Temperature at which loss tangent = 1.

⁸ Level of intact α_{s1} -CN as a percentage of the level at 1 d.

⁹ Accumulation β -CN (f1-189/192) fraction as the percentage of intact β -CN at 1 d.

6.4.3 Proteolysis

The primary proteolysis of cheeses during ripening, expressed as the level of pH 4.6 soluble N, is shown in Fig. 6.2a. Treatment and cheese age had significant effects on the level of proteolysis (Table 6.5). Levels of proteolysis increased for all treatments during ripening and similar levels of proteolysis were found during the first 14 d. After 30 d, RF cheeses exhibited slightly higher levels of proteolysis than LF treatments. In addition, fat content WD or LS treatments did not impact pH 4.6 soluble N levels. Similar trends were also observed when secondary proteolysis was assessed by 12% TCA soluble N (Fig. 6.2b; Table 6.5). At the end of ripening, LF and RF cheeses made from LS treatments exhibited lower levels of 12% TCA SN, when compared to cheeses made with WD ($P < 0.05$).

Urea-PAGE electrophoretograms of cheese samples clearly showed an increase in the breakdown of CNs during ripening (Fig. 6.3). Degradation of α_{s1} -CN (Fig. 6.4a) significantly increased during ripening ($P < 0.05$); however, there were no significant differences among treatments ($P > 0.05$; Table 6.5). We also observed a significant increase in levels of β -CN (f1-189/192) during the first 30 d of ripening for all treatments (Fig. 6.4b; $P < 0.05$). The cheeses made with LS exhibited higher accumulation of β -CN (f1-189/192), when compared to WD treatments ($P < 0.05$; Fig. 6.4b).

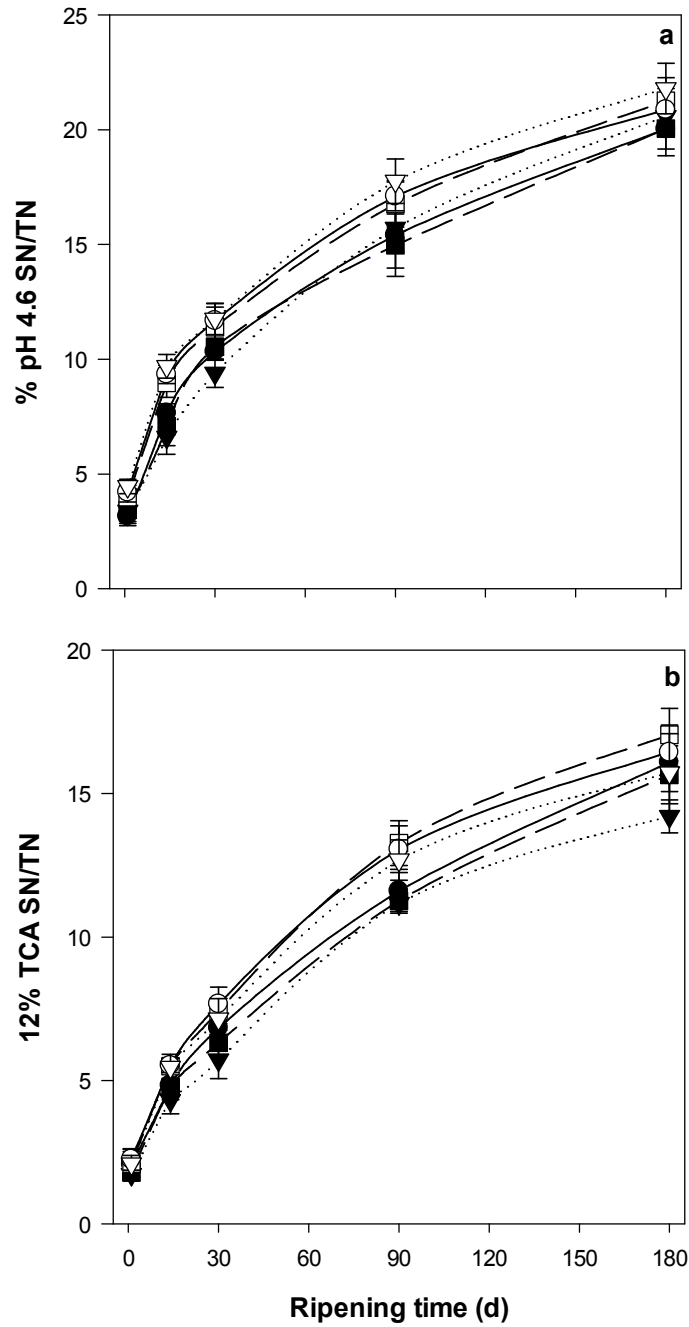


Fig. 6.2 Levels of proteolysis expressed as pH 4.6-soluble N (%pH 4.6 SN/TN; a) and 12% trichloroacetic acid-soluble N (%12 TCA SN/TN; b) found in of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization of cheesemilk by ultrafiltration (▼ ▽) during 180 d of ripening. Values represent mean and standard deviation of four replicate trials.

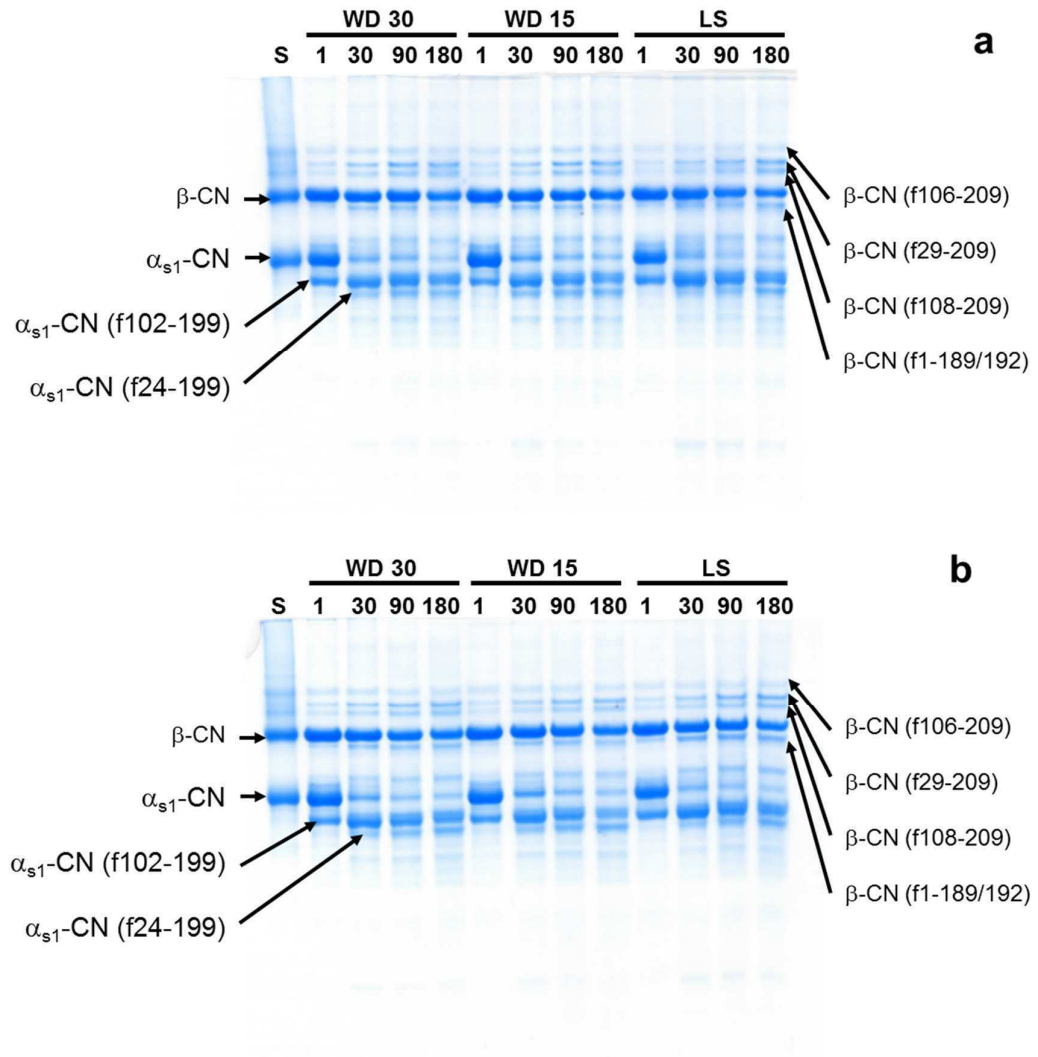


Fig. 6.3 Urea-Polyacrylamide gel electrophoresis of low fat (a) and reduced fat (b) Gouda-style cheeses at 1, 30, 90 and 180 d of ripening manufactured with 30% of whey dilution (WD30), 15% of whey dilution (WD15) and lactose standardization (LS). Sodium caseinate (S) was used as standard for each gel.

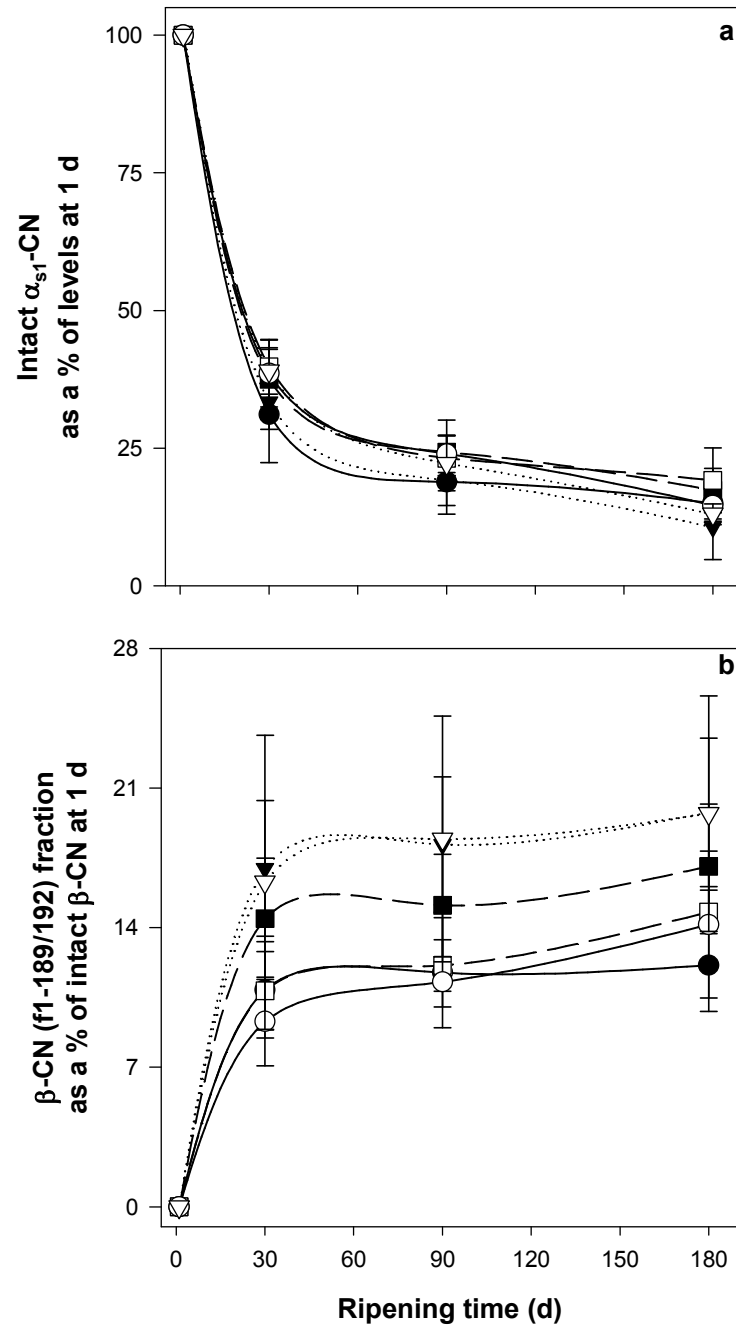


Fig 6.4 Changes in level of intact α_{s1} -CN as a percentage of the level at 1 d (a) and accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 1 d (b) for low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening. Values represent mean and standard deviation of four replicate trials.

6.4.4 TPA hardness

The TPA hardness values of cheese during ripening are shown in Fig. 6.5. A significant decrease in cheese hardness was observed during ripening (Table 6.5). In general, LF cheeses were harder when compared with RF samples (Fig. 6.5). Cheeses made with LS exhibited the lowest hardness values when compared to other treatments ($P < 0.05$), within a fat level.

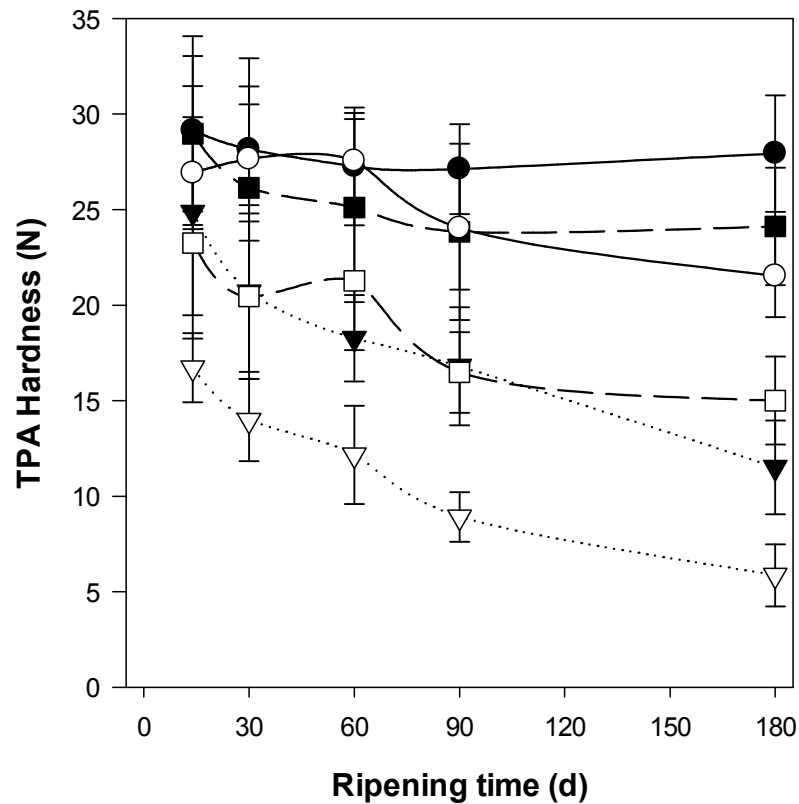


Fig. 6.5 TPA hardness of low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening. Values represent mean and standard deviation of four replicate trials.

6.4.5 Rheological properties

During the heating of cheese, all samples showed a decrease in the G' values (results not shown). Treatment, age and their interactions had a significant effect (Table 6.5) on the temperature where the $LT=1$ or at the crossover point (Fig. 6.6). All treatments showed a decrease of the crossover point during ripening ($P < 0.05$). During the first 60 d of ripening, only RF-LS cheese exhibited lower crossover point compared to other treatments ($P < 0.05$). At 180 d of ripening, the two WD15 treatments exhibited the highest crossover temperatures, followed by the two WD treatments ($P < 0.05$). The LS cheeses had the lowest crossover temperature after 180 d of ripening ($P < 0.05$).

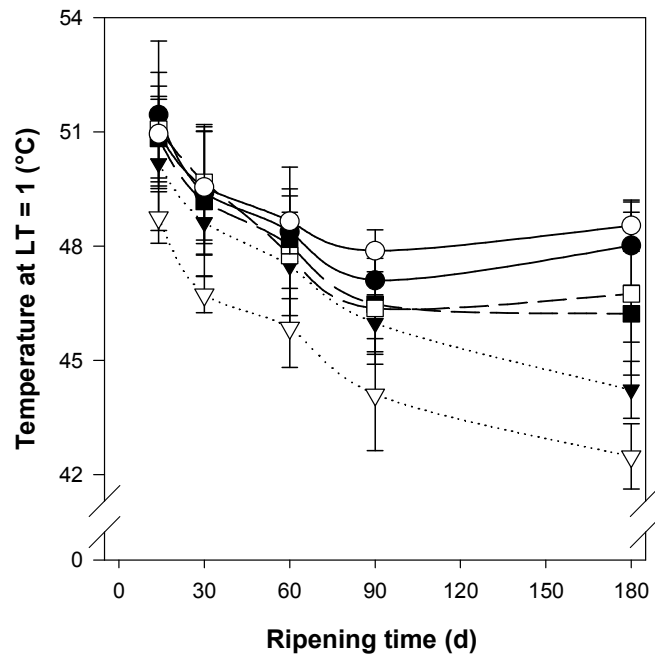


Fig. 6.6 Temperature of the crossover point of the storage and loss moduli (or when $LT = 1$) obtained from low fat (filled symbols) and reduced fat (open symbols) Gouda-style cheeses made with 30% of whey dilution (■ □), 15% of whey dilution (● ○) or lactose standardization (▼ ▽) during 180 d of ripening. Values represent mean and standard deviation of four replicate trials.

6.4.6 Sensory analysis

Sensory texture and flavour attributes of the cheeses during ripening are shown in Table 6.6. Attributes of particle size, sweetness, milkfat, brothiness, cardboard, burn and astringent were not significantly different among treatments ($P > 0.05$; results not shown), whereas other attributes had significant effect in treatments and ripening time (Table 6.7). RF cheeses exhibited lower hand firmness compared to LF and most cheeses exhibited a decrease during ripening ($P < 0.05$). Cheeses made with LS were significantly softer than other treatments during ripening. The cohesiveness significantly increased during ripening for cheese samples and was found to be higher in RF, when compared to LF cheeses ($P < 0.05$). LF cheese made with LS had lower cohesiveness scores than other treatments at all timepoints ($P < 0.05$). The chewiness was significantly lower in RF compared to LF cheeses and all cheeses showed a slight decrease (<1 unit) in chewiness during ripening ($P < 0.05$). LS cheeses tended to have slightly lower salt and acid scores than the other treatments ($P < 0.05$). A similar trend than acidity was also found in sourness. Bitterness values were low during the initial ripening period, but significantly increased at 180 d of ripening for all treatments ($P < 0.05$).

Table 6.6 Sensory analysis results of low-fat (LF) and reduced-fat (RF) Gouda-style cheeses manufactured using 30% of whey dilution (WD30), 15% of whey dilution (WD15) and standardization of cheesemilk lactose by ultrafiltration (LS) treatments during 180 d of ripening.

Attribute	Time (d)	Low-fat			Reduced-fat		
		WD30	WD15	LS	WD30	WD15	LS
Hand firmness	30	12.56 ^{a,A}	12.74 ^{a,A}	12.28 ^{ab,A}	11.16 ^{c,A}	11.71 ^{bc,A}	9.09 ^{d,A}
	60	11.95 ^{a,AB}	12.28 ^{a,A}	11.11 ^{bc,B}	10.78 ^{c,AB}	11.69 ^{ab,A}	8.84 ^{d,AB}
	90	11.89 ^{a,AB}	12.41 ^{a,A}	10.74 ^{b,B}	10.41 ^{b,B}	11.73 ^{a,A}	8.40 ^{c,B}
	180	11.70 ^{b,B}	12.54 ^{a,A}	9.40 ^{d,C}	10.37 ^{c,B}	11.80 ^{ab,A}	6.35 ^{e,C}
Cohesiveness	30	5.94 ^{b,C}	6.27 ^{b,C}	4.11 ^{c,C}	8.66 ^{a,C}	9.18 ^{a,C}	8.39 ^{a,C}
	60	6.67 ^{c,B}	6.86 ^{c,C}	5.04 ^{d,BC}	10.04 ^{ab,B}	10.65 ^{a,B}	9.14 ^{b,BC}
	90	7.80 ^{b,A}	8.30 ^{b,B}	6.04 ^{c,B}	10.39 ^{a,B}	10.65 ^{a,B}	9.90 ^{a,B}
	180	10.22 ^{b,A}	10.21 ^{b,A}	8.95 ^{c,A}	11.85 ^{a,A}	12.07 ^{a,A}	11.51 ^{a,A}
Chewiness	30	6.92 ^{a,A}	6.76 ^{a,A}	7.01 ^{a,A}	5.92 ^{b,A}	6.19 ^{b,A}	5.37 ^{c,A}
	60	6.61 ^{a,AB}	6.65 ^{a,A}	6.69 ^{a,AB}	5.80 ^{b,A}	6.03 ^{b,AB}	5.07 ^{c,A}
	90	6.49 ^{a,B}	6.55 ^{a,A}	6.45 ^{a,BC}	5.80 ^{b,A}	6.17 ^{ab,A}	5.26 ^{c,A}
	180	6.29 ^{ab,B}	6.72 ^{a,A}	6.23 ^{b,C}	5.38 ^{c,B}	5.66 ^{c,B}	4.54 ^{d,B}
Salt	30	4.32 ^{a,A}	4.06 ^{a,AB}	4.40 ^{a,A}	4.25 ^{a,A}	4.43 ^{a,A}	4.05 ^{a,A}
	60	3.58 ^{a,B}	3.55 ^{a,B}	3.43 ^{a,B}	3.61 ^{a,A}	3.62 ^{a,B}	3.22 ^{a,B}
	90	3.64 ^{a,AB}	3.76 ^{a,AB}	3.34 ^{ab,B}	3.72 ^{a,A}	3.83 ^{a,AB}	3.12 ^{b,B}
	180	4.14 ^{a,AB}	4.33 ^{a,A}	3.70 ^{b,AB}	4.30 ^{a,A}	4.35 ^{a,A}	3.72 ^{b,AB}
Acid	30	4.06 ^{abc,A}	4.30 ^{ab,A}	3.28 ^{c,A}	4.29 ^{ab,A}	4.66 ^{a,A}	3.69 ^{bc,A}
	60	3.71 ^{ab,A}	3.93 ^{ab,A}	3.25 ^{b,A}	4.03 ^{ab,A}	4.42 ^{a,A}	3.40 ^{b,A}
	90	3.86 ^{ab,A}	4.08 ^{ab,A}	3.34 ^{b,A}	4.09 ^{ab,A}	4.40 ^{a,A}	3.39 ^{b,A}
	180	4.14 ^{ab,A}	4.43 ^{a,A}	3.32 ^{b,A}	4.36 ^{a,A}	4.85 ^{a,A}	3.53 ^{b,A}
Bitter	30	0.57 ^{a,B}	0.73 ^{a,B}	0.63 ^{a,B}	0.67 ^{a,B}	0.90 ^{a,B}	0.81 ^{a,B}
	60	0.70 ^{a,B}	0.73 ^{a,B}	0.72 ^{a,B}	0.80 ^{a,B}	0.87 ^{a,B}	1.24 ^{a,B}
	90	0.79 ^{a,B}	0.77 ^{a,B}	0.86 ^{a,B}	0.87 ^{a,B}	0.82 ^{a,B}	1.09 ^{a,B}
	180	2.62 ^{b,A}	2.77 ^{ab,A}	2.67 ^{b,A}	2.93 ^{ab,A}	2.81 ^{ab,A}	3.28 ^{a,A}

Continued

Table 6.6 (Continued)

Attribute	Time (d)	Low-fat			Reduced-fat		
		WD30	WD15	LS	WD30	WD15	LS
Sour	30	3.06 ^{abc,AB}	3.63 ^{a,AB}	2.27 ^{c,B}	3.50 ^{ab,A}	3.88 ^{a,A}	2.63 ^{bc,AB}
	60	2.97 ^{bc,B}	3.36 ^{ab,B}	2.35 ^{c,B}	3.19 ^{abc,a}	3.94 ^{a,A}	2.49 ^{bc,B}
	90	3.17 ^{abc,AB}	3.35 ^{ab,B}	2.39 ^{c,AB}	3.34 ^{ab,A}	3.75 ^{a,A}	2.67 ^{bc,AB}
	180	3.83 ^{ab,A}	4.39 ^{a,A}	3.22 ^{b,A}	4.01 ^{ab,A}	4.10 ^{ab,A}	3.41 ^{b,A}

^{a,b,c} Means within the same row not sharing a common lowercase superscript differ ($P < 0.05$; comparing the effect of treatment at a single storage time).

^{A,B,C} Means within the same column (for a particular attribute) not sharing a common uppercase superscript differ ($P < 0.05$; comparing the effect of storage time at a single treatment).

Values represent mean of four replicate trials.

Table 6.7 Probabilities and R^2 values for sensorial properties of low-fat (RF) and reduced-fat (RF) Gouda-style cheeses during 180 d of ripening.

Factors ¹	df	Hand Firmness	Cohesiveness	Chewiness	Salt	Acid	Bitter	Sour
<i>Whole plot</i>								
Day of cheese manufacture (D)	3	0.006	0.019	<0.001	0.158	0.716	0.012	<0.001
Treatment (T)	5	<0.001	<0.001	<0.001	<0.001	<0.001	0.012	<0.001
Error (D x T)	15							
<i>Subplot</i>								
Age (A)	3	<0.001	<0.001	<0.001	<0.001	0.024	<0.001	<0.001
T x A	15	<0.001	0.010	0.033	0.803	0.999	0.913	0.944
Error	54							
R^2		0.97	0.96	0.94	0.68	0.73	0.95	0.81

¹Split-plot design with the six treatments (LF and RF cheeses made with 30% of whey dilution, 15% of whey dilution and cheesemilk lactose standardization by ultrafiltration) were analyzed as a discontinuous variable and day of cheese manufacture (D) was blocked. Subplot included the effect of aging of cheese (A) and the interaction age x treatment (A x T) as variables.

6.5 Discussion

6.5.1 Cheese composition

Cheeses made with decreased levels of fat exhibit an increase of other components, such as moisture and protein and also reductions in the levels of MNFS and S/M (Fenelon and Guinee, 2000; Guinee et al., 2000; Johnson et al., 2009). This agrees with the data from our study, as LF cheeses had higher contents of moisture and protein and lower levels of MNFS when compared to RF cheeses. However, we obtained LF and RF cheeses with similar levels of S/M, which is attributed to different levels of salting during the cheese manufacturing process (Table 6.2).

Concentration of milk by LCF-UF, followed by the addition of permeate and water to standardize the lactose content of LF-LS and RF-LS, led to a reduction in the levels of total Ca in these cheesemilks (Table 6.1). This could be attributed due to a combined effect of concentration and partial dilution with water, as concentration of milk leads to an increase in the levels of colloidal calcium phosphate, whereas dilution with water leads to decreased levels (Fox et al., 2015). In contrast, preacidification with lactic acid and the addition of CaCl_2 to cheesemilks prior cheesemaking (Table 6.2), along with similar pH values at critical points during cheese manufacture, could contribute to similar levels of total Ca between cheese treatments when expressed in mg per 100 g of protein.

Cheeses made with reduced fat content often develop excessive acidity due to decreased levels of S/M in comparison to their full-fat counterparts (Fenelon and Guinee, 2000). During the traditional manufacture of Gouda cheese (i.e., stirred curd and brine salted) most of the lactose is depleted by the starters cultures, reaching levels <100 mg per 100g of cheese after pressing and pH values ranging 5.4-5.5 (Lolkema, 1991; McSweeney and Fox, 2004; Van den Berg et al., 2004). The cheesemaking protocol used in this study may have resulted in similar levels of lactose in the curd during milling and salting as pH these steps were performed at pH 5.5. Despite very low levels of residual lactose was found at 1 d of ripening (Table 6.4), cheeses made with LS had the lowest values of residual lactose as their levels were initially reduced in milks prior cheesemaking. The use of whey dilution and curd washing has been used during cheese manufacture to reduce the amount lactose and hence control the acidity of cheese (Lawrence et al., 1987; Shakeel-Ur-Rehman et al., 2004; Upreti and Metzger, 2006; Lee et al., 2005, 2011; Hou et al., 2014). However, we found that increased levels of WD led to slightly higher levels of lactic acid among RF cheeses; in contrast, LF and RF cheeses made with LS exhibited the lowest levels during ripening (Table 6.4). In addition, low levels of residual lactose at 1 d of ripening in cheeses made with WD treatments, led to a slight increase in the content of lactic acid at 7 d of ripening that remained constant thereafter, whereas in LS the content of lactic acid remained constant during ripening as levels of residual lactose were close to zero.

Levels of lactic acid produced in cheese have an inverse relation to pH and lactate levels could also be associated with the initial levels of lactose in milk. Lawrence et al. (1987) found that the pH values of 5 mo Gouda cheeses made mid- and

end-lactation milks periods were ~5.4 and ~5.9, respectively. This could be explained due to a progressive and significant decrease in the concentration of milk lactose towards the end of lactation, along with an increase of the casein content (Fox et al., 2015) reducing the L:CN ratio of milk, increasing the final pH of cheese. Due to the changes in the composition of milk during lactation, Lolkema (1991) suggested that the levels of WD applied during cheese manufacture should be based on the composition of cheesemilk to target the desired pH, composition and yield of cheese. This is a critical factor, as in our study we found little effect in removing lactose LF-WD30 and LF-WD15, leading to similar levels of lactic acid and pH values. In contrast, the use of LS in milk by UF would allow cheesemakers to control the L:CN prior processing and target a final cheese pH and thus avoiding the use of WD during cheese manufacture.

An increase in cheese pH during ripening is associated with a reduction in the levels of INSOL Ca (Fig. 6.1), which relates to the release of phosphate ions from the colloidal calcium that combines with hydrogen ions (Hassan et al., 2004), leading to a buffering effect (Lucey et al., 1993). The buffering effect of cheese may be affected by the protein content (Upreti and Metzger, 2007). However, the occurrence of higher levels of INSOL Ca at 1 d of ripening in RF cheeses within each individual treatment (WD30, WD15 or LS) could also be related to lower extent of preacidification, in comparison with LF (Table 6.2). Metzger et al. (2001) found a decrease in the water insoluble Ca content of low-fat Mozzarella cheese when using preacidification of milk. The use of curd washing has successfully reduced the amount of INSOL Ca in different cheese varieties, such as Cheddar (Lee et al., 2005) and Colby (Lee et al., 2010, 2011). As previously stated, the similar levels of INSOL Ca in LF-WD30 and LF-WD15 could

be attributed to insufficient addition of water to solubilize Ca and remove lactose. On the other hand, lowest levels of INSOL Ca found in cheeses made with LS may be related to the addition of water to cheesemilks during standardization that depleted some Ca (Table 6.1). This could also have reduced the ionic strength of the cheeses and hence significantly increase solubilization of CCP (Lucey et al., 2003).

6.5.2 Proteolysis

Differences in the levels of the pH 4.6 SN between cheeses with different fat content relates to increased levels of MNFS found in RF cheeses (Table 6.4). This agrees with Fenelon and Guinee (2000) who found that lower amount of MNFS in cheeses with reduced fat content led to decreased retention of chymosin. Similarities in the primary proteolysis of WD and LS among LF and RF cheeses are also in agreement with the studies from Shakeel-Ur-Rehman et al. (2004) and Lee et al. (2011), who reported that different levels of cheese acidity obtained by whey dilution had no effect on primary proteolysis. In contrast, a significant increase in levels of proteolysis has been observed in cheeses with high acid development (Watkinson et al., 2001; Lee et al., 2005; Upreti et al., 2006; Lee et al., 2010). Upreti et al. (2006) reported that higher proteolysis in high acidity cheese was attributed to higher levels of MNFS and lower content of S/M.

The 12% TCA-SN fraction is produced by the contribution of residual chymosin and starter culture proteinases and peptidases (Rank et al., 1985). As occurred with

primary proteolysis, higher concentrations of 12% TCA-SN in RF cheeses could be related to increased MNFS content that would lead to higher retention of chymosin in the curd. However, LF and RF cheeses made from LS treatments exhibited lower levels 12% TCA-SN when compared to WD treatments, which may be due to decreased growth of starter cultures during cheese manufacture that contributes with proteolytic activity, as the content of lactose of LS milks prior processing was reduced around 40-45%, when compared to cheesemilks from WD treatments (Table 6.2). Sheehan and Guinee (2004) found that the amount of 5% phosphotungstic acid-soluble N (an indicator of the concentration of small peptides and amino acids) was lower in reduced-fat Mozzarella cheeses made with pre-acidification, in comparison with cheeses made with starter cultures added, due to an absence of proteinases and peptidases from starter cultures. Shakeel-Ur-Rehman et al. (2004) studied the effect of reduction and fortification of the lactose content in the curd modified by whey dilution in the manufacture of Cheddar cheese and found that the counts of starter cultures were not affected by modifying the lactose content in the curd. These authors suggested that reduction in levels of lactose obtained by WD left sufficient substrate to support the growth of starter bacteria during cheesemaking. Therefore, similar levels of 12% TCA-SN, obtained between different levels of WD among LF and RF cheeses, could be attributed to similar levels of proteases and peptidases from starter cultures.

Similar degradation patterns of α_{s1} -CN among cheese treatments could be attributed to similar levels of MNFS that would result in similar residual chymosin activity. Hou et al. (2014) found similar activity of residual chymosin when the pH of Cheddar cheeses made with different types of whey dilution increased from 5.2 to 5.6.

As occurred with our study, a rapid breakdown of α_{s1} -CN occurs in standard Gouda cheeses, in which 70-80% is hydrolyzed during the first 60 d of ripening (Van den Berg et al., 2004). Similarities could also be attributed to levels of S/M among treatments. After 28 d of ripening, Cheddar cheeses containing 3% S/M exhibited only 5% of casein intact, whereas with 6% S/M they had 30% of casein intact (Lawrence et al., 1987).

The formation of β -CN (f1-189/192) due to cleavage at Leu₁₉₂-Tyr₁₉₃ is also associated with the formation of the hydrophobic peptide β -CN (f193-209) that contributes to bitterness (Visser et al., 1983). We found higher degradation of β -CN to β -CN (f1-189/192) in LF and RF cheeses made with LS. These treatments exhibited the highest pH values, when compared to WD. Increased pH values of cheese relates to higher degradation of β -CN by plasmin activity due to its proximity with its optimum pH, that occurs at pH 7.5 (McSweeney and Sousa, 2000). However, an increase in the formation of β -CN (f1-189/192) is associated with chymosin activity (Feeney et al., 2002). It has been reported that greater solubilization of CCP during ripening might increase the susceptibility of caseins to hydrolysis (O'Mahony et al., 2005). Therefore, as LF and RF cheeses made with LS treatments had the highest solubilization of CCP during the first 30 d of ripening (Fig. 6.1), this could have enhanced formation of β -CN (f1-189/192).

6.5.3 TPA hardness

As expected, cheeses with reduced fat content had higher TPA hardness, due to a higher protein content and a decrease in the number of fat globules in the cheese matrix that leads to a more compact structure (Bryant et al., 1995; Fenelon and Guinee, 2000; Johnson et al., 2009). The occurrence of a softening of cheese during ripening is attributed due to an increment of proteolysis, mainly due to degradation of α_{s1} -CN (Creamer and Olson, 1982) and solubilization of CCP (Lucey et al., 2003; O'Mahony et al., 2005). Despite an increased degradation of α_{s1} -CN was observed during ripening, there were no differences among treatments (Table 6.5), suggesting that differences in TPA hardness could be attributed to different levels of INSOL Ca (Fig. 6.1). These data agree with O'Mahony et al. (2005) who reported that TPA hardness of Cheddar cheese during the first stages of ripening was more correlated with a reduction in the levels of INSOL Ca than hydrolysis of α_{s1} -CN.

In our study, increasing levels of whey dilution from 15 to 30% in RF treatments led to RF cheeses with lower amount of residual lactose, lower amount of lactic acid (which relates to higher pH) and higher solubilization of CCP (i.e., lower levels of INSOL Ca) that resulted in cheeses with a softer texture. In contrast, these levels of WD used in the manufacture of LF cheeses had the same effect in the content of residual lactose, lactic acid and INSOL Ca, therefore these treatments showed similar TPA hardness. The use of LS in LF and RF cheesemilks seemed more efficient than using WD at levels of 30 and 15% during cheesemaking, as LS cheeses exhibited the lowest amount of residual lactose, lactic acid and INSOL Ca, resulting in highest pH values

(5.49 and 5.38 at 180 d for LF and RF, respectively) and the softest texture (<15 N and < 10 N at 180 d for LF and RF, respectively). On the other hand, several studies have shown that increasing cheese pH leads to opposite effects on texture. Visser (1991) observed increased firmness of Gouda cheeses with similar composition as pH varied from ~5.2 to ~5.6. Watkinson et al. (2001) found a similar trend in model cheeses varying in pH from 5.2 to 6.2. Hou et al. (2014) evaluated the use of different levels of WD in the manufacture of Cheddar cheese and found that cheeses had similar composition, decreased levels of lactic acid and higher pH values as levels of WD increased; however, cheeses had higher firmness. A possible explanation of these results speculated by the authors related to insufficient acidification of the curd that may lead to reduced solubilization of CCP that contributed to a firmer texture. In contrast, in our study, we found reduced levels of INSOL Ca and hence softer texture in RF cheeses made with increased WD.

6.5.4 Rheological properties

The crossover point or temperature where $LT=1$ is defined as the transition where cheese changes from solid to viscous-like material and it is also considered as the melting temperature (Gunasekaran and Ak, 2003). A reduction of the melting temperature during cheese ripening is probably attributed due to breakdown of the protein matrix caused by proteolysis and solubilization of the colloidal calcium phosphate (Lucey et al., 2003; Govindasamy-Lucey et al., 2005). We found an increase in the primary proteolysis during the ripening of all cheese treatments, although RF

exhibited higher levels than LF. Hence, melting properties of cheese could be more related to changes in the proportion of INSOL Ca than to proteolysis (Lucey et al., 2005).

A decrease in the levels of INSOL Ca was also observed during ripening. Decreased temperature at LT=1 found in LF-LS and RF-LS cheeses could be attributed to lower contents of INSOL Ca than in WD cheeses, whereas the latter had similar levels of INSOL Ca after 90 d of ripening and thus had a similar melting temperature.

Cheese melting may also be influenced by pH as decreased levels of whey dilution or the use of lactose standardization led to lower (5.12 – 5.22) or higher (5.38 – 5.49) pH values, respectively. This agrees with Brickley et al. (2008) who found decreased melting as pH values of directly acidified non-fat Mozzarella cheeses were reduced from 5.4 to 5.0, even though the cheese with pH 5.0 exhibited lower content of total and insoluble Ca. The authors elucidated that a decrease of cheese pH would approach caseins closer to their isoelectric point (pH 4.6), leading to extensive aggregation of proteins, resulting in reduced melt. Lee et al. (2005) reported the occurrence of limited melting in Colby cheeses with pH <5.0 due to decreased repulsion of charges and an increase in electrostatic repulsion. Similar results were also observed by Pastorino et al. (2003), who found reduced heat-induced flowability when the pH of directly acidified cheese injected with 40% CaCl₂ solution dropped below 5.0. These authors also reported that the melting of cheeses with similar content of total Ca was not affected when pH decreased from 5.8 to 5.3, although they did not analyze whether the proportion of INSOL Ca was changed. On the other hand, Guinee et al. (2002) found a

decrease in melting when the pH of Mozzarella cheeses with similar composition and levels of total Ca ranged from 5.42 to 5.93 at 1 d of ripening. Nevertheless, differences in melting were explained by increased levels of intact casein in cheeses that exhibited higher pH. Therefore, several variables along with their interactions have to be considered, in order to understand cheese melting.

6.5.5 Sensory properties

The results obtained by hand firmness were in agreement with the results obtained by TPA hardness (Fig. 6.5), in which textural differences were mainly attributed to composition, different levels of INSOL Ca caused by treatments of WD or LS (Fig. 6.1) and an increase of primary proteolysis during ripening (Fig. 6.2a). An increase of the cohesiveness of the mass (degree to which a masticated sample holds together) during ripening could be associated due to an increase of primary proteolysis in all treatments, whereas lower cohesiveness found in all LF chesses, could also be attributed to lower proteolysis than RF cheeses (Fig. 6.2a). It seemed that the cohesiveness and chewiness of cheese samples were also affected by levels of INSOL Ca, in which lower levels were associated with decreased scores of these attributes, as occurred with cheeses made with LS treatments. A higher protein content in LF cheeses led to increased cohesiveness, when compared with RF samples.

The use of water in LS cheesemilks could reduce the concentrations of minerals in cheese, leading to reduced perception of saltiness. This agrees with the results of

McMahon et al. (2014) who reported that lower levels of native minerals in cheese is associated with decreased saltiness. Reduced scores of acidity found in cheeses made with LS related to lower levels of lactic acid, when compared with WD cheeses (Table 6.4). No changes in acidity during ripening were also in agreement with levels of lactic acid, which only exhibited a slight increase during the first 7 d of ripening, remaining constant thereafter. Despite that β -CN (f1-189/192), an indicator of the formation of a bitter peptide, was formed during the first 30 d of ripening and remained constant thereafter (Fig. 6.4b), panelists only found a significant increase of bitterness after 180 d of ripening. Singh et al. (2005) reported that breakdown of bitter peptide β -CN (f193-209) leads to the formation of others low molecular mass peptides that are relatively more bitter. A possible explanation in our results could be associated with the formation of these types of peptides at the end of ripening, leading to increased bitterness. Increased perception of bitterness could also associate with decreased masking effect caused by reduced perception of saltiness (Singh et al., 2005). In Gouda cheeses, pyruvate intermediates from lactose fermentation may lead to the formation of small amounts of diacetyl, acetoin, acetaldehyde or acetic acid that may contribute to sour flavours (Van den Berg et al., 2004), whereas a reduction in the initial levels of lactose of cheesemilks by LS probably contributed with decreased levels of previous compounds, affecting on cheese sourness.

6.6 Conclusions

The results of this study showed that the concentration of milk by LCF-UF along with the dilution with water can be used as a tool to standardize the lactose content. In addition, this technique also leads to losses of total Ca. The use of LS of cheesemilks at a L:CN of ~1.1 avoided the use of WD during cheese manufacture and led to LF and RF cheeses with similar composition than cheeses made from milks with no LS and WD at levels of 30 and 15%. However, cheeses made from LS had reduced content of residual lactose and galactose at 1 d of ripening, along with lower content of lactic acid that led to increased pH values, when compared with WD treatment. On the other hand, the use of different levels of WD only led to slight differences in these parameters, suggesting that WD has to be based in function of milk composition. As expected, levels of primary proteolysis (expressed by pH 4.6-soluble N) was found higher in RF cheeses in comparison with LF and no differences were found between LS or WD treatments within cheeses varying in fat content. A similar trend was also observed in secondary proteolysis, when expressed by 12% TCA-soluble N, but cheeses made with LS exhibited lower levels when compared by WD treatments. An important observation from this study was that the use of LS led cheeses with significantly lower content in the levels of INSOL Ca, affecting on texture and functional properties of cheeses. The TPA hardness was higher in cheeses with lower fat content. In addition hardness was also significantly lower in cheeses made with LS that had lower levels of INSOL Ca, when compared to cheeses made with WD treatment. The melting properties of cheeses analysed by dynamic small amplitude oscillatory rheology showed that the temperature at which $LT=1$ was significantly reduced in cheeses made with LS,

probably due to decreased levels of INSOL Ca, when compared to treatments with WD. Cheeses made with WD15 had higher softening point than WD30, probably due to a combination of higher INSOL levels and also lower pH values that may lead to increased electrostatic repulsions. Sensory analyses performed by trained panelists were in agreement with the findings of this study. The use of LS led to LF and RF cheeses that were softer, less cohesive, with reduced chewiness, less salty, less acidic and sour. These results suggest that the use of LS could be used as an alternative not only in the manufacture of cheeses with reduced fat content, as may control lactose prior manufacture and hence the development of acidity, but also influences in the proportion of cheese Ca, affecting on texture and functionality of cheese. Further investigation will be required to evaluate, in Gouda and other cheese varieties, the optimum L:CN necessary to obtain desirable cheese pH, along with texture, functionality and sensory properties.

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

This thesis includes three independent sections that involved: (1) the study of one of the most common defects found in low fat cheeses in terms of appearance: cheese translucency (**Chapters 2, 3 and 4**); the exploration of new strategies to improve the properties of low-fat cheeses, based on (2) the addition of ingredients (**Chapter 5**) and (3) the modification of cheesemaking protocols (**Chapter 6**).

A common defect in the appearance of cheeses with reduced fat content is associated with a translucent appearance, which has been extensively estimated by measurements of whiteness based on the CIELAB system (L^* values). However, the appearance of translucent materials is highly affected by size of the sample, especially thickness, which may interfere with loss or reflection of light from the borders or surface, respectively, leading to inaccurate determination of colour. The estimation of cheese translucency based on the Kubelka-Munk theory of reflectance (**Chapter 2**) avoided this problem, since colour measurements were performed in thin layers of samples positioned under black and white background that leads to a reduction of noise from light reflection, leading to reflectance values represented in terms of infinite thickness. An inverse relationship was found in the Kubelka-Munk (K/S) values when estimated by whiteness, i.e., low K/S values should represent low translucency and vice versa. To avoid this problem, Little (1964) proposed, in a first step, to calculate K/S values from tristimulus values (CIE 1931 colour space: X, Y and Z) as reflectance units, and secondly transform the obtained values into the CIELAB colour space (L^* , a^* and b^* values). The Kubelka Munk values obtained from cheeses varying in fat content during heating and cooling temperatures were highly correlated ($r \geq 0.800$) with direct measurements of cheese whiteness by mean of CIE L^* values. However, we did not

establish a relationship between cheese translucency by mean of instrumental and sensory analysis. Dave et al. (2001) reported the transition point between opaque-translucent appearance of non-fat Mozzarella cheese when estimated by visual observations and whiteness measurements (CIE L^* values). Nevertheless, the authors of this study did not define properly, from a sensory point of view, cheese translucency and cheese opacity, or share if visual observations of cheese appearance were performed under standardized conditions of illumination necessary to estimate translucency (MacDougall, 2002), or whether evaluators were trained for sensory analysis.

Despite the fact that the estimation of the K/S by L^* values was useful to estimate cheese translucency, there is incomplete information of the actual appearance of cheese when other variables (CIE a^* and b^* values) are not considered. This is the reason we evaluated the K/S analysis in cheeses by mean of whiteness and chroma (L^* and C^* , respectively) to evaluate how this technique may detect significant differences when whiteness, colour development and a combination of both, were modified by the addition of titanium dioxide, annatto and homogenisation of cheese milk (**Chapter 4**), respectively. The addition of titanium dioxide and annatto led to big impacts in the reduction of translucency and colour development, respectively that also impacted in a reduction of colour intensity (titanium dioxide) and increase of translucency (annatto). In these treatments, K/S were highly correlated with CIELAB measurements. However, in cheeses made with different levels of homogenisation of cheese milks, K/S analysis exhibited significant differences among cheese treatments when expressed by L^* and C^* values. In contrast, CIELAB measurements only showed significant differences in

L* values. In addition, there was no correlation between K/S C* and CIE C*. In this scenario, the use of K/S expressed in C* values was more efficient in finding small differences among treatments than direct measurements of C* in the surface of cheese samples, which could be associated with interference of light reflection from the borders and/or the bottom, whereas the use of K/S was originally developed to reduce this noise effect.

Changes of cheese translucency during the heating of cheese has been previously studied and has been associated with increased of hydrophobic interactions of proteins that lead to the formation of aggregates and increasing light scattering. In this thesis (**Chapter 2**), we developed the measurement of the Temperature Soluble Nitrogen of cheese (TSN) as an indicator of protein aggregation during heating. However, several studies have suggested that during a heat treatment, colloidal calcium phosphate becomes insoluble, leading to the formation of new structures that may also increase light scattering. By studying the effect of heating cheese at different combinations of high temperatures and holding times (**Chapter 3**), we were able to observe that an increase in cheese whiteness was affected by these two variables. By combining the estimation of insoluble colloidal calcium phosphate content and changes in the rheological properties of cheese during heating, we determined that an increase in cheese whiteness was also related to the formation of heat-induced colloidal calcium phosphate that occurred at $\geq 70^{\circ}\text{C}$. Under the same conditions ($\geq 70^{\circ}\text{C}$), the rheological measurements of cheese suggested the formation of heat-induced structures, due to an increase of G' values as holding time increased. However, a decrease of cheese whiteness was observed in those treatments heated at $\geq 70^{\circ}\text{C}$ with extended holding

times, even though levels of insoluble calcium phosphate exhibited a significant increase during holding time and rheological measurements showed that cheeses became stiffer. A possible explanation may be associated with the interaction between reducing sugars, such as lactose and or galactose, and free amino groups, formed by proteolysis, in the cheese matrix which leads to the formation of high molecular compounds that develop browning development (Maillard reaction). These new compounds may also contribute to an increase in G' . With these results, we can update the model system proposed by Metzger et al. (2000), who indicated that changes in cheese appearance during heating only associated with fat melting and protein-protein interactions. Therefore, formation of heat-induced colloidal calcium phosphate and Maillard reaction may also be included.

Addition of hydrocolloids as fat replacer in cheese are associated with an increase in the moisture content, due to their water holding capacity. One of the main limitations in the use of hydrocolloids is associated with the degree of association that may form with milk proteins. In the case of pectin (**Chapter 5**), addition of levels of 0.1 – 0.2% in cheese milk led to destabilization of the casein micelles and their further precipitation. In our study, levels of three commercial pectins (amidated, high methoxy and low methoxy) were selected based in the highest concentration added without the occurrence of casein destabilization. However, this criteria is not recommended, especially for comparison purposes: rennet coagulation curves obtained by dynamic small amplitude oscillatory rheology were completely different among treatments, since nature and concentration of individual pectins were different. In addition, cheese manufacture protocols were similar for all treatments, leading to cheeses differing in

moisture content. In order to obtain comparable results, all cheeses should have similar composition. On the other hand, Ca-gelation mechanisms from amidated- and low-methoxy pectins may lead to solubilization of colloidal calcium phosphate in cheese, which may enhance texture and melting properties of cheese. However, improvement in these properties may be a combined effect between increase moisture content and solubilization of calcium phosphate content. Cheese made with amidated pectin had highest levels of proteolysis and showed higher production of β -CN (f1-189/192), an indicator of bitter peptides, which could be associated with excessive solubilization of colloidal calcium phosphate, probably due to gelation mechanisms between Ca and pectin.

A new approach was proposed (**Chapter 6**), which involved the use of ultrafiltration of milk and water addition to standardize the lactose content, in order to control the content of residual lactose and galactose, along with the development of lactic acid of cheese to potentially replace the use of whey dilution during cheese manufacture, as reduced- and low-fat cheeses may develop excessive acidity. Since water is added into milk, levels of insoluble calcium of cheeses were considerably lower, leading to an improvement in the textural and melting properties of cheese, when compared to those treatments made with whey dilution. Levels of lactose selected for standardization by the ultrafiltration-water addition approach were based on a preliminary study of a full-fat Gouda cheese. However, we did not account for increasing levels of protein as occurred in low- and reduced-fat Gouda cheeses, leading to very high cheese pH, due to low levels of lactic acid, and excessive solubilization of

insoluble colloidal calcium phosphate, which led to extremely soft cheeses that even lost their block shape during 6 mo of ripening at refrigeration storage.

Based on our research, the following recommendations for future work are suggested:

1. Apply the Kubelka-Munk theory of reflectance could be applied to predict changes of appearance based on cheese composition, the addition of ingredients, heat treatment or modification of processing steps.
2. Establish the relationship between cheese translucency based on instrumental and sensory analysis.
3. Evaluate the effect of the reversible effect of cooling on the translucency of cheese after heating and the influence of the stability of heat-induced colloidal calcium phosphate, along with its effect on the rheological properties of cheese.
4. Development of a model system to understand the role of soluble calcium ions on cheese translucency.
5. Establish the influence of Maillard reaction during the heating of cheese, along with changes of colour based on protein-protein interactions, proportion of insoluble colloidal calcium phosphate and rheological properties.
6. Increase moisture content of reduced-fat cheeses by adding hydrocolloids as a good alternative to improve their properties. However, correction factors should be made at cutting the gel at similar levels of firmness to achieve similar moisture content to make a proper comparison among treatments.

7. Add of pectin to cheesemilks at levels near the point in which casein micelles are destabilized is not recommended. As an example, treatment made with amidated pectin had a considerably weaker gel at cutting, levels of insoluble calcium in cheese was the lowest, had a low pH and developed bitter peptides. Therefore, evaluation of the effect of individual pectins at different concentrations levels is suggested.
8. Addition of pectin into cheesemilk may not be the most convenient alternative to manufacture cheese. Some of the pectin may be lost in the whey and therefore, further separation steps would have to be considered in order to remove hydrocolloids before whey processing. As an alternative, pectin could be microencapsulated and then incorporated into cheesemilk. In that scenario, pectin would not interact with casein micelles and the majority of pectin will be retained in the curd. However, cost of ingredients and processing could be a limiting factor. On the other hand, pectin could be directly incorporated in further steps during cheese manufacture, such as dry salting (i.e., direct application to cheese curds) as shown in other studies from our group (Marchiani et al., 2016).
9. Evaluation of the effect of pectin on the sensory properties of cheeses is recommended, as the literature has indicated that hydrocolloids may develop off-flavours.
10. Lactose to casein ratios should be standardized based on the (expected) composition of cheese, in order to obtain optimum values of cheese pH, insoluble calcium phosphate content and functionality.

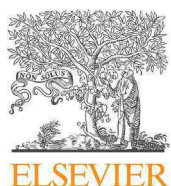
11. Levels of insoluble calcium in cheeses made with lactose standardization were lower due to the excessive addition of water. As an alternative, the use of nanofiltration permeate could be used to adjust the lactose content, without altering levels of insoluble colloidal calcium phosphate.
12. The approach of standardizing the lactose to casein ratio of cheese milk can also be applied in other cheese varieties to reduce levels of lactic acid in cheese and control the formation of calcium lactate crystals.
13. Reducing levels of residual lactose and galactose may have an effect on browning development of cheese and reduce some undesired defects in appearance of heated cheese.

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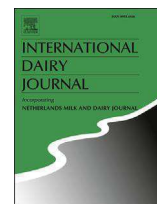
**APPENDIX:
PUBLICATIONS**

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Effect of fat content and temperature on the translucency of Cheddar cheese



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ABSTRACT

Cheese translucency is influenced by fat content, ripening and temperature and is traditionally determined by CIELAB whiteness. However, translucency is also affected by thickness of materials. The Kubelka-Munk method (K/S) estimates the ratio of absorbance to scatter from whiteness measurements on thin layers of materials above white and black backgrounds. The effect of fat content and temperature on Cheddar cheese translucency was evaluated using K/S and CIELAB methods. Cheddar cheeses varying in fat composition were made and translucency was measured during heating/cooling at 2 and 180 d of ripening. Low fat content in cheese was found to be related to high translucency at <30 °C. At 40 °C, translucency increased due to fat melting. At >60 °C, a reversible protein aggregation led to a reduction of translucency. Translucency increased as cheeses aged. CIELAB and K/S methods were highly correlated; K/S could therefore be useful for estimating cheese translucency.

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1. Introduction

Colour is considered one of the most important attributes in food appearance; it is associated with certain parameters of quality, such as composition and deterioration and has a big impact on consumers preference (MacDougall, 2002). In cheese, colour is highly influenced by processing conditions, addition of colouring agents, composition, pH, age and temperature (Dave, McMahon, Broadbent, & Oberg, 2001; Joshi, Muthukumarappan, & Dave, 2003; Metzger, Barbano, & Kindstedt, 2001; Wadhwani & McMahon, 2012). The influence of fat content in cheese not only relates to texture and flavour properties, but also has a big impact on colour and appearance. One of the major appearance defects found in reduced-fat cheeses is associated with an increase in translucency, due to different light scattering properties of fat and protein (Johnson, Kapoor, McMahon, McCoy, & Narasimmon, 2009), which are temperature-dependent (Metzger, Barbano, Rudan, Kindstedt, & Guo, 2000).

The measurement of whiteness has been used extensively to determine the degree of cheese translucency (Dave et al., 2001; Joshi et al., 2003; Metzger et al., 2000, 2001; Rudan & Barbano, 1998; Wadhwani & McMahon, 2012). The level of whiteness is represented on the CIELAB colour scale as the L^* value, which

ranges from 0, which represents black, to 100, representing white (i.e., a perfect reflecting diffuser; Hunterlab, 2012). However, in the study of translucent materials, the use of traditional methods of colour measurement may not represent the actual colour of samples as perceived by the human eye, due to reflection of light from below the surface (Gullett, Francis, & Clydesdale, 1972) and is greatly influenced by the thickness of samples (Calvo & Salvador, 1997; Little, 1964). Independent of the type of material (translucent or opaque), the reflection of light is highly dependent of the ratio of absorption to scatter, which is affected by levels of pigmentation, refractive index and light-scattering properties of the material (MacDougall, 2002). The importance of the study of the ratio of absorption to scatter coefficients on translucent materials has to be considered to obtain an adequate concept of colour appearance (Judd & Wyszecki, 1975; MacDougall, 2002).

The Kubelka-Munk index (K/S) is calculated from an empirical formula that determines the ratio of absorbance, K , to scatter, S , based on the measurement of reflectivity, i.e., the reflectance of a certain material at infinite thickness (Judd & Wyszecki, 1975). This analysis has been applied extensively to predict the optical properties of materials used in paint, textile, plastics and other industries (MacDougall, 2002). The application of the Kubelka-Munk theory of reflectance has also been used in different food products such as fruit gels, orange juice, meat products, milk and coffee (Calvo & Salvador, 1997; Gullett et al., 1972; Huang, Francis, & Clydesdale, 1970; MacDougall, 2002).

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This study aimed to evaluate the optical properties of Cheddar cheeses by means of the Kubelka-Munk analysis; in particular, the effect of fat content, cheese age and heating and cooling were studied. A relationship between this methodology and the measurement of whiteness by the L^* values was also established.

2. Materials and methods

2.1. Cheese manufacture

A standard Cheddar cheese manufacturing procedure was conducted at the pilot plant facilities of the School of Food and Nutritional Sciences, University College Cork, Ireland. Experimental cheeses were manufactured on a 50 kg scale, from cheesemilk standardised to fat levels of 3.50%, 2.63%, 1.75% or <0.1% (maintaining casein to fat ratios of 0.70, 1.05, 1.40 and ≥ 24.50 , respectively), to obtain four different cheeses: full fat (FF), reduced fat (RF), half fat (HF) and non-fat (NF). Each standardised cheesemilk was pasteurised at 73.5 °C for 15 s and cooled to 31 °C. A concentrated starter culture (R-604Y Cheddar culture starter, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into the vats at a level of 0.03% (w/w) and left to ripen for 30 min under continuous stirring, then 0.09% (v/w) of 1 M CaCl_2 was added and equilibrated for 2 min. Chymosin (Maxiren 180, DSM Food Specialties, Delft, Netherlands) at a strength of 180 International Milk-Clotting Units (IMCU mL^{-1}) was added to each cheesemilk at a level of 0.03% (v/w), diluted 1 to 4 with distilled water to aid dispersion. Once the coagulum developed enough firmness after 45–50 min, curds were cut and cooked from 31 to 39 °C over a period of 30 min. The temperature was maintained at 39 °C until the pH of curds decreased to 6.2 and the whey was drained from the vats. The curd was cut into blocks and inverted every 15 min until the pH reached 5.4. Curd blocks were milled, salted at a level of 2.5% (w/w) NaCl and equilibrated for 20 min. The salted curd was transferred to 5 kg rectangular moulds and pressed for 14 h at 2.5 kg cm^{-2} at 20 °C. The experimental cheeses were vacuum-packed and ripened for 6 months at 8 °C.

2.2. Chemical analysis

Composition of experimental cheeses was determined after 14 d of ripening. Moisture content was measured by the oven drying method (IDF, 1982), fat by the Gerber method (IIRS, 1955), protein by the macro-Kjeldahl method (IDF, 1986) and salt content by potentiometric determination (Fox, 1963). Total calcium was determined by atomic absorption spectroscopy (IDF, 2007) on the cheesemilk, rennet whey and experimental cheeses. The proportion of insoluble calcium in cheese (INSOL Ca) was determined by the acid-base titration method after 2 and 180 d of ripening as described by Hassan, Johnson, and Lucey (2004). The pH of experimental cheeses was measured at 20 °C on a homogenised mixture of 10 g of cheese and 10 mL of deionised water using a glass pH electrode (Madkor, Fox, Shalabi, & Metwalli, 1987). Proteolysis during ripening was determined by the pH 4.6-soluble N (Kruchoo & Fox, 1982).

2.3. Translucency during heating

The degree of translucency of experimental cheeses during heating and cooling was evaluated by the determination of the Kubelka-Munk values and the measurement of CIE L^* values at 2, 14, 30, 40, 120 and 180 d of ripening. Measurements were performed with a Konica-Minolta colorimeter CR-400 attached to a data processor DP-400 (Konica Minolta Optics Inc., Osaka, Japan). The instrument was set on the CIELAB system based on illuminant D65

and a visual angle of 2°. A white calibration plate CR-A43 (Y: 87.1, x: 0.3186, y: 0.3364) was used to calibrate the instrument before analysis. All the measurements were performed through an 8 mm diameter diaphragm containing a glass light projection tube.

2.3.1. Kubelka-Munk index

The Kubelka-Munk formula relates the coefficient of absorption K and scatter S to the reflectance of a material of infinite thickness R_∞ , as described in Equation (1):

$$K/S = (1 - R_\infty)^2 / 2 R_\infty \quad (1)$$

where

$$R_\infty = a - b \quad (2)$$

$$a = 1/2 [R + (R_0 - R + R_g)/R_0 R_g] \quad (3)$$

$$b = (a^2 - 1)^{1/2} \quad (4)$$

From Equations (2)–(4), it is possible to establish R_∞ based on the reflectance R of a sample positioned above a white background with a known reflectance R_g and the reflectance of the sample placed on a black background R_0 (Judd & Wyszecki, 1975). Previous studies have shown that K/S values can be expressed on the CIE system (Calvo & Salvador, 1997; Gullett et al., 1972; Huang et al., 1970). In the present study, the K/S values were based on L^* values ($K/S L^*$) obtained from samples placed above black and white backgrounds (L_B^* and L_W^* , respectively). The contrast ratio (ratio between L_B^* and L_W^* : CR L^*), which correspond to an indicator of opacity (Judd & Wyszecki, 1975) was also estimated. A black ceramic tile (Y: 3.8, x: 0.3129, y: 0.3294) was used as a black background and the white calibration tile (previously described) was used as a white background.

Cheese samples were cut into discs (35 mm diameter and 2 mm height), placed in plastic petri dishes (90 mm internal diameter), coated with a liquid paraffin layer (Fisher Scientific UK Limited, Loughborough, UK) and covered with the petri dish lid to prevent loss of moisture. All the samples were preincubated for 30 min in a cold room at 4 °C. Samples were then incubated at 4, 20, 30, 40, 60 and 80 °C for 30 min. After treatment, the CIE L^* value was measured in the middle of each cheese disc placed on black and white backgrounds. Five cheese discs were incubated at each temperature and two measurements per cheese disc were made on each background. After measurements, samples were incubated at their respective lower temperature for an additional 30 min and colour was measured again on each background. Petri dishes coated with a layer of liquid paraffin were also preincubated and incubated under the same conditions as experimental cheeses to obtain L^* values on black and white backgrounds and used them in Equation (3) as R_0 and R_g , respectively.

2.3.2. CIE L^* values

Samples were prepared according the method described by Dave et al. (2001) with some modifications. Cheese samples were cut into cylinders (26 mm of diameter and 28 mm of height), placed into 10 mL Pyrex® beakers ensuring that cheese samples were in contact with the walls and the bottom of the glass. Beakers were sealed with rubber stoppers to prevent loss of moisture during treatment. The preincubation and incubation stages were made under the same conditions as in cheese discs. After heat treatment, the CIE L^* values were measured through the bottom of the beakers.

Five cheese cylinders were incubated at each temperature and two colour measurements were performed per cylinder.

2.4. Temperature soluble nitrogen

Nitrogen solubility in a heat-treated aqueous phase model of experimental cheeses was determined to evaluate the role of protein aggregation on cheese translucency at 2 and 180 d of ripening. Synthetic Cheddar cheese aqueous phase (SCCAP) solutions were prepared for each experimental cheese according to the method of Broome and Limsowtin (2002) as described by O'Mahony, McSweeney, and Lucey (2006) with some modifications. The pH of SCCAP was adjusted to the values found for the experimental cheeses and the calcium content was adjusted with calcium chloride to obtain the calcium concentration found in the soluble phase of each experimental cheese at 2 and 180 d of ripening. Fifty grams of grated cheese was mixed with 100 mL of SCCAP at 10,000 revolutions s^{-1} for 5 min with an Ultra-Turrax® homogeniser (T25 Basic with S25N-18G dispersing element; IKA-Labortechnik, Staufen, Germany). The mixture was stored overnight at 4 °C with continuous stirring and centrifuged at $3,000 \times g$ for 30 min at 4 °C. The supernatant obtained was filtered at 4 °C through glass wool and Whatman 113 filter paper. Aliquots of the filtered supernatants were incubated under the same conditions as of experimental cheeses during heat treatment in a waterbath. After incubation, samples were centrifuged at $15,000 \times g$ for 5 min at 20 °C, placed for additional 5 min at the incubation temperature and centrifuged again under the same conditions previously described for additional 5 min. Samples were then filtered through a 0.45 μm filter material (Millex®; Millipore Corporation; Bedford, MA, USA). The nitrogen content of the filtered sample was determined by the Kjeldahl method to estimate the temperature soluble nitrogen (TSN) content as a percentage of the total nitrogen (TN) found on cheese (%TSN/TN), as described in Equation (5):

$$\% TSN/TN = 2 TNS (MAP + MC/2)/TNC \quad (5)$$

where TNS corresponds to the total nitrogen content of the treated sample, MAP is the moisture content of the SCCAP, MC is the moisture content of the cheese sample and TNC is the total nitrogen of the cheese sample.

To evaluate the reversible effect of cooling on aggregation, samples were heated at 80 °C for 30 min, cooled to 4 °C for 24 h and the content of %TSN/TN was also estimated. In addition, urea-polyacrylamide gel electrophoresis (Andrews, 1983) was performed on these treated samples to characterise the protein profile of the soluble aqueous phase model from cheese. Gels were stained with Coomassie blue G250 as described by Blakesley and Boezi (1977).

2.5. Experimental design and statistical analyses

Four treatments based on different fat levels (FF, RF, HF and NF) were used in three independent trials, based on a randomised 4×3 block design. Analysis of variance (ANOVA) was performed on cheese composition at a significance level of $P < 0.05$. A split-split-plot design (Montgomery, 2013) was used to evaluate the effect of treatment (fat content: FF, RF, HF and NF), ripening time, temperature and their interactions on the translucency of cheese determined by the K/S index, CIE L^* values and TSN. A split-plot design (Montgomery, 2013) was used to evaluate the effects of treatment, ripening and their interactions on proteolysis, L^*_B values, L^*_W values and CR L^* . The ANOVA for both the split-split-plots and the split-plots designs was performed using a general linear model (GLM) procedure. When significant differences ($P < 0.05$) were found, the treatments means were analysed by the Tukey's multiple

Table 1

Composition (14 d), percentage of insoluble calcium as a percentage of total cheese Ca and pH values (2 and 180 d) of Cheddar cheeses with various fat levels.^a

Parameter	Treatment				SEM
	FF	RF	HF	NF	
Moisture (%)	37.81 ^c	39.54 ^{cb}	42.34 ^c	47.74 ^a	1.21
Fat (%)	32.44 ^a	23.89 ^b	19.00 ^c	nd	1.98
Protein (%)	24.47 ^d	27.84 ^c	30.46 ^b	41.50 ^a	1.94
Salt (%)	1.69 ^a	1.60 ^a	1.73 ^a	1.82 ^a	0.063
MNFS (%)	55.95 ^a	51.96 ^b	52.25 ^b	—	0.921
FDM (%)	52.17 ^a	39.54 ^b	32.96 ^c	—	2.84
S/M (%)	4.46 ^a	4.05 ^a	4.09 ^a	3.81 ^a	0.158
Total calcium (mg 100 g ⁻¹)	761 ^d	888 ^c	985 ^b	1412 ^a	75.9
INSOL Ca/total Ca					
2 d	65.05 ^b	66.38 ^b	71.81 ^a	65.03 ^b	1.15
180 d	52.46 ^b	55.81 ^b	60.68 ^{ab}	64.71 ^a	1.68
pH					
2 d	5.21 ^{ab}	5.17 ^b	5.20 ^b	5.31 ^a	0.019
180 d	5.13 ^c	5.22 ^b	5.24 ^b	5.44 ^a	0.035

^a Abbreviations are: MNFS, moisture in the non-fat substance; FDM, fat content on a dry basis weight; S/M, salt in the moisture phase of the cheese; INSOL Ca, insoluble calcium. Data are means of three replicate trials; means within the same row not sharing a common superscript letter differ ($P < 0.05$); nd, not detected (by the Gerber method).

comparison test. Pearson correlation coefficients were determined among the translucency values measured with K/S L^* , CR L^* and L^* values at different temperatures during heating and cooling ($P < 0.05$). All analyses were performed using Minitab® 16 (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Cheese composition, pH and level of insoluble calcium

Means of three independent trials for composition, percentage of insoluble calcium and pH from experimental cheeses are shown in Table 1. As expected, significant differences were found in composition of cheeses as the fat content was reduced ($P < 0.05$), leading to a marked increase in moisture, protein and calcium content ($P < 0.05$). A reduction in the fat content of cheese leads to a shift in the compositional balance of most of the other cheese

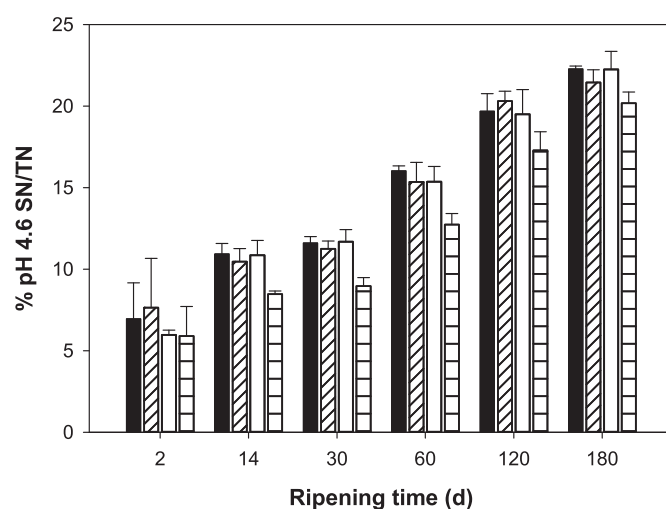


Fig. 1. Changes in proteolysis expressed as pH 4.6 soluble N as a percentage of total N (% pH 4.6 SN/TN) of full fat (■), reduced fat (▨), half fat (□) and non-fat (▤) Cheddar cheeses during ripening. Values represent mean and standard deviations of three replicate trials.

Table 2
Mean squares, probabilities (in parenthesis) and R^2 values for proteolysis, CIE whiteness (L^*) on black and white backgrounds and contrast ratio based on whiteness during ripening for full fat (FF), reduced fat (RF), half fat (HF) and non-fat (NF) Cheddar cheeses.^a

Factors	df	Proteolysis	L^*_B	L^*_W	CR L^*
Whole Plot					
Day of cheese manufacture (D)	2	2.959(0.306)	75.410*(0.029)	42.960*(0.031)	0.002*(0.047)
Treatment (T)	3	21.425***(0.008)	2757.420***(<0.001)	851.62***(<0.001)	0.154***(<0.001)
Error (D $\times$ T)	6	2.036	11.09	6.600	0.0004
Subplot					
Age (A)	5	392.140***(<0.001)	328.800***(<0.001)	151.670***(<0.001)	0.009***(<0.001)
T $\times$ A	15	0.844(0.694)	14.840*(0.018)	4.090(0.200)	0.002***(<0.001)
Error	40	1.088	6.440	2.950	0.0003
R^2		0.98	0.98	0.97	0.98

^a Split-plot design with the 4 treatments (fat content: FF, RF, HF and NF) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of age and treatment $\times$ age as variables. Significance levels are indicated by single and double asterisks: *, $0.01 < P \leq 0.05$; **, $P \leq 0.01$. Proteolysis determined by % pH 4.6 SN/TN. Abbreviations are: df, degrees of freedom; L^*_B , whiteness (CIE L^* values) determined on a black background at 20 °C; L^*_W , whiteness (CIE L^* values) determined on a white background at 20 °C; CR L^* , contrast ratio based on L^* values at 20 °C.

components (Mistry, 2001). At 2 d of ripening, levels of INSOL Ca ranged about 65%–70%, although HF exhibited a higher percentage than other treatments ($P < 0.05$). A decrease of INSOL Ca was observed at 30 d of ripening ($P < 0.05$; data not shown) and remained constant until 180 d, except for NF cheese that remained constant during ripening ($P > 0.05$). Hassan et al. (2004) reported that the proportion of INSOL Ca in Cheddar cheese decreases during the first 30 d of ripening and remains constant thereafter. On the other hand, Metzger et al. (2001) found no changes in the proportion of water-insoluble calcium and an increase in pH during

90 d of storage of low-fat Mozzarella cheese (6% fat). The pH values at 2 d were slightly different, ranging between 5.2 and 5.3 ($P < 0.05$). The pH at 180 d remained constant for FF ($P > 0.05$) and increased in RF, HF and NF cheeses ($P < 0.05$). This increase is associated with a reduction of INSOL Ca, causing the release of phosphate ions from colloidal Ca phosphate, leading to their combination with hydrogen ions and hence an increase in pH (Hassan et al., 2004). This increase was also higher in those cheeses with a lower fat content, given by a higher buffering effect due to a higher protein and calcium content.

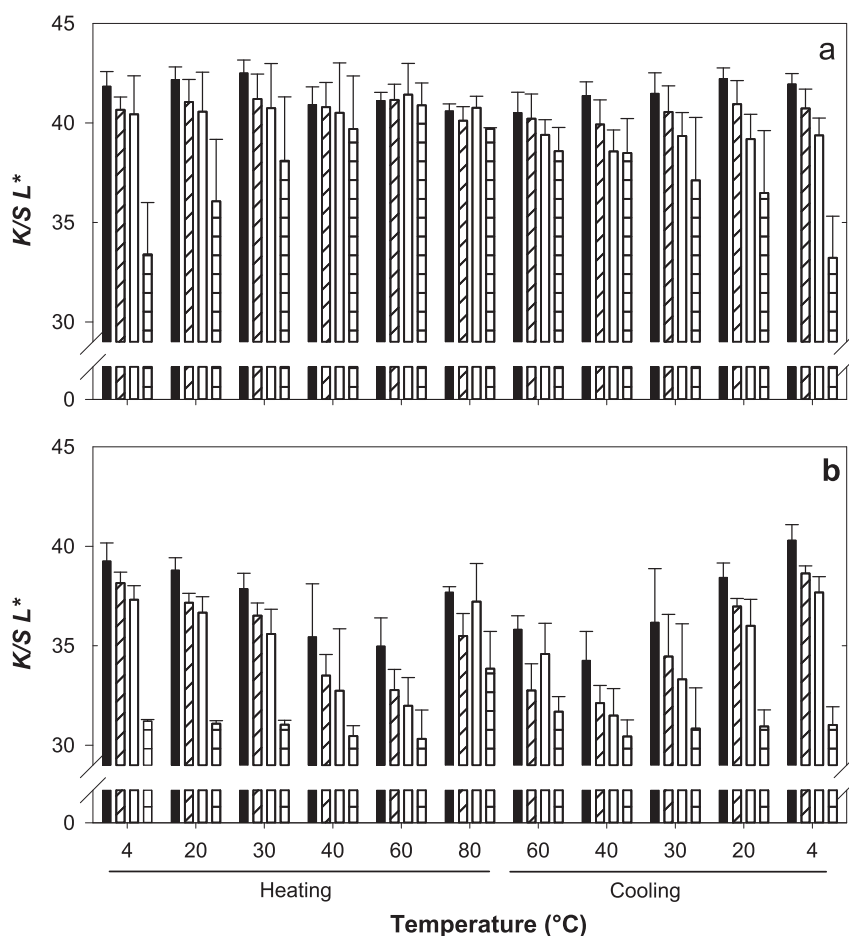


Fig. 2. Changes in the Kubelka-Munk index determined by L^* values ($K/S L^*$) during heating and cooling of full fat (■), reduced fat (▨), half fat (□) and non-fat (▤) Cheddar cheeses at 2 (a) and 180 d of ripening (b). Values represent mean and standard deviations of three replicate trials.

Table 3Mean squares, probabilities (in parenthesis) and R^2 values for $K/S L^*$ index and CIE L^* whiteness for experimental cheeses.^a

Factor	df	$K/S L^*$	CIE L^*	df	TSN
Whole-Plot					
Day of cheese manufacture (D)	2	75.107(0.085)	49.360(0.630)	2	14.53(0.636)
Treatment (T)	3	302.330**(<0.001)	3399.64**(<0.001)	3	70.260*(0.045)
Whole-Plot error D $\times$ T	6	9.042	135.860	6	14.06
Subplot					
Ageing (A)	1	1822.922**(<0.003)	13554.690**(<0.001)	1	6333.62**(<0.003)
T $\times$ A	3	10.441(0.229)	510.590*(0.012)	3	107.810*(0.042)
Subplot error					
D $\times$ A	2	6.310	3.600	2	20.250
D $\times$ T $\times$ A	6	5.471	55.970	6	20.810
Sub-subplot					
Temperature (H)	10	13.714**(<0.001)	344.640**(<0.001)	6	380.570**(<0.001)
T $\times$ H	30	8.544**(<0.001)	167.490**(<0.001)	18	2.570**(<0.001)
A $\times$ H	10	26.594**(<0.001)	367.800**(<0.001)	6	20.970**(<0.001)
T $\times$ A $\times$ H	30	0.917(0.313)	15.970*(0.045)	18	19.160**(<0.001)
Sub-subplot error	160	0.815	10.300	96	1.670
R^2		0.97	0.96		0.99

^a Split-Split-plot design with the 4 treatments (fat content: FF, RF, HF and NF) were analysed as a discontinuous variable and cheese manufacture day was blocked. Subplot included the effect of ageing of cheese (A) and treatment $\times$ age as variables. Sub-subplot included the effect of temperature of heating and cooling (H), treatment $\times$ heat, age $\times$ heat and treatment $\times$ age $\times$ heat as variables. Significance levels are indicated by single and double asterisks; *, $0.01 < P \leq 0.05$; **, $P \leq 0.01$. Abbreviations are: df, degrees of freedom (differed for $K/S L^*$, CIE L^* , and TSN); $K/S L^*$, translucency determined by the Kubelka-Munk analysis; CIE L^* , whiteness determined by the CIE L^* value; TSN, temperature soluble N as a percentage of total cheese N.

3.2. Proteolysis

Proteolysis expressed as the level of pH 4.6-soluble N of experimental cheeses is shown in Fig. 1. It was found that treatment and

age of cheese had a significant effect on the level of proteolysis (Table 2). During the first stages of ripening, similar levels of pH 4.6-soluble N were found in all treatments. Proteolysis significantly increased during ripening and it was found to be lower in NF after

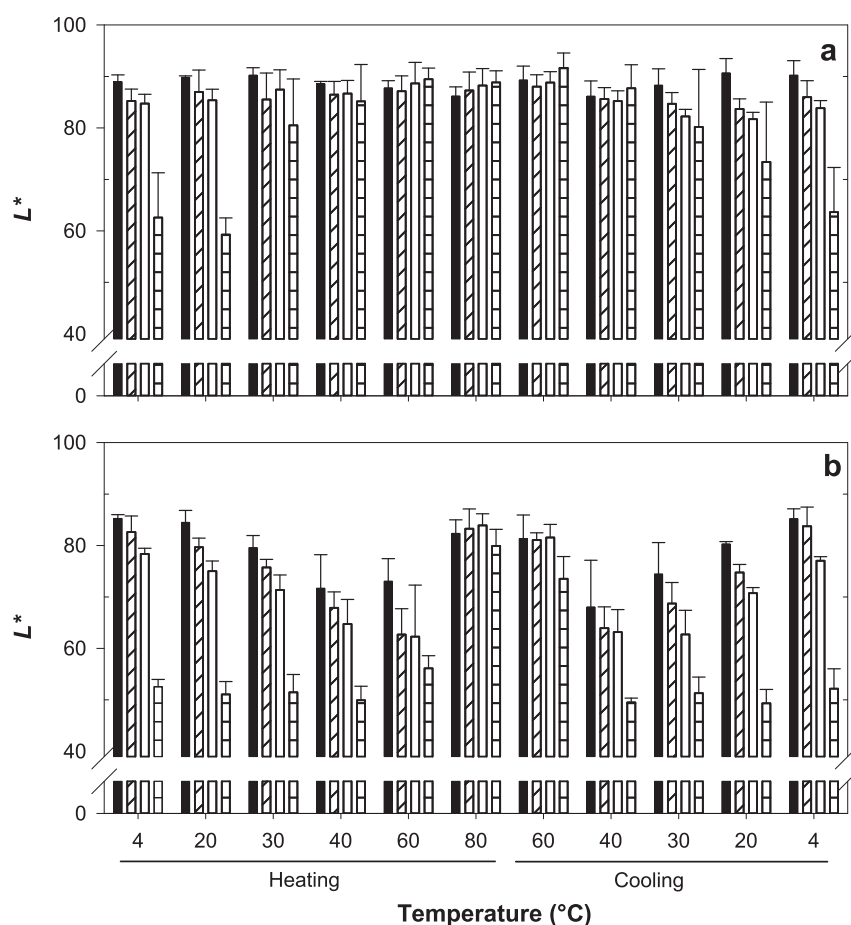


Fig. 3. Changes in whiteness (L^* values) during heating and cooling of full fat (■), reduced fat (▨), half fat (□) and non-fat (▤) Cheddar cheeses at 2 (a) and 180 d of ripening (b). Values represent mean and standard deviations of three replicate trials.

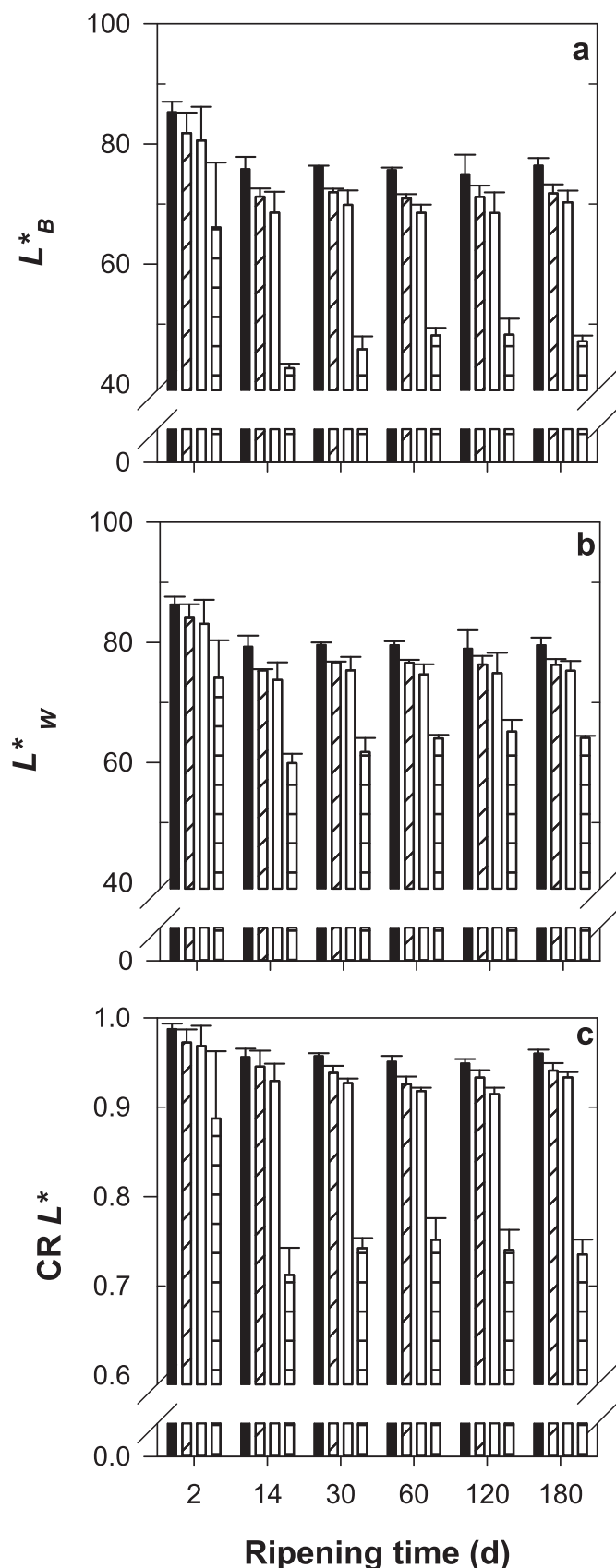


Fig. 4. Changes in whiteness measured above black (a) or white (b) backgrounds and contrast ratio (c) of full fat (■), reduced fat (▨), half fat (□) and non-fat (▩) Cheddar cheeses during ripening. Values represent mean and standard deviations of three replicate trials.

14 d of ripening and similar in other treatments ($P < 0.05$). This trend was also observed thereafter. At 180 d, no differences were found among cheeses ($P > 0.05$). A reduction in primary proteolysis in cheese with reduced fat content is attributed to a concomitant decrease in the level of moisture-in-non-fat-substances (MNFS; Table 1) and hence a reduction in the availability of water for microbial and enzyme activity (Fenelon & Guinee, 2000). In addition, the extent of proteolysis is highly dependent of the moisture and salt content of cheese (Mistry & Kasperson, 1998). In our study, the MNFS content was higher only in FF and no differences in the S/M content were found (Table 1), which may be related to the similar levels of proteolysis at the end of ripening.

3.3. Cheese translucency

3.3.1. Kubelka-Munk analysis

Fat content of experimental cheeses had a significant effect ($P < 0.05$) on the K/S L^* values during heating and cooling at different points of ripening (Fig. 2; Table 3). It was also found that K/S L^* values were affected ($P < 0.05$) by the interaction of treatment $\times$ temperature and age $\times$ temperature. The K/S index is defined as the ratio of the absorption (K) to the scattering (S) coefficient (Judd & Wyszecki, 1975). At 2 d of ripening K/S L^* values at 4 °C decreased as the fat content was reduced ($P < 0.05$; Fig. 2a). This finding was opposed to what was expected, as lower K/S values are related to a higher scattering and hence a lower translucency. However, a reversal of this relation has been previously observed when K/S is expressed in terms of whiteness due to a considerable increase of S and a reduction of K and also to the sensitivity of L^* value on scattering effects (Gullett et al., 1972; Huang et al., 1970). There were no variations in K/S L^* values during heating and cooling for FF, RF and HF cheeses ($P > 0.05$). On the other hand, the K/S L^* values of NF cheese increased as the sample was heated, reaching the same level as other cheeses at 40 °C ($P > 0.05$) and remaining constant thereafter. The same trend was observed when heated samples were cooled to their previous temperature stage and held for additional 30 min. We observed no changes in the translucency of FF, RF and HF cheeses, whereas NF exhibited a gradual decrease in K/S L^* as the temperature decreased.

During ripening, the K/S L^* value decreased for all cheeses ($P < 0.05$). As no significant differences were found for K/S L^* values at different temperatures from 14 to 180 d of ripening ($P > 0.05$; data not shown), this study only reports the translucency results obtained after 180 d of ripening (Fig. 2b). The K/S L^* values of experimental cheeses at 4 °C also decreased as the fat content was reduced ($P < 0.05$). During heating, a decrease in K/S L^* was observed for FF, RF and HF cheeses from 4 to 40 °C ($P < 0.05$), remaining constant until 60 °C. At 80 °C, K/S L^* values increased to the same levels found at 4 °C for each respective cheese ($P < 0.05$). On the other hand, K/S L^* values of NF were constant when heated from 4 to 60 °C and exhibited an increase only at 80 °C. During cooling, a decrease of K/S L^* was observed for FF, RF and HF from 80 to 40 °C to increase again to the same levels found before heating. On the other hand, cooling of NF cheese from 80 to 40 °C caused higher K/S L^* values than heating.

3.3.2. CIE L^* whiteness

Similar trends as K/S L^* values were obtained for the measurement of cheese translucency by L^* values (Fig. 3). A significant effect on the fat content ($P < 0.05$) was also observed on heating and cooling during ripening (Table 3). The L^* values were also affected by the interactions of treatment $\times$ age, treatment $\times$ temperature, age $\times$ temperature and treatment $\times$ age $\times$ temperature. A decrease in L^* values at 4 °C was found as the fat content was reduced after 2 d of ripening (Fig. 3a; $P < 0.05$). No differences ($P < 0.05$) in L^*

values were found for FF, RF and HF during heating. Only NF exhibited an increase of L^* value from 4 to 40 °C, remaining constant at higher temperatures. The L^* values of FF, RF and HF cheeses showed no differences when samples were cooled down, whereas NF exhibited a decrease.

The L^* values decreased at 180 d of ripening (Fig. 3b; $P < 0.05$). FF, RF and HF exhibited a decrease in L^* values from 4 to 40 °C, remaining constant at 60 °C and increased again at 80 °C to the same value as that of FF at 4 °C ($P > 0.05$). On the other hand, the L^* values of NF were constant from 4 to 40 °C, exhibiting a slight increase at 60 °C and at 80 °C values reached the same level of whiteness as other cheese samples ($P > 0.05$). We observed higher L^* values in those samples initially heated at 80 °C and then cooled to 60 °C in all cheeses ($P < 0.05$).

3.3.3. Contrast ratio

When translucency was calculated by the $K/S L^*$ method, we found differences in the individual measurements of whiteness obtained in cheese discs above black or white backgrounds. Fig. 4 shows the CIE L^* values of 2 mm cheese layers measured above black (L^*_B) or white (L^*_W) backgrounds and its contrast ratio ($CR L^*$) at 20 °C for different ripening times. For each background, whiteness was found to be higher in those cheeses with a higher fat content (Fig. 4a and b; $P < 0.05$). We also found a decrease in L^* values after 14 days of ripening (Table 2). The $CR L^*$ values decreased as the fat content of cheese was reduced and all samples showed a decrease in this parameter with cheese age (Fig. 4c; Table 2). Judd and Wyszecski (1975) indicated that a contrast ratio of 0.98 is equivalent to a completely opaque material, although it is influenced by the thickness of the sample. Under the conditions studied, only FF, RF and HF were found to be opaque at 2 d of ripening, which corresponds to $K/S L^* > 40$ and CIE L^* values > 85 . Our data agree with the results of Dave et al. (2001) who determined by visual observations that salted and unsalted non-fat Mozzarella cheeses were opaque when L^* values were higher than 85. These authors also indicated that a CIE L^* value of 82 corresponds to the transition point from opaque to translucent. However, visual observations may lead to some mistakes, even under standardised conditions of illumination (Judd & Wyszecski, 1975). In addition, the $CR L^*$ values showed a similar trend to $K/S L^*$ and L^* values during heating and cooling of cheese samples (data not shown).

3.3.4. Influence of cheese composition, temperature and ripening

As expected, a lower fat content in cheese is associated with a reduction in the number of light-scattering centres and an increase in the protein and moisture content lead to a translucent appearance (Johnson et al., 2009; Mistry, 2001). The $K/S L^*$, $CR L^*$ and CIE L^* parameters showed differences in translucency based on the fat content. At 2 d of ripening, none of the methods was able

to find differences in the translucency of FF, RF and HF during heating and cooling; however NF exhibited a decrease of translucency at ~30 °C. A significant increase in L^* values during heating at 30 °C was reported by Dave et al. (2001) for non-fat Mozzarella cheese at 1 d of ripening, probably due to protein–protein interactions. However, cheese fat could also influence the optical properties of FF, RF and HF cheeses. During heating, a high proportion of milk fat melts in the range of 10–20 °C and is completely liquid at about 40 °C (MacGibbon & Taylor, 2006), resulting in a reduction of light-scattering and hence an increase in translucency. At 180 d of ripening, the methods used showed an increase in translucency for all cheese samples. Translucency increased when FF, RF and HF cheeses were heated to 40 °C and was reduced again on heating at 80 °C, whereas NF exhibited a decrease in translucency at 60 °C. A reduction in cheese translucency during heating has been previously related to changes in the serum phase of the cheese. Metzger et al. (2000) found the formation of a white gel when the expressible serum of low-fat and full-fat low moisture part skim Mozzarella cheese was heated from 7 to 49 °C, which is associated with a decreased protein solubility as hydrophobic interactions are increased with temperature.

Translucency is also related to changes in cheese structure. A shrinkage of the cheese matrix during heating at high temperatures leads to the expulsion of fat and water (Guinee, Auty, & Fenelon, 2000). Pastorino, Dave, Oberg, and McMahon (2002) found a denser matrix when they observed, by electron microscopy, the microstructure of non-fat Mozzarella cheese heated at 50 °C, and found the formation of new serum pockets and protein aggregates of high density, suggesting that protein–protein interactions were promoted by an increase in temperature. We observed a reduction of $K/S L^*$, $CR L^*$ and CIE L^* values as cheese age increased, which might be influenced by an increase in proteolysis (Fig. 1) and a reduction of the proportion of INSOL Ca (Table 1). An increase in levels of cheese proteolysis with age is related to an increase in translucency and higher heating temperatures to produce a reduction in translucency (Dave et al., 2001; Metzger et al., 2000, 2001). The content of calcium in cheese had no effect on cheese translucency at 5 °C; however, a marked reduction was observed at 60 °C as the calcium content was increased (Joshi et al., 2003). In addition, a decrease in the proportion of water-insoluble calcium in low-fat Mozzarella cheese during ripening was associated with higher translucency during heating and cooling (Metzger et al., 2001).

An increase in cheese translucency during cooling has been reported previously. Metzger et al. (2000) attributed this phenomenon to an increase in the solubility of light scattering particles formed during heating from the cheese serum phase. At lower temperatures (< 40 °C) an increase of the cheese whiteness occurs due to solidification of cheese fat.

Table 4

Pearson correlation (r) and probabilities (in parenthesis) between the ratio of absorbance to scatter ($K/S L^*$), whiteness (CIE L^*) and contrast ratio ($CR L^*$) of experimental cheeses at different temperatures during heating and cooling.^a

Treatment	Temperature (°C)					
	4	20	30	40	60	80
Heating						
$K/S L^* - L^*$	0.990**(<0.001)	0.968**(<0.001)	0.968**(<0.001)	0.964**(<0.001)	0.937**(<0.001)	0.802**(<0.001)
$K/S L^* - CR L^*$	0.970**(<0.001)	0.957**(<0.001)	0.907**(<0.001)	0.873**(<0.001)	0.944**(<0.001)	0.790**(<0.001)
$L^* - CR L^*$	0.989**(<0.001)	0.970**(<0.001)	0.963**(<0.001)	0.927**(<0.001)	0.889**(<0.001)	0.765**(<0.001)
Cooling						
$K/S L^* - L^*$	0.989**(<0.001)	0.985**(<0.001)	0.954**(<0.001)	0.958**(<0.001)	0.922**(<0.002)	—
$K/S L^* - CR L^*$	0.973**(<0.001)	0.932**(<0.001)	0.917**(<0.001)	0.953**(<0.001)	0.914**(<0.001)	—
$L^* - CR L^*$	0.984**(<0.001)	0.964**(<0.001)	0.964**(<0.001)	0.897**(<0.003)	0.895**(<0.003)	—

^a Single and double asterisks indicate level of significance: *, $0.01 < P \leq 0.05$; **, $P \leq 0.01$.

3.3.5. Relationship between Kubelka-Munk, contrast ratio and CIE whiteness

A high Pearson correlation ($r > 0.900$; $P < 0.05$) was found among the measurement of cheese translucency by $K/S L^*$, $CR L^*$ and L^* whiteness (Table 4), suggesting that these methods could be useful to estimate cheese translucency during heating and cooling. However, a lower correlation was observed ($r \sim 0.800$; $P < 0.05$) when cheeses were heated at 80°C . In a preliminary study of $K/S L^*$ and $CR L^*$ (data not shown), we found loss of moisture when samples were heated at 60 and 80°C , that contributed to a decrease in the diameter of cheese discs. The addition of a layer of liquid paraffin avoided this problem and the $K/S L^*$ and $CR L^*$ values showed no differences when coated samples were compared with control samples (not shown).

The hydrophobic surface coating on a cheese pizza baking model was successfully used by Rudan and Barbano (1998) to reduce cheese dehydration and also prevented the formation of blisters. At elevated temperatures, cheese behaves as a viscous-like material mainly due to a reduction of the CN–CN interactions ($>40^\circ\text{C}$) and melting of cheese fat ($\leq 40^\circ\text{C}$; Lucey, Johnson, & Horne, 2003). We observed, however, no melting in our cheese samples following any heat treatment. Guinee et al. (2000) reported shrinkage of the cheese matrix when exposed to high temperatures. This phenomenon may present some limitations if shrinkage affects the thickness of cheese samples exposed at high temperatures. Even though the Kubelka-Munk method estimates the ratio of absorbance to scatter based on reflectivity, the thickness of the samples analysed is also an important parameter to consider.

Best (1987) established that to obtain accurate, reliable and reproducible results, the ratio of the aperture area of measurement to the thickness of the sample has to be greater than 10. In our study, we obtained a ratio (~ 25) much higher than that recommended, as the aperture diameter of measurement of the colorimeter used was 8 mm (area $\sim 50\text{ mm}^2$) and the thickness of cheese discs was 2 mm. Another explanation of differences observed at high temperatures, could also be attributed to the nature of translucent materials that may reflect light from below the surface (Gullett et al., 1972). To prevent this limitation, Little (1964) concluded that the best conditions to determine the optical properties of translucent materials is measurement of the colour of thin samples positioned above a white background. Calvo and Salvador (1997) found the use of K/S method (based on L^* values) as a better alternative than the measurement of CIE L^* values to estimate the translucency of fruit gels due to a higher correlation with sensorial lightness.

3.4. Nitrogen solubility during heating and cooling

We studied the proportion of temperature soluble-N (TSN) of experimental cheeses based on a cheese aqueous extract model to determine the role of temperature on the translucency of cheese. Fat content of cheeses had a significant effect ($P < 0.05$) on the TSN content during heating at different points of ripening (Fig. 5; Table 3). The TSN was also affected by the interaction of ageing $\times$ heating and fat content $\times$ ageing $\times$ heating ($P < 0.05$). At 2 d of ripening, levels of TSN were lower in NF cheeses (Fig. 5a; $P < 0.05$). These values remained constant between 4 and 40°C ($P > 0.05$). A strong decrease in the levels of TSN was observed in cheeses heated at $> 40^\circ\text{C}$, remaining constant at higher temperatures. A reverse effect on nitrogen solubility was observed in samples heated to 80°C and then cooled and stored at 4°C in a waterbath for 24 h, which had the same levels of TSN as untreated samples at 4°C . An increase in TSN levels was observed for all cheeses at 180 d of ripening (Fig. 5b; $P < 0.05$) and no differences were found among treatments ($P > 0.05$). We also found a decrease

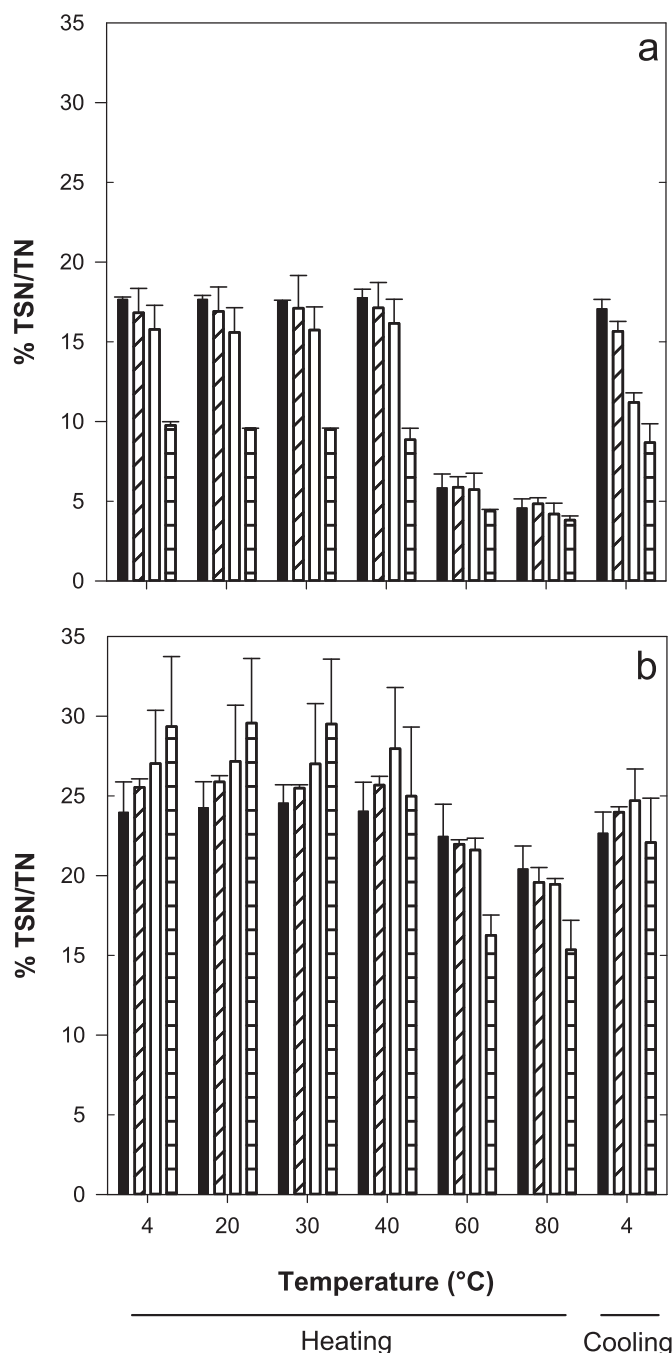


Fig. 5. Changes in the proportion of the temperature soluble N as a percentage of total cheese N (%TSN/TN) during the heating and cooling of full fat (■), reduced fat (▨), half fat (□) and non-fat (▤) Cheddar cheeses at (a) 2 d and (b) 180 d of ripening. Values represent mean and standard deviations of three replicate trials.

in TSN when samples were heated at $> 40^\circ\text{C}$, although in a lower intensity than occurred on cheeses at 2 d of ripening. A reversible effect was also observed on heated and cooled samples.

An electrophoretic characterisation of the soluble phase of cheese samples at 180 d of ripening (Fig. 6) showed that untreated samples at 4°C contained mainly intact α_{S1} -CN and β -CN and some of their large degradation products. Differences in band intensity of α_{S1} -CN and β -CN among samples can be related to differences in proteolysis caused by differences in pH (Table 1). Post, Arnold, Weiss, and Hinrichs (2012) observed a decrease in the solubility of α_{S1} -CN and β -CN in deionised water and

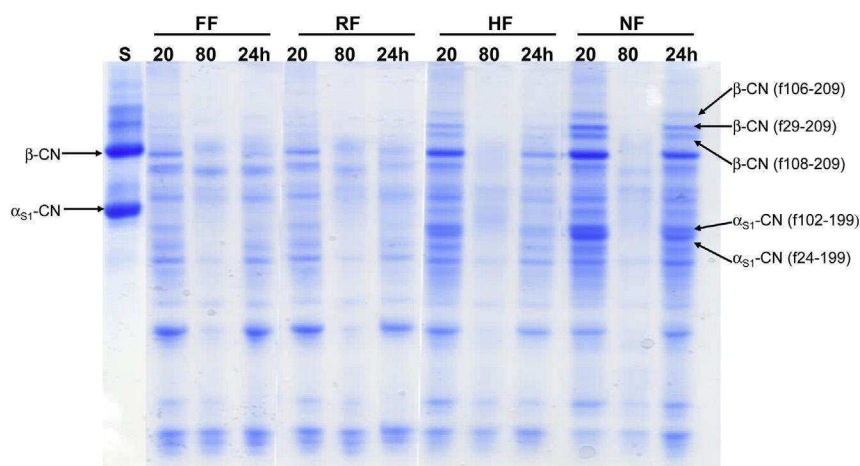


Fig. 6. Urea-polyacrylamide gel electrophoresis of aqueous phase extracts of full fat (FF), reduced fat (RF), half fat (HF) and non fat (NF) Cheddar cheeses obtained at 180 d of ripening from unheated samples at 4 °C (4), samples heat treated at 80 °C over 30 min (80) and samples heat treated at 80 °C for 30 min and cooled for 4 °C over 24 h (24 h). Sodium caseinate was used as standard (S).

ultrafiltration permeate of milk when pH decreased from 5.5 to 5.0, probably due to the pH approaching these proteins' isoelectric points. The majority of the bands precipitated due to heat treatment of the aqueous phase at 80 °C. However, a group of unidentified bands with high electrophoretic mobility remained soluble. Finally, a reverse effect was observed after heat treated samples were stored at 4 °C for 24 h.

Nevertheless, we observed no changes of translucency during heating of FF, RF and HF at 2 d of ripening, probably due to a counteracting effect of fat melting (Figs. 2a and 3a); NF exhibited a significant increase in both K/S and L^* values at temperatures higher than 40 °C. A decrease in the TSN levels at >40 °C was related to the formation of new aggregates that would increase the light scattering and hence reduce the translucency of cheeses. Kim, Lim, and Gunasekaran (2011) observed a decrease in the casein solubility when full-fat and reduced-fat Cheddar cheeses were maintained for different holding times at 60 °C, due to an increase of the surface hydrophobicity that led to the formation of aggregates. As previously stated, Metzger et al. (2000) suggested that hydrophobic heat-induced aggregates form a white gel at 49 °C, leading to a shift in cheese appearance. This gel however, was reversed when sample was cooled for several hours. Based on this observation, Dave et al. (2001) suggested that at low temperatures β -CN migrates from the casein micelle to remain soluble in the serum phase of cheese, due to a higher solubility at cold temperatures (Bingham, 1971) and during heating, β -CN precipitates. From the rheological point of view, hydrophobic interactions during heating might lead to a weakening of the cheese matrix due to a decrease in the contact area among particles and an increase in interactions within particles (Lucey et al., 2003). On the other hand, it has been demonstrated that hydrophobic interactions are greatest at 70 °C and that they decrease at higher temperatures (Bryant & McClements, 1998). In addition, the formation of heat-induced structures might also be related to changes on the proportion of INSOL Ca. Broome and Limsowtin (2002) observed a decrease in the solubility of Ca in SCCAP when heated at >40 °C. It has also been reported that heat treatment promotes the binding of α_{S1} -CN to Ca (Dalglish & Parker, 1980). Udayarajan, Lucey, and Horne (2005) elucidated that an increase of cheese stiffness during heating at high temperatures (>70 °C) is related to the formation of colloidal calcium phosphate that interacts with caseins from the soluble phase, which is believed to be reversible when cheese is cooled. We hypothesised the occurrence of these two phenomena during cheese heating

(i.e., hydrophobic interactions and formation of new colloidal calcium phosphate binding to soluble caseins) and its reversible effect during cooling resulted in changes in the proportion of soluble nitrogen (Fig. 5) and hence on translucency. This is confirmed by urea-PAGE (Fig. 6), where all the soluble fractions heated at 80 °C did not contain α_{S1} -CN, β -CN or their degradation products, although these became soluble when samples were cooled at 4 °C for 24 h. Differences in the profiles of TSN obtained at 2 and 180 d can be related to an increase of cheese proteolysis that might lead to a reduction on the proportion of intact α_{S1} -CN and β -CN. These data agree with those of Guo and Kindstedt (1995) who observed an increase in both the crude protein content and the proportion pH 4.6-soluble nitrogen on the expressible serum of Mozzarella cheese with ageing.

4. Conclusions

The results of this study showed that translucency of Cheddar cheese is highly influenced by composition (mainly fat content), temperature and ageing. A lower fat content in cheese resulted in a higher translucency. At low temperatures, cheese fat scatters light, reducing the translucent appearance. During heating, melting of cheese fat led to an increase of cheese translucency. In addition, a decrease in the proportion of soluble nitrogen, probably caused by increased hydrophobic interaction among the caseins, led to the formation of aggregates that would contribute to a decrease in cheese translucency. After cooling, aggregation was found to be reversible contributing again to an increase in cheese translucency, whereas solidification of cheese fat counteracts the effect on translucency. It was also believed that changes of translucency at high temperatures were also associated with the formation of new colloidal calcium phosphate; however, further investigation will be required to establish the role of calcium solubility on the optical properties of cheese at high temperatures. Independent of the fat content, cheese age led to an increase in translucency, probably due to an increase in levels of proteolysis and a reduction in the proportion of INSOL Ca. Measurement of cheese translucency by CIE whiteness, the ratio of absorbance to scatter (K/S L^*) and the contrast ratio (CR L^*) were useful to determine differences and were highly correlated up to 60 °C; however, exhibited a slight reduction in correlation at 80 °C, probably caused by shrinkage of cheese matrix when exposed to high temperatures that may influence in thickness of the samples analysed.

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Effect of pectin on the composition, microbiology, texture, and functionality of reduced-fat Cheddar cheese

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Abstract Hydrocolloids have been extensively studied in low-fat cheeses as a way to improve defects associated with fat reduction, which are often related to texture and functionality (meltability). Pectin is a polysaccharide obtained from plant cells and is commonly used as a stabilizer for acidified dairy beverages. This work aimed to evaluate the effect of three types of commercial pectins on the characteristics of reduced-fat Cheddar cheese during a ripening period of 180 days. Five Cheddar cheeses were made: full-fat control (FF), reduced-fat control (RF), and reduced-fat cheeses with amidated (RA), high-methoxy (RH), or low-methoxy (RL) pectin added to milk prior processing at concentrations of 0.175%, 0.100%, and 0.075% (w/w), respectively; levels were chosen to avoid phase separation of the casein micelles, due to depletion flocculation. Addition of amidated pectin markedly increased the moisture content of the experimental cheese (~49%), compared to RF (~45%; $P<0.05$). A significant reduction ($P<0.05$) in the proportion of insoluble calcium observed in RA and RL at 180 days (~40% versus ~56% in RF) was probably caused by calcium-induced gelation mechanisms of amidated and low-methoxy pectins. Texture profile analysis showed a softening of cheeses with added pectin (hardness <100 N versus >100 N in RF at 180 days; $P<0.05$). The melting properties of cheeses were significantly improved during ripening, although RF exhibited the lowest values (diameter increase >85 versus <70% at 180 days; $P<0.05$). These results suggest that pectin addition can be used to modify the moisture content, texture, and melting properties of reduced-fat Cheddar cheese.

Keywords Pectin · Fat replacers · Reduced-fat cheese · Cheddar cheese · Cheese texture

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1 Introduction

In recent years, consumers have shown an increasing interest in the consumption of cheeses with lower fat contents (Childs and Drake 2009). One of the main properties of reduced-fat cheeses is a higher protein to fat ratio that confers a more compact structure leading to a firmer and rubbery texture, lack of flavor, bitterness, development of off-flavors, poorer melting properties, and a translucent appearance (Mistry 2001; Johnson et al. 2009). To compensate for the increased proportion of protein in the matrix, it is possible to increase the moisture content of the cheese by various techniques, such as the modification of processing steps or the addition of ingredients that may increase the water-holding capacity (Johnson et al. 2009).

Hydrocolloids have been used to modify the composition, and hence the texture and functionality, of reduced or low-fat cheeses. Mistry (2001) and Johnson et al. (2009) extensively reviewed the manufacture of different types of cheeses supplemented with a variety of carbohydrate-based fat replacers, such as StellarTM, NovagelTM, microcrystalline cellulose, carrageenan, gum arabic, polyanionic gum, starch, β -glucan, and gum tragacanth.

Pectin corresponds to a class of anionic polysaccharide found in the middle lamella from the cell wall of higher plants and is responsible for the firmness and structure of plant tissues, acting as a hydrating agent and providing a cementing material for the cellulose network (Thakur et al. 1997). Although pectin is found in most plant tissues, the main commercial sources of pectins are extracted from apple pomaces and citrus peels. The structure consists of a homopolymer of α -(1 $\rightarrow$ 4)-*D*-galactopyranosyluronic acid with repeating α -(1 $\rightarrow$ 2)-L-rhamnosyl- α -(1 $\rightarrow$ 4)-*D*-galacturonosyl sections that may contain branched sections with neutral side chains. The carboxylic groups from the galacturonans contain varying degrees of methyl esterification (DM). Pectins with more than 50% DM are classified as high-methoxy pectin (HMP), whereas those ones with lower than 50% DM are known as low-methoxy pectin (LMP; Thakur et al. 1997). In addition, a modified LMP may contain degrees of amidation (DA) on its carboxyl groups and is referred as amidated pectin (AMP). One of the main characteristics of pectin is its ability to form gels. In general, HMP gels under acidic conditions ($\text{pH} \leq 3$) and the presence of high concentration of sugars ($\geq 55\%$), where the driving forces of gelation are hydrogen bonding and hydrophobic interactions, whereas LMP gels in the presence of Ca^{+2} , based on an egg-box model (Thakur et al. 1997). The main difference between LMP and AMP is that the latter gels at low concentrations of Ca^{+2} .

The main application of pectin in dairy industry is to stabilize caseins in acidified milks to avoid syneresis (Marozienne and de Kruif 2000; Harte et al. 2007). Some other applications have focused the use of pectin in rennet gels (Tuinier et al. 2002; Fagan et al. 2006; Acero Lopez et al. 2009). However, the concentration of pectin in milk plays an important role in stability, due to interactions between caseins and polysaccharides. Above a certain concentration of pectin, depletion interactions may cause destabilization of milk and hence a phase separation (Marozienne and de Kruif 2000; Tuinier et al. 2002; Acero Lopez et al. 2009). Lobato-Calleros et al. (2001) successfully used LMP in milk at a concentration of 0.2% (w/w) to increase moisture content and improve the texture of low-fat Mexican Manchego cheese, probably due to the formation of calcium pectate gels that may lead to an interruption of the compact *para*-casein matrix. The authors did not report the presence of phase separation in milk

due to the interaction of pectins and caseins. However, the proportion of calcium associated with caseins plays an important role determining the texture and melting properties of cheese, and it is highly influenced by pH and aging (Hassan et al. 2004; Lucey et al. 2003). As the gelation of pectin has an impact on the calcium equilibrium of milk (Harte et al. 2007), cheeses made with added pectin could potentially reduce the proportion of insoluble Ca of cheese, leading to a weakening of the *para*-casein matrix and hence modify its textural and melting properties. This study aimed to evaluate the effect of different types of pectins on the composition, proportion of insoluble Ca and rheological properties of reduced-fat Cheddar cheese during ripening. Pectins were added into milks at concentrations below this critical point to avoid destabilization.

2 Materials and methods

2.1 Pectin solutions

One day prior cheese manufacture, solutions of amidated (AMP; 34% DM and 15% DA) high-methoxy (HMP; 67% DM), and low-methoxy (LMP; 39% DM) pectins (Herbstreith and Fox KG, Neuenbürg, Germany) were prepared at levels of 4.55 (w/v), 2.60 (w/v), and 1.95% (w/v), respectively. Pectins were mixed with 1.5 L of deionized water at 65 °C for 2 h using an overhead stirrer to ensure complete solubilization. Pectin solutions were then cooled to 25 °C, adjusted to 2 L, stored overnight at 4 °C, and heated again at 65 °C using a waterbath before use.

2.2 Cheese manufacture

Cheddar-type cheese was manufactured in the pilot plant facilities of the School of Food and Nutritional Sciences, University College Cork, Ireland. Experimental cheeses were manufactured on a 50-kg scale based on cheesemilks standardized to a casein to fat ratio of 0.7 (full-fat, 35 g of fat per liter of cheesemilk) or 1.4 (reduced-fat, 17.5 g of fat per liter of cheesemilk) to obtain five different vats: full-fat Control (FF), reduced-fat control (RF), reduced-fat with AMP (RA), reduced-fat with HMP (RH), and reduced-fat with LMP (RL). Each cheesemilk was batch pasteurized at 63 °C for 30 min. During this step and when cheesemilks reached 63 °C, 2 L of each pectin solution at 65 °C was added to their corresponding vat. In order to maintain the same volume in all the treatments, 2 L of deionized water at 65 °C was added to control vats (FF and RF). The final concentration of AMP, HMP, and LMP in cheese milks were 0.175%, 0.100%, 0.075% (w/w), respectively. These levels were selected based on preliminary work performed on 10% (w/w) reconstituted low-heat skim milk and RF cheese milks, to maximize addition of pectin while preventing depletion interaction (Marozienne and de Kruif 2000). Once pasteurization was finished, milks were cooled to 31 °C. A Cheddar cheese starter culture (R-604Y, Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added to a level of 0.03% (w/w) and left to ripen for 30 min with continuous stirring. Cheesemilks were supplemented with 0.09% (v/w) of 1 mol.L⁻¹ CaCl₂ and equilibrated for additional 5 min. Chymosin (Maxiren 180, 180 IMCU.mL⁻¹, DSM Food Specialties, Delft, Netherlands) was added to each vat at a level of 0.03% (v/w) diluted one in four with distilled water to aid water dispersion. Once the curd developed

enough firmness after 45–50 min, the coagulum was cut and cooked from 31 to 39 °C in a period of 30 min and held to that temperature until the pH dropped to 6.2, and the whey was drained from the vats. The curd was then cut into blocks and inverted every 15 min until the pH decreased to 5.4. Curd blocks were milled, salted at a level of 2.5% (w/w) NaCl, and equilibrated for 20 min. The salted curds were transferred to 5-kg rectangular molds and pressed during 14 h at a pressure of 2.5 kg.cm⁻². Experimental cheeses were vacuum sealed and ripened for 6 months at a temperature of 8 °C.

2.3 Rennet coagulation properties

The rheological properties of cheesemilks during rennet coagulation were studied with a dynamic small amplitude oscillatory rheometer equipped with a Peltier concentric cylinder system and a conical rotor (28 mm diameter and 42 mm length; model AR-G2; TA Instruments, Waters LLC, Leatherhead, Surrey, UK) at a frequency of 1 Hz and 0.1% strain. Chymosin was added to cheesemilks previously heated at 31 °C at a level of 0.03% (w/v) and stirred for 1 min. Two additional min elapsed between the mixing of rennet in milks and the starting of oscillation. The storage modulus (G') and the loss modulus (G'') were measured during a gelation time of 45 min. The loss tangent (LT ; G''/G') was also estimated. Gelation time (GT) was defined as the time required for rennet gels to reach $G' \geq 1$ Pa. Each treatment was analyzed in triplicate.

2.4 Compositional analysis

Composition of cheeses was determined at 14 days of ripening. Moisture content was determined by the drying-oven method (IDF 1982), fat by the Gerber method (IIRS 1955), protein (%N $\times$ 6.38) by the macro-Kjeldahl methodology (IDF 1986), salt by potentiometric titration with AgNO₃ (Fox 1963), and calcium by atomic absorption spectroscopy (IDF 2007). The proportion of insoluble calcium (INSOL Ca) was estimated by the cheese juice extraction method at 2 and 180 days of ripening as described by Hassan et al. (2004). The pH was measured at 2, 30, 60, 120, and 180 days of ripening on a homogenized mixture of 10 g of cheese and 10 mL of water at 20 °C. All analyses were performed in triplicate.

2.5 Microbiological analysis

Samples were prepared as described by Fenelon et al. (2000). Starter lactic acid bacteria (LAB) were enumerated on LM 17 agar (Terzaghi and Sandine 1975) using aerobic incubation at 30 °C for 3 days and non-starter lactic bacteria (NSLAB) were counted on Rogosa agar (Rogosa and Mitchell 1951) incubated anaerobically at 30 °C for 5 days. Enumeration of LAB and NSLAB were performed in duplicate at 2, 14, 30, 60, 90, 120, and 180 days of ripening.

2.6 Proteolysis

Proteolysis was assessed by the pH 4.6 soluble N (pH 4.6 SN/TN) method (Kruchoo and Fox 1982) and the level of total free amino acids (FAA) by the trinitrobenzenesulphonic acid (TNBS) method (Kruchoo et al. 1983) at 2, 60, 120, and 180 days of ripening. Urea-polyacrylamide gel electrophoresis (urea-PAGE) was performed directly on cheese

samples as described by Andrews (1983) to monitor the breakdown of α_{s1} - and β -casein (CN) during ripening. Gels were stained with Coomassie blue G250 as described by Blakesley and Boezi (1977). Densitometric analysis of scanned gels was performed with an image processing software (ImageJ 1.48v, National Institutes of Health, Bethesda, MD, USA). All treatments were analyzed in triplicate.

2.7 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 14, 30, 60, 120, and 180 days of ripening. Cheese samples were cut into cylinders (20 mm diameter, 20 mm height) and stored overnight at 8 °C. Cheese cylinders were compressed to 75% of strain in two consecutive cycles at a rate of 1 mm.s⁻¹. Hardness, springiness, and cohesiveness were estimated as previously described by Bourne (1978). Five cheese cylinders were analyzed per treatment.

2.8 Meltability and release of free oil

Melting properties of cheeses during ripening was performed by the Schreiber meltability test as described by Altan et al. (2005), by heating cheese discs at 232 °C for 5 min. Meltability was calculated as the percentage of increase in diameter of unmelted samples. The release of free oil of experimental cheeses at 60 °C during ripening was determined by the modified Gerber method described by Kindstedt and Fox (1991). Results were expressed as the amount of free oil, as a percentage of the total cheese fat, released at 60 °C. Analyses were performed in triplicate at 7, 30, 90, and 180 days of ripening.

2.9 Dynamic small amplitude oscillatory rheology

The rheological properties of cheese samples were determined using a controlled stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) at 180 days of ripening. Serrated parallel plate geometry was used, and cheese discs (40 mm diameter, 2 mm height) were placed on the bottom plate at an initial temperature of 20 °C. A normal force of ~1.8 N was initially applied to the cheese disc. Liquid paraffin was used to cover the exposed layers of the sample to prevent loss of moisture. When the normal force decreased to ~0.7 N, sample was heated to 80 °C at a heating rate of 2 °C.min⁻¹. Analyses were performed using a total strain of 1% and a frequency of 1 Hz, which were found to be within the linear viscoelastic region (this was confirmed by performing strain sweep and frequency sweep tests). Storage modulus (G'), loss modulus (G''), and loss tangent (LT) were measured during heating. The maximum LT (LT_{max}), which is an indicator of melting, was also recorded. Each treatment was analyzed in triplicate.

2.10 Color analysis

Color of experimental cheeses was performed with a Konika-Minolta colorimeter CR-400 (Konika-Minolta Optics Inc., Osaka, Japan) at 2, 14, 30, 60, 120, and 180 days of ripening. The instrument was set on the CIELAB system based on illuminant D65 and a visual angle of 2°. Five random measurements were performed directly on a fresh cut of cheese block at 20 °C.

2.11 Experimental design and statistical analysis

Five treatments (fat content and pectin type: FF, RF, RA, RH, and RL cheeses) were manufactured in three independent trials, based on a 5×3 randomized block design. Analysis of variance (ANOVA) was performed on cheese composition, INSOL Ca, color, and rheological properties at a significance level of $P < 0.05$. A split-plot design (Montgomery 2013) was used to evaluate the effects of treatment, ripening time, and their interactions on pH, microbiological analysis, proteolysis, texture, melting, and free oil release. The ANOVA for the split-plot design was carried out using a general linear model (GLM) procedure. If significant differences were found ($P < 0.05$), the treatments means were analyzed by the Tukey multiple comparison test. All analyses were performed using Minitab 16® (Minitab Inc., State College, PA, USA).

3 Results

3.1 Rennet coagulation properties

The rheological properties of rennet-induced gels are shown in Table 1. The addition of pectins had an effect on the gelation time (GT) and the stiffness (G') of rennet curds. Cheesemilks containing pectin had a reduction of ~4 min in GT compared to controls ($P < 0.05$). During the first 15 min of renneting, cheesemilks containing pectin exhibited similar increases in G' as the time increased. Beyond this time, RA increased at a slower rate than other milks (data not shown). At 45 min, the highest G' values were found for RH and RL, followed by FF and RF and finally on RA milk ($P < 0.05$). Even though RA gel exhibited a lower LT at 45 min when compared to other treatments ($P < 0.05$), differences were lower than 0.003 units.

3.2 Cheese composition, levels of insoluble calcium, and pH

The composition and percentage of INSOL Ca of cheeses are shown in Table 2. As expected, a significant decrease in fat content was found between full-fat and reduced-fat cheeses ($P < 0.05$). Levels of salt of experimental cheeses ranged around 1.95–2.15%; however, a higher content of salt in the moisture phase of cheese (S/M) was found only in FF ($P < 0.05$), and no differences were found between reduced-fat cheeses. The addition of pectin led to an increase in the moisture content of RA, when compared to RF. No changes ($P > 0.05$) in the total Ca content was found between treatments when results were expressed per 100 g of protein. Similar levels of INSOL Ca were observed at 2 days of ripening for all treatments, which was around 60%–70% ($P > 0.05$). At 180 days, the INSOL Ca significantly decreased for all reduced-fat treatments ($P < 0.05$); RA and RL cheeses had a lower proportion of INSOL Ca when compared to RF ($P < 0.05$), and RH exhibited similar levels of INSOL Ca to RF, RA, and RL cheeses. The pH values of experimental cheeses during ripening are shown in Fig. 1. No significant differences ($P > 0.05$) were observed in the pH values of experimental treatments at 2 days of ripening. The pH of FF cheese showed no variations during 180 days of ripening ($P > 0.05$). An increase in the pH values was observed for other treatments after 60 days of ripening ($P < 0.05$). At 180 days, similar pH values

Table 1 Rheological properties of rennet-induced gels made from full-fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH), and low-methoxy pectin (RL) cheesemilks

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
Gelation time (min)	15.03 ^a	14.99 ^a	10.90 ^b	10.86 ^b	11.30 ^b	0.527
Storage modulus (G') at 45 min (Pa)	32.87 ^b	34.17 ^b	26.88 ^c	52.06 ^a	51.70 ^a	2.790
Loss tangent (LT) at 45 min	0.244 ^a	0.246 ^a	0.238 ^b	0.244 ^a	0.242 ^a	0.001

Gelation time corresponds to the point when gels had a storage modulus (G') of ≥ 1 . G' and LT were measured for 45 min after rennet addition. Data are means of three replicate trials. Means within the same row not sharing a common superscript differ ($P < 0.05$)

were similar between RF, RH, and RL cheeses, and they were higher than those found in FF and RA ($P < 0.05$).

3.3 Starter and non-starter lactic acid bacteria

The number of starter LAB exhibited a significant decrease of 2–4 log cycles for all cheeses over the ripening period of 180 days (Fig. 2a; Table 3). However, no significant differences were found between treatments (Table 3). The counts of NSLAB increased

Table 2 Composition (day 14) and proportion of insoluble Ca (days 2 and 180) of full-fat control (FF), reduced-fat control (RF), reduced-fat with amidated pectin (RA), high-methoxy pectin (RH), and low-methoxy pectin (RL) Cheddar cheeses

Item	Treatment					SEM
	FF	RF	RA	RH	RL	
Moisture (%)	38.95 ^c	44.47 ^b	49.11 ^a	45.86 ^b	46.25 ^{ab}	0.924
Fat (%)	31.33 ^a	18.78 ^b	15.00 ^c	17.66 ^b	17.77 ^b	1.550
Protein (%)	23.52 ^b	29.40 ^a	29.08 ^a	28.28 ^a	29.13 ^a	0.640
Salt (%)	2.12 ^a	2.03 ^{bc}	2.14 ^a	1.96 ^c	2.09 ^{ab}	0.034
MNFS (%)	56.72 ^a	54.76 ^a	57.78 ^a	56.47 ^a	55.50 ^a	0.415
FDM (%)	51.32 ^a	33.81 ^b	29.48 ^c	33.72 ^{bc}	32.04 ^{bc}	2.110
S/M (%)	5.46 ^a	4.56 ^b	4.37 ^b	4.36 ^b	4.43 ^b	0.132
a_w	0.964 ^b	0.968 ^a	0.969 ^a	0.970 ^a	0.969 ^a	0.0006
Total calcium (mg .100 g ⁻¹ protein)	2820 ^a	2869 ^a	2731 ^a	2832 ^a	2853 ^a	34.8
% INSOL Ca/total Ca						
day 2	69.89 ^a	65.85 ^a	61.83 ^a	60.82 ^a	58.65 ^a	1.38
day 180	57.33 ^a	55.89 ^{ab}	39.51 ^c	44.03 ^{bc}	42.35 ^c	2.30

Data are means of three replicate trials. Means within the same row not sharing a common lowercase superscript differ ($P < 0.05$)

MNFS moisture in the non-fat substance, FDM fat content on a dry weight basis, S/M salt in the moisture phase of the cheese, a_w water activity

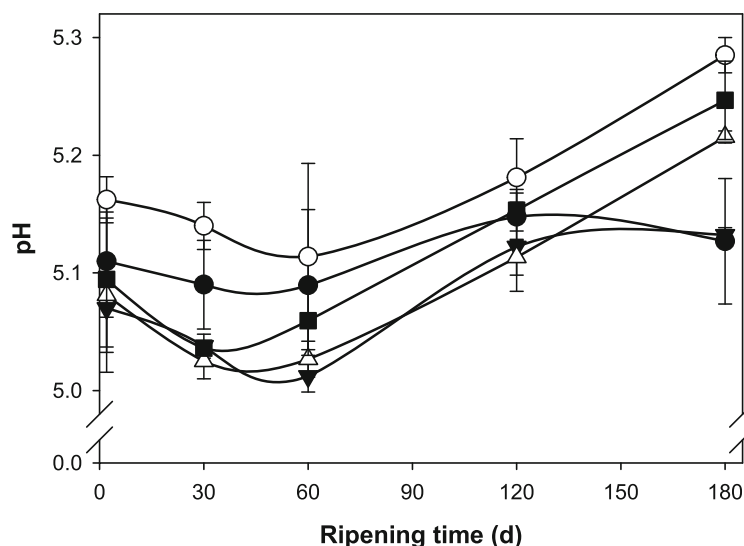


Fig. 1 Changes in pH during ripening of full-fat control (*filled circle*), reduced-fat control (*open circle*), reduced-fat with amidated pectin (*filled inverted triangle*), high-methoxy pectin (*open triangle*), and low-methoxy pectin (*filled square*) cheeses. Values are means of three replicates. Error bars indicate standard deviation

for all cheeses during ripening (Fig. 2b; Table 3). NSLAB in FF cheese increased and reached a maximum at 14 days ($P < 0.05$; Table 3), remaining constant beyond, whereas the number of NSLAB in RF and cheeses containing pectin also exhibited an increased during ripening, but at a slower rate of growth, where RF, RA and RL reached a maximum at 60 days and RH at 30 days ($P < 0.05$), remaining constant thereafter. The number of NSLAB were similar ($P > 0.05$) between treatments at 180 days of ripening

3.4 Proteolysis

The primary proteolysis expressed as the level of pH 4.6-soluble N of cheeses during ripening is shown in Fig. 3a, whereas the secondary proteolysis expressed as the

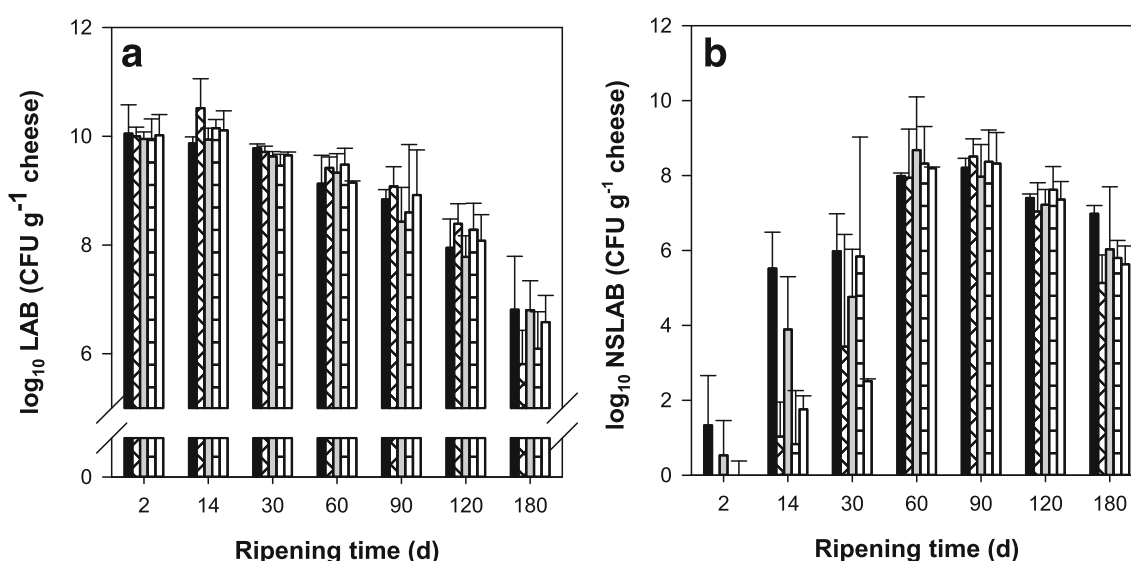


Fig. 2 Numbers of **a** starter (LAB) and **b** non-starter lactic acid bacteria (NSLAB) during ripening of full-fat control (*black bars*), reduced-fat control (*white bars filled with perpendicular lines*), reduced-fat with amidated pectin (*gray bars*), high-methoxy pectin (*white bars filled with parallel lines*), and low-methoxy pectin (*white bars*) Cheddar cheeses. Values are means of three replicates. Error bars indicate standard deviation

Table 3 Mean squares, probabilities (in parenthesis) and R^2 values for pH, microbiological analysis, and proteolysis for experimental cheeses during 180 days of ripening

Factor	<i>df</i>	pH	<i>df</i>	LAB	NSLAB	<i>df</i>	pH 4.6 SN/TN	FAA	β -CN (f1-189/192)	α_{s1} -CN
Whole plot										
Trial (T)	2	0.0039 (0.284)	2	0.6884 (0.161)	7.186* (0.048)	2	0.938 (0.690)	48,346 (0.078)	179.78** (<0.001)	273.50** (<0.001)
Treatment (t)	4	0.0222** (0.006)	4	0.0699 (0.911)	7.631* (0.028)	4	10.321* (0.039)	73,015* (0.021)	120.93** (<0.001)	218.40** (0.004)
Error (T $\times$ t)	8	0.0026	8	0.2972	1.575	8	2.418	13,549	6.32	23.60
Subplot										
Age (A)	4	0.0518** (<0.001)	6	25.4905** (<0.001)	135.257** (<0.001)	3	418.05** (<0.001)	1,935,034** (<0.001)	2061.11** (<0.001)	17168.3** (<0.001)
A $\times$ t	16	0.0028** (0.003)	24	0.1993 (0.489)	2.431 (0.078)	12	0.959 (0.441)	5850 (0.122)	17.15* (0.042)	33.70** (0.007)
Error	40	0.0009	60	0.2008	1.537	30	0.922	3482	7.89	11.30
R^2		0.91		0.93	0.91		0.98	0.98	0.97	0.99

Split-plot design with the five treatments (FF, RF, RA, RH, and RL) were analyzed as a discontinuous variable and trial was blocked. Subplot included the effect of aging of cheese (A) and the interaction age $\times$ treatment (A $\times$ t) as variables. Degrees of freedom (*df*) differed for pH, microbiology, and proteolysis measurements as the time points for the analyses were different.

LAB number of starter lactic acid bacteria, *NSLAB* number of non-starter lactic acid bacteria, *pH 4.6 SN/TN* pH 4.6 soluble N as a percentage of total N, *FAA* free amino acids expressed as mg of L-leucine per 100 g of cheese, *β -CN (f1-189/192)* accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 2 days, *α_{s1} -CN* level of intact α_{s1} -CN as a percentage of the level at 2 days

* $0.01 < P \leq 0.05$; ** $P \leq 0.01$

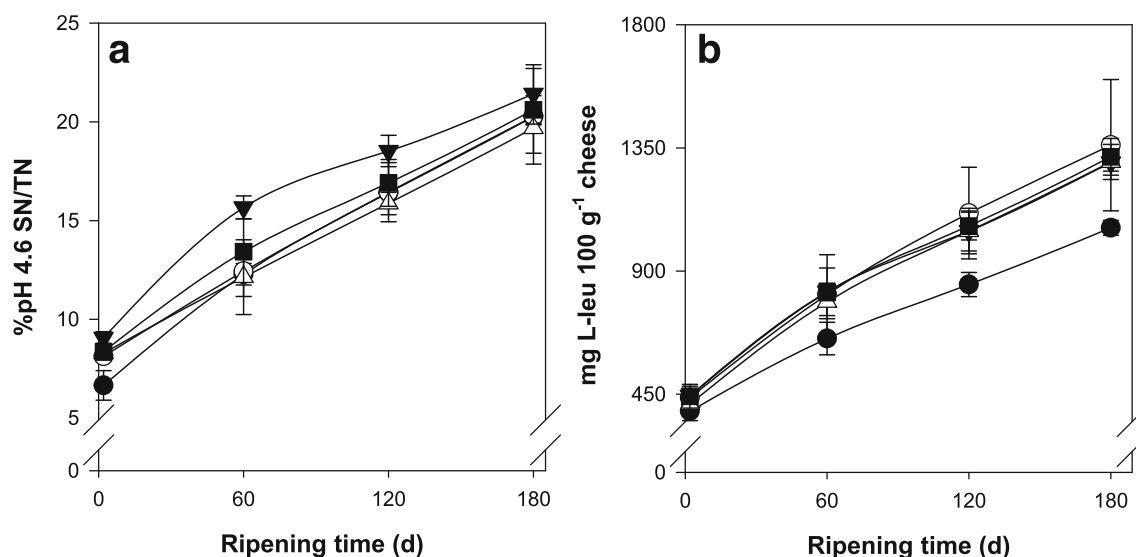


Fig. 3 Changes in **a** pH 4.6 soluble N as a percentage of total N and **b** free amino acids expressed as g of L-leucine per 100 g of cheese during ripening of full-fat control (filled circle), reduced-fat control (open circle), reduced-fat with amidated pectin (filled inverted triangle), high-methoxy pectin (open triangle), and low-methoxy pectin (filled square) Cheddar cheeses. Values are means of three replicates. Error bars indicate standard deviation

concentration of total FAA is shown in Fig. 3b. A significant effect on treatment and cheese age was observed for both parameters (Table 3). A lower level of pH 4.6 SN/TN was observed for FF cheese at 2 days of ripening. Proteolysis significantly increased during ripening for all cheeses ($P < 0.05$). RA exhibited an increment of pH 4.6 SN/TN at a higher rate than other treatments ($P < 0.05$). However, no differences between treatments were observed at 180 days. A significant increase in levels of FAA was observed during ripening for all the treatments ($P < 0.05$). In addition, FF contained lower amount of FAA than reduced-fat cheeses ($P < 0.05$).

Urea-PAGE electrophoretograms of cheese samples during ripening are shown in Fig. 4. Treatments and cheese age had an effect on the breakdown of β - and α_{s1} -CN. We observed a significant increase in levels of β -CN (f1-189/192) during ripening for all treatments (Fig. 5a; Table 3). FF cheese had significantly lower accumulation of β -CN (f1-189/192) than RF and all cheeses containing pectin ($P < 0.05$). RA exhibited higher amounts of β -CN (f1-189/192) than other treatments at 60 days ($P < 0.05$) and were similar to RF cheeses after 120 days ($P > 0.05$). Degradation of α_{s1} -CN significantly ($P < 0.05$) increased during ripening for all treatments (Fig. 5b; Table 3). RA and RL cheeses showed a higher proportion of intact α_{s1} -CN after 120 days ($P < 0.05$), when compared to FF and RF, whereas there were no differences ($P > 0.05$) between treatments after 180 days of ripening.

3.5 Texture profile analysis

The TPA properties of experimental cheeses are shown in Fig. 6. A significant decrease in hardness values was observed during ripening for all cheeses (Fig. 6a; Table 4). A reduction in the fat content of cheese led to an increase in hardness of RF, when compared to FF ($P < 0.05$). The hardness of cheeses containing pectin was lower than that of the RF cheese ($P < 0.05$).

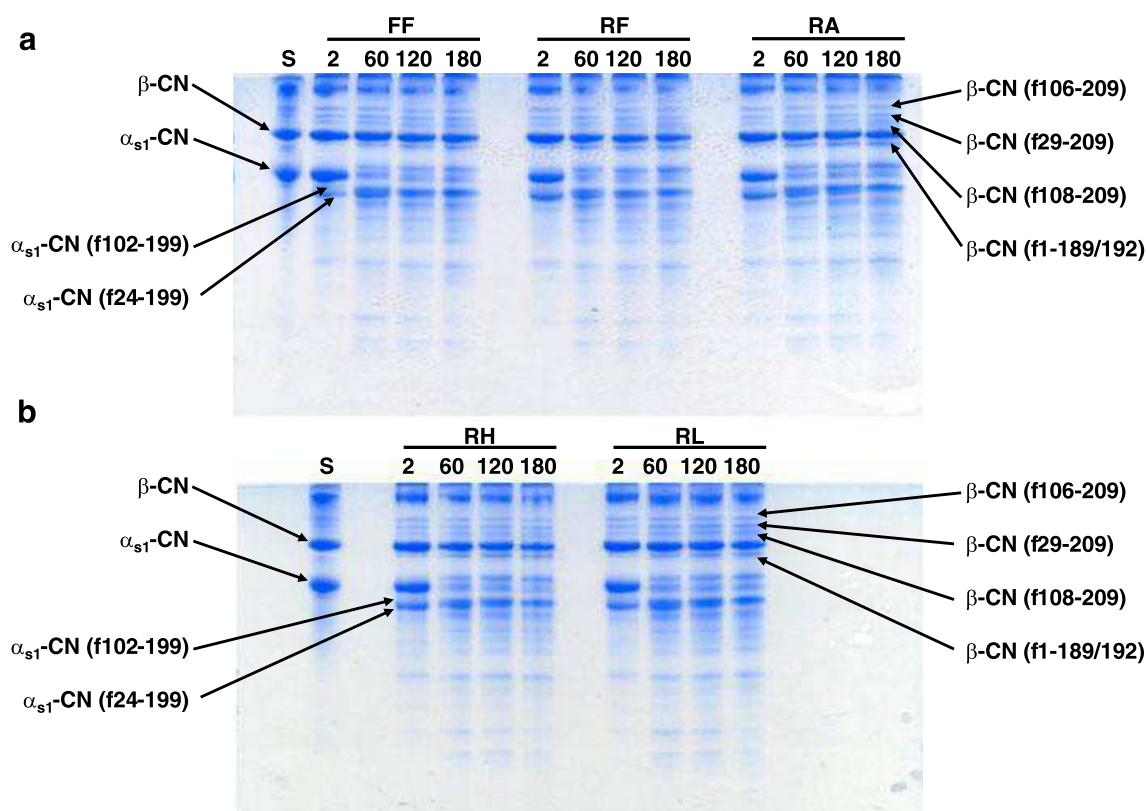


Fig. 4 Urea-Polyacrilamide gel electrophoresis of experimental Cheddar cheeses at 2, 60, 120, and 180 days of ripening of **a** full-fat (FF), reduced-fat (RF), and reduced-fat with amidated pectin (RA) and **b** reduced-fat with high-methoxy (RH) and low-methoxy pectin (RL) Cheddar cheeses. Sodium caseinate (S) was used as standard for each gel

The TPA springiness of experimental cheeses decreased during ripening for all treatments (Fig. 6b; Table 4). No differences between treatments were observed at 14 days of ripening ($P > 0.05$). RF exhibited a higher springiness than FF after 30 days ($P < 0.05$). Cheeses containing pectin had similar values than FF until 60 days ($P > 0.05$) and then became higher beyond ($P < 0.05$). However at 180 days, cheeses containing pectin exhibited similar values than RF ($P > 0.05$).

No differences were observed between treatments in TPA cohesiveness ($P > 0.05$). Cheese cohesiveness showed a decrease during ripening (Fig. 6c; Table 4).

3.6 Melt analysis and release of free oil

The melting determined by the Schreiber analysis of experimental cheeses is detailed in Fig. 7a. A significant increase in melting was observed for all treatments during cheese age ($P < 0.05$; Table 4). At 7 days of ripening, a higher meltability was observed in FF, when compared to reduced-fat cheeses ($P < 0.05$) and similar levels of melting were observed between RF and pectin-containing cheeses. At 90 days of ripening, RA showed similar meltability than FF. At 180 days of ripening, cheeses containing pectin exhibited a higher meltability than RF ($P < 0.05$).

The release of free oil was higher in FF than all other treatments ($P < 0.05$), and only FF and RH exhibited an increase during ripening ($P < 0.05$). There were no differences in the release of free oil between RF and cheeses containing pectin.

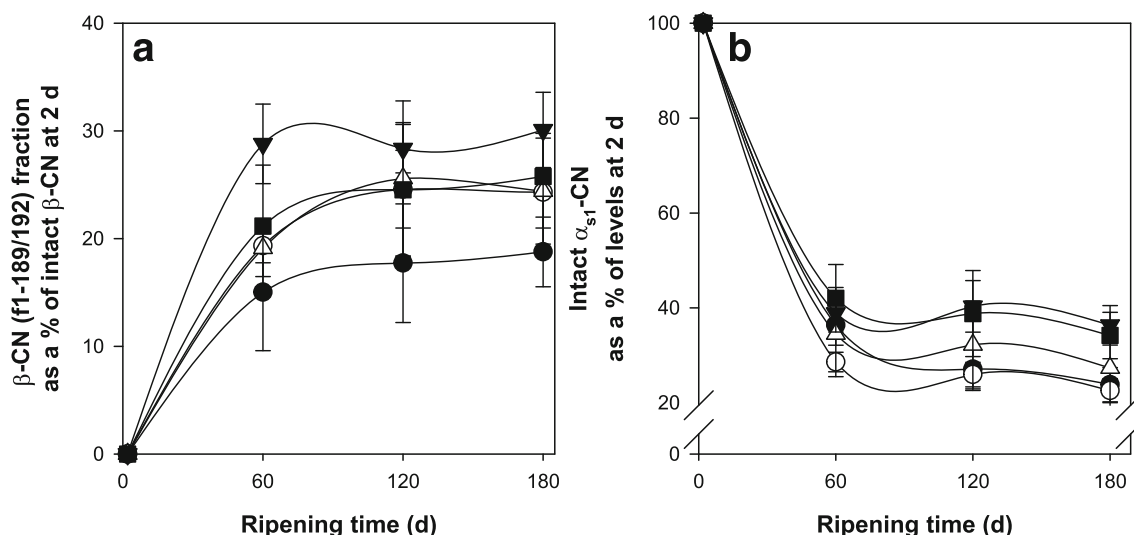


Fig. 5 Changes in **a** accumulation of β -CN (f1-189/192) fraction as a percentage of the intact β -CN level at 2 days and **b** level of intact α_{s1} -CN as a percentage of the level at 2 days for full-fat control (*filled circle*), reduced-fat control (*open circle*), reduced-fat with amidated pectin (*filled inverted triangle*), high-methoxy pectin (*open triangle*), and low-methoxy pectin (*filled square*) Cheddar cheeses during ripening. Values are means of three replicates. Error bars indicate standard deviation

3.7 Dynamic small amplitude oscillatory rheology

The G' values of experimental cheeses showed a decrease during heating (Table 5). At 20 °C, RF exhibited higher G' than FF and pectin-containing cheeses ($P < 0.05$). At 70 °C, no significant differences were found in G' values between FF and RF. RL exhibited lower G' than RA and RH ($P < 0.05$). RF exhibited lower LT_{max} than FF. RL cheese had similar LT_{max} than FF cheese, whereas RA and RH exhibited similar LT_{max} than RF. There were no differences in the temperature of LT_{max} between FF, RF, and RL; however, RA and RH exhibited the LT_{max} at a lower temperature ($P < 0.05$).

3.8 Color

The color of experimental cheeses at 14 days of ripening is detailed in Table 6. As expected, FF exhibited higher whiteness (L^* values) than reduced-fat treatments. At 2 days of ripening (data not shown), L^* values were ~ 88 for FF and ~ 85 for reduced-fat cheeses. At 14 days, a decrease in whiteness was observed for all treatments ($P < 0.05$), and this difference remained at longer ripening times. No differences were observed in L^* values of cheeses containing pectin, when compared to RF ($P > 0.05$). The greenness of RA and RL cheese was significantly lower (higher a^* values) than RF ($P < 0.05$) and similar to FF. The addition of pectin had no effect on cheese yellowness (b^* values; $P > 0.05$).

4 Discussion

A reduction on the GT during renneting of cheesemilks supplemented with pectins is associated with an increase in the viscosity of the aqueous phase of milk (Fagan et al. 2006). The concentration of pectin has a strong influence on the microstructure and

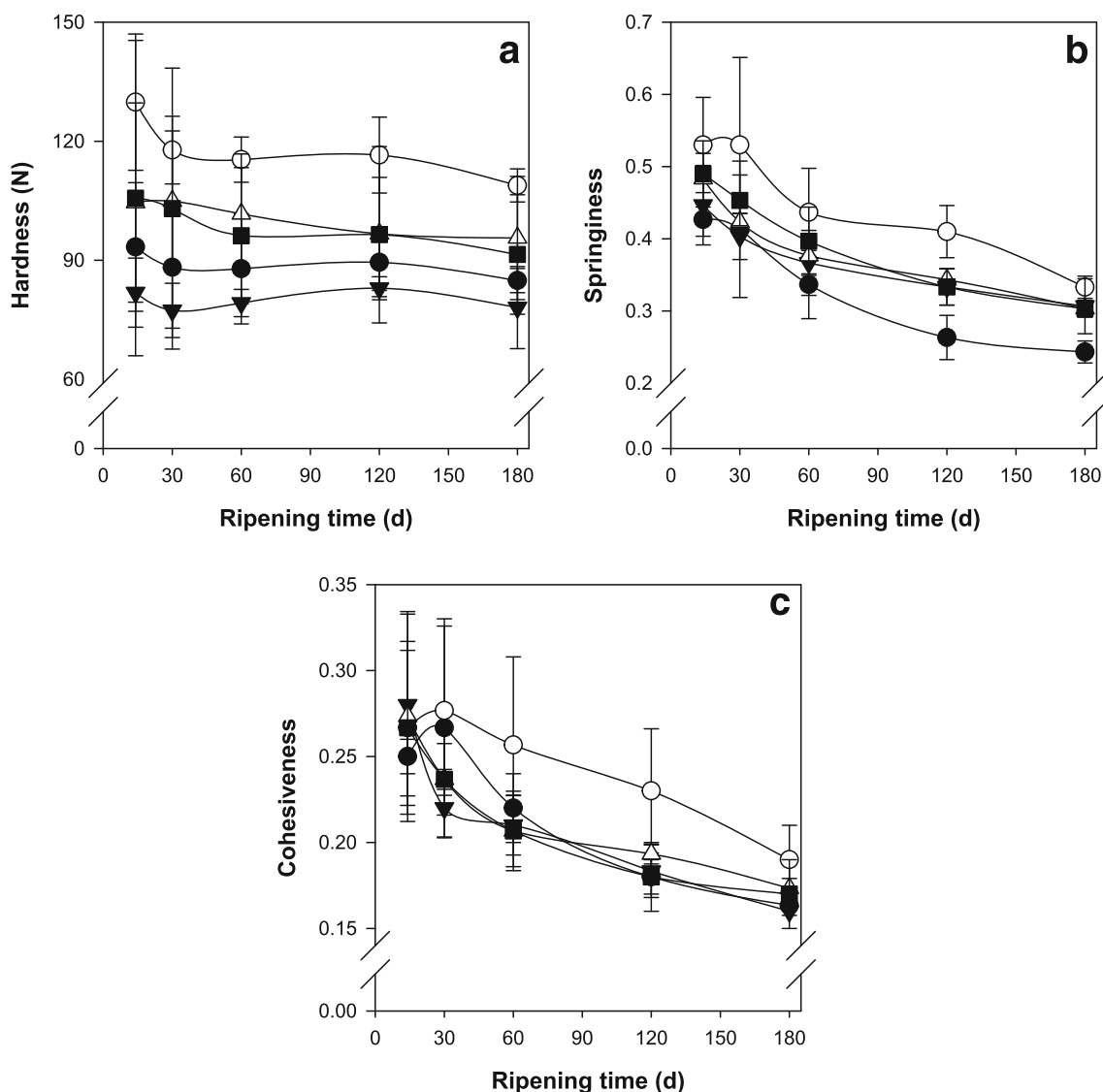


Fig. 6 Changes in TPA **a** hardness, **b** springiness, and **c** cohesiveness values during ripening full-fat control (filled circle), reduced-fat control (open circle), reduced-fat with amidated pectin (filled inverted triangle), high-methoxy pectin (open triangle), and low-methoxy pectin (filled square) Cheddar cheeses. Values are means of three replicates. Error bars indicate standard deviation

hence the rheological properties of rennet-induced gels. The results obtained from RH and RL are in agreement with Tan et al. (2007) who observed an increase in the stiffness of rennet gels and a reduction on syneresis as the concentration of HMP increased from 0% to 0.1% due to a more compact microstructure when observed by confocal laser microscopy. At higher concentrations of pectins (0.12%–0.15%), a more open network of depleted caseins led to the conformation of a weaker gel (Acero Lopez et al. 2009), as was observed in RA rennet gel.

A reduction in the fat content of cheese leads to a shift in the compositional balance such as in moisture and protein content (Mistry 2001). The addition of pectin only led to an increase in the moisture content of RA cheese; however, moisture content of RL cheese is similar to both RF and RA cheeses. The capacity of AMP and LMP to form gels could increase the moisture content of experimental cheeses. However, amidation of LMP improves the gel-forming ability of AMP, leading to a reduction of syneresis (Thakur et al. 1997) that could increase the moisture content of RA cheese. Another

Table 4 Mean squares, probabilities (in parenthesis) and R^2 values for textural analysis, melting properties, and release of free oil for experimental cheeses during 180 days of ripening

Factor	df	Hardness	Springiness	Cohesiveness	df	Melting	Free oil
Whole plot							
Trial (T)	2	4993.6* (0.024)	0.007109 (0.319)	0.0042893 (0.223)	2	44.27 (0.725)	7.44 (0.448)
Treatment (t)	4	3949.4* (0.028)	0.024635* (0.032)	0.0028280 (0.380)	4	548.21* (0.041)	1859.30** (<0.001)
Error (T×t)	8	810.9	0.005378	0.0023560	8	131.97	8.37
Subplot							
Age (A)	4	654.2** (<0.001)	0.081001** (<0.001)	0.0227513** (<0.001)	3	4656.97** (<0.001)	98.30** (<0.001)
A×t	16	68.8 (0.778)	0.000905 (0.764)	0.0005780 (0.508)	12	101.61** (0.005)	12.05* (0.017)
Error	40	94.4	0.001269	0.0005977	30	31.46	4.65
R^2		0.89	0.91	0.85		0.95	0.98

Split-plot design with the five treatments (FF, RF, RA, RH, and RL) were analyzed as a discontinuous variable and trial was blocked. Subplot included the effect of aging of cheese (A) and the interaction age x treatment (A×t) as variables. Degrees of freedom (*df*) differed for texture and functional properties as the time points for the analyses were different

*0.01 < $P \leq 0.05$; ** $P \leq 0.01$

explanation in the increment of moisture content could be associated with the amount of pectin added to cheesemilks, as HMP and LMP were added at $\leq 0.1\%$ (w/w), comparing with AMP that was added at 0.175% (w/w). Lobato-Calleros et al. (2001) attributed an increase in the moisture content of low-fat Manchego cheese made from cheesemilks supplemented with LMP at levels of 0.2% (w/w) to the water-binding

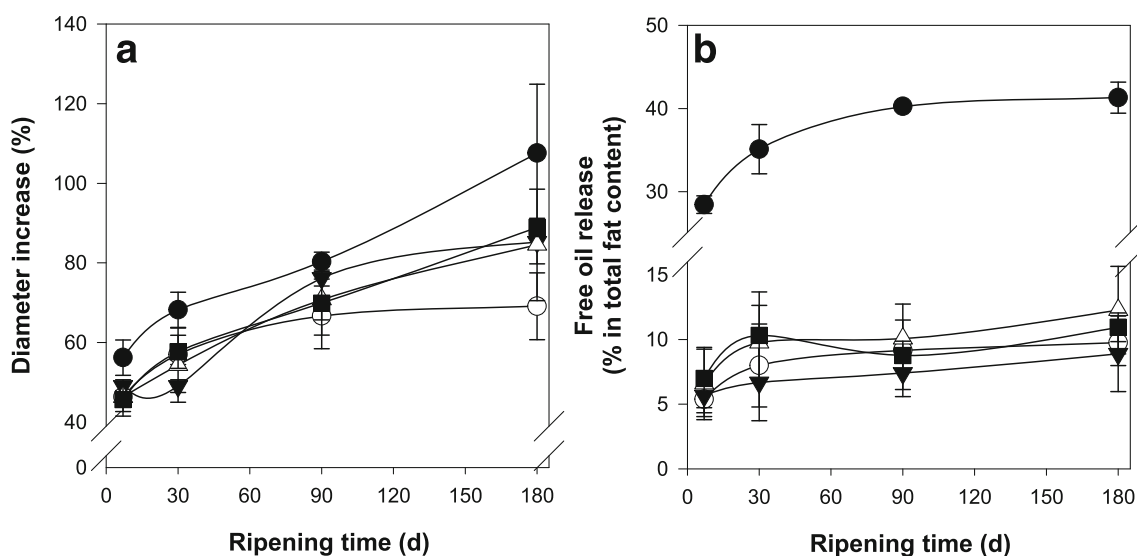


Fig. 7 Changes in **a** melting determined by the Schreiber test and **b** release of free oil values during ripening of full-fat control (filled circle), reduced-fat control (open circle), reduced-fat with amidated pectin (filled inverted triangle), high-methoxy pectin (open triangle), and low-methoxy pectin (filled square) Cheddar cheeses. Values are means of three replicates. Error bars indicate standard deviation

Table 5 Rheological properties of experimental cheeses at 180 days of ripening determined by dynamic small amplitude oscillatory rheology

Item	Treatment					
	FF	RF	RA	RH	RL	SEM
G' at 20 °C (Pa)	37159.1 ^{bc}	43236.0 ^a	38466.7 ^b	34079.8 ^c	39394.1 ^b	873.0
G' at 70 °C (Pa)	336.1 ^{bc}	538.5 ^{abc}	652.5 ^a	591.6 ^{ab}	290.2 ^c	43.1
LT _{max}	1.10 ^a	0.82 ^b	0.77 ^{bc}	0.73 ^c	1.17 ^a	0.05
Temperature at LT _{max} (°C)	67.18 ^a	65.89 ^a	62.42 ^b	62.89 ^b	66.12 ^a	0.52

G' corresponds to storage modulus. LT_{max} corresponds to loss tangent maximum. Data are means of three replicate trials. Means within the same row not sharing a common superscript differ ($P < 0.05$)

capacity of pectin due to the formation of gels in the presence of calcium. Similar levels of total Ca probably resulted from the pH values at critical points during cheese manufacture being similar between treatments.

The proportion of INSOL Ca in Cheddar cheese is reduced during the first 30 days of ripening and remains constant beyond (Hassan et al. 2004). Differences in the proportion of INSOL Ca between RF with RA and RL cheeses could be attributed to gelation mechanisms of AMP and LMP. Lobato-Calleros et al. (2001) found the presence of calcium pectate particles when they observed the microstructure of low-fat Mexican Manchego cheese supplemented with LMP. Harte et al. (2007) proposed a gelation model of LMP during the acidification of milk that could also be applied to cheese. As pH decreases during acidification, solubilization of Ca from the colloidal calcium phosphate interacts with LMP, promoting cross linking of chains and hence the formation of a gel structure. This phenomenon probably occurred during cheese manufacture due to acidification of RA and RL cheesemilks caused by action of starter LAB.

An increment of cheese pH during ripening is attributed to the buffering capacity of cheese due to a reduction in the levels of INSOL Ca (Hassan et al. 2004; Table 2). In addition, pH plays an important role in the interaction between pectins and casein micelles. As previously stated, pectin remains dispersed in the serum phase of milk at neutral pH, and it adsorbs onto casein micelles at pH 5.3. If the concentration of pectin is not enough to cover casein micelles, then pectin interacts with casein micelles by

Table 6 CIELAB color of experimental cheeses after 14 days of ripening

Item	Treatment					
	FF	RF	RA	RH	RL	SEM
L*	83.45 ^a	78.34 ^b	78.57 ^b	79.71 ^b	78.80 ^b	0.564
a*	-3.92 ^{ab}	-4.38 ^b	-3.40 ^a	-3.92 ^{ab}	-3.38 ^a	0.111
b*	36.91 ^a	30.88 ^{ab}	27.71 ^b	27.99 ^b	29.13 ^b	1.06

Data are means of three replicate trials. Means within the same row not sharing a common superscript differ ($P < 0.05$)

L* whiteness, a* greenness or redness, b* yellowness or blueness

mean of bridging flocculation, whereas if casein micelles are fully covered by pectins, then they are sterically stabilized. However, a further increase in the pectin concentration leads to depletion interaction (Marozienne and de Kruif 2000). In addition, below pH 5.0 pectin and casein micelles are associated by mean of electrosorption (Tuinier et al. 2002). These authors also indicated that renneting had no influence on the interaction of pectins and κ -casein depleted micelles. As the pH of pectin-added cheeses was close to 5.3, pectin may interact with the protein matrix by adsorption mechanisms, interrupting the compact protein structure, which will influence on water retention (i.e., higher moisture content, as occurred with RA cheese) and also cheese functionality. The use of carbohydrate-based fat replacers has shown a more open structure in low-fat cheeses, which might be caused by the presence of amorphous carbohydrate particles that interrupts the compact protein matrix (McMahon et al. 1996; Lobato-Calleros et al. 2001), altering the composition, texture, and functionality (Mistry 2001).

Similarities in the counts of LAB between treatments could be explained due to similar levels of MNFS (Table 2). Fenelon et al. (2000) found a reduction in the counts of starter LAB, which was attributed to a decrease in the MNFS as fat content was reduced. As NSLAB likely use the milk fat globule membrane as a source of carbon for their growth (Fox et al. 1998), it would have been expected that propagation of NSLAB would be lower in reduced-fat cheeses.

Similar levels of primary proteolysis between FF and reduced-fat cheeses can be related to their similar levels of MNFS (Table 2). A significant increment in the levels of pH 4.6 SN/TN as the fat content of cheese was increased has been associated with a concomitant increment of MNFS that may lead to a higher retention of chymosin (Guinee et al. 2000; Fenelon et al. 2000). An increase in the levels of FAA has been previously reported for low-fat cheese (Fenelon et al. 2000), which is attributed to a concomitant increase of the protein content as the fat content is reduced (Guinee et al. 2000).

The hydrophobic peptide β -CN (f193-209) formed by cleavage at Leu₁₉₂-Tyr₁₉₃ which also forms β -CN (f1-189/192), contributes bitterness to cheese (Visser et al. 1983). The concentration of salt to moisture content (S/M) has a direct influence on the residual activity of chymosin. We observed lower levels of S/M in reduced-fat cheeses, which might increase the accumulation of β -CN (f1-189/192) due to an enhancement of the residual activity (Fenelon and Guinee 2000). As previously stated, higher accumulation of β -CN (f1-189/192) in RA can also be influenced by its lower pH. Fenelon and Guinee (2000) found a lower degradation of α_{s1} -CN as the fat content of cheese is reduced, due to a decrease of residual activity of chymosin. In our study, similar levels of intact α_{s1} -CN between treatments could be associated with similar levels in the MNFS (Table 2), which influences in the residual activity of chymosin.

The softening of cheese during ripening is partly associated to the extent of proteolysis, mainly caused by the degradation of α_{s1} -casein (Fig. 5b; Creamer and Olson 1982). In addition, the reduction of the INSOL Ca levels during ripening (Table 2), which corresponds to the release of colloidal calcium phosphate (CCP) to the serum phase, has a strong influence on cheese hardness, causing a weakening of the protein matrix (Lucey et al. 2003). An increase in TPA hardness as the fat content is reduced is attributed to a concomitant increase in the protein content (Fenelon and Guinee 2000) and also to a decrease in the interruption of the cheese matrix by fat globules, leading to a more compact structure (Bryant et al. 1995; Johnson et al. 2009).

As previously stated, the addition of pectin may cause an interruption of the cheese matrix, probably due to protein polysaccharide interactions (Marozienne and de Kruif 2000; Tuinier et al. 2002) and or the formation of calcium pectate gels (Lobato-Calleros et al. 2001), conferring a less compact structure and hence reduce hardness. A similar effect has been reported by McMahon et al. (1996) who observed the presence of amorphous carbohydrate-based fat replacers immersed in the cheese matrix. In addition, lower levels of INSOL Ca, when compared to RF, was found in RA and RL cheeses (Table 2), probably due to the formation of calcium pectate gels (Lobato-Calleros et al. 2001; Harte et al. 2007) that might contribute to a softer texture. The addition of AMP was more efficient in reducing hardness, leading to similar values than FF, probably caused by higher concentrations of pectin added than in the other treatments. An increase in springiness as the fat content is reduced was also observed by Lobato-Calleros et al. (2001). These authors also found a reduction in the springiness of reduced-fat Manchego cheese with LMP added when compared to control which was attributed to an interruption of the protein matrix by pectin particles. The same effect in the protein matrix might have caused a reduction of springiness in RA, RH, and RL cheeses, when compared to RF. Bryant et al. (1995) observed an increase in the cohesiveness of Cheddar cheese as the fat content was lower than 21%. On the other hand, Tunick et al. (1991) found that higher moisture content in Mozzarella cheese is associated to higher cohesiveness values. In our study, we found no differences in the cohesiveness of experimental cheeses, which could be explained by higher moisture content of reduced-fat cheeses, when compared to FF (Table 2) and also with the presence of fat replacers, i.e., pectin, that would reduce casein-casein interactions, leading to a loosening of the protein matrix (Lobato-Calleros et al. 2001).

An increase of melting during ripening for all cheeses is associated to the extent of proteolysis (Fig. 3) and changes in levels of INSOL Ca (Table 2; Lucey et al. 2003). A lower melting in reduced-fat cheeses is associated to a higher protein content that leads to a more compact structure (Guinee et al. 2000). In addition, levels of free oil release might also be influenced by the melting properties (Fig. 7b). Rudan and Barbano (1998) observed the formation of a thick skin and/or blisters during the baking of low-fat Mozzarella cheese that prevented its softening, and hence flow, due to dehydration of the surface caused by a lack of free oil release that may act as a hydrophobic surface coating. To avoid this problem, the authors found that covering the surface of cheese with a small amount of a hydrophobic surface coating considerably improved its melting properties. As previously stated, a more open structure due to the addition of pectin may eventually increase melting (McMahon et al. 1996). A higher meltability found in cheeses containing pectin could also be attributed to lower levels of INSOL Ca at 180 days of ripening, when compared to control (Table 2), due to a lower amount of CCP crosslinks, that leads to a reduction in the attractive interactions of the cheese matrix (Lucey et al. 2003).

A decrease in storage modulus (G') during heating of cheese indicates a weakening of the casein matrix structure (Lucey et al. 2003). A higher G' value in RF at 20 °C is probably associated to higher protein content. Lower G' values found in cheeses containing pectin could be associated with a more open structure (McMahon et al. 1996; Lobato-Calleros et al. 2001) and lower levels of INSOL Ca (Table 2). During the heating of cheese at high temperatures ($>$ temperature at LT_{max}), Udayarajan et al. (2005) observed an increase in G' probably caused by a heat-induced formation of INSOL Ca that may

interact with caseins to form new structures. This same phenomenon might have caused increments of G' values in RA and RH cheeses at 70 °C, due to a lower temperature at LT_{max} . The LT values at temperatures <40 °C (results not shown) were constant for all treatments (0.3–0.4), indicating the presence of a solid-like matrix. At higher temperatures, the LT values increased to a maximum (LT_{max}) reached at 60–70 °C (Table 5) to then decrease at higher temperatures (results not shown). Higher LT values relate to a higher melting (Lucey et al. 2003). As expected, RF had a lower LT_{max} than FF. Only RL exhibited a similar LT_{max} than FF cheese, whereas RA and RH had similar values to RF. A lower temperature at LT_{max} found in RA and RH cheeses could be associated to a combined effect in the levels of INSOL Ca and the extent of proteolysis. However, Udayarajan et al. (2005) found that LT_{max} is highly frequency dependent, and hence, these authors suggest caution in using this parameter as melting index. We observed differences in the results obtained between the Schreiber melting test and dynamic small amplitude rheology. Cooke et al. (2013) studied the melting properties of full-fat and reduced-fat Cheddar cheese made from milk supplemented with gum tragacanth and attributed these differences to a higher extent of fat liquefaction in the Schreiber test due to the high temperatures of exposure of cheese samples. In addition, these authors suggested the presence of interactions between casein and gum tragacanth that inhibited cheese melting at the temperatures of dynamic small amplitude rheology. This type of interactions may also inhibit cheese melting in pectin-containing cheeses.

In unmelted cheese, fat acts to scatter light and a reduction in fat content leads to a translucent appearance, which is reflected by a reduction in whiteness (Johnson et al. 2009). A decrease of cheese whiteness during ripening could be attributed to an increase in proteolysis (Fig. 3) and also a decrease in INSOL Ca levels (Table 2). The addition of pectin had no effect on cheese whiteness; however, greenness of RA and RL cheeses was reduced (Table 6). This finding was not expected as we assumed that pectin would act as a light scatter center and hence increase whiteness.

5 Conclusions

Depending on the type of pectin, its addition to milks for the manufacture of reduced-fat Cheddar cheese had to be below certain concentration in order to prevent phase separation due to depletion flocculation. Amidated pectin had an impact on pH and composition of experimental cheeses, by increasing the moisture content due to its water holding capacity. The levels of INSOL Ca were modified by the use of AMP and LMP, probably due to the formation of calcium pectate structures that influenced on texture and meltability. The addition of pectin had no effect in the number of LAB and NSLAB during ripening, when compared to reduced-fat control. The TPA hardness was significantly reduced in cheeses supplemented with pectins, especially RA. Melting properties of reduced-fat cheeses were modified when analyzed by Schreiber melting test and dynamic small amplitude rheology. Pectin had no effect in the whiteness of cheeses, when compared to RF. These results suggest that the use of pectin might be a useful strategy to modify the composition, texture, and functionality of reduced-fat Cheddar cheeses. However, interactions of protein-polysaccharides also have to be considered as a disadvantage, due to phase separations prior rennet coagulation.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

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