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The Effect of Ripening and Cooking Temperatures on Proteolysis and Lipolysis in Manchego Cheese

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ABSTRACT

The influence of ripening temperature on proteolysis and lipolysis was studied on four lots of raw ewe's milk Manchego cheese held for 60 days at 5, 10, 15 or 20°C. Mean levels of pH 4·6, trichloroacetic acid and phosphotungstic acid soluble N in 20°C cheeses were 52%, 78% and 95% higher than the respective levels in 5°C cheeses at the end of the ripening period. Free fatty acids content after 60 days was 90% higher in 20°C cheeses than in 5°C cheeses. Significant effects of the cooking temperature of the curd (30, 36, 38 or 40°C) on pH, moisture and NaCl content were recorded, but levels of nitrogenous fractions or free fatty acids in 60-day cheeses were not affected.

INTRODUCTION

Manchego cheese, with a production exceeding 30 000 tonnes/year, is the most important cheese variety manufactured in Spain. Chemical and microbiological changes occurring during ripening of dairy- and farm-made Manchego cheeses were studied by Román (1975) and Nuñez & Martínez-Moreno (1976), respectively. Considerable variations in the chemical composition of Manchego cheese samples at the retail level have been reported (Marcos *et al.*, 1976).

Manufacturing procedures differ between Manchego cheese producers: the curd is cooked at 36–38°C for 10–15 min in dairy-made

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cheese, whereas it is not heated in farm-made cheese. Differences in curd temperature may affect the ripening process as a consequence of variable whey retention.

Seasonal variations in ewe's milk production and market fluctuations are responsible for differences in both age and characteristics of Manchego cheese when marketed. Increases or decreases in ripening temperature are currently used to control the development of cheese flavour, by accelerating or retarding maturation.

The effects of manufacturing and ripening parameters on the microbial quality of raw ewe's milk Manchego cheese were recently investigated (Nuñez *et al.*, 1985). Complementary information dealing with the influence of these parameters on protein and fat breakdown during maturation would be useful in order to standardize Manchego cheesemaking and final product characteristics. Manufacturing and curing procedures establish the environmental conditions which regulate microbial growth and enzymatic activities and, consequently, the rate of chemical reactions occurring during ripening and the type and balance of end products formed. With this objective, the effects of curd cooking temperature and cheese curing temperature on chemical aspects of Manchego cheese ripening have been investigated.

MATERIALS AND METHODS

Cheesemaking

Refrigerated raw ewe's milk from the Institute herd was used to manufacture Manchego cheese according to the following procedure, unless otherwise specified. Milk was heated to 30 °C and inoculated (0·2%) with a skimmed milk culture of *Streptococcus lactis* INIA 12. Animal rennet was added after 30 min; coagulation took place in 40 min. The curd was cut into 6–8 mm cubes, the temperature of the vat was raised to 37 °C and the curds stirred for 15 min. Cheeses, 20 cm in diameter and 8 cm high, were pressed overnight, brine-salted for 48 h at 10 °C and ripened for 60 days at 10 °C.

Cooking temperatures of 30, 36, 38 and 40 °C (four lots of each temperature) were used to study the effects of curd cooking on proteolysis and lipolysis. The influence of ripening temperature was investigated on cheeses held at 5, 10, 15 or 20 °C (four lots at each temperature).

Total nitrogen and soluble nitrogenous fractions

Homogenates were prepared from representative (40 g) cheese samples and pH 4·6, 12% trichloroacetic acid (TCA)- and 1% phosphotungstic acid (PTA)-soluble N obtained as described by Gripon *et al.* (1977). Nitrogen in homogenates and soluble nitrogenous fractions was determined in duplicate by the Kjeldahl method. Nitrogen in the various fractions was expressed as percentages of total N.

Free fatty acids (FFA)

Cheese (duplicate 10-g samples) was macerated with 6 g anhydrous Na_2SO_4 in a mortar and transferred with 60 ml diethyl ether to a 100-ml screw-capped bottle. The homogenate was stirred for 1 h, with ultrasonification for 30 s at 15-min intervals, decanted and the supernatant filtered through Whatman No. 1 paper. The precipitate in the bottle was resuspended in three successive 20-ml portions of diethyl ether, decanted and filtered. The total solvent was titrated with 0·1N ethanolic KOH solution. After titration the solvent was evaporated *in vacuo* to dryness and fat was weighed. FFA in cheese were expressed as mequiv/100 g fat.

To verify the recovery rate of the procedure, internal standards (approximately 1 mequiv FFA/sample) of acetic, lactic, propionic and C_4–C_{18} fatty acids were added to samples (four replicates per acid).

Moisture, pH and NaCl content

Analyses were performed as described by Horwitz (1980).

Statistical treatment of data

Analyses of variance, significance of differences between means by the Student–Newman–Keuls test and regression equations were calculated according to Steel & Torrie (1980).

RESULTS AND DISCUSSION

Influence of ripening temperature on proteolysis and lipolysis

Mean levels of pH 4·6-, TCA- and PTA-soluble N in curds were 10·1%, 2·75% and 2·20%, whereas FFA present in curds reached, on average,

TABLE 1
Influence of Ripening Temperature on Chemical Characteristics of Manchego Cheese* after 60 Days of Maturation

Chemical determination	*Ripening temperature*			
	5°C	*10°C*	*15°C*	*20°C*
pH 4·6-soluble N†	14·5[a]	15·9[a]	18·6[b]	22·0[c]
TCA-soluble N†	9·05[a]	10·3[b]	12·7[c]	16·1[d]
PTA-soluble N†	5·51[a]	6·39[a]	8·15[b]	10·8[c]
Free fatty acids**	9·28[a]	10·4[a]	12·9[b]	17·7[c]
pH	5·05[a]	5·07[a]	5·18[b]	5·28[c]
H_2O (%)	38·7	40·2	39·5	39·3
NaCl (%)	2·21	2·39	2·52	2·40

* Mean of the data from four lots (two replicates per lot).
† Nitrogenous fractions are expressed as percentage of total N.
** Free fatty acids are expressed as mequiv/100 g fat.
[a,b,c,d] Means on the same line with different superscripts differ significantly ($P < 0·05$).

8·19 mequiv/100 g fat. Chemical characteristics of 60-day old Manchego cheeses ripened at different temperatures are summarized in Table 1.

In a previous study on ten vats of Manchego cheese analysed throughout maturation, Nuñez & Martínez-Moreno (1976) reported mean levels of 20·28%, 8·82% and 4·67% for pH 4·6-, TCA- and PTA-soluble N after 8 weeks of ripening. Their data fitted well linear regressions of the levels of soluble nitrogenous fractions on cheese age, with coefficients of determination (r^2) over 0·55 in all cases.

In the present work, linear regressions of the levels of nitrogenous

TABLE 2
Regression Equations of the Levels of Soluble Nitrogenous Fractions* and Free Fatty Acids† on Ripening Temperature (T), in Manchego Cheese after 60 Days of Maturation

Chemical determination	*Regression equation*	r^2
pH 4·6-soluble N (x_1)	$\log x_1 = 0·012\ T + 1·089$	0·646
TCA-soluble N (x_2)	$\log x_2 = 0·017\ T + 0·857$	0·841
PTA-soluble N (x_3)	$\log x_3 = 0·020\ T + 0·626$	0·848
Free fatty acids (x_4)	$\log x_4 = 0·019\ T + 0·839$	0·666

* Nitrogenous fractions expressed as percentage of total N.
† Free fatty acids expressed as mequiv/100 g fat.

fractions or FFA on ripening temperature had r^2 ranging from 0·63 to 0·82. Equations shown in Table 2 were obtained after a log transformation of the levels of nitrogenous fractions and FFA.

Mean levels of pH 4·6-, TCA- and PTA-soluble N after 60 days in 20 °C cheeses were 52%, 78% and 95% higher, respectively, than in 5 °C cheeses. Values of pH in 15 °C and 20 °C cheeses significantly exceeded those of 5 °C and 10 °C cheeses (Table 1), due to increased proteolysis.

Both pH 4·6- and TCA-soluble N result from the action of rennet and streptococcal endopeptidases on casein, whereas PTA-soluble N originates from the hydrolysis of larger peptides to low molecular weight peptides and free amino acids mainly due to streptococcal exopeptidases (Reiter *et al.*, 1969; Gripon *et al.*, 1977; O'Keeffe *et al.*, 1978). Native milk protease contributes to proteolysis in Cheddar cheese less than rennet or streptococcal endopeptidases (Reiter *et al.*, 1969).

Fox (1969) studied the influence of temperature on the proteolytic activity of rennet at pH 6·0, reporting increases in soluble N from 4·3% after 6 days at 4 °C to 10·6% after 2 days at 21 °C or 10·4% after 1 day at 32 °C. At lower temperatures, β-casein was relatively more susceptible to proteolysis than α_{s1}-casein. Streptococcal dipeptidases remain active at low temperatures, intracellular dipeptidases retaining a comparatively higher activity at low temperatures (5 °C:30 °C ratio in the range 55–65%) than extracellular dipeptidases (5 °C:30 °C ratio in the range 4–8%), according to Law *et al.* (1974). However, few data are available on the activity of streptococcal proteases and peptidases at cheese ripening temperatures, as they have been studied generally at their optimal temperatures (30 °C–50 °C). We may conclude, nevertheless, from Tables 1 and 2 that ripening temperature exerts a greater influence on exopeptidase activity in cheese than on the activity of rennet or bacterial endopeptidases, as the increase in PTA-soluble N from 5 °C cheeses to 20 °C cheeses was higher than the increases in pH 4·6- and TCA-soluble N.

The method used for FFA determination permitted a high recovery of individual acids added to cheese samples, with values ranging from 90·6% for C_2 to 109·6% for C_{10}. An average recovery of 98·6% was recorded for the ten fatty acids studied, whereas lactic acid recovery was low (20·3%). Variation coefficients (four replicates) ranged from 0·11 for C_3 to 4·55 for C_2, values similar to those previously reported (Gray, 1975; Horwood & Lloyd, 1980; Woo & Lindsay, 1982).

Lipolytic activity in Manchego cheese was enhanced by increasing

maturation temperature. Levels of FFA after 60 days were 90% higher in 20°C cheeses than in 5°C cheeses (Table 1). Native milk lipase is responsible for FFA liberation in raw milk Cheddar cheese according to Reiter *et al.* (1969), although FFA levels in cheeses made with starter were higher than those found in cheeses made with δ-gluco-lactone. Lipases of Gram-negative bacteria contribute significantly to Cheddar cheese lipolysis (Law *et al.*, 1976) and may also be involved in FFA liberation during Manchego cheese ripening, as a high incidence of lipolytic Gram-negative psychrotrophs in raw ewe's milk has been reported (Nuñez *et al.*, 1984).

Moderate acceleration of protein and fat breakdown in Cheddar cheese by the use of higher ripening temperatures was achieved by Kosikowski & Iwasaki (1975), who recorded levels of soluble protein and volatile fatty acids 31·2% and 9·6% higher, respectively, in cheeses with no enzyme preparations added, cured at 20°C, than in those cured at 10°C after 30 days of maturation. Ripening temperature has been claimed as the most important factor for typical Cheddar flavour intensity (Law *et al.*, 1979). These authors detected highly significant differences in flavour intensity between cheeses ripened at 6°C and 13°C and observed that flavour quality could also be improved by higher curing temperatures.

In the present work, cheeses ripened at 15°C received the highest mean score (7·13 points on a 1–9 points hedonic scale) after sensory evaluation by ten trained panelists whilst 20°C cheeses obtained the lowest score (6·34 points), although the difference was not statistically significant. Accelerated ripening of Manchego cheese by higher maturation temperatures thus seems feasible, provided that 15°C is not significantly surpassed.

Influence of cooking temperature on proteolysis and lipolysis

Levels of soluble nitrogenous fractions or FFA did not differ significantly between cheeses manufactured from curds heated at 30, 36, 38 or 40°C for 15 min (Table 3). Cheeses from 30°C curds exhibited significantly lower pH values, as insufficient whey drainage of the curd favoured lactose retention and acid production by streptococci. Higher moisture in 30°C curds facilitated salt diffusion into the cheese, with significantly higher NaCl contents (Table 3).

Moisture, NaCl concentration and pH value influence the rate of casein proteolysis by rennet in cheese. High moisture levels are known to

TABLE 3
Influence of Cooking Temperature of the Curd on Chemical Characteristics of Manchego Cheese* after 60 Days of Maturation at 10 °C

Chemical determination	*Cooking temperature*			
	30 °C	*36 °C*	*38 °C*	*40 °C*
pH 4·6-soluble N†	17·7	18·0	17·3	18·4
TCA-soluble N†	12·2	10·8	10·5	10·1
PTA-soluble N†	5·91	6·00	5·61	5·84
Free fatty acids**	8·85	7·94	8·42	8·46
pH	$4·89^a$	$5·14^b$	$5·16^b$	$5·16^b$
H_2O (%)	$42·8^a$	$38·8^b$	$38·0^b$	$38·2^b$
NaCl (%)	$2·84^a$	$2·18^b$	$2·13^b$	$1·96^b$

* Means of the data from four vats (two replicates per vat).
† Nitrogenous fractions are expressed as percentage of total N.
** Free fatty acids are expressed as mequiv/100 g fat.
^{a,b} Means on the same line with different superscripts differ significantly ($P < 0·05$).

accelerate proteolysis, hydrolysis of β-casein being more favoured by this factor than hydrolysis of α-casein (Creamer, 1970). Proteolysis of α_{s1}-casein, by rennet in cheese, reaches its maximum rate at 4% NaCl-in-moisture, whereas β-casein proteolysis is higher in unsalted cheeses and decreases rapidly with salt content, even at low NaCl concentrations (Noomen, 1978). An optimum of rennet activity at pH 5·8 was reported by Fox (1969) after electrophoretic examination of samples incubated 1 day at 32 °C or 6 days at 4 °C, but changes in non-protein N showed that activity at 32 °C increased with decreasing pH from pH 7·0 to pH 3·5, whereas two optima (pH 3·5 and pH 5·2) were recorded at 4 °C. Maximum degradation of α_{s1}- and β-casein by rennet in cheese was detected at pH 5·05 and pH 5·50, respectively, the highest levels of soluble N occurring at pH 4·9–5·0 (Noomen, 1978). Streptococcal proteases and peptidases are affected by low pH values, their optima having been recorded generally at pH 6·5–8·0 (Law & Kolstad, 1983). Information on the influence of moisture or NaCl concentration on the activity of these enzymes is lacking.

Proteolysis by rennet in Manchego cheeses, manufactured from 30 °C curds, may be enhanced by higher moisture and lower pH values, although it will probably be retarded by NaCl concentration which reaches NaCl-in-moisture levels over 6·50%. Proteolysis by streptococcal

enzymes in cheeses from 30 °C curds should also be favoured by high moisture levels, but reduced by low pH values. As a result of these favourable and unfavourable factors, no significant differences in the levels of soluble nitrogenous fractions between cheese from cooked and uncooked curds were detected (Table 3).

Milk lipase has its maximum activity at pH 8·5–9·0, whilst microbial lipases generally exhibit pH optima in the alkaline region (Deeth & Fitz-Gerald, 1983), with decreasing activity at low pH values (Law *et al.*, 1976). The remaining activity of milk lipase is considerably reduced by increasing NaCl concentrations in the range 0·5–1·0M (Castberg *et al.*, 1975). Therefore, conditions present in cheeses from 30 °C curds seem less favourable for lipolysis than those found in cheeses from cooked curds. However, analysis of variance did not show any significant difference in FFA levels between cheeses from 30 °C curds and cheeses from curds cooked at 36, 38 or 40 °C.

REFERENCES

Castberg, H. B., Egelrud, T., Solberg, P. & Olivecrona, T. (1975). Lipases in bovine milk and the relationship between the lipoprotein lipase and tributyrate hydrolysing activities in cream and skim-milk. *J. Dairy Res.*, **42**, 255–66.

Creamer, L. K. (1970). Protein breakdown in Gouda cheese. *New Zealand J. Dairy Sci. Technol.*, **5**, 152–4.

Deeth, H. C. & Fitz-Gerald, C. H. (1983). Lipolytic enzymes and hydrolytic rancidity. In: *Developments in dairy chemistry*, Vol. 2. (Fox, P. F. (Ed.)), London, Applied Science Publishers, 193–239.

Fox, P. F. (1969). Influence of temperature and pH on proteolytic activity of rennet extract. *J. Dairy Sci.*, **52**, 1214–18.

Gray, I. K. (1975). The quantitative analysis of free fatty acids in dairy products. *New Zealand J. Dairy Sci. Technol.*, **10**, 158–62.

Gripon, J. C., Desmazeaud, M. J., Le Bars, D. & Bergere, J. L. (1977). Role of proteolytic enzymes of *Streptococcus lactis*, *Penicillium roqueforti* and *Penicillium caseicolum* during cheese ripening. *J. Dairy Sci.*, **60**, 1532–8.

Horwitz, W. (Ed.) (1980). *Official methods of analysis of the Association of Official Analytical Chemists* (13th edn), Washington DC.

Horwood, J. F. & Lloyd, G. T. (1980). A reliable gas chromatographic method for the determination of the lower free fatty acids in cheese. *Australian J. Dairy Technol.*, **35**, 30–2.

Kosikowski, F. V. & Iwasaki, T. (1975). Changes in Cheddar cheese by commercial enzyme preparations. *J. Dairy Sci.*, **58**, 963–70.

Law, B. A. & Kolstad, J. (1983). Proteolytic systems in lactic acid bacteria. *Antonie van Leeuwenhoek*, **49**, 225–45.

Law, B. A., Sharpe, M. E. & Reiter, B. (1974). The release of intra-cellular dipeptidase from starter streptococci during Cheddar cheese ripening. *J. Dairy Res.*, **41**, 137–46.

Law, B. A., Sharpe, M. E. & Chapman, H. R. (1976). The effect of lipolytic Gram-negative psychrotrophs in stored milk on the development of rancidity in Cheddar cheese. *J. Dairy Res.*, **43**, 459–68.

Law, B. A., Hosking, Z. D. & Chapman, H. R. (1979). The effect of some manufacturing conditions on the development of flavour in Cheddar cheese. *J. Soc. Dairy Technol.*, **32**, 87–90.

Marcos, A., Esteban, M. A., Fernández-Salguero, J., Mora, M. T. & Millán, R. (1976). Componentes nitrogenados del queso tipo Manchego. I. Análisis químico de las principales fracciones. *Anal. Bromatol.*, **28**, 57–68.

Noomen, A. (1978). Activity of proteolytic enzymes in simulated soft cheeses (Meshanger type). 2. Activity of calf rennet. *Neth. Milk Dairy J.*, **32**, 49–68.

Nuñez, M. & Martínez-Moreno, J. L. (1976). Flora microbiana del queso Manchego. I. Evolución de la flora microbiana de quesos Manchegos artesanales. *Anales INIA Ser. Gen.*, **4**, 11–31.

Nuñez, J. A., Chavarri, F. J. & Nuñez, M. (1984). Psychrotrophic bacterial flora of raw ewe's milk, with particular reference to Gram negative rods. *J. Appl. Bacteriol.*, **57**, 23–9.

Nuñez, M., Gaya, P. & Medina, M. (1985). Influence of manufacturing and ripening conditions on the survival of *Enterobacteriaceae* in Manchego cheese. *J. Dairy Sci.*, **68**, 794–800.

O'Keeffe, A. M., Fox, P. F. & Daly, C. (1978). Proteolysis in Cheddar cheese: Role of coagulant and starter bacteria. *J. Dairy Res.*, **45**, 465–77.

Reiter, B., Sorokin, Y., Pickering, A. & Hall, A. J. (1969). Hydrolysis of fat and protein in small cheeses made under aseptic conditions. *J. Dairy Res.*, **36**, 65–76.

Román, M. (1975). Etude de la flore microbienne du fromage espagnol 'Manchego'. I. Son évolution au cours de la fabrication et de l'affinage. *Le Lait*, **55**, 401–13.

Steel, R. G. D. & Torrie, J. H. (1980). *Principles and procedures of statistics.* (2nd edn), New York, McGraw-Hill Book Co., Inc.

Woo, A. H. & Lindsay, R. C. (1982). Rapid method for quantitative analysis of individual free fatty acids in Cheddar cheese. *J. Dairy Sci.*, **65**, 1102–9.