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Study, characterization and recovery of bioactive components from milk and whey

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CHAPTER I

Milk: A complex source of nutrients

1.1. Introduction

Milk is an opalescent biologic liquid, with a sweetish taste and a delicate flavor, with a very complex composition. His extraordinary richness in nutrients make this matrix “the complete food”.

Its composition is divided in: water which represents 87%, to 3.6% from lipids (triglycerides, phospholipids, sterols; mainly are formed from saturated fatty acids, because of animal origin), for 3.5% proteins (they are very concentrated in the "skins" that forms on the surface when the milk is heated), for the 5% from carbohydrates (mainly lactose) and 0.8% mineral salts (such as calcium and phosphorus).[1]

Milk is also a source growt factors, hormones, cytochines, and other bioactive compounds. Milk proteins possess a high biologic value, which is second only to egg proteins. Historically, milk proteins have been considered only as food proteins for young mammals because they are their principal source of amino acids.

Cheese is the food that better than others concretized the concept of transforming and store for long periods the nutritional values. The transformation of the milk in cheese allows, in fact, to retain protein and fat, essential elements for life. One of by-products of cheese production is Whey. Whey is the fraction that is separate from milk when happens the curd, by the addition of rennet, through heating, leads to the formation of the ricotta cheese, a healthy and nutritious cheese. The production of ricotta process involves the separation of a further waste products rich in bioactive components which is called “scotta”. The wastewater of dairy industries have always been regarded as products with high levels of disposal costs for companies, but new technologies have made it possible to reconsider these rich arrays of components of great interest to pharmaceutical companies.

1.2. Dairy industry

An high percent of the food sector is represented from the dairy industry, which is based on milk and his derivatives.[2] Dairy products are defined as all the milk based product.[3] Those product are typical of temperate regions and from the presence of animals such as cows, goats and sheeps. The area of major production is Europe. In Italy the cow milk production has been valued in $1{,}05\times10^8$ quintals in 2015, whereas the whey production was $3{,}37\times10^8$, the 22,15% of which has been reutilized for ricotta cheese production, the 67,62% for livestock feeding, the 7,06%v in concentrated form and the 3,17% in powder or tablets.[4]

The food industry at large, and the dairy industry in particular have been searching for innovative modes of conversion of their effluents into added-value products, following economically feasible routes, also reducing disposal costs. One booming market is that of health-promoting foods—generically known as functional foods. These foods are able to promote health and reduce the risk of diseases, if regularly included in balanced diets—besides being useful to correct nutritional deficiencies.

Addition of whey components, and their derivatives bearing physiological roles has responded to demand by specific consumer groups—and the possibility of releasing biopeptides encrypted in their proteins has motivated several studies in recent decades. Development of membrane separation techniques has been crucial in food processing—and the dairy industry pioneered development of equipment for membrane filtration, thus taking advantage of its easy scale-up and low-cost when compared with more involved preparative chromatographic techniques.[5] Moreover, heat treatment is no longer a requirement, so the nutritional properties and the bioactive components remain essentially intact (or are only marginally affected) when undergoing said membrane treatments.

1.2.1. Whey

Whey is a greenish-yellow liquid and it is a by-product of dairy industries, particularly the watery portion that is formed during the coagulation of milk casein in cheese making or in casein manufacture.[6] Whey is produced in large amounts and has a high polluting load, therefore representing a significant environmental problem.[7]

The amount of whey resulting from the production of mozzarella is remarkable, for every kg of cheese products are about 4 liters of whey.[8]

World production of whey in 2008 was over 160 million tons per year , in 2010 the world production of whey was over 180 million tons per year (estimated at 9 times the production of cheese) . In Italy the production of whey is estimated to be about six million tonnes a year.[6]

There are two main varieties of whey, that are derived from different technologies[9]:

- The sweet whey , which is derived from the production of pressed cheeses and pasta , cheese, and casein obtained by the action of rennet . One liter of this whey contains 65 g of dry residue, of which 48 g of lactose , 8 g protein, 7 g of salt and about 1 g of fat .
- The acid whey, which is a byproduct of cheese and fresh springs and the production of lactic casein . The acidification of the milk causes increased demineralization of the curd ; consequently this whwy is characterized by a higher mineral content of the sweet whey and lower pH .

The composition of whey is variable depending on the milk used , but all of the different types of whey are valuable because they contain a large part of the nutrients of the milk;[10] Whey represents about 85–95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5–5% w/v), soluble proteins (0.6–0.8% w/v), lipids

(0.4–0.5% w/v) and mineral salts (8–10% of dried extract). Whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins.[11]

Because of its high level of organic substances , mainly lactose, whey exhibits a biochemical oxygen demand (BOD) of 30–50 g/L[12] and a chemical oxygen demand (COD) of 60-80 g/L. Protein recovery reduces the COD of whey only by about 10 g/L.[13]

However, in spite of the high content in sugars and proteins, whey has always been considered a deviation of the dairy chain. The utilization of whey has been a challenge since man started making cheese. As cheese production increased, the volume of whey also grew and many cheese factories were built near waterways so that most of the whey was diverted to these streams or rivers. Modern times brought the awareness of the polluting problem that whey represents and the consequent regulations prohibiting its dumping into waterways and even into municipal sewage systems, whose conventional treatments are not appropriate to sufficiently reduce whey polluting load.[14] Even today, there being no plans for the use of whey in many countries of the world are created problems of pollution.[15] Moreover, even if there were measures prohibiting its discharge into water courses and sewage networks, conventional treatments are not suitable enough to reduce the pollution load of the whey. [6] Whey is produced in large amounts and has a high polluting load, therefore representing a significant environmental problem. On the other hand, however, whey retains much of the milk nutrients, including functional proteins and peptides, lipids, lactose, minerals and vitamins and therefore has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource and not only as a waste problem. Proteins whey account for about 20% of the milk proteins and are a protein source competitive enough compared to soybeans. Protein separation of whey

is typically achieved by ultrafiltration to produce the "whey protein concentrate" (WPC = whey protein concentrates), which have many applications in the food industry as supplements. Whey protein also have applications in cosmetics and pharmaceuticals industries.[16] The whey is also partly used in animal feed as feed for rearing pigs either as such or after suitable treatment to improve the shelf life is that the nutritional value.[11] The 50% of the total whey production is treated and transfromed in different food products[17], such as whey powder, which possesses a better shelf life and an easier transport. Powdered whey is employed especially as livestok feeding and bakery products. In order to develop integrated solutions, the whey should be considered as a resource and not only as a substance waste, in view of its great potential as a source of value-added products.

1.2.2.Scotta

Scotta is the main by-product in the making of ricotta cheese. It is widely produced in southern Europe and particularly in Italy where it represents a serious environmental pollutant due to its high lactose content.

Scotta is the main by-product of ricotta cheese production and is widely produced in southern Europe, with 1 million tons per year being produced in Italy alone. It is obtained after the flocculation of whey proteins and their separation as ricotta cheese induced by thermal treatment of cheese-whey at 85–90°C for about 20 min. Scotta from bovine whey contains proteins (0.15–0.22%), salts (1.0–1.13%), and lactose (4.8–5.0%), and has a biological oxygen demand of 50 g/L and a chemical oxygen demand of 80 g /L.[18]

Scotta from ovine milk is generally characterized by a higher protein content.[19]

Thus, similar to what is observed for cheese-whey, the disposal of scotta represents a serious environmental problem and its biological treatment is an economically demanding step for the dairy industries, particularly for cheese-

makers.[20] On the other hand, scotta may be considered as a source of lactose and other nutrients with possible biotechnological applications. For all of these reasons, the use of scotta as a raw material for alternative processes is recommended by the European Commission. In fact, the bioconversion of scotta into valuable products, besides representing an appealing approach to the reduction of its environmental impact, would allow the exploitation and valorization of this by-product.[21]

1.3 Bioactive components of waste dairy industries

1.3.1. Lactose

Lactose is a disaccharide sugar, produced by the mammary glands of mammals, which gives the milk the soft sweet taste. It consists of two monosaccharides, glucose and galactose, and is present in variable concentration in milk and derived products.

The most abundant component in whey is lactose (ca. 5% w/v), which represents a significant environmental problem. This amount decreases in yogurt and fresh cheese, until it disappears in the cheeses seasoned hard paste.

The hydrolysis product is widely used in the food industry for making soft drinks, but, beyond its direct applications in the food sector, lactose is a valuable raw material for upgrading by fermentation or chemical transformation. In the pharmaceutical industry, lactose is used as excipient for most tablet drugs because it is inert, non-hygroscopic, and available with high purity and having good binding properties.[22] The lactose is used for the formulation and development of delivery systems and the controlled release. The fermentation of whey lactose to ethanol, using selected yeasts, has been frequently referred in the literature.[6]

1.3.2. Riboflavin

Milk is a good source of riboflavin containing 1.83 mg of the vitamin for liter milk.[23] Riboflavin (vitamin B2 or 7,8-Dimethyl-10- (D-ribo-2,3,4,5-tetraidrossipentil) isoalloxazine) is a vitamin photosensitive, was isolated for the first time in 1927 by Paul Gyorgy. Riboflavin is a heterocyclic compound obtained from a molecule of isoalloxazine which is linked to a chain formed by Ribitol.[24]

It is a compound of yellow color slightly soluble in water, stable to heat (baking determines the inactivation of only 10-20% of the total quantity).The riboflavin is found in foods mainly as a phosphorylated form, and after being absorbed, from the blood it reaches the liver and other tissues where it is later turned flavin-mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the two coenzyme forms.[25] It is involved in various redox reactions of metabolism of carbohydrates, proteins and lipids. Being involved in many metabolic reactions which may include other vitamins, a state of riboflavin deficiency can lead to a state of deficiencies of other vitamins. Riboflavin can then be recovered from the whey in order to be reused, for example enriching foods, by means of suitable formulations, to reduce deficiency states.

1.3.3. Lipids

Milk contains about 33 g total lipid (fat)/L. Triacylglycerols, which account for about 95 % of the lipid fraction, are composed of fatty acids of different length (4-24 C-atoms) and saturation. Each triacylglycerol molecule is built with a fatty acid combination giving the molecule a liquid form at body temperature. Other milk lipids are diacylglycerol (about 2% of the lipid fraction), cholesterol (less than 0.5%), phospholipids (about 1%), and free fatty acids (FFA) accounting to less than 0.5% of total milk lipids. Increased levels of FFA in milk might result in off-flavours in milk and dairy products,

and the free volatile short-chain fatty acids contribute to the characteristic flavours of ripened cheese. [26]

More than half of the milk fatty acids are saturated, accounting to about 19 g/L whole milk. The specific health effects of individual fatty acids have been extensively studied. Butyric acid (4:0) is a wellknown modulator of gene function, and may also play a role in cancer prevention. Caprylic and capric acids (8:0 and 10:0) may have antiviral activities, and caprylic acid has been reported to delay tumour growth. Lauric acid (12:0) may have antiviral and antibacterial functions, and might act as an anti caries and anti plaqueagent. Interestingly, Helicobacter pylori can be eradicate by this fatty acid. Capric and lauric acid are, moreover addressed for inhibition of COX-I and COX-II. Finally, stearic acid (18:0) does not seem to increase serum cholesterol concentration, and is not atherogenic. [27]

Oleic acid is the single unsaturated fatty acid with the highest concentration in milk accounting to about 8 g/litre whole milk. Accordingly milk and milk products contribute substantially to the dietary intake of oleic acid in many countries. Oleic acid is considered to be favourable for health, since diets with high amounts of this monounsaturated fatty acid reduce plasma cholesterol, LDL-cholesterol as well triacylglycerol concentrations. Moreover, replacement of saturated fatty acids with cis unsaturated fatty acids reduces risk for coronary artery disease. Several studies also indicate a cancer protective effect of oleic acid, but the data are not fully persuasive. Milk fat is rich in oleic acid (about 25%) containing also a very high ratio of oleic acid/polyunsaturated fatty acids. A diet rich in milk fat therefore may be helpful to increase this ratio in the total dietary fatty acids. [28]

Bovine milk, milk products and bovine meat are the main dietary sources of the cis-9, trans-11 isomer of conjugated linoleic acid (CLA). In most cases this isomer is the most abundant CLA-isomer in bovine milk. Minor amounts of other geometrical and positional isomers of CLA also occur in milk, with

different biological effects. Milk content of cis-9, trans-11 CLA vary considerably, but may constitute about 0,6% of the fat fraction. The biochemical role of CLA effects in metabolism added to its reported anti-proliferative and pro-apoptotic effect on various types of cancer cell, makes CLA potential therapeutic agent in nutritional cancer therapy. [29]

The main trans 18:1 isomer in milk fat is vaccenic acid, (18:1, trans-11, VA), but trans double bounds in position 4 to 16 is also observed in low concentrations in milk fat. The amount of VA in milk fat may vary, constituting 1.7%, or 4-6% of the total fatty acid content. Typically, the concentration of VA may be about 2-4% when the cows are on fresh pasture and about 1-2% on indoor feeding. Normally, increase in cis-9, trans-11 CLA in milk also results in increased concentration of VA. VA has a double role in metabolism as it is both a trans fatty acid and a precursor for cis-9, trans-11 CLA. [30]

Phospholipids and glycosphingolipids accounts to about 1% of total milk lipids. These lipids contain relatively larger quantities of polyunsaturated fatty acids than the triacylglycerols. They have different functional roles, such as cations binding, emulsions stabilization, enzymatic activity regulation on the globule surface, cell-cell interactions, differentiation, proliferation, immune recognition, transmembrane signaling, hormones receptors and growth factors. Gangliosides are one of these components found in milk. Gangliosides (with more than one sialicacid moiety) are mainly found in nerve tissues, and they have been demonstrated to play important roles in neonatal brain development, receptor functions, allergies, for bacterial toxins. [31]

1.3.4. Protein

Milk proteins have a high biological value, second only to the one owned by egg proteins. Milk proteins with biological activity are represented by three main groups: casein (α, β and κ), whey proteins (immunoglobulins, β-

lactoglobulin, α-lattalbumine, lactoferrin and lysozyme) and growth factors (prolactin, transforming growth factor [TGF], insulin-like growth factor [IGF].[32] In addition to the ability to provide amino acids, such proteins are also precursors of biologically active peptides, capable of an effect on the organism additional to that nutritional.[33]

These peptides are inactive if they are encrypted in the sequence of the parent protein and may become available during gastrointestinal digestion, or following technological processes and / or bacterial fermentations. In recent years it has been isolated a large number of biologically active peptides, that depending on the amino acid sequence, may exert a number of different activities in vivo, for example by influencing the cardiovascular system, endocrine, immune and nervous systems.[34] A particularly important resource of biologically active peptides is the whey. The whey, for a long time regarded as a troublesome waste product, thanks to new technologies, can be a huge source of proteins and protein derivatives, of great food and pharmaceutical industries. The whey protein (SP), present in the milk of cow, buffalo, goat and sheep, are the most used compound of whey. In light of recent studies emerge important new scientific information demonstrating the high value nutraceutical, pharmaceutical and nutritional, and commercial of the protein component. The SP also have the function of "fat replacer", that is to replace the taste of fat and enhance the taste and aroma of food in general.[35] These properties have made it possible to employ whey derivatives in the food (pasta, chocolate, cookies, mayonnaise, sauces, baby products etc...).

1.4. Bioactive peptides from milk and whey

The peptides are primarily derived from the degradation of proteins , and are naturally in foods , which confer functional properties , sensory and nutritional. In the last two decades a number of studies have been performed on bioactive peptides that are present in the amino acid sequence of milk

proteins. The increasing attention that the pharmaceutical industry to show that the peptides formed following the enzymatic hydrolysis of proteins in foods has made it necessary to development of suitable systems to the separation and characterization.

1.4.1. Physico-chemical properties

Peptides represent a class of compounds very heterogeneous, share being constituted by more or less long chains of amino acids, linked together through the formation of amide bonds (peptide bond), for which the characteristics of a given peptide will depend greatly the amino acidic composition and the length of the chain. The acid-base behavior of the peptides is determined by the α-amino group of the free N-terminal residue (pKa slightly lower than that of the corresponding free amino acid), from the α-carboxyl group of the free C-terminal residue (pKa slightly higher than that the corresponding amino acid free), and the ionizable groups of the side chains of residues in the chain. The reactivity of the amino groups and carboxyl terminals in a peptide is substantially similar to that of the amino acids free (acylation, esterification, etc.), in particular that of the amino group with some reagents (eg. Ninhydrin), it is exploited for the purpose of revelation and quantification. In the presence of carbonyl compounds such as sugars, for heating some peptides take part in the Maillard reaction, with formation of a series of pigments melanoidinici contributing to the development of flavors, aromas and colors you want or unwanted food.

The peptides, finally, they play a very important role in determining the rheological properties of the foods, as a result of the hydrolysis during processing of the same foods (fermentation, curing, cooking, etc.).[36]

1.4.2. Sensory properties

The peptides are generally tasteless or bitter, with the exception of some peptides containing glutamic acid or aspartic acid which have a characteristic sweet taste; the latter are of great importance in the food industry, because, unlike sucrose, do not cause dental caries, and do not contribute to obesity and diseases such as diabetes mellitus, for which it is increasingly frequent development of sweeteners of a peptidic nature to use as an additive in low-calorie food. In this regard the dipeptide Aspartame (L-aspartyl-L-phenylalanine methyl ester) is a common alternative to sucrose, although it presents some drawbacks, such as low stability at high temperatures and at neutral pH, low solubility, sensitivity to enzymatic hydrolysis, and possible side effects such as phenylketonuria and accumulation of methanol; to solve these problems have been developed a series of dipeptide analogues that have shown that there is a strong correlation between structure and flavor.[37]

Following the proteolytic activity in the proteins it can also be generated peptides with a bitter taste; The bitter taste has been related mainly to the content in hydrophobic amino acids[38] and the length of the chain: in general, the bitter taste increases with the number of hydrophobic residues, although more than a certain length of the peptide chain is found no longer perceptible. It was also demonstrated that[39] the presence of a hydrophobic amino acid from the C-terminal side, and of a basic residue from the N-terminal side results in an increase of the bitter taste of di- and tripeptides.

The peptides of low molecular weight play an important role also in the flavor of meat products, in particular, was seen as some of them confer desirable sensory properties, and are greatly responsible for the characteristic flavor of some sausages.[40]

1.4.3. Functional properties

Studies performed in recent years have established that the functional properties of the proteins, intended as physical-chemical properties, are greatly changed during the enzymatic hydrolysis, so that their hydrolysates are frequently used as food additives in order to improve the quality of ' food, given the better preparation than the native proteins. An extensive hydrolysis of food proteins can, however, result in the formation of peptides with a strong bitter taste, so it is important that the degree of hydrolysis is not excessive, and it is also very important the choice of the protease with which they will carry out this process.[41] Some protein hydrolysates are used for their antioxidant properties: a typical case is represented from egg white hydrolisate, whose addition to foods with a high lipid content is able to slow down oxidative phenomena that may occur in their cargo; similarly, it is seen that some dipeptides are capable of slowing down the auto-oxidation of linolenic acid.[42]

1.4.4. Physiological and nutritional properties

Many of the oligopeptides originated from the degradation of milk proteins were characterized as biologically active. Thanks to the ability of the protein to release, after gastrointestinal digestion, or processing of the product, peptides with biological activity, the criterion of qualitative assessment of dietary protein on a purely nutritional properties, has been reconsidered.

Bioactive peptides are molecules that are inactive when they are present in the sequence of the native protein, but which assume pharmacological activity at the time when they are released. From this point of view the milk proteins, caseins[43] in particular, represent a very important source of bioactive peptides; Numerous are the proteins of food origin which, subjected to enzymatic hydrolysis, release peptides with ACE-inhibitory activity, especially proteins of the milk. [44] Although many of the peptides responsible for the antihypertensive activity of these hydrolysates have been identified,

have not yet entirely clear the structural properties necessary for an activity of this type: in almost all cases, it comes to peptides of low molecular weight, rich in idrofobic amino acids. [45]

Milk proteins are also a significant source of peptides with antimicrobial activity. A structural feature common to the peptides with antimicrobial activity is the presence of a cationic head, is required for interacting with the negative charges of membrane phospholipids. Another important aspect is the minor antigenicity that the peptides of low molecular weight are compared to the native proteins: the hydrolysates of milk proteins are characterized by a low allergenicity, and as such can be used to formulate hypoallergenic foods intended for children. [46] From a nutritional point of view, the peptides represent a most bioavailable form of essential amino acids than the protein; In fact, protein hydrolysates are widely used in clinical applications such as parenteral nutrition. [47]

1.5. Aim of work

The main objective of my PhD was essentially the recovery, analysis and activity evaluation of the main biological bioactive components of dairy industry waste products. The research focused on the analysis of bio-macromolecules recovered products and derived from waste of the milk and dairy industry, using powerful analysis tools such as liquid chromatography, mass spectrometry with high resolution and membrane separation systems. In this regard, more matrices were considered. In particular, whey and scotta from processing, respectively, mozzarella and ricotta buffalo, in order to study the degradation of the protein fraction and identify biologically active peptides, potentially useful for the production of innovative added-value products and, at the same time, reduce the amount of food waste and its environmental impact. Also made from milk, which is considered a waste, in

collaboration with the Centrale del Latte di Salerno, it has been developed a pilot plant which provides for the presence of semipermeable membranes, in order to recover components made from milk and other wastewaters by dairy industries. The results obtained show that the effluent of dairy industries can not be regarded as waste which entail high disposal costs as well as an environmental problem, but rather a source of potential peptides which involve salutary effects, which may be used in functional foods and nutraceuticals.

1.6. The membrane separation technologies

Technological advances related to the development of new membranes, improvements in process engineering and better understanding of the functionality of milk constituents have extended the range of membrane separation processes to cover the entire compositional spectrum of milk and whey. Advanced membrane processes allow the recovery and purification of valuable constituents and have become an integral part of an increasing number of dairy processes. Current applications are aimed at enhancing the manifestation of desired functional properties of milk proteins, fractionating caseins and whey proteins, enhancing the microbial quality of dairy fluids, upgrading the quality of low-quality whey, and standardizing milk. Combinations of membrane processes with traditional milk-processing practices are being used to enhance the quality attributes of various dairy products.[48]

Conncentration and/or separation technologies based on the employment of membranes constitute a new application that could replace the traditional approaches, and be a solution to valorize waste products.

The most popular approaches are based on hydraulic pressure or menrane based technologies, the latter are known as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and inverse hosmosis (Figure 1).

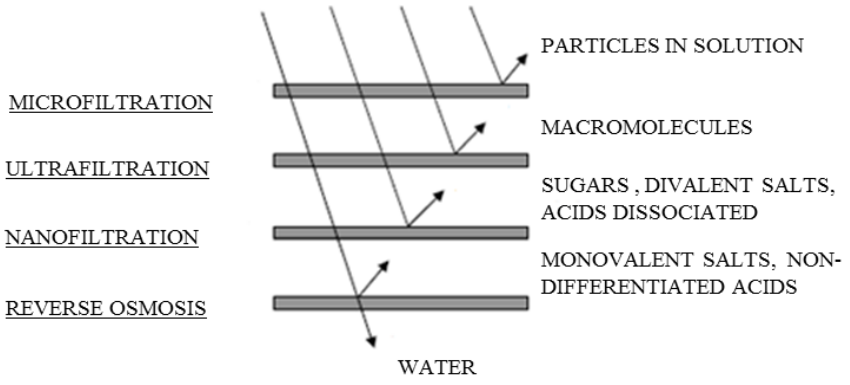


Figure 1. *Separation performed by various processes.*

Those technologies can be distinguished on the basis of the employed membrane and on the pressure conditions. Although not to be considered as innovative technologies strictly, as they are existing already long and their application is already widespread in some countries (particularly in North America), introducing them industrially on a large scale in Italy, could help a significant step forward towards a better use of energy, more than scientific level and as a total savings potential.

The efficiency of the technology is to obtain an effective and immediate reduction of energy consumption (thermal and electrical) in comparison with the traditional technologies, moreover, it makes use of unique characteristics for production purposes or for environmental quality.

1.6.1. Markets

The technology of membrane filtration is particularly used in the U.S.A, by making the significant investments that have produced following a significant gain for the companies. Consider that the market for membrane systems, that is, the operating equipment that uses membranes, has a worldwide turnover of at least 60-80 times that for the cost of the membrane itself. This technique is exploited in various fields, but mainly for food purposes or to reduce the environmental contamination (Figure 2).

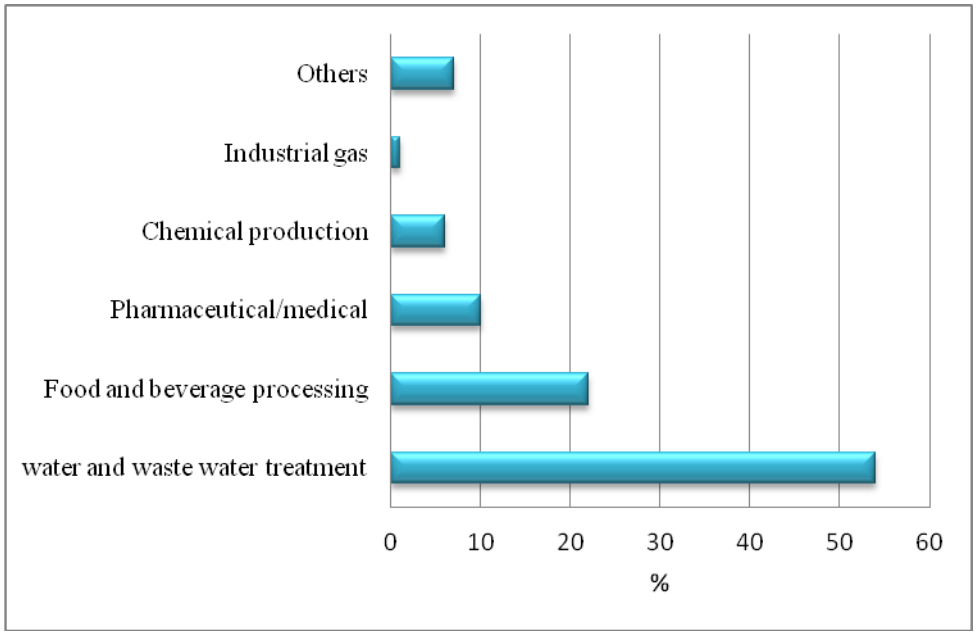


Figure 2. *Main application of the membranes separation technique in the USA.*

While a complete plant membrane has an average life time of the order of tens of years, the membrane modules have operating times of the order of years, therefore they must be replaced in time not only for their loss of efficiency but also because they are continuously developed new best-performing membranes and also more resistant to chemical agents.[49]

1.6.1.2. The Italian market

Although these techniques are widely used in the countries of northern Europe and the U.S.A., and in spite of industrial plants have been made which demonstrate the validity of the technique and above all the economic returns on investment, in Italy the situation does not seem to change and dairy wastewater continue to be illegally disposed of or used as feed. Moreover Italy continues to import from other European countries important quantities of whey-derived, in particular whey protein and lactose, with very high costs. Italy does not produce membranes but has a significant industrial representation of companies producing pilot plants and industrial. There are now thousands of businesses that employ these technologies and are at least thirty industries marketing membrane systems in various application areas. In recent times in our country the membrane technologies are considered more carefully and on average accounted for purification and concentration of "clean technologies", with economic advantages in terms of energy consumption, the system simplification (modular technology) and low-impact environmental (recovery of dispersed substances, the pollutant load reduction).[49]

1.6.1.2. Application areas

Specific applications have been developed in the treatment of dairy industry effluents, in particular whey, patents as well as for the treatment of waste water of the oil industry, the so-called vegetation waters, and for the paper industry, where the water consumption is very important and where the process water recovery is still not entered into industrial practice.

In the food industry the membrane technologies allow a series of operations for the cold sterilization of some matrices such as milk, to the clarification of wine industry products, as the must, wine, vinegar, or the concentration of purees of fruit or other horticultural products such as tomatoes. For the

concentration of these raw materials, membrane technology is particularly effective because they avoid the use of heat, then allow to qualify the finished products, not heat-damaged, and at the same time ensure a strong energy saving against evaporation. The membrane technologies are well established on the market because ever more competitive in respect of the conventional techniques of separation, concentration, in terms of increased separation efficiency, modular plant, of reduced energy consumption and environmental sustainability. However, it should be mentioned that recent advances in membrane technology have led to improvements in this regard, thus allowing the application of membrane processes in an efficient and economical manner. For some processes, severe fouling phenomena have prevented or limited the implementation of attractive processes on an industrial scale. Although much has been accomplished, further developments in improving membrane characteristics and operations are needed. [50, 51]

1.6.2. Description of the Technique

The membrane separation technologies (TSM), called also tangential filtration technologies, are based on the use of semipermeable filters or ion-selective by means of which it is possible to obtain the separation of the organic and inorganic solutes at the molecular and ionic level and solvents in which they are dissolved. As opposed to the perpendicular traditional filtration, the membrane technologies operate tangentially facilitating the removal of solutes by the membrane surface (Figure 3).

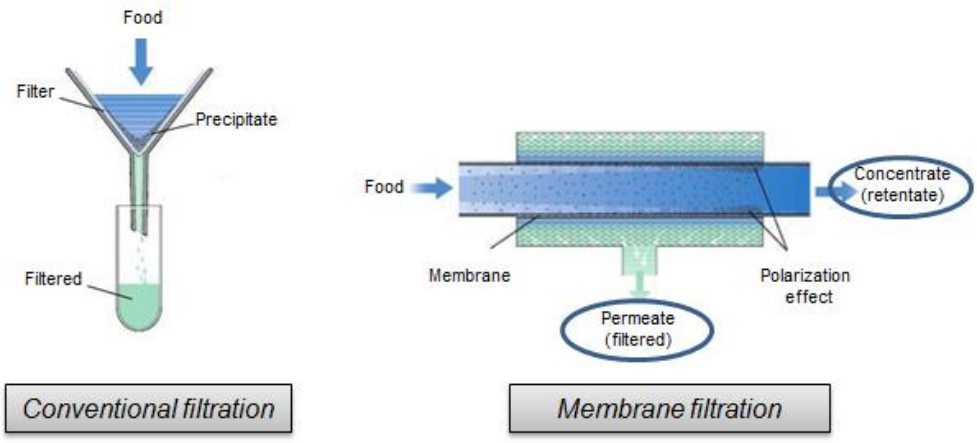


Figure 3 *Difference between classical filtration and membrane filtration.*

The separations are for a physical process, selective filtration through a special filter (membrane) in which the "driving force" is represented by the hydraulic pressure, or by a concentration gradient between the two sides of the membrane, or by a temperature difference; therefore they do not require chemicals to operate the required separations.

Membrane processes are used to concentrate or fractionate a liquid to yield two liquids that differ in their composition. The separation process is based upon the selective permeability of one or more of the liquid constituents through a membrane.[52] The mechanisms governing mass transport in different membrane processes vary as a function of the membrane type, process conditions and equipment configuration. The four major membrane processes are ultrafiltration (UF), reverse osmosis (RO), microfiltration (MF) and nanofiltration (NF).[50] In a classic separative the filtration process allows to recover the effluent permeate and the concentrate, and heat is not used, then the filtration may also take place at low temperatures so as not to cause damage to thermolabile molecules.

The TSM using only electrical energy for running a pump must ensure that an adequate hydraulic flow to allow the filtration. Every single separative

technique requires defined hydrodynamic conditions, for more employees from the engineering features of the membrane module.[53]

The fluid dynamic conditions are the key to good operation and process optimization separative (Table 1).

	(MF)	**(UF)**	**(NF)**	**(OI)**
Pressur (bar)	idrostatic 1- 5	idrostatic 1- 10	idrostatic 20- 40	idrostatic 15- 60
Mechanism	physical filtration	filtration, absorption	solubility / diffusion	solubility / diffusion
Cut off	0.1-20 µm	1 - 100 kD	100 – 250 D	10 – 100 D 1 – 10 Å
Membrane type	ceramic or polymer (symmetrical) (thick 10-150 µm)	polymer or ceramic (Asymmetric) (Separation layer: 0.1-1 µm)	polymer (Asymmetric) or composite (Separation layer: 0.1-1 µm)	polymer (Asymmetric) or composite (Layer separation: 0.1-1 µm)
Configuration of membrane	spiral wound, fiber hollow, tubular, ceramics, inorganic	spiral wound, fiber hollow, tubular	spiral wound,tubular	spiral wound,tubular

Table 1. *Scheme of the technical characteristics of the different membrane technologies.*

The membranes mainly used at industrial level may be polymeric or ceramic:

- The polymer membranes in turn comprise different types of membrane, including the spiral membranes, hollow fiber and flat (plate and frame), all made of organic materials. The polymeric spiral membranes thanks to the wide surface of the membrane for each element guarantee the realization of more compact and less expensive systems. The cleaning of this type of membranes is however complex, and the duration of life of polymer membranes is therefore relatively short. Since the polymeric membranes are available in a wide range of pore sizes, they are usable for many filtration applications in the dairy industry.

- The ceramic membranes include several membranes made all in inorganic materials. Since the ceramic membranes are highly resistant to temperature and chemicals, they are easy to clean. The duration of life of ceramic membranes is superior to that of polymeric membranes. However, by virtue of the limited surface area of the membrane for each element, the ceramic membranes are relatively expensive. The ceramic membranes are available in a limited range of pore sizes, and are usually used only for microfiltration processes and in some cases for ultrafiltration processes.

1.6.2.1. Fouling

The processing of dairy fluids in pressure-driven membrane operations is characterized by a progressive decline in flux with increasing run time. There are a number of phenomena that act simultaneously to reduce the permeate flowrate. The initial rapid decline in the first fewminutes of processing is largely attributed to concentration polarization (CP). This describes the

increased solute concentration adjacent to the membrane, resulting from the balance between: (a) the convective transport of solutes to the membrane carried by the solvent flux, (b) solute rejection and, hence, solute accumulation at the membrane, and (c) backdiffusion of solute into the bulk phase driven by the concentration gradient that has been established[54]. After the initial drop in flux due to CP, the flux continues to decline due to membrane fouling. This decline in flux is initially rapid, and then eventually stabilizes to give a quasi-steady state flux, typically after 1–2 h. This value is often used to characterize the membrane's response to differing environmental and operating conditions. However, the flux continues to decrease slowly with continued filtration; after several hours of operation, as occurs in commercial processes, the final flux may be significantly lower than this initial quasi-steady state value. Chemical cleaning is an integral part of a membrane process operation that must be regularly carried out to remove fouling and ensure continued satisfactory operation and product safety. [55]

Fouling comprises the matter that has left the liquid phase to form a deposit on either the membrane surface or inside its porous structure. The latter is especially important for UF or MF membranes, because they have a more open structure compared to RO and NF membranes. Membrane fouling is influenced by the hydrodynamics of the filtration process, the interactions between the membrane and foulants in the feed stream, and between the fouling layer and foulants. Its consequences are a modification of the transport properties of the membrane, causing a reduction in both membrane permeability (flux) and membrane selectivity (or molecular weight cut-off; MWCO).[56, 57]

Key phenomena in membrane fouling by dairy solutions are: (a) protein adsorption, (b) protein or particle deposition (including casein or cheese fines, microorganisms, and protein aggregates), and (c) deposition of fats and minerals (especially calcium phosphate). Adsorption refers to molecules in

direct contact with the membrane surface, while deposition (also called aggregation) refers to all material forming a cake or deposit on the membrane due to convection-driven sieving, protein-protein interactions, and further adsorption onto initial adsorption layers.[58]

Proteins and minerals play a key role in fouling for most membrane applications in dairy processing. Lactose has been shown to be of little importance and lipids are generally regarded to be of lesser importance, except for feed streams where the lipid content is initially high.[59] Various pre-treatments of the feed to remove particulates, lipids, and minerals are recommended to minimize fouling from these sources.[60]

Protein adsorption occurs rapidly on a clean membrane surface and is largely irreversible, this layer is tightly bound and can only be removed by chemical cleaning. Static or dynamic methods can be used to characterize the extent of adsorption, and both the membrane surface properties (charge and degree of hydrophobicity) and the state of the protein as determined by pH, temperature, and ionic composition affect the degree of protein adsorption and deposition. In UF, the amount of protein deposited within the membrane pores is small compared with that on the membrane surface. However, in MF, there is a greater deposition within the pores, and internal fouling appears to dominate as the nominal pore size increases.[61]

Various operating strategies may be adopted to reduce the extent of flux decline and prolong the operating time for the membrane process. These may include stepwise or continuous increases in the operating pressure, the use of a constant (low) pressure driving force achieved by permeate recycle or the use of tapered membranes, operation at constant (sub-critical) flux, or hydrodynamic methods for removing the reversible component of the fouling. Examples of the latter include the placement of turbulence promoters on or above the membrane surface, periodic reversal of the permeate flow (back-pulsing), reducing or oscillating the feed pressure, or surface rinsing of the

membrane.[58] However, regardless of the temporary effectiveness of these procedures, periodic chemical cleaning of membrane systems is necessary to maintain the membrane performance at an acceptable level. Given the large variety of possible combinations of membrane materials, feed materials, and system specifications, it is not surprising that each situation could require unique cleaning and sanitizing procedures.[62]

Cleaning is defined "a process where material is relieved of a substance that is not an integral part of the material." Disinfection implies the destruction of all pathogenic microorganisms and a marked reduction of the number of spoilage microorganisms that could adversely affect product quality. The aim of cleaning and disinfection procedures is to obtain a structure that is physically clean (providing adequate flux and separation performance in subsequent operations), chemically clean (free from residues which might contaminate subsequent batches of product), and biologically clean (where adequate reduction of the microbial load has been achieved).[63]

To achieve these goals, chemical cleaning regimes must frequently use aggressive chemicals under conditions that will test the pH and temperature tolerances of the membrane material. The cleaning process and cleaning frequency must, therefore, be optimized in order to minimize the adverse effects of cleaning agents on membrane life, the cost of purchasing and disposing of cleaning chemicals, the volumes of water consumed and wastewater produced, and the disruption to normal manufacturing operations.[55]

The optimization of membrane cleaning protocols requires indepth understanding of the complex interactions between the foulants, the feedstock, and the membrane. It also requires knowledge of how operating conditions affect cleaning and subsequent performance, as well as the stability of cleaning agents.[64]

1.6.3. Valorization of the whey through the membrane separation technology

Membrane processes have been employed in the dairy industry for many years. Whey processing represents one of the first fields of application for membrane processes in the dairy industry. UF and RO have been extensively used in whey concentration and have allowed the development of a broad array of whey protein concentrates (WPC). Among the promising current applications for membrane technologies in whey processing are those aimed at increasing the protein content of WPC, fractionating whey proteins, and enhancing the manifestation of specific functional properties of whey proteins.(Figure 4)

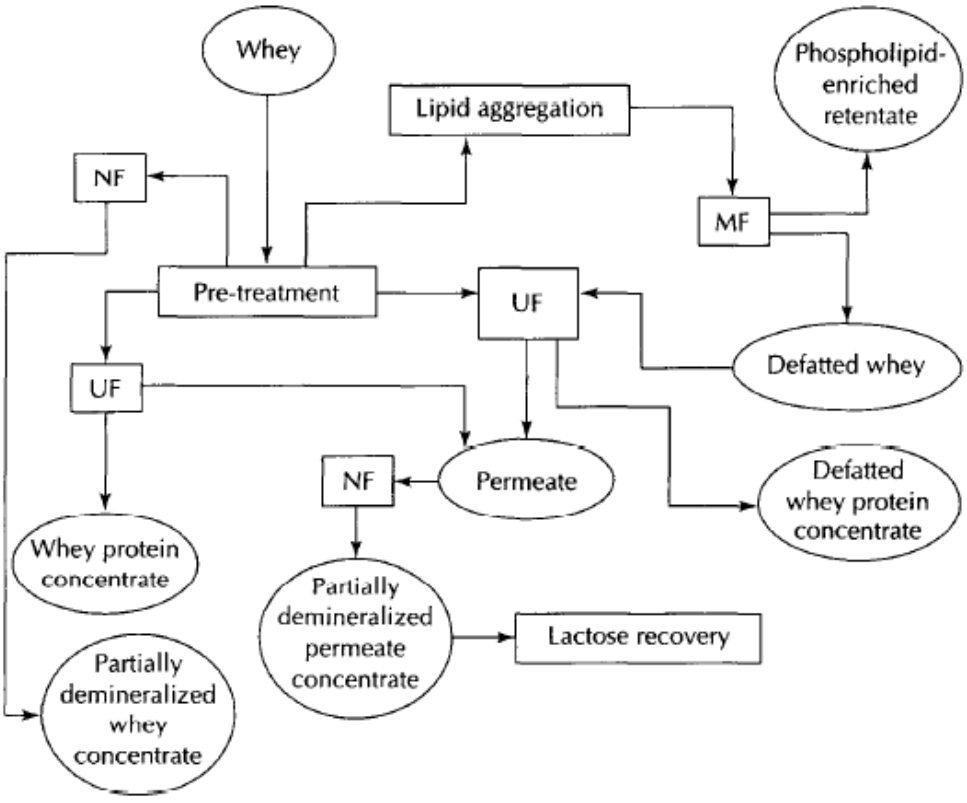


Figure 4. *Applications of UF, MF and NF in whey processing.*

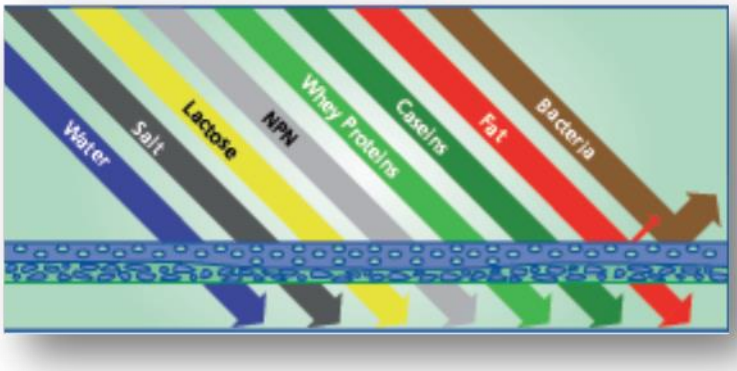
In addition to their nutritional properties, whey proteins possess functional properties that make them useful ingredients in dairy and food products. One of the main problems related to the functionality of whey protein products is variations in their functionality caused by differences in milk and whey composition, and by differences in cheesemaking and whey-processing conditions. Compositional factors that have been shown to affect whey protein functionality include lipids, mineral content (Ca, Mg), and the ratio of individual protein components. Additionally, the hydrophobicity of whey proteins determines their functionality.[48] Lipids and fragments of milk-fat globule membranes associated with whey products adversely affect the functionality of whey proteins, impair the UF membrane flux during the manufacture of WPC, and promote the development of off-flavor in WPC products.[65] Several whey treatment methods have been developed in order to enhance the removal of lipids from whey by membrane processes. A thermocalcic aggregation of lipoproteins has been developed based on the tendency of lipoproteins to form aggregates through calcium bridging when subjected to moderate heat treatment.[66] The aggregation of lipoproteins by this method and removal of the aggregates by MF (pore size 0.2 pm) has proven efficient in producing lipid-free whey protein products. The method has been further improved by using UF whey retentate instead of untreated whey for the first stage, maintaining the MF retentate pH at 7.5, and treating with heat at 55°C for 8 min; the modified process results in improved flux during MF and in improved protein recovery. Lipoprotein-Ca aggregates can also be removed by centrifugation, but the defatting efficiency is inferior to that obtained by MF.[65] The application of an MF membrane with a pore size of 0.2 p,rn has also been reported to allow the removal of a significant proportion of the microbial load from the treated whey.[66]

A pilot plant system that decouples the transmembrane pressure from the inlet pressure allows the removal of lipoproteins while limiting the extent of

membrane fouling. It seems that future developments in industrial MF systems will enable the direct removal of lipid-like material from whey. The foaming properties of defatted whey protein products have been reported to be superior to those of untreated products, and are affected by the pore size of the MF membrane used. Gels prepared from the UF retentate of MF-treated whey exhibited better fracture and shear strength than gels prepared from UF whey retentate. [65] The application of pH adjustment and heat treatment during or before UF allow control of the Ca content of the retentate and hence provide a way to enhance WPC gel strength. Processes for lipid removal described result in two fractions: a lipid-rich retentate and a protein-rich permeate. Owing to its high content of phospholipids, whey MF retentate has potential as an effective emulsification agent for food and cosmetic applications after purification and, probably, fractionation of the retentate. Additional applications in the food industry, exploiting the nutritional value of the phospholipids, are likely to be developed in the future.

1.6.3.1. Microfiltration

The microfiltration and a membrane filtration process that uses a low pressure. It is based on an open structure membrane that allows the passage of the dissolved components, while it prevents most of the undissolved components, which are blocked by the membrane.



Traditionally, the removal of microorganisms from dairy fluids such as milk, whey and brine has been based on heat treatment. Developments in MF membranes and process design have enabled the use of MF in removing bacteria from such fluids.[67] Early studies of the removal of bacteria from milk with 3.9% fat, using a ceramic MF system and backflush/back pulse operation mode, suggested that fat separation before MF is needed in order to increase the concentration factor while maintaining high flux. Similar membrane and operation conditions were also reported for brine treatment.[68]

With the implementation of the uniform transmembrane pressure (UTMP) concept in MF, a system capable of removing 99.7% of the bacterial load from skim milk containing 30000 colony-forming units (cfu) for ml has been introduced.[69] The UTMP concept consists of adding a filtrate-circulating system, in addition to the usual concentrate-circulating system that characterizes an MF module. Using a relatively small but uniform transmembrane pressure of 30-40 kPa, which has to be slightly increased throughout the process, high flux can be maintained. A process combining high-temperature treatment (130°C for 4s) and MF (MF/HTT) for the removal of bacteria from raw milk has been introduced and implemented on an industrial scale.[67]

The processing of skim milk by MF, using membranes with a pore size of 0.2 pm diameter, enables the separation of milk into permeate (consisting of the major whey proteins, free of lipid-like substances and casein macropeptide) and retentate (consisting of native caseins).[70] By manipulating the MF process design, the retentate can be standardized to a desired ratio of casein to total protein, for use in cheesemaking. .

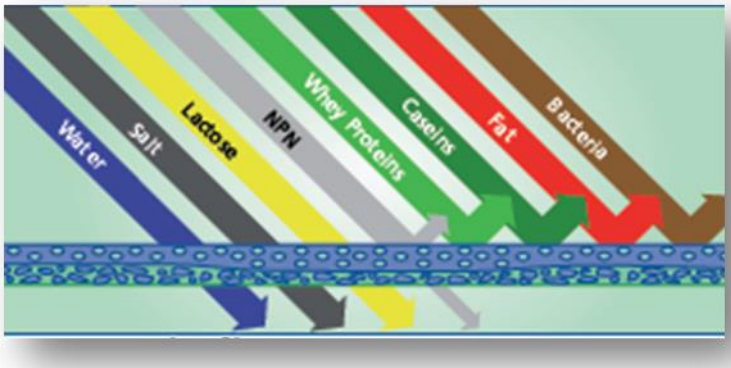
The application to skim milk of MF in combination with other membrane processes, enzymatic processes and/or chromatography processes opens up possibilities in isolating and purifying caseins, as well as bioactive peptides derived from them that can find application in the pharmaceutical industries.

Of special interest in this regard are peptides derived from B-casein that have been shown to exhibit morphine-mimicking, cardiovascular and immunostimulating activities.[71]

The separation of casein micelles from all other milk constituents by membrane filtration methods has been developed at the laboratory scale.

1.6.3.2. Ultrafiltration

Ultrafiltration is a membrane filtration process that uses a medium pressure. It is based on a membrane with average-open structure, which allows the passage of most dissolved components and a part of non-dissolved components, while the components of larger size are retained by the membrane.



Ultrafiltration offers the possibility of adjusting the mass ratios of different milk constituents without adversely affecting their physicochemical characteristics, and can be used in milk protein standardization. The ultimate relative levels of milk proteins, lactose and minerals in the retentate depend on the extent to which milk is processed by UF and the conditions (pH and temperature) used. The application of UF can be used to standardize the

nutritional value of consumer milk or to prepare standardized milk powders, thus overcoming natural variations in milk composition.[72]

The use of UF to standardize protein and total solids levels in milk used in the manufacture of fermented products has been shown to result in products whose quality and characteristics are superior to those of products prepared from milk fortified with milk powder or evaporated milk. The use of membrane processes in the manufacture of fermented dairy products can allow improved control of quality attributes such as consistency, post-processing acidification, and extent of syneresis.

Wise selection of membranes, separation conditions and the extent of heat treatment can allow the development of a broad array of new product characteristics. [73]

However, it should be noted that compositional changes that result from treating milk by UF may have implications that require the selection of appropriate starter cultures and adjustment of the fermentation conditions. Such implications include the effects of UF on lactose and mineral concentrations, osmotic pressure, ionic strength, and the concentrations of substances known to inhibit the development or alter the activity of starter culture bacteria. Such compositional changes can affect the nature and rate of the fermentation as well as the propagation of bacteria.

Milk protein concentrates prepared by membrane processes have gained increasing interest during recent years. The terms milk protein concentrate (MPC) and milk protein (MP) are currently being used for products containing 50-85% and more than 85% protein, respectively.[74] By using different UF membranes, a combination of UF, MF and RO , and correct adjustment of pH and temperature conditions, the physicochemical and functional properties of the MPC and MP products obtained can be designed for different applications. The changes in composition, rheological characteristics and heat stability that are associated with the manufacture of MPC are of practical importance owing

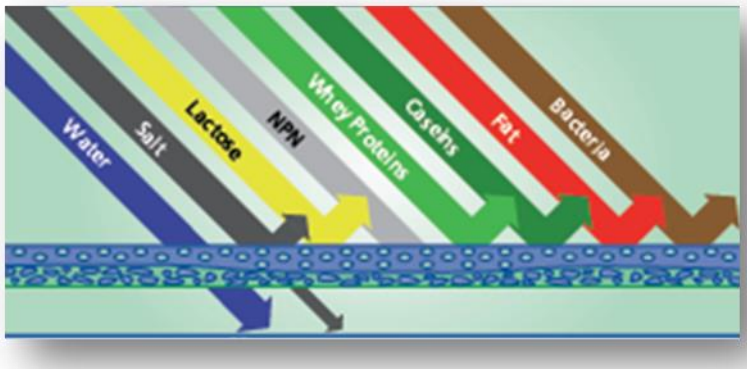
to their influence on further processing of the concentrate as well as on the properties of the final products.

Control of the rheological characteristics of MPC can be achieved by proper adjustment of the heat treatment conditions to favor a minimum degree of whey protein denaturation, by carrying out the UF process at 50-60°C and using high flow rates. Combining the milk-protein fractionation capabilities offered by MF with advanced UF processes enables the production of MPC and MP with modified mass ratios between different caseins, between caseins and whey proteins, and between proteins and minerals and/or lactose. Such approaches offer new possibilities in tailoring the functional properties of milk constituents to different desired extents, and thus enable the development of value-added, milk derived functional ingredients for food and other applications.[74, 75]

Solubility and water-holding capacity, fat binding, emulsification characteristics, whippability and heat stability are among the functional properties of interest.

1.6.3.3. Nanofiltration

Nanofiltration is a membrane filtration process that uses a medium to high pressure. Substantially it is a type of reverse osmosis, in which the membrane has a structure slightly more open, allowing monovalent ions to pass through the membrane. Divalent ions are largely blocked by the membrane.



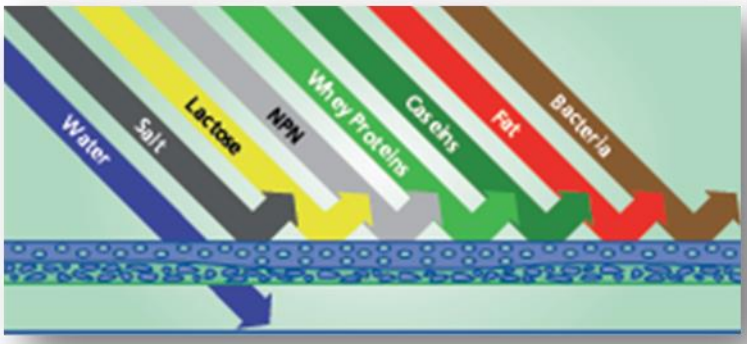
Acid whey (pH 4.5-5) is produced in significant quantities during the manufacture of cottage cheese or soft creamy cheese. Salt-containing whey is generated during the production of Cheddar cheese at stages following the application of salt to the curd. Acid and salty whey streams represent difficulties related to the functionality of the whey proteins they contain as well as to their adverse effects on the environment. NF offers simultaneous separation and concentration of minerals, and has been developed into applications such as the removal (84%) of salt from salty whey, the partial (42%) removal of acid from acid whey, and the partial demineralization of sweet whey in the manufacture of lactose or demineralized whey. Other applications involve the concentration and partial demineralization of whey UF permeate in lactose-recovery processes, and the partial demineralization and concentration of whey produced in the manufacture of caseinates.[76]

The electrochemical properties of the membranes are of great importance to the effectiveness of NF applications. NF membranes are available with various rejection profiles that allow the specific rejection of desired ions, and are characterized by their chemical and structural complexity.

1.6.3.4. Reverse osmosis

The reverse osmosis is a membrane filtration process that uses a high pressure and is based on a very dense membrane. In principle, from the layer of the membrane can only pass water.

In the dairy industry, reverse osmosis is used normally for concentration applications or volume reduction of the milk and whey, for the recovery of the solid substance content in the milk and for the regeneration of process water.



1.6.3.5. Microparticulation

In developed countries, high intake of fat has led to an increasing prevalence of medical conditions such as obesity, coronary artery disease and certain types of cancer. Full or partial replacement of fat in food can offer the consumer organoleptic characteristics similar to those of its full-fat counterpart, while helping to maintain a healthier lifestyle. Fat replacers, substitutes, extenders and mimetics, are common terminologies used to describe ingredients that lower the calorific value of high-fat foods. Fat mimetics in particular, have received much attention and can be derived from starch, fibre, proteins and gums.[77]

An example of the use of proteins as fat replacers is the process of microparticulation , whereby protein aggregates ranging in size from 0.1 to 3

μm are created to impart a creamy perception in the mouth similar to that produced by fat globules.[78] Microparticulated protein is a natural ingredient produced from whey protein concentrate (WPC, typically with 35 or 55% protein) and thus compositionally similar to traditional WPC, ensuring that the same regulatory standards of identity apply. The principal proteins in whey are b-lactoglobulin (50% of total whey protein) and a-lactalbumin (20% of total whey protein) and, to a lesser extent, blood serum albumin, various immunoglobulins and other minor milk proteins. The process of producing microparticles involves heating and shearing the protein dispersion for a pre-selected holding period conducive to formation of aggregates.[79]

The unfolding, denaturation and aggregation of whey proteins during heating affects functional properties such as water immobilisation and heat-induced gel formation. Irreversible aggregate formation is primarily due to the ability of β-lactoglobulin to denature at temperatures above 70°C at neutral pH, while α-lactalbumin with its more ordered structure does not form aggregates. Hence, formation of microparticulates is dependent on the presence of b-lactoglobulin.[80] In the microparticulation process itself, lactose and calcium content, as well as pH and heating temperature have been shown to influence aggregate size and structure.

A recently developed patented thermal process, was used for the production of microparticulates from WPC containing low (15.0 g/100g) solids content. This study evaluated the addition of the dietary fibre inulin as a replacement for lactose and as a potential mechanism for a further reduction in solids content.[81]

Inulin is a nondigestible carbohydrate found in many plants, such as chicory and artichoke, as a storage carbohydrate.[82] Inulin has a number of important attributes that make it a novel functional food ingredient including, its crystalline structure which gives it potential as a fat replacer[83], its low calorific value[84], and its pre-biotic properties due to its resistance to digestion

in the upper gastro-intestinal tract.[85] While there may be potential benefits to replacing the lactose in WPC with inulin in terms of health claims, consideration should be given to thermodynamic compatibility between the two polymers and the potential effect on the formation and stability of the protein aggregates.[86]

1.6.3.6. Experimental application of membrane technologies

Are currently being experiments study to try to separate and isolate the protein of whey with different molecular weights by means of membrane technology, in particular immunoglobulins, lactoferrin and peroxidase with higher molecular weights in the BSA, lactoglobulin and a-lactalbumin in lower molecular weights.

Another line of activities involves the enzymatic hydrolysis of UF in an enzyme membrane reactor into the protein concentrate for the production of bioactive peptides and concentrated NF carbohydrates to produce monosaccharides (glucose and galactose) with a greater sweetening power of lactose and galacto -oligosaccharides with strong prebiotic properties.[87, 88]

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CHAPTER II

Bioactive peptides derived from simulated gastro-intestinal 1 digestion of "Stracchino" soft cheese: characterization, antioxidant activity and bioavailability.

Abstract

Stracchino cheese samples were subjected to in vitro gastro-intestinal digestion. The digest was fractionated by preparative reversed-phase liquid chromatography and tested for its antioxidant potential in intestinal epithelial cell line (IEC-6). The most active peptide fraction was characterized by UHPLC-MS-IT-TOF, showing the presence of two abundant β-casein derived hexapeptides. The synthetized peptides analogues showed an antioxidant activity involving reactive oxygen species reduction, superoxide dismutase increase and Nuclear factor (erythroid derived 2)-like 2 antioxidant response activation. Hence, bioavailability studies were carried out through Caco-2 monolayers cell model on the two hexapeptides, which revealed resistance to peptidase hydrolysis and a relative concentration in the basolateral side of 8.2% and 5.8% respectively.

Keywords

Antioxidant / Gastrointestinal digestion / Intestinal epithelial cells / IT-TOF / Peptides / Stracchino

Abbreviations

G.I., gastrointestinal; SOD, superoxide dismutase; ROS, reactive oxygen species; NQO1, NAD(P)H quinone oxidoreductase-1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1.

2.1. Introduction

Free radicals are the physiological products of endogenous metabolic processes in aerobic organisms. Even if these molecules are normally involved in cell signaling and defense [1], excessive levels of these species can lead to deleterious effects such as lipid peroxidation, DNA mutations and damages to proteins and other macromolecules [2]. Reactive oxygen species (ROS) are normally controlled by antioxidant endogenous enzymatic systems, such as superoxide dismutase and glutathione peroxidase. Failure of this regulation, which can be related to ageing and mitochondrial dysfunctions [3], causes oxidative stress, increasing the possible onset of chronic pathologies, such as cardiovascular, neurodegenerative and intestinal diseases. In order to prevent and counteract these effects, the employment of natural antioxidants has been suggested, in particular those derived from food and plants, since they are safer than synthetic compounds and they can be included nutraceuticals and functional foods [4]. Food proteins, and especially milk derived, have gained attention as a source of bioactive peptides. These compounds are encrypted in the sequences of proteins, and become active after hydrolysis, showing several activities including antihypertensive, antimicrobial, immunomodulatory and antioxidant [5]. The antioxidant properties of peptides are related to their aminoacidic sequence and also to their molecular weight. Multiple mechanisms have been attributed to explain the antioxidant activity of peptides, such as radical scavenging, inhibition of lipid peroxidation and chelation of transition metals ions [6]. Milk and dairy products like cheese contain many potential antioxidant peptides [7], these compounds can be introduced with the diet and exert their activity after gastro-intestinal (G.I.) digestion. Several studies on different kind of cheese showed the release after G.I. digestion of bioactive peptides, in particular with ACE inhibitory as well

as antioxidant activity [8-10]. To perform their activity peptides must be absorbed in the intestinal tract and reach the systemic circulation. Different mechanisms can be used such as paracellular and transcellular routes, lymphatic system, basolateral and via carrier transports [6]. Nevertheless, if not absorbed, they can exert their functions on the local tissue or by binding to intestinal receptors [11]. Among Italian dairy products, Stracchino, is a widely consumed soft cheese made from cow milk, it has origin in Northern Italy and is characterized by a soft, creamy consistency with a typical flavor, has a fast ripening and is consumed usually fresh. In this work we investigated the release of potential antioxidant peptides from Stracchino soft cheese, which has not been reported so far. The cheese was firstly subjected to simulated G.I. digestion, the obtained peptide mixture was fractionated by preparative reversed phase liquid chromatography (prep-RPLC) and peptides were identified by ultra high performance liquid chromatography-ion trap-time of flight mass spectrometry (UHPLC-IT-TOF). Thus peptide fractions were tested in intestinal epithelial cell line (IEC-6) in order to evaluate their antioxidant potential by influencing ROS release, superoxide dismutase (SOD) induction and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway activation, also evaluating the detoxifying and cytoprotective enzymes NAD(P)H quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1) expression. Furthermore an individual study of the most abundant peptides in the fraction showing the highest antioxidant activity was carried out. Peptides were synthetized by F-Moc chemistry, and individually tested. Moreover, their bioavailability was investigated by assessing their permeation through Caco-2 monolayer cells. This work evidences the content of bioactive peptides in this product, highlighting the nutraceutical value of this simple and cheap food, which could provide health benefits to consumers.

2.1.1. Aim of work

This study was aimed to investigate the release of bioactive peptides from Stracchino soft cheese after in vitro gastro-intestinal digestion and their ability to reduce the oxidative stress in intestinal epithelial cells.

2.2. Materials and methods

2.2.1. Chemicals

Ultra pure water (H_2O) was obtained by a Direct-8 Milli-Q system (Millipore, Milan, Italy), LC-MS grade acetonitrile (ACN) and additives formic acid (HCOOH), trifluoroacetic acid (TFA), were all purchased from Sigma-Aldrich (St. Louis, Mo, USA). N^{α}97 -Fmoc-protected amino acids, Fmoc- Lys(Boc)-Wang resin, Fmoc-Gln(Trt)-Wang resin, HBTU, HOAt, DIEA, piperidine and trifluoroacetic acid were purchased from Iris Biotech (Germany). Unless stated otherwise, all other reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy).

2.2.1.1. Sampling and sample preparation

Samples of Stracchino cheese (50 g) were kindly donated by Centrale del latte di Salerno (CdLdS, Salerno, Campania, Italy). Sample was freeze-dried and, then, 3 g of lyophilized were subjected to lipid extraction according to AOAC (1995) method 948.16. Pellets were dried and weighed and kept at −80°C until analyses.

2.2.2. In vitro gastrointestinal digestion

The procedure was performed according to Tenore et al. [12]. Defatted samples (500 mg) were solubilized in 10 mL of deionized water and the pH was adjusted to 2 with HCl 0.1 M. The mixture was incubated with pepsin (1:100

enzyme/protein ratio, w/w) at 37°C for 2 hours in a Thermomixer comfort (Eppendorf, Hamburg, Germany) and the reaction was stopped by heating the solution at 95°C for 10 minutes. The digest were lyophilized and further reconstituted in a solution of HCOONH4 10 mM adjusted to pH 7.5 and further incubated with trypsin, pancreatin, chymotrypsin and bile salts (all in 1:100 enzyme/protein ratio, w/w) at 37°C for 2 hours, the reaction was stopped bringing the solution to pH 2. The mixture was lyophilized stored at −80°C and filtered prior LC analyses on 0.45 μm filters (Phenex RC membrane, Phenomenex, Bologna, Italy)

2.2.3. Gastrointestinal digest fractionation by prep-RPLC

The obtained digest was fractionated by preparative reversed phase liquid chromatography (prep- RPLC) on a Prominence LC system (Shimadzu, Milan, Italy) consisting of two LC-20 AP pumps, a SIL-10 AP, a fraction collector FRC-10 AP and a SPD-20A UV detector. For the separation a Kinetex C18 150 mm × 21.2 mm, 5 μm (Phenomenex, Bologna, Italy) column was employed. Analysis was carried out with a flow rate of 20 mL/min with the following gradient: 0-5 min 1% B, 5-45 min, 1-30% B, 45-55 min, 30-70% B, 55-59 min, 70-95% B, then five minutes for column requilibration. Mobile phase were: (A) 0.1% TFA in H2O v/v, (B) 0.1% TFA in ACN v/v, 1 mL was injected. Four fractions were collected (Fig. 1), on the basis of their elution times and thus hydrophobicity. Fractions were lyophilized and tested for their antioxidant properties.

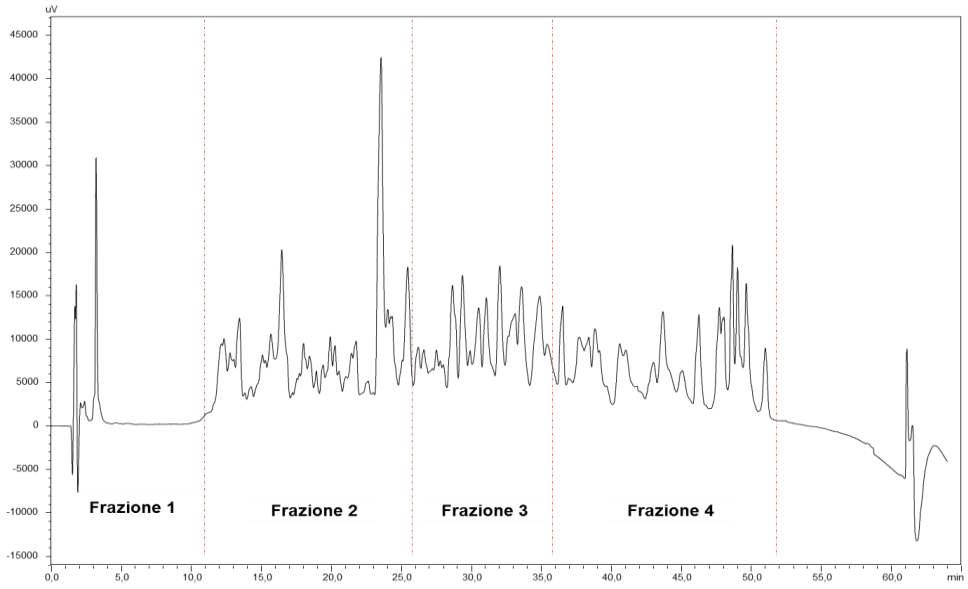

Figure 1. *Prep-RPLC separation of Stracchino $^{G.I.}$ digest.*

2.2.3.1. Peptide identification by UHPLC-MS-IT-TOF

The fraction which exhibited the highest activity was subjected to UHPLC-MS/MS in order to identify peptide sequences. Analyses were performed on a Shimadzu Nexera UHPLC (Shimadzu), consisting of a CBM-20A controller, four LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A PDA detector (equipped with 2.5 μL detector flow cell volume), a CTO-20AC column oven, a SIL-30AC autosampler. The instrument was coupled online with a hybrid LCMS-IT-TOF mass spectrometer (Shimadzu), by an electrospray (ESI) source. The peptide fraction was separated on a Kinetex C18 150 mm × 2.1 mm, 2.6 μm (Phenomenex) column at a flow rate of 0.5 mL/min with the following gradient: 0-1.50 min, 0% B, 1.50-20 min, 0-35% B, 20- 21 min, 35-70% B, 35-70 min, 70-90% B, hold for 2 min., column temperature was set to 50°C and 2 μL were injected. MS was operated in positive ESI mode, interface and curve desolvation line (CDL) temperature

250°C. Nebulizer and drying gas 1.5 L/min and 10 L/min. Full scan 250-2000 m/z, ion trap accumulation time 25 ms. MS/MS was conducted in Data dependent acquisition, ion trap accumulation time 50 ms, the minimal signal required for precursor ion selection was set to an absolute threshold of 100,000. To identify peptides sequences MS/MS datafile were converted into mzXML format by LCMS solution (Shimadzu), and a free trial of PEAKS 7.0 software (Bioinformatics Solutions Inc., Waterloo, Canada) was employed for sequence determination. Search was performed using a database search tool, by searching against SwissProt/UniProt database (taxonomy: Bos taurus), with an improved algorithm that validates and assists the database search with de novo sequencing results (detailed conditions in supporting material). The most abundant peptides were selected for synthesis and in vitro assays.

2.2.4. Peptides Synthesis and quantification in Stracchino G.I. digest

Synthesis of analogues P1 and P2 (EAMAPK, AVPYPQ) was performed according to the solid phase approach using standard Fmoc methodology, by Biotage Initiator + Alstra automated microwave synthesizer and purified by RP-HPLC, detailed conditions are reported in supporting material

2.2.4.1. In vitro intestinal transepithelial transport studies

The assay was performed as previously described [13]. The human colon carcinoma cell line Caco- 2 (HTB-37) was obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were cultured (17–21 passages) in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and supplemented with 12.5% fetal calf serum (FCS), 1% nonessential amino acids, 5 mM L-glutamine, 40 U/mL penicillin, 100 μg/mL gentamycin and 40 μg/mL streptomycin (DMEMc). Cells were maintained at 37°C in a humidified atmosphere of CO2/air (5:95, v/v) and

passaged every 7 days by trypsinisation. They were seeded in Transwell (Transwell® inserts of 3 μm pore size and 24 mm diameter) at 6×10^4 cells/cm^2 . The medium (15 mL DMEM containing 12.5% FCS) was changed every 2 days until cells reached confluence (7–8 days). The integrity of the monolayers (cultured for 14–15 days) was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Zug, Switzerland). Only Caco-2 monolayers showing TEER higher than 300 $\Omega \times cm^2$ were used for the experiments. The integrity of the monolayers was checked before, during and after the experiment. Then, Caco-2 cells monolayers were gently rinsed twice with PBS, medium was removed from the apical and basal sides of the cultures, transport medium (TM, Hank's balanced salt solution supplemented with 25 mM glucose and 10 mM HEPES) was added to the apical (2 mL) and to the basolateral (2 mL) compartments, and pH was adjusted to 6 or 7.4. After 30 min of incubation, medium at the apical side was replaced with fresh TM containing peptides P1 and P2 (EAMAPK and AVPYPQ) at 0, 0.5, 1, 2 or 4 mM. After 4 h of incubation at 37°C, apical and basal solutions were collected, and aliquots (5 mL) were filtered on Phenex-PVDF 17 mm Syringe Filter 0.45 μm (Phenomenex, Torrance, CA). Samples were stored at −20°C until UHPLC MS/MS analyses to measure the concentration of peptides in both compartments.

2.2.5. Measurement of ABTS radical scavenging activity

ABTS method is based on the reduction of the $ABTS^{\bullet+}$ activity by the antioxidants contained in the sample. A solution of 7.4 mM $ABTS^{\bullet+}$ 180 (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 μL) was prepared, stabilized for 12 h at 4°C and then mixed with ethanol (1:88, v/v). Subsequently, 100 μL of supernatant obtained from the above extraction were added to 1 mL of diluted $ABTS^{\bullet+}$182 , incubated for 2.5 min and the absorbance was read at 734 nm.

The standard curve was linear between 0 and 20 μM Trolox. Results were expressed as μmol of TE/100 g FW

2.2.6. Cell culture

The IEC-6 cell line (CRL-1592) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). IEC-6 cell originated from normal rat intestinal crypt cells [14]. This non-tumorigenic cell line was cultured using Dulbecco's modified Eagle's medium (4 g/L glucose) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2 mM L-glutamine, 1.5g/L $NaHCO_3$, and 0.1 unit/mL bovine insulin. Cells were used at the 17^{th}–21^{st} passage. Before the experiments cells were plated and allowed to adhere for 24 h; thereafter, the medium was replaced with fresh medium and cells were incubated with G.I. digest ($^{G.I.}$ digest; 250-5 μM) or fractions 2, 3 and 4 (2,3,4) or sub-fractions (2A, 2B) and peptides (P1 and P2; 150-5 μM) for 1 h before and simultaneously to H2O2 (1 mM) for 1 h.

2.2.6.1. Cell Viability Assay

Cells (5×10^3) were plated in 96-well microtiter plates and allowed to adhere for 24 h. Cells were exposed to the G.I. digest ($^{G.I.}$ D; 250-5 μM) or fractions 2, 3 and 4 (2,3,4) or sub-fractions (2A, 2B) and peptides (P1 and P2; 150-5 μM) for 24/48 or 72 h. Cell viability was then assessed as previously reported using the MTT assay [15-16]. Briefly, 25 mL of MTT (5 mg/mL) were added and the cells were incubated for 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 mL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. IEC-6 viability

in response to treatment with NIV and DON was calculated as: % dead cells= 100-(OD treated/OD control)×100.

2.2.6.2. Measurement of intracellular ROS

ROS formation was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF209-DA) as previously reported [17]. H_2DCF-DA is a non-fluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, IEC-6 cells were plated at a seeding density of 8×10^3 cells/well into 24-well plates and treated as described above. After incubation period cells were then collected, washed twice with phosphate buffer saline (PBS) buffer and then incubated in PBS containing H_2DCF-DA (10 mM) at 37°C. After 45 min, cells fluorescence was evaluated using a fluorescence-activated cell sorter (FACSscan; Becton Dickinson) and elaborated with Cell Quest software

2.2.6.3. Immunofluorescence analysis with confocal microscopy for Nrf-2 activation

For immunofluorescence assay, IEC-6 cells (2×10^5 219 for well) were seeded on coverslips in 12 well plate and treated with H_2O_2 as described above. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% saponin in PBS for 15 min. After blocking with BSA and PBS for 1 h, cells were incubated with nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antibody (sc-722 Santa Cruz Biotechnology) for 2 h at room temperature. The slides were then washed with PBS three times and fluorescein-conjugated secondary antibody (FITC) was added for 1 h. 4′,6-diamidin-2-phenylindol (DAPI) was used for counterstaining of nuclei.

Coverslips were finally mounted in mounting medium and fluorescent images were taken under the Laser Confocal Microscope (Leica TCS SP5) as previously described [18].

2.2.6.4. Measurement of HO-1, NQO1 and SOD expression by cytofluorimetry

IEC-6 cells were plated into 96-well plates (1×10^4 229 cells/well) and treated with H_2O_2 as described. IEC-6 were then collected, washed twice with PBS and then incubated in Fixing Solution for 20 min at 4°C and then incubated in Fix PermSolution for 30 min at 4°C. Anti-HO-1 antibody (sc-10789, Santa Cruz Biotechnology) or anti-NQO1 (sc-376023, Santa Cruz Biotechnology) anti-SOD (sc-30080, Santa Cruz Biotechnology) were then added for further 30 min. The secondary antibody was added in Fix Solution and cells fluorescence was evaluated using a fluorescence-activated cell sorter (FACSscan; Becton Dickinson) and elaborated with Cell Quest software as previously described [19].

2.2.7. Data analysis

Data are reported as mean ± standard error mean (s.e.m.) values of at least three independent experiments, each in triplicate. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test. A P-value less than 0.05 was considered significant.

2.3. Results and discussion

2.3.1. Fractionation of Stracchino $^{G.I}$ 245 Digest and peptide identification by UHPLC-IT-TOF

Crude $^{G.I.}$ digest was fractionated by prep-RPLC, on the basis of elution order and thus hydrophobicity, four fraction were collected (Fig. 1). Fraction 1 was

discarded, due to the absence of peptides, whereas fraction 2,3,4 were tested for their antioxidant potential. Based on these results (see afterwards), fraction 2 was recognized as the most active, and subjected to UHPLC-MS/MS to identify peptide sequences. Thus it was sub-fractionated into fraction 2A and 2B. The UV (220nm) and total ion chromatograms (TIC) are reported in Fig. 2.

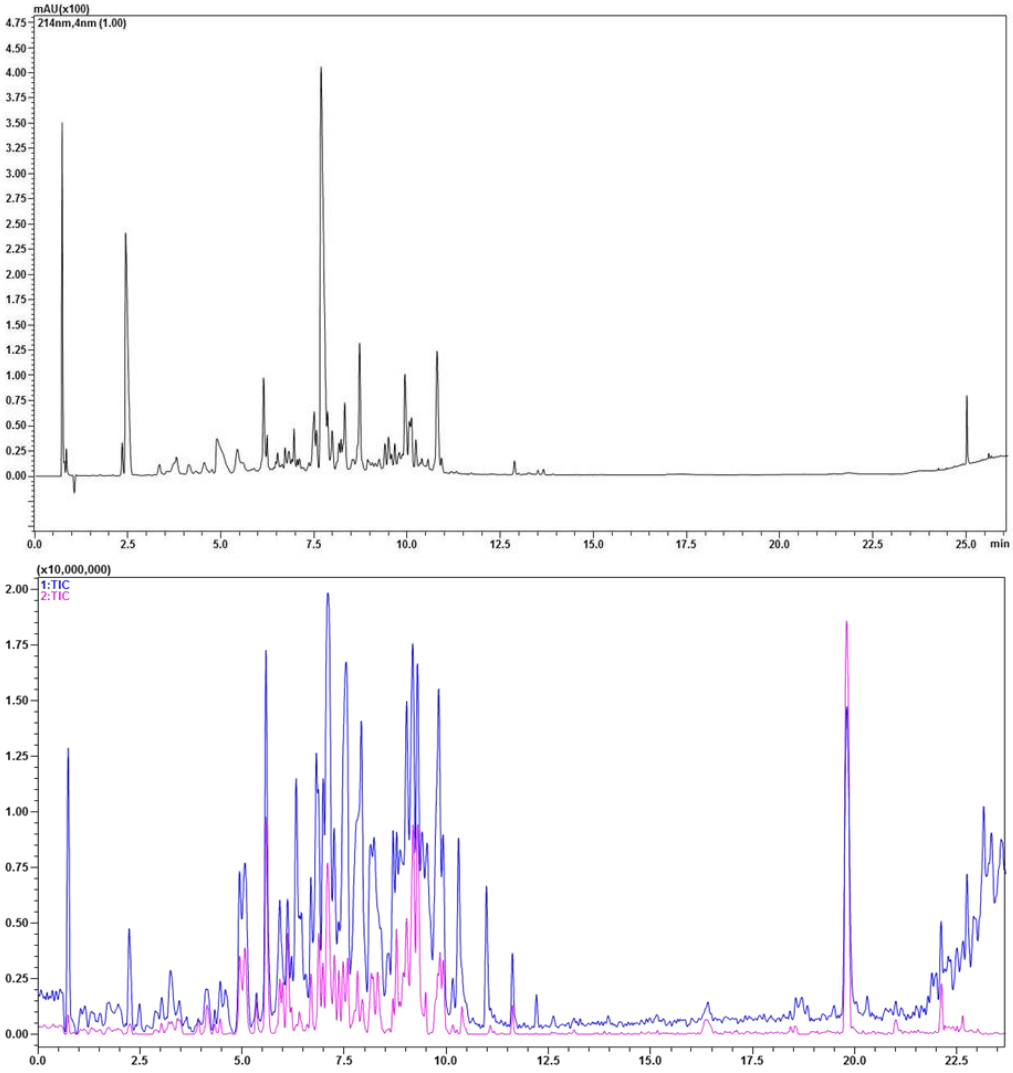

Figure 2. *Uv and Total ion chromatogram of fraction 2.*

Several peptides were identified, derived mainly from β-casein and αs2-casein (Table 1).

Peptide	-10lgP	Mass	Error (ppm)	m/z	z	Rt	Protein
K.EAMAPK.H	31.25	645.3156	-0.5	646.3225	1	7.10	P02666|CASβ_BOVIN
K.VKEAMPK.H	19.85	872.4789	-5.6	437.2443	2	8.23	P02666|CASβ_BOVIN
R.DMPIQ.H	12.46	602.2734	-2.5	603.2792	1	9.90	P02666|CASβ_BOVIN
K.AVPYPQ.R	8.37	673.3435	2.2	674.3523	1	9.83	P02666|CASβ_BOVIN
K.ITVDDK.H	22.78	689.3596	-1.2	690.3660	1	8.10	P02663|CASα2_BOVIN
K.TKLTEEEK.N	14.74	976.5007	-25.2	489.2488	2	7.77	P02663|CASα2_BOVIN
L.NEINQ.F	8.41	616.2816	-14.0	617.2803	1	6.03	P02663|CASα2_BOVIN
K.ITVDD.K	6.79	561.2646	-38.7	562.2501	1	8.41	P02663|CASα2_BOVIN
R.NAVPITPT.L	6.19	811.4440	-5.0	812.4472	1	11.09	P02663|CASα2_BOVIN
R.NAVPIT.P	5.56	613.3435	-1.3	614.3503	1	10.16	P02663|CASα2_BOVIN

Table 1. *Sequences of main peptides identified in fraction 2.*

Since this fraction showed the main activity, our attention was thus focused on the most abundant analytes of this fraction, which can contribute considerably to the bioactivity. In this regard two intense peaks, both belonging to sub-fraction 2B, possessing strongest UV absorbance and thus highest area percent, were selected. These hexapeptides P1 and P2, namely EAMAPK (β-CN f: 115-120) and AVPYPQ (β-CN f: 192-197) both detected as $[M+H]^+$, where selected for solid-phase synthesis and further tested, MS/MS spectra and fragmentation pattern are reported in Fig. 3.

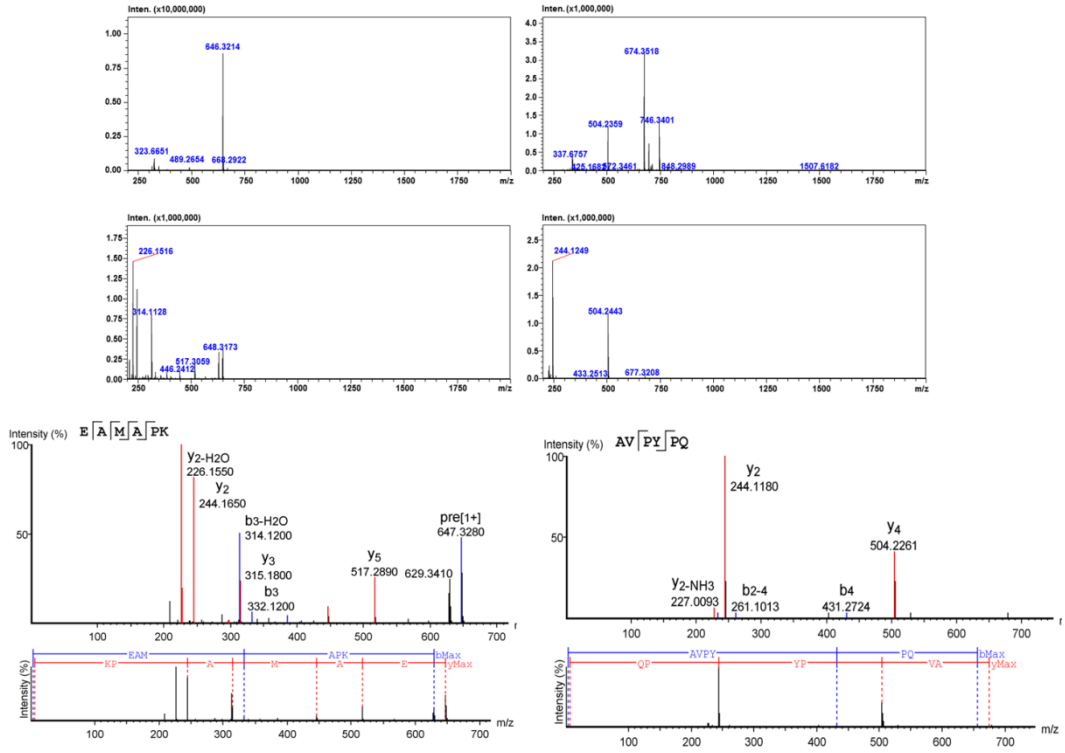

Figure 3. *Full scan, MS/MS and fragmentation pattern of peptides P1 and P2.*

In order to quantify the amount of these peptides in the [G.I.] digest a standard curve of each analogue peptide was prepared in water in a with five point concentration range and quantification carried out by external calibration. Results showed that peptides P1 and P2 were present respectively in 5.9 and 0.6 mg/100mg of [G.I.] digest. Even if present, free aminoacids were not detected by MS/MS (lowest m/z value 250), since we focused mainly on small-medium peptides.

2.3.2. Peptide Fractions and analogues didn't affect IEC-6 viability

In order to evaluate if [G.I.] digest, fractions and analogues should affect IEC-6 viability, cells were treated with the analytes for 24, 48 and 72 h. Our data

indicated that viability of IEC-6 cells was not affected by analytes at the concentrations used for our experiments.

2.3.3. Antioxidant effects on H_2O_2-treated IEC-6 cells

In order to evaluate the anti-oxidant potential of Stracchino peptides at intestinal level, they were tested in a model of H_2O_2-induced oxidative stress IEC-6 cells.

Effects on ROS release. To investigate whether $^{G.I.}$ digest, fractions and synthetic peptides influenced oxidative stress induced by H_2O_2 in IEC-6 cells, their effect on intracellular ROS release were firstly evaluated. Treatment with H_2O_2 (1 mM) induced a significant increase in ROS release of after 1 h ($P<0.001$ vs control; Fig. 4A, B). When $^{G.I.}$ digest and fractions 2,3,4 (250-5 μg/mL) were added to IEC-6 cells, 1 h before and simultaneously to H_2O_2 stimulation, ROS production resulted inhibited ($P<0.001$ vs H_2O_2, Fig. 4A). In particular among tested fractions, fraction number 2 exerted the highest effect in inhibiting ROS release in IEC-6 cells ($P<0.001$ vs H_2O_2, $P<0.001$ vs $^{G.I.}$ digest; Fig. 4A). In the same experimental conditions, it was observed that sub-fractions 2A and 2B and peptides 1 and 2 also inhibited ROS release ($P<0.001$ vs H_2O_2): in particular 2B sub fraction showed the highest effect in inhibiting ROS release in IEC-6 at the lower concentrations ($P<0.001$ vs fraction 2; Fig. 4B). Among the peptides, P2 (AVPYPQ) exerted the highest effect in inhibiting ROS release in IEC-6 cells ($P<0.01$ vs fraction 2; Fig. 4B).

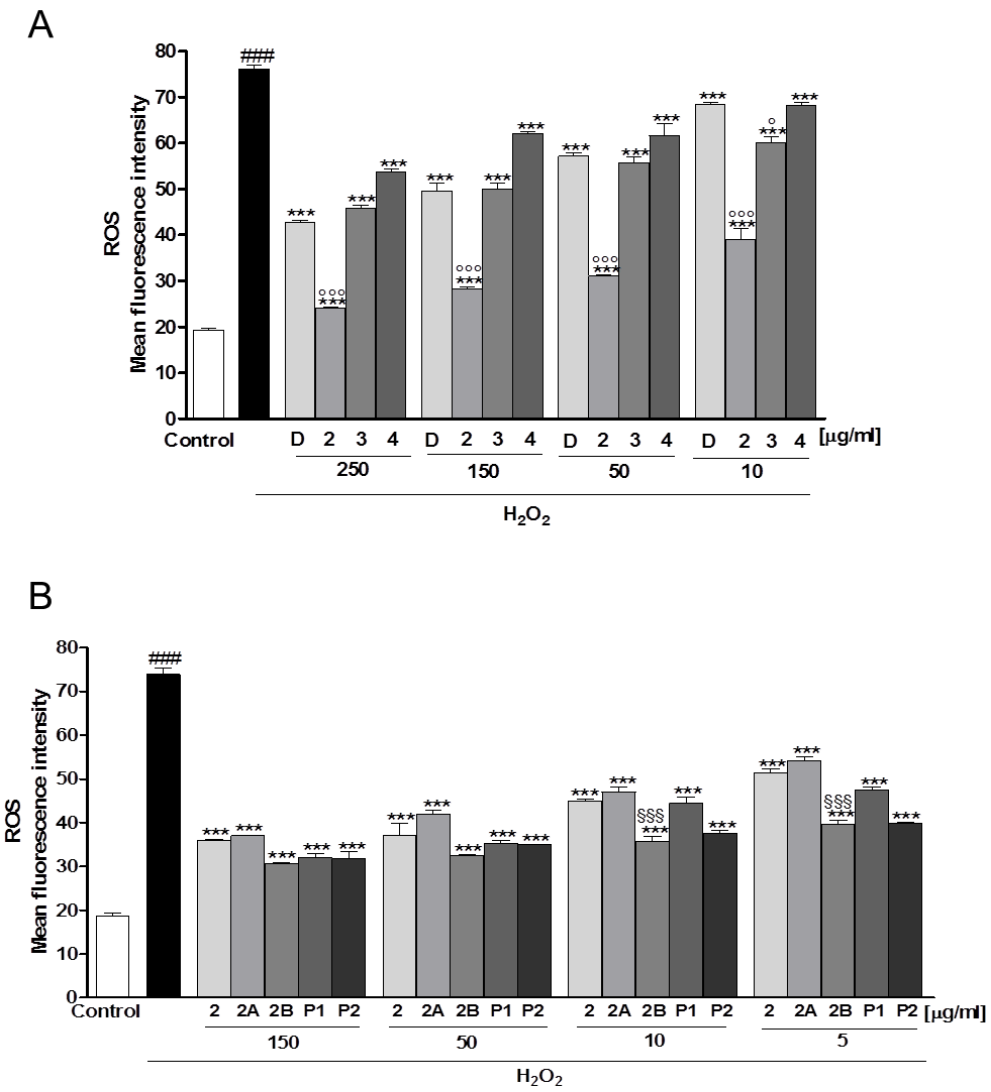

Figure 4. *Effect of Stracchino soft cheese fractions (G.I.D; fractions 2,3,4; 250–10 µg/mL; A) and of Stracchino soft cheese sub-fractions (2A and 2B; 150-5 µg/mL; B) and peptides (1 and 2; 150-5 µg/mL; B) on ROS formation, evaluated by means of the probe 2',7' dichlorofluorescein-diacetate (H2DCF-DA), in IEC-6 cells stimulated with H_2O_2. Values, mean ± s.e.m., are expressed as mean fluorescence intensity. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ### denotes P<0.001 vs control; *** denotes P<0.001 vs IEC-6 treated with H_2O_2. °°° and ° denote P<0.001 and P<0.05 vs gastrointestinal digest. §§§ denotes P<0.001 vs fraction 2.*

Effects on SOD expression. SOD is metal ion cofactor-requiring enzyme that catalyze dismutation of $O_2^{\bullet}$ into O_2 and H_2O_2 and its increased levels resulted beneficial [20]. SOD was significantly increased by H_2O_2 ($P<0.001$ vs control; Fig. 5A, B). G.I. digest significantly increased SOD expression ($P<0.05$ vs H_2O_2). Between tested fractions, fraction 2 showed again the highest activity also on SOD expression ($P<0.001$ vs H_2O_2, $P<0.01$ vs G.I. D; Fig. 5A). Regarding sub-fractions and peptides, 2B and P1 (EAMAPK) showed respectively the highest activity on SOD expression at the lower concentrations ($P<0.001$ vs H_2O_2, $P<0.05$ vs fraction 2; Fig. 5B).

Effects on Nrf2 activation. In order to evaluate Nrf-2 activation, we labeled Nrf-2 with a green fluorescence to track the influence of G.I. digest fractions and peptides P1 and P2 on its activation. As shown in Fig. 6, among tested fractions, fraction 2 and P1 (EAMAPK) showed the stronger effect in activating Nrf2. The effects on Nrf2 products revealed a significant effect also on NQO1 expression ($P<0.001$ vs H_2O_2, Fig. 7A). Among sub-fractions and peptides, 2B and P1 (EAMAPK) showed the highest activity on NQO1 (Fig. 7B). Another product of Nrf2 activation is HO-1. HO-1 resulted significantly increased by H_2O_2 ($P<0.001$ vs control; Fig. 7C, D). Between tested fractions, fraction 2 showed the highest activity on HO-1 expression ($P<0.05$ vs H_2O_2, $P<0.001$ vs G.I. D; Fig. 7C). Regarding sub-fractions and peptides, 2B and P1 showed again the highest activity on HO-1 expression ($P<0.001$ vs H_2O_2 , Fig. 7D).

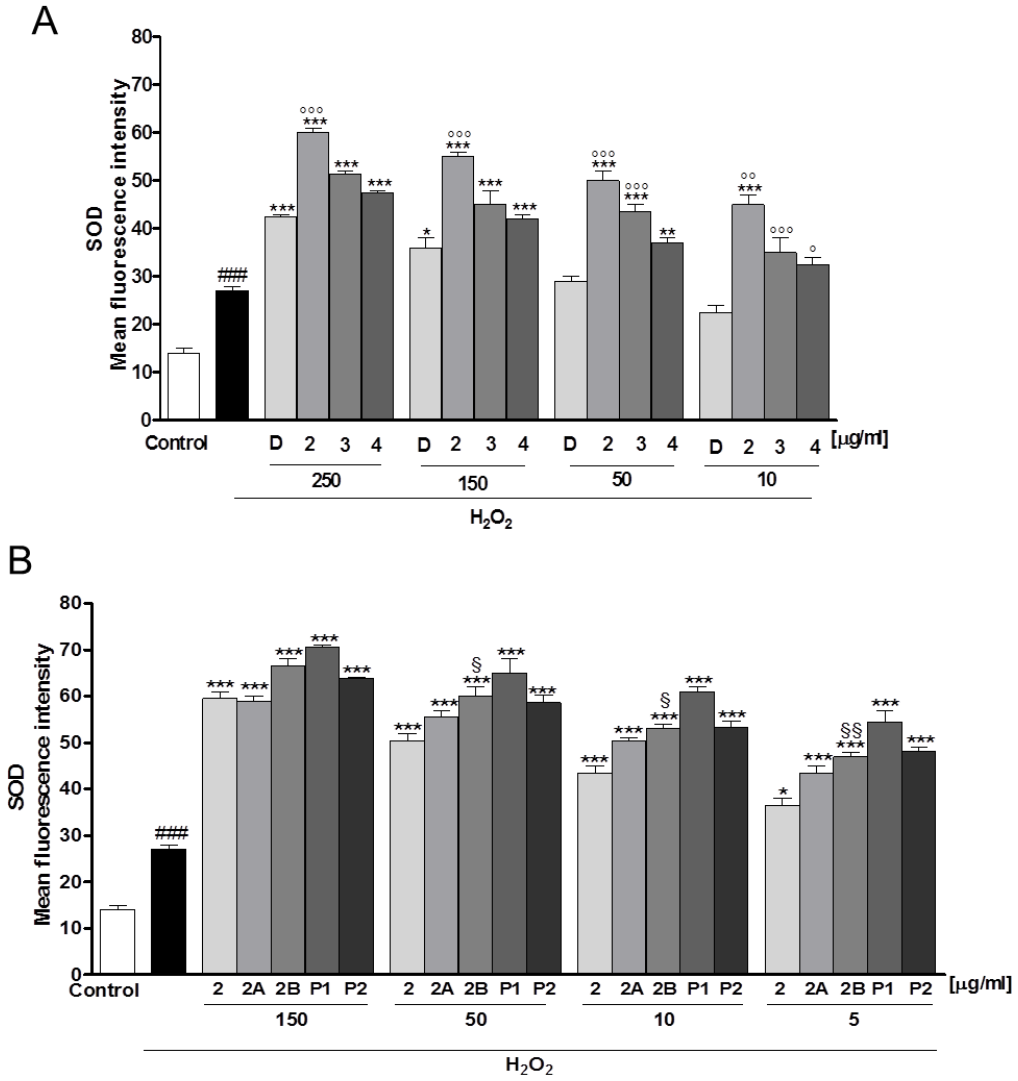

Figure 5. *Effect of Stracchino soft cheese fractions (G.I.D; fractions 2,3,4; 250–10 µg/mL; A) and of Stracchino soft cheese sub-fractions (2A and 2B; 150-5 µg/mL; B) and peptides (P1 and P2; 150-5 µg/mL; B) on SOD expression in IEC-6 cells stimulated with H_2O_2. Values, mean ± s.e.m., are expressed as mean fluorescence intensity. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ### denotes P<0.001 vs control; ***, ** and * denote P<0.001, P<0.01 and P<0.05 vs IEC-6 treated with H_2O_2. °°°, °° and ° denote P<0.001, P<0.01 and P<0.05 vs G.I.D. § denotes P<0.05 vs fraction 2.*

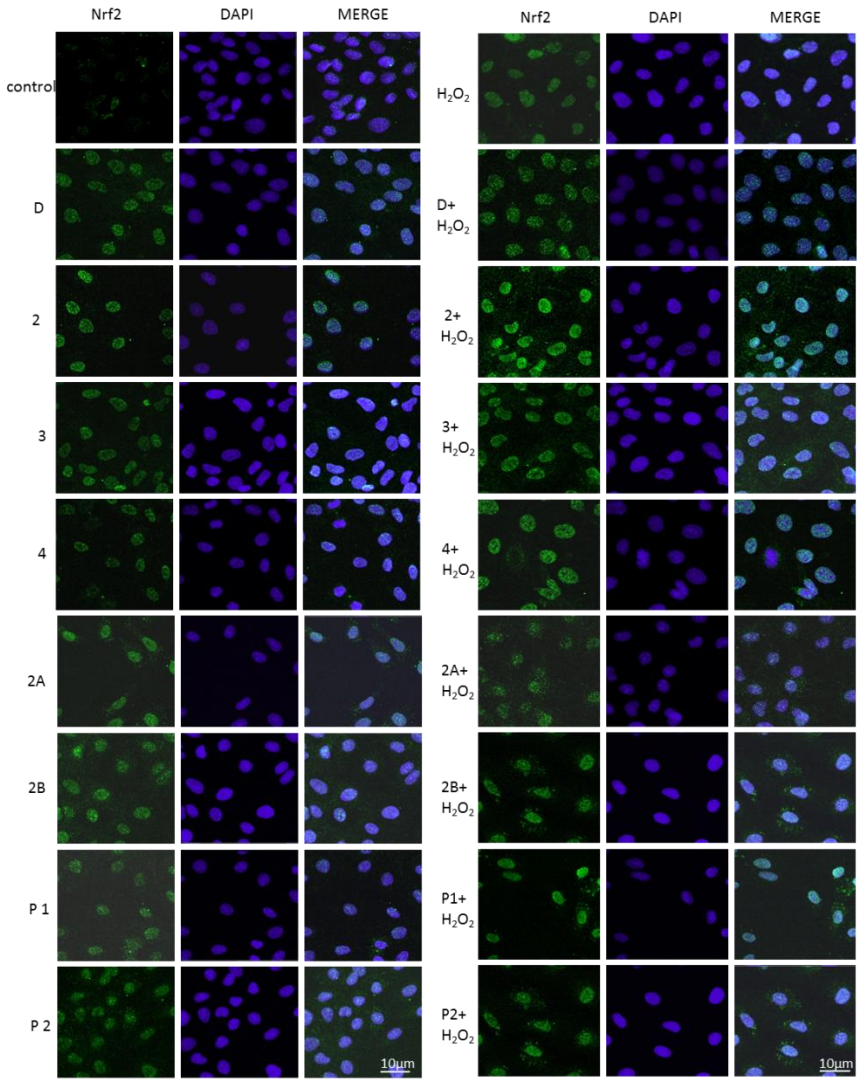

Figure 6. *Effect of Stracchino soft cheese fractions (G.I.D; fractions 2,3,4; 250–10 µg/mL) and of Stracchino soft cheese sub-fractions (2A and 2B; 150-5 µg/mL) and peptides (1 and 2; 150-5 µg/mL; B) on H_2O_2-induced Nrf2 nuclear translocation in IEC-6 cells. Nuclear translocation of Nrf2 was detected using immunofluorescence assay at confocal microscopy. Scale bar, 10µm. Blue and green fluorescences indicate localization of nucleus (DAPI) and Nrf2 respectively. Analysis was performed by confocal laser scanning microscopy.*

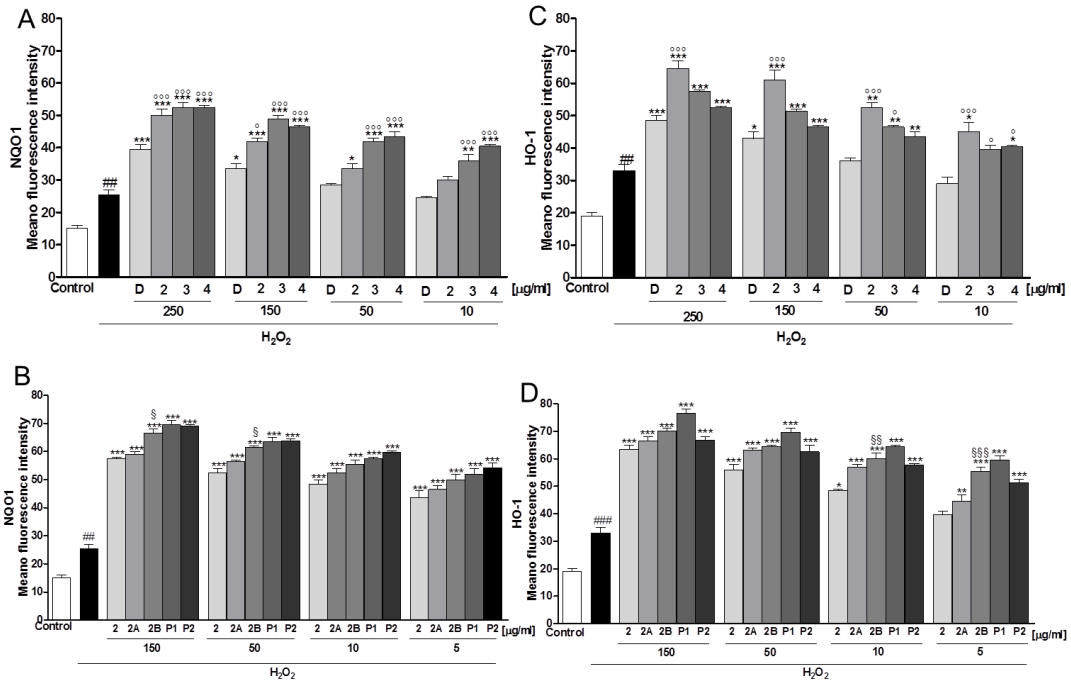

Figure 7. *Effect of Stracchino soft cheese fractions (G.I.D; fractions 2,3,4; 250–10 µg/mL; A, C) and of Stracchino soft cheese sub-fractions (2A and 2B; 150-5 µg/mL; B, D) and peptides (1 and 2; 150-5 µg/mL; B, D) on NQO1 (A, B) and HO-1 (C, D) expression in IEC-6 cells stimulated with H_2O_2. Values, mean ± s.e.m., are expressed as mean fluorescence intensity at least 3 independent experiments with three replicates each. Comparisons were performed using one-way analysis of variance and multiple comparisons were made by Bonferroni's test. ### denotes P<0.001 vs control; ***, ** and * denote P<0.001, P<0.01 and P<0.05 vs IEC-6 treated with H_2O_2. °°° and ° denote P<0.001 and P<0.05 vs G.I.D. §§§ denotes P<0.001 vs fraction 2.*

Evaluation of permeation through Caco-2 cells. In order to evaluate the stability and bioavailability of peptides P1 and P2 single layers of Caco-2 cells were employed. Peptides were quantified by UHPLC-MS/MS with the same condition reported previously. UHPLC–MS/MS analysis revealed no significant hydrolysis (less than 20%) of both peptides in the apical solution by the brush border peptidases after 120 min incubation (Fig. 8 A, B).

Moreover, the analysis of basolateral solution showed that P1 and P2 were absorbed through Caco-2 monolayer with a concentration-dependent transport following a saturable pattern described by a linear curve (Fig. 8 C, D). The amount transported was estimated as 8.2% and 5.8% respectively. It is noteworthy to observe that these concentration have been employed for antioxidant assays.

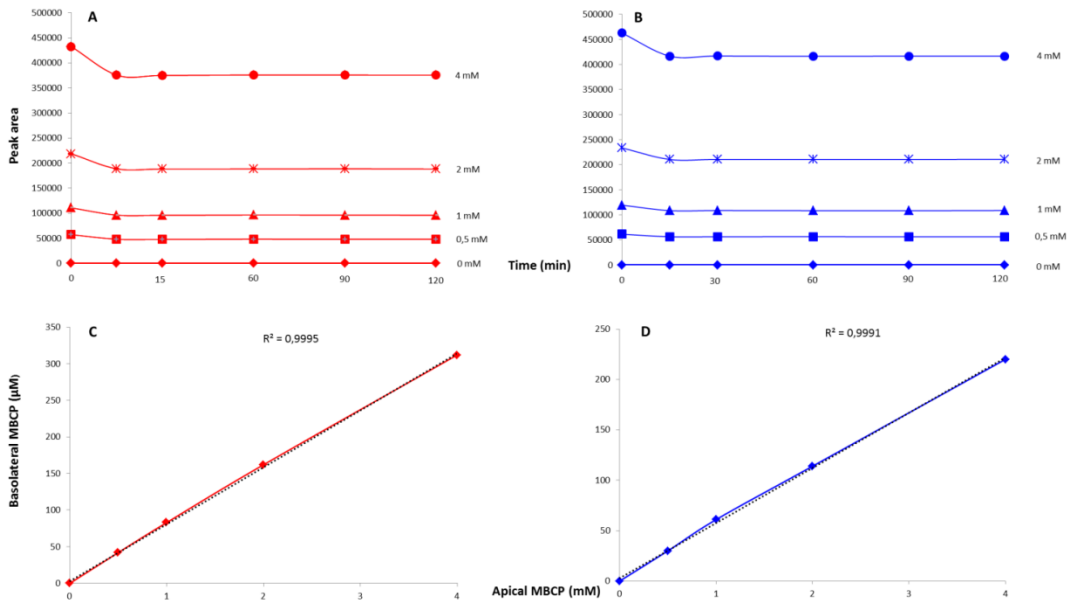

Figure 8 (A, B): *Stability of peptides P1 and P2 to the brush border exopeptidases measured as change in UHPLC-PDA-MS/MS chromatogram peak areas of P1 and P2 introduced at different concentrations in the apical compartment of CaCo-2 cell monolayer.*
Figure 8 (C, D): *Calibration curve relative to the quantification of peptides P1 and P2 in apical and basolateral solutions.*

2.4.Conclusions

The release of bioactive peptides from dairy products after gastro-intestinal digestion has been highlighted by several papers [21], but never investigated in Stracchino cheese so far. Among peptides resulting from Stracchino G.I. digestion two abundant hexapeptides, possessing sequence EAMAPK and AVPYPQ, identified by MS/MS as β−CN residues, showed the most interesting activity. The formation of these peptides have been previously reported during digestion of caseins and other dairy products [22]. In particular the peptide EAMAPK is probably derived by a subsequent cleavage of the residue β−CN f113-120 (VKEAMAPK), which showed antioxidant activity in vitro against the oxidation of linolenic acid [23]. The ABTS radical scavenging assay measures the potential of an antioxidant to inhibit the formation of a colored $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm [24]. Peptides P1 and P2 showed ABTS radical scavenging activity with a trolox equivalent value of 23 ± 4.1 and 19.5 ± 2.4 μmol / mg, our results were in good agreement with previous in vitro antioxidant measurements on casein peptides [25]. The activity of these peptides could be ascribed to the presence of a methionine residue, which can be oxidized as sulfone, and to tyrosine, which can be oxidized in the corresponding quinone methide, as well as to triptophane and proline [26-27]. The gastrointestinal tract is a key source of ROS. Despite the protective barrier provided by the epithelial layer, ingested materials and pathogens can cause inflammation by activating the epithelium, polymorphonuclear neutrophils and macrophages to produce inflammatory cytokines and other mediators that further contribute to oxidative stress. The pathogenesis of various disease also at G.I. level including peptic ulcers, gastrointestinal inflammatory bowel disease and G.I. cancers are in part due to excessive oxidative stress [28] thus ROS regulation

resulted beneficial in these conditions. Our results indicated that $^{G.I.}$ digest, fractions 2,3,4 and P1 and P2 are able to reduce ROS release in H_2O_2-treated IEC-6. The reduction of ROS release can be also linked related to SOD expression. $^{G.I.}$ digest, fractions 2,3,4 and P1 and P2 in our experimental conditions are also able to increase the expression of this antioxidant defence system, a similar effect has been reported in macrophages by casein glycomacropeptide hydrolysates [29]. A recurrent theme in oxidant signalling and antioxidant defence is reactive cysteine thiol-based redox signalling. In this regard Nrf2 is an emerging regulator of cellular resistance to oxidants, the activation of Nrf2 pathway. Nrf2 controls the basal and induced expression of an array of antioxidant response element-dependent genes that regulate the physiological and physiopathological outcomes of oxidant exposure such as NQO1 and HO-1 [30-31]. NQO1 is a flavoprotein that catalyzes the metabolic detoxification of quinones and their derivatives to hydroquinones, using either NADH or NADPH as the electron donor. This protects cells against quinone-induced oxidative stress, cytotoxicity, and mutagenicity [32]. HO-1 is the rate limiting enzyme in the conversion of haem into biliverdin/bilirubin, iron and carbon monoxide (CO); all of them can potentially function as antioxidants [33]. It is strongly suggested that HO-1 provides a potent cytoprotective effect, as shown in various in vitro and in vivo models of cellular and tissue injury [34-35]. HO-1 is inducible by a variety of oxidative stresses and is thought to play an important role in the protection of tissues from oxidative injuries. In this study we report that $^{G.I.}$ digest, fractions 2,3,4 and P1 and P2 are able to activate Nrf2 antioxidant response. Our data are also in according with the effect of β−casein phosphopeptides and casein peptides containing glutamic acid residues [36-37]. These results show that stracchino peptides were able both to reduce ROS release and to activate a strong antioxidant response, increasing SOD expression and Nrf2 activation, thus highlighting their potential use as

antioxidants. Although many peptides possess in vitro antioxidant activity, it is highly favorable that they should be absorbed and reach the systemic circulation [38]. In this regard sequence and molecular weight play a key role [39]. It has been reported that small peptides (2-6 residues) are absorbed more rapidly with respect to larger peptides [40]. For this purposes we assessed the bioavailability of most abundant peptides P1 and P2, by using single layers of Caco-2 cells as a model of absorption in the small intestine, which possesses microvillus structure, tight junction at the apical side, carrier-mediated transport systems, but also expresses brush border membrane peptidases, as the human intestinal epithelium. UHPLC–MS/MS analysis of basolateral solution revealed that P1 and P2 were absorbed almost intact through Caco-2 monolayer, with no significant degradation. The amount of P1 and P2 transepithelially transported were respectively 8.2% and 5.8%, which is higher than reported previously for similar studies [41]. The resistance of P1 and P2 to hydrolysis in the intestinal tract could be related to the presence of residues such as proline and glutamic acid in their sequences as reported by other observations [42]. In this case several mechanism can be hypothesized, such as hydrogen bonds with lipid phosphates of cell membranes, or by passive paracellular transport via tight junctions has been usually reported to be normally applicable to the absorption of water soluble low molecular weight and short-chain peptides. Our results are in accordance with other studies which evidenced both the stability of several β-Casein sequences as well as their ability to pass through Caco-2 cells monolayers [41,43]. Despite this, the potential antioxidant effects could be also exerted by the not absorbed peptides amount at local level. The present study highlights Stracchino soft cheese as a source of potential bioactive peptides released after G.I. digestion. The tested peptide fractions showed significant effects in reducing the oxidative cellular stress, inhibiting ROS release and increasing an antioxidant response, as SOD

expression and Nrf2 pathway activation. Among the characterized peptides, two hexapeptides, namely EAMAPK and AVPYPQ, showed the most significative action. Those peptides were abundant in the G.I. digest, and were characterized by a discrete bioavailability, tested by crossing Caco-2 cells. The in vitro G.I. digestion together with the Caco-2 absorption can provide many informations for in vivo correlations. Obviously other parameters, which are present in vivo, should be carefully considered, such as the presence of other food components, as lipids and sugars, as well as the pharmacokinetic and stability of the peptides, which are capable to enter in the systemic circulation. The results indicate how this cheese can be considered for his healthy properties, as well as his peptides as an "ingredient" for nutraceuticals formulations and functional and personalized foods.

2.5. References

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CHAPTER III

Qualitative and Quantitative Evaluation of the Variation of the Lipid Fraction Composition After the Expiration Date in Commercial Samples of Bovine Milk from Centrale del Latte di Salerno

Abstract

The alteration of both organoleptic characteristics and nutritional properties of milk is caused by the enzymatic activities of endogenous and microbial lipoprotein lipases, which catalyze the lipolysis process yielding the release of free fatty acids from triglycerides.

In this study, 15 commercial samples of bovine milk (whole, semi-skimmed and skimmed) were analyzed, at different weeks after their expiration date, in order to qualitatively and quantitatively evaluate the variation of the lipid fraction composition. The lipase activity was evaluated spectrophotometrically, by measuring the amount of p-nitrophenol released from p-nitrophenyl butyrate used as the substrate of milk lipase.

GC-MS analysis showed a decrease in the amount of unsaturated fatty acids over time, whereas the hydrolysis of triglycerides is time-independent, but dependes on the values of pH and total titratable acidity of the milk sample.

Keywords

bovine milk, free fatty acids, total activity lipase, total titratable acidity

Abbreviations

Fatty acids, FFA; Lipoprotein lipase, LPL; Polyunsaturated fatty acids, PUFAs; Tris buffered saline, TBS; Liquid-liquid extraction, LLE; Centrale del latte di Salerno, CdLdS; Fatty acid methyl esters, FAME; Mass Spectrometry, MS; Gas Chromatography, GC; Total titratable acidity, TA; Ethylenediaminetetraacetic acid, EDTA;Soxhlet-Henkel degree, °SH; Total lipase activity, TLA; Ethylenediaminetetraacetic acid, EDTA; Phenylmethanesulfonyl fluoride, PMSF; Thiobarbituric acid reactive

substances, TBARS; malonaldehyde, MDA; thiobarbituric acid, TBA; 1,1,3,3-tetraethoxypropane, TEP; milk fat globule membrane, MFGM.

3.1. Introduction

The main reactions taking place in the development of off-flavours in milk are lipid oxidation and lipolysis. These are the main phenomena that participate in the modification of the overall flavor and can reduce shelf life and quality of pasteurized fluid milk.[1] The oxidative stability of milk is affected by the presence of pro-oxidative factors (such as oxygen, light, endogenous and exogenous metals, and enzymes such as xanthine oxidase and lactoperoxidase) and antioxidative factors (i.e, ascorbic acid, tocopherols, carotenoids, thiols, and enzymes such as superoxide dismutase), and by the balance between them.[2,3] Cholesterol, fatty acids in triacylglycerols, partial glycerides, and free fatty acids, particularly unsaturated fatty acids, are vulnerable to oxidation and give rise to unstable hydroperoxides, which decompose into a wide range of carbonyl compounds, such as saturated and unsaturated aldehydes followed by lesser amounts of unsaturated ketones, saturated and unsaturated hydrocarbons, semialdehydes, and saturated and unsaturated alcohols.[2] Lipolysis in milk consists in the hydrolysis of triglycerides into free fatty acids (FFA) and partial glycerides. This process can be mediated by the lipoprotein lipase (LPL) naturally occuring in milk, or by the microbial lipases from psychrotrophic bacteria contaminating raw milk during cold storage.[4-6] Since LPLs are relatively unstable to heat, pasteurization inactivates most of the enzyme, and therefore, lipolysis does not occur in pasteurized homogenized milk.[7] The release of short- and medium-chain FFA (from C_4 to C_{10}) and their subsequent conversion to other acids and/or ethyl esters by microbial lipases produces detrimental features such as rancid smell and taste and

functionality defects.[8] Many strategies have been adopted in the attempt to adjust the balance between various fatty acids in milk fat to higher nutritional levels and to increase the benefits for human health. As a matter of fact, dairy cows have been fed on fats rich in unsaturated fatty acids to increase the content in polyunsaturated fatty acids (PUFAs) of milk fat.[9-10] Nevertheless, any increase in PUFAs content had negative effects on milk, making it more susceptible to oxidation.[11] In fact, lipid oxidation of milk is highly influenced by content of long-chain unsaturated fatty acids, which are particularly vulnerable to oxidation and can give rise to development of off-flavors at a rate which especially depends on the extent of unsaturation.[12,13] Therefore, dairy products from milk of cows fed on diets rich in PUFAs are prone to oxidation if no precautions against oxidation are taken, such as adding tocopherol to the raw milk.[14,15] In dairy products with a highly unsaturated fatty acid profile oxidation causes , especially after storage, oxidized, metallic, oily, or stale flavors and a paler color.[16,17] Additionally, cheese with a highly unsaturated fatty acid profile tends to taste flat.[18-19]

3.1.1. Aim of work

In the second year of my PhD, the degradation of the lipid fraction of milk samples stored at room temperature, after expiration date, was analyzed in order to identify a time window for the recovery of bioactive molecules.

The objective of this study was to assess the qualitative and quantitative variation of single fatty acids in cow milk samples before expiration date and within 28 days after expiration date in order to monitor how the profile of the lipid fraction is influenced by different physicochemical parameters.

3.2. Materials and methods

3.2.1. Chemicals

Acetonitrile, clarifying reagent for dairy products, ethylenediaminetetraacetic acid, N,N-dimethylformamide, n-exane, 4-nitrophenol, 4-nitrophenyl butyrate, phenolphthalein, phenylmethanesulfonyl fluoride, 2-propanol, sodium sulfate, sodium hydroxide and Tris buffered saline (TBS) were purchased from Sigma-Aldrich (Milan, Italy).

3.2.2. Sample preparation

Commercial samples of bovine milk (skimmed, semi-skimmed and whole) from Centrale del latte di Salerno (CdLdS) (Salerno, Campania, Italy), were analyzed; samples were preserved at room temperature and the corresponding fatty acids were extracted at different weeks after expiration date. The lipid fraction was obtained from milk by liquid-liquid extraction (LLE).[20] An aliquot of 15 mL of milk was treated with 50 mL of a mixture of isopropanol and *n*-hexane (3:2 v/v). This solution was stirred vigorously for 10-15 min and the hexane phase was recovered,. The extraction procedure was repeated two times and the combined hexane fractions were dehydrated first with 15 mL of 0.47 M Na_2SO_4 solution, then treated with Na_2SO_4 (3 g) for final dehydration. After solvent removal under vacuum, the lipid extract was dried at 40 °C to constant weight.

An aliquot of 50 μg of lipid extract was dissolved in dry toluene (1 mL), then treated with 1 M sodium methoxide in dry methanol (1 mL), to convert fatty acids in complex lipids into their corresponding methyl esters (FAMEs), and analyzed by GC-MS.

3.2.3. GC-MS conditions

GC–MS analysis was performed on an Agilent 6850 Series II apparatus, fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm i.d.), 0.33 μm film thickness, coupled to an Agilent Mass Selective Detector MSD 5273; ionization voltage, 70 eV; electron multiplier energy, 2000 V; scan range, 40–550. Gas chromatographic conditions were as follows. Injector and detector temperatures, 250 and 280 °C, respectively. Column temperature was set at 140 °C for 10 min, followed by a first ramp at 15 °C min^{-1} until 200 °C for 1 min. The second ramp was at 10 °C min^{-1} until 230 °C for 1 min, the third ramp at 0.4 °C min-1 until 233 °C for 3 min and the fourth ramp at 0.5 °C min^{-1} until 238 °C for 2 min. Total analysis time was 41.50 min. Gases flow rates (White Martins) were 30 mL min^{-1} for hydrogen, 30 mL min^{-1} for nitrogen and 250 mL min^{-1} for synthetic air. Injections of 1.2 μL were performed in duplicate. The peak areas of FAMEs were determined by ChromQuest 4.1 software.

3.2.4. Acidity and pH analysis

The pH value and total acidity were determined in commercial milk samples (skimmed, semi-skimmed and whole milk) at different weeks after their expiry date. The pH values were measured using a digital pH meter of a glass electrode (Basic 20, Crison). Prior to use, the pH meter was calibrated with standard buffer solution (pH 4 and 7). Total titratable acidity (TA) was determined according to the method of AOAC.[21] TA was determined by measuring the volume (mL) of 0.25 N sodium hydroxide required to titrate 100 mL of milk sample, using phenolphthalein as an indicator (five drops). The milk sample was titrated to a faint pink color that persisted after vigorous shaking (end-point). The volume of titrant consumed corresponds to the

amount of lactic acid present in the sample. The results were expressed in Soxhlet-Henkel degree (°SH) and are reported in Table 1.

	Samples Milk	**T0**	**I Week**	**II Week**	**III Week**	**IV Week**
pH value[a]	Skimmed Milk	6.71 ± 0.010	4.33 ± 0.006	4.23 ± 0.006	4.91 ± 0.012	5.85 ± 0.021
	Semi-Skimmed Milk	6.72 ± 0.015	5.51 ± 0.015	4.55 ± 0.010	4.28 ± 0.010	4.41 ± 0.006
	Whole Milk	6.72 ± 0.015	5.38 ± 0.046	4.61 ± 0.007	5.22 ± 0.021	4.73 ± 0.006
Total acidity [a,b]	Skimmed Milk	6.36 ± 0.029	35.57 ± 0.492	58.15 ± 0.460	28.93 ± 0.404	16.70 ± 0.001
	Semi-Skimmed Milk	6.17 ± 0.029	23.41 ± 0.402	52.22 ± 0.076	37.00 ± 0.500	41.66 ± 0.306
	Whole Milk	6.17 ± 0.100	30.20 ± 0.050	56.75 ± 0.035	20.56 ± 0.475	45.45 ± 0.189

[a] Samples Milk were analized in triplicate.
[b] The data were expressed as Soxhlet Henkel degree.

Table 1. *Titratable acidity and pH values of samples of milk skimmed, semi-skimmed and whole, at several weeks after their expiration date.*

3.2.5. Total lipase activity assay

In this work a spectrophotometric method was used in order to determine the total activity of the lipase (TLA) in milk samples, thus determining the amount of free fatty acids (FFA) liberated from the triglycerides contained in bovine milk.[22]

Through this method it is possible to quantify the level of p-nitrophenol (A_{max} 400 to 420 nm) released after the lipase-catalyzed hydrolysis of p-nitrophenyl butyrate, used as a substrate. The analysis was performed on different milk samples (skimmed, semi-skimmed and whole milk) as well as at different weeks after the expiration date.

The lipase activity was assayed by detecting the product, p-nitrophenol, spectrophotometrically with a Genesys 10s UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.) equipped with xenon lamp and quartz cuvettes 1 cm length. The analyses were carried out at room temperature and the

absorbance was measured at 420nm. The p-nitrophenol was liberated by incubating 500 µl of milk sample with 50 µl of 0.05 mM p-nitrophenol butyrate standard solution and 2 mL of 0.05 M Tris buffer, pH 7.6 at 37 °C for exactly 30 min. Results of this test are based on the yellow color reaction of p-nitrophenol. The reaction was terminated by incubation at 37 °C for 10 min with 400 µL of a solution composed by 3:1 (v/v) 0.06 M EDTA pH 7.6 in NaOH 2N, and Phenylmethanesulfonyl fluoride (PMSF) 0.06 M in dimethylformamide. This solution was diluted with 2 mL of clarifying reagent solution at 37 °C for 5 min, to render casein micelles and fat globules soluble and allow direct spectrophotometric measurements without preliminary separation. The final solution was spectrophotometrically analyzed at 420 nm against corresponding solvent blank, which was prepared in the same assay conditions, except that lipase activity inhibitors were added to the milk sample before adding the substrate solution. Samples were tested in triplicate (Table 2).

	Samples Milk	**T0**	**I Week**	**II Week**	**III Week**	**IV Week**
Total acidity [a, b]	Skimmed	6.36 ± 0.029	35.57 ± 0.492	58.15 ± 0.460	28.93 ± 0.404	16.70 ± 0.001
	Semi-Skimmed	6.17 ± 0.029	23.41 ± 0.402	52.22 ± 0.076	37.00 ± 0.500	41.66 ± 0.306
	Whole	6.17 ± 0.100	30.20 ± 0.050	56.75 ± 0.035	20.56 ± 0.475	45.45 ± 0.189
Lipase activity [a,c]	Skimmed	0.024 ± 0.011	0.0044 ± 0.0013	0.0004 ± 0.00015	0.0137 ± 0.006	0.017 ± 0.005
	Semi-Skimmed	0.015 ± 0.006	0.0037 ± 0.0011	0.0008 ± 0.00016	0.0011 ± 0.0014	0.001 ± 0.0004
	Whole	0.006 ± 0.001	0.0012 ± 0.0003	0.0007 ± 0.00015	0.0054 ± 0.0012	0.001 ± 0.0001

[a] Samples Milk were analized in triplicate.
[b] The data were expressed as Soxhlet Henkel degree.
[c] The results were expressed as µmoli p-nitrophenol / ml reaction mixture.

Table 2. *Total lipase activity present in samples milk (skimmed, semi-skimmed and whole) at several weeks after their expiration date.*

Lipase activity was determined by comparing sample A_{420} values to those of a standard curve prepared with p-nitrophenol (Figure 1).

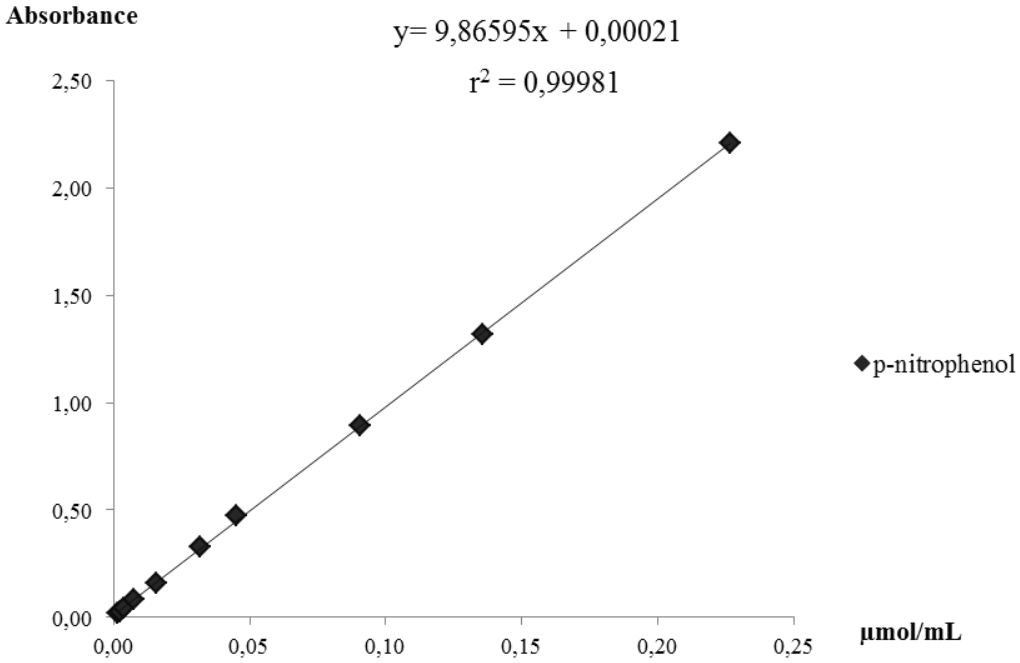

Figure 1. *Calibration curve of p-nitrophenol using UV–Vis spectrophotometer set at λ_{max} = 420nm.*

3.2.6. Determination of TBA reactive substances in lipid extract

The method for determination of 2-thiobarbituric acid reactive substances (TBARS) in milk according to AOCS (2005) was used, and the analysis was performed on whole milk samples at different weeks after the expiration date. Spectrophotometric analysis was carried out by Genesys 10s UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.) and the results were expressed as nmol/mL of malonaldehyde (MDA).

In this assay the MDA, indicator of the lipid oxidation process, reacts with thiobarbituric acid (TBA) to form a pink MDA:TBA complex (1:2) that is measured spectrophotometrically at its absorption maximum at 532 nm.

Briefly, 10 mg of lipid extract sample were dissolved in 5 mL 1-butanol and an ultrasonic water bath was used to ensure uniform mixing. 1.0 mL sample solution was incubated and shaked for 2 hr in 95°C with 1.0 mL of 0.2% TBA in 1-butanol. The solution was cool under running tap water for 10 min, until they reach room temperature. The final solution was spectrophotometrically analyzed at against corresponding solvent blank, 1-butanol and 0.2% TBA at the same time was prepared. 1,1,3,3-tetraethoxypropane (TEP) was used in the construction of a TBARS.

3.3. Results and discussion

3.3.1. Fatty acid profile

The fatty acid composition of skimmed, semi skimmed and whole milk before their expiry date (T0) is reported in Table 3.

Fatty acids		Whole milk[b]	Semi-skimmed milk[b]	Skimmed milk[b]
Saturated[a]		589.04	111.31	2.992
Butyric	4:0	34.25	6.39	0.17
Caproic	6:0	21.29	3.97	0.11
Caprylic	8:0	11.77	2.19	0.06
Capric	10:0	22.46	4.19	0.11
Lauric	12:0	25.52	4.76	0.13
Tridecanoic acid	13:0	0.84	0.16	0.004
Myristic	14:0	93.78	17.51	0.48
Pentadecenoic	15:0	13.80	2.58	0.07
Palmitic	16:0	251.70	46.99	1.28
Margaric	17:0	8.07	1.51	0.04
Stearic	18:0	103.90	19.4	0.53
Arachidic	20:0	1.66	1.66	0.008
Monounsaturated[a]		283.68	110.13	3.606
Myristoleic	14:1	8.99	3.49	0.09
Palmitoleic	16:1	12.13	4.70	0.12
Heptadec-10-enoic acid	17:1	5.60	2.17	0.05
Elaidic	18:1n-9t	25.51	9.89	0.27
Oleic	18:1n-9c	216.70	84.05	2.92
Vaccenic acid	18:1n-7t	9.47	3.78	0.10
Cis vaccenic acid	18:1n-7c	5.28	2.05	0.056
Polyunsaturated[a]		42.88	13.97	0.377
Linolelaidic	18:2n-6t	5.56	1.81	0.05
Trans-9, cis-12 acid octadienoic	18:2t9c12	2.54	0.83	0.02
Gamma-linoleic	18:2n-6	8.01	2.61	0.07
Alpha-linolenic	18:3n-3	3.82	1.24	0.03
Dihomo-gamma-linolenic acid	20:3n-6	11.99	3.91	0.11
Conjugated linoleic acid	CLAc9t11	10.18	3.32	0.09
Conjugated linoleic acid	CLAt10c12	0.78	0.25	0.007

[a] Data were presented as mg g^{-1}.
[b] Commercial samples of bovine milk, from Centrale del latte di Salerno, were analyzed to T0.

Table 3. *Fatty acids composition of the commercial samples of bovine milk (skimmed, semi-skimmed and whole) from Centrale del Latte di Salerno.*

The saturated fatty acids weight percentage (w/w) of total fatty acids was approximately 64.3 in whole milk, 47.3 in semi-skimmed milk and 42.9 in skimmed milk. Although there was a different percentage of saturated fatty acids in the three samples of milk, from a quantitative point of view the most important fatty acid was palmitic acid (16:0), which accounted for approximately 43% by weight of saturated fatty acids. Myristic acid (14:0) and stearic acid (18:0) made up 16 and 17.5% by weight of saturated fatty acids, respectively. Of the saturated fatty acids, 15% were short-chain fatty acids (C4:0–C10:0). The amount of mono-unsaturated fatty acids by weight of the total fatty acids was 31.0%, 46.8%, 51.7% in whole, semi-skimmed and skimmed milk, respectively. Oleic acid (18:1) was the main mono-unsatured fatty acid, accounting approximately for 77.5%. Poly-unsaturated fatty acids account for about 4.7%, 5.9%, 5.4% (w/w) of the total fatty acids contained in whole, semi-skimmed and skimmed milk, respectively. Among them, the most important are dihomo-gamma-linolenic acid (20:3n-6) and conjugated linoleic acid (CLAc9t11) accounting for 28.4 and 23.8% by weight of the poly-unsaturated fatty acids. The ratio between omega-6 and omega-3 fatty acids in whole and semi-skimmed milk was 2,1:1 while in skimmed milk is 2,3:1.

3.3.2. Fatty acid profile of milk after expire date

Analysis of whole milk samples carried out at different weeks after the expiration date showed a gradual decrease in the amount of unsaturated fatty acids, while unchanged quantities of saturated fatty acids were detected. After first, second, third and fourth week, the percentage of degradation of mono-unsaturated and poly-unsaturated fatty acids of whole and semi-skimmed milk was 25%, 44%, 58% and 69%, respectively (Table 4 and 5).

Whole milk	**T0**	**I Week**	**II Week**	**III Week**	**IV Week**
Monounsaturated[a]					
Myristoleic	8.99	6.74	5.06	3.79	2.84
Palmitoleic	12.13	9.10	6.82	5.12	3.84
Heptadec-10-enoic acid	5.60	4.20	3.15	2.36	1.77
Elaidic	25.51	19.13	14.35	10.76	8.07
Oleic	216.70	162.53	121.89	91.42	68.57
Vaccenic acid	9.47	7.10	5.33	4.00	3.00
Cis vaccenic acid	5.28	3.96	2.97	2.23	1.67
Polyunsaturated[a]					
Linolelaidic	5.56	4.17	3.13	2.35	1.76
Trans-9, cis-12 acid octadienoic	2.54	1.91	1.43	1.07	0.80
Gamma-linoleic	8.01	6.01	4.51	3.38	2.53
Alpha-linolenic	3.82	2.87	2.15	1.61	1.21
Dihomo-gamma-linolenic acid	11.99	8.99	6.74	5.06	3.79
Conjugated linoleic acid	10.18	7.64	5.73	4.29	3.22
Conjugated linoleic acid	0.78	0.59	0.44	0.33	0.25

[a] Data were presented as mg g^{-1}.

Table 4. *Fatty acid profile of whole milk at several weeks after their expiration date.*

Semi-skimmed milk	**T0**	**I Week**	**II Week**	**III Week**	**IV Week**
Monounsaturated[a]					
Myristoleic	3.49	2.62	1.96	1.47	1.10
Palmitoleic	4.70	3.53	2.64	1.98	1.49
Heptadec-10-enoic acid	2.17	1.63	1.22	0.92	0.69
Elaidic	9.89	7.42	5.56	4.17	3.13
Oleic	84.05	63.04	47.28	35.46	26.59
Vaccenic acid	3.78	2.84	2.13	1.59	1.20
Cis vaccenic acid	2.05	1.54	1.15	0.86	0.65
Polyunsaturated[a]					
Linolelaidic	1.81	1.36	1.02	0.76	0.57
Trans-9, cis-12 acid octadienoic	0.83	0.62	0.47	0.35	0.26
Gamma-linoleic	2.61	1.96	1.47	1.10	0.83
Alpha-linolenic	1.24	0.93	0.70	0.52	0.39
Dihomo-gamma-linolenic acid	3.91	2.93	2.20	1.65	1.24
Conjugated linoleic acid	3.32	2.49	1.87	1.40	1.05
Conjugated linoleic acid	0.25	0.19	0.14	0.11	0.08

[a] Data were presented as mg g^{-1}.

Table 5. *Fatty acid composition of semi-skimmed milk at several weeks after their shelf life.*

The degradation process of each unsaturated fatty acid present in the skimmed milk was not constant as that observed for the samples of semi-skimmed and whole milk (Table 6).

Skimmed milk	**T0**	**I Week**	**II Week**	**III Week**	**IV Week**
Monounsaturated[a]					
Myristoleic	0.09	0.07	0.05	0.04	0.03
Palmitoleic	0.12	0.09	0.07	0.05	0.04
Heptadec-10-enoic acid	0.05	0.04	0.03	0.02	0.02
Elaidic	0.27	0.20	0.15	0.11	0.09
Oleic	2.92	2.19	1.64	1.23	0.92
Vaccenic acid	0.10	0.08	0.06	0.04	0.03
Cis vaccenic acid	0.056	0.04	0.03	0.02	0.02
Polyunsaturated[a]					
Linolelaidic	0.05	0.04	0.03	0.02	0.02
Trans-9, cis-12 acid octadienoic	0.02	0.02	0.01	0.01	0.01
Gamma-linoleic	0.07	0.05	0.04	0.03	0.02
Alpha-linolenic	0.03	0.02	0.02	0.01	0.01
Dihomo-gamma-linolenic acid	0.11	0.08	0.06	0.05	0.03
Conjugated linoleic acid	0.09	0.07	0.05	0.04	0.03
Conjugated linoleic acid	0.007	0.005	0.004	0.003	0.002

[a] Data were presented as mg g^{-1}.

Table 6. *Fatty acid profile of skimmed milk at several weeks after their shelf life.*

The amount of trans-9 cis-12 octadienoic acid after 7 days was unchanged while the alpha-linolenic remained constant up to 2 weeks. At 21 day, the rate between gamma-linoleic and alpha-linolenic is 3:1. The reduction of linolelaidic, heptadec-10-enoic and vaccenic acids was 20% at first week, 40% at second week, 60% at third and fourth week.

On the basis of the abovementioned data, it is evident that the amount of unsaturated fatty acids decreased over time, apparently independently of pH, titratable acidity, microbial load and type of milk. On the contrary, a decisive factor for fatty acid degradation is the time during which the samples are

exposed to atmospheric oxygen, at room temperature. In fact, these conditions induce an oxidation of unsaturated fatty acids by molecular oxygen.

3.3.3. Relationship between total lipase activity, TA and pH

Three biochemical factors appear largely responsible for determining the susceptibility of milk to spontaneous lipolysis: I) the amount of lipase activity; II) the integrity of the milk fat globule membrane (MFGM); III) the balance of lipolysis-activating and -inhibiting factors.[23-24] Lipases are an important group of enzymes, being associated with fat metabolism.[25] Therefore, the rate of lipase activity in milk is, indeed, the major out of the three aforementioned factors. These enzymes hydrolyse the glycerol esters of fatty acids at the oil/water interface and are responsible for the production of undesirable rancid flavors.[26]

The activity of lipase in milk is highly correlated to the acidity, a determining factor for assessing the quality of milk products. There are two fundamentally different conventions for expressing acidity in dairy products: titratable acidity and pH. Titratable acidity plays a fundamental role in all phases of milk coagulation. This includes the aggregation rate of para-casein micelles and the reactivity of rennet. Titratable acidity also influences the rate of syneresis and determines the suitability of milk for cheese making. The pH value of milk affects not only the enzymatic reaction, but also aggregation, as lowering the pH decreases the colloidal stability of milk.[27] Table 1 shows the pH values and TA of 15 commercial milk samples analyzed at several weeks after their expiry date. The results show that pH values were in the range of 6.71–4.23 in skimmed milk, 6.72–4.28 in semi-skimmed milk and 6.72–4.61 in whole milk. The values of titratable acidity were in the range of 6.36–58.15 in skimmed milk, 6.17–52.22 in semi-skimmed milk and 6.17–56.75 in whole milk. This

decrease in pH was attributed to increased lactic acid levels produced by fermenting bacteria. Titratable acidity also increased for the same reasons.

Both the pH values and the degree of acidity did not change in a regular manner over time. This could probably be abscribed to a different rate of fermentation of the various milk samples, since the initial microbial load is heterogeneous.

However, as showed in Table 2, the total acidity was inversely correlated with the lipase activity level that has been demonstrated to be highly sensitive to the pH values. Most of lipases in milk have pH optimum values of 8.5–9.0, while other lipases have pH optimum values ranging from 6.5 to 7.9.[28] In the milk samples analyzed, lipase activity was significantly low at acidic pH values. In fact, at pH values of 6.71, 5.85, 4.91, 4.33 and 4.23, the amount of p-nitrophenol generated in skimmed milk was 70.8%, 57.1%, 18.3% and 1.7%, respectively. Conversely, the percentage of lipase activity in whole milk decreased by 10.0%, 80.0%, 83.3% and 98.3%, corresponding to increasing TA values.

This increase in the TA values stimulates a deterioration process in samples milk which is characterized by four distinct and successive phases. First, acid production by Lactococcus lactis occurs, followed by a further acid production due to the growth of acid-resistant organisms, such as Lactobacillus. Then, yeasts and moulds, the dominant population in this conditions, degrade the lactic acid accumulated, with gradual reduction of acidity. Finally, the inactivation of lipase by yeasts and moulds determines the low levels of free fatty acids.

In contrast to other enzymes, the rate at which a lipase-catalysed reaction proceeds is not governed by the substrate concentration at the interface between the lipid substrate and the aqueous phase of an emulsion,[29-30] but it is

dependent on the amount of free fatty acids, which are released by disruption of the milk fat globule and exposing the lipid substrate to the lipase.

3.4. Conclusions

This study intended to evaluate both qualitative and quantitative profile changes of the lipid fraction and the physicochemical properties (pH, TA and TLA) of 15 commercial samples of bovine milk (skimmed, semi-skimmed and whole). These parameters were monitored for 28 days after expiration date.

The analysis carried out by GC-MS showed that the amount of unsaturated fatty acids decreased over time. Contrary to the degradation of fatty acids, the hydrolysis of triglycerides (triacylglycerols), catalyzed by lipases of milk, is a process independent of time but dependent on the values of pH and TA. An acidic environment is not favorable for the enzymatic activity of milk lipase. Therefore, low pH values result in a reduction of the lipase overall activity.

The correlation existing between the TA and TLA provides data more significant than the information derived from the pH values. In fact, while the pH expresses only the concentration of hydrogen ions present in the solution, the titratable acidity is influenced by several parameters, comprising the acid functions of casein, the presence of organic and inorganic acids and mineral salts. Therefore, the information that could be obtained on the lipolytic activity of milk by a single pH measurement are quite unsatisfactory, whereas the determination of total titratable acidity provides a detailed description of the changes that occur in milk during the fermentation period. For this reason, it may be a useful tool to monitor the good quality and storage of bovine milk.

3.5. References

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CHAPTER IV

Detailed peptide profiling of “Scotta” by high resolution mass spectrometry: From a dairy waste to a source of health-promoting compounds.

Abstract

"Scotta" is a liquid waste deriving from Ricotta cheese production, which is wrongly considered only a dairy by-product and it is not recovered. In this work, with the aim to elucidate the presence of valuable bioactive compounds in this matrix, a peptide fraction under 3000 Dalton was isolated by ultra-filtration, purified by solid phase extraction and, subsequently, characterized in detail by liquid chromatography coupled to Orbitrap mass spectrometry. Analytical results revealed a complex profile, leading to the identification of 226 peptides, belonging to alpha, beta and kappa caseins. A database driven search approach was used to assess the biological effects of some of the identified peptides. A wide range of healthy properties was found, comprising antihypertensive, antimicrobial, immunomodulating, opioid, antioxidant and antithrombotic. The peptidomic profile of scotta was highlighted in depth for the first time, the obtained results revealed how this matrix cannot be considered only a mere by-product, but is a source of potential healthy promoting peptides, which can be recovered and employed in nutraceuticals formulations or functional foods.

Keywords

bioactive peptides, LC-HRMS, Orbitrap, peptidomics, Scotta

Abbreviations

Biochemical Oxygen Demand, (BOD); Liquid-Chromatography-High Resolution Mass Spectrometry, (LC-HRMS); Solid Phase Extraction, (SPE); Tandem Mass Spectrometry, (MS/MS); Nominal Molecular Weight Limit, NMWL; Fourier Transform Mass Spectrometry, FTMS; Mass Spectrometry, MS; Collision Induced

Dissociation; CID; Liquid Chromatography-Tandem Mass Spectrometry, LC-MS/MS; ; Total Ion Current, TIC;

4.1. Introduction

Food waste and by-products represent one of the main challenges for agro-food industries, which must face demanding economic costs for their treatment and disposal. Moreover, they can be a risky source of pollution, especially when illegal methods are employed. In this regard dairy industry produces, yearly, tons of by-products, whose main component is whey, which correspond to the liquid fraction remaining after milk clotting.[1] Whey constitutes a huge problem for disposal due to its elevated content of organic matter and associated high biochemical oxygen demand (BOD).[2] On the other hand whey has been recognized as a source of functional and bioactive compounds such as lactose, minerals, and, especially, proteins. [3] Whey proteins and the peptides arising from their hydrolysis have been extensively investigated for their healthy properties such as anti-oxidant and anti-ACE. [4, 5, 6] In Italy, a small percent of whey is employed for the production of Ricotta cheese, obtained by heating of the cheese whey at 85-90 °C thus leading to the precipitation and separation of whey proteins. The liquid fraction that remains after the cheese separation is called "Scotta", which is the main by-product of Ricotta production process. Scotta is composed by lactose (4.8–5.0%), salts (1.0-1.13%) and proteins (0.15-0.22%).[7] This by-product is considered, as well as whey, a significant source of pollution, possessing high values of BOD. While the interest in the recovery of Scotta has been focused mainly on his conversion in bio-fuel and biotechnological substrate for fermented products,[8, 9] little is known about the low-medium peptide fraction of this matrix, which is mainly composed by casein peptides coming from the action

of rennet enzymes and released in whey, such as in the Mozzarella production. With the aim to elucidate the caseomic profile of scotta, in particular the one deriving from buffalo ricotta-cheese processing, we carried out an in-depth characterization through a liquid-chromatography-high resolution mass spectrometry (LC-HRMS) approach. A peptide fraction of 3000 Dalton was isolated and purified through ultrafiltration and solid phase extraction (SPE), subsequently the peptides were identified by LC-Orbitrap based tandem mass spectrometry (MS/MS) with the help of online driven database search for bioactivity assessment. A large number of peptides has been identified, many with established healthy properties. Our results highlight the considerable content of bioactive peptides in scotta, and could drive the dairy industry towards its recovery, in order to turn this waste product into a possible source of healthy compounds for nutraceutical purposes.

4.1.1. Aim of work

In the last year of the course, a sample of Scotta, a dairy wastewater resulting from buffalo ricotta processing, was analyzed with the aim to highlight the content of potential bioactive peptides. A large number of peptides was identified, mainly in the low-medium molecular weight range, many with recognized healthy promoting properties, thus underlining that this matrix cannot be considered only a waste but represent a rich source of bioactive peptides which may be used in the formulation of functional foods and cosmetics.

4.2. Materials and methods

4.2.1. Chemicals

Ultra pure water (H2O) was obtained by a Milli-Q system (Millipore, Milan, Italy). The following chemicals were all purchased from Sigma Aldrich (Milan, Italy): acetonitrile (ACN), and formic acid LC–MS grade (HCOOH). Centrifugal Filter Devices Amicon® Ultra-4 3 K and 10 K and Strata-XTM polymeric reversed phase SPE (500 mg/3 mL) cartridge were purchased by Millipore© and Phenomenex® (Castel Maggiore, Bologna, Italy), respectively, while filter paper Whatman® 540 from Sigma–Aldrich.

4.2.2. Sampling and sample preparation

Scotta samples (500 mL each) were kindly donated by Di Lascio dairy factory (Capaccio, SA, Campania, Italy). Samples were prepared by separating the residual fat fraction: 50 mL of scotta were subjected to centrifugation at 4000 × g for 30 min at 4 °C (Mikro 220R, Hettich, Germany) and further filtered by filter paper. Ultrafiltration was carried out by Millipore's Amicon® Ultra-4 centrifugal filter devices at different cutoffs. A preliminary filtration was carried out for all the samples using filters with 10,000 NMWL (Nominal Molecular Weight Limit) and subsequently 4 mL of permeate were loaded on devices with 3000 NMWL. The devices were centrifuged for 40 min at 6000 rpm at 25 °C, using a centrifuge with fixed angle rotor (35°). In order to recover the peptides, the devices were washed with 4 mL of acidified water at pH 3.0 by formic acid. A peptide fraction, roughly 3,5 mL, with molecular weight ≈ 3000 Da was collected, filtered through a 0.45 μm pore cellulose membrane (Millipore©) and lyophilized for 24 h (LyoQuest-55, Telstar Technologies, Spain). This fraction was solubilized in 0.1% (v/v) aqueous TFA, and purified from salts and sugars with a Strata-XTM reversed phase

polymeric SPE cartridge (Phenomenex®), previously equilibrated in 0.1% TFA, then eluted with 70/30/0.1 ACN/water/TFA (v/v/v) and finally re-lyophilized and stored at -20 °C. Sixteen milligrams of the lyophilized were solubilized in 1 mL of water and injected.

4.2.3. LC-HRMS analysis of peptide fraction

Peptide separation and identification was performed on an Accela 600 LC system coupled on-line to an LTQ-Orbitrap-XL-ETD mass spectrometer (Thermo Scientific, Bremen, Germany) through an electrospray source. For peptide separation an Ascentis Express Peptide ES C18 150 mm × 2.1 mm (L × I.D), 2.7 μm 160 Å (Supelco, Bellefonte, PA, USA) was employed. Mobile phases were (A) 0.1% HCOOH in H2O v/v and (B) ACN plus 0.1 % HCOOH v/v. LC gradient was the following: 0-22 min, 0-30 % B, 22-27 min, 30-70 % B, 27-28 min, 70-95 % B, isocratic for 1 min, 29-34 min, returning to 0 % B. Flow rate was set to 0.3 mL/min. Column oven was set to 45 °C. For the MS part spray voltage was set at +3.5 kV, sheath gas arbitrary units 30, auxiliary gas arbitrary units 10, capillary temperature 250 °C. Spectra were collected in data dependent mode, over the m/z range of 300–2000, at 30,000 resolution. All MS/MS spectra were collected using a normalized collision energy of 35%, and an isolation window of 2 m/z, minimum signal threshold 150, monoisotopic precursor enabled. Ion trap and Orbitrap maximum ion injection times were set to 50 and 100 ms, respectively. Automatic gain control was used to prevent over-filling of the ion traps and was set to 2×10^5 for full FTMS scan, and 3×10^4 ions in MS/MS mode for the linear ion trap. Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s, list size 50, with exclusion duration of 30 s. All parameters were optimized by infusion of a standard peptide mixture at 20 μL/min.

4.2.4. Database search and peptide identification

Raw MS/MS data files from Xcalibur software (version 2.2 SP1.48, Thermo Fisher Scientific) were converted in and mzXML format and a free trial of PEAKS 7.5 software (Bioinformatics Solutions Inc., Waterloo, Canada) was employed for sequence determination. Search was performed using a database search tool, by searching against SwissProt/UniProt database with an improved algorithm that validates and assists the database search with *de novo* sequencing results For database search the following settings were chosen: digestion with no enzyme, peptide charges from +1 to +4. Precursor Mass Search Type: monoisotopic. Fragmentation Mode: CID (y and b ions). Precursor mass tolerance: 15 ppm, fragment mass tolerance of 0.5 Da; oxidation (M) and phosphorylation (S, T, Y) were used as dynamic modifications; no static modification was selected. Peptide identifications were accepted if the peptides were detected in at least two of the three replicates. To assess the bioactivity, all identified peptides were searched with the following online available databases: BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), milkAMP (http://milkampdb.org/home.php) and EROP-Moscow (http://erop.inbi.ras.ru/).

4.3. Results and discussion

4.3.1. The peptidomic profile of Scotta

The growing interest in healthy promoting molecules from food matrices pushes the research in the recovery of these bioactive compounds also from food processing materials and by-products. MS based peptidomic approaches are the best strategies to characterize and monitor dairy bioactive peptides.[10] The interest in "Scotta" has been mainly restricted to the possible conversion

of this matrix as bioethanol or for lactic acid production. Contrariwise, this study was aimed to identify the peptides present in this dairy by-product and investigate, through database driven approach, their potential bioactivity, in order to re-evaluate this waste product and drive the dairy industry to its recovery. After the isolation and purification of the peptide fraction (figure 2A), identification was performed through LC-MS/MS, the total ion chromatogram (TIC) relative to the separation of the isolated peptide fraction is depicted in figure 1.

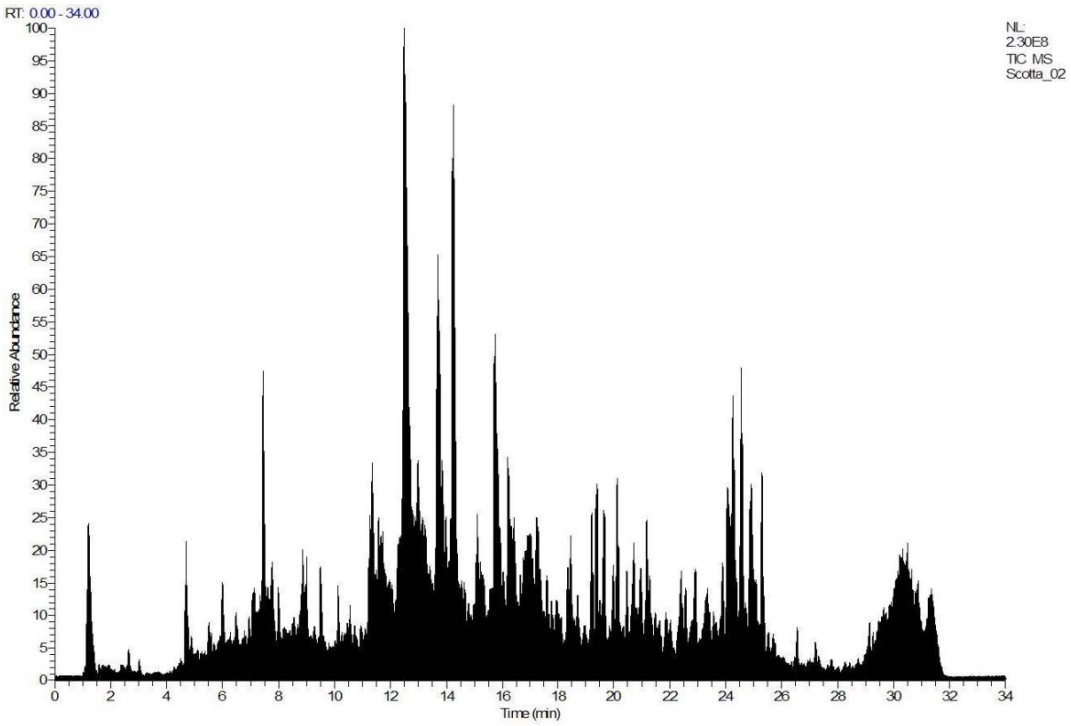

Figure 1. *Total ion chromatogram(TIC) relative to the analysis of peptide fraction ≤ 3000 Da of Scotta. Column: Ascentis Express Peptide ES C18 150 mm × 2.1 mm, 2.7 μm,. Detection: ESI$^+$-MS.*

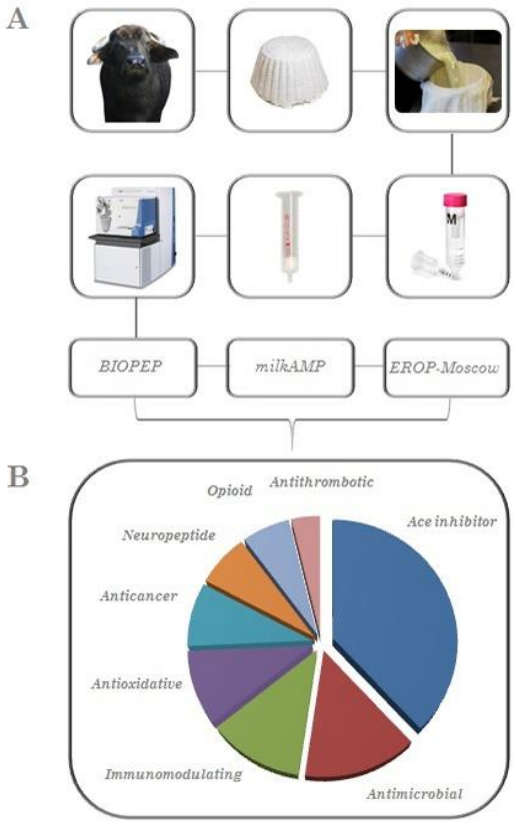

Figure 2. *Workflow of scotta peptide fraction isolation (a) and bioactivities of identified peptides (b)*

The complete list of peptides including retention time, peptide sequence, precursor protein, position and mass is reported in table 1. As can be appreciated from the total ion chromatogram a very complex profile was obtained, despite this, the employment of a fused-core particle column provided a satisfactory and fast separation with an analysis time of 34 minutes, that led to the identification of 226 peptides, belonging to buffalo caseins α_{s1}, β and κ. No peptides belonging to α_{s2}-casein were detected. It is noteworthy that the only previous investigation on scotta, led to the identification of only 29 peptides.[11]

n°	*t_r (min)*	*Mass*	*Error ppm*	*Casein*	*Amino acid*	*Peptide containing the sequence*	*Activity*	*References*
1.	2.87	926.4644	-3.2	β	115-122	K.**EAM**(+15.99)**APK**HK.E (oxidation)	Antioxidative	Gupta et al., 2009
2.	3.18	616.3908	-0.9	β	94-99	M.**GVSKVK**.E	Neuropeptide, Antioxidative	Yamamoto et al., 1994
3.	3.49	669.3268	-3.1	β	116-121	E.**AM**(+15.99)**APK**H.K (oxidation)	Antioxidative	Gupta et al., 2009
4.	4.61	910.4694	-1.8	β	100-107	K.**EAMAPK**HK.E	Neuropeptide, Antioxidative	Gupta et al., 2009
5.	4.64	798.3694	-3.0	β	115-121	K.**EAM**(+15.99)**APK**H.K (oxidation)	Antioxidative	Gupta et al., 2009
6.	4.66	1002.6086	-2.4	α_{s1}	1-8	A.**RPKQPIKH**.Q	Antimicrobial, Immunomodulating	Hayes et al., 2006
7.	4.93	1016.5688	0.5	β	112-120	S.KVK**EAM**(+15.99)**APK**.H (oxidation)	Antioxidative	Gupta et al., 2009
8.	4.99	1130.6672	0.8	α_{s1}	1-9	A.R**PKQPIKHQ**.G	Antimicrobial, Immunomodulating	Hayes et al., 2006
9.	5.06	865.5497	-1.3	α_{s1}	1-7	A.**RPKQPIK**.H	Antimicrobial, Immunomodulating	Hayes et al., 2006
10.	5.20	891.4297	-1.9	α_{s1}	186-194	P.**IGSEN**SGKT.T	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
11.	5.32	816.4705	-2.7	β	94-101	M.**GVSKVK**EA.M	Neuropeptide, Antioxidative	Yamamoto et al., 1994
12.	5.41	992.4774	0.4	α_{s1}	186-195	P.**IGSEN**SGKTT.M	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
13.	5.46	782.3745	0.00	β	100-106	K.**EAMAPK**H.K	Neuropeptide, Antioxidative	Gupta et al., 2009
14.	5.67	1000.5739	-0.1	β	97-105	S.K**VKEAMAPK**.H	Neuropeptide, Antioxidative	Gupta et al., 2009
15.	5.86	1009.5378	-0.7	β	98-106	K.**VKEAMAPK**H.K	Neuropeptide, Antioxidative	Gupta et al., 2009
16.	5.92	645.3156	-0.1	β	100-105	K.**EAMAPK**.H	Neuropeptide, Antioxidative	Gupta et al., 2009
17.	6.08	1396.7496	-2.9	β	109-121	M.**GVSKVKEAM**(+15.99)**APK**H.K (oxidation)	Antioxidative	Gupta et al., 2009; Yamamoto et al., 1994
18.	6.13	1087.6060	-1.7	β	96-105	V.SK**VKEAMAPK**.H	Neuropeptide, Antioxidative	Gupta et al., 2009
19.	6.17	1089.5302	-2.6	α_{s1}	185-195	N.**PIGSEN**SGKTT.M	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
20.	6.35	1041.5641	-2.0	κ	127-135	F.**M**(+15.99)**AIPPKKN**Q.D (oxidation)	Antimicrobial, Antithrombotic	Malkoski et al., 2001
21.	6.41	806.4399	-2.3	α_{s1}	7-13	I.**KHQGLPQ**.G	Antimicrobial, Immunomodulating	Hayes et al., 2006
22.	6.48	1259.6907	-2.0	β	109-120	M.**GVSKVKEAM**(+15.99)**APK**.H (oxidation)	Antioxidative	Gupta et al., 2009; Yamamoto et al., 1994
23.	6.78	913.5055	-1.9	κ	127-134	F.**M**(+15.99)**AIPPKKN**.Q (oxidation)	Antimicrobial, Antithrombotic	Malkoski et al., 2001
24.	7.12	1336.6735	-1.6	α_{s1}	80-90	K.**HIQKEDVPSER**.Y	Neuropeptide, Antioxidative	Gupta et al., 2009

25.	7.26	1380.7548	-2.1	β	94-106	M.**GVSKVKEAMAPK**H.K	Neuropeptide, Antioxidative	Gupta et al., 2009; Yamamoto et al., 1994
26.	7.27	671.3676	-0.3	κ	127-132	F.**M**(+15.99)**AIPPK**.K (oxidation)	Antimicrobial, Antithrombotic	Malkoski et al., 2001
27.	7.28	1051.5298	-2.8	α_{s1}	80-88	K.**HIQKEDVPS**.E	Neuropeptide, Antioxidative	Gupta et al., 2009
28.	7.45	1525.8840	0.1	α_{s1}	1-13	A.RPKQPI**KHQGLPQ**.G	Antimicrobial, Immunomodulating	Hayes et al., 2006
29.	7.68	646.3286	-0.5	β	53-58	F.**AQTQSL**.V	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
30.	7.75	1180.5724	-0.6	α_{s1}	80-89	K.**HIQKEDVPSE**.R	Neuropeptide, Antioxidative	Gupta et al., 2009
31.	7.76	1243.6958	-0.07	β	94-105	M.**GVSKVKEAMAPK**.H	Neuropeptide, Antioxidative	Gupta et al., 2009; Yamamoto et al., 1994
32.	8.26	897.5106	-2.3	κ	106-113	F.**MAIPPKKN**.Q	Antimicrobial, Antithrombotic	Malkoski et al., 2001
33.	8.27	607.3693	-3.1	β	63-68	F.**PGPIPK**.S	Ace inhibitor, Neuropeptide	Miguel et al., 2006; Yamamoto et al., 1994
34.	8.44	1043.5134	-0.7	α_{s1}	81-89	H.**IQKEDVPSE**.R	Neuropeptide, Antioxidative	Gupta et al., 2009
35.	8.66	1600.7134	0.7	β	44-55	K.KIEKFQS(+79.97)EEQQQ.M (phospho)	Ace inhibitor	Yamamoto et al., 1994
36.	8.95	1072.4858	0.00	α_{s1}	130-138	N.LAEEQLHSM(+15.99).K (oxidation)	Ace inhibitor	Yamamoto et al., 1994
37.	9.01	1025.5692	-2.6	κ	106-114	F.**MAIPPKKNQ**.D	Antimicrobial, Antithrombotic	Malkoski et al., 2001
38.	9.15	801.4385	-1.8	β	176-182	Q.K**AVPYP**Q.R	Ace inhibitor, Antioxidative	Hayes et al., 2007
39.	9.32	763.3574	-0.4	β	123-128	K.**EM(**+15.99)**PFPK**.Y (oxidation)	Ace inhibitor	Yamamoto et al., 1994
40.	9.41	1126.5784	-1.2	κ	97-105	T.**RHPHPHLSF**.M	Antioxidative	Korhonen et al., 2007
41.	9.64	942.3583	-0.4	κ	168-175	L.EAS(+79.97)SEVIE.S (phospho)	Antimicrobial	Malkoski et al., 2001
42.	9.65	757.4082	-1.2	α_{s1}	17-22	L.**NENLLR**.F	Anticancer, Antimicrobial, Immunomodulating	Hayes et al., 2006
43.	9.75	1184.5859	-0.1	α_{s1}	115-124	N.LAEEQLHSMK.E	Ace inhibitor	Yamamoto et al., 1994
44.	9.82	901.4505	-1.5	β	1-7	A.**RELEELN**.V	Immunomodulating	Hayes et al., 2007
45.	10.17	947.4448	-1.2	κ	132-140	T.**STPTTEA**IE.N	Antimicrobial	Malkoski et al., 2001
46.	10.38	2026.8425	2.3	β	48-63	K.FQSEEQQQM(+15.99)EDELQDK.I (oxidation)	Ace inhibitor	Yamamoto et al., 1994

47.	10.42	732.4018	-1.9	κ	163-169	A.**QVTST**VV	Antithrombotic	López-Expósito et al., 2006
48.	10.44	1027.5087	-1.0	β	49-57	K.**IHPFAQTQS**.L	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
49.	10.50	2525.1592	-1.7	β	44-63	K.KIEKFQSEEQQQM(+15.99)EDELQDK.I (oxidation)	Ace inhibitor	Yamamoto et al., 1994
50.	10.53	940.4766	-1.8	β	49-56	K.**IHPFAQTQ**.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
51.	11.09	1669.8423	-2.3	α_{s1}	80-93	K.**HIQKEDVPSER**YLG.Y	Neuropeptide, Antioxidative	Gupta et al., 2009
52.	11.45	1795.0580	1.4	α_{s1}	1-16	A.RPKQPI**KHQGLPQ**GVL.N	Antimicrobial, Immunomodulating	Hayes et al., 2006
53.	11.55	904.4866	-1.4	κ	161-169	N.**TAQVTST**VV	Antithrombotic	López-Expósito et al., 2006
54.	11.61	1612.8209	-2.7	α_{s1}	80-92	K.**HIQKEDVPSER**YL.G	Neuropeptide, Antioxidative	Gupta et al., 2009
55.	11.63	1056.4910	-2.8	α_{s1}	115-123	N.LAEEQLHSM.K	Ace inhibitor	Yamamoto et al., 1994
56.	12.07	1055.4424	0.4	κ	167-175	T.LEAS(+79.97)SEVIE.S (phospho)	Antimicrobial	Malkoski et al., 2001
57.	12.11	2154.1355	-3.6	κ	127-145	F.**M**(+15.99)**AIPPKKNQDKTEIPTINT**.I (oxidation)	Antimicrobial	Malkoski et al., 2001
58.	12.17	1574.7312	4.2	κ	127-141	V.SVEP**TSTPTTEA**IEN.T	Antimicrobial	Malkoski et al., 2001
59.	12.21	2053.0876	-0.3	κ	127-144	F.**M**(+15.99)**AIPPKKNQDKTEIPTIN**.T (oxidation)	Antimicrobial	Malkoski et al., 2001
60.	12.33	914.5073	-2.6	κ	116-123	D.**KTEIPTIN**.T	Antimicrobial	Malkoski et al., 2001
61.	12.34	1119.5771	1.6	κ	159-169	E.TN**TAQVTST**VV	Antithrombotic	López-Expósito et al., 2006
62.	12.36	885.4080	1.2	β	4-11	L.**EELNVPGE**.I	Immunomodulating	Hayes et al., 2007
63.	12.37	2037.0928	-1.3	κ	106-123	F.**MAIPPKKNQDKTEIPTIN**.T	Antimicrobial, Antithrombotic	Malkoski et al., 2001
64.	12.39	1157.5928	-0.9	κ	114-123	N.**QDKTEIPTIN**.T	Antimicrobial	Malkoski et al., 2001
65.	12.74	844.4542	0.7	κ	124-131	N.TIVSVEPT.S	Antimicrobial	Malkoski et al., 2001
66.	12.84	1440.6021	-2.5	κ	169-181	E.AS(+79.97)SEVIESVPETN.T (phospho)	Antimicrobial	Malkoski et al., 2001
67.	13.01	651.3955	-1.8	β	170-175	K.**VLPVPQ**.K	Ace inhibitor	Yamamoto et al., 1994
68.	13.03	754.4377	-2.5	β	62-68	P.**FPGPIP**K.S	Ace inhibitor, Neuropeptide	Miguel et al., 2006; Yamamoto et al., 1994

69.	13.08	1258.6405	-2.2	κ	114-124	N.Q**DKTEIPTINT**.I	Antimicrobial	Malkoski et al., 2001
70.	13.12	2509.1641	-3.2	β	29-48	K.KIEKFQSEEQQQMEDELQDK.I	Ace inhibitor	Yamamoto et al., 1994
71.	13.49	1869.7881	-0.1	κ	168-184	L.EASS(+79.97)EVIESVPETNTAQ.V (phospho)	Antimicrobial	Malkoski et al., 2001
72.	13.53	1079.5651	-2.4	α_{s1}	28-36	P.**FPEVFGK**EK.V	Ace inhibitor; Anticancer	Robert et al., 2004
73.	13.59	658.3326	-2.1	α_{s1}	25-30	F.**VAPFPE**.V	Ace inhibitor; Anticancer	Robert et al., 2004
74.	13.60	2010.8477	1.3	β	33-48	K.FQSEEQQQMEDELQDK.I	Ace inhibitor	Yamamoto et al., 1994
75.	13.84	1035.5059	-2.4	α_{s1}	206-214	N.**SGKTTM**(+15.99)**PL**W (oxidation)	Ace inhibitor, Antmicrobial	Yamamoto et al., 1994
76.	13.95	2589.1306	0.7	β	44-63	K.KIEKFQS(+79.97)EEQQQMEDELQDK.I (phospho)	Ace inhibitor	Yamamoto et al., 1994
77.	14.10	1283.6357	-1.5	β	1-11	A.**RELEELNVPGE**.I	Immunomodulating	Hayes et al., 2007
78.	14.37	2090.8140	-0.3	β	48-63	K.FQS(+79.97)EEQQQMEDELQDK.I (phospho)	Ace inhibitor	Yamamoto et al., 1994
79.	14.45	1281.7292	-2.0	β	164-175	L.SLSQSK**VLPVPQ**.K	Ace inhibitor	Yamamoto et al., 1994
80.	14.55	953.5658	-1.6	κ	66-74	A.**AVRSPAQIL**.Q	Antimicrobial	López-Expósito et al., 2006
81.	14.79	2461.0356	3.2	β	45-63	K.IEKFQS(+79.97)EEQQQMEDELQDK.I (phospho)	Ace inhibitor	Yamamoto et al., 1994
82.	14.85	2257.9839	-0.6	κ	168-188	L.EAS(+79.97)SEVIESVPETNTAQVTST.V (phospho)	Antimicrobial, Antithrombotic	López-Expósito et al., 2006
83.	15.14	1178.6335	-3.0	α_{s1}	28-37	P.**FPEVFGK**EKV.N	Ace inhibitor; Anticancer	Robert et al., 2004
84.	15.22	1247.6549	-2.3	α_{s1}	26-36	V.**APFPEVFGK**EK.V	Ace inhibitor; Anticancer	Robert et al., 2004
85.	15.38	822.4276	-2.4	α_{s1}	28-34	P.**FPEVFGK**.E	Ace inhibitor; Anticancer	Robert et al., 2004
86.	15.56	1434.8235	-3.5	β	170-182	K.**VLPVPQ**K**AVPY**PQ.R	Antioxidative	Hayes et al., 2007; Yamamoto et al., 1994
87.	15.57	1632.7817	-0.5	α_{s1}	200-214	N.P**IGSENS**GKTTM(+15.99)PLW (oxidation)	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
88.	16.22	1014.5538	-3.3	β	60-68	V.**YPFPGPIP**K.S	Ace inhibitor, Opioid	Yamamoto et al., 1994
89.	16.39	1346.7234	-1.5	α_{s1}	25-36	F.**VAPFPEVFGKEK**.V	Ace inhibitor; Anticancer	Robert et al., 2004
90.	16.60	904.4766	-2.0	α_{s1}	17-23	L.**NENLLRF**.F	Anticancer, Antimicrobial, Immunomodulating	Hayes et al., 2006
91.	16.68	2277.0776	-3.0	β	106-124	K.**HKEMPFPKYPVEPFTE**SQS.L	Ace inhibitor	Yamamoto et al., 1994
92.	16.79	1019.5110	-2.2	α_{s1}	191-199	N.**SGKTTMPL**W	Ace inhibitor, Antmicrobial	Yamamoto et al., 1994

93.	16.97	1479.7391	-2.1	α_{s1}	111-123	E.IVPNLAEEQLHSM.K	Ace inhibitor	Yamamoto et al., 1994
94.	17.01	2246.2244	-2.6	β	170-189	K.**VLPVPQ**K**AVPY**PQRDMPIQA.F	Antioxidative	Hayes et al., 2007; Yamamoto et al., 1994
95.	17.13	990.5174	-2.4	α_{s1}	26-34	V.**APFPEVFGK**.E	Ace inhibitor; Anticancer	Robert et al., 2004
96.	17.23	1113.6222	0.0	β	59-68	L.**VYPFPGPIP**K.S	Ace inhibitor, Opioid	Yamamoto et al., 1994
97.	17.31	1624.8308	-2.7	β	1-14	A.**RELEELNVPGEIVE**.S	Immunomodulating	Hayes et al., 2007
98.	17.43	1557.8250	0.5	κ	114-127	N.**QDKTEIPTINTIV**S.V	Antimicrobial	Malkoski et al., 2001
99.	17.48	1412.7664	0	α_{s1}	103-114	K.KY**NVPQ**LEIVPN.L	Ace inhibitor	Yamamoto et al., 1994
100.	17.54	2327.0781	5.3	κ	169-190	E.AS(+79.97)SEVIESVPETNTA**QVTST**VV (phospho)	Antimicrobial, Antithrombotic	López-Expósito et al., 2006
101.	17.55	2256.0410	4.00	κ	170-190	A.S(+79.97)SEVIESVPETNTA**QVTST**VV (phospho)	Antimicrobial, Antithrombotic	López-Expósito et al., 2006
102.	17.61	1445.7917	-3.3	α_{s1}	25-37	F.**VAPFPEVFGK**EKV.N	Ace inhibitor; Anticancer	Robert et al., 2004
103.	17.89	805.4010	-2.1	α_{s1}	24-30	F.**FVAPFPE**.V	Ace inhibitor; Anticancer	Robert et al., 2004
104.	17.95	2933.3137	-0.5	β	33-56	K.FQSEEQQQMEDELQDK**IHPFAQTQ**.S	Ace inhibitor	Yamamoto et al., 1994
105.	17.98	694.3326	-2.6	α_{s1}	28-33	P.**FPEVFG**.K	Ace inhibitor; Anticancer	Robert et al., 2004
106.	18.04	1616.7869	-2.2	α_{s1}	185-199	N.**PIGSENSGKTTMPL**W	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
107.	18.19	2390.1616	-2.1	β	106-125	K.**HKEMPFPKYPVEPFTE**SQSL.T	Ace inhibitor	Yamamoto et al., 1994
108.	18.24	990.5498	-2.4	α_{s1}	93-100	L.**GYLEQ**LLR.L	Opioid	Loukas et al., 1983
109.	18.30	1956.9615	2.7	α_{s1}	197-214	D.IPNP**IGSENS**GKTTM(+15.99)PLW (oxidation)	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
110.	18.32	1017.5607	-3.3	α_{s1}	16-23	V.L**NENLLRF**.F	Anticancer, Antimicrobial, Immunomodulating	Hayes et al., 2006
111.	18.33	1227.7074	-2.2	κ	116-126	D.**KTEIPTINTI**V.S	Antimicrobial	Malkoski et al., 2001
112.	18.36	1089.5858	1.4	α_{s1}	25-34	F.**VAPFPEVFGK**.E	Ace inhibitor; Anticancer	Robert et al., 2004
113.	18.37	1470.7930	-0.8	κ	114-126	N.**QDKTEIPTINTI**V.S	Antimicrobial	Malkoski et al., 2001
114.	18.45	1218.6284	-2.8	α_{s1}	25-35	F.**VAPFPEVFGK**E.K	Ace inhibitor; Anticancer	Robert et al., 2004
115.	18.51	1421.7991	-1.4	α_{s1}	10-22	Q.**GLPQGVLNENLLR**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007
116.	18.52	1549.8577	-1.1	α_{s1}	9-22	H.**QGLPQGVLNENLLR**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007

117.	18.53	1555.7617	-3.2	β	2-15	R.**ELEELNVPGEIVES**.L	Immunomodulating	Hayes et al., 2007
118.	18.57	1263.6975	-1.80	β	195-206	Q.**EPVLGPVRGPF**P.I	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
119.	18.64	571.3370	-0.4	β	84-88	V.**VPPFL**.Q	Ace inhibitor	Yamamoto et al., 1994
120.	18.66	2159.0205	1.7	α_{s1}	195-214	F.SDIPNP**IGSENS**GKTTM (+15.99)PLW (oxidation)	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
121.	18.70	2670.2524	0.1	κ	166-190	A.TLEASS(+79.97)EVIESV PETNTA**QVTST**VV (phospho)	Antimicrobial, Antithrombotic	López-Expósito et al., 2006
122.	18.71	1342.7344	-2.8	κ	115-126	Q.**DKTEIPTINTI**V.S	Antimicrobial	Malkoski et al., 2001
123.	18.80	637.3112	-1.6	α_{s1}	28-32	P.**FPEVF**.G	Ace inhibitor; Anticancer	Robert et al., 2004
124.	18.85	1503.8297	-0.7	β	69-82	K.SLPQNIPPL**TQTP**V.V	Ace inhibitor	Yamamoto et al., 1994
125.	18.89	2595.3367	-1.5	α_{s1}	118-139	K.KY**NVPQ**LEIVPNLAEE QLHSM(+15.99)K.E (oxidation)	Ace inhibitor	Yamamoto et al., 1994
126.	19.03	1530.8154	-0.1	α_{s1}	8-21	K.**HQGLPQGVLNENLL**. R	Antimicrobial, Immunomodulating	Hayes et al., 2006
127.	19.08	1495.7057	-1.7	β	123-134	K.**EM**(+15.99)**PFPKYPVE PF**.T (oxidation)	Ace inhibitor	Yamamoto et al., 1994
128.	19.15	1493.7917	-3.3	α_{s1}	24-36	F.**FVAPFPEVFGK**EK.V	Ace inhibitor; Anticancer	Robert et al., 2004
129.	19.19	1226.7063	-1.8	β	58-68	S.**LVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
130.	19.22	1116.6292	-1.8	α_{s1}	15-23	G.**VLNENLLRF**.F	Antimicrobial	Hayes et al., 2006
131.	19.29	1984.0364	-0.4	κ	114-131	N.**QDKTEIPTINTI**VSVEP T.S	Antimicrobial	Malkoski et al., 2001
132.	19.30	1741.9402	-0.8	β	53-68	F.**AQTQSLVYPFPGPIP**K. S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
133.	19.36	2304.1782	-2.3	α_{s1}	120-139	Y.**NVPQ**LEIVPNLAEEQL HSM(+15.99)K.E (oxidation)	Ace inhibitor	Yamamoto et al., 1994
134.	19.38	1670.9031	-1.2	β	54-68	A.**QTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
135.	19.48	1441.7969	-2.6	β	56-68	T.**QSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
136.	19.49	1542.8446	-1.1	β	55-68	Q.**TQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994

137.	19.51	2123.1204	2.9	β	50-68	I.HPF**AQTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
138.	19.58	998.5648	-1.3	κ	118-126	T.**EIPTINTI**V.S	Antimicrobial	Malkoski et al., 2001
139.	19.60	1855.9778	-1.2	κ	115-131	Q.**DKTEIPTINTI**VSVEPT.S	Antimicrobial	Malkoski et al., 2001
140.	19.65	1313.7383	0.8	β	57-68	Q.**SLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
141.	19.88	2479.3262	-1.7	β	47-68	Q.DK**IHPFAQTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
142.	19.89	1937.9734	-2.7	α_{s1}	83-98	Q.**KEDVPSER**YLGYLEQL.L	Neuropeptide, Antioxidative	Gupta et al., 2009
143.	20.06	2467.2417	-1.4	α_{s1}	118-138	K.KY**NVPQ**LEIVPNLAEEQLHSM(+15.99).K (oxidation)	Ace inhibitor	Yamamoto et al., 1994
144.	20.10	1592.8602	-0.8	α_{s1}	24-37	F.**FVAPFPEVFGK**EKV.N	Ace inhibitor; Anticancer	Robert et al., 2004
145.	20.11	2579.3418	-2.9	α_{s1}	103-124	K.KY**NVPQ**LEIVPNLAEEQLHSMK.E	Ace inhibitor	Yamamoto et al., 1994
146.	20.16	2368.1758	-1.6	β	7-28	L.**NVPGEIVESLSSSEESITH**INK.K	Ace inhibitor, Immunomodulating	Hayes et al., 2007
147.	20.19	1940.9666	1.6	α_{s1}	182-199	D.**IPNPIGS**ENSGKTTMPLW	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
148.	20.21	2320.2063	-1	α_{s1}	103-122	K.KY**NVPQ**LEIVPNLAEEQLHS.M	Ace inhibitor	Yamamoto et al., 1994
149.	20.32	2849.5115	-1.0	β	44-68	D.ELQDK**IHPFAQTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
150.	20.33	1904.9132	-0.8	β	16-31	A.**RELEELNVPGEIVES**(+79.97)**L**.S (phospho)	Immunomodulating	Hayes et al., 2007
151.	20.37	1725.9301	-1.1	α_{s1}	103-117	K.KY**NVPQ**LEIVPNLAE.E	Ace inhibitor	Yamamoto et al., 1994
152.	20.43	1889.0087	-2.1	β	52-68	P.F**AQTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
153.	20.46	2236.2043	3.2	β	49-68	K.**IHPFAQTQSLVYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
154.	20.49	2143.0256	-0.8	α_{s1}	180-199	F.**SDIPNPIGSENS**GKTTMPLW	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
155.	20.56	1173.6506	-2.8	α_{s1}	14-23	Q.GVL**NENLLRF**.F	Antimicrobial, Immunomodulating	Hayes et al., 2006
156.	20.59	1350.6682	-3.5	β	109-119	E.**MPFPKYPVEPF**.T	Ace inhibitor	Yamamoto et al., 1994

157.	20.61	1833.9849	-0.8	α_{s1}	8-23	K.**HQGLPQGVLNENLLR F**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007
158.	20.65	2176.0835	-1.5	α_{s1}	120-138	Y.**NVPQ**LEIVPNLAEEQL HSM(+15.99).K (oxidation)	Ace inhibitor	Yamamoto et al., 1994
159.	20.67	2288.1833	-1.2	α_{s1}	105-124	Y.**NVPQ**LEIVPNLAEEQL HSMK.E	Ace inhibitor	Yamamoto et al., 1994
160.	20.74	1265.6979	-3.5	α_{s1}	10-21	Q.**GLPQGVLNENLL**.R	Antimicrobial, Immunomodulating	Hayes et al., 2007
161.	20.78	2029.0480	-2.1	α_{s1}	105-122	Y.**NVPQ**LEIVPNLAEEQL HS.M	Ace inhibitor	Yamamoto et al., 1994
162.	20.82	1995.9651	-1.8	β	109-125	E.**MPFPKYPVEPFTE**SQS L.T	Ace inhibitor	Yamamoto et al., 1994
163.	20.93	2964.5383	-1.0	β	43-68	E.DELQDK**IHPFAQTQSL VYPFPGPIP**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
164.	21.02	1986.0614	3.4	β	51-68	H.PF**AQTQSLVYPFPGPI P**K.S	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
165.	21.05	1738.9657	1.7	β	57-72	Q.**SLVYPFPGPIP**KSLPQ. N	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
166.	21.06	961.4908	-1.3	α_{s1}	25-33	F.**VAPFPEVFG**.K	Ace inhibitor; Anticancer	Robert et al., 2004
167.	21.08	2561.2107	1.2	α_{s1}	191-214	D.APSFSDIPNP**IGSENS**G KTTM(+15.99)PLW (oxidation)	Ace inhibitor, Antmicrobial	Hayes et al., 2006; Yamamoto et al., 1994
168.	21.18	1236.6542	-0.6	α_{s1}	24-34	F.**FVAPFPEVFGK**.E	Ace inhibitor; Anticancer	Robert et al., 2004
169.	21.19	1504.8401	-0.7	β	194-207	Y.**QEPVLGPVRGPFPI**.I	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
170.	21.20	1093.6648	-3.4	β	200-209	G.**PVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
171.	21.21	1365.6968	-1.4	α_{s1}	24-35	F.**FVAPFPEVFGK**E.K	Ace inhibitor; Anticancer	Robert et al., 2004
172.	21.23	1150.6862	-2.4	β	199-209	L.**GPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
173.	21.26	2240.0808	2.9	β	7-27	L.**NVPGEIVESLSSSEESI T**HIN.K	Ace inhibitor, Immunomodulating	Hayes et al., 2007
174.	21.29	1376.7815	-0.5	β	195-207	Q.**EPVLGPVRGPFPI**.I	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
175.	21.40	1896.0720	-1.7	β	69-86	K.SLPQNIPPL**TQTPVVVP P**.F	Ace inhibitor	Yamamoto et al., 1994
176.	21.48	2451.2468	-3	α_{s1}	104-124	K.Y**NVPQ**LEIVPNLAEEQ LHSMK.E	Ace inhibitor	Yamamoto et al., 1994
177.	21.49	2339.1467	-0.3	α_{s1}	119-138	K.Y**NVPQ**LEIVPNLAEEQ LHSM(+15.99).K (oxidation)	Ace inhibitor	Yamamoto et al., 1994
178.	21.57	1640.8602	-1.2	α_{s1}	23-36	R.**FFVAPFPEVFGKEK**.V	Ace inhibitor; Anticancer	Robert et al., 2004
179.	21.65	2451.2468	1.1	α_{s1}	103-123	K.KY**NVPQ**LEIVPNLAEE QLHSM.K	Ace inhibitor	Yamamoto et al., 1994

180.	21.73	1539.8237	-3.8	α_{s1}	22-34	L.**RFFVAPFPEVFGK**.E	Anticancer	Robert et al., 2004
181.	21.75	2431.1602	0.3	β	1-22	A.**RELEELNVPGEIVESL**SSSEES.I	Immunomodulating	Hayes et al., 2007
182.	21.77	1667.9034	-0.8	β	193-207	L.**YQEPVLGPVRGPFPI**.I	Ace inhibitor	Yamamoto et al., 1994
183.	21.83	1824.9469	-2.5	β	1-16	A.**RELEELNVPGEIVESL**.S	Immunomodulating	Hayes et al., 2007
184.	21.87	1511.8459	-2.4	α_{s1}	11-23	G.**LPQGVLNENLLRF**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007
185.	21.94	684.4210	-1.3	β	204-209	G.**PFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
186.	22.02	2585.3601	-3,3	α_{s1}	80-100	K.H**IQKEDVPSER**YLGYLEQLLR.L	Neuropeptide, Antioxidative	Gupta et al., 2009
187.	22.12	2046.0455	4.3	α_{s1}	106-123	N.**VPQ**LEIVPNLAEEQLHSM.K	Ace inhibitor	Yamamoto et al., 1994
188.	22.21	1550.8344	-2.3	β	78-91	L.**TQTPVVVPPFLQPE**.I	Ace inhibitor	Yamamoto et al., 1994
189.	22.22	1696.9260	-2.1	α_{s1}	9-23	H.**QGLPQGVLNENLLRF**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007
190.	22.26	1739.9286	-3.7	α_{s1}	23-37	R.**FFVAPFPEVFGK**EKV.N	Ace inhibitor; Anticancer	Robert et al., 2004
191.	22.31	1568.8674	-2.5	α_{s1}	10-23	Q.**GLPQGVLNENLLRF**.F	Antimicrobial, Immunomodulating	Hayes et al., 2007
192.	22.39	2160.0884	-1.5	α_{s1}	105-123	Y.**NVPQ**LEIVPNLAEEQLHSM.K	Ace inhibitor	Yamamoto et al., 1994
193.	22.46	1995.0254	-0.9	β	50-67	I.HPF**AQTQSLVYPFPGPIP**.K	Ace inhibitor, Opioid	Miguel et al., 2006; Yamamoto et al., 1994
194.	22.65	1202.6482	-2.1	β	183-192	Q.**RDMPIQ**AFLL.Y	Ace inhibitor, Antioxidative	Hayes et al., 2007
195.	22.81	1263.7703	-2.0	β	198-209	V.**LGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
196.	22.85	2207.1584	-1.6	α_{s1}	83-100	Q.**KEDVPSER**YLGYLEQLLR.L	Neuropeptide, Antioxidative	Gupta et al., 2009
197.	22.93	2429.2590	-3.3	α_{s1}	80-99	K.**HIQKEDVPSER**YLGYLEQLL.R	Neuropeptide, Antioxidative	Gupta et al., 2009
198.	22.96	1663.9185	-2.1	β	77-91	P.**LTQTPVVVPPFLQPE**.I	Ace inhibitor	Yamamoto et al., 1994
199.	23.10	2323.1519	1.9	α_{s1}	104-123	K.Y**NVPQ**LEIVPNLAEEQLHSM.K	Ace inhibitor	Yamamoto et al., 1994
200.	23.18	1489.8656	-2.2	β	195-208	Q.**EPVLGPVRGPFPII**.V	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
201.	23.36	1156.6968	-2.3	β	132-141	E.NL**HLPLP**LLQ.S	Ace inhibitor	Miguel et al., 2006
202.	23.49	1810.9539	0.30	β	93-108	L.**TQTPVVVPPFLQPE**IM(+15.99).G (oxidation)	Ace inhibitor	Yamamoto et al., 1994
203.	23.50	2545.2158	-2.2	α_{s1}	176-199	D.**APSFSDI**PNPIGSENSGKTTMPLW	Ace inhibitor	Yamamoto et al., 1994
204.	23.52	1780.9875	-0.2	β	193-208	Y.**QEPVLGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
205.	23.63	1383.7227	-0.5	α_{s1}	23-34	R.**FFVAPFPEVFGK**.E	Ace inhibitor; Anticancer	Robert et al., 2004
206.	23.81	2051.0574	0.30	α_{s1}	83-99	Q.**KEDVPSER**YLGYLEQLL.R	Neuropeptide, Antioxidative	Gupta et al., 2009
207.	23.91	1108.5593	-1.8	α_{s1}	24-33	F.**FVAPFPEVFG**.K	Ace inhibitor; Anticancer	Robert et al., 2004

208.	23.95	1857.9811	-2.6	β	177-192	K.AVPYP**QRDMPIQ**AFLL.Y	Ace inhibitor, Antioxidative	Hayes et al., 2007
209.	23.99	1588.9341	-0.01	β	195-209	Q.**EPVLGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
210.	24.00	1459.8915	-2.4	β	196-209	E.**PVLGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
211.	24.19	1678.8929	0.9	α_{s1}	86-99	D.VPSE**RYLGYLE**QLL.R	Neuropeptide, Antioxidative, Opioid	Loukas et al., 1983
212.	24.21	1880.0559	-1.2	β	193-209	L.**YQEPVLGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
213.	24.30	2380.2578	-0.5	α_{s1}	17-36	L.**NENLLRFFVAPFPEVFG**KEK.V	Anticancer	Robert et al., 2004
214.	24.41	2043.1404	2.6	β	69-87	K.SLPQNIPPL**TQTPVVVPPF**.L	Ace inhibitor	Yamamoto et al., 1994
215.	24.44	2479.3262	-1.9	α_{s1}	17-37	L.NENLL**RFFVAPFPEVFGKEKV**.N	Anticancer	Robert et al., 2004
216.	24.63	2085.1509	-0.4	β	73-91	Q.**NIPPLTQTPVVVPPFLQPE**.I	Ace inhibitor	Yamamoto et al., 1994
217.	24.70	2510.3784	-0.1	β	69-91	K.SLPQNIPPL**TQTPVVVPPFLQ**PE.I	Ace inhibitor	Yamamoto et al., 1994
218.	24.88	2123.1204	-2.4	α_{s1}	17-34	L.**NENLLRFFVAPFPEVFG**K.E	Anticancer	Robert et al., 2004
219.	24.91	2106.2241	-2.0	β	191-209	F.**LLYQEPVLGPVRGPFPIIV**	Ace inhibitor, Immunomodulating	Yamamoto et al., 1994
220.	25.13	2926.5876	0.6	β	84-110	K.SLPQNIPPLTQTPVVVPPFLQPEIM(+15.99)GV.S (oxidation)	Ace inhibitor	Yamamoto et al., 1994
221.	25.21	2811.5244	1.1	β	69-94	K.SLPQNIPPL**TQTPVVVPPFLQ**PEIMG.V	Ace inhibitor	Yamamoto et al., 1994
222.	25.25	2997.6248	0.1	β	69-96	K.SLPQNIPPLTQ**TPVVVPPFLQP**EIMGVS.K	Ace inhibitor	Yamamoto et al., 1994
223.	25.27	2754.5029	0.6	β	69-93	K.SLPQNIPPL**TQTPVVVPPFLQ**PEIM.G	Ace inhibitor	Yamamoto et al., 1994
224.	25.33	2329.2756	3.2	β	73-93	Q.NIPPL**TQTPVVVPPFLQ**PEIM.G	Ace inhibitor	Yamamoto et al., 1994
225.	25.42	2215.2327	2.6	β	74-93	N.IPPLTQ**TPVVVPPFLQ**PEIM.G	Ace inhibitor	Yamamoto et al., 1994
226.	25.49	2910.5928	4.5	β	69-95	K.SLPQNIPPLTQ**TPVVVPPFLQP**EIMGV.S	Ace inhibitor	Yamamoto et al., 1994

Table 1. *Peptides identified to buffalo caseins αs1, β and κ.*

This significant difference cannot be solely explained by a different processing method used by the factory, but should be ascribed to a different analytical set-up. The suitability for peptidomics of the Orbitrap-MS analyzers has been, in fact, widely described.[12] Most peptides originated from β-casein (47%) followed by α_{s1} (40.5%) and κ (12.5%). These data confirm a major degradation of β-casein, in particular in the C-Terminal portion with L^{192}-Y^{193} and Y^{193}-Q^{194} residues together with the N-terminal portion with A^{1}-R^{2} residues, which are more susceptible to cleavage, probably by amino- and carboxy-peptidases, as observed previously.[13] Also α_{s1}-casein was prone to degradation at the N-terminal portion, within the region comprising residues A^{1}-R^{2} and, in particular, F^{23}-F^{24} and F^{24}-V^{25} which are known to be subject of cleavage by chymosin and cathepsin B respectively. Kappa casein was mainly represented by peptides deriving from κ-casein macropeptide f(106-169), especially in the region containing residues N^{113}-Q^{114}. The sequence coverage was 83% for β-CN, 49% for α_{s1}-CN and 41% for κ-CN. Peptides were comprised in the range of 500-3000 Da.

4.3.2. Bioactive peptides of Scotta

As showed in figure 2B, a wide range of healthy properties can be attributed to scotta peptides, many of them showing multifunctional activity. Some bioactive peptides are summarized in table 2.

Activity	***Amino acid***	***Casein***	***Bioactive sequence***	***References***
Ace inhibitor	176-199	α_{s1}	**APSFSDI**PNPIGSENSGKTTMPLW	Yamamoto et al., 1994
	170-175	β	**VLPVPQ**	Yamamoto et al., 1994
	78-91	β	**TQTPVVVPPFLQPE**	Yamamoto et al., 1994
	106-124	β	**HKEMPFPKYPVEPFTE**SQS	Yamamoto et al., 1994
	132-141	β	NL**HLPLP**LLQ	Miguel et al., 2006
Antimicrobial	15-23	α_{s1}	VL**NENLLR**	Hayes et al., 2006
	132-140	κ	**STPTTEA**IE	Malkoski et al., 2001
	66-74	κ	**AVRSPAQIL**	López-Expósito et al., 2006
Opioid	93-100	α_{s1}	**GYLEQ**LLR	Loukas et al., 1983
Antithrombotic	163-169	κ	**QVTST**VV	López-Expósito et al., 2006
Ace inhibitor, Antimicrobial	185-199	α_{s1}	P**IGSENS**GKTTMPLW	Hayes et al., 2006; Yamamoto et al., 1994
Ace inhibitor, Anticancer	25-37	α_{s1}	VAP**FPEVFG**KEKV	Robert et al., 2004
Ace inhibitor, Immunomodulating	193-209	β	**YQEPVLGPVRGPFPIIV**	Yamamoto et al., 1994
	7-28	β	**NVPGEIVESLSSSEESIT**HINK	Hayes et al., 2007
Ace inhibitor, Opioid	58-68	β	**LVYPFPGPIP**K	Yamamoto et al., 1994
Ace inhibitor, Antioxidative	176-182	β	K**AVPYP**Q	Hayes et al., 2007
	183-192	β	**RDMPIQ**AFLL	Hayes et al., 2007
Neuropeptide, Antioxidative	80-90	α_{s1}	**HIQKEDVPSER**	Gupta et al., 2009
	100-107	β	**EAMAPK**HK	Gupta et al., 2009
	94-99	β	GVSK**VK**	Yamamoto et al., 1994
Ace inhibitor, Neuropeptide	63-68	β	**PGPIP**K	Miguel et al., 2006; Yamamoto et al., 1994
Antimicrobial, Immunomodulating	7-13	α_{s1}	**KHQGLPQ**	Hayes et al., 2006
	10-22	α_{s1}	**GLPQGVLNENLLR**	Hayes et al., 2007
	106-113	κ	**MAIPPKKN**	Malkoski et al., 2001

Table 2. *Main examples of bioactive peptides*

It should be pointed out that several sequences are still encrypted in precursor oligopeptides, but they can be released by the action of peptidases, as often occur in gastrointestinal digestion process. Numerous recognized anti-ACE peptides have been identified, among them the αs_1-casein peptide with sequence NENLLRFFVAPFPEVFGK f(17-34) has been reported to exhibit strong ACE inhibitory activity[14] together with a large number of β-casein derived peptides, such as NLHLPLPLLQ f(132-141) and SLPQNIPPLTQTPVVVPP f(69-86)[15, 16] which were not detected in the previous investigation. Also antimicrobial peptides (AMPs) were found in the sample, in this regard particularly useful was the search against the milkAMP database.[17] Several fragments belong to α_{s1} casein peptide Isracidin f(1-23), which has been recognized as antimicrobial[18], and it is known to be subjected to chymosin cleavage. The shorter peptides, such as the Caseicin B with sequence VLNENLLR f(15-23), showing activity against *Escherichia Coli* at very low concentration[19], could derive from both lactic acid bacteria peptidases as well as from rennet enzymes action. The αs1 buffalo variant fragment 1-23, presents two amino acid substitutions, therefore the activity of the interested sequences should be confirmed. Among potential antimicrobial peptides numerous κ-casein derived fragments were found. In particular, the peptide AVRSPAQIL f(66-74) and the N-terminal portion f(106-123) of peptide Kappacin f(106-169) were detected [20], together with the C-terminal QVTSTVV f(163-169), which has been reported to possess antimicrobial activity.[21] The antimicrobial activity of the C-terminal peptide should be further investigated, since the buffalo variant possesses an amino-acid variation (V^{168}-A). Moreover, an antithrombotic function was reported for the κ-CN peptide MAIPPKKNQ f(106-114) [22], K-casein fragments f(66-74) and f(106-114) had not been detected before in scotta. Several peptides belonging to β-CN , identified in the present investigation, are characterized by a

multifunctional activity. A clear example is casecidin 17, β-CN f(193-209), which exerts both anti-ACE [16] and immunomodulating activity.[23] In addition, also the β-CN peptide VYPFPGPIPK f(58-68) showed both anti-ACE and opioid activity, similarly to the α_{s1}-CN f(86-99) known as α-Casomorphin 6, the latter has not been previously reported in "Scotta".[24] Also present were antioxidant peptides such as the β-CN sequences f(100-107) and f(176-182) [25] Many other sequences have been identified in the sample, that are reported to exert other relevant healthy functions. In this regard, the activity of the identified peptides has been only partially described, in particular *in vivo* and bioavailability studies are necessary to understand in depth their mechanisms. Furthermore some peptides present amino acid substitutions, in comparison to the bovine variant, thus their activity should be further investigated. The isolation and purification of different peptide fractions are the following steps to elucidate and confirm the predicted biologic activity.

4.4. Conclusions

Similarly to whey, Scotta cannot be considered only a mere waste product of dairy industry. Our approach, based on the isolation of a peptide fraction ≤ 3000 Dalton, and subsequent LC-HRMS based method has revealed a high complex profile. The peptidomic analysis highlighted a wide presence of valuable multifunctional peptides, with recognized healthy properties, which could be recovered from the matrix and potentially employed in nutraceutical formulations and functional foods.

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CHAPTER V

Conclusions

The main objective of my PhD has been focused to the analysis and characterization of milk and dairy products derived from "Centrale del latte di Salerno" in order to assess their potential use as dietary supplements and functional foods.

The purpose of my work was essentially the recovery and biological evaluation of the main bioactive components belonging to the dairy industry and its by-products. Starting from milk, with the aid of a pilot plant equipped with spiral semipermeable membranes, was formulated a soft cheese with a high protein content, which, besides its nutritional also possesses strong antioxidant capabilities. Moreover, was investigated the qualitative and quantitative variations of the individual fatty acids after several weeks from the expiration date, by evaluating the action of various factors present in milk that influence the degradation.

Finally, the potential of bioactive peptides present in the liquid dairy waste, known as Scotta, which derives from the production of ricotta cheese was in depth investigated. Very little is known about this waste product, our results led to the identification of 226 peptides with potential biological activity, thus allowing to reconsider Scotta no longer as a waste product, but rather as a source of peptides with many known healthy properties.

Based on our results, we can conclude that dairy by-products cannot considered only waste, since from these matrices many health promoting compounds can be recovered and re-employed in nutraceutical formulations or functional foods, as well as cosmeceuticals products. The developed techniques could be useful to drive the dairy industry to recover and valorize their by-products in order to obtain potential economic benefits from them as well as reduce the impact on the environment.