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Highly Proteolyzed Novel and Traditional Foods

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Highly Proteolyzed Novel and Traditional foods

by

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A thesis for the Philosophiae Doctor degree
in
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To my family

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Preface

Amino acids, peptides and proteins are important constituents of food both from nutritional and functional point of view.

They supply the required building blocks for protein biosynthesis and they directly contribute to the flavour of food and are precursor for aroma compounds and colours during thermal or enzymatic reactions in production, processing and storage of food. Proteins also contribute significantly to the physical properties of food through their ability to build or stabilize gels, foams, emulsion and fibrillar structure.

The main sources of proteins in human diet can be from animal (meat, fish, milk and eggs) or from vegetables origin (cereals, pulses, nuts, beans and soy proteins).

Not all proteins have the same nutritional value, since protein quality strongly depends on their amino acids composition and digestibility. Generally, animal proteins have an higher biological value than vegetable ones, which are generally characterized by an unbalanced amino acidic composition.

Milk and dairy products are considered a good source of high quality dietary protein for humans, because of the balanced amino acids content in their proteins. Indeed, casein the major milk protein, was proposed by the FDA as the standard when expressing the percentage of reference daily intake (RDI) that a food protein source supplies, and also serves as reference to calculate the protein efficiency ratio (PER). Milk composition depends on the origin, but casein is always the more abundant protein. The rest of the proteins (20%) are whey proteins that have the highest biological value (BV) in comparison with any known proteins, but heat treatments of the milk proteins can produce undesired reactions, giving rise to the unavailability of essential amino acids, such as cysteine and tryptophan. Whey proteins also constitute a good source of lysine, a dietary essential amino acid that is sometime problematic because it limits the nutritional value for human, particularly in diets rich in cereal.

Meat proteins are also considered high quality proteins, because of their balanced content in amino acids, especially in the essential ones necessary for physical and mental well-being. Anyway, not all meat proteins are of high quality, some, like the connective tissue proteins (collagen, elastin, etc.), are poor in essential amino acids.

Proteins are present in almost any food, albeit in different amounts, different kinds and different forms. Intact or minimally degraded proteins are mostly common in foods, but, in some of them, proteinaceous compounds can be present in proteolyzed form, giving rise to mixtures of amino acids and peptides. Some proteolyzed foods rely on “natural” proteolytic process and are part of the food tradition in many countries, such as cheeses and ripened sausages.

Some dietary proteins cause specific effects going beyond nutrient supply. In recent year extensive scientific evidence has been provided for the existence of biological active peptides and proteins derived from foods that might be have beneficial effect upon human health. Proteins and peptides originating from a range of different food sources, including animal, fish, plant and bacteria, have been found to be physiologically active or bioactive, either in a direct manner through their presence in the food itself or after their release from the respective host proteins either during food processing and/or during gastrointestinal digestion.

Bovine milk, cheese and dairy products seem by far to be the greatest source of bioactive proteins and peptides derived from food. Peptides produced by the hydrolysis of milk proteins during technological processes and storage greatly influence the functional and biological properties of dairy products. For instance, in cheese, proteolysis during ripening is the most important biological event that decisively contributes to texture and flavour development through the degradation of the protein network and formation of savoury peptides and substrates for secondary catabolic changes. In addition several studies have demonstrated the presence of peptides with biological activity, that are hidden in the latent state within the precursor protein sequence, but can be released by proteolysis occurring during milk fermentation and cheese ripening. For these reasons, modification of milk proteins based on enzymatic hydrolysis has a broad potential for designing hydrolyzates containing peptides with the required functionality for specific applications. There is also the possibility to influence the proteolytic process in order to produced peptides for hypoallergenic formula, special diets or clinical nutrition.

The sequence of the amino acids and the length of the peptides are the crucial characteristics which influence the biological activity, thus the proteolytic processes can be engineered and exploited in order to produce novel protein-derived food ingredients, also starting from leftovers of food production.

Thus, mixtures of peptides and free amino acids, of any origin, have become of particular interest in food science, because they have been shown to play physiological roles that can have an impact on body function, or condition, and ultimately, health.

The present Ph.D. thesis concerns the molecular characterization and the study of the biological properties of proteolyzed foods, both traditional and novel. In particular, “Culatello” sausages and “Parmigiano Reggiano” cheese have been studied as traditional foods. Moreover, food ingredients obtained from the proteolytic recovery of poultry by-products have been characterized as potential novel foods, also focusing on their functional properties and therefore their bioactive potential. The high protein degradation, which is the common tract in all these food materials, is due to natural proteolytic phenomena occurring during ripening in the former cases, while in the latter case it is obtained by a suitably designed enzymatic digestion process.

The Ph.D. thesis is organized in two main parts. The first part is devoted to the traditional Italian highly proteolyzed food, focusing on the identification of potential bioactive compounds and the study of their relationship with the technological production processes, including the long ripening period. The second part is mainly concerned with the evaluation of the nutritional properties of novel foods obtained from the recovery of poultry by products and to the assessment of their biological activity. For each chapter a brief introduction is provided, in order to allow an immediate contextualization of the subject treated.

Part I

Traditional Highly Proteolyzed Foods

Chapter 1
Small Nitrogen Compounds as Technological Markers in Culatello Sausages

1.1 "Culatello"

"Culatello" is one of the most famous Italian dry pork meat products; its traditional origins are linked to the particular climatic conditions, characterized by a high level of humidity, typical of the Parma and Piacenza areas close to the Po River. Some years ago, "Culatello" was known only in the production area, but after the acquisition of PDO (as "Culatello di Zibello", named after a small village close to the Po river), it became one of the most valuable products of the Italian gastronomy. "Culatello di Zibello" is prepared with meat from sufficiently mature heavy pigs, using the central part of the thigh, previously boned and trimmed, to remove most of the fat. Then the salting period follows, which can last up to six days. For this operation only sodium chloride and sodium or potassium nitrate, at a maximum level of 195 ppm, are allowed by the disciplinary of production. The product is then cased in natural gut materials and ripened for 14-16 months under proper environmental conditions. Maturation, as in similar dry salted or cured meat products, is characterized by important biochemical events involving the lipidic and proteic fractions [1]. The hydrolytic processes which affect these components, also leading to the formation of volatile molecules, thanks to subsequent enzymatic reactions, constitute the basis of the qualitative characteristics of the finished products, particularly as far as the aromatic profile is concerned [2]. The duration of ripening is the main factor affecting the peptidic profile, though several conditions may affect the enzymatic activity, such as the raw material composition, the production process, the amount of salts or the use of microbial starters.

As stated before, differently from the more famous "Parma ham", the production of "Culatello di Zibello" allows the use of nitrates, beside sodium chloride, to be used as preservatives. In addition to their antimicrobial properties, nitrates have many functions in cured meats, including the formation of the characteristic cured meat red colour and the contribution to the flavour and texture. Moreover, nitrates are converted in the meat products into nitrites, the primary use of which as antimicrobial compounds is the inhibition of *Clostridium botulinum* growth and its toxin production. The effectiveness of

nitrate conversion into nitrites depends on pH, salt concentration, presence of ascorbate and isoascorbate, storage and processing temperatures, and the amount of bacterial contamination [3]. However, the presence of nitrites in cured meats is suspected to promote the formation of nitrosamines, carcinogenic compounds formed by reactions of nitrous acid with secondary or tertiary amines [4]. As indicated before, the amount of sodium or potassium nitrate salts used during the production of Culatello is limited to 195 ppm (mg/kg).

The traditional Italian Culatello is known for its sensory properties, which derive mainly from a long dry curing period, in which proteolysis plays a critical role. This phenomenon takes place straight after the death of the animal and continues during all the ripening period by the action of muscle endogenous enzymes, first generating high molecular weight peptides and then small peptides and free amino acids [5].

Several peptides are naturally present in the muscle: Carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine) and balenine (β-alanyl-L-3-methylhistidine), which are dipeptides which display certain physiological properties in the muscle such as buffering, antioxidant activity, neurotransmission and vasodilatory action [6]. In meat, they are related to the sensory perception and generation of meat flavour compounds [7].

Other characteristic constituents of the muscle tissue are Creatine and its degradation product Creatinine, usually assayed in order to detect the presence of meat extract in food products. Creatine in muscle is mainly present as its phosphorylated form with energy reservoir function due to the high phosphoric group transfer potential. The amount of creatine phosphate is in equilibrium with ATP. Especially in the post-mortem muscle the supply of creatine phosphate will became depleted by maintaining ATP concentration [8]. Once creatine phosphate is exhausted, ATP rapidly drops within a few hours of the postmortem being converted into ADP and AMP [9], which act as intermediate compounds and decrease to a negligible value. The final products are Inosine and hypoxanthine, that experience a substantial increase as the muscle ages [10].

Studies regarding the amount of these compounds in cured meat are poor or absent in the literature. The objective of this work was to study the presence of inosine, hypoxanthine, creatine, creatinine and histidine dipeptides in “Culatello” products manufactured with and without nitrates and nitrites by means reversed phase liquid

chromatography coupled with electrospray mass spectrometry detection (RP-LC-ESI/MS).

1.2 Aim of the work

The aim of this work is to study by RP-HPLC/MS the main low molecular weight components, beside amino acids, present in “Culatello”, in order to evidence eventual differences existing in the product manufactured with or without nitrate salts.

1.3 Experimental Procedures

1.3.1 Reagents

- Hydrochloric acid 0.1 mol/l (Normadose™ PROLABO)
- Diethyl-ether (Riedel-de Häen)
- Formic acid >99% (Acros Organics)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Deionised water obtained by using Millipore Alpha Q system
- Deuterated methanol (SIGMA)
- Deuterated water (SIGMA)
- (L,L)-phenylalanilphenylalanine, L-carnosine, L-anserine, creatine, creatinine, inosine and hypoxanthine (SIGMA)

1.3.2 Instrumentation and Materials

- Ultraturrax T50 Basic (IKA-WERKE)
- 4237R centrifuge (ALC)
- Paper filters
- 47 mm Steril Aseptic System (Millipore)
- HVLP filters cut-off 5 and 0.45 µm (Millipore)
- Amicon Micropartition kit with PLGC1400 filters with nominal cut-off 10 KDa
- 4206 centrifugette (ALC)
- Rotavapor (BÜCHI), 461 water bath (BÜCHI)
- MULTIVAP nitrogen evaporator
- V-Vial 10-425 screw 12X32 limited volume 1.5 ml-15 µl (Phenomenex)
- HPLC Alliance 2695 (Waters)

- Jupiter 5 µ C18 300 Å (250X4.6 mm) analytical column (Phenomenex)
- SQD mass spectrometer (Waters)
- Quattro Micro^{TM} API Mass Spectrometer (Waters)
- HPLC 510 binary system solvent, Automated Gradient Controller, 486 Tunable Absorbance Detector, 745 Data module (Waters)
- Jupiter 5 µ C18 300 Å (250X10 mm) semi preparative column (Phenomenex)
- NMR AVANCE 300 (Bruker)
- MassLynx software (Waters)
- QuanLynx software (Waters)
- SPSS 17 software

1.3.3 Procedures

1.3.3.1 Preparation of standard solutions

All standard solutions were prepared with suitable concentration by dissolving the required weight of each compound in deionised water.

1.3.3.2 Sample preparation

5 g of Culatello sample were finely minced and homogenized by Ultraturrax with 45 ml of HCl 0.1N and 250 µl of Phe-Phe 1 mM, added to the mixture as internal standard. The homogenate was then centrifuged at 3000*g* for 45 min at 4 °C and the surnatant was filtered through paper filters. The lipophilic fraction of the resulting solution was extracted with diethyl ether (three times) and the residual water phase was filtered again through 5 µm filters and then through 0.45 µm filters. 4 ml of the extract obtained was ultrafiltered at 10 KDa by using Millipore PLGC 01400 filters (nominal cut-off 10 KDa) and the filtrate was dried by rotavapor. The residue was dissolved in 1 ml of water, transferred into a vial and dried again under nitrogen flow. Finally, the residue was dissolved in 200 µl of deionised water with 0.1 % of formic acid to be analyzed by RP-HPLC-ESI/MS.

1.3.3.3 HPLC/ESI-MS analysis

The separation was achieved by using an Alliance 2695 separation system (Waters, Milford, MA, USA) with a Jupiter (Phenomenex, Torrance, Ca, USA) analytical column (5 µm C18, 300 Å, 250x4.6 mm). The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.2% H_2O + 0.1% HCOOH (eluent B).

Gradient elution was performed according to the following steps: isocratic 100% A for 5 min, from 100% A to 30% B by linear gradient in 30 min, 10 min of a washing step with 100% B, plus reconditioning. Flow rate: 1 ml/min. column temperature: 30°C. Detection was performed by using Waters SQ mass spectrometer (Waters, Milford, MA, USA) with the following conditions: flow entering in the ESI source 0.2 ml/min (obtained by T split), positive ionization mode, capillary voltage 3 kV, cone voltage 30V, source temperature 120°C, desolvation temperature 350°C, cone gas flow (N_2): 50 l/h, desolvation gas flow (N_2): 650 l/h, full scan detection (50-400 m/z), scan duration 1s, interscan delay 0.1 s.

1.3.3.3.1 Data analysis

Each HPLC/ESI-MS chromatogram was analyzed in order to determine the molecular weight and the characteristic ions of the main peaks. In the case of molecular weights higher than 1kDa, reconstructed mass peaks were obtained by MaxEnt 1, application of Masslynx 4.0 software. The integration was performed in an automatic way on the eXtract Ion Chromatograms (XICs), determined by the characteristic ions of each compound, by QuanLynx software, using as integration parameters: automatic ApexTrack peak integration and noise measurement, 1 smooth ± 1 scan with the Savitski Golay algorithm. The integrated areas of each compound in each sample were determined and semiquantified by dividing for the Phe-Phe (internal standard) area in the same sample. A moisture correction was performed, based on the moisture data of each sample.

1.3.3.3.2 Statistical analysis

Normalized data were analysed by using the SPSS 17 software. Principal component analysis (PCA) was performed by factorial data reduction extracting 2 factors, with an unrotated factor solution in the space of the observed variables. The first and second PCA components were calculated as independent variables on data by linear regression analysis.

1.3.3.4 HPLC-ESI/MS/MS analysis of the most abundant compounds

The separation was achieved by using the same condition described above. Detection was performed by using a triple quadrupole Quattro Micro™ API mass spectrometer (Micromass UK, Manchester, UK) with the following condition: flow entering in the ESI

source 0.2 ml/min (obtained by T split), positive ionization mode, capillary voltage 3.2 kV, cone voltage 30V, source temperature 120°C, desolvation temperature 350°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 600 l/h. Products scan detection (40-300 m/z) by using as parent the protonated molecular ions, scan duration 0.5 s, interscan delay 0.05 s, Collision Energy from 14 to 40 on the basis of compounds analysed. Data analysis was carried out by using MassLynx 4.0 software.

1.3.3.5 Purification of unknown compounds to NMR analysis

The first step of purification was carried out by semipreparative RP-HPLC/UV by using Jupiter Phenomenex 5 µ C18 300 Å (250X10 mm) semi preparative column and detection at 246 nm. Gradient elution was performed in isocratic condition at 100% A for 15 min at flow rate 4 ml/min and column at ambient temperature. A second step of purification was achieved by using Jupiter Phenomenex 5 µ C18 300 Å (250X4.6 mm) analytical column in the same elution conditions.

Sample extract was injected several times until about 1 mg of the unknown compounds was collected. The purified compounds were dried first by nitrogen flow and then by vacuum. The solid residue was dissolved in 600 µl of deuterated methanol with small amount of deuterated water. NMR analysis was carried out by a 300 MHz instrument.

1.4 Results and Discussion

Samples of Culatello sausages, produced with or without nitrate salts, obtained from the "Stazione Sperimentale per l'Industria delle Conserve Alimentari" (Parma, Italy) are described in Table 1.1.

Table 1.1. Sample of Culatello used in this work.

Sample	NO_3^-	pH	RH%	NaCl %	Fats %	Prot. Index	Proteins %
1	0	5.9	46.4	6.9	19.6	23.7	27.2
2	0	5.9	46.4	6.9	19.6	23.7	27.2
3	0	5.8	46.8	6.8	15.0	22.6	31.5
4	0	5.8	46.8	6.8	15.0	22.6	31.5
5	0	5.8	45.9	6.8	18.2	25.6	29.0
6	0	5.8	45.9	6.8	18.2	25.6	29.0
7	0	5.9	48.5	6.2	17.4	28.9	27.9
8	0	5.9	48.5	6.2	17.4	28.9	27.9
9	0	5.9	49.4	7.6	11.1	24.0	32.1
10	0	5.9	49.4	7.6	11.1	24.0	32.1
11	0	5.8	44.9	6.7	18.7	23.5	29.7
12	0	5.8	44.9	6.7	18.7	23.5	29.7
13	195	5.8	46.6	6.4	18.2	26.6	28.8
14	195	5.8	46.6	6.4	18.2	26.6	28.8
15	195	5.9	48.3	8.2	13.4	24.4	30.1
16	195	5.9	48.3	8.2	13.4	24.4	30.1
17	195	5.8	47.5	6.7	14.9	25.9	30.9
18	195	5.8	47.5	6.7	14.9	25.9	30.9
19	195	5.8	45.5	7.0	15.5	22.2	32.0
20	195	5.8	45.5	7.0	15.5	22.2	32.0
21	195	5.9	51.4	7.0	13.9	27.5	27.7
22	195	5.9	51.4	7.0	13.9	27.5	27.7
23	195	5.9	48.4	7.3	12.2	24.7	32.1
24	195	5.9	48.4	7.3	12.2	24.7	32.1

In order to identify the most abundant peptides and low molecular weight nitrogen containing molecules in the "Culatello" samples, RP-HPLC/ESI-MS analysis of the fractions extracted, as described in the experimental part, was performed in order to calculate the molecular weight of the associated molecules. These molecules were then semiquantified by extracting the chromatogram defined by their characteristic ions, integrating the corresponding chromatographic peak and calculating the ratio between its area and the area of a suitable internal standard (Phe-Phe) added at the beginning of

the sample preparation. Typical Total Ion Chromatogram (TIC) of the low molecular weight nitrogen fraction of a Culatello sample is shown in Figure 1.1.

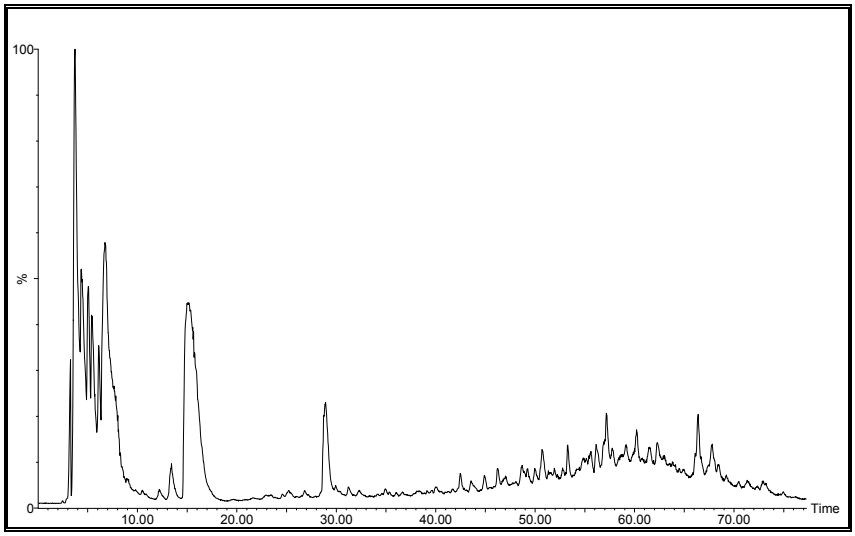

Figure 1.1. Typical RP-HPLC/ESI-MS Total Ion Chromatogram of the low molecular weight nitrogen fraction extracted from a sample of Culatello sausages.

The detailed analysis of all chromatograms allowed for the detection of about 100 compounds, with molecular weight ranging from 200 Da to 9 kDa, beside the free amino acids.

Semiquantitative values of each peptide in each sample, corrected taking into account the relative humidity of each sample, were used as variables to perform Principal Components Analysis (PCA) in order to see if the use of nitrate salt during Culatello production may be a factor that affects data variability. The total variance explained was 70.3%: 57.9% by PC1 and 12.4% by PC2. The loading plot and the score plot, with the samples marked according to the presence of nitrate salts, are reported in Figure 1.2.

As can be seen, although a clear discrimination between samples produced with or without nitrate salts is not visible, the score plot clearly indicates that the nitrogen fraction is affected by the use of nitrates, and in particular, by comparison with the loading plot, some compounds of low MW seem to be highly related to this factor.

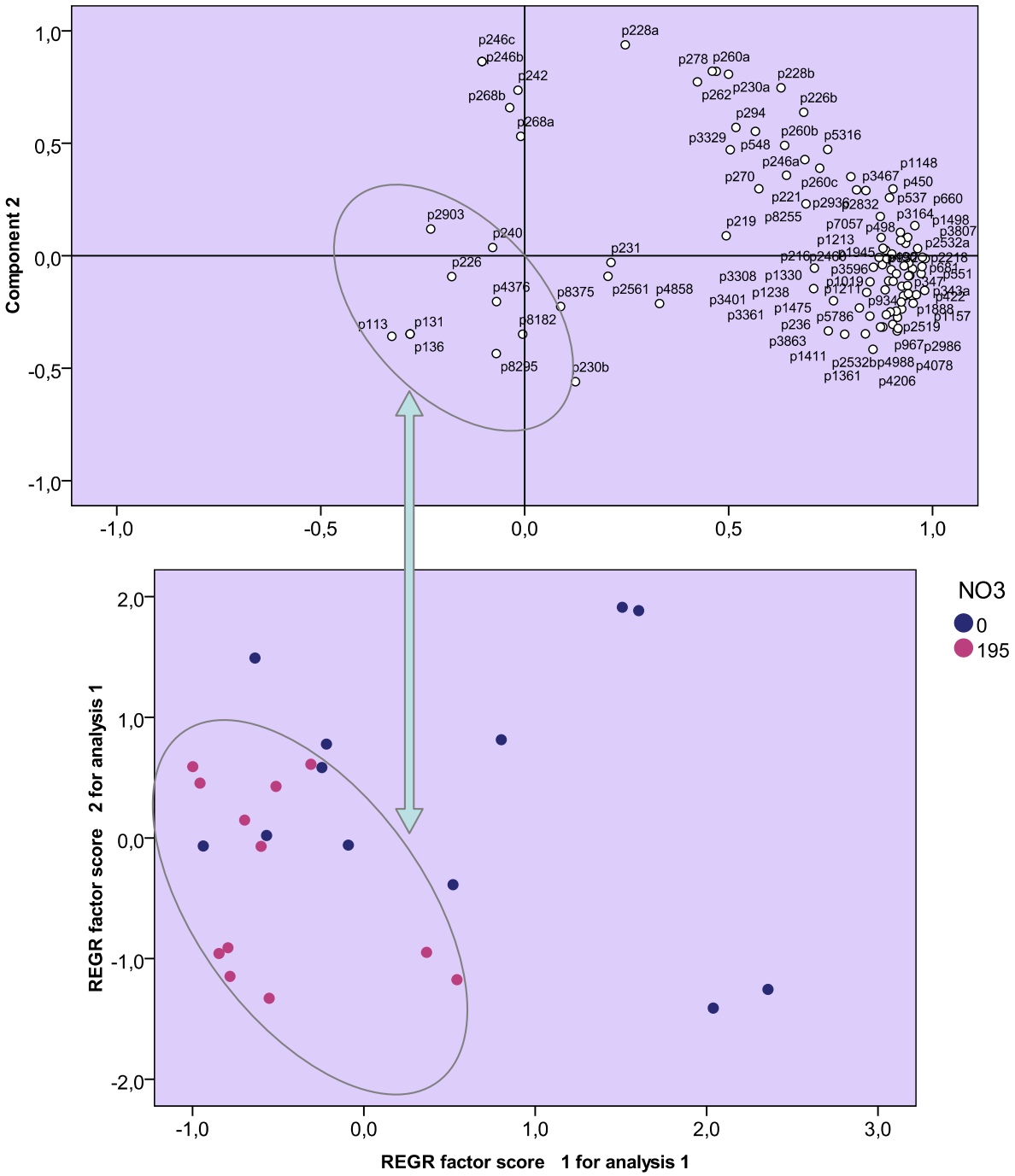

Figure 1.2. Loading plot (above) of peptides, labelled by MW, detected in Culatello samples and Score plot (below) including all the analyzed samples. Samples are marked according to the use of nitrate salts during production.

A more detailed study of the molecular profile of each sample showed that the molecules influenced by the presence of nitrates, indicated in the loading plot, were the most abundant compounds of the nitrogen fraction in “Culatello”, as shown in Figure 1.3 in which it is evident that the compounds having MW 226, 240, 113 and 131 Da

represent more than 50% of the nitrogen fraction, together with another compound, having MW 268 Da, with a very high relative abundance.

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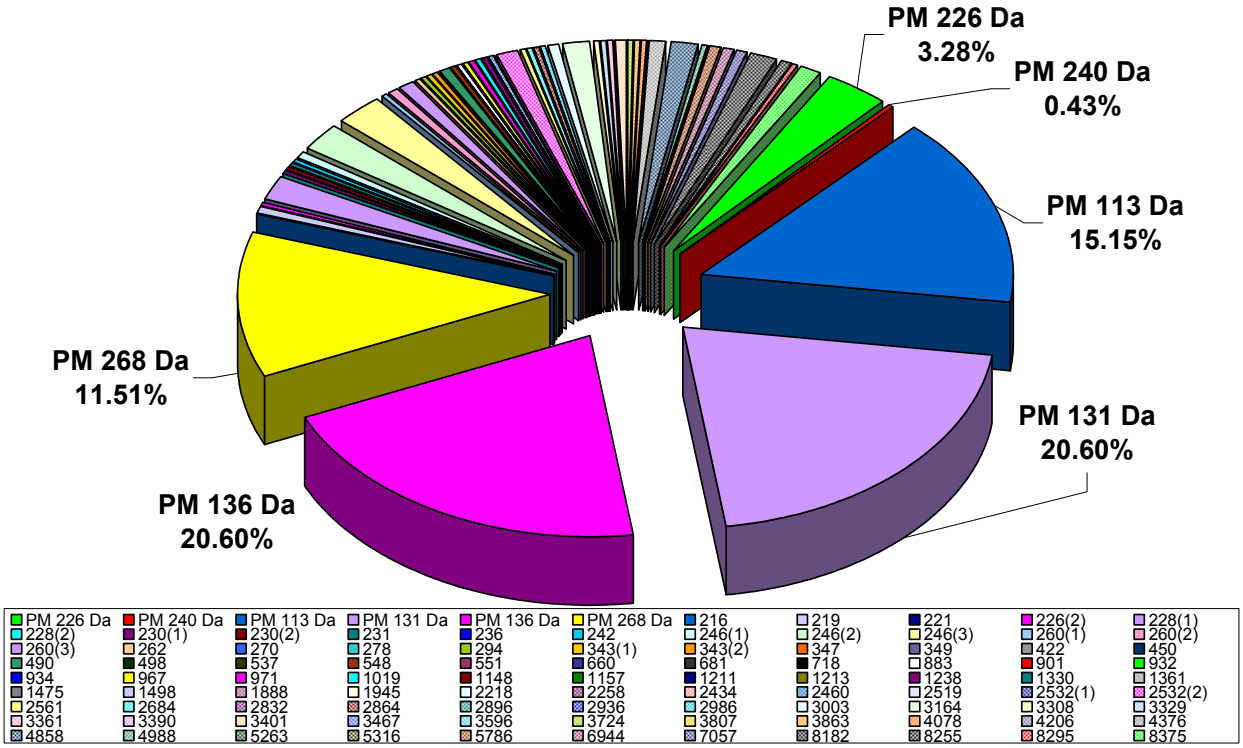


Figure 1.3. Relative amount of each compound detected in the extract of Culatello sausages.

The molecular characterization of these abundant compounds detected in “Culatello”, which seemed to be related to the presence of nitrates, was then studied in detail.

1.4.1 Molecular characterization of the most abundant compounds

1.4.1.1 Compounds with molecular weight 131 Da and 113 Da

According to their molecular weight, these compounds were tentatively identified respectively as creatine and creatinine, molecules involved in the energy metabolism of the muscles. In order to confirm this hypothesis, HPLC/ESI-MS/MS analyses, performing daughter scan experiments of the unknown compounds and of the authentic standards of creatine or creatinine, were carried out. In Figure 1.4 and Figure 1.5, respectively, the product ions spectra of creatine and creatine standard and the product ion spectra of the compounds detected in Culatello samples are reported.

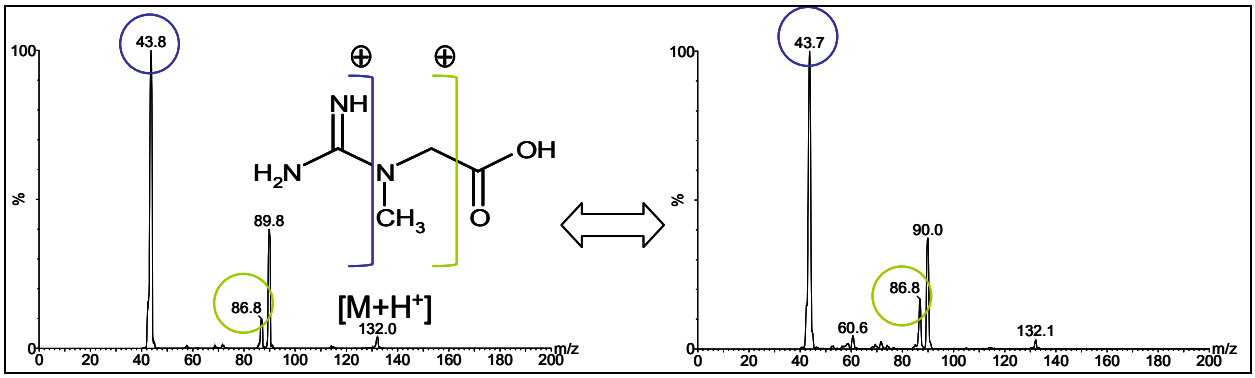


Figure 1.4. Comparison of product ion spectra of Creatine (left) and product ion spectra of compounds with MW 131 detected in Culatello (right).

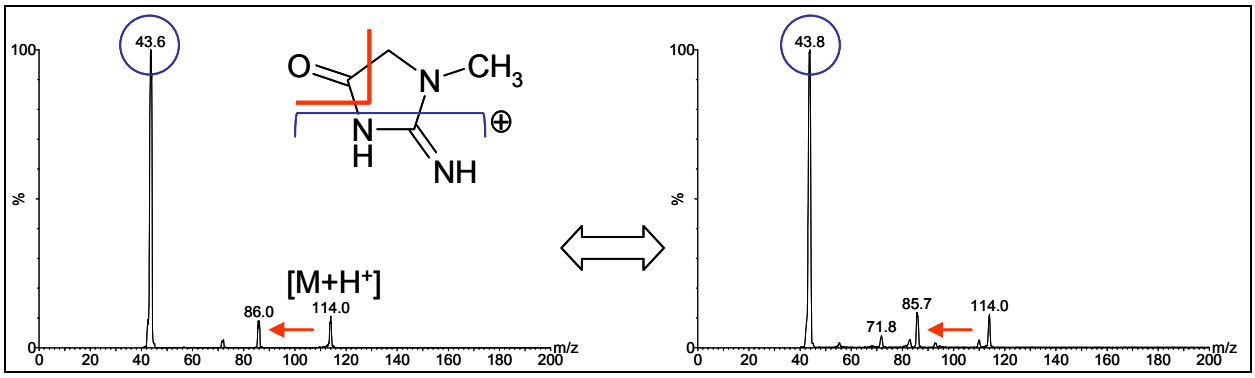


Figure 1.5. Comparison of product ion spectra of Creatinine (left) and product ion spectra of compounds with MW 113 detected in Culatello (right).

In both cases, a very good consistence between the spectra was observed, supporting the proposed identification. In order to definitely confirm the sequence, Culatello extracts were spiked with standard solutions of creatine and creatinine at increasing concentrations. The chromatographic signals corresponding to the two compounds consistently increased (Figure 1.6 and Figure 1.7) confirming that compounds with MW 131 Da and 113 Da detected in Culatello samples were actually creatine and creatinine. By applying the standard addition method, on the basis of the linear regressions obtained (Figure 1.8), it was also possible to estimate the absolute amount of those compounds in Culatello. Creatine and creatinine content in Culatello resulted to be around 175-358 mg/100g and 140-335 mg/100g respectively, as reported in Figure 1.9.

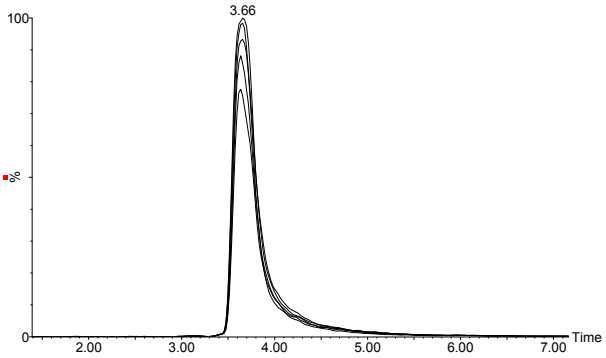


Figure 1.6. Overlaid XIC chromatograms of the ion 114 m/z obtained by the analysis of spiked sample with different creatinine amount.

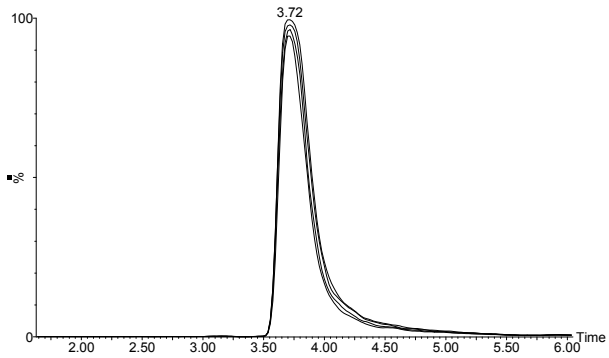


Figure 1.7. Overlaid XIC chromatograms of the ion 132 m/z obtained by the analysis of spiked sample with different creatine amount.

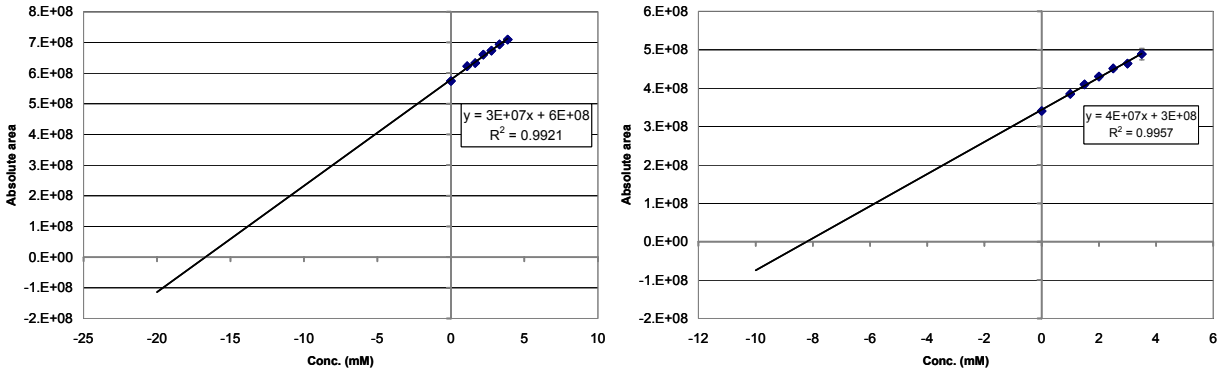


Figure 1.8. Linear regressions obtained after addition of a standard solution of Creatine (left) and Creatinine (right) to the Culatello extract.

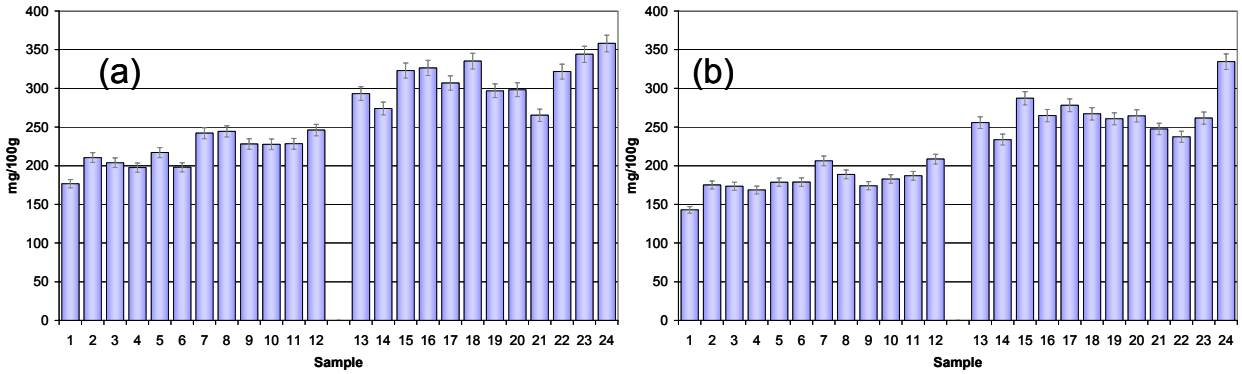


Figure 1.9. Amount of creatine (a) and creatinine (b) estimated in several Culatello samples.

The amount of creatine was in good agreement with the literature data (300-400 mg/100 g of meat depending on the muscle analysed) while the amount of creatinine resulted significantly higher than the data reported (5-10 mg/100g of meat, depending on the muscle analysed). This differenceis likely to be due to an increasing concentration of

creatinine during ripening, caused by the degradation of creatine and creatine phosphate.

1.4.1.2 Compound with molecular weight 226 Da

According to the molecular weight and the product ion spectra obtained performing the daughter scan experiment in comparison with the standard, this compound was identified as carnosine. In Figure 1.10 the comparison between the product ion spectra of carnosine and the product ion spectra of the compound identified in Culatello is reported.

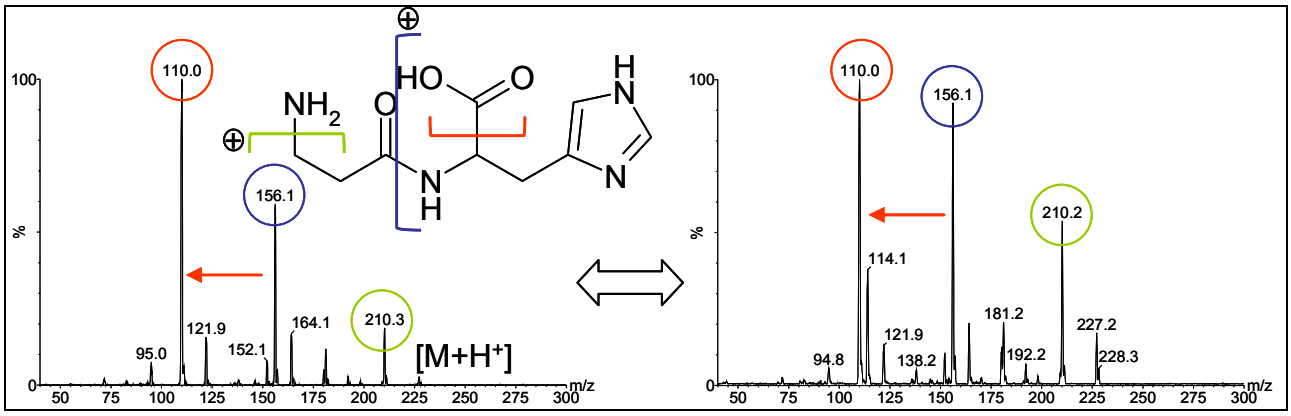


Figure 1.10. Comparison of the product ion spectrum of Carnosine (left) and the product ion spectrum of the compounds with MW 226 detected in Culatello (right).

Also in this case, in order to confirm this attribution, the samples of Culatello were spiked with the corresponding standard solutions at increasing concentration (Figure 1.11).

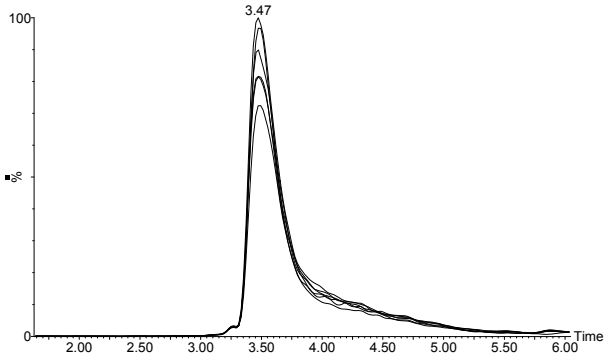


Figure 1.11. Overlaid XIC chromatograms of the ion 227 m/z obtained by the analysis of spiked sample with different carnosine amount.

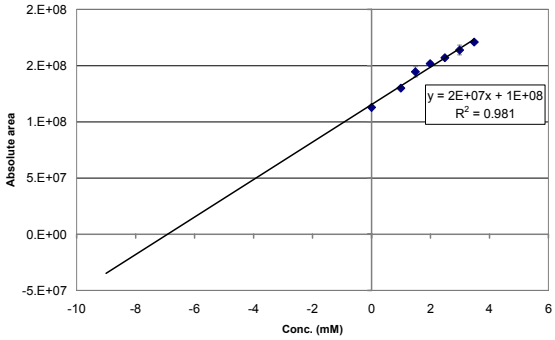


Figure 1.12. Linear regressions obtained after addition of the standard solutions of Carnosine to the Culatello extract.

By using the calibration curves obtained (Figure 1.12), the amount of carnosine in Culatello was estimated to be around 120-280 mg/100g, in agreement with the literature data (100-300 mg/100 g): detailed results are reported in Figure 1.13.

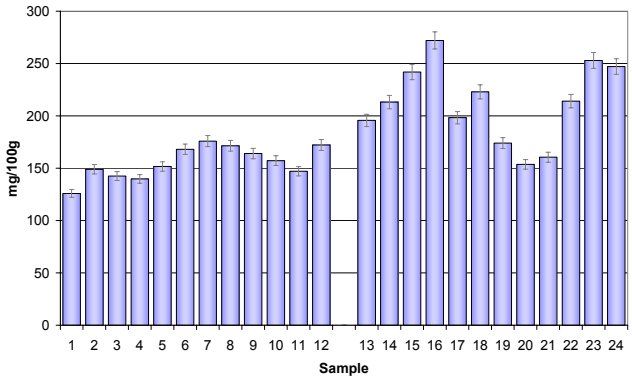


Figure 1.13. Amount of carnosine estimated in several Culatello samples.

1.4.1.3 Compound with molecular weight 240 Da

Other compounds which might be naturally present in muscle tissue, corresponding to the molecular weight 240 Da, are anserine and balenine. Due to the commercial non-availability of balenine, the daughter scan experiment and spiking of the sample were performed only by using an anserine standard solution. The results obtained are reported in Figure 1.14, Figure 1.15 and Figure 1.16.

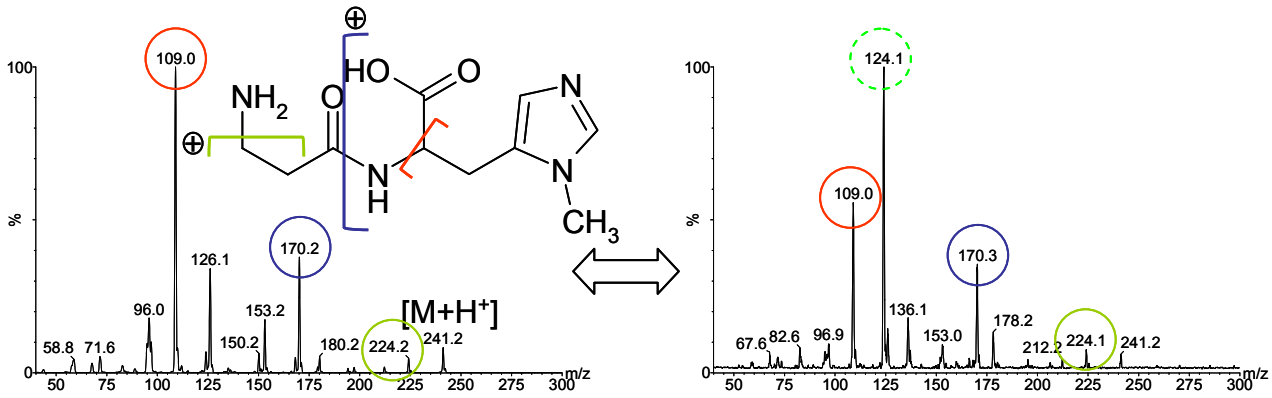


Figure 1.14. Comparison of the product ion spectrum of Anserine (left) and the product ion spectrum of compounds with MW 240 detected in Culatello (right).

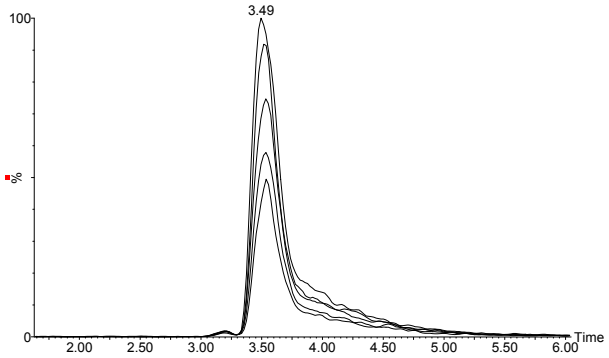


Figure 1.15. Overlaid XIC chromatograms of the ion 241 m/z obtained by the analysis of spiked sample with different anserine amount.

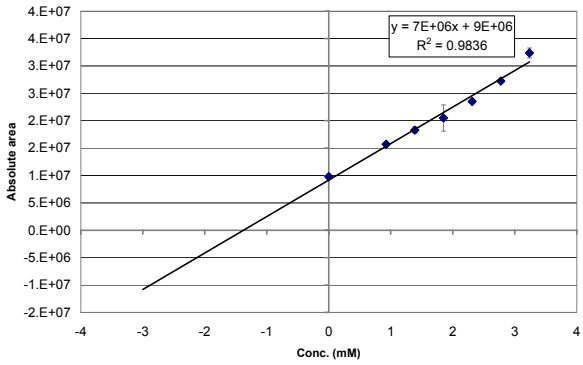


Figure 1.16. Linear regressions obtained after addition of the standard solutions of Anserine to the Culatello extract.

Although the spiking experiment with anserine seemed to indicate that this compound was that actually present in the samples, it can be seen that its product ions spectrum did not completely correspond to that of anserine. The ion at m/z 126 probably corresponding to the loss of CO_2 from the protonated 1-methyl-histidine, obtained by fragmentation of anserine, was replaced, in the sample, by an ion with m/z 124, probably due to the loss of formic acid from the protonated 3-methyl-histidine. This difference, not negligible, may be due to the coelution of balenine.

In order to gain evidence for this hypothesis, the spiked solutions were analysed again performing MRM experiments recording both the reactions: from 241 m/z to 124 m/z and from 241 m/z to 126 m/z. By integrating the respective peaks and correlating them to the standard solution concentrations the regression equations reported in Figure 1.18 were obtained.

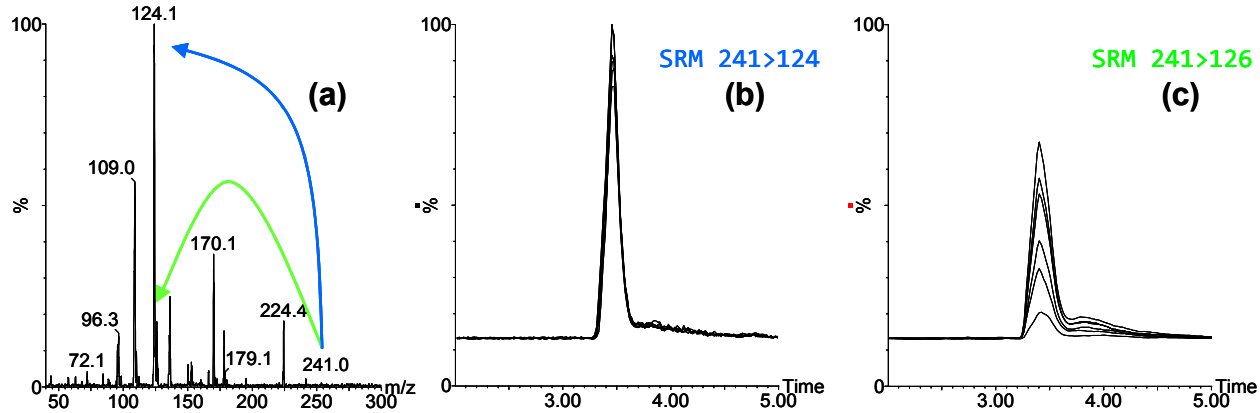


Figure 1.17. MRM experiment (a) and overlaid chromatograms obtained for each reaction monitored (b, c) after addition of anserine at different concentration to the sample.

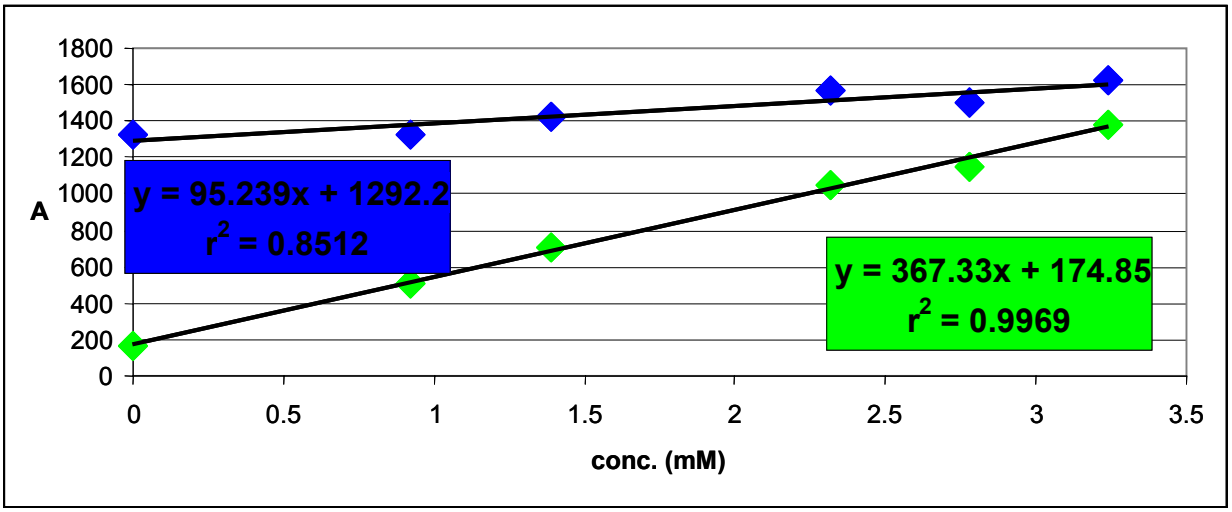


Figure 1.18. Linear regression calculated for the two reactions monitored (241>124 blue, 241>126 green).

Only in one case, SRM 241>126, characteristic transition of anserine, the absolute area increased linearly with the concentration, whereas in the other case the transition was nearly insensitive to the increase of anserine concentration, supporting the hypothesis that it could be due to a very similar, coeluting, albeit different compound, which was tentatively assigned to balenine. Therefore, in this case, from the standard addition method it was only possible to estimate the content of the sum of anserine and balenine in “Culatello” samples, which resulted around 30-50 mg/100g, an amount slightly higher than the data reported in literature (7-16 mg/100 for anserine and 18 mg/100 g for balenine). Detailed results are reported in Figure 1.19.

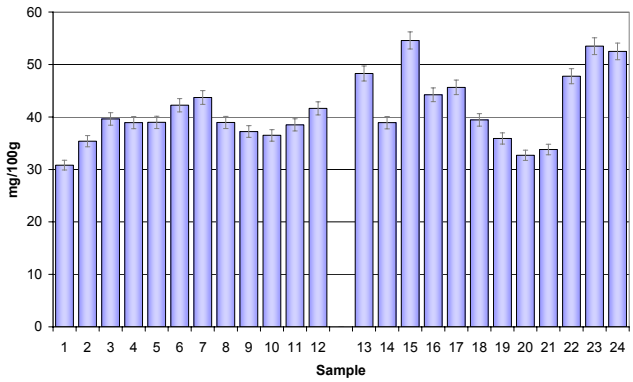


Figure 1.19. Amount of anserine and balenine estimated in several Culatello samples.

1.4.1.4 Compounds with molecular weight 136 and 268 Da

A more complex procedure was necessary in order to identify compounds with molecular weight 136 and 268 Da.

Daughter scan experiments, reported in Figure 1.20, Figure 1.21 and Figure 1.22, showed that the main ion generated by the fragmentation of the compound with molecular weight 268 Da corresponded exactly to the molecule having a molecular weight 136 Da. Moreover, fragmentation of this ion generated ions exactly corresponding to the ions produced by the compound with MW 136 Da. These experiments indicated that the compound with MW 136 Da, was present as a free form and bound to another moiety to give a molecule with MW 268 Da. This moiety, with a mass of 132 Da, exactly corresponds to the MW of a pentose residue.

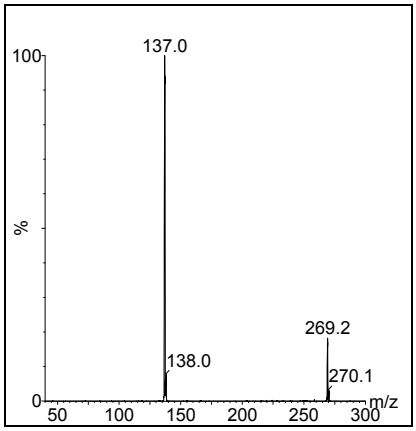


Figure 1.20. Product ion spectrum of the compound with MW 268 Da.

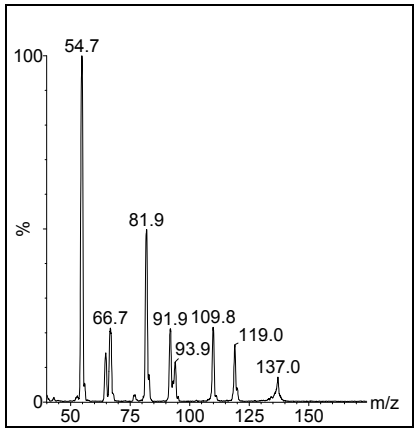


Figure 1.21. Product ion spectrum of ion 137 m/z, daughter of compound with MW 268

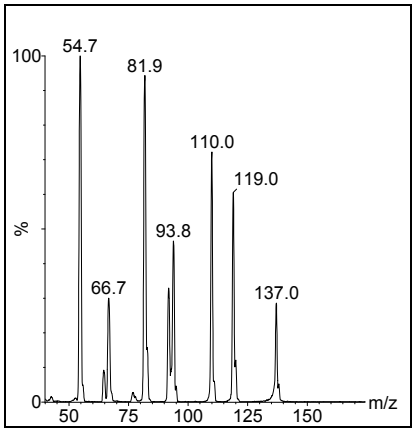


Figure 1.22. Product ion spectra of the compound with MW 136 Da.

This compound was then purified by semipreparative RP-HPLC/UV to be analysed by NMR. The NMR spectrum, obtained at 300 MHz, is reported in Figure 1.23, in which the regions of interest are indicated.

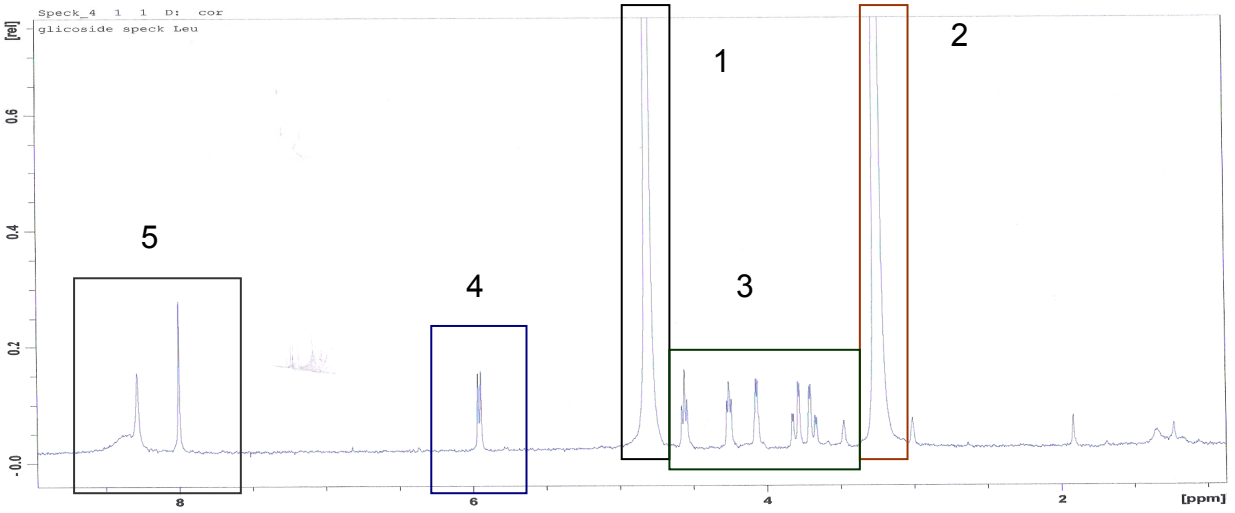


Figure 1.23. NMR spectrum at 300 MHz of the compound having molecular weight 268 Da.

Peak 1 and peak 2 represent protons of water and methanol present as contaminant in deuterated water and methanol used to dissolve the purified compound. In the region 3 the characteristic profile of a pentose is present: specifically peaks between 4 and 4.5 ppm correspond to protons bonded to C2, C3 and C4, while of 3 to 4 ppm the peaks of geminal protons on C5 are present. Peaks present at 6 ppm, region 4, can be attributed to the proton bound to the anomeric carbon. Around 8 ppm, region 5, two aromatic protons are present, clearly not belonging to the monosaccharide residue. Summarizing, the compound with molecular weight 268 Da appears to be a glicosylated aromatic compound. One molecule compatible with those characteristics, known in meat, is the nucleoside inosine, the product of ATP degradation, consisting of hypoxanthine linked to ribose. The molecular structure of inosine is shown in Figure 1.24

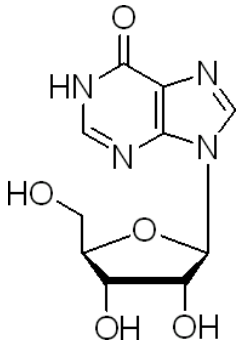


Figure 1.24. Molecular structure of inosine.

Daughter scan experiments and spiking of the sample with standard solution of inosine definitely confirmed this hypothesis, and also allowed to confirm that the compound with

molecular weight 136 was the free nucleotide hypoxanthine. Finally the amount of these compounds was estimated in Culatello samples by the internal standard method. The results obtained are shown in Figure 1.25.

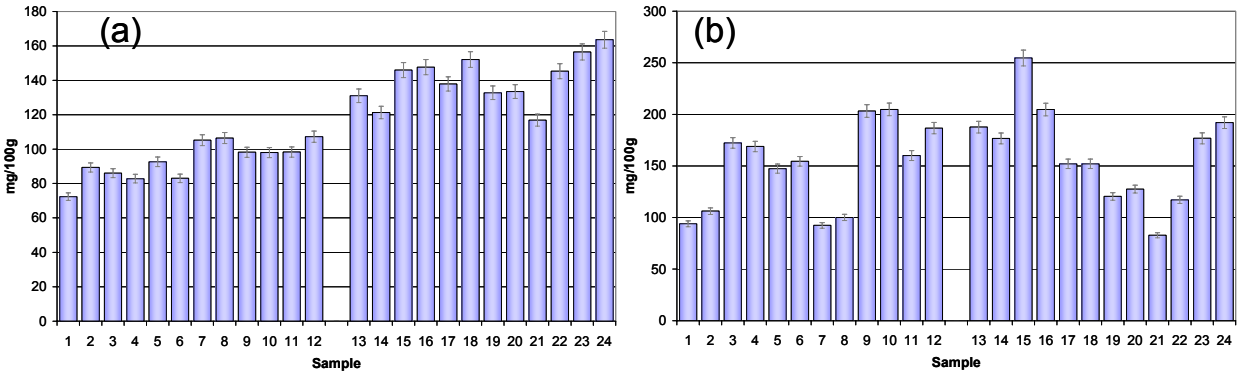


Figure 1.25. Amount of hypoxanthine (a) and inosine (b) estimated in several Culatello samples.

1.4.2 Correlation of the most abundant compounds with the use of nitrates during Culatello production

Carnosine, anserine, creatine, creatinine, inosine and hypoxanthine were more accurately studied with particular attention to their correlation with the use of nitrate salts during Culatello production. Anova tests clearly showed that all compounds, with the exception of inosine, were significantly higher in the sample manufactured using nitrate salts; Creatine, with the highest Person coefficient, resulted to be the compound mainly influenced by the presence of nitrate salts.

1.5 Conclusion

In this work an extensive study of the low molecular weight nitrogen fraction of Culatello sausages, manufactured with or without nitrate salts, has been performed. This study showed that the main components of this fraction are carnosine, anserine, creatine, creatinine, hypoxanthine and inosine, compounds that have been studied for the first time in cured meat products. Their amounts result to be in a good agreement with the literature data on generic fresh meat, only creatinine resulting higher, indicating that these compounds are not influenced by degradative phenomena occurring during ripening. All these compounds, beside inosine, have to be strongly linked to the proved technological treatment, resulting significantly higher in Culatello manufactured with nitrates salts. In particular creatine was the component most affected by the presence of

nitrate. It might be speculated that enzymatic processes leading to degradation of low molecular weight nitrogen compounds are inhibited by the presence of nitrate salts.

References

[1] Toldra, F.; Flores, M.; Sanz, Y., Dry-cured ham flavour: Enzymatic generation and process influence. *Food Chemistry* **1997,** 59, (4), 523-530.

[2] Toldra, F.; Aristoy, M. C.; Flores, M., Contribution of muscle aminopeptidases to flavor development in dry-cured ham. *Food Research International* **2000,** 33, (3-4), 181-185.

[3] Sanz, Y.; Vila, R.; Toldra, F.; Nieto, P.; Flores, J., Effect of nitrate and nitrite curing salts on microbial changes and sensory quality of rapid ripened sausages. *International Journal of Food Microbiology* **1997,** 37, (2-3), 225-229.

[4] Tricker, A. R.; Preussmann, R., Carcinogenic N-Nitrosamines in the Diet - Occurrence, Formation, Mechanisms and Carcinogenic Potential. *Mutation Research* **1991,** 259, (3-4), 277-289.

[5] Toldra, F.; Flores, M.; Sanz, Y., Dry-cured ham flavour: Enzymatic generation and process influence. *Food Chemistry* **1997,** 59, (4), 523-530.

[6] Quinn, P. J.; Boldyrev, A. A.; Formazuyk, V. E., Carnosine - Its Properties, Functions and Potential Therapeutic Applications. *Molecular Aspects of Medicine* **1992,** 13, (5), 379-444.

[7] Gianelli, M. P.; Flores, M.; Toldra, F., Interactions of soluble peptides and proteins from skeletal muscle on the release of volatile compounds. *Journal of Agricultural and Food Chemistry* **2003,** 51, (23), 6828-6834.

[8] Belitz, H. D.; Grosch, W.; Schieberle, P., *Food chemistry*. Springer: **2004**, p 547.

[9] Huff-Lonergan, E., *Chemistry and Biochemistry of Meat*. Wiley-Blackwell: **2010** p 3-24.

[10] Tikk, M.; Tikk K.; Tørngren M. A.; Meinert L.; Aaslyng M. D.; Karlsson A. H.; Andersen H. J. Development of Inosine Monophosphate and Its Degradation Products during Aging of Pork of Different Qualities in Relation to Basic Taste and Retronasal Flavor Perception of the Meat. *Journal of Agricultural and Food Chemistry* **2006,** 54, 7769-7777.

Chapter 2
Parmigiano Reggiano Cheese: Molecular Characterization and Biological Properties

Parmigiano Reggiano cheese is a well known Italian hard cheese, long ripened, made from raw and partially skimmed cow's milk. It is included in the list of Italian cheeses bearing the Protected Designation of Origin (PDO, EU regulation 2081/92).

The cheese-making procedure of Parmigiano-Reggiano starts with the addition of a natural whey starter, followed by heating at 33°C and the addition of calf rennet. After the coagulation has started, the curd, continuously stirred, is heated up to 55°C within 5 to 10 min. After this phase, a resting period follows, in which the curd consolidates at the bottom of the vat in about 1 hour. After this time, the curd is placed in a mould to complete whey drainage and cooled at room temperature for 2-3 days, then placed in saturated brine for 20-25 days, and finally ripened for a period of at least 12 months, but usually lasting two years or even more [1,2].

Characteristic elements of Parmigiano Reggiano cheese technology are:

1. the use of natural whey culture as starter obtained from the previous day's cheese making whey and constituted mainly by thermophilus Lactic Acid Bacteria coming from *Lb. Helveticus* and *Lb. Delbruekii bulgaricus* species;
2. the high temperature of curd cooking, which determines the relative humidity and lactose contents and allows suitable conditions for the growth of thermophili Lactid Acid Bacteria;
3. the long maturation period, in which proteolysis and other reactions, such as lipolysis and lactic and propionic acid fermentation, influence the organoleptic properties of the final product. In particular, proteolysis is the most complex event in cheese ripening as it contributes to textural changes (breaking down of caseins), it decreases the water activity through water binding to the free amino acids carboxyl and amino groups, it increases the pH by producing NH_3 by deamination of free amino acids. Moreover, proteolysis directly contributes to flavour, by releasing peptides and amino acids, and off-flavours, by releasing bitter peptides [3].

In the ripened cheeses, the nitrogen fraction is mainly constituted by caseins, which are the most abundant proteins in milk and are concentrated during the cheese-making

process, and the derived peptides [4-8]. Whey proteins are usually considered to be lost in the liquid whey fraction during curdling, although it is well known that during the cheese-making process a small, but significant, part of the whey proteins (up to 15% of the total whey proteins, according to different cheese-making technologies) can be trapped in the curd, thus being incorporated in the final cheese product [9].

The casein fraction is mainly composed by: α-S1 casein, mainly present in variant B, constituted by 199 amino acid residues and having a molecular weight of about 23 kDa, presenting in its sequence 8 phosphorylated serine residues, 7 of them localized in the portion 43-80; β-casein (variant A) made by 209 amino acid residues and with a molecular weight of about 24 kDa, presenting in its amino terminal region (1-40) 5 phosphorylated serine residues while the carboxyl region (136-209) is mainly composed by non polar residues. K-casein and α-S2 casein are also present in smaller amounts [10].

The most abundant whey proteins are β-Lactoglobulin (β-Lg) and α-Lactoalbumin (α-La). The former protein is usually present in milk as two isoforms with almost the same abundance, named variant A and variant B. The two isoforms differ only in two amino acids: aspartic acid at residue 64 in variant A, glycine in variant B, and valine at residue 118 in variant A, alanine in variant B [11]. The relative abundance of the two Lg isoforms varies according to the genetics of the cow breeds providing the milk used for cheese making. The B variant has been associated with an improved cheese making quality of the milk [12].

Although many methods can be found in the literature for producing whey protein-enriched cheeses, even in the case of Parmesan-like cheeses [13], a sound study of the amount and the behaviour of the whey proteins which are “naturally” incorporated in the traditional Parmigiano-Reggiano cheese, as well as their fate during ageing, is virtually absent in the literature. The presence of a consistent amount of whey proteins in other traditionally made cheeses, such as Cheddar and other similar cheeses, is well acknowledged and is usually linked to the heat-induced whey protein unfolding which leads to their co-precipitation in the curd [14]; nevertheless, the data on the matter are also quite scarce. Quite interestingly, β-Lg in Cheddar has been reported to be proteolysis-resistant during ripening [15] and in general whey proteins have been found to be very resistant to proteolytic enzymes at slightly acidic pH [16]. It is to be noted that previous papers reporting the identification of peptides in Parmigiano-Reggiano never

reported the presence of whey protein-derived peptides [5-8], and when studying proteolysis-derived peptides, usually whey proteins are not taken into consideration [17].

The maturation period of Parmigiano Reggiano cheese is characterized by a significant solubilization of the casein fraction, that leads to important structural modifications. Casein breakdown is very intensive during the first 6 months and then sensibly decreases, mainly during the final stage of ripening. On the other hand, the soluble fraction initially increases and then becomes stable after the sixth month leading to a balance between peptons and low molecular weight peptides or amino acids. Ammonium nitrogen, being the last step of nitrogen catabolism, progressively increases during all the ageing period [18].

Moreover, soluble nitrogen compounds of non proteolytic origin have recently been characterized in aged Parmigiano Reggiano cheeses. These peptide-like molecules seem to originate from the coupling of some apolar free amino acids with pyroglutamic acid, lactic acid or with the γ-carboxyl group of glutamic acid. The amount of these compounds, unlike that of proteolytic peptides deriving from casein degradation, increases progressively during the ageing giving a concentration of up to about 50 mg/100g in long ripened cheese [19].

The importance of peptides is not limited to their contribution to the development of flavour and texture in the ripened cheeses, since they also show a certain bioactivity [20-23]. Different health effects have been attributed to casein-derived peptides, including antimicrobial properties, blood pressure lowering (ACE inhibitory) effects, antithrombotic and antioxidant activities, enhancement of mineral absorption or bioavailability, cyto- or immunomodulatory effects and opioid activities [24-26]. Bioactive peptides may be encrypted in the amino acid sequence of larger proteins and become active only after they are released. Most of them possess typical structural features, that include a relatively short length of the chain (2-9 amino acid residues), the presence of hydrophobic amino acid residues in addition to proline, lysine or arginine, and the resistance to gastrointestinal digestion [27].

Another biological aspect affected by proteolysis may be the allergenicity, since milk and whey proteins, as intact units, can trigger allergic reactions in sensitive subjects [28].

In Parmigiano Reggiano cheese, due to its manufacture technology, the allergenicity potential of caseins and whey proteins may be subjected to a decrease during the long ripening period. However, data concerning the study of milk protein allergenicity during cheese dairying or ripening are quite poor or absent in the literature.

2.1 Aim of the work

The objective of the present work is the molecular characterization of the water soluble nitrogen fraction of the traditional Italian cheese Parmigiano Reggiano, with the aim of indentifying and semiquantifying proteins, peptides and peptide-like molecules with potential biological properties. The fraction will be characterized in a number of Parmigiano Reggiano cheeses coming from different cheese factories at different months of ripening, in order to obtain a general overview of their molecular composition taking into account also the natural variability of this traditional food. The oligopeptides identified will be compared with the sequence of bioactive peptides previously reported in the literature and the effective bioaccessibility, as defined by Oomen et al. [29], of these compounds will be evaluated. Finally, two biological aspects will be studied: the antioxidant activity and the allergenic properties of Parmigiano Reggiano cheese, with particular attention to the possible influence of the proteolytic process on allergenicity.

2.2 Experimental Procedures

2.2.1 Reagents

- Deionised water obtained by using Millipore Alpha Q system
- Formic acid >99% (Acros Organics)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Methanol HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Hydrochloric acid 37% (Carlo Eba)
- Acetic acid (Carlo Eba)
- Ammonium bicarbonate (Carlo Erba)
- Potassium persulfate (FLUKA)
- calcium chloride (Carlo Erba)
- Sodium chloride (Carlo Erba)
- Potassium chloride (Carlo Erba)

- Disodium hydrogen phosphate dodecahydrate (SIGMA)
- Potassium dihydrogen phosphate (SIGMA)
- Sodium hydroxide anhydrous pellets (Carlo Erba)
- Phosphoric acid (Carlo Erba)
- Phosphate Buffer Saline (PBS) prepared as follow:
  - 800 ml deionised water
  - 8 g NaCl
  - 0.2 g KCl
  - 2.86 g Na_2HPO_4•12H2O
  - 0.24 g KH_2PO_4
  - adjust pH to 7.4 by addition of phosphoric acid;
  - take to final volume 1 l with deionised water
- Quant-it Protein Assay reagents (Invitrogen)
- SDS-PAGE standard Broad Range, XT Reducing Agent, XT Sample buffer 4X, XT MES Running buffer 20X, Comassie Brillant Blue R-250 (BIO-RAD)
- α-lactoalbumin from bovine milk, β-lactoglobulin from bovine milk, α-casein from bovine milk, β-casein from bovine milk, k-casein from bovine milk, pepsin from swine gastric tissue, α-chymotripsin from swine pancreas, trypsin from swine pancreas (SIGMA)
- ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Fluka)
- Trolox, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (SIGMA)

2.2.2 Instrumentation and materials

- Analytical balance (Gibertini)
- Technical balance (Orma)
- Ultraturrax T50 Basic (IKA-WERKE)
- ALC 4206R centrifugette
- Universal 320R centrifuge (Hettich)
- Vortex SA6 (Stuart Scientific)
- Timer (Oregon Scientific)
- Paper filters
- 47 mm Steril Aseptic System (Millipore)

- HVLP filters cut-off 0.45 µm (Millipore)
- Vivaspin 2 filters with nominal cut-off 10 KDa (Sartorius)
- HPLC Alliance 2695 (Waters)
- Quattro Micro™ API Mass Spectrometer (Waters)
- ACQUITY UPLC (Waters)
- SQD mass spectrometer (Waters)
- HPLC 510 binary system solvent, Automated Gradient Controller, 486 Tunable Absorbance Detector, 745 Data module (Waters)
- Jupiter 5 µ C18 300 Å (250X2.0 mm) analytical column (Phenomenex)
- Jupiter 5 µ C18 300 Å (250X10 mm) semi preparative column (Phenomenex)
- ACQUITY BEH 1.7 µ C18 (150X2.1 mm) UPLC column (Waters)
- MassLynx 4.0 Software (Waters)
- QuanLynx software (Waters)
- SPSS 17 software
- Qubit Fluorometer (Invitrogen)
- Power Pac Universal power supply, Midi Format vertical electrophoresis Criterion Cell, Criterion™ XT Precast gels 12% Bis-Tris 18 wells, GS-800 Calibrated Densistometer with Quantity One software (BIO-RAD)
- Thermocycler (Thermo Hybaid)
- Gyro-rocker SSL3 shaker, Reciprocating SSL3 shaker, Incubator SI60D (Stuart)
- Microprocessor pHmeter (Hanna Instruments)
- UV/VIS Spectrophotometer LAMBDA BIO 20 (Perkin Elmer)
- Disposable cuvettes (PLASTIBRAND®)

2.2.3 Procedures

2.2.3.1 Water Soluble Extracts of Parmigiano-Reggiano cheese preparation

Water soluble extracts (WSE) of Parmigiano-Reggiano cheese were prepared by homogenizing 20 g of finely grated cheese by Ultraturrax T50 Basic for 1.5 minutes at 4000 rpm in 90 ml of deionised water. The mixture was then filtered through filter paper to obtain a limpid extract. For UPLC analysis, 900 µl of WSE were transferred into a vial and dried under nitrogen flow.

2.2.3.2 Water Soluble Extracts of Parmigiano-Reggiano cheese ultrafiltrated at 10 KDa

The WSE was filtered under vacuum trough a 0.45 μm filter. 1.5 ml of the resulting solution were filtered again through a filter with nominal molecular cut-off of 10 kDa, by using Universal 320R centrifuge at 5400 g at 23°C for 30 minutes. Ultrafiltration was followed by 3 washing step with 2 ml of a 0.1% formic acid solution, and all the filtrates were collected together with the first filtrate. In order to obtain the WSE fraction <10kDa,the ultrafiltrate was then dried by rotavapor, redissolved in 1.4 ml of 0.1% formic acid solution and transferred into a vial to be dried under nitrogen flow. In order to obtain the WSE fraction >10 kDa, the retentate was dissolved in 700 μl, transferred into a vial and dried under nitrogen flow.

2.2.3.3 UPLC/ESI-MS analysis

Total WSEs, WSE fraction <10 kDa and WSE fraction >10 kDa, were respectively dissolved in 150 μl, 250 μl and 250 μl of 0.1% formic acid solution.

UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C_{18} column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 7 min, from 100% A to 50% A by linear gradient in 43 min and 2.6 mins at 50% A plus the washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.2 ml/min, injection volume 2 μl, column temperature 35°C and sample temperature 6°C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30V, source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition mode from 100 to 2000 m/z, scan duration 1s.

2.2.3.3.1 Data analysis

Each ULPC/ESI-MS chromatogram was analyzed in order to determine the molecular weight and the characteristic ions of the main peaks.

In the case of molecular weights higher than 1kDa, reconstructed mass peaks were obtained by MaxEnt 1, application of Masslynx 4.0 software. The integration was performed in an automatic way on the eXtract Ion Chromatograms (XICs), determined

by the characteristic ions of each compound, by QuanLynx software, using as integration parameters: automatic ApexTrack peak integration and noise measurement, 1 smooth ± 1 scan with the Savitzki Golay algorithm. In order to obtain a semiquantitative value, the integrated areas of each compound in each sample were determined and normalized to the Total Ion Chromatogram (TIC) area.

2.2.3.3.2 Statistical analysis

Normalized data were analysed by using the SPSS 17 software. Principal component analysis (PCA) was performed by factorial data reduction extracting 2 factors, with an unrotated factor solution in the space of the observed variables. The first and second PCA components were calculated as independent variables on data by linear regression analysis.

2.2.3.4 WSE fractionation by semipreparative HPLC/UV

500 µl of 10 different WSEs were mixed and dried under nitrogen flow. Solid residue was redissolved in 500 µl of deionised water.

HPLC/UV fractionation was achieved by using an HPLC 510 binary system solvent coupled with Waters Automated Gradient Controller and a Waters 745 Data module with a Jupiter Phenomenex 5 µ C18 300 Å (250X10 mm) semipreparative column.

The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 12 min, from 100% A to 50% A by linear gradient in 65 min and 4 mins at 50% A plus the washing step at 0% A (100% B) and reconditioning. Flow rate was 5 ml/min; injection volume 500 µl, column and sample were at ambient temperature. Detection was performed by using Waters 486 Tunable Absorbance Detector monitoring the absorbance at 214 nm. 1 fraction per minute was collected starting from 1 minute after the injection. Each fraction was dried by nitrogen flow and redissolved in 1 ml of 0.1% formic acid solution to be analysed by UPLC/ESI-MS and HPLC/ESI-MS/MS.

2.2.3.5 UPLC/ESI-MS analysis of WSE fractions

UPLC/ESI-MS analysis of WSE fractions was performed as previously described at paragraph 1.1.3.3.

2.2.3.6 HPLC/ESI-MS/MS analyses of WSE fractions

HPLC/ESI-MS/MS analyses of WSE fractions were carried out by using an Alliance 2695 separation system with a Jupiter Phenomenex 5 µ C18 300 Å (250X2.0 mm) analytical column. Mobile phase and gradient elution were the same as those applied during fractionation by HPLC/UV. Flow rate was 0.2 ml/min, column temperature was set at 30°C and injection volume was 40 µl. Detection was performed by using a Waters triple quadrupole Quattro Micro^{TM} API mass spectrometer applying the following conditions: positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h; collision energy:10, 20, 30 eV or collision energy ramp from 10 to 40 eV, dwell time: 0.1 s. Daughter scan detection starting from 40 m/z.

2.2.3.6.1 Data analysis

Data analysis for peptides sequence identification was carried out by using MassLynx 4.0 software in order to identify the molecular weight of the main fragment, a Microsoft Excel spreadsheet previously set up by our group [30] in order to identify all the possible casein sequences compatible with that molecular weight and “Proteomics ToolKit” software (available on line) in order to calculate the theoretical fragments.

2.2.3.7 Quantification of soluble whey proteins in Parmigiano-Reggiano cheeses

The quantification of α-La and β-Lg in the extracts was performed with the external standard method. Calibration curves were obtained by injecting in the UPLC/ESI-MS systems standard solutions of α-La and β-Lg at different concentrations. All standards and samples were analyzed by extracting from the full scan chromatograms the selected ion chromatograms for the two proteins, by using the following ions: m/z 1418.3, 1575.8 for α-La; m/z 1143.0, 1219.1 (variant B) and 1148.3, 1224.9, (variant A) for β-Lg. The chromatographic peaks were integrated in all the extracted chromatograms, and the standard solutions were used as reference for the quantification of the same proteins in the samples. The relative percentages of the two Lg isoforms were calculated by the relative intensities of the corresponding mass peaks in the reconstructed mass spectra.

2.2.3.8 SDS-PAGE of WSE

2.2.3.8.1 Reducing sample buffer preparation

For 500 µl of reducing sample buffer, 50 µl of XT reducing agent (5% of final volume), 125 µl of XT sample buffer 4X and 350 µl of deionised water were mixed.

2.2.3.8.2 Molecular marker preparation

For 20 µl of molecular marker, 19 µl of reducing sample buffer, prepared as previously described, and 1 µl of SDS-PAGE standard Broad Range were mixed and incubated at 95°C for 5 minutes.

2.2.3.8.3 Sample preparation

100 µl of WSE were dried by nitrogen flow, redissolved in 25 µl of reducing sample buffer, prepared as previously described, and incubated at 95°C for 5 minutes.

2.2.3.8.4 Standard preparation

A few mg of α-lactoalbumin from bovine milk, β-lactoglobulin from bovine milk, α-casein from bovine milk, β-casein from bovine milk and k-casein from bovine milk were dissolved in 1 ml of deionised water and the volume corresponding to 5 µg of protein was transferred into a 200 µl tube and dried under nitrogen flow. Solid residue was dissolved in 25 µl of reducing sample buffer, prepared as previously described, and incubated at 95°C for 5 minutes.

2.2.3.8.5 Running buffer preparation

For 1 l of running buffer 1X 50 ml of XT MES running buffer 20X were diluted with 950 ml of deionised water.

2.2.3.8.6 Comassie destaining solution preparation

For 1 l of destaining solution 500 ml of deionised water, 400 ml of methanol and 100 ml of acetic acid were mixed.

2.2.3.8.7 Comassie staining solution preparation

1g of Comassie Brillant Blue R-250 was dissolved in 1 l of comassie destaining solution prepared as previously described.

2.2.3.8.8 Electrophoretic run

25 µl of sample or standard and 5 µl of molecular marker were loaded in each well of the Criterion™ XT Precast gels 12% Bis-Tris. Criterion Cell was filled with running buffer 1X and 150V was applied. The run lasts about 55 minutes. 1% of comassie blue in the sample buffer runs as indicator.

2.2.3.8.9 Gel staining

The gel was transferred into a glass-made vessel with the staining solution covering it and placed in swelling for at least 1 hour. After that, the gel was rinsed with destaining solution in order to achieve the desired contrast.

2.2.3.8.10 Data analysis

Gel was acquired by GS-800 Calibrated Densistometer with Quantity One software.

2.2.3.9 In vitro gastrointestinal digestion of WSE

2.2.3.9.1 Pepsin stock solution preparation

10 mg of pepsin were dissolved in 10 ml of hydrochloric acid solution at pH 4.4 and stored at -20°C.

2.2.3.9.2 Trypsin stock solution preparation

10 mg of trypsin were dissolved in 10 ml of hydrochloric acid solution 1 mM at pH 3.0 and stored at -20°C.

2.2.3.9.3 Chymotrypsin stock solution preparation

10 mg of chymotrypsin were dissolved in 10 ml of hydrochloric acid solution 1 mM containing $CaCl_2$ 2mM and stored at -20°C.

2.2.3.9.4 Gastrointestinal digestion simulation

5 ml of WSE were acidified to pH 2.2 by the addition of hydrochloric acid 1 mM and 50 µl of pepsin stock solution were added (enzyme:protein ratio 1:100). The mixture was incubated in horizontal swelling for 3 hours at 37°C in order to simulate the gastric transit.

The solution was then neutralized by the addition of NH_4HCO_3 to pH 7.5 and 50 µl of trypsin and 50 µl of chymotrypsin stock solutions (enzyme:protein ratio 1:100) were then

added. The mixture was incubated in horizontal swelling for 4 hours at 37°C in order to simulate intestinal transit. In order to stop the digestion at the end of the simulation, 1 ml of acetonitrile was mixed with the solution. Finally, 1 ml of digested WSE was centrifuged at 16000 g for 15 minutes.

In order to monitor only the gastric step 600 µl of the neutralized solution after gastric simulation were directly analysed by UPLC/ESI-MS.

Two controls were also performed, the first one by substituting the enzymes with the same volume of deionised water and the second one by substituting WSE with the same volume of deionised water.

2.2.3.10 UPLC/ESI-MS analysis of digested WSE

UPLC/ESI-MS analysis of WSE digested was performed as previously described at paragraph 2.2.3.3.

2.2.3.11 Determination of the antioxidant capacity of WSE by ABTS assay

2.2.3.11.1 ABTS radical cation preparation

ABTS radical cation was generated according to Re et al. [31]. Strictly before using, 100 µl of 2 mM potassium persulfate were added to 10 ml of 2 mM ABTS solution in PBS and the mixture was incubated overnight in the dark.

The $ABTS^{\bullet+}$ stock solution obtained was further diluted with PBS to reach the final absorbance at 734 nm equal to 0.7.

2.2.3.11.2 UV-VIS determination

1.8 ml $ABTS^{\bullet+}$ 2 mM PBS solution freshly prepared were added, directly in cuvette, to 0.2 ml of WSE diluted 100 times. The absorbance is measured at 734 nm after reaction time of 60 minutes.

In order to calculate the Trolox Equivalents Antioxidant Capacity (TEAC), standard calibration solutions of Trolox in the range from 100 µM to 10 µM were prepared.

For each sample or standard 4 tests were analysed: MAX, Bmax, MIN and Bmin, where MAX is the absorbance at 734 nm of 1.8 ml of $ABTS^{\bullet+}$ + 0.2 ml of PBS (maximum absorbance), Bmax is the absorbance at 734 nm of 2 ml of PBS (blank control of maximum absorbance), MIN is the absorbance at 734 nm of 1.8 ml of $ABTS^{\bullet+}$ + 0.2 ml of antioxidant (minimum absorbance) and Bmin is the absorbance at 734 nm of 1.8 ml of PBS + 0.2 ml of antioxidant (blank control of minimum absorbance).

The calibration standard curve was obtained by plotting trolox concentration against MAX-MIN value, and slope, intercept and least-squares fit were calculated. Standard curve slope and intercept were then used to compute the corresponding concentration of trolox in the sample. To obtain the TEAC value (mol/g) the ratio between trolox concentration obtained (mol/l) and sample concentration (g/l) was calculated.

2.2.3.12 Determination of antioxidant capacity of digested WSE by ABTS assay

1 ml of digested WSE was dried by nitrogen flow and solid residue obtained was redissolved in 1 ml of PBS and diluted 100 times with the same solvent. Antioxidant capacity was then determined as previously described for WSE of Parmigiano Reggiano cheese (paragraph 2.2.3.11).

2.3 Results and Discussion

2.3.1 Characterization of whey proteins in aqueous extracts of Parmigiano Reggiano cheese

Water soluble extracts (WSE) of Parmigiano Reggiano cheese samples (n = 8) obtained from 8 different cheese factories and having different ageing times (from 6 to 36 months of ageing) were first studied in order to evaluate the whey protein content. In addition 8 Parmigiano-Reggiano cheese samples, aged from 6 to 24 months, were obtained from wheels produced in the same factory, on the same day, starting from the same batch of milk and sampled at regular intervals during the following two years, these samples were also analyzed in order to reduce the variability usually present in Parmigiano-Reggiano cheeses. Finally, 12 curd samples taken immediately before moulding and coming from 6 different dairies were also analyzed, in order to assess whey proteins immediately after curding. All samples were provided by the "Consorzio del Parmigiano-Reggiano" (Reggio Emilia, Italy). The cheese wheels had been ripened following the standard ripening conditions applied for the production of Parmigiano-Reggiano cheese.

2.3.1.1 Identification of α-La and β-Lg in Parmigiano-Reggiano cheese extracts

In the SDS-PAGE analysis of WSE of Parmigiano-Reggiano cheeses aged from 6 to 36 months, bands corresponding to the β-Lg standard were found to be the most abundant in all the extracts and bands corresponding to the α-La standard were also found in smaller amounts. The comassie blue stained gel including all the samples is shown in Figure 2.1.

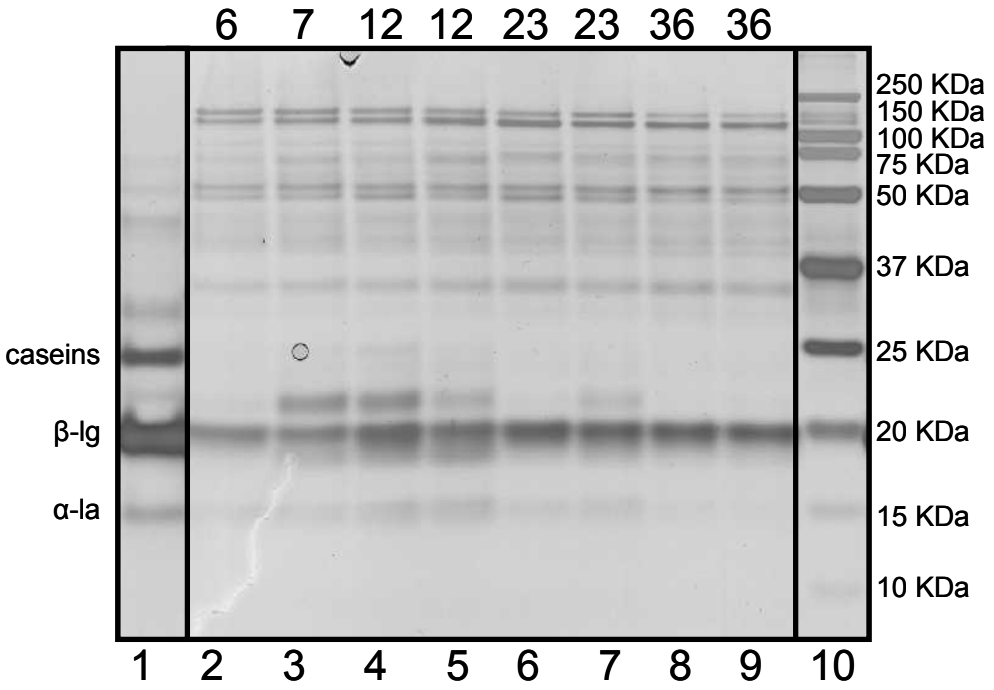


Figure 2.1. SDS-PAGE separation of the aqueous extracts of differently aged Parmigiano Reggiano cheeses. Lane 1, standard solution containing α-lactoalbumin (α-La), β-Lg(β-Lg) and mixture of αS1, β and k casein; lanes 2-9, WSE of Parmigiano Reggiano cheeses, above each line is indicated the ageing time (months); line 10, protein mass marker.

The presence of whey proteins, even after a long period of ripening, was confirmed by an UPLC/ESI-MS analysis: a typical chromatographic profile is reported in Figure 2.2.

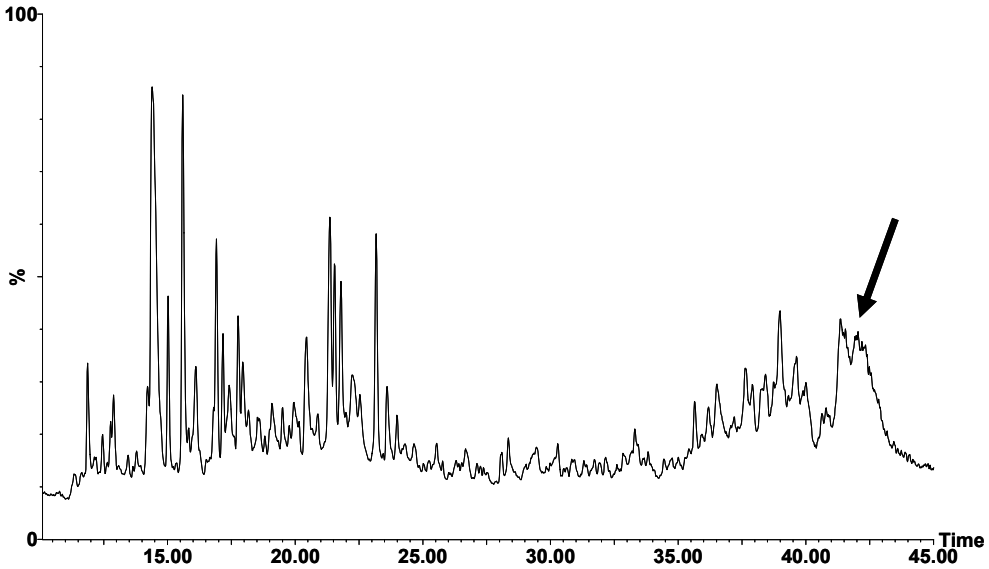


Figure 2.2. UPLC/ESI-MS profile (Full Scan acquisition) of water soluble extract of Parmigiano-Reggiano cheese sample ripened for 12 months. Chromatographic peaks corresponding to α-La and β-Lg are indicated.

The raw ESI mass spectra associated with the last eluting components (retention time from 41 to 45 min, indicated with an arrow in the figure) are reported in Figure 2.3, together with the reconstructed mass spectra showing the molecular mass of the different compounds. The presence of three different proteins is evident, with respectively MW 14180 Da (±2), 18280 Da (±2) and 18366 Da (±2).

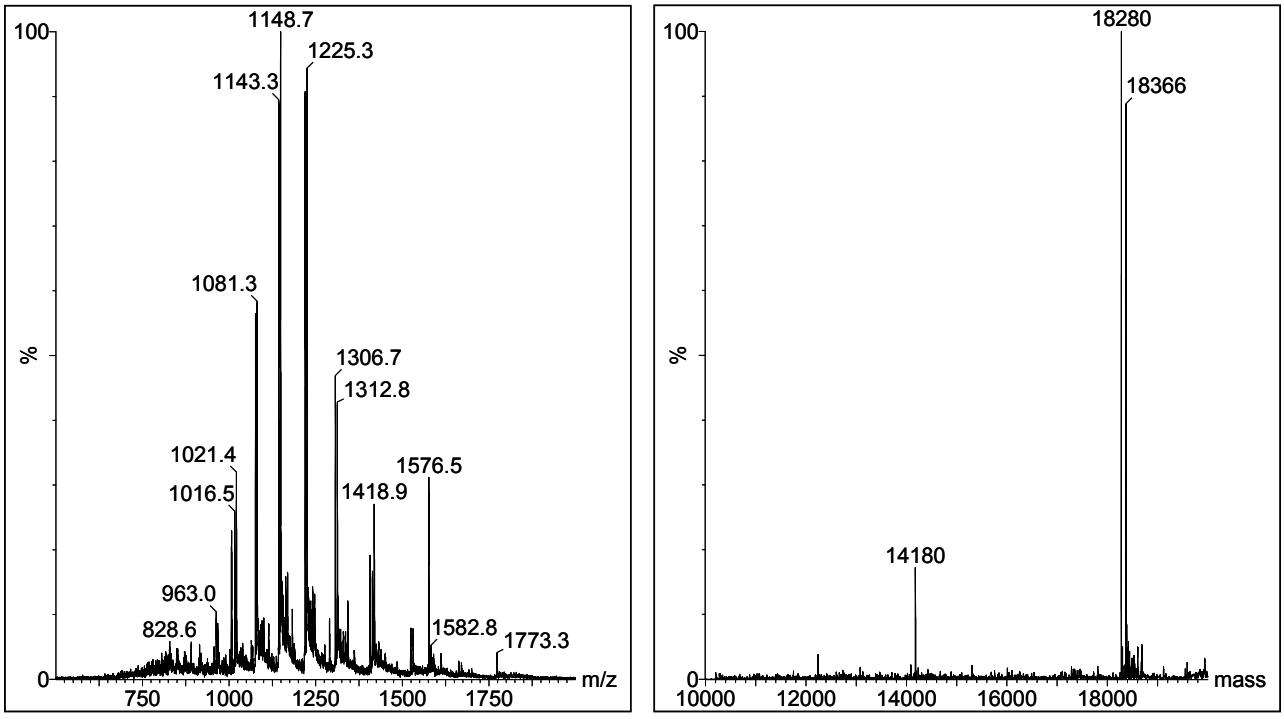


Figure 2.3. Original (left) and reconstructed (right) ESI-MS spectrum associated to the compounds eluting from 41 to 45 min. Three different multicharged patterns corresponding to three different molecular weights can be evidenced.

Comparison with milk protein databases allowed to identify these molecules as α-La (theoretical MW=14178, including four disulfide bridges) and variant B and A, respectively, of β-Lg (theoretical MW respectively, of 18281 Da and 18367 Da). UPLC/ESI-MS analysis of the corresponding commercial standards allowed to confirm the attributions (data not shown).

Calvo et al. considered the presence of whey proteins, determined by FPLC analysis, in Cheddar cheese obtained from pasteurized milk as the result of their co-precipitation with the curd following denaturation [15]. However, the MS data indicated the presence of intact disulfide bridges in α-La, and no sign of cross-linking between β-Lg and caseins. According to these data, the detection of soluble non-denatured whey proteins in Parmigiano-Reggiano cheese can be considered in this case the result of the ability

of the casein network to trap part of the whey and its components, among them the whey proteins.

From the intensities in the reconstructed ESI-MS spectra of the two Lg isoforms, their relative amounts could be calculated in the first set of eight cheese samples. The relative percentage is given in Table 2.1.

Sample	*Ageing time*	*Variant A*	*Variant B*
A	6 months	57%	43%
B	7 months	47%	53%
C	12 months	60%	40%
D	12 months	43%	57%
E	23 months	55%	45%
F	23 months	54%	46%
G	36 months	53%	47%
H	36 months	54%	46%

Table 2.1. Relative percentages of variant A and B of beta-Lg in Parmigiano-Reggiano cheese samples with different ageing time and produced in different cheese factories.

Although the relative percentages were always around 50%, a quite evident variability was found, with both variants ranging between 40-43% and 57-60%. Raw and reconstructed ESI-MS spectra extracted from the sample having the lowest and the highest content of variant A (and thus respectively the highest and the lowest content of variant B) are shown in Figure 2.4 for comparison.

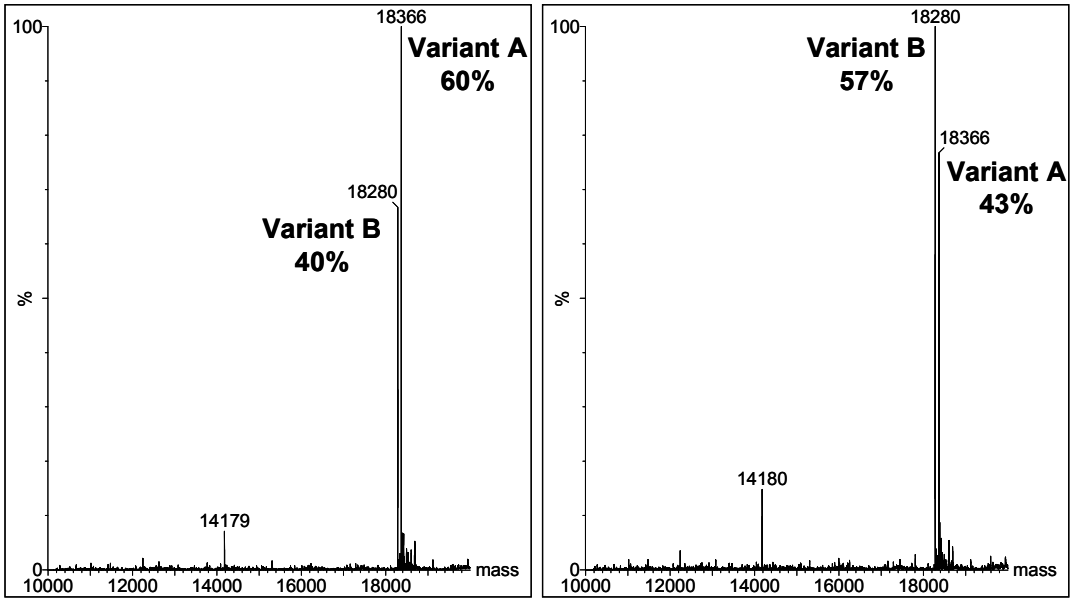


Figure 2.4. Reconstructed ESI-MS spectra of β-Lg in the samples with the lowest (right) and the highest (left) content of variant A.

The variability was clearly not related to the ageing time and was most likely due, according to the literature data [12], to the genetics of the cow breeds. In order to confirm this hypothesis, eight samples of Parmigiano-Reggiano were further studied, with different ageing times but produced in the same factory, on the same day, starting from the same batch of milk. Results are reported in Table 2.2.

Sample	*Ageing time*	*Variant A*	*Variant B*
1	4 months	57%	43%
2	6 months	57%	43%
3	8 months	58%	42%
4	10 months	57%	43%
5	12 months	58%	42%
6	16 months	59%	41%
7	20 months	54%	46%
8	24 months	55%	45%

Table 2.2. Relative percentages of variant A and B of β-Lg in Parmigiano-Reggiano cheese samples with different ageing time but produced in the same cheese factory, on the same day and starting from the same batch of milk.

It is immediately evident that, in spite of the different ageing times, the relative percentages for the two isoforms are much more similar in all samples, evidencing the strict relation existing between the relative percentage and the milk used for production, thus the corresponding cow breed.

2.3.1.2 Quantification of whey proteins in Parmigiano-Reggiano at different ageing times

In order to study in more detail the relative amounts of the two whey proteins during the ageing time, the aqueous extracts of the two sets of Parmigiano Reggiano cheese samples were concentrated by ultrafiltration and the retentate was then reanalysed by SDS-PAGE. Comassie stained gels (Figure 2.5) showed a progressive decrease of α-La during cheese ripening, whereas the amount of β-Lg seemed to be less affected by the ripening time.

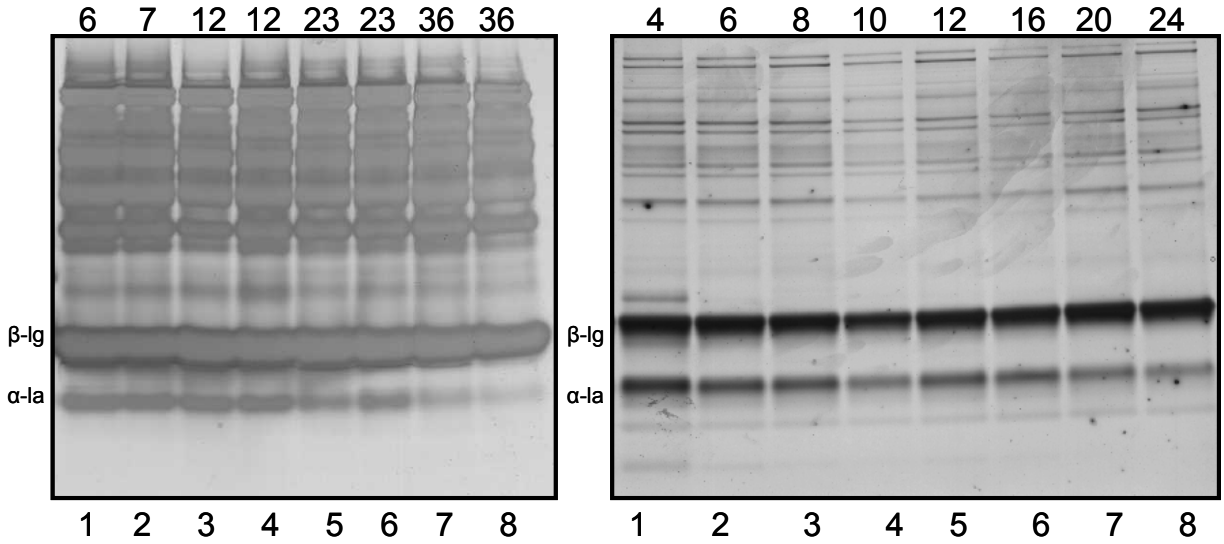


Figure 2.5. SDS-PAGE separation of the aqueous extracts (fraction with molecular weight above 10 kDa) of differently aged Parmigiano Reggiano cheeses obtained from different factories (left) or from the same factory and manufactured the same day (right). Above each line the ageing time (months) is indicated.

In order to obtain quantitative results, the two proteins were analysed by using the UPLC/ESI-MS method previously developed, with external standard calibration. The extract ion chromatograms corresponding to α-La and β-Lg were obtained from every sample and from the standard by extracting the corresponding ions (details in the experimental section). The two β-Lg isoforms were obviously quantified together as a single protein, since no standard of the pure isoforms was commercially available.

The results corresponding to the first sets of samples, those obtained from different cheese factories (Figure 2.6), indicated a content of β-Lg in the range of 25-40mg/100g of cheese in all samples, and a content of α-La around 3mg/100g of cheese in the younger samples, which decreased to 1mg/100g in the older cheeses.

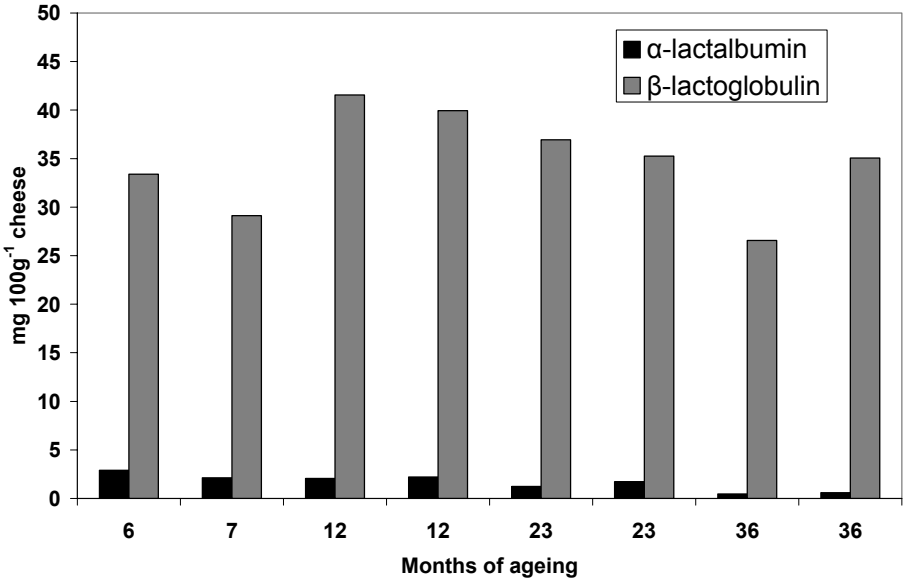


Figure 2.6. Amounts of α-La and β-Lg (mg/100g cheese) in the aqueous extracts of Parmigiano Reggiano cheeses having different ripening times, as measured by UPLC/ESI-MS. Cheese samples were produced in different factories. The value reported for every sample is an average of two independent analyses (average standard deviations 10%).

The same analysis repeated for the second set of samples, those obtained in the same factory and on the same day (Figure 2.7), confirmed the previous findings. The results showed a ratio β-Lg to α-La ranging from 8:1 after 6 months of ripening to a 20:1 after 36 months: both ratios are higher than those usually found in milk.

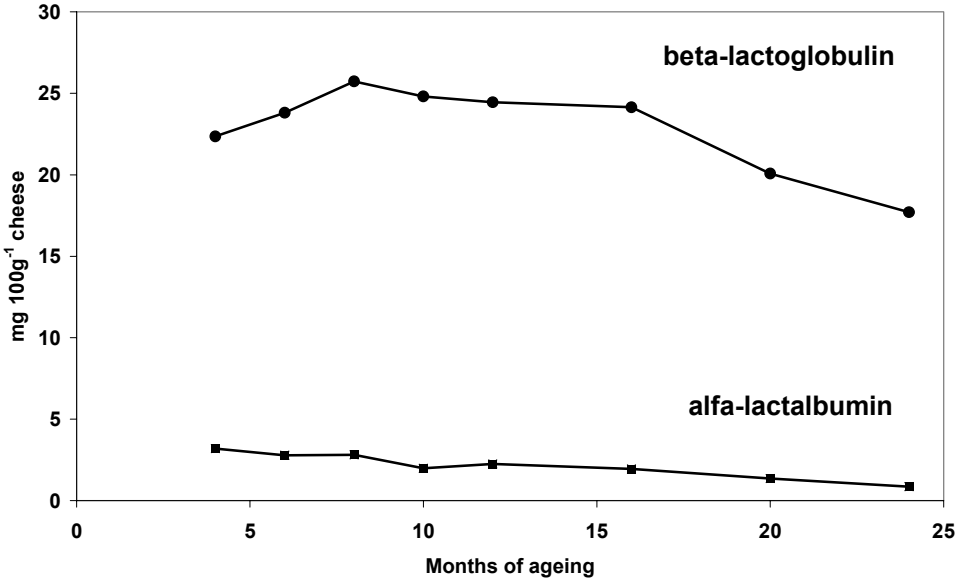


Figure 2.7. Amounts of α-La and β-Lg (mg 100g-1 cheese) in aqueous extracts of Parmigiano Reggiano cheeses with different ripening times, as measured by UPLC/ESI-MS. Cheese samples were produced in the same factory, on the same day and starting from the same batch of milk. The value reported for every sample is an average of two independent analyses (average standard deviations 10%).

With the aim of explaining this behaviour, fresh curd samples of Parmigiano-Reggiano cheese, taken immediately before moulding, were also analyzed, in order to estimate the whey protein content in the water soluble fraction of fresh cheeses. The results are reported in Table 2.3.

Dairy	*α-La (mg 100 g^{-1})*	*β-Lg (mg 100g^{-1})*
A	4.6	85.2
A	3.4	77.0
B	5.8	60.2
B	4.6	79.4
C	5.7	51.6
C	3.3	65.9
D	5.9	64.2
D	4.0	68.5
E	3.3	98.4
E	6.3	67.1
F	5.4	52.8
F	4.5	53.9

Table 2.3. Whey protein content in curd samples taken immediately before moulding.

The contents of β–Lg and α-La were respectively 68.7±14.1 and 4.7±1.1 mg/100g, with a ratio β–Lg to α-La 15.6±6.3. These data indicate that the amount of β–Lg is drastically reduced from the curd to the few month aged samples, and thereinafter the reduction becomes slower or absent; on the other hand, the α-La content is already quite low in the curd, and it decreases slowly during the ageing time.

An estimation of the theoretical content of α-La and β –Lg in 24 h Parmigiano Reggiano cheese yields values ranging from 32 to 56 mg/100g and from 110 to 192 mg/100, respectively, according to the actual values of milk total proteins, the percentage of whey proteins, and the percentage of α-La or β –Lg [1,32-34]. Such values can be further affected by the curd moisture content. It is immediately evident that the measured values of α-La and β–Lg in curd are definitely lower than the estimated ones, particularly those of α-La. Considering that the cooked curd remains under hot whey at 55°C for more than 60 min and that the internal temperature of Parmigiano Reggiano cheese curd, due to its low heat conductivity, is at that value for several hours [2], it appears likely that such conditions are sufficient to partly unfold the whey proteins (α-La more than β-Lg). Thus, the content of the soluble whey proteins in Parmigiano Reggiano cheese, lower than that expected, and the unbalanced ratio of β-Lg to α-La

may thus be considered the result of an asymmetric partial denaturation, caused by heat and the environment changes, followed by a slow differential proteolytic degradation occurring during the ageing time.

2.3.1.3 Conclusion

In this section it has been demonstrated, both by SDS-PAGE and UPLC/ESI-MS methodologies, that soluble whey proteins, in particular α-La and β-Lg, are present in a non negligible amount in Parmigiano-Reggiano cheeses, even after long ageing times. The UPLC/ESI-MS method used for their identification also allowed to detect separately the two main β-Lg isoforms and to measure their relative abundance, which appeared to be mainly determined by the cow breeds, allowing the potential use of this ratio, even in long aged cheese, as molecular marker for the assessment of the cow breeds providing the milk used for the cheese production.

The content of α-La and β-Lg in cheeses and their behaviour during the ageing time has been studied in detail. The content of whey proteins in Parmigiano-Reggiano cheese curds was generally found to be less than that which might be calculated by theoretical considerations, and also the β-Lg to α-La ratio was found to be higher than that calculated or normally found in milk. These differences could suggest that the cheese making technology of Parmigiano Reggiano cheese caused an initial thermal unfolding, highly consistent for α-La, followed by a proteolytic degradation, which in the first weeks mainly affects β-Lg and then, more slowly, α-La.

Further general studies, also performed on different types of cheeses, will be necessary in order to further elucidate the quantitative details of whey protein incorporation into the cheese curd.

2.3.2 Peptides characterization in Parmigiano Reggiano cheese

Water soluble extracts of Parmigiano Reggiano cheese were analysed by UPLC/ESI-MS obtaining a typical chromatographic profile shown in Figure 2.8.

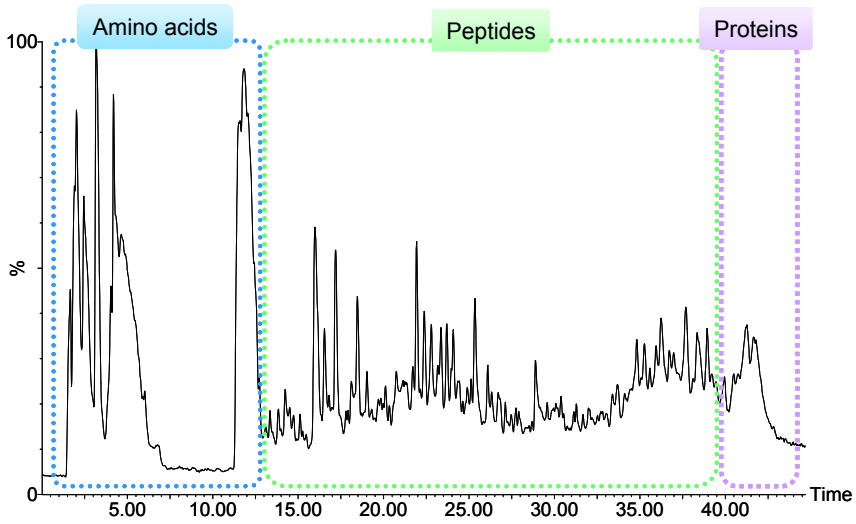


Figure 2.8. Total Ion Chromatogram of WSE of Parmigiano Reggiano cheese.

Due to the very high number of compounds present in the mixture, in order to focus the attention on the most abundant peptides, it has been decided to analyze in each chromatogram only the peaks having a relative intensity higher than the 30 % of the Total Ion Chromatogram. In Figure 2.9 an example of the chromatogram obtained by considering only the peaks exceeding this limit is reported.

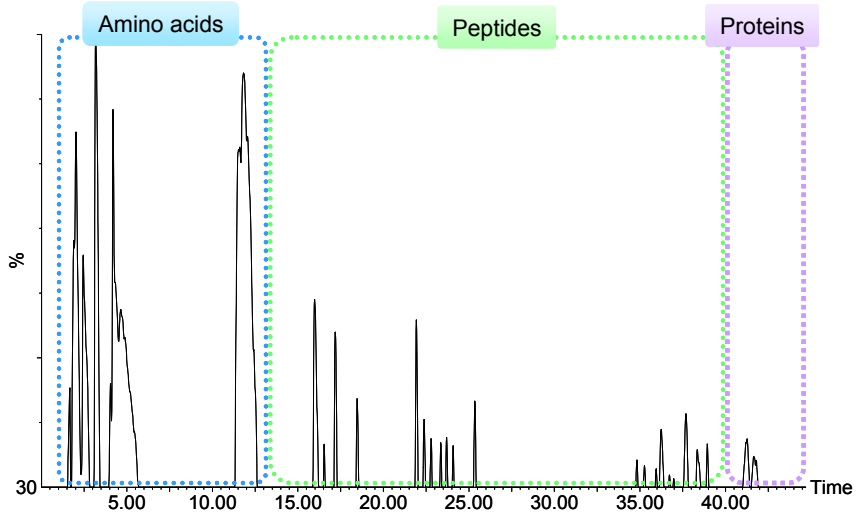


Figure 2.9. Total Ion Chromatogram in which the baseline has been set at the 30% of the total relative intensity.

As can be seen, only a small number of peaks have a relative intensity higher than this threshold, and thus only the mass spectra of these peaks have been taken into account. By applying this approach, the peptide profile was analyzed in several WSE of Parmigiano Reggiano cheese obtained from different cheese factories and at different stages of ripening, from 6 to 40 months. Sample details are reported in Table 2.4.

Dairy	*Months of ripening*
A	6-12-16-24-36
A	6-12-16-24-36
B	6-12-18-25-36
B	6-12-18-24-36
C	6-14-18-24-38
C	6-14-18-24-38
D	7-12-20-26-41
D	7-12-20-26-41
E	6-12-24-37-41
F	11-24-33

Table 2.4. Description of the samples of Parmigiano Reggiano Cheese analyzed.

The complete list of peptides traced and identified in different Parmigiano Reggiano cheese, identified according to their different molecular weight and characteristic ions, is reported in Attachment Table 1.

2.3.2.1 Peptide sequences identification

Identification of peptide sequences has been carried out by HPLC/ESI-MS/MS analysis performing daughter scan experiments on mono- or dicharged ions at different collision energies. Once determined the molecular weight of peptides and their possible sequences, theoretical fragmentations were determined and compared with those obtained in product ion spectra. Two different examples of identification of peptide sequences are reported in figures here below.

(a)

Possible sequences with MW 801 Da:

- α s1 f(73-79) PNSVEQK
- αS1 f(82-88) QKEDVPS
- αS1 f(113-119) PNSAEER
- αS1 f(120-126) LHSMKEG
- αS1 f(121-127) HSMKEGI
- αS1 f(122-128) SMKEGIH
- αS1f (131-137)QKEPMIG
- β f(140-145) LQSWMH
- β f(174-180) PQKAVPY
- β f(175-181) QKAVPYP
- β f(176-182) KAVPYPQ
- β f(186-192) PIQAFLL

(b)

Fragment Ion Calculator Results

Sequence: **KAVPYPQ**, pI: **8.59095**

Fragment Ion Table, average masses

Seq	#	A	B	C	X	Y	Z	# (+1)
K	1	101.17197	129.18206	146.21263	-	802.94875	785.91819	7
A	2	172.25077	200.26086	217.29143	700.76888	674.77467	657.74411	6
V	3	271.38333	299.39343	316.42399	629.69008	603.69587	586.66531	5
P	4	368.50001	396.51011	413.54067	530.55752	504.56330	487.53274	4
Y	5	531.67597	559.68607	576.71663	433.44085	407.44662	390.41607	3
P	6	628.79265	656.80275	673.83331	270.26489	244.27067	227.24011	2
Q	7	756.92337	784.93346	-	173.14820	147.15399	130.12342	1

(c)

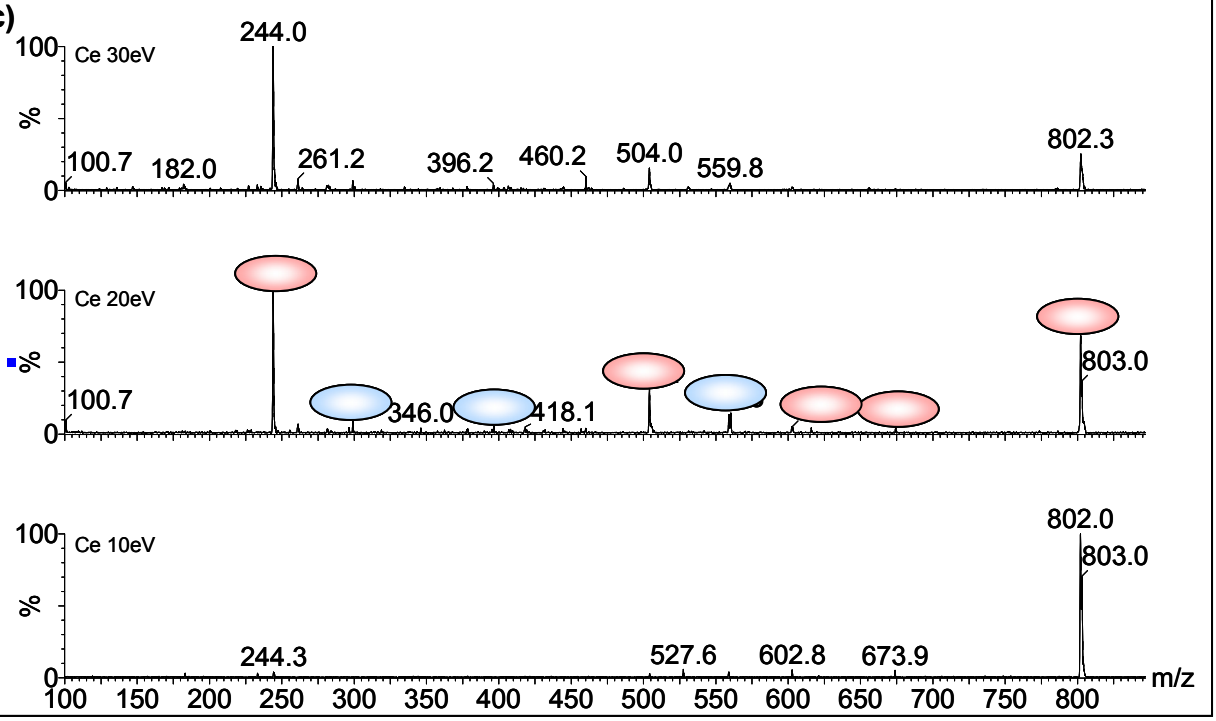


Figure 2.10. Example of peptide sequence identification. (a) Casein-derived peptides with molecular weight 552; (b) theoretical fragmentation of β f(176-182); (c) product ions spectra obtained by fragmentation of the molecular ion at different collision energies. Matching fragments have been indicated.

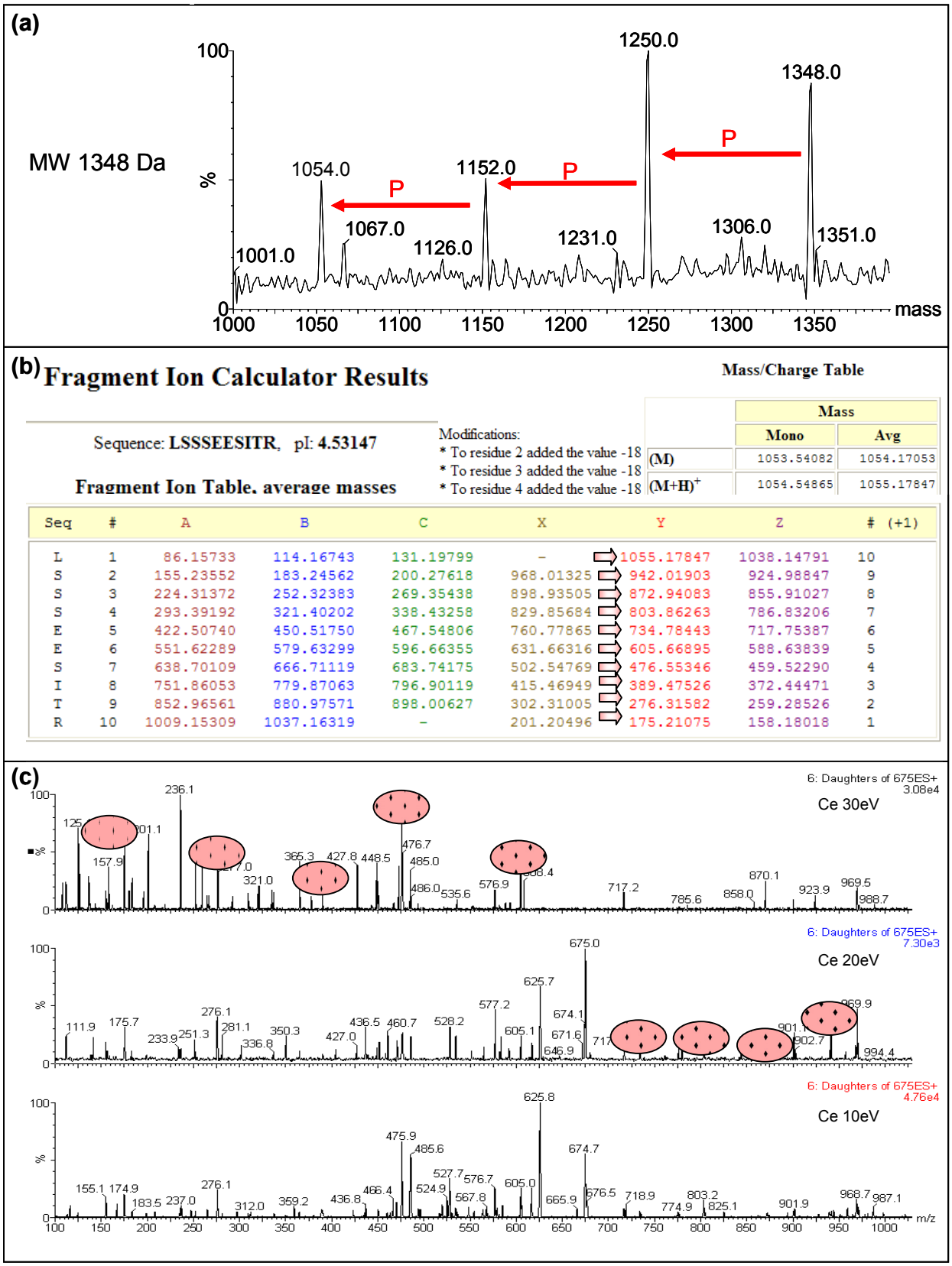


Figure 2.11. Example of identification of peptides containing phosphorilated residues in its sequence. (a) reconstructed ESI-MS/MS spectrum in which the loss of 3 phosphate groups is evident; (b) theoretical fragmentation of the dephosphorilated molecular ion. Matching fragments have been indicated.

In this way, excluding the peptides represented only by multicharged ions, 80% of the unknown compounds were identified.

2.3.2.2 Analysis of the peptide profile of Parmigiano Reggiano cheese

All identified peptides were then semiquantified in all samples and the values obtained were analyzed by Principal Component Analysis, with the purpose of providing a general overview of the factors affecting the data variability. The loading plot obtained is reported in Figure 2.12; peptides are represented in a 2 dimensional plan generated by the two calculated main factors (Principal Component 1, PC1, and Principal Component 2, PC2) which explain, respectively, 37.4 and 18.6% of the variance.

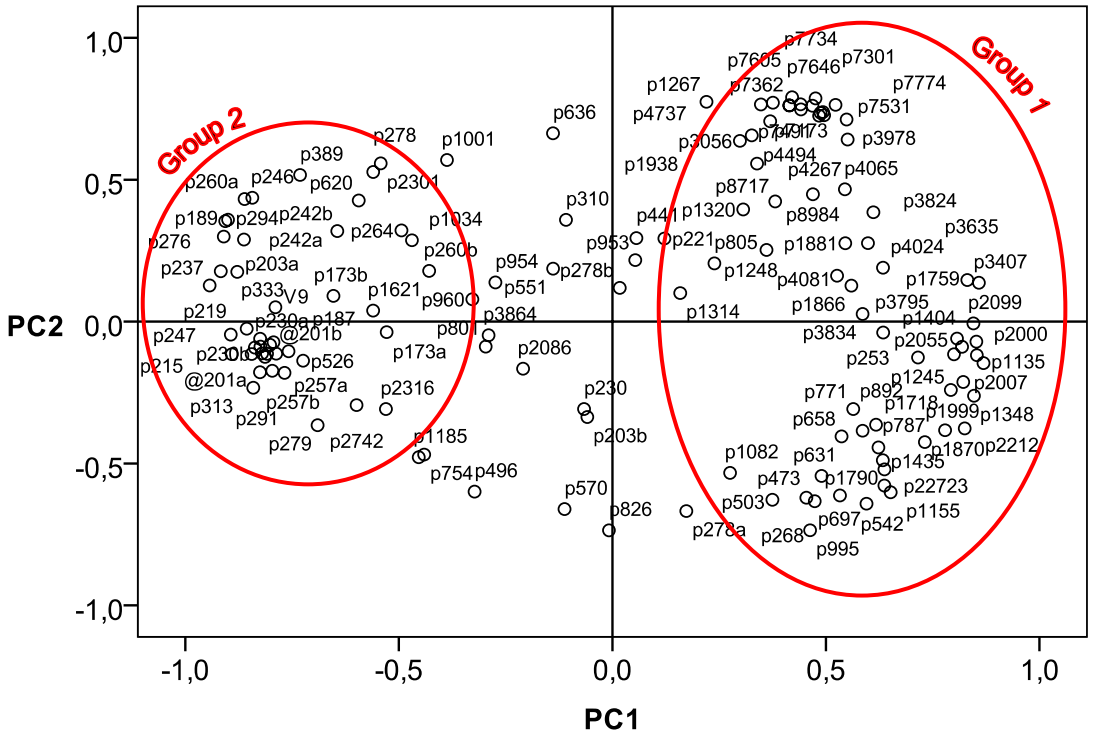


Figure 2.12. Loading plot of peptides in Parmigiano-Reggiano cheese.

As can be seen from the loading plot, peptides appeared to be clustered into two main groups, positively or negatively correlated to the PC1. In order to investigate which factors may affect the peptide profile, the score plot including all the analyzed samples has been analyzed. Samples were labelled according to the dairy, score plot in Figure 2.13, and according by their ageing time, score plot in Figure 2.14.

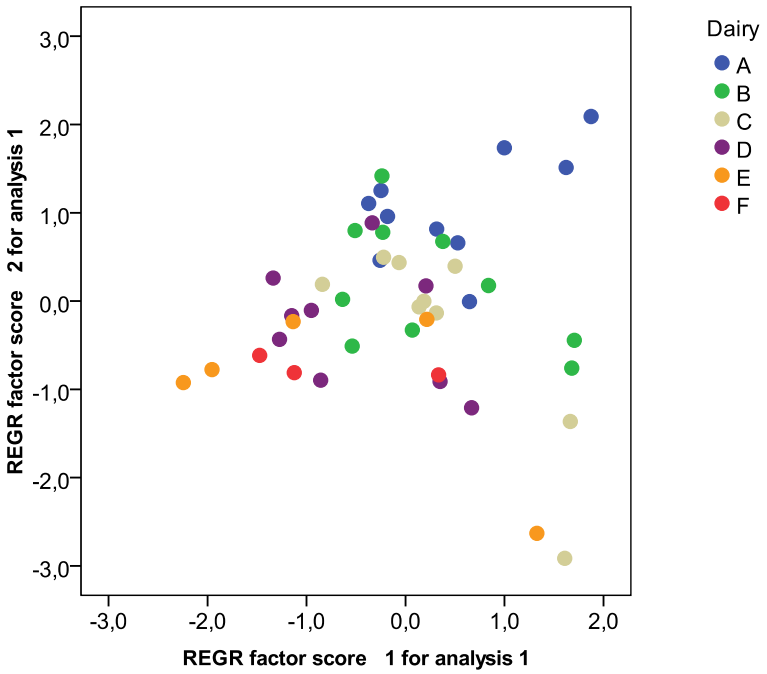


Figure 2.13. Score plot of the Parmigiano Reggiano cheese samples, labelled according to the dairy factory.

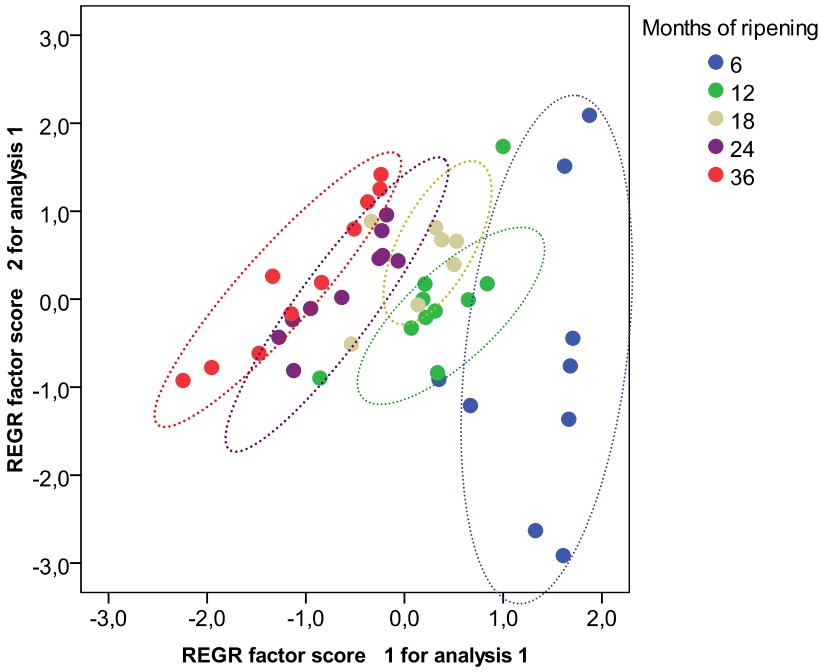


Figure 2.14. Score plot of the Parmigiano Reggiano cheese samples, labelled according to their ageing time.

It is quite evident that the dairy factory of production does not affect significantly the peptidic profile of cheese samples. Peptides, as can be seen in Figure 2.14, are mainly influenced by ripening time.

The peptidic profile during ripening has been investigated in detail by studying the trend of each peptide in the different samples. By this analysis, 3 main groups of peptides have been recognized: examples of their respective trends during ripening are reported in Figure 2.15.

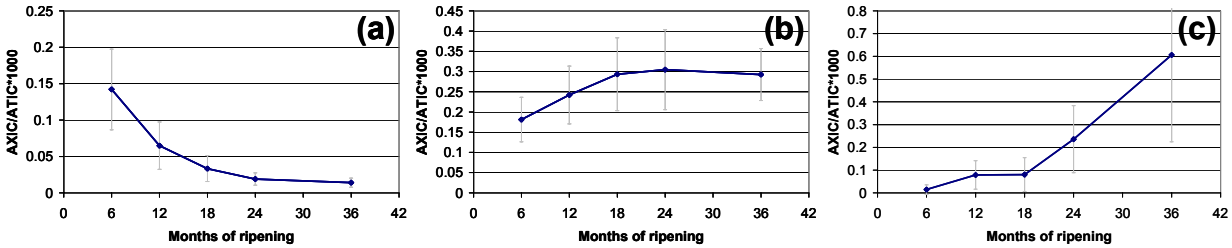


Figure 2.15. Example of typical trend of peptides included in the group 1 (a), group 2 (b) and group 3 (c).

In the first group, peptides present in high amount at 6 months, which decrease during ripening, are present. Peptides which increase during ripening until 24 months, and then remain quite constant belong to the second group. In the third group peptides which constantly increase up to 36 months are determined.

2.3.2.2.1 Group 1 peptides (highest amount at the beginning of the ripening time)

In Table 2.5 the list of the group 1 peptides, which have been identified, is reported.

Table 2.5. Casein fragments, amino acidic sequences and qualifier ions of group 1 peptides identified.

MW	Casein fragment	Amino acidic sequence	Qualifier ions
230	---	Ile-Val	231(MH^+); 117.7(V C-term); 85.9(I-HCOOH); 69.2(I-HCOOH-NH_3)
278	---	Tyr-Pro	279.0(MH^+); 115.9(P C-term); 261.9(MH^+-NH_3); 118.9(a1- NH_3); 136.0(a1); 90.5(a1-HCOOH)
503	αS1 f(55-58)	EDIK	504(MH^+); 130.0(b1); 147.2(y4); 244.9(b2); 260.1(y3); 357.5(b3)
441	αS1 f(71-74) αS1 f(111-114)	IVPN	442(MH^+); 329(y2); 230(y3); 133(y4); 86(a1); 185(a2); 312(z2)
1245	αS1 f(101-110)	LKKYKVPQLE	1246(MH^+); 567(Y2); 129(E C-term$^+$-H_2O); 101(E C-term$^+$- HCOOH)
551	αS1 f(94-97)	YLEQ	276.8(MH^{2+}); 136.0(a1); 249.0(a2); 423.2(c3); 535.5(z1); 406.1(b3); 389.3(y2);147.1(y4); 276.9(y3); 378.7(a3)
1154	β f(17-25)2P	SLSSSEESITR	1155(MH^+); 578(MH^{2+}); 605(y5); 476(y6); 389(y7); 276(y8);175(y9); 484(b5); 226(b3); 529.2((M;2H^+)/2)-H_3PO_4); 480.0((M+2H^+)/2)-2H_3PO_4)
473	αS1 f(14-17)	ELVN	474(MH^+); 229(b2); 342(b3); 456(b4); 346(y3);133(y4)
1248	αS1 f(167-178)1P	VPLGTQYTDAPS	665.2((M+2H^+)/2); 616.3((y2+2H^+)/2); 607.1((z2+2H^+)/2);566.3((y3+2H^+)/2)
1320	β f(187-197)	IQAFLLYQEPV	661(M2H^+); 611.5(c10+2H^+); 603.8 (b10+2H^+); 562.9 (c9+2H^+); 86.1(a1)
542	αS1 f(10-14)	GLPQE	543(MH^+); 485.8(y2); 373.2(y3); 273.7(y4); 225.7(y3-E)
1348	β f(16-25)3P	LSSSEESITR	1348(MH^+); 675((M+2H^+)/2); 175.1(y10); 276.4(y9); 390.1(y8); 475.9(y7); 604.6(y6);734.2(y5); 803.1(y4); 872.4(y3); 941.4(y2)
496	αS1 f(183-187)	PINPIG	497.7(MH^+); 440.0(c4); 230.1(c2); 85.8(I-HCOOH); 69.8(a1); 309.0(b3); 213.0(b2); 115.9(c1); 268.0(z4)
570	αS1 f(70-74) / αS1 f(110-114)	EIVPN	571(MH^+); 243.1(b2); 439.4(b4); 132.8(y5); 230.1(y4) ;328.9(y3); 341.9(b3)
754	β f(47-52)	DKIHPE	756(MH^+); 400(y4); 263(y5); 166(y6); 494 (b4) ; 321((y2+2H^+)/2)
1435	β(15-25)3P	SLSSSEESITR	718.2((M+2H^+)/2); 475.9(y8); 276.4(y10); 173.1(a2); 605.1(y7); 389.1(y9); 201.2(x11)
954	β f (136-143)	PLPLLQSW	954.3(MH^+); 205.2(y1); 231.1(x1); 726.0(z3)
1034	αS1 f(67-74)2P	SSEEIVPN	1035(MH^+); 397.3(b4); 510.2(b5); 608.9(b6); 707.6(b7)
1314	αS1 f(65-74)3P	ISSSEEIVPN	1314.9(MH^+); 1085.1(b8); 987.1(b8); 889.5(c6); 872.6(b6); 571.6(y6); 443.0(y7); 328.6(y8); 2299(y2); 133.1(y10)
953	αS1 f(67-74)1P	SSEEIVPN	954(MH^+); 133.0(y8); 230.0(y7); 328.9(y6); 441.3(y5); 513.2(b4); 627.0(b5); 724.9(b6)
697	αS1 f(106-111)	VPQLEI	698.5(MH^+); 115.0(z6); 158.2(x1); 484.5(z3); 528.2(x3)
173	β f (1-6)	RELEEL	657.4(b5); 528.4(b4)

MW	Casein fragment	Amino acidic sequence	Qualifier ions
1790	β f (15-28)3P	SLSSSEESITRINK	1791 (MH^+); 847 (($M+2H^+$)/2)-H_3PO_4); 798 (($M+2H^+$)/2)-$2H_3PO_4$); 749 (($M+2H^+$)/2)-$3H_3PO_4$)
631	β f (2-6)	ELEEL	632(MH^+); 501 (b4); 372(b3); 243(b2); 102(a1); 215(a2); 147(c1); 260(c2); 389(c3); 390(y3); 261(y4); 132(y5)
658	αS1 f(25-30)	VAPFPE	659(MH^+); 560(Yy2); 489(y3); 393(y4); 245(y5); 148(y6); 171(b2); 268(b3); 415(b4)

2.3.2.2.2 Group 2 peptides (highest amount at 24 months of ripening)

In Table 2.6 the list of the group 2 peptides characterized in Parmigiano Reggiano cheese is reported.

Table 2.6. Casein fragments, amino acidic sequences and qualifier ions of non proteolytic peptides belonging to group 2 peptides.

MW	Amino acidic sequence	Qualifier ions
246	γ-Glu-Val	247(MH^+); 117.5(V C-term); 129.9(E N-term); 230.0(MH^+-H_2O); 71.6(V C-term- HCOOH); 83.5(E N-term-HCOOH); 184.1(MH^+-NH_3-HCOOH); 155.8(MH^+- 2HCOOH)
278	γ-Glu-Met	279(MH^+); 130(E N-term); 150 (M C-term); 132.9(M C-term-NH_3); 83.4(E N-term- HCOOH); 103.5(M C-term-HCOOH)
310	γ-Glu-Tyr	311.0(MH^+);182..0 (Y C-term)
226	**pyroglutamyl-Pro***	227.1(MH^+); 181.0(MH^+-HCOOH); 209.1(MH^+-H2O); 115.8(P C-term)
260	γ-Glu-Ile	261.2(MH^+); 130.0 (E N-term); 132.0 (I C-term); 244.0 (MH^+-NH_3); 85.8 (I C-term-HCOOH); 83.6 (E N-term-HCOOH); 198.1 (MH^+-NH_3-HCOOH); 69.0(I C-term-HCOOH-NH_3)
260	γ-Glu-Leu	261.2(MH^+); 130.0 (E N-term); 132.0 (L C-term); 244.0 (MH^+-NH_3) ; 85.8 (L C-term-HCOOH); 83.6 (E N-term-HCOOH); 198.1 (MH^+-NH_3-HCOOH)
221	lactoyl-Met	222.1(MH^+) ;150.0 (M C-term); 103.6 (M C-term-COOH); 175.8(MH^+-HCOOH); 132.6 (M C-term-H_2O)
253	lactoyl-Tyr	254(MH^+);208(MH^+-HCOOH);136(i2)
294	γ-Glu-Phe	295.0(MH^+);166.1 (F C-term); 130.0(E N-term); 83.7 (E N-term-COOH); 149.1(F C-term-OH)
189	lactoyl-Val	190(MH^+); 118.0(V C-term); 144.1(MH^+-HCOOH); 71.8 (V C-term-HCOOH); 172.2(MH^+H_2O)
242	pyroglutamyl-Ile	243(MH^+); 197c; 132(I C-term)
333	**γ-Glu-Trp***	334(MH^+); 205.2(W C-term); 130.1(E N-term); 188.0(W C-term-NH_3); 84.1(E N-term-HCOOH); 159.0 (W C-term-HCOOH); 317.0(MH^+-NH_3)
242	pyroglutamyl-Leu	243(MH^+); 196.8(MH^+-HCOOH); 132.1(L C-term) ;226.3(MH^+-H_2O); 86.0(L C-term- HCOOH)
276	pyroglutamyl-Phe	277.2(MH^+); 231.1(MH^+-HCOOH); 166(F C-term); 120(i2)
203	lactoyl-Ile	204.2(MH^+); 158.2(MH^+-HCOOH)
203	lactoyl-Leu	204.2(MH^+); 158.2(MH^+-HCOOH)
237	lactoyl-Phe	238.2(MH^+); 192.1(MH^+-HCOOH); 120.1(i2)

**never previously identified*

This group, in which peptides increase during ripening until 24 months, was mainly composed by non proteolytic peptide-like molecules, as previously reported by Sforza et al. [19]. Moreover, 2 new non proteolytic peptide-like molecules have been identified for the first time: the product ion spectra and hypothetical structures are shown in Figure 2.16 and Figure 2.17.

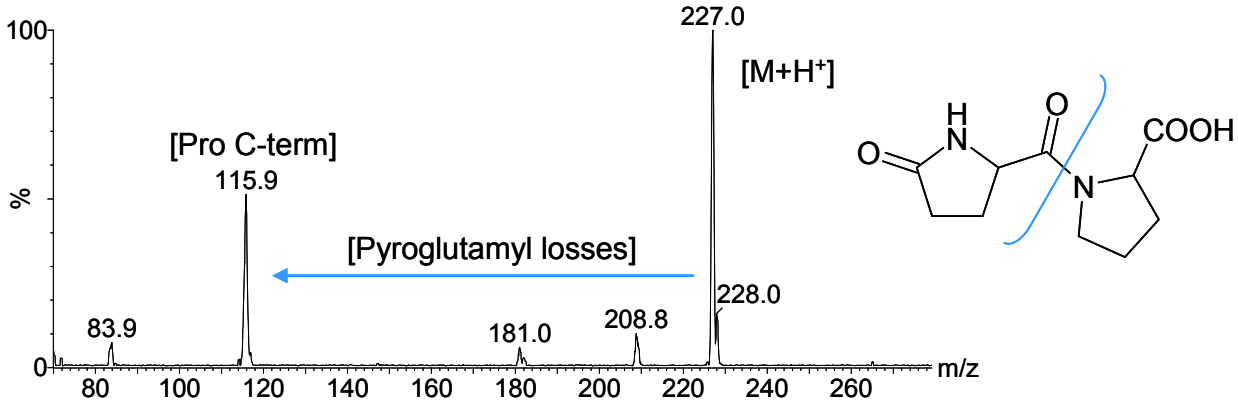


Figure 2.16. Product ion spectrum and molecular structure of Pyroglutamyl-Proline.

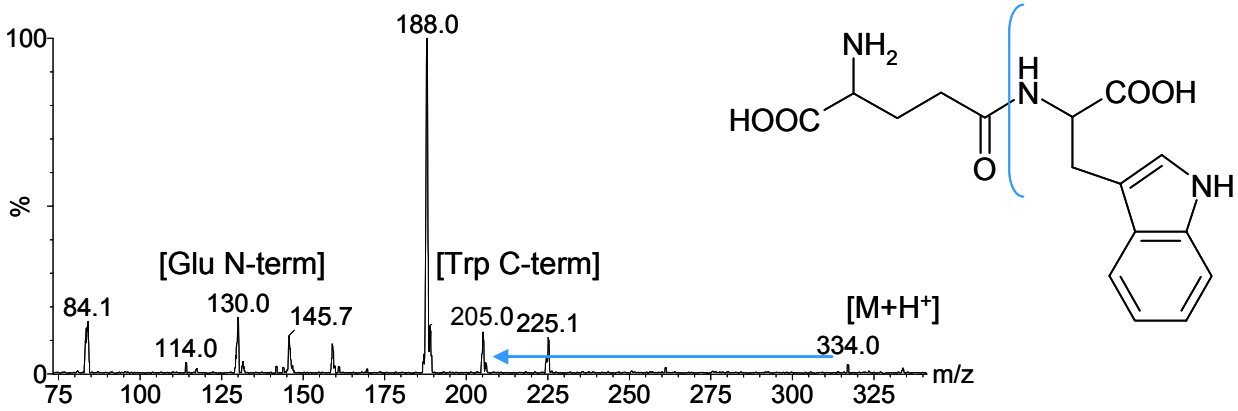


Figure 2.17. Product ion spectrum and molecular structure of γ-Glu-Trp.

The identification has been performed by taking into account the particular characteristics of these compounds. In the product ion spectrum of pyroglutamyl-proline the C-terminal ion of proline and the neutral loss, calculated from the molecular ion are evident, corresponding to a pyroglutamyl residue; moreover, the retention time is in good agreement with those of the other non proteolytic peptide-like molecules.

Consistently with the product ion spectrum reported in Figure 2.17, the compound with molecular weight 333 Da was proposed to correspond to the dipeptide Glu-Trp; the high

retention time and its accumulation during ripening, a typical characteristic of non proteolytic peptides, suggested that it was a γ-glutamyl derivative.

Other proteolytic peptides showing the same trend during ripening, for which it has been possible to identify the amino acidic sequence, are reported in Table 2.7

Table 2.7. Casein fragments, amino acidic sequences and qualifier ions of proteolytic peptides belonging to group 2 peptides

MW	*Casein fragment*	*Amino acidic sequence*	*Qualifier ions*
264	Val-Phe	VF	265(MH^+); 166.4(F C-term); 119.7(F C-term-HCOOH)
636	αS1 f(89-93)	ERYLG	637(MH^+); 561.9(b4); 449.0(b2); 591.4(a5); 534.2(a4); 421.2(a3); 544.2 (MH^+-H_2O- E C-term); 431.1(MH^+-H_2O- G C-term)
801	β f(176-182)	KAVPYPQ	802.1(MH^+); 673.9(c6); 559.0(b5); 244.0(y6); 504.7(y4); 656(b6); 396(b4); 299(b3); 674(y2); 603(y3); 407(y5); 147(y7); 657(z2); 487(z4); 390(z5); 227(z6)
389	β f(4-6)	EEL	390.2(y1); 261.0(y2);132.1(y3); 129.9(b1); 258.9(b2)

It is interesting to note the presence of the fragment β (4-6), probably coming from the proteolysis of β f(1-6) identified in the first group and having a decreasing trend during ripening.

2.3.2.2.3 Group 3 peptides (highest amount at 36 months of ripening)

This group is constituted by nitrogen compounds with a non conventional structure. In every product ion spectrum analyzed the presence of non polar amino acid residue, such as Ile, Leu, Val, Met, Tyr and Phe was evident, although their molecular weight does not correspond to those of di- or tripeptides. The relative retention time of these molecules was very high, indicating an apolar molecule, and they all present common neutral losses. On the basis of these considerations, and consistently with the molecular weights, these molecules were supposed to be composed by an amino acid residue linked to fatty acid residues: *acetyl*-, *butiryl*-, *caproyl*- and *capryl*- residues. A representative product ion spectrum obtained is shown in Figure 2.18.

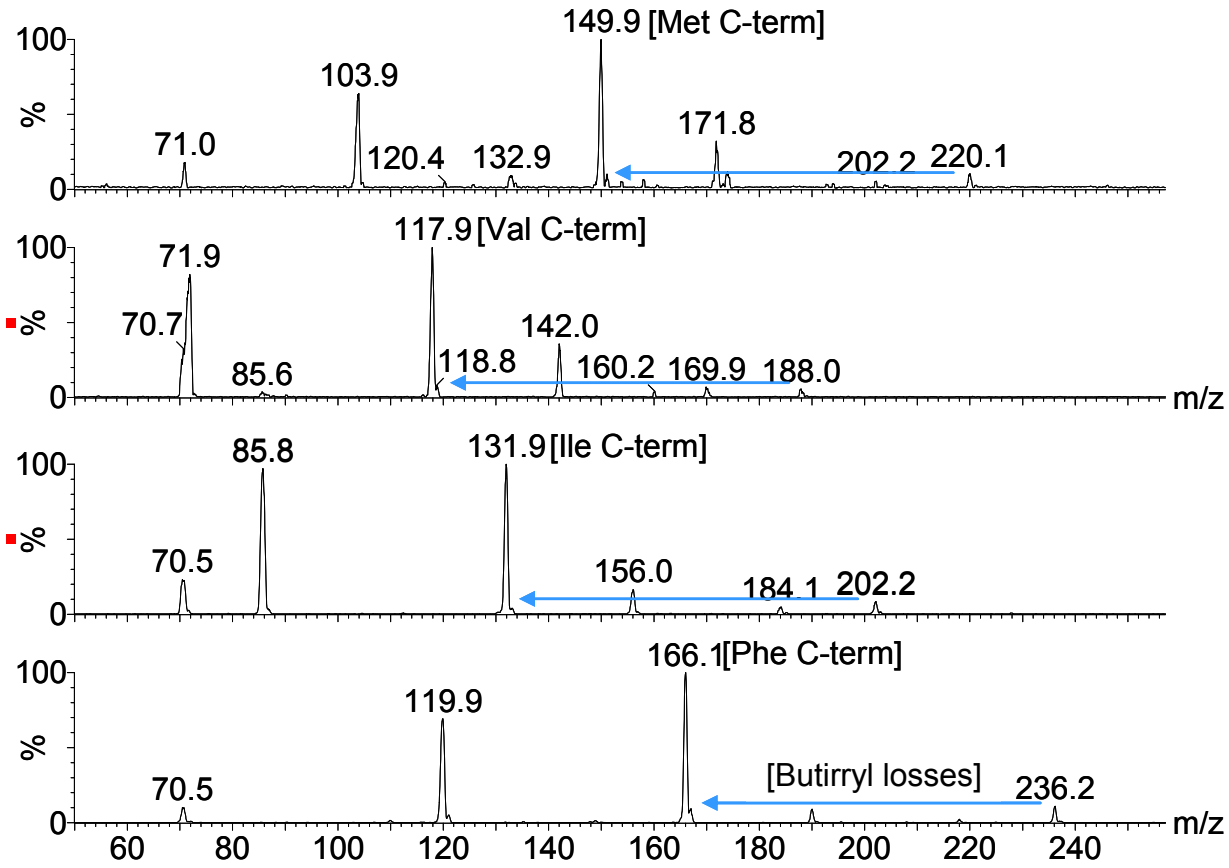


Figure 2.18. Characteristic product ion spectra of a group 3 compound.

In Figure 2.19 the proposed molecular structures of group 3 compounds, listed in Table 2.8 are reported.

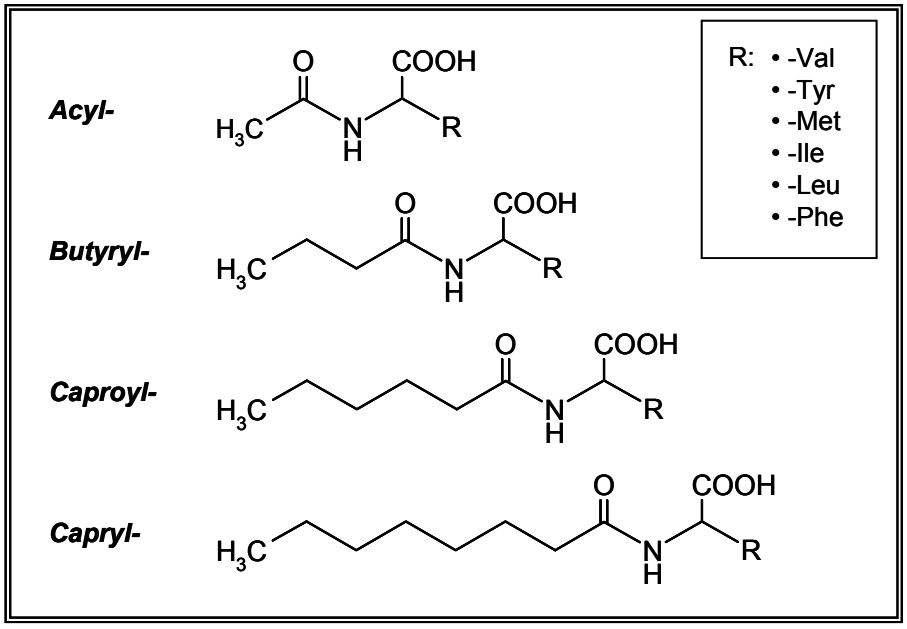


Figure 2.19. Proposed molecular structure of the group 3 compounds.

Table 2.8. Non proteolytic compounds identified in Parmigiano Reggiano cheese (group 3 peptides)

MW	*Amino acidic sequence*	*Qualifier ions*
173	Acetyl-Ile	85.6 (I C-term-HCOOH); 131.9(I C-term); 69.0(I C-term-HCOOH- NH_3)
173	Acetyl-Leu	85.6 (I C-term-HCOOH); 131.9(I C-term)
207	Acetyl-Phe	208.0 (MH^+); 166.0 (F C-term); 120.0 (i2)
187	Butyryl-Val	187.8(MH^+); 170.1(MH^+-H_2O); 142.0(MH^+-HCOOH); 118.1(V C-term); 71.8(V C-term-HCOOH)
219	Butyryl -Met	219.90 (MH^+); 150.1(M C-term); 201.2(MH^+-H_2O); 103.8(M C-term-HCOOH); 174.2(MH^+-HCOOH)
201	Butyryl -Ile	202.0 (MH^+); 184.2(MH^+-H_2O); 85.6 (I C-term-HCOOH); 131.9(I C-term); 69.0(I C-term-HCOOH- NH_3)
201	Butyryl -Leu	202.0 (MH^+); 184.2(MH^+-H_2O); 85.6 (I C-term-HCOOH); 131.9(I C-term)
235	Butyryl l-Phe	236.1(MH^+); 166.0(F C-term); 120.0(F C-term-HCOOH); 190.0(MH^+-HCOOH)
215	Caproyl-Val	118.1(V C-term); 71.8(V C-term-HCOOH)
247	Caproyl-Met	103.8(M C-term-HCOOH); 150.1(M C-term)
279	Caproyl-Tyr	182.0 (Y C-term); 136.0 (Y C-term-HCOOH)
229	Caproyl-Ile	85.6 (I C-term-HCOOH); 131.9(I C-term); 69.0(I C-term-HCOOH- NH_3)
229	Caproyl-Leu	85.6 (I C-term-HCOOH); 131.9(I C-term)
263	Caproyl-Phe	166.0(F C-term); 120.0(F C-term-HCOOH)
275	Capryl-Met	103.8(M C-term-HCOOH); 150.1(M C-term)
243	Capryl-Val	118.1(V C-term); 71.8(V C-term-HCOOH)
257	Capryl-Ile	85.6 (I C-term-HCOOH); 131.9(I C-term); 69.0(I C-term-HCOOH- NH_3)
257	Capryl-Leu	85.6 (I C-term-HCOOH); 131.9(I C-term)
291	Capryl-Phe	166.0(F C-term);120.0(F C-term-HCOOH)

The presence of N-Acetyl-L-amino acids in water soluble extracts of Parmigiano Reggiano cheese has been confirmed by spiking the sample with the respective commercial standards, while further studies will be necessary in order to confirm the molecular structures of the other compounds and to define their origin.

This group of compounds could be used to define the ageing time in highly ripened cheese, due to their particular trend during ripening, because they are present in a very small amount until the 18th month of ripening and after that they increase progressively.

2.3.3 Bioactive Peptides in Parmigiano Reggiano cheese

Milk proteins are well recognized as one of the most important sources of bioactive peptides, released from caseins or whey proteins by different digestion procedures. In this section the bioactive potential of peptides identified in Parmigiano Reggiano cheese has been investigated by comparison of their amino acidic sequence with those of known bioactive peptides. The results obtained are reported in Table 2.9.

Table 2.9. Comparison between bioactive peptides and peptides identified in Parmigiano Reggiano cheese. Matching sequences are indicated.

Bioactive peptides	*Biological activity*	*Peptides identified in P-R cheese*
αS1 f(43-58) DIGSESTEDQAMEDIK	Caseinphosphopeptide	αS1 f(55-58) EDIK
αS1 f(59-79) QMEAESISSSEEIVPNSVEQK αS1 f(66-74) SSSEEIVPN αS1 f(106-119) VPQLEIVPNSAEER	Caseinphosphopeptide	αS1 f(71-74) IVPN αS1 f(111-114) IVPN
αS1 f(102-109) KKYKVPQ αS1 f(106-119) VPQLEIVPNSAEER	antihypertensive caseinphosphopeptide	αS1 f(101-110) LKKYKVPQLE
αS1 f(90-96) RYLGYLE	opioid	αS1 f(94-97) YLEQ
β f(1-28) RELEELNVPGEIVESLSSSEESITRINK	immunomodulatory	β f(17-25)2P SLSSSEESITR
αS1 f(1-23) RPKHPIKHQGLPQEVLNENENLLRF	antimicrobic	αS1 f(14-17) ELVN
β f(193-202) YQEPVLGPVRGPFPI β f(191-193) LLY β f(193-209) LLYQEPVLGPVRGPFPIIV	antihypertensive immunomodulatory	β f(187-197) IQAFLLYQEPV
αS1 f(1-23) RPKHPIKHQGLPQEVLNENLLRF	immunomodulatory	αS1 f(10-14) GLPQE
β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28) ERELEELNVPGEIVESLSSSEESITRINK	caseinphosphopeptide	β f(16-25)3P LSSSEESITR
β(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28) ERELEELNVPGEIVESLSSSEESITRINK	immunomodulatory caseinphosphopeptide	β f(4-6) EEL
αS1 f(59-79) QMEAESISSSEEIVPNSVEQK αS1 f(66-74) SSSEIVPN	caseinphosphopeptide	αS1 f(70-74) EIVPN αS1 f(110-114) EIVPN
β(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28) ERELEELNVPGEIVESLSSSEESITRINK	immunomodulatory	β(15-25)3P SLSSSEESITR
αS1 f(66-74) SSSEEIVPN	caseinphosphopeptide	αS1 f(67-74)2P SSEEIVPN
αS1 f(66-74) SSSEEIVPN	caseinphosphopeptide	αS1 f(65-74)3P ISSSEEIVPN
αS1 f(66-74) SSSEEIVPN	caseinphosphopeptide	αS1 f(67-74)1P SSEEIVPN
β(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28) ERELEELNVPGEIVESLSSSEESITRINK	immunomodulatory caseinphosphopeptide	β f (1-6) RELEEL

Bioactive peptides	*Biological activity*	*Peptides identified in P-R cheese*
β(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28) ERELEELNVPGEIVESLSSSEESITRINK	immunomodulatory caseinphosphopeptide	β f (15-28)3P SLSSSEESITRINK
β(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(1-25) RELEELNVPGEIVESLSSSEESITR β f(1-28) RELEELNVPGEIVESLSSSEESITRINK β f(2-28)ERELEELNVPGEIVESLSSSEESITRINK	immunomodulatory caseinphosphopeptide	β f (2-6) ELEEL
αS1 f(23-27) FFVAP	antihypertensive	αS1 f(25-30) VAPFPE
β f(193-209) LLYQEPVLGPVRGPFPIIV β f(193-202) YQEPVLGPVRGPFPI	immunomodulatory antihypertensive	β f(193-209) LLYQEPVLGPVRGPFPIIV
β f (134-139) HLPLPL	antihypertensive	β f (136-143) PLPLLQSW

As can be seen, in most cases the peptides identified in Parmigiano Reggiano cheese match only with fragments of known bioactive peptides, indicating, as expected, that the proteolytic process occurring during ripening causes an extensive degradation of proteins that results in a release of shorter peptides than those obtained after digestion by the endoproteases usually used to release bioactive peptides.

In some cases, in which most of the sequence of the bioactive peptide was preserved, the biological property may eventually be maintained.

The bioaccessibility of these potential bioactive peptides has also been investigated by UPLC/ESI-MS analysis of WSE after simulated gastrointestinal digestion. All potential bioactive peptides were found to be resistant to digestive enzymes, indicating the possibility to reach the gut lumen in the intact form, a fundamental characteristic of bioavailability for the human organism.

Also all non proteolytic peptides proved to be resistant to the action of the gastrointestinal enzymes, probably due to their non conventional structure. Future studies will have to determine their potential as bioactive molecules.

Antioxidant activity of Parmigiano-Reggiano cheese

2.4 Aim of the work

Proteolysis is the most complex of all the primary events during the ripening of cheese, which results in the formation of various peptides. These peptides not only contribute to the development of flavour and texture in the ripened cheese but also show a potential bioactivity. The main objective of the present study is the evaluation of the antioxidant activity of water-soluble extracts of Parmigiano Reggiano cheese produced by different factories and in different stages of ripening (from 6 to 36 months).

Antioxidant activity will also be evaluated after simulated gastrointestinal digestion in order to verify the effective bioaccessibility of the antioxidant constituents of Parmigiano Reggiano cheese.

2.5 Results and Discussion

The antioxidant capacity of several samples of Parmigiano Reggiano cheese, coming from different factories and at different ageing time was evaluated. The Trolox Equivalent Antioxidant Capacity (TEAC) values were determined according to the capacity of each sample to scavenge the $ABTS^{\bullet+}$ up to 60 min of reaction time relative to Trolox, and the results were expressed in terms of μmoles of Trolox/g of cheese. The TEAC of the WSE for all cheeses is reported in Table 2.10.

Parmigiano Reggiano cheese, with an average TEAC value of about 60 μmol/g, were found to have a quite good antioxidant capacity. In comparison with other foods with known antioxidant capacity, one portion (about 30 g) of Parmigiano Reggiano cheese has, *in vitro*, the same antioxidant potential of 2 oranges or 1 kg of tomatoes or 1.5 glassfuls of red wine, according to the TEAC values reported by Pellegrini et al. [35].

TEAC values were not found to differ significantly among factories, indicating that the antioxidant capacity of Parmigiano Reggiano cheese is not strongly affected by the variability of the cheese making process.

Table 2.10. TEAC value of all WSE analysed (for each sample a code is provided; capital letter indicates the factory, the following number specifies the months of ripening and I or II distinguish different cheese coming from the same factory) .

Sample	TEAC (µmol/g)	Sample	TEAC (µmol/g)	Sample	TEAC (µmol/g)
A6-I	63.7	C6-II	62.4	E6-I	56.2
A6-II	51.7	C6-II	61.0	E12-I	64.6
A12-I	64.6	C14-I	71.6	E24-I	67.9
A12-II	56.4	C13-II	62.1	E37-I	71.3
A16-I	58.1	C18-I	67.4	E41-I	66.8
A16-II	47.7	C18-II	62.9		
A24-I	51.1	C24-I	70.0		
A24-II	45.1	C23-II	60.2		
A36-I	52.9	C36-I	47.5		
A36-II	51.3				

Sample	TEAC (µmol/g)	Sample	TEAC (µmol/g)	Sample	TEAC (µmol/g)
B6-I	66.7	D7-I	72.0	F11-I	60.0
B6-II	55.3	D6-II	60.6	F24-I	68.3
B12-I	63.0	D12-I	70.0	F33-I	67.9
B12-II	60.3	D12-II	66.2		
B18-I	61.4	D20-I	67.2		
B18-II	59.5	D26-I	59.6		
B25-I	51.8	D24-II	64.8		
B24-II	59.2	D41-I	59.0		
B36-I	59.1	D36-II	70.1		
B36-II	62.1				

The antioxidant activity was also evaluated at different stages of ripening (6, 12, 18, 24 and 36 months) and the results obtained are shown in Figure 2.20: TEAC values are reported as the mean of TEAC value determined in 10 different cheeses for each stage of ripening.

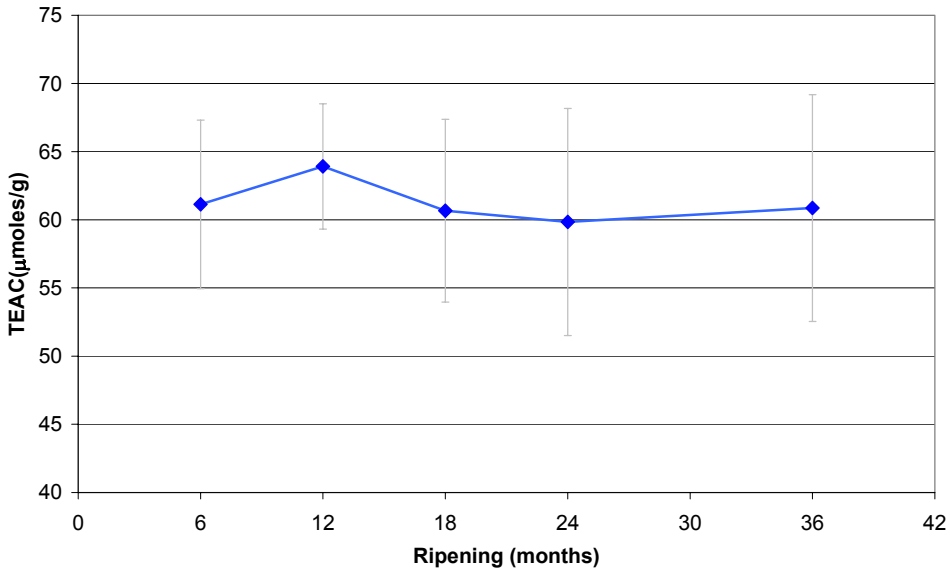


Figure 2.20. Antioxidant activity of WSE of Parmigiano Reggiano cheese during ripening.

It is evident that the antioxidant activity in Parmigiano Reggiano cheese remains quite constant during ripening, indicating that the proteolysis does not significantly affect the antioxidant capacity. Statistical analysis has confirmed that no significant correlation is present between the antioxidant capacity and cheese ripening.

2.5.1 Antioxidant activity of Parmigiano Reggiano cheese and peptide composition

The correlation between the oligopeptide fraction and the antioxidant capacity of Parmigiano Reggiano cheese was evaluated by performing bivariate correlation statistical tests, applying the Pearson correlation coefficient. Variables used were the months of ripening, TEAC values and the semiquantitative amount of each peptide in each samples (N=46). Only significant results obtained are reported (Table 2.11).

Table 2.11. Peptides statistically correlated with TEAC value.

Molecular weight	*Pearson coefficient*	*Sequence*
310	+291*	n.i.
1267	-344*	n.i.
260(b)	+295*	γ-Glu-Leu
960	-354*	n.i.
496	+291*	αS1 f(183-187) PINPIG
1938	-440*	n.i.
1881	-301*	β f(193-209) LLYQEPVLGPVRGPFPIIV
279	+520**	*Caproyl-Tyr*[(a)]
313	+410**	n.i.
4494	-491**	n.i.
4737	-376*	n.i.
229(b)	+302*	*Caproyl-Leu*[(a)]
3978	-319*	n.i.
4454	-503*	n.i.
275	+352*	*Capryl-Met*[(a)]
243	+327*	*Capryl-Val*[(a)]
257(a)	+412**	*Capryl-Ile*[(a)]
257(b)	+421**	*Capryl-Leu*[(a)]
291	+406**	*Capryl-Phe*[(a)]

**significant level higher than 95%*
***significant level higher than 99%*
n.i. *not identified*
[(a)] *proposed identification*

As can be seen, about 20 peptides or peptide-like molecules were found to be statistically correlated with the antioxidant capacity. It is interesting to note that peptides with higher molecular weight were negatively correlated to the antioxidant capacity while smaller molecules were positively correlated, indicating that the length of the chain plays a fundamental role in the determination of the antioxidant activity.

Quite interestingly, several non proteolytic peptides, also constituted by acyl residues, were found to be positively correlated. The reason for this correlation will have to be further investigated.

2.5.2 Bioaccessibility of antioxidant components

In order to evaluate whether Parmigiano Reggiano cheese may be a possible functional food, its antioxidant property was evaluated also after simulated gastrointestinal digestion, with the aim of verifying the bioaccessibility of the antioxidant components, a fundamental point in the assessment of bioavailability.

Several WSE of Parmigiano Reggiano cheese were submitted to a simulated gastrointestinal digestion taking into account gastric and intestinal phases and the antioxidant activity of the digests was determined by the ABTS assay. The TEAC values obtained, in comparison with those of the corresponding WSE, are reported in Figure 2.21.

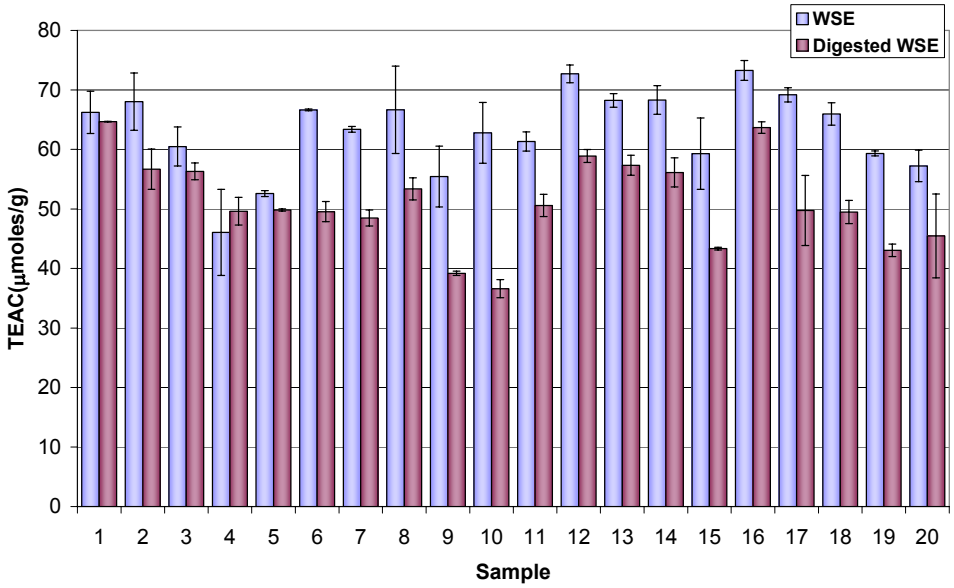


Figure 2.21. Comparison between the antioxidant activity of WSE and digested WSE of Parmigiano Reggiano cheese.

After gastrointestinal digestion about 20% of the antioxidant capacity of Parmigiano Reggiano cheese was lost in all compounds, indicating that a small amount of active compounds were inactivated by gastrointestinal enzymes. However the TEAC value remains quite good demonstrating that Parmigiano Reggiano may possess functional characteristics. Molecular composition analysis has confirmed the presence, in the

digested WSE, of the compounds positively correlated to the total antioxidant activity, showing their complete bioaccessibility.

2.6 Conclusion

The antioxidant capacity of Parmigiano Reggiano cheese from different factories and at different stages of ripening has been determined by the ABTS assay. The results obtained showed that Parmigiano Reggiano cheese possesses a quite good antioxidant capacity, comparable with that of other known food, such as oranges, tomatoes and red wine. The analysis of the total antioxidant activity of Parmigiano Reggiano cheese during ripening has shown that it is not particularly affected by the proteolytic process, indicating a fairly constant content in antioxidant components. Some peptides were found to be statistically correlated to the antioxidant capacity of cheese and deserve further investigation

Finally, it has been demonstrated, by analysing the antioxidant activity of gastrointestinal digested WSE, that Parmigiano Reggiano cheese maintains most of its antioxidant activity also after digestion, indicating its high potential as food with natural antioxidant properties.

Allergenic properties of Parmigiano-Reggiano cheese

The allergenic potential of Parmigiano Reggiano cheese has been evaluated with the aim of verifying whether allergenicity may decrease during cheese ripening, on account of the high degradation of proteins caused by proteolysis. Indeed it is known that several patients with demonstrated allergenicity to milk proteins can consume aged Parmigiano Reggiano cheese without evident clinical reactions.

The allergenic potential has been evaluated in collaboration with the *Center for Molecular Allergology, IDI-IRCCS (Rome, Italy)*. The Allergenic test has carried out by the Immuno Solid-phase Allergen Chip (ISAC). Proteomic microarray ISAC is a miniaturized immunoassay platform that allows for multiplex measurement of specific IgE antibodies to many allergen components using only few µl of serum or plasma. Purified natural or recombinant allergen components are immobilized on a solid support (biochip). In a two step assay, IgE antibodies from the patient serum bind to the immobilized allergen components. After a short washing step, allergen-bound IgE antibodies are detected by a fluorescence-labelled anti-IgE antibody. Test results are measured with a biochip scanner and evaluated using proprietary software. ISAC is a semiquantitative test and results are reported as a percentage of IgE inhibition.

Immunological tests on water soluble extracts of Parmigiano Reggiano cheese at different stages of ripening were carried out. IgE inhibition was determined by ISAC test performed on the serum of patients with milk allergy after its incubation with WSE of Parmigiano Reggiano cheese. Allergenic proteins spotted on the ISAC microarray were: α-lactoalbumin (Bos d 4), β-lactoglobulin (Bos d 5.0101, Bos d 5.0102), bovine serum albumin (Bos d 6), αS1-casein, β-casein and k-casein (Bos d 8), lactoferrin (Bos d).

The ISAC test results obtained are reported in Figure 2.22.

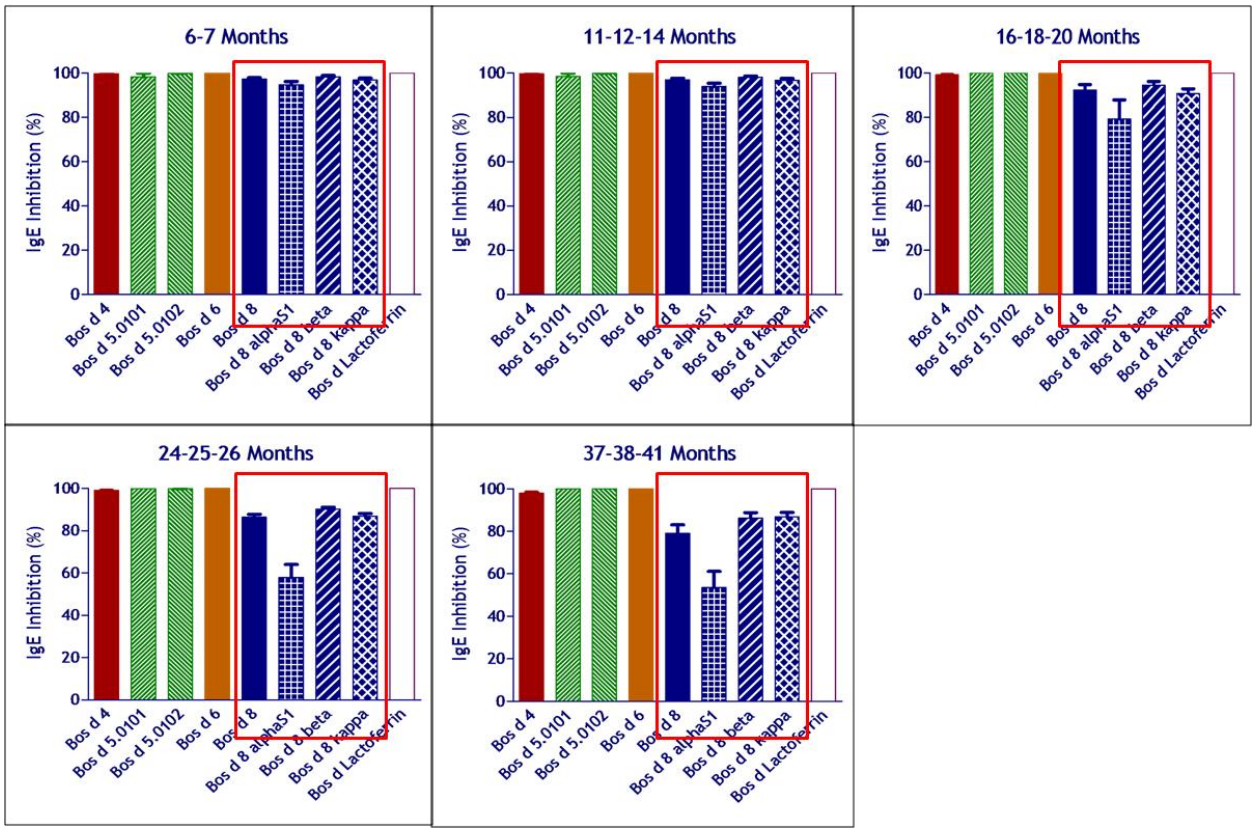


Figure 2.22. IgE inhibition of WSE of Parmigiano Reggiano cheeses at different stages of ripening.

It is evident that all samples have given positive results, meaning that in all WSE proteins identified from the sera of the patients as allergens are present. It is interesting to note that, during ripening, the IgE inhibition caused by caseins, the main allergens of milk, decrease, almost certainly due to their high degradation occurring during the maturation of cheese (only a small amount of them probably remains in intact form after 24 months of ripening). The same is not true for whey proteins: IgE inhibition remains constant at about 100% during all the ripening period. According to the results previously reported from the molecular characterization, these proteins were only slightly susceptible to the proteolytic phenomena, and can be found in intact form also after 36 months of ripening.

References

[1] Malacarne, M.; Summer, A.; Fossa, E.; Formaggioni, P.; Franceschi, P.; Pecorari, M.; Mariani, P., Composition, coagulation properties and Parmigiano-Reggiano cheese yield of Italian Brown and Italian Friesian herd milks. *Journal of Dairy Research* **2006,** 73, (2), 171-177.

[2] Mucchetti, G., & Neviani, E., Microbiologia e tecnologia lattiero casearia. Qualità e sicurezza. Tecniche Nuove Editore **(2006)**. Milano, Italy.

[3] McSweeney, P. L. H., Biochemistry of cheese ripening. *International Journal of Dairy Technology* **2004,** 57, (2-3), 127-144.

[4] Mayer, H. K.; Rockenbauer, C.; Mlcak, H., Evaluation of proteolysis in Parmesan cheese using electrophoresis and HPLC. *Lait* **1998,** 78, (4), 425-438.

[5] Addeo, F.; Chianese, L.; Salzano, A.; Sacchi, R.; Cappuccio, U.; Ferranti, P.; Malorni, A., Characterization of the 12-Percent Trichloroacetic Acid-Insoluble Oligopeptides of Parmigiano-Reggiano Cheese. *Journal of Dairy Research* **1992,** 59, (3), 401-411.

[6] Addeo, F.; Chianese, L.; Sacchi, R.; Musso, S. S.; Ferranti, P.; Malorni, A., Characterization of the Oligopeptides of Parmigiano-Reggiano Cheese Soluble in 120 G Trichloroacetic Acid/L. *Journal of Dairy Research* **1994,** 61, (3), 365-374.

[7] Ferranti, P.; Itolli, E.; Barone, F.; Malorni, A.; Garro, G.; Laezza, P.; Chianese, L.; Migliaccio, F.; Stingo, V.; Addeo, F., Combined high resolution chromatographic techniques (FPLC and HPLC) and mass spectrometry-based identification of peptides and proteins in Grana Padano Cheese. *Lait* **1997,** 77, (6), 683-697.

[8] Sforza, S.; Galaverna, G.; Neviani, E.; Pinelli, C.; Dossena, A.; Marchelli, R., Study of the oligopeptide fraction in Grana Padano and Parmigiano-Reggiano cheeses by liquid chromatography-electrospray ionisation mass spectrometry. *European Journal of Mass Spectrometry* **2004,** 10, (3), 421-427.

[9] Hinrichs, J., Incorporation of whey proteins in cheese. *International Dairy Journal* **2001,** 11, (4-7), 495-503.

[10] *www.uniprot.org*

[11] Alexander, L. J.; Hayes, G.; Pearse, M. J.; Beattie, C. W.; Stewart, A. F.; Willis, I. M.; Mackinlay, A. G., Complete sequence of the bovine beta-lactoglobulin cDNA. *Nucleic Acids Research* **1989,** 17, (16), 6739.

[12] Caroli, A. M.; Chessa, S.; Erhardt, G. J., Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science* **2009,** 92, (11), 5335-5352.

[13] Govindasamy-Lucey, S.; Jaeggi, J. J.; Bostley, A. L.; Johnson, M. E.; Lucey, J. A., Standardization of milk using cold ultrafiltration retentates for the manufacture of Parmesan cheese. *Journal of Dairy Science* **2004,** 87, (9), 2789-2799.

[14] Law, A. J. R.; Banks, J. M.; Horne, D. S.; Leaver, J.; West, I. G., Denaturation of the Whey Proteins in Heated Milk and Their Incorporation into Cheddar Cheese. *Milchwissenschaft-Milk Science International* **1994,** 49, (2), 63-67.

[15] Calvo, M. M.; Leaver, J.; Law, A. J. R.; Banks, J. M., Analysis of the Whey-Protein Fraction During Ripening of Cheddar Type Cheese Containing Heat Denaturated Beta-Lactoglobulin. *Milchwissenschaft-Milk Science International* **1992,** 47, (6), 343-347.

[16] Candioti, M. C.; Hynes, E. R.; Perotti, M. C.; Zalazar, C. A., Proteolytic activity of commercial rennets and pure enzymes on whey proteins. *Milchwissenschaft-Milk Science International* **2002,** 57, (9-10), 546-550.

[17] Sousa, M. J.; Ardo, Y.; McSweeney, P. L. H., Advances in the study of proteolysis during cheese ripening. *International Dairy Journal* **2001,** 11, (4-7), 327-345.

[18] Pecorari M., Fossa E., Sandri S., Mariani P., Proteolysis progress during the ageing of Parmigiano Reggiano cheese. *Scienza e tecnica lattiero casearia* **1997,** 48, (1), 61-72.

[19] Sforza, S.; Cavatorta, V.; Galaverna, G.; Dossena, A.; Marchelli, R., Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening. *International Dairy Journal* **2009,** 19, (10), 582-587.

[20] De Noni, I.; Cattaneo, S., Occurrence of beta-casomorphins 5 and 7 in commercial dairy products and in their digests following in vitro simulated gastro-intestinal digestion. *Food Chemistry* 119, (2), 560-566.

[21] Lignitto, L.; Cavatorta, V.; Balzan, S.; Gabai, G.; Galaverna, G.; Novelli, E.; Sforza, S.; Segato, S., Angiotensin-converting, enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. *International Dairy Journal* 20, (1), 11-17.

[22] Pritchard, S. R.; Phillips, M.; Kailasapathy, K., Identification of bioactive peptides in commercial Cheddar cheese. *Food Research International* 43, (5), 1545-1548.

[23] Nielsen, M. S.; Martinussen, T.; Flambard, B.; Sorensen, K. I.; Otte, J., Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time. *International Dairy Journal* **2009,** 19, (3), 155-165.

[24] Meisel, H., Bioactive peptides from milk proteins: a perspective for consumers and producers. *Australian Journal of Dairy Technology* **2001,** 56, (2), 83-92.

[25] Madureira, A. R.; Tavares, T.; Gomes, A. M. P.; Pintado, M. E.; Malcata, F. X., Invited review: Physiological properties of bioactive peptides obtained from whey proteins. *Journal of Dairy Science* 93, (2), 437-455.

[26] Korhonen, H., Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods* **2009,** 1, (2), 177-187.

[27] Moller, N. P.; Scholz-Ahrens, K. E.; Roos, N.; Schrezenmeir, J., Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition* **2008,** 47, (4), 171-182.

[28] Restani, P.; Ballabio, C.; Di Lorenzo, C.; Tripodi, S.; Fiocchi, A., Molecular aspects of milk allergens and their role in clinical events. *Analytical and Bioanalytical Chemistry* **2009,** 395, (1), 47-56.

[29] Oomen, A. G.; Rompelberg, C. J. M.; Bruil, M. A.; Dobbe, C. J. G.; Pereboom, D.; Sips, A., Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. *Archives of Environmental Contamination and Toxicology* **2003,** 44, (3), 281-287.

[30] Sforza, S.; Ferroni, L.; Galaverna, G.; Dossena, A.; Marchelli, R., Extraction, semi-quantifcation, and fast on-line identification of oligopeptides in Grana Padano cheese by HPLC-MS. *Journal of Agricultural and Food Chemistry* **2003,** 51, (8), 2130-2135.

[31] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* **1999,** 26, (9-10), 1231-1237.

[32] Schopen, G. C. B.; Heck, J. M. L.; Bovenhuis, H.; Visker, M.; van Valenberg, H. J. F.; van Arendonk, J. A. M., Genetic parameters for major milk proteins in Dutch Holstein-Friesians. *Journal of Dairy Science* **2009,** 92, (3), 1182-1191.

[33] Hill, J. P., The Relationship between Beta-Lactoglobulin Phenotypes and Milk-Composition in New-Zealand Dairy-Cattle. *Journal of Dairy Science* **1993,** 76, (1), 281-286.

[34] Summer, A.; Franceschi, P.; Bollini, A.; Formaggioni, P.; Tosi, F.; Mariani, P., Seasonal variations of milk characteristics and cheesemaking losses in the manufacture of Parmigiano-Reggiano cheese. *Veterinary Research Communications* **2003,** 27, 663-666.

[35] Pellegrini, N.; Serafini, M.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F., Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *Journal of Nutrition* **2003,** 133, (9), 2812-2819.

Part II

Novel Highly Proteolyzed Foods

Chapter 1
Development and validation of an LC/MS/MS method for the direct analysis of underivatized amino acids in food matrices

Amino acid analysis by liquid chromatography is of paramount importance in many biomedical and food applications, but, due to their peculiar polarity and absorption characteristics, their derivatization before or after chromatographic separation is usually mandatory.

The most used methods for determining amino acid contents in food and other biological matrices are usually based on ion-exchange liquid chromatography (IE-LC) coupled with UV detection, after post-column derivatization, usually with ninhydrin [1] [2], or, more commonly, on reversed phase liquid chromatography (RP-LC) with pre-column derivatization [3-4]. By using RP-HPLC the most common pre-column derivatizing agents are phenylisothiocyanate (PITC), o-phthalaldehyde (OPA), 9-fluorenylmethylchloroformate (Fmoc), 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl-Cl) and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ). Their use in RP-HPLC is essential because they allow for the spectroscopic detection of amino acids (UV or fluorescence) and make the amino acids hydrophobic enough to be easily separated in an RP-HPLC system.

On the other hand, most of the pre-column derivatization methods also bring about several drawbacks. PITC amino acid derivatives are only moderately stable at room temperature (1 day), the excess reagent must be removed before the analysis and short column life can result due to unreacted PITC injected into the column [5-12]. The main disadvantage of OPA is the instability of its derivatives (a few minutes) and its inability to react with secondary amines, thus making the proline detectable only if previously converted to primary amine by oxidazing reagents. Moreover, cysteine is not efficiently detected because of its propensity to react via the thiolic side chain, and can be reliably determined only after conversion of the thiol group by oxidation or alkylation [4,12-15]. Tryptophan cannot be detected by Fmoc derivatization, because the derivative is not fluorescent, due to the quenching effect exerted by the indole ring, whereas histidine and cyst(e)ine derivatives fluoresce weakly. Excess reagent must be extracted or

converted into a non interfering adduct before the injection [4,10,14-18]. By using Dabsyl-Cl the completeness of reaction can be adversely affected by the presence of various salts. Because the reaction efficiency is highly matrix dependent and variable for different amino acids, for an accurate calibration standard amino acid solution should be derivatized under conditions similar to those found in the matrix [19].

Pre-column derivatization with ACQ overcomes many of the drawbacks associated with the above derivatizing reagents: derivatives are very stable at room temperature and can be formed from both primary and secondary amines. Unreacted ACQ is not fluorescent and the product of the hydrolysis, 6-aminoquinoline (AMQ), is only weakly fluorescent. However, the method associated with the use of the commercial ACQ kit does not allow the detection of tryptophan, because the fluorescence of its corresponding derivative is very poor, and also Gln and Asn are missed [18,20-27].

Beside the lack of detection of many important amino acids by using these existing methods, underestimation of their content is often a problem in complex matrices, due to incomplete derivatization. Finally, these methods are also time consuming and labour intensive.

1.1 Aim of the work

The aim of this work is the development of a sensitive, reproducible, accurate and specific analytical procedure, based on liquid chromatography coupled with mass spectrometry detection, for the quantification of underivatized free amino acids in complex food matrices in order to overcome the main drawbacks present in the quantification of amino acids due to the derivatization procedure.

1.2 Experimental Procedures

1.2.1 Reagents

- Formic acid >99% (Acros Organics)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Deionised water obtained by using Millipore Alpha Q system
- L-Alanine, L-Arginine hydrochloride, L-Asparagine, L-Aspartic acid, L-Cysteine, L-Cystine, L-Glutamic acid, L-Glutamine, Glycine, L-Histidine hydrochloride, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine, D/L-Norleucine (Fluka)
- Amino Acid Hydrolysate Standard Mixture (SIGMA)
- AccQ Fluor™ reagent KIT (Waters)
- AccQ.Tag eluent A (Waters)

1.2.2 Instrumentation

- WB-OD 24 thermostatic bath (FALC)
- 1.5 ml disposable tubes (Eppendorf)
- HPLC Alliance 2695 (Waters)
- Quattro Micro™ API Mass Spectrometer (Waters)
- Spherisorb 5 µm (2.1X250 mm) CN narrow bore column (Waters)
- Proteo 4 µm C18 90 Å (250X2.0 mm) analytical column (Phenomenex)
- 470 Fluorescence detector (Waters)
- AccQ.Tag (3.9mmX150 mm) amino acid analysis column (Waters)
- MassLynx software (Waters)
- QuanLynx software (Waters)

1.2.3 Procedures

1.2.3.1 Standard solution preparation to LC-ESI/MS/MS analysis

All amino acid standard stock solutions were prepared at 2.5 mM concentration, by dissolving the required weight of each compound in deionised water. A mixed working standard solution, containing all 20 proteic amino acids, cystine and NorLeu was

prepared by suitable dilutions of the stock solution in the same solvent to obtain a final concentration at about 0.5 mM of each analyte. All stock solutions and working solution were then stored at −20°C.

1.2.3.2 Standard solution preparation to HPLC/FLD analysis

Calibration standard solutions were prepared by mixing 40 µl of D/L-Norleucine (2.5 mM in HCl 0.1 N), 40 µl of Amino Acid Hydrolysate Standard Mixture and 920 µl of deionised water.

1.2.3.3 HPLC/ESI-MS/MS analysis

The separation was achieved by using an Alliance 2695 separation system with a Spherisorb narrow bore column followed by a Proteo analytical column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 15 min, from 100% A to 30% B by linear gradient in 30 min, 10 min of a washing step with 100% B, plus reconditioning. Flow rate: 0.22 ml/min; first column temperature: 30°C; second column temperature: 25°C; Injection volume: 5 µl. Detection was performed by using a triple quadrupole Quattro MicroTM API (Micromass, Manchester, UK) mass spectrometer applying the following conditions: positive ionization mode, capillary voltage 3.0 kV, cone voltage 25 V, source temperature 130°C, desolvation temperature 350°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 600 l/h; collision energy 14 eV, dwell time: 0.1 s, Selected Reaction Monitoring (SRM) detection.

All data were acquired and processed by the software MassLynx 4.0 (Waters).

Quantitative analysis was set up by the internal standard method, with D/L-norleucine as internal standard. Amino acid contents were estimated as follows: $AAC = Rf(A_S/A_{IS}) \times nmoles_{IS}$, where: AAC, amino acid content in nmoles; A_S, amino acid area in the sample obtained by the integration of the SRM chromatogram; A_{IS}, internal standard area obtained in the same way.

1.2.3.4 HPLC/FLD analysis

The derivatization step was carried out by using AccQ Fluor^{TM} reagent KIT, 10 µl of standard solution were transferred into a 1.5 ml tube, 70 µl of borate buffer (included in the reagent kit) were added and the solution was briefly vortexed. Then, 20 µl of

reconstituted AccQ.Fluor reagent was added and the mixture was immediately vortexed for several seconds. The tube was closed and left to stand for one minute at room temperature. It was then heated in a heating bath at 55°C for 10 min. The resulting derivatized standard solution was diluted with 400 µl of deionised water before injecting. Standard solution were analysed using an Alliance 2695 with a Waters AccQ.Tag amino acid analysis column (3.9mmX150 mm). The column was thermostated at 37°C and the flow rate was 1.0 ml/min. The injection volumes of standard calibration solution were 5. 10, 15, 20, 25 and 30 µl, corresponding to 10, 20, 30, 40, 50, 60 pmoles injected.
The mobile phase A consisted of AccQ.Tag eluent A (100 ml AccQ.Tag A concentrate+1L deionised water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionised water respectively. Gradient elution was performed statring from 100% eluent A according to the following steps:1 min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 Fluorescence detector (λ excitation 250 nm and λ emission 395 nm). Quantitative analysis was performed by the internal standard method, with D/L-norleucine as internal standard. Amino acid contents were estimated as follows: $AAC = Rf(A_S/A_{IS}) \times pmoles_{IS}$, where: AAC, amino acid content in pmoles; A_S, amino acid area; A_{IS}, internal standard area.

1.3 Results and Discussion

1.3.1.1 Set up of the MS conditions for SRM monitoring

In order to set up the optimal instrumental parameters for maximizing the molecular ions intensity, a solution containing about 0.1 mM of each amino acid in acidified water, was first directly infused into the mass spectrometer (data not shown).
Then, several collision parameters were tested in order to obtain a product ion spectrum containing both the molecular ion and the characteristic fragments of each amino acid.
Optimal collision energies (CE) for amino acid fragmentation were found ranging between 12 and 16 eV; at CE lesser than 12 eV the fragmentations were too poor and at more than 16 eV the molecular ions were absent. CE at 14 eV resulted in nearly every case the optimal value. This collision energy was then used to perform SRM experiments, in which the reaction from the molecular ion to the most abundant fragment was monitored. SRM parameters are given in Table 1.1. For most amino acids

analyzed, the strongest transition was the loss of the COOH moiety from the neutral amino acid.

Amino acids	*Parent ion*	*Quantifier ion*	*Qualifier ions*
Gly	76.0	29.7	
Ala	90.0	43.4	
Ser	106.0	59.6	69.6-87.8
Thr	120.0	73.7	55.6-101.9
Cys	122.0	75.9	86.8-58.8
Asn	133.0	73.7	87.9-115.9
Asp	134.0	73.7	87.8-69.6
Gln	147.0	83.9	129.9-101.8
Glu	148.0	83.9	101.9-129.9
Cyss	241.0	152.0	122.0
Pro	116.0	69.8	115.8
Val	118.0	71.7	54.6
Lys	147.0	83.9	129.9
His	156.0	110.0	94.9
Arg	175.0	69.8	116.1-59.7
Leu	132.0	85.9	
Ile	132.0	85.9	68.9
Norleu	132.0	85.9	
Met	150.0	103.9	55.5-132.9
Tyr	182.0	136.0	123.0-165.0
Phe	166.0	119.9	131-102.9
Trp	205.0	188.0	146.0

Table 1.1. Parent ions and product ions of each amino acid.

The use of MS/MS experiments allowed to obtain for every amino acid specific transitions, except in the case of compounds having the same molecular weight and giving the same product ion spectra; this was obviously the case of leucine and isoleucine, but also of lysine and glutamine, isobaric in a low resolution mass spectrometer. The product ion spectra of these compounds are shown in Figure 1.1. This problem made the chromatographic separation of Ile/Leu and Lys/Gln a fundamental step for the correct quantification of all free amino acids.

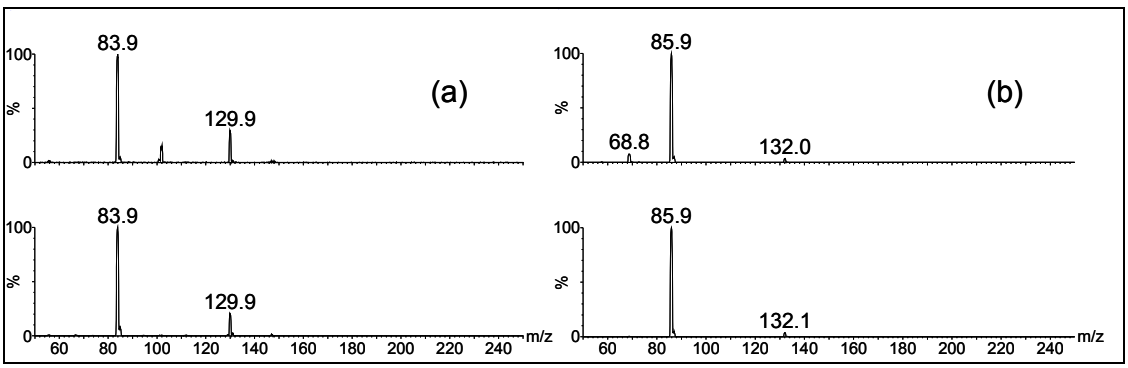


Figure 1.1. (a) Product ion spectra of Ile (above) and Leu (below); (b) Product ion spectra of Gln (above) and Lys (below).

Conditions including an apolar stationary phase, constituted by a C_{18} analytical column, and a mixture of acidified water with an organic volatile solvent, optimal for RP-HPLC separation, and also optimal for ESI/MS in the positive ion mode, were first tested. By using this column, leucine and isoleucine were nicely resolved, but lysine and glutamine coeluted and moreover, not surprisingly, most polar amino acids eluted with the dead volume, a condition better to be avoided in complex mixtures, since polar components like salts give rise to ionic suppression phenomena (Figure 1.2).

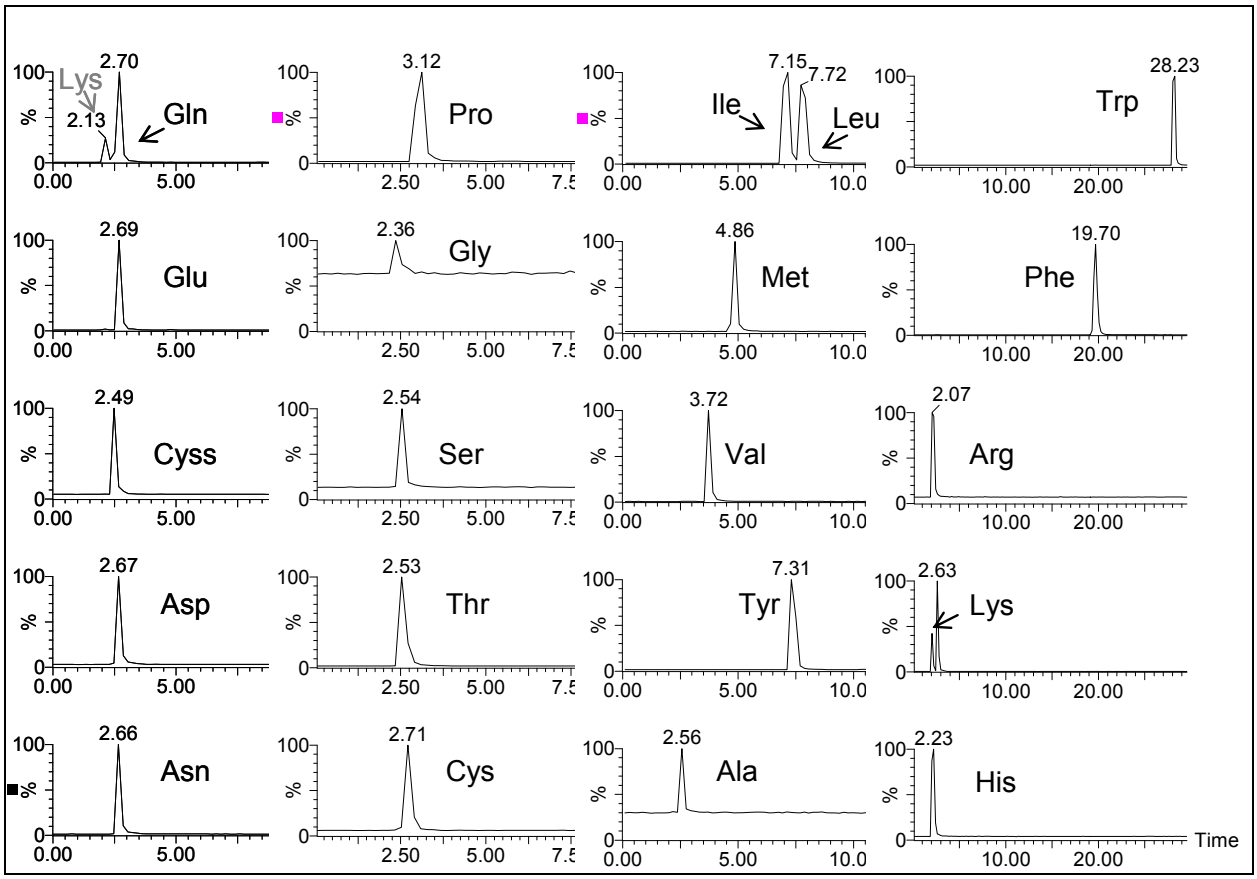


Figure 1.2. SRM chromatograms obtained after analysis of standard solution containing all proteinaceous amino acids, cystine and D/L-norleucine by using a C_{18} analytical column.

Therefore, a more polar stationary phase (a cyano column) was tested. In this case, most of the amino acids were well retained, glutamine and lysine were resolved, but leucine and isoleucine coeluted (Figure 1.3).

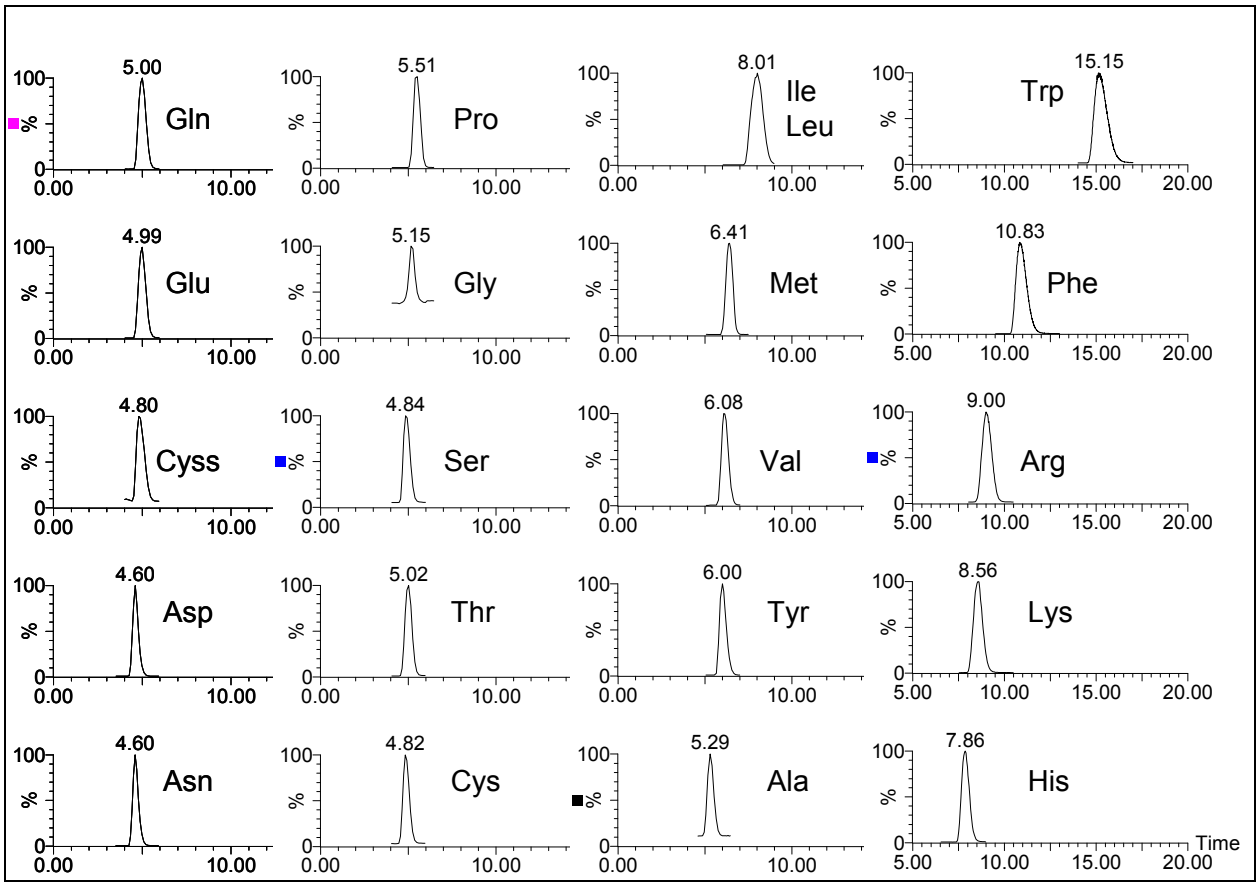


Figure 1.3. SRM chromatograms obtained after analysis of amino acids standard solution containing all proteinaceous amino acids, cystine and D/L-norleucine by using CN column.

In order to overcome these problems, a separation system with the two columns, C_{18} and cyano, linked in series was set up. In this system the polar stationary phase first allowed to obtain a good retention of each amino acid and to resolve glutamine and lysine completely, whereas the second apolar stationary phases allowed the resolution of leucine and isoleucine, making the use of Nor-leucine also possible as internal standard. Even if not all the amino acids were perfectly resolved, the use of SRM detection allowed to specifically detect every desired compound.

The Single Reaction Monitoring (SRM) chromatograms obtained by the simultaneous use of two different stationary phases in series are shown in Figure 1.4.

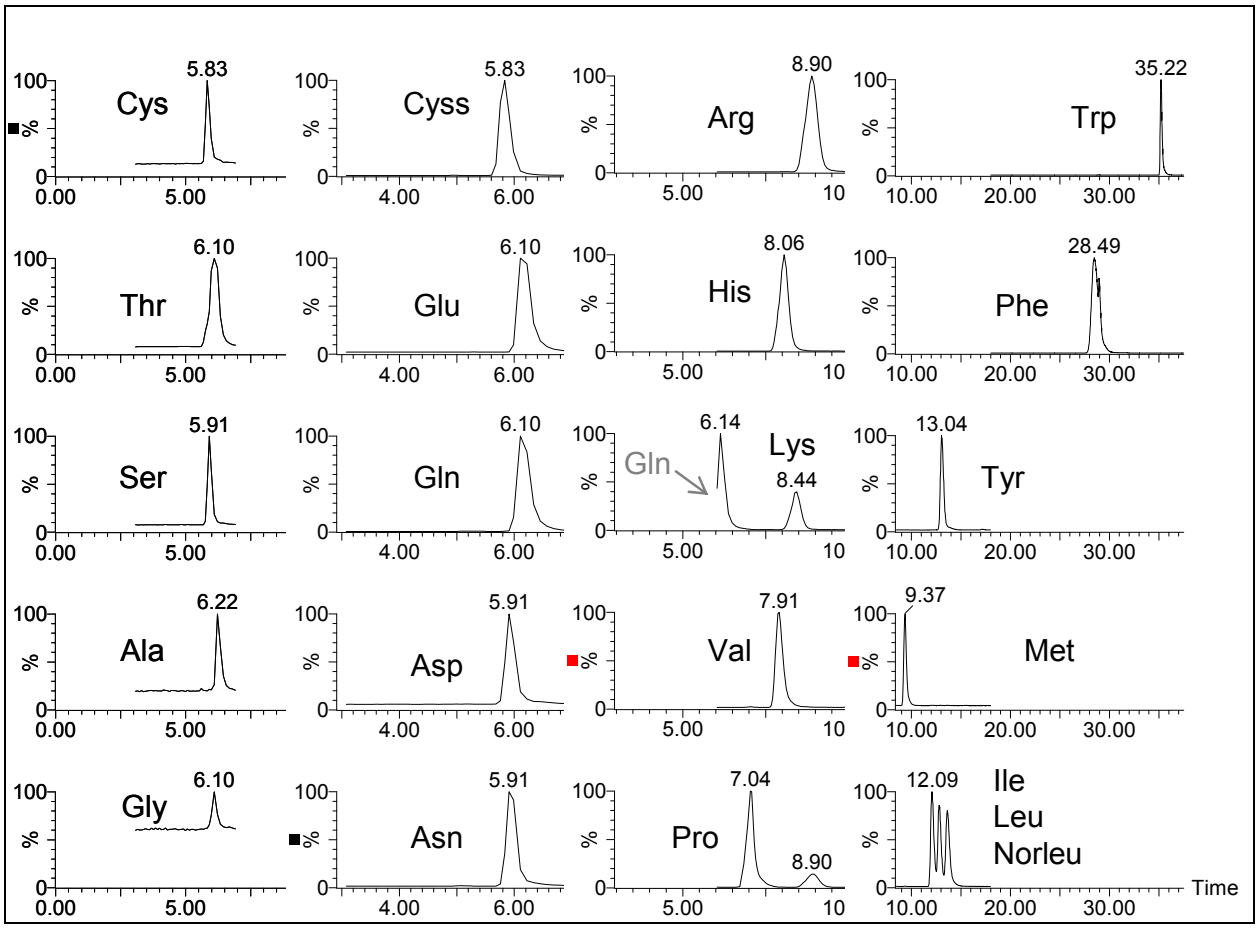


Figure 1.4. SRM chromatograms obtained after analysis of standard solution containing all proteinaceous amino acids, cystine and D/L-norleucine.

The most serious problem potentially arising from the use of two columns in series, the excessive back pressure, was in this case limited by the use of narrow bore columns with low flow rate. The system back pressure in that case was about 180 Bar, totally compatible with the HPLC system.

1.3.1.2 Quantitative analysis

The quantitative analysis was set up by the internal standard method, with D/L-norleucine as internal standard.

1.3.1.3 Linearity range

The linearity range was verified between 0.1 and 1 nmole injected by analysing in triplicate standard solutions, containing Nor-Leucine as internal standard, at 10 different concentrations. The regression equation parameters and linearity correlation coefficients obtained are reported in Table 1.2.

Amino acids	m	q	r^2
Gly	199.180	-0.112	0.9812
Ala	33.528	-0.064	0.9982
Ser	12.986	-0.082	0.9979
Thr	6.349	-0.082	0.9948
Cys	19.792	-0.099	0.9830
Asn	2.601	-0.050	0.9925
Asp	10.025	-0.107	0.9944
Gln	0.880	-0.015	0.9981
Glu	2.6629	-0.0359	0.9983
Cyss	1.092	-0.001	0.9833
Pro	0.494	-0.054	0.9985
Val	1.903	-0.035	0.9968
Lys	1.443	-0.041	0.9908
His	0.409	-0.011	0.9932
Arg	0.586	0.009	0.9802
Leu	1.036	-0.008	0.9964
Ile	1.099	-0.012	0.9963
Norleu	4.237	0.009	0.9976
Met	1.564	-0.004	0.9970
Tyr	0.320	0.017	0.9995
Phe	1.151	0.032	0.9921
Trp	1.151	0.032	0.9921

Table 1.2. Linear regression curve parameters and correlation coefficients of each amino acid analyzed.

1.3.1.4 Quantification limit (LOQ)

The signal-to-noise ratio criterion was used to estimate the limit of quantification, corresponding to the analyte amount for which the signal-to-noise ratio was equal to 10. By this approach the LOQ of the method was found to be 100 pmoles injected for Gly and Ala and 50 pmoles injected for all the other amino acids in a standard solution.

The values obtained showed that the sensitivity of the method, albeit lower than that of the fluorescence detection, was adequate for the determination of the amino acid content in many food matrices.

1.3.1.5 Repeatability

The repeatability of the method is reported in Table 1.3, in terms of coefficients of variation calculated for three injections of one standard solution containing all 21 amino acids, which were found to range between 0.46 and 3.4%.

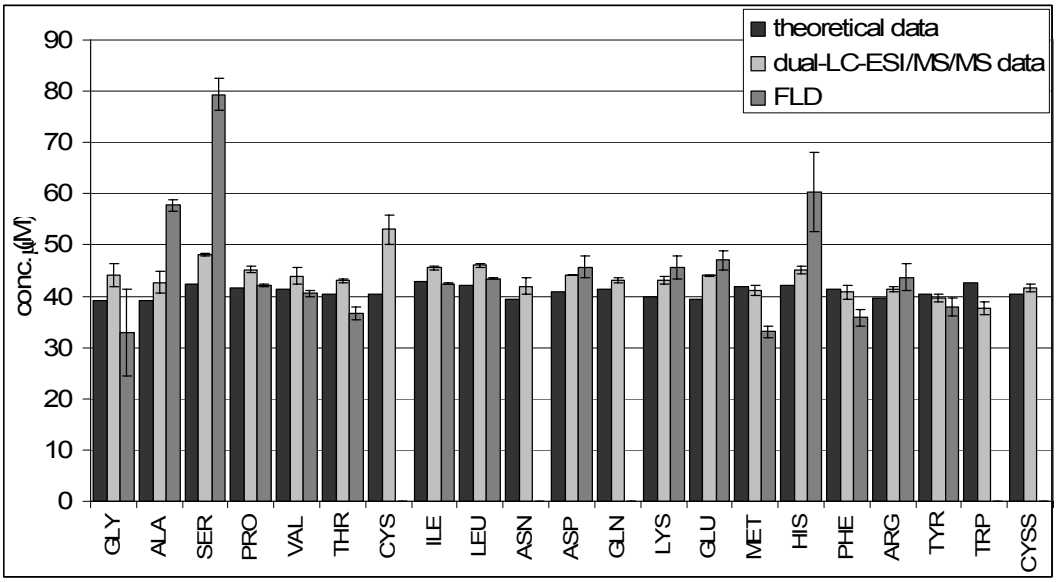
Amino acids	*Instrumental (VC%)*
Gly	3.39%
Ala	2.04%
Ser	0.91%
Thr	0.46%
Cys	1.6%
Asn	1.45%
Asp	1.46%
Gln	0.71%
Glu	1.56%
Cyss	1.50%
Pro	1.19%

Amino acids	*Instrumental (VC%)*
Val	1.63%
Lys	1.71%
His	1.25%
Arg	1.91%
Leu	1.77%
Ile	0.56%
Nor-Leu	0.91%
Met	3.23%
Tyr	0.77%
Phe	0.77%
Trp	0.77

Table 1.3. Precision values expressed as coefficient of variation (calculated in standard solution containing all free amino acids).

1.3.1.6 Accuracy

A standard solution containing all amino acids at known concentrations was analyzed and quantified in order to confirm the accuracy of this new approach. In Figure 1.5 the concentrations of the single amino acids in the standard solution obtained from *dual*-LC-ESI/MS/MS analysis compared with the theoretical data are reported. The same standard solution was also analysed by a standard method using fluorescence detection after pre-column derivatization with ACQ.



	Recovery (%)	
	MSMS	FLD
GLY	113	84
ALA	109	148
SER	113	187
PRO	108	101
VAL	106	98
THR	106	91
CYS	131	0
ILE	106	99
LEU	109	103
ASN	107	0
ASP	108	112
GLN	104	0
LYS	108	114
GLU	112	120
MET	98	79
HIS	107	143
PHE	99	87
ARG	105	110
TYR	98	94
TRP	89	0
CYSS	103	0

Figure 1.5. Comparison between the quantification of free amino acid in a standard solution obtained by dual-HPLC/MS/MS analysis and standard HPLC/FLD method with theoretical data. For each amino acid the percentage recovery is also reported.

The results obtained show very good agreement between the data obtained by LC/MSMS and the theoretical ones and between the two methods for most compounds,

except Ala, Ser and His. It is clear, given the results reported in Figure 1.5, that the LC/FLD method overestimates these compounds, although the reasons for this overestimation have not been investigated. It is also to be noted that several amino acids not detectable with the standard LC/FLD method (asparagine, glutamine, cysteine, cystine and tryptophan) can be quantified by the present dual-LC/MSMS method.

1.3.1.7 Application of the method to real food matrices

In order to demonstrate the applicability of this new method, free amino acids in several proteinaceous hydrolyzates obtained from by-products of the poultry industry were analyzed (sample details and data obtained are reported in Part II 2.3.1). The data were in good agreement with the different hydrolyzate digestion processes, showing in every case an amino acidic amount correlated with the hydrolytic degree.

The same samples spiked with an amino acid standard solution containing increasing amounts of all 21 amino acids showed linear regression curves with coefficients not significantly different from those obtained from standard solutions, showing the absence of possible interferences due to the matrix effect.

Finally one of the poultry protein hydrolyzate was analysed by a standard method using fluorescence detection after pre-column derivatization with ACQ. The results obtained, reported in Figure 1.6, show good agreement between the two methods for most compounds.

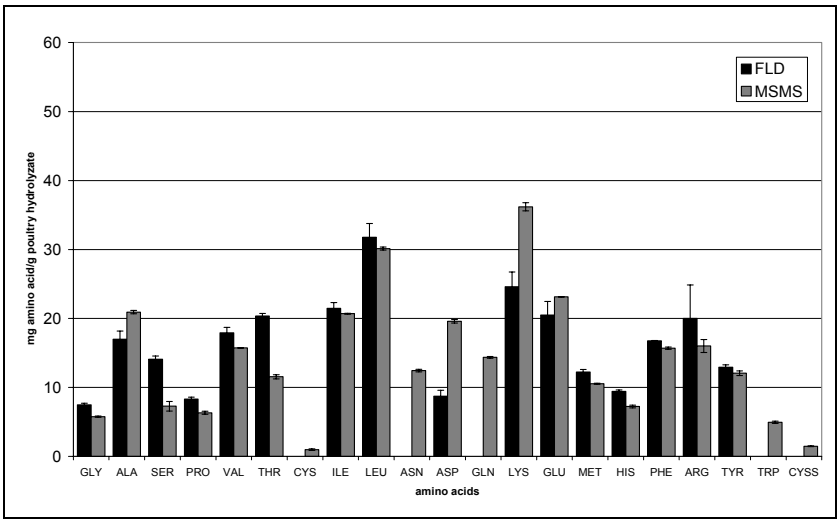


Figure 1.6. Comparison between the quantification of free amino acid in a poultry protein hydrolyzate obtained by dual-HPLC/MS/MS analysis with those obtained by using a standard HPLC/FLD method.

1.4 Conclusion

The new *dual*-LC/MS/MS method for the analysis of underivatized amino acids here presented, which combines two different stationary phases placed in series on the separation system, a polar cyano column followed by a non polar C_{18} column, has yielded accurate results. This new method, without performing any derivatization procedure, allowed the detection of every single proteinaceous amino acid, including Trp, Gln, Asn, Cys and Cyss. The application of the method to samples of hydrolyzates derived from poultry leftovers allowed to determine all proteic amino acids rapidly and accurately.

References

[1] Friedman, M., Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agricultural and Food Chemistry* **2004,** 52, (3), 385-406.

[2] Fontaine, J.; Eudaimon, M., Liquid chromatographic determination of lysine, methionine, and threonine in pure amino acids (feed grade) and premixes: Collaborative study. *Journal of Aoac International* **2000,** 83, (4), 771-783.

[3] Fekkes, D., State-of-the-art of high-performance liquid chromatographic analysis of amino acids in physiological samples. *Journal of Chromatography B-Biomedical Applications* **1996,** 682, (1), 3-22.

[4] Schwarz, E. L.; Roberts, W. L.; Pasquali, M., Analysis of plasma amino acids by HPLC with photodiode array and fluorescence detection. *Clinica Chimica Acta* **2005,** 354, (1-2), 83-90.

[5] Sarwar, G.; Botting, H. G., Evaluation of Liquid-Chromatographic Analysis of Nutritionally Important Amino-Acids in Food and Physiological Samples. *Journal of Chromatography-Biomedical Applications* **1993,** 615, (1), 1-22.

[6] Woo, K. L., Determination of amino acids in foods by reversed-phase HPLC with new precolumn derivatives, butylthiocarbamyl, and benzylthiocarbamyl derivatives compared to the phenylthiocarbamyl derivative and ion exchange chromatography. *Molecular Biotechnology* **2003,** 24, (1), 69-88.

[7] Checa-Moreno, R.; Manzano, E.; Miron, G.; Capitan-Vallvey, L. F., Revisitation of the phenylisothiocyanate-derivatives procedure for amino acid determination by HPLC-UV. *Journal of Separation Science* **2008,** 31, (22), 3817-3828.

[8] Shang, S. F.; Wang, H., Sensitive determination of amino acids in kelp by reversed phase high performance liquid chromatography with precolumn derivatization using phenylisothiocyanate. *Chromatographia* **1996,** 43, (5-6), 309-312.

[9] Woo, K. L.; Hwang, Q. C.; Kim, H. S., Determination of amino acids in the foods by reversed-phase high-performance liquid chromatography with a new precolumn derivative, butylthiocarbamyl amino acid, compared to the conventional phenylthiocarbamyl derivatives and ion-exchange chromatography. *Journal of Chromatography A* **1996,** 740, (1), 31-40.

[10] Lai, F.; Mayer, A.; Sheehan, T., Matrix Effects in the Derivatization of Amino-Acids with 9-Fluorenylmethyl Chloroformate and Phenylisothiocyanate. *Biotechniques* **1991,** 11, (2), 236-244.

[11] Puigdeu, M.; Buxaderas, S., Analytical Conditions for the Determination of 23 Phenylthiocarbamyl Amino-Acids and Ethanolamine in Musts and Wines by High-Performance Liquid-Chromatography. *Journal of Chromatography A* **1994,** 685, (1), 21-30.

[12] Avino, P.; Campanella, L.; Russo, M. V., High-performance liquid chromatography intercomparative study for amino acid analysis in two tissues by PITC- and OPA-derivatizations. *Analytical Letters* **2001,** 34, (6), 867-882.

[13] Pappa-Louisi, A.; Sotiropoulos, S.; Balkatzopoulou, P., Mobile phase pH, column temperature, and eluent flow rate effects on separation and fluorescence-electrochemical detection of OPA derivatives of amino acids in reversed-phase liquid chromatography. *Journal of Liquid Chromatography & Related Technologies* **2008,** 31, (10), 1434-1447.

[14] Herbert, P.; Barros, P.; Ratola, N.; Alves, A., HPLC determination of amino acids in musts and port wine using OPA/FMOC derivatives. *Journal of Food Science* **2000,** 65, (7), 1130-1133.

[15] Butikofer, U.; Fuchs, D.; Bosset, J. O.; Gmur, W., Automated Hplc-Amino Acid Determination of Protein Hydrolysates by Precolumn Derivatization with Opa and Fmoc and Comparison with Classical Ion-Exchange Chromatography. *Chromatographia* **1991,** 31, (9-10), 441-447.

[16] Jambor, A.; Molnar-Perl, I., Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride Literature overview and further study. *Journal of Chromatography A* **2009,** 1216, (15), 3064-3077.

[17] Melucci, D.; Xie, M.; Reschiglian, P.; Torsi, G., FMOC-Cl as derivatizing agent for the analysis of amino acids and dipeptides by the absolute analysis method. *Chromatographia* **1999,** 49, (5-6), 317-320.

[18] Bernal, J. L.; Nozal, M. J.; Toribio, L.; Diego, J. C.; Ruiz, A., A comparative study of several HPLC methods for determining free amino acid profiles in honey. *Journal of Separation Science* **2005,** 28, (9-10), 1039-1047.

[19] Chen, Y. H.; Shih, L. L.; Liou, S. E.; Chen, C. C., Analysis of dabsyl-Cl derivated amino acids by high performance liquid chromatography and tandem mass spectrometry. *Food Science and Technology Research* **2003,** 9, (3), 276-282.

[20] Strydom, D. J.; Cohen, S. A., Comparison of Amino-Acid Analyses by Phenylisothiocyanate and 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate Precolumn Derivatization. *Analytical Biochemistry* **1994,** 222, (1), 19-28.

[21] vanWandelen, C.; Cohen, S. A., Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate-derivatized amino acid mixtures. *Journal of Chromatography A* **1997,** 763, (1-2), 11-22.

[22] Deantonis, K. M.; Brown, P. R.; Cohen, S. A., High-Performance Liquid-Chromatographic Analysis of Synthetic Peptides Using Derivatization with 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate. *Analytical Biochemistry* **1994,** 223, (2), 191-197.

[23] Cohen, S. A.; Deantonis, K. M., Applications of Amino-Acid Derivatization with 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate - Analysis of Feed Grains, Intravenous Solutions and Glycoproteins. *Journal of Chromatography A* **1994,** 661, (1-2), 25-34.

[24] Pappa-Louisi, A.; Nikitas, P.; Agrafiotou, P.; Papageorgiou, A., Optimization of separation and detection of 6-aminoquinolyl derivatives of amino acids by using reversed-phase liquid chromatography with on line UV, fluorescence and electrochemical detection. *Analytica Chimica Acta* **2007,** 593, (1), 92-97.

[25] Bosch, L.; Alegria, A.; Farre, R., Application of the 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2006,** 831, (1-2), 176-183.

[26] Hernandez-Orte, P.; Ibarz, M. J.; Cacho, J.; Ferreira, V., Amino acid determination in grape juices and wines by HPLC using a modification of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) method. *Chromatographia* **2003,** 58, (1-2), 29-35.

[27] Diaz, J.; Lliberia, J. L.; Comellas, L.; BrotoPuig, F., Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection. *Journal of Chromatography A* **1996,** 719, (1), 171-179.

Chapter 2 Novel Highly Proteolyzed foods: Molecular Characterization

In the process of production of meat for human consumption approximately 50% of the animal is turned into animal by-products. The rendering and fat industry provides the vital outlet for these materials by transforming them into a wide variety of products, while reducing pollution loads. Unfortunately, most of the left-overs are subjected to incineration or used as compost for soil fertilization [1]. Only about 22% is used for pet food and 2-3% is converted into food and animal feeding stuffs. Thus a major portion of a pool of animal protein with naturally balanced amino acid composition is discarded.

Moreover, today, many people, principally in the third world countries are suffering protein deficiency. Worldwide, the poultry industry is the most important source of inexpensive meat-derived proteins for human nutrition.

Poultry industry by-products are mainly represented by meat trimmings, bones and feathers and contribute in a substantial way to the pollution loads [2,3]. Chicken meat and bone residues containing both collagens and myofibril proteins could be considered as valuable raw material for the production of multifunctional food-grade protein ingredients for high value-added products. Bones and meat trimmings contain up to 10% of valuable and easily accessible proteins, fit even for human consumption. Nowadays, the valorisation of animal by-products ranges high on the economic agenda in various regions of the world.

Traditional rendering technologies are based on long-term heating of the mixed residues (bones, meat trimmings, visceral residue, blood clots, legs and heads, feathers): this approach provides for the inactivation of infectious agents (viruses and bacteria), somewhat improves digestibility and allows separation of the input material into a solid fraction and a liquid tallow/fat which are utilized separately. The drawback of this technology is the elevated energy input required. Besides, cooking causes degradation of biologically valuable components as well as the appearance of abnormal and potentially biologically harmful compounds in the liquid and gas states; on account of these factors the balanced proteins of the input material are converted to a meal with compromised alimentary and functional properties and a low commercial price. In short, high energy consumption and the low quality of the end product determine economic inefficiency of the cooker rendering technology. New biocatalytic technologies of

conversion of poultry meat and bone residues into proteic hydrolyzates of high nutritional value have been elaborated [4,5]. The approach proposed is based on the deep controlled bioconversion of meat and bone residues by enzymatic hydrolysis carried out at neutral pH and relatively low temperature (50-55°C), thus providing high retention of biologically valuable compounds such as amino acids, peptides and unsaturated fatty acids [6,7]. Functional and biological properties of the hydrolyzates produced have proved to be affected by the hydrolysis conditions [4,5]. A detailed description of the molecular composition of the peptide hydrolyzates, the correlation with the process conditions, a thorough assessment of the nutritional value, are nevertheless still lacking. In the framework of a European project named “PROSPARE” (PROgress in Saving Proteins And Recovering Energy), proteic hydrolyzates (also called “Functional Animal Proteins” or FAP), have been produced by new enzymatic technologies and are planned to be used as a food protein substance in instant foods and processed meat products. Mild conditions of short-term hydrothermal and enzymatic hydrolytic steps, finely tuned to the concrete types of the poultry slaughtering raw materials, were set in order to ensure high preservation of the biologically valuable compounds.

Feathers, which contain 85% keratin (calculated on the basis of the dry weight), a poorly soluble and heat resistant protein with high content of cysteine and multiple intramolecular cross-links, have also been processed separately by a short term extra-heating of the native feathers under simultaneous mechanical pressure, which results in the breaking of the hydrogen bond network and a partial non-enzymatic hydrolysis of the protein main chains. The resulting feather hydrolyzates (also called “Functional Feather Proteins” or FFP) can be used for the development of feed products [8].

In the present chapter a detailed molecular characterization of the nitrogen fraction of these proteic hydrolyzates, including total and soluble nitrogen determination, free and total amino acid content, racemisation, low molecular weight peptides and other nitrogen compounds, together with the assessment of their nutritional value, is reported.

2.1 Aim of the work

The aim of the current work is to provide the molecular characterization of poultry protein hydrolyzates and poultry feather hydrolyzates produced under different hydrolytic conditions and different scales of the process by which the samples were obtained (lab scale or pilot scale).

2.2 Experimental Procedures

2.2.1 Reagents

- Deionised water obtained by using Millipore Alpha Q system
- Hydrochloric acid 37% (Carlo Erba)
- Formic acid >99% (Acros Organics)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Amino Acid Hydrolysate Standard Mixture (SIGMA)
- AccQ Fluor™ reagent KIT (Waters)
- AccQ.Tag eluent A (Waters)
- Hydrogen peroxide 30% in water (Carlo Erba)
- Hydrobromic acid (Carlo Erba)
- Performic acid prepared as follow:
  - Formic acid and hydrogen peroxide solution 9:1 (V/V)
  - 1h at room temperature
  - 1 h at 0°C
- Sulphuric acid 96% (Carlo Erba)
- Trichloroacetic acid (Carlo Erba)
- Sodium hydroxide anhydrous pellets (Carlo Erba)
- Hydrogen chloride, pure 5 to 6 N solution in 2-propanol (Acros Organics)
- Dichloromethane (distilled in the Department)
- Trifluoroacetic anhydride (SIGMA-ALDRICH)
- L-Alanine, L-Arginine hydrochloride, L-Asparagine, L-Aspartic acid, L-Cysteine, L-Cystine, L-Glutamic acid, L-Glutamine, Glycine, L-Histidine hydrochloride, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-

Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine, D/L-Norleucine, D/L- Methionine sulfone, L-Cysteic acid monohydrate (Fluka)

- D-Alanine, D-Arginine hydrochloride, D-Asparagine, D-Aspartic acid, D-Cysteine, , D-Glutamic acid, D-Glutamine, D-Histidine hydrochloride, D-Isoleucine, D-Leucine, D-Lysine hydrochloride, D-Methionine, D-Phenylalanine, D-Proline, D-Serine, D-Threonine, D-Tryptophan, D-Tyrosine, D-Valine (Fluka)
- N-Acetyl-L-Tryptophanamide (SIGMA)
- (L,L)-phenylalanilphenylalanine, L-carnosine, L-anserine, creatine, creatinine, inosine, hypoxanthine, guanine, guanosine (SIGMA)

2.2.2 Instrumentation and Materials

- Technical balance (Orma)
- Analytical balance (Gibertini)
- Vacuum desiccator
- Ultraturrax T50 Basic (IKA-WERKE)
- Rotavapor RE 111 (BÜCHI), 461 water bath (BÜCHI)
- Filter paper
- HVLP filters cut-off 0.45 µm (Millipore)
- MULTIVAP nitrogen evaporator
- 4237R centrifuge (ALC)
- WB-OD 24 thermostatic bath (FALC)
- Kjeldahl apparatus (Velp)
- SA6 Autovortex (Stuart Scientific)
- 18 ml (15 mm) glass tube with teflon-lined screw cap (PIREX)
- 7 ml (13 mm) glass tube with teflon-lined screw cap (PIREX)
- 5 ml, 10 ml and 250 ml volumetric flask
- 1.5 ml disposable tubes (Eppendorf)
- V-Vial 10-425 screw 12X32 limited volume 1.5 ml-15 µl (Phenomenex)
- HPLC Alliance 2695 (Waters)
- AccQ.Tag (3.9mmX150 mm) amino acid analysis column (Waters)
- Waters 470 Fluorescence detector (Waters)
- Spherisorb 5 µm (2.1X250 mm) CN narrow bore column (Waters)

- Proteo 4 µm C18 90 Å (250X2.0 mm) analytical column (Phenomenex)
- Quattro Micro™ API Mass Spectrometer (Waters)
- ACQUITY UPLC (Waters)
- SQD mass spectrometer (Waters)
- ACQUITY BEH 1.7 µ C18 (150X2.1 mm) UPLC column (Waters)
- MassLynx 4.0 Software (Waters)
- QuanLynx software (Waters)
- UV/VIS Spectrophotometer LAMBDA BIO 20 (Perkin Elmer)
- Disposable cuvettes (PLASTIBRAND®)
- 6890 N gas chromatograph (Agilent Technologies)
- 5973 mass spectrometer (Agilent Technologies)
- CHIR-L-VAL, 25 m*0.25 mm I.D., film thickness: 0.12 µm, chiral capillary column (Varian)

2.2.3 Procedures

2.2.3.1 Determination of free amino acids in FAP by HPLC/FLD after ACQ-derivatization

0.03 g of dried proteinceous hydrolyzate were dissolved in 10 ml of deionised water and 340 µl of Norleucine (5 mM in deionised water) were added to the mixture as internal standard. In order to prepare a calibration standard solution, 40 µl of Norleucine (2.5 mM in HCl 0.1 N), 40 µl of Amino Acid Hydrolyzate Standard Mixture (SIGMA) and 920 µl of deionised water were mixed. Then 10 µl of proteinaceous hydrolyzate sample or standard solution were transferred into a 1.5 ml tube, 70 µl of borate buffer were added, in order to keep the optimal pH range for derivatization (8.2–9.7), and the solution was briefly vortexed. 20 µl of reconstituted AccQ.Fluor reagent was finally added and the mixture was immediately vortexed for several seconds. The tube was closed and left to stand for one minute at room temperature, then heated in a heating bath at 55°C for 10 min. The resulting derivatized standard solution was diluted with 400 µl of deionised water before injecting in the HPLC system.

2.2.3.1.1 HPLC/FLD analysis

All samples and standard solutions were analysed by using an Alliance 2695 separation system with a Waters AccQ.Tag amino acid analysis column (3.9mmX150 mm). The

column was thermostated at 37°C and the flow rate was set at 1.0 ml/min. The injection volume of samples was 5 µl, while standard calibration solution was injected at several volumes: 5. 10, 15, 20, 25 and 30 µl, corresponding to 10, 20, 30, 40, 50, 60 pmoles injected. Mobile phase A consisted of AccQ.Tag eluent A (100 ml AccQ.Tag A concentrate+1L deionised water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionised water respectively. Gradient elution was performed according to the following steps: 0 min 100%A, 1 min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 Fluorescence detector (λ excitation = 250 nm and λ emission = 395 nm).

2.2.3.1.2 Quantification of free amino acids

Quantitative analysis was carried out by using the internal standard method. Amino acid contents were estimated as follows: $AAC = Rf(A_S/A_{IS}) \times pmoles_{IS}$, where: AAC, amino acid content in pmoles; A_S, amino acid area; A_{IS}, internal standard area.

2.2.3.2 Determination of free amino acids in FAP by dual-HPLC/ESI-MSMS

0.03 g of dried proteinaceous hydrolyzate were dissolved in 10 ml of deionised water and D,L-Norleu (340 µl of a 5 mM solution) was added as internal standard and the mixture was directly analyzed by the LC/MS/MS system. All amino acid standard stock solutions were prepared at 2.5 mM concentration, by dissolving the required weight of each compound in deionised water. A mixed working standard solution, containing all 20 proteic amino acids, cystine, NorLeu, cysteic acid and methionine sulfone was prepared by suitable dilution of the stock solutions in the same solvent, in order to obtain a final concentration at about 0.5 mM of each analyte. In order to obtain a standard calibration curve, the final solution was diluted 2, 3, 4, 5 and 6 times.

2.2.3.2.1 HPLC/ESI-MS/MS analysis

All samples and standard solutions were analysed by using an Alliance 2695 separation system with a Spherisorb narrow bore column followed by a Proteo C_{18} analytical column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 15 min, from 100% A to 30% B by linear gradient in 30 min, 10 min of a washing step with 100% B, plus reconditioning. Flow rate: 0.22 ml/min; first column temperature: 30°C; second column temperature:

25°C; Injection volume: 5 µl. Detection was performed by using a triple quadrupole Quattro Micro™ API mass spectrometer applying the following conditions: positive ionization mode, capillary voltage 3.0 kV, cone voltage 25 V, source temperature 130°C, desolvation temperature 350°C, cone gas flow (N2): 100 l/h, desolvation gas flow (N2): 600 l/h; collision energy 14 eV, dwell time: 0.1 s. Selected Reaction Monitoring (SRM) detection. All data were acquired and processed by the software MassLynx 4.0.

2.2.3.2.2 Quantification of free amino acids

Quantitative analysis was set up by the internal standard method, with D/L-norleucine as internal standard. Amino acid contents were estimated as follows: AAC = $Rf(A_S/A_{IS})\times nmoles_{IS}$, where: AAC, amino acid content in nmoles; A_S, amino acid area in the sample obtained by the integration of the SRM chromatogram; A_{IS}, internal standard area obtained in the same way.

2.2.3.3 Determination of free amino acids in FFP

5 g of FFP sample were dissolved in 50 ml of 0.1 N hydrochloric acid and homogenized for 1 minute at 4000 rpm by Ultraturrax. The mixture was first filtered by paper filter and then by 0.45 µm filter and the filtrate was dried by rotavapor. Solid residue was redissolved in 1 ml of deionised water and the free amino acids content was determined by HPLC/ESI-MS/MS analysis as previously described for free amino acids in FAP.

2.2.3.4 Determination of total amino acids in FAP and FFP by dual-HPLC/ESI-MSMS

0.05 mg of dried proteinceous hydrolyzate were weighed into a 18 ml Pyrex glass tube fitted with teflon-lined screw caps. 6 ml of 6N HCl were added and mixed. The tube was flushed with nitrogen for 1 min in order to remove air. Hydrolysis was then carried out at 110°C for 23 h. After letting the tubes cool at room temperature, the internal standard (7.5 ml of Norleucine 5 mM in water) was added, the mixture was filtered through paper filter and collected into a 250 ml volumetric flask.

Acid hydrolysis was used for the determination of all amino acids except tryptophan (Trp), cysteine (Cys) and methionine (Met). For Cys and Met performic acid oxidation followed by acid hydrolysis was used. In this case, an amount of 0.05 g of dried proteinceous hydrolyzate sample was weighed in a 18 ml Pyrex glass tube fitted with teflon-lined screwcap. After adding 2 ml of neat performic acid freshly prepared,

samples were kept in an ice bath for 16 h at 0°C. Then 0.3 ml of hydrobromic acid was added in order to remove excess performic acid. The bromine formed during the reaction was removed by drying with nitrogen flow. The acid hydrolysis procedure using 6N HCl as described above was then performed.

2.2.3.4.1 HPLC/ESI-MS/MS analysis

HPLC-ESI/MS/MS analysis was performed as previously described for free amino acid determination. Met and Cys were determined as methionine sulfone and cysteic acid, SRM parameters are reported in Table 2.1.

Table 2.1. Parent ion and product ions of cysteic acid and methionine sulfone.

Amino acids	Parent ion	Quantifier ion	Qualifier ions
Cysteic acid	170.1	124.0	106.1
Methionine Sulfone	182.1	55.5	135.8

2.2.3.4.2 Quantification of total amino acids

Quantitative analysis was set up as previously described for free amino acid quantification.

2.2.3.5 Determination of total tryptophan in FAP and soluble FFP by derivative spectrophotometry

Quantification of total tryptophan was carried out as previously described by Fletouris et al. [9].

2.2.3.5.1 Sample preparation

20 mg of dried sample were dissolved in 15 ml NaOH 0.1 N only before the spectrophotometric analysis.

2.2.3.5.2 N-Acetyl-L-Tryptophanamide calibration standard solution preparation

N-Acetyl-L-Tryptophanamide 0.2 mM in NaOH 0.1 M stock solution was prepared and diluted 1, 2, 3, 4, 5 and 6 times with NaOH 0.1 M to obtain working calibration standard solutions.

2.2.3.5.3 UV determination

The fourth derivative UV spectra of both samples and working standard solutions, from 250 to 350 nm, were recorded and the difference between the peak at 290 nm and the

trough at 285 nm, was plotted against the concentration of the working solutions (µg/ml). Slope, intercept and least-squares fit of standard curve were calculated and standard curve slope and intercept were used to compute concentration of tryptophan in sample. Tryptophan content in sample was determined by the following formula: mg Trp/g of sample=*C*V/W* where *C* is tryptophan concentration determined in µg/ml according to calibration curve, *V* is the volume (ml) of sodium hydroxide sample solution in cuvette and *W* is the weight of sample (mg) in cuvette.

2.2.3.6 Determination of total tryptophan in FFP by LC/ESI-MS after alkaline hydrolysis

2.2.3.6.1 Sample preparation

The total tryptophan in FFP sample was determined as previously described by Just et al. [10] 1 g of dried FFP sample, was weighed into a 7 ml Pyrex glass tube fitted with teflon-lined screw caps. 4 ml of 4N NaOH were added and mixed. Hydrolysis was then carried out at 100°C for 4 h. After letting the tubes cool at room temperature, the solution was neutralized by adding 37% HCl, filtered through 0.45 µm filter and collected into a 10 ml volumetric flask to reach the final volume with deionised water.

2.2.3.6.2 Tryptophan calibration standard solution preparation

The above procedure was also applied to tryptophan standard solutions in order to obtain tryptophan stock solution at final concentration 1 µM.
Tryptophan calibration standard solutions were obtained by diluting stock solution 2, 4, 6, 8 and 10 times.

2.2.3.6.3 UPLC/ESI-MS analysis

UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C_{18} column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 1.8 mins, from 100% A to 50% A by linear gradient in 11.4 mins and 0.8 mins at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.25 ml/min, injection volume 2 µl, column temperature 35°C and sample temperature 6°C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30V,

source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition mode from 100 to 2000 m/z, scan duration 1s.

2.2.3.6.4 Data analysis

The integration of tryptophan was performed in an automatic way on the eXtract Ion Chromatograms (XICs), determined by the characteristic ions, 205.0 m/z and 188.0 m/z, by QuanLynx software, using as integration parameters: automatic ApexTrack peak integration and noise measurement, 1 smooth ± 1 scan with the Savitzkyi Golay algorithm. Quantitative analysis was set up by the external standard method.

2.2.3.7 Determination of amino acid racemisation degree by GC/MS analysis

40 ml of solution coming from standard acidic hydrolysis of hydrolyzate were dried by rotavapor and the solid residue was then redissolved in 2 ml of 2N hydrogen chloride in 2-propanol. The resulting solution was totally transferred into a 13 mm Pyrex glass tube fitted with teflon-lined screw caps and heated at 90°C for 1 hour. The sample was then dried under nitrogen flow, redissolved in 1ml of dichloromethane and 0.5 ml of trifluoroacetic anhydride and incubated 30 min at 50°C. The derivatized mixture was dried under nitrogen flow and, only before the GC/MS analysis, redissolved in 1 ml of dichloromethane.

2.2.3.7.1 GC/MS analysis

The GC/MS analyses were performed with an HP-5890 Series2 gas chromatograph coupled with an HP-5971 mass selective detector (Hewlett-Packard, Palo Alto, CA). The injector and detector temperatures were 250 and 260 C, respectively. Samples (2.0 µl) were injected in the splitless mode. The flow rate of helium, as carrier gas, was set at 1.3 ml/min in constant flow mode. The separation of D-/L-amino acids was performed by using a chiral capillary column (CHIR-L-VAL, 25 m*0.25 mm I.D., film thickness: 0.12 µm, Varian). The oven temperature program started at 80°C, and increased at 3°C/min up to 190°C, then was held for 4 min. MS conditions were as follows: ion source temperature: 230°C; electron impact: 70 eV; acquisition mode: full scan (m/z 40–400).

2.2.3.7.2 Determination of D/L amino acid ratio

The racemisation degree (in percentage) was calculated as follow: 100-[(A_L - A_D)/(A_D+A_L)]*100, where A_D and A_L corresponds to the chromatographic area of D or L enatiomer respectively.

2.2.3.8 Determination of total nitrogen by Kjeldahl method

0.5 g of hydrolyzate sample were put into a Kjeldahl digestion flask, and the catalyst mixture and 7 ml of 96% sulphuric acid were added. Total nitrogen was determined by the Kjeldahl method [11].

In order to obtain the percentage of total proteins, a conversion factor taking into account the composition in free and proteinaceous amino acid of every sample, was used.

2.2.3.9 Determination of TCA soluble nitrogen (SN) by Kjeldahl method

180 ml of deionised water were added to 0.5 g of hydrolyzate sample. The mixture was centrifuged at 3000 g at 5 °C for 40 minutes and filtered over a paper filter. 50 ml of filtrate were centrifuged again after the addition of 10% of trichloroacetic acid. TCA soluble nitrogen on 40 ml of the resultant solution was determined by the Kjeldahl method.

2.2.3.10 Quantification of small nitrogen compounds in hydrolyzate samples by UPLC/ESI-MS analysis

2.2.3.10.1 Sample preparation

100 mg of sample exactly weighted in 5 ml volumetric flask were dissolved to reach the final volume with deionised water, containing Phe-Phe 1 µM as internal standard, and then filtered through 0.45 µm Durapore Membrane Filters type HV (Millipore).

2.2.3.10.2 Small nitrogen compounds identification by HPLC/ESI-MS/MS

All samples were analysed using an Alliance 2695 separation system (Waters, Milford, MA, USA) with a Jupiter (Phenomenex, Torrance, Ca, USA) analytical column (5 µm C18, 300 Å, 250x2.00 mm). The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 12 min, from 100% A to 50% A by linear gradient in 65 min, isocratic step at 50% A for 4 min and final washing

step with 0% A for 8 min, plus reconditioning. Flow rate: 0.2 ml/min; column temperature: 35°C. Detection was performed using a triple quadrupole Quattro Micro API (Micromass, Manchester, UK) mass spectrometer with the following condition: ESI positive ion, capillary voltage 3.2 kV, cone voltage 30V, source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2) 100 l/h, desolvation gas flow (N_2) 650 l/h. Data were acquired in full scan mode in the range 100-2000 *m/z* (scan duration 1 s, interscan delay 0.1 s). Product ion scan experiments (40-300 m/z) were performed on the most intense signals present in the full scan spectra. Scan duration was 0.5 s, interscan delay was 0.05 s, collision energy ranged from 14 to 40 eV on the basis of analysed compounds.

2.2.3.10.3 Calibration standard curve preparation

25 ml of solution containing Phe-Phe 1 µM, carnosine 0.1 mM, anserine 0.1 mM, creatine 0.4 mM, creatinine 0.4 mM, hypoxanthine 0.4 mM, inosine 0.2 mM, guanine0.1 mM and guanosine 0.2 mM was prepared as stock solution and then diluted 2, 4, 6, 8 and 10 times in order to obtain working calibration standard solutions.

2.2.3.10.4 UPLC/ESI-MS analysis

UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C_{18} column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 7 min, from 100% A to 50% A by linear gradient in 43 min and 2.6 mins at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.2 ml/min, injection volume 2 µl, column temperature 35°C and sample temperature 6°C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30V, source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition mode from 100 to 2000 m/z, scan duration 1s.

2.2.3.10.5 Data analysis

The integration of small nitrogen compounds previously identified in hydrolyzate was performed in an automatic way on the eXtract Ion Chromatograms (XICs), determined

by the characteristic ions of each compound, reported in Table 2.2, by QuanLynx software, using as integration parameters: automatic ApexTrack peak integration and noise measurement, 1 smooth ± 1 scan with the Savitzki Golay algorithm.

Quantitative analysis was set up by the internal standard method, with Phe-Phe as internal standard. Small nitrogen compound contents were estimated as follows: SNCC = $(A_S/A_{IS})\times nmoles_{IS}$, where: SNCC, small nitrogen compound content in nmoles; A_S, small nitrogen compound area in the sample obtained by the integration of the XIC chromatogram; A_{IS}, internal standard area obtained in the same way.

Compound	*Characteristic ions*
Phe-Phe	313.0, 166.0, 120.0
Carnosine	227.0, 212.0, 156.0, 110.0
Anserine	241.0, 170.0, 109.0
Creatine	132.0
Creatinine	114.0
Hypoxanthine	137.0,
Inosine	137.0, 269.0
Guanine	152.0
Guanosine	152.0, 284.1

Table 2.2. Characteristic ions of small nitrogen compounds used as quantify trace to perform XIC chromatograms.

2.3 Results and Discussion

2.3.1 Sample description

The flow sheet describing the processes developed under the PROSPARE project for poultry leftovers recovery [12] is reported in Figure 2.1.

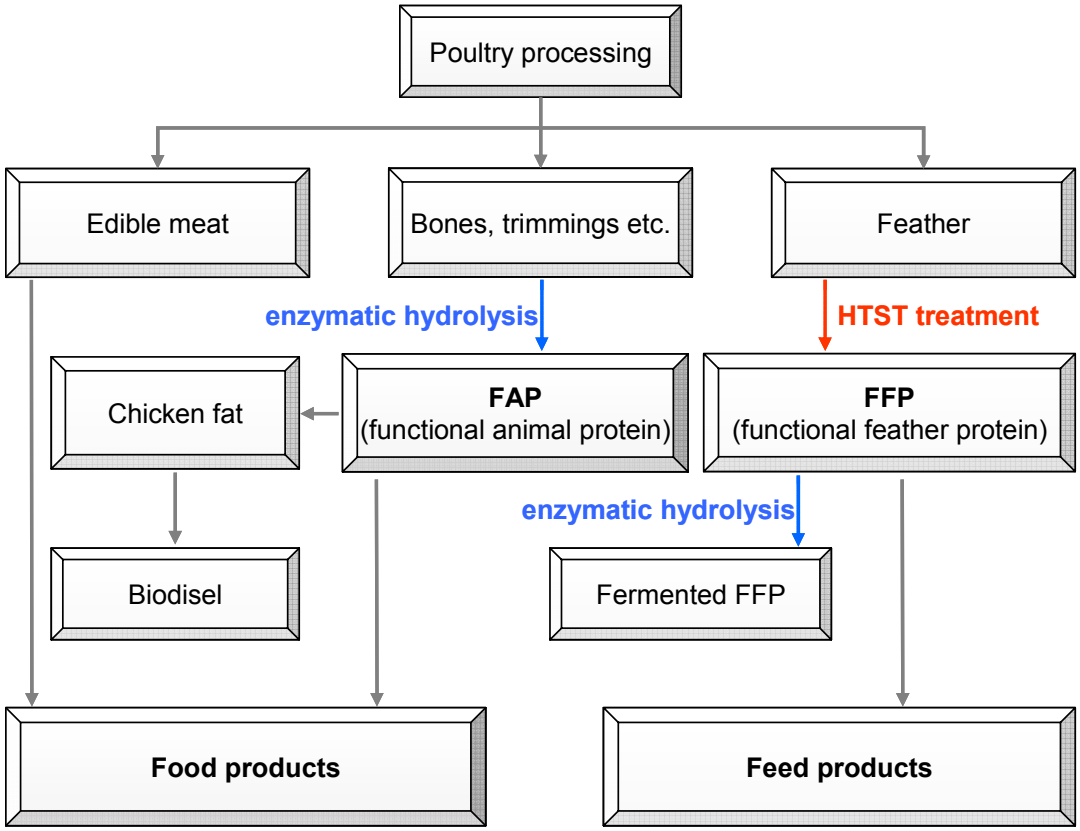


Figure 2.1. Schematical description of the poultry by-products recovery.

2.3.1.1 Functional Animal Protein (FAP) samples

Functional Animal Proteins, FAP, are protein hydrolyzates produced by a new technology, based on an enzymatic hydrolysis of poultry leftovers, in particular bones and meat trimmings. The crucial point of FAP making process is the enzymatic digestion based on deep controllable conversion of poultry meat and bone residues using multienzyme mixture containing four commercially available enzyme preparations. This step is carried out at different conditions, such as different enzyme weight ratio, different hydromodulus and starting from different raw materials, in order to evaluate the optimal ones.

In Table 2.3 the FAP samples obtained from VNIIPP (State Institution All-Russian Research Institute for Poultry Processing Industry of Russian Academy of Agricultural Sciences, RF) are listed.

For every sample an identification code is provided, together with the scale of the process by which the sample has been obtained (lab scale or pilot scale), the type of raw material used, the enzyme percentage used for its production, pH and temperature of the enzymatic hydrolysis, duration and hydromodulus, defined as the ratio between the raw material and water added before the hydrolysis.

Table 2.3. FAP scheme and production parameters.

Sample code	58T	78T	83T	81T	82T	100T	100AT	101T
scale	lab	lab	lab	lab	lab	pilot	pilot	pilot
raw materials	broiler backs	broiler backs	broiler backs	necks	necks	necks	necks	necks
E/W (%)	0.15	0.25	0.20	0.20	0.20	0.25	0.25	0.25
pH	7	7	7	7	7	7	7	7
T° (C°)	55	55	55	55	55	55	55	55
Duration (h)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Hydromodulus	1:1.5	1:2.5	1:2.0	1:2.0	1:2.0	1:2.0	1:2.0	1:2.0

2.3.1.2 Functional Feather Protein (FFP) samples

Functional Feather Proteins, FFP, are protein hydrolyzates produced by a new technology, based on hydrothermal treatment carried out on poultry feathers in non drastic conditions.

In Table 2.4 the FFP samples obtained from SYMBOL (Moscow, RF) are listed. FFP samples have been produced by hydrothermal hydrolysis, except the last one wich was produced by enzymatic hydrolysis starting from sample 5L. For every sample an identification code is provided, together with the scale of the process by which the sample has been obtained (lab scale), the type of raw material used (the raw material for the last one is the sample 5L), the temperature of the heat carrier, the temperature during the hydrolysis, the duration of the hydrolysis, the enzyme percentage used for the production of the sample 6L, the pH and the temperature of the enzymatic hydrolysis.

Table 2.4. FFP samples scheme and production parameters.

sample code	1L	2L	3L	4L	5L	6L
Scale	lab	lab	lab	lab	Lab	lab
raw materials	wet broilers feathers	wet broilers feathers	wet broilers feathers	dry broilers feathers	dry broilers feathers	sample 5L
Heat carrier T (C°)	220-240	290-320	250-270	290-300	240-250	-a
Hydrolysis T (°C)	150-160	190-200	170-180	225	200	-a
Duration (sec)	60	90	60	60	90	-a

a- this sample has been enzymatically hydrolyzed according to parameters similar to those used for FAP production.

2.3.2 Soluble and total protein in FAP and FFP hydrolyzates

The soluble (in 12% TCA) and total proteins in FAP and FFP hydrolyzates was first determined by the Kjehldal method.

As can be see in Figure 2.2 the percentage of total protein content in FAP samples was in the range from 75 to 90% of the weight of the material (raw data obtained are reported in Attachment Table 2).

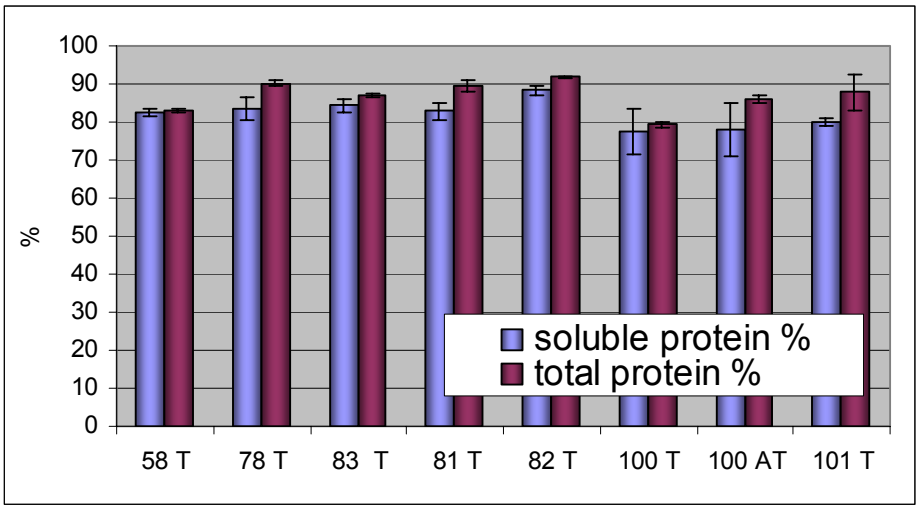


Figure 2.2. Total and soluble proteins content in FAP samples.

The FAP samples appeared to be a food material very rich in proteinaceous compounds, with a protein content around 80-90% of the total weight. Samples from the pilot plant (100T, 100AT, 101AT) appear to be, on average, slightly less rich in proteins than samples coming from laboratory scale, but in general the protein content was found to be fairly stable. The observed variations were not linked to the different technologies used, but were more likely due to the raw material variability. Most of the proteins appeared to be present in soluble form, indicating a very high digestibility of the FAP samples.

The total and soluble protein content of FFP samples are shown in Figure 2.3 (raw data obtained are reported in Attachment Table 3).

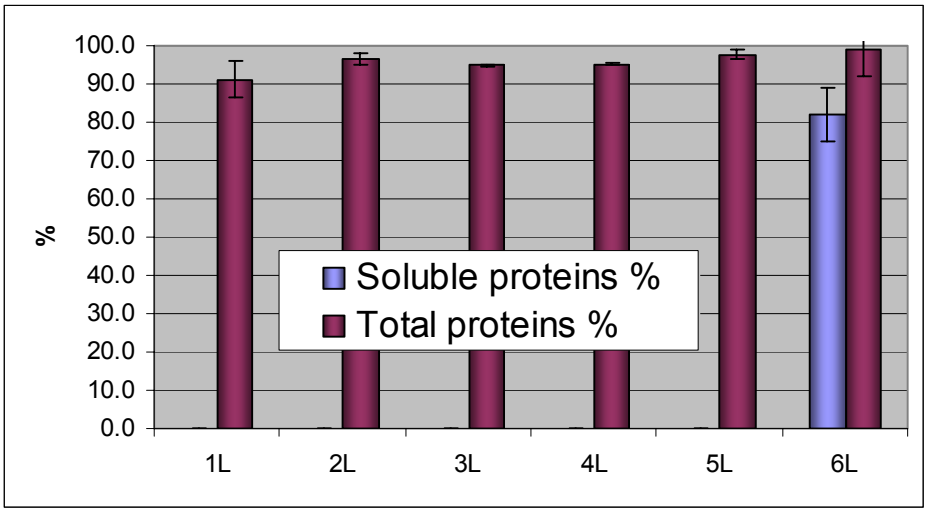


Figure 2.3. Total and soluble proteins content in FFP samples.

FFP samples resulted very rich in protein, even more than FAP samples (more than 90% of the total weight in all samples), but proteins were found to be present in a totally insoluble form. A notable exception was represented by sample 6L, prepared by enzymatic hydrolysis of the hydrothermally treated 5L, using a protocol similar to the one of the FAP samples, which showed a very high content of soluble proteins. The more protein-rich samples proved to be samples 2L, 5L and 6L, in which the duration of the hydrothermal hydrolysis was longer, showing that this parameter highly affects the final protein content.

2.3.3 Free and total amino acid composition of FAP and FFP

A standard commercial method for the determination of the amino acids, based on a pre-column derivatization procedure with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by the determination of the amino acid content by RP-HPLC with fluorescence detection (details are reported in the experimental section, Part II - 1.2.3.4), was first applied for the determination of free and total amino acids of FAP obtained on the laboratory scale.

The results of the free amino acid content in the five FAP samples are reported in Figure 2.4 (raw data obtained are reported in Attachment Table 4).

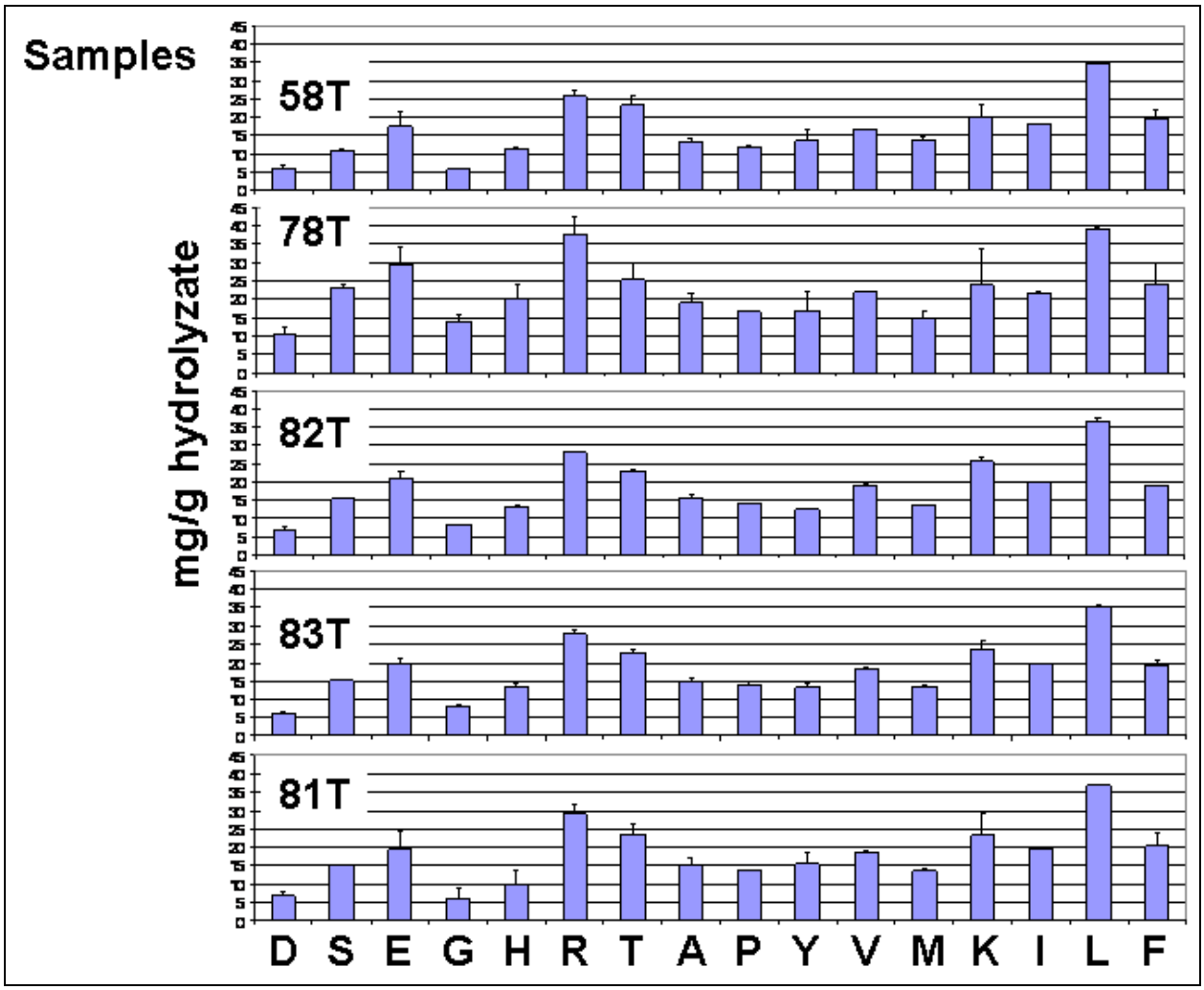


Figure 2.4. Free amino acids in hydrolyzate samples produced on a laboratory scale as determined by the commercially available LC/FLD method.

It can be seen that a few amino acids are missing: actually, this method, developed for amino acid determination after acidic hydrolysis, does not allow to determine Trp, Cys, Gln and Asn (amino acid lost during amino acid hydrolysis), even when addressing free amino acid determination.

In general, it was evident that the distribution of the free amino acids was nearly the same in all samples, thus the composition of the free amino acidic fraction was not affected by the proteolytic conditions, as was expected. Leu, Arg, Glu were the dominant amino acids in the free amino acidic pool.

The total amounts of the free amino acids in the different samples are reported in Figure 2.5. This parameter is obviously strictly linked to the hydrolysis degree, with the highly proteolyzed sample (78T) being the richest in free amino acids and the lowest

proteolyzed sample (58T) being the poorest in their content, with the middle ones lying in between.

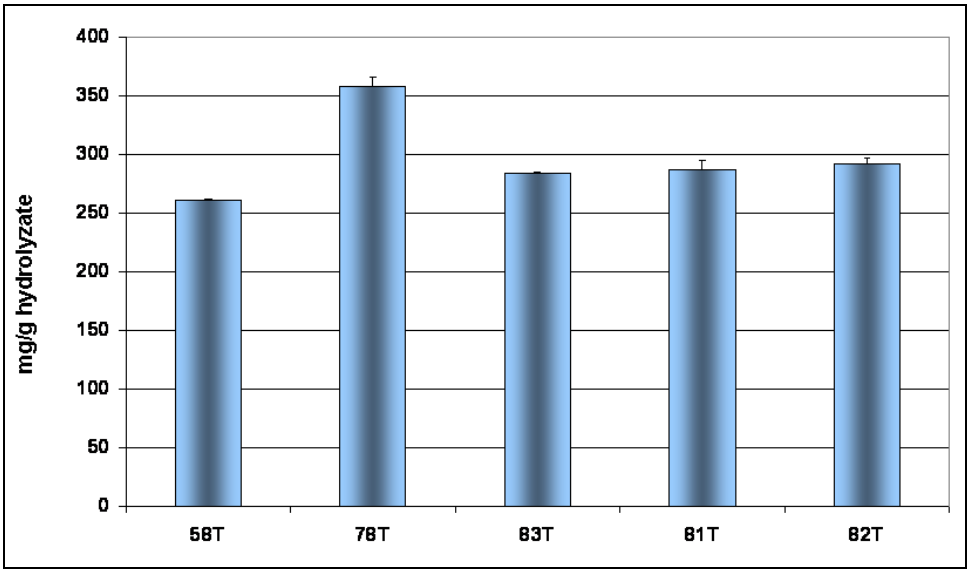


Figure 2.5. Total amount of free amino acids in FAP samples produced on the laboratory scale determined by the commercially available LC/FLD method.

The total amino acids in these FAP samples were also determined by the same method, after acidic hydrolysis in 6N HCl performed for 24h at 110°C, in the presence of performic acid, in order to convert Cys and Met in cysteic acid and methionine sulfone. The results concerning the single amino acids are given in Figure 2.6 (raw data obtained are reported in Attachment Table 5).

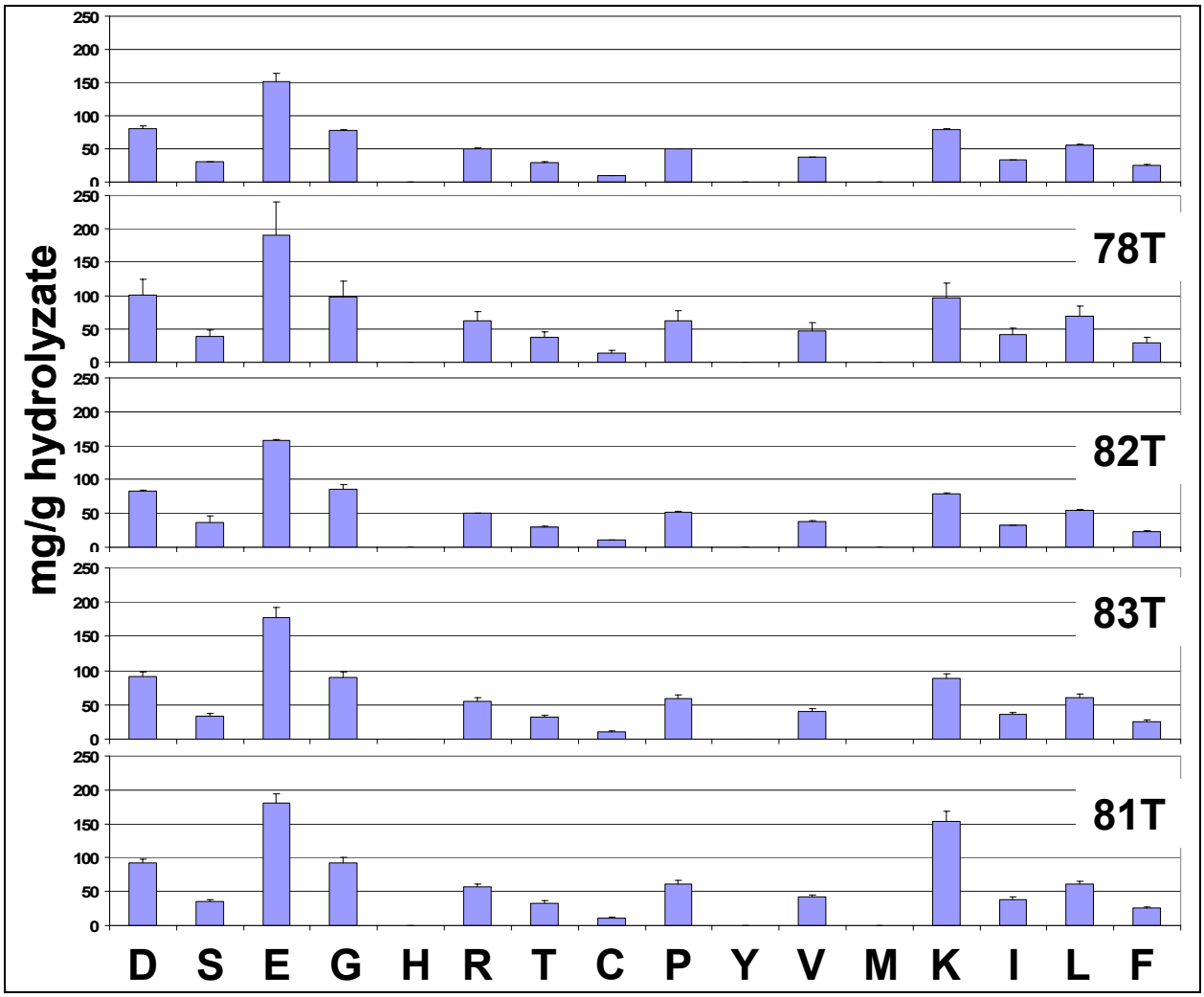


Figure 2.6. Total amino acids in FAP samples produced on the laboratory scale determined by the commercially available LC/FLD method.

Again, several amino acids could not be detected by using this analysis, both because the method did not allow for their detection (as shown before) and also because the hydrolytic conditions degraded some amino acids. Asn, Gln and Trp were degraded by the acidic hydrolysis. Tyr and His were not found in the mixture, very likely degraded by the oxidizing environment. Finally, unreliable results could be obtained for Ala and Met, due to their coelution in the gradient used, which allowed Met to be determined as such, but made methionine sulfone coelute with alanine. As far as the other amino acids were concerned, the distribution of the total amino acids was nearly the same in all samples, indicating that the total amino acidic fraction was not affected by the proteolytic conditions, as was expected. Lys and Glu were the dominant amino acids in the total amino acidic pool.

The amounts of the total amino acids are reported in Figure 2.7. The variability observed in these cases is likely to be related to the different raw materials used as starting ingredients.

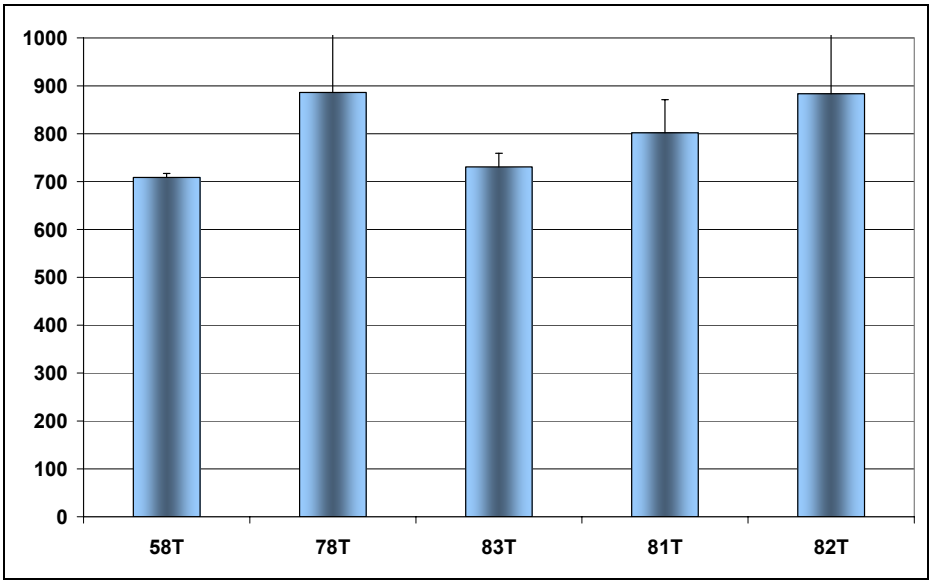


Figure 2.7. Total amount of amino acids in FAP samples produced on the laboratory scale determined by the commercially available LC/FLD method

However, these preliminary experiments clearly showed that many important amino acids were missing by using the standard methods. Moreover, underestimation of the actual content could also be possible due to the use of a procedure with a derivatization step. In order to overcome all these problems, a new dual LC/ESI-MS/MS method has been developed (all the details are described in Part II -Chapter 1) in order to determine all the amino acids without any derivatization step. The method is based on the simultaneous use of two columns in series, one with a polar stationary phase (CN) and the other with an apolar side chain (C18), by eluting with water/acetonitrile acidified mixtures. Every single amino acid was detected in the ESI-MS detector (triple quadrupole) by monitoring specific SRM transitions. This new method was validated and the linearity range was verified between 0.1 and 1 nmole injected. A LOD value was found to be less than 100 pmoles for Gly and Ala and less than 10 pmoles for all the other amino acids. By this method, every single amino acid (including Trp, Gln, Asn, Met, as sulfone, Cys, as cysteic acid) was easily detected.

The method was compared with the commercial one, and the results are reported in Figure 2.8 (free amino acids) and Figure 2.9 (total amino acids).

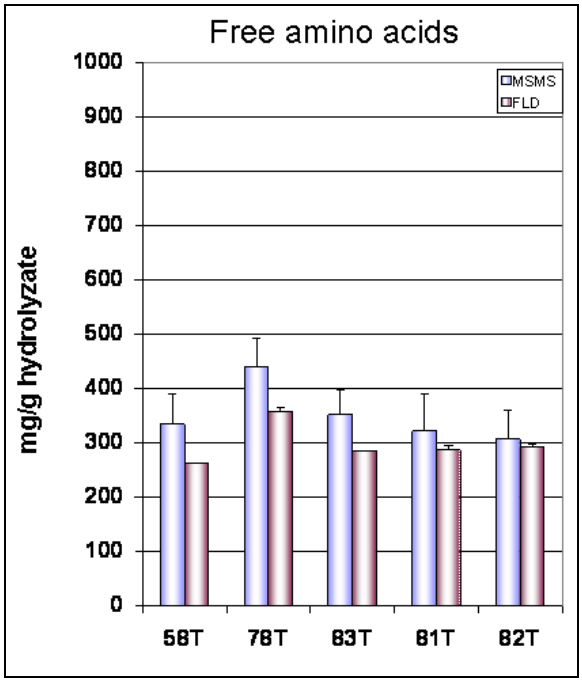


Figure 2.8. Free amino acids in the different samples as determined by the commercial LC/FLD (red) and by the newly developed LC/ESI-MS/MS (blue) method.

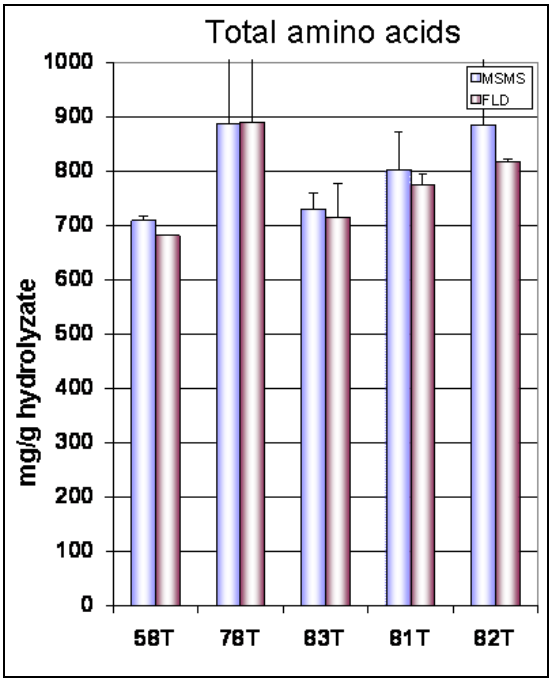


Figure 2.9. Total amino acids in the different samples as determined by the commercial LC/FLD (red) and by the newly developed LC/ESI-MS/MS (blue) method

It was evident that the two methods were perfectly consistent, with the obvious advantages of the easier sample preparation and the complete determination of all the amino acids in the case of the MS-based method. Anyway, even with the new method the amino acids degraded by the hydrolysis were obviously still missing: His and Tyr, degraded by the oxidizing conditions and Trp, degraded by the acidic environment.

For these reasons all samples, FAP and FFP, were analysed by HPLC/ESI-MS/MS after acid hydrolysis for the determination of all amino acids except tryptophan (Trp) cysteine (Cys) and methionine (Met). For Cys and Met, performic acid oxidation followed by acid hydrolysis was used and the determination was done by HPLC/ESI-MS/MS for these two amino acids only. For Trp, a standard spectrophotometric essay was performed, exploiting the peculiar absorption characteristics of this amino acid. Due to the insolubility of FFP samples 1L-5L, in these cases the spectrophotometric method could not be applied and alkaline hydrolysis followed by LC/ESI-MS analysis was performed. Anyway, this method yielded very low amounts of Trp, indicating a partial degradation during the hydrolysis step. Thus, the total Trp content in samples 1L-5L has been assumed to be the same as in sample 6L (which is produced from sample 5L by

enzymatic hydrolysis and thus accessible for Trp determination by spectrophotometric determination).

Free and total amino acid contents in FAP samples determined by the HPLC/ESI-MS/MS method are reported in Figure 2.10 (raw data obtained are reported in Attachment Table 6 and Attachment Table 7).

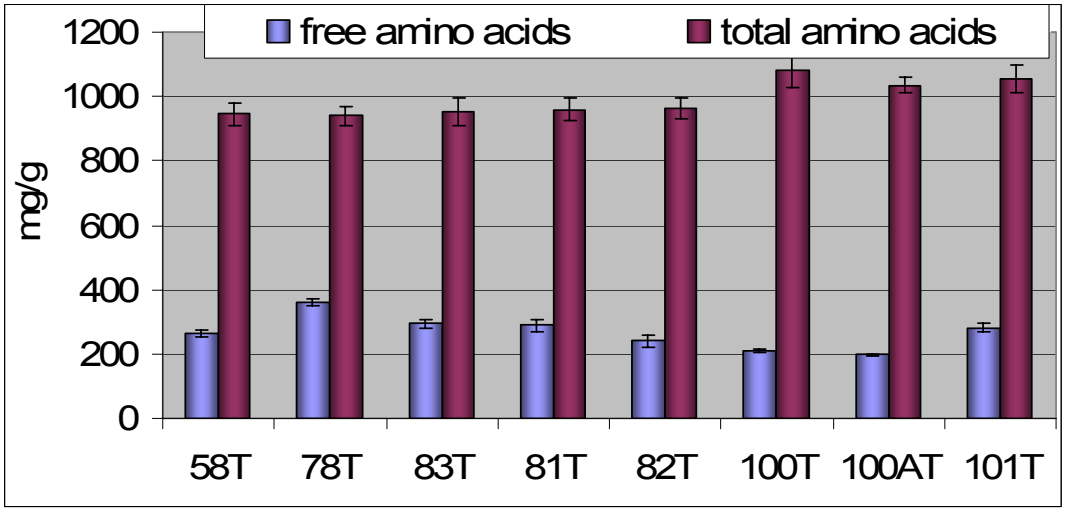


Figure 2.10. Free and total amino acid contents in FAP samples.

The data indicated, consistently with nitrogen content determinations, that FAP samples are essentially composed of proteinaceous derivatives. Both total and free amino acid contents appear to be fairly stable, indicating a high stability of the production process, even when going from a lab to a pilot scale. Quite interestingly, although soluble proteins are always around 95% of the total protein, free amino acids constitute only about 30% of total amino acids, indicating that most of the soluble proteinaceous fraction is actually composed by peptides.

Free amino acids present in FAP samples are reported in Figure 2.11.

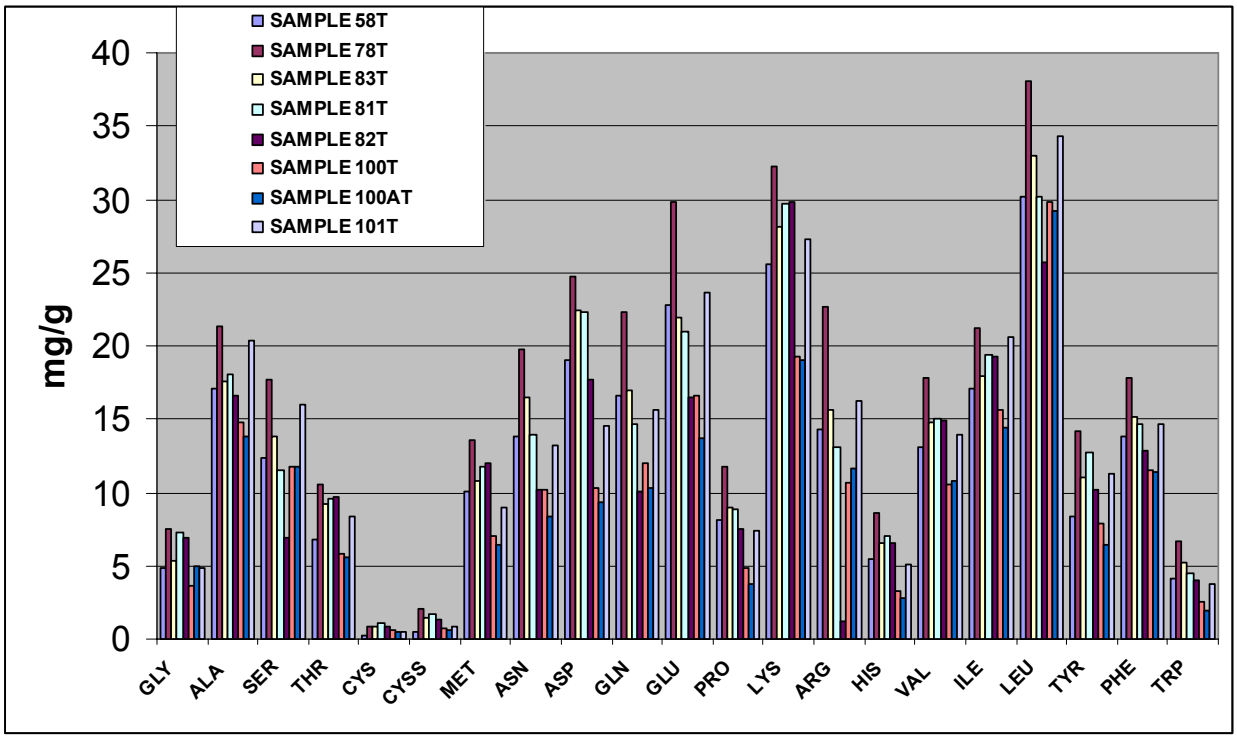


Figure 2.11. Free amino acid composition in hydrolyzate samples.

As can be seen, the composition of the free amino acidic fraction is fairly constant, and it is dominated by Leu, Lys, Glu, Gln, Asp, Asn, Ala, Ile, with a very low content in Cys and Cyss. The variability observed for every single amino acid content seems to be quite random and clearly related neither to the process conditions nor to the scale. An exception is represented by sample 78T, the one produced in the highest proteolyzing conditions (higher amount of enzyme and water), in which almost all amino acids are present in the highest amount. The rest of the observed variability, albeit small, can be traced back to the variability of the raw materials.

Total amino acids present in FAP samples are reported in Figure 2.12.

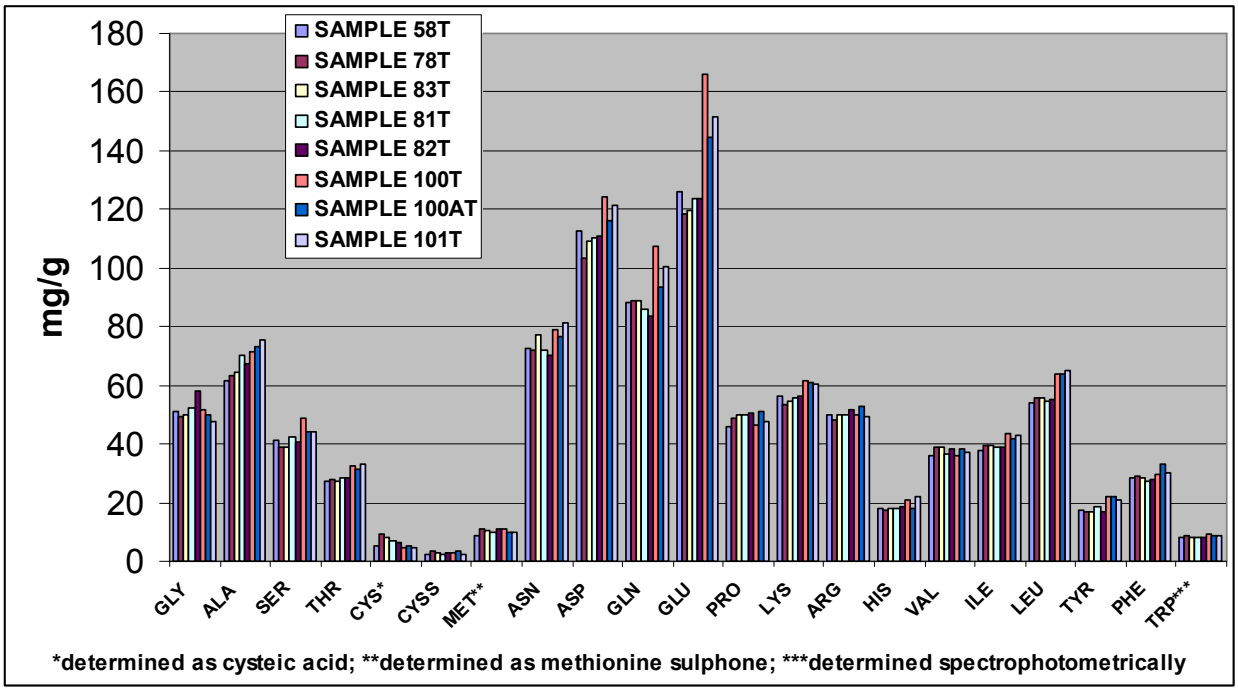


Figure 2.12. Total amino acid composition in hydrolyzate samples.

It is easy to observe that total amino acids are even more stable from batch to batch than free amino acids. The total amino acidic fraction is dominated by Glu, Gln, Asp, with a very low content of sulphorated amino acids and Trp. The only variation observed is clearly related to the scale of the process: for many amino acids (in particular Glu, Gln, Asp and Leu) the amount is higher in the samples produced in the pilot plant, indicating that in this case there is a more efficient release of these amino acids in the free form. Anyway, the variations observed are quite small, indicating again a very reproducible process.

Free and total amino acid contents in FFP samples are reported in Figure 2.13 (raw data obtained are reported in Attachment Table 8).

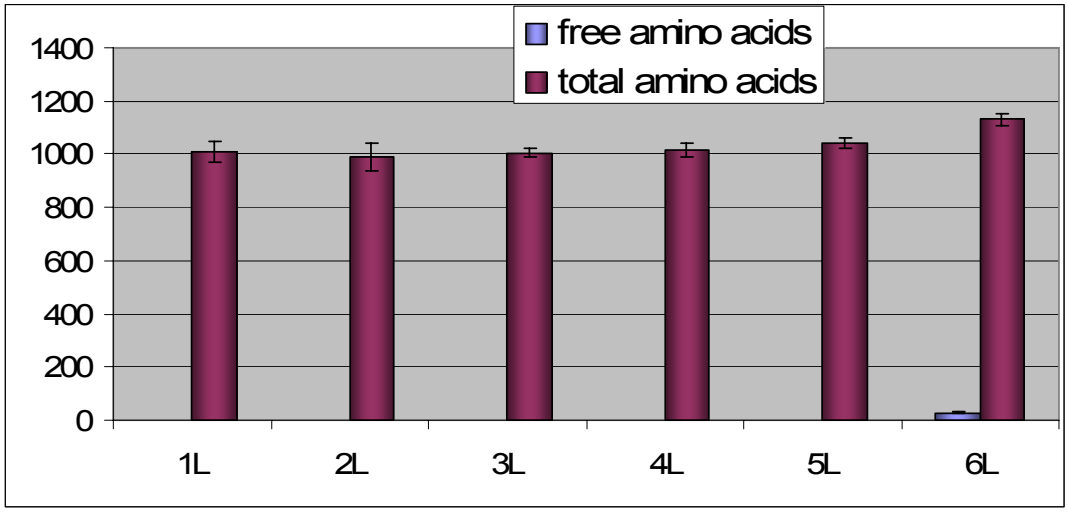


Figure 2.13. Free and total amino acid contents in FFP samples.

The data indicated, consistently with nitrogen content determinations, that FFP samples are very rich in proteinaceous derivatives, but, for samples 1L-5L, consistently with their insolubility, free amino acids are practically absent. Even sample 6L, obtained from sample 5L through enzymatic hydrolysis and quite rich in soluble nitrogen compounds, appears to be quite poor in free amino acids, being less than 3% of the total mass and about ten times less than FAP samples. This indicates that in sample 6L almost the totality of the soluble nitrogen fraction is actually composed by peptides.

Free amino acids present in FFP sample 6L are reported in Figure 2.14 (raw data obtained are reported in Attachment Table 9).

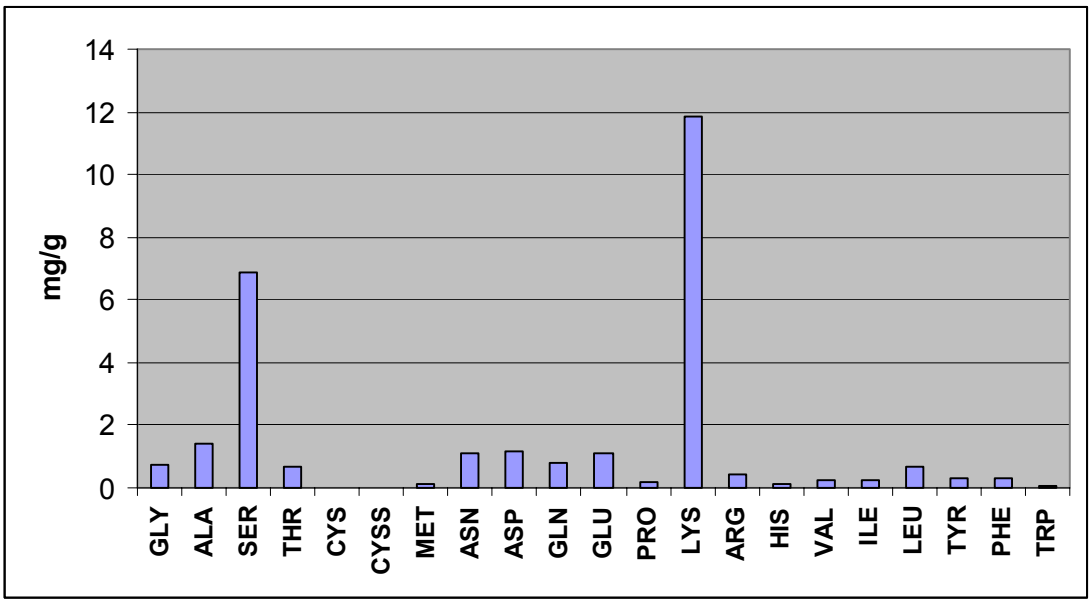


Figure 2.14. Free amino acids in FFP sample 6L.

As can be seen, the composition of the free amino acidic fraction is dominated by Lys and Ser, whereas sulphorated amino acids and Trp are practically absent. If the high amount of Ser can be justified by the type of raw material used (feathers, thus keratin-rich proteins), the high percentage of Lys is likely to be due to the specificity of the enzymes used for the production of this FFP sample. It is to be remembered, however, that the reported amounts are very low, if compared to the FAP samples or to the total mass of the FFP sample.

The total amount of amino acids present in FFP samples are reported in Figure 2.15.

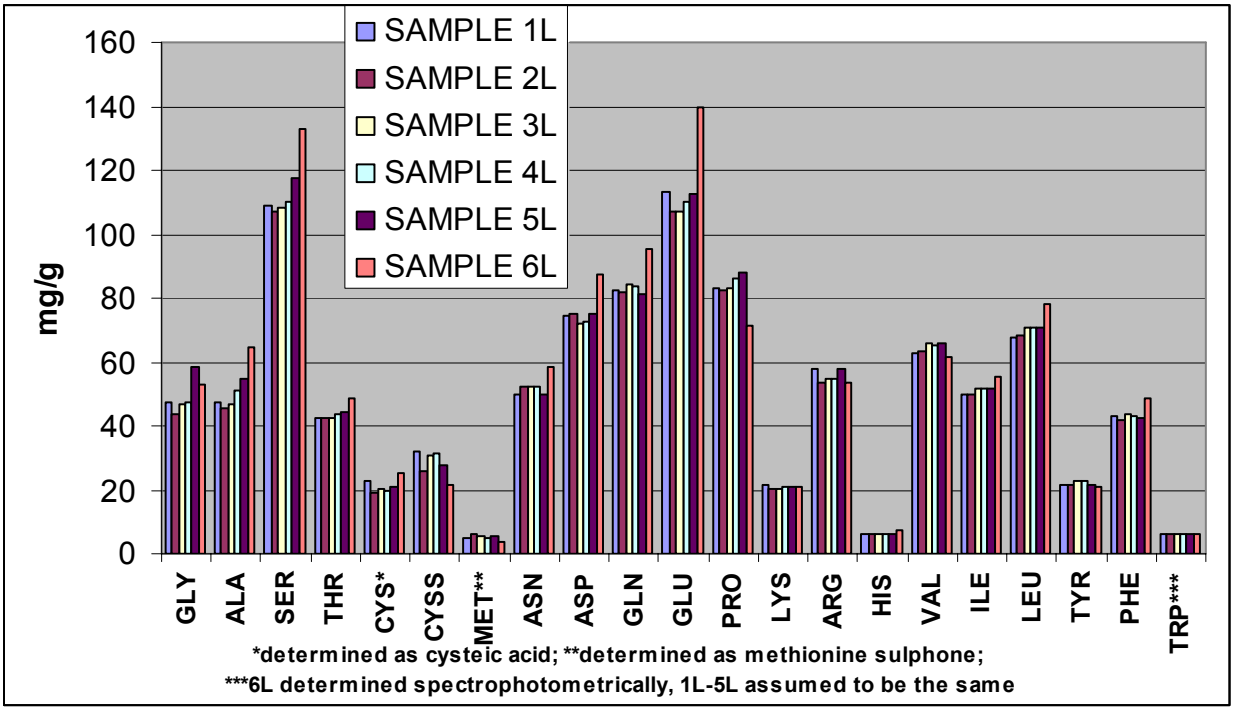


Figure 2.15. Total amino acid composition in FFP samples.

The total amino acidic fractions seem to be very similar in all samples. Sample 5L, in which the duration of the hydrolysis was longer, and sample 6L, derived from it, presented the highest amounts for several amino acids, though the differences were quite small. The pattern was found to be dominated by Ser and Glu, whereas Met, His and Trp were present in lesser amounts.

2.3.4 Chemical score of FAP and FFP samples

The nutritional values of FAP and FFP samples were determined by calculation of the chemical score, using as reference egg white proteins. FAP samples were assumed to be 100% digestible, whereas for FFP samples, chemical scores were corrected

assuming that sample 1L was 50% digestible, samples 2L-5L 90% digestible and sample 6L 100% digestible as resulted from digestibility studies carried out on the same samples from the research group of Agricola 3Valli (data not shown).
Chemical scores of essential amino acids for FAP samples are reported in Table 2.5.

Table 2.5. Chemical scores of essential amino acids for FAP samples.

	58T	*78T*	*83T*	*81T*	*82T*	*100T*	*100AT*	*101T*
Thr	76.1	77.0	75.5	77.6	77.1	79.5	78.9	82.5
Val	55.5	60.5	59.0	55.7	57.8	48.4	53.5	51.4
Ile	71.2	74.3	73.3	71.5	71.9	71.5	72.2	72.8
Leu	66.1	68.9	68.0	66.1	66.2	68.4	71.5	71.5
Phe+Tyr	49.6	50.8	48.4	49.3	47.7	49.2	54.8	50.0
Trp	79.6	89.2	81.1	77.7	78.6	79.5	77.7	75.9
Lys	86.9	83.0	83.9	84.9	85.3	83.4	86.0	83.5
Met+Cys	25.2	36.6	33.6	30.2	30.9	24.4	24.3	23.0

The limiting amino acids were clearly the sulphur-containing amino acids Met and Cys, and their content was even lower in the samples obtained from the pilot plant, indicating that the samples had probably experienced a higher thermal stress during sample preparation in a larger scale.
On the other hand, nutritional values for Trp, Thr, Lys and Ile and Leu were very good, outlining the potentialities of the hydrolyzate samples as nutritionally rich ingredients.
Chemical scores of essential amino acids for FFP samples are reported in Table 2.6.

Table 2.6. Chemical scores of essential amino acids for FFP samples.

	1L	*2L*	*3L*	*4L*	*5L*	*6L*
Thr	54.7	100.1	98.4	99.9	99.2	112.9
Val	44.8	83.1	84.8	83.4	81.8	78.7
Ile	43.9	80.2	81.5	80.7	78.4	86.8
Leu	38.7	71.7	73.0	72.2	70.0	79.8
Phe+Tyr	32.8	58.9	60.8	59.4	56.1	63.0
Trp	29.1	53.4	52.6	51.9	50.6	52.1
Lys	15.7	26.8	26.4	27.2	26.1	27.1
Met+Cys	23.2	38.9	38.7	37.2	39.0	43.7

The limiting amino acid was in this case Lys, even if samples 2L-5L were clearly improved as compared to sample 1L, due to their improved digestibility, whereas no further improvement was observed in sample 6L obtained by enzymatic hydrolysis.
On the other hand, nutritional values for Thr, Val and Ile and Leu were very good, also outlining the potentialities of FFP samples as nutritionally rich ingredients.

2.3.5 Amino acid racemisation degree in FAP and FFP samples

Amino acid racemisation was evaluated on the total pool, after acidic hydrolysis in non-oxidizing conditions by GC/MS analysis, after derivatization of the amino acid pool as isopropyl esters and trifluoroacetyl anhydrides and separation on a Chirasil-Val column. GC/MS chromatograms of a standard amino acidic mixture and of a FAP sample are reported in Figure 2.16.

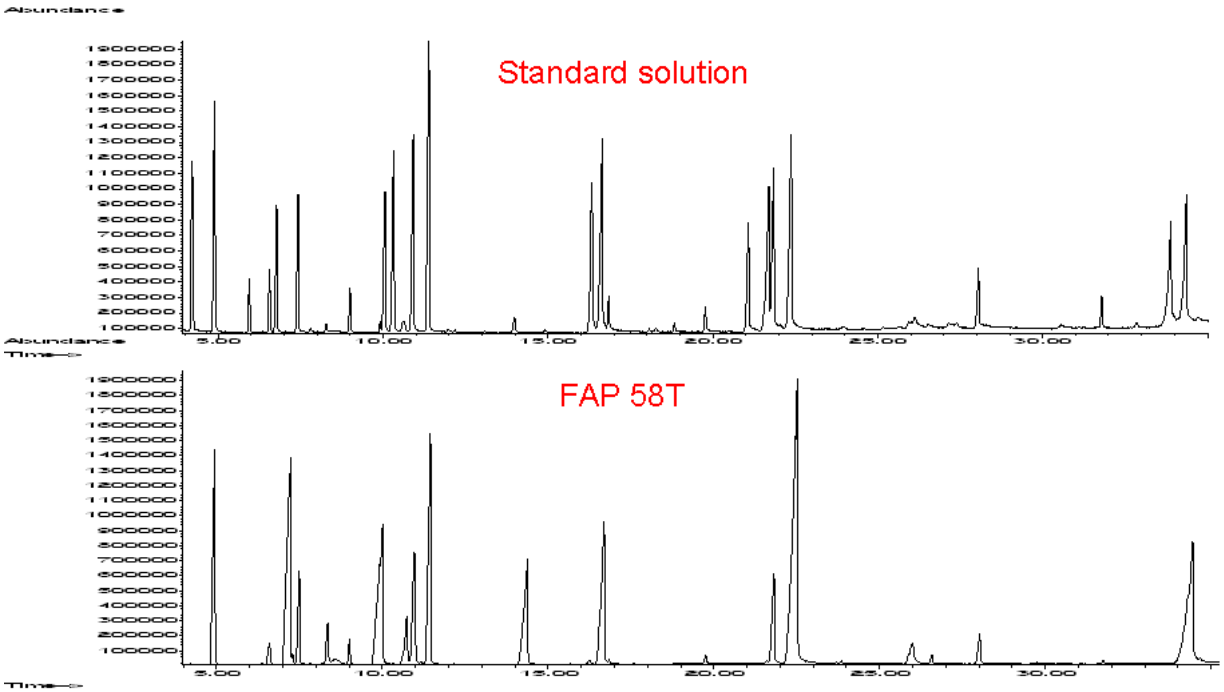


Figure 2.16. GC/MS chromatogram of amino acid standard solution (above) and FAP 58T (below) after derivatization as isopropyl esters and trifluoroacetyl anhydrides.

In FAP samples, the only amino acid found to be slightly racemized was Asp, which is known to be the amino acid most liable to racemization. This data indicates a very mild process and the absence of bacterial contamination. Anyway, the racemisation percentage for Asp was found to be 2.6% (±1.2) in FAP samples from the lab scale and 7.3 %(±1.4) in FAP samples from the pilot scale, again outlining slightly higher thermal stress during preparation of the latter samples. As expected, racemisation was found to be more consistent in FFP samples, prepared by hydrothermal hydrolysis. Racemisation of Asp was the most consistent (up to 30%) but also other amino acids were found to be slightly racemized, as represented in Figure 2.17 (raw data obtained are reported in Attachment Table 10 and Attachment Table 11).

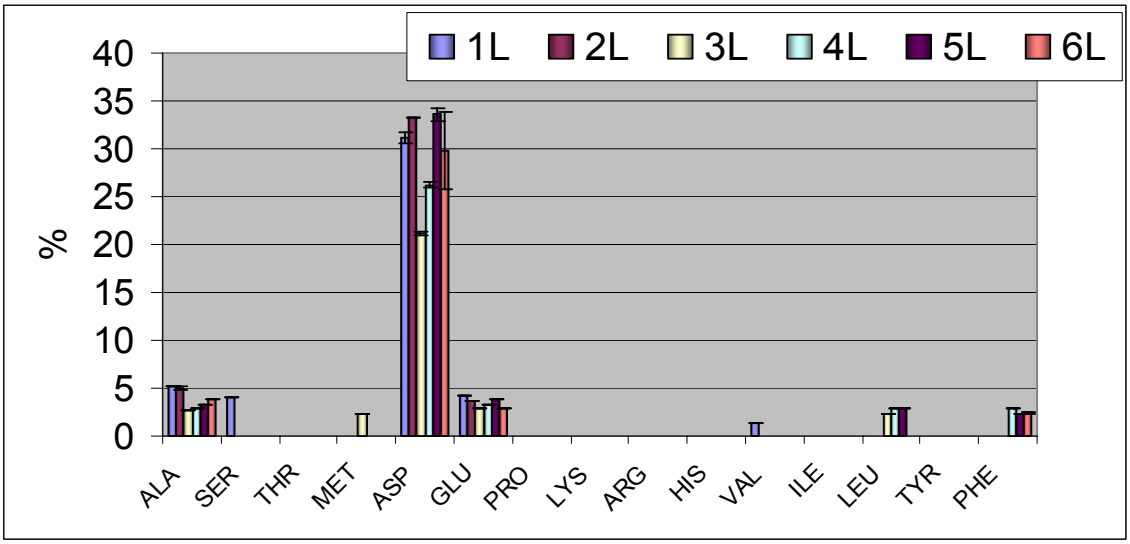


Figure 2.17. Amino acid racemisation in FFP samples.

Anyway, given the scarce relevance of Asp from the nutritional point of view and the limited racemisation for the other amino acids, this racemisation does not constitute a nutritional problem. Samples 2L and 5L, which underwent more thermal stress due to the longer hydrolysis duration, were consistently found to be slightly more racemized.

2.3.6 Small nitrogen compounds in FAP and FFP samples

FAP samples, at a concentration of 20mg/ml in an aqueous solution of formic acid (0.1%), were also analysed by UPLC/MS in order to determine the most abundant nitrogen compounds with low MW present in the hydrolyzates. The detection was performed by a single quadrupole ESI mass spectrometer in the full scan mode. A representative TIC chromatogram is reported in Figure 2.18.

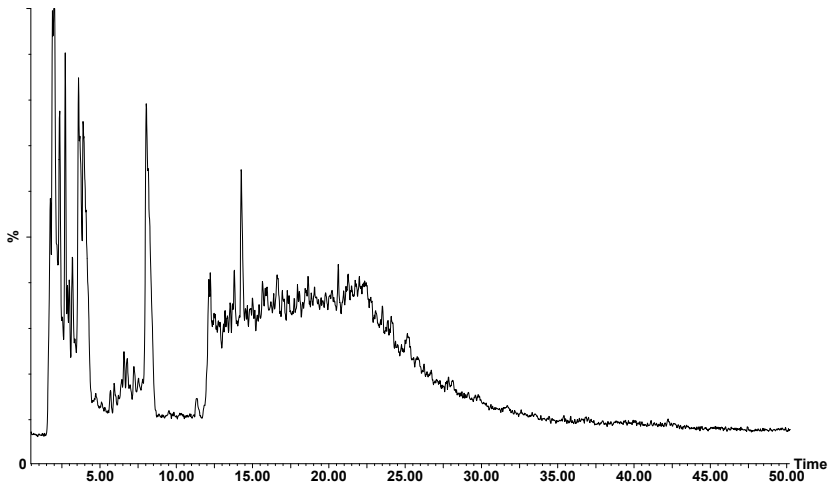


Figure 2.18. Typical chromatographic profile of poultry hydrolyzate

Mass spectra of the main chromatographic peaks were used in order to calculate the molecular weight of the associated compounds; the most abundant compounds were also analyzed by MS tandem technique in order to identify their structure.

The fragmentations obtained by performing daughter scan experiments confirmed the presence of carnosine, anserine, creatine, creatinine, inosine and hypoxanthine, characteristic meat compounds previously determined in Culatello sausages (Part I - Chapter 1), and also allowed to identify two other small nitrogen compounds: guanine and guanosine, products of guanosine monophosphate degradation [13]. In Figure 2.19 the daughter scan spectra of the unknown compounds (distinguished by MW) together with the daughter mass spectra of guanine and guanosine are reported.

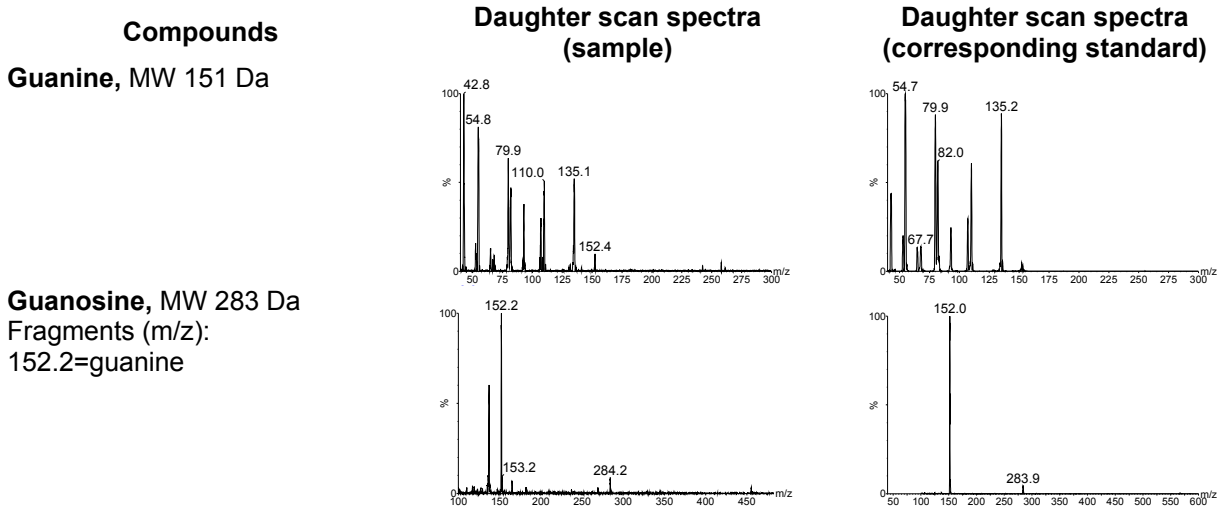


Figure 2.19. Daughters scan spectra of compounds identified in FAP samples in comparison with those of guanine and guanosine.

Anserine, carnosine, creatine, creatinine, guanine, guanosine, hypoxanthine and inosine were then quantified in FAP samples applying an UPLC/ESI-MS method suitably developed, which employed a reverse phase separation and a single ion monitoring detection of the different species, quantified with the internal standard method by using the dipeptide Phe-Phe as internal standard.

The data for FAP samples coming from the lab scale and coming from the pilot scale are shown in Figure 4.15 and 4.16 respectively (raw data obtained are reported in Attachment Table 12)

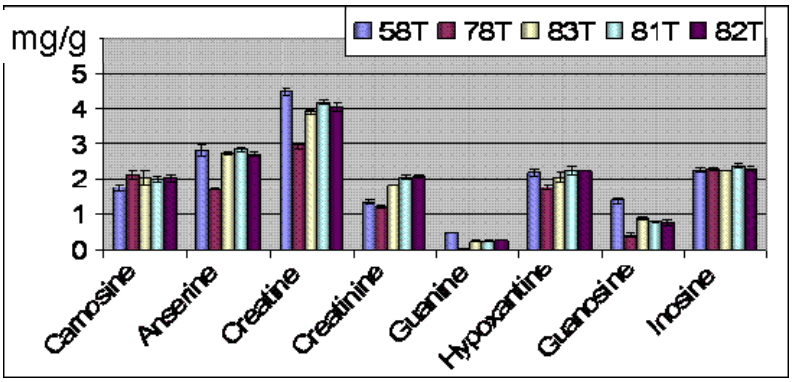


Figure 4.15. Small nitrogen compounds determined in FAP samples from lab scale.

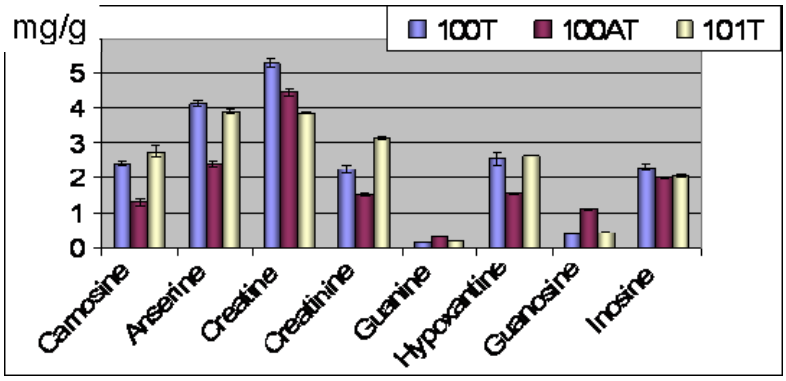


Figure 4.16. Small nitrogen compounds determined in FAP samples from pilot scale

The amount was found to be few mg/g, and quite interestingly samples coming from pilot scale were found to be generally richer in carnosine, anserine, creatine, creatinine than samples coming from lab scale.

The same determination was also performed on FFP sample 6L (the only soluble one), but, analogously to what has been found for free amino acids, sample 6L, on average, contained only traces of these compounds about 0-0.2 mg/g (raw data are reported in Attachment Table 13).

2.3.7 Peptides in FAP and FFP samples

The oligopeptide identification turned out to be hampered by the complexity of the mixtures. In order to overcome this complexity a different approach for the peptide identification was employed in collaboration with the Interdepartimental Center for Mass Spectrometry of the University of Florence (also participating in the PROSPARE project). Five FAP samples coming from lab scale plant and FFP sample 6L (the only soluble one) were prefractionated on a polar stationary phase (ZIC-HILIC column) and twenty seven fractions were collected and then singularly analyzed on a reversed phase

PepMap C18 column hyphenated with an LTQ-OrbiTrap detector allowing to perform MS/MS experiments in high resolution mode.

Data dependent scan experiments were used for data acquisition, BioWorks 3.3 software was used for data interpretation and protein/peptides identification. A specific chicken protein database was created using entries from UNIPROT Tremble.

The acquired data were searched using the Sequest programme (ThermoFisher) against a specific chicken protein database created using entries reporting *Gallus Gallus* from UNIPROT Tremble (downloaded from http://expasy.org/, September 2009)

The research was carried out using a tolerance value of 10 ppm for the monoisotopic precursor and of 0.5 AMU for monoisotopic product ions; "no enzyme" was selected for the database indexing and no missed cleavages were included in the research parameters.

Methionine oxidation was the only post-traductional modification allowed as variable.

SEQUEST parameters for protein identification were: P (pep) value lower than 0.001, cross-correlation values higher than 2.5 for triply charged and 3.0 for tetra charged ions, ion coverage percentage higher than 30%. A protein probability value lower than 0.001 was set as the threshold for identification.

Identification of the sequences of the most abundant peptides in the mixtures was performed with the main purpose of providing a detailed molecular characterization which could eventually be linked to desirable functional and technological properties (digestibility, antioxidant activity, bifidogenic activity, etc).

MS spectra were matched with a chicken protein database (also including protein sequences derived from genomic database) in order to identify the peptide sequences. The most abundant peptides identified for every sample belonged to the protein reported in Table 2.7.

Table 2.7. Protein identified in the FAP and FFP samples according to the most abundant peptides detected.

58T	*78T*	*81T*	*82T*	*83T*	*6L*
actin	alpha 1 globin	alpha 1 globin	apolipoprotein A1	Actin	actin
myosin	elongation factor	myosin	actin	myosin	thymidine kinase
	RNA polymerase II	troponin	albumin collagen	alpha 1 globin	keratin
	Beclin 1	actin	myosin	tyrosine kinase	feather keratin
		tropomiosin		exonuclease	cytokeratin

In FAP samples peptides coming from chicken actin and myosin are mostly represented among the most abundant peptides. It is interesting to note that FAP sample 78T, that

obtained in the strongest proteolytic conditions, does not present peptides coming from actin and myosin. In FFP sample 6L, obtained from feather, keratin is mostly represented.

2.4 Conclusion

The nitrogen fraction of several proteic hydrolyzates, obtained from the recovery of poultry by-products, produced at lab scale under different hydrolysis conditions and under optimal hydrolysis conditions at lab scale and pilot scale, was extensively studied.
Soluble and total proteins of Functional Animal Proteins (FAP), derived from meat and bone residues by enzymatic hydrolysis, and Functional Feathers Proteins (FFP), derived from feathers by hydrothermal hydrolysis, were first determined by the Kjeldahl method. FAP samples turned out to be very rich in proteins (total protein 80-90%), mostly present in water-soluble form. The protein content was found to be fairly stable, also in the sample obtained at pilot level.
FFP samples were even richer in proteins, the protein fraction being more than 90% of the total mass, but it was present in a totally insoluble form. Anyway, enzymatic hydrolysis of the FFP samples turned out to yield a very soluble protein-rich material.
The free amino acid content, determined by a new LC/MS/MS method suitably developed, was found to be not more that 35% of the FAP samples, slightly affected by the proteolysis conditions. Pilot level samples were found to be less rich in free amino acids, with a content of about 20%, but no significant variations in the amino acidic composition were observed. The free amino acid fraction was dominated in all samples by Glu and Asp (including Gln and Asn), with a quite low content of sulphorated amino acids and Trp.
Free amino acids in FFP samples, consistently with their almost total insolubility, were found to be nearly absent, but also in the digested FFP sample, which was much more soluble, their amount was found to be very low, less than 3% of the total mass.
Nutritional properties and biological value of FAP could be evaluated by calculating the amino acid score (AAS) and the racemisation degree. AAS of essential amino acids were calculated based on the FAO/WHO (1991) recommended protein standard. The chemical score, calculated assuming a total digestibility of the FAP hydrolyzates, was found to be quite high for Ile, Lys, , Leu, Thr, Trp , whereas it was slightly lower for Val

and Tyr+Phe (90-97%) and much lower for the sulphorated amino acids Met+Cys. Therefore, the biological value of FAP is limited by the content of Met+Cys.

D-amino acids were found to be nearly absent (only Asp was slightly racemized at 6-9%) in FAP, indicating at the same time the mildness of the proteolytic treatment and the absence of bacterial contamination, supporting the good nutritional value of the hydrolyzates.

On the other hand, the chemical score of essential amino acids calculated for FFP hydrolyzates identified Lys in all samples as limiting amino acid. The racemisation degree of Asp resulted quite high, about 30%, and also Ala and Glu were slightly racemized, probably due to the thermal stress during the hydrolytic process.

FAP was demonstrated to be mainly composed by peptides. By means of High Resolution Mass Spectrometry technique it was possible to identify a great number of peptides present in the hydrolyzates. Most of them originated from muscle tissue: peptides from actin and myosin were the most represented in the FAP sample. On the other hand, in the FFP sample, as expected, keratin was the most representative protein identified.

Small nitrogen compounds (non proteolytic peptides, non proteic amino acids, and nucleotides), were also identified and quantified in all soluble hydrolyzates and their content was found to be affected by the proteolysis degree: the highest proteolyzed sample showed the lowest value for every compound, indicating that increasing the harshness of treatment induced a higher degradation of the nitrogen compounds. The amount of each compound in the different samples indicates that guanine is the most sensitive to the proteolytic conditions (90% of the amount present in the lowest proteolyzed sample is lost in the highest proteolyzed one), followed by guanosine (80% loss) and anserine (50% loss).

Concluding, in this work it has been demonstrated that enzymatic hydrolysis and hydrothermal hydrolysis are very promising technologies for the recovery of poultry by-products, providing mild processing conditions which ensure higher retention of labile biologically active compounds. The evident advantages of these technologies are the deep bioconversion of raw materials into hydrolyzates with an high nutritional value.

In particular the FAP sample, obtained by a very mild enzyme-based technology, due to its higher soluble peptide content, may be a food ingredient with potential bioactive properties, such as antioxidant or antihypertensive.

References

[1] I. Arkhipchenko, I. A.; Salkinoja-Salonen, M. S.; Karyakina, J. N.; Tsitko, I., Study of three fertilizers produced from farm waste. *Applied Soil Ecology* **2005,** 30, (2), 126-132.

[2] Arvanitoyannis, I. S.; Ladas, D., Meat waste treatment methods and potential uses. *International Journal of Food Science and Technology* **2008,** 43, (3), 543-559.

[3] Montavalli J., The trouble with meat. *Environ. J*. **1998,** 9, 28.

[4] Nikolaev I.V., Stepanova E.V., Eremeev N.L., Ismailova D.Y., Zaichik B.T., Ruzhitskii A.O., Khotchenkov V.P., Kostyleva E.V., Sinitsyn A.P., Volik V.G., Koroleva O.V., Optimization of enzymatic hydrolysis of animal raw material for obtaining functional meat protein preparation. *Biotechnology-Moscow*, **2008**, 5, 59.

[5] Semenysheva A.I., Nikolaev I.V., Mashentseva N.G., Stepanova E.V., Mitaseva L.F., Koroleva O.V., Approach for rational conversion of raw materials of poultry processing industry. *Fleischwirtsch. Int. Rus.*. **2009**, 2, 50.

[6] Neklyudov, A. D.; Ivankin, A. N.; Berdutina, A. V., Properties and uses of protein hydrolysates - (Review). *Applied Biochemistry and Microbiology* **2000,** 36, (5), 452-459.

[7] Severin, S.; Xia, W. S., Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry* **2006,** 30, (1), 77-97.

[8] Eremeev, N. L.; Nikolaev, I. V.; Keruchen'ko, I. D.; Stepanova, E. V.; Satrutdinov, A. D.; Zinov'ev, S. V.; Ismailova, D. Y.; Khotchenkov, V. P.; Tsurikova, N. V.; Sinitsyn, A. P.; Volik, V. G.; Koroleva, O. V., Enzymatic hydrolysis of keratin-containing stock for obtaining protein hydrolysates. *Applied Biochemistry and Microbiology* **2009,** 45, (6), 648-655.

[9] Fletouris, D. J.; Botsoglou, N. A.; Papageorgiou, G. E.; Mantis, A. J., Rapid-Determination of Tryptophan in Intact Proteins by Derivative Spectrophotometry. *Journal of Aoac International* **1993,** 76, (6), 1168-1173.

[10] Yust, M. M.; Pedroche, J.; Giron-Calle, J.; Vioque, J.; Millan, F.; Alaiz, M., Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. *Food Chemistry* **2004,** 85, (2), 317-320.

[11] Wiles, P. G.; Gray, I. K.; Kissling, R. C., Routine analysis of proteins by Kjeldahl and Dumas methods: Review and interlaboratory study using dairy products. *Journal of Aoac International* **1998,** 81, (3), 620-632.

[12] *http//www.prospare.eu*

[13] Urich, K. Small nitrogen compounds. In: *Comparative Animal Biochemistry.* Springer-Verlag, Berlin, **1990**, p 403.

Chapter 3
Novel Highly Proteolyzed foods: Biological properties

Meat leftovers management has been, and continues to be, one of the most important issues related to pollution management and, at the same time, a potential solution for problems related to protein deficiency [1]. Animal processing by-products may be defined as carcasses or parts of animals including unmarketable animal residues not intended for direct human consumption. The fact that these by-products are rich in proteins, makes them very prospective as secondary protein sources. Nowadays, the emphasis is placed on reusing the discarded materials for production of food and feed grade protein ingredients.

Moreover, shifts which have occurred in the dietary habits to more healthy-like nutrition have led to a considerable increase in the consumption of poultry meat and consequently to elevated amounts of the corresponding residues [1]. Therefore, among all the potential raw material sources, leftovers of the poultry processing industry (meat and bone residues, backs, carcasses, necks etc.) seem to be highly promising. Chicken meat is considered to be a valuable source of biologically active compounds, including essential fatty and amino acids, as well as low molecular weight bioactive peptides of proteolytic and non-proteolytic origin [2,3]. Among the components of chicken meat and bone residues, collagenous and myofibrillar proteins are the most abundant ones, and it has been recently demonstrated that they could be converted into protein hydrolyzates exhibiting different biological activities [2,4]. Thus, protein hydrolyzates obtained from poultry processing by-products could also be used as bioactive food ingredients for high value-added products.

The existing technologies of protein recovery from complex natural matrices are mainly based on different types of hydrolysis – hydrothermal, acidic, alkaline, and enzymatic. Among them, enzymatic hydrolysis is the most promising one, providing mild processing conditions (e.g. pH and temperature) along with higher retention of labile biologically active compounds [5]. The evident advantages of enzyme-based technologies are the deep bioconversion of raw materials with a high level of protein recovery, the high

bioavailability of hydrolyzates produced, and the possibility to control and/or regulate both the functional properties and biological activity of the final products [6].

Functional and biological properties of protein hydrolyzates of animal origin have been intensively studied recently with particular emphasis on milk whey and fish-bone formulae [7,8]. Protein hydrolyzates have been demonstrated to exhibit a wide spectrum of biologically important properties, including immunomodulatory, antihypertensive, anticarcinogenic, bifidogenic, mineral-binding and antioxidant activities.

A wide spectrum of animal low-rank proteic by-products have been shown to be a valuable source of bioactive peptides, in particular showing antihypertensive, antioxidant and immunomodulatory properties. Carnosine and anserine, two peptides widely diffused in meat products, were identified as antioxidant by Wu and co-workers [3,9]. Fujita et al. [10] isolated seven peptides from chicken muscle hydrolyzate possessing antihypertensive properties, expressed by their ability to inhibit the Angiotensin Converting Enzyme (ACE). These peptides were shown to originate from actin, myosin, creatine kinase and aldolase, the most abundant proteins of muscle tissue. Saiga and co-workers isolated four bioactive peptides from chicken collagen hydrolyzate: these peptides derived from both α1 and α2 collagens exhibited ACE-inhibitory activity [4].

Protein hydrolyzates obtained from different sources were found to exhibit antioxidant properties both *in vitro* and *in vivo* [11]. Moreover, the potential of the utilization of poultry processing by-products into protein hydrolyzates possessing antioxidant properties was demonstrated [12]. There has been great interest in recent years in using the food protein hydrolyzates as a source of bioactive peptide compositions, especially those possessing antioxidant and antihypertensive activities [2,13]. Nevertheless, in spite of the potential prospectives mentioned above, little is known about the biological activity of enzymatic hydrolyzates originating from poultry processing leftovers.

Antioxidant capacity of poultry hydrolyzates

3.1 Aim of the work

The aim of the work presented in this chapter is the evaluation of the biological properties of the protein hydrolyzates enzymatically produced from poultry leftovers, also in relationship with their molecular composition. In particular proteolyzed samples were assayed for their antioxidant capacity against DDPH, ABTS radical-cation (TEAC) and peroxyl radical (ORAC), the methods most widely used for the characterization of food antioxidants [14].

3.2 Experimental Procedures

3.2.1 Reagents

- Deionised water obtained by using Millipore Alpha Q system
- Formic acid >99% (Acros Organics)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Methanol HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Potassium persulfate (FLUKA)
- Sodium chloride (Carlo Erba)
- Potassium chloride (Carlo Erba)
- Disodium hydrogen phosphate dodecahydrate (SIGMA)
- Potassium dihydrogen phosphate (SIGMA)
- Sodium hydroxide anhydrous pellets (Carlo Erba)
- Phosphoric acid (Carlo Erba)
- Phosphate Buffer Saline (PBS) prepared as follow:
  - 800 ml deionised water
  - 8 g NaCl
  - 0.2 g KCl
  - 2.86 g Na_2HPO_4•12H2O
  - 0.24 g KH_2PO_4
  - adjust pH to 7.4 by addition of phosphoric acid;

~ take to final volume 1 l with deionised water

> DPPH, 2,2-Diphenyl-1-picrylhydrazyl (SIGMA)

> ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Fluka)

> Trolox, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (SIGMA)

3.2.2 Instrumentation and Materials

> Technical balance (Orma)

> Analytical balance (Gibertini)

> Vacuum desiccator

> Paper filters

> WB-OD 24 thermostatic bath (FALC)

> Vortex SA6 (Stuart Scientific)

> Timer (Oregon Scientific)

> HPLC Alliance 2695 (Waters)

> Quattro Micro^{TM} API Mass Spectrometer (Waters)

> HPLC 510 binary system solvent, Automated Gradient Controller, 486 Tunable Absorbance Detector, 745 Data module (Waters)

> Jupiter 5 µ C18 300 Å (250X2.0 mm) analytical column (Phenomenex)

> Jupiter 5 µ C18 300 Å (250X10 mm) semi preparative column (Phenomenex)

> MassLynx 4.0 Software (Waters)

> Microprocessor pHmeter (Hanna Instruments)

> UV/VIS Spectrophotometer LAMBDA BIO 20 (Perkin Elmer)

> Disposable cuvettes (PLASTIBRAND®)

3.2.3 Procedures

3.2.3.1 Determination of antioxidant capacity by DPPH assay

The antioxidant capacity of FAP samples was determined as described by Sherer et al. [15] with slight modification. 0.2 g of a FAP sample were dissolved in 10 ml of deionised water and vacuum filtered with paper filter. 200 µl of filtered solution were transferred into the UV cuvette where 2 ml of DPPH 0.1 mM (in H_2O:CH_3OH solution 30:70 V/V) were directly added. The mixture was then incubated for 7 min in the dark. UV-VIS analysis was carried out from 600 to 450 nm and absorbance at 520 nm was recorded.

In order to calculate the Trolox Equivalents Antioxidant Capacity (TEAC), standard calibration solutions of Trolox were prepared starting from 250 µM Trolox stock solution then diluted 2, 3, 4 and 5 times.

For each sample or standard, 4 parameters were determined: MAX, Bmax, MIN and Bmin, where MAX is the absorbance at 520 nm of 2 ml of DPPH + 0.2 ml of deionised water (maximum absorbance), Bmax is the absorbance at 520 nm of 2 ml of H_2O:CH_3OH solution 30:70 + 0.2 ml of deionised water (blank control of maximum absorbance), MIN is the absorbance at 520 nm of 2 ml of DPPH + 0.2 ml of antioxidant (minimum absorbance) and Bmin is the absorbance at 520 nm of 2 ml of H_2O:CH_3OH solution 30:70 + 0.2 ml of antioxidant (blank control of minimum absorbance).

The calibration standard curve was obtained by plotting trolox concentration against (MAX-Bmax)-(MIN-Bmin) value, and slope, intercept and least-squares fit were calculated. Standard curve slope and intercept were then use to compute the corresponding concentration of trolox in the sample. To obtain the TEAC value (mol/g), the ratio between trolox concentration obtained (mol/l) and sample concentration (g/l) were calculated.

3.2.3.2 Determination of antioxidant capacity by ABTS assay

3.2.3.2.1 ABTS radical cation preparation

ABTS radical cation was generated according to Re et al. [16]. Strictly before using, 100 µl of 2 mM potassium persulphate were added to 10 ml of 2 mM ABTS solution in PBS and the mixture was incubated overnight in the dark.

The $ABTS^{\bullet+}$ stock solution obtained was further diluted with PBS to reach the final absorbance at 734 nm equal to 0.7.

3.2.3.2.2 UV-VIS determination

1.8 ml $ABTS^{\bullet+}$ 2 mM PBS solution freshly prepared was added, directly in cuvette, to 0.2 ml of 0.25 mg/ml solution of FAP samples in PBS. The absorbance is measured at 734 nm after a reaction time of 60 minutes.

In order to calculate the Trolox Equivalents Antioxidant Capacity (TEAC), standard calibration solutions of Trolox in the range from 100 µM to 10 µM were prepared.

For each sample or standard 4 tests were analysed: MAX, Bmax, MIN and Bmin, where MAX is the absorbance at 734 nm of 1.8 ml of $ABTS^{\bullet+}$ + 0.2 ml of PBS (maximum

absorbance), Bmax is the absorbance at 734 nm of 2 ml of PBS (blank control of maximum absorbance), MIN is the absorbance at 734 nm of 1.8 ml of $ABTS^{\bullet+}$ + 0.2 ml of antioxidant (minimum absorbance) and Bmin is the absorbance at 734 nm of 1.8 ml of PBS + 0.2 ml of antioxidant (blank control of minimum absorbance).

The calibration standard curve was obtained by plotting trolox concentration against MAX-MIN value, and slope, intercept and least-squares fit were calculated. Standard curve slope and intercept were then used to compute the corresponding concentration of trolox in the sample. To obtain the TEAC value (mol/g) the ratio between trolox concentration obtained (mol/l) and sample concentration (g/l) were calculated.

3.2.3.3 Hydrolyzate fractionation by semipreparative HPLC/UV

50 mg of poultry hydrolyzate sample exactly weighted were dissolved in 1.5 ml of deionised water.

HPLC/UV fractionation was achieved by using an HPLC 510 binary system solvent coupled with Waters Automated Gradient Controller and Waters 745 Data module with a Jupiter Phenomenex 5 µ C18 300 Å (250X10 mm) semipreparative column.

The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 12 min, from 100% A to 50% A by linear gradient in 65 min and 4 mins at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was 5 ml/min, injection volume 500 µl, column and sample were at ambient temperature. Detection was performed by using Waters 486 Tunable Absorbance Detector monitoring the absorbance at 214 nm.

10 different fractions were collected: 0-10 mins, 10-20 mins, 20-25 mins, 25-30 mins, 30-35 mins, 35-40 mins, 40-45 mins, 45-50 mins, 50-55 mins and 55-70 mins, each fraction was then dried by rotavapor. Several injections were performed until 200 mg of the sample were Collected.

3.2.3.4 Determination of antioxidant capacity of hydrolyzate fractions by DPPH assay

The solid residue of each fraction was dissolved in 10 ml of deionised water in order to maintain for each fraction the same concentration that had in the sample. The antioxidant capacity of fractions was determined as previously described for FAP sample.

3.2.3.5 HPLC/ESI-MS/MS analysis of hydrolyzate fractions

HPLC/ESI-MS/MS analyses of hydrolyzate fractions were carried out by using an Alliance 2695 separation system with a Jupiter Phenomenex 5 µ C18 300 Å (250X2.0 mm) analytical column. Mobile phase and gradient elution were the same applied during fractionation by HPLC/UV. Flow rate was 0.2 ml/min, column temperature was set at 30°C and injection volume was 20 µl. Detection was performed by using a Waters triple quadrupole Quattro Micro™ API mass spectrometer applying the following conditions: positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 100°C, desolvation temperature 150°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h. Full scan acquisition followed by a daughter scan experiment of most abundant ions identified in each fraction was performed. Data analysis was carried out by using MassLynx 4.0 software.

3.2.3.6 Identification of the most abundant antioxidant components

This part of the work has been carried out at the A.N. BAKH Institute of Biochemistry of Russian Academy of Science (INBI).

3.2.3.6.1 Fractionation by SE-HPLC

Fractionation of the lowest and the highest hydrolyzed FAP samples was achieved by high-performance size-exclusion chromatography; by using a BioSep-SEC-S 2000 column (600×2.1 mm) from Phenomenex (Torrance, Ca, USA) installed into the ProStar HPLC chromatographic device (Varian Inc., USA). FAPs were dissolved (2 mg/ml) in 50 mM sodium phosphate buffer, pH 6.8, filtered through 0.45 µm hydrophilic filters (Sartorius AG, Germany), and the resultant FAP solutions were injected (5 ml) into the system with subsequent isocratic elution with 25 mM NaH_2PO4:Na_2HPO_4 (1:1) at a flow rate of 5 ml/min. The UV-Vis detector was set at 280 nm. One fraction per minute was collected starting from 15 minutes.

3.2.3.6.2 Fractionation by IE-HPLC

Fractionation of the highest antioxidant SE-fractions derived from FAP samples was achieved by high-performance ion-exchange chromatography; by using a Ge Healthcare Source (10.3X10 mm) column installed into the ProStar HPLC chromatographic device (Varian Inc., USA). 1 ml of solution was injected into the system with subsequent linear gradient elution of buffer A (15 mM NaH2PO4 +25mM

H3PO4+15% ACN) and buffer B (buffer A + 0.25 mM Na2SO4 +15% ACN) at a flow rate of 2 ml/min. The UV-Vis detector was set at 280 nm. One fraction per 1.5 minutes was collected starting from 3 minutes.

3.2.3.6.3 Determination of antioxidant capacity by ABTS assay

Antioxidant capacity (AOC) of the highest and the lowest hydrolyzed FAPs, of the SE-fractions and of the IE-fractions was measured.

The radicals were generated according to Re et al. (1999) as previously described (Part II -3.2.3.2.1). Trolox solutions (10-100 μM) were used for calibration. The reaction was initiated by mixing 20 μl of sample or solvent blank with 180 μl of $ABTS^{\bullet+}$, followed by measurement of the absorbance at 734 nm for 40 minutes using Synergy 2 plate reader (BioTek, USA). TEAC values were determined at final FAP concentration, in the reaction mixture, of 15 μg/ml. All measurements were performed in 4 replicates. TEAC values of FAP were calculated based on the linear regression equation between the trolox concentration and the decrease in the absorbance of $ABTS^{\bullet+}$ (ΔD=Dblank-Dstandard) (ΔD=0.0012[trolox]). AOC was expressed as an amount of trolox molar equivalents (TE, μM) per g of FAP. AOC of each fraction was expressed as a percentage.

3.2.3.6.4 Determination of antioxidant capacity by ORAC-FL assay

ORAC-FL assay of the highest and the lowest hydrolyzed FAPs, of the SE-fractions and of the IE-fractions was carried out on a Synergy 2 plate reader (BioTek, USA) at 37°C. The procedure was based on the previous report of Ou et al. (2001) [17] with slight modifications. Briefly, AAPH was used as a source of peroxyl radicals and fluorescein sodium salt as a fluorescent probe. All solutions were prepared at 75 mM sodium phosphate buffer, pH 7.4. Trolox solutions (5-75 μM) were used for calibration.

The assay was performed in 96-well plates with 4 replicates for each sample or standard solution or FAP fractions. The final mixture contained 115 μL of fluorescein sodium salt solution (8.16×10^{-8} M), 15 μl of sample or standard solution and 15 μl of 0.6 M AAPH solution. The AAPH solution was added directly before the reading with subsequent vigorous orbital shaking of the plate for 30 s at 1400 rpm. Fluorescence intensity was monitored for 1 hour under the following working parameters of the reader: excitation at 485 nm, emission filter at 528 nm, reading interval 60 s. The final ORAC-FL

values of FAP were calculated by using the linear regression equation between the trolox concentration and the net area (net AUC) of the fluorescein decay curve (net AUC=Rf [trolox]. AOC of each fraction was expressed as a percentage.

3.2.3.6.5 Identification of the most abundant antioxidant components

Most antioxidant active components of the selected fractions were identified by INBI by RP-HPLC-MS/MS analysis.

3.3 Results and discussion

Two methods were optimized in order to test hydrolyzate antioxidant capacity, based on the scavenging of ABTS and DPPH radicals. The free radical scavenging capacity of FAP samples, 0.25 mg/ml dissolved in Phosphate Buffer Saline (PBS), was evaluated against chemically generated ABTS radical. The scavenging power was determined by measuring the absorbance at 734 nm after a reaction time of 60 minutes. The radical DPPH scavenging capacity of FAP samples produced at lab level (samples description in Part II -2.3.1.1) was determined following a standard procedure suitably modified in order to accommodate the low solubility of the FAP samples in organic solvents. Briefly, 20 mg/ml of FAP sample in deionised water were added to a freshly prepared 0.1 mM DPPH hydroalcoholic solution (H_2O:MeOH 70:30 V/V). The absorbance was measured at 520 nm after a reaction time of 7 minutes.

In both cases Trolox Equivalents Antioxidant Capacity (TEAC) was calculated using a standard curve prepared with trolox and expressed in µmoles of Trolox per gram of FAP sample. The results obtained are shown in Figure 3.1 and Figure 3.2.

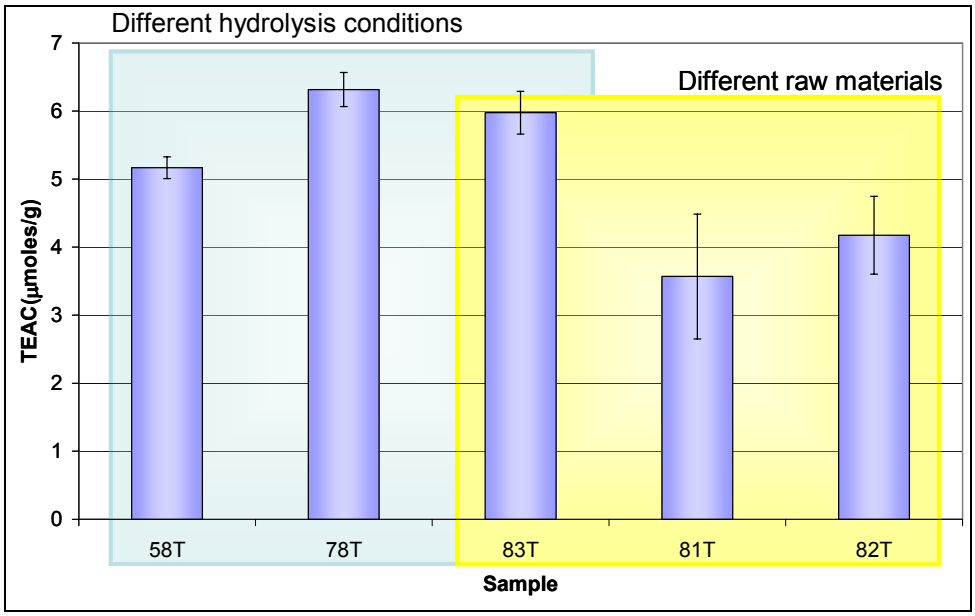


Figure 3.1. Antioxidant capacity of poultry protein hydrolyzates determined by DPPH• assay.

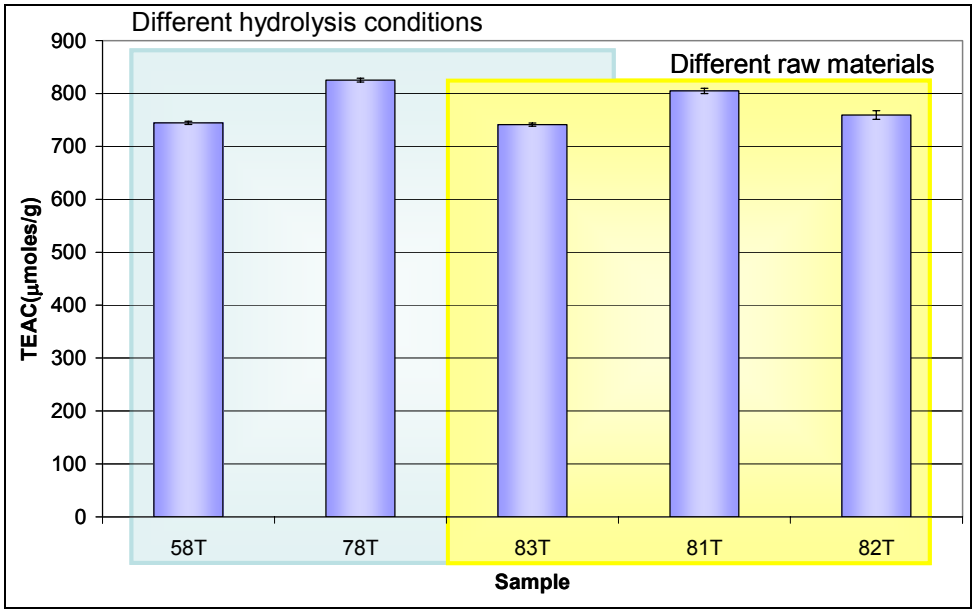


Figure 3.2. Antioxidant capacity of poultry protein hydrolyzates determined by ABTS•$^{+}$ assay.

It can be easily seen that the values obtained by DPPH assay are much lower than the ones obtained using ABTS. This is consistent with literature data, which indicate that antioxidants react faster with ABTS than with DPPH, due to its higher steric hindrance. An alternative hypothesis might be that the addition of water to the reaction mixture, a procedure seldom applied for DDPH, might lead to side reactions which disturb the quenching activity, eventually leading to the observed underestimation. Obviously, this observation stresses the necessity of combining more than one method in order to determine antioxidant capacity.

In spite of the differences between the TEAC values, the comparison among the different samples, both in the ABTS and DPPH assay, showed that the antioxidant capacity of FAP hydrolyzates was strictly related to the hydrolysis degree of the samples. Moreover, the difference observed among samples prepared with the same procedures also suggested a certain dependence on the composition of the raw material used as ingredients.

In order to better evaluate the antioxidant capacity of the samples, ten fractions obtained from hydrolyzates produced with different digestion conditions, purified by semipreparative HPLC, were also tested by DPPH assay. Their relative antioxidant activity compared with the total one is shown in Figure 3.3.

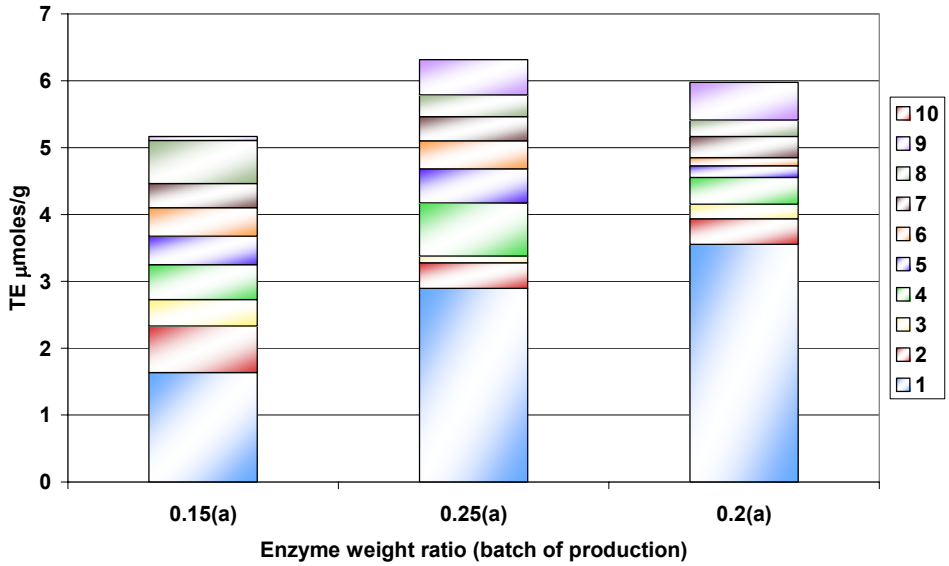


Figure 3.3. Effects of the different fractions on the total FAP antioxidant capacity.

It is clear that the main activity is due to the first fraction, which is quite obvious, this fraction also being the most abundant, but it is also evident that in all the samples, a major percentage of the antioxidant activity is also exerted by the other fractions.

The single fractions were also analysed by UPLC/ESI-MS, the chromatograms obtained are shown in Figure 3.4, allowing for the identification of some of the components contained.

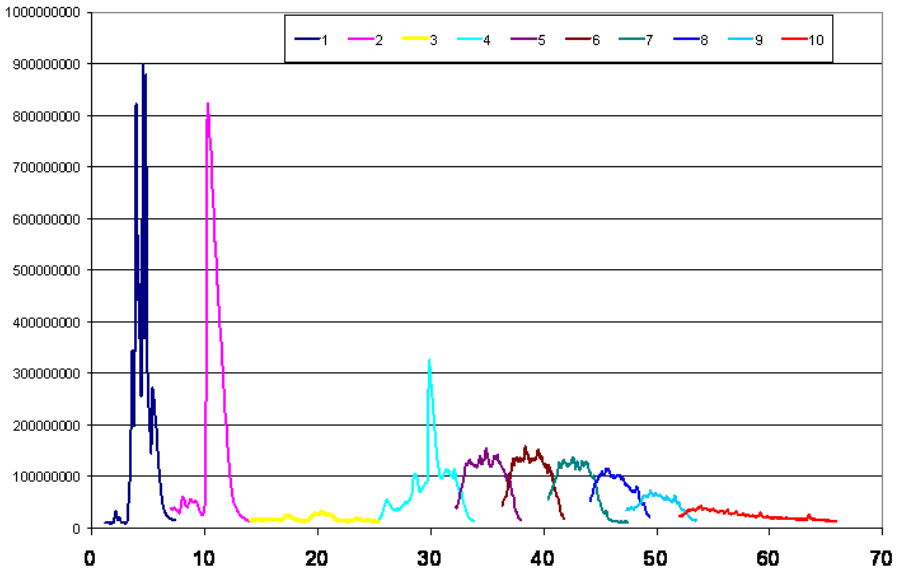


Figure 3.4. UPLC/ESI-MS chromatograms (superimposed) of the different fractions purified by the preparative HPLC.

In the first fraction (0-10 min), beside several amino acids, creatinine, creatine, hypoxanthine, guanine, carnosine and anserine were identified. The second fraction was constituted almost only by the amino acid Phe. In the fourth fraction Trp is the main component but inosine and guanosine were also identified, together with several peptides: Phe-Arg, Ala-Leu. Fractions from fifth to tenth are made by peptides with low molecular weight, less than 5 KDa.

In conclusion, the fraction showing the higher antioxidant activity is that containing the free amino acids, but it also proved evident that in all the samples a non-negligible percentage of the antioxidant activity was also exerted by the fractions containing peptides. As expected, these fractions were found to give a more important relative contribution to the antioxidant capacity in the less proteolyzed sample.

The antioxidant activities of FAP samples obtained at pilot level and of FFP sample 6L (the only soluble one, sample description in Part II -2.3.1) have also been determined by ABTS tests. The data, compared with the FAP sample obtained at lab level in an optimized hydrolysis condition, are reported in Figure 3.8.

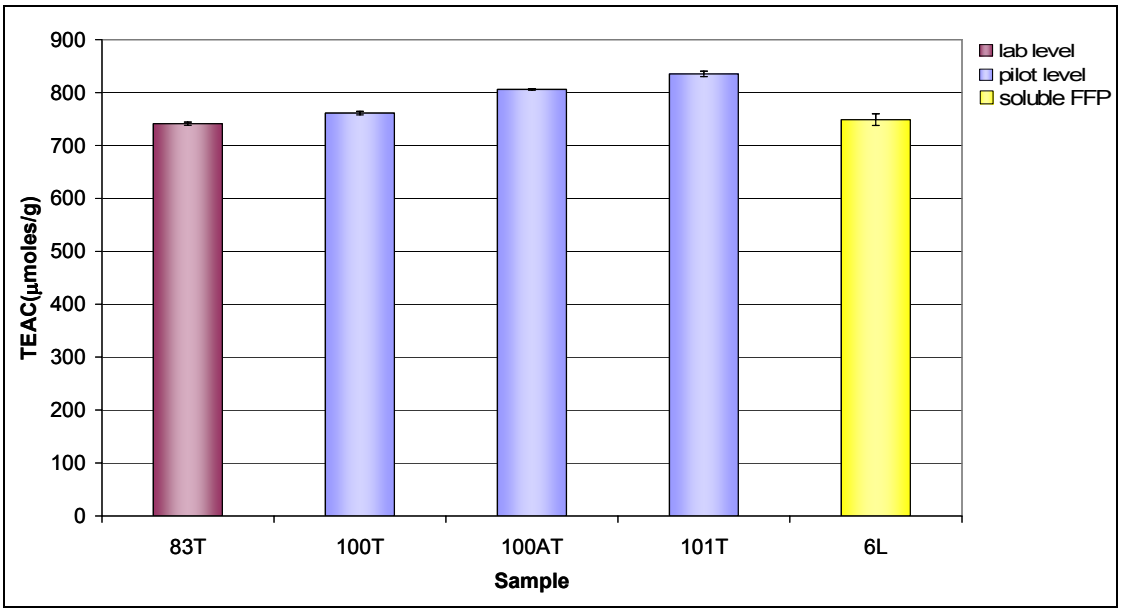


Figure 3.5. TEAC value calculated for the FAP samples produced at pilot level and for the soluble FFP sample.

Antioxidant activity turned out to be very similar in all compounds, with values around 750-830 μmoles of trolox per g of FAP/FFP.

The results above reported show that all these samples have a marked antioxidant capacity: 10 g of FAP are equivalent to 0.8 g of ascorbic acid or 2 g of alfa-tocopherol, calculated on the basis of literature data [16].

The TEAC values of FAP were also compared to common natural preservatives, the results are reported in Figure 3.6.

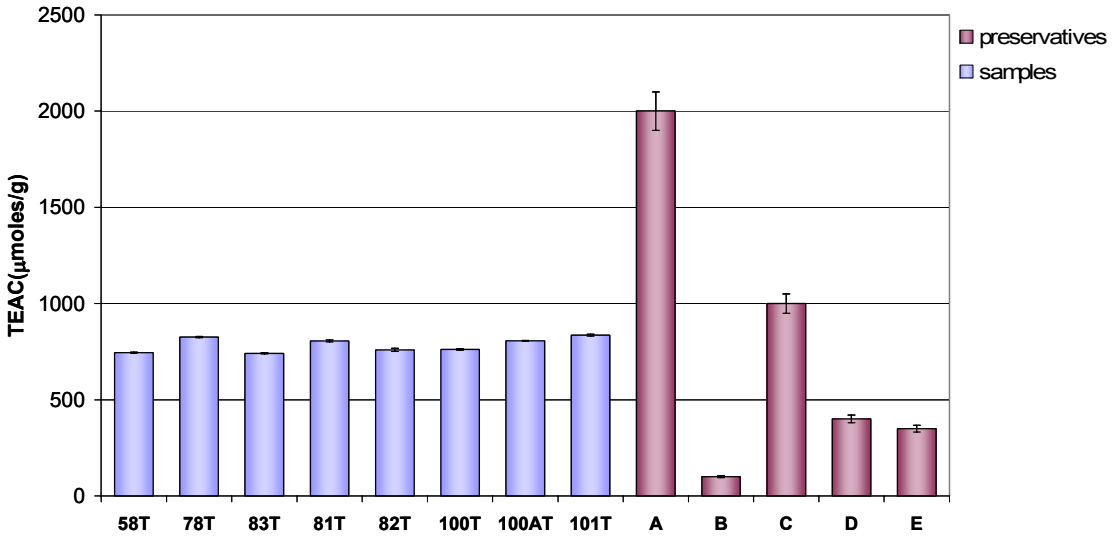


Figure 3.6. TEAC values calculated for the FAP samples in comparison with natural preservatives commonly used in meat industry.

As can be seen, except for the preservative coded “A”, the FAP values are comparable, and in some cases even better than the values observed for the already used meat preservatives.

The results obtained provide evidence that FAP could be used as a natural antioxidant ingredient in food matrices.

3.3.1 Identification of the most abundant antioxidants components

In order to identify the main antioxidant constituents presents in poultry proteins hydrolyzates, the lowest (58T) and the highest (78T) proteolyzed FAP samples were first fractionated by SE-chromatography and the antioxidant capacity of each fraction was determined by ORAC and ABTS assays (Figure 3.7).

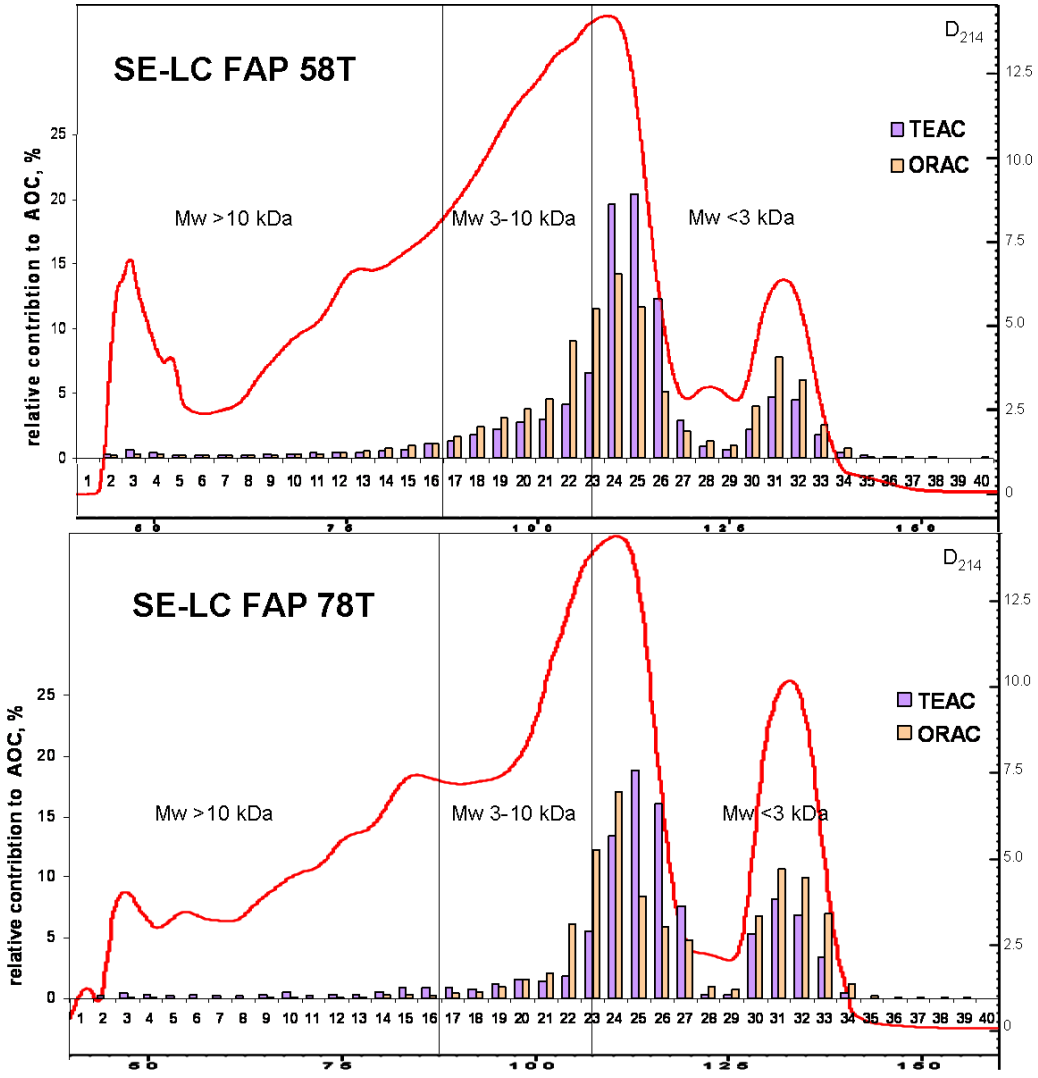


Figure 3.7. SE-LC fractionation of FAP samples, for each fraction its relative contribution of antioxidant capacity is reported.

The distribution of the antioxidant capacity among FAP fractions determined by ORAC and TEAC assays are very similar, with two main regions (fractions 23-26 and 30-33) providing the highest relative contributions to the antioxidant power. Antioxidant properties of FAP were mainly related to low molecular weight compounds (Mw <3 kDa). Their contribution to the antioxidant capacity amounted to 73% for the less hydrolyzed FAP (58T) and to 86% for the more hydrolyzed FAP (78T). The increase of the relative antioxidant power of low molecular weight compounds was accompanied by a decrease in the antioxidant power of the fractions with medium molecular weight constituents (3-10 kDa).

The most active fractions (23, 25, 31, and 32) were successively subjected to further subfractionation by IE-HPLC and the antioxidant capacity of each subfraction was tested again both by ORAC and ABTS assay. Active constituents were finally identified by RP-HPLC/MS/MS (Figure 3.7).

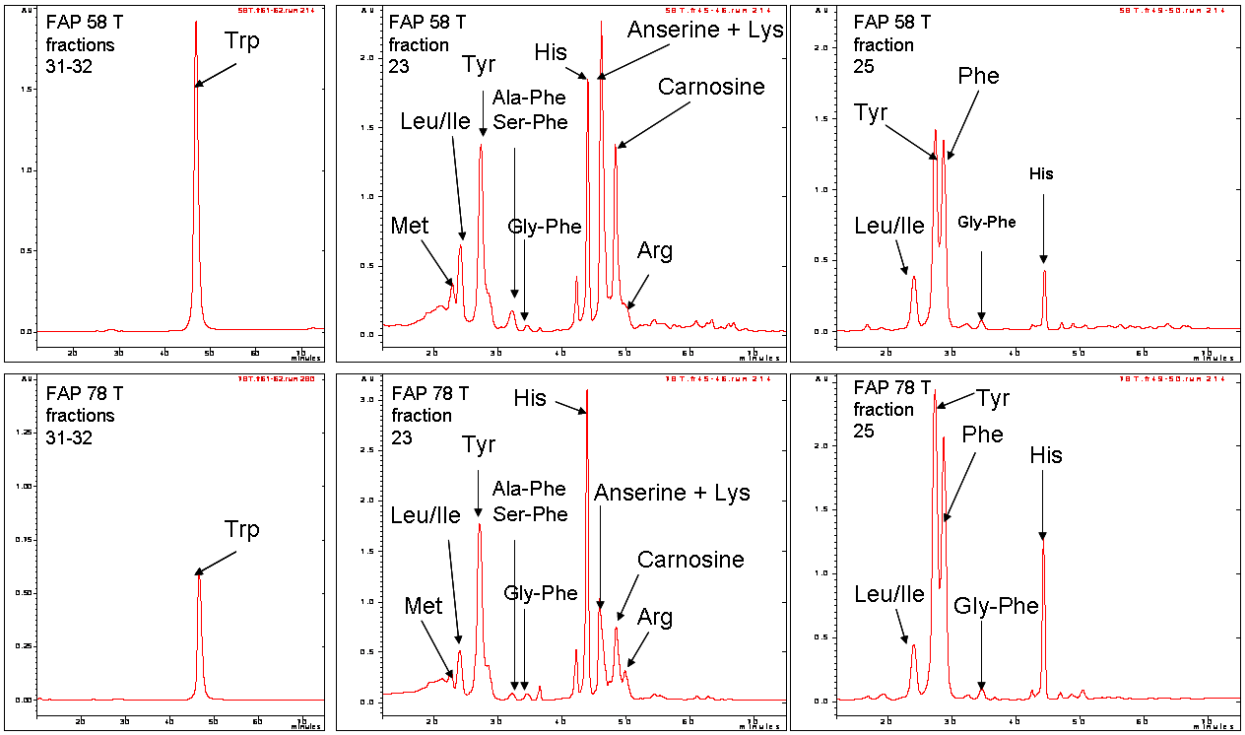


Figure 3.8. IE-HPLC subfractionation of FAP and identification of active constituents.

Fractions which corresponded to the first active region (fractions 23-26) included the free amino acids Met, Leu/Ile, Tyr, His, Lys, Arg, Phe, the phenylalanine-containing dipeptides Gly-Phe, Ala-Phe, Ser-Phe and the His-containing dipeptides anserine and carnosine. The last two constituents could be considered as characteristic markers of

meat-originating products. The second active region (fractions 30-33) was completely attributed to free tryptophan, which alone accounts for 20% of the total antioxidant capacity.

Free His, Met and Tyr contributed on average 1.5, 10 and 18% of FAP antioxidant capacity determined by ORAC-Hydro assay, while free tyrosine contributed to more than half (54%) FAP´s antioxidant capacity determined by TEAC assay. The relative input of carnosine and anserine could be considered negligible.

3.4 Conclusions

The antioxidant capacity of FAP hydrolyzates and of the enzymatically digested FFP sample have been evaluated by DPPH and ABTS assays. The results obtained showed that all the analyzed samples possessed a quite good antioxidant capacity, comparable to those of other protein hydrolyzates of plant and animal origin, and the scale up of the process does not significantly affect this property, which remains constant also in the samples produced by the pilot plant. In general, antioxidant capacity was linked to the proteolysis degree (the most antioxidant being also the most proteolyzed sample), and small variations could be observed, probably linked to the different raw materials used.

Chromatographic fractionation of the highest proteolyzed sample, possessing the highest antioxidant capacity, allowed to determine that, more than 50% of total antioxidant activity was due to the content of free tyrosine and tryptophan. Their contribution in the lower proteolyzed sample was slightly less, but in this case the contribution of the peptides was not negligible. The fact that the total antioxidant activity of the sample was less probably indicates that the antioxidant capacity of the amino acids is less if they are included in the peptide form.

The data show that poultry hydrolyzates, obtained by this new biocatalytic approach, have an high potential to be used as natural antioxidant food ingredients and that their antioxidant capacity may be easily tailored for different purposes by adjusting the harshness of the digestion treatment.

References

[1] Arvanitoyannis, I. S.; Ladas, D., Meat waste treatment methods and potential uses. *International Journal of Food Science and Technology* **2008,** 43, (3), 543-559.

[2] Toldrá, F.; Arihara, K.; Ohata, M., Bioactive Compounds in Meat. In *Meat Biotechnology*, Springer New York: **2008**; pp 231-249

[3] Wu, H. C.; Pan, B. S.; Chang, C. L.; Shiau, C. Y., Low-molecular-weight peptides as related to antioxidant properties of chicken essence. *Journal of Food and Drug Analysis* **2005,** 13, (2), 176-183.

[4] Saiga, A.; Iwai, K.; Hayakawa, T.; Takahata, Y.; Kitamura, S.; Nishimura, T.; Morimatsu, F., Angiotensin I-Converting Enzyme-inhibitory Peptides Obtained from Chicken Collagen Hydrolysate. *Journal of Agricultural and Food Chemistry* **2008,** 56, (20), 9586-9591.

[5] Neklyudov, A. D.; Ivankin, A. N.; Berdutina, A. V., Properties and uses of protein hydrolysates - (Review). *Applied Biochemistry and Microbiology* **2000,** 36, (5), 452-459.

[6] Klompong, V., Benjakul, S., Katanchote, D., Shahidi, F., 2007. Antioxidant activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102, 1317-1327.

[7] Erdmann, K.; Cheung, B. W. Y.; Schroder, H., The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry* **2008,** 19, (10), 643-654.

[8] Moller, N. P.; Scholz-Ahrens, K. E.; Roos, N.; Schrezenmeir, J., Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition* **2008,** 47, (4), 171-182.

[9] Wu, H. C.; Shiau, C. Y.; Chen, H. M.; Chiou, T. K., Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *Journal of Food and Drug Analysis* **2003,** 11, (2), 148-153.

[10] Fujita, H.; Yokoyama, K.; Yoshikawa, M., Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science* **2000,** 65, (4), 564-569.

[11] Sarmadi, B. H.; Ismail, A., Antioxidative peptides from food proteins: A review. *Peptides* **2010,** 31, (10), 1949-1956.

[12] Flaczyk, E.; Amarowicz, R.; Korczak, J., Antioxidant activity of protein hydrolyzates from by-products of the food industry. *Journal of Food Lipids* **2003,** 10, (2), 129-140.
[13] Gilmartin, L.; Jervis, L., Production of cod (Gadus morhua) muscle hydrolysates. Influence of combinations of commercial enzyme preparations on hydrolysate peptide size range. *Journal of Agricultural and Food Chemistry* **2002,** 50, (19), 5417-5423.
[14] Moon, J. K.; Shibamoto, T., Antioxidant Assays for Plant and Food Components. *Journal of Agricultural and Food Chemistry* **2009,** 57, (5), 1655-1666.
[15] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* **1999,** 26, (9-10), 1231-1237.
[16] Ou, B. X.; Hampsch-Woodill, M.; Prior, R. L., Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry* **2001,** 49, (10), 4619-4626.

Antihypertensive properties of poultry hydrolyzates

High blood pressure or hypertension is the major controllable risk factor associated with cardiovascular disease events, such as myocardial infarction, stroke, heart failure, and end-stage diabetes [1]. Angiotensin Converting Enzyme (ACE) is a dipeptidyl carboxypeptidase that plays an important role in regulating blood pressure. In the rennin-angiotensin system, ACE cleaves the C-terminal dipeptide portion of the peptide angiotensin I, producing the potent vasopressor peptide angiotensin II, which induces the release of aldosterone, and causes the retention of sodium ions by the kidneys and elevates the blood volume, thus increasing blood pressure [2]. In addition, ACE also inactivates the vasodilator, bradykinin [3] (Figure 3.9). Consequently, ACE inhibitors may exert an antihypertensive effect.

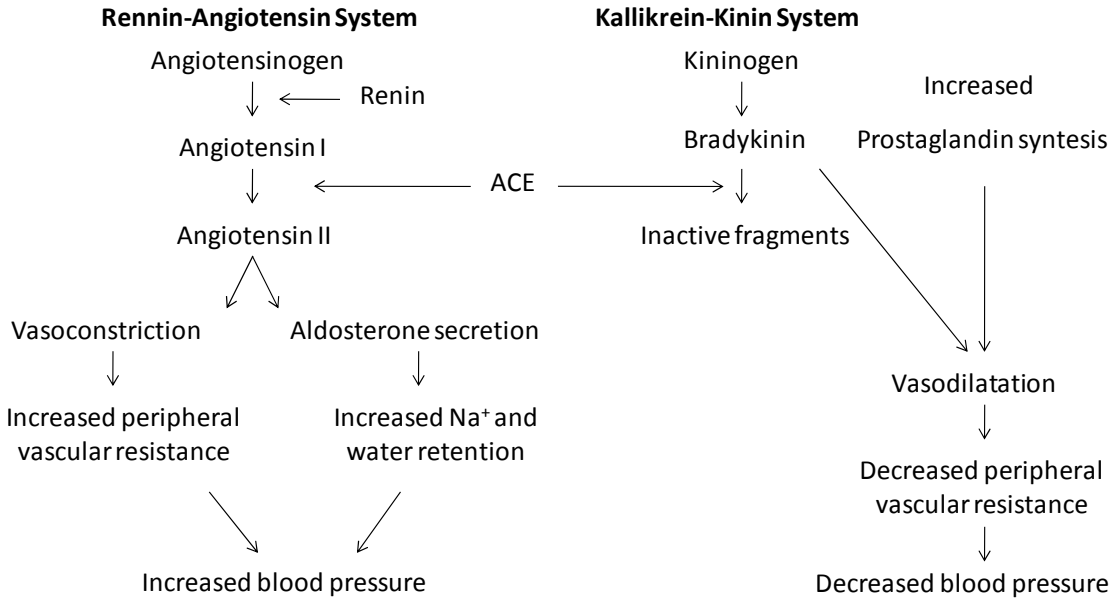


Figure 3.9. Angiotensin Converting Enzyme role in the regulation of blood pressure [4].

Several ACE inhibitor drugs have been designed in order to fight hypertension; the most potent inhibitor is D-3-mercapto-2-methylpropanoyl-L-proline, or Captopril, that contains a sulfhydril function, a group able to sequester the zinc ion from the active site of ACE, thus inhibiting its activity. Captopril and its derivatives with antihypertensive effects are reported in Figure 3.10.

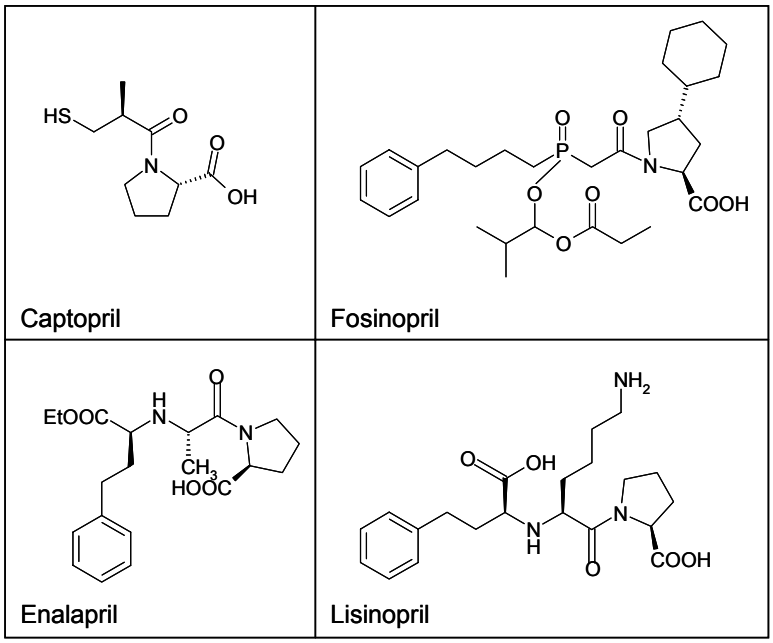


Figure 3.10. Molecular structure of Captopril and its derivatives [5]

In spite of their high efficiency, the oral administration of these compounds presents several side effects on body functions.

Many other peptides, naturally present in some foods (peptides derived from milk proteins have been particularly studied from this point of view), may exert a slight ACE-inhibiting effect, with the advantage of not presenting side effects [6].

For this reason, to moderately control the blood pressure level, even as prevention in healthy subjects, without bringing a sharp decrease of the pressure or other side effects, the nutraceutical approach of consuming peptides derived from natural food sources may be used.

Several food protein sources including fish, gelatine, maize and animal muscle proteins contain ACE-inhibitory peptides as reviewed by Ariyoshi, 1993 [7] and Vercruysse [8] and also milk proteins are precursors for a range of peptides which inhibit ACE activity [9-10]. Several methods to assay Angiotensin Converting enzyme activity have been published [11].

In the present work the Chusman and Cheung assay [12] was used to monitor the antihypertensive activity in proteic hydrolyzates derived from poultry leftovers. Briefly, this method is based on the production of hippuric acid by the Angiotensin Converting Enzyme (ACE), starting from the substrate Hyppuryl-Hystidyl-Leucine (HHL), reaction reported in Figure 3.11.

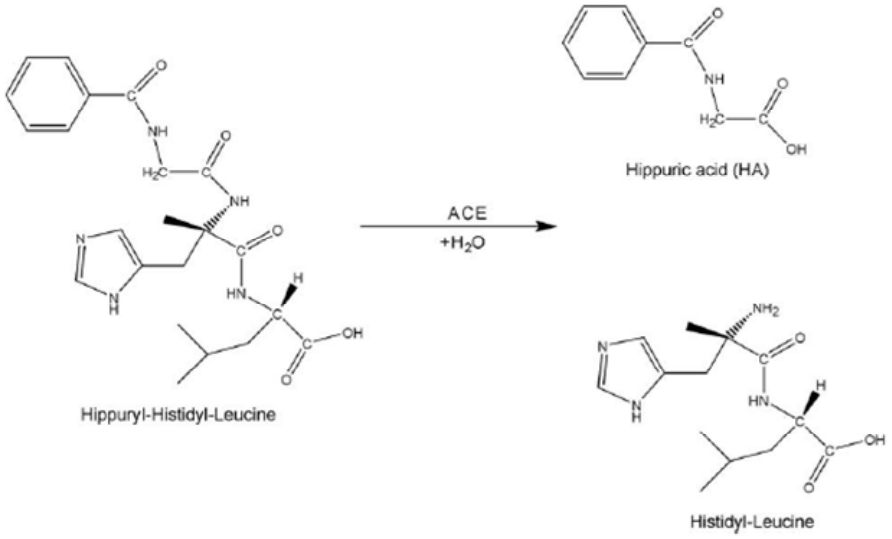


Figure 3.11. Reaction of the Angiotensin Converting Enzyme with the synthetic substrate HHL.

In this spectrophotometric assay the increase of hippuric acid released from hippuryl-histidyl-leucine (substrate) is related to the ACE activity. The sample (inhibitor) has to be incubated with ACE at 37°C in the presence of the substrate. The hippuric acid formed by the action of ACE has to be extracted with ethyl acetate, and after removal of ethyl acetate by evaporation, the amount of hippuric acid is measured spectrophotometrically at 228 nm. The inhibitory activity can be expressed as a percentage of ACE inhibition per gram of sample or as the grams of the sample needed to inhibit 50% of the ACE activity (IC_{50}).

Although highly selective for ACE, this procedure has low sensitivity and reproducibility due to the incomplete inhibition of ACE at the end of incubation time and/or the presence of interfering substances that are extracted in the standard liquid-liquid extraction procedure with ethyl acetate, resulting in high blank values. In order to overcome these problems, the original method, based on the spectrophotometric determination of hippuric acid, was improved by using LC/MS for its determination.

3.5 Aim of the work

In the present work the Angiotensin Converting Enzyme inhibition activity of poultry protein hydrolyzates very rich in peptides, obtained from the recovery of poultry by-products in different conditions, has been evaluated.

3.6 Experimental Procedures

3.6.1 Reagents

- Deionised water obtained by using Millipore Alpha Q system
- Formic acid >99% (Acros Organics)
- Hydrochloric acid 37% (Carlo Erba)
- Boric acid (Carlo Erba)
- Phosphoric acid (Carlo Erba)
- Sodium hydroxide anhydrous pellets (Carlo Erba)
- Potassium hydroxide anhydrous pellets (Carlo Erba)
- sodium chloride (Carlo Erba)
- Sodium borate decahydrate (SIGMA)
- Potassium dihydrogen phosphate (SIGMA)
- Acetonitrile HPLC grade (CHROMASOLV® SIGMA-ALDRICH)
- Ethyl acetate (distilled in the Department)
- Acetone (distilled in the Department)
- Angiotensin-I-Converting Enzyme, *ACE* (SIGMA)
- Hippuryl-L-Hystidyl-L-Leucine, *HHL* (SIGMA)
- Enalapril (SIGMA)
- 0.1 M Sodium borate buffer (NaBB) pH 8.3 prepared as follow:
  - 90 ml of deionised water;
  - 3.8 g of $Na_2B_4O_7 \cdot 10H_2O$;
  - 1.7 g of NaCl;
  - adjust pH to 8.3 by addition of boric acid;
  - take to final volume 100 ml with deionised water
- 0.01 M potassium phosphate buffer (KPB)pH 7 prepared as follow:
  - 90 ml of deionised water
  - 0.136 g of KH_2PO_4
  - 2.9 g of NaCl
  - adjust pH to 7 by addition of H_3PO_4;
  - take to final volume 100 ml with deionised water

3.6.2 Instrumentation and Materials

- Technical balance (Orma)
- Analytical balance (Gibertini)
- Vacuum desiccators
- Paper filters
- WB-OD 24 thermostatic bath (FALC)
- Ultrasonic bath 57X (Ultrasonik)
- Vortex SA6 (Stuart Scientific)
- Timer (Oregon Scientific)
- Disposable 5 ml glass tubes
- Microprocessor pHmeter (Hanna Instruments)
- 4206 centrifugette (ALC)
- MULTIVAP nitrogen evaporator
- HPLC Alliance 2695 (Waters)
- 996 Photo Diode Array (Waters)
- Quattro Micro™ API Mass Spectrometer (Waters)
- Jupiter 5 µ C18 300 Å (250X2.0 mm) analytical column (Phenomenex)
- ACQUITY UPLC (Waters)
- SQD mass spectrometer (Waters)
- ACQUITY BEH 1.7 µ C18 (50X2.1 mm) UPLC column (Waters)
- MassLynx 4.0 Software (Waters)
- UV/VIS Spectrophotometer LAMBDA BIO 20 (Perkin Elmer)
- Disposable cuvettes (PLASTIBRAND®)
- SPSS 17 software

3.6.3 Procedures

3.6.3.1 Evaluation of the optimal conditions to determine Angiotensin-I-Converting-Enzyme (ACE) activity

In Table 3.1 all reagents with their corresponding used volumes in order to determine the optimal condition to monitor the ACE activity are reported.

200 µl of 5 mM HHL solution in sodium borate buffer (NaBB) were placed in 5 ml glass tubes and heated at 37°C in a water bath. Then, the required volumes of sodium borate

buffer (NaBB) 0.1 u/ml ACE solution in potassium phosphate buffer were added. The mixtures were incubated following the times reported in the table and, after the due time, the reaction was stopped with 250 µl of 1N HCl.

Hippuric acid was extracted with the addition 1.75 ml of ethyl acetate and centrifugation at 1200g for 10 minutes. 1.5 ml of organic phase were collected into another glass tube and dried by nitrogen flow.

Table 3.1. Volumes and reagents used to determine the ACE kinetic activity.

Time (min)→	*5*	*10*	*15*	*20*	*30*	*45*	*60*
Test ↓							
Blank control	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE	200 µl HHL 100 µl NaBB 0 µl ACE
1	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE	200 µl HHL 90 µl NaBB 10 µl ACE
2	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE	200 µl HHL 90 µl NaBB 20 µl ACE
3	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE	200 µl HHL 70 µl NaBB 30 µl ACE
4	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE	200 µl HHL 60 µl NaBB 40 µl ACE

3.6.3.1.1 UV-determination

Solid residues were dissolved in 2 ml of deionised water and the solutions were transferred into disposable cuvette. UV analysis was carried out from 220 to 300 nm and absorbance at 230 nm was recorded.

ACE activity was determined by plotting the absorbance value at 230 nm against the reaction time.

3.6.3.1.2 UPLC/ESI-MS determination

Solid residues were dissolved in 1 ml of deionised water. UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C_{18} column. The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 2 min, from 100% A to 0% A by linear gradient in 6 min plus washing step at 0% A (100% B) and reconditioning. Flow

rate was set at 0.25 ml/min, injection volume 5 µl, column temperature 35°C and sample temperature 6°C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in negative ionization mode, capillary voltage 2.0 kV, cone voltage 30V, source temperature 100°C, desolvation temperature 350°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition mode from 80 to 1000 m/z, scan duration 0.5 s.
ACE activity was determined by plotting the XIC area of Hippuric acid, obtained by use as quantify traces the characteristic ions 178 m/z (molecular ion) and 134 m/z (decarbossilated molecular ion), against the reaction time.

3.6.3.2 HPLC/ESI-UV-MS/MS analysis of Hippuric acid and HHL

HPLC/ESI-UV-MS analyses were carried out by using an Alliance 2695 separation system with a Jupiter Phenomenex 5 µ C18 300 Å (250X2.0 mm) analytical column.
The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 15 min, from 100% A to 0% A by linear gradient in 25 min, washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.2 ml/min, injection volume 10 µl, column temperature 30°C.
UV-VIS detection was performed from 210 to 400 nm by 996 PDA and mass analysis by Waters triple quadrupole Quattro Micro^{TM} API mass spectrometer applying the following conditions: ESI source in negative ionization mode, capillary voltage 2.0 kV, cone voltage 30V, source temperature 100°C, desolvation temperature 350°C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition mode from 80 to 1000 m/z, scan duration 0.5 s.

3.6.3.3 Determination of antihypertensive activity of Enalapril by UPLC/ESI-MS

Antihypertensive activity was determined on several Enalapril NaBB solutions at different concentrations obtained starting from 1mM Enalapril stock diluted as described in Table 3.2.

Table 3.2. List of Enalapril working solutions.

	µl stock solution	µl NaBB	Concentration Enalapril (M)
1			1.00E-03
2	100	900	1.00E-04
3	100	900	1.00E-05
4	100	900	1.00E-06
5	500	500	5.00E-07
6	500	500	2.50E-07
7	500	500	1.25E-07
8	500	500	6.25E-08
9	500	500	3.13E-08
10	500	500	1.56E-08
11	500	500	7.81E-09
12	500	500	3.91E-09
13	500	500	1.95E-09
14	100	900	1.95E-10
15	100	900	1.95E-11
16	100	900	1.95E-12
17	100	900	1.95E-13
18	100	900	1.95E-14

ACE maximum activity was determined by using 20 µl of 0.1 u/ml solution and 60 minutes of incubation time. ACE activity was evaluated in the presence of 80 µl of all Enalapril solution in order to obtain the sigmoid curve by plotting sample concentration against the percentage of activity inhibition calculated as follows: (*MAX-MIN)/MAX**100, where *MAX* is the XIC area of hippuric acid without inhibitor corrected for the area of hippuric acid in the ACE blank control and *MIN* is the area of hippuric acid in the presence of inhibitor corrected for the area of hippuric acid in the sample blank control. Details of these parameters are reported in Table 3.3. The sigmoid curve equation was finally applied in order to compute the IC_{50} value.

Table 3.3. Parameter considered for the determination of ACE inhibition activity.

Maximum ACE Activity	200 µl HHL + 80 µl NaBB + 20 µl ACE
ACE blank control	200 µl HHL + 80 µl NaBB + 20 µl KPB
Minimum ACE activity	200 µl HHL + 80 µl inhibitor + 20 µl ACE
Sample blank control	200 µl HHL + 80 µl inhibitor + 20 µl KPB

3.6.3.4 Determination of antihypertensive activity of poultry hydrolyzates by UPLC/ESI-MS

Antihypertensive activity was determined on several hydrolyzate NaBB solutions at different concentrations ranging from 100 to 1 mg/ml as described in Table 3.4.

Table 3.4. List of hydrolyzate working solutions.

	µl stock solution	µl NaBB	Concentration mg/ml
1			100.0
2	100	20	83.5
3	100	50	66.6
4	50	50	50.0
5	50	75	40.0
6	50	125	28.6
7	50	200	20.0
8	50	450	10.0
9	50	600	7.7
10	50	950	5.0
11	50	1950	2.5
12	50	4950	1.0

The ACE inhibition activity of hydrolyzate samples was determined as previously described for Enalapril.

3.7 Results and discussion

3.7.1.1 Optimal conditions for determining Angiotensin-I-Converting-Enzyme activity

In order to evaluate the optimal conditions to carry out the assay, the activity of 0.1 u/ml ACE solution at different volumes was monitored after 5, 10, 15, 20, 30, 45 and 60 minutes of reaction. ACE activity was evaluated on the basis of the absolute absorbance value at 230 nm (Figure 3.12).

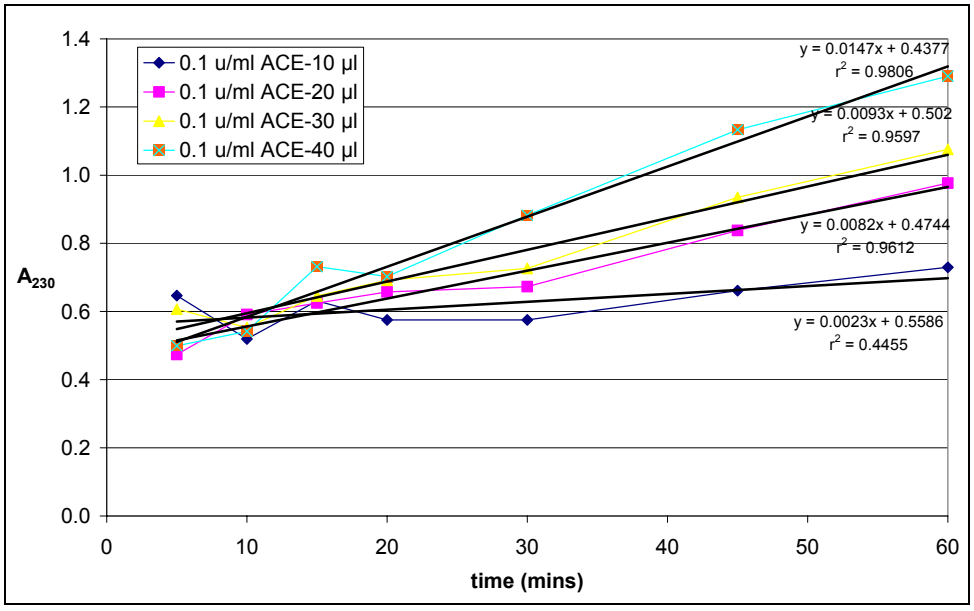


Figure 3.12. ACE kinetic activity spectrophotometrically determined.

As can be seen in Figure 3.12 during the first 15-20 minutes of reaction, there is no apparent accumulation of hippuric acid, while after 20 minutes hippuric acid increases progressively during reaction time.

20 µl of ACE solution and 60 minutes were chosen as optimal conditions, because of the good accumulation of hippuric acid which led to an optimal absorbance value at 230 nm, slightly lower than 1 In order to correct the absorbance at 230 nm taking into account eventual interference, ACE activity was evaluated on the basis of the absorbance value at 230 nm corrected taking into account the absorbance of a blank control at the same wavelength (Figure 3.13).

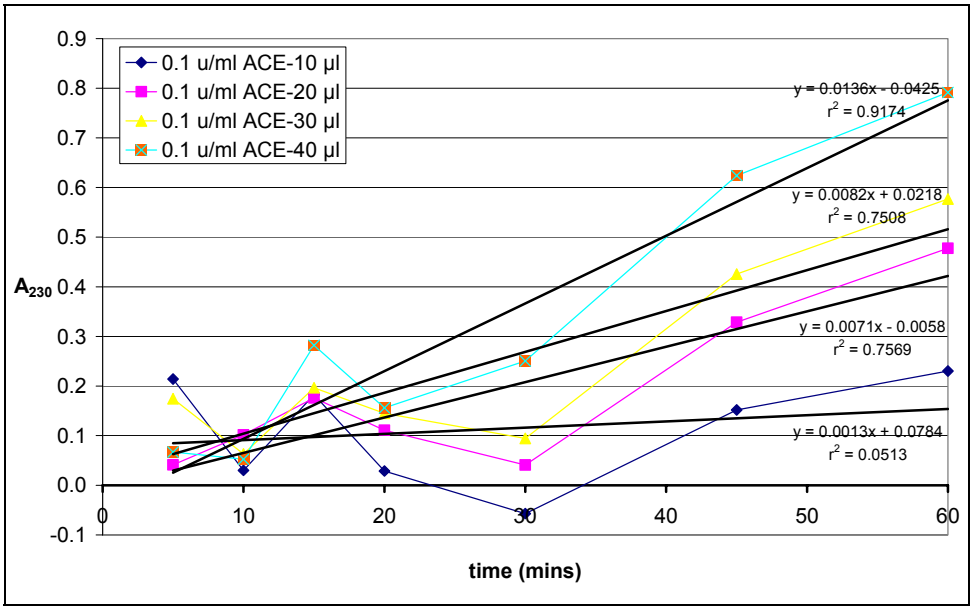


Figure 3.13. ACE kinetic activity spectrophotometrically determined corrected for the blank control absorbance value.

By this approach the results obtained were quite similar to those previously described but, quite unexpectedly, a higher variability of the data was observed, including some cases in which the absorbance of the blank control was higher than that obtained by using the enzyme, indicating that other compounds were extracted in ethyl acetate.

The same extracts were then reanalysed by HPLC-UV-MS: the chromatograms obtained are reported in Figure 3.14 and Figure 3.15.

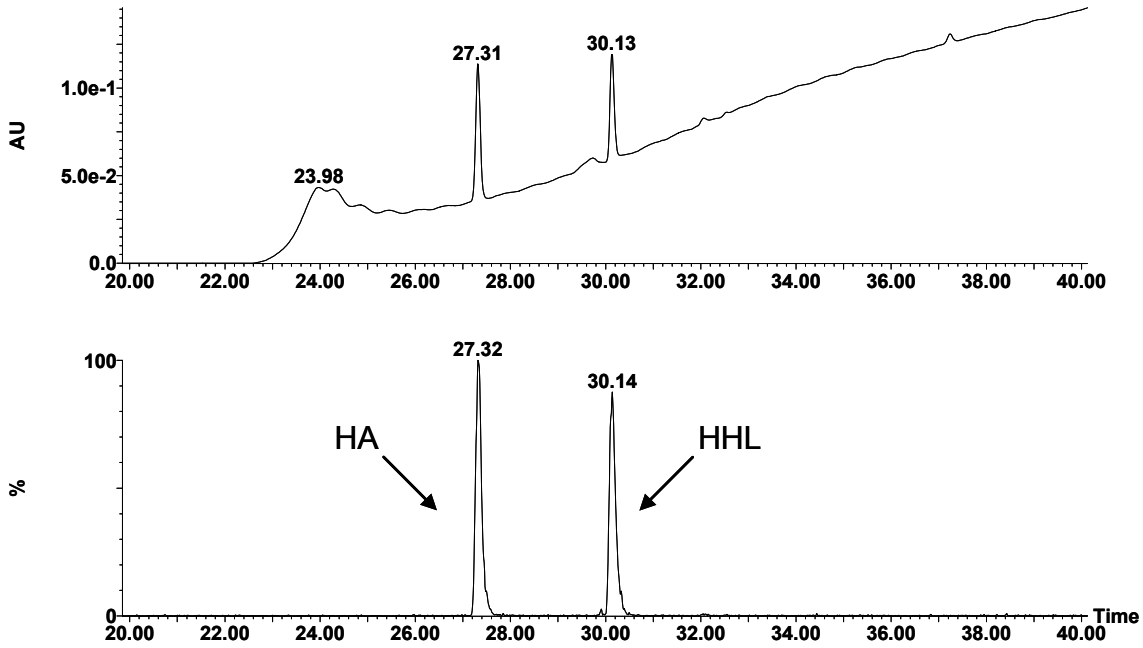


Figure 3.14. UV chromatogram at 228 nm (above) and XIC of molecular ion of hippuric acid (178 m/z) and HHL (428 m/z) obtained by the analysis of 20 µl of 0.1 u/ml ACE solution incubated with HHL for 60 minutes at 37 °C.

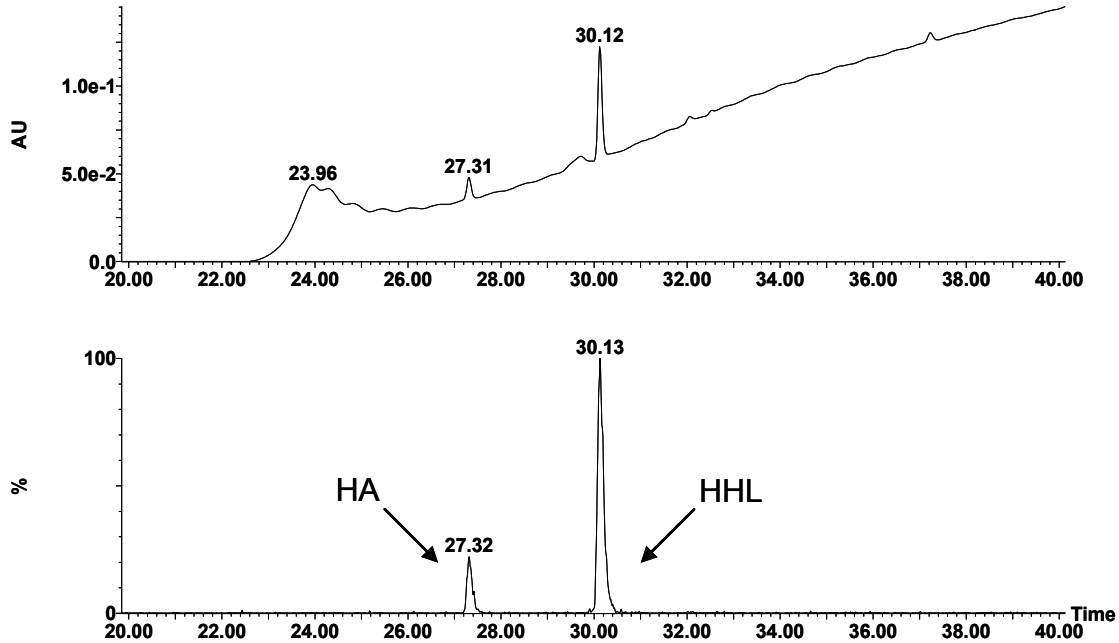


Figure 3.15. UV chromatogram at 228 nm (above) and XIC of molecular ion of hippuric acid (178 m/z) and HHL (428 m/z) obtained by the analysis of 20 µl of buffer solution incubated with HHL for 60 minutes at 37 °C (blank control).

It is evident, observing the UV chromatogram, that two different molecules absorbing at 230 nm were present in the ethyl acetate extracts. Mass analysis allowed to identify these molecules as hippuric acid and HHL, indicating that the main interference present in the extract was the unreacted substrate, thus the absorbance values measured at 230 nm were due both to the product and the substrate, as shown in Figure 3.16.

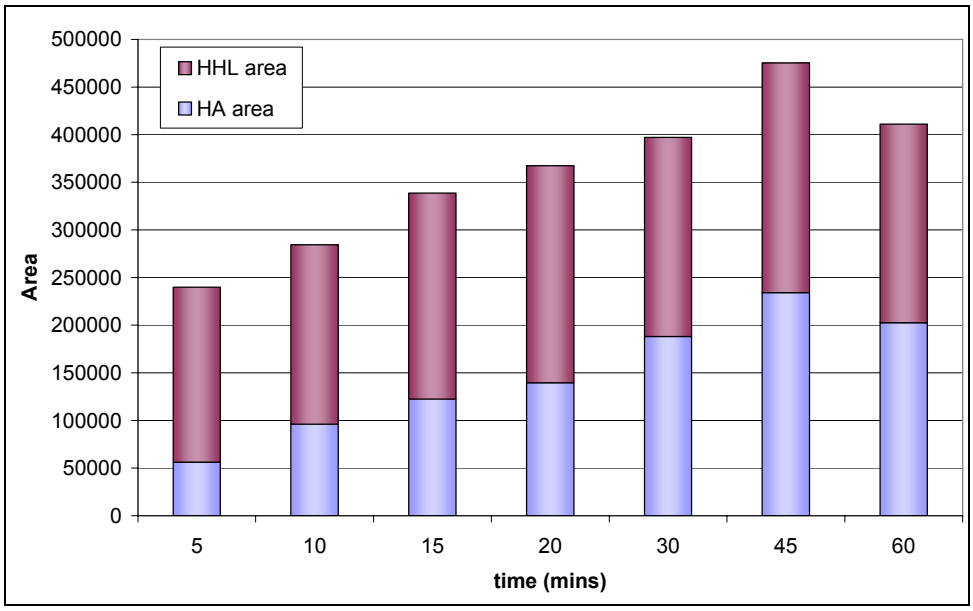


Figure 3.16. Areas of the two chromatographic peaks detected at 228 nm: blue bar represent the contribution of HA, obtained by using 10 µl of 0.1 u/ml ACE solution, and the red one the contribution of HHL.

It is also interesting to note that, in spite of the absence of ACE in the blank control, a small amount of hippuric acid was nevertheless produced, probably due to a chemical hydrolysis of the substrate.

These data indicated that UV analysis of the ethyl acetate extracts was not an accurate method for correctly evaluating ACE activity.

3.7.1.2 Angiotensin-I-Converting-Enzyme activity determination by UPLC/ESI-MS analysis

The activity of 0.1 u/ml ACE solution at different volumes was monitored after 5, 10, 15, 20, 30, 45 and 60 minutes of reaction. In order to avoid spectrophotometric analysis of the hippuric acid produced and the high time consumption due to the chromatographic separation achieved by means HPLC system, a very fast suitable UPLC/ESI-MS method was developed, a representative chromatogram obtained is reported in Figure 3.17.

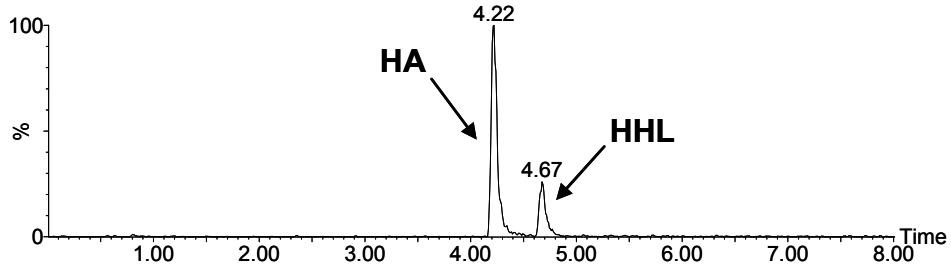


Figure 3.17. Example of chromatogram obtained by UPLC/ESI-MS

In this case, ACE activity was evaluated on the basis of the XIC area of hippuric acid in the test, corrected for the XIC area of hippuric acid in the blank control. The results obtained are shown in Figure 3.18.

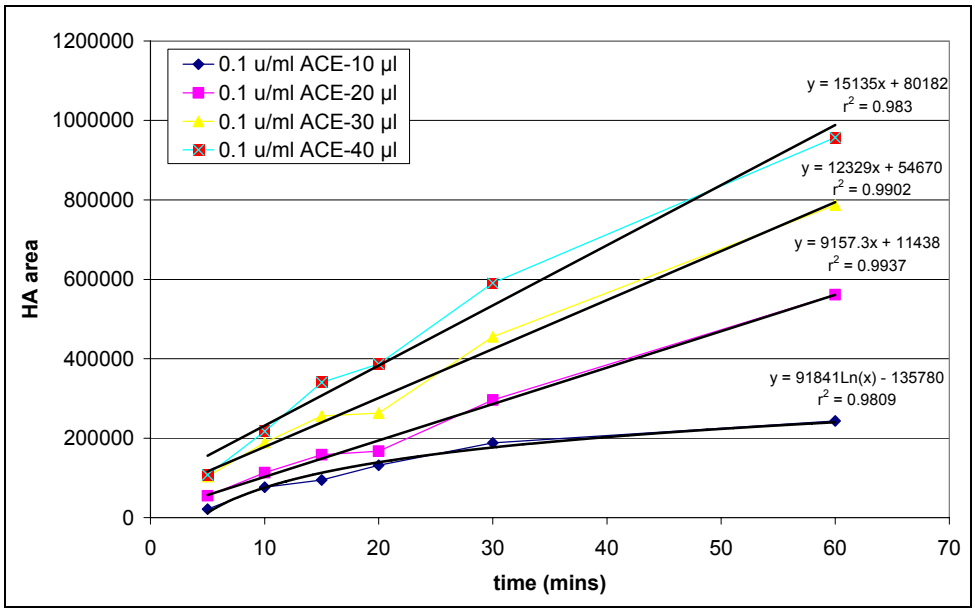


Figure 3.18. ACE kinetic activity determined by UPLC/ESI-MS.

As can be seen, by UPLC/ESI-MS, more precise results were obtained, and this method also seemed to be more sensitive than the spectrophotometric determination, also showing the small increases of hippuric acid during the first 20 minutes of reaction.

20 µl of ACE solution and 60 minutes of reaction were confirmed as the optimal conditions to carry out the inhibition assay.

In order to verify the accuracy of the new method, the ACE inhibitory activity of Enalapril was evaluated. As described in the experimental section, the inhibition of the ACE activity caused by several Enalapril solutions was determined and plotted against the Enalapril concentration: a sigmoid curve was obtained and the IC_{50} value calculated was 2.5 µM, in good agreement with literature data [13].

3.7.1.3 Determination of Angiotensin-I-Converting-Enzyme inhibitory activity of poultry hydrolyzates

The ACE inhibitory activity of poultry hydrolyzates produced under different conditions (sample description in Part II -2.3.1.1) was evaluated. In Figure 3.19, as an example, the sigmoid curves obtained by plotting the inhibitory activity against the concentration of the lowest and the highest inhibitory hydrolyzates are reported.

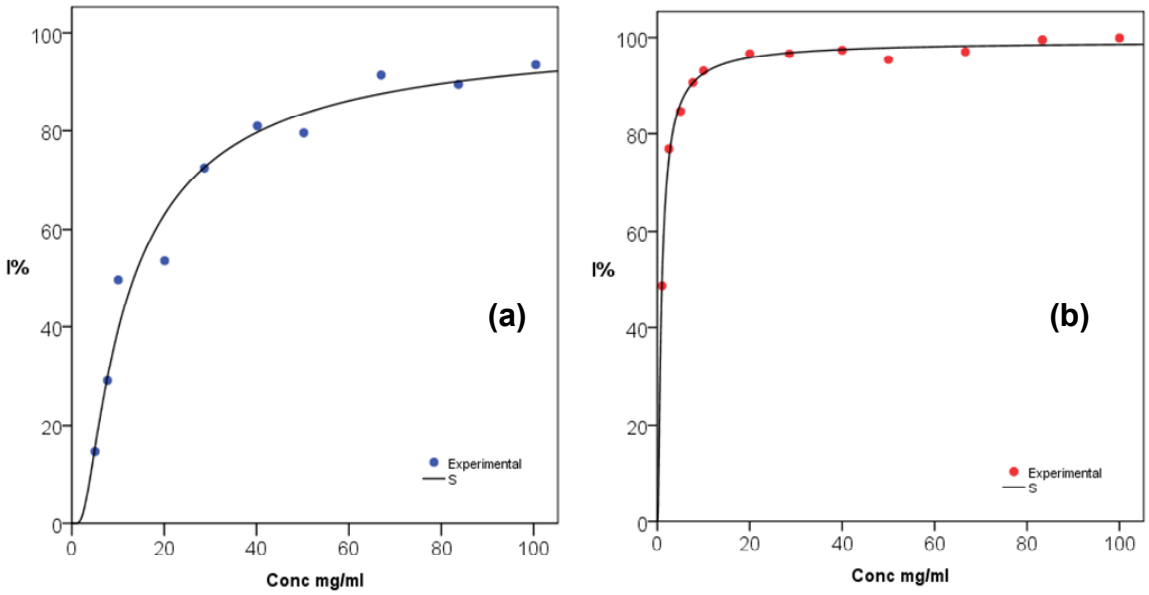


Figure 3.19. Sigmoid curve obtained by the analysis of the sample with the lowest ACE inhibitory activity (a, sample 78T) and Sigmoid curve obtained by the analysis of the sample with the highest ACE inhibitory activity (b, sample 100T)

In Figure 3.20 the ACE inhibitory activity of the various hydrolyzates, expressed as IC_{50}, is reported.

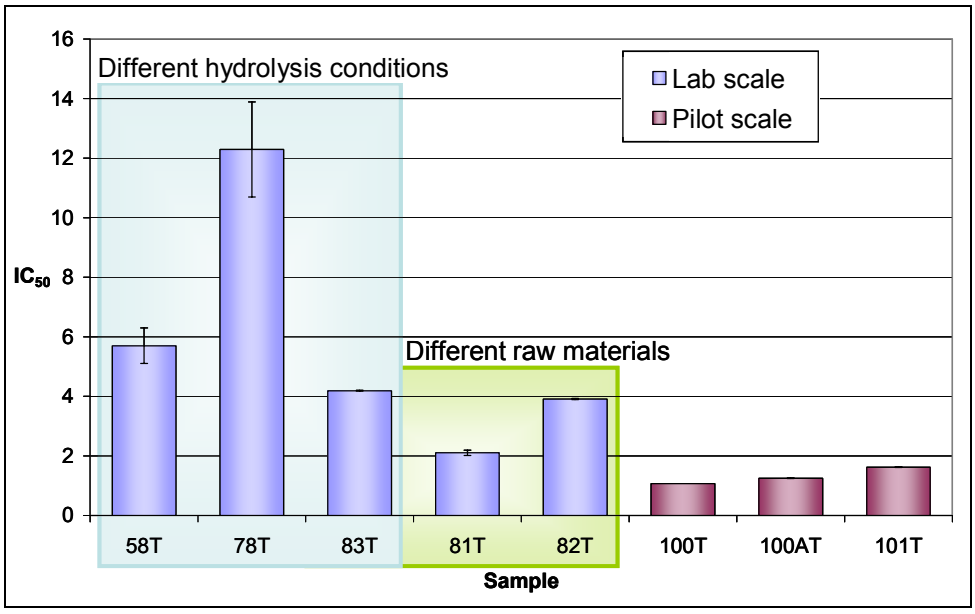


Figure 3.20. ACE inhibitory activity of poultry hydrolyzates.

It is interesting to observe that the samples from the pilot plant, richer in peptides, exerted the highest activity, and samples from lab scale 78T, highly proteolyzed, thus less rich in peptides, exerted the lowest activity, indicating, as expected, that most of the ACE inhibitory activity was due to the peptidic fraction.

By comparison with a drug (IC50 value in the range 1-10 ng/ml) the antihypertensive activity seems to be very low, but protein hydrolyzates are to be considered as possible food ingredients.

The IC50 value of the sample with the highest ACE inhibitory activity was considerably lower than that observed in milk derived peptides like, as an example, the peptides in Manchego cheese with an IC50 value from 7.7 to 63 µg/ml depending on the casein fragment [14], or other peptides obtained after the tryptic digestion of casein or whey proteins [15], but is comparable to the antihypertensive activity of egg proteins hydrolyzates that possess an IC50 value about 1mg/ml depending on the digestion procedure [16].

3.8 Conclusions

The antihypertensive activity of FAP hydrolyzates has been evaluated by applying the Cushman and Cheung assay, one of the most widely used methods, based on the spectrophotometric determination of the hippuric acid released by ACE from the synthetic substrate HHL. ACE activity was first studied in order to determine the optimal

condition in which to perform the assay. However, the data obtained showed problems due to the high blank value. By HPLC/UV/MS analysis it has been confirmed that, in the ethyl acetate extracts, also the unreacted HHL, beside hippuric acid was present, absorbing at 228 nm and interfering with the spectrophotometric determination. In order to overcome this problem a suitable UPLC/ESI-MS method for the analysis of the ethyl acetate extracts, has been developed and applied to evaluate the ACE inhibitory activity of poultry protein hydrolyzates. The results obtained have shown that the ACE inhibitory capacity of hydrolyzates is affected by the proteolysis degree, but also the starting raw materials also have an influence. Poultry hydrolyzates obtained at pilot level presents the highest ACE inhibitory activity, due to their higher content in peptides.

The ACE inhibitory activity of these samples proved quite comparable to those of other proteinaceous hydrolyzates from food matrices, indicating that the hydrolyzates obtained from the recovery of poultry leftovers may be exploited for this biological property.

References

[1] Sowers, J. R.; Epstein, M.; Frohlich, E. D., Diabetes, hypertension, and cardiovascular disease - An update. *Hypertension* **2001,** 37, (4), 1053-1059.

[2] Skeegs L.T.; Kahn J. E.; Shumway N. P., The preparation and function of the angiotensin-converting enzyme. *Journal of Experimental Medicine* **1956**, 103, 295-299.

[3] Erdo; s, E. G.; Yang, H. Y. T., An enzyme in microsomal fraction of kidney that inactivates bradykinin. *Life Sciences* **1967,** 6, (6), 569-574.

[4] Li, G. H.; Le, G. W.; Shi, Y. H.; Shrestha, S., Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research* **2004,** 24, (7), 469-486.

[5] Cohen, M. L., Synthetic and Fermentation-Derived Angiotensin-Converting Enzyme-Inhibitors. *Annual Review of Pharmacology and Toxicology* **1985,** 25, 307-323.

[6] FitzGerald, R. J.; Meisel, H., Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition* **2000,** 84, S33-S37.

[7] Ariyoshi, Y., Angiotensin-Converting Enzyme-Inhibitors Derived from Food Proteins. *Trends in Food Science & Technology* **1993,** 4, (5), 139-144.

[8] Vercruysse, L.; Van Camp, J.; Smagghe, G., ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. *Journal of Agricultural and Food Chemistry* **2005,** 53, (21), 8106-8115.

[9] Meisel, H.; Schlimme, E., Bioactive peptides derived from milk proteins: Ingredients for functional foods? *Kieler Milchwirtschaftliche Forschungsberichte* **1996,** 48, (4), 343-357.

[10] FitzGerald, R. J.; Murray, B. A.; Walsh, D. J., Hypotensive peptides from milk proteins. *Journal of Nutrition* **2004,** 134, (4), 980S-988S.

[11] Meng, Q. C.; Oparil, S., Purification and assay methods for angiotensin-converting enzyme. *Journal of Chromatography A* **1996,** 743, (1), 105-122.

[12] Cushman, D. W.; Cheung, H. S., Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology* **1971,** 20, (7), 1637-1648.

[13] Vermeirssen, V.; Van Camp, J.; Verstraete, W., Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods* **2002,** 51, (1), 75-87.

[14] Gomez-Ruiz, J. A.; Ramos, M.; Recio, I., Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. *International Dairy Journal* **2002,** 12, (8), 697-706.

[15] Ricci, I.; Artacho, R.; Olalla, M., Milk Protein Peptides With Angiotensin I-Converting Enzyme Inhibitory (ACEI) Activity. *Critical Reviews in Food Science and Nutrition* **2010,** 50, (5), 390-402.

[16] Yoshii, H.; Tachi, N.; Ohba, R.; Sakamura, O.; Takeyama, H.; Itani, T., Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **2001,** 128, (1), 27-33.

Part III

Concluding Remarks

In the present Ph.D. thesis different nitrogen components in proteolyzed novel and traditional foods, present in different form and amount, have been investigated.

Characteristics meat nitrogen compounds, such as β-alanyl histidine peptides (carnosine, anserine and balenine), guanidine compounds (creatine and creatinine) and nucleotides or nucleosides (hypoxanthine and adenosine), widely studied in fresh meat, have been studied for the first time, in cured meat after a long period of ripening. The amounts of these molecules were found to be, beside creatinine, in a good agreement with the literature data reported on fresh meat, indicating that they are not significantly affected by the ageing process. On the other hand, these compounds were found to be significantly affected by the use of nitrate salts during the production process, resulting in a lower amount when the nitrate salts were not used. On the basis of these results, taking into account also the safety of the final product, the exclusion of nitrate salts from the manufacturing of Culatello sausages in order to obtain an additive-free product might lead to a partial loss of small nitrogen compounds usually considered beneficial. On the other side, the amount of these compounds, especially creatine, may be a good molecular marker to recognize the use of nitrate salts during the production of cured meat.

The second aspect investigated in the present work was the molecular characterization of highly proteolyzed foods rich in proteins, with particular attention to the influence on their biological properties exerted by residual proteins, oligopeptides and amino acids. Two different kinds of foods have been considered: those derived from a natural proteolysis, considering as a model system a traditional Italian food product, Parmigiano-Reggiano cheese, and those obtained by a proteolytic process due to an innovative biocatalytic digestion procedure, generating proteic hydrolyzates from the recovery of poultry industry by-products.

The nitrogen fractions of proteinaceous hydrolyzates coming from these different food matrices, both traditional and produced according to novel technologies, have been extensively studied by means of mass spectrometry coupled with liquid chromatography. This technique has been demonstrated to be a valid approach to monitor the composition of complex amino acid, peptide and protein mixtures, because of its high sensibility and selectivity.

Parmigiano Reggiano cheese is a natural product with a potential high biological value, mainly due to its high digestibility that results in an available source of amino acids for

normal growth and maintenance. Moreover, proteolysis occurring during the ageing time leads to the release of a large number of peptides from caseins, which have been demonstrated to be potential biologically active by in vitro studies. Anyway, in the present work has been demonstrated that known bioactive peptides are quite scarce in aged Parmigiano Reggiano cheeses, due to the extensive proteolytic breakdown. Since data of their effective bioavailability are still quite scarce, in the present work also the effective bioaccessibility of the oligopeptide fraction derived from Parmigiano-Reggiano cheese after simulated gastrointestinal digestion has been taken into account, showing that the oligopeptide fraction is quite resistant to digestive enzymes. In addition, the presence of unbroken whey proteins in Parmigiano Reggiano cheese, also after a long ripening period, established by UPLC/ESI-MS analysis, may be a source of encrypted bioactive peptides that are released during gastrointestinal digestion, On the other side, the presence of intact whey proteins makes Parmigiano Reggiano cheese a source of these milk allergens, also after a long ageing period.

Finally, free and total amino acid analysis of the biosynthetic proteinaceous hydrolyzates derived from poultry leftovers have shown that this potential novel food has an high nutritional value

Furthermore these hydrolyzates have demonstrated, in vitro, to be biologically active, showing an high antioxidant capacity and a quite good antihypertensive activity, both affected, as expected, by the molecular composition. Amino acidic composition, and overall oligopeptide fractions, have been found to be strictly related to the proteolytic conditions used for food production, thus bioactive compound amounts may be tailored according to the desired features.

The results here presented demonstrated that novel foods obtained by enzymatic proteolysis may be a source of proteinaceous mixtures with high nutritional, functional and biological properties, comparable or even better to those present in traditional proteolyzed foods.

Attachment

Attachment Table 1. Peptides mainly present in WSE of several Parmigiano Reggiano cheese samples. For each ion the state of charge is indicated.

Rt	*MW*	*Characteristic ions (m/z)*
12.40	246	247.2(1)+118.1(1)
12.40	278(a)	279(1)+150(1)
13.35	268	269.1(1)
13.38	1001	334.7(3)+501.7(2)
13.70	310	311.0(1)+182.0(1)
14.22	226	227.2(1)
15.03	230	231(1)
15.10	278b	279(1)
15.10	503	504.3(1)
15.99	441	442.3 (1)
15.99	1245	416.1(3)+623.7(2)
16.40	1267	423.5(3)+634.2(2)
16.40	260(a)	261.2(1)+132.2(1)
16.50	551	552.3(1)
16.52	1155	578.4(2)+1155.6 (1)
16.52	620	310.8(2)+620.5 (1)
16.52	473	474.2 (1)
17.00	636	637(1)+562(1)+544(1)+449(1)+431(1)
17.10	260(b)	261.2(1)+132.2(1)
17.98	221	222.1(1)+176.1(1)
17.98	771	386.9(2)
18.00	1248	665.4(1)
18.00	1320	660.9(1)
18.10	253	254(1)+208(1)+136(1)
18.12	542	543.3(1)
18.30	294	295.2(1)+166.2(1)
18.35	1404	468.9(3)+702.7(2)
18.37	801	401.9(1)+802.6(1)
18.38	960	480.8(2)
18.38	892	446.8(2)+299.0(3)
18.46	1348	1348.7(1)+674.5(2)
18.60	496	497.3(1)
18.81	1185	593.1(2)
18.82	389	390(1)
18.90	189	190.2(1)+144.1(1)
19.30	570	571.5
19.60	754	378.8(2)+756.5(1)
19.65	230(a)	231.2(1)+158.2(1)
19.70	264	265(1)
19.70	1435	718(2)
19.90	954	478(1)+ 954.5(1)
20.10	242	243(1)+197(1)+132(1)
20.11	1034	518.2(1)+1035(1)
20.20	333	334(1)+205(1)+188(1)
20.40	1314	658.0(2)+1314.9
20.60	230b	231.1(1)
20.70	953	954
20.97	173	174(1)+132(1)+128(1)
21.00	697	698.5(1)
21.20	242	243.2(1)+197.1(1)+132(1)

Rt	*MW*	*Characteristic ions (m/z)*
21.57	1999	667.4(3)+1000.4(2)
21.86	173	174(1)+132(1)+128.2(1)
21.93	787	394.8(2)+788.5(1)+657.4(1)+528.4(1)
21.93	1759	587.4(3)+880.5(2)
21.93	3407	1136.5(3)+852.5(4)+1704.5+568.5
22.00	2055	686.1(3)+1028.7(2)
22.37	2272	758.5(3)+1137.3(2)
22.38	2000	1000.6(2)+667.6(3)
22.38	1870	624.5(3)+936.0(2)
22.38	1082	541.9(2)+1082.7(1)
22.38	995	498.7(2)
22.79	2099	1050.3(2)+700.5(3)
22.79	2007	1004.3(2)+669.8(3)
22.79	1790	597.8(3)+896.1(2)
22.79	1575	788.3(2)
22.90	1621	811.7(2)
22.90	276	277.2(1)+231.1(1)+166(1)+120(1)
23.13	203	158.2(1)+204.2(1)
23.35	2340	780.9(3)+1171.0(2)
23.46	203	158.2(1)+204.2(1)
23.60	631	632.3(1)
23.60	826	414.0(2)
23.68	187	188(1)+118(1)
23.68	219	220(1)+150(1)
23.70	1866	623.1(6)+934.0(4)
23.70	278	279.3(1)
24.03	2212	1106.8(2)+738.3(3)
24.03	1135	568.1(2)+1134.9(1)
24.03	658	659.4(1)
25.02	237	238.2(1)+192.1(1)+120.1(1)
25.36	2301	768.2(3)
25.36	1938	647.1(3)+485.6(4)
26.10	3056	1019.3(3)+764.8(4)+612.0(5)
28.11	201a	202(1)+132(1)
28.86	201b	202(1)
28.88	805	403.8(2)+806.4(1)
30.72	235	236(1)
31.11	1718	859.8(2)+573.6(3)
33.90	215	216(1)
33.90	247	248(1)
34.83	8984	642.6(14+692.2(13)+749.6(12)+817.7(11)+ 899.4(10)+ 999.3(9)+ 1124.0(8)+1284.3(7)+1498.1(6)
35.24	7301	1826(4)+1461.0(5)+1217.8(6)+1043.8(7)+913.6(8)
35.60	1881	941.7(2)+628.2(3)
35.95	7173	1196.3(6)+1435.3(5)+1794.0(4)
35.95	8717	1246.2(7)+1090.6(8)+ 969.4(9)+ 873.1(10)+793.5(11)+727.3(12)+1454.0(6)
36.22	7531	837.7(9)+942.3(8)+1076.9(7)+1256.1(6)+1507.2(5)
36.22	7774	972.7(8)+1111.6(7)+1296.7(6)+1555.7(5)
36.22	2086	696.4(3)+1044(2)
36.25	7491	1249.5(6)+1499.3(5)+1873.9(4)+1071.1(7)+937.5(8)+833.3(9)
36.70	7261	1211.2(6)+1453.2(5)+1816.0(4)+1038.3(7)+908.6(8)+807.8(9)
36.70	7318	1220.7(6)+1464.6(5)+1830.4(4)+1046.4(7)+915.4(8)
36.98	7404	1235.0(6)+1481.6(5)+1851.6(4)+1058.5(7)+926.3(8)

Rt	*MW*	*Characteristic ions (m/z)*
36.98	7646	1275.3(6)+1093.6(7)+956.7(8)+1530.1(5)+1912.2(4)
36.98	3635	909.8(4)+1212.7(3)
37.30	279	280(1)
37.68	3834	959.7(4)+1279.1(3)+1918.3(2)
37.68	7734	860.3(9)+ 967.7(8)+ 1105.9(7)+ 1290(6)+ 1547.7(5)+ 1934.5(4)
38.19	313	314(1)
38.29	229	230(1)
38.37	3864	773.0(5)+967.1(4)+1289.1(3)
38.37	7362	1228.1(6)+1473.4(5)+1841.6(4)
38.37	7605	1268.7(6)+1522.0(5)+1902.1(4)
38.37	4494	1124.2(4)+1498(3)
38.37	2742	915.2(3)+1372.2(2)
38.37	2316	773.3(3)+1159.8(2)
38.37	4737	1185.2(4)+1579.9(3)
38.90	229	230(1)
38.93	4065	813.9(5)+1017.1(4)+1355.9(3)
38.93	4308	1078.0(4)+1436.8(3)
38.93	3978	995.4(4)+1326.8(3)
39.96	3795	949.8(4)+1266.0(3)
40.27	263	264(1)+166(1)
40,70	3824	1276.0(3)+957.0(4)
40.74	4454	1114.4(4)+1485.4(3)
41.27	4024	1007.3(4)+1342.5(3)+806.0(5)
41.27	4081	1361.3(3)+1021.1(4)
41.27	4267	1068.0(4)+1423.9(3)
41.27	526	527.4 (1)
43.88	275	276(1)
44.22	243	244(1)
47.50	257(a)	258(1)
47.90	257(b)	258(1)
48.60	291	292(1)

Attachment Table 2. Soluble and total nitrogen of FAP samples determined by Kjeldahl method.

sample code	58T	78T	83T	81T	82T	100T	100AT	101T
Total N %	11.9	12.7	12.4	12.7	13.1	11.4	12.5	12.5
Soluble N %	11.8	11.8	12.1	11.8	12.7	11.2	11.3	11.4

Attachment Table 3. Soluble and total nitrogen of FFP samples determined by Kjeldahl method.

sample code	1L	2L	3L	4L	5L	6L
Total N %	13.4	14.2	13.9	14.0	14.4	14.5
Soluble N %	0.0	0.0	0.0	0.0	0.0	12.0

Attachment Table 4. Free amino acids amount (mg/g) of FAP samples determined by HPLC/FLD method.

Sample code	58T	78T	83T	81T	82T
GLY	5.9±0.1	14.0±1.6	8.3±0.2	6.0±2.8	8.2±0.1
ALA	13.3±0.9	19.2±2.6	14.9±0.7	15.3±1.8	15.9±0.5
SER	11.0±0.3	23.1±1.2	15.1±0.1	15.2±0.0	15.4±0.1
THR	23.3±2.7	25.3±4.6	22.7±1.1	23.5±2.7	23.1±0.2
CYS	missed	missed	missed	missed	missed
CYSS	missed	missed	missed	missed	missed
MET	13.7±0.8	14.9±1.8	13.4±0.6	13.5±1.0	13.4±0.0
ASN	missed	missed	missed	missed	missed
ASP	5.9±0.9	10.3±2.3	6.1±0.7	6.8±1.	6.8±0.7
GLN	missed	missed	missed	missed	missed
GLU	17.4±3.8	29.2±5.1	19.5±1.5	19.4±4.8	21.1±2.0
PRO	11.6±0.6	16.6±0.4	14.1±0.6	13.7±0.2	14.2±0.0
LYS	20.3±3.0	24.0±9.6	23.8±2.3	23.3±6.2	25.6±0.9
ARG	25.7±1.4	37.6±5.0	27.8±1.3	29.0±2.9	28.0±0.2
HIS	11.5±0.1	20.1±3.8	13.5±0.8	9.6±4.2	13.3±0.2
VAL	16.5±0.1	22.0±0.4	18.3±0.3	18.8±0.1	19.0±0.1
ILE	18.0±0.2	21.8±0.4	19.4±0.3	19.9±0.0	20.1±0.1
LEU	34.4±0.0	39.0±0.5	35.1±0.9	36.8±0.1	36.5±0.8
TYR	13.6±2.8	16.9±5.0	13.2±1.3	15.6±3.0	12.5±0.0
PHE	19.5±2.3	24.4±5.6	19.4±1.4	20.4±3.6	19.0±0.0
TRP	missed	missed	missed	missed	missed

Attachment Table 5. Total amino acids amount (mg/g) of FAP samples determined by HPLC/FLD method.

Sample code	58T	78T	83T	81T	82T
GLY	77.5±1.9	97.9±22.9	85.7±6.9	90.4±7.8	92.8±7.3
ALA	missed	missed	missed	missed	missed
SER	30.7±0.5	38.2±10.3	37.1±8.7	34.1±3.2	34.8±2.8
THR	29.4±1.0	36.9±8.6	29.4±1.5	32.3±3.0	33.2±2.9
CYS ac.	9.1±0.5	14.3±3.3	10.3±0.0	11.4±1.2	11.4±1.0
MET sulf.	missed	missed	missed	missed	missed
ASN	missed	missed	missed	missed	missed
ASP	79.9±4.7	100.9±23.5	82.3±2.2	90.9±7.3	92.5±6.0
GLN	missed	missed	missed	missed	missed
GLU	152.1±11.7	190.0±51.0	157.6±1.8	177.4±15.1	181.2±13.7
PRO	49.8±0.5	62.8±15.0	52.1±1.1	58.6±5.3	60.8±6.2
LYS	79.7±1.5	96.8±22.5	78.7±0.9	88.7±7.3	153.4±9.5
ARG	50.0±1.8	61.8±14.0	49.7±0.6	55.6±5.0	56.5±4.2
HIS	missed	missed	missed	missed	missed
VAL	37.2±1.0	47.6±11.5	37.6±2.2	40.6±3.3	42.1±3.3
ILE	33.0±0.7	40.8±9.6	32.3±0.9	35.7±3.1	37.8±4.2
LEU	55.6±1.3	68.6±16.2	54.4±1.2	60.5±5.3	61.1±3.5
TYR	missed	missed	missed	missed	missed
PHE	24.5±1.2	29.6±7.0	23.2±0.7	25.6±2.5	25.8±1.7
TRP	missed	missed	missed	missed	missed

Attachment Table 6. Free amino acids amount (mg/g) of FAP samples determined by HPLC/MS/MS method.

	58T	78T	83T	81T	82T	100T	100AT	101T
GLY	4.8±0.1	7.5±1.0	5.3±0.4	7.3±1.1	7.0±0.1	3.6±0.3	4.9±0.6	4.8±0.1
ALA	17.1±0.6	21.4±0.8	17.6±0.1	18.0±0.4	16.6±0.2	14.7±0.0	13.9±0.0	20.4±1.3
SER	12.4±0.7	17.6±0.6	13.8±1.3	11.5±1.0	7.0±3.7	11.7±0.0	11.7±0.1	16.0±0.6
THR	6.7±0.2	10.5±0.2	9.2±0.5	9.5±0.1	9.7±0.3	5.8±0.1	5.6±0.1	8.4±0.4
CYS	0.3±0.0	0.9±0.0	0.8±0.2	1.0±0.2	0.9±0.1	0.6±0.0	0.5±0.0	0.5±0.0
CYSS	0.5±0.0	2.0±0.1	1.5±0.0	1.8±0.0	1.3±0.0	0.7±0.1	0.6±0.0	0.8±0.0
MET	10.1±0.3	13.6±0.2	10.8±0.1	11.7±0.8	12.0±0.1	7.0±0.1	6.4±0.3	9.0±0.6
ASN	13.9±0.4	19.8±0.1	16.5±0.3	14.0±0.9	10.1±0.1	10.2±0.1	8.4±0.1	13.2±0.5
ASP	19.0±0.3	24.7±0.1	22.5±1.1	22.3±0.9	17.7±0.2	10.3±0.3	9.3±0.0	14.6±0.8
GLN	16.6±0.1	22.3±0.0	17.0±0.3	14.7±0.7	10.1±0.1	12.0±0.6	10.3±0.2	15.6±0.7
GLU	22.8±0.3	29.8±0.5	22.0±0.2	21.0±0.3	16.5±0.0	16.6±0.1	13.7±0.2	23.6±0.9
PRO	8.1±0.4	11.7±0.9	9.0±0.0	8.8±0.3	7.5±0.3	4.8±0.1	3.8±0.1	7.3±0.3
LYS	25.5±1.9	32.3±1.0	28.1±1.4	29.7±2.6	29.9±0.5	19.3±0.5	19.1±0.7	27.2±1.5
ARG	14.3±1.1	22.7±1.2	15.7±0.1	13.0±1.8	1.3±0.9	10.7±0.0	11.7±0.0	16.3±0.4
HIS	5.4±0.2	8.6±0.2	6.5±0.4	7.0±0.3	6.6±0.2	3.3±0.1	2.8±0.0	5.1±0.3
VAL	13.1±0.1	17.8±0.3	14.8±0.2	15.0±0.4	14.9±0.0	10.5±0.7	10.8±0.2	13.9±0.6
ILE	17.0±0.3	21.2±0.8	17.9±0.6	19.5±1.9	19.3±0.1	15.6±0.8	14.5±0.1	20.7±1.3
LEU	30.2±0.2	38.0±1.0	32.9±1.1	30.2±2.6	25.7±0.2	29.8±1.6	29.3±0.3	34.3±2.0
TYR	8.4±0.1	14.2±0.3	11.1±0.4	12.7±0.1	10.2±0.3	7.8±0.0	6.4±0.0	11.2±0.6
PHE	13.8±0.5	17.8±0.3	15.1±0.6	14.6±0.1	12.9±0.1	11.5±0.1	11.4±0.0	14.6±1.1
TRP	4.2±0.2	6.6±0.2	5.2±0.2	4.5±0.0	4.0±0.1	2.5±0.1	2.0±0.1	3.8±0.4
TOTAL	264.2±9.7	361.2±11.4	293.3±11.6	288.0±16.7	241.0±19.3	209.2±6.5	197.1±4.5	281.2±13.9

Attachment Table 7. Total amino acids amount (mg/g) of FAP samples determined by HPLC/MS/MS method (*,Cys and Met were determined as Cysteic acid and methionine sulfone after oxidation step, *** Trp was determined spectrophotometrically).**

	58T	78T	83T	81T	82T	100T	100AT	101T
GLY	51.2±0.6	49.3±1.6	49.9±1.6	52.3±13.8	58.3±0.1	51.5±2.0	49.8±1.2	47.9±0.5
ALA	61.6±2.3	63.1±1.1	64.5±1.7	70.2±0.4	67.2±0.5	71.4±0.7	73.4±1.4	75.4±1.6
SER	41.1±1.4	39.2±0.7	38.7±1.9	42.6±0.9	40.8±2.8	48.8±2.7	44.1±0.1	44.4±1.4
THR	27.5±0.4	27.6±0.5	27.5±0.4	28.5±0.3	28.4±0.5	32.8±1.6	31.2±1.0	33.3±1.6
CYS*	5.0±0.8	9.1±1.3	8.2±2.8	7.0±0.6	6.6±1.9	4.6±0.3	5.0±0.1	4.4±0.3
CYSS	2.4±0.0	3.3±0.1	2.9±0.0	2.2±0.0	2.8±0.1	3.0±0.0	3.7±0.2	2.6±0.1
MET**	8.9±0.2	11.0±3.2	10.5±3.6	9.9±0.4	10.8±1.2	10.8±0.6	9.6±1.7	9.8±1.0
ASN	72.3±4.0	72.1±0.2	77.2±0.9	72.0±2.0	70.2±1.1	79.1±2.8	76.6±1.3	81.4±1.0
ASP	112.9±0.5	103.6±0.9	108.9±0.5	110.0±2.0	110.9±3.1	124.5±6.2	116.3±1.2	121.6±0.9
GLN	88.0±5.7	88.8±0.0	88.6±1.6	85.9±0.3	83.7±0.3	107.6±5.3	93.7±0.6	100.3±5.4
GLU	125.9±2.6	118.6±3.1	119.4±0.2	123.9±2.5	123.7±0.7	166.1±7.0	144.8±0.8	151.8±1.4
PRO	45.7±0.4	48.8±0.8	49.7±0.3	50.1±0.7	50.7±0.6	46.2±0.8	51.3±0.7	47.4±0.3
LYS	56.3±1.0	53.5±1.2	54.9±0.5	55.9±1.2	56.3±0.2	61.8±3.1	61.1±0.6	60.5±7.9
ARG	49.7±1.7	48.2±0.5	49.7±0.6	49.7±2.7	51.5±0.5	50.2±0.9	53.1±0.4	49.6±2.7
HIS	17.8±2.2	17.2±0.1	18.2±0.1	18.1±0.4	18.7±0.5	20.7±2.4	18.0±0.5	22.2±1.2
VAL	36.1±1.7	39.2±0.1	38.7±0.3	36.8±1.9	38.3±0.2	36.0±1.3	38.1±0.8	37.4±0.5
ILE	37.9±2.1	39.3±0.3	39.3±0.4	38.6±0.8	38.9±0.1	43.4±1.2	42.1±0.2	43.2±0.4
LEU	54.0±2.7	56.0±0.2	56.0±0.2	54.8±0.7	55.1±0.4	63.9±2.5	64.0±0.7	65.2±0.6
TYR	17.3±0.1	17.1±0.1	16.6±0.3	18.9±0.5	16.9±0.3	22.2±0.6	21.9±0.1	21.0±1.9
PHE	28.3±0.5	29.3±0.2	28.3±0.2	27.2±0.6	27.8±0.4	29.4±4.7	33.2±0.0	30.3±2.0
TRP***	8.0±0.1	8.9±0.2	8.2±0.3	7.9±0.3	8.0±0.1	9.1±0.4	8.6±0.2	8.5±0.3
TOTAL	945.4±35.9	939.8±31.3	952.9±43.3	960.4±36.6	962.6±31.3	1080.0±51.0	1035.8±24.3	1055.5±43.6

Attachment Table 8. Total amino acids amount (mg/g) of FFP samples determined by HPLC/MS/MS method (*,Cys and Met were determined as Cysteic acid and methionine sulfone respectively after oxidation step, *** Trp was determined spectrophotometrically).**

	1L	2L	3L	4L	5L	6L
GLY	47.4±2.9	43.8±1.7	46.8±0.1	47.7±6.2	58.3±3.2	53.1±0.6
ALA	47.6±1.4	45.4±2.4	46.8±2.2	51.0±1.3	54.8±2.0	64.9±0.7
SER	109.2±1.9	107.2±5.4	108.6±1.1	110.0±2.0	117.3±3.1	132.7±1.1
THR	42.4±0.3	42.3±2.0	42.2±0.3	43.4±0.4	44.2±0.6	48.9±0.2
CYS*	22.7±1.0	18.8±0.1	20.1±0.0	19.6±0.5	21.2±0.1	25.3±1.2
CYSS	31.8±0.5	26.1±1.0	30.5±0.6	31.3±0.8	27.8±0.1	21.7±0.7
MET**	4.7±0.8	6.3±2.0	5.3±0.3	5.2±0.0	5.4±0.3	3.6±0.1
ASN	50.0±0.6	52.3±1.4	52.4±1.4	52.2±2.2	49.6±1.3	58.3±1.8
ASP	74.4±3.7	75.1±3.4	72.2±1.3	72.5±0.9	75.2±1.7	87.3±0.8
GLN	82.2±0.1	81.8±2.0	84.4±0.8	83.8±1.7	81.1±0.9	95.2±1.5
GLU	113.4±0.3	107.2±4.7	107.3±1.1	109.9±1.9	112.6±0.7	139.8±3.4
PRO	83.3±3.8	82.6±4.4	83.3±2.4	86.0±0.7	87.8±0.1	71.1±1.2
LYS	21.8±0.2	20.3±0.8	20.3±0.3	21.2±0.6	20.9±0.6	21.1±0.6
ARG	57.9±0.1	53.8±2.0	54.7±1.1	55.1±0.7	57.9±2.2	53.3±2.0
HIS	6.2±0.4	6.3±0.3	6.2±0.2	6.4±0.2	6.2±0.1	7.6±0.1
VAL	62.5±4.4	63.2±1.9	65.6±0.1	65.3±1.0	65.7±1.0	61.3±1.3
ILE	50.1±2.0	49.9±1.5	51.5±0.2	51.6±1.0	51.5±0.6	55.3±0.8
LEU	68.0±3.7	68.5±2.6	70.9±0.6	71.0±1.4	70.6±0.4	78.1±0.3
TYR	21.6±0.7	21.6±1.1	23.0±0.0	22.5±1.3	21.3±0.3	20.7±0.1
PHE	43.2±1.1	41.7±1.2	43.4±0.7	43.2±0.7	42.3±0.0	48.7±1.3
TRP***						6.3±0.3
TOTAL	1015.1±2.6	994.3±66.5	1011.2±29.0	1023.9±39.4	1049.9±27.2	1132.5±23.2

Attachment Table 9. Free amino acids amount (mg/g) of water soluble FFP samples determined by HPLC/MS/MS method.

	6L
GLY	0.7±0.2
ALA	1.4±0.2
SER	6.9±0.6
THR	0.7±0.1
CYS	0.0±0.0
CYSS	0.0±0.0
MET	0.1±0.0
ASN	1.1±0.0
ASP	1.2±0.3
GLN	0.8±0.0
GLU	1.1±0.1
PRO	0.2±0.0
LYS	11.9±0.8
ARG	0.4±0.2
HIS	0.1±0.0
VAL	0.3±0.1
ILE	0.2±0.1
LEU	0.7±0.1
TYR	0.3±0.1
PHE	0.3±0.1
TRP	0.1±0.0
TOTAL	28.5±

Attachment Table 10. Racemization degree (%) of FAP samples determined by GC/MS analysis.

	58T	78T	83T	81T	82T	100T	100AT	101T
GLY	nd	nd	nd	nd	nd	nd	nd	nd
ALA	nd	nd	nd	nd	nd	nd	2.25	nd
SER	nd	nd	nd	nd	nd	nd	nd	nd
THR	nd	nd	nd	nd	nd	nd	nd	nd
MET	nd	nd	nd	nd	nd	nd	nd	nd
ASP	1.4±0.0	3.5±0.0	2.2±0.1	1.5±0.0	4.2±0.0	6.7±0.0	6.3±0.0	8.9±0.0
GLU	nd	nd	nd	nd	nd	nd	nd	nd
PRO	nd	nd	nd	nd	nd	nd	nd	nd
LYS	nd	nd	nd	nd	nd	nd	nd	nd
ARG	nd	nd	nd	nd	nd	nd	nd	nd
HIS	nd	nd	nd	nd	nd	nd	nd	nd
VAL	nd	nd	nd	nd	nd	nd	nd	nd
ILE	nd	nd	nd	nd	nd	nd	nd	nd
LEU	nd	nd	nd	nd	nd	nd	nd	nd
TYR	nd	nd	nd	nd	nd	nd	nd	nd
PHE	nd	nd	nd	nd	nd	nd	nd	nd

(nd = not detectable)

Attachment Table 11. Racemization degree (%) of FFP samples determined by GC/MS analysis.

	1L	2L	3L	4L	5L	6L
ALA	5.2±0.0	5.0±0.2	2.6±0.0	2.87±0.0	3.22±0.0	3.90±0.0
SER	4.1±0.0	nd	nd	nd	nd	Nd
THR	nd	nd	nd	nd	nd	Nd
MET	nd	nd	2.3±0.0	nd	nd	Nd
ASP	31.2±0.6	33.2±0.0	21.2±0.1	26.23±0.32	33.58±0.7	29.78±4
GLU	4.2±0.0	3.7±0.0	2.9±0.0	3.25±0.02	3.81±0.0	2.94±0.01
PRO	nd	nd	nd	nd	nd	Nd
LYS	nd	nd	nd	nd	nd	Nd
ARG	nd	nd	nd	nd	nd	Nd
HIS	nd	nd	nd	nd	nd	Nd
VAL	1.3±0.0	nd	nd	nd	nd	Nd
ILE	nd	nd	nd	nd	nd	Nd
LEU	nd	nd	2.3±0.0	2.93±0.0	2.94±0.02	Nd
TYR	nd	nd	nd	nd	nd	Nd
PHE	nd	nd	nd	2.89±0.01	2.35±0.01	2.41±0.03

(nd = not detectable)

Attachment Table 12. Small nitrogen compounds amount (mg/g) of FAP samples determined by UPLC/ESI-MS analysis.

	58T	78T	83T	81T	82T	100T	100AT	101T
Carnosine	1.76±0.1	2.13±0.1	2.06±0.2	2.02±0.1	2.04±0.1	2.42±0.1	1.31±0.1	2.76±0.2
Anserine	2.83±0.1	1.70±	2.74±0.0	2.84±0.0	2.72±0.1	4.13±0.1	2.42±0.1	3.89±0.1
Creatine	4.48±0.1	2.95±0.1	3.90±0.1	4.22±0.1	4.05±0.2	5.25±0.1	4.45±0.1	3.84±0.0
Creatinine	1.33±0.0	1.20±0.0	1.84±0.0	2.06±0.1	2.08±0.0	2.25±0.1	1.55±0.0	3.14±0.0
Guanine	0.47±0.0	0.04±0.0	0.23±0.0	0.24±0.0	0.25±0.0	0.15±0.0	0.32±0.0	0.18±0.0
Hypoxanthine	2.19±0.1	1.75±0.1	2.06±0.1	2.25±0.1	2.22±0.0	2.55±0.2	1.56±0.0	2.64±0.0
Guanosine	1.37±0.1	0.42±0.1	0.87±0.0	0.79±0.0	0.77±0.1	0.41±0.0	1.12±0.0	0.46±0.0
Inosine	2.27±0.1	2.31±0.0	2.25±0.0	2.39±0.1	2.31±0.1	2.31±0.1	2.01±0.0	2.06±0.0

Attachment Table 13. Small nitrogen compounds amount (mg/g) of water soluble FFP sample determined by UPLC/ESI-MS analysis.

	6L
Carnosine	0.19±0.0
Anserine	0.00±0.0
Creatine	0.09±0.0
Creatinine	0.18±0.0
Guanine	0.00±0.0
Hypoxanthine	0.15±0.0
Guanosine	0.19±0.0
Inosine	0.00±0.0

Summary

The present Ph.D. Thesis concerns the molecular characterization of highly proteolyzed foods, with particular attention to the influence on their biological and functional properties exerted by oligopeptides and amino acids. Different kinds of foods containing low molecular weight nitrogen compounds have been considered: traditional food products derived from a natural proteolytic processes, , and in particular Parmigiano-Reggiano cheese and Culatello sausages, and novel foods obtained by a proteolytic digestion through an innovative biocatalytic processes, considering in particular proteic hydrolyzates obtained from the recovery of poultry industry leftovers.

The nitrogen fractions of the proteinaceous hydrolyzates extracted from the different food matrices, both traditional and produced according to novel technologies, have been extensively studied by means of mass spectrometry coupled with liquid chromatography. This technique has been demonstrated to be a valid approach to monitor the composition of complex peptide mixtures, because of its high sensibility and selectivity. In particular a new LC/MS/MS methods to quantify free and total amino acids without any derivatization step has been developed and validated, and improved LC/MS methodologies have been set up also for the analysis of other nitrogen components

Cheese is a natural product with a potential high biological value. Proteolysis occurring during ageing time leads to the release of a large number of peptides from caseins, which have been demonstrated to be biologically active by in vitro studies. The composition of the oligopeptide fraction of different Parmigiano-Reggiano cheeses at different ageing times has been extensively studied in a very detailed way by means of LC/MS methodologies. Antioxidant and antihypertensive properties of these peptide fractions have been studied. Moreover, since data on the effective bioavailability of these peptides are still quite scarce, the work of the present PhD. thesis also addressed the study of their bioaccessibility through simulated gastrointestinal digestion.

Low molecular weight nitrogen compounds in Culatello sausages, one of the most famous Italian dry pork meat products, have also been studied. For the first time data on the amount of characteristic's meat compounds, such as carnosine, anserine, creatine and creatinine, in cured meat are reported and their amount was found to be strictly related to the technological production process.

Finally, free and total amino acid analysis of the biosynthetic proteinaceous hydrolyzates derived from poultry leftovers have shown that this potential novel foods posses an high nutritional value, due to the high content of bioavailable essential amino acids. These proteic hydrolyzates showed interesting antioxidant and antihypertensive properties. Amino acidic composition, and overall oligopeptide fractions, have been found to be strictly related to the proteolytic conditions used for food production, thus bioactive compound amounts may be regulated on the basis of the desired features. For example, samples with the highest hydrolysis degree may be used as functional food ingredients in virtue of their high antioxidant capacity.

In conclusion, the present thesis demonstrated that proteolyzed foods can be considered high quality food and food ingredients, either if traditional food obtained by natural proteolytic processes or novel foods obtained through innovative technologies. In the latter case, it has been demonstrated that leftover of the food industry can be transformed in new food and feed not only having a high nutritional value, but also possessing biofunctional properties.

Keywords: amino acids, peptides, proteolysis, hydrolyzates, antioxidant, antihypertensive, poultry leftovers, parmigiano reggiano, culatello

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Publications

F. Lambertini, B. Prandi, S. Sforza, R. Virgili, G. Galaverna, A. Dossena, R. Marchelli

INOSINE, IPOXANTHINE, CREATINE AND HISTIDINE DIPEPTIDES IN "CULATELLO" SAUSAGES, *"Food for the Future- the contribution of chemistry to improvement of food quality"*.

Proceedings of EuroFoodChem XV, 2009, Department of Life Sciences, Faculty of Life Sciences, University of Copenhagen, Volume 1, 118-121, ISBN: 978-87-993033-4-2.

F. Lambertini, S. Sforza, G. Galaverna, C. Dall'Asta, R. Marchelli, A. Dossena

PROSPARE: AN EUROPEAN PROJECT FOR THE VALORIZATION OF THE LEFTOVERS OF THE POULTRY INDUSTRY, *"Food for the Future- the contribution of chemistry to improvement of food quality"*.

Proceedings of EuroFoodChem XV, 2009, Department of Life Sciences, Faculty of Life Sciences, University of Copenhagen, Volume 2, 215-218, ISBN: 978-87-993033-5-9.

F. Lambertini, S. Sforza, A. Dossena

EMERGING PROPERTIES OF OLIGOPEPTIDES IN HIGLY PROTEOLIZED NOVEL AND TRADITIONAL FOODS.

Proceedings of 15th WORKSHOP on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, Faculty of Agriculture, University of Napoli, pp 89-92, ISBN: 978-88-95028-62-0.

S. Sforza, G. Galaverna, C. Dall'Asta, F. Lambertini, A. Dossena

NUOVE RISORSE DALLA FILIERA AVICOLA

Rivista delle Tecnologie Alimentari, **2009**, 3, 26-29.

V. Cavatorta, S. Sforza, F. Lambertini, M. Bellini, F. Stefani, G. Galaverna, A. Dossena, R. Marchelli

EVOLUZIONE DELLA FRAZIONE AMINO ACIDICA E PEPTIDICA DEL PARMIGIANO REGGIANO FINO ALL'OTTEVO MESE DI STAGIONATURA.
Scienza e Tecnica Lattiero Casearia, **2007**, 58, 183-203, ISSN 0390-6361.

Poster communications

F. Lambertini, S. Sforza, A. Faccini, G. Galaverna, C. Dall'Asta, E. Giocaliere, G. Moneti, G. Pieraccini, R. Marchelli, A. Dossena

CHARACTERIZATION BY MASS SPECTROMETRY TECHNIQUES OF PEPTIDE HYDROLYZATES OBTAINED FROM THE RECOVERY OF POULTRY LEFT-OVERS

International Conference on FoodOmics, Cesena, Italy, **2009**, pp70-71.

S. Sforza, F. Lambertini, A. Faccini, G. Galaverna, C. Dall'Asta, R. Marchelli, E. Giocaliere, G. Moneti, G. Pieraccini, A. Dossena

PROSPARE: CHARACTERIZATION OF PEPTIDE HYDROLYZATES OBTAINED FROM THE RECOVERY OF POULTRY SECONDARY RESOURCES BY MASS SPECTROMETRY TECHNIQUES

XXIII Congresso Nazionale della Società Chimica Italiana, **2009**, DSM-PO-11, p345.

F. Lambertini, B. Prandi, S. Sforza, R. Virgili, G. Galaverna, A. Dossena, R. Marchelli

INOSINE, IPOXANTHINE, CREATINE AND HISTIDINE DIPEPTIDES IN "CULATELLO" SAUSAGES

EuroFoodChem XV, Copenhagen, Denmark, **2009**, p46.

F. Lambertini, S. Sforza, G. Galaverna, C. Dall'Asta, R. Marchelli, A. Dossena

PROSPARE: AN EUROPEAN PROJECT FOR THE VALORIZATION OF THE LEFTOVERS OF THE POULTRY INDUSTRY

EuroFoodChem XV, Copenhagen, Denmark, **2009**, p188.

S. Sforza, T. Tedeschi, A. Calabretta, M. Bencivenni, F. Lambertini, G. Galaverna, A. Dossena, R. Marchelli

DNA AND PEPTIDES AS MOLECULAR MARKERS FOR FOOD AUTHENTICITY

4th International Symposium on Recent Advances in Food Analysis, Prague, **2009**, J-5, P447.

G. Galaverna, F. Lambertini, S, Sforza, A. Dossena, R. Marchelli

ANTIOXIDANT CAPACITY OF PEPTIDE HYDROLYZATES OBTAINED BY POULTRY LEFTOVERS

4th International Symposium on Recent Advances in Food Analysis, Prague, **2009**, N-30, P573.

Elisa Giocaliere, Giuseppe Pieraccini, Gloriano Moneti, Stefano Sforza, Francesca Lambertini, Arnaldo Dossena, Rosangela Marchelli.

MOLECULAR CHARACTERIZATION OF FOOD-GRADE MEAT PROTEIN HYDROLYZATES

1st Mass Spectrometry Food Day, Parma, Italy, **2009**, p267.

Oral communications

Lambertini F., Manzini L., Sgarbi E., Neviani E., Marchelli R., Dossena A., Gatti M., Sforza S.

DERIVATI AMMINOACIDICI DI ORIGINE NON PROTEOLITICA NEI FORMAGGI

VIII Congresso Nazionale di Chimica degli Alimenti, *Marsala (TP), Italy,* **2010**, C11

F. Lambertini, S. Sforza, A. Dossena

EMERGING PROPERTIES OF OLIGOPEPTIDES IN HIGLY PROTEOLIZED NOVEL AND TRADITIONAL FOODS.

15th WORKSHOP on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, Faculty of Agriculture, *Portici (NA), Italy*, **2010**.