How milk type, coagulant, salting procedure and ripening time affect the profile of free amino acids in *Picante da Beira Baixa* cheese

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Abstract: The concentration of total free amino acids (FAA) in *Picante* cheese increased with ripening time irrespective of the particular protocol used for manufacture (ie ratio of caprine to ovine milks, animal or plant rennet and number of salting steps). The experimental cheeses manufactured with 20% (v/v) caprine milk, coagulated with animal rennet and salted only once exhibited the highest content of total FAA by 120 days of ripening. All four manufacture parameters were statistically significant on the 0.5% level of significance in terms of total concentration of FAA. The dominating free amino acids present in the various experimental cheeses throughout the ripening period were valine, leucine and phenylalanine, each one representing more than 10% (w/w) of the total concentration of FAA. All four manufacture parameters were, in general, statistically significant with respect to the content of every single FAA, with particular emphasis on salting and ripening time.

Keywords: dairy foods; maturation; ovine and caprine milk; animal and plant rennet; proteolysis

INTRODUCTION

Increasing interest in scientific and technological aspects of traditional food products has been observed recently, driven by their unique organoleptic characteristics on the one hand, and queries raised on their safety and sustained quality on the other. *Picante* cheese is one such food product; it is an artisanal cheese manufactured on the farm level only from mixtures of ovine and caprine raw milks using commercial calf rennet without any starter culture, and is ripened for a long period in rooms without temperature or humidity controls, on layers of wheat straw and sand.[1] As happens with other Portuguese traditional cheeses, little data are available on *Picante* cheese, and only recently was some light shed onto its physicochemical and microbiological aspects.[2–4]

It is known that proteolysis is one major phenomenon that takes place during ripening of cheese,[5] and that the degradation products thereof, which include amino acids and peptides, have a considerable influence on the sensory characteristics of the cheese.[6] Changes in the free amino acids content during ripening in different types of cheese have been reported (eg Polo *et al*,[7] Fresno *et al*,[8] Barcina *et al*[9]). Resmini *et al*[10,11] have used the free amino acid profiles in *Parmigiano-Reggiano* and *Provolone* cheeses in an attempt to monitor proteolysis and assess the stage of ripening. Proteolysis in *Picante* cheese has been characterized via assays of lumped breakdown products (nitrogen fractions soluble in water, trichloroacetic acid or phosphotungstic acid) throughout an 180-day ripening period using cheeses produced from different combinations of ovine and caprine milks,[4] as well as via assays of free amino acids (FAA).[12] This research effort complements these two studies by considering two extra technological parameters relevant for cheese technology (namely type of coagulant and NaCl content). With respect to type of coagulant, flowers of the thistle (*Cynara cardunculus* L) have been used since ancient times for manufacture of various types of Portuguese traditional ovine cheeses,[13] so this plant coagulant was considered as a rennet in alternative to its animal counterpart. On the other hand, since the NaCl content of *Picante* cheese may reach such high final values as 10–12% (w/w),[2] which are inhibitory or

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even lethal to a wide variety of micro-organisms, studies on the effects of addition of NaCl are in order.

MATERIAL AND METHODS

Cheese manufacture and sampling

The experiments were laid out as 2^4 complete factorial designs replicated twice; hence, cheeses were produced from two alternative milk blends (20–80% and 40–60% (v/v) of caprine–ovine milks, respectively), and coagulated by plant rennet or regular calf rennet; one half of the cheeses was salted only once immediately after manufacture, whereas the other half was salted twice, and cheeses were ripened for 30 or 120 days. Shorthand codes are used hereafter to denote each experimental cheese; the meanings of these codes, in terms of experimental conditions, are set out in Table 1. The manufacture of the aforementioned 32 cheeses was based on the traditional protocol,[3] ie mixtures of raw ovine and caprine milks were coagulated with rennet, without addition of a starter culture, at around 29°C for about 50 min, salted and duly ripened.

Chromatographic analysis

The cheese samples generated were assayed for each FAA using the Pico-Tag™ method for sample preparation and analysis, according to Alonso *et al*[14] with some modifications.

Sample preparation

Samples of 10 g of cheese were dissolved in 40 ml of 0.6 M perchloric acid and homogenised in a Sorvall Omni-mixer (Waterbury, CT, USA) for 2 min. The mixture was centrifuged at 1790 × *g* for 5 min in a Heraeus Christ centrifuge (Osterode, Germany), the supernatant was filtered through No 54 filter paper (Whatman, Maidstone, UK) and its pH was adjusted to 6.0 with 1 M potassium hydroxide. The filtrate was placed in an ice bath for about 20 min, filtered again through No 54 filter paper, and concentrated at 40°C in a rotavapor R-Büchi (Switzerland). The dry extract was dissolved in 20 ml of 0.5 M sodium bicarbonate (pH 8.5). The mixture was finally filtered through Millex-Gs 0.45 μm filter (Millipore, Molsheim, France) and frozen for later analysis by HPLC. Derivatisation of amino acids with phenylisothiocyanate solution (PITC) was performed according to Alonso *et al*;[14] 50 μl of extract were evaporated to dryness at 37°C under liquid nitrogen; the residue, corresponding to the derivatised dried samples, was dissolved in 500 to 1000 μl of solvent (prepared by dissolving 710 mg disodium hydrogen phosphate in 1 l of water-acetonitrile (19 : 1) and by adjusting pH to 7.40 with phosphoric acid) depending on the expected total FAA concentration of the sample. All reagents were obtained from Panreac (Barcelona, Spain) except triethylamine (liquid chromatography grade), PITC (protein sequencing grade) and amino acid standards (liquid chromatography grade), which were purchased from Sigma (St Louis, MO, USA).

Table 1. Codes used to denote the various experimental cheeses

Code	*Meaning*
20	Mixture of 20% caprine and 80% ovine milk
40	Mixture of 40% caprine and 60% ovine milk
P	Plant rennet as coagulant
An	Animal rennet as coagulant
A	Single addition of salt
B	Double addition of salt
30d	30 days of ripening
120d	120 days of ripening

Chromatographic conditions

A liquid chromatography system consisting of a ternary pump model SP-8800 and a column heater model SP-8792 from Spectra-Physics (San José, CA, USA), an injector model 7125 from Rheodyne (Cotati, CA, USA), a UV-spectrophotometer model 730 S LC from Kontron Uvikon (Middlesex, UK) and an integrator model SP-4290 from Spectra-Physics were used to perform the FAA analysis. Elution was performed in a C_{18} reversed-phase Brownlee™ column (25 cm × 0.46 cm) from Applied Biosystems (Foster City, CA, USA). The gradient conditions are depicted in Table 2, the column temperature was 50°C and detection was by absorbance at 254 nm. The amounts of FAA in the various cheese samples were calculated using peak area

Table 2. Solvent gradients used in the HPLC method

Run time (*min*)	*Solvent A* (%)	*Solvent B* (%)	*Flow rate* (*ml min*$^{-1}$)
0.0	100	0	0.90
5.0	100	0	0.80
20.0	78	22	0.75
40.0	54	46	0.80
42.0	0	100	1.00
43.0	0	100	1.00
44.0	100	0	1.00
46.0	100	0	1.50
47.5	100	0	0.90

Solvent A: 19 g sodium acetate trihydrate were dissolved in 1 l water; to this solution, 500 μl of triethylamine were added; the pH was then adjusted to 6.40 with glacial acetic acid, and 940 ml of this solution were finally mixed with 60 ml of pure acetonitrile
Solvent B: 600 ml acetonitrile plus 400 ml of water

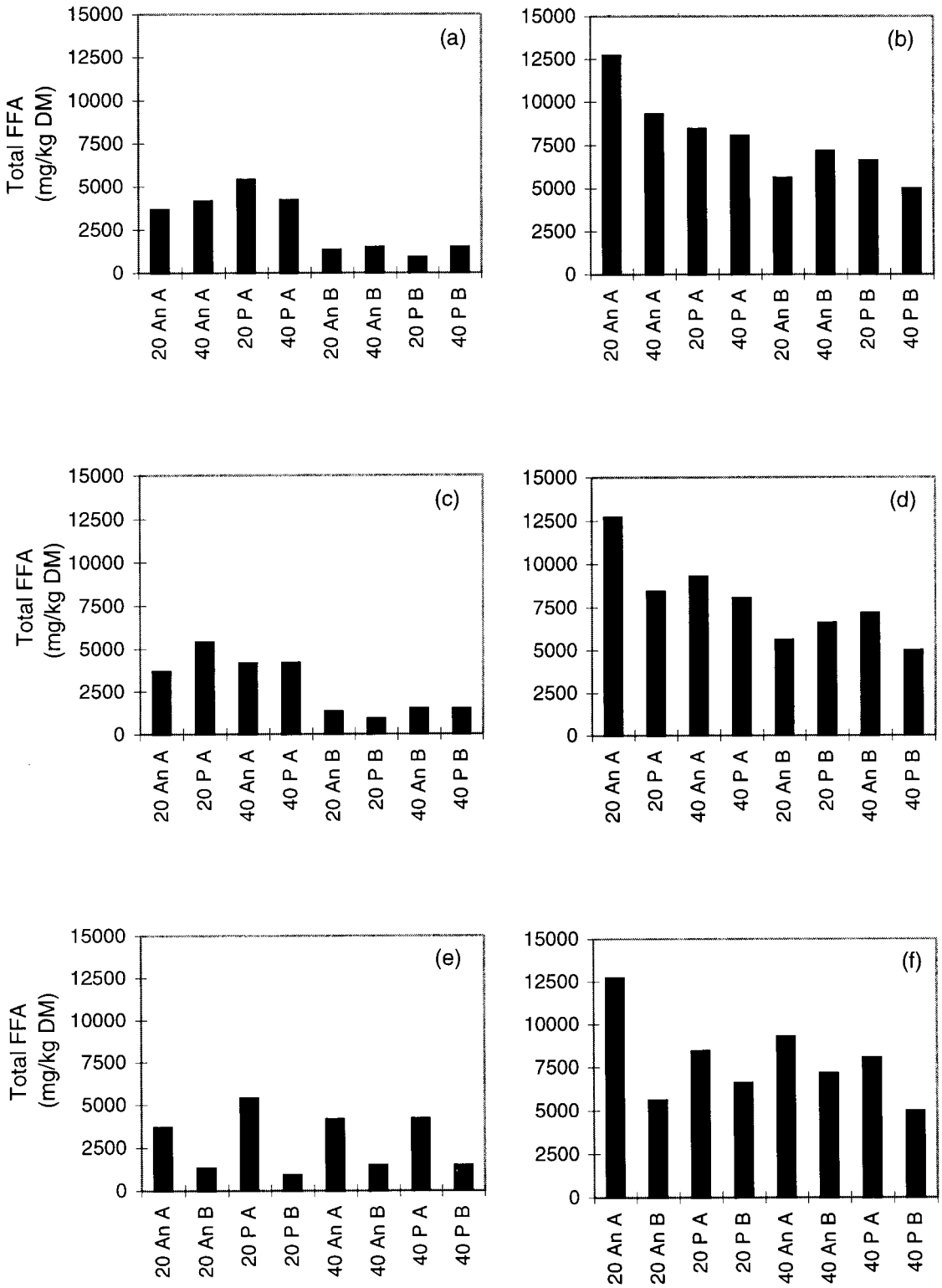

Figure 1. Total concentration of free amino acids (referred to dry matter, DM) for the various experimental cheeses ripened for 30 and 120 days as a function of milk type, coagulant type and salting extent: (a) 30 days, milk type; (b) 120 days, milk type; (c) 30 days, coagulant type; (d) 120 days, coagulant type; (e) 30 days, salting extent; (f) 120 days, salting extent. 20 and 40 means cheeses manufactured with 20% or 40% of caprine milk, respectively, P and An refers to cheeses coagulated with Plant or Animal rennet, respectively, A and B means cheeses that were salted via a single or double additions of salt, respectively, and 30d and 120d refers to cheeses ripened for 30 or for 120 days, respectively.

values from duplicate analytical samples, and peak areas were converted to concentration using calibration curves of amino acid standards (Sigma) dissolved in 0.5 M sodium bicarbonate.

Statistical analysis

The experimental data were statistically analysed using the ANOVA methodology. The technological variables manipulated were expressed in normalised

Table 3. Free amino acid contents in cheeses manufactured with caprine (and ovine) milk after 30 and 120 days of ripening

Amino acid (mg/kg DM)	*Cheese code*							
	20 An A 30d	*20 An A 120d*	*20 An B 30d*	*20 An B 120d*	*20 P A 30d*	*20 P A 120d*	*20 P B 30d*	*20 P B 120d*
20% caprine, 80% ovine milk								
Aspartic acid	99.8	717.9	57.2	184.3	163.5	499.8	18.1	194.5
Glutamic acid	1414.0	766.3	59.5	305.1	169.6	468.9	25.5	319.2
Glutamine	8.6	ND	6.1	ND	ND	ND	ND	ND
Serine	8.5	194.8	15.7	42.7	40.9	115.5	11.2	68.8
Asparagine	55.8	77.5	29.4	57.8	43.3	139.3	20.0	86.9
Glycine	41.1	78.6	8.6	21.0	56.6	61.1	7.9	20.9
Histidine	62.8	465.8	45.3	170.8	184.5	225.8	28.1	208.1
Taurine	ND	ND	15.1	ND	ND	ND	16.2	ND
γ-aminobutyric acid	66.4	46.3	13.8	47.1	31.4	52.8	9.0	48.2
Threonine	24.1	131.7	15.7	47.2	47.2	111.1	15.4	61.5
Arginine	ND	ND	ND	ND	ND	ND	ND	ND
Alanine	329.7	696.9	105.9	277.7	598.8	308.6	70.3	267.2
Proline	231.1	660.7	176.6	136.4	409.3	650.3	108.3	175.1
Tyrosine	78.5	526.0	69.5	187.5	104.3	225.9	60.5	249.5
Valine	627.6	2056.8	164.8	1128.9	845.3	1330.2	111.5	1344.4
Methionine	150.0	366.6	40.6	75.2	110.0	241.0	21.6	115.0
Cysteine	8.7	9.4	5.0	13.2	22.1	5.0	7.0	21.5
Isoleucine	177.2	911.3	58.5	574.6	331.1	541.4	30.3	618.5
Leucine	610.8	1708.3	165.1	949.6	941.7	1310.5	163.8	1170.8
Phenylalanine	646.9	1125.8	172.6	690.9	807.5	979.3	107.2	861.3
Tryptophan	159.1	739.3	42.3	287.4	239.9	442.2	24.3	336.0
Lysine	138.0	1425.7	72.2	379.5	239.0	720.2	42.1	412.3
40% caprine, 60% ovine milk								
Aspartic acid	124.1	477.6	46.5	184.8	159.4	365.4	31.2	130.0
Glutamic acid	178.1	375.9	43.6	354.1	141.4	400.2	47.3	233.2
Glutamine	4.0	ND	4.5	ND	ND	ND	ND	ND
Serine	23.4	102.3	14.8	71.5	16.1	90.6	21.2	36.0
Asparagine	49.5	73.1	29.2	82.7	42.9	86.0	30.0	43.9
Glycine	54.6	48.4	7.9	20.2	47.7	52.2	8.9	17.4
Histidine	109.5	427.7	53.2	201.5	97.3	243.0	44.6	130.4
Taurine	14.1	ND	ND	ND	15.2	ND	22.1	ND
γ-aminobutyric acid	88.5	28.9	17.4	20.3	49.9	69.6	20.7	26.9
Threonine	41.6	95.7	15.0	55.2	39.5	108.3	22.1	42.9
Arginine	ND	ND	ND	ND	ND	ND	ND	ND
Alanine	273.5	322.1	120.5	247.2	238.2	369.3	119.9	173.7
Proline	295.6	543.4	207.0	140.1	435.8	517.2	162.5	141.1
Tyrosine	77.0	289.1	68.1	206.1	79.3	245.2	49.0	156.4
Valine	633.6	1461.3	196.8	1394.0	554.2	1432.9	187.4	1041.6
Methionine	139.7	348.2	48.6	88.2	128.6	218.2	36.5	61.5
Cysteine	19.5	11.9	8.3	30.8	41.8	31.6	6.4	57.8
Isoleucine	183.4	794.4	72.4	834.1	223.7	553.9	60.1	459.6
Leucine	921.7	1326.9	193.3	1219.6	810.0	1361.7	300.5	951.8
Phenylalanine	665.3	895.3	213.9	862.8	714.5	934.0	205.1	717.2
Tryptophan	130.7	544.6	47.4	398.9	176.3	366.8	54.2	269.9
Lysine	121.1	1120.8	80.7	738.4	185.8	581.7	47.9	278.5

ND, Not detected

form according to: x_1 (effect of milk type), equal to $(30 - C)/10$ where C is volumetric percentage of caprine milk in the cheese milk blend; x_2 (effect of coagulant), equal to -1 for animal rennet and $+1$ for plant rennet; x_3 (effect of salting), equal to -1 for single addition of salt and $+1$ for double addition of salt; and x_4 (effect of ripening time), equal to $(t - 75)/45$, where t is ripening time expressed in days. Estimates of the effects of all processing parameters were obtained by linear regression. Statistical analyses based on linear regression, or, equivalently, minimisation of the squares of the residuals, are valid if the experimental errors are independent and normally distributed, and if they possess a constant variance; for most variables, the original experimental data had to be transformed using a

Table 4. Estimated effects, and 99.5% confidence intervals, for the linear and interaction effects of the four manufacture parameters tested upon the concentration of free amino acids (effects significant at the 0.5% level are denoted in bold)

FAA (mg kg^{-1} DM) effects	*Total FAA*	*Aspartic acid*	*Glutamic acid*	*Serine*	*Asparagine*	*Glycine*	*Histidine*	*γ-aminobutyric acid* $\lambda = 0.45$	*Threonine* $\lambda = 0.15$	*Alanine*
Average	**5341 ± 203**	**216 ± 6**	**252 ± 8**	**55 ± 3**	**59 ± 4**	**35 ± 1**	**169 ± 6**	**35 ± 2**	**59 ± 2**	**277 ± 12**
1 (Composition)	**460 ± 244**	**52 ± 7**	**60 ± 10**	**15 ± 4**	**9 ± 4**	**5 ± 1**	**11 ± 7**	0 ± 2	0 ± 2	**87 ± 15**
2 (Clotting agent)	**−690 ± 244**	**−41 ± 7**	**−52 ± 10**	**−9 ± 4**	**5 ± 4**	**−1 ± 1**	**−47 ± 7**	−2 ± 2	**5 ± 2**	**−40 ± 15**
3 (Salting)	**−3314 ± 244**	**−220 ± 7**	**−157 ± 10**	**−39 ± 4**	**−23 ± 4**	**−41 ± 1**	**−117 ± 7**	**−27 ± 2**	**−34 ± 2**	**−231 ± 15**
4 (Ripening time)	**5034 ± 244**	**257 ± 7**	**302 ± 10**	**71 ± 4**	**43 ± 4**	**11 ± 1**	**181 ± 7**	**8 ± 2**	**48 ± 2**	**89 ± 15**
1 × 2	195 ± 244	−5 ± 7	**−20 ± 10**	3 ± 4	**13 ± 4**	0 ± 1	**22 ± 7**	**−7 ± 2**	**3 ± 2**	**−24 ± 15**
1 × 3	**−636 ± 244**	**−37 ± 7**	**−52 ± 10**	**−17 ± 4**	**−7 ± 4**	**−4 ± 1**	−5 ± 7	**7 ± 2**	−1 ± 2	**−95 ± 15**
1 × 4	**470 ± 244**	**58 ± 7**	**64 ± 10**	**15 ± 4**	**10 ± 4**	**6 ± 1**	7 ± 7	**13 ± 2**	**7 ± 2**	−1 ± 15
2 × 3	**285 ± 244**	**17 ± 7**	**18 ± 10**	**7 ± 4**	**−9 ± 4**	0 ± 1	**32 ± 7**	2 ± 2	−1 ± 2	−13 ± 15
2 × 4	**−1023 ± 244**	**−53 ± 7**	**−43 ± 10**	**− 16 ± 4**	**12 ± 4**	**−3 ± 1**	**−68 ± 7**	**14 ± 2**	**−6 ± 2**	**− 89 ± 15**
3 × 4	**−263 ± 244**	**−122 ± 7**	**−43 ± 10**	**−32 ± 4**	−3 ± 4	1 ± 1	**−46 ± 7**	15 ± 2	−2 ± 2	**25 ± 15**
1 × 2 × 3	**497 ± 244**	**15 ± 7**	**44 ± 10**	**10 ± 4**	2 ± 4	0 ± 1	3 ± 7	1 ± 2	−1 ± 2	8 ± 15
1 × 2 × 4	−119 ± 244	−6 ± 7	**−27 ± 10**	**−4 ± 4**	**17 ± 4**	**−5 ± 1**	**−9 ± 7**	−2 ± 2	−1 ± 2	**−92 ± 15**
1 × 3 × 4	**−276 ± 244**	**−41 ± 7**	**−53 ± 10**	**−12 ± 4**	−3 ± 4	**−5 ± 1**	**11 ± 7**	**4 ± 2**	−1 ± 2	**25 ± 15**
2 × 3 × 4	**838 ± 244**	**55 ± 7**	**24 ± 10**	**13 ± 4**	**−12 ± 4**	**2 ± 1**	**66 ± 7**	**−10 ± 2**	**− 20 ± 2**	**54 ± 15**
1 × 2 × 3 × 4	**1020 ± 244**	**28 ± 7**	**70 ± 10**	**23 ± 4**	3 ± 4	**6 ± 1**	**38 ± 7**	**5 ± 2**	**10 ± 2**	**93 ± 15**

FAA (mg kg^{-1} DM) effects	*Proline*	*Tyrosine* $\lambda = 0.31$	*Valine*	*Methionine*	*Cysteine*	*Isoleucine* $\lambda = 0.56$	*Leucine*	*Phenylalanine*	*Tryptophan* $\lambda = 0.39$	*Lysine* $\lambda = 0.50$
Average	**312 ± 22**	**242 ± 10**	**907 ± 41**	**137 ± 8**	19 ± 11	**472 ± 15**	**882 ± 37**	662 ± 28	**350 ± 10**	**467 ± 14**
1 (Composition)	13 ± 26	**24 ± 12**	**88 ± 49**	6 ± 10	**−15 ± 13**	2 ± 18	−8 ± 45	23 ± 34	**14 ± 12**	**18 ± 16**
2 (Clotting agent)	26 ± 26	**−21 ± 12**	**−102 ± 49**	**−41 ± 10**	11 ± 13	**−63 ± 18**	−11 ± 45	7 ± 34	**−27 ± 12**	**−109 ± 16**
3 (Salting)	**−312 ± 26**	**−55 ± 12**	**−422 ± 49**	**− 152 ± 10**	0 ± 13	**− 145 ± 18**	**−485 ± 45**	**−368 ± 34**	**−157 ± 12**	**−234 ± 16**
4 (Ripening time)	**117 ± 26**	**166 ± 12**	**984 ± 49**	**105 ± 10**	8 ± 13	**470 ± 18**	**737 ± 45**	**441 ± 34**	**281 ± 12**	**483 ± 16**
1 × 2	9 ± 26	3 ± 12	15 ± 49	4 ± 10	−6 ± 13	**35 ± 18**	**49 ± 45**	24 ± 34	5 ± 12	**43 ± 16**
1 × 3	**−27 ± 26**	−9 ± 12	**−106 ± 49**	−2 ± 10	0 ± 13	**−38 ± 18**	**−46 ± 45**	**−65 ± 34**	**−44 ± 12**	**−57 ± 16**
1 × 4	**57 ± 26**	**14 ± 12**	44 ± 49	**14 ± 10**	−6 ± 13	0 ± 18	**78 ± 45**	**39 ± 34**	**14 ± 12**	5 ± 16
2 × 3	**−44 ± 26**	**14 ± 12**	**52 ± 49**	**36 ± 10**	−2 ± 13	−14 ± 18	25 ± 45	−19 ± 34	4 ± 12	1 ± 16
2 × 4	−25 ± 26	**− 22 ± 12**	**−121 ± 49**	**− 20 ± 10**	2 ± 13	**−93 ± 18**	**−92 ± 45**	−27 ± 34	**−50 ± 12**	**−127 ± 16**
3 × 4	**133 ± 26**	**−16 ± 12**	**79 ± 49**	**−57 ± 10**	**16 ± 13**	**93 ± 18**	**131 ± 45**	**166 ± 34**	**41 ± 12**	**−69 ± 16**
1 × 2 × 3	−5 ± 26	**26 ± 12**	**116 ± 49**	**11 ± 10**	2 ± 13	**27 ± 18**	**46 ± 45**	**42 ± 34**	10 ± 12	**47 ± 16**
1 × 2 × 4	5 ± 26	**−13 ± 12**	−48 ± 49	**13 ± 10**	−5 ± 13	17 ± 18	−35 ± 45	10 ± 34	**15 ± 12**	**34 ± 16**
1 × 3 × 4	**−28 ± 26**	−2 ± 12	−8 ± 49	2 ± 10	−7 ± 13	10 ± 18	**−49 ± 45**	−12 ± 34	2 ± 12	**−30 ± 16**
2 × 3 × 4	**63 ± 26**	**31 ± 12**	**102 ± 49**	**31 ± 10**	7 ± 13	**58 ± 18**	**54 ± 45**	**52 ± 34**	**45 ± 12**	**82 ± 16**
1 × 2 × 3 × 4	11 ± 26	**25 ± 12**	**201 ± 49**	5 ± 10	−1 ± 13	**64 ± 18**	**184 ± 45**	**84 ± 34**	**33 ± 12**	**54 ± 16**

Note λ-maximum likelihood estimator in the Box-Cox transformation of y to $(y^{\lambda} - 1)/(\lambda \dot{y}^{\lambda-1})$, where $\dot{y}$ is the geometric mean of the original data

Box–Cox transformation to stabilize their variance[15] before the ANOVA could be applied. Plots of the residuals of the transformed data (not shown) have indicated that their behaviour is in good agreement with the aforementioned claim, so the model-fitting routines and computation of the confidence intervals using the pooled replicate variance as estimator were statistically accepted.

RESULTS

The total concentrations of FAA for each experimental cheese are displayed in Fig 1. The concentrations of each individual FAA for each experimental cheese produced are listed in Table 3. Finally, the estimates of the linear and interaction effects of the four manufacture parameters tested are depicted in Table 4.

DISCUSSION

The total concentration of FAA increased with ripening time for every combination of manufacture parameters (see Fig 1); according to Ordoñez and Burgos,[16] the total concentration of FAA increases throughout maturation irrespective of the source of proteases used. From Table 4 it can be concluded that ripening time, besides being a statistically significant factor at the 0.5% level, has a major impact when compared with the other three factors (milk composition, clotting agent and salting); proteolysis of *Picante* cheese is indeed shown to be faster between 3 and 6 months of ripening.[4] Cheeses manufactured with 20% caprine milk, coagulated with animal rennet and salted only once exhibited the highest content of total FAA by 120 days of ripening.

The effect of milk type is easily grasped from inspection of Figs 1(a) and (b); a higher percentage of ovine milk gave, in general, an increasing effect in terms of total FAA, especially by 120 days of ripening, and this factor appeared to be statistically significant at the 0.5% level (see Table 4). This disagrees with previous experimental data obtained with *Picante* cheese, in which the ANOVA indicated that the ratio of addition of caprine milk to ovine milk had significant effects on the total FAA content;[12] a higher proportion of caprine milk was in fact equivalent to a higher level of total FAA.

The effect of type of coagulant used in the manufacture of cheese can be seen in Figs 1(c) and (d); slightly higher contents of total FAA were apparent by 30 days of ripening when plant rennet was

employed, especially in cheeses salted just once (ie cheeses with lower content of NaCl); cheeses coagulated with animal rennet had, in general, higher levels of total FAA by 120 days. As happened with the other three manufacture parameters, type of coagulant was a statistically significant factor, and the negative sign associated with it (see Table 4) is *per se* an indication of a lower contribution of plant than animal rennet to FAA release. According to Freitas and Malcata,[17] gradual proteolysis occurs during ripening in cheeses manufactured with animal rennet, whereas in cheeses manufactured with plant rennet no significant differences are found for the water soluble nitrogen between 30 and 120 days. Cheeses manufactured with plant rennet exhibited significantly higher levels of water soluble nitrogen than cheeses manufactured with animal rennet, although the former showed significantly lower levels of thricloroacetic acid soluble nitrogen and lower levels of phosphotungstic acid soluble nitrogen.[18] According to Heimgartner *et al*,[19] plant rennet proteinases cleave casein into high molecular weight peptides, but significant peptidase activity cannot be observed; these reports could explain the differences found for the total content of FAA between cheeses manufactured with animal rennet and plant rennet and ripened for 120 days. Our data generated by HPLC for individual FAA contrasts with experimental data obtained by Kjeldahl or spectrophotometric methods,[17] which have suggested that plant rennet use results in increasing the pool of small peptides and FAA.

Assessment of the salting effect is shown in Figs 1(e) and (f); cheeses salted twice (ie cheeses with higher content of NaCl) possessed lower levels of total FAA. This realisation could be extrapolated to every single FAA, and Table 4 shows that NaCl was a statistically significant factor in terms of release of every FAA but cysteine. The differences in NaCl content between the cheeses associated with the two patterns of salt addition remained approximately constant at around 6.8% (w/v in moisture) throughout ripening;[17] the relatively higher levels of FAA contents in cheese with low NaCl contents than in cheeses with high NaCl contents can be attributed to a considerable activity of the native microflora under those favourable environmental conditions;[20] furthermore, Freitas and Malcata[17] claimed that NaCl content had negative effects upon all microbiological groups (ie higher values of NaCl led to lower microbiological viable counts).

The FAA profile was not significantly different between the various experimental cheeses, ie the relative proportions of each FAA were, in general, similar for most of them. According to Polo *et al*,[7] the pattern associated with FAA release depends on the routes followed by the enzymatic degradation of peptides, as well as FAA interconversion, affected by exocellular enzymes or endocellular enzymes of lysed cells. In the present work all four manufacture parameters studied possessed, in general, statistically significant effects on the FAA contents (see Table 4), with a particular emphasis on salting and ripening time, most likely due to microbial-related activity. In terms of milk composition and clotting agent, the level of statistical significance was not so low for some of the FAA. The former observation could derive, at least partially, from the fact that 20% of caprine in the cheese milk was not sufficient to permit significant differences to build up; although caprine and ovine milks have different protein composition, such differences did not apparently play a role in our experiments. With respect to clotting agent, plant rennet proteinases (cardosins) are characterised by extreme ability to cleave casein into high molecular weight peptides, but by a poor peptidase activity; according to Freitas and Malcata,[17] the molecular weight distribution of peptides produced by plant rennet exhibits lower electrophoretic mobilities than the animal rennet counterparts.

The major FAA present in the several experimental cheeses produced throughout the ripening period were valine, leucine and phenylalanine; each one represented, individually, more than 10% of the total FAA inventory, which is consistent with results presented elsewhere by Freitas *et al*.[12] Valine, leucine and phenylalanine have also been listed as major amino acids in several varieties of cheeses manufactured with milks from small ruminants, eg *Idiazabal*,[9] *Toscano*[21] and *Manchego*.[16] All four manufacture parameters were statistically significant at the 0.5% level for valine, whereas neither milk composition nor clotting agent were statistically significant factors for leucine and phenylalanine. The absence of a significant difference in terms of leucine and phenylalanine content in cheeses manufactured with both coagulants could probably be ascribed to their enzymatic action as rennet. The primary site for animal rennet action on α_{s1}-casein is Phe_{23}-Phe_{24}[22] or Phe_{24}-Val_{25},[23] although Pélissier *et al*[24] have reported that hydrolyses of purified α_{s1}-casein by chymosin yielded more than 20 peptides from preferential hydrolyses of peptide bonds involving the carboxyl groups of phenylalanine and leucine residues. According to Macedo *et al*,[25] the primary cleavage site of bovine α_{s1}-casein by cardosin is also Phe_{23}-Phe_{24}, whereas of bovine β-casein it is Leu_{192}-Tyr_{193}, which is also the most susceptible peptide bond to be attacked by chymosin and other milk-clotting enzymes.[24] According to Macedo *et al*,[25] cardosins show a clear preference for bonds linking hydrophobic, bulky amino acids, and cleave easily four consecutive peptide bonds in bulky, hydrophobic regions of both bovine α_{s1}-casein (Ala_{163}-Val_{167}) and β-casein (Ala_{189}-Tyr_{193}); since these bonds are less attacked by chymosin in various experimental conditions, a tentative rationale for the significant difference found for valine in terms of type of coagulant may thus be available.

Proline, one of the most abundant amino acid resi-

dues present in β-casein,[9] was also present in more than 10% of the whole FAA pool in cheeses ripened for 30 days, salted twice and manufactured with either coagulant (ie the 20 An B 30d, 20 P B 30d, 40 An B 30d and 40 P B 30d cheeses); however, in cheeses ripened for 120 days, proline was present as a minor fraction. Studies on *Kaskhawal* cheese[26] have shown a marked increase of free proline during the first three months of ripening and decreases in its relative concentration towards the end of ripening. The higher concentration of free proline in cheeses with higher content of NaCl contrasts with the higher resistance of β-casein to hydrolysis in those cheeses;[17] as emphasised by Guinee and Fox,[27] NaCl and similar salts cause conformational changes in β-casein that render its chymosin- or pepsin-susceptible bonds less accessible to enzymatic attack. Isoleucine, which appeared among the major FAA at later stages of ripening of *Picante* cheese manufactured with various proportions of ovine and caprine milks,[12] was a part of the set of amino acids representing *per se* more than 10% of the total FAA in the 20 An B 120d and the 40 An B 120d cheeses. This fact could somehow be characteristic of traditional *Picante* cheese because the classical protocols of manufacture of this type of cheese involve coagulation of mixtures of ovine and caprine milks with animal rennet coupled with double salting of the cheese. Milk composition, in terms of free proline and isoleucine, and clotting agent, in terms of free proline, were not statistically significant factors. According to Favier[28] and Parkash and Jeness,[29] proline, glutamic acid, leucine and isoleucine represent approximately 40% of the total FAA inventory of caprine casein.

Arginine, which has been claimed to be responsible for bitter tastes,[30,31] appeared at such low concentrations that it could not be detected, so it is not expected that off-flavours in cheese were produced by arginine. The γ-aminobutyric acid, generally present in low-quality cheeses,[32] was found at concentration levels of 9–89 mg kg^{-1} DM, or 1.0–2.3% of total FAA, which again are low enough values to be of a lesser importance.

CONCLUSIONS

The increase in concentration of the total FAA with ripening time was observed for every combination of manufacture parameters. In fact, the ripening time effect had a major impact when compared with milk composition, clotting agent and salting effects. Milk type also showed to be a statistically significant effect making ovine milk to display an increasing effect of total FAA. In terms of type of coagulant, plant rennet showed to be a poorer contributor to release of FAA than animal rennet. Higher content of salt is associated with lower FAA release, which can be related to the native microflora. The FAA profile is not significantly different between the various experimental cheeses; the major FAA present in several experimental cheeses throughout the ripening period are valine, leucine and phenylalanine. All four manufacture parameters are statistically significant at the 0.5% level for valine, whereas neither milk composition nor clotting agent are statistically significant factors for leucine and phenylalanine. Isoleucine, which appears among the major FAA in the later stages of ripening of *Picante* cheese, represents *per se* more than 10% of the total FAA in the 20 An B 120d and the 40 An B 120d cheeses.

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