ELSEVIER

Food Chemistry 75 (2001) 287–291

Food Chemistry

www.elsevier.com/locate/foodchem

Effect of temperature on evolution of free amino acid and biogenic amine contents during storage of Azeitão cheese

Olívia Pinho [a], Isabel M.P.L.V.O. Ferreira [b,*], Eulália Mendes [b], Bruno M. Oliveira [a], Margarida Ferreira [b]

[a] *Assistente na Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, R. Dr. Roberto Frias, 4200-465 Porto, Portugal*

[b] *CEQUP/Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, R. Aníbal Cunha 164, 4050 Porto, Portugal*

Received 3 October 2000; received in revised form 19 December 2000; accepted 19 December 2000

Abstract

A study on the evolution of free amino acids and biogenic amines in Azeitão cheese during 4 weeks at different temperatures of storage (4 and 25°C) was performed. Free amino acids and biogenic amines were determined by RP-HPLC with visible detection, following extraction from the cheese and derivatization with dabsyl chloride. The method presented a linear relation between peak area and concentration from 2–200 mg/l. The detection limit value was less than 1.5 mg/l. The average repeatability was less than 4%. The major free amino acids were proline, valine, isoleucine and leucine and the major amines were tyramine, cadaverine and histamine. Room temperature (25°C) promoted a significant increase of the contents of valine, leucine, tyramine and putrescine, expressed as g/kg of dry matter. These two free amino acids and two biogenic amines may serve as indicators of temperatures changes in ripened cheese. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Free amino acids; Biogenic amines; Cheese; HPLC

1. Introduction

Cheeses represent an ideal environment for the formation of proteolytic products, namely, free amino acids and biogenic amines, directly influenced by bacterial activity, pH, salt concentration and, indirectly, by water availability, storage temperature, and ripening time (Vale & Gloria, 1997). These breakdown products result from the proteolysis of casein by the action of the proteases and peptidases present in the cheese and are also important to achieve the characteristic texture and aroma of the cheese (Leuschner, Kurihara, & Hammes, 1998). Thus, every type of cheese has its own characteristic free amino acid and biogenic amine profile, resulting from its specific degradation, interconversion and synthesis (Polo, Ramos, & Sanchez, 1985).

Many studies have been undertaken to determine the biogenic amine contents of cheeses. A variety of amines, such as tyramine, histamine, putrescine, cadaverine, tryptamine and 2-phenylethylamine, have been found in many types of cheeses (Santos, 1996). Histamine and tyramine occur at levels varying extensively, not only between different cheese varieties, but within the actual varieties (Stratton, Hutkins, & Taylor, 1991). The presence of low levels of biogenic amines in cheeses and other foods is not considered a serious risk. However, if normal routes of catabolism of amines are inhibited, or the amount consumed thereof is large, various physiological effects may result, such as hypertensive crises, migraine headache and even death from cerebral haemorrhage in patients treated with monoamino oxidase inhibitor drugs (Reuvers, Pozuelo, Ramos, & Jimenez, 1986). Thus, knowledge on the levels of biogenic amines in cheese is necessary to assess the health hazards arising from consumption of these products. Furthermore, these could be useful as indicators of freshness and

* Corresponding author. Tel.: +351-2-200-564; fax: +351-2-200-3977.

E-mail address: bromato@ffup.pt (I.M.P.L.V.O. Ferreira).

PII: S0308-8146(01)00109-1

hygienic quality of the raw materials and the manufacturing conditions used within cheese manufacture.

With respect to the influence of temperature on the synthesis of biogenic amines, there are reports indicating that storage temperature may affect the content of biogenic amines in food. Some authors found that storage temperature did not significantly influence maximum content of certain biogenic amines in anchovies, though refrigeration temperatures delayed the onset of such increase (Santos, 1996). Other authors disagree with the above statement (Diaz-Cinco, Fraijo, Grajeda, Lozano-Taylor, & Gonzalez de Mejía, 1992), and find that histamine and tyramine concentrations increase with the time and storage temperature of Chihuahua cheese. Therefore, the influence of temperature on amine formation during cheese storage (period that includes transportation from the cheese factory, commercial distribution, purchase, and eventual consumption) should be determined in order to limit accumulation in cheese.

The objective of the present study was to determine the contents of free amino acids and biogenic amines in Azeitão cheese, as well as to assess the possible influence of storage temperature on the formation of these compounds. This cheese bears the status of "Protected Denomination of Origin" (PDO) D.N. No. 293/93 and 47/97. It is made from raw milk of ovine origin, using an extract of cardoon flower (*Cynara cardunculus* L.) as rennet; it undergoes a minimum of 20 days of ripening, and its consumption has increased over the past few years. Studies related to its composition are quite scarce, yet are necessary for the maintenance or improvement of quality of this cheese.

Some modifications of the method of Krause, Bockhardt, Neckermann, Henle, and Klostermeyer (1995) were directed to optimize derivatization with dabsyl chloride and chromatographic separation in cheese samples. Several advantages of the dabsyl method over pre or post-column derivatization with *o*-phthaldialsehyde, 9-fluorenyl methyl chloroformate, phenylisothiocyanate or fluorescamine were reported (Krause et al., 1995), namely, stability of dabsyl derivatives at room temperature and detection in the visible region ($\lambda = 436$–460) with high specificity and sensitivity.

2. Materials and methods

2.1. Chemicals and supplies

Amino acids (aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, glycine, arginine, alanine, proline, valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, cysteine, ornithine, lysine and histidine) and biogenic amines (ethylamine, tyrosine, tyramine, tryptamine, β-phenylethylamine, histamine, cadaverine, putrescine and spermidine), as their corresponding crystalline hydrochlorides, as well as dabsyl chloride dimethylamino-benzene-1-sulphonyl, were purchased from Sigma. Spermine was obtained from Aldrich.

The stock solution standards were prepared at a concentration of 1 g/l. Double distilled water was used, for this purpose.

2.2. Samples

Four cheeses of the same batch, previously ripened for a 30-day period (254 ± 10 g) were purchased from a manufacturing establishment approved by the Entity that certifies the "Protected Denomination of Origin" for Azeitão cheese.

Two experimental situations were generated, in order to simulate different ways of acquisition by consumers. One group (A) was chilled at 4°C throughout its transportation (2 days), whereas the second group of cheeses (B) was transported, without refrigeration, at temperatures ca. 25 ± 3°C. Upon arrival at the laboratory, a representative sample of each cheese was taken to determine dry matter, free amino acid and biogenic amine contents. The remaining portions of each of the four cheeses (from both groups) were stored at refrigeration temperatures (4°C) during 2 weeks and subsequently were removed and placed in a clean storage room (25°C) for a further 2-week period. Thus, the shelf-life period evaluated was equivalent to a total of 4 weeks.

Representative samples of each cheese were taken at 7, 14, 21 and 28 days, for analysis. For that purpose, samples of ca. 40 g were grated, thoroughly homogenized, and the dry matter, free amino acid and biogenic amine contents analyzed immediately. Each analysis was performed in duplicate.

The determination of moisture was performed by oven-drying at 100–105°C to constant weight, in order to express all the concentrations of free amino acids and biogenic amines with respect to dry matter.

2.3. Analysis of biogenic amines in the cheeses

The method of simultaneous extraction and separation of the free amino acids and biogenic amines present in Azeitão cheese was used under specific conditions with slight modifications, as mentioned in Krause et al. (1995).

In order to simultaneously extract the free amino acids and biogenic amines, 4 g of cheese sample was suspended in 15 ml 0.2 M perchloric acid. The mixture was homogenized in an Ultra Turrax blender for 2 min and kept in an ultrasonic bath for 30 min and then centrifuged at 4000 *g* for 20 min. Any topping fat layer was removed. The supernatant was filtered through a 0.45 μm membrane.

Derivatization was carried out by using dabsyl chloride at 70°C for 15 min. The reaction was stopped by

Table 1
Total free amino acid and biogenic amine contents during storage of Azeitão cheese[a]

Storage temperature (°C)	Storage time (days)	Group A transport 4°C (2 days)		Group B transport 25°C (2 days)	
		Total free amino acids content	Total biogenic amine content	Total free amino acids content	Total biogenic amine content
Transport temperature	0	478±17a	838±34a	890±27c	1078±10d
4	7	465±29a	907±12a	929±18c	1416±22c
4	14	432±38a	869±21a	869±33c	1408±19c
25	21	607±18b	989±8b	842±14c	1469±32c
25	28	847±20c	1393±15c	871±27c	1442±10c

[a] Values are expressed as mean±standard deviation of two determinations (mg/kg dry matter). a,b,c,d, means in columns without common letters are significantly different ($P<0.05$; $n=4$) when analyzed by ANOVA methodology.

placing the vials in an ice bath for 5 min. The dabsylated derivatives were diluted in a mixture of acetonitrile and ethanol and, after centrifugation, separations were performed by using high-pressure liquid chromatography (HPLC).

The HPLC equipment consisted of two Gilson (Gilson Medical Electronics, Villiers le Bel, France) type 305 pumps, a type 7125 Rheodyne Injector with a 20-μl loop, a Gilson model (temperature control module) oven, a Gilson 118 variable wavelength UV/vis detector. The integrator used was a Gilson 712 HPLC System Controller Software.

The separations were performed at 50°C on a 150×4.6 mm I.D. Spherisorb ODS C 18 column, 3 μm. Dabsylated amino acids and amines were eluted at a flow-rate of 1 ml/min using a gradient made with solution A, consisting of 9 mM sodium dihydrogenphosphate, 4% dimethylformamide and 0.1% triethylamine, titrated to pH 6.55 with phosphoric acid and solution B, 80% (v/v) aqueous acetonitrile. The gradient applied comprised an initial range with 8% B in A during the first 2 min, followed for four different linear gradients: from 8% B in A to 20% B in A within 5 min, from 20% B in A to 35% B in A within 28 min, from 35% B in A to 50% B in A within 10 min, and from 50% B in A to 100% B in A over a 21-min period, maintaining this upper limit condition during 11 min and returning to initial conditions within 13 min. The detector was set at 436 nm. Quantification was carried out using a mixture of amino acids and biogenic amine standards.

2.4. Statistical analysis

Data are represented as the mean±standard deviation. Analysis of variance (ANOVA) was used to determine the effects of storage time and temperature on the content of total free amino acids and biogenic amines. Fisher's protected least significant difference *t*-test (PLSD) at the 5% significance level was applied to assess intrapair significant differences. ANOVA were done with the Statview TM 4.0 statistical package (Abacus Concepts, Berkeley, CA 94704-1038, USA).

The calculations of the correlation coeficients between the levels of amino acids and biogenic amines of the two groups of cheeses (A and B) and the storage time and temperature were those of Pearson, using Microsoft Excel 97.

3. Results and discussion

A linear relationship between concentrations of aspartic acid, glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine, ornithine, lysine, histidine, ethylamine, tyrosine, putrescine, phenylethylamine, tyramine, cadaverine and spermidine in Azeitão cheese and absorbance at 436 nm was obtained. This linearity was maintained over the concentration range of 2–200 mg/l. The detection limit value was less than 1.5 mg/l. The precision and accuracy of the analytical method, including the extraction and derivatization steps, were also evaluated. Relative standard deviations less than 4% and good recoveries (>90%) were obtained. The results were in good agreement with those reported by Knecht and Chang (1986) and Krause et al. (1995).

Table 1 shows the total free amino acid and biogenic amine contents during storage of Azeitão cheese. Table 1 shows that when group A cheeses were stored at 4°C the content of free amino acids and biogenic amines remained almost the same; no significant differences were found between the total content of free amino acids and biogenic amines upon arrival at the laboratory and after storage at 4°C during the two week period, when analyzed by ANOVA methodology. However, the placement of group A cheeses at room temperature significantly increased ($P<0.05$) the total content of free amino acids and biogenic amines.

Analysis of the collected data revealed significant differences ($P<0.05$) between the initial contents of total free amino acids and biogenic amines of the two groups

Table 2
Monitoring of free amino acids and biogenic amines in cheeses of groups A and B (mg/kg dry matter) during storage

Amino acids and biogenic amines	Group A					Group B				
	Storage time (days)					Storage time (days)				
	Start	7	14	21	28	Start	7	14	21	28
Glycine	14.9	19.0	17.3	17.2	17.3	21.4	25.8	23.8	18.5	18.3
Alanine	37.0	46.4	39.0	42.1	62.1	59.5	68.8	69.7	62.8	57.3
Proline	85.8	73.2	74.6	98.8	98.3	105	109	111	101	107
Valine	81.2	72.9	69.9	105	173	120	137	133	153	178
Methionine	23.8	40.5	25.2	17.2	50.0	145	175	175	167	164
Arginine	11.9	15.5	12.1	21.4	25.9	41.5	39.3	43.0	45.0	37.8
Isoleucine	133.3	123	123	175	205	166	189	184	177	168
Leucine	35.1	33.3	32.0	60.1	67.5	63.0	69.0	69.4	75.6	83.5
Ornithine	n.d.	n.d.	n.d.	1.3	3.1	4.0	0.8	0.9	traces	0.4
Lysine	10.1	5.6	14.8	36.7	56.6	8.0	9.3	24.3	21.8	26.1
Histidine	45.3	36.1	24.3	32.4	88.4	157.1	107	35.5	20.1	31.6
Tyramine	122	72.3	126	285	358	195	293	287	407	445
Putrescine	15.0	20.7	29.1	50.8	110	n.d.	35.1	45.6	50.0	137
Cadaverine	181	215	195	193	231	260	217	240	162	161
Histamine	458	517.6	438.4	414	644	566	821	780	818	682
Spermine	61.4	81.6	80.2	47.5	48.8	57.5	50.0	55.8	32.4	17.2

Table 3
Pearson correlation coefficients (ρ) for cheeses of groups A and B

Amino acids and biogenic amines	$\rho_{(A,\ time)}$	$\rho_{(B,\ time)}$	$\rho_{(A,\ B)}$
Glycine	−0.18	−0.80	0.32
Alanine	0.39	−0.22	−0.79
Proline	0.48	−0.25	−0.71
Valine	0.77	0.97*	0.88*
Methionine	−0.01	−0.06	−0.79
Arginine	0.79	0.13	0.10
Isoleucine	0.61	−0.13	−0.48
Leucine	0.68	0.86**	0.92*
Lysine	0.91*	0.89*	0.76
Histidine	0.33	−0.85**	0.18
Tyramine	0.89*	0.96*	0.86**
Putrescine	0.85**	0.91*	0.95*
Cadaverine	0.34	−0.76	−0.15
Histamine	0.37	−0.75	−0.06
Spermine	−0.56	−0.77	0.80

*$P < 0.05$.
**$P < 0.01$.

of cheeses. Group A showed lower contents when compared with its counterpart in group B. Interestingly, at the end of the experiment, similar contents of total free amino acids and biogenic amines were obtained for both groups of cheeses. This observation was due to the removal of refrigeration temperatures in group A cheeses; in fact, an increase in the storage temperature of ca. 21°C promoted a 37% increase in the biogenic amine content.

Table 2 presents the individual contents of amino acids and biogenic amines quantified in cheeses of groups A and B. The major free amino acids in cheeses of group A, upon arrival at the laboratory, were proline (85.8 mg/kg of dry matter), valine (81.8 mg/kg dry matter) and isoleucine (133 mg/kg dry matter) and the major amines were tyramine (122 mg/kg dry matter), cadaverine (181 mg/kg dry matter), histamine (458 mg/kg dry matter) and spermine (61.4 mg/kg dry matter). The remaining free amino acids and biogenic amines were below 50 mg/kg dry matter. Some amino acids and biogenic amines were not detected, namely aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, tryptophan, phenylalanine, cysteine, ethylamine, tyrosine, tryptamine and phenylethyamine.

The major free amino acids in cheeses of group B, upon arrival at the laboratory, were proline (105 mg/kg dry matter), valine (120 mg/kg dry matter), alanine (59.5 mg/kg dry matter), isoleucine (166 mg/kg dry matter), methionine (145 mg/kg dry matter) and leucine (63 mg/kg dry matter). Tyramine (195 mg/kg dry matter), cadaverine (260 mg/kg dry matter) and histamine (566 mg/kg dry matter) were again the main amines present, although at higher concentrations. The remaining free amino acids and biogenic amines were below 50 mg/kg of dry matter, and aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, tryptophan, phenylalanine, cysteine, ethylamine, tyrosine, tryptamine and phenylethyamine were not detected.

Pearson correlation coeficients between the levels of amino acids and biogenic amines of the two groups of cheeses and the storage time at different temperatures were determined for each amino acid and biogenic amine. As shown in Table 3, for cheeses from group A, lysine, tyramine and putrescine were the only compounds to report significant increases ($P < 0.05$ for the first two and $P < 0.01$ for the last one, respectively). Despite no statistical significance, valine, arginine, isoleucine and leucine increased slightly under the experimental

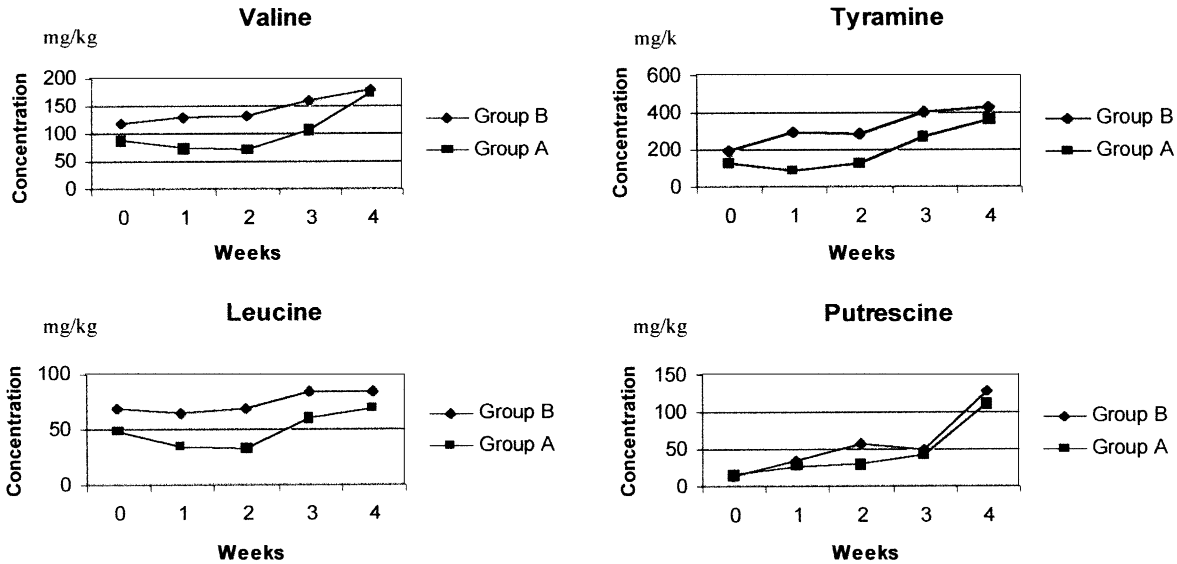

Fig. 1. Evolution of the mean values of valine, leucine, tyramine and putrescine (expressed in mg/kg dry matter) under the conditions described in the text.

conditions, during storage. Spermidine content declined over the whole assay.

During the evolution of free amino acids and biogenic amines in group B cheeses throughout the whole experiment, only valine, leucine, lysine, tiramine and putrescine showed significant increases (Table 3). Histidine, cadaverine, histamine and spermine showed a declining trend over the storage period but without statistical significance.

The Pearson correlation coefficient between the concentrations of free amino acids and biogenic amines in cheeses of group A compared with that in group B showed that only the amino acids, valine and leucine, and the biogenic amines, tyramine and putrescine, presented positive correlations with statistical significance. From inspection of Fig. 1, which depicts the evolution of the mean content values of valine, leucine, tyramine and putrescine (expressed in mg/kg dry matter), it may be observed that, in general, group A cheeses started with lower levels of those compounds than group B cheeses; when stored at 4°C the levels oscillated slightly; when stored at room temperature, their contents increased in both cheeses groups.

On the basis of these results, it appears that these two free amino acids (valine and leucine) and two biogenic amines (tyramine and putrescine) may serve as indicators of temperature changes in ripened cheese.

In conclusion, the results presented herein appear to support the need for the implementation of a well-designed refrigeration scheme throughout transportation, commercial distribution, purchase and storage by consumers, in order to guarantee low levels of biogenic amines in the cheese (and therefore, less potential for health hazards) by the time of consumption.

References

Diaz-Cinco, M. E., Fraijo, O., Grajeda, P., Lozano-Taylor, X., & Gonzalez de Mejía, E. (1992). Microbial and chemical analysis of chihuahua cheese and relationship to histamine and tyramine. *Journal of Food Science, 57*(2), 355–365 (D.N. No. 293/93 and 47/97).

Knecht, R., & Chang, J. Y. (1986). Liquid chromatographic determination of amino acids after gas-phase hydrolysis and derivatization with (dimethylamino)azobenzenesulfonyl chloride. *Analytical Chemistry, 58*, 2375–2379.

Krause, I., Bockhardt, A., Neckermann, H., Henle, T., & Klostermeyer, H. (1995). Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. *Journal of Chromatography, A*(715), 67–79.

Leuschner, R. G. K., Kurihara, R., & Hammes, W. P. (1998). Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during Gouda cheese ripening. *International Journal of Food Microbiology, 44*, 15–20.

Polo, M. C., Ramos, M., & Sanchez, R. (1985). Free amino acids by high performance liquid chromatography and peptides by gel electrophoresis in Mahon cheese during ripening. *Food Chemistry, 16*, 85–96.

Reuvers, T. B. A., Pozuelo, M. M., Ramos, M., & Jimenez, R. (1986). A rapid ion-pair HPLC procedure for the determination of tyramine in dairy products. *Journal of Food Science, 51*, 84–86.

Santos, M. H. S. (1986). Biogenic amines: their importance in foods. *International Journal of Food Microbiology, 29*, 213–231.

Stratton, J. E., Hutkins, R. W., & Taylor, S. L. (1991). Biogenic amines in cheese and other fermented food. A review. *Journal Food Protection, 54*, 460–470.

Vale, S. R., & Gloria, M. B. A. (1997). Determination of biogenic amines in cheese. *Journal of AOAC International, 60*(4), 651–657.