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# Free Amino Acids by High Performance Liquid Chromatography and Peptides by Gel Electrophoresis in Mahon Cheese During Ripening*

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*ABSTRACT*

*The free amino acids and peptides contents of Mahon cheese during four months of ripening were studied using high performance liquid chromatography and gel electrophoresis, respectively. The total content of free amino acids generally increased throughout the ripening period, except in one batch. Phenylalanine, valine, proline, glutamic acid and isoleucine were the most abundant free amino acids in all the tests throughout the four month period. They accounted for between 67 and 80% of the free amino acids. No difference was found between the pattern of traditionally made and industrially made Mahon cheeses. Electrophoresis of soluble nitrogen showed the main whey proteins as well as other bands corresponding to peptides.*

## INTRODUCTION

Mahon cheese is a variety with peculiar characteristics, manufactured in Spain from cow's milk. Its production, including the amount used for processed cheese, is 3500 tons/year. Some studies on this type of cheese have been published (Iñigo *et al.*, 1982; Ramos & Jiménez-Pérez, 1982; Marcos *et al.*, 1983). Recently, we have studied the evolution of the microbial flora and the breakdown of caseins during the ripening process in self-made Mahon cheese (Ramos *et al.*, 1982).

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Proteolysis is the major event in the ripening of most varieties of cheese. It affects both the flavour and the texture of the finished cheese.

Although many papers have been published on proteolysis of caseins, few data are available on the nature of peptides in water-soluble extracts of different varieties of cheese.

The present study was undertaken to follow the evolution of free amino acids by HPLC and the characterization of the soluble nitrogen fraction by electrophoresis in three batches of traditionally made cheese and one batch of industrially made Mahon cheese during ripening.

## MATERIAL AND METHODS

Three batches of Mahon cheese were prepared by the usual traditional procedure (Ramos *et al.*, 1982). One batch was prepared by the usual industrial procedure: (a) pasteurization of cow's milk, (b) addition of starters, (c) renneting at 32 °C with calf rennet for 45 min, (d) cooking at 35 °C, (e) cutting the curd mechanically to rice size pieces, (f) salting in brine over 48 h, and (g) ripening at 12 °C and 97 % humidity.

Samples were analysed at different times of ripening.

### Preparation of soluble extract

Soluble extracts of the cheese were obtained by the Skirs procedure (Stadhouders, 1960) and freeze-dried.

Ultrafiltration was carried out under $N_2$ pressure in an Amicon Corp UF cell equipped with a DIAFLO PM-10. The PM-10 retentate was dialized and the PM-10 ultrafiltrate was freeze-dried.

### Gel electrophoresis

Samples of freeze-dried soluble extracts, PM-10 retentate and PM-10 ultrafiltrate, were analysed by vertical polyacrylamide gel electrophoresis, following the method of Hillier (1976).

### Thin layer chromatography

Thin layer chromatography (TLC) was performed according to Kuchroo & Fox (1982*a*).

## High performance liquid chromatography of free amino acids

The free amino acids fraction was obtained following the procedure of Reiter *et al.* (1969). Amino acids in sulphosalicylic acid (SSA) were determined by the ninhydrin method (Saifer *et al.*, 1960) and expressed as mg leucine/100 g dry weight.

In addition the individual free amino acids in the SSA-soluble fraction were determined by high performance liquid chromatography (HPLC).

*Instrumentation*

All separations were performed on a Waters Associates instrument with two 6000 A pumps, a 720 system controller, radial compression module RCM-100 and a U6K injector. Fluorescence was detected using a fluorimeter 420 AC with standard flow-cell and standard filters (excitation filter 340 ± 6 nm and emission filter 425 nm (long pass)). A reversed phase column (10 cm × 8 mm id) with Radial Pak $C_{18}$ (10 μm) and Bondapak C-18/Corasil (37–50 μm) guardcolumn was used.

*Chemicals and buffers*

Methanol was HPLC grade from Scharlau, Madrid, Spain. All buffers were prepared from analytical grade chemicals and MilliQ (Millipore Corp., Bedford, MA) water. Before use, all buffers were filtered, using a Millipore Type HA filter with a pore diameter of 0·45 μm, and degassed.

Dns-amino acid standard and amino acids were obtained from Sigma (St. Louis, MO) and Dns-Cl from Fluka Buchs, Switzerland.

*Dansyl derivatization*

Dansyl derivitization was carried out under conditions similar to those used by Tapuhi *et al.* (1981). The SSA-soluble fraction was raised to a pH of around 9 and an aliquot containing approximately 15 ng amino nitrogen was taken. 1 ml dansyl chloride (5·56 mM, i.e. 1·5 mg in 10 ml acetone without water) and 1 ml lithium carbonate buffer (40 mM, pH 9·5) were added. The reaction was allowed to take place for 1 h in the dark at room temperature and the internal standard (25 μl Dns-norvaline 0·1 mg/ml) was added. The mixture was concentrated to dryness at room temperature and was dissolved again in 0·3 ml methanol. It was filtered through a Millipore type FH filter with a pore diameter of 0·50 μm before injection. The injected quantities corresponded to approximately 100 pM of each amino acid.

*Chromatographic separation*

Dansyl amino acids separation was carried out in conditions similar to those described by Martin *et al.* (1984). Gradient elution was used, mobile phase: (A), methanol: 0·01 M sodium phosphate buffer, pH 6·3 (15:85 w/w); (B), methanol: 0·01 M sodium phosphate buffer, pH 6·3 (35:65 w/w), 30 min, flow rate 2 ml/min.

## RESULTS AND DISCUSSION

### Free amino acids

The total content of free amino acids determined by the ninhydrin reaction increases, in general, through the ripening period. This increase was higher during the first month (Fig. 1). In batch number 2 a faster increase of free amino acids was observed reaching, in the third month of ripening, a value of 3·29 mg/g dry weight. This value was higher than that of other batches even in the fourth month. Nevertheless a decrease in the free amino acid content in batch number 2 between the third and the fourth month was observed. This could be due to the decarboxylation,

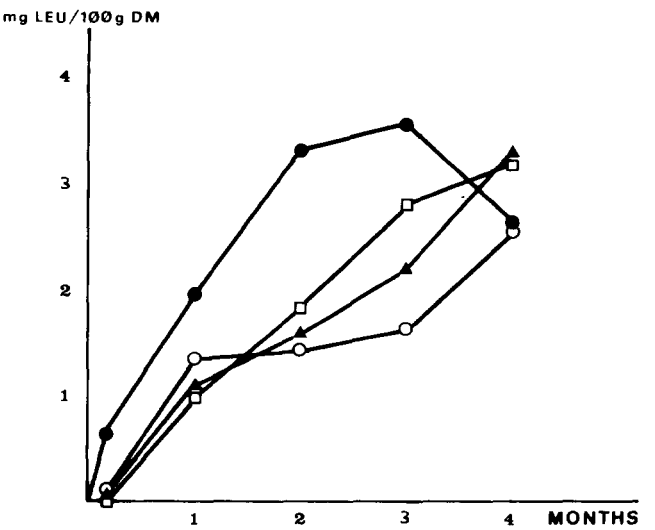


**Fig. 1.** Evolution of total content of free amino acids during ripening. ▲, Batch 1; ●, batch 2; ○, batch 3; □, batch 4.

deamination and transamination reactions which take place in cheeses with a high level of proteolysis (Marcos & Mora, 1982).

Figure 2 shows the chromatogram of Dns-amino acids from the industrial Mahon cheese at the third month of ripening.

Table 1 sums up the results obtained from four replicated analyses of the individual amino acids of a sample of cheese. Acceptable values of variation coefficients were obtained (from 1·8% for valine to 5·7% for aspartic acid) even though the internal standard is in a 'crowded' portion of the chromatogram. The short analysis time and the reproducibility of the results makes this method suitable for the determination of the free amino acid content in cheeses.

Table 2 shows the content of individual free amino acids of four batches of Mahon cheese during ripening. Results are the mean of two or more analyses of every sample.

In general, amino acids increased during the whole process except for batch number 2, in agreement with the above results for total amino acids. Phenylalanine, valine, proline, glutamic acid and isoleucine were the most

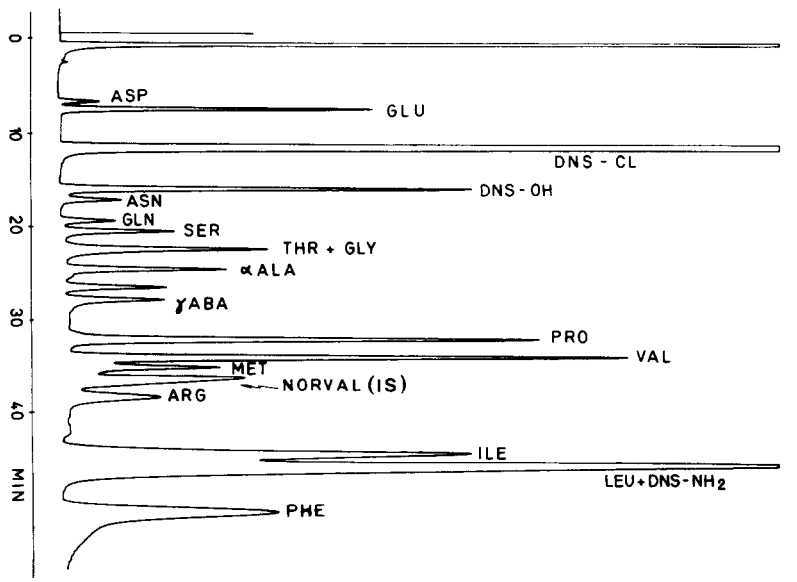


**Fig. 2.** Cheese chromatogram at 3 months of ripening (batch number 4), after Dns-derivatization, gradient elution, mobile phase: (A), methanol: 0·01 M sodium phosphate buffer, pH 6·3 (15:85 w/w); (B), methanol: 0·01 M sodium phosphate buffer, pH 6·3 (35:65 w/w), 30 min, flow rate 2 ml/min.

**TABLE 1**
Variability of the Data Obtained from Four Replicates of a Sample of Cheese

| *Amino acids* | *Mean of the data* ($\bar{X}$) (*mg/100 g of dry matter*) | *Standard deviation of* $\bar{X}$ (*s*) | *Coefficient of variation* (*v*) |
|---|---|---|---|
| Aspartic acid | 17·0 | 0·97 | 5·7 |
| Glutamic acid | 100·8 | 3·96 | 3·9 |
| Asparagine | 29·7 | 1·45 | 4·9 |
| Glutamine | 26·3 | 0·74 | 2·8 |
| Serine | 28·8 | 1·55 | 5·4 |
| Threonine + glycine | 33·5 | 1·59 | 4·7 |
| Alanine | 25·0 | 0·99 | 4·0 |
| $\alpha + \gamma$ amino butyric acid | 15·5 | 0·72 | 4·6 |
| Proline | 102·8 | 4·16 | 4·0 |
| Valine | 96·1 | 1·75 | 1·8 |
| Methionine | 37·4 | 1·38 | 3·7 |
| Arginine | 29·6 | 1·36 | 4·6 |
| Isoleucine | 129·7 | 6·49 | 5·0 |
| Phenylalanine | 296·1 | 15·55 | 5·3 |

abundant amino acids in all the tests throughout the four month period, and together accounted for between 67 and 80% of the free amino acids. Some of them have been reported as the most abundant by other authors in other cheese varieties. Also, in Teleme cheese (Polychroniadou & Vlachos, 1979), leucine, phenylalanine, valine and lysine accounted for 50% of free amino acids. However, each type of cheese has its own characteristic pattern. This pattern results from the enzymatic degradation of peptides by various microorganisms and also from amino acid interconversion, excretion and degradation. Thus glutamic acid, which is the major amino acid in many varieties of cheese, is found in small amounts in Manchego cheese after long ripening periods (Ordoñez & Burgos, 1980). Proline, which according to Kosikowski (1951) is found in small amounts in ripe cheese, is a major amino acid in Emmental cheese and contributes to its sweet flavour (Law, 1981). Studies on Kaskhawal cheese (Buruiana & Zeidan, 1982) showed a marked increase of proline during the three first months, decreasing at the end of ripening.

Arginine, which has been reported to be responsible for an unpleasant or bitter-sweet taste (Scormuler, 1968), was found in the four batches

**TABLE 2**

Changes in Free Amino Acids during Ripening of Mahon Cheese (mg/100 g of Dry Matter)

| | *1 Month* | | | | *2 Months* | | | | *3 Months* | | | | *4 Months* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Batch no.* | | | | *Batch no.* | | | | *Batch no.* | | | | *batch no.* | | | |
| | *1*[a] | *2*[a] | *3*[a] | *4*[b] | *1*[a] | *2*[a] | *3*[a] | *4*[b] | *1*[a] | *2*[a] | *3*[a] | *4*[b] | *1*[a] | *2*[a] | *3*[a] | *4*[b] |
| Aspartic acid | 2·8 | 15·7 | 17·0 | 9·4 | 13·5 | 43·5 | 26·3 | 5·5 | 26·7 | 30·5 | 23·7 | 16·9 | 52·0 | 24·7 | 52·6 | 14·7 |
| Glutamic acid | 12·6 | 64·3 | 47·7 | 41·9 | 47·5 | 117·6 | 74·6 | 56·7 | 85·4 | 151·2 | 87·9 | 100·7 | 148·3 | 94·6 | 100·0 | 170·1 |
| Asparagine | 1·1 | 0·6 | 2·3 | 12·1 | 3·2 | 5·6 | 2·3 | 20·7 | 3·7 | 8·0 | 2·3 | 25·8 | 5·3 | 2·0 | 3·1 | 18·5 |
| Glutamine | 1·9 | 3·3 | 10·3 | 12·1 | 8·6 | 3·2 | 18·3 | 24·0 | 12·4 | 3·2 | 14·8 | 38·3 | 16·3 | 2·8 | 33·0 | 43·2 |
| Serine | 4·2 | 6·2 | 7·4 | 9·4 | 6·6 | 35·8 | 17·5 | 23·8 | 19·9 | 38·8 | 26·2 | 30·9 | 47·4 | 30·7 | 46·5 | 42·5 |
| Threonine + glycine | 3·5 | 14·0 | 9·9 | 6·8 | 16·2 | 48·8 | 18·1 | 14·4 | 31·1 | 37·7 | 25·1 | 33·6 | 48·9 | 26·1 | 37·2 | 61·7 |
| Alanine | 3·0 | 17·9 | 9·4 | 9·1 | 11·5 | 70·6 | 11·0 | 8·0 | 17·8 | 45·6 | 27·2 | 24·9 | 29·0 | 27·8 | 25·8 | 35·3 |
| $\alpha + \gamma$ Amino butyric acid | 7·8 | 41·8 | 14·1 | 4·9 | 24·7 | 69·8 | 12·9 | 4·8 | 22·2 | 55·8 | 15·3 | 15·4 | 37·9 | 42·0 | 20·7 | 6·5 |
| Proline | 14·9 | 48·2 | 17·4 | 15·7 | 19·9 | 168·8 | 30·9 | 58·9 | 65·1 | 230·3 | 64·4 | 102·8 | 83·5 | 212·7 | 94·4 | 100·5 |
| Valine | 15·6 | 64·3 | 32·8 | 26·6 | 59·3 | 122·9 | 64·4 | 44·9 | 81·0 | 111·6 | 113·0 | 95·9 | 118·9 | 97·1 | 109·5 | 141·7 |
| Methionine | 0 | 0 | 0 | 3·0 | 0 | 0 | 0 | 18·1 | 0 | 0 | 0 | 40·4 | 0 | 0 | 0 | 32·6 |
| Arginine | 1·1 | 3·7 | 2·2 | 3·0 | 6·3 | 0 | 3·0 | 4·8 | 6·8 | 0 | 4·2 | 29·7 | 6·4 | 0 | 7·8 | 11·7 |
| Isoleucine | 5·1 | 23·7 | 7·0 | 3·0 | 6·6 | 402·9 | 18·8 | 3·5 | 47·5 | 282·0 | 38·1 | 131·6 | 78·2 | 248·5 | 16·2 | 209·9 |
| Phenylalanine | 15·9 | 75·2 | 25·1 | 89·9 | 62·7 | 433·5 | 80·7 | 156·8 | 101·6 | 348·7 | 66·4 | 296·1 | 191·2 | 259·3 | 169·4 | 226·4 |

[a] Traditionally made.

[b] Industrially made.

studied in very small amounts. Methionine was only detected in the industrial batch.

The relative proportions of glutamic acid, serine, alanine, valine and arginine were kept constant through the ripening. The other amino acids studied showed different variations in the four batches. Thus the relative proportion of proline decreases from 16·6 to 9·7 in batch number 1 and there is a considerable increase (from 1·2 to 18%) in isoleucine in the industrial batch.

These results agree with those of Gooda *et al.* (1983) who showed changes in the relative distribution of the free amino acids in Cheddar cheese. Ismail & Hansen (1972) achieved similar results studying some types of Danish cheese. The authors found that the relative quantities of asparagine, glutamine, leucine and phenylalanine declined while the quantities of valine and isoleucine increased. Nevertheless in a study of free amino acids determined by gas chromatography in Cheddar cheese (Jarret *et al.*, 1982), it was stated that amino nitrogen levels increased during cheese ripening but the relative proportions of individual amino acids remained essentially constant.

In batch number 2 there was a decrease of free amino acids from the second month, leading in the fourth month to the lowest content of glutamic acid and a high content of $\gamma$-amino butyric acid, the latter presumably formed by decarboxylation of glutamic acid. This batch of cheese was the one in which the ratios of soluble nitrogen/total nitrogen and non-protein nitrogen/total nitrogen were higher. In the sensory evaluation of the samples carried out in a previous work (Ramos *et al.*, 1982) this batch had the worst qualifications. The relation between the poor quality of cheese and the high content of $\gamma$-amino-butyric acid/low content glutamic acid was also found in other cheeses (Ismail & Hansen, 1972; Resmini *et al.*, 1969).

No difference between the pattern of the traditionally made cheese and that of the industrial cheese was found.

**Characterization of water-soluble nitrogen by gel electrophoresis**

Electrophoretograms of the soluble nitrogen during ripening in the industrial batch showed the presence of seven to eight protein bands (Fig. 3). $\beta$-Lactoglobulins A and B, $\alpha$-lactoalbumin and seralbumin were identified by comparing them with electrophoretograms of isolated whey proteins. These proteins were not hydrolysed during ripening. This fact

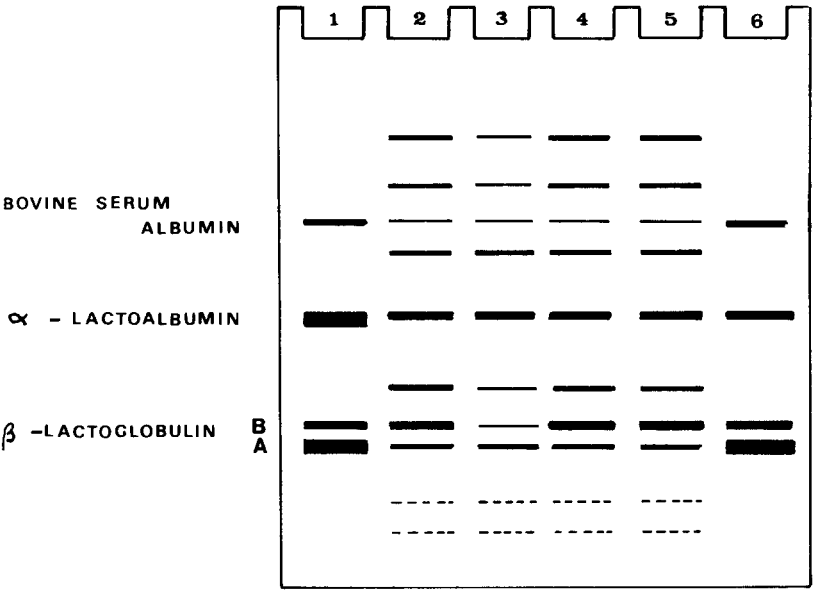


**Fig. 3.** Diagrammatic representation of polyacrylamide gel electrophoresis patterns of soluble nitrogen in the industrial batch during ripening. (1) and (6), whey proteins; (2), cheese at 5 days of ripening; (3), cheese at 20 days of ripening; (4), cheese at 60 days of ripening; (5), cheese at 120 days of ripening.

has been observed in other types of cheese (O'Keefe *et al.*, 1978). In cheeses manufactured from ultrafiltered milk, proteolysis of whey proteins was not observed (Koning *et al.*, 1981).

In addition to whey proteins, four to six bands showing different mobilities corresponding to peptides were also observed. The intensities of these bands were variable during ripening. The pattern of the main bands of the traditionally made cheese was similar to that of the industrially made one.

Figure 4 shows the electrophoretograms of the soluble nitrogen (PM-10 retentate) at the end of ripening of the four batches. The four whey proteins, two bands with mobilities lower than seralbumin, another between $\alpha$-lactoalbumin and $\beta$-lactoglobulin and two more bands with mobilities higher than that of $\beta$-lactoglobulin can be observed. Although no peptides were detected in the soluble nitrogen (PM-10 ultrafiltrate) by electrophoresis, TLC of this fraction showed seven to ten ninhydrin-positive components.

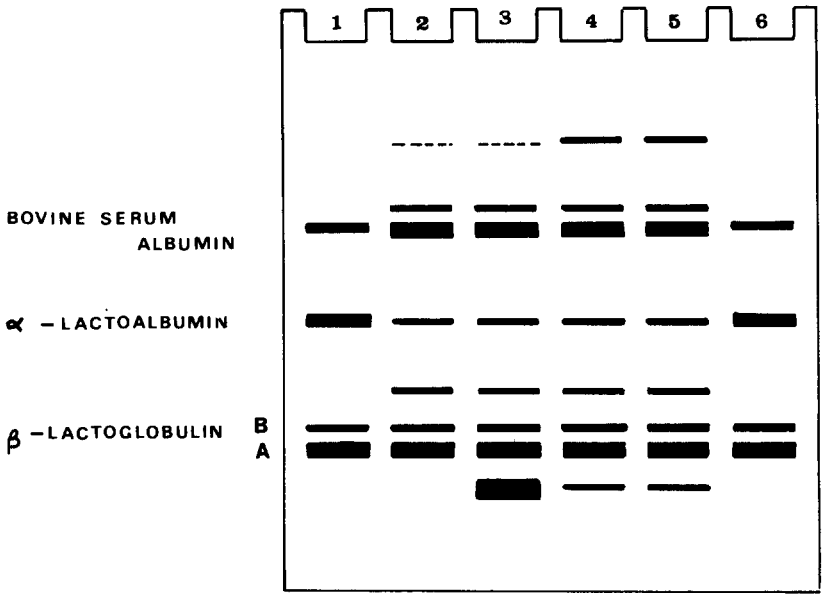


**Fig. 4.** Diagrammatic representation of polyacrylamide gel electrophoresis patterns of soluble nitrogen PM-10 retentate of four batches of Mahon cheese at the end of ripening. (1) and (6), whey proteins; (2), batch number 4; (3), batch number 3; (4), batch number 2; (5) batch number 1.

This fraction is very complex, as has been reported for Cheddar cheese by Kuchroo & Fox (1982*b*; 1983*a*; 1983*b*) who emphasised the difficulty of isolating the small peptides in homogeneous form.

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