

Amino acid and soluble nitrogen evolution throughout ripening of Serra da Estrela cheese

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Abstract

Four batches of Serra da Estrela cheese originating from as many dairy farms were sampled throughout the ripening period, and assayed for the evolution of free amino acid (FAA) content, total nitrogen content (TN), water-soluble nitrogen content (WSN), trichloroacetic acid-soluble nitrogen content (TCASN) and phosphotungstic acid-soluble nitrogen content (PTASN). The WSN content increased from 1% (on the day of manufacture) up to 43% of TN by 180 d of ripening, thus reflecting the intense proteolytic activity of the enzymes contributed by the plant coagulant utilized. The TCASN was also found to be high in this cheese by the end of ripening (16–20%), which suggests a high extent of FAA release throughout maturation. The major FAA by 180 d of ripening were Glu, Val, Leu and Lys, representing 56–70% of the total in all four dairies sampled. Cheeses produced from refrigerated milk possessed higher amounts of γ -amino-n-butyric acid (Gaba) and lower amounts of Glu when compared with those manufactured with non-refrigerated milk.

Introduction

Proteolysis is referred to by many authors (Fox, Law, McSweeney, & Wallace, 1993) as the most important set of biochemical events taking place during cheese ripening, owing to their major impact on texture and flavor development. During proteolysis, proteins are degraded to primary products (polypeptides), and subsequently to secondary products such as small and medium-size peptides, and eventually free amino acids (FAA; Desmazeaud & Gripon, 1977). Proteolysis leads to substances that are either important for flavor per se, or which act as aroma precursors (Adda, Gripon, & Vassal, 1982). Certain FAA are extremely important in flavor development, e.g. Arg is related to bitterness, while Pro, Ser and Asn are related to sweetness (Izco & Torre, 2000). Although the direct contribution of FAA

to the typical cheese flavors may be somewhat limited, they are associated with the volatile fraction of cheese (Salles et al., 2000) because they are precursors of many volatile aroma compounds (e.g. amines, carboxylic acids, thiols, esters, alcohols, aldehydes and thioesters).

Techniques for assessment of cheese proteolysis have been extensively reviewed (Rank, Grappin, & Olson, 1985; McSweeney & Fox, 1993; Fox, McSweeney, & Singh, 1995). Various extraction protocols are available, using different solvents according to solubility patterns of the proteolysis products under scrutiny. Primary proteolysis is believed to cause softening of the cheese texture early during ripening, via disruption of its three-dimensional protein matrix (Fox et al., 1993). The secondary proteolysis generates peptides that are small enough to be detected by the human taste receptors, to influence background flavor often in a negative fashion (e.g. bitterness); in addition, they may serve as substrates for microbial proteinases and peptidases, that lead in turn to smaller peptides and FAA. The alteration of cheese texture also influences release of aroma compounds during mastication (Fox et al., 1993). The FAA quantitative profile in cheese is usually complex, as

Abbreviations: DM, dry matter; FAA, free amino acids; TN, total nitrogen; WSN, water soluble nitrogen; PTASN, phosphotungstic acid-soluble nitrogen; TCASN, trichloroacetic acid-soluble nitrogen

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it results from the enzymatic degradation of short peptides by various microorganisms coupled with FAA interconversion. Unlike reports by Bütikofer and Fuchs (1997) pertaining to Appenzeller, Emmentaler, Gruyère, Raclette, Sbrinz and Tilsiter cheeses, such a profile was difficult to predict in our case owing to lack of standardization of the cheesemaking procedure.

Serra da Estrela cheese is manufactured artisanally from raw ewe's milk and coagulated using an aqueous extract of the wild thistle (*Cynara cardunculus*). Manufacture is dependent on the seasonal availability of ovine milk, and usually takes place only from December to May. Hence, a number of factors are believed to affect milk composition, including climate, nutritional and physiological status of the flock, and lactation stage. Consequently, variation in the physicochemical and microbiological composition of milk is expected to exert an influence on the final characteristics of the cheese. In previous studies pertaining to proteolysis of Serra da Estrela cheese (Macedo & Malcata, 1997), the contents of water-soluble nitrogen (WSN) and of nitrogen soluble in 2% TCA, in 12% TCA and in 5% PTA were followed up to 35 d of ripening during the cheesemaking season, for only one single dairy and using only non-refrigerated milk. Sousa and Malcata (1997) compared these parameters between cheeses manufactured with *C. cardunculus* using ovine, caprine and bovine milk. However, both of these studies did not consider dairy-to-dairy variation, the ripening period was relatively short and no experimental results were generated encompassing FAA.

The aim of this study was thus to ascertain major trends in evolution of the nitrogenous fractions of Serra da Estrela cheese, with a particular emphasis on FAA, in cheeses originating from a number of selected dairies in the official demarcated region, throughout a ripening period of up to 180 d. Such a study is relevant toward assessment of the roles of the coagulant and the microflora upon flavor and texture generation in this type of cheese, as well as in testing a set of measurements that can lead to technological standardization of such a complex and heterogeneous product.

Materials and methods

Cheese manufacture

In order to assess the effects of ripening time and location of the processor, 20 cheeses were manufactured on the same day (in January 1998) according to the traditional protocol (Macedo, Malcata, & Oliveira, 1993), from raw milk immediately following collection in each of four well spaced dairies (located in Oliveira do Hospital, Penalva, Nelas and Gouveia, all within the Appellation d'Origine Protégée region in Portugal). The

cheeses were ripened at 7–10°C and 85–95% relative humidity in all dairies, and brought to our laboratory at each of 10 selected times (0, 3, 7, 18, 35, 60, 90, 120, 150 and 180 d following manufacture) for chemical analyses. Two independent cheeses selected at random were used as true replicates for each analysis; the resulting experimental design was thus a 4 (dairies) × 10 (ripening times) full factorial design, replicated twice. The milk pH and temperature were measured prior to cheesemaking, and were in 6.3–6.6 and 29–32°C ranges, respectively.

Additionally, in order to assess the effect of using refrigerated or non-refrigerated milk in the cheese manufacture, 10 extra cheeses were produced in one of the dairies (located in Oliveira do Hospital) from refrigerated milk (kept at 4°C for 12 h), and allowed to ripen for 60, 90, 120, 150 and 180 d (with sampling as above). Studies were only carried out encompassing ripening times greater than 60 d because previous experience had indicated that at earlier times, the pool of FAA is still incipient.

Sample preparation and analyses

The total nitrogen (TN) content was determined by the micro-Kjeldahl method (FIL-IDF; 1993) using a Kjeltex system 1002 distilling unit (Tecator; Höganäs; Sweden). Fractionation of the cheese nitrogen was according to FIL-IDF (1991). The WSN was obtained by homogenization of a cheese sample with water following Kuchroo and Fox (1982). The TCASN was based on a fraction prepared by adding 7.5 ml of an aqueous solution of 48% (w/v) TCA (Merck; Germany) to 22.5 mL of water-soluble extract; the mixture was allowed to stand for 30 min at room temperature, and then filtered through Whatman No. 42 filter paper. The PTASN was based on a fraction prepared by adding 14 ml of 3.95 M sulfuric acid (Pronalab; Lisbon; Portugal) and 6 ml of 33.3% (w/v) PTA (Merck; Germany) to 20 ml of water-soluble extract. The mixture was then allowed to stand overnight at 4°C, and subsequently filtered through Whatman No. 542 filter paper. Aliquots from both fractions were then analysed by the micro-Kjeldahl method. All determinations were made in duplicate.

Using the Pico-Tag™ (Waters, Milford MA, USA) method for sample preparation, the FAA in cheese samples were analysed using two replicates according to Alonso, Alvarez, and Zapico (1994) with some modifications: the gradient and flow rate are shown in Table 1; the column temperature was 50°C; and absorbance was recorded at 254 nm. The chromatograms were processed using the Millenium 2010 software package (Waters). In order to quantify the FAA, 22 solutions of pure standards (Sigma) were prepared as 12.5 mM for Tyr and 25 mM for the other 21 amino acids.

Table 1
Gradient conditions used for HPLC elution

Time (min)	Flow rate (mL min ⁻¹)	% (v/v) eluant A	% (v/v) eluant B	Curve type
0	0.90	100	0	—
5	0.80	100	0	Concave
20	0.75	78	22	Slightly concave
40	0.80	54	46	Slightly concave
42	1.00	0	100	Slightly concave
43	1.00	0	100	Slightly concave
44	1.00	100	0	Slightly concave
46	1.50	100	0	Slightly concave
47	0.90	100	0	Slightly concave

Eluant A: 94:6 (v/v) of anhydrous sodium acetate solution (11.48 g in 1 L H₂O, pH = 6.65) and acetonitrile (HPLC grade).

Eluant B: 40:60 (v/v) H₂O and acetonitrile (HPLC grade).

Calibration was carried out using hydroxy-proline and nor-leucine as internal standards. The dry matter of the cheese samples was determined according to FIL-IDF (1982), for the results be expressed on a dry matter basis.

Statistical analyses

The effects of ripening time, dairy farm and refrigeration status of the milk (as well as analytical replication) on the contents of TN (per 100 g cheese), WSN (as % of TN), TCASN (as % of TN) and PTASN (as % of TN), and on the concentration of every FAA were assessed using analysis of variance (ANOVA); for each (either linear or interactive) significant effect, the corresponding critical difference was computed as a basis for the least significant difference; the multiple comparison test was set at the 5% level of significance. In one set of tests the main factors were farm and ripening time; in the other set, these were ripening time and refrigeration status. All statistical analyses were carried out with the software Statistica for Windows (StatSoft, 1996).

Results and discussion

Soluble nitrogen assays

The evolution of soluble nitrogen (mean values and associated standard deviations) in cheeses produced in the various dairies is shown in Fig. 1, whereas comparison of cheeses manufactured from non-refrigerated and refrigerated milk in one dairy is shown in Fig. 2. The WSN represented ca. 11% of TN at the time of manufacture, and increased to 43% by 180 d of ripening. The TCASN accounted for ca. 1.8% of TN by day 0, reaching 16% by 180 d. The PTASN ranged from 1% at the day of manufacture up to 12% of TN by 180 d of ripening. Therefore, quite high values of WSN were recorded for Serra da Estrela cheese from the very start of ripening when compared to Manchego (Fontecha, Peláez, & Juárez, 1994) or Los Pedroches (Fernández-

Salguero & Sanjuán, 1999) cheeses, as a likely result of the intense proteolytic activity of the enzymes contributed by the plant coagulant (*C. cardunculus*).

The water-soluble fraction is very heterogenous in terms of composition, and includes whey proteins, and high-, medium- and low-molecular weight peptides, as well as FAA. The set of reactions normally designated as secondary proteolysis result from the action of residual rennet and indigenous milk proteinases, in addition to adventitious microflora. Similar trends have been reported by Vioque et al. (2000) in a ewe's milk cheese coagulated with *C. cardunculus*, and by Macedo and Malcata (1997) for Serra da Estrela cheese. The latter authors reported 34.6% WSN, 5.8% TCASN and 1.2% PTASN for 35 d-old cheeses, similar to our average values pertaining to the four dairies (i.e. 23.8%, 5.43% and 1.3%, respectively). Fernández-Salguero and Sanjuán (1999) reported values of 33.4% for WSN in ewe's milk cheese after 60 d of ripening that had been coagulated with aqueous extracts of *C. cardunculus*. In La Serena cheese by the same time of ripening, WSN was 38.8%, whereas non-protein nitrogen leveled off at 14.5% (Fernández del Pozo, Gaya, Medina, Rodríguez-Marín, & Núñez, 1988; Fernández-Salguero, Matos, & Marsilla, 1978); other researchers (Núñez, Fernández del Pozo, Rodríguez-Marín, Gaya, & Medina, 1991) reported even higher values for WSN and non-protein nitrogen, thus confirming that cheeses coagulated with plant rennets undergo extensive proteolysis.

The values for the non-protein nitrogen fraction (TCASN) were lower than those of WSN, which is expected considering that Kuchroo and Fox (1982) showed this fraction to contain mainly small peptides (2–20 residues) and FAA. Such TCASN fraction is thus traditionally regarded as a “ripening depth” index, as most peptides that are a part of the WSN fraction will precipitate in the presence of 12% TCA. Our TCASN values were in the 16–20% range, hence confirming the conclusion by other authors who have studied plant coagulants (Macedo & Malcata, 1997;

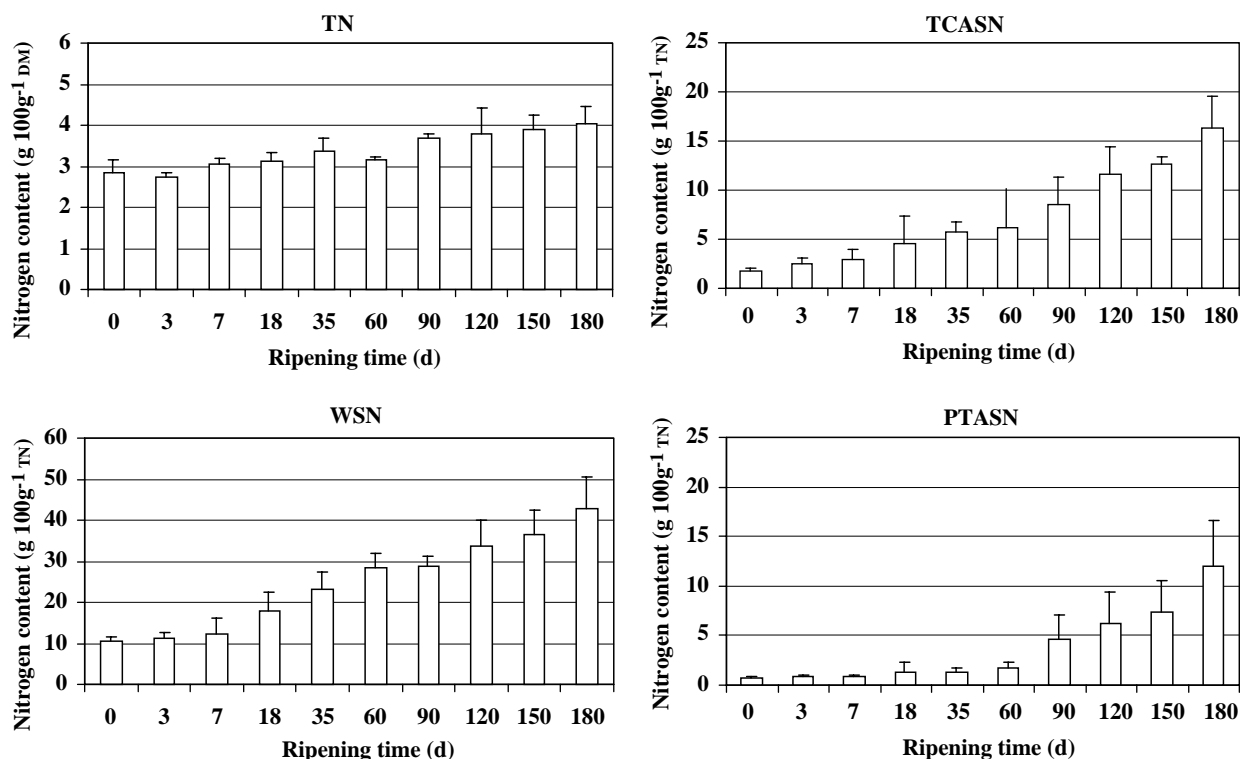


Fig. 1. Mean ($n = 4$ dairies) and corresponding standard deviation for total nitrogen (TN) as % of dry matter (DM); and for water-soluble nitrogen (WSN), TCA-soluble nitrogen (TCASN) and PTA-soluble nitrogen (PTASN), all as % TN, in Serra da Estrela cheese during the ripening process.

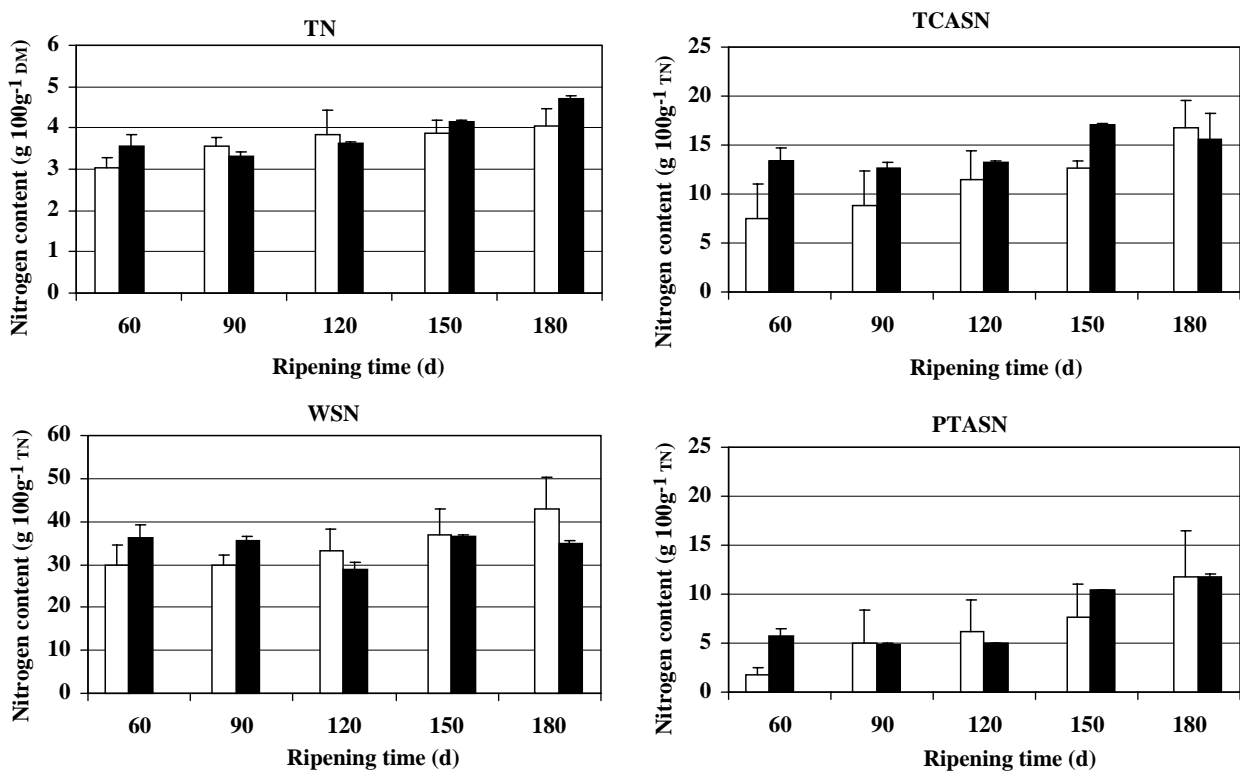


Fig. 2. Mean ($n = 2$ replicates) and corresponding standard deviation for total nitrogen (TN) as % of dry matter (DM); and for water-soluble nitrogen (WSN), TCA-soluble nitrogen (TCASN) and PTA-soluble nitrogen (PTASN), all as % TN in Serra da Estrela cheese produced from non-refrigerated milk (□) and refrigerated milk (■) during the ripening process.

Fernández-Salguero & Snjuan, 1999), and further reinforcing the claim that the strong proteolytic action of *C. cardunculus* expands considerably the inventory of fermentable, nitrogen-containing substrates in this cheese. The PTASN fraction (which is a measure of the concentration of FAA) increased by 175% between 60 and 90 d of ripening. The sharpest increase per day was observed between 7 and 18 d of ripening (6.1%), and between 60 and 90 d and 150 and 180 d of ripening (2.0%), hence coinciding with the exponential increase in the numbers of viable lactic acid bacteria during ripening (Dahl, Tavarıa, & Malcata, 2000). These pieces of evidence suggest that the plant coagulant possesses little peptidase activity, in agreement with Sousa and Malcata (1997) and Reis et al. (2000), hence reinforcing the hypothesis that the peptidases synthesized by the adventitious microorganisms, the only ones present in this type of cheese, are the main vectors of FAA release.

Based on the ANOVA results, dairy farm and ripening time were both found to be statistically significant ($p < 0.05$) toward determination of all nitrogen fractions, so they can be claimed to be directly associated with the heterogeneity observed among the cheeses. The remaining factor (milk refrigeration) also played a significant role ($p < 0.05$) except for TCASN. Least significant differences between non-refrigerated

and refrigerated milk cheeses (at the 5% level of significance) showed significant differences for WSN, PTASN and TCASN, but only at later maturation times (from 150 d on). Refrigeration apparently affected the TN content by 60 d but not afterwards (Fig. 2); this is believed to arise from the development of a psychrotrophic flora during milk storage at 4°C, which initially may bring about a slight pH decrease (6.0 vs. 6.6, data not shown), and in turn promotes action of the coagulant (a plant rennet containing aspartic proteases). Refrigeration of milk is known to induce changes that can affect cheese quality; Lalos and Roussis (2000), when studying the quality of Kefalograviera cheese produced from raw and refrigerated ewe's milk, concluded that cold storage accelerated flavor development and enhanced flavor of the low-fat variety. Witting (1981) reported increased coagulation time for refrigerated milk, thus producing a less firm coagulum, as well as promoting growth of psychrophilic bacteria that release lipases and heat-resistant proteinases.

The flavor impact caused by the water-soluble fraction of cheese is well established, since it contains the majority of such taste compounds as salts, amino acids and low-molecular weight peptides, in addition to some volatiles (Engels, Dekker, de Jong, Neeter, & Visser, 1997; Salles et al., 2000). However, the variety of

Table 2

Evolution of FAA during ripening in Serra da Estrela cheese manufactured from refrigerated milk (mean \pm standard deviation within one batch, expressed as mg 100 g⁻¹ DM)

Amino acid	Ripening time (d)				
	60	90	120	150	180
Asp	3.30 \pm 0.06 ^a	37.89 \pm 2.45 ^{ab}	70.57 \pm 1.53 ^b	53.47 \pm 1.92 ^b	46.97 \pm 1.08 ^b
Glu	25.12 \pm 0.08 ^{a*}	87.74 \pm 1.13 ^a	110.34 \pm 3.34 ^{a*}	299.58 \pm 7.40 ^b	230.60 \pm 13.81 ^{b*}
Asn	ND ^a	0.15 \pm 0.00 ^a	ND ^a	ND ^a	ND ^a
Ser	3.01 \pm 0.04 ^a	12.76 \pm 0.08 ^a	8.84 \pm 0.27 ^a	4.72 \pm 0.18 ^a	15.78 \pm 1.27 ^b
Gln	46.80 \pm 1.18 ^a	77.03 \pm 0.81 ^{b*}	89.11 \pm 1.39 ^{b*}	105.78 \pm 0.98 ^c	103.14 \pm 9.06 ^c
Gly	25.98 \pm 0.59 ^{a*}	25.14 \pm 1.97 ^{a*}	40.54 \pm 1.68 ^{b*}	31.28 \pm 0.80 ^a	34.23 \pm 1.85 ^{a*}
His	0.41 \pm 0.05 ^a	6.03 \pm 0.03 ^a	ND ^a	ND ^a	28.50 \pm 2.15 ^b
Tau	19.26 \pm 0.13 ^a	22.45 \pm 0.24 ^{ab}	38.06 \pm 1.08 ^{abc}	40.78 \pm 0.93 ^{bc}	49.57 \pm 3.63 ^c
Gaba	23.81 \pm 0.18 ^a	34.98 \pm 0.02 ^a	58.90 \pm 1.95 ^{ab}	141.89 \pm 2.07 ^{ab}	163.89 \pm 9.15 ^b
Arg + Thr	56.82 \pm 0.53 ^{a*}	72.70 \pm 0.65 ^{ab*}	128.85 \pm 4.11 ^{c*}	104.17 \pm 1.80 ^{bc*}	99.03 \pm 5.74 ^{abc*}
Ala	27.67 \pm 0.18 ^a	26.87 \pm 0.24 ^a	95.78 \pm 2.43 ^{b*}	116.06 \pm 1.41 ^{bc}	142.00 \pm 1.90 ^{c*}
Pro	26.46 \pm 0.24 ^a	16.35 \pm 0.19 ^a	50.23 \pm 1.41 ^{ab}	39.47 \pm 1.31 ^a	97.04 \pm 9.25 ^b
Tyr	ND ^a	19.36 \pm 11.32 ^{ac}	68.80 \pm 2.11 ^{b*}	74.43 \pm 1.57 ^{b*}	39.29 \pm 1.14 ^c
Val	330.61 \pm 3.33 ^{a*}	334.92 \pm 1.13 ^{a*}	494.26 \pm 15.89 ^{b*}	413.28 \pm 8.09 ^{ab}	494.06 \pm 37.47 ^b
Met	34.47 \pm 0.28 ^{ab*}	27.38 \pm 0.10 ^a	54.33 \pm 1.65 ^{ab}	57.14 \pm 0.80 ^{ab}	67.74 \pm 2.88 ^b
Cys	ND ^a	ND ^a	ND ^a	ND ^a	63.48 \pm 1.20 ^b
Ile	64.74 \pm 0.81 ^{a*}	55.92 \pm 0.75 ^a	100.27 \pm 3.24 ^{b*}	105.94 \pm 2.23 ^b	110.40 \pm 11.42 ^b
Leu	477.09 \pm 6.79 ^a	506.11 \pm 2.94 ^a	759.58 \pm 22.91 ^b	543.70 \pm 9.54 ^a	612.93 \pm 34.96 ^{ab}
Phe	124.41 \pm 3.85 ^{a*}	129.71 \pm 1.19 ^a	167.12 \pm 5.81 ^a	144.31 \pm 3.03 ^a	154.50 \pm 11.30 ^a
Trp	29.53 \pm 0.91 ^a	33.63 \pm 0.14 ^a	24.50 \pm 0.71 ^{a*}	26.69 \pm 0.64 ^{a*}	40.28 \pm 0.97 ^{a*}
Lys	97.44 \pm 3.07 ^a	178.80 \pm 0.96 ^b	200.09 \pm 6.36 ^b	307.56 \pm 5.08 ^{cd}	397.43 \pm 22.45 ^d
Total	1416.90 \pm 13.28 ^{a*}	1705.91 \pm 4.51 ^{a*}	2560.17 \pm 77.86 ^a	2610.25 \pm 49.76 ^a	2990.87 \pm 182.67 ^a

Means in the same row without a common letter are significantly different ($p < 0.05$).

*Significant differences between cheeses manufactured from non-refrigerated and refrigerated milk.

ND—not determined.

Table 3
Evolution of FAA during ripening in Serra da Estrela cheese (mean \pm standard deviation of eight replicates-two for each of four dairy farms, expressed as mg 100 g⁻¹ DM)

Amino acid	Ripening time (d)									
	0	3	7	18	35	60	90	120	150	180
Asp	0.95 \pm 0.63 ^a	0.28 \pm 0.22 ^a	1.09 \pm 1.76 ^a	1.03 \pm 1.53 ^a	0.66 \pm 0.59 ^a	3.67 \pm 0.59 ^a	11.25 \pm 9.18 ^{ab}	33.27 \pm 27.26 ^c	25.52 \pm 9.460 ^b	51.37 \pm 32.22 ^c
Glu	2.06 \pm 3.70 ^a	3.30 \pm 2.94 ^a	14.50 \pm 19.26 ^{ab}	14.71 \pm 9.93 ^{ab}	21.27 \pm 5.62 ^{ab}	70.40 \pm 57.98 ^{bc}	105.29 \pm 57.30 ^c	318.42 \pm 33.92 ^{de}	261.90 \pm 71.98 ^d	360.33 \pm 53.17 ^c
Asn	ND ^a	0.60 \pm 1.10 ^a	3.08 \pm 3.57 ^a	1.66 \pm 0.97 ^a	4.84 \pm 2.57 ^{ab}	2.85 \pm 2.91 ^a	2.15 \pm 2.76 ^a	2.46 \pm 2.14 ^a	6.49 \pm 12.03 ^a	7.12 \pm 15.03 ^a
Ser	0.19 \pm 0.18 ^a	0.41 \pm 0.18 ^a	0.51 \pm 0.94 ^a	0.48 \pm 0.85 ^a	0.17 \pm 0.11 ^a	0.74 \pm 0.98 ^a	0.44 \pm 0.62 ^a	19.44 \pm 14.37 ^{ab}	35.36 \pm 32.52 ^b	37.73 \pm 35.29 ^b
Gln	1.14 \pm 2.11 ^a	0.74 \pm 1.37 ^a	3.18 \pm 4.20 ^a	6.20 \pm 4.03 ^a	10.32 \pm 6.39 ^a	12.88 \pm 7.24 ^a	13.91 \pm 11.04 ^a	25.16 \pm 48.54 ^a	79.11 \pm 51.66 ^b	79.39 \pm 28.29 ^b
Gly	1.49 \pm 0.34 ^a	1.44 \pm 0.19 ^a	2.22 \pm 1.20 ^a	0.93 \pm 0.67 ^a	6.48 \pm 2.56 ^{ab}	7.37 \pm 1.79 ^{ab}	11.25 \pm 7.05 ^{bc}	16.44 \pm 5.48 ^{cd}	20.99 \pm 6.02 ^d	11.32 \pm 11.77 ^{bc}
His	0.18 \pm 0.34 ^a	0.13 \pm 0.24 ^a	3.63 \pm 4.95 ^a	2.92 \pm 2.19 ^a	4.39 \pm 1.75 ^a	7.11 \pm 6.66 ^a	12.79 \pm 6.63 ^a	24.73 \pm 16.41 ^a	24.23 \pm 21.52 ^a	69.01 \pm 92.19 ^b
Tau	4.50 \pm 2.89 ^a	3.91 \pm 0.79 ^a	3.78 \pm 1.94 ^a	2.97 \pm 1.11 ^a	7.28 \pm 2.92 ^a	8.41 \pm 1.79 ^a	14.49 \pm 5.61 ^a	30.83 \pm 11.49 ^b	35.47 \pm 14.56 ^b	47.88 \pm 11.28 ^c
Gaba	3.28 \pm 3.65 ^a	3.22 \pm 2.60 ^a	6.36 \pm 6.44 ^a	6.59 \pm 3.17 ^a	19.27 \pm 9.24 ^a	27.36 \pm 11.24 ^{ab}	55.41 \pm 13.35 ^{ab}	90.53 \pm 16.41 ^{bc}	131.03 \pm 88.45 ^c	101.57 \pm 137.18 ^c
Arg + Thr	1.65 \pm 2.34 ^a	2.47 \pm 1.32 ^a	6.64 \pm 4.45 ^a	4.63 \pm 2.29 ^a	9.91 \pm 7.45 ^{ab}	14.03 \pm 1.86 ^{ab}	24.47 \pm 13.03 ^{bc}	27.10 \pm 11.17 ^c	38.98 \pm 13.92 ^{cd}	49.71 \pm 27.07 ^d
Ala	2.26 \pm 2.96 ^a	2.04 \pm 1.22 ^a	2.59 \pm 1.53 ^a	2.48 \pm 1.38 ^a	4.83 \pm 8.12 ^a	9.76 \pm 6.41 ^a	17.21 \pm 9.13 ^a	62.98 \pm 9.01 ^b	83.01 \pm 27.26 ^b	82.48 \pm 27.59 ^b
Pro	4.02 \pm 6.70 ^a	2.84 \pm 3.03 ^a	5.15 \pm 6.17 ^a	2.70 \pm 2.70 ^a	7.34 \pm 8.60 ^a	8.51 \pm 5.59 ^a	9.03 \pm 2.95 ^a	30.44 \pm 10.31 ^a	62.34 \pm 28.21 ^b	98.87 \pm 49.29 ^c
Tyr	12.86 \pm 3.75 ^{ab}	17.23 \pm 5.53 ^{ab}	14.98 \pm 14.37 ^{ab}	2.22 \pm 2.16 ^a	4.29 \pm 3.65 ^a	4.58 \pm 3.43 ^a	8.87 \pm 6.98 ^a	19.82 \pm 21.63 ^{ab}	13.63 \pm 15.22 ^{ab}	30.04 \pm 15.49 ^b
Val	8.44 \pm 11.71 ^a	9.66 \pm 4.24 ^a	17.63 \pm 14.03 ^a	18.62 \pm 8.82 ^a	64.97 \pm 37.01 ^{ab}	118.72 \pm 14.35 ^{bc}	148.15 \pm 8.68 ^c	316.26 \pm 28.63 ^d	390.60 \pm 94.73 ^e	456.37 \pm 57.64 ^f
Met	1.38 \pm 0.92 ^a	1.17 \pm 0.25 ^a	1.92 \pm 1.29 ^a	1.42 \pm 0.77 ^a	3.46 \pm 2.51 ^{ab}	7.92 \pm 1.04 ^{ab}	18.47 \pm 26.53 ^{bc}	27.36 \pm 6.77 ^c	45.16 \pm 23.36 ^d	56.00 \pm 17.73 ^d
Cys	3.10 \pm 4.01 ^a	5.15 \pm 3.53 ^a	6.97 \pm 7.27 ^a	9.62 \pm 5.30 ^a	32.56 \pm 10.37 ^{ab}	26.36 \pm 7.30 ^{ab}	31.98 \pm 12.81 ^{ab}	20.43 \pm 29.58 ^a	51.94 \pm 48.88 ^b	57.56 \pm 15.30 ^b
Ile	0.56 \pm 1.03 ^a	0.15 \pm 0.28 ^a	0.29 \pm 0.53 ^a	0.28 \pm 0.49 ^a	4.89 \pm 8.77 ^a	11.82 \pm 2.60 ^a	24.97 \pm 16.20 ^{ab}	47.76 \pm 13.65 ^{bc}	77.79 \pm 42.20 ^{cd}	104.62 \pm 49.45 ^d
Leu	12.75 \pm 15.93 ^a	15.72 \pm 7.59 ^a	30.82 \pm 21.04 ^a	36.37 \pm 12.63 ^{ab}	113.39 \pm 46.17 ^b	228.79 \pm 28.87 ^{cd}	295.28 \pm 44.09 ^d	502.66 \pm 37.23 ^e	569.81 \pm 118.46 ^{ef}	599.66 \pm 64.33 ^f
Phe	2.75 \pm 4.26 ^a	3.53 \pm 1.55 ^a	6.44 \pm 4.70 ^a	8.61 \pm 3.21 ^a	33.38 \pm 15.56 ^{ab}	64.49 \pm 9.64 ^{bc}	86.84 \pm 22.73 ^{cd}	119.51 \pm 8.76 ^{de}	148.50 \pm 55.04 ^{ef}	169.45 \pm 24.06 ^f
Trp	8.10 \pm 7.77 ^a	6.90 \pm 3.28 ^a	15.75 \pm 18.43 ^a	13.69 \pm 4.77 ^a	21.22 \pm 5.86 ^{ab}	50.34 \pm 38.17 ^{ab}	77.55 \pm 36.17 ^{ab}	103.65 \pm 45.58 ^{bc}	165.26 \pm 110.68 ^c	186.48 \pm 108.66 ^c
Lys	2.27 \pm 4.20 ^a	1.86 \pm 1.48 ^a	7.04 \pm 12.50 ^a	3.51 \pm 2.97 ^a	12.93 \pm 8.51 ^a	33.93 \pm 17.64 ^b	91.21 \pm 48.65 ^b	224.59 \pm 63.24 ^c	292.11 \pm 116.64 ^c	429.81 \pm 156.71 ^d
Total	75.47 \pm 85.74 ^a	82.73 \pm 32.49 ^a	154.56 \pm 137.13 ^a	141.64 \pm 64.04 ^a	387.86 \pm 172.67 ^{ab}	720.05 \pm 160.80 ^{bc}	1072.99 \pm 174.71 ^c	2060.20 \pm 244.63 ^d	2559.21 \pm 776.18e	3086.75 \pm 383.32 ^f

Means in the same row without a common letter are significantly different ($p < 0.05$).

ND—not determined.

cheese peptides makes it difficult to correlate flavor with specific peptides. As suggested by Engels and Visser (1994), large and medium-sized peptides are probably not major contributors to cheese flavor. These authors concluded that low-molecular weight compounds (<500 Da) are responsible for flavor in the water-soluble fraction of several cheeses, as accounted for by small peptides, FAA, free fatty acids, or breakdown products of such compounds; however, their direct contribution to flavor is rather limited.

Free amino acid assays

The FAA composition of Serra da Estrela cheese varied quantitatively, increasing with ripening time up to $3250 \text{ mg } 100 \text{ g}^{-1} \text{ DM}$ as apparent in Tables 2 and 3, as well as qualitatively along the maturation process. Early in maturation (by 1 d), the major FAA were Tau (which originates from Cys), Tyr, Val, Leu and Trp, comprising 51–80% of the total on a molar basis, whereas the major FAA by 180 d were Glu, Val, Leu and Lys, which constituted 56–70% of the total. Val and Leu alone accounted for ca. 20% of the total by 1 d, and ca. 34% by the end of ripening. These four FAA tend to be present at higher levels in refrigerated milk cheeses (Fig. 3) up to 120 d of ripening, after which they tend to be present in highest amounts in the non-refrigerated milk counterparts; this is quite evident in the case of Leu and Val. However, these differences were not always

significant, as can be inferred from Table 2. As discussed before, refrigeration overall lowers the pH, therefore favoring release of certain amino acid residues (although this effect may not be equally apparent throughout the whole ripening period, as such other factors as moisture content, a_w and E_h come into play). By the end of ripening, these differences are less notorious because the developing microflora are then mainly responsible for amino acid release from small- and medium-sized peptides. The numbers of viable lactic acid bacteria are known to remain high (10^8 – 10^9 CFU g^{-1} cheese) up to 180 d of ripening in Serra da Estrela cheese (Dahl et al., 2000), being higher in cheeses manufactured from refrigerated milk. It can then be concluded that this effect is associated with the refrigeration process itself, rather than with the intrinsic microbial activity. The greatest rate of increase in FAA was observed between 3 and 7 d following manufacture ($21.7\% \text{ d}^{-1}$), although the major overall increase was observed between 18 and 35 d ($174\% \text{ d}^{-1}$). This fact is associated with the exponential increase in the numbers of viable lactic acid bacteria in Serra da Estrela cheese (Dahl et al., 2000), which reinforces the likely role of these microorganisms in flavor generation via FAA conversion into volatile compounds.

The FAA profile of several cheeses throughout ripening is available in the literature (e.g. Marcos & Mora, 1982; González de Llano, Polo, Ramos, & Martín-Alvarez, 1991; Fresno, Carballo, Bernardo, &

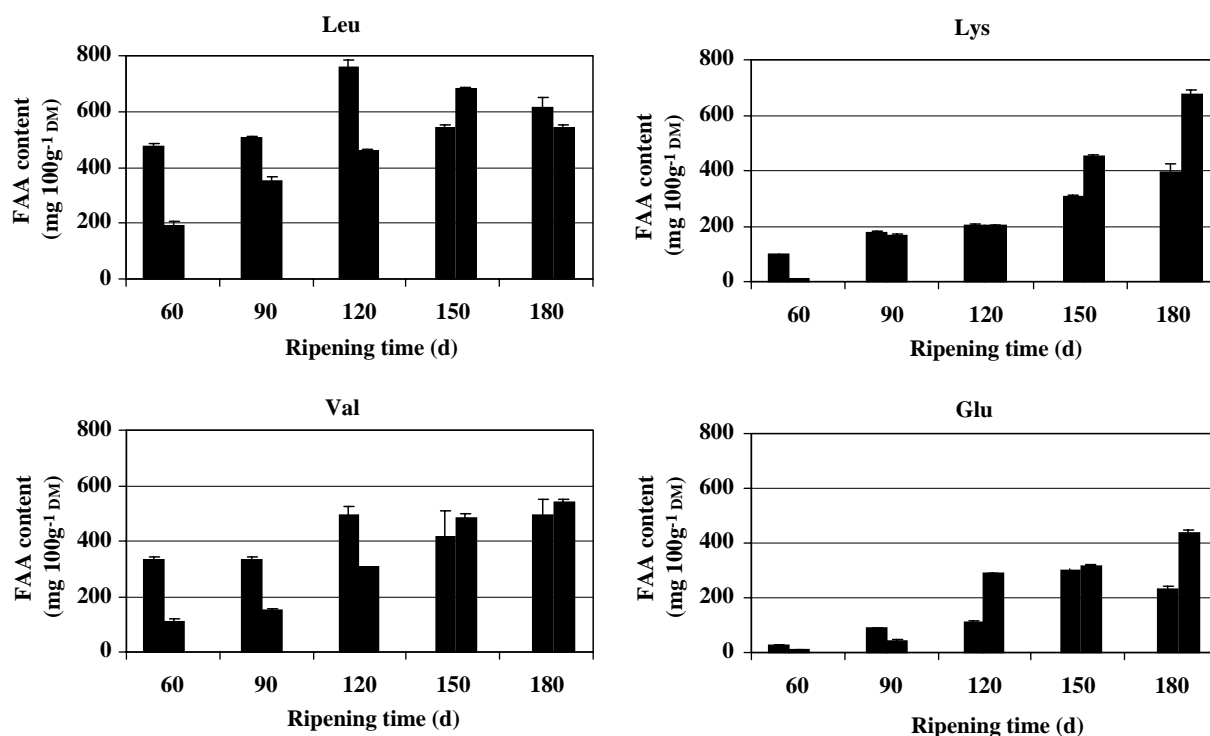


Fig. 3. Evolution in concentration of the four dominant free amino acids, FAA (leucine, Leu; valine, Val; lysine, Lys; glutamic acid, Glu), in Serra da Estrela cheese produced from non-refrigerated (□) and refrigerated (■) milk during the ripening process.

González, 1993; Barcina et al., 1995; Freitas, Fresno, Prieto, Franco, & Carballo, 1998). The major FAA found in La Serena cheese were Leu, Val, Lys and Arg. In Idiazábal cheese, increases in the total FAA concentration from 200 in 1 d-old cheeses to 1300 mg 100 g⁻¹ DM in 12 month-old cheeses have been reported (Barcina et al., 1995); the dominant FAA were Glu, Val, Leu and Phe. The FAA in Manchego cheese add typically to 1728 mg 100 g⁻¹ DM, in Cheddar cheese to 1592 mg 100 g⁻¹ DM, in Edam cheese to 1056 mg 100 g⁻¹ DM, and in Romano cheese to 650 mg 100 g⁻¹ DM (Fernández-Salguero & Sanjuán, 1999).

Serra da Estrela cheese exhibits high values of FAA when compared with the aforementioned cheeses, which reflects the strong peptidasic activity of the developing microflora. The major FAA found between 60 and 180 d in cheeses obtained from non-refrigerated milk (Table 3) were similar to those found in cheeses manufactured from refrigerated milk (Table 2) by the same ripening time. However, major differences can be pinpointed in the amount of Glu and γ -amino-*n*-butyric acid (Gaba); cheeses produced from refrigerated milk possess higher amounts of Gaba (164 vs. 46 mg 100 g⁻¹ DM) by 180 d, as well as lower amounts of Glu (231 vs. 437 mg 100 g⁻¹ DM) than those manufactured with non-refrigerated milk. This may reflect the growth of psychrotrophic bacteria that were present in the refrigerated milk at 10⁵–10⁶ CFU mL⁻¹ (data not shown), which are known to favor decarboxylation of Glu to Gaba (Hemme, Bouillanne, Métro, & Desmazeaud, 1982; Christensen, Dudley, Pederson, & Steele, 1999), or arise from the higher pH encountered in refrigerated milk (Witting, 1981). This, in turn, favors the decarboxylation reaction forming Gaba, while concomitantly decreasing the amount of Glu. Although the role of FAA in flavor development is well recognized, their direct contribution is probably limited. Specific FAA act as precursors of specific cheese flavor compounds in various cheese varieties through both enzymatic and chemical transformations (Visser, 1993), whereas total FAA contribute to the background flavor which is typical of cheese in general. Therefore, there is not necessarily a relation between typical cheese flavor and total FAA content (Engels et al., 1997).

The dairy farm and the ripening time were found to be highly significant factors for all FAA concentrations as concluded from the ANOVA, except His and Arg+Thr (for which the dairy farm factor was not significant at the 5% level of significance). Refrigeration of milk seems to lead to higher FAA content (Table 2), although such a difference was found to be not significant (at the 5% level); these higher values are in agreement with those found for the PTASN of the same samples. Milk refrigeration was a statistically significant factor in determining FAA content, except for Val, Glu and Phe.

Conclusions

From the values obtained for the nitrogen fractions in Serra da Estrela cheese, it can be concluded that this cheese undergoes proteolysis that is high in extent, but low to moderate in depth. Polypeptides and large peptides are released preferentially during ripening, which is reflected by the high values obtained for WSN and TCASN. The highest rates of release of both fractions were attained in the first 3 d following manufacture: there was a 3.2% d⁻¹ increase in WSN, and a 14.3% d⁻¹ increase in TCASN. The PTASN showed the highest increase between 7 and 18 d of ripening (i.e. 6% d⁻¹), which coincides with the exponential growth of lactic acid bacteria (LAB) at this stage of ripening. The increase in FAA was shown to be the highest between 18 and 35 d of ripening, eventually reaching 174%. The outgrowth of LAB (including their enzyme activities), coupled with the pool of FAA (which act as substrates) provide conditions that may promote flavor development via amino acid conversion into volatile compounds.

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