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NMR BASED FOODOMICS TO INVESTIGATE THE DIGESTIBILITY OF PROTEIN-RICH FOOD PRODUCTS

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PREFACE

Nowadays, in developed countries, the excessive food intake, in conjunction with a decreased physical activity, has led to an increase in lifestyle-related diseases, such as obesity, cardiovascular diseases, type -2 diabetes, a range of cancer types and arthritis.

The socio-economic importance of such lifestyle-related diseases has encouraged countries to increase their efforts in research, and many projects have been initiated recently in research that focuses on the relationship between food and health. Emphasis should be placed on preventing non-communicable diseases by delaying the initiation process, that is, preventing rather than curing.

Thanks to these efforts and to the growing availability of technologies, the food companies are beginning to develop healthier food and modifying their recipes by removing and/or modifying existing ingredients and their quantities. The necessity of rapid and affordable methods, helping the food industries in the ingredient selection and the evaluation of the improvement so far reached during the different phases along the development of new products, has stimulated the development of in vitro systems that simulate the physiological functions to which the food components are submitted when administrated in vivo. One of the most promising tool now available appears the *in vitro* digestion, which aims at predicting, in a comparative way among analogue food products, the bioaccessibility of the nutrients of interest. The validation of such *in vitro* systems is made by using existing standard food products as the models, better if produced according to strict protocols, provided that an extensive characterization precisely defines the products, better if based on the foodomics approach. The adoption of the foodomics approach has been chosen in this work to evaluate the modifications occurring during the in vitro digestion of selected protein-rich food products. The measure of the proteins breakdown, both its extent and kinetics, was performed via NMR spectroscopy, the only techniques capable of observing, directly in the simulated gastric and duodenal fluids, the soluble oligo- and polypeptides released during the in vitro digestion process. The overall approach pioneered along this PhD work, has been discussed and promoted in a large scientific community, with specialists networked under the INFOGEST COST Action, which recently released a harmonized protocol for the *in vitro* digestion. NMR spectroscopy, when used in tandem with the *in vitro* digestion, generates a new concept, which provides an additional attribute to describe the food quality: the comparative digestibility, which measures the improvement of the nutrients bioaccessibility.

1. NUTRITIONAL VALUE OF THE BALANCE BETWEEN PROTEIN, FATS AND CARBOHYDRATES IN THE DIET

The oldest expression of the human energy human intake is seen in the Book of Exodus, where it is written about the manna on desert.

Hippocrates, too, had described the relationship between age and energy requirements more or less in these terms: “The growing bodies need higher inside energy, thus, they require a maximum food quantity, otherwise they died. In the elderly people, indeed, body heat is less, thus, their fuel requirement is always less”.

Ancient Greeks had employed diet as a form of therapy, but they never really understood the nature of food chemistry. Around 1670, an English Doctor, Sydenham, got a hint of how iron could help in the treatment of chlorosis, and one century after this, in 1753, Lind discovered the relation between the scurvy and poor vegetables and fruits consumption. In 1816, Magendie validated the hypothesis that fats and carbohydrates alone were not able to allow life in dogs fed with no proteins. After some years, Prout, proposed that human nutrition should be based on three fundamental pillars: proteins, carbohydrates and fats. Through the scientific work of Boussingalt, Chossat and Liebig, in the half of the XIX century, six essential elements (Ca, P, Mg, K, Cl and Fe) were identified. In 1906, Willcock and Hopkins discovered the importance of tryptophan as an essential molecule in human nutrition. Osburne and Mendel, in 1910, by studying rats, demonstrated the different nutritional values for proteins[1]. The above-mentioned studies and the research efforts of the two last centuries have set the foundation for the modern nutrition.

Food nutritional values do not depend solely on the composition in carbohydrates, fats and proteins, but on the ability of a food to release nutrients that are essential for human physiology.

There is thus a wide difference between the terms diet and nutrition: diet means food (egg, jam, ice cream), while nutrition means molecules. A nutritional component is a molecule naturally present in food, which can be released from the latter during digestion processes, and then absorbed in the small intestine, where finally become available for the performance of the required functions.

On a genetic level, nutrients are able to influence the processes of transcription, translation or even post-translational reactions, changing in this way the individual’s genotype. In other words, nutrients can influence genetic expression, RNA and proteins synthesized. Just as an example, the translation for the synthesis of ferritin increases with iron content. Thus, by influencing gene expression inside cells, nutrient are capable to influence the synthesis of functional and structural

proteins. For those reasons, the nutrient requirements of the body are always dictated by health or diseases states of different organs and systems[2].

Nowadays, more than 50 nutrients and a lot more chemicals are known to influence the health and the functionality of the human body. Since nutrients, though, exists usually in a composite form, together with other nutrients and molecules, unless we talk about water or some medicines, they will interact with each other during digestion, fermentation and absorption in the gut.

For this reason, each nutrient is usually studied together with other nutrients and in a global perspective of the bodily functions, in order to understand its true behaviour.

The study of nutrition then also tries to determine the specific nutritional requirements for different people and different categories of population and monitors the nutritional status with the measurements of body parameters (anthropometry), food intakes, biomarkers of nutritional status and signs of malnutrition.

The first studied type of malnutrition is the insufficient intake in energy. An average human adult is known to consume about 1 million calories per year, and even though this number is surprising, the majority of healthy subjects remains in balance between the energy consumed and the energy expended. When this energetic balance happens, body weight and body energy storage remain constant; this balance is kept and achieved in the long term, so the body can afford daily fluctuations in energy intakes and expenditures. On the other side, if the energy intake is always more or less than the energy expenses, then the same person will gain or lose weight, respectively.

The energy intake is the food content in calories (or energy), as provided by the four main caloric nutrients: carbohydrates (16.8 kJ/g), proteins (16.8 kJ/g), fats (37.8 kJ/g), and alcohol (29.4 kJ/g).

The energy consumed with food and drinks has various fates: it can be stored as fat (the main storage of energy), as glycogen (reserve of carbohydrates and short-term energy) or as protein (used as energy just in extreme starvation conditions) or it could be used for bodily functions that require energy.

In effect, the body needs this energy from food to perform metabolic, cellular and mechanical functions, like muscular work, heart beats and breathing[3]. The main use of the consumed energy is dedicated to the so-called BMR (basal metabolic rate), the energy employed for basic physiological functions: this is the minimum level of energy used by the body to keep it alive and functioning in the awake state.

Together with the BMR, after food ingestion, energy is employed in what is called the thermic effect of the meal. This is the energy employed for the digestion, metabolism and storage of the

macronutrients ingested. This thermic effect generally amounts for the 10% of the energy content of the consumed meal.

Another important source of energy expenditure is physical activity in any form. This, as it can be imagined, is the most variable part of the daily energy expenditure and it also varies a lot between individuals.

Finally, our body uses energy in other three ways: for keeping body temperature constant, for growth (important especially in the first months of our lives) and for thermogenesis, due to other factors such as smoking or the intake in stimulants like coffee.

As mentioned before, four are the main macronutrients and sources of energy from food: carbohydrates, fats, proteins and alcohol. It was stated how fats are the most energy dense nutrient, with 37.8 kJ in each gram.

The macronutrients composition of a food is generally assessed as the contribution (percentage) of each macronutrient to the total energy content, i.e. the total number of calories of that food.

It is clear how a balanced nutrition will involve the correct intake of all macro and micronutrients and many attempts in the generation of golden standards for this intake have been made by nutritionists over the years.

In 1943 the Food and Nutrition Board of the USA National Research Council published the first RDAs (recommended daily allowances), whilst the British Medical Association published its version in 1950. After that, many countries have generated their nutritional standards and, in the years, since more has been discovered about human need and nutrient effects and functions, the documents describing those RDAs have increased their size incredibly and it is clear how this knowledge is continuously expanding.

As stated, the major and most studied groups of macronutrients, that will be briefly described, are:

- Carbohydrates
- Lipids
- Protein

Carbohydrates are the single most abundant and economic source of energy in the human diet, constituting between 40 and 80 % of the total energy intake of the human population. They play several important roles in all life forms: they represent the major source of fuel for metabolism since our birth and they can be used both as an energy source (especially glucose, the most important carbohydrate in nature) and for biosynthesis.

Carbohydrates are mainly found in foods of plant origin and in processed foods. Little amount of

carbohydrates are found in animal foods, such as meat, fish or poultry. Sources of carbohydrates in plant food are grains, seeds, legumes, tubers but also in some vegetables and fruits.

Carbohydrates can be found in two differ dietary form: simple (monomeric and dimeric) and complex (polymeric). Those two forms have different behaviour after ingestion. Indeed, the humans digest the simple form, whilst the complex one, represented by dietary fibre is not digested.

Glucose, fructose, galactose or their derivates are the principal sugars in carbohydrates. The bonds between those sugars are mostly α-1,4 or α-1,6 with the exception of lactose, while in fibres those bonds tend to be β-1,4 (cellulose and pectins) and since digestive enzymes are not secreted for these bonds, fibres are not digested. The last aspect in the nutritional value of carbohydrates regards their bioavailability. While monosaccharide units (previously hydrolysed) can be absorbed by the human small intestines in a range of 95% , there is a big variation in the bioavailability between different carbohydrate classes and between different foods.

The second main macronutrient class, the lipids, are organic compounds made of a carbon skeleton saturated with hydrogen atoms that make the molecular chain hydrophobic and mostly immiscible with water. Nitrogen, sulphur, and phosphorus are also present in some lipids. For nutritionists, there are four categories of lipids:

- Simple: esters of fatty acids with various alcohols such as glycerol or cholesterol (i.e. triacylglycerols = TAG, neutral fats and oils, waxes, colesterlyl esters, and vitamin A and D esters);
- Complex: esters of fatty acids in combination with both alcohols and other groups (phospholipids, glycolipids, cerebrosides, sulfolipids, lipoproteins, and lipopolysaccharides);
- Derived: products from the hydrolysis of simple or complex lipids, including fatty acids monoacylglycerols and diacylglycerols, straight-chain and ring-containing alcohols, sterols, and steroids;
- Miscellaneous: wax lipids, carotenoids, squalene, and vitamins E and K.

Lipids are necessary compounds for human health. However, their ratio within the diet should not exceeded 26 % of total intake. They have also important structural roles in membranes.

Finally, proteins represent the third class of macronutrients, the main nitrogen-containing compound in the human diet. They are large molecules with complex conformations and their most important role is to release amino acids useful for several body functions. Amino acids have the same central structure with the exception of proline.

For most of the past 65 years, nutritionists classified amino acid in two general groups: essential and non-essential. These two terms have been referred to the necessity to be introduced by the diet (the first) and to the ability of our bodies to produce them.

Protein are necessary for the constitution of tissues and many have enzymatic activity. Food proteins provide 10-20% of the daily energy, they are the major source of amino acids and they supply for essential amino acids [1]. In general, every day, endogenous sources release 20-30 grams of proteins that reach the small intestine. A small quantity (about 2 grams) could came from the plasmatic proteins that go through the intestinal lumen. In conclusion, the guideline of the World Health Organization (WHO) summarizes the daily nutrient intake, by taking into account the established rules for prevention of death and disability (Table 1):

Dietary Factor (food or nutrient)	Recommended goal (range)
Total Fat	15-30% of total energy
Saturated fatty acids	<10% of total energy
Polyunsaturated fatty acids (PUFAs)	6-10 %of total energy
N-6 PUFAs	5-8 %of total energy
N-3 PUFAs	1-2% of totsl energy
Total carbohydrate	55-75% of total energy
Free sugars	<10% of total energy
Protein	10-15% of total energy
Cholesterol	<300 mg per day
Sodium Chloride	<5 g per day (<2 g per day)
Fruits and vegetables	>400 g per day
Total dietary fibers	>25 g per day
Non-starch polysaccharides	>20 g per day

Table 1. Daily nutrient intake taking into account the guideline of the World Health Organization (WHO) established for prevention of death and disability.

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2. PROTEIN – RICH FOODS: THEIR CONSUMPTION IN DIFFERENT DIETS (MEDITERRANEAN VS. NORTH – ATLANTIC DIET)

The intake of proteins varies among different dietary habits. Many factors such as environment, culture, history and economics caused those differences. As an example, there is historical proof of dietary differences caused by the ecological environment between Southern (Greek and Roman) and Northern (German and Celtic) populations. In effect, the first one was relying on agriculture, especially on the production of wheat, grapes and olives, developing the so-called 'Mediterranean' diet, mainly based on vegetable consumption. Northern populations, instead, was more focused on hunting, fishing and rearing of livestock. Thus, meat, fish and dairy were the main components of their diet [1].

Afterward, economics had an influence in the development of these habits. After the World War II the European diet was experienced one of the main transformation, when a diffused homogenisation of food consumption occurred and every country increased the percentage of animal proteins and fats in their diet. A multitude of factors caused this change: increased productivity in agriculture, less differences between national incomes, a great boost in food trades and globalisation of dietary habits.

Nonetheless, nationwide peculiarities still exist, rooted in traditional values. Therefore, different countries still exhibit some nutritional discrepancies in protein consumption, also between the Northern and Southern part of Europe.

A study from de Boer et al. [2] investigated on the different protein intakes in EU-15 countries through analysis of national food consumption surveys, EUROSTAT national household budget surveys and FAOSTAT supply data.

This research shows that, in Europe, plant-derived proteins represent the smallest fraction of protein intake. Cereals and wheat above all, are the largest source of plant-protein in Europe. As expected, Mediterranean countries, like Greece or Italy, are the ones with the highest consumption of plant-proteins, Italians mainly basing their diet on cereals. Other plant sources are vegetables and potatoes, but their contribution on the total intake is quite low, with some exceptions. In the Mediterranean area, tomatoes are great suppliers of proteins; Portugal consumes high amounts of potatoes while Spain of pulses.

As expected, meat is the main supplier of animal protein and has a similar but higher contribution to the total protein intake than that of cereals. Meat varieties differ in their preference among EU countries: pork is the main source of meat protein, but still shows a great range of consumption

values among the different countries, with a threefold span between the highest intake in Austria and the lowest in UK.

Dairy products are the second main source of animal proteins; other sources include offal, mainly consumed in Ireland for traditional reasons, fish and seafood. This last group shows a great variation between the different countries, exhibiting a higher intake in Southern countries like Portugal.

A study from Karamanos et al. [3] investigated the eating habits of Mediterranean countries and compared them to the traditional Mediterranean diet. As also found by de Boer et al., the modern diet in Mediterranean countries differs from the traditional one: nowadays, this diet sees a lower contribution of fats to the total energy intake, especially due to a decrease in the use of plant fats. In addition, a great increase in consumption of meat and cheese was seen, proof that the animal protein intake is greater nowadays even in Southern Europe.

Another research by Slimani et al. [4], studying dietary patterns through 24 hour recalls in 27 Western European centres, highlighted differences in the protein intake of the investigated countries. Again, it was seen how Mediterranean country like Italy and Greece have a higher consumption of plant foods and a lower consumption of animal and processed foods, whilst Nordic and Northern countries have a higher intake in animal-derived products. The involved so-called Mediterranean centres showed an evolution towards a diet closer to the 'Western' one; in the last 30-40 years all the countries have increased their consumption of animal foods, though Southern European countries have done it in a more rapid way in comparison with Nordic countries, where this intake was already quite high.

Nordic countries in Europe, in effect, have among the highest intakes of meat in the world [5] and over the years this intake has almost doubled [6].

For these reasons, for example, Danish nutritionists developed a so-called New Nordic Diet, in order to obtain a healthier, more sustainable and plant-based nutrition.

The employment of modern analytical approaches, like metabonomics, provided proofs of cultural differences in the diet. Lenz et al., studying urine samples from healthy British and Swedish subjects, observed that samples of Swedish urine exhibited metabolites of a predominantly fish-based diet (i.e. TMAO), proving again that the protein intake varies among nationwide diets [7].

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3. MAIN DIETARY PROTEINS: DAIRY, MEAT/FISH, GRAINS/PULSES

It is of common knowledge that proteins are essential macronutrients for humans' survival and health, since an appropriate amount of amino acids is indispensable for our bodily functions. Another important feature of dietary proteins is their quality, depending on the content of specific amino acids and their availability and absorption after digestion. This quality varies with the source of protein, possible processing procedures and interaction with other nutrients or foods among the individual diet. [1]

To evaluate the quality of proteins, one of the main points is the essentiality of amino acids. In effect, amino acids are essential for the provision of nitrogen and they are divided into three subcategories according to their value in this sense:

- indispensable amino acids: isoleucine, leucine, lysine, valine, methionine, histidine, phenylalanine, threonine, tryptophan;
- conditionally indispensable amino acids: tyrosine, cysteine, arginine;
- dispensable amino acids: aspartic acid, glutamic acid, glutamine, asparagine, glycine, serine and proline.

In order to carefully evaluate the nutritional value of proteins, many different methodologies have been developed in the years. The main definitions employed also by FAO/WHO can give two information on the analysed proteins: their resistance to food processing and their percentage of amino acids and digestibility, the latter more related to their effect on humans. The most commonly employed methods are:

- Limiting Amino Acid: essential amino acid contained in a protein showing the greatest concentration difference in high-quality protein of reference. A reference protein is a protein with high biological value, (i.e. lactalbumin) containing a specified pattern of amino acids.
- Amino Acid/Chemical Score: (mg of amino acid in 1 g of test protein)/(mg of amino acid in reference protein).
- Amino Acid Availability (percentage): (total amino acid intake – fecal excretion of amino acid)/ (total intake of amino acid) x 100.
- Digestibility.
- Nitrogen requirement: endogenous nitrogen excreted in urine + endogenous nitrogen lost in feces + nitrogen lost in sweat, skin, etc + nitrogen required for growth.
- Plasma Amino Acid Ratio: variation in concentration of free essential amino acids in plasma

after the consumption of a food containing protein.

- Protein Efficiency Ratio (PER): weight gain of test group/total proteins consumed. Low quality proteins are described by a PER value below 2, whilst good quality proteins have a PER value above 2. Many studies, though, employ the Adjusted PER, where PER is corrected for the PER measured for casein (standard value of 2.5). [2]
- Protein digestibility-Corrected Amino Acid Score (PDCAAS): it is the amino acid score corrected for the digestibility of the protein. This was introduced by FAO/WHO in 1991 and is currently the international method for the assessment of protein quality [3].

As explained, the quality of a protein, intending its nutritional value, can vary according to many factors, thus every source of protein will have a varying quality.

Cereals are still a great ratio of the daily diet in many populations and wheat is the main grain consumed. Its protein are considered of low quality, due to the insufficient percentage of lysine and threonine. However, new varieties with a high protein content supplemented with lysine can have a potential similar to that of casein. [1]

However, it must be considered that in many bakery products, such as the bread, crust ha less nutritive value than crumb or the whole bread, due to the Maillard reaction destroying lysine or glutamyl-lysine in crust, whilst still leaving it present in crumb [4 – 5 – 6].

Rice, another widespread grain, has a protein content (5-7%) lower than that of most cereals, but it has a 50% greater value of lysine than wheat and a better amino acid balance. Many efforts have been made to improve its protein content, due to its great consume all over the world, and although most attempts have been unsuccessful, some developments have been made.

For example, Gastanduy et al. [7] analysed an infant formula made of a high protein rice flour fortified with lysine and threonine for male infants suffering of malnourishment. It was reported that this product had equivalent nutritional value to the highest quality cow-milk formula and was thus a good product, especially for children with allergies, since it is a hypoallergenic food.

Corn is another grain with a great consumption, especially in South America, Africa and Asia, where it is employed to cover up to 50% daily energy intake. In the last two decades of the 20th century, maize varieties with high lysine content were discovered and studied. Those varieties, called opaque-2-maize, could give a similar amount of absorbable nitrogen to milk and eggs and were therefore studied for human nutrition[8].

Pulses are another main source of plant proteins; they provide for almost 10% of the world's total dietary protein and have between 2 and 4 times the protein percentage of grains[1]. They

represent the sole source of dietary protein in many countries of the world, where animal proteins are not available or too expensive; only in the last 20-30 years their employment as food products has been investigated for their possible beneficial effects, thanks to their nutritional value and bioactive components.

Proteins are contained in grain legumes from 20% (peas, beans) up to 40% (soybeans) of dry weight and thus represent a rich source of proteins and amino acids. These proteins usually lack sulphur-containing amino acids, such as cysteine, methionine, and tryptophan. On the other side, they have a higher percentage in lysine than cereals.

Legumes contain also so-called anti-nutritional compounds (ANCs), like hydrolase inhibitors and lectins, since those mainly represent a form of protection for the plant. Hydrolase inhibitors can affect the actions of many enzymes like amylase, trypsin or chymotrypsin, but this effect is avoided if seeds or legume flour are cooked and, when this happens, those inhibitors can also play a nutritionally beneficial role, due to their amino acidic content. Lectins, instead, are glycoproteins that can reversibly bind carbohydrates and some can have haem-agglutinating activities. Their toxicity is expressed with growth inhibition in lab animals and with diarrhea, bloating, nausea and vomiting in humans; this can be avoided, again, with heating, but insufficient cooking might retain some symptoms of toxicity.

Despite all these cons, legumes are increasingly showing beneficial effects on health and some of these are: inhibition of enzymes involved in negative physio-pathological events, immuno-modulation, glucose and plasma lipids homeostasis, blood pressure control, thus helping in the prevention of common diseases such as CVDs, diabetes, hypertension or obesity [9]. The main legumes consumed by the human population are:

Chickpeas: are legume seeds with a low fat content, widely consumed in southern Europe, North Africa and Asia; they are considered to have a good protein quality.

Beans: are employed mainly in India and South America as an energy and protein source. Dry beans, however, have a low nutritional value due to many factors: low digestibility, insufficient bioavailability of essential amino acids, low quantity of sulphur amino acids and presence of anti-nutritive elements, even though these might still be reduced by heating, as mentioned.

Lentils: are another type of pulse that is widely consumed. They have a high content of saponins and this could have health benefits since it could help reducing cholesterol. Nevertheless, lentils contain anti-nutritive factors such as flatus-inducing carbohydrates, trypsin inhibitors (though destroyed by boiling) and lectins (growth inhibitors and toxic at high levels).

Soybeans: are used especially in East Asia through many different food products such as soy sauce, tofu (soybean protein curd), natto (fermented whole soybeans), fermented soybean paste (miso) and soy milk. Soy proteins are considered nutritionally equivalent to those deriving from animal sources, thus they are studied in order to be more widely employed in human nutrition. However, remains still the fact that, as pulses, soybeans contain anti-nutritional elements such as enzyme inhibitors and lectin. Processing employing heating and fractionation can improve their quality, but to destroy all inhibitor activity a long heating treatment is needed, which in turn damages other nutritional properties of soybeans proteins. Other methods [10] are now employed for this reason [1]. As stated before, animal proteins are one of the greatest source of proteins in terms of relative abundance, especially meat proteins. In addition, animal proteins are usually considered of higher nutritional quality than those from plant sources because of the greater balance of essential amino acids. Of all animal proteins, milk and eggs are considered the best sources, and are considered the reference to evaluate the quality of other proteins. Meat and poultry muscles, together with fish muscle proteins, are still considered very nutritious [11].

Actin, myosin and collagen are the main proteins contained in meat products and are present at different content in the different meat products. Some bioactive amino acids, such as methylhistidine or hydroxymethyllisine, are present in meat proteins and rarely in plant proteins.

The processing procedures on meat (and fish) products can generate bioactive peptides, for example by fermentation or enzymatic hydrolysis, which have been shown health benefits to the human population, such as ACE inhibition [12].

As shown in table 2 [13 – 14], animal proteins have a greater amount of most essential amino acids.

Fish and seafood are other sources of animal proteins. Most fish proteins have similar or better nutritional values than casein, thus they are considered better than meat proteins [1]. Shellfish have amino acid scores from 68 to 95, providing high-quality proteins and having leucine or valine as the first limiting amino acid. [1]

There are slight differences in fish muscle compared to the one from other animals. Since fish needs less support, as they are supported by water, their meat has less connective tissue and it is thus tenderer. In addition, due to their typical movement, their muscle fibres have a different structural arrangement and, finally, most fish are cold adapted and their proteins are more heat sensitive [11].

Food Source	Lysine	Sulphur amino acids	Theonine	Tryptophan
Cereals	31 ± 10	37 ± 5	32 ± 4	12 ± 2
Pulses	64 ± 10	25 ± 3	38 ± 3	12 ± 4
Nuts/seeeds	45 ± 14	46 ± 17	36 ± 3	17 ± 3
Fruits	45 ± 12	27 ± 6	29 ± 7	11 ± 2
Animal products	85 ± 9	38	44	12

Table 2. Amino acid content of different food protein sources. Data were based on FAO (Amino acid content of foods and biological data on proteins. Rome: Food and Agriculture Organization. 1985. (FAO nutritional studies no 24.)) and on the US Department of Agriculture (Agricultural handbook no. 8-1 (1976); 8-2 (1977); 8-5 (1979); 8-6 (1980), 8-8 (1982); 8-9 (1982); 8-10 (1983); 8-1 1 (1984); 8-12 (1986) and 8-14 (1986). Washington, DC: Agriculture Research Service.) Based on data from FAO (reference 12) and US Department of Agriculture (reference 13).

Dairy proteins, having origin from milk, are mainly: α-lactalbumin, β-lactoglobulin, immunoglobulin, albumin, κ-casein, β-casein, α-caseins, lactoferrin and lactoperoxidase. Rennet, also employed for the production of cheese, contains a glycomacropeptide that chymosin cleaves from κ-casein in order to start forming curd from the precipitation of caseins.

Cheese is thus a sort of precipitate of milk caseins. Thus, caseins are more concentrated in cheese than in milk, but the relative proportions of casein fractions are almost unaltered [15].

Dairy proteins have an amino acid composition close to the ideal “average” protein, calculated averaging the values of 207 unrelated proteins with a known sequence [16].

For these reasons, usually dairy proteins are considered the ones of better nutritional quality. Whey proteins are usually employed for their functional properties for the preparation of different food products: as fat and water binders in meat; for the production of yogurt and other dairy products; as milk replacers in ice creams; as egg replacement in bakery and confectionery products. B-lactoglobulin is a rich source of the essential amino acid cysteine, stimulant for the synthesis of glutathione, which is considered anticarcinogenic. Albumin is the same as the one contained in human blood and binds insoluble free fatty acids, and helps again the production of glutathione.

Lactoferrin and lactoperoxidase are contained in minor quantities but are considered bioactive proteins. Lactoferrin helps iron absorption in the gut and together with lactoperoxidase can help reducing chronic diarrhea [17].

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4. FOOD TECHNOLOGIES IN THE PREPARATION OF PROTEIN–RICH FOODS

Since the beginning of the human civilization, humans have always tried to find the best way to preserve food as long as possible. At first, by using the energy from the sun, men learned how to dehydrate the hunted and fished meat. In the prehistorical time, a great discover changed the entire human future: the fire. The fire allowed ways of cooking food and their preservation.

Also, salt was another great discovery that helped food preservation, in particular for meat. Thanks to this, salt became a prime item of trade until modern time.

“But man’s greatest change came with the innovation of agriculture. It forced him to cooperate with the neighbour and to protect his community from conflicts with animals and other human beings. It also forced men to learn about their environment and to begin to think in terms of energy. Agriculture throughout man’s history has been the primary form of technology.” [1].

During the decades, food technologies have improved their ability in preserving food quality, but some of the main points were kept the same. For example, the basis for cheese production, milk fermentation, wine and beer distillation, bread leavening, dehydration, salting and smoking of food remained the same.

A new chapter in food technology was reached the 2nd September of 1802 when Nicolas Appert, a French inventor, started a little pilot plant for the production of canned food. Appert realized that the bottles filled with cooked food, if sealed and pasteurized, could keep food fresh. Appert never submitted the patent for his invention, thus in 1810 Peter Duran, another English inventor, took his idea, realizing a patent for food in metal sheets.

During those years, another man allowed other important changes in food technology. The French chemist Louis Pasteur discovered of the principles of vaccination, microbial fermentation and pasteurization. Pasteur's research showed that the growth of microorganisms was responsible for the spoiling of beverages, such as beer, wine and milk. Therefore, he created a process in which liquids, such as milk, were heated to a temperature between 60 and 100°C, so that the most part of bacteria and moulds present in the matrix were killed. Pasteur patented the process, to fight the "diseases" of wine, in 1865. The method became known as pasteurization and was soon applied to beer and milk [2].

In 1870, the production of pasteurized food started in the United States. In less than 20 years, the number of food companies arose extremely fast. Food technologies were born in this historical period.

The available technologies for food preservation can be summarized as follows:

- Drying: one of the oldest preserving techniques used to hamper the decomposition of food products;
- Refrigeration: used to shoot down the growth and reproduction of micro-organism and the action of enzymes that cause food spoilage;
- Freezing: one of the most used processes, suitable for a wide range of foods, from the raw matrix to the prepared food;
- Heating: methods commonly used, both commercially and domestically, for killing microorganism;
- Salt: salting or curing is a very old technique used in particular with meat and fish. This process prevents, using salt, the growth of microorganism;
- Sugar: commonly used for the storage of many fruits. Sugar is considered as a preservative because it allows the dehydration of microbial cells, with the consequence of their death;
- Smoking: commonly subjected to this method of preservation are meat and fish. With smoking, foods are dehydrated and it is possible to extend their shelf life. Historically this effect was achieved by exposing food to smoke from different kind of burning wood, depositing a wide number of pyrolysis products on the food matrix;
- Pickling: it can be divided in two categories: chemical and fermentation pickling. In the first one, food is placed in an edible liquid that inhibits or kills bacteria and other microorganism. In the fermentation pickling the food itself, by a process that generates lactic acid, produces the preservation agent;
- Lye: this method is possible thanks to the Sodium hydroxide by which the bacterial growth is arrested;
- Canning and bottling: these technological techniques involve the cooking of food, its sealing in sterile cans or jars, and the boiling of the containers as a form of sterilization to kill the bacteria;
- Jellying: gelatine, maize flour, arrowroot flour and agar are some of the possible materials in which food can be cooked and preserved thanks to the formation of a gel.
- Curing: is very close to salting technique. The combination of salt, spices, nitrates and nitrite with raw meat or fish and the ripening time allow the preservation and flavouring of food product;
- Fermentation: by using specific microorganisms, able to fight spoilage from other less-benign organisms, it is possible to preserve many foods such as cheese, milk, beer, wine and others.

Other important food preservation techniques that have been developed in modern age, in

research laboratories, and only for commercial application are:

- Pasteurization
- Vacuum packing
- Artificial food additives
- Irradiation
- Pulsed electric field electroporation
- Modified atmosphere
- Non-thermal plasma
- High-pressure food preservation
- Bio-preservation
- Hurdle Technology

In conclusion, the processing of food can improve nutrition, quality, safety, and taste. Occasionally, lead to the formation of anti-nutritional and tossic compounds [3 – 4].

4.1 BRESAOLA DELLA VALTELLINA

Figure 1. An Example of Bresaola della Valtellina. It is a typical product of Northern Italy (Valtellina), and is produced by curing the intact beef muscle through different processing steps including a careful selection and skilful trimming cuts of meat, a dry salting and a slow maturation

Bresaola is a typical product of Northern Italy (Valtellina), and is produced by curing the intact beef muscle through different processing steps including a careful selection and skilful trimming cuts of meat, a dry salting and a slow maturation (Figure 1). The processing steps of the “Bresaola Valtellina PGI” (Protected Geographical Indication) must follow strict rules, which guarantee the

authenticity of the product and the respect for the traditional processing:

1. Selection and trimming cuts of meat

- Every single piece of meat is subjected to careful selection. The specialist operator shall evaluate the potential of the piece and trimming, which must be done with skill and precision to remove fat and tendon external parts without affecting the pulp. The non-compliant raw material (e.g. too mottled or discolored) is discarded.

2. Dry salting

- During dry salting, the salt solution is formed with the juice of the meat. During this processing step, wine, spices, sugar (with the aim of encouraging microbial phenomena responsible for much of the aging of the product), nitrites and nitrates of sodium and/or potassium, ascorbic acid and its sodium salt can be added to. Upon the “wise” dosage of the used spices, the product begins to acquire its own sensorial properties.
- Salting lasts not less than 10 days, and it is interrupted by the operation of massage (churning) to allow the uniform migration of the salt and spices into the meat.

3. Maturation

- After salting, the meat is stuffed into natural or artificial casings and sent to the next stage of drying in special cells. The drying lasts one week on average and it should allow rapid dehydration of the product in the first days of treatment.
- The drying phase follows the maturing conducted at an average temperature between 12 and 18 °C. The aging, including the drying time, lasts 4-8 weeks.
- The Regulations prohibit the adoption of techniques that provide for an accelerated dehydration of the product. Given the favorable climatic conditions of the production area, natural ventilation and exposure to moisture have been allowed.

4. Packaging

- The cured product that passes quality controls provided by the Regulations is marked with the IGP EU-trademark.
- Bresaola of the Valtellina can be sold whole, in bulk or in a vacuum, in pieces or sliced, vacuum packaged or under modified atmosphere.
- Each package must report the specific label.

4.2 RICOTTA CHEESE

Ricotta is an Italian cheese and probably the oldest and well-known whey cheese (Figure 2). Ricotta cheese has a soft matrix and it can be produced by using sheep, goat, cow and buffalo milk.

However, it can be produced using cheese whey proteins, whey, milk or mixtures of both.

“Whey protein was discovered some 3000 years ago when calves' stomachs were used to store and transport milk. Throughout the action of the naturally occurring enzyme chymosin (rennet) found in the calves' stomachs, the milk coagulated during storage and transport resulting in curds and whey and as such spawned the start of the cheese (and whey) industries” [6].

Ricotta is knows as fresh cheese. Indeed its initial pH is above 6 and this is the main cause of the faster microbiological spoilage. Thus, even under refrigeration conditions, the shelf life is less than one week for the handmade product and less than one month for the industrial one. To the detriment, the flavour plays a central role in influencing consumer preference. For example, goat-milk products have a specific odour and flavour, which can be strongly appreciated or not and which have been subject of several investigations [7 – 8].

Figure 2. Ricotta is an Italian cheese and probably the oldest and well known whey cheese. Ricotta cheese has a soft matrix and it can be produced by using sheep, goat, cow and buffalo milk. But it can be produced using cheese whey proteins, whey, milk or a mixtures of both.

Sometimes a relatively strong goaty flavour is desirable, but it can commonly represent an off-flavour affecting goat milk. A strong flavour in the milk causes a strong flavour in the product when making white cheese, as well as brown whey cheese [9]. Nevertheless, such variations in product properties are undesirable in modern dairy production. Therefore, with the increased consumption and popularity of goat-milk products, an investigation and resolution of some of the factors influencing the sensory properties of goat’s milk have become economically important. The fat fraction of cheese is important for the perception and development of flavour [9].

In general, to determine the quality of Ricotta cheese flavour, rheological properties, visual appearance and nutritional features are parameters to be considered [10 – 11 – 12].

4.2.1 Technological procedure to make Ricotta cheese from cow's milk

The technological process for the making of Ricotta cheese can be synthesized as follows:

After the pasteurization of whole cow's milk, it is necessary to mix the milk with the whey protein in a proportion of 60:40. Then the temperature is raised up to 30-32°C, and S*treptococcus thermophylus* is added as a starter. The importance of these gram-positive bacteria is that it is able to increase total acidity to 0.24% of lactic acid. The temperature is then brought up to 85 °C and it is kept that way for minutes. The final acidity is adjusted to 0.3% by adding lactic acid.

The sample is collected in moulds to partially remove whey proteins. Then the ricotta is placed in a tank where it is stirred until the final moisture reaches 70%. At the end, the Ricotta cheese is packaged in polyethylene bags at a temperature between 65 and 68 °C.

4.3 SPREADABLE CHEESE

The spreadable cheese is a soft, fresh cheese typical of Northern Italy (Figure 3). The most famous spreadable cheese in Italy is the "Crescenza" cheese [13]. In general, this kind of cheese has a high water content (55-60%) and fat content (50%). It is rich of calcium and its matrix is homogeneous, creamy, smooth and spreadable. One of the most important parameter for the production of this kind of cheese is the nature of milk [14]. Whole milk in particular, has to have a minimal fat content between 3.4-3.6%. The protein content has to be high too, because the final fat/protein ratio has to be 1:10.

Figure 3. The spreadable cheese is a soft, fresh cheese typical of Northern Italy. It is rich of calcium and its matrix is homogeneous, creamy, smooth and spreadable.

The calcium content is a critical point; milks with a low calcium quantity give flawed cheese and

low performance.

4.3.1 Technological procedure to make Spreadable cheese from cow's milk

The technological processes by which obtaining a "crescenza" [15 – 16] style spreadable cheese can be summarized as follows:

- Whole milk is pasteurized between 72-75 °C for 15-20 minutes. During the process the acidity has to have values around 3.4-3.6 °SH/50.
- When the milk has achieved the temperature of 36-40°C in the boiler, lactic ferments such as the *Streptococcus thermophilus* are added.
- After 10 minutes, the rennet is added. The enzymatic coagulation gives a relatively hard curd and the time for the entire process is between 1-1.5 hours.
- At the end of this phase, the draining phase starts. The latter is relatively slow: 7-8 hours; the temperature has to be between 25 and 27°C and the moisture around 90%.
- The salting phase is carried out by adding 0.5-1% of salt in the final mixture.
- After that, the cheese is left to ripen, usually in pots, for about 8 days.

The final characteristic of the soft cheese are:

- Moisture: 55-60%
- Fat: >50%
- Salt: 1%
- pH: 5.2-5.4

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5. THE EFFECT OF pH AND IONIC STRENGTH ON THE PROTEIN STRUCTURE

> "Food can deteriorate during processing and storage due to both the enzymatic and Maillard-type reactions of primary amino group with reducing sugars and other enzymatic and non-enzymatic browning reactions with non-reducing carbohydrates. High pH values induce racemisation of L-amino acid, residues to D-isomers" [1 – 2 – 3 – 4]

The above effects of pH on food stability are not the only ones. Proteins stability is affected as well, because pH affects the protein surface charges. pH is modified by changing the concentration of acids or bases. According to Arrhenius and Ostwald, acids are substances that placed in aqueous solutions dissociate in an anionic radical and in a hydrogen ion/positive proton (H^+). The acidic taste and the salt formations (when an acid substance attacks a metal) are the typical acids actions. On the contrary, bases are substances which produce hydroxide ions (OH^-) in aqueous solution.

The balance between and within proteins are deeply affected by the pH values. Indeed, proteins change their net charge in a positive and negative way, depending if the value is below or above their isoelectric point. The net charge is important also in the regulation of the solubility of proteins.

"The major force responsible for the stability of protein structures is the hydrophobic interaction between nonpolar side chains [6]".

When the proteins are at their isoelectric point, the surface charge reaches the neutrality, and the molecules tend to associate because of the attractive hydrophobic interactions, which predominates.

Two are the possible ways in which ions affect the proteins conformation:

- their effect on hydrophobic forces influencing the structure of water;
- their electrostatic interaction with the charged groups [7];

When the concentrations of salt is low, the solubility of proteins firstly increases and, after reaching the maximum, decreases again.

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6. ENZYMATIC HYDROLYSIS: SITES OF ATTACK OF DIGESTIVE ENZYMES

The term “enzyme” comes from the Greek language and it means “inside yeast”. When it was discovered that yeast extract, although with dead cells, was able to ferment sugar, this word was in effect coined. In reality, enzymes are catalysts, they are produced by the same cells that is then going to use them and their action is deeply connect with the kinetic property of a reaction. Thus, they can catalyze reactions in both directions, while, the real reaction direction depends on thermodynamic characteristics, ΔG. When reagents became products (or vice-versa), they pass through an intermediate state which could possess a slow formation time, due to the fact that not all the molecules have the energy needed. This kind of energy is called activation energy, and it is necessary to reach the active state, from which reagents will become products [1].

Often the molecular size of enzymes is bigger than that of the substrates in which they act. That part in which the enzymatic molecule links and acts is called active site. Usually these sites are in the form of a cavity surrounded by reactive groups able to link their substrates by chemical bonds.

In general, enzymes are divided in 6 classes, on the basis of an international convention.

- Oxidoreductase (catalyze redox reactions)
- Transferase (catalyze groups transfers)
- Hydrolase (catalyze hydrolysis reactions)
- Lyase (catalyze addiction reactions)
- Mutase or Isomerase (catalyze isomerisation reactions)
- Ligase or Synthetase (build a bond thanks to cofactors that give energy to the system) [2].

6.1 DIGESTIVE ENZYMES

The aim of digestion is to hydrolyse proteins in little peptides and amino acids able to be absorbed by human body. Every day the small intestine digests and absorbs a high quantity of proteins, some of them coming from diet and the other released in gastric tract (digestive enzymes, sieroalbumin, mucoprotein). Then, for 100 g of protein ingested, 160 g are the protein absorbed and 10 g the quantity of proteins excreted. It is estimated that almost 50 % of the nitrogen loss is due to bacteria fixation of nitrogen substances such as urea, ammonia, etc.

Protein digestion requires the action of two specifics enzymes:

- Endopeptidase
- Exopeptidase [3]

Enzyme	Precursor	Product	Activator	Substrate	Action
Stomach					
Pepsin	Pepsinogen	Polypeptides and amino acid	Acidic pH	Proteins	Hydrolyzes bonds near aromatic amino acids
Intestine					
Trypsin	Trypsinogen	Oligo-peptides	Enterokinase, trypsin	Proteins, poly-peptides	Cuts the bond in which are involved lysine, arginine
Chymotrypsin	Chymotrypsinogen	Oligo-peptides	Trypsin	Proteins, poly-peptides	Cuts the bond in which are involved neutral and aromatic amino acid
Elastase	Proelastasi	Oligo-peptides	Trypsin	Elastin and thers proteins	Cuts the bond in which are involved aliphatic amino acid (alanine, glycine, serine)
Carboxypeptidase A	Procarboxypeptidase A	Aromatic and peptic amino acid	Trypsin	Poly-peptides	Cuts aromatic amino acid from carboxyl end
Carboxypeptidase B	Procarboxypeptidase B	Arginine, lysine and peptides	Trypsin	Poly-peptides	Cuts arginine or lysine form carboxyl end

Table 3. Proteolityc enzyme present in the gastrointestinal tract

The first ones breaks, at different extents depending on the presence of particular amino acids, the polypeptides chains in little fragments, while exopeptidase breaks protein chains at the amino or carboxylic terms. It is important to consider that, often, the bonds that need to be hydrolysed are not in direct contact with enzymes, but inside the proteins; for this reason the action of endopeptidases is so important. These endopeptidases and the denaturant environment allow the hydrolytic enzymes to carry out their functions. The environment is, in effect, also very important for the action of enzymes. For example, in the neonatal phase, intestinal barrier is not strong enough and allows the absorption of whole proteins or bigger fragments. In the stomach, the

acidic pH allows proteins denaturation exposing them to the action of pepsin, which will start cutting the proteins.[4 – 5] On the other hand, pancreatic endopeptidases (trypsin, chymotrypsin and elastase) hydrolyze the fragments arriving from the stomach in smaller peptides that are then successfully hydrolyzed by carboxypeptidase. The pepsin released into the stomach is not the active form, but pepsinogen and pH are able to change its form, from inactive to active. In table 3 are reported other examples of hydrolytic enzymes.

All proteolytic enzymes are digested from the human body at the end of their activity. This system makes possible a powerful proteins hydrolysis avoiding the hydrolysis of intestinal structures and glands [6]. The final products of digestion are absorbed from the enteric mucosa, and it is possible that half of the amino acids present are absorbed in the form of di- and tri-peptides. Then, due to the presence of peptidase within the enterocytes, those are further hydrolysed into free amino acids. The part of amino acids that are absorbed will go through the portal vein and will reach the liver.

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7. PROTEIN AS SOURCE OF BIOACTIVE PEPTIDES

Several research works have demonstrated that the ingestion of protein-rich foods is health enhancer, thanks to the biologically and protecting functions carried out by protein digestion. The hydrolysis of food proteins start at the beginning of the gastrointestinal tract, releasing the hydrolysed products to be absorbed, as di- and oligo-peptides, amino acids, later into small intestine.

The important biological properties of proteins are related to their possibility to originate bioactive peptides, evaluated in recent years, as functional food ingredient aimed at health maintenance.

The definition of bioactive peptides can be summarized as “Food derived components (genuine or generated) that, in addition to their nutritional value exert a physiological effect in the body” [1]. Bioactive peptides are usually absorbed through the intestine and through after into the blood stream to exert various effects. The discovery of the first food derived bioactive peptide was attributed to Mellander, who understood that casein phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants [2].

However, bioactive peptides has been suggest as nutraceuticals and functional food ingredients thanks to their important role in metabolic regulation and modulation. In general, three ways exist by which these peptides can be released:

- through hydrolysis by digestive enzymes;
- through hydrolysis by proteolytic microorganism;
- through the action of proteolytic enzymes derived from microorganism or plants [3].

Depending on their amino acid sequence, bioactive peptides may affect the main body functions (Figure 4)

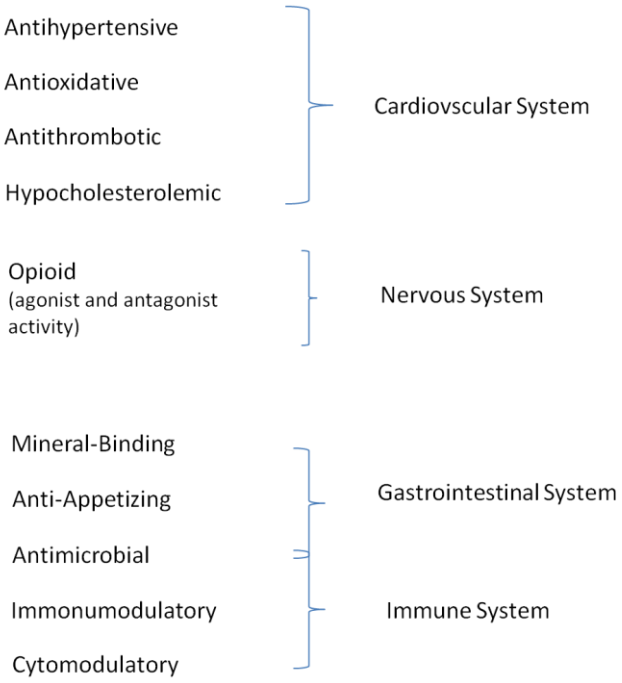

Figure 4. Depending on their amino acid sequence, bioactive peptides may affect the main body systems.

For these reasons, more studies are being performed exploring the sources, bioavailability, and the potential role to promote human health by reducing the risk of chronic diseases or boosting natural immune protection.

Many beneficial effects may be attributed to numerous known peptide, for example, antimicrobial, antioxidative, antithrombotic and, immunomodulatory [4].

Nowadays, it has been ascertained that the greatest sources of bioactive proteins are cheese, bovine milk, and dairy. This find sense in the purpose to which milk is the first form of nutrition in young mammals inspiring the concept that "bioactive substance in milk and colostrums is the mother language on a substrates basis". Recent studies have also dedicated efforts for finding other food bioactive, and meat and fish proteins have demonstrated offering a huge potential as novel sources of bioactive peptides [5 – 6].

Studying bioactive peptides is the prerogative not only of scientific and pharmaceutical field but of food market too, because these peptides could also be added in commercial food product. Indeed, numerous products are already available on the market, or under development by food companies, that include casein hydrolysed. It is clear that the first objective to reach, for all workers involved in food and nutrition field, is the best knowledge about bioaccessibility and bioavailability.

In general, optimizing design of healthy products by using bioactive peptides is a technologic and scientific challenge. In effect, until now, there has been little concern about the safety issue on using bioactive peptides since they are normally produced by digestive enzymes and food-grade enzymes, whilst different processes may be used for industrial production of peptides [7].

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8. BIOACCESIBILITY AND BIOAVAILABILITY

The FDA has defined bioavailability as the rate and extend to which the active substance or therapeutic moieties contained in a drug are absorbed and become available at the site of action [1]. This definition is also useful to define the active substances present in food. More specifically, bioavailability can be defined as the fraction of a nutrient contained in the indigested food that can be release during digestion process and may be metabolized into the small intestine and found in the bloodstream. Bioavailability is a concept strictly connected to the nutritional field, in particular for its role in the realization of nutritional tables, for the selection of substances useful for food fortification, and the formulation of food for childhood food.

"The main component of bioavailability refers to the digestion and absorption of nutrients in the gut, which is the main rate-limiting factor [2]". The study of intestinal availability is quite difficult, in particular for ethical reasons and wide inter-individual variations. For these reasons, in the last decades, experimental models have been evolved for overcoming these problems and to obtain reliable results. The principal techniques are based on:

- In vitro digestion of homogenized foods, using a closed system for determination of the soluble nutrient fraction [3];
- In vitro digestion and dialyzeability of soluble nutrients [4];
- Usage of Caco-2 cells and measurement of cellular uptake of nutrients after transmembranous dialysis during intestinal digestion of foods [5].

The common weakness in these models concerns the absence of the complex mucosa barrier and the calculation of the fractional transport and flux rate are not accurate due to the static transport conditions. Bioavailability is influenced by several factors such as the chemical state of the nutrient, the interactions with others food components, the presence of cofactors or suppressors, formation of stable compounds that are slowly metabolized, and so on.

Some food technological/manufacturing processes could affect positively or negatively the nutritional availability. For example, the wheat milling removes the external layer containing high quantity of phytic acid, which is able to decrease the bioavailability of iron, copper and zinc. Other simple technological processes for reducing phytic acid content are fermentation and germination, while others such as polishing techniques, which produces the Maillard reaction, have negative effect on some nutrients bioavailability. Furthermore, the gastrointestinal environment is very variable for bioavailability determination. Bioavailability of nutrients is usually measured in the

blood in vivo, but individual factors such as variability, physiological state, dose and presence of other meal components play an important role [6].

Another term often associated to bioavailability is bioaccessibility, typically used for in vitro procedures, that can be defined as the fraction that is release from food matrix and is available for intestinal absorption, after digestion [7].

The presence of food is one of the problem that can affect bioaccessibility, because food can increase the period during which mobilization can take place, being a critical point for those compounds for which dissolution in rate limiting [7].

The bioaccessible fraction has the major importance due to the facts that can be used by the organism as nutriment. If the amount of recovered nutrient after digestion is relevant, the better term to use is bioaccessibility.

In conclusion, in order to make compounds bioavailable for human body it is necessary that these nutrients are bio accessible [8 – 9].

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9. DEFINITION AND METHODS FOR THE MEASUREMENT OF DIGESTIBILITY

9.1 NUCLEAR MAGNETIC RESONANCE (DESCRIPTION OF SPECTRA, RANGE OF CHEMICAL SHIFTS FOR AMINO ACIDS

9.1.1 Metabolomics

The term metabonomics is often confused with the term metabolomics and sometimes both are used interchangeably. Metabonomics can be defined as "*the technology geared towards providing an essentially unbiased, comprehensive qualitative and quantitative overview of all the metabolites present in an organism. Even if they include approaches as metabolite profiling, metabolite target analysis and metabolic fingerprinting, they explain different concepts*" [1- 2].

Metabolomics obtains a *"complete set of metabolites/low-molecular-weight intermediates, which are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism"* [3].Metabonomics is defined as *"the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"* [6].

Even if the basic concept is different, metabolomics and metabonomics approaches can easily coexist in same study and one is strictly connected to the other in a double sense: the metabolomics exploration (metabolomics profile) can help understanding the response of a biological system to external stimuli (metabonomics profile) and *vice versa.* For this reason, the metabolomics term will be used along the whole text for the sake of simplicity.

Thus, **metabolomics** is the study of metabolites and their role in various physiological states. It is a holistic approach aimed to detect, quantify and catalogue the time related metabolic processes of an integrated biological system. Ultimately, it relates such processes to the trajectories of the physiological and pathophysiological events. However, a metabolomics study can provide significant results only if the metabolic changes in a target group is significantly different from the biological variation of the relative control group [7]. Complementary to proteomics and genomics, first metabolomics has been widely applied to a wide range of problems in diverse biomedical research areas to understand the metabolites' behaviour under certain exogenous conditions.

In recent times, due to the increasing interest in studying the effects of foods on the biochemistry of human and associated microflora, metabolomics also finds a role in nutritional science and in food chemistry, also leading to the birth, of other "omics" sciences such as transcriptomics, and a

new science called **foodomics** [1]. Thus, metabolomics becomes a key tool in human nutrition, helping in the study of different aspects of ‘‘molecular nutrition’’ more strictly connected to **food quality**, including:

- food component analysis;
- food quality/authenticity detection;
- food consumption monitoring;
- physiological monitoring in food intervention or diet challenge studies;

In fact, traditionally, food component analysis involves identifying and classifying food constituents into very broad categories such as proteins, fats, carbohydrates, fibres, vitamins, trace elements, solids and/or ash. However, with the advent of metabolomics, foods and beverages are now being analyzed with considerably more chemical detail [9] with hundreds or even thousands of distinct chemical identities being detected and/or identified in certain foods.

9.1.2 HR ^{1}H-NMR and Chemometrics in food science

In food science, and in general in metabolomics study, high-resolution proton nuclear magnetic resonance (HR ^{1}H-NMR) spectroscopy is uniquely suited to detect a large range of endogenous low molecular weight metabolites in an organism. This technique is rapid and rich in structural and quantitative information and allows the metabolites to be analyzed simultaneously [4]. In particular, the NMR technique has the advantage to supply detailed information on the molecular structure of the biological material observed, reflecting at the end the metabolic status of a biological living system, without losing important information on the system. This spectroscopic technique is generally used to detect hydrogen atoms in metabolites (^{1}H-NMR). Thus, in a typical sample (biological fluid in medical research or organic extract in food research) all hydrogen-containing molecules (almost all metabolites) will give a ^{1}H-NMR spectrum, as long as they are present in concentrations above the detection limit. In this way, the NMR spectrum is the superposition, also commonly called **fingerprint** [12], of the spectra of all of the metabolites in the sample (Fig. 5). This fingerprint evaluates, in one shot, the entire metabolites’ pattern variation due to external factors for example, considering food processing, storage time and temperature.

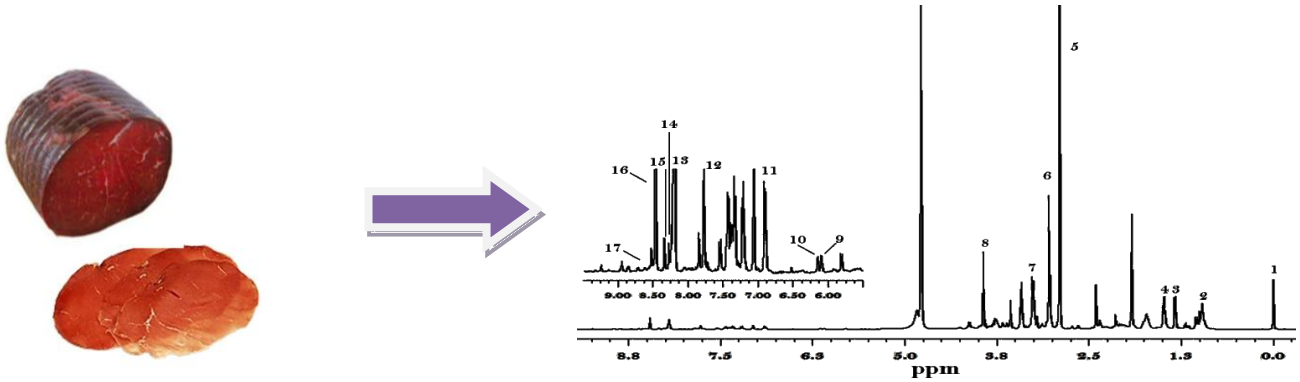

Figure 5. Example of a spectrum of Bresaola This fingerprint evaluates, in one shot, the entire metabolites' pattern variation due to external factors for example, considering seafood stuff, storage time and temperature.

Nuclear magnetic resonance has also other unique advantages over other metabolomics techniques such as chromatography and MS methods. First, it is a more uniform detection system and can be used directly to identify and to quantify metabolites, even in vivo. The most promising features of NMR are its non-destructive nature and its relatively shorter time or even direct measurement of the samples, e.g. urine. Another major advantage of NMR is that quantification is easy for all compounds as with a single internal standard all the detected metabolites can be quantified without the need of calibration curves for each single compound. At the end, NMR sample does not require any physical or chemical treatment prior to the analysis, but only the solution conditions such as the temperature, pH and salt concentration have to be adjusted. In this last case, the preparation of a generic sample involves three fundamental steps:

1) the first step is to rapidly collect and freeze the sample to quench metabolism and preserve the metabolites. Samples are typically stored a -80°C to prevent any decay;
2) the second step involves the extraction methods. The choice depends on the polarity of the metabolites that are required. Solvents that extract only polar metabolites include perchloric acid, methanol or acetonitrile. If both polar and lipophilic metabolites are desired, then extraction using methanol and chloroform can be used to fractionate the metabolite classes;
3) the third step is to optimize the solution for high resolution NMR spectroscopy. This typically entails buffering the sample pH to minimize variation in the chemical shifts of the NMR resonance (e.g 100 mM phosphate buffer, pH = 7.0), adding D2O to provide a frequency lock for the spectrometer and adding an internal chemical shift (and intensity) standard like sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP).

Since nearly no sample pre-treatment is required in NMR spectroscopy, the inherent properties of the sample are well kept.

On the other hand, the preparation of the NMR sample does not require particular procedures; but its intrinsic dynamic nature leads to the choice of an appropriate pulse sequence able to extract from the sample as much information as possible. Generally, 1D 1H-NMR with water presaturation, commonly called PRESAT or zgpr sequence, is a standard pulse sequence used in the NMR metabolomics approach (Figure 6). It is a simple two-pulse experiment that utilizes a relatively long, low power RF pulse to selectively saturate a specific frequency, typically water, and a non-selective 45-90° pulse to excite the desired resonances (H). This pulse sequence is particularly useful for aqueous samples or those with a single large solvent signal. With proper optimization, the resulting spectrum can be mostly free of the solvent signal and lead to improved Signal-to-Noise (S/N) ratio for solute resonances due to the reduction in dynamic range and subsequent increase in available gain.

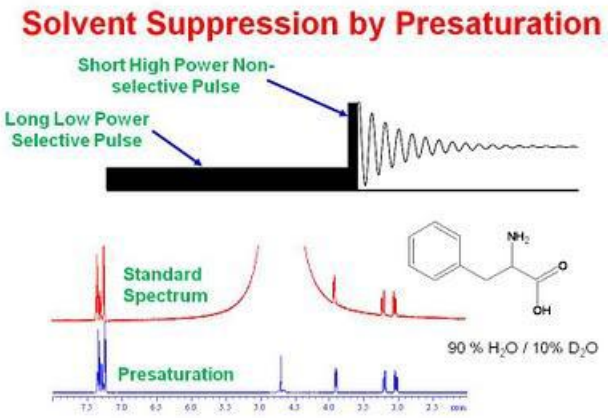

Figure 6. solvent (water) suppression by presaturation

HR 1H-NMR has been used to perform quantitative measurements of water soluble metabolites ,[2 – 12], but also a total of n-3 Fatty Acids (FA) and of the DHA levels. This analysis can be carried out with a high degree of automation and gives a rapid fingerprint of the polar and lipid profile. Other important information derives from the 13C-NMR experiment, which gives information about FA composition and the positional distribution of polyunsaturated fatty acids (PUFA) in triacylglycerols and phospholipids.

Different information, but yet interesting, comes from the 31P-NMR experiments used to evaluate degree of freshness of loach muscle depending on metabolic changes of the high-energy phosphate compounds.

9.1.3 Chemometrics

NMR spectroscopy is capable to detect all metabolites in a sample. The spectrum is full of information (variables), part of which results to be redundant most of the time. For this reason, it is important in a metabolomics research to compress these variables in order to have only those that contain the useful information. This kind of approach is commonly called chemometrics (approach) and it can be defined as “How to get chemically relevant information out of measured chemical data, how to represent and display this information, and how to get such information into data” [13].

Chemometrics is the field of extracting information from multivariate chemical data using the tools of statistics and mathematics. It is typically used for one or more of three primary purposes:

1) to explore patterns of association in data;
2) to track properties of samples;
3) to prepare and use multivariate classification models.

Exploratory data analysis can reveal hidden patterns in complex data by reducing the information to a more comprehensible form. This chemometrics analysis can expose possible outliers and indicate whether there are patterns or trends in the data. Exploratory algorithms such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are designed to reduce large complex data sets into a series of optimized and interpretable views. These views emphasize the natural groupings in the data and show which variables greatly influence those patterns. Formally, PCA is a way of identifying patterns in data, expressing them in such a way as to highlight their similarities and/or [14]. The advantage of this technique is the capability to reduce multidimensional data set (a data matrix) into a new set of uncorrelated (i.e., orthogonal) variables by performing a covariance analysis (ANCOVA) between factors. The PCA works by decomposing the X-matrix (containing the original data set) as the product of two smaller matrices, which are called loading and score matrices.

The loading matrix (V) contains information about the variables: it is composed of a few vectors (Principal Components, PCs) which are (obtained as) linear combinations of the original X-variables.

The score matrix (U) contains information about the objects. Each object is described in terms of its projections onto the PCs, (instead of the original variables).

The information not contained in these matrices remains as "unexplained X-variance" in a residual matrix (E) which has exactly the same dimensionality as the original X-matrix. The PCs, among many others, have two interesting properties:

1. they are extracted in decreasing order of importance. The first PC always contains more information than the second, the second more than the third and so on;
2. they are orthogonal to each other. There is absolutely no correlation between the information contained in different PCs.

In PCA, is possible to decide how many PCs should be extracted (the number of significant components, i.e. the dimensionality of the model). Each new PC extracted further increases the amount of information (variance) explained by the model. However, usually the first four or five PCs explain more than 90% of the X-variance. Anyway, there is not a simple nor unique criterion that decides how many PC to extract, so two kinds of considerations should be taken into account. From a theoretical point of view, it is possible to use cross-validation techniques to decide the number of PCs to include. Anyway, PCA helps in reducing the dimensionality of the variance space by eliminating redundancy. In ^{1}H-NMR, redundancy means that some of the variables are correlated each with another because they are measuring the same construct (different peaks for the same molecule). Therefore, this redundancy of the observed variables can be reduced into a smaller number of artificial variables (principal components or latent factors) that are a linear combination of the original ones and will account for most of the variance in the observed variables without losing information.

In this way, by using a few components, each sample (spectrum) can be represented by relatively few numbers instead that through the values of thousands of variables (spectral data points). Then, samples can be plotted making it possible to visually assess similarities and differences between samples and determine whether samples can be grouped [16]. As a clustering technique, PCA is most commonly used to identify how one sample is different from another, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated) or independently (i.e. uncorrelated) from each other. In contrast to PCA, **PLS** and **PLS-DA** [15] are supervised classification techniques that can be used to enhance the separation between groups of observations by rotating PCA components so that a maximum separation among classes is obtained [17]. The purpose of Discriminant Analysis is to classify objects (people, customers, foods, genes, things, etc.) into one of two or more groups based on a set of features that describe the objects (e.g. gender, age, income, weight, preference score, genotypes, metabolites' content etc.). In general, an object is assigned to one of a number of predetermined groups based on observations made about the object. For example, if one wants to know whether a soap product is good or bad, this judgment is based on several measurements of the product

such as weight, volume, people's preferential score, smell, colour contrast etc. The object here is soap. The class category or the group “good” and “bad”) is what it is looked (it is also called dependent variable). Each measurement on the product is called feature describing the object (it is also called independent variable). Thus, in discriminant analysis, the dependent variable (Y) is the group and the independent variables (X) are the object features that might describe the group.

The dependent variable is always category (nominal scale) variable while the independent variables can be any measurement scale (i.e. nominal, ordinal, interval or ratio). Partial Least Squares (PLS) is useful when a (very) large set of independent variables have to be predicted. It originates in the social sciences but becomes popular also in all branches basing on chemometrics methods, including food science [18]. It is a multivariate regression method allowing the establishment of a relationship between one or more dependent variables (U) and a group of descriptors (T). T- and U-variables are modelled simultaneously to find the latent variables (LVs) in T that will predict the latent variables in U and at the same time account for the largest possible information present in T; Figure 2.6 gives a schematic outline of the method. The overall goal (shown in the lower box of Figure 2.6) is to use the factors to predict the responses in the population. This is achieved indirectly by extracting latent variables T and U from sampled factors and responses, respectively.

The extracted factors T (also referred to as X-scores) are used to predict the Yscores U, and then the predicted Y-scores are used to construct predictions for the responses [11]. So, in this case the latent variables are selected on the basis of explaining contemporarily both descriptors and predictors. These latent variables are similar to the principal components calculated from PCA. The first one accounts for the largest amount of information followed by the other components that account for the maximum residual variance. As for PCs, the last LVs are mostly responsible for random variations and experimental error. The optimal number of LVs, i.e. modelling information in X useful to predict the response Y but avoiding overfitting, is determined on the basis of the residual variance in prediction.

9.1.4 NMR data pre-processing for chemometrics data analysis

The discipline of chemometrics originates in chemistry; thus, typical applications of chemometrics methods are the development of quantitative structure activity relationships or the evaluation of analytical - chemical data. The data flood generated by modern analytical instrumentation (like spectroscopic technologies) is one reason that analytical chemists, in particular, develop applications of chemometrics methods. While most other types of spectroscopic data can be

subjected to chemometrics analysis directly from the spectrometer, NMR data often need to be pre-processed in several ways in order to conform to the prerequisites for chemometrics data analysis:

- **Fourier transformation** [19] (Figure 7).

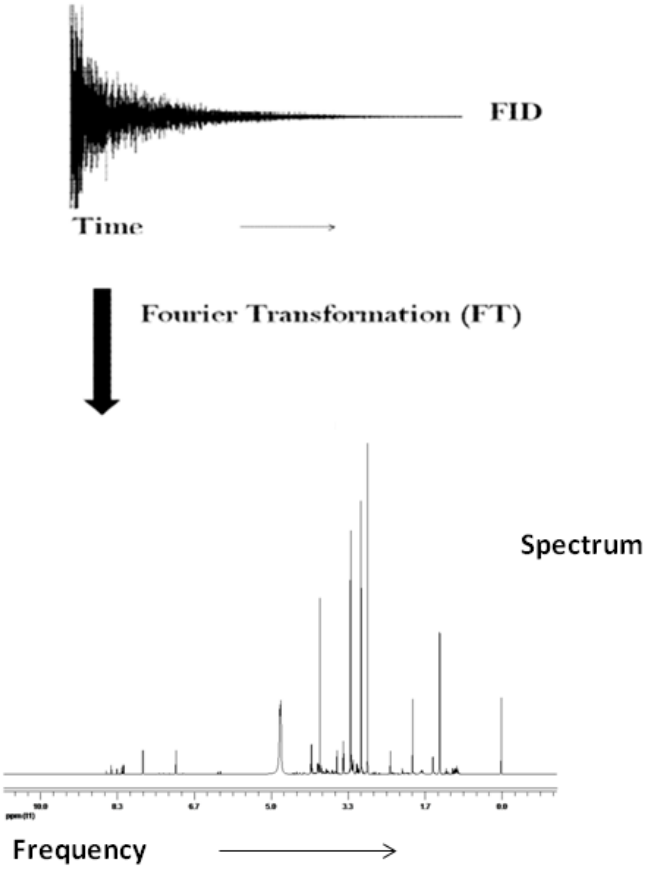

Figure 7. Fourier transformation of a FID obtained from a fish muscle (Bogue, *Boops boops*) sample's extract. The FT process takes the time domain function (the FID) and converts it into a frequency domain function (the spectrum). Free induction decay (FID) was Fourier transformed, with the MestReC Software (http://www.mestrec.com/), by performing an exponential multiplication with a 0.5 Hz line broadening.

In NMR spectroscopy, a Fourier transformation (FT) is required to convert the time domain data (free induction decay or FID, an electrical signal oscillating at the NMR frequency), obtained from the spectrometer, to the frequency domain (NMR spectrum). Naturally, quantitative methods require that parameter settings for the Fourier transform (choice of zero-filling and apodization function) are equal for all samples to be evaluated, since they may influence the finer details in the spectra.

- **Phase errors** [19] (Figure 8).

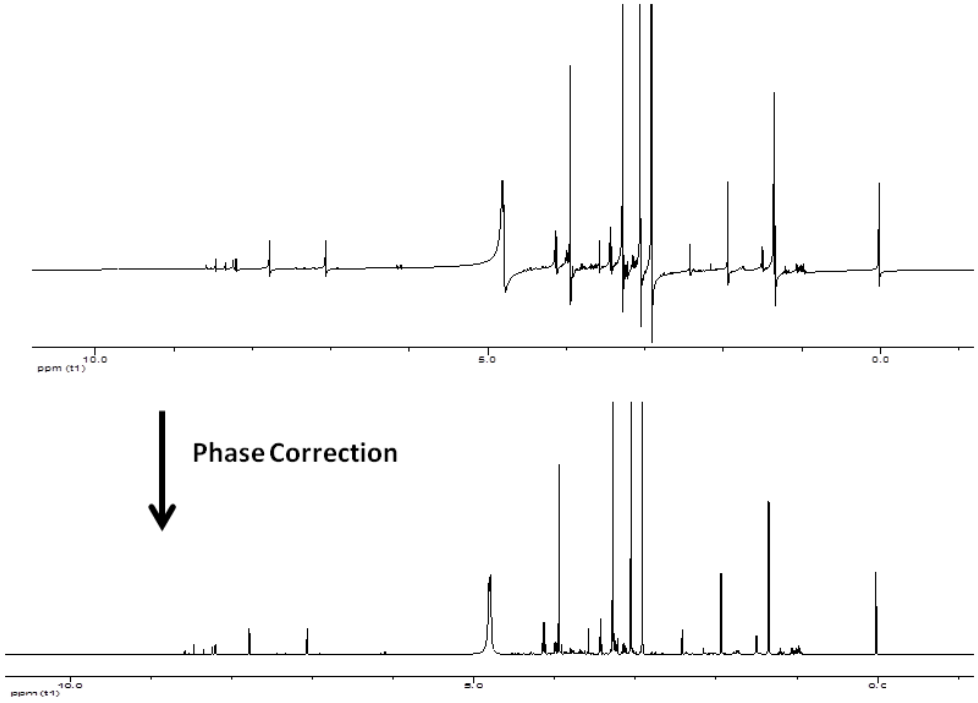

Figure 8. Phase Correction of a spectrum obtained from a fish muscle (Bogue, *Bops boops*) sample's extract. The phase error correction was made with the MestReC Software by performing a manual correction on both zero and first errors orders (http://www.mestrec.com/)

A difficult problem encountered with NMR data is the existence of phase errors of two orders: one and zero. In the real experiment, after FT, the spectrum line shapes are a mixture of absorption and dispersion signals. They are related to the delayed FID acquisition that is commonly called first order phase. The delayed acquisition is a consequence of the minimum time required to change the spectrometer from transmit to receive mode. During this delay, the magnetization vectors process according to their chemical shift frequencies. The zero order phase error arises because of the phase differences between the magnetization vectors and the receiver. Manual phase correction is usually implemented in the instrument software, but this process is very time consuming, especially for the large data sets that are often analyzed using chemometrics. More importantly, manually phase-correcting a series of spectra will lead to suboptimal results due to the subjective evaluation of the correction necessary for individual spectra.

- **Data normalization** (Figure 9).

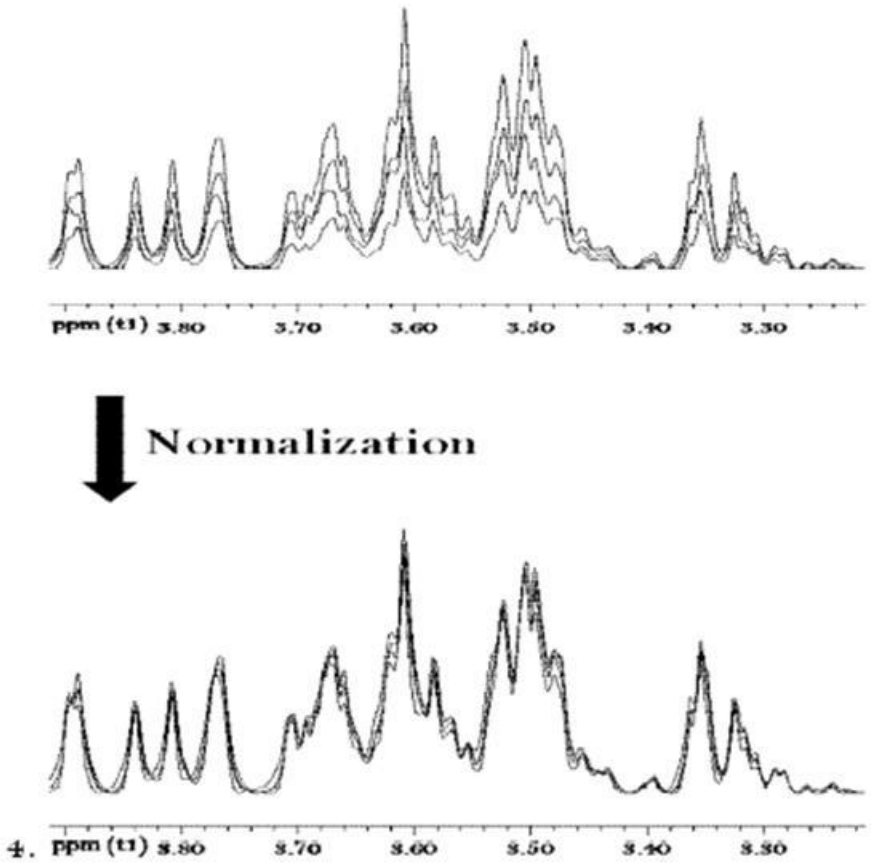

Figure 9. normalization of a set of spectra obtained from a fish muscle (Bogue, *Boops boops*) samples' extract. In this Figure is shown part of the midfield 1H NM region from 4.0 to 3.3 ppm). The normalization was made with the MestReC Software (http://www.mestrec.com/)

Data normalization is an important step for any statistical analysis. The objective of data normalization is to allow meaningful comparisons of samples within the dataset. It is a row operation that is applied to the data from each sample and comprises methods to make the data from all samples directly comparable with each other [20]. In this way it is possible to minimize most of the differences introduced with the effect of variable dilution and spectral data acquisition and processing. Normalization can be done using an internal "housekeeping" metabolite for example, an inner standard like TMS or, in this case, normalize each spectrum to (divide each variable by) the sum of the absolute value of all variables for the given sample. It returns a vector with an unit area (area = 1) "under the curve" [21]. One of its common applications is to remove or to minimize the effects of variable dilution of the samples [10].

- **Chemical shift variations**

The last pre-processing problem to be mentioned here, and which occurs only in HR-NMR spectroscopy, is the chemical shift variations that may occur from sample to sample or even from

peak to peak. The overall sample-to-sample variations are due to small variations in spectrometer frequency, while the peak to peak chemical shift variations are due to variations in, for example, pH. In this last case, a data reduction in the form called binning [20] is a pragmatic solution to the problem.

9.2 CAPILLARY ZONE ELECTROPHORESIS (CZE)

Capillary electrophoresis is a physical method of analysis based in the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field" [22].

Against Capillary Electrophoresis, the CZE has a more widespread mode, it has been used for analytes as diverse as sodium ions, drugs, and protein molecule. Analyte species can be separated by CZE if they migrate at different velocities in the electric field.

The base principle in CZE is that analytes are separated in a capillary containing only buffer without any anti-convective medium. The migration of different components of the samples as discrete bands with different velocities makes the separation possible. Each band has an own velocity depending on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary. One way to increase the separation capacity is by using coated capillary with fused-silica surfaces.

By using this criteria is possible identify molecules in a range between 2000<Mr<100 000.

Through this technique it is possible to separate chiral compounds by addiction in the separation buffer of chiral selectors.

In case of these techniques the main optimization factors to be considered are the following:

- Voltage: Separation time is inversely proportional to applied voltage. An increase in the voltage can cause a heat increase, and consequently the higher temperature causes band broadening and decreases the resolution.
- Polarity: Electro-osmotic flow moves toward the cathode if the electrode polarity is normal (anode at the inlet and cathode at the outlet). If the electrode polarity is reversed, only the electro-osmotic flow will pass to the outlet.
- Temperature: Changes in temperature can affect buffer viscosity and electrical conductivity and therefore the migration velocity.
- Capillary: Both length and internal diameter of capillary influence the analysis time, efficiency of separations and load capacity. An increasing of both can decrease the electric fields which increase migration time.

9.3 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Size exclusion chromatography is also known as gel filtration, gel permeation or molecular sieve chromatography. Molecules in solution are separated by their size and in some cases molecular weight by this method. The pioneers of this techniques were Grant Henry Lathe and Colin R. Ruthven who started this method for separation of analytes of different size with starch gels as the matrix, while later, Jerker Porath and Per Flodin introduced dextran gels. [23]

In general, depending on the different transport solutions, it is possible to distinguish between gel-filtration chromatography (aqueous system) and gel permeation chromatography (organic system). Usually SEC is used to analyze complex hydrocarbon mixtures. “In the absence of methods able to access the entire mass range of the sample, SEC allows estimating, rather than determining, mass ranges of sample above the GC range, about 300-350 u for aromatic compounds and about 500 u for aliphatic compounds.” [24]

One of the main advantages of this methods is the good separation of large molecules from smaller one using little volume of eluate.

Other advantages regard time separations. These are short, well defined, there is a good sensitivity and since there is no interaction of samples with the stationary phase, there is no sample loss. While only a limited number of bands can be accommodated, being the chromatogram time scale short, to obtain a good resolution the difference in molecular mass should be at least of 10%.

A critical point in SEC theory regards the choice of proper molecular size parameters by which molecules are separated. To solve this calibration problem, Benoit and colleagues found in 1967 an algorithm able to gain good correlation between elution volume and a demonically molecular size, called hydrodynamic volume that became the principle base of universal SEC calibration [25].

Otherwise, in general, samples that are analyzed with SEC are not constituted with an homogeneous molecular size, therefore these elution curves have a distribution similar to the Gaussian one. Retention time may be influenced by undesirable interaction of stationary phase with particle, and to minimize this problem it is important to check that the column is manufactured using inert stationary phase, in order not to compromise the analysis. Such as for other chromatography techniques, also for SEC the increase of length column allows better resolution and the increase of column diameter allows better column capacity.

In general, SEC has found a wide use in the purification step, because it is not able to discern very well between similar species and for this reason it is considered a low resolution chromatography. SEC can determine the tertiary and quaternary structures of purified proteins, if their time

exchange is slow. Other use regard synthesised polymers in which is possible determine the size and polydispersity. For quantitative measurements, before sample analysis it is necessary to run the standard molecules of interest for the construction of a calibration curve. In this way, it is possible to determine the size. Furthermore, if in the sample there are two or more molecules with identical molecular weights, the absolute determination method is more desirable [26].

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10. IN VITRO MODEL

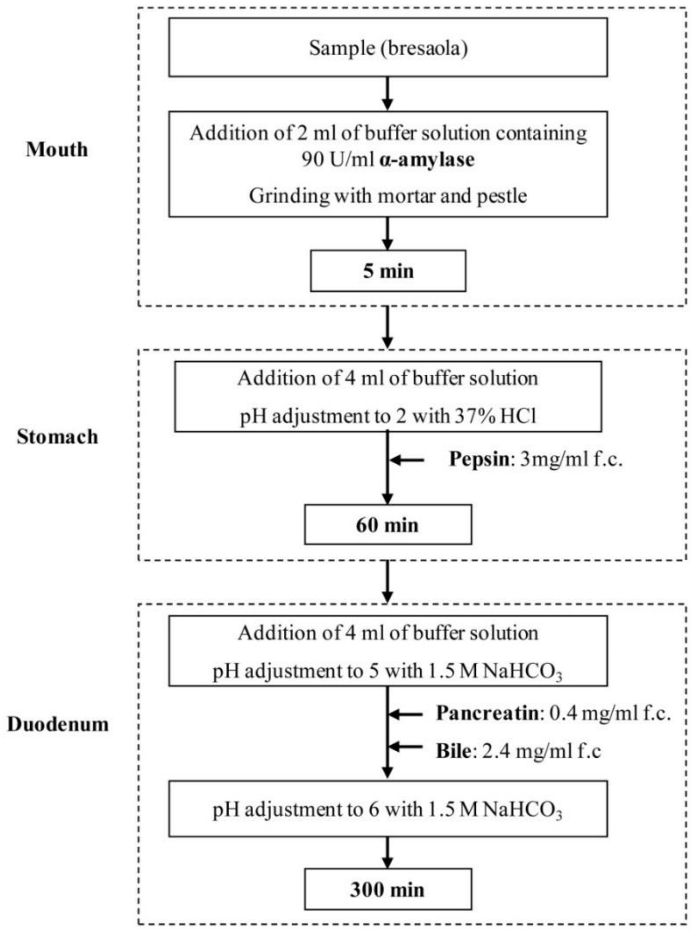

Figure 10. Within the Bordoni's model the human digestion was simulated in vitro inside a 100 ml flask kept at 37° C by means of a water bath on a magnetic stirrer equipped with a heating plate.

Within the Bordoni's [1] model the human digestion was simulated in vitro inside a 100 ml flask kept at 37° C by means of a water bath on a magnetic stirrer equipped with a heating plate. The digestive fluid, pH, enzymes were adjusted to better mimic the physiological condition in mouth, stomach and small intestine. In particular a buffer solution (120 mM NaCl, 5 mM KCl, 6mM $CaCl_2$ – pH 6.9) was added in proper volumes in every step. For 1 gram dry matter of cheese, 2:4:4 ml of buffer was added to resemble saliva, gastric juice and intestinal juice, respectively. To simulate mastication and oral digestion 15 grams of cheese (10 grams of dry matter) was chopped and subjected to amylase digestion for 5 minutes adding 20 ml of buffer solution with 90 U ml-1 alpha amylase. Then, 40 ml of buffer solution was added and the pH was decrease to 2.0 by drop wise addition of 375 HCL. The adding of pepsin to a final concentrations made started the gastric phase. After 60 minutes the gastric phase was stopped by adding 40 ml of buffer solution and the pH was

increase to 5 with 1.5 M NaHCO3. The last digestion phase was started by adding the pancreatin in the flask, (0.4 mg ml-1 final concentration). In order to mimic the physiological condition, lipid hydrolysis was guaranteed by pancreatic lipase, present in pancreatin, together with amylase, trypsin ribonuclease and protease.

The second in vitro model that has been used in this experimental work was that one developed by the Infogest Cost Network. [2]

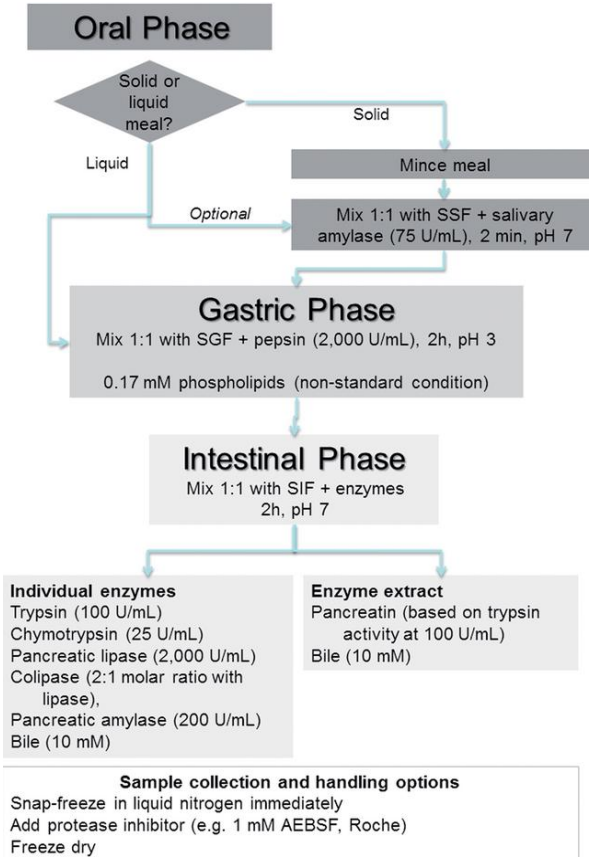

Figure 11. Within the Cost Network Model the human digestion was simulated in vitro inside a 100 ml flask kept at 37° C by means of a water bath on a magnetic stirrer equipped with a heating plate.

In the oral phase, five grams of sample were minced with Eddingstons Mincer Pro and put in a Falcon tube. To these simulated salivary fluid (SSF), human salivary α-amylase (75 U/ml in the final mixtures) following by CaCl2 (0.75mM in the final mixtures) were added to achieve a final ratio of 50:50 (w/v). For the gastric phase, five parts of oral bolus was mixed with 4 parts of Simulated Gastric Fluid (SGF) to obtain a final ratio of 50:50 (v/v). Porcine pepsin was added to achieve 2000 u/ml in the final digestion mixtures following by CaCl2 to achieve 0.075mM in the final digestion mixtures. 1 M of HCl was added to reduce the pH to 3.0. The gastric phase was digested for 2 h at

37 °C.

Five parts of gastric chime was mixed with 4 parts of Simulated Intestinal Fluid (SIF) to obtain a final ratio of 50:50 (v/v). 1 M NaOH was added to neutralized the mixture to pH to 7. Pancreatine from porcine pancreas was used to achieve 100 u/ml in the final digestion mixtures followed by the bile salt to give a final concentration of 10 mM and CaCl2 to achieve 0.3 mM in the final digestion mixture. The pH was re-adjusted to 7. The intestinal phase was digested for 2 h at 37 °C. At the end of the intestinal phase, Pefabloc (1mM in the final volume) was used to stop the pancreatin activity.

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11. RESEARCH OBJECTIVES

The purpose of this research thesis was to develop, for the first time, a system of evaluation of the in vitro digestibility of protein-rich foods, through the analysis of digestive fluids, based on NMR spectroscopy.

It was a new approach and, therefore, it was necessary to consider a long preparatory phase to establish the most suitable experimental design and to optimize the flow of operations so as not to introduce artefacts. The method to be developed had to be simple and rapid, using reagents compatible with the environment, and replicable in other laboratories.

Among the different operations to be optimized, it was important to determine how to measure the quantity of digested product by a technique, such as the high-resolution MMR spectroscopy, which does not directly measure the disappearance of food from the heterogeneous phase in its original form, because NMR only observes molecules in solution.

Then, the most critical step was standardizing the quantity of product to be digested, which is not actually visible to the spectroscope as it is, to be put in relation with the amount of molecules so far released by its digestion.

In addition, to develop the digestion system, it was necessary to make the digestion protocol suitable for NMR recording, by using procedures, which stop the process at the proper moment, that do not interfere with the detection technique. This last detail occurs when digestion is blocked with enzyme inhibitors that show interference in the spectrum.

Furthermore, it was important to recognize the spectral features that are diagnostic of the digestion phenomenon, in the series of spectra acquired during the different phases of digestion. For example, the region around 4 ppm of the spectrum is useful for measuring the amount of amino acids, free and/or linked to soluble peptides, released during the gastric and duodenal phases.

The research activity has had the purpose, for each selected food, to adapt the harmonized protocol digestion, released from the COST INFOGEST network of scientists, and in particular the simulated chewing conditions, the volumes of food and digestion fluids, the concentration of the buffers and the duration of digestion.

As far as the spectra processing concerns, the accuracy of the spectrum had to be verified and the correspondence of spectra to a quality standard evaluated. This point was made difficult by the fact that currently there are not recognized standards, as the method is still in the pioneering

phase. Nevertheless, one of the simplest and preliminary step, consisting of removing noisy regions, had to be applied to improve the quality of the information represented in the NMR spectra.

Maximizing the overlap between the spectra of different samples, at the same digestive stage, was the purpose of choosing proper calibration algorithms, in order to optimize the operation of comparison between products, and preliminary to the integration of the areas of the diagnostic signals.

At last, the development of a comparison algorithm for determining the technological effect of food processing on the digestibility was the final goal of this thesis, together with a molecular description to explain the different digestibility observed among analogue food products.

12. MATERIAL AND METHODS

Equipment

12.1.1 Laboratories supplies

- Pipettes Standard – Gilson's Pipetman® P (P10, P20, P100, P200, P1000, P5000 and P10000. Range of volumes from 5 µl to 10000 µl) with suitable tips (Diamond ® precision tip);
- Eppendorf ® safe lock microcentrifuge tubes volumes 0.5, 1.5 and 2 ml;
- Bottle in polypropylene (PP) from 100-250-500 and 1000 ml,
- Laboratory ceramic mortar grinder with pestle;
- Laboratory glassware;
  - Beakers, low form, with spout (50-250-500 ml) by Simax;
  - Beute from 50 ml (SCHORR DURAN);
  - Cylinders from 50-1000 ml (PIREX);
  - Quartz cuvets from 3 ml (EXACTA)
  - Corning® Disposable Pasteur Pipettes, Bulk Pack, Non sterile (SIGMA ALDRICH®);
  - Graduated pipette from 5 to 20 ml (class A);
- Inox steel Spatulas;
- Inox steel Scalpels;
- Syringe filter in cellulose with diameter 0.20 µm;
- Pipette controller;
- AMPOL NMR sample tuber for use up to 700 MHz NMR (203, round bottom);

12.1.2 Safety and protection supplies

- Safety Eyewear Glasses;
- Natural Latex Powdered and Powder – Free Exam Gloves;
- White lab coat;

12.1.3 Reagents

- Trichloroacetic acid (CCl3COOH, 163.39 g/mol, TCA) 6.1 N, SIGMA ALDRICH;
- Potassium hydroxide (KOH, 56.11 g/mol), SIGMA ALDRICH;
- Sodium sulfate (Na2SO4, 142.04 g/mol) granular anhydrous, SIGMA ALDRICH;
- Potasssium dihydrogen phospate (KH2PO4, 136.09 g/mol), PANREAC;

- 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt, ((CH3)3SiCD2CD2CO2Na, 127.27 g/mol TSP), 98 atom % D, BRUKER
- Potasssium hydrogen phospate anhydrous (K2HPO4, 174.18 g/mol), PANREAC;
- Hydrochloric Acid (HCl, 36.01 g/mol) 37% purity, SIGMA ALDRICH;
- Milliq demineralized water, SIGMA-ALDRICH
- Deuterium oxide (D2O, 20.04 g/mol, 99.9% purity), BRUKER;
- Deuterated Chloroform ($CHCl_3$, 99.96% purity) SIGMA ALDRICH;
- Pancreatin (Sigma-Aldrich, P7545)
- Human salivary α-amylase (Sigma-Aldrich, 1031)
- Porcine pepsin (Sigma -Aldrich, P7012)
- Porcine trypsin (Sigma-Aldrich, T0303)
- Bile (Sigma-Aldrich, P8631)
- $CaCl_2(H_2O)_2$ (Merck 2382)
- NaOH (Merck 9141)
- HCl (J. T. Baker 6081)
- KCl (Merck 4936)
- KH_2PO_4 (J. T. Baker 0240
- $NaHCO_3$ (Merck 6329)
- $MgCl_2(H_2O)_6$ (Merck 5833)
- $(NH_4)_2CO_3$ (Sigma-Aldrich, 207861)
- Pefabloc SC (4-(2-Aminoetyl)benenesulfonyl fluoride) (Fluka, Sigma-Aldrich, Ref: 76307)
- Ultrapure type I water, generated by a Milli-Q system (referred in text as water)

12.1.4 Solution

- **KOH (25% w/w):** Carefully and with stirring dissolve 25g KOH in 75 ml distilled water;
- **Trichloroacetic acid (7.5%)**: Dissolve 7.5 g TCA in 92.5 ml distilled water;
- **Solution HCl (1 M)**: Dissolve 82,85 ml of HCl (37% or 12.07 M) in 917.15 ml distilled water;
- **500 mM Pefabloc**:
  - Weigh 119.85 mg of Pefabloc and dilute it in 1 mL of Milli-Q water.
- **0.3M $CaCl_2(H_2O)_2$**:
  - Weigh 2.205 g of $CaCl_2 \cdot 2H_2O$ and dilute it in 50 mL of Milli-Q water.
- **0.5M KCl**:

- Weigh 7.456 g of KCl and dilute it in 200 mL of Milli-Q water.

❖ **0.5M KH_2PO_4**:

- Weigh 6.804 g of KH_2PO_4 and dilute it in 100 mL of Milli-Q water.

❖ **1M $NaHCO_3$:**

- Weigh 16.802 g of $NaHCO_3$ and dilute it in 200 mL of Milli-Q water.

❖ **2M NaCl**:

- Weigh 23.378 g of NaCl and dilute it in 200 mL of Milli-Q water.

❖ **0.15M $MgCl_2(H_2O)_6$**:

- Weigh 1.525 g of $MgCl_2(H_2O)_6$ and dilute it in 50 mL of Milli-Q water.

❖ **0.5M $(NH_4)_2CO_3$** :

- Weigh 2.4025 g of $(NH_4)_2CO_3$ and dilute it in 50 mL of Milli-Q water.

❖ **1M NaOH and 1M HCl**: for pH adjustment of stock solutions of simulated digestion fluids

12.1.5 Buffer Solution

❖ Phosphate Buffer, 0.1 M and pH 7.00

12.1.6 Instruments

❖ Eletronic digital tecnica Balance (max 2200 g, d=0.01 g), SCALTEC (SBA 52) ;

❖ Eletronic digital analytical Balance (max 220g, d=0.0001g), SCALTEC (SBA 31) ;

❖ IKA ® ULTRA-TURRAX® homogenizer T18, basic, AC input 115 V;

❖ JENWAY Model 3310 pH Meter with glass bodied combination electrode swing arm electrode holder & ATC;

❖ Heating magnetic stirrer mod. ARE, VELP Scientifica®;

❖ Beckman Coulter TM Microfuge® 18 Microcentrifuge (max 14000 rpm adjustable in 500 increments)

❖ Spectrophotometer UV-Vis (UV-1601-Shimadzu);

❖ FT-NMR Avance Bruker AvIII (600 MHz) spectrometer Ultra Shield Plus equipped with:

- The electronic 3-channel RF consisting amplifiers from 100
- Watt 1H and broadband for X 300 Watt
- Control unit gradients GCU
- Control system of the temperature BVT3000
- Probe 5 mm with Z grad 1H-13C-15N

- Autosampler with 60 holders
- Software Topspin 3.0

Software

- ❖ NMR data processing

  Mestrec (www.mestrec.com), Magnetic Resonance Companion, "is a software package that offers state-of-the-art facilities for data processing, visualization, and analysis of high resolution nuclear magnetic resonance (NMR) data, combined with a robust, user-friendly graphical interface that fully exploits the power and flexibility of the Windows platform. The program provides a variety of conversion facilities for most NMR spectrometer formats and includes all the conventional rocessing, displaying, and plotting capabilities of an NMR program, as well as more advanced processing techniques". A pdf format tutorial designed to help to become familiar with Mestrec's features is available at http://nmr-aci.unihd.de/Anleitungen/mestrec/mestec.pdf

- Chemometrics data processing

  The multivariate statistical analysis were carried out using different statistical software due to thje different kind of analysis: R program, Matlab and Latentix were the software used for these porpoises.

  R (http://www.r-project.org) is a language and environment for statistical computing and graphics. The language provides a wide variety of statistical (linear and nonlinear modeling, classical tatistical tests, time-series analysis, classification, clustering, etc) and graphical techniques, and is highly extensible. One of its strengths is the ease with which well-designed publication-quality plots can be produced, including mathematical symbols and formulae where needed. R is available as Free Software under the terms of the Free Software Foundation's GNU General Public License in source code form.

  Matlab (http://www.mathworks.com) is a high-performance language for technical computing. The name stands for matrix laboratory for tecnica computing. The name stands for matrix laboratory and was originally written to provide easy access to matrix software. It integrates computation, visualization, and programming in an easy-to-use environment where problems and solutions are expressed in familiar mathematical notation; this allows to solve many technical computing problems, especially those with matrix and vector

formulations, in a fraction of the time it would take to write a program in a scalar non interactive language such as C or Fortran.

13. CASE STUDIES WITH EXPERIMENTAL DESIGNAND RESULTS

13.1 BRESAOLA DELLA VALTELLINA

In this work, the effect of pH on the solubility and in turn the bioaccessibility of carnosine was assessed in Bresaola, a beef-based product typical of Northern Italy (Valtellina), which is produced by curing the intact beef muscle. As this substrate is consumed crude, the evaluation of carnosine content is not affected (unlike what would occur with other beef products) by alterations due to the cooking process, which can cause a reduction of carnosine up to 50%, depending on the cooking procedure [1]. Human digestion of Bresaola was simulated using an *in vitro* static system [17]Bordoni et al. (2011), and the soluble free compound in the digestion fluid was evaluated by coupling ^{1}H Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR) and Capillary Zonal Electrophoresys (CZE). These techniques were operated at two different pH values to obtain a global description of the free fraction of the molecule, at two key points of digestion, which is the end of the gastric and duodenal phases.

This research work represents the first study on the estimation of the effects of pH on bioaccessibility of carnosine as modulated by its interactions with the meat matrix. This information is a preliminary key factor that should be determined for any bioactive compound food source, in order to assess its potential significance in human health.

The *in vitro* digestion model used in this study closely mirror the conditions and processes that actually occur in vivo and normally consider three main stages [9] [12]: (i) processing in the mouth (oral digestion), (ii) processing in the stomach (gastric digestion) and (iii) processing in the duodenum (duodenal digestion). *In vitro* systems are widely used to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions, and many recent studies indicate that they are common and useful tools for food analysis

13.1.1 Material and Methods

Salts and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO), except where indicated. The two types of commercial Bresaola, named X and Y, were bought in a local market.

13.1.2 Experimental design

Bresaola meat samples were prepared for carnosine quantification by grinding them with a common mincer. Five g of minced meat where then homogenized with 10 mL of distilled water for 4 min at 25K rpm using an Ultraturrax (…). After centrifugation for 30 min at 11500 rpm at 4 °C, the

supernatant was filtered through a 0.45 µm filter, boiled for 10 min, centrifuged for 20 min at 11500 rpm, 4 °C and filtered again using a 0.22 µm filter. For CZE analysis the filtered sample was diluted 1:20 with 100 mM phosphate buffer pH 2.5 (BioRad Laboratories, California). For ^{1}H-NMR spectroscopy 160 µL of 10 mM D_2O phosphate buffer, containing TSP 10 mM, were added to 1 mL of filtered sample and the pH was adjusted to 2.5.

13.1.3 Cured meat sample preparation for carnosine pH dependence evaluation

The pH dependence of carnosine in Bresaola meat was analysed by ^{1}H-NMR in samples prepared as described in the previous section, in which the pH was adjusted to the following values: 2, 2.5, 3.5, 5, 6, 7 and 8.

13.1.4 The in vitro digestion method

The samples of cured meat were digested in triplicate according to Bordoni et al., 2011, following the scheme reported in Figure 12.

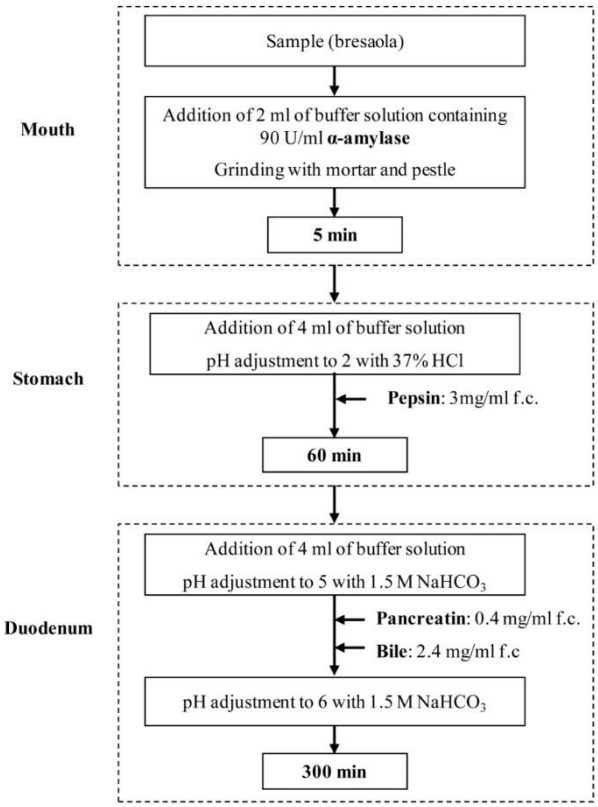

Figure 12.

Human digestion was simulated *in vitro* inside a 100 mL flask, kept at 37 °C in a water bath on a magnetic stirrer equipped with a heating plate. Chemical composition of the digestive fluid, pH and

residence periods were adjusted to mimic the physiological conditions. Three samples were collected during digestion: P1, at the beginning of gastric phase (after the decrease of pH to 2, before the addition of pepsin), and P2, P3 respectively after 180 and 300 min from the beginning of the duodenal phase. In samples P1 the pH was increased to 8 with 35 % NaOH to prevent possible modifications induced by acidic conditions. Samples P2 and P3 were acidified to pH 2 with 37 % HCl to stop pancreatic hydrolysis and to avoid bias caused by different pH values. P1, P2 and P3 samples were stored at -80 °C before CZE and ^{1}H-NMR experiments.

For CZE, 500 µl of samples P1-P3 were boiled for 10 min, immediately cooled and then centrifuged for 30 min at 10000 rpm and 4 °C; the supernatant was filtered through a 0.22 µm before analysis.

Samples P1-P3 were prepared for ^{1}H-NMR by adding to 1 mL of each sample 160 µL of 100 mM phosphate buffer in deuterium oxide (D_2O), containing 10 mM 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. After adjusting the pH to 7.00, the samples were centrifuged at 14000 rpm for 5 min in order to further remove impurities.

13.1.5 Carnosine quantification by CZE

CZE analysis and data processing were performed on a Biofocus 2000 from BioRad (BioRad Laboratories, California). Uncoated fused-silica capillaries with a diameter of 50 µm and effective length of 51 cm, were used for the analysis. The temperature during the analysis was maintained constant at 38 °C and samples were analyzed in a 110 mM phosphate buffer at pH 2.5 (BioRad Laboratories, California). The voltage was kept at 15 KV and the separated components were detected at 200 nm (UV), without derivatization. The quantification was performed in duplicate.

13.1.6 ^{1}H NMR evaluation of carnosine concentration and carnosine pH dependence

All ^{1}H-NMR spectra were recorded at 25 °C on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). The spectra were collected with a 90° pulse of 14 µsec with 10 W of power, a relaxation delay of 5 sec and an acquisition time of 2.28 sec. The spectra were registered by means of the first increment of a NOESY sequence, designed to suppress the HOD residual signal, while giving, for each proton, peaks proportional to the concentration of the substance they belong.

Each sample was analyzed once, as preliminary experiments showed that the error of the analytical method (1.2 % for CZE and 2.2 % for NMR) was lower than the error made in the replication of the *in vitro* digestion.

13.1.7 Statistical analysis

Data homoskedasticity and homogeneity of the variances were assessed by means of Shapiro-Wilks and Barlett tests respectively. Sample and pH effects on carnosine concentration were investigated through ANOVA test. Every calculation was performed by means of the correspondent packages implemented in R computational language. A trim value of 0.05 was considered in each case.

13.1.8 Results and discussion

- **Carnosine quantification in Bresaola meat by CZE and ^{1}H-NMR spectroscopy**

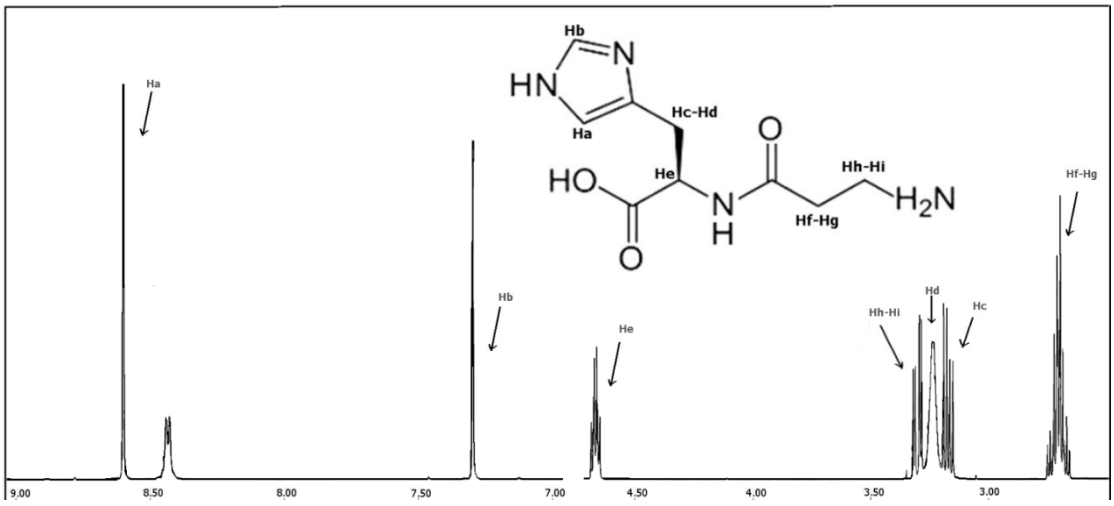

Figure 13. Spectrum of carnosine standard obtained by ^{1}H-NMR at pH 2.5, which shows the signals from L-histidine (Ha, Hb, Hc, Hd e He) and β-alanine (Hh, Hi, Hf, Hg) residues.

Carnosine [5 – 6] content was measured in two commercial Bresaola samples at pH 2.5 by both CZE and ^{1}H-NMR, using the purified compound from Sigma as reference standard. The selected pH optimizes carnosine resolution through CZE [2], and allows a direct comparison between data obtained on raw samples and gastric digestates. The content of carnosine in the former was estimated to be 628 mg/100 g and 671 mg/100 g in Bresaola X and Y respectively.

A typical ^{1}H-NMR spectrum of carnosine at pH 2.5 is reported in Figure 13. For quantification of carnosine, the signals at 7.30 and 8.60 ppm, corresponding to hydrogens H_a and H_b of the histidine residue, were considered, because singlets (thus granting the highest signal to noise ratio), and well resolved from peaks ascribable to other molecules. In all the registered spectra it was possible to observe a 1:2 ratio between the area of these signals and those from β-alanine appearing in the

range 2.65 - 2.80 ppm, corresponding to the two protons H_f and H_g. This convinced that the amount of free histidine in all the samples was negligible. [7 -8]. The amounts of carnosine measured by ^{1}H-NMR for Bresaola X and Y were calculated as 623.3 and 664.1 mg/100 g respectively, with a difference from the CZE values of 0.7 % and 1.0 % for the two samples, indicating a good agreement between the two techniques. The values of carnosine found in Bresaola are higher than those reported in literature for beef and other animal species, independently from tissue type. Beef generally contains the highest value of this metabolite with carnosine content ranging from 32.6 mg/100 g in heart to 452 mg/100 g in semitendinosus muscle [1], being generally higher in anaerobic, glycolitic, white muscle than in red, aerobic muscle [3]. The even higher carnosine content found in Bresaola is probably related to the dehydration process that raw meat undergoes during transformation to the final product, that increases the protein content from 20.5 to 32 g / 100g (http://www.inran.it/646/tabelle_di_composizione_degli_alimenti.html).

- **Effect of pH on carnosine quantification in Bresaola meat**

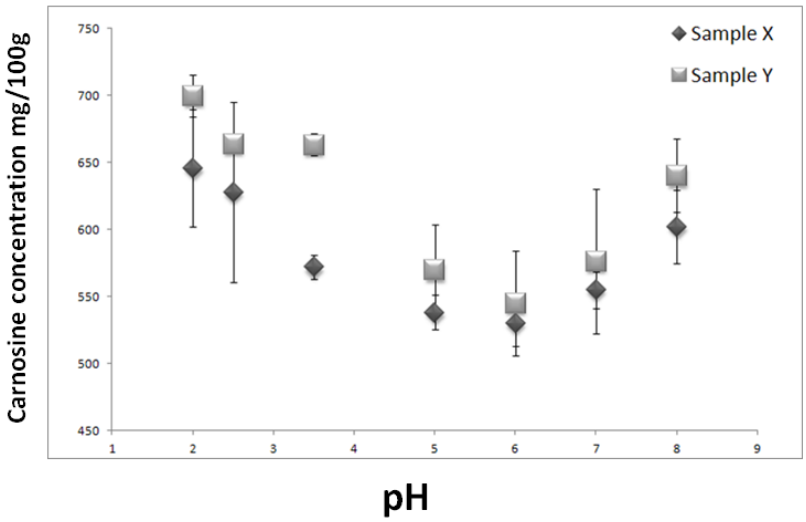

Figure 14. Carnosine concentration as assessed by ^{1}H-NMR in meat samples X (circles and dashed line) and Y (squares and full line) at different pH values. To help visualizing the values trends, smoothed curves were superimposed to the experimental points. The error bars indicate the standard deviation calculated on 3 replicates.

Main effects (values vs. pH) and interactions of two different sample of Bresaola were tested by two-way ANOVA. The LSD was used for mean separations and to compare individuals treatments. Significant values ($P<0.05$) were obtained between pH of: 2-5, 2-6, 2-7,2.5-6, instead ($P<0.1$) were

obtained between pH of 2.5-5, 6-8. The relationship between carnosine bioaccessibility and pH was investigated by means of its quantification with ^{1}H-NMR in the 2-8 pH range on both cured meat samples X and Y. The graph in Figure 14 shows that the amount of free carnosine detected was maximum below pH 2.5, and was characterized by a curvilinear relationship with pH, with a minimum around pH 5.3, corresponding to the meat proteins isoelectric point.

- **Carnosine bioaccessibility during in vitro Bresaola digestion**

A

X	CZE (pH 2.5) mg/100 g			^{1}H NMR (pH 7) mg/100 g			ΔS CZE/^{1}H NMR
	X1	620.30		X1	514.70		
P1	X2	601.00	ΔP1 = 600.73 ± 19.70	X2	574.66	ΔP1 = 543.82 ±30.02	10.50 %
	X3	580.90		X3	542.09		
	X1	638.94		X1	519.57		
P2	X2	658.76	ΔP2 = 654.43 ± 13.85	X2	584.29	ΔP2 = 557.81 ± 33.93	17.32 %
	X3	665.60		X3	569.58		
	X1	608.89		X1	524.70		
P3	X2	637.50	ΔP3 = 631.78 ± 20.64	X2	580.85	ΔP3 = 555.80 ± 28.56	13.67 %
	X3	648.96		X3	561.86		

B

Y	CZE (pH 2.5) mg/100 g			^{1}H NMR (pH 7) mg/100 g			ΔS CZE/^{1}H NMR
	Y1	595.32		Y1	519.30		
P1	Y2	599.02	ΔP1 = 607.18 ± 17.44	Y2	513.01	ΔP1 = 518.02 ± 4.50	17.21 %
	Y3	627.20		Y3	521.74		
	Y1	712.726		Y1	519.57		
P2	Y2	588.64	ΔP2 = 659.57 ± 63.92	Y2	584.29	ΔP2 = 542.02 ± 10.52	21.60 %
	Y3	677.34		Y3	569.58		
	Y1	681.68		Y1	524.7		
P3	Y2	575.12	ΔP3 = 640.74 ± 57.41	Y2	580.85	ΔP3 = 555.02 ± 24.03	15.32 %
	Y3	665.42		Y3	561.86		

Table 4. Carnosine concentration of Bresaola X (A) and Y (B), measured by CZE and ^{1}H-NMR, at the beginning of the gastric digestion (P1) and after 180 and 300 min from the beginning of the duodenal phase (P2 and P3). The values are expressed in mg/ 100 g of meat. ΔP1, ΔP2 and ΔP3 represent the average values of three replicates of digestion. ΔS CZE/^{1}HNMR is the percentage difference between the two analytical methods.

In order to study carnosine bioaccessibility during human digestion, its concentration was measured in the soluble fraction of samples collected at different phases of an *in vitro* static digestion process, where chemical composition of the digestive fluid, pH and residence periods were adjusted in series to simulate the physiological conditions in mouth, stomach and small intestine [4]. CZE and ^{1}H-NMR were used in parallel at pH 2.5 and 7 respectively, so that the former

gave information about the total carnosine released from the bolus mass undergoing digestion, while the latter allowed to focus on the carnosine fraction suitable for duodenal absorption, at pH 7. The values obtained from three different digestions for each sample are reported in Table 4 Carnosine concentration at P1 corresponds to the amount of compound brought into solution after the oral step, during which the meat is chewed in the presence of saliva. The amounts measured by CZE for Bresaola X and Y were the 17.21% and the 21.60% higher than those measured by ^{1}H-NMR. This was then the gap between total carnosine in solution at gastric pH and carnosine with suitable characteristics for duodenal digestion.

13.2 THE SECOND STUDY WITH INFOGEST'S PROTOCOL

13.2.1 Biological Material

Three samples, "Time 0" (after 10 days of salting), "Time 2" (after 2 weeks of ripening), "Time 4" (after 4 weeks of ripening), of Bresaola, each cured for different lengths of time were subjected to simulated gastric and intestinal digestion following the Infogest protocol reported in the paper (A standardised static *in vitro* digestion method suitable for food-an international consensus). The Bresaola samples (Time 0, Time 2 and Time 4 in duplicate) were sent from Cesena, University of Bologna to Teagasc institute. In total fourteen digestions were carried out, both with and without substrates as control. The enzymes assay and the samples digestions were carried out following the Infogest *in vitro* digestion protocol.

13.2.2 Material and Methods

- **Size-Exclusion HPLC/One dimensional electrophoresis**

The Molecular weight distribution profiles of 32 samples were estimated by size exclusion chromatography using a series connection column with TSK G 2000 SW (7.5 mm 60 cm , 10 m, 131 Japan) fitted to a TSK guard column (7.5 mm 7.5 cm). 20 µL of samples were injected at the concentration of 2.5 g L-1. And 30 % acetonitrile in 0.1%TFA Milli-Q water was used as the eluent at a flow rate of 0.5 mL/min. Absorbances were monitored at 214 nm. Seven standards were used bovine Blue Dextran (2000 kDa), Carbonic anydrase (29 kDa), β-lactoglobulin (18.276 kDa), Bacitracin (1.423kDa), Leu-Try-Met-Arg (604.77Da), Asp-Glu (262.22 Da), L-Leucine (131.22 Da), as MW calibration standards (log MW= -0.1508x + 8.61, with retention time, expressed in minutes, R2=0.9697). Samples were analyzed by sodium dodecyl sulfate-polyacrilamide gel electrophoresis

(SDS-PAGE) in order to determine the MW of the protein and/or peptide separated on gels. Amersham ECL™ precast gel (12%, GE Healthcare Bio-Sciences Corp, Piscataway, USA) were use on a Amersham ECL™ box according to the manufacturer's instructions. Sample volumes were optimized in order to get the best separation and quantification of proteins. Samples were prepared under reducing (with β-mercaptoethanol) condition. Protein was visualized by staining with the Coomasie blue (Bio-Safe Coomassie Stain G-250, Bio-Rad). An Amersham Low Molecolar Weight Calibration kit (14.4 to 97 kg/mol, GE Healthcare UK Limited, UK) was used as the molecular weight standard.

13.2.3 Results and Discussion

- **One dimensional electrophoresys (SDS-PAGE)**

At the end of each digestive phase all the samples were split in two aliquots. The first was immediately snap-frozen, while a 10 kDa cut off and a snap-frozing was performed on the second aliquot. In this way two different sample, one at high molecular weight (HMW) and the other one at low molecular weight (LMW) were obtained. A multi-analytical approach was used for the analysis of the samples, and in particular the HMW samples were performed with monodimensional electrophoresis and the LMW samples were performed with size exclusion HPLC. In order to separately focuse on high-, medium- low- weight proteins during digestions, SDS-PAGE analysis of samples at different steps was performed, and is reported below. According to the Infogest in vitro protocol, sampled proteins and peptones were those released in the aqueous /saline simulated intestinal fluid, as protein digestion progressed. Briefly, the first gel represent the Initial Bresaola, the second gel the gastric phase and the third the intestinal phase. The main difference that characterized the first gel depend on the inter variance of the samples. For example the band between 55 e 43 kDa is present in all samples except in time 0; two other bands between 43 and 34 kDa, troponin and fructose biphosphate aldolanse [14], are present only in time 2 and time 4. (The different color of the three sample is not depending on the dilution, but on a different destaining level). Following the simulated gastric digestion the bands from 72 to 43 kDa showed the substantial degradation of some sarcoplasmic protein bands. Simultaneously, additional polypeptides covering the 130-72 kDa range appeared, due to the release and partial breakdown of myosin heavy chain (MHC). Furthermore, the band at 43 kDa (that could be enolase) is more marked in time 2 and time 4 than time 0. As expected, all the bands present in the gel of substrates digestion with enzymes, belonging to the digestion and solubilization of HMW's proteins

and not to the enzymes (as a previous gel of control with gastric enzymes and without substrates showed). Indeed, in the other half of the gel the substrate's digestion without enzymes is showed. In time 0 the band between 43 e 34 kDa and those between 26 e 10 kDa are absent in time 2 and time 4. The fact that in the ripening products (time 2 and 4) the quantity of HMW's proteins are lower is probably due to the typical autolysis mechanics that has an effect on the soluble proteins. Whereas the others bands present both in time 0 and time 2 and 4 belonging to the autolysis resistant proteins, i.e myoglobin. Following the simulated intestinal digestion, the proteins bands are belonging to enzymes digestion (as a previous gel of control with intestinal enzymes and without substrates showed). As expected, after 2 hours of intestinal digestion all the HMW's proteins are digested. Thus, the simulated gastric phase digestion contributed to a massive release of myofibrillar proteins by the muscle tissue, making them to predominate the protein fraction at the final stage of simulated digestions. The comparison between the intestinal and gastric phases of the substrate digestion without enzyme gel, figured out a few difference in the bands presence and intensity, this could be due to the different proteins isoelectric point. In conclusion, the SDS-PAGE allowed identifying both the difference due to the three matrices and the difference due to the presence or not of enzymes. The further analysis on the same samples will allow exactly quantifying and qualifying not only the soluble hydrolyzed composition of protein but also which proteins were made soluble during the digestive phase.

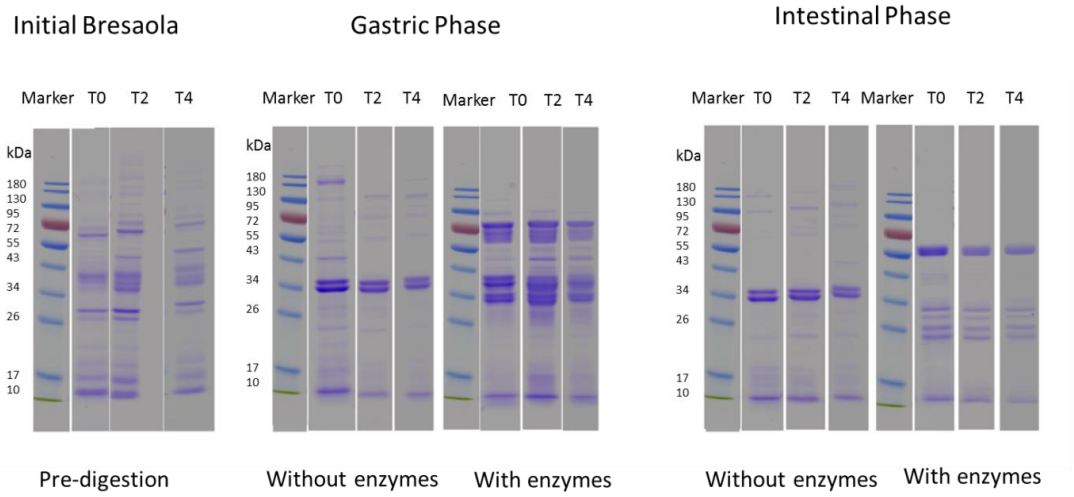

Figure 15. In conclusion the SDS-PAGE allowed to identify both the difference due to the three matrices and the difference due to the present or not of enzymes. The further analysis on the same samples will allow to exactly quantify and qualify not only the soluble hydrolyzed composition of protein but also that proteins that were made soluble during the digestive phase.

- **Size-exclusion high performance liquid chromatography (HPLC)**

As reported above, the 10 kDa cut off samples was analyzed by size-exclusion hplc . Through size-exclusion hplc technique was possible both quantify the percentage of low molecular weight (LMW) peptides (1500-100 kDa) and highlights how the proteolysis kinetic was linked by digestion. The chart representing the area's percentage of LMW peptides of time 0, time 2 and time 4 release during in the in vitro digestion, is showed below. The main difference here, is due to the massive presence of small peptides (<100 kDa) in the intestinal phase against in the gastric phase. Indeed, the orange area, corresponding to those peptides with a molecular weight lower than 100 kDa, is increased in the intestinal phase, to the detriment of red, green and purple areas, corresponding to peptides with a molecular weigh between 1500 - 250 kDa, that were higher in the gastric phase. No relevant differences were recorded between the three Bresaola samples. The chart representing the area's percentage of LMW peptides of time 0, time 2 and time 4 release during in the in vitro digestion of substrates without enzymes, are showed below. The main differences were recorded between the three Bresaola samples (time 0, time 2, time 4) and were to be ascribed to the ripening process. In particular, with the increases of ripening time, the amount of small peptides, grows. For example in time 0 the orange area is less abundant of that one in time 2, the same phenomena is present from time 2 to time 4. This, is due both to the natural presence, in meet products of microorganism that affecting the meet with degradation's mechanism and to the autolysis mechanics that has an effect on the soluble proteins. Only minor differences were recorded inside the same Bresaola time of ripening, from gastric phase and intestinal phase (i.e from GF0 to IF0 …) due to a different isoelectric protein point (pH condition and salts presence).

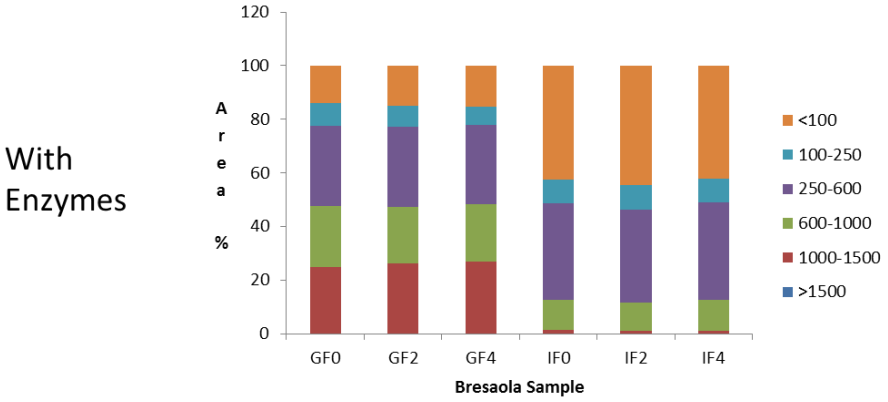

Figure 16. SDS-PAGE allowed to identify both the difference due to the three matrices and the difference due to the present or not of enzymes. The further analysis on the same samples will allow to exactly quantify and qualify not only the soluble hydrolyzed composition of protein but also which proteins were made soluble during the digestive phase.

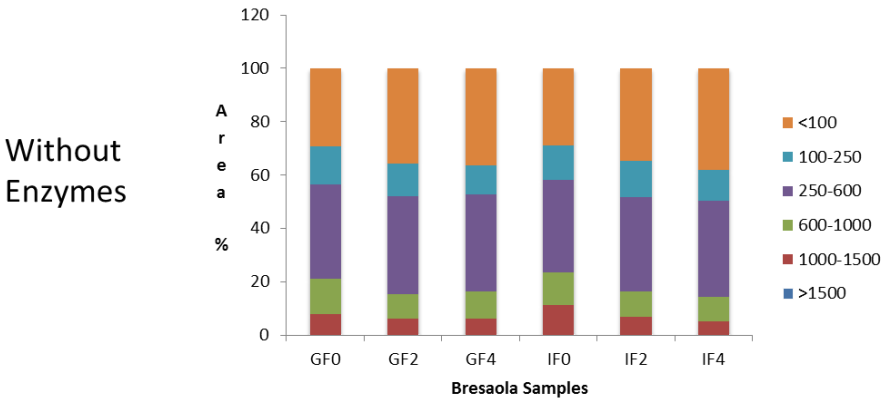

Figure 17. The SDS-PAGE allowed identifying both the difference due to the three matrices and the difference due to the present or not of enzymes. Further analysis on the same samples will allow exactly quantifying and qualifying not only the soluble hydrolyzed composition of protein but also those proteins that were made soluble during the digestive phase.

13.3 RICOTTA CHEESE

Thanks to a static in vitro system developed in the Campus of food science of the University of Bologna, the research group has performed the study and analysis of dairy products digestibility. Within the NMR experiment the bioaccessibility of nutrient and molecules release can be studied during the principal endpoints (mouths, stomach and small intestine) of a simulate digestion [12]. Through the NMR spectroscopy, the molecular profile of soluble aqueous extract was obtained. The molecular profile is constitute by a graph (spectrum) where, in the horizontal axis, the resonance frequency of hydrogen atom, present in the molecule constituting the sample, is expressed. Instead in the vertical is reported the intensity of respective signals, proportional to the atom concentration. Every region of the spectrum is characterized for a functional group of molecule.

This research study was carried out with the aim of compare he digestibility of two kind of spreadable cheese (produced by an important Italian food company), one commercial and the other a prototype (light formulation). Each analysis was performed via NMR; in particular, the three endpoints were collected: oral phase, gastric phase, intestinal phase. The conventional (commercial) samples have been identified with the label C1, C2, while the prototype in the light formulation with the label C3, C4. The samples collected during the entire digestion have been indicated as P1 oral phase, P2 starting of gastric phase; P3 end of gastric phase, P4 after 1 hour from intestinal phase, P5 at the end of intestinal phase. Only the P1, P3, P5 and an aliquot of P5

with 3kDa cut off are used for NMR analysis.

13.3.1 Materials and Methods

Salts and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO), except where indicated. The two types of Ricotta cheese, named C1, C2 and C3, C4 were obtained from an important Italian food company.

13.3.2 Experimental design in vitro digestion method

The samples of Ricotta cheese were digested in duplicate according to [4], following the scheme reported in Figure 18.

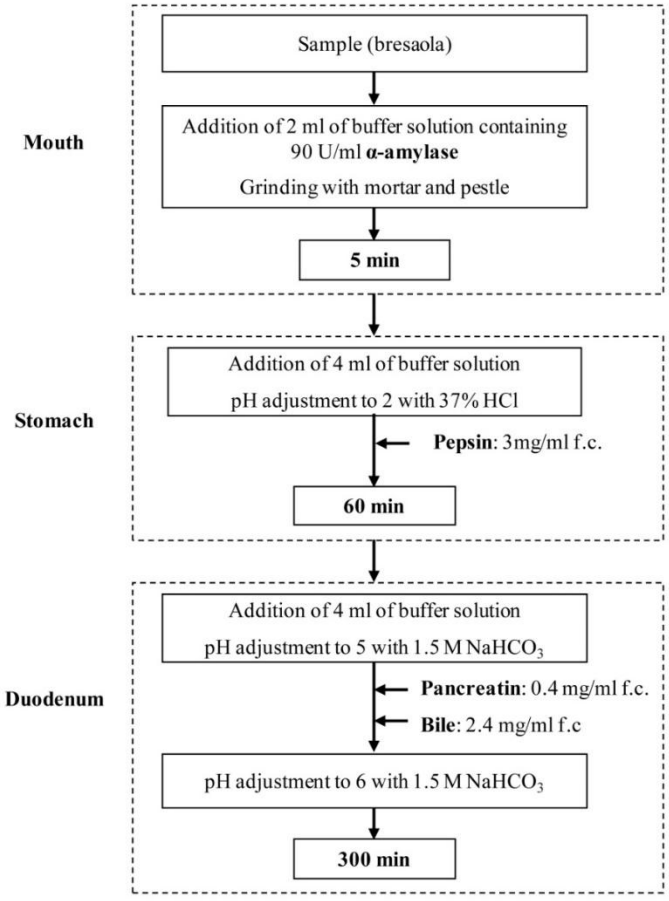

Figure 18. In conclusion the SDS-PAGE allowed to identify both the difference due to the three matrices and the difference due to the present or not of enzymes. The further analysis on the same samples will allow to exactly quantify and qualify not only the soluble hydrolyzed composition of protein but also that proteins that were made soluble during the digestive phase.

Human digestion was simulated *in vitro* inside a 100 mL flask, kept at 37 °C in a water bath on a

magnetic stirrer equipped with a heating plate. Chemical composition of the digestive fluid, pH and residence periods were adjusted to mimic the physiological conditions. Three samples were collected during digestion: P1, at the beginning of gastric phase (after the decrease of pH to 2, before the addition of pepsin), and P3, P5 respectively at the end of gastric phase and after 300 min from the beginning of the duodenal phase. In samples P1 the pH was increased to 8 with 35 % NaOH to prevent possible modifications induced by acidic conditions. Samples P2 and P3 were acidified to pH 2 with 37 % HCl to stop pancreatic hydrolysis and to avoid bias caused by different pH values. P1, P2 and P3 samples were stored at -80 °C ^{1}H-NMR experiments.

Samples P1-P3-P5-P5<3kDa were prepared for ^{1}H-NMR by adding to 800 µL of each sample 800 µL of deuterated chloroform ($CHCl_3$)[13]. The sample was mixed for 5 minutes with vortex and centrifuged at 14000 rpm for 15 minutes. Then 700 µL of surnatant were adding on a 1.5 mL eppendorf with 70 µL of phosphate buffer in deuterium oxide (D_2O), containing 10 mM 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. After adjusting the pH to 7.00, the samples were centrifuged at 14000 rpm for 5 min in order to further remove impurities and put in NMR tube.

13.3.3 ^{1}H NMR evaluation proteins kinetics during in vitro digestion

All ^{1}H-NMR spectra were recorded at 25 °C on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). The spectra were collected with a 90° pulse of 14 µsec with 10 W of power, a relaxation delay of 5 sec and an acquisition time of 2.28 sec. The spectra were registered by means of the first increment of a NOESY sequence, designed to suppress the HOD residual signal, while giving, for each proton, peaks proportional to the concentration of the substance they belong.

13.3.4 Results and discussion

Below are shown 4 parts of molecular profile of four sample of Ricotta cheese (C1-4) in P1 phase. Spectra showed different signal present in 4 region of the spectra. In particular, the signals that resonate in the region between 0.7 e 1.1 ppm (aliphatic methyl), 1.6 e 2.5 ppm (aliphatic methyl) 3.5 e 4.0 ppm (sugars) and 6.7 e 7.5 ppm (aromatic proton).

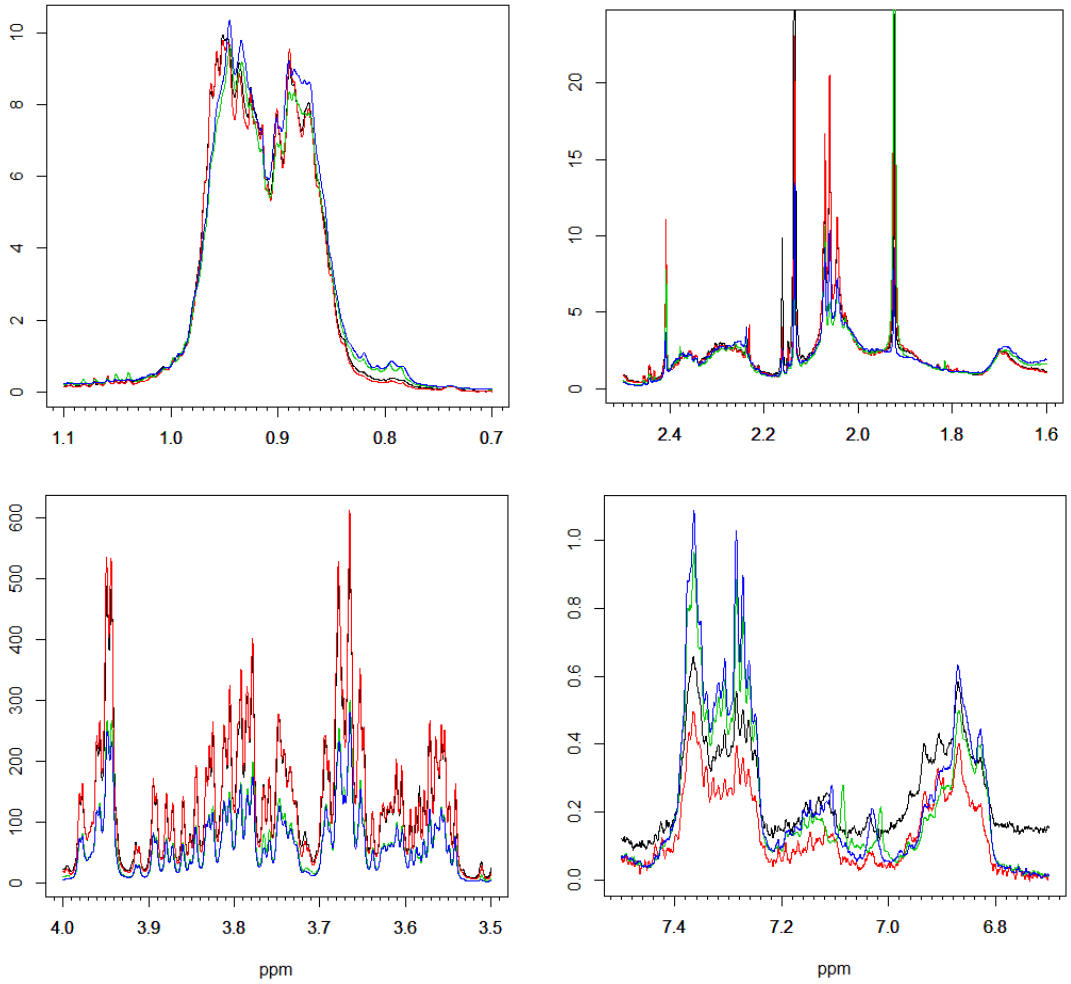

Figure 19. Overlapping of 4 Ricotta samples of the 2different typologies (convenctional: C1 and C2 green and blu; light: C3 and C4 black and red). The different region are shown with different vertical size.

The first pre-processing step accounts for the effect of dilution factors. Although the digestion process had been the same for all samples, it has been shown, by the match of the signals in specific areas, that the concentration can change for two reasons:

1) different content in dry matter. The two types of Ricotta cheese were produced by the same food company, but following two different protocols (light and conventional) and due to this different water content was determining a different concentration in soluble molecules.
2) collecting point in different digestion phases.

For these reasons is important find an algorithm able to account variations of the overall concentrations of samples caused by different dilutions. This second critical pre-processing step is called normalization.

The best normalization algorithm that was used in this work was the Probabilistic Quotient Normalization (PQN) by which was obtained the best overlap of the spectra in P1, when the proteins digestion was not started.

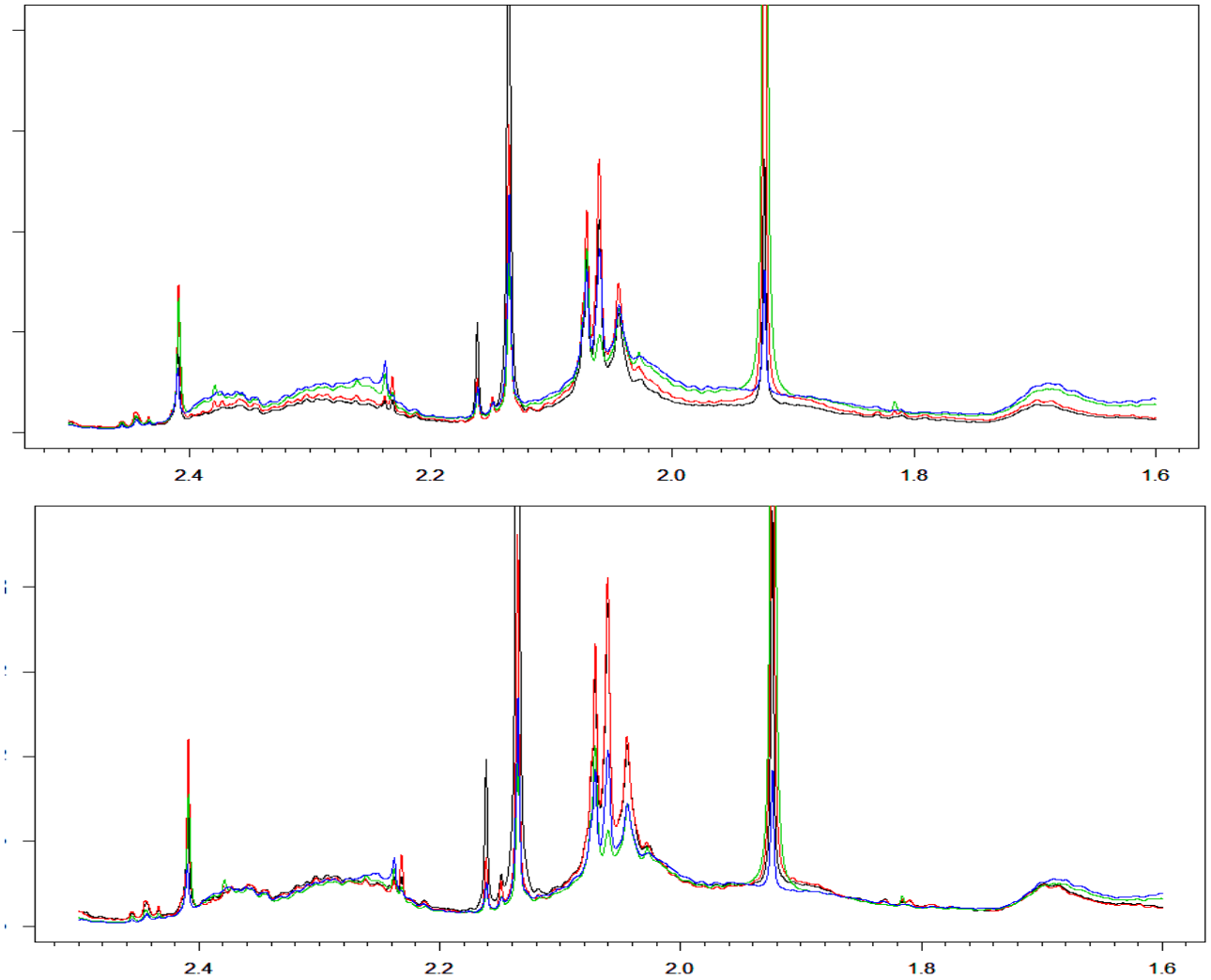

Figure 20. Spectra region between 1.6-2.5 ppm of C1-4 samples, before and after normalization process, at the digestive step P1.

As the quantity of soluble substance changes during the digestive phases, this kind of normalization cannot be chosen in all digestive steps. Thus, lactose anomeric signals (5.22, 5.26 ppm) was used as constant content molecule, after we had checked the absence of lactase enzymes. Indeed in P1, the signal of lactose was found remaining stable in all the four spectra. For this reason, the area under the lactose signals was measured and used to normalize all NMR spectra in all digestions phases.

Below normalization results obtained on lactose signal, are shown.

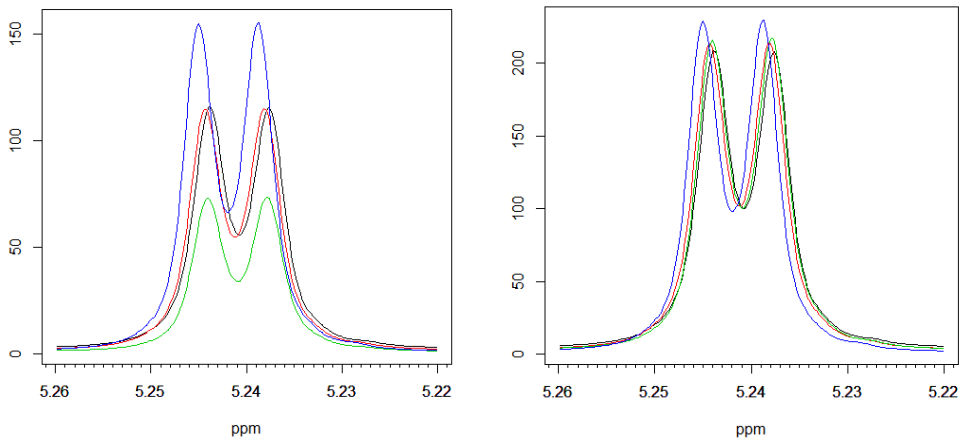

Figure 21. Before and after normalization step in lactose signals in Ricotta C1 (conventional).

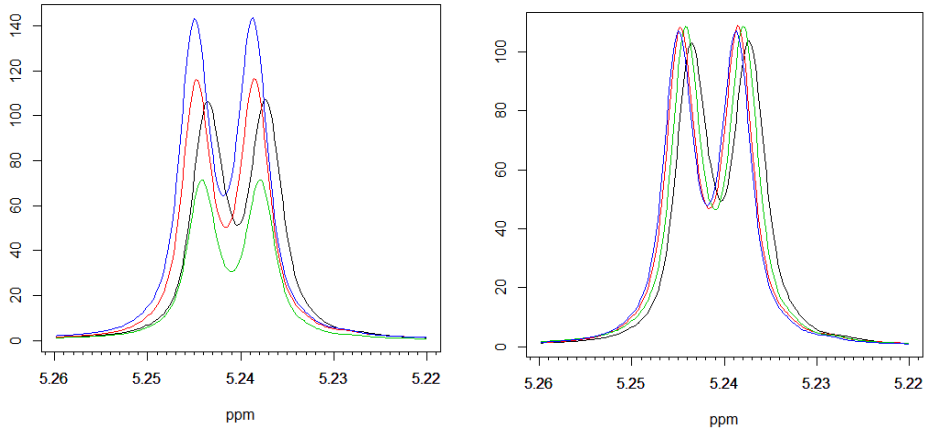

Figure 22. Before and after normalization step in lactose signals in Ricotta C3 (light).

After has been applied the normalization protocol to all samples and to all digestive phases, have been measured the areas of two interested spectra regions. All the values collected were subjected to ANOVA and LSD test. The regions that were taken into account were:

- Aliphatic area, between 0,85 a 1.05 ppm, where are recorded molecules such as branched-chain amino acid in free amino acid, di- and oligo-pepdides, as well as short chain fatty acids;
- Aromatic area between 6.68 to 6.92, where are recorded molecules with low molecular weight such as aromatic amino acid (phenylalanine, tryptophan, ect.)

Taking into account the increase in both two regions, aromatic and aliphatic, the quantity of low molecular weight molecules that had been released during the digestion process has been determined, and the existing differences between the two Ricotta samples have been verified.

The results obtained from ANOVA analysis showed a meaningful difference between light and conventional Ricotta cheese. In particular conventional products released the double quantity of

soluble molecules during in vitro digestions, and these result is clear both in the aliphatic and aromatic regions.

	Aromatic Region Conventional	Aromati Region Light	Aliphatic Region Conventional	Aliphatic Region Light
P1	852,93±341,9 ^{a,d}	1004,52±74,61 ^{b,d}	5640,16±123,22 ^{a,d}	5859,72±312,35 ^{b,d}
P2	16394,95±1869,01 ^{a,c}	8990,29±869 ^{b,c}	60714,92±4461,98 ^{a,c}	39086,36±8391,93 ^{b,c}
P5	27834,16±3295,53 ^{a,b}	12907,6±1056,86 ^{b,b}	105254,9±8209,17 ^{a,b}	52232,77±5911,39 ^{b,b}
P5_3kDa	20892,88±1821,65 ^{a,a}	9772,45±13,72 ^{b,a}	88312,84±1656,34 ^{a,a}	42709,26±3268,37 ^{b,a}

Table 6. Incremental area of NMR signals in different regions of the spectra during the three different steps of the digestion of Ricotta cheese, both conventional and light products. In addition, the area of signals after ultrafiltration (<3KDa) has been reported to show the proportion of low molecular weight metabolites at the end of the duodenal digestion.

13.4 SPREADABLE CHEESE

The study and analysis of dairy products digestibility was made possible by using the static in vitro system developed by the nutritional group of the Campus of food science by the university of Bologna. The NMR spectroscopy allows the study of the bioaccessibility of nutrient and molecules release during the principal endpoints (mouths, stomach and small intestine) of a simulate digestion. NMR spectroscopy obtained the molecular profile of soluble aqueous extract. The molecular profile is constitute by a graph (spectrum), where the resonance frequency of hydrogen atom, present in the molecule of the sample, constitutes the horizontal axis. The intensity of the respective signals, proportional to the atom concentration, is reported in the vertical axix. Every region of the spectrum is characteristic for each functional group in each molecule.

This research study was carried out with the aim of compare he digestibility of two kind of spreadable cheese (produced by an important Italian food company), one commercial and the other a prototype (light formulation). Each sample was analysed by NMR. In particular, the three endpoints were collected: oral phase, gastric phase, intestinal phase.

In particular, the conventional (commercial) samples have been identified by the label C1, C2, while the prototype with light formulation by the label C3, C4.

The samples collected during the entire digestion have been indicated as P1 oral phase, P2 starting of gastric phase; P3 end of gastric phase, P4 after 1 hour from intestinal phase, P5 at the end of intestinal phase. Only the P1, P3, P5 and an aliquot of P5 with 3kDa cut off are used for NMR analysis.

13.4.1 Materials and Methods

Salts and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO), except where indicated. The two types of spreadable cheese, named C1, C2 and C3, C4 were obtained from an important Italian food company.

13.4.2 Experimental design in vitro digestion method

The samples of spreadable cheese were digested in duplicate according to [4], following the scheme reported in Figure 23.

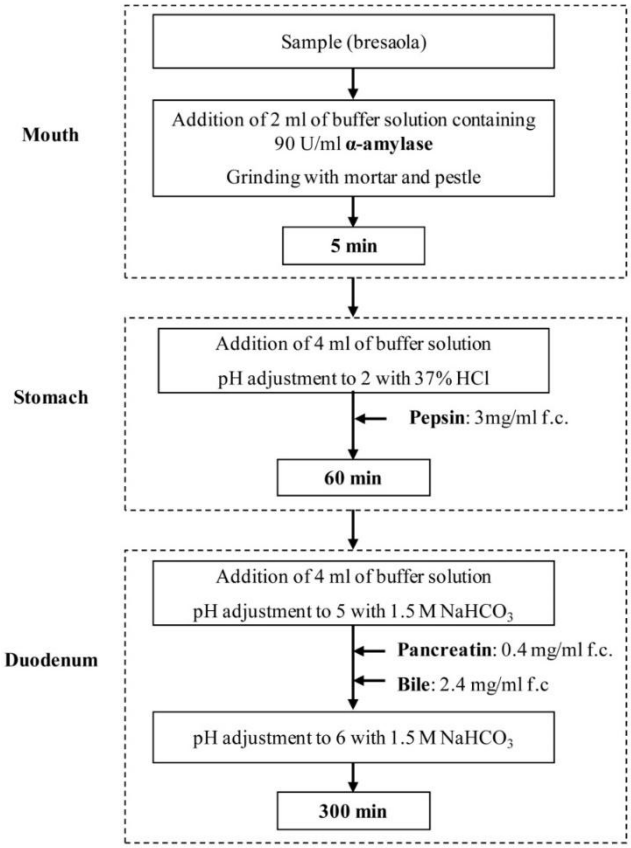

Figure 23. The simulated in vitro digestion protocol applied to the spreadable cheese, according to the protocol published in Bordoni et al. 2011 [4].

Human digestion was simulated *in vitro* inside a 100 mL flask, kept at 37 °C in a water bath on a magnetic stirrer equipped with a heating plate. Chemical composition of the digestive fluid, pH and residence periods were adjusted to mimic the physiological conditions. Three samples were collected during digestion: P1, at the beginning of gastric phase (after the decrease of pH to 2,

before the addition of pepsin), and P3, P5 respectively at the end of gastric phase and after 300 min from the beginning of the duodenal phase. In samples P1 the pH was increased to 8 with 35 % NaOH to prevent possible modifications induced by acidic conditions. Samples P2 and P3 were acidified to pH 2 with 37 % HCl to stop pancreatic hydrolysis and to avoid bias caused by different pH values. P1, P2 and P3 samples were stored at -80 °C ^{1}H-NMR experiments.

Samples P1-P3-P5-P5<3kDa were prepared for ^{1}H-NMR by adding to 800 µL of each sample 800 µL of deuterated chloroform ($CHCl_3$)[13]. The sample was mixed for 5 minutes with vortex and centrifuged at 14000 rpm for 15 minutes. Then 700 µL of surnatant were adding on a 1.5 mL eppendorf with 70 µL of phosphate buffer in deuterium oxide (D_2O), containing 10 mM 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. After adjusting the pH to 7.00, the samples were centrifuged at 14000 rpm for 5 min in order to further remove impurities and put in NMR tube.

13.4.3 ^{1}H NMR evaluation proteins kinetics during in vitro digestion

All ^{1}H-NMR spectra were recorded at 25 °C on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). The spectra were collected with a 90° pulse of 14 µsec with 10 W of power, a relaxation delay of 5 sec and an acquisition time of 2.28 sec. The spectra were registered by means of the first increment of a NOESY sequence, designed to suppress the HOD residual signal, while giving, for each proton, peaks proportional to the concentration of the substance they belong.

13.4.4 Results and discussion

Below are shown 4 parts of molecular profile of four sample of spreadable cheese (C1-4) in P1 phase. Spectra showed different signal present in 4 region of the spectra. In particular, the signals that resonate in the region between 0.7 e 1.1 ppm (aliphatic methyl), 1.6 e 2.5 ppm (aliphatic methyl) 3.5 e 4.0 ppm (sugars) and 6.7 e 7.5 ppm (aromatic proton).

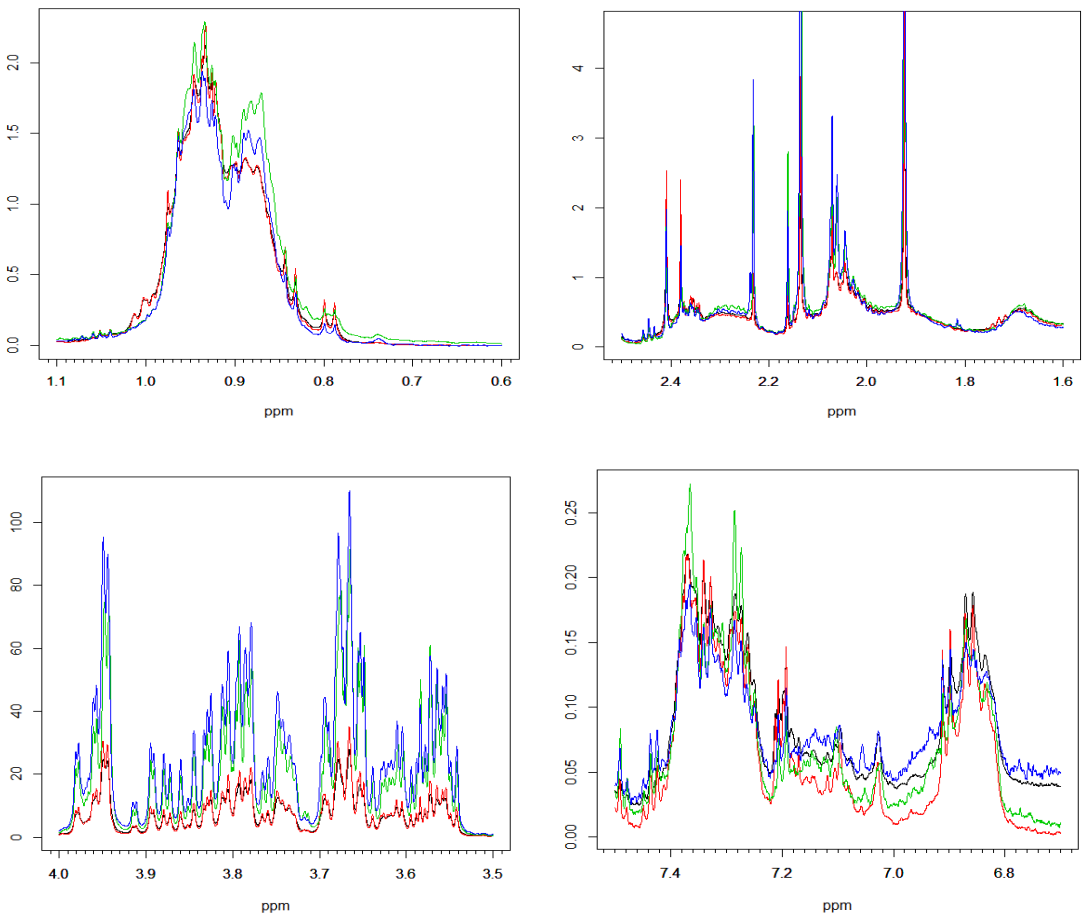

Figure 24. Overlapping of the NMR spectra recorded on the spreadable cheese samples of the two different typologies (convenctional: C1 and C2 green and blu; light: C3 and C4 black and red). The different regions are shown with different vertical size.

The first pre-processing step corrects the effect of dilution factors. Although the digestion process had been the same for all samples, it has been shown that, by matching the signals in specific areas, the concentration can change for two reasons:

1) different content in dry matter. The two spreadable cheese were produced by the same food company, but following two different protocols (light and conventional) and due to this fact different water content was possible, determining different concentration in soluble molecules.
2) the collecting point in different digestion phases

For these reasons, an algorithm correcting the variations of the overall concentrations of solutes, caused by different dilutions, has been applied. This second critical pre-processing step is called normalization.

The best normalization algorithm that was used in this work was the Probabilistic Quotient Normalization (PQN) by which was obtained the best overlap of the spectra in P1, when the proteins digestion was not started.

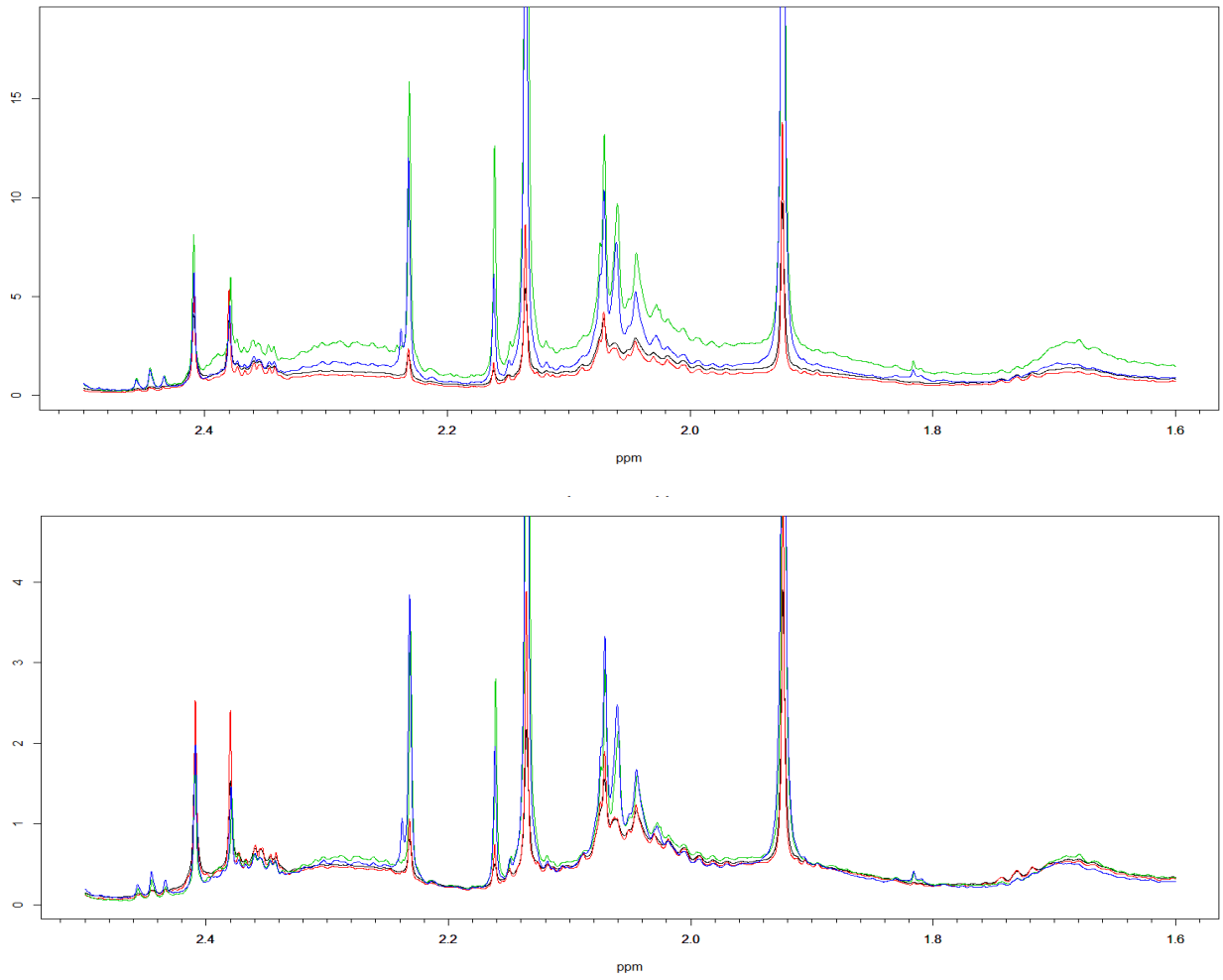

Figure 25. Spectra region between 1.6-2.5 ppm of C1-4 samples, before and after normalization process, at the digestive step P1.

As the quantity of soluble substance changes during the digestive phases, this kind of normalization cannot be chosen in all digestive steps. Thus, the lactose signals were used to keep constant the concentration of such molecule along the digestion steps, provided the absence of lactase enzymes was checked. The lactose signals, indeed, remained constant in all 4 spectra after P1. For this reason, the area under the triplet lactose signals (3.26, 3.33 ppm) was measured and used to normalize each NMR spectra in all digestions phases. The results of normalization obtained for lactose signal are shown below.

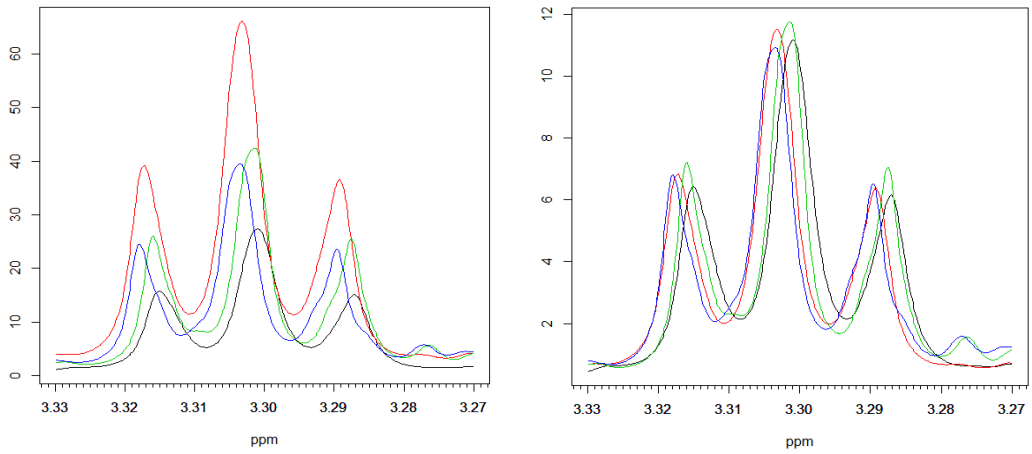

Figure 26. Before and after normalization step in lactose signals in spreadable C1 (conventional).

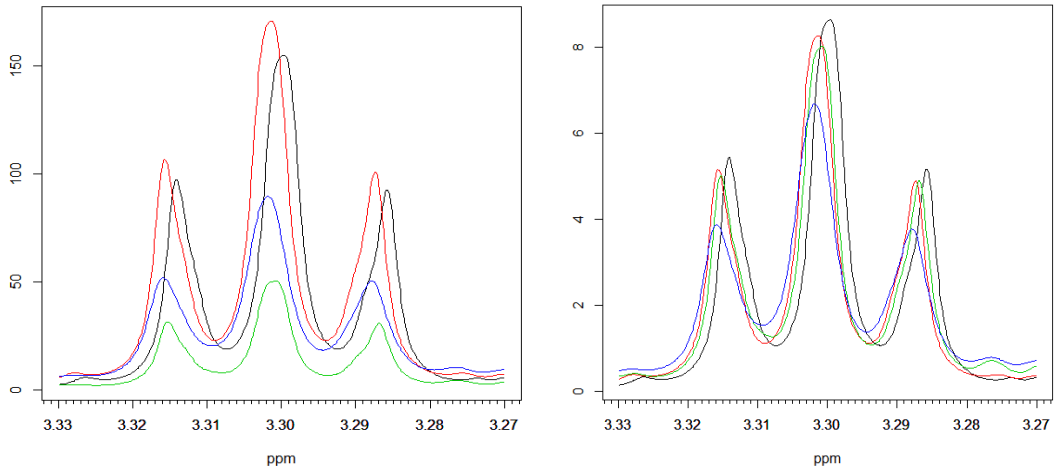

Figure 27. Before and after normalization step in lactose signals in spreadable C3 (light).

After the normalization protocol has been applied to all samples and to all digestive phases, the areas of two interesting spectral regions have been measured. All the values collected were subjected to ANOVA and LSD test. The region that were taken into account were:

- Aliphatic area, between 0,85 a 1.05 ppm, where are recorded molecules such as branched-chain amino acid as free amino acid, di- and oligo-pepdides, as well as short chain fatty acids;
- aromatic area between 6.68 to 6.92, where aromatic molecules with low molecular weight such as amino acid phenylalanine, tryptophan, ect are recorded.

Taking into account the increase in both regions, aromatic and aliphatic, the quantity of low molecular weight molecules that had been released during digestion process has been

determined, and the existing differences between the two Ricotta samples have been verified and reported in Table 6.

	Aromatic Region	Aromati Region	Aliphatic Region	Aliphatic Region
	Conventional	Light	Conventional	Light
P1	115,04±30,3 ^{a,d}	103,88±39,9 ^{b,d}	1076,903±112,8134 ^{a,c}	1044,206±10,91 ^{b,c}
P2	1441,918±262,2 ^{a,c}	666,67±3,5 ^{b,c}	14522,11±2384,57 ^{a,b}	6837,55±93,82 ^{b,b}
P5	3072,47±508,4 ^{a,b}	1095,165±56,97 ^{b,b}	29995,7±7003,5 ^{a,a}	10311,4±456,669 ^{b,a}
P5_3kDa	2255,042±539,87 ^{a,a}	849,474±53,54 ^{b,a}	24471,22±6873,57 ^{a,ab}	7630,71±303,16 ^{b,ab}

Table 7. Incremental area of NMR signals in different regions of the spectra during the three different steps of the digestion of spreadable cheese, both conventional and light products. In addition, the area of signals after ultrafiltration (<3KDa) has been reported to show the proportion of low molecular weight metabolites at the end of the duodenal digestion.

The results of the ANOVA analysis showed a meaningful difference between light and conventional spreadable cheese. In particular, conventional products released a double quantity of soluble molecules with low molecular weight (P5_3kDa) during in vitro digestions, and these results is clear for both aliphatic and aromatic region.

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14. CONCLUSION AND FUTURE PROSPECTIVE

In the last decades, the awareness that an healthy diet is a main determinant of human health has hugely increased nutritional studies, in order to optimize food production and consumption matching human needs. Although the effects of nutrients on health are deeply investigated by nutritionists, and the effects of processing on nutrients by food technologists, foods are often evaluated in terms of quality and quantity of components without considering the supra-molecular organization that makes foods with similar composition actually different. This supra-molecular organization, often referred to as the "matrix effect", indicates the nature of aggregation. As for the fat micelles, or the cellular compartmentalization of the heterogeneous mixtures of components constituting foods, it can deeply affect the bioavailability of molecules. Bioavailability can be defined as the proportion of a nutrient that can be utilized for normal physiological functions. The main component of bioavailability refers to the digestion and absorption of nutrients in the gut, which is the main rate-limiting factor. As a first approximation, we can consider as bioaccessible those molecules that, once made accessible to the solvent through the digestive process, are extracted from the matrix and mobilized in order to freely diffuse to the absorption site. While changing supra-molecular organization and the network of molecular interactions and their localization within compartments normally inaccessible to the solvent, processing can deeply modify nutrient bioavailability and therefore the nutritional value of foods. Therefore, the nutritional evaluation of a food must also consider its behaviour upon digestion. Particularly, given that meat-based food are nutritionally valuable mainly as protein source, the actual bioavailability of peptides and amino acids after digestion is an important parameter.

Quality is a key concept in this thesis; for food products, it is a tricky element to be described, because foods are complex heterogeneous mixtures that cannot be simply described by their chemical composition (even the most precise). A digestion pathway may be the additional attribute of quality to explain food matrix complexity.

Spectroscopic techniques (IR, RAMAN, NMR) are suitable to follow digestion directly on digestates, and chemometry applied to NMR spectra provides digestion pathways which could be used as descriptors of food quality.

NMR spectroscopy, when used in tandem with the in vitro digestion, generates a new concept, namely the digestion pathway, which provides an additional attribute to describe the food quality, the digestibility, which measures the improvement of the nutrients bioaccessibility.

The results of this research work represents a first embryo of using the in vitro simulation of a

physiological process as a tool to distinguish, in a comparative way, among complex foodstuffs, a task otherwise difficult by the direct observation of such heterogeneous mixtures.

In the future, this approach could be used more and more, taking advantage from consolidation of in vitro digestion protocols and from the availability of standard digestion pathways that can be used as terms of comparison for new developed food products.