Effect of production factors and ripening conditions on the characteristics of Serra cheese

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Summary The individual and interactive effects of four production factors (amount of vegetable rennet, temperature of coagulation, pressing and salting of the fresh cheese) and two ripening factors (temperature and relative humidity) on microbiological, physico–chemical, biochemical, textural and sensory characteristics of Serra cheese were simultaneously studied using a 2^{6-1}_{VI} factorial design. Highly significant effects of salting and ripening relative humidity upon the characteristics of the cheese were detected. Addition of salt to the surface of the fresh cheese reduced microbial growth, water activity, moisture and lactic acid contents, proteolysis, lipolysis, aroma and softness of the cheese. Conversely, increase of the relative humidity during ripening increased these charactereristics. Pressing had no statistically significant effect on cheese characteristics.

Keywords Ewes' raw milk, maturation, optimization methods, quality, rural technology, vegetable rennet.

Introduction

Serra cheese is the best representative of Portuguese farm cheeses produced by traditional methods from raw ewes' milk using a vegetable rennet (thistle flower). It is the most prized cheese on the national market and is an important source of income for the local farmers. Depending on the techniques used during coagulation, whey draining, pressing and salting, and the environmental conditions prevailing during ripening, different characteristics can be imparted to the cheese; for example, the rheological properties of the coagulum depend on the amount of coagulant enzyme, pH, acidification rate, coagulation temperature, intensity of mechanical work during cutting and rate of whey drainage (Brule & Lenoir, 1987). Furthermore, the texture, taste and aroma of the cheese are dependent on the composition (moisture, protein and fat) and pH of the curd, as well as on the maturation conditions (temperature and relative humidity), and on the extent of microbial contamination in the bulk and on the surface of the cheese (Brule & Lenoir, 1987).

For centuries Serra cheese has been farm-produced under poor hygienic conditions following a wide array of practices which vary from one local cheesemaker to another (Macedo *et al.*, 1993). In addition, the ripening conditions vary over the lactation season and between the various geographical locations within the Serra mountains due to the direct effect of the weather upon the environment of the ripening chambers and its indirect effect upon the quantity and nutritional quality of the pastures. Governmental inspection of farm cheese factories and enforcement of minimum quality standards have, nevertheless, provided an impetus for the improvement of traditional cheesemaking technology encompassing *Appélation d'Origine Controllée* cheeses in

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recent years. Good examples of this trend are (i) thermometers instead of the cheesemaker's finger tips to aid control of temperature during coagulation and provision of a warm water bath instead of unstable heating power from the fireplace, (ii) plastic moulds and stainless sloped drainage tables instead of perforated metal plates and wooden sloped tables, (iii) controlled hydraulic pressing instead of a raw stone placed on top of the curd in the mould and (iv) ripening in chambers with controlled temperature instead of rooms with environmental conditions chiefly determined by the prevailing weather. Furthermore, the need for innovation and diversification in a market sector plagued by surplus makes it a very interesting product/process to study and promote widely because of the unusual use of a vegetable rennet with raw ewes' milk.

In order to achieve uniform cheese quality, production procedures must be as consistent and standardized as possible. Nonetheless, this optimization cannot be fully achieved until causal relationships between each major processing variable (or combination of variables) and relevant microbiological, physico–chemical and sensory properties of the final cheeses are established within known limits of statistical validity. The present study attempts to address this issue by applying adequate statistical methods to study the effect of the most important factors on the quality attributes of Serra cheese.

Materials and methods

Experimental design

The selection of the processing factors to be manipulated was the best compromise between *in loco* practical feasibility and *in vitro* analytical constraints. The final decision included production variables such as (i) the amount of vegetable rennet (thistle flower) added to the milk, (ii) the temperature of coagulation, (iii) the presence of absence of pressing and (iv) the addition or not of salt to the fresh cheese, and ripening variables such as (v) temperature and (vi) relative humidity. In order to generate as much statistically sound information as possible, a non-replicated two-level, half fractional factorial design in six variables with resolution VI (or 2^{6-1}_{VI} for short) was chosen (Box *et al.*, 1978), corresponding to a set of 32 independent experiments. The coding of the low and high levels of the original variables is given in Table 1. The defining relation for the sixth variable of the design was the (plus) five-variable interaction (i.e. $x_6 = +x_1x_2x_3x_4x_5$). Because the experimental design selected required the manufacture of 32 cheeses but the manufacture in a single day was limited to, at most, 20 cheeses due to the size of the farmhouse flock, a block variable (defined as $B = \pm x_1x_2x_5 = \pm x_3x_4x_6$) had to be introduced in order to account for day-to-day variations (Box *et al.*, 1978); hence, 16 cheeses were randomly manufactured in each of two consecutive days.

Cheesemaking and sampling

Cheeses were manufactured in a certified dairy farm in the *Denominação de Origem Protegida* region (Fornos de Algodres) using the most widespread protocol among dairy farmers. Milking was done manually by the shepherd into a small open vessel at sunset after the flock was returned to the stable. The milk batch (*c.* 40 L) was brought to the cheesemaking area in the house and stored overnight in a tank at 4 °C. A second milking was done at sunrise on the day of experimental cheesemaking, before the same flock was walked to the outer pasture, and the milk batch (*c.* 40 L) was mixed with that obtained in the pre-

Table 1 Coded variables and levels in the experimental design

Original variable	Coded variable	Level	Coded level
Amount of rennet	x_1	0.3 g L^{-1} milk	−1
		0.5 g L^{-1} milk	+1
Temperature of coagulation	x_2	27 °C	−1
		35 °C	+1
Pressing	x_3	no	−1
		yes	+1
Surface salting of fresh cheese	x_4	no	−1
		yes	+1
Ripening temperature	x_5	5 °C	−1
		10 °C	+1
Ripening relative humidity	x_6	80%	−1
		95%	+1

vious evening. The ovine milk used as feedstock for each day's experiments was characterized by 7.1% protein (w/w) and 7.0% (w/w) fat; standardization of the ratio of protein to fat was not necessary because experience in previous years had indicated that it only varied within the narrow range 0.95–1.03 throughout the whole lactation season. The raw ewes' milk (*c.* 80 L) was filtered through a fine, clean cloth and poured into the 16 coagulation vats. After the temperature of the milk had reached the desired value (27 or 35 °C), crude kitchen salt (12 g per litre of milk) was added and the milk was stirred until complete solubilization (this amount of salt was not a variable). Thistle (*Cynara cardunculus*) (0.3 or 0.5 g of ground dry flowers per litre of milk) was mixed with tap water (0.1 L) until a brown suspension was obtained. This suspension was filtered through a fine, clean cloth, and the clear filtrate was added to each vat of milk and gently stirred. The milk was then allowed to rest until complete coagulation had occurred. This state was empirically assessed by the cheesemaker via observation of the consistency of the curd upon gentle shaking; actual coagulation times ranged from 40 min (for the +1 level of variables x_1 and x_2) to 75 min (for the corresponding −1 level). The curd was manually cut by stirring with the bare hand. Ten minutes later, the curd pieces were poured into a fine cloth bag and lightly pressed so as to help in the expulsion of whey. The drainage of whey was (or was not, depending on the experiment in question) completed via pressing of the fresh cheese while in the plastic perforated mould by a 10-kg metal block for 12 h (after 6 h the weight was removed and the cheese was turned upside down and subjected to similar pressing once again). Salting, when used, was done by uniformly rubbing all the outer surface of the cheese with 15 g of kitchen salt. After 12 h of salting, the cheeses were appropriately distributed into four independent chambers with temperature and relative humidity controlled at the set points 5 °C and 80%, 5 °C and 95%, 10 °C and 80%, and 10 °C and 95%, respectively. The cheeses were turned upside down daily. After 15 d, every cheese was washed with warm water in order to remove the reddish slime on its surface. The duration of the whole ripening period was 45 d and the cheeses had a final weight of *c.* 0.8 kg.

Textural, sensory and microbiological analyses were performed on cheese samples after 45 d of ripening; chemical and biochemical analyses were performed on similar samples but after preliminary frozen storage at −30 °C in Whirl-pak™ vacuum packages (Cole-Parmer, Chicago IL, USA). Microbiological, chemical and biochemical analyses of homogenized rindless cheese samples were made in duplicate, and textural analyses of cheese with and without rind were made in triplicate, whereas only single samples of each were used for sensory assessment.

Microbiological analyses

Lactic acid bacteria (LAB) were measured according to the methods by Macedo *et al.* (1995) using Rogosa agar (RA; Oxoid, Basingstoke, UK) or M17 agar (M17; LabM, Bury, UK), coliforms using violet red bile agar (VRBA; LabM) and yeasts using potato dextrose agar (PDA; LabM). Enterococci were determined on KF streptococcus agar base (KF; Merck, Darmstadt, Germany) supplemented with a 1% (w/v) solution of 2,3,5-triphelytetrazolium chloride (Merck) at 37 °C for 1 d. Proteolytic and lipolytic microorganisms were determined on Skim milk agar (SKM; LabM) and Tributyrin agar (TBA; LabM), respectively, at 30 °C for 3 d. The results were expressed as decimal logarithms of the number of colony forming units (CFU for short) per g of cheese.

Chemical analyses

The pH, salt and moisture contents were determined according to the methods described by Case *et al.* (1985). Water activity was determined by a Protimeter Dewpoint Meter DP989M (Protimeter plc, Bucks, UK). The fat and total protein contents were determined by Van Gulik (Anon., 1983) and micro-Kjeldahl (Anon., 1993) methods, respectively. Lactose content was determined by a spectrophotometric method (Acton, 1977).

Biochemical analysis

Samples for water soluble nitrogen (WSN), total nitrogen content (TNC) and fat acidity assays

were prepared as described by Macedo *et al.* (1996). The WSN and TNC contents of the supernatants were both determined by micro-Kjeldahl method. The maturation index was calculated as the ratio of WSN to TN. Fat acidity (total free fatty acid content in fat) was measured by titration of the diethyl ether soluble fat extract with a 0.01 N ethanolic solution of potassium hydroxide (Merck) using phenolphthalein as indicator (Anon., 1991) and expressed as mmol per 100 g of fat. Lactic acid aqueous extracts were obtained and analysed by the method described by Macedo & Malcata (1997) and expressed as percentage of lactic acid in cheese.

Force–deformation curve analyses

Force–displacement curves were determined in puncture tests, at room temperature (23 °C), using a plunger of 5 mm at a constant penetration rate (50 mm min^{-1}) for a fixed height (25 mm) with an Instron Puncture Tester 4501 (High Wycombe, Bucks, UK).

Puncture tests have been applied to cheese (Chen *et al.*, 1979) because they are simple to perform and reproducible, although they neglect the viscoelastic nature of the product. They provide a joint measurement of the resistance of the original solid body to compression and to rupture, which depends on the measuring conditions. The force at the bio-yield, sometimes called fracture force, indicates the force required for rupturing under test conditions. The slope of the linear portion of the up-curve is also a useful parameter, which can be related to the apparent elasticity of the solid, although it is not the modulus of elasticity. These two parameters were chosen to describe the force–deformation relationship of the cheese samples. The fracture force of the rind and the slope of the linear portion of the up-force–deformation curve (hereafter considered as modulus) of the paste were taken as measures of the degree of shortness of the rind and the firmness of the paste, respectively (although correlation between test parameters and sensory perception was not assessed experimentally).

Sensory analyses

The aroma, texture, number of eyes and number of cracks on the rind were evaluated using the protocol encompassing the characteristics of the traditional Serra cheese as described in the Portuguese standard (NP-1922) and assessed by a four-member laboratory sensory panel after short-term previous training and screening with respect to Serra cheese assessment. Each day, each panel member individually evaluated, at the laboratory location, each of the sixteen 45-day old experimental cheeses and a traditional 50-day old Serra cheese, all of them cut in half and available at room temperature. Cheese aroma was assessed by the panel on a scale ranging from −2 (very bad) to +2 (very good) using as set point the value +1 for the aroma of a 50-day old Serra cheese. Cheese texture was judged by the panel on a scale ranging from −1 (hard) to +3 (extremely soft) using as set point the value +2 for the texture of the 50-day old reference Serra cheese. The number of eyes was determined on a scale defined as: −1 (none), 0 (few small eyes and no medium eyes), 1 (no small eyes and few medium eyes) and 2 (few small and medium eyes) using as set point the value 0 for the 50-day old reference Serra cheese. The number of cracks on the rind was determined in a scale defined as: −2 (several cracks), −1 (few cracks) and 0 (no cracks) using as set point the value 0 for the 50-day old reference Serra cheese.

Statistical analyses

The defining relation of the 2^{6-1}_{VI} design (given, in its canonical form, by $I = x_1x_2x_3x_4x_5x_6$) was used to find the confounded patterns (or alliases) naturally associated with the design owing to its fractional nature. The estimates of the effects associated with each combination of alliases were calculated by Yates' algorithm (Box *et al.*, 1978) using the whole dataset generated. Since the layout of the experiments did not allow for replicates of the cheeses themselves, the determination of which effects were statistically significant was done by plotting all effect estimates of sensory, textural, microbiological, chemical and biochemical properties of cheese on normal probability paper and identifying, for each type of property, those which were clear outliers from the median straight line, and so not easily explained as due to pure chance (Box *et al.*, 1978); those outliers (i.e., the significant effect estimates) were further

included in (simple forms of) a general polynomial model of the form

$$\hat{y} = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_5x_5 + \beta_6x_6 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \\ + \beta_{14}x_1x_4 + \beta_{15}x_1x_5 + \beta_{16}x_1x_6 + \beta_{23}x_2x_3 + \beta_{24}x_2x_4 + \beta_{25}x_2x_5 + \beta_{26}x_2x_6 + \\ + \beta_{34}x_3x_4 + \beta_{35}x_3x_5 + \beta_{36}x_3x_6 + \beta_{45}x_4x_5 + \beta_{46}x_4x_6 + \beta_{56}x_5x_6 + \beta_{123}x_1x_2x_3 + \\ + \beta_{124}x_1x_2x_4 + \beta_{125}x_1x_2x_5 + \beta_{126}x_1x_2x_6 + \beta_{134}x_1x_3x_4 + \beta_{135}x_1x_3x_5 + \\ + \beta_{136}x_1x_3x_6 + \beta_{145}x_1x_4x_5 + \beta_{146}x_1x_4x_5 + \beta_{156}x_1x_5x_6 + \beta_{234}x_2x_3x_4 + \\ + \beta_{235}x_2x_3x_5 + \beta_{236}x_2x_3x_6 + \beta_{245}x_2x_4x_5 + \beta_{246}x_2x_4x_6 + \beta_{256}x_2x_5x_6 + \\ + \beta_{345}x_3x_4x_5 + \beta_{346}x_3x_4x_6 + \beta_{356}x_3x_5x_6 + \beta_{1234}x_1x_2x_3x_4 + \\ + \beta_{1235}x_1x_2x_3x_5 + \beta_{1236}x_1x_2x_3x_6 + \beta_{1245}x_1x_2x_4x_5 + \beta_{1246}x_1x_2x_4x_6 + \\ + \beta_{1256}x_1x_2x_5x_6 + \beta_{1345}x_1x_3x_4x_5 + \beta_{1346}x_1x_3x_4x_6 + \beta_{1356}x_1x_3x_5x_6 + \\ + \beta_{1456}x_1x_4x_5x_6 + \beta_{2345}x_2x_3x_4x_5 + \beta_{2346}x_2x_3x_4x_6 + \beta_{2356}x_2x_3x_5x_6 + \\ + \beta_{2456}x_2x_4x_5x_6 + \beta_{3456}x_3x_4x_5x_6 + \beta_{12345}x_1x_2x_3x_4x_5 + \beta_{12346}x_1x_2x_3x_4x_6 + \\ + \beta_{12356}x_1x_2x_3x_5x_6 + \beta_{12456}x_1x_2x_4x_5x_6 + \beta_{13456}x_1x_3x_4x_5x_6 + \\ + \beta_{23456}x_2x_3x_4x_5x_6 + \beta_{123456}x_1x_2x_3x_4x_5x_6$$

which, given the aforementioned confounding patterns, is equivalent to

$$\hat{y} = (\beta_0 + \beta_{123456}) + (\beta_1 + \beta_{23456})x_1 + (\beta_2 + \beta_{13456})x_2 + (\beta_3 + \beta_{12456})x_3 + \\ + (\beta_4 + \beta_{12356})x_4 + (\beta_5 + \beta_{12346})x5 + (\beta_6 + \beta_{12345})x_6 + (\beta_{12} + \beta_{3456})x_1x_2 + \\ + (\beta_{13} + \beta_{2456})x_1x_3 + (\beta_{14} + \beta_{2356})x_1x_4 + (\beta_{15} + \beta_{2346})x_1x_5 + (\beta_{16} + \beta_{2345})x_1x_6 + \\ + (\beta_{23} + \beta_{1456})x_2x_3 + (\beta_{24} + \beta_{1356})x_2x_4 + (\beta_{25} + \beta_{1346})x_2x_5 + (\beta_{26} + \beta_{1345})x_2x_6 + \\ + (\beta_{34} + \beta_{1256})x_3x_4 + (\beta_{35} + \beta_{1246})x_3x_5 + (\beta_{36} + \beta_{1245})x_3x_6 + (\beta_{45} + \beta_{1236})x_4x_5 + \\ + (\beta_{46} + \beta_{1235})x_4x_6 + (\beta_{56} + \beta_{1234})x_5x_6 + (\beta_{123} + \beta_{456})x_1x_2x_3 + (\beta_{124} + \beta_{356})x_1x_2x_4 + \\ + (\beta_{125} + \beta_{346})x_1x_2x_5 + (\beta_{126} + \beta_{345})x_1x_2x_6 + (\beta_{134} + \beta_{256})x_1x_2x_4 + \\ + (\beta_{135} + \beta_{246})x_1x_3x_5 + (\beta_{136} + \beta_{245})x_1x_3x_6 + (\beta_{145} + \beta_{236})x_1x_4x_5 + \\ + (\beta_{146} + \beta_{235})x_1x_4x_5 + (\beta_{156} + \beta_{234})x_1x_5x_6$$

which, in turn, can be simplified to read

$$\hat{y} = \alpha_0 + \alpha_1x_1 + \alpha_2x_2 + \alpha_3x_3 + \alpha_4x_4 + \alpha_5x_5 + \alpha_6x_6 \\ + \alpha_{12}x_1x_2 + \alpha_{13}x_1x_3 + \\ + \alpha_{14}x_1x_4 + \alpha_{15}x_1x_5 + \alpha_{16}x_1x_6 + \alpha_{23}x_2x_3 + \alpha_{24}x_2x_4 + \alpha_{25}x_2x_5 + \alpha_{26}x_2x_6 + \\ + \alpha_{34}x_3x_4 + \alpha_{35}x_3x_5 + \alpha_{36}x_3x_6 + \alpha_{45}x_4x_5 + \alpha_{46}x_4x_6 + \alpha_{56}x_5x_6 + \\ + \alpha_{123}x_1x_2x_3 + \alpha_{124}x_1x_2x_4 + \alpha_{125}x_1x_2x_5 + \alpha_{126}x_1x_2x_6 + \alpha_{134}x_1x_3x_4 + \\ + \alpha_{135}x_1x_3x_5 + \alpha_{136}x_1x_3x_6 + \alpha_{145}x_1x_4x_5 + \alpha_{146}x_1x_4x_6 + \alpha_{156}x_1x_5x_6$$

where $\hat{y}$ denotes the estimated value of the quality parameter y and the α's denote adjustable parameters (i.e., one-half of the estimate effect). The outlier effects were in every case fewer than 20% of all effects estimated. The statistical analyses were validated by plotting the residuals (obtained as the differences between the experimental data and the values predicted by the empirical model containing only those effects considered significant) against a normal distribution (Box *et al.*, 1978).

Results

The average (α_0) and the remaining α's, i.e. those adjustable parameters in the empiric models for the microbiological, physico–chemical, biochemical, textural and sensory measurements, are shown in Tables 2 to 6, respectively.

Discussion

In order to effectively discuss the results obtained, the fifth order interactions were assumed to be negligible; this assertion is plausible in view of the smoothness and similarity of the response functions (Box *et al.*, 1978). The block variable (B) was not significant since estimates of the confounded effects ($x_1x_2x_5$ or $x_3x_4x_6$) could not be picked up as outliers in any of the normal probability plots mentioned above. Therefore, differences in milk composition, degree of extraction of enzymes from the thistle flower, degree of cutting or any other (lurking) process parameter did not significantly vary within the two consecutive days and were then eliminated from further consideration. Additionally, Tavaria & Malcata

Table 2 Polynomial equations best fitted to the microbiological data

Attribute	Standard Serra cheese	Equation fitted to experimental cheeses
LAB on M17 (log CFU g^{-1})	7.45	$\hat{y} = 6.92 - 0.19x_1 - 0.18x_4 - 0.18x_5 + 0.18x_6 + 0.13x_1x_3 + 0.15x_1x_5 + 0.15x_2x_3 + 0.18x_4x_5 - 0.18x_1x_2x_3 - 0.15x_1x_4x_5$
LAB on RA (log CFU g^{-1})	7.46	$\hat{y} = 7.08 - 0.33x_4 + 0.20x_6 - 0.20x_3x_6 + 0.23x_4x_5 - 0.23x_1x_2x_3$
Enterococci (log CFU g^{-1})	6.27	$\hat{y} = 5.76 - 0.09x_3x_4 + 0.10x_4x_6 - 0.10x_1x_2x_3$
Coliforms (log CFU g^{-1})	3.96	$\hat{y} = 4.72 - 0.55x_4 + 0.50x_6 + 0.25x_5x_6$
Proteolytic microorganisms (log CFU g^{-1})	8.14	$\hat{y} = 7.88 + 0.34x_1x_5 - 0.18x_3x_6 + 0.26x_4x_5 - 0.26x_1x_2x_3 - 0.19x_1x_2x_4 + 0.30x_1x_3x_6$
Lipolytic microorganisms (log CFU g^{-1})	3.96	$\hat{y} = 4.94 + 0.19x_4 + 0.19x_4x_6 - 0.19x_1x_2x_3$

LAB - lactic acid bacteria.

Table 3 Polynomial equations best fitted to the physicochemical data

Attribute	Standard Serra cheese	Equation fitted to experimental cheeses
pH	5.72	$\hat{y} = 5.48 + 0.12x_4 - 0.09x_4x_5 - 0.08x_4x_6 - 0.08x_5x_6 + 0.09x_1x_2x_3$
a_w	0.94	$\hat{y} = 0.96 - 0.02x_4 + 0.02x_6$
Salt (%, w/w)	2.51	$\hat{y} = 2.86 + 1.06x_4 - 0.27x_6 - 0.17x_4x_6$
Moisture (%, w/w)	47.35	$\hat{y} = 39.29 - 3.34x_4 + 4.53x_6 + 2.15x_4x_6$
Fat (%, w/w)	20.75	$\hat{y} = 23.64 + 1.92x_4 - 1.63x_6$
Protein (%, w/w)	20.32	$\hat{y} = 24.34 + 0.68x_2 + 1.58x_4 - 1.60x_6 - 1.14x_4x_6$
Lactose (%, w/w)	0.05	$\hat{y} = 0.43 + 0.25x_4 - 0.16x_5 - 0.16x_6$

Table 4 Polynomial equations best fitted to the biochemical data

Attribute	Standard Serra cheese	Equation fitted to experimental cheeses
Maturation index	0.39	$\hat{y} = 0.26 - 0.07x_4 + 0.06x_6$
Fat acidity (mmol/100 g_{fat})	7.12	$\hat{y} = 6.73 + 0.45x_2 - 1.12x_4 + 0.52x_6 - 0.55x_2x_3 + 0.69x_4x_5 + 0.85x_4x_6 - 0.69x_1x_2x_3 - 0.58x_1x_3x_6 + 0.55x_1x_4x_5 + 0.39x_1x_4x_6$
Lactic acid concentration (%, w/w)	1.02	$\hat{y} = 1.02 - 0.48x_4 + 0.18x_4x_6 - 0.12x_1x_2x_3$

Table 5 Polynomial equations best fitted to the force–deformation curve data

Attribute	Standard Serra cheese	Equation fitted to experimental cheeses
Rind (Fracture force; N)	12.43	$\hat{y} = 25.8 + 3.40x_2 + 14.3x_4 - 14.0x_6 - 9.05x_4x_6$
Paste (Modulus; N mm^{-1})	0.27	$\hat{y} = 3.56 + 3.30x_4 - 2.79x_6 - 2.71x_4x_6$

Table 6 Polynomial equations best fitted to the sensory data

Attribute	Standard Serra cheese	Equation fitted to experimental cheeses
Aroma	1	$\hat{y} = -0.38 - 0.38x_4 + 0.50x_5 + 0.44x_6 + 0.44x_4x_6$
Texture	2	$\hat{y} = 0.22 - 1.03x_4 + 0.47x_5 + 0.85x_6$
Eyes	0	$\hat{y} = 0.25 - 0.69x_4 - 0.50x_2x_5 + 0.38x_4x_5 + 0.38x_5x_6 + 0.50x_1x_3x_4$
Rind cracks	0	$y = -0.63 + 0.57x_5 + 0.32x_6 - 0.38x_5x_6$

(1997) claimed that the quantitative and qualitative microflora composition was not statistically altered throughout the lactation season and along the geographical area of Serra cheese production, so the results obtained in this research effort are, in principle, of relevance for the whole population of Serra cheeses processed under similar conditions.

Rennet concentration influences the rheological properties of the coagulum (Brule & Lenoir, 1987) and also affects proteolysis (Lawrence *et al.*, 1987). The influence of the rennet content on the rate of softening of relatively high moisture cheeses (such as Serra cheese) was reported by Jong (1978); in particular, rennet contents lower and higher than normal were claimed to lead to slower and faster softening, respectively. The two levels of thistle flower added to the milk yielded no significant differences in terms of the chemical, biochemical, textural and sensory characteristics of Serra cheese. Lactic acid bacteria enumerated on M17, mainly *Lactococcus, Enterococcus* and *Leuconostoc* (Macedo *et al.*, 1995), were the only microbial group whose numbers were influenced by the amount of rennet added to the milk (Table 2). This tendency might be explained considering that lactococci in general possess a proteolytic system able to ultimately hydrolyse caseins down to free amino acids (Fox & Law, 1991), on the one hand, and considering that the thistle flower catalyses primarily the hydrolysis of caseins into large peptides at higher rates during early stages of ripening (Macedo & Malcata, 1996), on the other hand.

Coagulation phenomena are strictly dependent on temperature. An increase of coagulation temperature from 20 to 40 °C accelerates the establishment of casein bonds, activates the lactic fermentation and increases the viscosity of the coagulum (Brule & Lenoir, 1987). From inspection of Tables 3–5, one verifies that protein content, fat acidity and fracture force of the rind were increased by coagulation temperature (x_2). Lower temperatures during coagulation lead to weaker three-dimensional casein networks which are less prone to retention of water and thus more prone to loss of proteins and enzymes (such as lipases) during curd cutting, as well as to surface strain. Apparently the range of temperature of coagulation was not large enough to observe a statistical effect of this variable on the microbiological and sensory characteristics of the cheese. Within the experimental range selected, pressing of the fresh cheese (x_3) with a 10-kg block for 24 h had a negligible effect on the microbiological, chemical, biochemical and textural characteristics of Serra cheese.

In general, the dominating effects of salt on cheese ripening are (Guinee & Fox, 1993): (i) control of microbial growth and activity, (ii) control of extracellular enzymic activities, (iii) control of syneresis of the curd and (iv) control of moisture (especially on the surface, where it contributes to formation of the rind). All these observable effects of salt result from its role in controlling the water activity of cheese (a_w). Table 2 shows that the log counts of lactic acid bacteria enumerated on M17, mainly *Lactococcus* and *Leuconostoc* (Macedo *et al.*, 1995), and on RA, mainly *Lactobacillus* and *Leuconostoc* (Macedo *et al.*, 1995), and the log counts of coliforms, mainly *Hafnia alvei* (Macedo *et al.*, 1995), were reduced by surface salting of the fresh cheese (x_4); on the other hand, yeasts, mainly *Sporobolomyces roseus* (Macedo *et al.*, 1995), and lipolytic microorganisms were increased by variable x_4, and this variable also increased pH and salt, fat, protein and residual lactose contents, and reduced water activity and moisture content (Table 3) and maturation index, fat acidity and lactic acid content (Table 4). These latter results can be explained due to the addition of salt to the surface of the cheese, that leads to a decrease in a_w since it is known that low a_w values inhibit bacterial growth (Sperber, 1983), viz. of those strains which produce lactic acid (Table 4). Following the generally accepted observations that there is an approximately linear negative relationship of the salt level with the moisture level in cheese (Marcos *et al.*, 1981), and since salt enters the cheese matrix mainly by diffusion from the surface within time frames of the order of the ripening period (Hardy, 1987), a decreasing salt gradient from the surface to the centre of the cheese should lead to a decreasing moisture gradient in the opposite direction, which should in turn lead to a thicker rind for higher salting levels (as is apparent from inspection of Table 5). The fat and protein contents were increased by the addition of salt due to the negative effect on

the moisture content (the two components coupled with water make up most cheese weight). As discussed above, proteolysis and lipolysis extents are affected by the salting procedure (Table 4), as apparent from the reducing effects of variable x_4 upon the maturation index and the fat acidity. These observations can be rationalized by the role played by salt upon the ionic strength of the cheese matrix, and hence upon the intra-enzymatic quality and quantity of salt-bridging, which in turn directly affects the activity of proteases and lipases contributed by milk itself, the rennet and/or the primary and secondary microflora (Fox *et al.*, 1993). Since proteolysis is inhibited by high salt levels, the body of high-salt cheeses tends to be firmer (Guinee & Fox, 1993), and this assertion agrees with the higher modulus values measured (Table 5).

Curds of different cheese varieties are recognizably differently by the end of coagulation (mainly as a result of compositional and textural differences arising from differences in milk composition and processing factors), so the unique characteristics of the individual cheeses developed during ripening (and hence the flavour and texture of the mature cheeses) are largely predetermined by the composition of the curd and by the type of native microorganisms in the curd (Fox, 1993). During ripening, an extremely complex set of biochemical changes occurs as a result of an equally complex set of enzymic and metabolic pathways effected by the rennet, the indigenous milk enzymes and the microflora. Therefore, all factors that interfere with enzyme activity and microbial growth also interfere with the biochemical processes throughout ripening. Temperature and relative humidity during ripening are two such factors, which play a critical role in the growth and action of microorganisms, as well as in the activity of enzymes present in cheese.

In general, cheese ripening temperature is lower than the optimal temperature for microbial proliferation and enzyme activity, and an increase of temperature will thus cause an accelerated maturation. In Serena cheese (also manufactured from raw ovine milk and *Cynara cardunculus* following an identical technology), González *et al.* (1990) reported that a ripening temperature in the range 10–15 °C had a significant effect on pH, moisture and protein contents, as well as on the ratio of 12% trichloroacetic acid-soluble nitrogen to total nitrogen, but no significant differences were found between the ratio of nitrogen soluble at pH 4.6 to total nitrogen and the viable numbers of coliforms; they also reported that cheeses ripened at 10 °C had better sensory quality than those ripened at 15 °C. The ripening temperature (x_5) was found to be generally irrelevant for Serra cheese characteristics; only the log counts of lactic acid bacteria enumerated on M17 (Table 2) and the residual lactose content (Table 3) were reduced by variable x_5. This apparently unexpected observation in view of the above reasoning could be partly explained by the fact that the two levels of temperatures chosen (5 and 10 °C) were probably not far enough from one another in order to cause major variations in the microbiological and biochemical properties of Serra cheese. It could be argued that a larger pacing could lead to different conclusions; however, preliminary tests done in our laboratory using very low ripening temperatures (i.e. below 5 °C) and very high ripening temperatures (i.e. above 10 °C) have yielded unripened cheeses and excessively bitter cheeses, respectively, after the normal ripening period, and both cases are completely devoid of practical interest. Therefore, the range of temperatures of interest was sufficiently narrow to make the actual value of less concern, which poses an additional difficulty since the ripening chambers ought to be cooled in summer and warmed in winter.

The relative humidity during ripening affects the final moisture content of cheeses by controlling the rate of water evaporation. In low ripening relative humidities the moisture content of cheese will decrease quickly with ripening time, and so a_w will quickly approach the value imposed by the salt content in cheese (although such salt content also depends on the amount of water available in the cheese matrix itself); therefore, low relative humidities during ripening will constrain the extent and rate of biochemical reactions catalysed by enzymes present in the curd. The relative humidity (x_6) exhibited a significant effect on Serra cheese characteristics, the higher level increasing growth (Table 2), moisture content and water activity (Table 3), and the lower level reducing salt, fat, protein and residual lactose contents (Table 3). High ripening relative

humidities reduce evaporation of water, thus leading to relatively high percent moisture contents, and consequently decreasing the percent content of the remaining components. The effect of high ripening relative humidity in increasing maturation index (Table 4), which is a measure of the extent of proteolysis breakdown, can be explained by the fact that the activity of proteases and peptidases is enhanced by high a_w values. The cheese texture is determined primarily by its pH and the ratio of intact casein to moisture, and changes of texture are mainly attributable to hydrolysis of α_s-casein to peptides brought about by rennet (Lawrence *et al.*, 1987); hence, the firmness of the cheese body is reduced by the high relative humidity during ripening, a conclusion that is in agreement with the above rationale. It was observed that a higher relative humidity during ripening produces cheeses with a higher moisture content, and consequently a lower salt content, and that lower amounts of surface salt added to the fresh cheese (i.e. lower salt contents of cheese) lead to higher fat acidity. Therefore, the extent of lipolysis in Serra cheese seems to be affected by the relative humidity via its negative effect on the salt content.

Although the estimated effects produced by variables x_4, x_5 and x_6 should be considered in association with their second or higher order interactions, it was decided to discuss such factors independently from the interactions in an attempt to facilitate understanding of the microbiological, chemical and biochemical transformations occurring in the cheese. Furthermore, if variables caused statistically significant effects on Serra cheese, their second and third order interactions would more likely be significant than the associated alliases (which are third and fourth order interactions involving the other, tentatively not such significant variables). The second order interactions x_4x_5 (salting–ripening temperature) and x_5x_6 (ripening temperature–ripening relative humidity) increased microbial growth. Therefore, the independent negative effects of variables x_4 and x_5, and the positive effect of variable x_6, on growth of microorganisms (previously discussed) should be carefully addressed because some degree of over- or underestimation of the global effects of these variables could arise. Similar considerations apply to the chemical characteristics: from inspection of Table 3, one finds significant estimates for the interactions x_4x_5, x_4x_6 and x_5x_6, so the effect of salting the fresh cheese (x_4) did not affect pH, and salt, protein and moisture contents as strongly as would be expected from consideration of this effect alone, because an opposing effect exists for both interactions salting–ripening temperature (x_4x_5) and salting–relative humidity of ripening (x_4x_6). From inspection of Table 4, it is concluded that the second order interactions x_4x_5 and x_4x_6 have a positive effect on lipolysis extent, as well as on lactic acid production. It is interesting to note that salting of the fresh cheese and ripening relative humidity produce independent negative effects, but their interaction is significantly positive. The firmness of the paste, which is a direct result of proteolysis, was also considerably affected by interaction x_4x_6 (Table 5). The third order interaction $x_1x_2x_3$ (or $x_4x_5x_6$) significantly reduces the number of lactic acid bacteria (Table 2), and so increases the pH (Table 3) and reduces the lactic acid content (Table 4). The number of lipolytic microorganisms (Table 2), as well as fat acidity (Table 4), are reduced by interaction $x_4x_5x_6$. The number of proteolytic microorganisms (Table 2) is reduced by interaction $x_4x_5x_6$, although no significant effects of this interaction upon proteolysis were observed (Table 4).

Interactions other than x_4x_5, x_4x_6 and x_5x_6 (and their corresponding alliases) were found to be statistically significant from microbiological and biochemical standpoints (Tables 2 and 4). Considering that only a half fraction of a 2^6 factorial design was run, some intrinsic difficulties arise in the determination of which interactions are statistically significant due to the existence of confounding patterns. However, if one assumed that interactions involving negligible main effects (such as pressing, x_3) are also negligible, then one would eliminate alliases to some extent, and hence might be able to justify statistical conclusions on the basis of the principles of dairy science. The log counts of lactic acid bacteria enumerated on M17 would then be increased by interactions $x_2x_4x_5x_6$ and $x_1x_4x_5x_6$ if one assumed that these interactions are more relevant than x_1x_3 and x_2x_3. By the same token, lipolysis would be reduced by interaction $x_1x_4x_5x_6$ (instead of x_2x_3), the growths of proteolytic microorganisms and

lactic acid bacteria on M17 increased by interaction x_1x_5, the growths of proteolytic microorganisms and lactic acid bacteria enumerated on RA (mainly *Lactobacillus* and *Leuconostoc*) reduced and increased, respectively, by interaction $x_1x_2x_4x_5$ (instead of x_3x_6), and growth of enterococci reduced by interaction $x_1x_2x_5x_6$ (instead of x_3x_4).

Third order interactions $x_1x_3x_6$, $x_1x_4x_5$ and $x_1x_4x_6$ (and corresponding alliases) were found to be statistically important on the lipolysis extent (measured by fat acidity): interactions $x_1x_4x_5$ and $x_1x_4x_6$ increased fat acidity, whereas interaction $x_1x_3x_6$ decreased it. The log counts of proteolytic microorganisms were increased by interaction $x_1x_3x_6$ and decreased by interaction $x_1x_2x_4$. The growth of lactic acid bacteria on M17 was decreased by interaction $x_1x_4x_5$.

The amount of plant rennet added to the milk, temperature of coagulation and pressing did not lead to significant effects upon the sensory characteristics (Table 6). The aroma of Serra cheese was increased by (in decreasing order) ripening temperature, ripening relative humidity and interaction between salting and ripening relative humidity (x_4x_6), and reduced by salting of the fresh cheese. This can be explained by the fact that proteolysis, lipolysis and glycolysis (which are the principal phenomena in cheese responsible for formation of aroma compounds) are affected similarly by these variables as already discussed (salting reduced microbial growth and enzymatic activity in Serra cheese, whereas both ripening conditions increased them).

The texture of Serra cheese was influenced significantly by ripening temperature (x_5) and ripening relative humidity (x_6). As mentioned above, cheese texture is determined primarily by its pH and the ratio of intact casein to moisture, and considering that pH, moisture content and maturation index were affected by the salting of the fresh cheese (as discussed before), the importance of salting upon texture is apparent. The ripening relative humidity and, to a lesser extent, the ripening temperature were also important factors for the development of texture in Serra cheese: both these variables contribute to the softening of the cheese matrix. Serra cheese is characterized by having none or very few small eyes in the paste. The formation of pin-hole eyes is usually caused by the multiplication of bacteria that are capable of producing gas such as coliforms, *Leuconostoc* and heterofermentative *Lactobacillus* (Choisy *et al.*, 1987). The largest effect of salting the fresh cheese was to reduce the number of eyes, an observation that agrees with previous discussion of the salt effect on the numbers of microorganisms and with consideration that high levels of coliforms and heterofermentative bacteria are present in Serra cheese (Macedo *et al.*, 1995). The number of eyes was also reduced by interaction x_2x_5, but such effect cannot be explained by higher rates of growth of bacteria (Table 2). Interaction $x_1x_3x_4$ (or $x_2x_5x_6$) produced the largest increase in the number of eyes, followed, to a lesser extent, by interactions x_4x_5 and x_5x_6. Finally, the number of cracks on the rind which was studied because it is a common and recurrent problem faced by manufacturers seeking certification status (and thus a higher market price, for their cheeses) was statistically reduced by increasing the ripening temperature and, to a lesser degree, by increasing the ripening relative humidity (Table 6), although interaction x_5x_6 exhibited an opposite effect.

Conclusions

From the reported results, one concludes that surface salt addition to the fresh cheese and relative humidity during ripening are the technological factors with the strongest influence on Serra cheese characteristics, although other manufacture and ripening parameters also play minor roles in assurance of good cheese quality standards. Hence, lower levels of salt and higher levels of ripening relative humidity may become valuable parameters in attempts to improve the quality of Serra cheese, and should be so considered in further optimization work.

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