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Evolution of free amino acid content during ripening of Mahon cheese

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Evolution of free amino acids (FAA) during ripening (10–300 days) of five batches of Mahon cheese was studied; three batches were made from raw milk and two from pasteurized milk. Major FAA were GLU, VAL, LEU, LYS and PHE (43-67% of total FAA). Relative THR, SER, GLU and GLY contents significantly increased during ripening whereas LEU, PHE and ORN decreased. GLU, ILE, SER, THR, GLY and PHE varied during ripening according to a zero order reaction ($r^2 > 0.99$). Principal component analysis showed that the first principal component could be considered representative of the ripening time. The most ripened cheeses showed high GLU, GLY, SER and THR contents and the less ripened cheeses presented high ORN, LEU, PHE and ALA contents. The second component distinguished between cheeses made with pasteurized milk (with high ASN and GLN contents) and raw milk. Indigenous microflora of raw milk showed a strong influence on the proteolytic activity in Mahon cheese. © 1997 Elsevier Science Ltd

INTRODUCTION

Free amino acids affect the characteristics of cheese and its organoleptic properties (Resmini *et al.*, 1993; Engels and Visser, 1994; Wilkinson *et al.*, 1994) and are, therefore, an important attribute of quality. Every type of cheese has its own characteristic free amino acid pattern, resulting from its specific degradation, interconversion and synthesis (Polo *et al.*, 1985). Therefore, through chemometric models and/or establishing limits or characteristic values for the concentrations of total free amino acids or of certain amino acids, it is possible to characterize different cheeses (Resmini *et al.*, 1988, 1993; Bertacco *et al.*, 1994; Prieto *et al.*, 1994). The concentrations of the different amino acids in a cheese are related to the manufacturing technology (type of curd, addition of proteinases, starters, ripening conditions, etc.) duration of ripening and the extent and type of proteolysis (Mariani *et al.*, 1993; Christensen *et al.*, 1995).

Principal component analysis (PCA) is a useful technique in exploratory data analysis and has been widely employed in research on dairy products with different objectives such as the study of the influence of different factors on the final characteristics of Comté cheese: manufacturer (Berdagué and Grappin, 1987), time of immersion in brine (Cabezas *et al.*, 1993), ripening time (Collin *et al.*, 1987; Guichard *et al.*, 1987). Moreover, PCA has been used to determine which are the peptides which better distinguish between different types of cheeses (Mohler and Nakai, 1990). Linear regression analysis applied to the free amino acid content of Idiazabal cheese showed that there is a good relationship between the major amino acid content (GLU, LEU, VAL, PHE, LYS and ALA) and ripening time (Barcina *et al.*, 1995).

Mahon cheese, a non-cooked pressed type of cheese, salted in brine, is manufactured on Menorca island (Spain) from cows' milk. The cheese is produced in the form of parallelepiped shapes of approximately 0.2 m × 0.2 m × 0.08 m with round edges and *ca* 2.5 kg mass. The cheese is manufactured under the methodology described by the 'Mahon' cheese Appellation of Origin, whose regulation distinguishes between four different kinds of Mahon cheese in relation to their ripening time: fresh (less than 10 days of ripening), half-ripened (from 2 to 5 months of ripening), ripened (from 5 to 10 months of ripening) and old-ripened (more than 10 months of ripening).

The aim of this work was to evaluate, on the one hand, whether the free amino acid contents could be used as a parameter indicative of the ripening time in Mahon cheese and, on the other hand, the influence of the indigenous microflora of milk on the former parameter.

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MATERIALS AND METHODS

Samples

Five batches of Mahon cheese were made by different manufacturers belonging to the 'Mahon' cheese Appellation of Origin. Three of these batches were made from raw milk (batches I, III and IV) and two from pasteurized milk (batches II and V). Three samples of each batch were analyzed at 10, 60, 150 and 300 days of ripening. These ripening times were considered as representative of the four types of Mahon cheeses; namely, fresh, half-ripened, ripened and old-ripened.

Free amino acids determination

Free amino acids were determined following the method of Resmini *et al.* (1993) with some modifications. The measurements were performed using an amino analyzer (LKB Alpha Plus model, Pharmacia, Sweden) by ion-exchange chromatography on a sulphonated polystyrene column with post-column ninhydrin derivation. Soluble extracts were obtained after dissolving 2.5 g cheese in 100 ml 0.05 M HCl. Aliquots (0.5 ml) were filtered through a 10-kDa membrane (Ultrafree, Millipore, France) and 100 μl × 25 ml of internal standard solution (400 μM) of norleucine were injected on a column of 250 × 4 mm. Amino acids were sequentially eluted with the following lithium citrate buffers: 0.2 M (pH 2.8, 16 ml, 35°C); 0.3 M (pH 3.0, 40 ml, 35°C); 0.5 M (pH 3.15, 24 ml, 35°C); 0.5 M (pH 3.15, 32 ml, 57°C); 1.0 M (pH 3.45, 16 ml, 75°C); 1.65 M (pH 3.55, 64 ml, 75°C), followed by 0.3 M LiOH (16 ml, 75°C) and, finally, 0.2 M lithium citrate buffer (pH 2.8, 8 ml, 75°C).

Variability in amino acid determinations on one sample was 2.5%, while this parameter between samples at each analysis time was 5.2%.

Statistical treatment

Statistical analysis was performed on pooled data by using the BMDP statistical software package (Dixon, 1992). 7D (ANOVA) and 4M (Principal Component Analysis) were employed.

RESULTS AND DISCUSSION

In all, 27 amino acids and derived compounds were identified in this work. Results obtained for the free amino acid determinations carried out on samples of each batch at 10, 60, 150 and 300 days of ripening are shown in Table 1 (mg total free amino acids per 100 g protein) and Table 2 (g amino acid per 100 g total free amino acids). Important differences in the total free amino acid contents were found between samples of every batch (Table 1). Nevertheless, this figure increased in the five batches throughout ripening.

Table 1. Evolution of the total free amino acid contents during the ripening time (mg 100 g^{-1} protein)

	Time (days)			
	10	60	150	300
Batch I	—	2285	3825	6024
Batch II	1583	2734	4370	4678
Batch III	1089	1126	1836	6575
Batch IV	250	397	507	1559
Batch V	1123	1404	1519	1855

As shown in Table 2, the major free amino acids in Mahon cheese were GLU, VAL, LEU, LYS and PHE. These amino acids accounted for 43 to 67% of the total free amino acid content. Lavanchy and Sieber (1993) found the same predominant free amino acids in different hard and semi-hard cheeses. According to different authors, PHE and GLU seem to be the major free amino acids in Mahon cheese (Polo *et al.*, 1985), Saint Paulin cheese (Do Ngoc *et al.*, 1971) and Vaccino Ragusano cheese (Nicolosi *et al.*, 1981), while LEU and GLU are the major free amino acids in Provolone (Resmini *et al.*, 1988) and Comté cheeses (Do Ngoc *et al.*, 1971). Results in Table 2 show that the amino acid content varied at different rates. Similar behaviour was found for Gruyere and Sbrinz cheeses (Lavanchy and Sieber, 1993).

The low TYR and TRP contents in Mahon cheese, in relation to other cheeses made from cows' milk (Marcos *et al.*, 1979), could be attributed, as pointed out by Alcalá *et al.* (1982), to the low level of hydrolysis of β-casein.

Very small amounts of AAAA, AABA, CYS, 1M-HIS and 3M-HIS were detected (less than 2 mg per 100 g cheese). 1M-HIS and 3M-HIS were identified only in samples of batch II, and CYS was detected in all batches but only at 10 and 60 days of ripening. Therefore, these amino acids and derived compounds were not used in the statistical analysis.

Samples of fresh cheese (10 days of ripening) had high values of LEU (19%), PHE (14%) and VAL (7%) as percentages of total free amino acids. These free amino acids are generated via different biochemical processes which take place. It should be pointed out that extensive proteolysis of α_{S1}-casein (which has a high content of LEU, PHE and VAL) occurs during the first days of ripening, which predominates over the β-casein proteolysis, the latter being limited by the high salt concentration. Therefore, during the early stages of ripening, proteolysis is due mainly to chymosin, which hydrolyzes both ARG1-PHE23 and ARG1-PHE24 peptides. The results of this hydrolysis are a peptide with terminal PHE and a macropeptide containing VAL (situated in a terminal position or next to the point of hydrolysis). Next, bacterial peptidases could act by releasing these amino acids. Moreover, there are high leucine-aminopeptidase and valine-aminopeptidase activities of *Lactococcus plantarum* and *Lactobacillus casei*, in Mahon cheese during the first days of ripening (Suárez *et al.*, 1984).

Table 2. Change in the free amino acid composition during ripening (mg 100 g^{-1} of total free amino acids)

	Batch I				Batch II				Batch III				Batch IV				Batch V			
Time (days)	60	150	300	10	60	150	300	10	60	150	300	10	60	150	300	10	60	150	300	
AAAA	0.27	0.08	0.08	0.00	0.08	0.00	0.16	0.86	0.28	0.41	0.03	0.00	0.11	0.00	0.05	0.00	0.33	0.00	0.06	
AABA	0.39	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	
ALA	4.53	2.77	2.61	2.40	1.70	1.75	1.85	2.16	3.05	1.89	2.75	5.43	6.29	3.22	2.88	3.31	3.11	2.57	2.86	
ARG	0.48	0.62	0.42	3.68	4.19	3.87	3.72	0.66	0.78	1.92	1.18	1.26	1.45	0.92	0.30	0.73	0.30	0.33	0.00	
ASN	0.00	0.00	0.00	13.02	11.40	7.33	6.83	0.00	0.00	3.03	2.28	0.56	0.00	0.00	0.43	1.49	2.72	6.58	9.43	
ASP	3.31	5.04	4.29	2.23	1.92	2.56	2.30	1.98	2.62	3.47	4.45	2.11	2.40	5.12	5.46	0.97	1.39	1.65	2.31	
CIT	3.26	5.38	3.38	3.41	2.17	3.61	3.83	4.43	0.00	2.43	2.93	3.07	2.86	5.13	4.18	2.58	1.56	2.67	3.77	
CYS	0.21	0.00	0.00	0.00	0.36	0.00	0.00	1.02	0.58	0.00	0.00	0.22	0.00	0.00	0.00	0.10	0.27	0.00	0.00	
GABA	6.55	2.33	1.34	0.11	0.28	0.00	0.00	0.33	2.04	1.12	0.76	0.00	2.26	1.95	0.55	1.63	1.43	1.60	1.51	
GLN	0.00	0.00	0.00	6.45	10.34	4.95	3.54	0.00	0.00	0.00	0.00	0.00	1.81	0.93	1.15	2.04	3.34	3.40	3.02	
GLU	11.37	16.37	23.02	13.92	12.23	14.51	17.31	9.41	11.60	20.99	22.65	8.30	18.47	17.63	20.78	4.76	10.22	13.30	18.47	
GLY	2.11	1.81	1.85	0.89	0.95	1.66	1.49	0.36	0.76	1.62	2.06	0.40	1.40	1.62	1.82	0.45	1.16	1.78	2.59	
HIS	0.97	0.98	1.42	1.23	1.35	1.41	1.36	0.82	0.74	1.53	2.26	1.41	2.05	1.63	2.21	1.73	1.03	1.14	1.70	
ILE	3.45	3.94	5.55	1.31	1.56	2.24	3.46	1.58	2.86	2.85	4.40	1.23	2.67	3.15	4.70	3.10	4.64	5.90	3.79	
LEU	16.29	16.96	12.27	13.81	13.03	16.75	12.95	23.83	26.00	15.16	11.57	19.32	18.60	16.08	11.96	22.30	18.30	17.16	11.90	
LYS	7.04	7.35	10.97	5.63	5.76	6.55	7.99	3.13	3.11	6.44	10.26	7.99	3.45	6.43	9.14	8.46	11.06	9.66	8.57	
MET	3.74	3.78	3.75	1.61	2.13	2.80	2.74	2.68	3.58	2.85	3.74	0.81	2.38	3.39	4.02	3.23	2.83	2.45	3.31	
ORN	5.47	2.66	1.67	2.32	2.03	1.05	0.76	8.47	5.30	3.58	1.63	7.05	3.37	2.29	1.44	7.78	3.92	1.76	1.84	
PHE	10.84	8.64	6.61	9.60	8.05	8.43	6.87	18.43	15.54	8.92	5.65	21.81	14.34	11.19	7.63	14.84	6.53	8.25	7.00	
PRO	3.90	4.05	3.97	3.83	1.69	2.66	3.28	3.21	2.92	3.23	3.77	4.46	3.75	2.31	2.92	2.26	2.56	3.06	2.75	
PSE	3.76	4.48	3.79	4.64	7.52	4.20	7.06	3.69	7.89	7.01	2.54	2.27	2.03	5.08	3.21	9.62	9.10	3.77	1.89	
SER	2.22	2.46	2.92	1.66	1.65	2.50	2.07	0.84	0.44	1.55	2.66	0.79	1.51	1.37	2.89	0.65	1.40	1.62	2.92	
THR	2.47	3.15	3.03	1.12	1.17	1.89	1.60	0.14	1.06	2.91	3.90	1.03	1.67	2.39	3.41	0.07	1.27	1.87	2.43	
TYR	0.32	1.21	0.94	3.11	4.31	4.00	3.18	0.25	2.05	1.94	2.78	0.63	0.00	2.25	2.89	0.42	5.37	1.34	1.62	
VAL	7.03	5.96	6.11	4.01	4.12	5.25	5.63	11.72	6.80	5.13	5.74	9.85	7.13	5.91	5.81	7.48	6.15	8.14	6.27	

In order to study the relative amounts of the different amino acids and their changes throughout ripening time, ANOVA analysis was carried out. From the experimental results and their statistical treatment, it was concluded that THR ($p > 99.9\%$), SER ($p > 99\%$), GLU ($p > 99\%$), GLY ($p > 99.9\%$) and ILE ($p > 95\%$) increased significantly during ripening, whereas LEU, PHE and ORN decreased (Table 2 and Table 3). Negative correlations were found between SER and LEU, PHE and ORN contents and between GLY and LEU, PHE and ORN contents. These results could be indicative of the conversion of LEU, PHE and ORN in GLY and SER.

The former results do not totally agree with those presented by Polo *et al.* (1985). These authors found that the ILE content in Mahon cheese increased during ripening time, whereas GLU and SER content remained constant. Nevertheless, the change in amino acids was studied in fewer samples (four) and a shorter ripening time (120 days) was considered.

The multiple range test analysis showed the existence of significant differences ($p > 99.5\%$) in the concentrations of PHE and ORN between samples of 10 days (fresh cheese) and samples with longer ripening time. Moreover, significant differences were detected in THR ($p > 99.9\%$), SER ($p > 99.5\%$), GLU ($p > 99.5\%$), GLY ($p > 99.9\%$) and ORN ($p > 99.5\%$) contents in 300 day-old cheeses (old-ripened) and of 10 and 60 day-old (fresh and half-ripened cheeses, respectively). Results of the former analysis are summarized in Table 3.

The average GLU content increased from 9 ± 3 to $21 \pm 2\%$ throughout ripening. This increase could be due to the contribution of different factors. Among them could be the high GLU content in casein and the formation of GLU from GLN, VAL, LEU and ILE owing to the glutaminase and transaminase activities. Although PRO is one of the major amino acids in β-casein, the free PRO content in Mahon cheese was low, less than 5%. No significant increase in the content of this amino acid was detected during ripening. Therefore, this result could be indicative of the low β-casein hydrolysis.

Table 3. ANOVA and multiple range test analysis of free amino acid content (g $100\,g^{-1}$ free amino acids) vs ripening time (Tuckey test)

Time (days)	10	60	150	300	Significance level
ALA	—	—	—	—	ns
ARG	—	—	—	—	ns
ASN	—	—	—	—	ns
ASP	—	—	—	—	ns
CIT	—	—	—	—	ns
GABA	—	—	—	—	ns
GLN	—	—	—	—	ns
GLU	a	ab	bc	c	0.0011
GLY	a	ab	bc	c	0.0002
HIS	—	—	—	—	ns
ILE	a	ab	b	b	0.0230
LEU	a	a	ab	b	0.009
LYS	—	—	—	—	ns
MET	—	—	—	—	ns
ORN	a	b	bc	c	0.0011
PHE	a	b	b	b	0.0046
PRO	—	—	—	—	ns
PSE	—	—	—	—	ns
SER	a	ab	bc	c	0.0011
THR	a	ab	bc	c	0.0008
TYR	—	—	—	—	ns
VAL	—	—	—	—	ns

ns: non-significant. Values in the same row with different letters showed significant differences ($p > 99.5\%$).

ORN content was higher than ARG content in all batches except batch II. The proportion of ORN in the total free amino acid amount, initially $5 \pm 2\%$, decreased *ca* 75% during ripening. Extensive hydrolysis of ARG could take place during the early stages of ripening, by means of arginase activity, generating ORN and urea. ORN could then be hydrolyzed, releasing each molecule, two amino groups which subsequently would turn into NH_4^+ or into CIT (by transference of one carbonyl group due to the ornithine transcarbamylase activity).

With regard to ORN and CIT contents, two stages could be distinguished: during the first stage, the concentration of ORN was higher than that of CIT, seemingly attributed to the high transformation of ARG into ORN; in the second stage, from 150 days onwards, ORN content was lower than CIT content, possibly due to the prevalence of the ORN degradative process.

ANOVA analysis was carried out in order to determine the influence of the type of milk used in cheesemaking, either raw or pasteurized. There were significant differences between cheeses made from raw or pasteurized milk in the concentration of ASP ($p > 99.5\%$), ASN ($p > 99.9\%$) and GLN ($p > 99.9\%$). ASP content was higher in cheeses made from raw milk whereas ASN and GLN content were lower in these cheeses. Similar results were found by Polo *et al.* (1985) between these two types of cheeses. The higher concentrations of ASN and GLN in pasteurized cheeses could be due to the asparaginase and glutaminase denaturalization during the pasteurization process.

GLU, ILE, SER, THR, PHE and GLY varied during ripening according to zero order kinetics (eqn 1), k being the zero order reaction rate constant, t, the ripening time, C, the concentration (mg $100\,g^{-1}$ protein) and Co, the initial concentration (mg $100\,g^{-1}$ protein) of free amino acid. The confidence level of the correlation was in all cases higher than 99%.

$$C - Co = kt \tag{1}$$

Principal component analysis (PCA)

PCA was applied to pooled data in order to establish the relationship between the different variables and to detect the most important causes of variability.

Squared multiple correlation coefficients and communalities among the different free amino acid contents were high (ranging from 0.63 to 1.00 and from 0.79 to 0.98, respectively), where the great correlation between the free amino acid contents is notable. Thus, principal component analysis could be applied to our data without deterioration or loss of information.

PCA of the 22 free amino acids on the 19 cheeses resulted in six principal components with eigenvalues greater than 1.0, a common statistical cut-off point (Dixon, 1992).

The eigenvalues explained by the different components are shown in Table 4. It can be observed that the six selected components accounted for 87.1% of the total variance. Thus, the dimensionality of the data was reduced from 22 free amino acid contents to six uncorrelated principal components with 12.9% loss of variation. The first principal component ($k = 7.84$) condensed 35.6% of variance in data space and 40.0% in factor space.

The principal components matrix is shown in Table 5. The principal component that best summarized the information contained in the original data matrix was the first component, and the second component better summarized the remaining information. These two principal components are independent of one another.

The first principal component was high in SER, THR, GLU, GLY and ASP (positive values) and in LEU, PHE, ORN and VAL (negative values). The second component was high in GLN, ASN and ARG (positive values) and in MET and ASP (negative values).

Free amino acids were represented as a function of both first (PC1) and second (PC2) principal components (Fig. 1). Different groups of free amino acids can be observed in this figure. SER, GLY, GLU and THR were grouped near the positive side of the horizontal axis, whereas LEU, PHE, ORN and VAL were placed on the negative side. ANOVA analysis showed that the concentrations of these amino acids varied significantly during ripening. From these results, it could be concluded that the first principal component could be considered as representative of the ripening time.

It can be observed in Fig. 1 that the second component distinguished between cheeses made from pasteurized milk, with high ASN and GLN contents, from

Table 4. Principal component analysis; characteristics of the selected component

Component	Eigenvalue (k)	Cumulative proportion of variance	
		In date space	In component space
1	7.8	35.6	40.9
2	5.3	59.6	68.5
3	2.4	70.6	81.1
4	1.4	76.8	88.2
5	1.2	82.2	94.3
6	1.1	87.1	100.0

Table 5. Principal component analysis; loading components rotated and sorted

	PC1	PC2	PC3	PC4	PC5	PC6
ALA	−0.20	−0.26	−0.58	−0.08	0.08	0.67
ARG	0.11	0.72	0.21	−0.49	−0.21	−0.08
ASN	0.13	0.93	0.17	0.01	−0.03	−0.12
ASP	0.73	−0.53	−0.11	−0.15	−0.08	−0.22
CIT	0.33	−0.11	−0.45	−0.08	−0.14	−0.62
GABA	0.10	−0.35	−0.16	0.02	0.83	0.28
GLN	0.03	0.87	0.35	−0.08	−0.05	0.01
GLU	0.88	−0.15	−0.11	0.03	−0.21	−0.04
GLY	0.83	−0.04	−0.16	0.33	0.29	−0.04
HIS	0.52	−0.10	−0.13	0.08	−0.61	0.40
ILE	0.41	−0.33	0.10	0.77	0.17	0.00
LEU	−0.81	−0.34	0.11	−0.25	0.18	0.07
LYS	0.30	0.00	0.04	0.87	−0.15	0.01
MET	0.51	−0.64	0.30	0.18	0.31	−0.15
ORN	−0.83	−0.38	−0.13	−0.16	0.10	0.09
PHE	−0.77	−0.26	−0.35	−0.40	−0.12	0.14
PRO	0.08	−0.16	−0.77	−0.01	0.07	0.02
PSE	−0.33	0.03	0.86	−0.01	0.10	0.07
SER	0.84	0.10	−0.20	0.31	0.04	−0.22
THR	0.89	−0.28	−0.15	0.19	0.01	−0.03
TYR	0.27	0.47	0.65	0.10	−0.20	−0.06
VAL	−0.71	−0.39	−0.46	0.12	−0.04	−0.14

those made from raw milk, which presented the opposite characteristics. Therefore, it could be assumed that the second principal component was representative of the type of milk used in the manufacture, either raw or pasteurized. Similar results were proposed by McSweeney *et al.* (1993). These authors found significant differences among the amino acid contents in Cheddar cheese, and especially among the free amino acid contents depending on this parameter. Therefore, it could be concluded that the indigenous microflora of milk had a strong influence on the free amino acid content in Mahon cheese made from raw milk.

Cheeses with different ripening times, on the one hand, and cheeses made with pasteurized or raw milk, on the other hand, were differentiated by PC1 and PC2, as can be observed in Fig. 2, corroborating the conclusions mentioned above. However, in this figure (Fig. 2) it can be observed that samples of batch II (enclosed in the ellipse) were clearly different from the remaining samples. In this case, the variable manufacturer seemed to be more important than the variable ripening time.

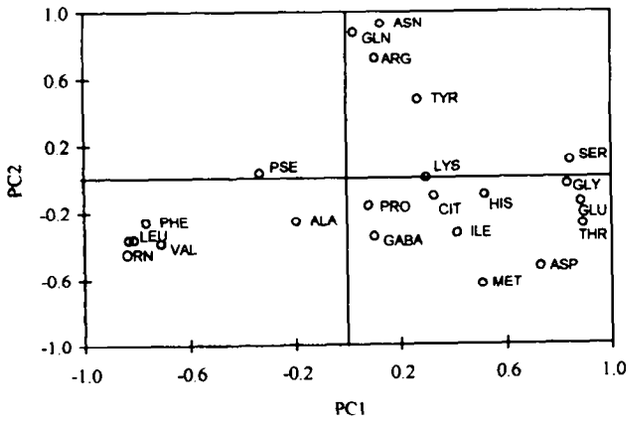

Fig. 1. Principal component analysis. Representation of the amino acid as a function of the first (PC1) and second (PC2) principal components.

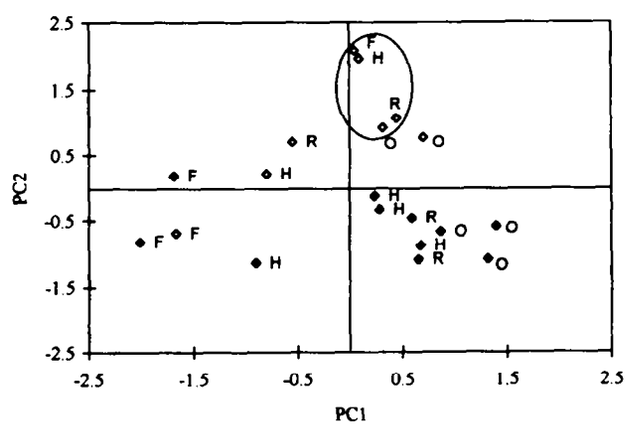

Fig. 2. Principal components analysis. Representation of the four types of Mahon cheese vs the first (PC1) and second (PC2) principal components: F (fresh); H (half-ripened); R (ripened); O (old-ripened).

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