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Amino acid profile in the ripening of Grana Padano cheese: a NMR study

S. de Angelis Curtis [a,*], R. Curini [a], M. Delfini [a], E. Brosio [a], F. D'Ascenzo [b], B. Bocca [c]

[a] *Department of Chemistry, University La Sapienza, Box 34-Roma 62, P. le A. Moro, 5, 00185 Rome, Italy*
[b] *Institute of Economics, Faculty of Economics, University La Tuscia, Viterbo, Italy*
[c] *Istituto Superiore di Sanità, Rome, Italy*

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Abstract

Among typical Italian cheeses, Grana Padano is the most famous, with the biggest production. The final step in the cheesemaking is the natural ripening, which involves many modifications of high complexity. In the present work, thermoanalytical and spectroscopic techniques are applied to Grana Padano cheese as a new analytical approach. These are thermogravimetry (TG) and derived thermogravimetry (DTG) to determine different types of water present in the cheese matrix, low-resolution nuclear magnetic resonance (NMR) to provide information on the water localization and displacements within the foodstuff and high-resolution nuclear magnetic resonance (NMR) to evaluate all the amino acids present in the cheese. Modifications in both water and free amino acid contents are observed, as a function of the ripening time and of the distance from the centre of the cheese wheel.

Keywords: Grana Padano cheese; Ripening; Thermoanalytical techniques; NMR spectroscopy

1. Introduction

Grana Padano is the most popular Italian cheese. Its typical features are due to a specific processing technique, a long ripening, and, especially, the quality of milk.

The cheese contains 32% water and 28.4% fat, which corresponds to 42% by weight of the dry product. Its protein content has a high biological value and the amino acids profile is unique (Resmini, Hogenboon, Pazzaglia & Pellegrino, 1993). Grana Padano cheese represents a valuable source of calcium, magnesium, phosphorus, potassium, and is rich in water and fat-soluble vitamins.

The final step in the production of Grana Padano cheese is the natural ripening which requires that the cheese be stored at a fixed temperature and humidity (15–22°C and 85–88%, respectively).

The ripening time must not be less then 9 months; generally it is at least 12–14 months.

The ripening process implies many modifications of high complexity which take place simultaneously or successively. The cheese components undergo important physico-chemical and enzymatic modifications. The biochemical transformations impart new characteristics: the paste of the cheese is modified in its composition and structure and, consequently, in its appearance, consistency and colour. At the same time, flavour and typical taste develop. Such processes ensure a constant excellent quality of the product and control of these can be achieved by examining the phases of the production process and/or the finished goods.

The enzyme digestion of the curd represents a phase of ripening; coagulation and drainage produce a substrate consisting essentially of caseins, fat and some of the soluble components of the milk. The substrate, rich in micro-organisms, during the ripening, will be transformed by action of the enzymes originally present in the curd or produced by bacterial synthesis.

The enzymes, which are ripening agents, are of different origins: milk natural enzymes, lipases and proteases being the most important; milk-clotting enzymes, both rennet and substitutes of bacterial origin (the group of carboxypeptidases); and the enzymes of the microbial

* Corresponding author. Tel.: +39-6-49913830; fax: +39-6-490631.

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population of the cheese (particularly lactic acid enzymes streptococci and lactobacilli).

The ripening process is also affected by various factors: the state of water, the proportions of free and bound water, the structure of the casein micelles and the fat.

Basically, these modifications are due to proteolysis and lipolysis, salt diffusion and water migration.

In the present work we have considered:

- the content of water in the cheese, including: (a) determination of the total water; (b) determination of the different types of water present in the cheese matrix, that is *free* and *bound* water;
- the modification of the free amino acid contents in the ripening process and their quantitative analysis.

Water plays an essential role in determining the structure of proteins, polysaccharides and lipid aggregates, influencing the texture and stability of the final product. This is why it is important to study the dynamic properties of water and its interactions with other components in the system.

Water can be bonded to the matrix with different energies and so, when the matrix is heated, the water is lost in successive stages, depending on the amount of activation energy required to break the bonds (hydrogen bonds, van der Waals forces, London forces, etc.) formed between the water and the matrix.

Water activity is a very important factor in microbial growth and enzyme action and exerts a major influence on the ripening process (Esteban & Marcos, 1990; Ross, 1975). In fact, as already described in a previous work (de Angelis Curtis, Curini, D'Ascenzo, Sagone, Fachin & Bocca, 1999), the presence of strong bonds in the water contained in a foodstuff can mean that several different kinds of water are bonded to the matrix. These interactions are so stable that, under the conditions used for analysis by official methods, it is impossible to attain a sufficiently high activation energy to break the bonds in question.

The thermoanalytical and low-resolution NMR techniques were employed for the determination of total water, the distribution and the motion property of water in the cheese matrix.

NMR relaxation and diffusion measurements have been shown to give detailed information on the state of water, to be a sensitive probe in the study of structure changes associated with protein and/or lipid components and to provide insight into the dimensions and geometries of diffusive domains. The transverse relaxation time is generally used to investigate the state of water in heterogenous systems (Brosio & Barbieri, 1996).

The proteins are pre-digested to peptides, peptones and amino acids. The free amino acids are the most important final parts of the proteolytic activity. Both qualitatively and quantitively, it is possible to evaluate all the enzymatic activities that occur in the course of ripening. They are also relatable to the milk used and to the productive technologies.

The free amino acid content, expressed as a percentage of the total proteins, is an index of the solubilization of caseins and of the digestibility of the product (Choisy, Desmazeaud, Gripon, Lamberet, Lenoir & Tourner, 1984; Hemme, Bouillanne, Metro & Desmazeaud, 1982). In particular, in Grana Padano cheese, the β-casein is the primary source of free amino acid production.

Significant variations in the levels of free amino acids, both as a function of the ripening time and of the distance from the centre of wheel, are found. So, it is important to utilize a technique capable of detecting amino acid levels which avoids the many steps of analysis.

Nuclear magnetic resonance is a non-destructive, multinuclear, multiparametric and often non-invasive technique, successfully employed in plant and mammalian biochemistry (Fan, 1996). In fact, free metabolites have been detected in organs such as the brain, and quantitatively evaluated (Aureli et al., 1990).

The advantages of this technique are illustrated by the possibility of detecting all the substances in the sample at the same time, avoiding treatment repetitions and avoiding artefacts due to demolition of macromolecular structures.

In the present study, aqueous Grana Padano extracts, obtained by the Bligh–Dyer technique (Miccheli et al., 1988), have been examined by high-resolution NMR. All the amino acids were identified, and a quantitative absolute analysis was carried out.

2. Materials and methods

2.1. Instrumentation

The thermogravimetric analyses were performed on a TGA 7 Perkin Elmer, operated by a UNIX 433 DX LP computer.

Low resolution NMR analyses were performed with a Bruker Minispec PC120 low resolution spectrometer operating at 20 MHz. The spin–spin relaxation times (T_2) were measured by the Carr–Purcell–Meiboom–Gill (CPMG) sequence. The spectra were performed by using the following conditions:

- Pulse width = 90° flip angle (4 μs)
- Filter 100 kHz
- Attenuation (ATT) = 30 dB
- Repetition time (RD) = 1 s
- Number of scans = 144
- Sample temperature = 18°C.

The ^{1}H NMR spectra were carried out on a Bruker AM-500 spectrometer operating at 500.137 MHz. The spectra were performed by using the following conditions:

- Pulse width = 30° flip angle (3 μs)
- Acquisition time = 4.06 s
- Acquisition delay = 0.94 s
- Number of scans = 80
- Spectral width = 8100 Hz
- Number of points = 64 k
- Temperature = 25 ± 0.5°C.

The spectra were relative to external dioxane and compared to tetramethylsilane.

The water signal was suppressed by a presaturation technique, irradiating by decoupling the frequency of the solvent. The time of irradiation was 5 s at a power of 1 W.

2.2. Sampling

Cheese samples were kindly gifted from Prof. Bruno Battistotti, University Cattolica del Sacro Cuore, Piacenza (Italy).

For the present research, Grana Padano round cheeses, produced during the winter season (same day), were used and samples taken at different stages of ripening (6, 12 and 18 months).

A wedge-shaped sample was taken from each whole cheese; this wedge was obtained by cutting the cheese along the equatorial axis so as to obtain two symmetrical disks, and then cutting one of the two disks radially into eight equal parts. To avoid errors, the wedges were sampled at 30 different points 2 cm apart, lying on the plane of symmetry of the wedges. The 30 samples were obtained by subdividing the surface into a grid of horizontal lines parallel to the base of the cheese and vertical lines running at right angles to the base and parallel to the rind side. Then, using a special probe, "cores" were removed at each point of intersection on the grid, excluding the outermost points, which were subject to shocks during cutting and preparation.

The samples thus obtained were placed in special containers and kept in liquid nitrogen until used in order to maintain their characteristics and avoid degradation. In order to verify interactions, preliminary tests were carried out using fresh cheese. This treatment was validated by comparing the consistency of the thermoanalytical results, using fresh samples, with those obtained using refrigerated samples.

Untreated samples were employed for the TG analyses: the cheese samples, in the form of small disks, each weighing about 20 mg, were placed in the thermobalance sample pan and heated over a temperature range of 30–750°C, at a scanning rate of 10°C min^{-1}, in a flow of air of 50–100 ml min^{-1}.

For low resolution NMR experiments, untreated samples were examined as extrudates of cylindrical form (7 mm in diameter × 10 mm high).

Samples for the high resolution NMR were subjected to an extraction process.

2.2.1. Extraction technique

A methanol–chloroform–water extraction of cheese samples was performed on the basis of the procedure previously described by Bligh and Dyer and subsequently modified (Miccheli et al., 1988); a mixture of cold chloroform : methanol (1:2), 3 ml/g of cheese was added, in a steel or ceramic mortar, to cheese samples frozen in a liquid nitrogen bath. The samples were vortexed with 1 ml chloroform and 1 ml water for each gram of cheese sample. The phases were separated by centrifuging at 10,000×g for 20 min at 4°C and drying under a nitrogen flow.

The extracts were dissolved in 0.4 ml deuterium oxide (D_2O—99.996%, Sigma Chemical Company) for ^{1}H NMR spectroscopy.

3. Results

3.1. Thermogravimetry and low resolution NMR experiments

Observation of the thermogravimetric curves and their first derivatives shows that water loss always occurs through two main, partially overlapping processes, due to interactions between the water and different components of the matrix over the temperature range 30–200°C. Two different types of water can thus be identified: *free water* and *bound water*.

Free water, that is water bound with less energy to the matrix, is released over the temperature range between 30 and 90–110°C, corresponding to the first slight thermogravimetric step. *Bound water*, that is water more strongly linked to the matrix, is lost through a more extensive process over the temperature range 110–200°C. The anhydrous sample then decomposes thermally until ashes are obtained at a temperature exceeding 700°C.

The mean values of total water, both *free* and *bound*, were calculated and are shown in Table 1, which gives information on the water distribution in the samples and their modifications as a function of the ripening time and of the distance from the centre of the wheel.

During the ripening process, the total amount of water decreases. This is due to a migration–evaporation process, much stronger at the periphery of the wheel. The trend, of the *bound water,* is similar to that of the total water. The amount of *bound water*, at different points of the wedge, diminishes as the distance from the centre of the wheel increases (de Angelis Curtis et al., 1999).

Table 1
Mean values, as a percentage, of *free*, *bound* and *total* water in Grana Padano cheese at different ripening stages and at different points on the wheel. Data obtained by thermogravimetric techniques (TG and DTG)

	6 Months			12 Months			18 Months		
Sample point[a]	Free water	Bound water	Total water	Free water	Bound water	Total water	Free water	Bound water	Total water
A2	5.3	27.3	32.6	5.8	23.6	29.4	7.1	20.6	27.7
A8	5.8	29.2	35.0	6.4	26.3	32.7	7.5	22.4	29.9
A14	5.9	30.4	36.3	6.4	27.5	34.9	6.6	25.1	31.7
C2	4.6	27.7	32.3	4.9	25.5	30.4	5.2	23.6	28.8
C8	5.5	30.7	36.2	5.6	28.7	34.3	5.9	26.6	32.5
C14	5.7	30.7	36.4	5.8	29.4	35.2	6.0	27.1	33.1
E2	5.1	26.9	32.0	5.4	25.7	31.1	5.5	25.3	30.8
E8	5.3	30.2	35.5	5.6	28.6	34.2	5.6	25.8	31.4
E14	5.7	30.8	36.5	5.9	29.1	35.0	6.1	27.5	33.6

[a] A–C–E: samples points at 2–5–8 cm from the base of the wheel, respectively, 2–8–14: samples points at 2–8–14 cm from the rind side of the wheel, respectively.

The amount of *free water* does not diminish during the ripening process, but, unexpectedly, it increases. The number of parameters which influence the water distribution over the different portions of the wheel is higher (evaporation, diffusion, proteolysis) for the *free water* than for the *bound water*.

The trends relative to the distribution of *free* and *bound water* can be explained on the basis of the following phenomena:

- proteolysis and lipolysis;
- salt diffusion;
- water migration.

Proteolysis increases during the ripening process, reaching a stable value after about 14 months (Addeo & Chianese, 1995; Resmini, Pellegrino, Pazzaglia & Hogenboom, 1985).

The hydrolysis of proteins brings about the release of the *bound water*: this explains the decrease in time (between 6 and 18 months). The percentage of free amino acids — which reflects the proteolytic process — increases in the cheese portions closer to the centre of the wheel. This explains the data for the *bound water* which is more abundant at the centre, due to the proteolytic process being more effective at the centre of the wheel than at the periphery.

Another process causing the decrease of the *bound water* is lipolysis, but this is of secondary importance with respect to proteolysis.

Adding salt to the cheese should result in an inhibition of the enzyme activity, thereby preventing protein degradation, so much so that the release of the *bound water* is reduced in higher salt content portions of the cheese, where proteolysis is less effective. After about 10 months, when the diffusion process of the salt is completed, its inhibiting effect on protein degradation becomes negligible. The lower salt concentration causes the amounts of free ions, that are capable of binding water, to diminish, and this, in turn, causes the value of the *free water* to increase, allowing it to migrate (Hardy, 1984).

The model suggested here is consistent with low resolution NMR data: the transverse relaxation times of the water protons provide information on the water localization and displacements within the foodstuff. Transverse magnetization decay curves showed the presence of four different kinds of water, two of which can be attributed to *interstitial water* (water present between the micelles), and to *water present inside the micelles themselves*, together with two more portions related to

Table 2
Data obtained by low resolution nuclear magnetic resonance in Grana Padano cheese at different stages (6, 12 and 18 months of ripening)[a]

Fraction		6 Months	12 Months	18 Months
Component 1	%	8.6±0.1	10.9±0.1	11.0±0.4
	T_2 (ms)	110±1.0	124.0±1.0	103.8±0.9
Component 2	%	22.5±0.1	26.8±0.4	26±1
	T_2 (ms)	35.8±0.4	39.1±0.4	33.3±0.3
Component 1/ Component 2		0.38	0.4	0.42
Component 3	%	62.4±0.4	55.0±0.3	54±2
	T_2 (ms)	8.6±0.1	8.1±0.1	7.3±0.1
Component 4	%	6.5±0.2	7.3±0.3	8.99±0.1
	T_2 (ms)	2.5±0.1	2.5±0.1	2.6±0.1
Component 3/ Component 4		9.6	7.5	6.1

[a] Component 1: unsaturated lipid fraction. Component 2: saturated lipid fraction. Component 3: intra-micelle aqueous fraction. Component 4: inter-micelle aqueous fraction. T_2: spin–spin relaxation times measured by the Carr–Purcell–Meiboom–Gill sequence (CPMG). The measurements were carried out on a Bruker Minispec PC 120 low-resolution spectrometer.

saturated and unsaturated lipid fractions. The values of the decay times suggest that the *interstitial water* — characterised by shorter relaxation times than the *water inside the micelles themselves* — is less free to migrate and interacts with the macromolecules present in the surroundings, establishing bonds with –OH, –SH, $–NH_2$ groups. The NMR low resolution data are reported in Table 2.

During the ripening process of Grana Padano cheese, the amount of *interstitial water* increases whereas that of the *water inside the micelles themselves* decreases, due to the shrinkage of the casein micelles, which lets out water which, in turn, becomes *interstitial water*.

3.2. High resolution NMR experiments

High resolution NMR experiments allow all the amino acids present in the cheese to be quantitatively and qualitatively evaluated.

The ^1H spectrum of Grana Padano cheese is shown in Fig. 1. The assignments were performed on the basis of chemical shift, comparison with model compounds and pH variation. All NMR parameters are reported in Table 3. ^1H NMR spectra are very complex and a strong overlap of the resonances occurs. The unequivocal assignment was obtained by 2D NMR COSY experiments where scalar interactions between adjacent groups of the same molecules are revealed.

Significant variations in the levels of the amino acids were found; Table 4 shows, as an example, the free amino acid contents (as a percentage of total amino acids) in three cheese samples at different ripening stages: 6, 12 and 18 months, respectively. The sample point (E14) for, both, was at 8 centimetre from the base and 14 centimetre from the rind side of the wheel, respectively. From the values it can be seen that, as a function of the ripening time, in particular, serine, alanine, methionine and phenylalanine increase, while glutamate, leucine and valine decrease. Such a variation is related to the proteolysis and to metabolic processes during the storage period and indicates the role played by the enzyme system present in the samples.

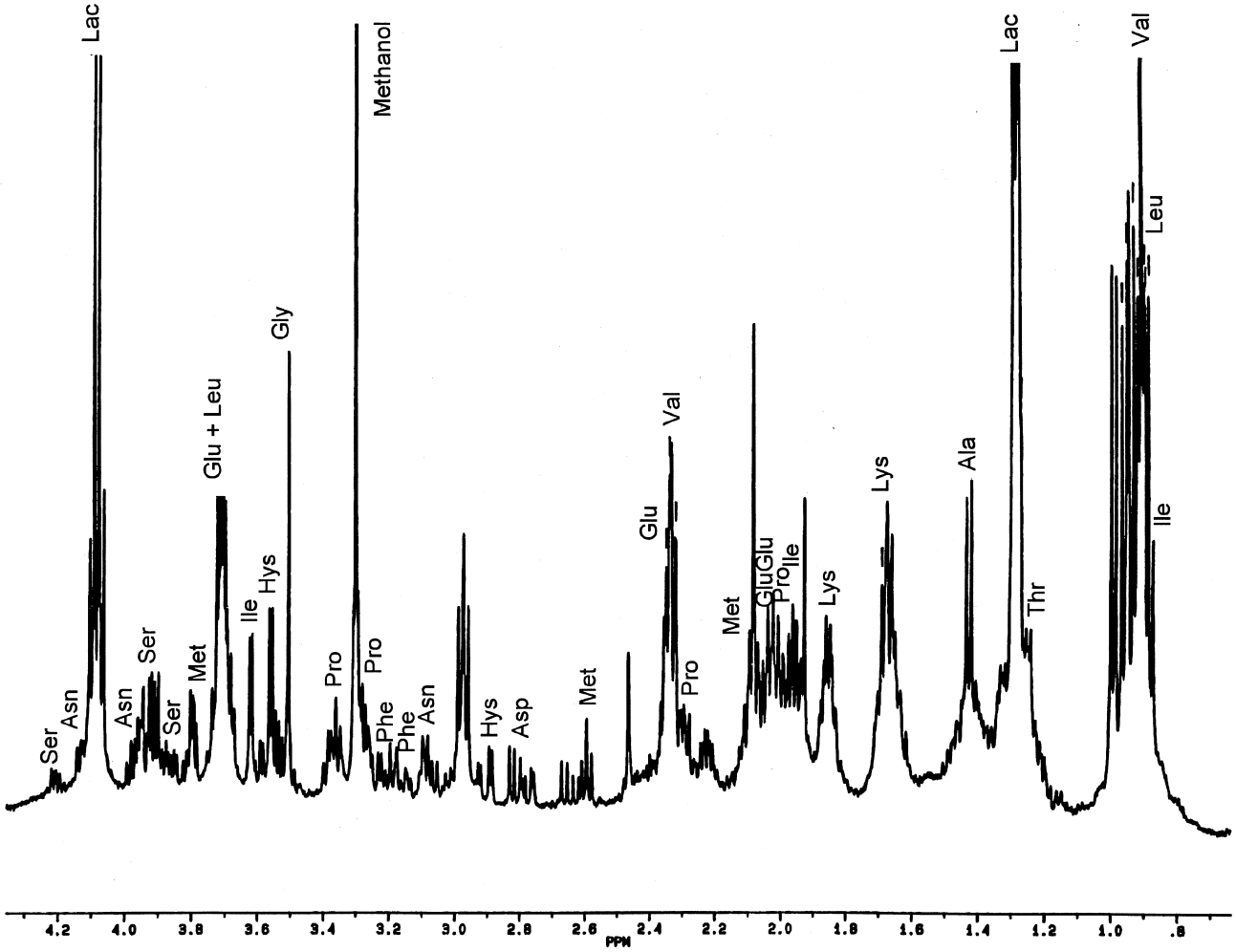

Fig. 1. ^1H spectrum of free amino acids in the aqueous extract of Grana Padano cheese, at 12 months of ripening, obtained by high-resolution nuclear magnetic resonance.

Table 3
^{1}H NMR data of aqueous extracts in Grana Padano cheese, at 12 months of ripening, obtained by high-resolution nuclear magnetic resonance. The unequivocal assignment was obtained by 2D NMR COSY experiments and carried out on a Bruker AM-500 spectrometer

Compounds	Resonances	Multiplicity	Chemical shift (ppm)
Aspartic acid	α-CH	dd	2.75
Glutamic acid	β-CH	t	2.05
	β′-CH	t	2.05
Lactate	α-CH	q	4.05
	-CH_3	d	1.30
Asparagine	α-CH	dd	4.20
	β-CH	dd	3.02
	β′-CH	dd	3.02
Phenylalanine	β-CH	dd	3.25
	β′-CH	dd	3.15
Glycine	α-CH	s	3.50
Glutamine	α-CH	t	3.70
	γ-CH_2	t	2.35
Isoleucine	α-CH	m	3.62
	β-CH	m	1.95
	-CH_3	d	1.00–0.9
Histidine	α-CH	dd	3.60
	β-CH	dd	3.00
	α-CH	t	3.70
	γ-CH	m	1.70
Leucine	β-CH_2	m	1.70
	δ-CH_3	d	0.98
	δ′-CH_3	d	0.96
	β-CH_2	m	1.90
Lysine	δ-CH_2	m	1.70
	γ-CH_2	m	1.50
	α-CH	t	3.80
Methionine	γ-CH_2	t	2.60
	-CH_3	s	2.15
	δ-CH	t	3.38
Proline	δ′-CH	t	3.25
	β-CH	m	2.20
	γ-CH_2	m	2.05
	α-CH	dd	4.25
Serine	β-CH	dd	3.90
	β-CH	dd	3.95
	-CH_3	d	1.30
Threonine	α-CH	d	3.50
	β-CH	m	4.25
	β-CH	m	2.37
Valine	γ-CH_3	d	1.00–0.9
	γ′-CH_3	d	1.00–0.9

Table 4
Free amino acid contents as a percentage of total amino acids in Grana Padano cheese at different stages (6, 12 and 18 months), at the same point (E14)[a] of the wheel, calculated by high-resolution nuclear magnetic resonance.

Free amino acid	6 Months	12 Months	18 Months
Aspartic acid	3.2±0.2	3.4±0.3	3.8±0.2
Glutamic acid	16.9±0.6	16.8±0.7	15.3±0.9
Asparagine	4.2±0.2	3.9±0.2	4.1±0.3
Serine	4.9±0.3	5.9±0.3	5.7±0.4
Histidine	3.3±0.1	3.8±0.2	3.6±0.3
Glycine	3.3±0.1	2.6±0.2	3.1±0.1
Threonine	4.2±0.3	3.6±0.3	4.1±0.3
Alanine	3.5±0.2	4.1±0.3	4.2±0.2
Tyrosine	2.6±0.1	2.8±0.1	2.8±0.2
Methionine	2.9±0.1	3.4±0.3	3.5±0.1
Valine	8.9±0.3	8.1±0.6	8.5±0.5
Phenylalanine	4.9±0.2	5.5±0.3	5.6±0.2
Isoleucine	4.8±0.2	4.2±0.2	4.7±0.4
Leucine	9.8±0.5	7.5±0.5	9.2±0.8
Lysine	13.6±0.7	13.4±0.8	13.6±0.8
Proline	8.7±0.4	8.2±0.2	8.4±0.3

[a] E14: sample point at 8 cm from the base and 14 cm from the rind side of the wheel, respectively.

Table 5
Free amino acid contents, as a percentage of total amino acids, in the same sample of Grana Padano cheese (12 months), at different points of the wheel, calculated by high resolution nuclear magnetic resonance

Free amino acid acids	12 Months A8[a]	12 Months E14[b]
Aspartic acid	3.6±0.2	3.4±0.3
Glutamic acid	15.4±0.4	16.8±0.7
Asparagine	4.4±0.1	3.9±0.2
Serine	5.7±0.3	5.9±0.3
Histidine	3.4±0.2	3.8±0.2
Glycine	3.0±0.2	2.6±0.2
Threonine	3.9±0.2	3.6±0.3
Alanine	4.4±0.2	4.1±0.3
Tyrosine	2.9±0.1	2.8±0.1
Methionine	3.5±0.2	3.4±0.3
Valine	8.6±0.4	8.1±0.6
Phenylalanine	5.8±0.2	5.5±0.3
Isoleucine	4.4±0.2	4.2±0.2
Leucine	9.1±0.6	7.5±0.5
Lysine	13.8±0.6	13.4±0.8
Proline	8.5±0.4	8.2±0.2

[a] A8: sample point at 2 cm from base and 8 cm from the rind side of the wheel, respectively.
[b] E14: sample point at 8 cm from the base and 14 cm from the rind side of the wheel, respectively.

Furthermore, when observing the same sample at different points on the wedge, important variations in the levels of free amino acids are found as a function of the distance from the centre of the wheel. Table 5 shows, as an example, the free amino acid contents (as a percentage of total amino acids) in the same sample of 12 months, at different points on the wheel. The sample was taken, for the point A8, at 2 cm from the base and 8 cm from the rind side of the wheel, respectively, while, for the point E14, it was at 8 cm from the base and 14 cm from the rind side of the wheel, respectively. From the values it can be seen that, as a function of the distance from the centre of the wheel, aspartate, glutamate and histidine increase, while important decreases are found for asparagine, valine and leucine, thus providing a further index for the course of the proteolysis process.

Table 6
Amino acid contents, expressed as mol/g, in Grana Padano cheese, at different stages and different points, obtained by high resolution nuclear magnetic resonance. The quantitative analysis was obtained from the ^{1}H NMR signal areas

Samples	Mol
6 Months A8[a]	$1.79\times10^{-3}\pm0.11$
12 Months A8	$2.09\times10^{-3}\pm0.20$
18 Months A8	$2.10\times10^{-3}\pm0.18$
6 Months E14[b]	$1.93\times10^{-3}\pm0.20$
12 Months E14	$2.21\times10^{-3}\pm0.20$
18 Months E14	$2.23\times10^{-3}\pm0.20$

[a] A8: sample point at 2 cm from the base and 8 cm from the rind side of the wheel, respectively.
[b] E14: sample point at 8 cm from the base and 14 cm from the rind side of the wheel, respectively.

A quantitative analysis was performed from the ^{1}H NMR signal areas. The results, expressed in mol, are listed in Table 6. The analysis was carried out on the basis of the ripening time and the distance from the centre of the wheel, respectively. The data show that the proteolytic process, which involves variations of total amino acids, is substantial in the first months, while the data at 18 months are the same as at 12 months. This confirms the results obtained with different techniques (Addeo & Chianese, 1995; Resmini, Hogenboom, Pellegrino & Pazzaglia, 1995).

A quantitative absolute analysis was carried out by adding dimethylsulphoxide. The values obtained indicate an increase in the amino acid levels as a function of the ripening process, as expected, and as a function of the distance from the centre of the wheel.

4. Conclusions

A new analytical approach to the study of the modifications in Grana Padano cheese during ripening is described. The investigations have been performed using the thermoanalytical and spectroscopic techniques.

The thermoanalytical techniques (TG and DTG) represent a simple and quick method to investigate the modifications in the interactions between the water and the Grana Padano matrix, providing information on the stage reached by the cheese ripening process. The qualitative and quantitative determinations of all kinds of water present in the matrix, and of the water interactions with it, is relevant for both analysis and production. More specifically, these techniques provide a way to verify the effect of the ripening process on foodstuffs.

Moreover, the low resolution nuclear magnetic resonance technique has been used to identify the water fractions present in different parts of the system in question. It does not require any sample preparation.

High resolution nuclear magnetic resonance allows quantitative determination of the free amino acids content. The possibility of using a technique to evaluate, simultaneously, all the free amino acids present in the same sample of Gran Padano cheese and at the same time, avoiding treatment repetitions, is very important in the dairy industry. The amino acid profile confirms the significant variations, as expected in ripening, giving an index of the proteolytic process.

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