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Innovative Nutritional Aspects of locally produced Italian cheeses

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Summary

Abstract

This study aimed to investigate about the not common known nutritional aspects of cheeses, which derive from their chemical components. In fact, in addition to the supply of macronutrient, cheeses are gaining interest as a source of bioactive peptides, of conjugated linoleic acid or for the new insight in the metabolic role of calcium. In vitro simulation of human gastrointestinal digestion revealed that cheeses have an higher digestibility of calcium than other foods, because of their casein-derived bioactive phosphopetides (CPPs) content has the ability to carry calcium minerals and avoid calcium precipitation, making it available for intestinal absorption. The in vitro calcium digestibility was calculated for different foods- cheeses, soya based foods and vegetables- to correct cover calcium requirements with an equilabrate energy intake.

Calcium digestibility was also assessed in different ripened time Italian locally produced, semi fat, hard cheeses, Grana Padano and TrentinGrana. The main difference between them is the use or not of lysozyme during manufacturing. In Grana Padano samples, produced using lysozyme, there is a positive relationship between aging and dCa ($r2 = 0.27$; $P<0.05$) when sample > 24 months aged are considered (Grana Padano dCa results of samples less 24 months aged are quite widespread) , while in TrentinGrana, produced without the use of lysozyme, no significant correlation has been detected.

RP-HPLC distribution analysis of oligopeptides molecular weight of these cheese showed that the only difference between them is that cheeses without lyzozyme, aged between 15 and 20 months, are more hydrolyzed than the same ripened time Grana Padano samples. Moreover the fraction of oligopeptides involved in calcium binding ranges between 1000 and 1500 D. SELDI analysis confermed CPPs presence in this range. Therefore changes in cheese peptidic profiles probably caused by the use of lysozyme do not infuence calcium digestibility because according to this study there is not a connection between change in peptidic profile and calcium digestibility results.The difference in calcium digestibility in Grana Padano samples aged over 24 month results should be probably ascribed also to the influence of other factors occuring during cheese manufacturing.

Moreover, ACE-inhibitory activity of bioactive peptides was tested on in vitro digested Grana Padano and TrentinGrana samples with different ripening times. Correlation was not found between ACE-inhibitory activity and proteolysis level in different ripened time samples nor the lysozyme influence in releasing ACE-inhibitory bioactive peptides.

1. Introduction

In Italy food culture is the sum of all local cultures: every region has its own typical food products, manufacturing procedures and village festivals. It's possible to see this regionalization also at linguistic levels, with the presence of specific and dialectal names. Moreover, every regional marketplace has its own unique regional products.

This extreme richness in variety includes many products ranging from wine to cheese.

The literature concerning cheese reveals that there are almost 2000 names applied to different cheeses, and periodically more names appear as new varieties are made.

Over the last few years, the interest in "locally produced foods" has increased. This attention was recognized by brands which certify an accordance with a series of standards. Included within these standards are a production zone, presence of inspections in all or part of the work producing procedure. The products with this identification have to respect a specific disciplinary approved by the European Community that specifies authority control methods.

Branded cheeses have both cultural and productive importance because they represent a continuity with our gastronomic traditions and a great economic potential.

In Italy in 2001, 2.736 million euros were spent on recognized denomination foods and 64% of these were cheeses, such as Parmigiano Reggiano, the Grana Padano. For this reason, knowledge of our local products means knowing our national identity. Furthermore they represent one of the most important food market outlets in Italy and in other foreign countries.

In this PhD study one of the best selling locally produced Italian cheeses in the world was examined for its nutritional features: the semi-fat, hard, granular cheese known as Parmesan . The most famous being the Parmigiano Reggiano and Grana Padano. The main difference between them is the use or not of lysozyme during manufacturing. In chapter 2 the two production techniques of the two cheeses are described. Cheese nutritional features derive from their chemical components. The chemical composition of Grana Padano and TrentinGrana are described in chapter 3. In addition to the supply of macronutrients, dairy products, like cheeses are gaining more and more interest as source of bioactive peptides, of conjugated linoleic acid (CLA) or for new insight in the metabolic role of calcium. These innovative nutritional aspects of cheese are described in chapter 4. These peptides encrypted in cheese proteins (caseins) are latent until released and activated by enzymatic proteolysis, e.g. during gastrointestinal digestion, ripening time. The presence of a proteolytic molecule, like lysozyme, affects peptides release and consequently the levels of bioactive peptides can be also modified. Specifically, the peptidic profile of Parmesan type cheese changes during

ripening time and only in Grana Padano cheese peptide release is also influenced by the presence of lysozyme molecule used in its manufacturing.

In this thesis the following subjects were focused in:
- differences in Ca digestibility among foods and their effects on human nutrition (chapter 5);
- relationship between peptides pattern and Ca digestibility (chapter 6);
- effect of aging and lysozyme on the presence of caseino-phosphopeptides in Grana Padano and TrentinGrana (chapter 6);
- effect of aging and lysozyme on the presence of ACE-inhibitory peptides in Grana Padano and TrentinGrana (chapter 7).

Another item widely dealt with in this PhD thesis has been the great importance of cheese as calcium source for humans. As a matter of fact cheese has an important calcium content and data from an Italian food consuption survey, completed by the National Institute of Nutrition in 1995, reported that milk and dairy products provide by far the greatest amounts of dietary calcium showing that around 50% of calcium intake derives from these foods. Both vegetables and cereals are also important contributors - with each providing 11% of the calcium intake. The contribution of water to daily intake of calcium, about 90 mg/die corresponds to 11% of the total daily calcium intake. In a industrialized country like Italy the deficiency of mineral calcium it is not common, but it might be inadequate in specific segments of the population (Van Dokkum, 1998). For example one of the major health corcerns related to the intake of calcium is osteoporosis. In Italy calcium RDA (recommended dietary allowance) has been established for sex and each age group. To fulfill these calcium needs it’s important to consider that it might be suitable to adopt a specific calcium digestibilty value for each food instead of a general value. In fact an initial calcium amount in foods is not completely available for human absorption. For example, in vegetables, calcium absorption is inhibited by oxalates and phitic acids. In dairy foods however, the casein-phosphopetide (CPP) content has the ability to carry calcium minerals and avoid calcium precipitation making it available for intestinal absorption. According to this, in the study described in chapter 6, calculation and comparison of calcium digestibility indexes % of different foods has been done. Moreover, calcium digestibility index % results have been useful for nutritional considerations and in the formulation of correct diets that are able to cover human daily calcium requirements with the adequate energy intakes. Besides the physiological factors that influence RDA values, calculation have been evaluated, assessing if the Italian recommended dietary allowances for calcium (LARN 1996) are set too high.

2. Grana Padano, TrentinGrana , Parmigiano Reggiano cheeses: manufacturing, compositional features and differences

Grana Padano, TrentinGrana and Parmigiano Reggiano are typical Northern Italy semi-fat hard cheeses with Protected Designation of Origin (PDO). To be qualified as PDO, the product must have qualities and characteristics which are essentially due to its region of production: Grana Padano cheese is made in five regions of the Po river valley, parts of Emilia-Romagna, Lombardy, Piedmont, Trentino and Veneto, while Parmigiano Reggiano can only come from the cities of Parma, Reggio-Emilia, Modena, Bologna and Mantua. TrentinGrana is a mountain milk, belonging to Grana Padano Consortium. It's made in the region Trentino.

The production method of Grana Padano and Parmigiano Reggano it's quite the same and differs mainly because :

- Grana Padano is made only with skimmed milk whereas Parmigiano Reggiano is made with a mix of skim and whole milk. The good quality milk arrives in the factories twice a day. According to Parmigiano Reggiano Production Disciplinary, milk from the evening milking is left to rest until morning in large vats, where the fat spontaneously rises to the surface. This fat is used for the butter. Instead, the remaining skimmed milk is added to the milk of the morning milking in order to made cheese. In Grana Padano, instead, both morning and evening milking are skimmed before making cheese. Therefore, in Grana Padano consortium factories, cheese is manufactured twice a day.

- Additives use is not allowed for Parmigiano Reggiano and TrentinGrana cheeses. Instead an antibacterial molecule (lysozyme) is added during Grana Padano manufacturing procedure. Lysozyme molecule is not used in TrentinGrana and Parmigiano Reggiano manufacturing. This is the most interest difference for the PhD work, because lysozyme can influence cheese peptidic profile during ripening time.

  In Grana Padano manufacturing the partly skimmed milk obtained is transferred into copper kettles and whey acidified with a mixture of *Lactobacillus helveticus*, *Lactobacillus Lactis*, *Lactobacillus bulgaricus* and *Lactobacillus fermentum* is added. At this moment lysozyme is added to prevent the growth of *Clostridium tyrobutyricum* which can cause gas production.

- The cows used graze on different pastures, resulting in different diets that create subtle nuance in flavor. There are more restriction on the diet for the cows of Parmigiano Reggiano. Parmigiano Reggiano cows must be fed with fresh or dried vegetable matter (i.e. grass and hay). Grana Padano cows are allowed to eat silage in their feed. For TrentinGrana cheese cows are fed, like Parmigiano Reggiano cheese, without silage.

2.1. Production process

When the milk from the evening milking arrives, is left to rest overnight for the skimming action (only for Parmigiano Reggiano manufacturing). Consequently the skimmed milk is poured into the typical bell-shaped copper cauldrons where calf rennet and fermented whey, which are rich in natural lactic ferments obtained from the processing of the day before, are added. The milk coagulates in around ten minutes, and the curd is broken down into minuscule granules using a traditional tool called “spino”. After resting for about thirty minutes, the cheese mass is removed, with deft movements, by the cheese maker. Once the cheese is cut into two parts and wrapped in its typical cloth, is placed in an mould for its final shape. Each cheese is provided with a unique progressive number which remains as a sort of identity card. A special marking bands engraves production month, year, dairy registration number as well as the unmistakeble dotted inscription. The cheese is then placed in a salt-saturated solution which induced the salt adsorption process necessary to start the ripening cycle. The cheese wheels are laid out in rows in a silent maturation rooms, on wooden tables where the outer part of the cheeses dries forming a natural crust. The experts of each consortium examine each cheese one by one. After inspection, if the cheese meets the requirement of the PDO, a mark is fire branded onto each cheese wheel. All identifying marks and the dotted inscriptions are remouved from any cheeses which do not meet the PDO requirements.

3. Cheese nutritional components

Cheese is a product obtained processing raw milk. The milk examined in this study is cow milk. Therefore cheese composition reflects features and the chemical components/nutrients of the starting raw milk. It is an excellent source of fat and particularly proteins. It is a rich source of fat soluble vitamins: A, D, E and K. Apart from serving as an important source of minerals calcium and phophorus, it is concentrated in energy. Each component is treated in details.

3.1. Cheese protein

The total protein component of cheese is composed of numerous specific proteins. The primary group of cheese proteins are caseins. All other proteins can be grouped together under the name of whey proteins.

3.1.1. Casein

Casein is the predominant phosphoprotein that accounts for nearly 80% of proteins in cheese. The principal casein fractions are •$_{S1}$, •$_{S2,}$ •, •. The 4 genetic variants of casein are distinct molecules but are similar in structure. The distinguishing property of all caseins is their low solubility at pH 4.6. The common compositional factor is that caseins are conjugated proteins, most with phosphate group(s) esterified to serine residues. Table 1-3.1.1 shows chemical and physical features of caseins.

Most, but not all caseins, cheeses structural components, exist in a colloidal particle, called casein micelle. In addition to casein molecules, the casein micelle contains water and salts, mainly calcium and phosphorous: calcium phosphocaseinate micelle structure (average diameter 150-200 nm). Besides casein protein, calcium and phosphate, the micelle also contains citrate, minor ions, lipase and plasmin enzymes, and entrapped milk serum. Casein micelle are composed of 70% of water and 30% of dry matter (8% mineral salts and 92% of caseins). Only 0,5% of water is bound to protein, the remaining part it’s trapped in protein reticule. Table 2-3.1.1 shows casein micelles average composition of caseins.

Table 1-3.1.1. caseins chemical-physical features

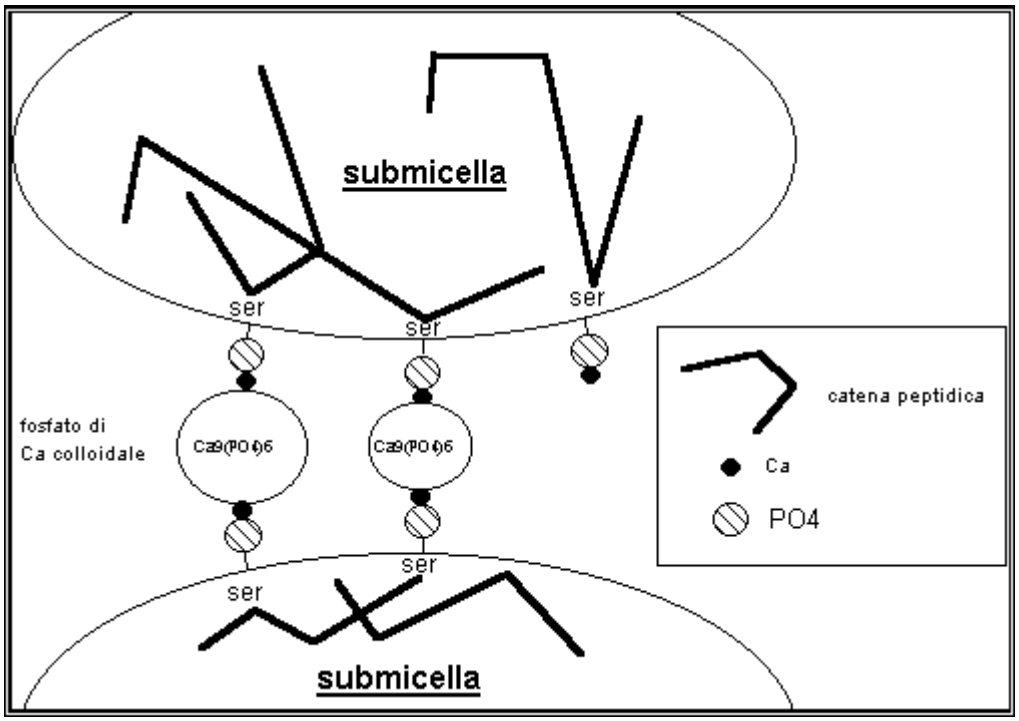
	S_1	S_2	•	•
Amino acid residues (n°/mol)	199	207	209	169
MolecularWeight (D)	23600	25200	24000	19000
Cystein residues	-	2	-	2
Phosphoserine groups	8,9	10,13	5	1,2
Glucides	-	-	-	+
Protease sensitivity	+	-	+	+++
Calcium sensitivity	++	+++	+	-

Table 2-3.1.1. casein micelles average composition (g/100g)

Caseins		Salt components	
S1	33	Calcium	2,9
S2	11	Magnesium	0,2
•	33	Inorganic Phosphate	4,3
•	11	Citrates	0,5
g	4		
Total caseins	**92**	**Total salt components**	**8,0**

Besides Schmidt (1980) model suggests casein micelles made up of sub-micelles; casein proteins associated through minerals (calcium phosphate and magnesium). These sub-micelle contains a hydrophobic core and are covered by a hydrophilic coat of casein phophoserinic residues and caseins COOH end part. Serine amino acids in casein primary structure are able to bind phosphoric groups. The number values of serine amino acid is different in the 4 caseins. The •-s_2 casein contains the wider number of serine residues. Specifically phosphoserine groups in sub-micelle outer surfaces make possible the aggregation between sub-micelles. The bond between calcium-calcium phosphate and phosphor-serine group establishes the formation of bridge between casein sub-micelles (figure 1-3.1.1).

Figure 1-3.1.1. Calcium- calcium phosphate and phosphoserin group bond between casein submicelles



In casein micelle, sub-micelles poor or completely without casein are arranged inside the structure, instead the sub-micelles rich in casein are in the outer coat. Casein, in N-terminal end (•-casein), is bound to an hydrophilic glucid and possesses a weak bond met 105-phe 106. Proteases selectively cleavage this bond, releasing the peptide 106-169 with the glucid bounded, called Casein Macropeptide (CMP). Casein without CMP is called calcium paracaseinate, which is hydrophobic and causes the coagulation

3.1.2. Whey proteins

Whey proteins are the proteins appearing in the supernatant of milk after precipitation at pH 4.6. These globular proteins are more water soluble than caseins and are subject to heat denaturation. Native whey proteins have good gelling and whipping properties. Denaturation increase their water holding capacity. The principal fractions are •-lactoglobulin, •-lactoalbumin, bovine serum albumin (BSA), and immunoglobulins (Ig). The most relevant protein in whey is •-lactoglobulin. It comprises 10% of the total milk protein or about 58% of the whey protein. It contains 162 amino acids with a molecular weight of about 18.300. The group of •-lactoglobulins, including eight genetic variants. The second most prevalent protein in whey is •-lactoalbumin which comprises about 2% of the total milk protein which is about 13% of the total whey

protein. The molecule consists of 123 amino acids and has a molecular weight of 14.146. This group of proteins contains eight cysteine groups, all involved in internal disulfide bonds, and four tryptophan residues.

3.1.3. Cheese protein traditional nutritional value

The nutritional importance of cheese arises from its high content of biologically valuable proteins. Good quality proteins are those that are readily digestible and contain the essential amino acids in quantities that correspond to human requirements. Humans have a requirements for essential amino acids, which are not synthesized by man and must be obtained by food .Animal protein (e.g. cheese) are more likely to contain these essential aminoacids than vegetable proteins, and therefore, have a better biological value (Table 1-3.1.3). The daily protein requirement per day for adults is approximately 1 g per kg of body weight. Cheese can contributes significantly to the supply of essential amino acid. In table 2-3.1.3., where the amino acid composition of milk and cheese proteins are compared to the reference protein, which indicates the ideal concentration of essential aminoacids in a dietary protein, it can be seen that cheese protein meets the requirements to the same extent as milk protein, except those for methionine cystine. The mean degree of utilization of the essential amino acids in cheese protein is 89.1%, i.e. greater than the corresponding value for milk protein (which is 85.7 %) and almost equal to the value for egg protein, which is 89.6%. The free amino acids of cheese, particularly aspartic and glutamic acids, are said to promote the secretion of gastric juice. It should be noted that a food allergy to cheese protein has never been described (Dillon 1984). During cheese ripening, part of the water-insoluble casein is converted in water-soluble nitrogenous compounds which include intermediate products of protein hydrolysis as well as aminoacids. Cheese ripening can be looked upon as a sort of predigestion whereby the digestibility of the proteins increase. Small peptides can pass through the wall of the intestine and it is possible that they penetrate even cell membranes so that they become directly available to the cell. An experiment with rats demonstrated that the rate of utilization of cheese protein was higher than the rate for casein. Besides, during cheese ripening time, the decarboxylation of free amino acids produces amines. The principal amines found in cheese are histamine, tyramine, tryptamine, putrescine, cadaverine, and phenylethylaminie. The concentration of individual amine in cheese show great variations and depend on the ripening period, on the intensity of flavor development and on the microbial flora. Physiologically active amines can affect the blood pressure, with tyramine and phenylethylamine having a hypertensive and histamine a hypotensive effect. However mono and diamine oxidases convert the biogenic amines that are consumed in foods relatively quickly into aldehydes and finally into carboxylic acids by oxidative deamination. Although opinions on the toxicity threshold values of amines widely diffused (for tyramine they are 10-80 mg, for histamine, 70-1000 mg), it is concluded that

healthy persons are able to metabolize the biogenic amines ingested, even when large amounts of cheeses are consumed, without adverse physiological reactions (Binder 1984).

Proteins content is also important for the energy intake and to provide protein material for the growth of human body as well as for replacement of tissue in the body. Protein content of different varieties rounds between 20-35%. Within all cheese, the protein content varies inversely with the fat content. The nutrient density (based on the energy content) for the protein content of different types of cheese is 2.9-6.2. A 100 g portion of soft cheese will provide 30-40% of the daily protein requirements of an adult and 100 g of a hard cheese will supply 40-50%. In cheese manufacture, the casein of milk is incorporated into the cheese while most of the biologically valuable whey proteins pass into the whey. Thus, 75-80% of the total protein and about 95% of the casein are transferred from the milk to the fresh cheese (Antila et al, 1982).

Table 1-3.1.3:Protein biological value of different foods

Foods	Protein Biological value
Milk	84,5
Cheeses	70,6
Egg (yolk+albumen)	97,3
Albumen of egg	83,0
Beef-veal	74,3
Pork	74,0
Chicken	74,3
Fresh fish	76,0
Wheat flour	52,0
Maize	59,4
Rice	64,0
Chickpeas	68,0
Bean	58,0
Lentils	44,6
Peas	65,2
Soya	72,8
Mushroom	66,7

TABLE 2-3.1.3.:Essential aminoacids in milk and casein(%), compared to the reference protein

Essential amino acid	Content (g/100 protein)		
	Reference protein FAO/WHO	Milk protein	Cheese protein
Tryptophan	1.0	1.4	1.4
Phenylalanine	6.0	10.5	10.9
Leucine	7.0	10.4	10.4
Isoleucine	4.0	6.4	5.8
Threonine	4.0	5.1	4.8
Methionine+ cystine	3.5	3.6	3.2
Lysine	5.5	8.3	8.3
Valine	5.0	6.8	6.8
Total	36.0	52.5	51.6

*Note: Arginine may not be essential for children, while histidine my be essential for children but not for adult.

3.1.3. Enzymes

Dairy enzymes are either natural extracts or produced by microbial fermentation. Their first functionality is to hydrolyse their respective specific substrate such as protein, peptide, lipid, carbohydrate in cheese. They are mainly used by cheese processors in order to bring texture and flavour. There are both indigenous and exogenous cheese enzyme. The indigenous ones are the same of raw milk used for cheese manufacturing. The most significant group are the hydrolases: lipoprotein lipase, plasmin, alkaline phosphatase. Lipoprotein lipase splits fats into glycerol and free fatty acids. This enzyme is found mainly in the plasma in association with casein micelles. Plasmin is a proteolytic enzyme; It attaks both • -casein and • -s_2 casein. It is very heat stable and play a role in the ripening and flavour development of certain cheeses. Phosphatease enzyme is able to split specific phosphoric acid esters into phosphoric acid and the related alcohols.

An important enzyme used in cheese manufacturing is rennet, also called rennin or chymosin. Its cleavage at the Phe105-Met106 bond eliminates the stabilizing the stabilizing activity, leaving a hydrophobic portion, para kappa-casein, and a hydrophilic portion called kappa-casein glycomacropeptide (GMP), or more accurately, caseinomacropeptide (CMP).

3.2. Cheese fat

By adjusting the fat content of milk , cheeses with widely different fat contents (usually expressed as % fat-in-dry-matter) are produced. Fresh cheeses have an absolute fat content of up to 12%, while ripened cheeses, in general, contain between 20 and 30% fat (table 1-3.2). Some consumers prefer high-fat cheeses because a high fat content contributes significantly to flavor quality. In some cheeses, for instance Cheddar, typical aromas develop only when the fat-in-dry matter content is at least 40-50%. This is because the aroma is mainly due to the breakdown products of fat formed during cheese ripening. Hydrolysis of lipids during cheese ripening is primarily caused by microbial lipases because the native lipase in milk is largely inactivated by pasteurization (except in cheeses in which rennet paste or pregastric esterase is used). As a result of lipolysis, the concentration of free fatty acids in cheese is usually 1-5 g/kg. There is a close link between free fatty acids content in a number of cheese varieties and their flavor. Also other components, such as cholesterol, depends on cheese fat content. Cholesterol has been often demonized, but it's only a cardiovascular diseases warning. Cholesterol it's important because it is a natural component of cells; i.e brain cells are not able to work without cholesterol. It feeds cells and eliminate from toxins them. It's also a bile, hormones, adrenalin, D vitamin component, a nervous impulse conductor, and essential factor for bone

Table 1-3.2: Average fat content, calcium and phosphorus in different cheeses

Cheese variety	Fat content		Protein content(%)	Ca content (g/kg)	P content(g/kg)
	Fat in dry matter(%)	Absolute			
Parmesan	35	26	36.5	13.0	8.5
Emmentaler	45	29.0	27.9	10.8	8.6
Brie	50	23.0	22.4	4.0	4.0
Camembert	45	22.3	22.0	4.0	4.0
Fresh cheese	40	11.8	11.8	0.7	1.5

It has to be emphasized that:

- The cholesterol content of cheese is rather low (0-100 mg/100g, depending on the fat content).
- Therefore, cheese contributes only 3-4% of total cholesterol intake.

- The cholesterol in a diet has only a limited effect on the level of blood cholesterol. The body has a control mechanism which ensures that the synthesis of cholesterol is reduced when the amount of consumed cholesterol increases.

The fat should be in such amounts that the body can utilize it satisfactory. To avoid arteriosclerosis the recommended daily intake of fat is from 25% (sedentary worker) to 35% (heavy manual worker) of the content of the diet. Since fat provides 9,0 kcal per g, it is suggested that fat must have a good proportion of *essential fatty acids*, i.e. linoleic, linolenic and arachidonic acids.These fatty acids are unsatured which means they are reactive at points along their structure where double bonds occur.

$CH_3(CH_2)CH=CHCH_2CH=CH(CH_2)_7COOH$

LINOLEIC ACID

$CH_3(CH_2)CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$

LINOLENIC ACID

$CH_3(CH_2)CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)CH_3COOH$

ARACHIDONIC ACID

Thus the reactive double bonds or linkages within the acid provide the unstable or unsatured aspect.The opinion points to *cis* isomer structure of the acid as being most important. Cheese from whole milk is known to contain most of these fatty acids but there has been an effort to introduce more unsatured fatty acids (linolenic) into the diet of cow in order to augment those in normal milk, but these extra unsatured fats in the milk quickly cause rancidity problems unless antioxidants are added immediately the milk is produced.

Negative health effects have been attributed to dairy products because their high levels of saturated fatty acids (SFA), considered risk factors for arteriosclerosis and coronary heart disease, weight gain and obesity (Insel et al., 2004; Lokuruka, 2007). Nevertheless, Haug et al. (2007) in a recent reviw discussed the healthy effects of bovine milk components. Conjugated linoleic acids (CLAs), a group of octadecadienoic acid isomers that occurnaturally in milk, dairy products and beef meat, deserve special attention because of the anticarcinogenic, antiatherogenic, antidiabetic, immune stimulatory and body fat-reducing properties that they have been reported to possess (Terpstra 2004; Nagao and Yanagita, 2005; Collomb et al., 2006; Kelley et al., 2007). The fatty acid composition and CLA content of Grana Padano cheeses were affected by the season and production area, and by the interaction of these two factors. CLA content in Grana Padano cheese can range between 5,29 and 9,47 mg/g fat (Prandini et al., 2009). Table 2-3.2. reports fatty acid composition of different sample of Grana padano cheese.

Table2-3.2.: Fatty acid composition of Grana Padano

Fatty acids	**g/100 g of Fatty acids**
Trans11-18:1 (vaccenic acid, TVA)	0.94±0.02-1.66±0.07
cis9-18:1 (oleic acid)	18.56±0.06-18.99±0.17
18:3 omega3 (linolenic acid, LNA)	0.39±0.01-0.61±0.02
Cis9, trans11CLA (mg/g fat)	5.29±0.13-9.47±0.35
Categories of fatty acids	**g/100g Fatty acids**
Short chain of fatty acids (SCFA)	7.21±0.02-7.43±0.05
Medium chain fatty acids (MCFA)	19.82±0.10-27.2±0.22
Long chain fatty acids (LCFA)	64.23±0.29-67.42±0.25
Satured fatty acids (SFA)	64.23±0.29-67.42±0.25
Monunsatured fatty acids (MUFA)	24.17±0.19-26.19±0.09
Polyunsatured fatty acids (PUFA)	3.97±0.07-4.38±0.08

3.3. Carbohydrate

Although milk does contain milk sugar (lactose)- a carbohydrate providing energy in a diet- cheese has been ripened (and some soft varieties) does not contain appreciable amounts of lactose since this is either lost in the whey during manufacture or is converted into lactic acid or lactate during processing. Therefore, cheese is suitable for the diets of persons suffering from lactose malabsorption and/or diabetics (Blanc B. 1982).

Those people who are allergic to lactose can normally eat cheese, except for those very soft fresh unripened cheeses which may still contain appreciable amounts of unfermented lactose. The average lactic acid content of a number of cheeses is as follow: Parmesan 0,7%, Cheddar 1,3%, Emmentaler 0,4%. Cheese usually contains both lactic acid isomers, L (+) and D (-), the relative proportion of the D-isomer depending on the type of the starter culture used and some other ripening factors. The content of D (-) lactic acid in different types of cheese can be very different (fresh cheese 4-14%; ripened cheeses 10-50%) (Krusch 1978). The human organism has only a limited capacity to metabolize D(-) lactic acid but from data available in the literature, a toxic effect of D(-) lactic acid cannot be derived for the adolescent or the adult.

As a logical conclusion, the WHO, has not limited the admissible intake foe adults, while for infants (up to 1 year of age), a D(-) lactic acid-free diet is recommended (Thomas T.D., 1983).

3.4. Other components

Cheese also contains appreciable amounts of minerals and salts. Among other, calcium (Ca), phosphorus (P) (for bones and teeth) are the most important ones.

Calcium and phosphorus contents of cheese are important as those of milk. Since 100 g of soft cheese will supply 30-40% of daily Ca requirement and 12-20% of the daily P requirement and 100 g of a hard cheese will meet the daily Ca requirement and contribute 40-50% of the P requirement. The Ca content varies in vary type of cheeses. It should be noted that when one variety of cheese is made with different fat contents, the higher the fat content the lower Ca and P.

Cheeses produced by rennet coagulation usually have higher calcium contents than those made from acid-coagulated milk (Lagrange 1982). The calcium, phosphorus and magnesium in cheese are as well utilized by the body as those in milk (Andlaw 1977). The ratio of calcium to phosphorus in cheese is also thought to be desiderable nutritionally. The contents (g/kg) of some other minerals in various types of cheese are: Sodium (Na), 0,3-18,5; Potassium (K), 0,5-3,8; Magnesium (Mg) 0,1-0,7.

The wide range of the Na contents is due to the different amounts of NaCl added to cheeses. Since a high sodium intake can induce hypertension, a restricted sodium intake is recommended to accommodate the diets of consumers under medical management for hypertension. Although even in countries with a high consumption, cheese contributes only about 5-8% to the total sodium intake, the manufacture of low – sodium cheese is recommended by using a brine containing mainly KCl (Demott 1984). It should be considered also that hypertension may be due to a deficiency of dietary calcium rather than to an excessive intake of sodium , since it has been observed that patients suffering from hypertension about 25% less Ca normotensive persons, because of a low consumption of milk and dairy products. (McCarron A. 1982)

The range of values for the concentrations (mg/kg) of some trace elements in cheese is listed below:

Fe	0,3-12,0
Cu	0,2-3,6
Mn	0,3-5,3
Mo	0,05-0,5
Zn	0,1-3,0
F	0,1-3,0
I	0,05-1,0
Co	0,004-0,038

Cheese contains most of the essential vitamins except vitamin C (ascorbic acid) which is lost during processing. Since cheese is rarely the only item in a diet, the small deficiencies in cheese are usually made good in the diet by other foods. For example green food (e.g. lettuce leaves) provide the vitamin C lacking in cheese. The concentration (80-85%) of fat soluble vitamins in cheese depends on its fat content. Most of the vitamin A contained in milk passes into the cheese. This is naturally lower for the water-soluble vitamins.

The values of thiamine, nicotinic acid, folic acid and ascorbic acid are 10-20%, for riboflavin and biotin, 20-30%, for pyridoxine and pantothenic acid, 25-45%, and for cobalamin, 30-60%; the rest remains in the whwy (Reif 1976)

However, milk contains such high concentrations of some B vitamins that cheese still contributes significantly to the supply of these vitamins.

Furthermore, concentrations of several of the B vitamins depend on the type of starter culture used and increase with time of storage. After a long ripening period, the concentrations of these vitamins in cheese are therefore increased (Zehren 1982). By isolation individual microorganisms from cheese it could be shown that they are able to synthesize nicotine acid, folic acid, biotin and pantothenic acid.

The synthesis of vitamin B_{12} by propionic acid bacteria in hard cheese has aroused great interest. The concentration of B vitamins change during ripening since these vitamins are used and synthesized by the cheese microflora. Propionic acid bacteria have, therefore, been added experimentally to cheese milk in the manufacture of many type of cheese with the result that in some cases, especially with *Propionicbacterium freudenreii*, the cobalamin content was doubled. Most of the ascorbic acid, on the other hand, is broken during cheese ripening.

4. Cheese newly discovered nutritional aspects

Cheese traditional components like minerals, calcium in particular, are revealing to possess important innovative nutritional aspects.

Moreover a large number of peptides derived from casein and free fatty acid as Coniugated Linoleic Acid (CLA) have numerous functional activities. For example CLA promotes muscle growth while reducing body fat, it's a potent cancer fighting substance, has been shown to lower cholesterol and to improve immune response. Therefore it's possible to consider cheese also as rich of nutrients food like a "functional food"

4.1. Calcium innovative function

Calcium is the most abundant mineral in the human body and its support several important functions. More than 99% of total body calcium is stored in bones and teeth where it functions to support their structure. The remaining 1% is found throughout the body, in blood, muscle, and the fluid between cells.

Calcium is needed for muscle contraction, blood vessel contraction and expansion, the secretion of hormones and enzymes, and sending messages through the nervous system, maintaining total body health. As well as physiological human functions, Calcium has important innovative functions: reducing blood pressure and the risk of hypertension, preventing colon cancer, reducing risk for kidney stones, contrasting calcium deficiency disease like osteoporosis and arthritis. Besides there are many studies about the impact of increased calcium intakes on body weight or composition.

4.1.1. Calcium and weight management. Role of dietary calcium and dairy products in the regulation of human adiposity

Recently, several studies detected inverse association between calcium intake and body weight or body fat (Carruth and Skinner J.D., 2001; Lin et al., 2000; Davies et al., 2000). Zemel and colleagues established a possible mechanism using an obese mouse model and cultured human adipocytes (Xue et al., 2001; Shi et al., 2002; Zemel et al., 2000).

Briefly, the demonstrated mechanisms is that low calcium diets lead to an increase in intracellular calcium concentrations, which act to promote body fat deposition, reduce lipolysis and reduce thermogenesis. High calcium diets reverse these trends, and it appears that calcium in the form of dairy products may be more effective than elemental calcium (Shi et al., 2002).

However, much work remains to be completed before the relevance of these findings to human nutrition is understood. In the meanwhile, some insights may be gained by reviewing results of published randomized studies in which dairy product intake or calcium intake was experimentally observed.

A Medline literature search conducted between the years 1996 and 2001 investigated the effect of increased dairy product intake and increased diet calcium intake on body weight or fat body composition. In eight studies participants were randomly assigned to increase dairy product intake or to maintain their “usual” diets (Chan et al.,1995; Cadogan et al., 199; Merrilees et al., 2000; Baran et al., 1989; Prince et al., 1995; Devine et al., 1996; Storm et al., 1998; Lau et al., 2001; Barr et al., 2000). One reported results of randomized crossover trial (Cleghorn et al., 2001). Three of the studies were conducted in adolescent girls (Chan et al., 1995, Cadogan et al., 1997; Merrilees et al., 2000), one in premenopausal women (Baran et al., 1989), one in women within 5y of menopause (Cleghorn et al., 2001) and the remainder in postmenopausal women (one of each also included men) (Prince et al., 1995; Devine et al., 1996; Storm et al., 1998; Lau et al., 2001; Stern et al., 2000).

Briefly, most studies did not detect group differences in the changes in weight, height, fat mass or lean mass. However, two studies conducted in older adults observed significantly greater weight gains in the dairy-supplemented groups (Lau et al., 2001; Barr et al., 2000).

The difficulty in interpreting studies of this nature lies in the ability of humans to regulate their energy intake (Schwartz & Seely, 1997). In fact subjects randomized to increase their intake of dairy prodicts, did not simply add these to their diets without compensating in some way for additional energy content provided by the dairy foods. Of the studies that monitored energy intake, two suggest that compensation may have been complete, given that energy intakes did not differ between the dairy and usual diet groups (Chan et al., 1995; Cadogan et al., 1997; Merrilees et al., 2000). Merrilees and colleagues suggest that, in their subject group,

this may have resulted from a reduced consumption of baked goods in the dairy product group (Merrilees et al., 2000). Partial compensation is suggested in several of the remaining studies. Cadogan and colleagues (Cadogan et al., 1997) detected a tendency for energy intake to increase in the milk group (P=0.065), Barr and colleagues (Barr et al., 2000) observed that energy intake increased in milk group, but not as much as would be expected by simply adding milk to the usual diet; and Lau and colleagues (Lau et al., 2001) found that energy intake decreased significantly in the control group. Although serious questions have arisen regarding the ability of diet records to reflect actual energy intake (Schoeller , 1995), the two studies that detected significant differences in body weight change between the dairy-supplemented and control groups also detected corresponding significant differences in reported energy intake (Lau et al., 2001; Barr et al., 2000).

In summary, the available data suggest that in most cases, body weight or composition do not change when dairy product intake is increased. Whether these observation support the concept that calcium or dairy products increase energy utilization depends on the extent to which compensation occurred for the additional energy contained on the dairy products. Because of the difficulty of accurately documenting energy intake in free living humans, it is unlikely that studies of this nature will address this issue in a definitive manner.

The same Medline search about increased calcium intake studies, reports twelve randomized trial of calcium supplementation that provided information on changes in body weight or composition (Dibba et al., 2000; Johnston et al., 1992; Lee et al., 1995; Lloyd et al., 1993; Bonjour et al., 1997; Nowson et al., 1997; Ricci et al., 1998; Recker et al., 1996; Perez-Jarauz et al., 1996; Elders et al., 1994), and for another five, information about group differences was obtained from study authors (Storm et al., 1998; Riggs et al., 1998; Dawson-Hughes et al., 1997; Kalkwarf et al., 1997; Prentice et al., 1995). Seven trial were conducted in children or adolescents (Dibba et al., 2000; Johnston et al., 1992; Lee et al., 1994; Lee et al., 1995; Lloyd et al., 1993; Bonjour et al., 1997; Nowson 1997),two in women during lactation (Kalkwarf et al., 1997; Prentice et al., 1995), one in perimenopausal women (Elders et al., 1994), five in postmenopausal women (one of these studies also included male subjects) (Storm et al., 1998; Recker et al., 1996; Perez-Jarauz et al., 1996; Riggs et al., 1998; Dwson-Hughes et al. 1997) and two were conducted during weight loss in pre-and postmenopausal women (Ricci et al., 1998; Jensen et al, 1994). The studies varied in length from 3 mo to 4 y, and included subjects that varied in ethnicity and baseline calcium intake.

In the large majority of studies, no differences in the changes in body weight or body composition were detected between the calcium and placebo/untreated group (Dibba et al., 2000; Johnston et al., 1992; Lee et al., 1994, 1995; Lloyd et al., 1993; Bonjour et al., 1997; Nowson et al., 1997; Ricci et al., 1998; Perez-Jarauz et al., 1996; Elders et al., 1994; Jensen et al., 2001; Kalkwarf et al., 1997; Prentice et al., 1995).

Only one of 17 studies, that of Recker and colleagues (Recker et al., 1996) detected a difference in body weight change. During the 4-y study, postmenopausal women receving 1.2 g calcium /d lost 0.35 kg/y more

than did the control group. (Davies et al., 2000). The only significantly different change in body composition was observed in the study of Riggs and colleagues (Riggs et al., 1998). In their 4-y study, women supplemented with 1.6 g calcium/d had a more negative change in lean mass than that of controls, although group differences in weight and fat were not observed (Riggs, unpublished results, personal communication, March 2002).

The reason for the discrepancy between the study of Recker and colleagues and the remaining studies, in which an increased calcium intake was not associated with increased weight loss (or less weight gain), is not readily apparent. It does not appear to be attributed to variables such as sample, size, baseline calcium intake, the amount or type of calcium administered, the age, relative weight or ethnicity of the subjects or study duration. Recker's study was somewhat unusual in that it was the only study of nonintentional weight loss in which the control group lost a significant amount of weight (Davies et al., 2000), but how this could contribute to the different results is not known.

Similarly, there is no ready explanation for the lower lean mass among the calcium-treated women studied by Riggs and colleagues (Riggs et al., 1998). Animal studies have suggested that a high calcium intake is associated with decreases in fat mass rather than lean tissue (Shi et al., 2000; Zemel et al.,2000).

Summarizing there are only little evidence to support the hypothesis that calcium or other components of dairy products have a small impact on body weight in generally healthy humans

4.1.2 Calcium and high blood pressure

There has been considerable interest in the role of dietary factors such as in hypertension (McCarron et al., 1984). In several (Harlan et al, 1984; Joffres et al., 1987; Witteman et al., 1989), (Gruchow et al., 1985), epidemiologic studies, hypertensive subjects had a lower calcium intake, than did nonhypertensive subjects (Ackley et al., 1983; Garcia-Palmieri et al., 1984; Reed et al., 1985; Iso, et al., 1991). In agreement with this, calcium supplementation has been reported to lower blood pressure (Hamet et al. 1992). Thus an adequate calcium intake to prevent not only osteoporosis but also hypertension has been advocated (Osborne et al., 1996).

A negative association between calcium intake and dairy products and blood pressure was reported in an epidemiologic study conducted in Tromsø, Northen Norway, in 1994-1995 Rolf (Kaare., 2000). The purpose of that study was therefore to use an patients habits database to relate calcium intake of calcium from dairy sources and blood pressure. Because the absorption of calcium is highly dependent on vitamin D status (Reichel et al., 1989), intake of vitamin D was also included in the analyses. A negative association was found between calcium intake from dairy sources and blood pressure that was significant in the 3-factors analysis of variance with sex, calcium intake, and vitamin D intake. Therefore significant linear decrease in blood pressure was found with increasing calcium intake. No significant interaction between sex and intake of calcium on blood pressure were found. Thus, in both sexes there was a weak negative correlation between calcium intake and blood pressure, but this was significant only for diastolic blood pressure. There was a weak positive correlation between vitamin D intake and blood pressure.

Several recent reviews addressed the issue of calcium ingestion and blood pressure. In 1990 Cuttler and Brittain found that the evidence for a role of calcium in hypertension was suggestive, Cappuccio et al., concluded in 1995 that there was a small, inverse association between dietary calcium intake and blood pressure, whereas Osborne et al, in 1996 firmly stated that dietary calcium plays an integral role in the maintenance of normal blood pressure and that an adequate intake may help reduce the risk of hypertension. This may be true, but is unlikely that the effect of dietary calcium on blood pressure is a major one. If it were, it would have been easily documented for both sexes as well as for systolic and diastolic blood pressure. In a study by Ackley et al, (1983), the effect was seen in men but not in women. Ascherio et al., (1992), the effect in men was seen in lean men only. In women, calcium intake was inversely related to blood pressure but not to the development of hypertension (Ascherio et al., 1996). Furthermore, Iso et al., (1991), found an effect on systolic but not on diastolic blood pressure, whereas Kesteloot and Joossens (1988) made the reverse observation. Finally, in all studies in which a negative association between calcium intake and blood pressure was found, the magnitude of the association was moderate. Similarly, in the analysis by Bucher et al., (1996), which included 33 studies with a total of 2412 patients, a statistically

significant reduction in systolic blood pressure for only 1,27 mm Hg and a nonsignificant reduction in diastolic blood pressure of 0,21 mm Hg were found. However, in some studies, like the one by Buonopane et al., (1992), the effect was more pronounced. In that study, supplementation with nonfat milk for 8 weeks caused a reduction in blood pressure of between 3% and 7%. Even if the effect of calcium on blood pressure is small, that does not rule out a major effect on cardiovascular disease. Thus, Cook et al., (1995), in their analysis based on data from the Framingham Study and the National Health and Nutrition Examination Survey, concluded that a 2-mm Hg reduction in distolic blood pressure could reduce the incidence of coronary heart disease by up 6% and the incidence of stroke and transiet ischemic attack by up to 13%. Although one cannot rule out the possibility that ingested calcium exerts its effect on blood pressure through release of gastrointestinal hormones or neutral reflexes, the absorbed calcium is more likely to be of importance.

4.2. Bioactive peptide

The nutritional value of milk and milk products is due to their major constituents: protein, lactose, lipids, mineral salts. Nevertheless, other minor constituents such as hormones, growing factors (Hagemeister et al., 1990) and immunoenhancing constituents (Bounous and Amer, 1990) may exert an important action in human nutrition especially in breast-fed babies.

In the last years, the physiological role played in human nutrition by a group of substances, the so called bioactive peptides, from milk proteins has been investigated. These peptides are produced by enzymatic proteolysis of bovine, casein human and whey protein (Yamauchi 1992). Some of them have been also found in different foods. These peptide can affect some biological functions of the body and therefore they are called bioactive peptides.

Until now bioactive peptides are phosphopetides, ACE-inhibitory peptides, immunomodulating peptides, opioid peptides and antithrombotic peptides.

4.2.1. Phosphopetides. Mineral binding property and bioavailabily

The casein phosphopeptides (CPPs) are phosphorylated casein-derived peptides that can be released from different form of casein, i.e.• $_{s1}$-, • $_{s2}$-, • - and • -casein. Many of them contain a common cluster sequence of three phosphoseryl groups followed by two glutamic acid residues (S(P)S(P)S(P)EE) representing the binding sites for minerals, especially calcium, iron and zinc. These peptides thus contribute to increase the solubility of such minerals at intestinal pH and play an important role in bioavailability (Fitzgerald RJ , 1998).

The term phosphopetides (CPP) have been first introduced by Mellander in 1950 to describe casein-derived phosphorylated peptides which enhanced vitamin D independent bone calcification in rachitic infants. Since then CPPs have been shown to possess the unique property of being able to bind macroelements such as Ca, Mg and Fe along with trace elements such as Zn, Ba, Cr, Ni, Co and Se. Milk and dairy products are an excellent source of calcium and it is thought that CPPs produced during the digestion of caseins enhanced the bioavailability of calcium by increasing the solubility of calcium in small intestine, where maximal passive absorption of dietary calcium is thought to take place. Analysis of the intestinal chyme of rats and minipigs has also shown that CPPs are formed during casein digestion *in vivo* (Naito et al., 1972; Meisel and Frister, 1988) and they have also been found in fermented dairy products such as cheese (Pause and Lembke, 1993; Roudot-Algaron et al, 1994). The large scale *in vitro* production of CPPs has expanded the potential use for these products in the food and pharmaceutical sectors.

Numerous CPPs are produced during the various stages of human digestion occurring in the stomach. Therefore casein can be partially hydrolyzed by pepsin and in the small intestine by trypsin, carboxypeptidase and chymotrypsin activities of pancreatin (Miquel E. et al., 2006). Fragments 43-58, 59-79, 66-74 from • $_{s1}$-casein, 46-70, 1-21, 2-21 from • $_{s2}$-casein, and 1-25, 1-28, 33-48 from • -casein have been reported (Kitt and Yuan, 1992). Due to their resistance to enzyme proteolysis, CPPs containing multiple phosphoseryl residues can be found in the gut where they form stable complexes with calcium phosphate (Reynolds, 1994). These complexes are very soluble and increase the calcium absorption by hindering calcium phosphate precipitation. In particular CPPs-Ca complexes may enhance calcium absorption in the distal small intestine where passive transport occurs. The passive transport is the main calcium absorption route under physiological conditions and it is effective in bone calcification and treatment of rickets (Kitts and Yuan, 1992). The addition of CPPs to toothpaste formula has been suggested in order to prevent enamel demineralization and to obtain anticariogenic effect (Reynolds, 1994). CPPs are suitable for these aims because they have not shown allergenicity. Moreover, they can be used as anticariogenic additives in food formulas because they have not been shown to be unpalatable.

The highly polar acidic domains confer mineral binding abilities to phosphopetides. Dephosphorilated peptides do not bind minerals (Sato et al., 1983; Gerber and Jost, 1986; Berrocal et al., 1989). The role of phosphorylated residues in mineral binding is further illustrated by the observation that chemical phosphorylation of •$_{s1}$- and ••-casein increased the binding capacity and the stability of these proteins in the presence of Calcium (Yoshikawa et al., 1981).

The ability of CPPs to bind minerals is intrinsic to their potential role as functional ingredients in foods. Binding and solubilization of Calcium in the presence of phosphate has many potential beneficial consequences. In binding Calcium, for example, CPPs inhibits the formation the formation of hydroxypatite crystal (Holt et al., 1996). Reynolds (1993) demonstrated that Na and Zn forms of a composite CPP sample and the Na form of specific peptides, i.e. ••-casein f(1-25).4P and •$_{s1}$-casein f(59-79).5P could cause a significant inhibition of hydroxyapatite formation using the so called seeded crystal growth inhibition assay. It was also reported, on a mole to mole basis at pH 9.0, that ••-casein f(1-25).4P and •$_{s1}$-casein f(59-79).5P could bind 24 and 17 moles of calcium and phosphate, respectively. Lee et al. (1980) reported that approximately one mole of CPP could bind 40 moles of calcium. Calcium/CPP complexes are reported to exist as tetramers (Reynolds, 1993). The calcium binding costant of CPPs are reported to be in order of 10^2-10^3 M^{-1} (Sato et al., 1983; Berrocal et al., 1989; Sato et al., 1991; Schlimme and Meisel, 1995). This rather low affinity should facilitate release of Ca^{2+} during intestinal absorption. However, fractioned CPPs have higher affinity for calcium, a fact which may retard their ability to release calcium for subsequent intestinal absorption (Meisel, unpubblished results).

While the anionic hydrophilic domain is important in mineral binding, it also been demonstrated that amino acid residues upstream and downstream from this region also play a role in binding. For example, the syntetic peptide corresponding to the phosphoseryl rich region of •$_{s1}$- casein, i.e. f(63-70) bind less calcium than the entire tryptic peptide f(59-63) and the corresponding C-terminal f(71-78) do not bind Calcium (Reynolds, 1994). It was postulated that the folded structure in f(59-79) may also play a role in efficient Calcium binding and solubilization (Perich et al., 1992). Further evidence for the contribution of flanking peptides on Calcium binding is evidenced by the observed difference in calcium binding abilities of CPPs prepared using different enzymatic activities (McDonagh and FitzGerald, 1998).

Calcium from milk has been attributed to phosphopetide mediate enhanced solubility of calcium in the small intestine (GammelgaardLarsen, 1991; Kitts and Yuan, 1992; Schaafsma, 1997).

There are three modalities of Calcium absorption in human, i.e. a saturable, vitamin D-dependent transport system located in the duodenum and jejunum and a passive, vitamin D-independent system located in the ileum and the distal small intestine (Bronner, 1987). It is thought that the passive transport route is the major mode of Calcium absorption from the diet (Schaafsma, 1997). A pinocytotic system for mineral absorption also exists, this system, thought present in adults, is thought important in mineral absorption in infants

(Walker and Isselbacher, 1974). Several factors affect the absorption of Ca from the diet including phytate, oxalate, phenol compounds, lactose, phosphorus and obviously vitamin D. Phytate, as present in many non-refined cereals and legumes, forms insoluble or poorly soluble complexes with Ca at intestinal pH (Rossander et al., 1992; Schaafsma, 1997).

Considerable controversy exists as to whether phosphopetides do in fact enhance the absorption of dietary Calcium. Studies with *in vitro* produced CPPs in chickens (Mykannen and Wasserman, 1980) and *in situ* tubal ligation experiments in rats (Naito et al., 1972; Kitts et al., 1992b) have confirmed their effectiveness in enhancing intestinal Ca solubility. However, a number of *in vivo* feeding trials involving Ca balance studies following administration of CPPs have failed to show a significant effect on the absorption of Ca in rats (Brommage et al., 1991; Kopra et al., 1992), piglets (Pointellart and Gueguen, 1989), minipigs and vitamin D-deficient rats (Scholz-Arhens et al., 1990). A 23% improvement in intestinal soluble calcium was reported from *in vivo* rats studies when comparing conventional CPPs to • -casein H derived CPPs (Han et al., 1996). The apparent disparities in the observed effects of CPPs may, in part, be attributed to differences in the methodology used to measure extrinsic and intrinsic absorption of Ca (Kitts and Yuan, 1992).

While numerous animal studies are available on the effect of CPPs on mineral bioavailability, very little information is available from human studies. It has been demonstrated that CPPs increase calcium and zinc absorption from a rice-based infant gruel in human adults by approximately 30%. However, no effect was seen when CPPs were ingested in either high or low phytate wholegrain cereal meals (Hansen, 1995).

These results serve to emphasize the complex interactions between meal constituents and mineral bioavailability (Rossander et al., 1992). The above findings are of significance to inhabitants in the Eastern Pacific who have rice-based diets containing little or very low quantities of dairy products.

4.2.2. ACE-inhibitory peptides

The angiotensin I-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) is involved in the renin –angiotensin system, which partially regulates peripheral blood pressure. This enzyme is responsible for the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor, and for the degradation of bradykinin, a vasodilatatory peptide.

Inhibition of ACE can therefore exert a hypotensive effect and may also influence different regulatory system involved in immunodefence and nervous system activity (Meisel 1997).

Food derived ACE inhibitors are of great interest since they are natural preventive measure for the control of hypertension and could lead to a decrease in the requirement of medicines which are known to exert strong side effects.

Biopeptides may also exert the antihypertensive function by means of:

- interaction with opioid receptors having vasodilatatory effects (Nurminen et al. 2000);
- inhibition of the release of endothelin -1, a 21 residues long peptide with vasoconstrictor properties (Maes et al. 2004);
- mineral carrier peptides that increase Calcium bioavailability (Seppo et al., 2003).

Active peptides must be absorbed in an intact way from intestine and, in addition, be resistant to degradation by plasma peptidases to obtain the physiological effect. In fact, using monolayer-cultured human intestinal Caco-2 cells, it has been demonstrated, that the ACE-inhibitory tripeptide Val-Pro-Pro can be transported intact through the intestinal wall into the blood via paracellular and transcellular routes, although a significant amount of the peptide is degraded to amino acids by intracellular peptidases (Vermeirssen et al. 2004).

To date, Walsh et al. (2004) indicated that • -lactoglobulin fragment f142-148, kwon as a potent inhibitor of ACE activity *in vitro* (Mullally et al. 1997), is degraded when it is incubated with gastrointestinal and serum proteinases and peptidases, simulating human digestion. This is of practical importance because not all potent peptide inhibitors of ACE that might be produced *in vitro,* may necessarily act as a hypotensive agent in humans *in vivo.*

Peptides with ACE inhibition action can derive both from caseins, named casokinins (Meisel and Schlimme, 1994) and whey proteins, named lactokinins (FitzGerald and Meisel, 2000). Highly active casokinins are present in the bovine • $_{s1}$-casein sequence 23-27 and in • -casein sequence 177-183.

Microbial proteases are capable of producing several ACE-inhibitors during fermentation (Yamamoto et al., 1999; Gobbetti et al., 2002; Ashar and Chand, 2004) and cheese-making (Addeo et al., 1992; Stepaniak et al., 2001)and the type of starter culture used is one of the main factors influencing their synthesis in dairy products. Potent ACE-inhibitory peptides VPP and IPP were purified from the fermented sour-milk "Calpis" and a significant reduction in blood pressure was recorded in mildly hypertensive patients after daily ingestion of 95 ml of "Calpis "for an eight-week period. Another fermented milk, "Evolus", proved effective in Spontaneously neously Hypertensive Rats; the treatment lasted 14 weeks and the calculated intake of IPP was 0.4 mg/die and 0.2 mg/die in the groups receiving fermented milk (A and B), respectively, whereas the corresponding amounts for VPP were 0.6 mg/die and 0.3 mg/die. At the end of the experiment, lower blood pressure was detected in the two groups (group A with greater effect than B), while the control group, fed simply skim milk, did not show any considerable change in blood pressure (Sipola et al., 2002). "Evolus" was also tested in two double-blind, placebo-controlled studies with mildly hypertensive subjects who ingested 150 ml if the product daily. It was found to decrease both diastolic to systolic pressure during the 8-week and 21-week treatment period, respectively. No such influence was reported in subjects with normal blood pressure (Seppo et al., 2002; Seppo et al., 2003). Minervini et al. (2003), prepared calcium caseinates from bovine, sheep, goat, pig, buffalo and human hydrolysates by a partially purified proteinase of *Lactobacillus Helveticus* PR4. Peptide in each hydrolysate were fractionated by reverse-phase fast-protein liquid chromatography (RP-FPLC). The fractions that showed the highest ACE-inhibitory activity were sequenced by mass spectrometry and Edman degradation analysis and the peptide profiles obtained differed according to the species. Bovine sodium caseinate hytdrolysates fractions showed the highest activity as the IC_{50} (peptide Concentration Inhibiting the activity of ACE by 50%) settled from 16.2 to 57.2 µg/ml ; this was slightly lower than sheep sodium caseinate hydrolysate wich had an IC_{50} 120.2 µg/ml; goat and buffalo fraction IC_{50} ranged from 112.6 to 210.5 µg/ml and human sodium caseinate also showed a considerable ACE-inhibitory activity (IC_{50} 228.1 µg/ml). This study gave evidence that milk of different species all have the potential to yield hypotensive peptides after enzymatic hydrolysis and the different bioactive peptides generated are related to the level of sequence identity and native conformation of protein.

Moreover the caseinate hydrolysates and related fermented milks may be considered as suitable functional foods, as the IC_{50} value of the tested peptides are consisted with IC_{50} (in order of µM) artificially synthesized by Maruyama at al., (1989), and compatible with the amount of bioactive peptides (10 to 60 mg) potentially produced during proteolysis of 1 g of caseins (Meisel. 1998), which are necessary to exert their action.

Besides the functional properties, the release of peptides in yoghuet fermentation is also interesting. It has been claimed that the traditional yoghurt starters *Lactobacillusud vulgaricus* and *Streptoccouccus thermophilus* act sinergistically , and symbiosis is promoted by the release of free amino acids and peptides (Bracquart and Lorient, 1979). Thus, some of the peptides also act as growth promoter of stimulatory peptides in the mixed starter culture (Van Boven et al., 1986).

In several ripened cheeses Meisel et al., (1997) have found the presence of low molecular mass peptides which ACE-inhibitory activity which increases as proteolysis develops, whereas the ACE inhibition index decrease when cheese ripening exceeds a certain level (e.g. the ACE-inhibitory activity detected in medium-aged Gouda was about double compared to the long-ripened Gouda cheese).

Many studies compared the ACE-inhibitory activity of specific cheese with different ripening degrees but only a few have tackled the search in Protected Denomination of Origin (DOP) cheeses elaborated with different technologies (mould-ripened, smoked, hard), starters and milk from different species.

4.2.2.1. Principal structure features of ACE-inhibitory peptides

Structure-activity correlations between different peptides inhibitors of ACE indicate that binding to ACE is strongly influenced by C- terminal tripeptide sequence of substrate.

Although the precise substrate specificity is not fully understood, ACE appears to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side-chains) amino acid residues at each of the three C-terminal positions (FitzGerald R.J. and Meisel H., 2000).

ACE inhibitions studies with dipeptides of varying structure, show that C-terminal tryptofan, tyrosine, phenylalanine or proline residues were most effective in enhancing substrate binding (Cheung et al. 1980)

All casokinins, i.e. casein-derived ACE-inhibitory peptides have proline, lysine or arginine as the C-terminal residues.

However, the presence of positively charged C-terminal lysine or arginine residues in casokinins, bradykinin and some synthetic inhibitors (Cheung at al., 1980) does not fit with the ACE active site model proposed by Ondetti & Cushman (1982).

Nevertheless, structure activity data suggest that the positive charge on the guanidine or •-amino group of C-terminal arginine and lysine side chains, respectively, contribute substantially to inhibitory potency.

For example, replacement of arginine at the C-terminus of bradykinin result in an essentially inactive analogue (Meisel, 1993).

It is postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site which is distinct from the catalytic site.

Given the above, it is expected that peptide conformation , i.e. the structure adopted in a specific environment, should contribute to ACE-inhibitory potency.

A detailed knowledge of the mechanism of action of ACE and the conformational behavior of ACE-inhibitory peptides should lead to a better understanding of the antihypertensive potential milk-protein derived.

4.2.3. Opioid peptides

The casein peptides with opioid agonist activity were the first and mainly studied opioid peptides (Brantl et al., 1979). Like other opioid peptides derived the exogenous opioid peptides have an N-terminal tyrosine residue which is critical for the opioid activity (H• llt et al., 1983). Nevetheless, the latter have an N-terminal sequence (i.e. Tyr-X-Phe or Tyr-X_1-X_2-Phe or Tyr) different from the sequence characterising endogenous peptides (Tyr-Gly-Glu-Phe) and therefore they are called “atypical” opioid agonist peptides. Opioid peptides from milk can be obtained by *in vitro* enzymatic hydrolysis of bovine and human •-caseins (Pihlanto-Leppälä et al., 1994) and in particular from their 60-70 fragment (Schlimme and Meisel, 1993). A similar amino acid sequence appears in the •-casein of sheep milk (Richardson and Mercier, 1979) and water buffalo milk (Petrilli et al, 1983). Such peptides, called casomorphins, have also been detected in the duodenal chyme of minipigs (Meisel, 1986) and in the human small intestine (Svedberg et al., 1985) as a consequence of *in vivo* digestion. Opioid peptides also arise from fragments: 90-96 of bovine •$_{s1}$-casein (exorphines) (Loukas, 1983), 40-44 of human •-casein (•-casorphins) (Chiba and Yoshikawa, 1986), and 399-404 of bovine blood serum albumin (serorphins) (Tani et al, 1994).

When opioid peptides are injected in the bloodstream they induce an analgesic and sedative effect due to their action on the nervous system (Teschemacher, 1987; Paroli, 1988) and affect gastrointestinal transit time (Hahn et al., 1994; Paakkari et al., 1994; Schulte-Frohlinde et al., 1994). Although *in vivo* formation of opioid agonist and opioid antagonist peptides has been proven, their absorption in the gut has not been detected in adults (Schulte-Frohlinde et al., 1994; Teschemacher et al., 1994). In this case opioid activity is limited to the gastrointestinal tract with important effects on transit time, amino acid absorption and water balance. Different effects may exist in sucklings due to their permeability of the intestinal wall which allows passive peptides transport (Maubois and Lèonil, 1989,Teschemacher et al., 1994). •-casomorphins were found in the plasma of pregnant or lactating women (Bicknell, 1985; Yen et al., 1985). These peptides arise from human milk, pass through the mammary tissue and affect the release of prolactine and oxitocine. Moreover they affect the activity of the central nervous system of the central nervous sysyetm (Teschmacher et al., 1994).

4.2.4. Immunomodulating peptides

Some peptides from tryptic and chymotryptic hydrolysate of •$_{s1}$- and •- casein can stimulate the macrophage activity against aging red-blood cells (Jolles et al., 1981-82; Parker et al., 1984). Such activity has been shown *in vitro* through specific receptors which have been found on human fagocitary cells. *In vivo* direct immunostimulating activity against *Klebsiella pneumoniae* has been reported for rats injected with casein or •-lactalbumin peptides (Migliore-Samour et al., 1989). More recently, *in vivo* antibacterial activity against *Staphylococcus aureus* and *Candida albicans* has been described for isracidin, the 1-23 fragment of •$_{s1}$-casein obtained from the action of chymosin (Lahov and Regelson, 1996). The injection of isracidin into the udder of sheep and cow gave protection against mastitis.

The intestinal absorption of these peptides is possible for suckling babies whereas there is no evidence for absorption in adults. Nevertheless, •-casokinins can exert indirect immunostimulating activity. These peptides inhibit ACE which is also responsible for bradykinin inactivation, a hormone with immunostimulating activity (Meisel and Schlimme, 1994).

A bacterial activity against gram negative bacteria and some yeasts (e.g. *Candida albicans*) has been reported for lactoferricin (Yamauchi et al., 1993), a pepsin–derived peptide from bovine lactoferrin. Like lactoferrin, lactoferricin inhibits microbial growth through nutritional deprivation of iron. In addition, it causes the release of lipopolysaccharide molecules from the outer membrane of the gram negative bacteria and directly acts as antibiotic. No effect has been detected against *Bifidobacterium. T*herefore lactoferricin may positively affects the intestinal flora.

4.2.5. Antithrombotic peptides

The clotting of blood and the clotting of milk show a number of similarities. In the final step of blood clotting the enzyme thrombin hydrolyses fibrinogen to insoluble fibrin clot.

Fibrinogen is also essential for platelet aggregation because the 400-411 sequence of its • -chain can bind the glycoprotein receptors on platelets.

During the first step of rennet milk coagulation, the enzyme chymosin hydrolyses the peptides bond between the 105-106 residues of •-casein. Structural and functional homologies were found between the dodecapeptide of fibrinogen and the 106-110 sequence of •-casein. The •-casein peptide, so-called casopiastrin, can be obtained from tryptic hydrolysates and shows antithrombotic activity by inhibiting fibrinogen binding on platelets (JollèS and Henschen, 1982, Jollès et al. 1986; Mazoyer et al., 1992). Also the 103-111 peptide of the •-casein sequence can prevent blood clotting by preventing platelet aggregation but it does not affect fibrinogen binding on platelets (Fiat et al, 1993).

5. *In vivo* and *in vitro* digestibility of the calcium contained in foods of animal and plant origin

Calcium (Ca) is an important nutrient which is present in bones and soft tissues. It can considerably affect metabolism by acting as a second messenger which activates coagulation or muscle contraction. Furthermore, the calcium contained in dairy products seems to speed up weight loss in obese subjects on a low-calorie diet.

Our dietary calcium intake is not fully digestible and the absorbable percentage varies from one food to another. In fact, there is evidence that the absorption values are higher for calcium contained in dairy products than in plant products (Ferraretto et al., 2001), probably because of the link between Ca and the casein phosphopeptides (CPP) released from casein. Conversely, in many foods of plant origin the link between this mineral and the naturally-occurring anti-nutritional factors of the plant content - such as phytic and oxalic acid – may hinder its absorption. Besides which, the calcium requirements refer to dietary calcium and not digestible calcium, and so assume that - independently of its origin - this mineral has only one digestibility value, which is rather unrealistic. All these factors have to be taken into account in diet formulation, in the choosing of foods (dairy products or vegetable) and their amount, in order to cover recommended calcium human daily allowance (RDA) with the adequate energy intake.

The absorption of this mineral in the gut may take place both via active (with low calcium concentrations) and passive transportation (when calcium reaches high concentrations in the intestinal lumen). Several factors affect the absorption of this mineral, such as age (digestibility tends to decrease with aging), the presence of anti-nutritional factors and plant fibre (same effect as with aging), while lactose (Allen, 1982, Spencer e Kramer , 1986) and Vitamin D increase digestibility, physiological losses (via urine, transpiration, bone turnover, endogenous fecal losses) . All these factors which influence RDA value calculations have been evaluated in order to assess whether the Italian recommended dietary allowances for calcium (LARN 1996) are adequate or possibly set too high.

All these factors make investigations on this mineral extremely important, above all when considering health concerns related to poor intakes of dietary calcium, i.e. osteoporosis in post menopausal women, development of rickets in infants, inadequate peak bone mass in young, etc.

Literature describing that digestibility of food can be assessed both *in vivo* and *in vitro*. In vitro methodologies are preferred because of being more practical and less expensive than tests on human volunteers or animal models. However, the results obtained *in vitro* must always be correlated with those *in vivo*.

5.1. Aim of work

The aim of our investigation was to assess calcium digestibility both with an *in vivo* and in *vitro* method in different cheeses, legumes and soy-derived foods, and evaluate the nutritional implications of using specific calcium digestibility values rather than a single value for all foods when formulating the diet. Moreover, physiological factors involved in calcium recommended daily allowance (RDA) setting have been analyzed to assess if calcium RDA are set too high.

5.2. In vitro digestion

5.2.1. Samples.

Seven different foods have been studied; i.e. Grana Padano, Emmenthaler, soya powder milk for babies, tofu, soya burgers, canned peas and canned beans.

The samples were purchased on the market and stored at 4°C, except for powder and tinned products, which were stored at room temperature.

5.2.2. Reagents.

Enzymes and bile salts were purchased from Sigma Chemical Co (St. Louis, Mo, USA): pepsin (porcine cat. n°. P-7000), 0.125 g of pepsin were suspended in 12 ml di HCl 0.1N; pancreatin (porcine cat. n°. P-1750) and bile extract (porcine cat no B-8631), 0.055 g of pancreatin and 0.310 g of bile extract were dissolved in 17.5 ml NaHCO3 0.1M. Enzymatic solutions were prepared immediately before use.

In all reactions, bidistilled water - obtained thorough the Millipor MilliQ filtration system (Millipore Iberica S.A., Barcelona, Spain) - was used.

5.2.3. Determination of Ca content.

The Ca content in the digested samples was determined with atomic absorption spectrophotometry (Varian spectrAA 50).

The Ca standard solution for atomic absorption spectrophotometry was prepared immediately before its use, dissolving a standard solution of 1000 mg/L CaCl2.6H2O (Carlo Erba, Rodano, Milan, Italy cat n°497481) in bi-distilled water. In order to avoid phosphate interferences during calcium quantification feeds and feces were dissolved in a lanthanum solution (2 g/L) obtained by dissolving a standard buffered cesium chloride - lanthanum chloride solution (Merck, Darmastadt, Germany cat n° 1.16755.1000).

5.2.4. Solubility method and enzymatic digestion.

The in vitro digestion methods described by Perales et al. (2006) have been used as reference: 80 ml of bi-distilled water were added to a food sample containing 40 mg of calcium, pH was adjusted to 2.0 with 6 N HCl. After 15 min, the pH value was checked, and, if necessary, readjusted to 2 with NaOH 0.1 N.

Then 0.02 mg of pepsin per sample mg were added. The sample was incubated in a shaking water bath (WTB Binder, Tuttlingen, Germany) at 37°C for 2 h. The gastric digest was kept in ice for 10 minutes to stop pepsin digestion.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5 by dropwise addition of NaHCO3 0.1 M. Then 0.05 mg of pancreatin and 0.30 mg of the bile extract mixture were added to each g of sample, continuing incubation for an additional 2 h. To stop the intestinal digestion, the sample was maintained in an ice bath for 10 min. The pH was adjusted to 7.2 by dropwise addition of 0.5 NaOH.

Aliquots of 20 g of the sample were transferred to polypropylene centrifuge tubes and centrifuged (Sorvall Instruments, Dupont, RC5C,Wilmington, DE, USA) at 3500 rpm for 1 h at 4°C.

Ca in supernatants (soluble fraction) was measured by atomic absorption spectrophotometry.

5.2.5. *In vitro* Ca digestibility (dCa)

The procedure starts from the assumption that solubilized Ca is also digestible at gut level. Therefore, dCa was calculated according to the following formula:

$$dCa(\%) = \frac{\text{Ca in supernatant (mg)}}{\text{Ca in sample (mg)}} \times 100$$

5.3. In vivo digestion

In vivo calcium digestion was assessed in rats.

Thirty-four Sprague-Dawley rats were raised in single metabolic cages fitted with stool-urine separator in conditions of controlled temperature and humidity. The animals received bi-distilled water ad libitum.

Each food was freeze-dried and given separately for a period 5 days during which the stools were collected on a daily basis. The protein content of all foods was at least 17% as recommended by Reeves et al (1993).

Endogenous Ca excretion was estimated by giving the animals the non-calcium-containing diet proposed.

Before receiving the tested food, each animal was left without food and only allowed to drink water for 36 hours so as to eliminate all food residues from the gut.

Apparent dry matter food digestibility was calculated as the ratio between Ca intake (mg) minus Ca in feces (mg) and Ca intake (mg).

The experiment was carried out in accordance with the Italian Law on protection and care of animals in biological experiments (DL 116/1992).

5.3.1. Statistical analysis

Statistical analysis was carried out using the SAS 9.1 statistical package, while the comparison of Ca digestibility between different foods was performed with the Tuckey test.

SAS PROC REG was used for the correlation between in vivo and in vitro results.

5.4. Results

5.4.1. *In vitro* digestion.

Seven different types of food were analyzed: Grana Padano, Emmenthaler, three soya-based foods - i.e. tofu, burgers, powder milk for babies- and vegetables, such as tinned green peas and beans.

Table 1-5.4.1. shows the calcium digestibility results in vitro. The results indicate that the highest calcium digestibility values were obtained in two Grana Padano samples. Showing difference (80.12 vs 73.05 %) between the two samples.

Table 1-5.4.1.: *In vitro* Ca digestibility (%) results.

Food	*In vitro* Ca digestibility (%)
Grana Padano 1	80.12
Grana Padano 2	73.05
Emmenthaler	58.54
Tofu	57.51
Soya powder milk for infants	50.91
Peas	23.04
Canned beans	18.7
Burger	16.11

Grana Padano was found to be the cheese with the highest digestibility level, while Emmenthaler and tofu showed similar digestibility. Calcium digestibility of the other foods appear inferior to the first three values.

5.4.2. In vivo digestibility

5.4.2.1. Dry matter digestibility (digDM).

The results are shown in Table 1-5.4.2.1, with high values for almost all foods, except for beans which features significantly lower digestibility level. The highest values are found in soya powder milk and Emmenthaler cheese.

Table 1-5.4.2.1: *In vivo* dry matter digestibility (%) of tested foods.

Food	*In vivo* dry matter digestibility (%)
Soya powder milk	96.76^{c}
Emmenthaler	92.70^{c}
Grana Padano	90.45^{c}
Soya burger	60.13^{b}
Tofu	86.44^{bc}
Peas	84.08^{bc}
Beans	31.61^{a}

5.4.2.2. *In vivo* Ca digestibility.

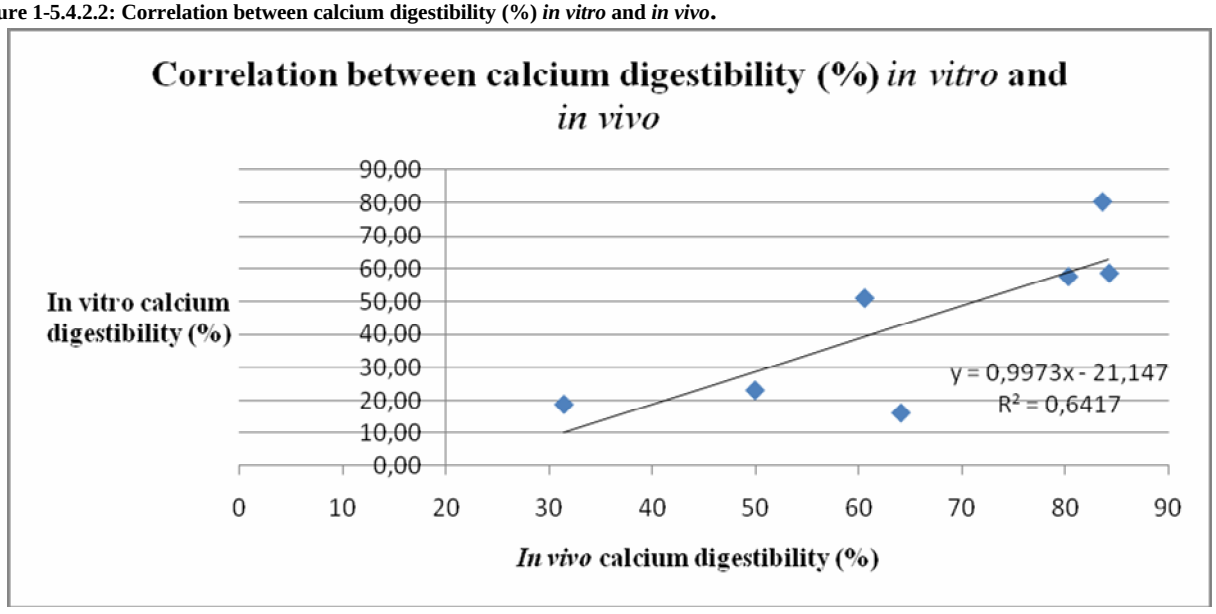
Also in the case of digCa the lowest values were those found in peas and beans, while the two cheeses and tofu featured the highest values. The digestibility levels of soya burgers lie in-between those of legumes and cheeses Table 1-5.4.2.2. show *in vivo* calcium digestibility % results compared with those *in vitro*: Grana Padano was found to be the cheese with the highest digestibility level, while Emmenthaler and tofu showed similar digestibility, as also observed in vivo. Confirming the results obtained *in vitro*, soya milk was a less good source of Ca than cheeses and tofu. Very similar data were observed for peas, legumes and soya burgers.

Table 1-5.4.2.2.: In vivo and in vitro Ca digestibility (%).

Food	*In vivo* Ca digestibility (%)	*In vitro* Ca digestibility (%)
Emmenthaler	84.26[d]	58.54
Grana Padano	83.61[d]	80.12
Tofu	80.30[d]	57.51
Soya burger	64.08[c]	16.11
Soya powder milk	60.56[c]	50.91
Peas	49.95[b]	23.04
Canned beans	31.44[a]	18.7

The correlation between *in vivo* and *in vitro* digCa digestibility is reported in Figure 1-5.4.2.2. As shown in this figure, the Ca digestibility values *in vitro* and *in vivo* are enough correlated with an R^2=0,6417. The better correlation between the two methods can be found for Grana Padano cheese.

Figure 1-5.4.2.2: Correlation between calcium digestibility (%) *in vitro* and *in vivo*.



5.5. Discussion

5.5.1. In vitro and *in vivo* Ca digestibility

The lower digestibility of the tested plant foods compared to the Grana Padano cheese may be explained by the fact that the foods of plant origin contain anti-nutritional factors such as oxalic and phytic acid which bind to calcium and make it unavailable for absorption. The negative impact of phytic acid on mineral digestibility has already been pointed out in pigs (Wovengo et al., 2009), in rats (Brink et al., 1992; Kumagai et al., 2004) and humans (Bohn et al., 2008). In dairy products, instead these compounds are physiologically absent and calcium, which is bound to phosphopeptidic molecules, is made more available to the action of the gut proteolytic enzymes. The presence of Ca in the casein phosphopeptide molecule favours its absorption, as observed by Ferraretto et al. (2001): this is the most likely cause of greater digestibility of this mineral in cheese. Tofu behaves differently from the other foods of plant origin, maybe because the technological process requires the solubilization of Ca which makes its absorption easier.

During production, tofu undergoes both protein hydrolysis and heat treatment with partial protein digestion accompanied by the production of lysinoalanine (LAL). As a consequence, the results are contrasting: on one hand the soy protein hydrolysates increase Ca uptake in the CaCO2 cells (Ly et al., 2008), on the other hand LAL production decreases the digestibility of the protein and mineral fractions (Friedman et al., 1999; Sarwar et al., 1999).Brink et al (1992) found less Ca digestibility in soya milk than in cow's milk, which confirms that the lower level of absorption of this mineral in soya than in milk. The apparent Ca digestibility values of cheese found in this study are higher than those obtained – also in rats - by Van Dael et al. (2005), equalling 56.0 % for casein and 61.1% for the mixture (40:60) of casein and whey proteins. In this 21-day trial, milk proteins were included in a synthetic diet containing also 5% cellulose, which most probably reduced the digestibility of the mineral. Furthermore, the weight of the animals used in our trial was approx. 100 g versus approx. 60 g in the experiment of Van Dael et al. (2005). Reduced gut development may have contributed to decreasing Ca digestibility, too.

The digestibility of the calcium contained in whole wheat flour chapattis given to adult rats weighing approx. 200 g was 42.0% (Ahmed et al., 2008), a value similar to what we obtained for peas and beans

5.5.2. Correlation between *in vivo* and *in vitro* data

A possible reason for the not complete linear correlation may be the CPPs which *in vivo* favour Ca absorption by the enterocyte, but cannot perform the same action *in vitro*. The body has two mechanisms for intestinal Ca absorption, an active and a passive one. These features cannot be reproduced *in vitro*.

5.6. Nutritional implications

One can discuss the nutritional implications of adopting specific Ca digestibility values for each food instead of a general average value. Ca requirements are currently calculated on the basis of the mineral losses and bone deposition attributing the same digestibility to all foods. Losses mainly occur via the following: urine, transpiration, bone turnover, endogenous fecal losses and are not all easily quantifiable.

The data of Hui et al. (1985) and Garn (1972) suggest that calcium accretion approaches zero in females by the age of 24 y, whereas males accumulate calcium beyond the age of 26 y, so in adults the daily Ca retention for accretion is 10 mg/day in females and 50 mg/day in males (Peacock 1991; Weaver et al. 1996).

Urinary and fecal losses depend on the total dietary Ca intake, so assuming an intake ranging from 683-1297 mg/d as calculated by Matkovic (1991), in females the urinary losses are 204 mg/day and the endogenous fecal losses 65 mg/day (Heaney and Skillman 1964), while in males the urinary losses are 162 mg/day (Matkovic, 1991) and the endogenous fecal losses 156 mg/day (Heaney and Skillman 1964).

Since in Italy the daily Ca RDA (Recommended Daily Allowance) is estimated at 1000 mg/day between the age of 18 and 29 years in both genders, if one considers that the requirements for this mineral have been estimated by Weaver (1994) at 342 mg/day in females and 431 mg/day in males, dietary Ca digestibility is thought to be equal to 34.2%. On the other hand, Weaver (1994) suggests that Ca digestibility is 30% in females and 30.7% in males. Using the current criteria, in order to cover the RDA one would require 84.2 grams of Grana Padano (950 mg Ca/100 g of food) or 1194 grams of peas (67 mg Ca/100 g of food). Instead, if one had to use the in vitro Ca digestibility values (dCa), the required amount of cheese would drop to 33.0 g while that of legumes would rise up to 1554.7 g. The adoption of the in vivo values (digCa) would only slightly change the situation for Grana Padano (35.0 grams) while the required amount of legumes would remain high anyway (664.8 grams).

One should not forget that the fecal losses depend on the Ca intake (Heaney and Skillman 1964). For this reason, a greater intake of this mineral in order to make up for the lower digestibility of plant foods would cause an increase in fecal losses.

It therefore becomes clear that the adoption of a single digestibility value for all foods tends to penalize those foods which are characterized by higher in vivo digestibility.

Since the RDAs have been established with the goal of avoiding nutritional deficiencies in 95% of the population, a certain surplus in nutrient intake remains unavoidable, though there are conditions in which a more accurate formulation of the dietary intake may be useful.

The Ca intake should also be considered in the light of the concurrent energy intake. Indeed, there are certain physiologic (pregnancy or lactation) or pathologic conditions (obesity) in which the Ca requirements must be met without excessive energy intake. If one assumes that Ca digestibility is the same for all foods (30%), then in order to make up for the physiologic losses (240 mg) one would need a 321 kcal intake for Grana Padano and a 633 kcal intake for peas. However, if one relies on the digCa values determined in this trial, then the consumption of Grana Padano and peas should be in the order of 133 kcal and 352 kcal respectively. This means lower energy intake but, above all, a different relationship between the two foods. With the same Ca intake, the ingestion of peas previously meant that the energy intake was two-fold if compared to cheese, while the adoption of specific values for each food implies that covering the mineral requirements with peas equals an energy intake which is 2.6 times that obtained with Grana Padano.

The assessment of energy intake per mg of digestible Ca is important when faced with major Ca requirements in settings where weight gains must be avoided (pregnancy, lactation, obesity).

The hypothesis of meeting the Ca requirements only with peas or cheese is unrealistic, but the same concepts are true when legumes or cheese are included in a complete diet together with other foods. However, a new approach based on the apparent digestibility of the minerals contained in the different foods requires considerable efforts, especially if the data are to be derived from tests on animal models. Furthermore, the broad range of currently available foods would make it very difficult to set up a relevant database. A similar undertaking could be accomplished with the availability of a reliable in vitro method, targeted towards assessing the digestibility of the main macro and micro-elements for each food category instead of each individual food.

5.7. Conclusion

The in vitro and in vivo digestibility of the Ca contained in legumes and soy derivatives is lower than that of cheeses. For this reason, the adoption – as in the current practice – of a single digestibility value for Ca intake tends to overestimate legumes as a source of this mineral. This study confirms that there is a risk of Ca deficiency if dairy products are entirely replaced by legumes.

The use of actual Ca digestibility values becomes particularly important in all those conditions (pregnancy, lactation, growth, obesity) in which the mineral requirements must be met without increasing or, better, decreasing the energy intake. The calculation of the actual digestibility of at least the most important minerals could improve the adequateness of the diets even if major efforts are required to establish these values.

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6. Casein phospho-peptidic profiles in different ripened time typical semi-fat hard cheeses *in vitro* digested. Potential effect of lysozyme presence on the peptidic profile structures and effects on Calcium digestibility

Cheese phosphopetides released from casein fraction, and called caseinphosphopetides (CPP), are important oligopeptide cheese fraction because of involved with their negatively charged side chains, in particular the phosphate groups, in binding minerals (Meisel, 1998). Phosphopetides can form soluble organophosphate salts and may function as carrier for different minerals, especially Ca (Sato et al., 1986).

Grana Padano, TrentinGrana, Parmigiano Reggiano are very similar Italian semi-fat hard cheeses. The important difference is the presence, in Grana Padano of the antibacterial molecule lysozyme.

Many analytical studies about the peptidic profile of these Italian typical semi-fat hard cheeses has been done. Ferranti et al., (1997) digested with trypsin a sample of Grana Padano cheese (14 months aged), in order to reduce phopsphopeptides size, and identified 45 phosphopetides. Ferranti et al., (1997) wrote that CPPs could be regarded as transient intermediate components susceptible to further hydrolysis by cheese enzyme to shorter peptides and free amino acids (FAA), including SerP. This degradation mechanism might explain the absence of CPPs in Parmigiano-Reggiano (PR) cheese older than 12 months (Addeo et al., 1992), in contrast with the results of Dulley & Kitchen (1973), who reported an increase in CPPs during cheese maturation without alteration of the Pi level. Sforza et al. (2003), conducted a semi-quantitative evaluation of the major oligopeptides in Grana Padano cheese and correlated their amount with the aging time (from 2 to 33 months). Studying only the peptides giving rise to strong signals in HPLC both by PDA and by MS detection, they divided all peptides in three groups: small peptides, phosphopetides and bigger apolar peptides. Sforza et al. (2004) reported that manufacturing of semi-fat hard cheeses with lysozyme strongly influence their peptidic profiles, especially in time and amount of phosphopetides appearance. Actually in Grana Padano cheese most of the phosphopeptides reach a maximum at 12 months of aging and then decrease, whereas in Parmigiano Reggiano cheese the phosphopetidic amounts are usually lower and have a less regular trend. The reason of this different behavior may be ascribed to the different production techniques and certainly to the use of lysozyme.

Therefore many studies analyzed peptidic profiles of these cheese but until now, no one study has been conducted about the peptidic profiles obtained after simulation of in vitro gastrointestinal human digestion of these type of cheeses at different ripening times. This kind of study could be useful to evaluate if lysozyme use in cheese manufacturing could influence the formation of bio-active/functional peptides also after in

vitro digestion. We focused on phosphopeptides due to their important role on Ca binding and consequently on intestinal absorption.

Therefore, the aim of the study was to assess the in vitro calcium digestibility (dCa) of Grana Padano and TrentinGrana at different ripening times. The potential role of lysozyme in modifying peptidic profile and in vitro dCa was also investigated. The dCa of each cheese samples were correlated to the molecular weight of peptides released during in vitro digestion.

Furthermore SELDI-TOF, RP-HPLC combined with MS analysis were carried out to know more about the phosphopeptides profile and investigate the relationship between dCa and peptides pattern.

6.1. Methods and Materials

6.1.1. Cheese samples and reagents

20 samples of Grana Padano, 6 of Parmigiano Reggiano e 11 of Trentingrana aged between 11 and 25 months were obtained from the main cheese-factories of the respective Consortium (Table1-6.1.1.).

Samples were first mineralized to determinate the initial Ca amount in each samples with flame atomic absorption method. In vitro Ca digestibility has been determined incubating cheese with digestive enzymes and bile salts. After digestion the sample were centrifuged and the supernatant was collected and analyzed for Ca content and peptide pattern.

Enzyme and bile salts were purchased from Sigma Chemical Co (St. Louis, Mo, USA): pepsin (porcine cat. n° P-7000), 0.125 g of pepsin was suspended in 12 ml di HCl 0.1N; pancreatin (porcine cat. n° P-1750) and bile extract (porcine cat no B-8631), 0.055 g of pancreatin and e 0.310 g of bile extract were dissolved in 17.5 ml of $NaHCO_3$ 0.1M. Enzymatic solutions are immediately been prepared before their use.

Distilled water was used for all analytical steps.

Table 1-6.1.1: Cheese samples of Grana Padano (GP), Parmigiano Reggiano (PR) and TrentinGrana (TN) :cheese consortium identification letters and cheese sample number, cheese ripening time

Cheese	Ripening time (months)
GP28	10
GP30	11
GP31	11
GP32	12
GP34	13
GP35	13
GP37	15
GP38	16
GP39	16
GP40	16
GP42	17
GP43	20
GP44	20
GP45	24
GP46	26
GP47	25
GP48	39
GP50	11
GP53	27
GP55	34
PR56	17
PR57	19
PR58	20
PR59	20
PR60	21
PR61	25
TN49	10
TN51	11
TN52	11
TN72	24
TN73	24
TN74	18
TN77	11
TN78	18
TN79	18
TN80	25
TN82	24

6.1.2. Mineralization method

Cheese samples were first mineralized prior to Calcium determination by flame atomic absorption analysis. Mineralization was carried out on about 0,200-0,400 g of cheese using a 1ml H2O2 and 5 N HNO3 mixture. A microwave oven MarsXepress (CEM corp., Matthews, NC, USA) equipped with temperature control device, was used.

6.1.3. Atomic absorption spectrophotometric Calcium determination

Atomic Absorption Spectrophotometry (Varian spectrAA 50) was used for Ca determination on samples and on supernatant after in vitro digestion.

The Ca standard solution for atomic absorption spectrophotometry measurements has been prepared immediately before its use, dissolving standard solution of 1000 mg/L CaCl2 .6H2O (Carlo Erba, Rodano, MI, Italy cat no.497481) in water MilliQ. The Lanthanum solution utilized (2 g/L) has been obtained dissolving a standard buffered cesium chloride lanthanium chloride solution (Merck, Darmastadt, Germany cat n° 1.10755.1000). Lanthanium Chloride utilization eliminate phosphates interferences during calcium quantification.

6.1.4. *In vitro* digestion method

The procedure described by Perales et al. (2005). Distilled water (80 ml; Millipore) was added to un amount of food samples containing 40 mg of calcium; pH was adjusted to 2.0 with 6 N HCl. After 15 min the pH value was checked, and if is necessary it was readjusted to 2 with NaOH 0,1 N. Then 0,02 mg of pepsin per sample mg was added. The sample was incubated in a shaking water bath (WTB Binder, Tuttlinge, Germany) at 37°C for 2 h. The gastric digest was kept in ice for 10 minutes to stop the pepsin digestion.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5 by addition of NaHCO3 0,1 M. Then 0,05 mg of the pancreatin and 0,30 mg of bile extract mixture per g of sample was added and the incubation continued for an additional 2 h. To stop the intestinal digestion the sample was maintained for 10 min in an ice bath. The pH was adjusted to 7,2 by dropwise addition of 0,5 NaOH.

Aliquots of 20 g of the sample were transferred to polypropylene centrifuges tubes and centrifuged (Sorvall Instruments, Dupont, RC5C,Wilmington, DE, USA) at 3500 rpm for 1 h at 4°C.

6.1.5. Gel filtration HPLC oligopeptides molecular weight distribution method

Exclusion chromatography (Shodex Protein Kw- 802,5, Jasco) was used to analyzed samples after in vitro digestion and separate oligopeptides according to their molecular weight.

The eluent solution was composed by “HPLC grade” water, phosphate buffer a pH 7, Na2SO4 150 mM, EDTA 1 mM, SDS (sodium dodecil sulphate) 0,1 M. Two liter of eluent was prepared. The column was at room temperature (20-25 °C). Flow 0.5 ml/min. Detector UV-Vis, lecture 215 nm. The run lasted 45 min with isocratic elution.

6.1.6. Statistical analysis

Analysis of variance of foods digestibility was carried out with GLM procedure of the SAS statistical software (SAS 9.1) using the Tukey-Kramer test.

The correlation between variables was carried out with the PROC CORR of SAS 9.

6.1.7. Surface enhanced Laser desorption (SELDI) peptidic profile investigation method

SELDI is a ionization method combined with time-of-flight mass spectrometry used to detect analytically macromolecules. The protein mixture is spotted on a surface chip modified with a chemical functionality. Different kind of chips are available, in which the chemically loaded ProteinChip contain hydrophobic, hydrophilic, anion exchange, cation exchange and immobilized-metal affinity surface. Protein with affinity for the chips surface characteristic will band, while the other are removed by washing. After washing the spotted sample, the matrix is applied to the surface and allowed to crystallize with the sample peptides. Binding to the SELDI surface acts as separation step and the subset of proteins that bind to the surface are easier to analyze.

To investigate on semi-fat hard typical Italian cheeses phosphopeptidase profile the IMAC 30 (metal-binding surface) was used.

Several time analysis were carried on with FeCl2, iron and zirconium dioxide mineral solution, to enrich the phosphopeptides as suggested by literature. Also different pH buffer and ionization matrix were tested to improve the isolation method. Then samples spotted on SELDI surface are typically analyzed using time-of-flight mass spectrometry. A laser ionizes peptides from crystals on the sample/matrix mixture. Ions are accelerated through an electric potential and down a flight tube. A detector measures ions as they reach the end of tube. The mass-to-charge ratio of each ion can be determined from the length of the tube, the kinetic energy given to ions by the electric field, and the time taken to travel the length of the tube. Because the ionized molecules have the same energy the time of flight is direct related to the mass of the molecules. The smaller the molecules the greater the speed of the molecules.

6.1.8. IMAC30-SELDI method to enrich phophopetides

The in vitro digested sample is first centrifuged, to be clarified, acidified adding some drops of TFA to separated phosphopeptides that should be bound together, and filtrated to isolate phosphopetides < 3000 Da.

The IMAC30 analysis with FeCl2 0.1 M mineral solution provides to:

- Place the ProteinChip array cassette in the bioprocessor and add 50 µl 0.1 M of the mineral solution;
- Incubate for 10' at room temperature with shaking;
- Remove metal solution from the wells;
- Add 150 µl of de-ionized water and incubate for 1' at room temperature with shaking;
- Remove de-ionized water and add 150 µl of buffer Sodium Acetate pH 4.0, 0.1 M for 5';
- Remove buffer solution and add 150 µl of binding buffer (sodium Phosphate, 0.5 M NaCl pH 7.0) incubate for 10'. Two times;
- Remove binding buffer and add 1 µl of sample and 99 of binding buffer, and incubate for 1 h;
- Remove the sample and add binding buffer, 150 µl and incubate 10, two times;
- Remove binding buffer and wash with 150 µl de-ionized water;

To desorption/Ionization 0,80 µl on the spot, two times of SPA- 200 µl ACN 100% + 200 µl TFA 1% or DHB- 20µg/ml DHB, 50% ACN, 1% Phosphoric acid.

The same method was tested also changing the Sodium Acetate buffer with Formic Acid buffer (pH 2.70 about) to optimize phosphopeptides enrichment;

In this case before the inclusion on Protein Chip the sample must be processed as follow:

- Put 4,5 mg ZrC02 into an Eppendorf tube;
- Wash three times with 100 • l 80% acetonitrile in 0.1% (v/v) TFA (100 • l);
- Condition with 100 • l 0.1% TFA;
- Centrifuge;
- Remove supernatant;

- Add 90 • l of sample (20 • g/mg ZrO2);
- Incubated at room temperature, 30’with gentle shaking ;
- Remove supernatant;
- Add 10 • l 80% acetonitrile in 0.1% TFA;
- Centrifuge;
- Remove supernatant (to eliminate non phosphopeptides);
- Wash 5 times with 200 • l 80% acetonitrile in 0.1% TFA;
- Centrifuge;
- Remove supernatant;
- Add 20 • l of diammonium phosphate 100 mM (pH 9), to elute bound phosphopeptides;
- Centrifuge;
- Take the supernatant with phosphopeptides and acidify it with 2 • l TFA 10% (v/v).

The phosphopeptides enriched sample is spotted (1 • l) on the normal phase ProteinChip,NP20.

For this method the ionization/ desorption solution is HCCA (Cyano-4-hydroxycinnamic acid): 2 mg/ml in 50% acetonitrile:0.1% (v/v) TFA (1:1). Then the ProteinChip were ready to be analyzed in time-of-flight spectrometer. Every ProteinChip contains 10 spots, one for each sample.

In the phosphopeptides analysis different sample dilutions were tried. Each dilution were tested with and without mineral solution to compare the results and prove the presence of phosphopeptide peaks.

6.1.9. RP-HPLC-MS and pre-column method to analyze samples phosphopetidic profile.

In order to reduce the salt interference in the chromatographic analysis and during the mass spectrometry chromatogram reading, the pre-column method for the removal of injected salt described by Pringle et al. (2007) was used.

RP-HPLC was performed on a Pepswift monolithic PS-DVB column (200µm x 50 mm, Dionex) on a Ultimate 3000 (Dionex Corporation, Sunnyvale, USA).

To desalt the samples, they were first trapped on a monolithic Trap PS-DVB column (200µm x 5 mm, Dionex). The columns were maintained at 60°C during separation. The solvents used were Millipore water, containing 0.05 % Formic Acid (solvent A) and Acetonitril, containing 0.04% Formic Acid (solvent B). The elution was as follows: from 0 to 5 min, 0% B (isocratic, desalting); from 5 to 12 min, 0% B (isocratic, start separation); linear gradient up to 35% B until 30 min; linear gradient up to 100% B until 37 min; isocratic elution with 100% B for 3 min; isocratic elution with 0% B for 10 min. All HPLC operations and data processing were controlled by Chromeleon version 6.7 software (Dionex Corporation, Sunnyvale, USA).

The flow rate was 2.5 µl/min, injection volume 0.5 • l. The column outlet was directly after the UV detector (214 nm) also connected to a ESI TOF Spectrometer.(Synapt High Defenition Mass Spectrometry system, Waters Corp. USA) controlled by Masslynx 4.1 software (Waters Corp. USA). A detailed description of the instrument is presented by Pringle et al (2007).

The data are acquired in ESI mobility TOF mode and the positive ion V mode between 450 - 2000 Da. The instrumental configuration was as follows: capillary was set to 3 kV, trap collision energy at 4V, transfer collision energy at 6 V, sampling cone voltage at 50V and a source temperature of 80°C . Mass spectra were acquired over a m/z range of 400-2000 Da. MS/MS data are acquired when the TIC rises above 20 counts/sec between 100-2000 Da during 4 sec.

Mass accuracy was ensured by calibration with 100 fmol/µL glu-fibrinopeptide (GFP, 785.8 Da).

6.1.10. Phosphopetides enrich kit method for pre-column combined RP-HPLC-MS-MS analysis method

Four Grana Padano samples, one of Parmigiano Reggiano and three of TrentinGrana were in vitro digested (Table 1-6.2.10) and analyzed to identify their phosphopetides pattern.

The aim of this study was to assess potential mineral chelating properties of CPPs identified in relation to their amino acid sequences and the presence of the phosphorylated cluster: S(P)S(P)S(P)EE .

Table1-6.1.10 :Cheese samples chosen for phosphopeptides identification and their amino acids sequence with ProteoExtract® Phosphopetide Enrichment TiO2 Kit , pre-column RP-HPLC-MSMS and Biopharmalynx v.1.1. software

Cheese	Aging (months)
GP30	11
GP40	16
GP45	23
GP55	34
PR56	17
TN52	11
TN79	18
TN80	25

It was used the ProteoExtract® phosphopetide Enrichment TiO2.

This kit enriches phosphorylated peptides from complex samples using a solid phase, highly selective, titanium oxide.

The enriched phosphopetide fraction is directly compatible with downstream analysis by MALDI and ESI mass spectrometry.

It is an efficient enrichment strategy help to compensate for the low stoichiometry of phosphopeptides relative to their unphosphorylated counterparts and for poor ionization and ion suppression effects inherent to MS analysis.

The ProteoExtract® Phosphopetide Enrichment TiO2 Kit uses a novel dioxide material to enable identification of a large number of phosphorilated species from complex protein mixtures.

The titanium dioxide id highly selective for phosphorylated peptides in the presence of abundant non-phosphorylated peptides.

Enrichment and selectivity for phosphopeptides is further improved by using a 2,5-DHB "displacer"concentration that is compatible with LC-MS and MALDI analysis.

The detailed protocol provides to:

- Warm all the reagents to room temperature;
- Centrifuge samples (100 ul);
- Weigh 1g Dihydroxybenzoic Acid and add into the vial of TiO2 Phosphobind buffer 200 •l and mix well;
- Dilute digested samples at least 1:4 with the solution of DHB-TiO2 phosphobind buffer (50 •l sample + 150 •l of solution);
- TiO2 phosphobind Resin: Mix 50 •l the TiO2 phosphobind resin by vortexing until completely suspended;
- Transfer 50 •l of the homogeneous suspension (resin) to a microcentrifuge tube, centrifuge the tube for 3 min at 2000-2500 x g. Completely remove the supernatant and discard.
- Add the diluted sample to the Resin, mix carefully, and incubate with gentle agitation for 10 min at room temperature. 1100 rpm is recommended;
- Centrifuge the tube for 3 min at 2000-2500 x g , remove the supernatant completely, and discard.
- Add 100 •l wash buffer 1 to the tube, mix carefully and centrifuge for 3 min at 2000-2500 g. Remove the supernatant and discard it. Repeat for a total of 2 washes;
- Add 100 ul wash buffer 2 to the tube, mix carefully and centrifuge for 3 min at 2000-2500 g. Remove the supernatant and discard it. Repeat for a total of 2 washes;
- Add 30 •l Elution Buffer to the tube, mix carefully by pipetting up and down, and incubate with gentle agitation for 10 min at room temperature. Mixer at 1100 rpm is recommended;
- Centrifuge the tube for 5 min at 10000 g and carefully transfer the supernatant to a new microcentrifuge tube;
- Centrifuge the tube containing supernatant for 3 min at 10000 x g. Collect the supernatant in a new tube and save it for a further analysis of the captured and purified phosphopeptides. Avoid transferring the resin.

- Each sample where mass spectrometry m/z separation revealed the possibility of phosphopeptides presence, was determined with Biopharmalynx v.1.1. software.

6.2. Results

6.2.1. *In vitro* Ca digestibility of cheese as a function of ripening time.

In table 1-6.2.1 are reported the in vitro Ca digestibility (dCa) values and in figures 1-6.2.1 and 2-6.2.1 are shown the relationship between dCa and ripening time. In Grana Padano samples (Figure 1-6.2.1), produced using lysozyme, there is a positive relationship between aging and dCa ($r2 = 0.27$; $P<0.05$) while in TrentinGrana (Figure 2-6.2.1), produced without the use of lysozyme, no significant correlation has been detected. Grana Padano dCa results of samples less 24 months aged are quite widespread and this weakens the relationship. However when data of more aged samples are considered, we can obtain a significant equation. A larger number of cheese samples is probably necessary in order to well understand the relationship between aging and Ca digestibility.

Table 1-6.2.1: Cheese samples Calcium digestibilty % indexes. Cheese samples and ripening times, Calcium amount before and after digestion, cheese samples Calcium digestibility % indexes.

Cheese	Ripening time	Start amount of Ca (mg/100 g)	After digestion Ca amount (mg/100g)	Ca digestibility %
GP28	10	0,997	0,592	59,41
GP30	11	1,063	0,750	70,52
GP31	11	0,933	0,768	82,32
GP32	12	0,879	0,767	87,27
GP34	13	0,908	0,696	76,59
GP35	13	0,961	0,678	70,6
GP37	15	1,282	0,841	65,6
GP38	16	0,961	0,631	65,75
GP39	16	0,757	0,442	58,42
GP40	16	0,890	0,691	77,59
GP42	17	0,748	0,573	76,68
GP43	20	0,948	0,533	56,27
GP44	20	0,987	0,686	69,45
GP45	24	0,891	0,814	91,34
GP46	26	0,866	0,631	72,85
GP47	25	0,954	0,908	95,2
GP48	39	0,781	0,698	89,4
GP50	11	1,165	0,689	50,19
GP53	27	0,922	0,775	84,11
GP55	34	0,965	0,805	83,35
PR56	17	0,889	0,557	62,73
PR57	19	1,032	0,468	45,35
PR58	20	0,915	0,422	46,14
PR59	20	0,843	0,393	46,59
PR60	21	0,965	0,851	88,23
PR61	25	1,016	0,606	59,67
TN49	10	0,768	0,605	78,83
TN51	11	0,927	0,603	65,12
TN52	11	0,865	0,577	66,73
TN72	24	0,900	0,694	77,20
TN73	24	0,869	0,6164	70,88
TN74	18	0,869	0,7013	80,64
TN77	11	0,7923	0,626	79,02
TN78	18	0,891	0,591	66,38
TN79	18	0,879	0,602	68,49
TN80	25	0,894	0,571	63,83
TN82	24	0,964	0,691	71,69

Figure 1-6.2.1.: Grana PadanoCalcium digestibility % with increased ripening times**.**

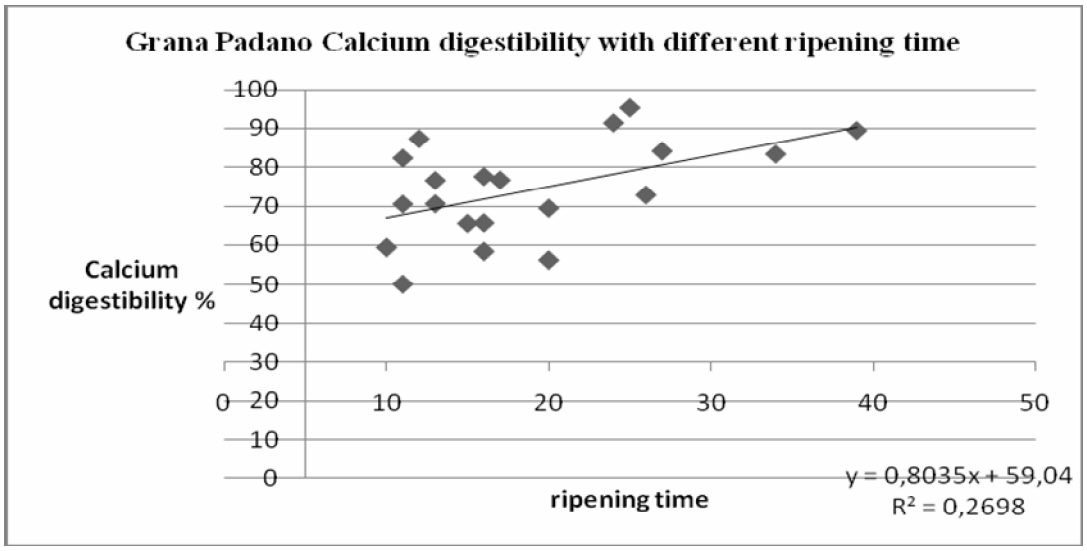
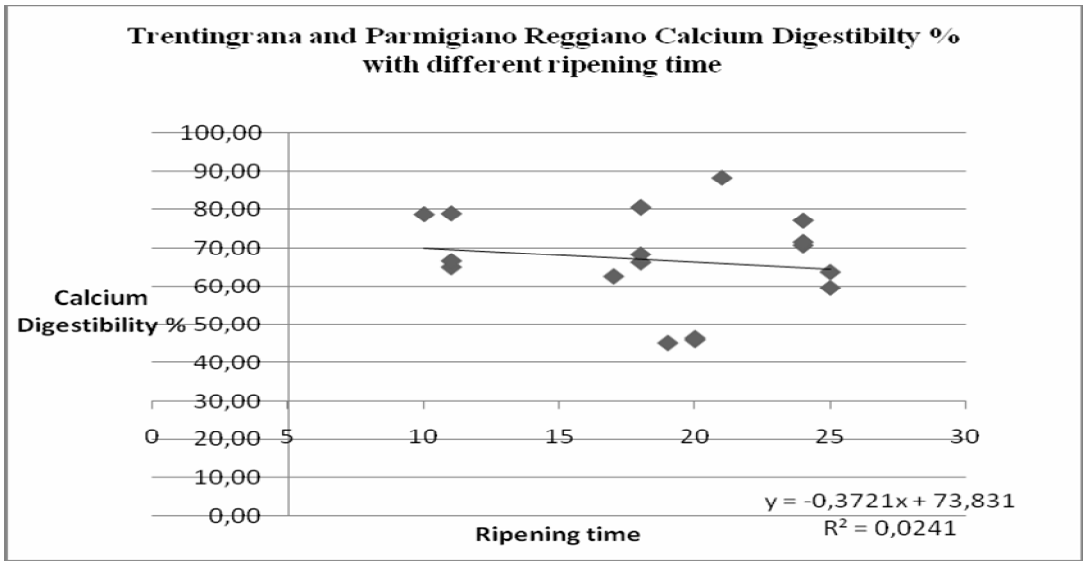


Figure 2-6.2.1: TrentinGrana and Parmigiano Reggiano Calcium digestibility %.



The in vitro Ca digestibility variance analysis between the two cheeses is reported in table 2-6.2.2. Until 20 months of aging no differences were observed between Grana Padano and TrentinGrana, while for more aged products the dCa of Grana padano was better than TrentinGrana one. Since Ca is almost entirely associated with caseins, the difference in dCa could be due to a different proteolysis mediated by the presence of lysozyme. The previously cited work of Sforza et al. (2004) confirm the existence of a different peptide pattern during cheese ripening determined by lysozyme, this support the hypothesis that lysozyme can affect dCa of cheese.

Table 2-6.2.1. *In vitro* Calcium digestibility variance analysis between Grana Padano and TrentinGrana with different ages.

Ripening time	Grana Padano	TrentinGrana	P
< 12 month	71.75 (SE 5.02)	73.21 (SE 5.61)	0.8509
15 < month < 20	67.10 (SE 3.24)	63.70 (SE 3.24)	0.4731
> 24 month	86.04 (SE 3.04)	62.59 (SE 3.04)	0.003

6.2.2. Gel filtration HPLC oligopeptides molecular weight distribution results

To evaluate casein phosphopeptides (CPPs) involvement in Ca binding and their role on in vitro Ca digestibility (dCa), peptide pattern of different ripened time cheeses was evaluate through molecular weight oligopeptides distribution.

Oligopeptides chromatographic analysis cut off were chosen between 500 and 4000 D. Results are showed in Figure 1-6.2.2.

In Grana Padano cheese dCa was significantly correlated with molecular weight fraction between 2500-1500 D (r=0,65469, P=0,0209), and the fraction between 1500-1000 D (r=0,3766; P=0,0062. In cheeses without lysozyme the only relevant correlations were between calcium digestibility and the < 1500 D fraction (r=0,40017,P=0,0585) and between 1000 and 500 D (r=0,4386, P=0,0782) (Table 2-6.2.2). The HPLC-MS analysis of peptides coming from in vitro cheese digestion can support these findings. In table 3-6.2.2. are reported the molecular weight of peptides having the S(P)S(P)S(P)EE common cluster binding Ca (CPP sequence) in their primary structure. In Grana Padano cheeses at MW of 1400 and 2450 D there are peptides with S(P)S(P)S(P)EE common cluster sequence, while other S(P)S(P)S(P)EE common cluster sequences can be find in more heavy peptides (MW 2635-2829 and 4038) that did not showed any relationship with dCa. They are probably hydrolyzed, during in vivo digestion, into the molecular weight peptide of significant range correlated with dCa (1500-1000 D).

In TrentinGrana the CPP sequences were found in peptides with Mw of 2450-2655-2829-3958 and 5999. In this case the amino acidic identification of CPPs (based on molecular weight studies) is not useful to conferm the correlation between dCa and peptide molecular weight distribution. Moreover it is important to underline that the correlation did not have high significant value i.e. p=0.07. Besides should be considered that in vitro could be subjected to some imperfections.

The important result is the pronounced difference between Grana Padano and the two cheeses without lysozyme. Grana Padano shows the most significant correlations between dCa and oligopeptides of 1500-1000 D r (r=0.3766, p=0,00062) and 2500-1500 D range cut off (r=0,65469 and p=0.0209).

Table 3-6.2.2:Molecular weight of peptides containing the S(P)S(P)S(P)EE common cluster sequence in Grana Padano and TrentinGrana.

Grana Padano			TrentinGrana	
MW	N° of P group		MW	N° of P group
1400	3		-	-
2450	3		2450	3
2655	4		2656	4

2829	4		2829	4
4039	5		3958	4
-	-		5999	4

The MW of 3 peptides is almost the same in both the two types of cheese. In Grana Padano we can find a smaller peptides, while the heavier peptides is obtained from the digestion of trentinGrana only.

Figure 1-6.2.2.: Cheese samples oligopeptides molecular weight distribution results.

Cheese	Aging	dCa	MW<4000	4000<Mw<3500	MW<3500	3500<Mw<3000	Mw< 3000	3000<Mw<2500	MW<2500 Da	2500< Mw <1500	< 1500 Mw	1500 < Mw < 1000	<1000	1000 < Mw < 500	Mw < 500	1000
GP 40	16	77,59	83,81	79,64	74,32	70,90	65,21	61,46	56,4	20	36,4	9,4	27	9,7	17,3	0,3957
GP 42	17	76,63	87,38	82,21	77,05	73,69	68,22	64,29	59,2	20,5	38,7	9,8	28,9	10,7	18,2	0,4309
GP 43	20	56,27	82,88	80,05	74,97	72,20	67,22	63,45	58	19,8	38,2	9,2	29	10,3	18,7	0,4264
GP 44	20	69,45	83,71	81,52	77,16	73,94	68,58	64,87	60	19,8	40,2	9,4	30,8	11	19,8	0,4248
GP 45	23	91,34	89,43	85,44	80,31	76,82	72,20	68,80	62,4	21,5	30	10,9	31	11,6	18,4	1,8779
GP 46	26	72,85	82,84	80,55	75,96	72,62	67,05	63,23	58,2	20,2	38	9,5	28,7	10,5	18,2	0,4123
GP 47	25	95,20	91,35	86,70	81,99	79,26	74,39	71,13	65	21	44	11	31,8	10	22	0,91
GP 53	27	84,11	99,96	99,96	99,94	99,94	99,93	99,93	63,2	21,7	41,5	11,3	25,2	11,7	18,5	2,5799
GP 53	27	84,11	99,96	99,96	99,94	99,94	99,93	99,93	63	22	41	11	30,2	11,5	18,5	0
GP 55	34	84,43	82,42	80,10	75,47	72,09	66,45	62,58	57,5	20,3	37,2	9,4	27,8	10,1	17,7	0,4056
GP32	12	87,27	88,00	85,18	81,27	77,30	72,17	68,81	63	22	41	12	29,00	10,5	18,5	0,92
GP48	39	89,40	89,32	86,88	80,87	78,78	72,44	70,39	66,2	20,5	45,7	11,2	34,5	13	21,5	2,0204
PR 56	17	67,78	99,83	99,80	99,77	99,76	99,74	99,72	58	21,65	36,35	1475,6	24,4	8,65	17,5	0
PR 57	19	53,25	99,90	99,88	99,85	99,84	99,81	99,79	59,5	21,4	30,1	9,7	28,4	12	16,4	0,3936
PR 58	20	51,26	86,94	83,71	79,41	75,61	70,73	67,01	61,9	21,4	40,5	11	29,5	11,1	18,4	0,444
PR 59	20	62,084	84,238	82,023	77,603	74,323	68,871	65,621	60,3	21,7	38,6	10,7	27,9	10,5	17,4	0,4202
PR 60	21	58,18	85,32	83,25	79,15	75,99	70,76	67,58	61,8	21,4	40,4	11	33,5	10,9	18,5	1,9635
PR 61	25	66,914	82,819	80,564	76,072	72,748	67,221	63,446	58,5	21	37,5	9,9	27,6	10,3	17,3	0,405
TN 72	24	72,57	90,88	87,63	83,39	80,75	76,04	72,24	66,8	23,1	43,7	11,9	31	12,4	19,4	1,6472
TN 73	24	69,62	90,32	88,43	84,71	81,58	76,41	72,64	67,4	23,1	44,3	12	30	12,3	20	1,6253
TN 74	18	76,67	92,94	90,35	86,84	83,68	79,58	74,73	69,7	23,5	46,2	12,7	33,5	12,8	20,7	0,5708
TN 78	18	90,23	85,62	83,48	77,05	77,06	68,46	66,37	60,5	21,5	39	10,3	30	10,5	18,2	1,6759
TN 79	18	80,59	88,82	86,25	81,68	79,01	74,24	70,50	65	22,5	42,5	11,9	31	11,6	19	1,7051
TN 80	25	62,07	85,02	82,86	78,60	75,44	70,16	66,52	61,6	20	41,6	9,1	32,5	10,3	22,2	0,4216
TN 81	25	56,02	83,63	81,29	76,65	73,20	67,45	63,48	58,1	21,3	36,8	9,3	27,5	10,1	17,4	0,403
TN 82	24	55,63	85,68	83,62	77,41	77,40	69,03	66,97	64,7	22,4	42,3	11,7	31	11,5	19,1	1,6667
TN 77	11	44,84	84,12	81,14	75,85	72,91	67,66	63,75	58,1	21,7	36,4	9,7		9,5	17,2	0,4103
TN 52	11	58,17	87,84	85,73	81,55	78,17	72,60	68,70	63,2	24	39,2	12,4	26,8	10,5	16,3	0,4831
TN 51	11	.	88,5555	85,9805	81,4024	78,5085	73,3842	69,4522	63,6	22,9	40,7	11,6	29,1	11,1	18	0,4742
TN 49	10	81,99	88,35	85,71	81,03	78,17	73,09	69,25	63,5	23	40,5	11,8	28,7	11,1	17,6	0,4742

Table 2-6.2.2. Relationship between *in vitro* Ca digestibility (dCa) and molecular weight (MW) of peptides released from digestion of cheese produced using lysozyme or not.

Use of lysozyme		MW (D) of peptides				
		2500-1500 D	1500 D	1500-1000 D	1000 D	1000-500 D
Yes	r	0.65469	0.16715	0.73766	0.49676	0.2980
	P	0.0209	0.6036	0.0062	0.1004	0.3464
No	r	0.12729	0.40017	0.22818	0.33498	0.43867
	P	0.5627	0.0585	0.2950	0.1488	0.0782

Analysis of variance has been performed between peptides MW distribution of the two cheeses, divided in ripening time classes has been done (Table 4-6.2.2.). The cheeses were classified as: 1) “Giovane”, under 12 month aged cheeses, 2) “Media”, between 15 and 20 months cheeses aged and 3) “Riserva”, over 20 months aged cheeses.

Table 4.-6.2.2.:Anova analysis of peptides distribution according to their molecular weight (MW) after in vitro digestion of two cheese (Grana Padano and TrentinGrana) at different aging.

	Type Giovane aging < 12 months.		Type media aging 15-20 months		Type Riserva: > 20 months aging	
MW peptides	Grana Padano	TrentinGrana	Grana Padano	TrentinGrana	Grana Padano	TrentinGrana
< 4000 D	88.00	86.62	83.16^{a}	88.55^{b}	90.75	87.10
4000-3500 D	6.72	7.57	8.14	7.20	5.82	7.06
<3500 D	81.27	79.04	75.01^{a}	81.34^{b}	84.92	80.04
3500-3000 D	9.10	8.36	8.82^{A}	7.68^{B}	6.01	8.15
<3000 D	72.17	70.68	66.19^{A}	73.66^{B}	78.91	71.88
3000-2500 D	9.17	9.00	8.89	9.01	16.69	8.87
<2500 D	63.00	61.00	57.30^{A}	64.65^{B}	62.21	63.01
2500-1500 D	22.00	22.52	20.00^{A}	22.33^{B}	21.02	21.60
<1500 D	41.00	39.16	37.30^{a}	42.31^{b}	39.62	41.41
1500-1000 D	12.00	11.10	9.31^{a}	11.21^{b}	10.61	10.44
1000 D	29.00	28.40	27.98	29.85	29.88	31.06
1000-500 D	10.50	10.54	10.00^{a}	11.63^{b}	11.20	11.23
<500 D	18.50	17.52	17.98^{a}	19.48^{b}	19.25	19.73

No significant differences can be detected between the two cheeses before 12 months of aging, while more differences were detected when ripening is between 15 and 20 months. In this case proteins of TrentinGrana appears more hydrolyzed than Grana Padano ones. In the more ripened cheeses no differences were detected. Although cheese's samples utilized for peptide analysis and in vitro Ca digestibility were not always the same, the higher proteolysis observed in TrentinGrana could account for its lower dCa because of a cleavage of linkage between peptides and Ca.

6.2.3. Identification of caseino-phospho-peptides (CPP) with ProteoExtract® phosphopetide Enrichment TiO_2 kit ,Pre-colum RP-HPLC-MS phosphopetides, Biopharmalynx v.1.1. software

The possible CPPs are characterized by the presence of a common cluster having the following amino acid sequence: SSSEE (Ser-Ser-Ser-Glu-Glu), where Ser is the insertion site for phosphoryl group. According to literature, they were isolated with ProteoExtract® phosphopeptide Enrichment TiO2 kit, pre-column combined RP-HPLC-MSMS and identified with Biopharmalynx v.1.1. software. The results are showed in Table 1-6.3.4.

Table 1-6.2.3.: Possible phosphopetides inside each cheese sample. Respectively GP30 (11 months), GP40 (16 months), GP45 (23 months), GP55 (34 month), PR56 (1 months), TN52 (11 months), TN79 (18 months), TN80 (25 months).

GP30 (11 months)

RT (Min)	Analyte m/z	Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
26,2	989,9374	4	3955,72	a-casein-S1	EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGY	61	94	Phosphoryl S(1)	5886
25	891,3971	3	2671,17	a-casein-S2	KENLCSTFCKEVVRNANEEEYS	32	53	Phosphoryl S(1)	1803
24,4	734,4037	2	1466,79	a-casein-S2	KQEKNMAINPSK	21	32	Phosphoryl S(1)	1551
23,2	972,5231	2	1943,03	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	840
23	790,7233	3	2369,15	a-casein-S2	TSEENSKKTVDMESTEVFTK	130	149	Phosphoryl S(1)	1109
22,2	1099,4025	2	2196,79	a-casein-S2	EENSKKTVDMESTEVFTK	132	149	Phosphoryl S(1)	2832
21,8	944,1225	3	2829,34	a-casein-S2	STSEENSKKTVDMESTEVFTKKTK	129	152	Phosphoryl S(1)	2008
20,9	972,4022	2	1942,79	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	531
17,5	801,3141	3	2400,92	a-casein-S1	EDIKQMEAESISSSEEIVPNS	55	75	Phosphoryl S(1)	9854
17,4	614,3111	2	1226,61	a-casein-S2	QEKNMAINPS	22	31	pryl S(1),Oxidati	900
17,3	988,7076	3	2963,1	a-casein-S2	CSTFCKEVVRNANEEEYSIGSSSEES	36	61	Phosphoryl S(1)	5157
15,1	1001,3578	3	3001,05	a-casein-S1	DIGSESTEDQAMEDIKQMEAESISS	43	67	Phosphoryl S(3)	1303
14,4	774,601	3	2320,78	a-casein-S1	SKDIGSESTEDQAMEDIKQM	41	60	Phosphoryl S(1)	598
14	983,304	2	1964,59	a-casein-S2	QEKNMAINPSKENLCS	22	37	Phosphoryl S(2)	953
12,1	983,3582	2	1964,7	a-casein-S2	SSEESIISQETYKQEK	9	24	Phosphoryl S(1)	1661
11,5	991,3228	2	1980,63	a-casein-S2	QEKNMAINPSKENLCS	22	37	Phosphoryl S(2)	6327
11	845,8332	2	1689,65	b-casein	FQSEEQQQTEDEL	33	45	Phosphoryl S(1)	1123

GP40 (16 months)

RT (Min)	Analyte m/z	Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
32,7	1137,1774	3	3408,51	b-casein	INKKIEKFQSEEQQQTEDELQDKIHPF	26	52	Phosphoryl S(1)	2529
32,4	1356,9453	3	4067,81	a-casein-S2	LTEEEKNRLNFLKKISQRYQKFALPQYLKTVY	153	184	Phosphoryl S(1)	4157
30,2	1308,2612	1	1307,25	a-casein-S1	QMEAESISSS	59	68	Phosphoryl S(3)	826
29,6	1056,1195	3	3165,33	a-casein-S1	EEIVPNSVEQKHIQKEDVPSERYLGY	69	94	Phosphoryl S(1)	1031
28,3	857,6031	5	4282,98	a-casein-S1	ESTEDQAMEDIKQMEAESISSSEEIVPNSVEQKHIQK	47	83	Phosphoryl S(1)	8114
27,6	1010,7288	4	4038,88	a-casein-S1	QMEAESI**SSSEE**IVPNSVEQKHIQKEDVPSER	59	90	Phosphoryl S(5)	3165
26,8	972,9797	2	1943,94	a-casein-S1	SEEIVPNSVEQKHIQK	68	83	Phosphoryl S(1)	728
26,3	1227,5264	2	2453,04	a-casein-S1	EAESISSSEEIVPNSVEQKHI	61	81	Phosphoryl S(2)	4371
25,9	972,4393	2	1942,86	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	656
24,1	817,7592	3	2450,25	b-casein	**SSSEE**SITRINKKIEKFQS	17	35	Phosphoryl S(3)	3971
12,2	1119,8673	2	2237,72	a-casein-S2	SEESAEVATEEVKITVDDK	58	76	Phosphoryl S(2)	1099
11,7	1079,8845	2	2157,75	a-casein-S2	SEESAEVATEEVKITVDDK	58	76	Phosphoryl S(1)	11678

GP45 (23 months)									
RT (Min)	Analyte m/z	Charge State	Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
26,3	1054,0026	2	2105,99	a-casein-S1	DIGSESTEDQAMEDIKQM	43	60	Phosphoryl S(1)	7561
24,7	932,3679	3	2794,08	a-casein-S2	SEESAEVATEEVKITVDDKHYQK	58	80	Phosphoryl S(2)	1722
21,4	944,0082	3	2829	a-casein-S2	SIG**SSSEE**SAEVATEEVKITVDDK	53	76	Phosphoryl S(4)	1228
19,4	791,3505	1	790,34	a-casein-S2	ESTEVF	142	147	Phosphoryl S(1)	2947
19,2	975,3334	2	1948,65	a-casein-S2	EVVRNANEEEYSIGSSS	42	58	Phosphoryl S(1)	612
1,5	1345,4373	1	1344,43	a-casein-S1	ESTEDQAMEDI	47	57	Phosphoryl S(1)	1068
1,5	990,3503	2	1978,68	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	1406
1,5	1071,3375	4	4281,32	a-casein-S2	EENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKK	132	166	Phosphoryl S(1)	1545
1,5	886,2888	3	2655,84	a-casein-S1	QMEAESI**SSSEE**IVPNSVEQK	59	79	Phosphoryl S(4)	2565
1,5	999,3764	2	1996,74	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	2542
1,5	1225,9797	2	2449,94	b-casein	**SSSEE**SITRINKKIEKFQS	17	35	Phosphoryl S(3)	1278
1,5	1053,9993	2	2105,98	a-casein-S1	DIGSESTEDQAMEDIKQM	43	60	Phosphoryl S(1)	729
1,5	1401,4728	1	1400,46	a-casein-S1	I**SSSEE**IVPNS	65	75	Phosphoryl S(3)	657

GP55 (34 months)									
RT (Min)	Analyte m/z	Charge State	Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
29,6	967,9237	2	1933,83	a-casein-S1	TEDQAMEDIKQMEAES	49	64	Phosphoryl S(1)	1312
27,6	847,3586	2	1692,7	a-casein-S1	HIQKEDVPSERYL	80	92	Phosphoryl S(1)	929
26,2	792,0948	5	3955,43	a-casein-S2	EKNRLNFLKKISQRYQKFALPQYLK	154	184	Phosphoryl S(1)	3092
23,1	972,4628	2	1942,91	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	610
21,9	1115,386	2	2228,76	a-casein-S2	QEKNMAINPSKENLCSTF	22	39	Phosphoryl S(2)	1066
21,8	944,0547	3	2829,14	a-casein-S2	SIG**SSSEE**SAEVATEEVKITVDDK	53	76	Phosphoryl S(4)	1171
21,4	742,3312	2	1482,65	b-casein	IHPFAQTQSLVY	49	60	Phosphoryl S(1)	2529
19,6	976,0475	3	2925,12	b-casein	IEKFQSEEQQQTEDELQDKIHPF	30	52	Phosphoryl S(1)	6200
18,9	764,3076	4	3053,2	b-casein	KIEKFQSEEQQQTEDELQDKIHPF	29	52	Phosphoryl S(1)	2083
18,4	976,0509	3	2925,13	b-casein	IEKFQSEEQQQTEDELQDKIHPF	30	52	Phosphoryl S(1)	1260
17,6	852,6442	3	2554,91	b-casein	FQSEEQQQTEDELQDKIHPF	33	52	Phosphoryl S(1)	6452
16,8	987,9755	3	2960,9	a-casein-S2	SSEESAEVATEEVKITVDDKHYQK	57	80	Phosphoryl S(3)	7777
16,6	893,2899	3	2676,85	a-casein-S2	EQLSTSEENSKKTVDMESTEVF	126	147	Phosphoryl S(2)	9544
16,2	1231,8403	2	2461,66	a-casein-S1	ESTEDQAMEDIKQMEAESIS	47	66	Phosphoryl S(2)	2372
15,8	803,6291	3	2407,86	b-casein	QSEEQQQTEDELQDKIHPF	34	52	Phosphoryl S(1)	3307
14,1	803,6326	3	2407,87	b-casein	QSEEQQQTEDELQDKIHPF	34	52	Phosphoryl S(1)	1206
13,3	1191,3602	2	2380,7	a-casein-S1	TEDQAMEDIKQMEAESISS	49	67	Phosphoryl S(3)	975
13,3	898,6134	3	2692,82	a-casein-S2	EQLSTSEENSKKTVDMESTEVF	126	147	Phosphoryl S(2)	3546
12,7	811,3047	3	2430,89	b-casein	IEKFQSEEQQQTEDELQDK	30	48	Phosphoryl S(1)	1446
10,9	845,7817	2	1689,55	b-casein	FQSEEQQQTEDEL	33	45	Phosphoryl S(1)	1115
10,9	1031,3634	2	2060,71	b-casein	FQSEEQQQTEDELQDK	33	48	Phosphoryl S(1)	957
1,7	1096,3562	2	2190,7	a-casein-S1	SSEEIVPNSVEQKHIQK	67	83	Phosphoryl S(3)	962
1,6	989,3316	3	2964,97	a-casein-S1	EDIKQMEAESISSSEEIVPNSVEQK	55	79	Phosphoryl S(2)	9551
1,5	893,6304	3	2677,87	a-casein-S2	EESIISQETYKQEKNMAINPS	11	31	Phosphoryl S(3)	1944
1,5	1278,4897	2	2554,96	b-casein	FQSEEQQQTEDELQDKIHPF	33	52	Phosphoryl S(1)	5166
1,5	1072,9767	2	2143,94	a-casein-S2	SSEESAEVATEEVKITVD	57	74	Phosphoryl S(3)	3964
1,4	987,981	3	2960,92	a-casein-S2	SSEESAEVATEEVKITVDDKHYQK	57	80	Phosphoryl S(3)	11496
1,4	1115,4066	2	2228,8	a-casein-S2	QEKNMAINPSKENLCSTF	22	39	Phosphoryl S(2)	2078
1,4	1031,3713	2	2060,73	b-casein	FQSEEQQQTEDELQDK	33	48	Phosphoryl S(1)	1375
1,4	829,3677	2	1656,72	a-casein-S1	EAESISSSEEIVPNS	61	75	Phosphoryl S(1)	782
1,4	1058,8577	2	2115,7	a-casein-S2	EESIISQETYKQEKNM	11	26	Phosphoryl S(2)	2049
1,4	1339,4816	1	1338,47	a-casein-S2	QEKNMAINPSK	22	32	Phosphoryl S(1)	1185
1,4	803,6343	3	2407,88	b-casein	QSEEQQQTEDELQDKIHPF	34	52	Phosphoryl S(1)	1127
1,4	784,35	1	783,34	a-casein-S1	NSVEQK	74	79	Phosphoryl S(1)	1645
1,4	852,6559	3	2554,94	b-casein	FQSEEQQQTEDELQDKIHPF	33	52	Phosphoryl S(1)	9976

PR56 (17 months)									
RT (Min	m/z	State	Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
33,6	1349,868	4	5395,44	b-casein	QDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM	46	93	Phosphoryl S(1)	3634
33,3	1500,707	4	5998,8	a-casein-S1	KDIGSESTEDQAMEDIKQMEAESI**SSSEE**IVPNSVEQKHIQKEDVPSERY	42	91	Phosphoryl S(4)	16881
33,3	1505,226	2	3008,44	a-casein-S2	NTMEHVSSSEESIISQETYKQEKNM	2	26	Phosphoryl S(1)	1529
32,6	1319,02	3	3954,04	a-casein-S2	TEVFTKKTKLTEEEKNRLNFLKKISQRYQKF	144	174	Phosphoryl S(1)	2451
32,3	808,9412	2	1615,87	a-casein-S1	VEQKHIQKEDVPS	76	88	Phosphoryl S(1)	1875
32,3	832,9517	4	3327,78	b-casein	EEQQQTEDELQDKIHPFAQTQSLVYPF	36	62	Phosphoryl S(1)	1816
32,2	1356,98	3	4067,92	a-casein-S2	LTEEEKNRLNFLKKISQRYQKFALPQYLKTVY	153	184	Phosphoryl S(1)	4173
31	1003,008	4	4008	a-casein-S2	KQEKNMAINPSKENLCSTFCKEVVRNANEEEYS	21	53	Phosphoryl S(2)	2137
30,8	1109,906	3	3326,7	a-casein-S2	IISQETYKQEKNMAINPSKENLCSTFCK	14	41	Phosphoryl S(1)	2599
30,8	1026,533	2	2051,05	a-casein-S2	NSKKTVDMESTEVFTKK	134	150	Phosphoryl S(1)	2250
30,5	1028,607	2	2055,2	a-casein-S1	PEVFGKEKVNELSKDIGS	29	46	Phosphoryl S(1)	1406
30,4	837,829	3	2510,46	a-casein-S1	VEQKHIQKEDVPSERYLGYL	76	95	Phosphoryl S(1)	712
29,7	1070,543	3	3208,6	b-casein	ELEELNVPGEIVESLSSSEESITRINKK	2	29	Phosphoryl S(1)	2020
29,7	1093,887	3	3278,64	a-casein-S1	EEIVPNSVEQKHIQKEDVPSERYLGYL	69	95	Phosphoryl S(1)	6034
29,2	951,8066	3	2852,4	a-casein-S2	SSEESIISQETYKQEKNMAINPS	9	31	Phosphoryl S(3)	4793
28,9	992,4803	3	2974,42	a-casein-S2	ENLCSTFCKEVVRNANEEEYSIGSSS	33	58	Phosphoryl S(1)	2305
28,2	857,6233	5	4283,08	a-casein-S1	ESTEDQAMEDIKQMEAESISSSEEIVPNSVEQKHIQK	47	83	Phosphoryl S(1)	26931
26,5	1336,667	2	2671,32	a-casein-S2	KENLCSTFCKEVVRNANEEEYS	32	53	Phosphoryl S(1)	11373
26,5	869,8853	2	1737,75	a-casein-S2	KTVDMESTEVFTKK	137	150	Phosphoryl S(1)	2545
26	792,1833	5	3955,88	a-casein-S1	EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGY	61	94	Phosphoryl S(1)	11663
25,7	972,4746	2	1942,93	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	928
25,1	1336,657	2	2671,3	a-casein-S2	KENLCSTFCKEVVRNANEEEYS	32	53	Phosphoryl S(1)	6324
25	907,0692	3	2718,18	a-casein-S1	SSEEIVPNSVEQKHIQKEDVPS	67	88	Phosphoryl S(3)	3434
24,4	961,1896	3	2880,54	a-casein-S1	VEQKHIQKEDVPSERYLGYLEQL	76	98	Phosphoryl S(1)	1806
23,6	848,7411	3	2543,2	b-casein	INKKIEKFQSEEQQQTEDEL	26	45	Phosphoryl S(1)	762
23,6	1272,113	2	2542,21	a-casein-S2	NMAINPSKENLCSTFCKEVVR	25	45	Phosphoryl S(2)	2111
22,8	791,422	3	2371,24	a-casein-S2	EESAEVATEEVKITVDDKHY	59	78	Phosphoryl S(1)	1368
22,7	790,754	3	2369,24	a-casein-S2	TSEENSKKTVDMESTEVFTK	130	149	Phosphoryl S(1)	1840
22,3	853,7686	3	2558,28	a-casein-S1	SSEEIVPNSVEQKHIQKEDVPS	67	88	Phosphoryl S(1)	8022
21,9	1174,596	3	3520,76	b-casein	IEKFQSEEQQQTEDELQDKIHPFAQTQS	30	57	Phosphoryl S(2)	3957
21,8	1109,852	3	3326,53	a-casein-S2	IISQETYKQEKNMAINPSKENLCSTFCK	14	41	Phosphoryl S(1)	964
21,7	935,1498	3	2802,43	a-casein-S2	IGSSSEESAEVATEEVKITVDDKHY	54	78	Phosphoryl S(1)	4074
20,8	972,4421	2	1942,87	a-casein-S1	LHSMKEGIHAQQKEPM	120	135	Phosphoryl S(1)	1758
18,6	835,0707	3	2502,19	a-casein-S2	IGSSSEESAEVATEEVKITVDDK	54	76	Phosphoryl S(1)	1449
12,3	1079,923	2	2157,83	a-casein-S2	SEESAEVATEEVKITVDDK	58	76	Phosphoryl S(1)	1542

TN52 (11 mesi)									
RT (Min)	m/z	State	Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
19,5	791,3568	1	790,35	a-casein-S2	ESTEVF	142	147	Phosphoryl S(1)	3647
19,2	975,3393	2	1948,66	a-casein-S2	EVVRNANEEEYSIGSSS	42	58	Phosphoryl S(1)	1477
19,2	975,3393	2	1948,66	a-casein-S2	EVVRNANEEEYSIGSSS	42	58	Phosphoryl S(1)	1477
1,5	1328,964	2	2655,91	a-casein-S1	QMEAESI**SSSEE**IVPNSVEQK	59	79	Phosphoryl S(4)	4431
1,5	990,3703	2	1978,72	a-casein-S2	NMAINPSKENLCSTFCK	25	41	Phosphoryl S(1)	708
1,5	999,3963	2	1996,78	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	1434

TN79 (18 month)									
RT (Min)	m/z	Charge State	Mass (Da	Protein	Peptide	Start	End	Modifiers	Intensity
34,1	1054,165	3	3159,47	a-casein-S1	KEGIHAQQKEPMIGVNQELA	121	146	Phosphoryl S(1)	1332
26,3	1054,004	2	2105,99	a-casein-S1	DIGSESTEDQAMEDIKQM	43	60	Phosphoryl S(1)	5547
20,6	999,343	2	1996,67	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	707
19,4	791,3501	1	790,34	a-casein-S2	ESTEVF	142	147	Phosphoryl S(1)	3920
19,2	975,327	2	1948,64	a-casein-S2	EVVRNANEEEYSIGSSS	42	58	Phosphoryl S(1)	1895
18	1074,438	1	1073,43	a-casein-S1	EDVPSERY	84	91	Phosphoryl S(1)	807
1,5	1225,975	2	2449,93	b-casein	**SSSEE**SITRINKKIEKFQS	17	35	Phosphoryl S(3)	1924
1,5	1053,997	2	2105,98	a-casein-S1	DIGSESTEDQAMEDIKQM	43	60	Phosphoryl S(1)	847
1,5	999,374	2	1996,73	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	1379
1,5	990,3442	2	1978,67	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	1112
1,5	843,3546	2	1684,69	a-casein-S1	SSEEIVPNSVEQK	67	79	Phosphoryl S(3)	1878
1,5	734,2974	2	1466,58	a-casein-S2	KQEKNMAINPSK	21	32	Phosphoryl S(1)	618

TN80 (25 months)									
RT (Min)	m/z	Charge State	Mass (Da)	Protein	Peptide	Start	End	Modifiers	Intensity
27,1	990,6625	4	3958,62	a-casein-S1	QMEAESI**SSSEE**IVPNSVEQKHIQKEDVPSER	59	90	Phosphoryl S(4)	2581
26,1	989,8724	4	3955,46	a-casein-S1	EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGY	61	94	Phosphoryl S(1)	2819
25,3	974,763	3	2921,27	a-casein-S1	DIGSESTEDQAMEDIKQMEAESISS	43	67	Phosphoryl S(2)	1564
24	817,6927	3	2450,05	b-casein	**SSSEE**SITRINKKIEKFQS	17	35	Phosphoryl S(3)	1381
17,5	975,3702	2	1948,72	a-casein-S2	EVVRNANEEEYSIGSSS	42	58	Phosphoryl S(1)	1913
17,5	939,8557	2	1877,7	a-casein-S2	SEESIISQETYKQEK	10	24	Phosphoryl S(1)	1406
17,5	852,6385	3	2554,89	b-casein	FQSEEQQQTEDELQDKIHPF	33	52	Phosphoryl S(1)	2714
17,2	614,2611	2	1226,51	a-casein-S2	QEKNMAINPS	22	31	Phosphoryl S(1)	868
1,4	755,2934	2	1508,57	a-casein-S2	KKTVDMESTEVF	136	147	Phosphoryl S(1)	1152
1,4	863,9806	3	2588,92	a-casein-S2	SIGSSSEESAEVATEEVKITVDDK	53	76	Phosphoryl S(1)	629
1,4	852,6182	3	2554,83	b-casein	FQSEEQQQTEDELQDKIHPF	33	52	Phosphoryl S(1)	1014
1,4	1062,385	2	2122,75	a-casein-S2	SEESIISQETYKQEKNM	10	26	Phosphoryl S(1)	3096
1,4	1053,865	2	2105,71	a-casein-S1	DIGSESTEDQAMEDIKQM	43	60	Phosphoryl S(1)	4075
1,4	1402,513	1	1401,5	b-casein	FQSEEQQQTED	33	43	Phosphoryl S(1)	680
1,4	907,0411	3	2718,1	a-casein-S1	SSEEIVPNSVEQKHIQKEDVPS	67	88	Phosphoryl S(3)	3082
1,4	791,3535	1	790,35	a-casein-S2	ESTEVF	142	147	Phosphoryl S(1)	944
1,4	990,3799	2	1978,74	a-casein-S2	TFCKEVVRNANEEEYS	38	53	Phosphoryl S(1)	1624
1,4	944,0176	3	2829,03	a-casein-S2	SIG**SSSEE**SAEVATEEVKITVDDK	53	76	Phosphoryl S(4)	2534
1,4	1226,001	2	2449,99	b-casein	**SSSEE**SITRINKKIEKFQS	17	35	Phosphoryl S(3)	1369

Phosphopeptides with the common cluster can be divided in 3 groups: peptides derived from • s1-casein(59-90); peptides obtained from • s2-casein(53-76) and ones from • -casein(17-35). The peptides pattern of Grana Padano and TrentinGrana is quite similar but the sequence • s1-casein(65-75) can be found in Grana Padano only, while a unique sample of TrentinGrana contained the • s1-casein(42-91) sequence.

In total 70 phospopeptides were indentified in the four Grana Padano samples and their partitioning between caseins was the following: 27 (38.57 %) derived from • s1-casein, 30 (42.86 %) were from • s2-casein and 13 (18.57 %) from • -casein.

Table 2-6.2.3 reports the number of phosphoril group and of phosphopeptides containing the common cluster mineral binding sequence of three phosphoseryl followed by two glutamic acid. The value of dCa is also included

Table 2-6.2.3: Number of single phosphopetides and CPP in some samples of Grana Padano (gp) and trentinGrana (pr, tn)

Cheese	Aging (months)	Cpps containing S(P)S(P)S(P)EE	Total number of CPPs	Calcium Digestibility%
gp30	**11**	**17**	**17**	**70,52495083**
gp40	**16**	**9**	**12**	**77,59420703**
gp45	**23**	**9**	**13**	**91,34106462**
gp55	**34**	**34**	**35**	**83,35329241**
pr56	**17**	**34**	**35**	**67,78341066**
tn52	**11**	**5**	**6**	**58,16934163**
tn79	**18**	**11**	**12**	**80,59223801**
tn80	**25**	**15**	**19**	**62,07305175**

There is not a linear relationship between single phosphoryl group and CPPS containing the common cluster S(P)S(P)S(P)EE, also the number of single phosphoryl group and phosphopetides containing the common cluster is not well related to aging. The best relationship observed between dCa and phosphopeptides was obtained with the number of single phopshoryl group, but it was not significant ($r = 0.312$; $P = 0.452$)

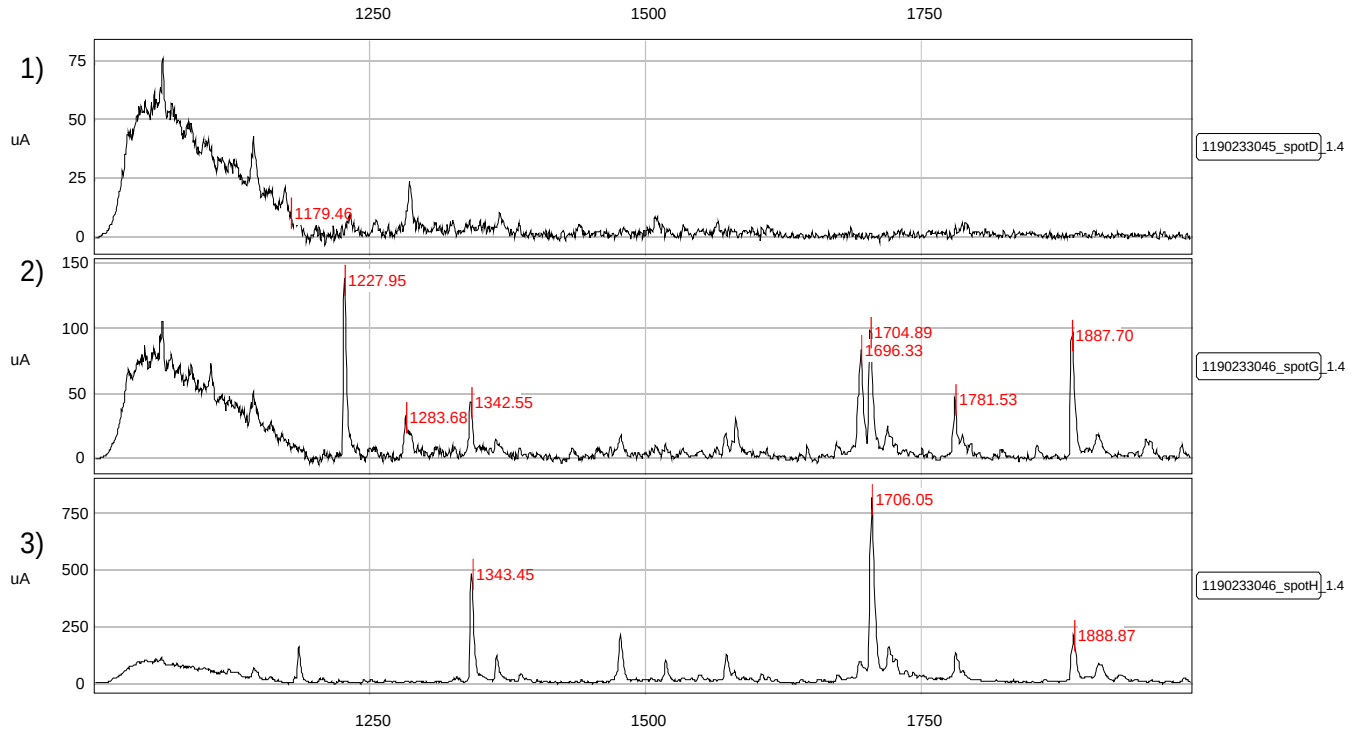
6.2.4. IMAC 30-SELDI method to enrich phophopetides results

In this paragraph are showed the results of Grana Padano (GP) and Trentin Grana (TN) phosphopetides enrichment SELDI analysis with different pH buffer and mineral solutions. Every graph contains 3 chromatograms, which are, respectively, the buffer (blanck), the acidified, filtrated cheese sample with mineral solution, and the acidified, filtrated cheese sample without mineral solution use. The mineral solution is added in order to selectively bind the phosphopeptides. Peaks retained by columns without the use of FeCl2 and shown in the third chromatogram, should not be phosphorilated.

The chromatogram x-axes is the mass to charge ratio (m/z) while the y-axes is the intensity. The three chromatograms of figure 1-6.2.4. are the result of TrentinGrana 77 (11 months aged) IMAC30 analysis with FeCl2 0.1 M mineral solution and Formic acid (more acid than Sodium Acetate). The second chromatogram of figure 1 contains three peaks at 1227.95 m/z, 1283,68 m/z and 1781,53 m/z, that should be the ones isolated through the affinity with iron.

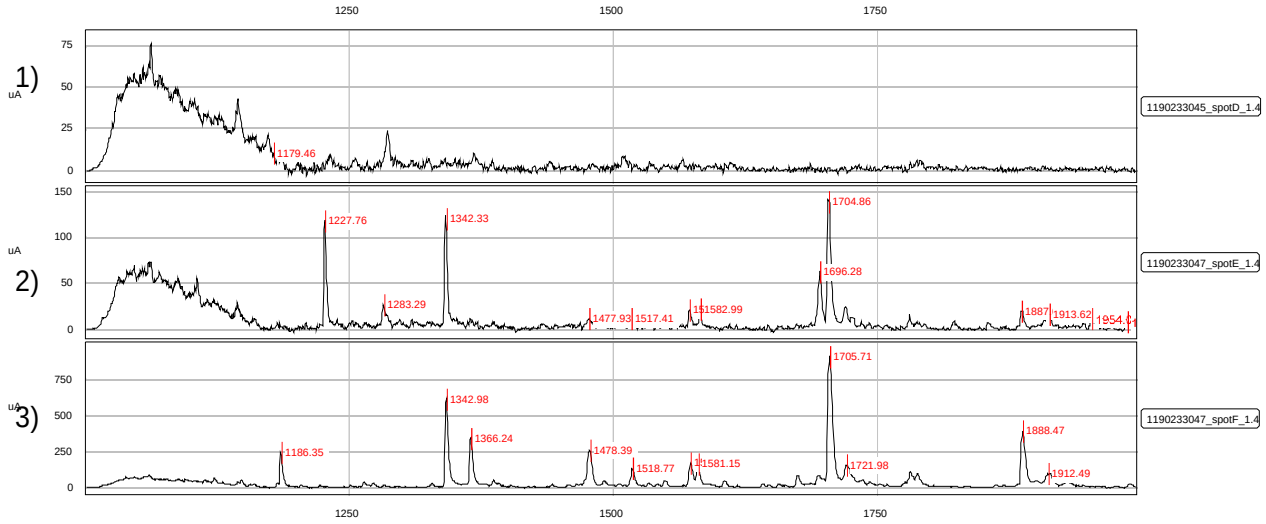
The other peaks in the third chromatogram should be not peaks of phospho-peptides.

Figure 1-6.2.4. : TrentiGrana 77 SELDI-TOF profile.1) Formic acid buffer;2)TN77 10x <3000 Da with Fe;3)TN77 10x <3000 Da without Fe



Another example of the same analysis is showed in figure 2-6.2.4.. with another sample: Grana Padano 44 (20 months aged) five time diluted. Also in this case in the second chromatogram, which is the one treated with iron to enrich the phosphopetides, there are peaks, at 1227.76 and 1283.29 m/z, not present in the third one, without iron.

Figure 2-6.2.4. : Grana Padano 44 (20 months) SELDI-TOF profile:1) Formic acid buffer; 2) GP44 <3000 Da 5x with Fe; 3) GP44 <3000 Da 5x without Fe



In figure 3-6.2.4. a comparisonof TrentinGrana 77 and Grana Padano 44 is shown. Samples were centrifuged but not filtered and the analysis were performed with and without the addition of the mineral solution. In both samples a peak at 1227.88 m/z (1228.04 m/z for GP44) is identified as a potential phosphorilated peptide. The addition of FeCl2 reduces the amount of peak at 1343 m/z, which can be found in both the chromatograms. It is not possible to exclude the presence of phosphorilated group in this peptide. SELDI analysis results of TrentinGrana 77 and Grana Padano 44 samples does not give the complete profile of m/z phospho-petides, but individualizes the protein fractions phosphorylated. In both samples, with and without lysozyme, appeare the peak at around 1227 m/z in y-axes. This result conferm that the oligopeptides moleculare weight cut off around 1500 and 1000 D, significantly correlated with calcium digestibility in both type of cheeses, is phosphorilated.

Figure 3-6.2.4. TN77-GP44, FA BUFFER, SPA SELDI-TOF profile comparing: 1) FA buffer with Fe; 2) TN77 only centrifuged with Fe;3) TN77 only centrifuged without Fe;4) GP44 only centrifuged with Fe; 5)GP44 only centrifuged without Fe.

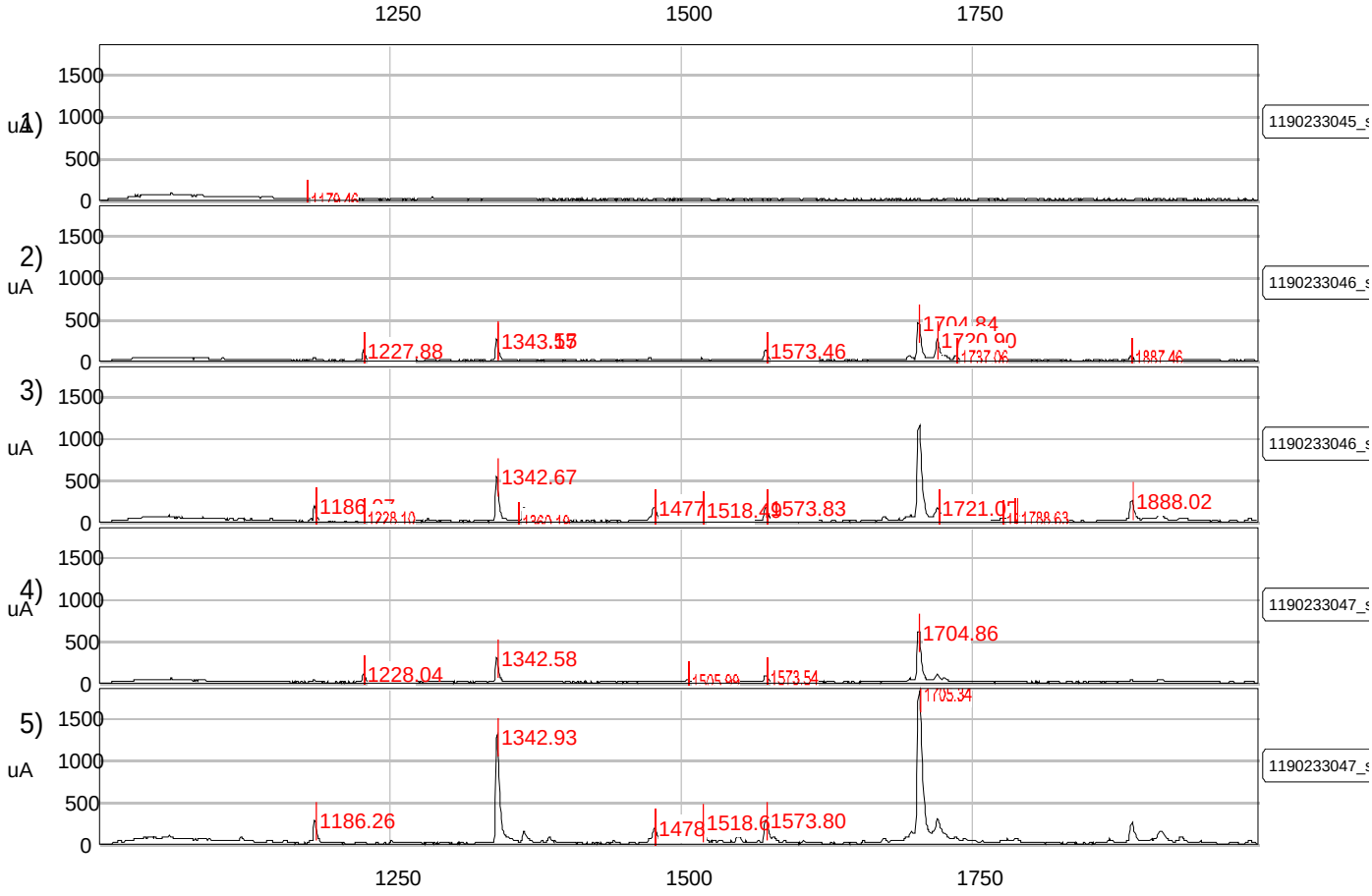
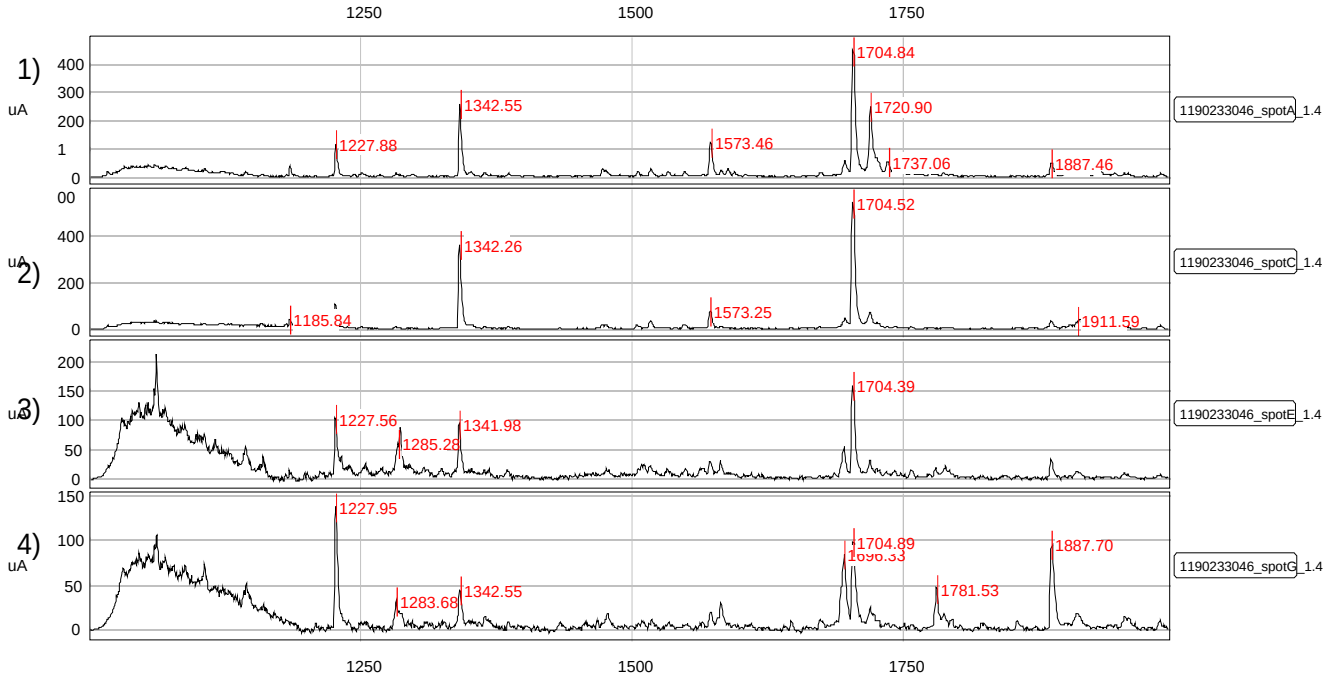


Figure 4-6.2.4.reports all TN 77 tests combination in each ProteinChip spot and the importance of the right dilution to observe the analysis results. The better dilution seems to be the five time and the ten time.

Figure 4-6.6.1.TN77 SELDI-TOF tests:1) TN77 only centrifuged with Fe; 2) TN77 filtrated <3000 Da with Fe; 3)TN77 < 3000 Da, 5x, with Fe; 4) TN77 <3000 Da, 10x, with Fe.



The best SELDI-TOF results were obtained combining a sample acidification to separate the potential stuck together peptides, followed by sample filtration, to isolate the < 3000 D protein fraction, and using 2,5-dihydroxybenzoic acid (DHB) solution to crystallize the bound peptides with the spot iron matrix, before time-of-flight spectrometer ionization and lecture. Figure 5-6.2.4. In this case many protein fraction phorilated have been isolated. Therefore between 1000 to 2000 D cut off there are phosphorilated protein fractions.

Figura 5-6.2.4. TN77 SELDI-TOF crystallizing DHB solution use: 1) TN77 acidified with Fe; 2) TN77 acidified, filtrated with Fe; 3) TN77 acidified, filtrated, 5x, with Fe.

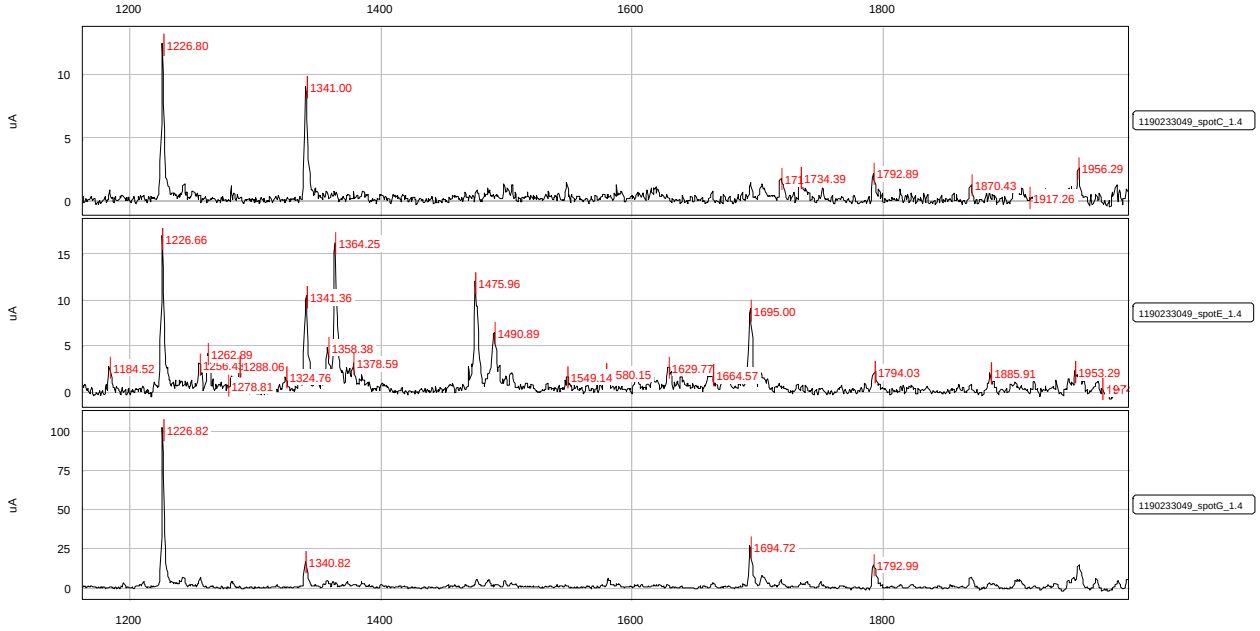
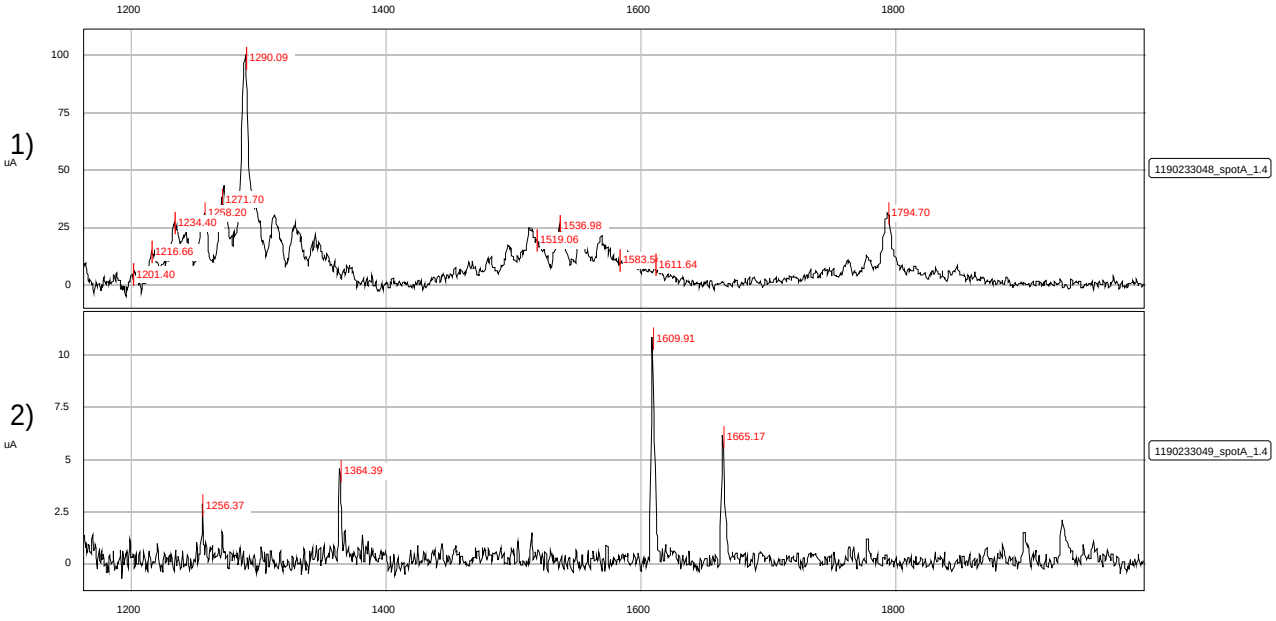


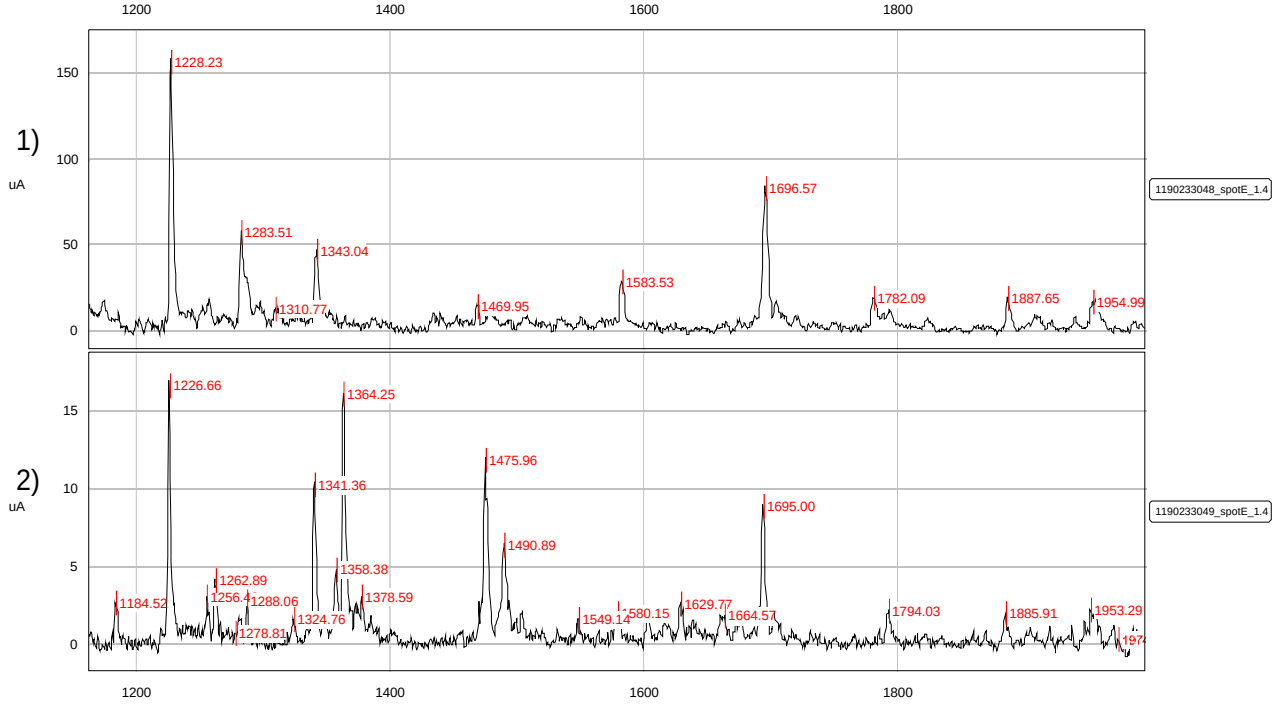
Figure 6-6.6.1 shows Formic acid buffer SELDI-TOF analysis using two different crystallizing solutions (Matrices), generally used for polypeptides MS: 3,5-dimethoxy-4-hydroxycinnamic acid (SPA) and 2,5-dihydroxybenzoic acid (DHB). The best results were obtained with DHB matrix.

Figure 6-6.6.1. Comparing SPA and DHB matrices, formic acid SELDI-TOF analysis with iron: 1) FA buffer with Fe and SPA; 2) FA buffer with Fe with Fe and DHB.



In figure 7-6.6.1. i.e. in TrentinGrana 77 SELDI-TOF resultd with DHB there are visualized more phosphorilated peaks than in the same sample with SPA.

Figure 7-6.6.1. TN77 SELDI-TOF results with SPA and DHB: 1) TN77 FA buffer, acidified, filtrated, with Fe and SPA; 2) TN77 FA buffer, acidified, filtarted, with Fe and DHB.



6.3. Discussion

Calcium digestibilities (dCa) % of different aged samples of Grana Padano (manufactured with lysozyme) and and TrentinGrana and Parmigiano cheeses (without lysozyme) were assessed.

Ca digestibility results of the three cheeses were quite scattered with a positive correlation found only between Grana Padano sample ripening times and their dCa when samples of a ripening time of over 24 months were included in the correlation analysis. In fact, the dCa results for Grana Padano samples under 24 months are also quite dispersed. The analysis of the distribution of oligopeptide molecular weights for each sample shows that the peptide range involved in calcium binding - and significantly correlated with dCa % - was between 2500-500 D. These values were as follows: 2500-1500 D and 1500-1000 D (the latter showing the most significant correlation with dCa , r=0,3766 , p=0,00062) in Grana Padano samples, and <1500 D and 1000-500 D ranges in cheeses manufactured without lysozyme.

The SELDI analysis results confirm the presence of phosphopetides within the 1000-2000 D range, and therefore the role of casein-phosphopetides (CPPs) in calcium binding .

Sforza et al. in their work on peptidic fractions (2004) concluded that the use of lysozyme in the production of Grana Padano modified its cheese peptidic profile (< 5000 D fraction) during ripening time. For example, Grana Padano and Parmigiano Reggiano presented a very similar composition. However, Grana Padano phosphopeptides levels increased and reached a maximum at 12 months of aging and then decreased, whereas in Parmigiano Reggiano cheeses, phosphopeptide amounts were usually lower and had a less regular trend. The lysozyme, in fact, is responsible for the different proteolitic evolution of the two cheeses. In Grana Padano cheese the proteolysis is faster than in Parmigiano Reggiano and TrentinGrana because of lysozyme induces Lactic Bacteria lysis and then the releasing of citoplasmatic proteolysis enzymes in advance.

In contrast with the work of Sforza et al, in this PhD study, the analysis variance results of oligopeptide distribution in the two cheeses with and without lysozyme showed differences only in samples with ripening times between 15 and 20 months,in which cheeses without lysozyme appear more hydrolyzed than in Grana Padano cheese without lysozyme. Therefore changes in cheese peptidic profiles probably caused by the use of lysozyme do not influence calcium digestibility because according to this study there is not a connection between change in peptidic profile and calcium digestibility results.

The increase of Grana Padano calcium digestibility results in samples over 24 month ripening time should be probably ascribed to other factors such as the production periods, temperature and humidity of aging room, bacterial strain used and development during acidification of milk and during cheese maturation, thermal treatments and cooking, percentage of salt, etc.

The second part of work, concerns amino acidic identification of isolated phosphopeptides of in vitro digested samples was done considering their molecular weights. Many difficulties arise in this part of work, because of initially phosphopetides amount was not possible to determinate and for the loss of phosphopetides during the analysis caused by the easily degradation of phosphopetides.

The • s1-casein(56-79) and (65-75) are quite similar to ones (61-79) identified by Ferranti et al. (1997a) in a 14 months aged Grana Padano not digested.

After tryptic digestion these authors obtained new peptides, one of them (• s1-casein 59-79) was obtained in our experiment also, this peptide is important because, according to FitzGerald (1998), it has a strong Ca-binding capacity. The cleavage site 79-80 is typical of • s1-casein in Grana Padano and can be attributed to endopeptidase (Sforza et al., 2003).

Another • s1-casein derived sequence (46-70) is partially overlapping to • s1-casein(42-91) observed in one of ours TrentinGrana samples. Sforza et al. (2003) detected a cleavage site of • s1-casein between 38-39 amino acid residue, very closed to the position 42 observed in one sample of TrentinGrana.

The 17-35 • -casein derived phosphopeptides is unusual, because this protein has a cleavage site in position 28-29 (Ferranti et al., 1997a; Sforza et al., 2003), perhaps this peptides comes from the hydrolytic activity of enzyme used during in vitro digestion.

If compared to results of Ferranti et al. (1997a) the importance of different caseins as source of phosphopeptides quite different: in undigested cheese • -casein was the main source of phosphopeptides (53.3%), followed by • s1-casein (35.6 %) and • s2-casein (11.1 %). The total number of phosphopeptides was also different 45 vs 4 . This lower number can be due to the enzymatic digestion underwent by our samples that has increase the amount of peptides release from caseins, the protein hydrolysis can have increased the number of phosphopeptides deriving from • s2-casein and reduced the importance of • -casein. Another difference between the two experiments is related to • -casein, which according to Ferranti et al. (1997) gave no contribution to phosphopeptides.

The same group (Ferranti et al., 1997b) observed that • -casein has a faster hydrolysis than • s1-casein and this could explain the high percentage of • -casein-derived phosphopeptides in undigested loafs. As a result of this fast disappearance of • -casein the enzymatic treatment has primarily digested • s1-and • s2-casein.

The difference could be due to the different proteolytic enzymes utilized, because while Ferranti et al. (1997) used only trypsin, our in vitro procedure requested others proteolytic enzymes and this could have modified the CPPs pattern.

The phosphopeptide pattern in undigested cheese was very different for • s2-casein (ranging from 7 to 21) while a partial overlapping was detected for • s1 and • -casein with phosphorylated sequences in 61-70 and 7-28 respectively.

7. Angiotensin-I-converting enzyme inhibition in locally produced semi-fat hard cheeses: Grana Padano and TrentinGrana.

Hypertension is a major risk factor for the development of cardiovascular diseases, which are the most important health problems worldwide.

Several food-derived peptides have been demonstrated to play a role in controlling the development in hypertension by interacting with the renin-angiotensin system. Angiotensin-I-converting enzyme (ACE) is a dipeptidyl-carboxypeptidase (EC 3.4.15.1) located in different tissues, that hydrolyses angiotensin I into angiotensin II, which has a vasoconstrictor effect. ACE also inactivates the vasodilatator bradikinin involved in the control of blood pressure (Ondetti et al., 1977).

Peptides with various bioactivities have been identified in several dairy-products, such as milk protein hydrolysates, fermented milks and many cheese varieties (Gobbetti et al., 2002; Korhonen and Pihlanto-Leppälä, 2004).

During cheese ripening, caseins are degraded by proteinases to large peptides and in a second steps by peptidases to smaller peptides and free amino acids. Some of the liberated peptides show biological activity in humans.

The degradation of milk proteins with proteinases from L. helveticus produced peptides with ACE – inhibiting activity that had a significant antihypertensive effect in spontaneously hypertensive rats (Yamamoto, Akino & Takano, 1994).

The same effect was observed in fermented milk containing L. helveticus (Nakamura, Yamamoto, Sakai, Okubo et al., 1995). Two tripeptides valyl-prolyl-proline (val-prol-prol; IPP) and isoleucyl-prolyl-proline (Ile-Pro-Pro; IPP) were identified as the bioactive peptides which are responsible for this effect (Nakamura, Yamamoto, Sakai, & Takano, 1995).

In several short- and long-term human studies, where VPP and IPP containing fermented milk products were ingested, a blood-pressure lowering effect was observed (Hata et al., 1996, Tuomilehto et al., 2004).

In vitro measurement of ACE-inhibitory activity in water soluble extracts of different cheese varieties such as Norvegia, Jarlsberg, Cheddar and Blue (Stepaniak, Jedrychowski, Wroblewska &Sorhaug, 2001) or Gouda, Emmental, Blue, Camembert, Edam and Havarti (Saito, Nakamura, Kitazawa, Kawai 6 Itoh, 2000) showed large differences in the IC50 values (concentration of cheese or an individual bioactive peptide that inhibits the ACE activity by 50% in an in vitro assay).

Nakamura, Yamamoto, Sakai, Okubo et al., (1995) found IC50 values of 9 and 5 µM for VPP and IPP respectively, whereas for Captropil a value of 0.007 µM was repoted (Pihlanto-Leppälä, Rokka, & Korhonem, 1998).

In further studies, a large number of individual peptides with ACE-inhibitory activity were isolated from cheese: a total of 22 and 75 peptides from a water-soluble extract of an eight month old Manchego manufactured from sheep milk (Gomez-Ruiz, Ramos & Recio, 2002, 2004), 41 ACE-inhibitorypeptides in the permeate >1000 Daof different Spanish cheeses analysed by HPLC-MS/MS and off-line MS/MS (Gomez-Ruiz, Taborda, Amigo, Recio & Ramos, 2006) and two of four peptides (• s1 – casein f(1-9) and • -casein f(60-68) in an eight month old Gouta (Saito et al., 2000).

Milk pre-treatment, processing and ripening time are to be considered as important key factors for the formation of such peptides: the ACE inihibition was stronger in cheese from raw milk than in cheese from pasteurised milk (Gomez-Ruiz et al., 2002) and it was strongly dependent on the degree of proteolysis and the age of the cheeses (Meisel , Goepfert & Günther, 1997).

Also Gouda aged for 8 months resulted in a significantly stronger reduction of blood pressure of spontaneously hypertensive rats than 24 months old Gouda (Saito et al., 2000).

However, a close correlation cannot be expected between the ACE-inhibitory activity of a cheese variety in vitro and its blood pressure lowering effect in vivo. On the one hand, most of the peptides released during the ripening of cheeses are further degraded during gastrointestinal digestion. On the other hand, new ACE-inhibiting peptides can be liberated during the same process as shown with Emmental using a simple in vitro protocol simulating gastrointestinal digestion (Parrot et al., 2003). Anyway enterocytes can absorb peptides of maximum length of 3 amino acids (Arienti, 1996) this limitation reduces the biological role of too long ACE-inhibiting peptides.

According to Saito et al. (2000) the systolic blood pressure was lowered in spontaneously hypertensive rats fed with isolated ACE-inhibiting peptides of various cheese varieties.

In several human studies, a dose dependent blood pressure lowering effect of the two tripeptides IPP and VPP has been proven (Hata et al., 1996; Seppo et al., 2002).

The quantification of these two well characterized ACE-inhibiting peptides is therefore a promising approach in order to assess the potential of a cheese variety for lowering blood pressure in humans.

The aim of work was to assess if the presence of lysozyme could influence casein peptides formation involved in ACE–inhibitory activity in Grana Padano (with lysozyme) and TrentinGrana (without lysozyme) in vitro digested samples. The ACE-inhibitory activity was also assessed in relation to the ripening time.

Cheese samples manufactured in the same factory were also injected in RP-HPLC-MS (pre-columnmethod) system to isolate and identify the possible ACE-inhibitory peptides in each sample.

Furthermore, the possible benefits of ACE activity, specifically in some very popular Italian cheeses often consumed even daily by many Italians, have also been taken into consideration as it may be of interest for dietary recommendations particularly in cases of high blood pressure.

7.1. Method and Material

7.1.1. Cheese samples

15 Grana Padano samples (cheese sample with lysozyme) and 13 TrentinGrana samples (without lysozyme molecule) ,obtained from Grana Padano Consortium and divided in three commercial groups:

1. 12 months aged, “giovane” (young) category, (9 months it’s the least time to commercialized Grana Padano Consortium cheeses),
2. 15-20 months aged, mean ripened time class,
3. 23 months aged, “riserva” typology,

and analyzed for their ACE-inhibiting activity

7.1.2. Cheeses *in vitro* digestion

The hydrolysis was carried out according the method of Hernàndez –Ledesma et al., 2004.

The hydrolysate was prepared from an aqueous solution of cheese (0.7%, w protein/v). Solution pH was adjusted to 2.0 with 6 N HCl. The 20 mg of pepsin per g of protein was added.

The sample was incubated in a shaking water bath at 37°C for 90 min, the kept in ice to stop the pepsin digestion. Prior to intestinal digestion step, pH solution was raised to 7-8 with NaOH 1 M. 40 mg of Corolase per g of protein was added. A new incubation in shaking water bath at 37 °C for 240 min was carried out . Then the solution pH was adjusted to 8. The solution was first centrifuged (10.000x g 4°C, 30 min) to eliminate fat, and after ultra centrifuged (6000x g, 10 min) to isolate the WSE (water solution extract) containing the <3000 D oligopeptides fraction.

7.1.3. Cheese samples soluble nitrogen on total nitrogen determination

Soluble N has been determined as described by Summer et al. (1997) and expressed as percentage on total N to know cheeses.

- 10 g of grated cheese were added to 40 ml of pH 7 citrate buffer and heated at 40°C;
- when this temperature is reached, sample is minced and leave to make cold;
- after adding of water ,solution pH is adjusted to 4,4 with 6 N HCl.;
- 20 ml of this solulion are preleved and miscelated with 5 ml of 60% TCA;
- after one hour, 20 ml of filtered solution are mineralized;
- 50 ml of distilled water and 10 ml of sodium tiosulphate;
- Follow distillation and titration of sulphuric acid with NaOH 0,1 N

7.1.4. Measurement of cheese samples ACE-inhibitory activity

ACE-inhibitory activity was measured by the spectrophotometric assay method of Cushman and Cheung (1971) with modifications.

Briefly a volume of 20 µl of each in vitro digested sample supernatant (inhibitory solution) were incubated at 37 °C for 30 min with 100µl of 0.1 M phosphate buffer, which consisted of 5mM hippuryl-histidyl-leucine, 0.1 M phosphate and 0.3 M NaCl (pH 8.3), and with 5mU of ACE (20 µl) (Sigma). The reaction was finished with 100 µl of 1 M HCl.

The hippuric acid formed by the action of ACE was extracted with ethyl acetate, and after removal of ethyl acetate by heat evaporation, the amount of the hippuric acid was measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

7.1.5. Statistical analysis

The comparison of ACE-inhibitory activity between cheeses was performed with the “t Student” test using the SAS statistical software (9.1). The time effect within the single cheese was assessed with the Tukey-Kramer test. For both statistical tests the PROC GLM of SAS statistical package (9.1) was used.

Correlation analysis between cheese samples ripening times and ACE-inhibitory activities was carried out with the PROC CORR of SAS statistical package (9.1).

7.1.6. RP-HPLC-MS pre-column method cheese samples oligopeptides isolation

To isolate and identify the potential active peptides, the Mr 3000 permeates of samples from a same factory, obtained after a simulated digestion process, were subjected to RP-HPLC-MS analysis.

Nine samples were chosen, six of Grana Padano and three of TrentinGrana. The analyzed samples, their ripening times, and their ACE-inhibitory activities % are showed in Table 1-7.2.2.

The method is the same described in the paragraph 6.1.9.

7.1.7. Cheese samples ACE-inhibitory peptides identification

The identification approach involved the search for the masses and partial sequences in a database of bovine milk proteins.

It was based on the principals structure features of the already known and identified ACE-inhibitory peptides. (described in chapter 4.2.2.1).

7.2. Results

7.2.1. ACE-inhibitory activity assay results of different ripened time cheese samples *in vitro* digested

Cheese samples in vitro digested ACE-inhibitory activity results assayed on WSE <3000 D are showed in table1-7.2.1.

Table1-7.2.1: Cheese samples ACE-inhibitory activity results

Cheese	Ripening time (months)	ACE-inhibitory %
Grana Padano 28	10	67,62
Grana Padano 29	11	72,86
Grana Padano 30	11	56,98
Grana Padano 31	11	68,09
Grana Padano 37	15	55,97
Grana Padano 39	16	88,82
Grana Padano 54	19	77,87
Grana Padano 43	20	62,17
Grana Padano 44	20	66,23
Grana Padano 47	25	67,94
Grana Padano 46	26	72,31
Grana Padano 53	27	71,45
Grana Padano 48	39	63,31
TrentinGrana49	10	73,23
TrentinGrana51	11	79,54
TrentinGrana77	11	64,47
TrentinGrana52	11	85,46
TrentinGrana78	18	76,06
TrentinGrana76	18	72,43
TrentinGrana74	18	62,88
TrentinGrana79	18	70,58
TrentinGrana72	24	65,69
TrentinGrana82	24	62,77
TrentinGrana81	25	77,14
TrentinGrana73	24	64,69
TrentinGrana 16	23,1	66,39

Statistical analysis ("T student test") shows that there is no significant difference between ACE-inhibitory activity % results of Grana Padano and TrentinGrana samples with the same ripening times.

The most evident difference between the two cheeses, manufactured with and without lysozyme, appears in "young" class cheese (<12 months) but it has not statistical significance (Table2-7.2.1).

Table2-7.2.1. Grana Padano and TrentinGrana ACE-inhibitory activity values (means ± DS of ACE-inhibitory activity %) in different cheeses ripening time classes.

Ripening time	Grana Padano	Trentingrana	P
< 12 months	66.39 ± 6.70	75.68 ± 8.99	0.149
15-20 months	70.21 ± 13.12	70.49 ± 5.56	0.977
> 24 months	68.84 ± 3.17	67.34 ± 5.65	0.767

The decreasing trend of ACE-inhibition activity in TrentinGrana samples is partially similar to the one observed in spanish sheep-goat cheeses (Silva et al., 2006) or swiss cow-based cheeses (Meyer et al., 2009). These Authors reported a curvilinear relationship between time and ACE-inhibiting activity, probably due to the proteolytic activity from milk or bacteria.

Both Grana Padano and TrentinGrana cheeses seem to have ACE-inhibitory activity values greater than the already assayed and reported sheep and goat cheese aged 45 days, (Silva et al., 2006) 12 months (Gòmez-Ruiz et al., 2002) and fermented milks (Hernàndez-Ledesma et al, 2004).

7.2.2. Cheese samples soluble nitrogen on total nitrogen determination results. Correlation with cheese samples ACE-inhibitory activity.

Table 1-7.2.2. shows the results of soluble Nitrogen % on total nitrogen content in Grana Padano and TrentinGrana samples. This analysis was done to evaluate the proteolysis level for each sample.

The correlation between cheese samples ripening times and soluble N/ tot N are showed in figure 1-7.2.2 and figure 2-7.2.2 for Grana Padano and TrentinGrana respectively.

As can be observed in figure 1-7.2.2., Grana Padano have not a clear relationship between aging and soluble N, while TrentinGrana shows a clear trend towards a higher soluble N in more ripened samples (Figure 2-7.2.2.).

If ACE-inhibition % is regressed against aging or soluble N the two cheese show different pattern. While in TrentinGrana an inverse trend ($P < 0.10$) can be detected between ACE inhibition and aging (see figure 3-7.2.2.) no relationship could be obtained for Grana Padano. When soluble N is considered, the relationship with ACE inhibition in TrentinGrana is not significant (r2 0.191, P 0.156; Figure 4-.7.2.2.) however if one plus-variant data is erased, the r2 become 0.39 ($P 0<0.5$). For Grana Padano cheese no relationship seems to exist between ACE-inibition and soluble N (Figure 5-7.2.2.).

Table 1-7.2.2. Soluble Nitrogen % on total nitrogen content in Grana Padano (GP) and TrentinGrana (TN) samples

Cheese	Ripening time	% Soluble N-Solub/%N-Tot
GP 28	10	18,77
GP 29	11	20,88
GP 30	11	20,51
GP 31	11	20,59
GP 37	15	20
GP 39	16	17,63
GP 54	19	17,19
GP 43	20	21,05
GP 44	20	14,6
GP 47	25	23,07
GP 46	26	24,28
GP13	26,7	16,25
GP 14	26,7	13,77
GP 53	27	17,76
GP 48	39	17,94
TN 49	10	15,85
TN 51	11	13,59
TN 52	11	17,47
TN 77	11	17,91
TN 74	18	20,76
TN 76	18	20,11
TN 78	18	19,3
TN 79	18	21,67
TN 72	24	21,16
TN 82	24	21,39
TN 73	25	20,42
TN 81	25	23,36

Figure 1-7.2.2. Correlation between Grana Padano samples with different ripening times and soluble N/ tot N content .

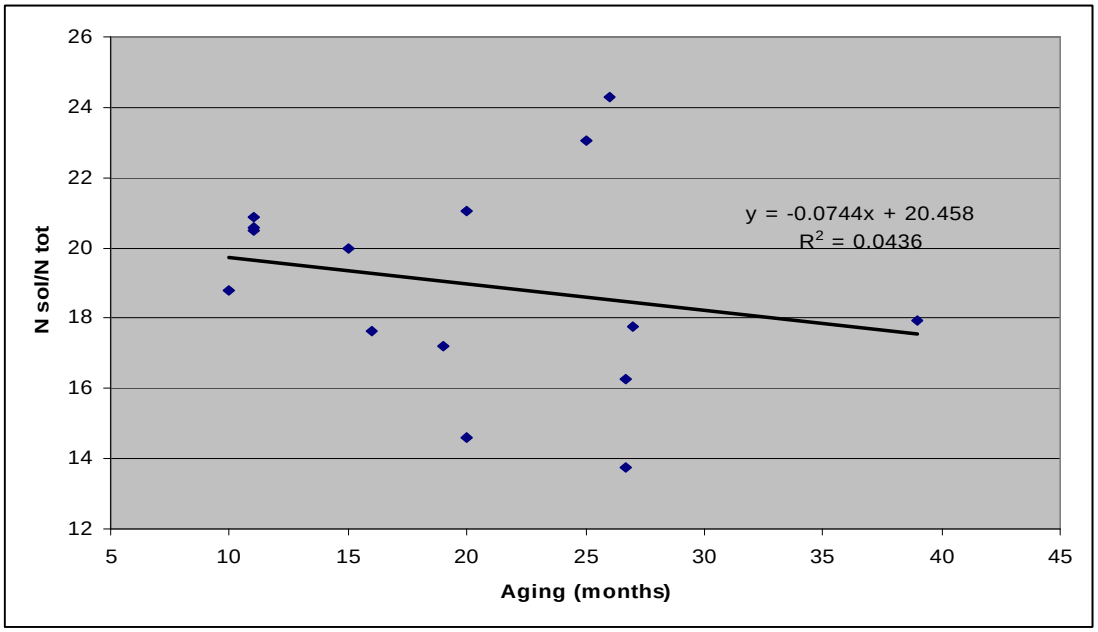


Figure 2-7.2.2. Correlation between ACE-inhibitory % activity and % soluble N/ tot N content inTrenTinGrana samples with different ripening times.

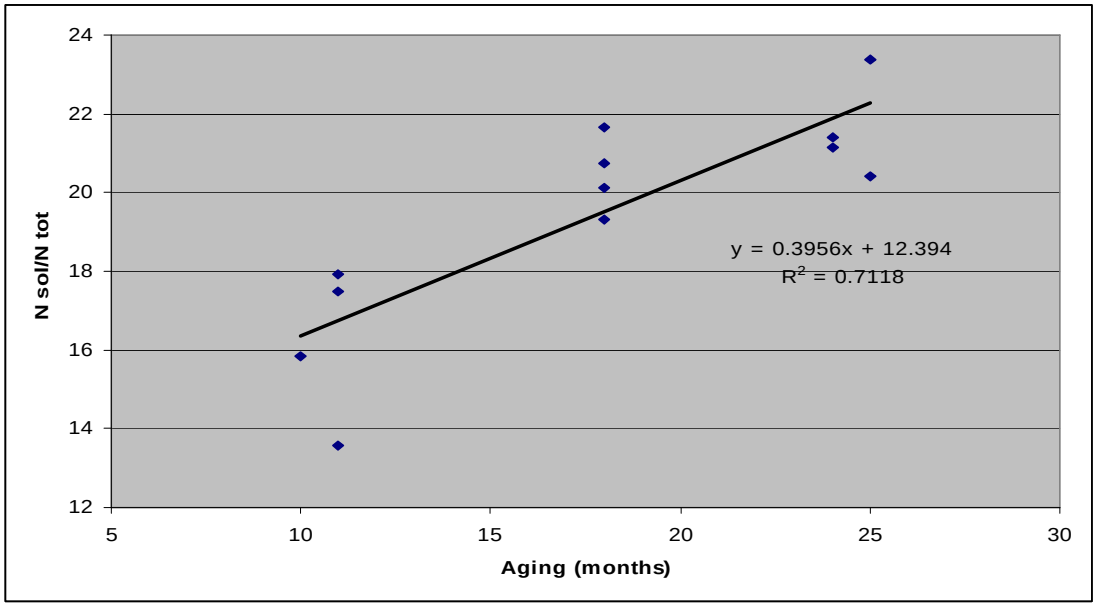


Figure 3-7.2.2. Regression between TrentinGrana samples ACE-inhibitory activity and their content of % soluble N/ tot N

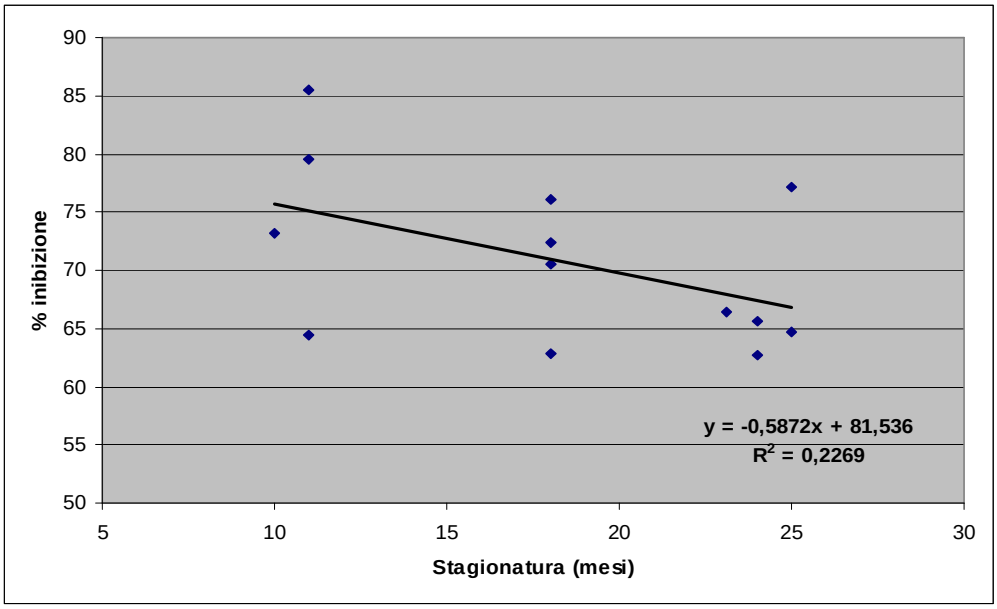


Figure 4-.7.2.2.Correlation between ACE-inhibitory activity and % soluble N/ tot N content in TrentinGrana

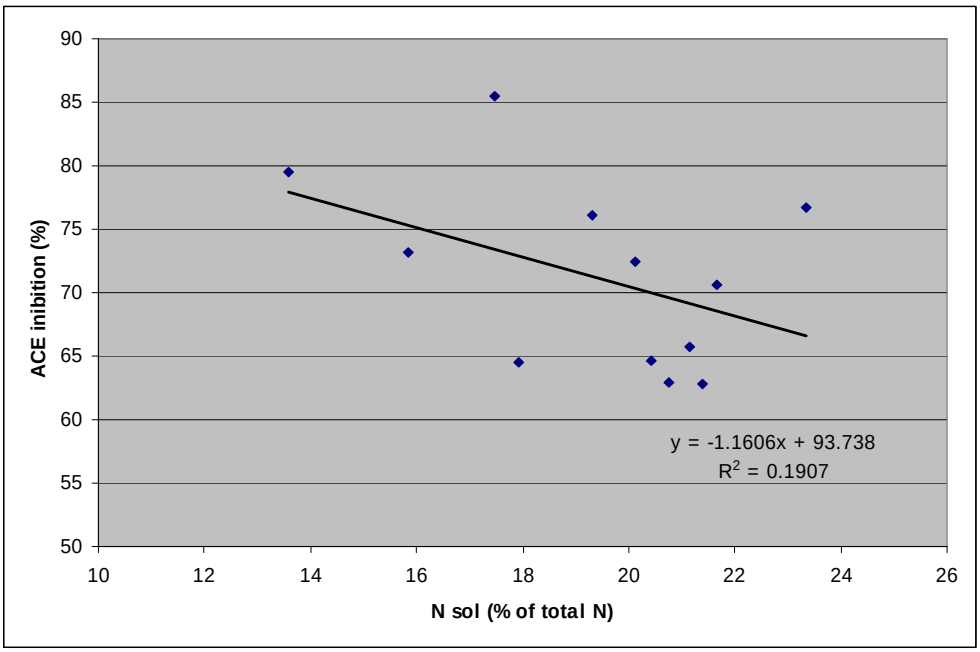
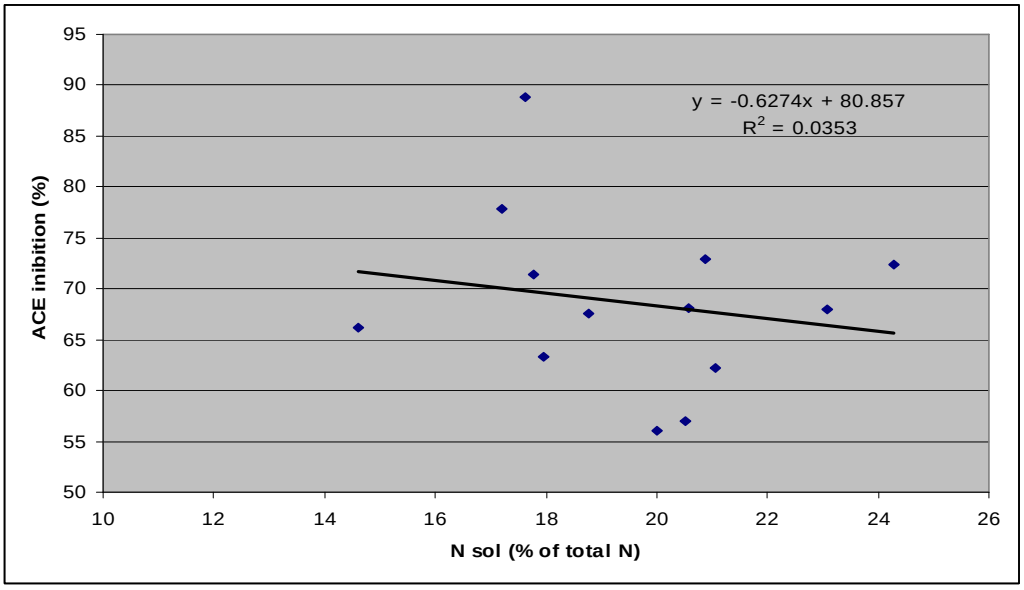


Figure 5-7.2.2. Correlation between ACE-inhibitory activity and % soluble N/ tot N content in Grana Padano



7.2.3. RP-HPLC-MS (pre-column method) same factory cheese samples oligopeptides isolation results and identification

In this chapter are showed all the peptides identified in each examined sample (table1-7.2.3.). All the peptides were eluted through the RP-HPLC –MS system, isolated by m/z ratio and processed using the Biopharmalynx v.1.1. software. The peptides identified (showed in tables 3-4-5-6-7-8-9-.7.2.3.) were also found in mass spectrometry chromatogram of their origin sample.(an example of identification of a peptide peak in its MS chromatogram is shoed in “Supplementary Material”).

The primary sequence identification of peptides through tandem mass spectrometry was difficult because of the signal was too low, in fact only few peptides peaks identified with Biopharmalynx v.1.1. software also appeared in MS/MS chromatogram. The few peptides revealed were identified by the instrument software, considering a, b or y, z fragments (Johnson R.S. et al, 1987), and their amino acids molecular weight sum. Cheese samples mass spectrometry chromatograms and MS/MS chromatograms were inserted in “Supplementary Materials”. The potential ACE-inhibitory peptides were selected by C-terminal tripeptidic composition criteria (described in chapter 4.2.2.1.). Proline amino acids in primary sequence of possible ACE-inhibitory peptides are indicated in boldface.

Table 1-7.2.3. Cheese samples from the same factories analysed.

Cheese	Ripening Time	ACE-inhibitory activity %
GP1	13,5	26,58
GP2	13,5	60,34
GP8	20,7	31,69
GP14	26,7	68,86
GP15	26,7	69,18
TN10	18,4	21
TN14	23,5	17,49
TN16	23,1	66,39

Table 2-7.2.3. Grana Padano 1 (13,5 months aged) results: Biopharmalynx v.1.1. software peptides results.

Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End
977,7609	3	2930,26	a-casein-S1	LHSMKEGIHAQQKEPMIGVNQELAY	120	144
1278,827	4	5111,28	a-casein-S2	EESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLY	59	100
1127,5458	2	2253,08	b-casein	LQSWMHQPHQPLPPTVMF	140	157
870,7292	3	2609,16	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157
1054,0157	2	2106,02	b-casein	LLYQEPVLGPVRGPFPIIV	191	209
1055,5184	2	2109,02	a-casein-S1	SDIPNPIGSENSEKTTMPL	180	198
940,942	2	1879,87	b-casein	YQEPVLGPVRGPFPIIV	193	209
859,4197	2	1716,82	b-casein	QEPVLGPVRGPFPIIV	194	209
488,2564	1	487,25	b-casein	PFPPK	110	113
742,3844	1	741,38	a-casein-S2	QHQKAM	185	190
819,4039	1	818,4	a-casein-S1	EKTTMPL	192	198

Table 3-7.2.3: Grana Padano 2 (13,5 months aged) results: Biopharmalynx v.1.1. software peptides results.

Analyte RT (Min)	Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide	Analyte Intensity (Counts)
37,8	657,5312	1	656,52	a-casein-S1	LRLKK	99	103	656,47	2136
34,9	746,5109	1	745,5	a-casein-S2	VIPYVR	200	205	745,45	3955
34,6	720,4973	1	719,49	a-casein-S2	TKVIPY	198	203	719,42	3916
33,8	689,5012	1	688,49	b-casein	HLPL**P**L	134	139	688,43	3023
31,7	634,4007	1	633,39	a-casein-S2	LNFLK	161	165	633,39	3084
29,1	1240,894	3	3719,66	b-casein	AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	177	209	3720,03	22272
29	1121,267	4	4481,03	a-casein-S2	YQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRY	171	206	4481,46	16253
28,6	1613,299	2	3224,58	a-casein-S2	KKISQRYQKFALPQYLKTVYQHQKAM	165	190	3224,76	2378
28,6	1023,259	5	5111,26	a-casein-S2	EESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLY	59	100	5111,51	8944
28,3	1166,272	4	4661,05	a-casein-S1	ISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKK	65	103	4661,41	4051
27,8	1500,502	4	5997,98	b-casein	INKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNI**PP**L	26	77	5998,08	8911
27,6	1127,546	2	2253,08	b-casein	LQSWMHQPHQPLPPTVMF	140	157	2253,03	4098
26,9	870,7297	3	2609,17	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	15254
26,3	1054,018	2	2106,02	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22	37468
26,3	1065,01	2	2128	a-casein-S1	DIPNPIGSENSEKTTM**P**LW	181	199	2128,01	2187
25,6	1152,885	2	2303,75	a-casein-S1	DIPNPIGSENSEKTTM**P**LW	181	199	2303,94	6314
25,4	997,479	2	1992,94	b-casein	LYQEPVLGPVRGPFPIIV	192	209	1993,14	27305
24,8	909,7068	5	4543,49	b-casein	PPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLL	152	192	4543,47	4340
24,8	940,9425	2	1879,87	b-casein	YQEPVLGPVRGPFPIIV	193	209	1880,06	12482
24,7	758,3184	1	757,31	a-casein-S2	QHQKAM	185	190	757,35	2347
24,6	1116,902	4	4463,58	b-casein	INKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY**P**F	26	62	4463,23	5667
24,3	859,4185	2	1716,82	b-casein	QEPVLGPVRGPFPIIV	194	209	1716,99	3323
23,6	970,5933	2	1939,17	b-casein	PIQAFLLYQEPVLG**P**VR	186	202	1939,09	4290
23,6	817,533	4	3266,1	b-casein	SLSQSKVLPVPQKAVPYPQRDMPIQAFLL	164	192	3265,79	3631
23	899,5586	1	898,55	a-casein-S1	EQLLRLK	96	102	898,56	7895
22,8	681,308	2	1360,6	a-casein-S2	KTVYQHQKAMK	181	191	1360,73	2440
22,6	1105,485	1	1104,48	a-casein-S2	TVYQHQKAM	182	190	1104,54	12858
22,4	1271,932	2	2541,85	a-casein-S2	NMAINPSKENLCSTFCKEVVR	25	45	2542,08	3146
22,3	758,3167	1	757,31	a-casein-S2	QHQKAM	185	190	757,35	2089
22,1	488,2577	1	487,25	b-casein	PF**P**K	110	113	487,28	4360
21,5	881,5695	4	3522,25	a-casein-S1	GTQYTDAPSFSDIPNPIGSENSEKTTM**P**LW	170	199	3522,4	4151
21,3	755,7924	2	1509,57	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	2009
21,1	742,387	1	741,38	a-casein-S2	QHQKAM	185	190	741,36	34695
20,8	802,4474	1	801,44	b-casein	HLPL**P**LL	134	140	801,51	2470
20,5	979,6115	3	2935,81	b-casein	LQPEVMGVSKVKEAMAPKHKEMPF**P**K	88	113	2935,55	23346
19,9	659,2581	1	658,25	a-casein-S1	GAWYY	162	166	658,28	3093
19,9	758,3192	1	757,31	a-casein-S2	QHQKAM	185	190	757,35	7347
19,5	902,4635	1	901,46	a-casein-S2	LYQGPIVL	99	106	901,53	5892
18,8	1003,58	3	3007,71	b-casein	HQPHQPLPPTVMFPPQSVLSLSQSKVL	145	171	3007,6	12668
18,8	996,255	3	2985,74	b-casein	QPEVMGVSKVKEAMAPKHKEMPF**P**KY	89	114	2985,53	24687
18,8	988,6017	3	2962,78	b-casein	QSKVLPVPQKAVPYPQRDMPIQAFLL	167	192	2962,65	16479
18,8	930,5175	3	2788,53	b-casein	PVEPFTESQSLTLTDVENLHLPL**P**L	115	139	2788,45	8558
18,5	819,4031	1	818,4	a-casein-S1	EKTTM**P**L	192	198	818,42	25986
18	1005,451	1	1004,44	a-casein-S1	EKTTM**P**LW	192	199	1004,5	3090
17,9	1243,278	2	2484,54	a-casein-S2	YQKFALPQYLKTVYQHQKAM	171	190	2484,3	25482
17,9	1254,265	2	2506,51	b-casein	VLPVPQKAVPYPQRDMPIQAFL	170	191	2506,38	10867
17,7	993,9337	3	2978,78	b-casein	QSKVLPVPQKAVPYPQRDMPIQAFLL	167	192	2978,64	3574
17,3	654,2956	1	653,29	a-casein-S1	YYV**P**L	165	169	653,34	4060
15,3	783,2722	5	3911,32	a-casein-S1	VPLGTQYTDAPSFSDIPNPIGSENSEKTTM**P**LW	167	199	3911,57	2415
14,2	507,2165	2	1012,42	b-casein	HKEMPF**P**K	106	113	1012,52	5280
2,1	507,2178	2	1012,42	b-casein	HKEMPF**P**K	106	113	1012,52	26851
1,8	788,3517	1	787,34	b-casein	HKEM**P**F	106	111	787,37	3603

Table 4-7.2.3.: Grana Padano 8 (20,7 months aged) results: Biopharmalynx v.1.1 software peptides results

Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide Mass (Da)	Analyte Intensity (Counts)
3735,61	b-casein	IEKFQSEEQQQTEDELQDKIHPFAQTQSLVY	30	60	3735,79	3614
5112,21	b-casein	SLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	164	209	5111,84	4535
2609,18	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	383318
2647,09	a-casein-S1	TDAPSFSDIPNPIGSENSEKTT**MPL**	174	198	2647,23	17050
1956,89	b-casein	LQPEVMGVSKVKEAMA**P**K	88	105	1957,04	30583
1840,85	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98	9643
1857,83	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98	3263
777,43	b-casein	PQN**IPPL**	71	77	777,44	23429
2045,93	a-casein-S2	ALPQYLKTVYQHQKAMK	175	191	2046,11	2615
2064,91	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08	5749
1639,72	b-casein	EAMAPKHKEMPF**P**K	100	113	1639,82	5498
1108,46	a-casein-S1	DAYPSGAWY	157	165	1108,39	45642
787,32	b-casein	HKEM**P**F	106	111	787,37	6478
561,24	a-casein-S1	TTM**P**L	194	198	561,28	3521
910,37	b-casein	EMPF**P**KY	108	114	910,43	3381
706,3	b-casein	PPTVMF	152	157	706,34	15793
2188,81	a-casein-S1	SDIPNPIGSENSEKTTM**P**L	180	198	2188,9	9337
949,38	b-casein	DMPIQAFL	184	191	949,46	3418
1362,55	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	5556
933,32	b-casein	LSQSKVL	165	171	933,4	58133
1509,6	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	38357
1086,44	a-casein-S2	PQYLQYLY	93	100	1086,54	4056
1271,49	a-casein-S1	DAYPSGAWYY	157	166	1271,45	3026
740,32	b-casein	SQSKVL	166	171	740,35	5875
801,44	b-casein	HLPL**P**LL	134	140	801,51	3298
1362,54	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	12986
1606,64	a-casein-S2	ENLCSTFCKEVVR	33	45	1606,69	8345
1509,6	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	48041
1104,46	a-casein-S2	TVYQHQKAM	182	190	1104,54	14548
551,32	b-casein	PL**P**LL	136	140	551,37	38212
2138,33	a-casein-S2	KKISQRYQKFALPQYLK	165	181	2138,24	2308
679,33	a-casein-S1	PSGAWY	160	165	679,3	30894
1362,54	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	15979
1100,48	b-casein	SLSQSKVL	164	171	1100,4	12580
757,33	a-casein-S2	QHQKAM	185	190	757,35	119300
561,23	a-casein-S1	TTM**P**L	194	198	561,28	7762
487,23	b-casein	PF**P**K	110	113	487,28	17270
562,23	a-casein-S1	AYFY	143	146	562,24	3896
2106,4	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22	4352
2128,38	a-casein-S2	KPWIQPKTKVIPYVRYL	191	207	2128,26	5097
777,43	b-casein	PQNI**PP**L	71	77	777,44	25079
1427,63	b-casein	PQRDMPIQAFLL	181	192	1427,76	14921
1421,61	a-casein-S1	SENSEKTTM**P**LW	188	199	1421,65	4752
2609,2	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	289974
1271,51	a-casein-S1	DAYPSGAWYY	157	166	1271,45	4081
2179,84	a-casein-S1	QFYQLDAYPSGAWYYVPL	152	169	2180,03	4154
1108,46	a-casein-S1	DAYPSGAWY	157	165	1108,39	118011
2188,83	a-casein-S1	SDIPNPIGSENSEKTTMPL	180	198	2188,9	15198
1617,77	a-casein-S1	PQEVLNENLLRFF	12	24	1617,85	15234
1588,63	a-casein-S1	QMEAESISSSEEIV**P**	59	73	1588,73	2091
1283,57	a-casein-S1	MIGVNQELAYF	135	145	1283,62	2978
1857,84	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98	2139
2161,92	a-casein-S1	QFYQLDAYPSGAWYYV**P**L	152	169	2162,01	2027
561,24	a-casein-S1	TTM**P**L	194	198	561,28	8759
706,31	b-casein	PPTVMF	152	157	706,34	12422
1509,61	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	105689
1840,87	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98	8370
2647,14	a-casein-S1	TDAPSFSDIPNPIGSENSEKTTM**P**L	174	198	2647,23	11966
637,29	b-casein	LVY**P**F	58	62	637,35	2909
1526,66	a-casein-S2	ENLCSTFCKEVVR	33	45	1526,72	12176
1737,68	b-casein	MHQPHQPLPPTVMF**P**	144	158	1737,85	9102
908,4	b-casein	AQTQSLVY	53	60	908,46	3048
2168,84	b-casein	TESQSLTLTDVENLHL**P**L	120	137	2168,96	42984
1362,56	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	31355

Table 5-7.2.3.: Grana Padano 14 (26,7 months aged) results: Biopharmalynx v.1.1. software peptides results, Synapt high definition mass

Analyte RT (Min)	Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	d Peptide Mass
28,1	1226,579	1	1225,57	b-casein	DMPIQAFLLY	184	193	1225,61
27	883,3768	3	2647,11	a-casein-S1	TDAPSFSDIPNPIGSENSEKTTM**P**L	174	198	2647,23
26,9	1305,603	2	2609,19	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33
26,9	979,4542	2	1956,89	b-casein	LQPEVMGVSKVKEAMA**P**K	88	105	1957,04
26,3	921,4391	2	1840,86	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98
26,2	929,9268	2	1857,84	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98
26,2	1054,016	2	2106,02	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22
25,9	778,4406	1	777,43	b-casein	PQNI**PP**L	71	77	777,44
25,8	802,4401	1	801,43	b-casein	HLPLPLL	134	140	801,51
25,4	998,4811	2	1994,95	b-casein	KVKEAMAPKHKEMPF**P**K	97	113	1995,08
25,3	1033,466	2	2064,92	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08
24,5	688,335	1	687,33	b-casein	PIQAFL	186	191	687,4
24,4	820,8708	2	1639,73	b-casein	EAMAPKHKEMPF**P**K	100	113	1639,82
24,3	861,4464	1	860,44	b-casein	LYQEPVL	192	198	860,46
24,3	989,492	1	988,48	b-casein	AQTQSLVY	53	60	988,43
23,4	603,2829	2	1204,55	a-casein-S1	AYFYPELFR	143	151	1204,59
23,4	562,2533	1	561,25	a-casein-S1	TTM**P**L	194	198	561,28
23,4	788,3297	1	787,32	b-casein	HKEMPF	106	111	787,37
23,4	1109,469	1	1108,46	a-casein-S1	DAYPSGAWY	157	165	1108,39
23,1	959,9408	2	1917,87	a-casein-S2	ALPQYLKTVYQHQKAM	175	190	1918,01
23	989,4902	1	988,48	b-casein	AQTQSLVY	53	60	988,43
22,8	1217,549	1	1216,54	b-casein	QPEVMGVSKVK	89	99	1216,65
22,7	911,3825	1	910,37	b-casein	EMPF**P**KY	108	114	910,43
22,7	835,3632	1	834,36	a-casein-S1	EKTTM**P**L	192	198	834,42
22,6	707,3138	1	706,31	b-casein	PPTVMF	152	157	706,34
22,4	667,2833	2	1332,55	a-casein-S2	ISQRYQKFAL	167	176	1332,66
21,5	730,6111	3	2188,81	a-casein-S1	SDIPNPIGSENSEKTTM**P**L	180	198	2188,9
21,3	688,355	1	687,35	b-casein	PIQAFL	186	191	687,4
21,2	909,4059	1	908,4	b-casein	AQTQSLVY	53	60	908,46
21,2	755,808	2	1509,6	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72
21,1	563,2578	1	562,25	a-casein-S1	AYFY	143	146	562,24
21	788,3294	1	787,32	b-casein	HKEM**P**F	106	111	787,37
20,9	641,2693	1	640,26	a-casein-S1	VAPF**P**E	25	30	640,32
20,8	574,3211	1	573,31	b-casein	QSKVL	167	171	573,35
20,8	802,4491	1	801,44	b-casein	HLPL**P**LL	134	140	801,51
20,6	1508,568	1	1507,56	a-casein-S1	EPMIGVNQELAYF	133	145	1507,7
20,5	852,4071	1	851,4	a-casein-S2	TEVFTKK	144	150	851,48
20,4	843,4071	1	842,4	a-casein-S1	PSGAWYY	160	166	842,36
20,1	682,7832	2	1363,55	a-casein-S2	LCSTFCKEVVR	35	45	1363,6
20,1	804,3293	2	1606,64	a-casein-S2	ENLCSTFCKEVVR	33	45	1606,69
20,1	755,8087	2	1509,6	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72
20	1105,47	1	1104,46	a-casein-S2	TVYQHQKAM	182	190	1104,54
19,1	843,3845	1	842,38	a-casein-S1	PSGAWYY	160	166	842,36
18,7	769,3828	1	768,37	a-casein-S2	YQKFAL	171	176	768,42
18,5	552,3278	1	551,32	b-casein	PL**P**LL	136	140	551,37
18,4	680,338	1	679,33	a-casein-S1	PSGAWY	160	165	679,3
18,3	1101,497	1	1100,49	b-casein	SLSQSKVL	164	171	1100,4
18,3	682,2798	2	1362,54	a-casein-S1	EPMIGVNQELAY	133	144	1362,65
18	758,3408	1	757,33	a-casein-S1	NENLLR	17	22	757,41
17,9	733,3301	1	732,32	a-casein-S2	CKEVVR	40	45	732,4
17,9	582,311	1	581,3	a-casein-S1	LRFF	21	24	581,33
17,1	654,2916	1	653,28	a-casein-S1	YYV**P**L	165	169	653,34
17,1	1307,577	1	1306,57	a-casein-S1	YYV**P**L	165	169	1306,68
16,3	789,3806	1	788,37	a-casein-S2	YQGPIVL	100	106	788,44
14,6	883,4122	1	882,4	a-casein-S1	RFFVA**P**F	22	28	882,48
13,8	488,2435	1	487,24	b-casein	PF**P**K	110	113	487,28
7	562,2401	1	561,23	a-casein-S1	TTM**P**L	194	198	561,28
3	562,2386	1	561,23	a-casein-S1	TTM**P**L	194	198	561,28

Table 6-7.2.3.: Grana Padano 15 (26.7 months aged) results: Biopharmalynx v.1.1. software peptides results.

Analyte RT (Min)	Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide Mass (Da)	Analyte Intensity (Counts)
28,2	1128,021	2	2254,03	a-casein-S1	EGIHAQQKEPMIGVNQELAY	125	144	2254,11	2133
28,1	1226,566	1	1225,56	b-casein	DMPIQAFLLY	184	193	1225,61	5825
26,9	979,4478	2	1956,88	b-casein	LQPEVMGVSKVKEAMAPK	88	105	1957,04	31530
26,3	921,4315	2	1840,85	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98	8633
26,2	929,9219	2	1857,83	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98	4665
25,9	553,2836	1	552,28	b-casein	NI**PP**L	73	77	552,33	3410
25,9	778,4312	1	777,42	b-casein	PQNI**PP**L	71	77	777,44	49497
25,4	1033,461	2	2064,91	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08	8657
24,4	809,8755	2	1617,74	a-casein-S1	PQEVLNENLLRFF	12	24	1617,85	13924
24,4	820,8649	2	1639,71	b-casein	EAMAPKHKEMPFPK	100	113	1639,82	6206
24,3	989,4854	1	988,48	b-casein	AQTQSLVY	53	60	988,43	59577
24,3	861,4418	1	860,43	b-casein	LYQEPVL	192	198	860,46	11217
23,4	585,2714	1	584,26	b-casein	QE**P**VL	194	198	584,32	18090
23,4	909,3862	1	908,38	b-casein	AQTQSLVY	53	60	908,46	3478
23,4	562,2497	1	561,24	a-casein-S1	TTMPL	194	198	561,28	7121
23,4	788,3216	1	787,31	b-casein	HKEM**P**F	106	111	787,37	17712
23,4	1109,461	1	1108,45	a-casein-S1	DAYPSGAWY	157	165	1108,39	142848
23,1	959,9355	2	1917,86	a-casein-S2	ALPQYLKTVYQHQKAM	175	190	1918,01	3784
22,8	1217,54	1	1216,53	b-casein	QPEVMGVSKVK	89	99	1216,65	4348
22,8	989,4829	1	988,47	b-casein	AQTQSLVY	53	60	988,43	2183
22,6	835,3573	1	834,35	a-casein-S1	EKTTM**P**L	192	198	834,42	3404
22,5	667,2783	2	1332,54	a-casein-S2	ISQRYQKFAL	167	176	1332,66	2156
22,5	707,3107	1	706,3	b-casein	PPTVMF	152	157	706,34	5650
21,5	1095,418	2	2188,82	a-casein-S1	SDIPNPIGSENSEKTT**M**PL	180	198	2188,9	11081
21,3	755,803	2	1509,59	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	14511
21,2	688,3466	1	687,34	b-casein	PIQAFL	186	191	687,4	18019
21,1	909,4001	1	908,39	b-casein	AQTQSLVY	53	60	908,46	3968
21	788,3236	1	787,32	b-casein	HKEM**P**F	106	111	787,37	9528
21	588,2476	1	587,24	b-casein	PVE**P**F	115	119	587,3	5330
20,8	574,3175	1	573,31	b-casein	QSKVL	167	171	573,35	9412
20,8	802,4421	1	801,43	b-casein	HLPL**P**LL	134	140	801,51	12917
20,7	741,3307	1	740,32	b-casein	SQSKVL	166	171	740,35	5587
20,5	911,3785	1	910,37	b-casein	EMPF**P**KY	108	114	910,43	18822
20,4	758,3131	1	757,31	a-casein-S2	QHQKAM	185	190	757,35	4531
20,2	923,3257	1	922,32	a-casein-S1	PSGAWYY	160	166	922,33	6005
20,1	682,7782	2	1363,54	a-casein-S2	LCSTFCKEVVR	35	45	1363,6	7379
20,1	804,3262	2	1606,64	a-casein-S2	ENLCSTFCKEVVR	33	45	1606,69	9345
20,1	755,8039	2	1509,59	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	48981
20	1105,467	1	1104,46	a-casein-S2	TVYQHQKAM	182	190	1104,54	110434
18,9	883,395	1	882,39	a-casein-S1	RFFVA**P**F	22	28	882,48	2137
18,8	654,3043	1	653,3	a-casein-S1	YYV**P**L	165	169	653,34	30394
18,5	1101,491	1	1100,48	b-casein	SLSQSKVL	164	171	1100,4	5674
18,3	682,2767	2	1362,54	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	6708
18,1	758,3378	1	757,33	a-casein-S2	QHQKAM	185	190	757,35	28542
18	923,3253	1	922,32	a-casein-S1	PSGAWYY	160	166	922,33	6304
17,8	580,262	1	579,25	a-casein-S1	FVA**P**F	24	28	579,31	13462
17,1	654,2875	1	653,28	a-casein-S1	YYV**P**L	165	169	653,34	78666
16,3	789,3773	1	788,37	a-casein-S2	YQGPIVL	100	106	788,44	23479
14,6	883,409	1	882,4	a-casein-S1	RFFVA**P**F	22	28	882,48	4220
6,9	563,2405	1	562,23	a-casein-S1	AYFY	143	146	562,24	3267
6,8	488,2416	1	487,23	b-casein	PF**P**K	110	113	487,28	4120
3,8	659,2203	1	658,21	a-casein-S1	GAWYY	162	166	658,28	3047
2,9	562,238	1	561,23	a-casein-S1	TTM**P**L	194	198	561,28	6804

Table 7-7.2.3: TrentinGrana 10 (21 months aged) results: Biopharmalynx v.1.1. software peptides results.

Analyte Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide Mass (Da)	Analyte Intensity (Counts)
3719,66	b-casein	AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	177	209	3720,03	6253
1225,57	b-casein	DMPIQAFLLY	184	193	1225,61	3421
2253,08	b-casein	LQSWMHQPHQPLPPTVMF	140	157	2253,03	2302
2647,13	a-casein-S1	TDAPSFSDIPNPIGSENSEKTTMPL	174	198	2647,23	7348
2609,18	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	169635
2647,15	a-casein-S1	TDAPSFSDIPNPIGSENSEKTTMPL	174	198	2647,23	3394
2609,2	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	198576
1956,91	b-casein	LQPEVMGVSKVKEAMAPK	88	105	1957,04	26689
2223,98	a-casein-S1	DIPNPIGSENSEKTTMPLW	181	199	2223,98	3589
1840,87	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98	5876
1842,88	b-casein	PQNIPPLTQTPVVVPPF	71	87	1843,02	2312
1857,85	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98	5384
777,44	b-casein	PQNIPPL	71	77	777,44	45307
1992,95	b-casein	LYQEPVLGPVRGPFPIIV	192	209	1993,14	9875
2064,93	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08	6563
1617,75	a-casein-S1	PQEVLNENLLRFF	12	24	1617,85	14165
1639,73	b-casein	EAMAPKHKEMPFPK	100	113	1639,82	5479
860,44	b-casein	LYQEPVL	192	198	860,46	28442
988,49	b-casein	AQTQSLVY	53	60	988,43	147887
561,25	a-casein-S1	TTMPL	194	198	561,28	9317
787,32	b-casein	HKEMPF	106	111	787,37	22279
1108,47	a-casein-S1	DAYPSGAWY	157	165	1108,39	125943
1917,88	a-casein-S2	ALPQYLKTVYQHQKAM	175	190	1918,01	5872
988,49	b-casein	AQTQSLVY	53	60	988,43	12094
1216,55	b-casein	QPEVMGVSKVK	89	99	1216,65	6998
834,36	a-casein-S1	EKTTMPL	192	198	834,42	4917
706,31	b-casein	PPTVMF	152	157	706,34	8549
1526,64	a-casein-S2	ENLCSTFCKEVVR	33	45	1526,72	9083
1332,56	a-casein-S2	ISQRYQKFAL	167	176	1332,66	7521
870,39	b-casein	LVYPFPGP	58	65	870,46	3640
732,36	a-casein-S2	CKEVVR	40	45	732,4	4292
1923,73	b-casein	QPEVMGVSKVKEAMAPK	89	105	1923,92	2784
2188,83	a-casein-S1	SDIPNPIGSENSEKTTMPL	180	198	2188,9	8159
1509,61	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	34129
1606,66	a-casein-S2	ENLCSTFCKEVVR	33	45	1606,69	3134
687,35	b-casein	PIQAFL	186	191	687,4	26079
908,41	b-casein	AQTQSLVY	53	60	908,46	5132
1362,55	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	8895
732,36	a-casein-S2	CKEVVR	40	45	732,4	3177
2059,8	b-casein	QSWMHQPHQPLPPTVMF	141	157	2059,98	2326
562,25	a-casein-S1	AYFY	143	146	562,24	9144
787,33	b-casein	HKEMPF	106	111	787,37	13301
740,33	b-casein	SQSKVL	166	171	740,35	10603
573,32	b-casein	QSKVL	167	171	573,35	15278
801,45	b-casein	HLPLPLL	134	140	801,51	19345
637,29	b-casein	LVYPF	58	62	637,35	2749
842,4	a-casein-S1	PSGAWYY	160	166	842,36	5818
757,31	a-casein-S2	QHQKAM	185	190	757,35	7274
838,37	b-casein	PPTVMFPP	152	159	838,44	30323
1362,55	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	16118
1606,65	a-casein-S2	ENLCSTFCKEVVR	33	45	1606,69	15953
1509,61	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	85157
1104,47	a-casein-S2	TVYQHQKAM	182	190	1104,54	152039
2106,39	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22	3717
2128,38	a-casein-S2	KPWIQPKTKVIPYVRYL	191	207	2128,26	4034
842,39	a-casein-S1	PSGAWYY	160	166	842,36	54352
860,38	b-casein	LYQEPVL	192	198	860,46	2278
882,4	a-casein-S1	RFFVAPF	22	28	882,48	3490
768,38	a-casein-S2	YQKFAL	171	176	768,42	3083
551,32	b-casein	PLPLL	136	140	551,37	18972
1362,55	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	11451
922,33	a-casein-S1	PSGAWYY	160	166	922,33	6483
726,32	a-casein-S1	FFVAPF	23	28	726,37	3825
658,26	a-casein-S1	GAWYY	162	166	658,28	3140
653,29	a-casein-S1	YYVPL	165	169	653,34	163661
788,38	a-casein-S2	YQGPIVL	100	106	788,44	36651
690,28	b-casein	PPTVMF	152	157	690,34	3054
882,41	a-casein-S1	RFFVAPF	22	28	882,48	6831
487,24	b-casein	PFPK	110	113	487,28	6564
561,23	a-casein-S1	TTMPL	194	198	561,28	10329
658,22	a-casein-S1	GAWYY	162	166	658,28	2939

Table 8-7.2.3: TrentinGrana 14 (23.5 months aged) results: Biopharmalynx v.1.1. software peptides results.

Analyte RT (Min)	Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide Mass (Da)	Analyte Intensity (Counts)
27	878,0561	3	2631,14	a-casein-S2	TEEEKNRLNFLKKISQRYQK	154	173	2631,35	94218
27	1305,598	2	2609,18	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	412796
26,9	979,452	2	1956,89	b-casein	LQPEVMGVSKVKEAMAPK	88	105	1957,04	32847
26,2	922,4383	2	1842,86	b-casein	PQNIPPLTQTPVVV**PP**F	71	87	1843,02	3694
25,9	553,2833	1	552,28	b-casein	NI**PP**L	73	77	552,33	3919
25,9	778,4365	1	777,43	b-casein	PQNI**PP**L	71	77	777,44	49771
25,4	1033,465	2	2064,91	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08	7398
24,5	809,8775	2	1617,74	a-casein-S1	PQEVLNENLLRFF	12	24	1617,85	19413
23,4	1109,47	1	1108,46	a-casein-S1	DAYPSGAWY	157	165	1108,39	116168
23,3	909,3886	1	908,38	b-casein	AQTQSLVY	53	60	908,46	3273
22,7	1217,548	1	1216,54	b-casein	QPEVMGVSKVK	89	99	1216,65	5799
22,7	989,4814	1	988,47	b-casein	AQTQSLVY	53	60	988,43	2424
22,6	835,3616	1	834,35	a-casein-S1	EKTTMPL	192	198	834,42	4350
22,4	733,3594	1	732,35	a-casein-S2	CKEVVR	40	45	732,4	2173
22,3	774,3457	1	773,34	b-casein	LVYPFPG	58	64	773,41	11073
21,2	755,8077	2	1509,6	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	19333
20,5	744,8353	2	1487,65	b-casein	APKHKEMPFPKY	103	114	1487,76	3860
20,5	638,2946	1	637,29	b-casein	LVYPF	58	62	637,35	2584
19,8	1065,188	2	2128,36	a-casein-S2	KPWIQPKTKVIPYVRYL	191	207	2128,26	3084
19,8	1054,197	2	2106,38	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22	2192
18,9	883,403	1	882,4	a-casein-S1	RFFVAPF	22	28	882,48	2917
18,5	552,3284	1	551,32	b-casein	PLPLL	136	140	551,37	14751
18,4	680,3377	1	679,33	a-casein-S1	PSGAWY	160	165	679,3	5091
18,3	682,2795	2	1362,54	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	9017
17,9	758,3413	1	757,33	a-casein-S1	NENLLR	17	22	757,41	51553
17,7	580,2649	1	579,26	a-casein-S1	FVAPF	24	28	579,31	22685
17,7	727,3191	1	726,31	a-casein-S1	FFVAPF	23	28	726,37	2995
17,5	659,2655	1	658,26	a-casein-S1	GAWYY	162	166	658,28	2832
17,1	654,2926	1	653,28	a-casein-S1	YYVPL	165	169	653,34	144531
16,3	789,3818	1	788,37	a-casein-S2	YQGPIVL	100	106	788,44	36111
14,5	883,4113	1	882,4	a-casein-S1	RFFVA**P**F	22	28	882,48	9252
14,3	488,2433	1	487,24	b-casein	PF**P**K	110	113	487,28	17404
6,7	563,2413	1	562,23	a-casein-S1	AYFY	143	146	562,24	5080
6,5	488,2418	1	487,23	b-casein	PF**P**K	110	113	487,28	6138
1,7	659,2174	1	658,21	a-casein-S1	GAWYY	162	166	658,28	3321

Table 9-7.2.3.: TrentinGrana 16(23.1 months aged) results: Biopharmalynx v.1.1. software peptides results,

Analyte RT (Min)	Analyte m/z	Analyte Charge State	Analyte Mass (Da)	Protein	Peptide	Start	End	Calculated Peptide Mass (Da)	Analyte Intensity (Counts)
29,1	1240,896	3	3719,66	b-casein	AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV	177	209	3720,03	8139
28,3	1128,032	2	2254,05	a-casein-S1	EGIHAQQKEPMIGVNQELAY	125	144	2254,11	4614
28,1	1226,582	1	1225,57	b-casein	DMPIQAFLLY	184	193	1225,61	8694
27	870,7341	3	2609,18	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	215479
26,9	1305,61	2	2609,2	b-casein	PLPLLQSWMHQPHQPLPPTVMF	136	157	2609,33	281805
26,9	979,4622	2	1956,91	b-casein	LQPEVMGVSKVKEAMA**P**K	88	105	1957,04	37485
26,9	844,407	2	1686,8	b-casein	LTLTDVENLHLPL**P**L	125	139	1686,96	2434
26,8	1324,584	2	2647,15	a-casein-S1	TDAPSFSDIPNPIGSENSEKTTM**P**L	174	198	2647,23	8970
26,5	808,7044	3	2423,09	b-casein	PVPQKAVPYPQRDMPIQAFLL	172	192	2423,3	2338
26,3	921,4414	2	1840,87	a-casein-S2	FPQYLQYLYQGPIVL	92	106	1840,98	15602
26,2	1093,515	2	2185,01	b-casein	DMPIQAFLLYQEPVLGPVR	184	202	2185,16	389858
26,2	1217,059	2	2432,1	a-casein-S1	FSDIPNPIGSENSEKTTM**P**L	179	198	2431,93	3302
26,1	929,9348	2	1857,85	b-casein	AVPYPQRDMPIQAFLL	177	192	1857,98	5158
26	778,4443	1	777,44	b-casein	PQNI**PP**L	71	77	777,44	104303
25,9	801,4371	1	800,43	b-casein	PIQAFLL	186	192	800,48	14235
25,4	997,4825	2	1992,95	b-casein	LYQEPVLGPVRGPFPIIV	192	209	1993,14	8529
25,3	1033,473	2	2064,93	a-casein-S2	FALPQYLKTVYQHQKAM	174	190	2065,08	13560
24,8	1403,789	1	1402,78	b-casein	IHPFAQTQSLVY	49	60	1402,72	4177
24,5	688,3369	1	687,33	b-casein	PIQAFL	186	191	687,4	18604
24,5	1474,775	1	1473,77	a-casein-S2	LKTVYQHQKAMK	180	191	1473,81	8844
24,4	809,8848	2	1617,75	a-casein-S1	PQEVLNENLLRFF	12	24	1617,85	41364
24,3	861,4501	1	860,44	b-casein	LYQE**P**VL	192	198	860,46	47809
24,3	989,501	1	988,49	b-casein	AQTQSLVY	53	60	988,43	221819
24,1	717,325	2	1432,63	a-casein-S1	QLDAYPSGAWYY	155	166	1432,63	3149
23,3	602,2832	2	1202,55	a-casein-S1	RQFYQLDAY	151	159	1202,57	60537
23,2	1109,477	1	1108,47	a-casein-S1	DAYPSGAWY	157	165	1108,39	140713
23,2	562,2554	1	561,25	a-casein-S1	TTM**P**L	194	198	561,28	6531
23,2	788,3321	1	787,32	b-casein	HKEM**P**F	106	111	787,37	14689
23,2	909,4013	1	908,39	b-casein	AQTQSLVY	53	60	908,46	9939
23	959,9492	2	1917,88	a-casein-S2	ALPQYLKTVYQHQKAM	175	190	1918,01	9766
22,7	1217,557	1	1216,55	b-casein	QPEVMGVSKVK	89	99	1216,65	11131
22,6	835,368	1	834,36	a-casein-S1	EKTTM**P**L	192	198	834,42	7839
22,4	1527,664	1	1526,66	a-casein-S2	ENLCSTFCKEVVR	33	45	1526,72	6033
22,4	707,3178	1	706,31	b-casein	PPTVMF	152	157	706,34	9473
22,3	1428,634	1	1427,63	b-casein	PQRDMPIQAFLL	181	192	1427,76	16615
22,3	1333,585	1	1332,58	a-casein-S2	ISQRYQKFAL	167	176	1332,66	2259
22,3	741,3227	1	740,31	b-casein	SQSKVL	166	171	740,35	3001
21,8	979,5061	1	978,5	a-casein-S2	FALPQYLK	174	181	978,55	8149
21,1	733,3595	1	732,35	a-casein-S2	CKEVVR	40	45	732,4	4388
21	934,3449	1	933,34	b-casein	LSQSKVL	165	171	933,4	3527
20,9	688,3566	1	687,35	b-casein	PIQAFL	186	191	687,4	42823
20,8	563,2623	1	562,25	a-casein-S1	AYFY	143	146	562,24	12858
20,8	788,3319	1	787,32	b-casein	HKEM**P**F	106	111	787,37	18416
20,5	923,3392	1	922,33	a-casein-S1	PSGAWYY	160	166	922,33	4981
20,4	742,3476	1	741,34	a-casein-S2	QHQKAM	185	190	741,36	5751
20,4	638,2975	1	637,29	b-casein	LVYPF	58	62	637,35	4788
20,4	645,7888	2	1289,56	b-casein	SQSKVLPVPQK	166	176	1289,67	3068
20,2	843,4127	1	842,4	a-casein-S1	PSGAWYY	160	166	842,36	8449
20,2	802,4502	1	801,44	b-casein	HLPL**P**LL	134	140	801,51	34725
20,1	574,3258	1	573,32	b-casein	QSKVL	167	171	573,35	25028
20,1	758,326	1	757,32	a-casein-S2	QHQKAM	185	190	757,35	12364
19,9	1336,083	3	4005,23	a-casein-S1	EPMIGVNQELAYFYPELFRQFYQLDAYPSGAWY	133	165	4004,88	2735

19,9	682,2881	2	1362,56	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	19995
19,8	804,3353	2	1606,65	b-casein	HQPHQPLPPTVMF**P**	145	158	1606,81	16843
19,8	755,8123	2	1509,61	a-casein-S1	EPMIGVNQELAYF	133	145	1509,72	81107
19,7	1105,486	1	1104,48	a-casein-S2	TVYQHQKAM	182	190	1104,54	187476
19,7	575,2214	2	1148,43	a-casein-S1	ENSEKTTM**P**L	189	198	1148,54	3094
19,6	1054,208	2	2106,4	b-casein	LLYQEPVLGPVRGPFPIIV	191	209	2106,22	3787
19,5	1065,199	2	2128,38	a-casein-S2	KPWIQPKTKVIPYVRYL	191	207	2128,26	3693
18,9	1316,613	1	1315,61	a-casein-S1	IGVNQELAYFY	136	146	1315,64	6211
18,5	680,3401	1	679,33	a-casein-S1	PSGAWY	160	165	679,3	4370
18,3	552,3311	1	551,32	b-casein	PL**P**LL	136	140	551,37	35367
18,3	1101,502	1	1100,49	b-casein	SLSQSKVL	164	171	1100,4	21462
18,1	682,2838	2	1362,55	a-casein-S1	EPMIGVNQELAY	133	144	1362,65	16728
18	1005,455	1	1004,45	a-casein-S1	EKTTMPLW	192	199	1004,5	2553
17,9	496,2333	1	495,23	a-casein-S1	GAWY	162	165	495,21	2070
17,8	582,2991	1	581,29	a-casein-S1	LRFF	21	24	581,33	3603
17,4	727,3262	1	726,32	a-casein-S1	FFVA**P**F	23	28	726,37	4562
17,1	586,2386	1	585,23	a-casein-S2	QYLY	97	100	585,28	2267
16,9	654,2964	1	653,29	a-casein-S1	YYV**P**L	165	169	653,34	163398
16,3	741,3531	1	740,35	b-casein	SQSKVL	166	171	740,35	2344
16,1	789,3854	1	788,38	a-casein-S2	YQGPIVL	100	106	788,44	35099
14,5	488,2466	1	487,24	b-casein	PF**P**K	110	113	487,28	30727
14,4	883,4169	1	882,41	a-casein-S1	RFFVA**P**F	22	28	882,48	24697
14,1	562,2448	1	561,24	a-casein-S1	TTM**P**L	194	198	561,28	12300
11,4	488,2467	1	487,24	b-casein	PF**P**K	110	113	487,28	18568
6,5	488,2456	1	487,24	b-casein	PF**P**K	110	113	487,28	11105
3,8	659,2284	1	658,22	a-casein-S1	GAWYY	162	166	658,28	10224
2,9	562,2438	1	561,24	a-casein-S1	TTM**P**L	194	198	561,28	19553
1,8	659,2276	1	658,22	a-casein-S1	GAWYY	162	166	658,28	8107

No correlation was found between the number of peptides containing proline amino acids or C-terminal sequences of hydrophobic amino acids, and ACE-inhibitory activity (Table 10-7.2.3.).

To establish which peptides have ACE-inhibitory activity, each peptides and their amount should be measured.

Moreover, because of three is the maximum number of amino acid absorbable by human enterocytes (Arienti, 1996), the potential ACE-inhibitory peptides identified (and showed in tables) during human digestion undergo to a further hydrolysis, with the consequent release of internal sequences of di-or tripeptidic. Therefore, if in the identified ACE-inhibitory peptides there are not IPP and VPP in the C-terminal sequences, during digestion their internal sequences of IPP and LPP could be released and absorbed in the intestine. Moreover the often found LPP sequence identified by the software, should be the IPP sequence, because both Leucine (L) and Isoleucine (I) have the same molecular weight of 131,17 .

Table 10-7.2.3.: Number of identified peptides containing potential C-terminal ACE-inhibitory sequence.

Cheese	Ripening Time	N°Peptides with one Proline	N° Pepetides with two Proline	N°of peptides containing all three C-termianl hydrophobic amino acids	Inhibition
tn3	12,1	1	.	7	.
tn14	23,5	13	3	26	17,4956
tn15	23	35	2	109	25,611805
gp10	20,7	16	1	40	.
gp14	26,7	21	1	36	68,86884
tn10	18,4	20	3	43	21,00352
gp15	26,7	18	4	37	69,18574
gp8	20,7	21	2	45	31,69374
gp2	13,5	19	3	31	60,34331
gp1	13,5	32	2	42	26,58613

7.3. Discussion

This study revealed the presence of ACE-inhibitory activity in Grana Padano and TrentinGrana Italian typical cheeses in permeate < 3000 D molecular weight. ACE-inhibitory activity indexes express the amount of casein protein involved in Angiotensin-I-converting enzyme inhibition.

Statistical analysis did not showed significant difference between the two cheeses ACE-inhibitory activity results. For this reason we can say that lysozyme molecule presence in Grana Padano cheese does not influence significantly the formation of ACE-inhibitory peptides.

The results show that there is no correlation between Grana Padano samples with different ripening times and their ACE-inhibitory activities, while there is an inverse linear trend for TrentinGrana samples ($p<0,10$). These findings are in accordance with Meisel H et al. 1997, which reported that there is not strict relationship between maturation time and ACE-inhibitory activity. Instead, other study reported that ACE-inhibitory activity increased as proteolysis progressed, and that decreased when proteolysis exceed a certain level. (Addeo et al., 1992). For this reason sample soluble N was calculate in order to evaluate samples proteolytic activities. Only in TrentinGrana samples was detected an inverse trend ($P<0,10$) between ACE-inhibition activity and soluble nitrogen, instead, no relationship was found between ACE-inhibition activity and ripening time in Grana Padano samples. Besides only TrentinGrana samples showed a clear trend towars a higher soluble nitrogen values in more ripened samples, as normally occur. Unusually, in Grana Padano samples soluble nitrogen values did not increase in more ripened time samples. This latter result could probably due to the many factors which are able to influence the specific enzymatic activities that take place during the whole ripening, such as the moisture content of cheese, pH values, salt content, free fatty acids, temperature, etc.

The identification of potential amino acidic sequences of ACE-inhibitory peptides was carried out using mass spectrometry techniques. After in vitro digestion the samples were directly injected into the ESI-HPLC combined with MS dispositive, without supernatant filtration. Instead, in many study about ACE-inhibitory activity in cheeses, i.e. in the Spanish ones (Gòmez-Ruiz et al., 2006), the amino acidic sequence identification of ACE-inhibitory peptides was focused under 1000D molecular weight. ACE-inhibitory peptides eluted were selected on the basis of their amino acid sequences, according with existing structure-activity relationship data. The number of ACE potential inhibitory peptides- containing one or two proline, or each three hydrofobic amino acids at C-terminal end- cannot be correlated with ACE-inhibitory activity. To evaluate the ACE-inhibitory activity of a peptide, it is necessary first to isolate and identified the peptide and then to quantify its amount necessary to inhibit the enzyme. In Grana Padano and TrentinGrana samples many potential ACE-inhibitory peptides derived from • s1-, • s2- and • - casein were identified. In Spanish cheeses, instead, there were not • s1-casein–derived ACE-inhibitory peptides (Gòmez-Ruiz et al., 2006).The

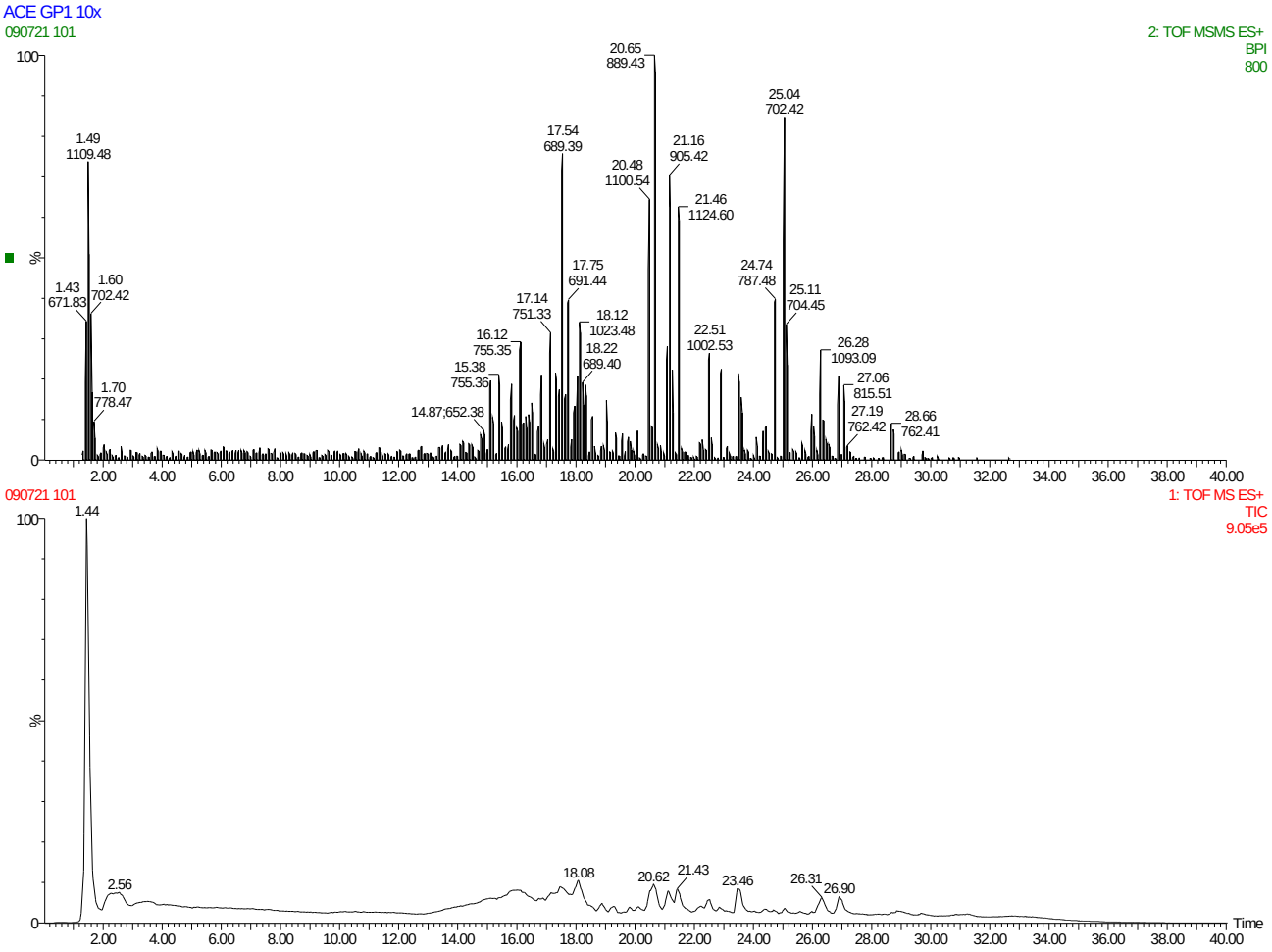
most frequent C-terminal tripeptidic sequence was constituted by hydrophobic amino acids, without Proline presence, or by sequence contained only one proline and two hydrophobic amino acids, like MPK, PKF, MPL, VPL,FPK, APF. Only few peptides for each sample contained two time proline, and the sequence was always of two time proline amino acid, followed by leucine: PPL. Leucine amino acid has the same molecular weight of Isoleucine (I), of the well kwon IPP ACE-inhibitory sequence. Moreover, probably the internal sequences of VPP and IPP (LPP) in the identified ACE-inhibitory peptides could be release when undergo to a further hydrolysis during human digestion, because of enterocytes are able to absorb only a maximum of three amino acids.

This results show that these typical cheese could be useful in a diet for the prevention of high blood pressure (BP) at a large population level. The salt content of Grana Padano and TrentinGrana could have a detrimental effect on BP but we have to consider that the amount of salt in cheese has been dramatically reducing over last years and, furthermore, the main contribution of salt to our diets come from ready-to-cook foods and from added salt during foods preparation.

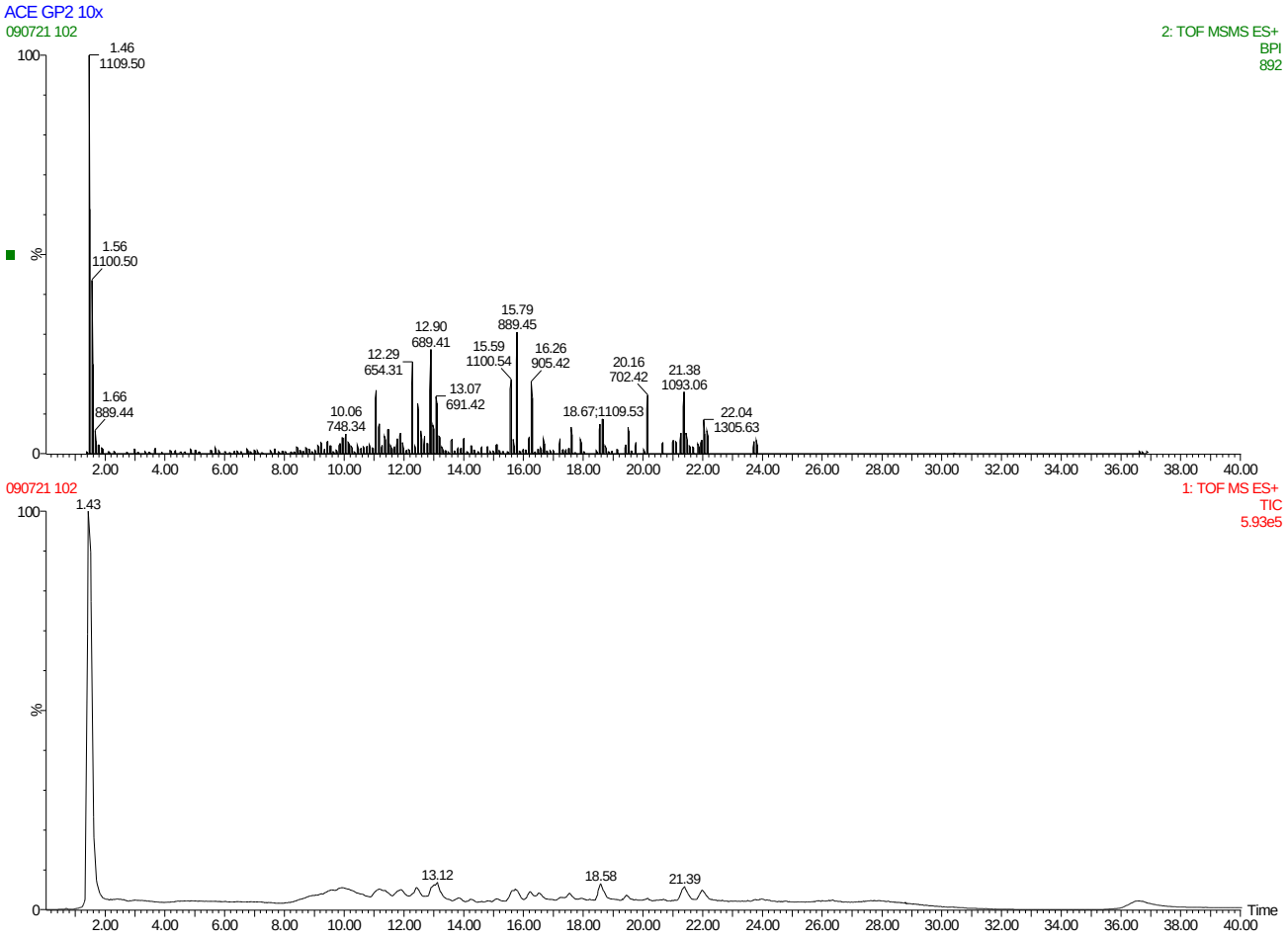
7.4. Supplementary Materials

In this chapter are showed mass spectrometry chromatrograms and MS/MS chromatograms obtained with Synapt high definition system of the analysed samples.

Grana Padano 1 (13,5 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram.



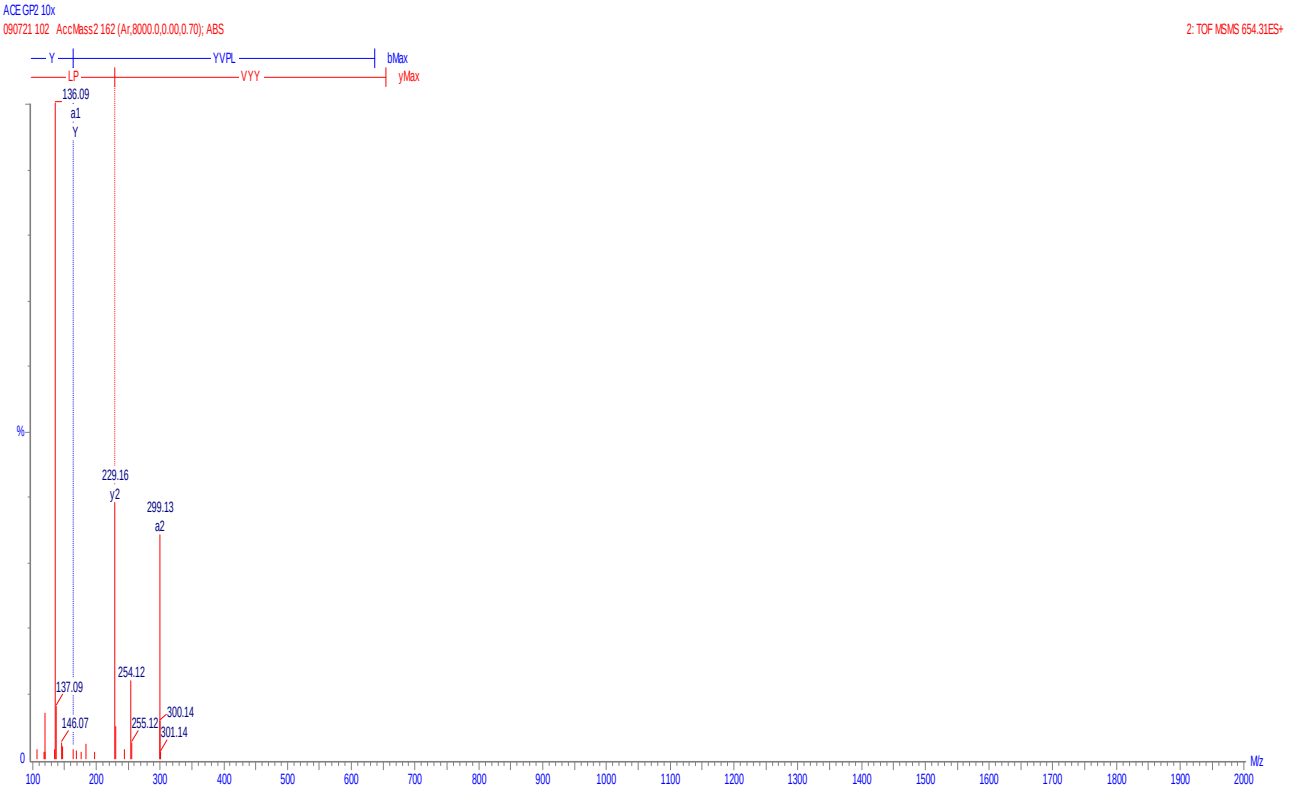
Grana Padano 2 (13,5 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering y and z fragments.



YYVPL Petide. Mw 653.2878. Rt 12.29

Observed MW: 653.2878 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 12 (0.750%)

a	136.08	299.14	398.21	495.26	608.34
	-0.01	0.01	---	---	---
b	164.07	327.13	426.20	523.26	636.34
	-0.00	---	---	---	---
	Tyr	Tyr	Val	Pro	Leu
y	654.35	491.29	328.22	229.16	132.10
	---	---	---	0.00	---
z	637.32	474.26	311.19	212.13	115.07
	---	---	---	---	---

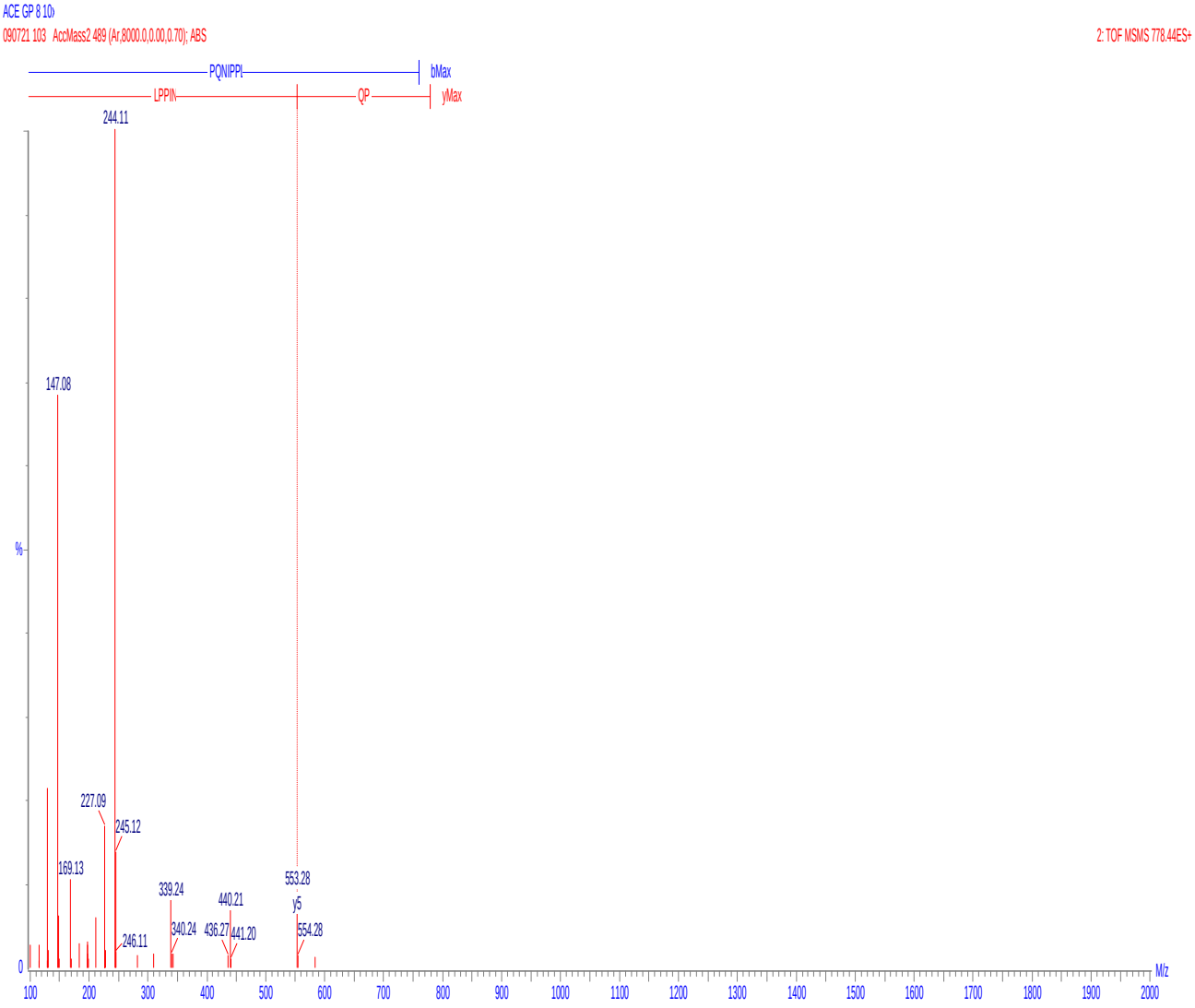

ACE GP2 10x
090721 102 AccMass2 162 (Ar,8000.0,0.00,0.70); ABS
2: TOF MSMS 654.31ES+
Y
YVPL
bMax
LP
VYY
yMax
136.09
a1
Y
%
229.16
y2
299.13
a2
254.12
137.09
146.07
255.12
300.14
301.14
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
M/z

Grana Padano 8 (20,7 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering a and b, y and z fragments.

PQNIPPL Peptide. Mw 777.4286. Rt 1.68

Observed MW: 777.4286 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 9 (0.750%)

a	70.07	**198.12**	312.17	425.25	522.30	619.36	732.44
	---	**-0.05**	---	---	---	---	---
b	98.06	226.12	340.16	453.25	550.30	647.35	760.44
	---	---	---	---	---	---	---
	Pro	**Gln**	**Asn**	**Ile**	**Pro**	**Pro**	**Leu**
y	778.45	681.39	**553.33**	439.29	326.21	229.16	132.10
	---	---	**0.05**	---	---	---	---
z	761.42	664.36	536.30	422.26	309.18	212.13	**115.07**
	---	---	---	---	---	---	**-0.02**

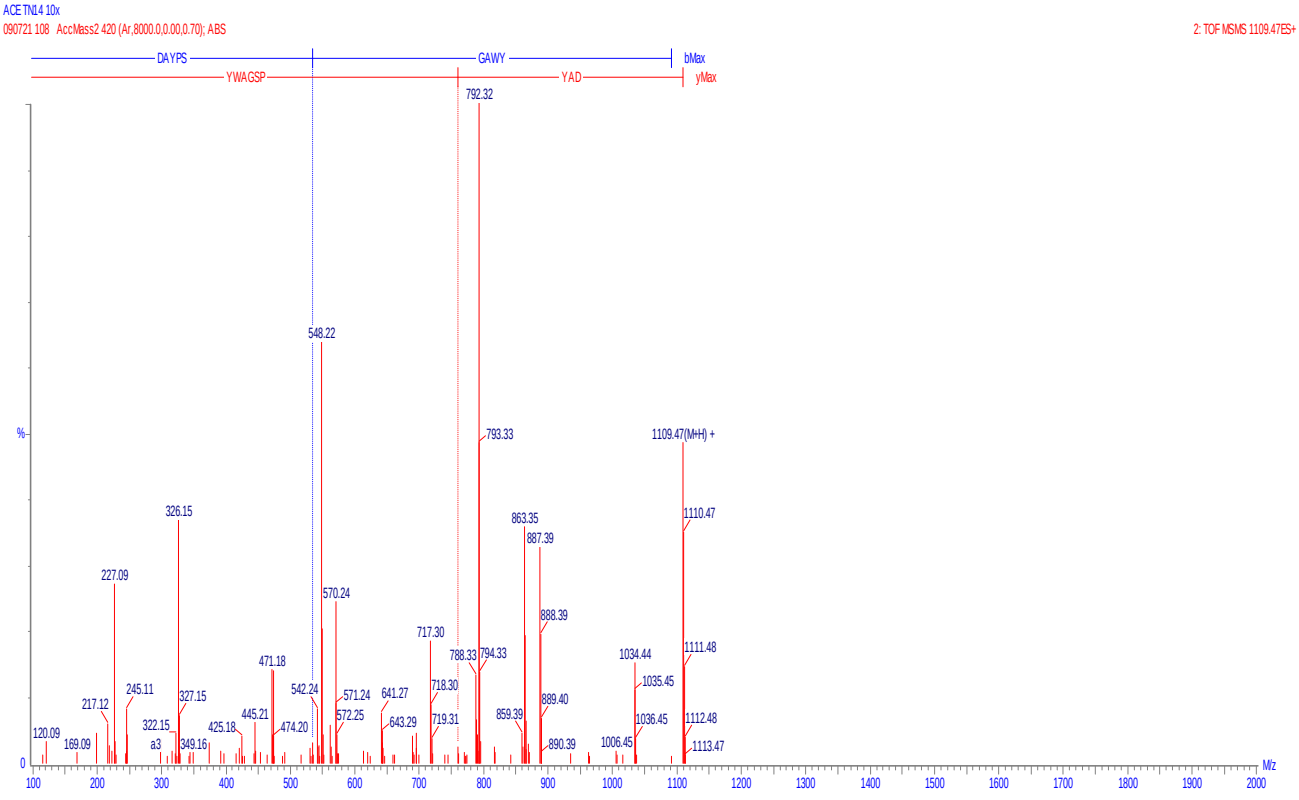


Grana Padano 14 (26,7 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering a and b, y and z fragments.

DAYPSGAWY Peptide. 1108.4612 Mw. Rt 23.31.

Observed MW: 1108.4612 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 67 (0.750%)

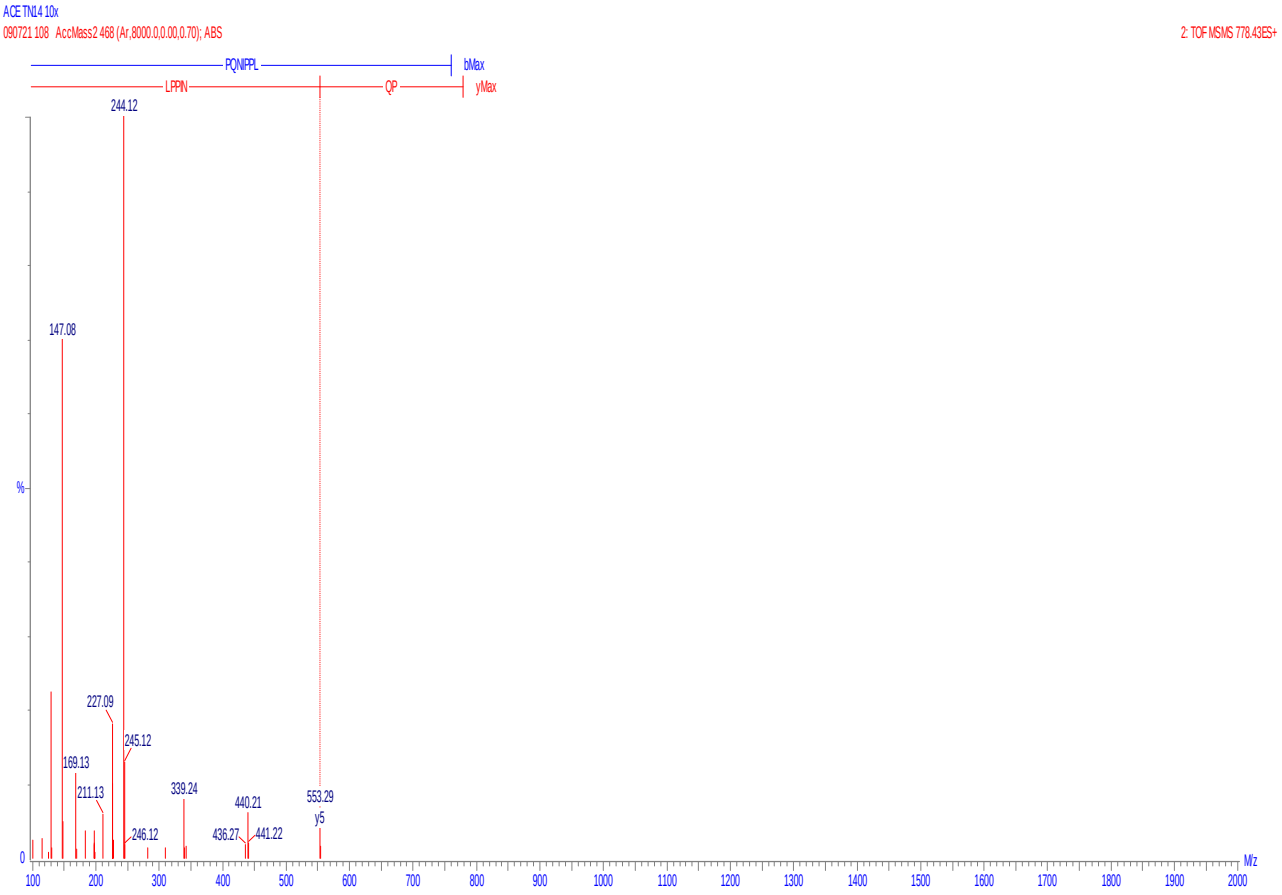
a	88.04	159.08	322.14	419.19	506.23	563.25	634.28	820.36	983.43
	---	---	-0.01	---	---	---	---	---	---
b	116.03	187.07	350.14	447.19	534.22	591.24	662.28	848.36	1011.42
	---	---	---	---	-0.04	---	---	---	---
	Asp	Ala	Tyr	Pro	Ser	Gly	Ala	Trp	Tyr
y	1109.47	994.44	923.40	760.34	663.29	576.26	519.24	448.20	262.12
	---	---	---	0.00	---	---	---	---	---
z	1092.44	977.41	906.37	743.31	646.26	559.23	502.21	431.17	245.09
	---	---	---	---	---	---	---	---	-0.03



PQNIPPL Pepetide. 777.4328 Mw. Rt 25.83

Observed MW: 777.4328 Precursor ion charge state: 1
Mz tolerance: 0.30 Intensity threshold: 13 (0.750%)

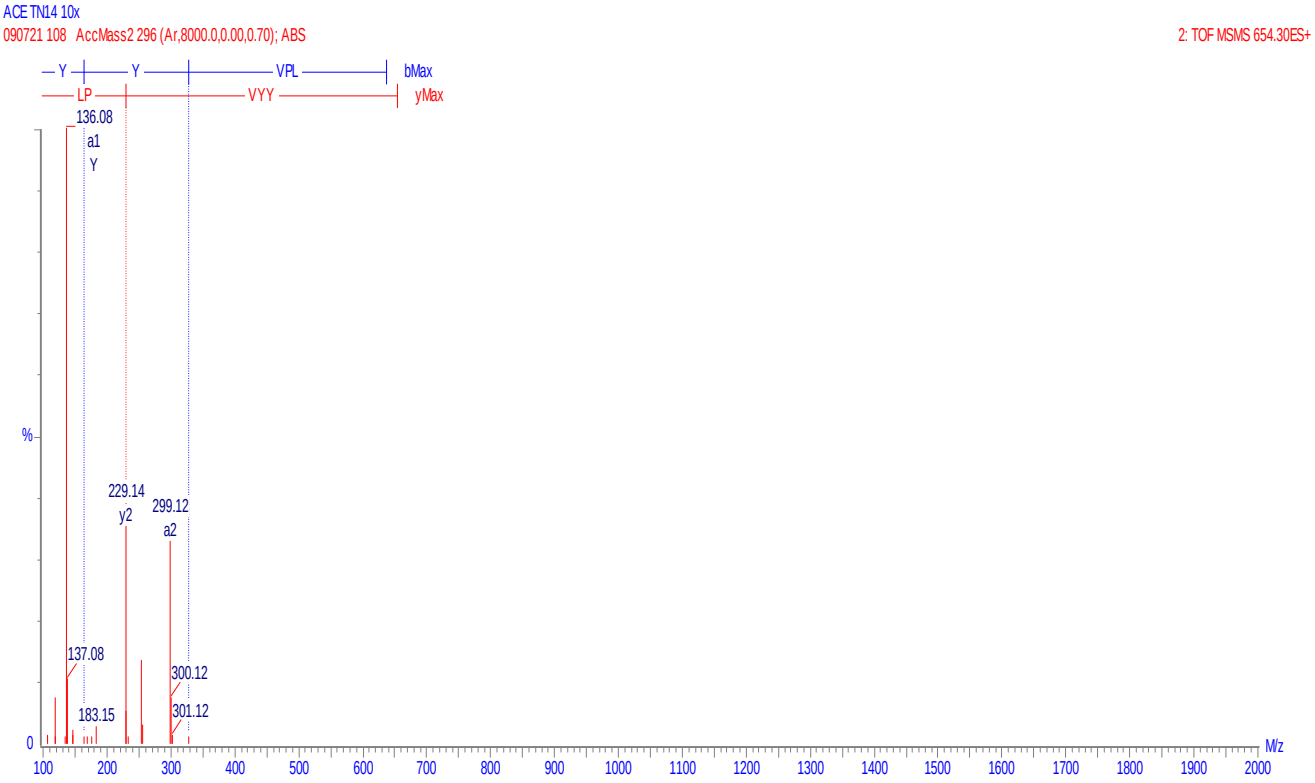
a	70.07	**198.12**	312.17	425.25	522.30	619.36	732.44
	---	**-0.05**	---	---	---	---	---
b	98.06	226.12	340.16	453.25	550.30	647.35	760.44
	---	---	---	---	---	---	---
	Pro	**Gln**	**Asn**	**Ile**	**Pro**	**Pro**	**Leu**
y	778.45	681.39	**553.33**	439.29	326.21	229.16	132.10
	---	---	**0.05**	---	---	---	---
z	761.42	664.36	536.30	422.26	309.18	212.13	**115.07**
	---	---	---	---	---	---	**-0.02**



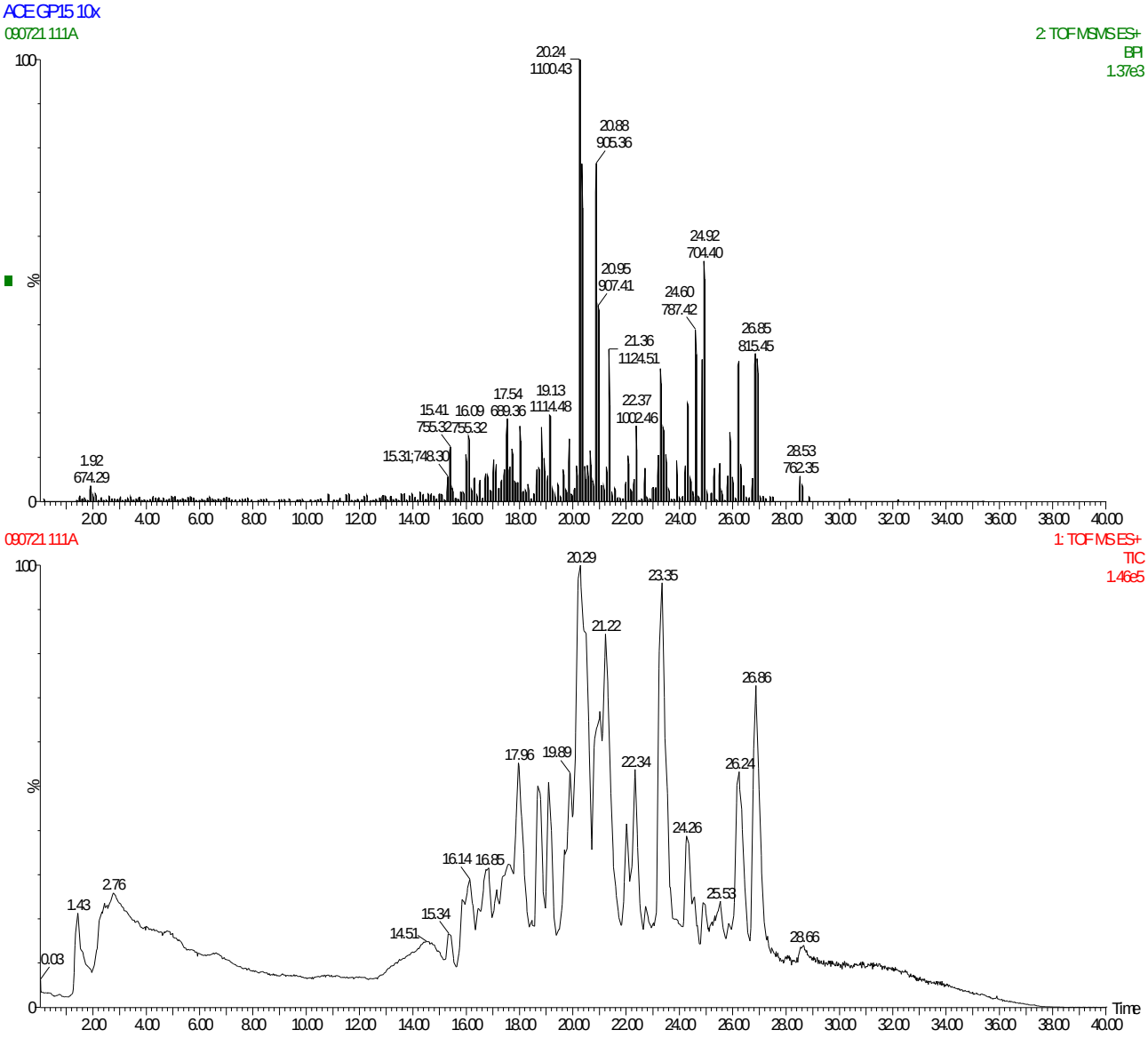
YYVPL Peptide. 653.2838 Mw. Rt 17.03

Observed MW: 653.2838 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 16 (0.750%)

a	**136.08**	**299.14**	398.21	495.26	608.34
	-0.00	**0.02**	---	---	---
b	**164.07**	**327.13**	426.20	523.26	636.34
	0.00	**0.03**	---	---	---
	Tyr	**Tyr**	**Val**	**Pro**	**Leu**
y	654.35	491.29	328.22	**229.16**	132.10
	---	---	---	**0.01**	---
z	637.32	474.26	311.19	212.13	115.07
	---	---	---	---	---



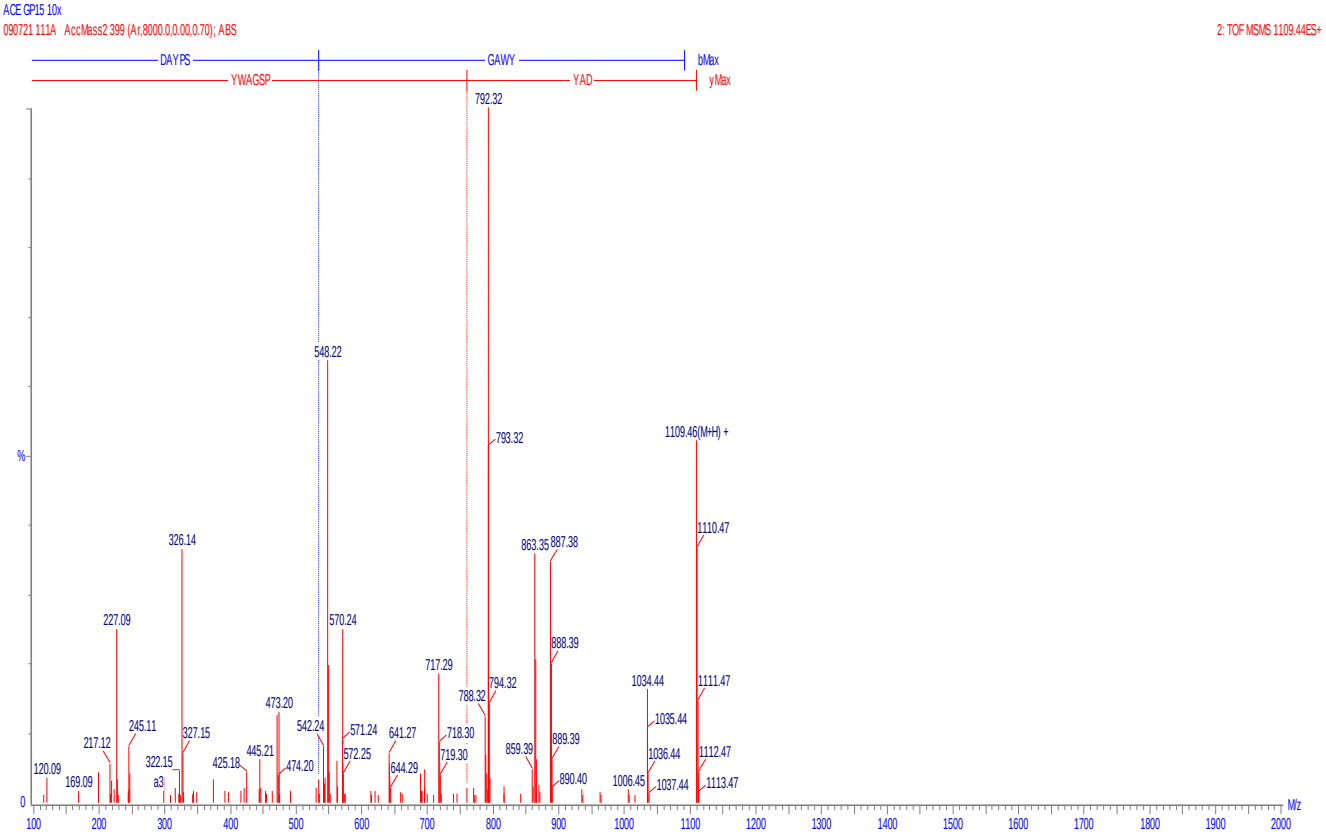
Grana Padano 15 (26.7 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering y and z fragments.



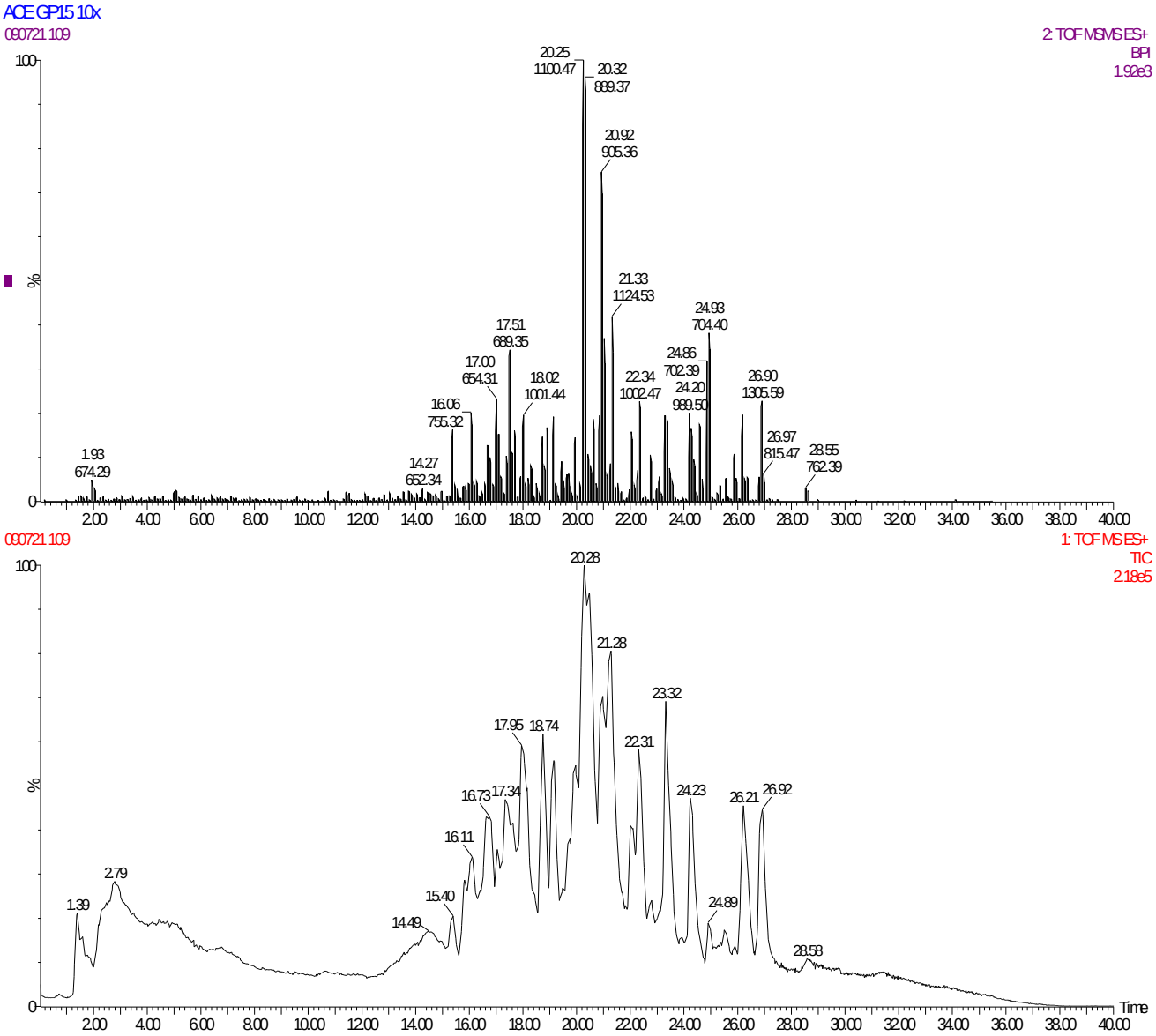
DAYPSGAWY Peptide. 1108.4352 Mw. 19.13 Rt

Observed MW: 1108.4532 Precursor ion charge state: 1
Mz tolerance: 0.30 Intensity threshold: 54 (0.750%)

a	88.04	159.08	322.14	419.19	506.23	563.25	634.28	820.36	983.43
	---	---	-0.01	---	---	---	---	---	---
b	116.03	187.07	350.14	447.19	534.22	591.24	662.28	848.36	1011.42
	---	---	---	---	-0.04	---	---	---	---
	Asp	Ala	Tyr	Pro	Ser	Gly	Ala	Trp	Tyr
y	1109.46	994.43	923.40	760.33	663.28	576.25	519.23	448.19	262.11
	---	---	---	0.00	---	---	---	---	---
z	1092.43	977.40	906.37	743.30	646.25	559.22	502.20	431.16	245.08
	---	---	---	---	---	---	---	---	-0.03



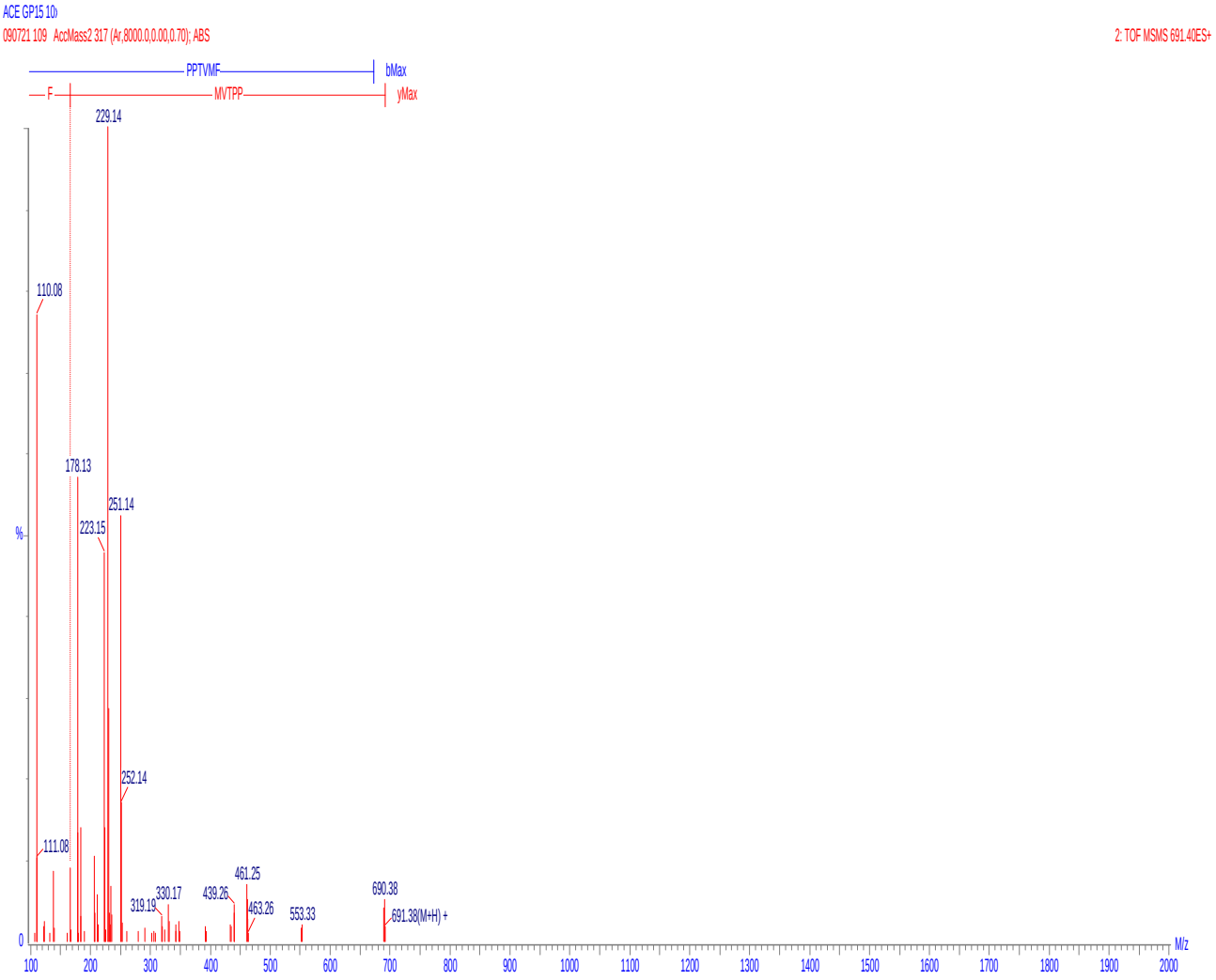
TrentinGrana 10 (21 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering y and z fragments.



PPTVMF Peptide. 690.2836 Mw. 17.51 Rt

Observed MW: 690.2836 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 23 (0.750%)

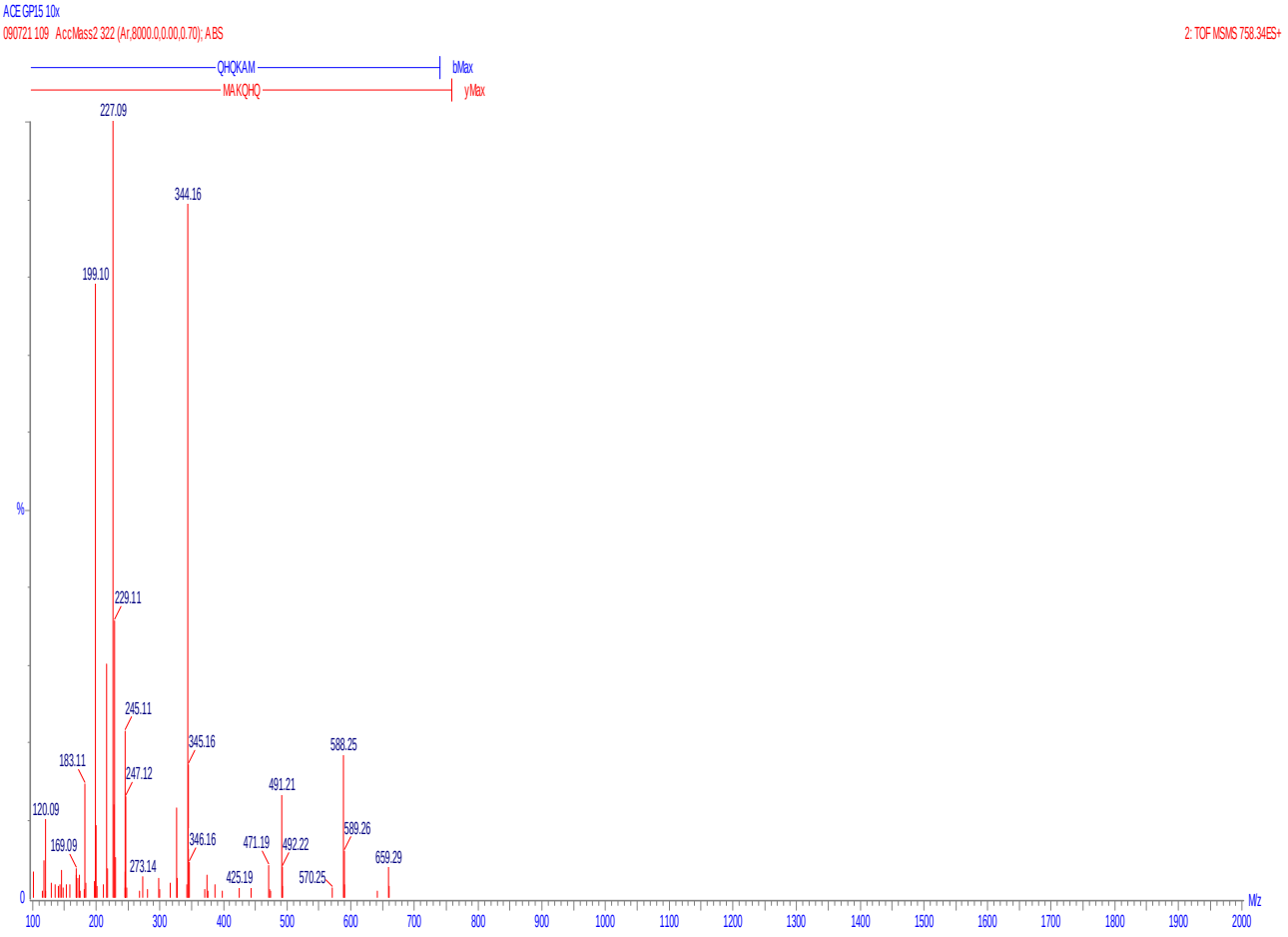
a	70.07	167.12	268.17	367.23	498.28	645.34
	---	---	---	---	---	---
b	98.06	195.11	296.16	395.23	526.27	673.34
	---	---	---	---	---	---
	Pro	**Pro**	**Thr**	**Val**	**Met**	**Phe**
y	691.35	594.30	497.24	396.20	297.13	**166.09**
	---	---	---	---	---	**0.03**
z	674.32	577.27	480.21	379.17	280.10	149.06
	---	---	---	---	---	---



QHQKAM Peptide. 757.3141 Mw. 16.06 Rt

Observed MW: 757.3141 Precursor ion charge state: 1
Mz tolerance: 0.30 Intensity threshold: 8 (0.750%)

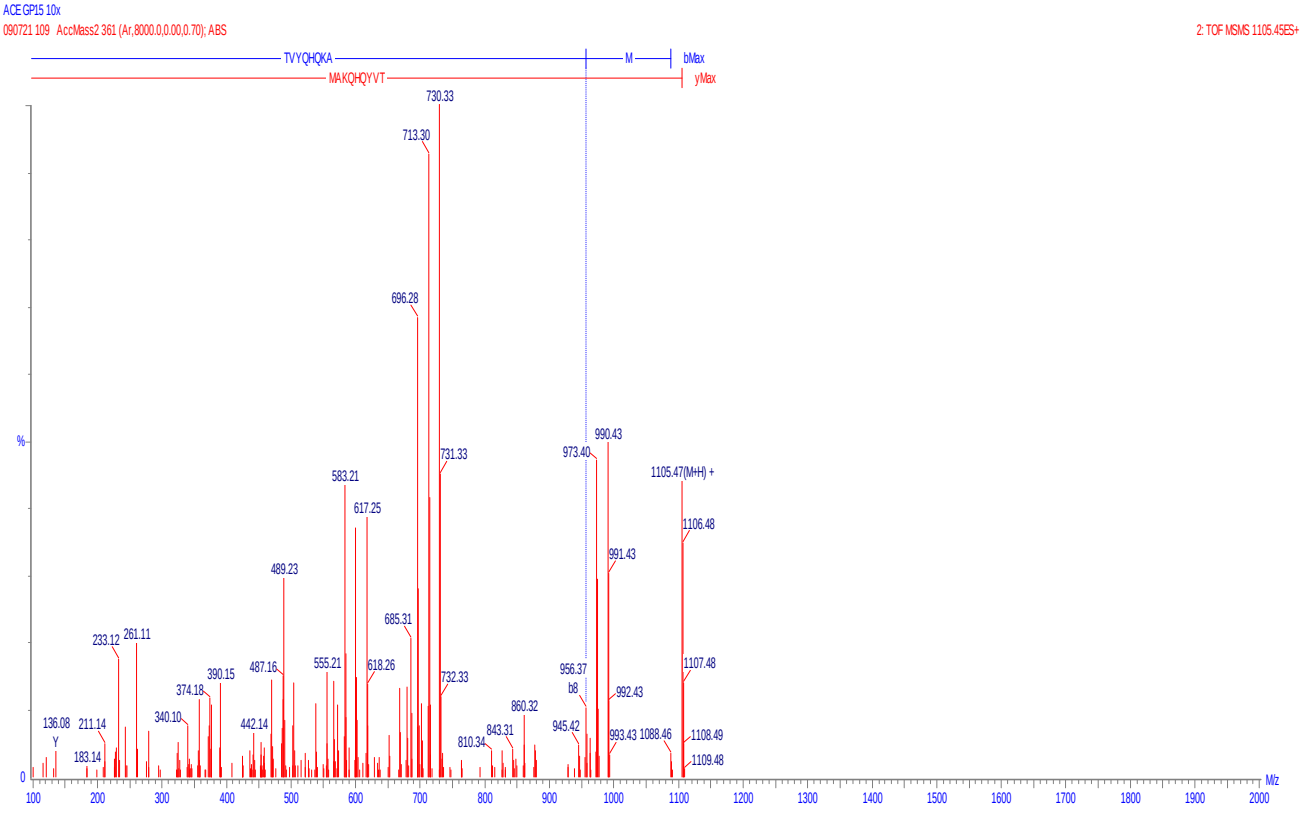
a	101.07	238.13	366.19	494.28	565.32	696.36
	---	---	---	---	---	---
b	129.07	266.13	394.18	522.28	593.32	724.36
	---	---	---	---	---	---
	Gln	His	Gln	Lys	Ala	Met
y	742.37	614.31	477.25	349.19	221.10	150.06
	---	---	---	---	---	---
z	725.34	597.28	460.22	332.16	204.07	133.03
	---	---	---	---	---	---



TVYQHQKAM Peptide. 1104.4672 Mw. 22.34 Rt

Observed MW: 1104.4672 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 35 (0.750%)

a	74.06	173.13	336.19	464.25	601.31	729.37	857.46	928.50	1059.54
	---	---	---	---	---	---	---	0.12	---
b	102.06	201.12	364.19	492.25	629.30	757.36	885.46	956.50	1087.54
	---	---	---	---	---	---	---	0.12	---
	Thr	Val	Tyr	Gln	His	Gln	Lys	Ala	Met
y	1105.55	1004.50	905.43	742.37	614.31	477.25	349.19	221.10	150.06
	---	---	---	---	---	---	---	---	---
z	1088.52	987.47	888.40	725.34	597.28	460.22	332.16	204.07	133.03
	---	---	---	---	---	---	---	---	---



YYVPL Peptide. 653.2898 Mw. 14.27 Rt

Observed MW: 653.2898 Precursor ion charge state: 1
M/z tolerance: 0.30 Intensity threshold: 25 (0.750%)

a	136.08	299.14	398.21	495.26	608.34
	-0.00	0.02	---	---	---
b	164.07	327.13	426.20	523.26	636.34
	-0.00	---	---	---	---
	Tyr	Tyr	Val	Pro	Leu
y	654.35	491.29	328.22	229.16	132.10
	---	---	---	0.01	---
z	637.32	474.26	311.19	212.13	115.07
	---	---	---	---	---

TrentinGrana 14 (23.5 months aged) results: Synapt high definition mass spectrometry system chromatogram and MS/MS chromatogram, tandem mass spectrometry peptides identification with software considering y and z fragments

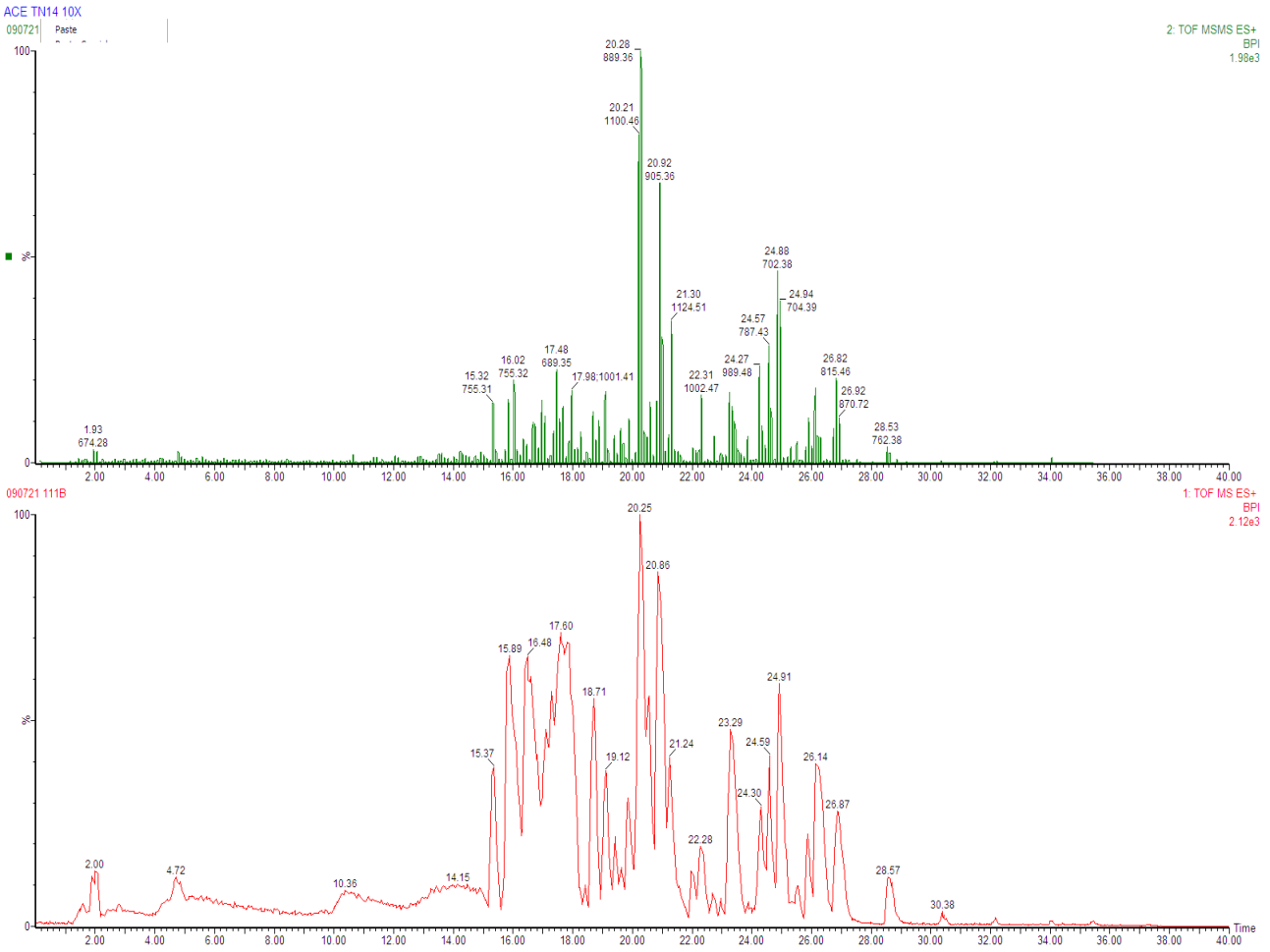
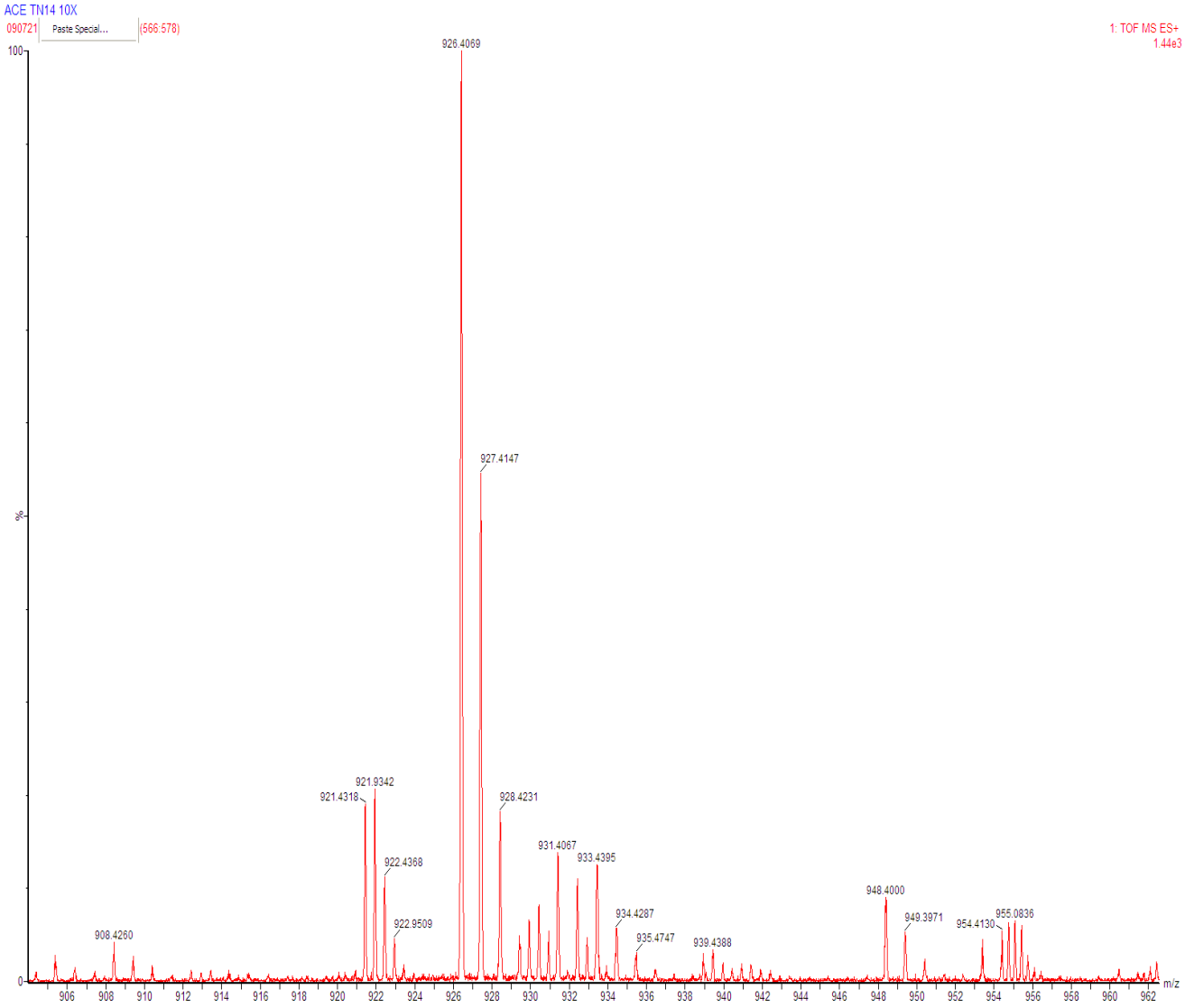
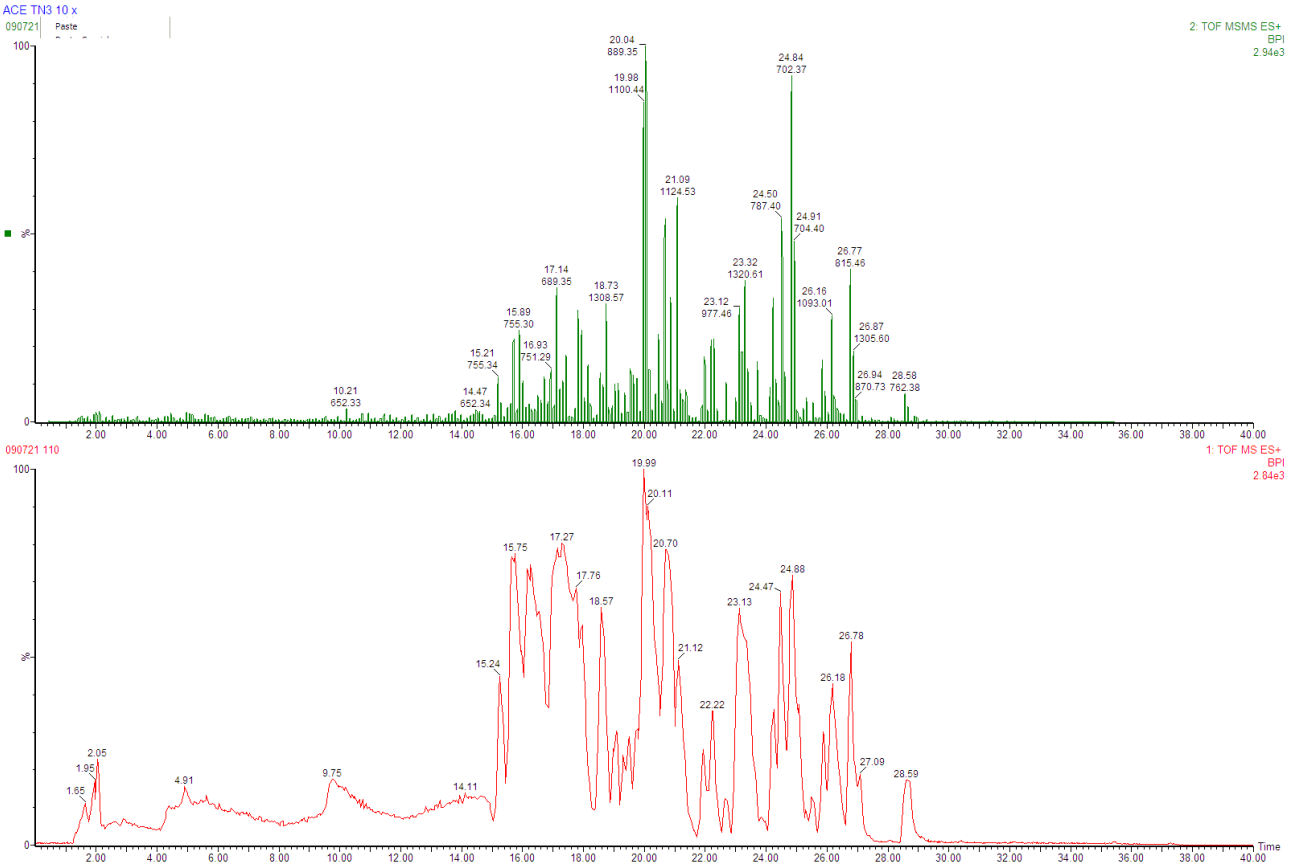


Figure1-7.6: Identification of the 922 m/z peak , 3 charged , in MS chromatogram.



TrentinGrana 16(23.1 months aged) results: Biopharmalynx v.1.1. software peptides results.



8. Final conclusion

Calcium digestibility analysis of different type of foods – cheeses, soya based foods and vegetable- conducted both in vivo and in vitro, showed that foods have different calcium digestibility values and cheeses have an higher calcium digestibility than other foods. The lowest in vivo calcium digestibility results were found in peas and canned beans instead the two analyzed cheeses, Emmenthaler and Grana Padano, and the soya based foods, tofu, burgher, powder milk, featured the highest values. Calcium digestibility in vitro of Emmenthaler cheese (58,54 %), Tofu (57,51%) and soya powder milk (50,91) have similar values. Grana Padano has calcium digestibility value, in vivo and in vitro, of 80%. In vitro calcium digestibility results confirmed that soya powder milk was a less good source of calcium than cheese and tofu. Peas and canned beans have the lowest calcium digestibility values both in vivo and in vitro, i.e.in vitro calcium digestibility of peas (23,04 %) and canned beans (31,34 %) are lower than in vivo values, peas (49, 95 %) and canned beans (31,44). Therefore calcium digestibility indexes results could be useful to cover calcium daily requirement optimazing energy intake.

Calcium digestibility assessed in different ripened times samples of Grana Padano (manufactured with lysozyme molecule) and Parmigiano Reggiano and TrentinGrana (manufactured without lysozyme) showed only significant trend between calcium digestibility and ripening time, in Grana Padano samples aged > 24 months. Grana Padano samples < 24 months aged and TrentinGrana samples calcium digestibility results are scattered. The RP-HPLC molecular weight distribution analysis of oligopetides demonstrated that the only difference between cheeses with and without lysozyme, was that cheese without lysozyme, with ripening times between 15 and 20 months, are more hydrolyzed than Grana Padano cheese. These events should probably due to lysozyme or other factors that occur during cheese manufacturing and cheese ripening.

RP-HPLC molecular weight distribution and SELDI analysis confirmed the presence of casein phophopetides in the most significantly involved calcium binding fraction of 1000-1500 D of Grana Padano and TrentinGrana cheeses.

ACE-inhibitory activity of bioactive peptides in Grana Padano cheese (manufactured with lysozyme) and TrentinGrana (manufactured without lysozyme) did not show any correlation with ripening time (and therefore proteolysis level) and was not influenced by lysozyme

9. Appendix

9.1. Amino acids table

Amino acid table used for quantification

Amino acid	**Abb.**	**Molecular weight**	**Conc. mg/100 ml**	**Solvent**	**Fluka Biochemica nr**
Alanine	A	89.09	44.5	20% ACN, 0.1% formic acid	5130
Arginine	R	210.66	105.3	20% ACN, 0.1% formic acid	11040
Asparagine	N	132.12	66.1	20% ACN, 0.1% formic acid	11150
Aspar tic acid	D	133.10	66.6	20% ACN, 0.1% formic acid 20 mM HCl	11190
Cysteine	C	121.16	60.6	20% ACN, 0.1% formic acid	30090
Glutamine	Q	146.14	73.1	20% ACN, 0.1% formic acid	49420
Glutamic acid	E	147.13	73.6	20% ACN, 0.1% formic acid 20 mM HCl	49450
Gl ycine	G	75.07	37.5	20% ACN, 0.1% formic acid	50050
Histidine	H	209.63	104.8	20% ACN, 0.1% formic acid	53370
Isoleucine	I	131.17	65.6	20% ACN, 0.1% formic acid	58880
Leucine	L	131.17	65.6	20% ACN, 0.1% formic acid	61820
Lysine	K	182.65	91.3	20% ACN, 0.1% formic acid	62930
Methionine	M	149.21	74.6	20% ACN, 0.1% formic acid	64320
Phenylalanine	F	165.19	82.6	20% ACN, 0.1% formic acid	78020
Proline	P	115.13	57.6	20% ACN, 0.1% formic acid	81710
Serine	S	105.09	52.5	20% ACN, 0.1% formic acid	84960
Threonine	T	119.12	59.6	20% ACN, 0.1% formic acid	89180
Tryptophan	W	204.23	102.1	20% ACN, 0.1% formic acid	93660
Tyrosine	Y	181.19	90.6	20% ACN, 0.1% formic acid 20 mM HCl	93830
Valine	V	117.15	58.6	20% ACN, 0.1% formic acid	94620

Bibliography

Adamson J., Reynolds E.R. (1994) Selective precipitation of multiple phosphoseryl-containing peptides. Biotechnology and bioengineering, vol 45, 3.

Addeo F., Chianes L., Salzano A., Scacchi R., Cappuccio U & Ferranti P., et al. (1992). Characterization of the 12% tricholoacetic acid-insoluble oligopeptides of Parmigiano Reggiano cheese. Journal of Dairy Research. 59: 401-411.

Addeo F., Chianese L., Sacchi R., Spagna Musso S., Ferranti P.&Malori A. (1994) Characterization of the oligopeptides of Parmigiano Reggiano cheese soluble in 120 g trichloroacetic acid/l. Journal of Dairy Research, 61: 365-374.

Addeo F., Chianese L., Sacchi R., Musso SS, Ferranti P., Malorni A. (1997) Characterization of the oligopeptides of Parmigiano Reggiano cheese soluble in 120 g trichloroacetic acid/l. Journal of Dairy Research.61 (3), 365-374.

Allen L.H. (1982) Calcium bioavailability and absorption: a review. Am. J. Clin. Nutr., 35: 783-808.

Anwaar A., Muhammad F.M. , Rehman S.U. , Randhawa M.A. , Farooq U. (2008) Bioavailability of calcium , iron and zinc fortified whole wheat flour Chapatti. Plant Foods Human Nutr., 63: 7-13.

Arienti G. (1996) Le basi molecolari della Nutrizione, Piccin Editore.

Ariyoshi Y. (1993) Angiotensin-converting enzyme inhibitors derived from proteins. Trends Food Sci. Tech. 4:139.

Baran D., Sorensen A., Grimes J., Lew R., Karellas A., Johnson B. & Roche J. (1989) Dietary modification with dairy products for preventing vertebral bone loss in premenopausal women: a three-year prospective study. J. Clin. Endocrinol. Metab. 70: 264-270.

Barr S.I., McCarron D.A., Heaney R.P., Dawson-Hughes b., Berga S., Stern J.S., & Oparil S. (2000) Effects of increased consumption of fluid milk on energy and nutrient intake, body weight, and cardiovascular risk factors in healthy older adults. J. Am. Diet. Assoc. 100: 810-817.

Belitz, H.-D., & Grosch, W. (1999) Food chemistry (2nd ed). Berlin:Springer.

Bernhart F. W, Savini S., Tomarelli R.M., (1969) Calcium and Phosphorus Requirements for Maximal Growth and Mineralizationof the Rat, J. Nutr., 98: 443-448

Berrocal R., Chanton S., Juillerat M.A., Pavillard B., Scherz J-C. and Jost R. (1989) Tryptic phosphopeptides from whole casein : II. Physicochemical properties related to the solubilisation of calcium. Journal of Dairy Research. 56: 335-341.

Bohn L., Meyer A.S., Rasmussen S.K. (2008) Phytate: impact on environment and human nutrition. A challenge for molecular breeding. J. Zhejiang Univ. Sci B, 9: 165-191.

Bonjour J.-P, Carrie A.-L., Ferrari S., Clavien H., Slosman D., Theintz G. & Rizzoli R. (1997) Calcium-enriched foods and bone mass in prepubertal girls: a randomized, double-bind, placebo-controlled trial. J. Clin. Invest. 99: 1287-1294.

Bounous G., Amer M.A. (1990) The immunoenhancing effect of dietary whey protein concentrate. In “Role of milk protein in human nutrition”. Bulletin of the International Dairy Federation, 253:3.

Brommage R., Juillerat M.A., Jost R. (1991) Influence of casein phosphopeptides and lactulose on intestinal calcium absoption in adult female rats. Lait, 1991. 71: 173-180.

Bronner F. (1987) Intestinal calcium absorption : Mechanism and apllications. Journal of nutrition. 117: 1347-1352.

Buttriss J (1990) The role of calcium in a balanced diet. Journal of the Socirty of Dairy Technology. 43: 1-3.

Cadogan J., Eastell R., Jones N. & Barker ME. (1997) Milk intake and bone mineral acquisition in adolescent girls : randomized, controlled intervention trial. Br. Med. J. 315:1255-1260.

Carini, S., & Lodi, R. (1997) Grana Padano. Consideration of starter culture and lysozyme. Latte, 22, 76–79.

Carruth B.R. & Skinner J.D. (2001) The role of dietary calcium and other nutrients in moderating body fat in preschool children. Int.J.Obes. 25: 559-566.

Chan G.M., Hoffman K. & McMurry M. (1995) Effects of dairy products on bone and body composition in pubertal girls. J. Pediatr. 126: 551-556.

Cheung H-S., Feng-Lai W., Ondetti MA, Sabo EF & Cushman DW. (1980) Binding of peptides substrates and inhibitors of angiotensin-converting-enzyme. Journal of biological Chemistry, 225: 401-407.

Clare D. A., Swaisgood H. E (2000) Bioactive milk peptides: A prospectus. J. Dairy Sci. 83: 1187-1195.

Cleghorn D.B., O’Loughlin P.D., Schroeder B.J. & Nordin B.E.C. (2001) An open, crossover trial of calcium-fortified milk in preventing of early postmenopausal bone loss. Med J. Austr. 175: 242-245.

Collomb M.A., Schimd A., Sieber R., Wechsler D. (2006) Comjugated linoleic acids in milk fat: Variation and physiological effects. Int Dairy J. 16: 1347-1361.

Cushman D.W., Cheung H.S (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20: 1637-1648.

Cushmann D.W., Cheung H.S., Sabo E.F., Ondetti M.A. (1977) Biochemistry 16: 5884-5491

EC (1996). Official Journal of the European Union, Regulation no. 1107/1996 of 21 June 1996.

Davies K.M., Heaney R.P., Recker R.R., Lappe J.M., Barger-Lux J., Rafferty K & Hinders S. (2000) Dairy calcium is related to changes in body composition during a two-year exercise intervention in young women. J. Am. Coll. Nutr. 19: 754-760.

Dawson-Hughes B., Harris S., Krall E.A. & Dallal G.E. (1997) Effect of calcium and vitamin D supplementation on bone density in men and women 65 yeras of age or olders. N. Engl. J. Med. 337:670-676.

DeLany J.P. & West D.B. (2000) Changes in body composition with conjugated linoleic acid. J.Am. Coll. Nutr. 19: 487S-493S.

Devine A., Prince R.L. & Bell R. (1996) Nutritional effect of calcium supplementation by skim milk powder or calcium tablets on total nutrient intake in postmenopausal women. Am. J. Clin. Nutr. 64: 731-737.

Dibba B., Prentice A., Ceesay M., Stirling D.M., Cole T.J., & Poskitt E.M.E. (2000) Effect of calcium supplementation on bone mineral accretion in Gambian children accustomed to a low-calcium diet. Am. J. Clin. Nutr. 71: 544-549.

Elders P.J.M., Lips P., Netelenbos J.C., Van Ginkel F.C., Khoe E., Van Der Vijgh W.J.F. & Van Der Stelt P.F. (1994) Long-term effect of calcium supplementation on bone loss in perimenopausal women. J. Bone Miner. Res. 9: 963-970.

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

Ferranti P., Barone F., Chianese L., Addeo F., Scaloni A., Pellegrino L., Resmini P. (1997) Phosphopeptides from Grana Padano cheese: nature, origin and changes duing ripening. Journal of Dairy Research. 64: 601-615.

Ferrretto A., Signorile A., Gravaghi C., Fiorilli A., Tettamanti G. (2001) Casein Phosphopeptides influence calcium uptake by cultured human intestinal HT-29 tumor cells. J. Nutr., 131: 1655-1661

Fitzgerald RJ (1998) Int Dairy J 8: 451-457

FitzGerald R.J. and Meisel H.(2000) Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. British Journal of Nutrition. Suppl. 1, S33-S37.

Gammelgaard-Larsen C. (1991) Casein phosphopeptide, CPP-a promising new way to enhance utilization and absorpition. Proceedings of food ingredients (Asia). Conference, Singapore, April.. Maarssen Expoconsult: 53-58.

GarnS. (1972) The course of bone gain and the phases of bone loss. Ortho. Clin. North Am., 3: 503-520.

Gobbetti M., Stepaniak L., De Angelis M., Corsettia A., Di Cagno R. (2002) Latent Bioactive Peptides in Milk Proteins: Proteolytic Activation and Significance inDairy Processing. Crit. Rev. Food Sci. Nutr. 42: 223-239.

Gòmez-Ruiz JA, Ramos M, Recio I (2002) Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. Int. Dairy J., 12: 697-706.

Gòmez-Ruiz J. A., Taborda G., Amigo L., Recio I., Ramos M. (2006) Identification of ACE-inhibitory peptides in different Spanish cheeses by tandem mass spectrometry. Eur Food Res Technol. 223:595-601.

Gruchow HW., Sobocinski KA., Barboriak JJ. (1985) Alcohol, nutrient intake, and hypertension in US adults. JAMA. 253: 1567-70.

Hagemeister H., Sick H., Barth C.A. (1990) Nitrogen balance in the human and effects of milk constituents. In: “Role of milk protein in human nutrition”.Bulletin of the International Dairy Federation, 253:3.

Harlan WR., Hull AL, Schmouder RL., Landis JR., Larkin FA., Thompson FE. (1984) High blood pressure in older Americans. The first National Health and Nutrition Examination Survey. Hypertension. 6: 802-9.

Haug A., Harstad O.M. (2007) Bovine milk in human nutrition- a rewie. Lipids Health Dis. 25: 6-25.

Heaney R.P., Skillman T.G. (1964) Secretions and excretion of calcium by the human gastrointestinal tract. J. Lab. Clin. Med., 64: 29-41.

Hernàndez-Ledesma B., Amigo Lourdes., Ramos M., Recio I.. (2004) Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. Journal of Chromatography A, 1049: 107-114.

Hui S.L., Johnston C.C. jr., Mazess R.R., (1985) Bone mass in normal children and young adults. Growth, 49: 34-43.

Holt C., Wahlgren N.M. and Drakenburg T. (1996) Ability of a •- casein phosphopetide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. Biochemical Journal. 314: 1035- 1039.

Insel P., Turner R.E., Ross D.(2004) American dietetic association . Jones and Bartlett, Boston, MA,USA.

Jensen L.B., Kollerup G., Quaade F. & Sorensen O.H. (2001) Bone mineral changes in obese women during a moderate weight loss and without calcium supplementation. J. Bone Miner. Res. 16: 141-147.

Joffres MR., Reed DM., Yano K. (1987) Relationship of magnesium intake and other dietary factors to blood pressure. The Honolulu heart study. AM.J. Clin Nutr. 45: 469-75.

Johnson R.S., Stephen A., Klaus Biemann, Stults J., Throck Watson J. (1987) Novel fragmentation process of peptides by collision-induced decomposition in a tandem mass spectrometer : differentiation of leucine and isoleucine. Anal. Chem.59, (21): 2621-2625.

Johnston C.C., Miller J.Z., Slemenda C.S., Reister T.K., Hui S., Christian J.C., & Peacock M. (1992) Calcium supplementation and increases in bone mineral density in children . N. Engl. J. Med. 327: 82-87.

Kelley N.S., Hubbard N.E., Erikson K. (2007) Conjugated Linoleic acid isomers and cancer. J. Nutr. 137: 2599-2607.

Kitts D. D., Yuan Y.V., Nagasawa T., Moriyama Y (1992b) Effect of casein, casein phosphopeptides and calcium intake on ileal 45 Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. British Journal of Nutrition 69, 765-781.

Korhonem H., Pihlanto A. (2004). Milk-derived bioactive peptides : formation and prospects for health promotion. In: C. shortt and J. O'Brien (eds) Handbook of functional foods and nutraceuticals series 6 , CRC Press, Boca Raton, FL, USA, pp 109-124.

Lalkwarf H.J., Specker B. L., Dianchi D.C., Ranz J., & Ho M. (1997) The effect of calcium supplementation on bone density during lactation and after weaning. N. Engl. J. Med. 337: 523-529.

LARN (1996). Livelli di assunzioni raccomandati di energia e nutrienti per la popolazione italiana. SINU-INN.

Lau E.M.C., Woo j., Lam V. & Hong A. (2001) Milk supplementation of the diet by postmenopausal Chinese women on a low calcium intake retards bone loss. J. Bone Miner. Res. 16: 1704-1709.

Lee Y.S., Noguchi T. and Naito H. (1980) Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. British Journal of Nutrition. 43: 457-467.

Lee Y.S., NouguchiT., Naito H. (1991) Intestinal absorption of calcium in rats given diets containing casein or aminoacid mixture ; the role of casein phosphopeptides. British Journal of Nutrition, 49 : 67-76.

Lee W.T.K., Leung S.S.F., Tsand H.S.Y., Lau K. & Cheung J.C.Y. (1995) A randomized, double-bind controlled calcium supplementation trial, and bone and height acquisition in children. Br. J. Nutr. 74: 125-139.

Lee W. T.K., Leung S.S.F., Wang S.-H., Wu Y.-C., Zeng W.-P., Lau j., Oppenheimer S. J. & Cheung J.C.Y. (1994) Double-bind, controlled calcium supplementation and bone mineral accretion in children accustomed to a low calcium diet, Am J. Clin. Nutr. 60: 744-750.

Li Y., Tome D. and Desjeux J.F. (1989) Indirect effect of casein phosphopetides on calcium absorption in rat ileum in vitro. Reproduction and Nutritional Development.29: 227-233.

Lin Y.C., Lyle R.M., McCabe L.D., McCabe G.P., Weaver C.M. &Teegarden D. (2000) Calcium intake and body weight. J. Clin. Endocrinol. Metab. 85:4635-4638.

Lloyd T., Andon M.B., Rollings N., Martel J.K., Landis J.R., Demers L.M., Eggli D.F., Kieselhorst K & Kulin H.E. (1993) Calcium supplementation and bone mineral density in adolescent girls. J. Am. Med. Assoc. 27:841-844.

Lokuruka, M.N.I. (2007) Role of fatty acids of milk and dairy products in cardiovascular diseases: a rewie. Afr. J. Food Agric. Nutr. Dev. 7(1):1-16.

Lombardi- Boccia G., Aguzzi A., Cappelloni M., Di Lullo G. (2000) Content of some trace elemnts and mineral in the italian total diet. J. Food composition and analysis. 13: 525-527.

Maes W., Van Camp J., Vermeissen V., Hemeryck M. (2004) Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg on the release of endothelial cells. Regul. Pept. 118: 105-109.

Matkovic V. (1991) Calcium metabolism and calcium requirements during skeletal modelling and consolidation of bone mass. Am. J. Clin. Nutr., 54: 245S-260S.

McCarron DA., Morris CD, Henry HJ., Stanton JL (1984) Blood pressure and nutrient intake in the United States. Science. 224: 1392-8.

McDonagh D. and FitzGerald R.J. (1998) Production of caseinophophopeptides (CPPs) from sodium caseinate using a range of protease preparations. International Dairy Journal. 8: 39-45

Meisel H and Frister H. (1988) Chemical characterization of a caseino phosphopetide isolated fron in vivo digestes of a casein diet. Biological Chemistry Hoppe-Seyler. 369: 1275-1279.

Meisel H. And Frister H. (1988) Chemical characterisation of a caseino-phosphopeptide isolated from in vivo digests of a casein diet. Biological Chemistry Hoppe-Seyler. 369: 1275-1279.

Meisel H. (1993) Casokinins as inhibitors of angiotensin-I- converting enzyme . In new Perspectives in Infant Nutrition. : 153-159. Stuttgart, New York: Theme.

Meisel H., Schlimme E. (1994) Inhibitors of angiotensin-converting enzyme derived from bovine casein (casokinins). In V. Brantl and H. Teschemacher (eds) •-Casomorphins and Related Peptides: Recent Developments. VCH-Weinheim, Germany pp 27-33.

Meisel H., Goepfert A., Gunther S. (1997) Bioactive peptides products: synthesis and interaction with proteolytic enzymes. Milchwissenschaft. 52: 307-311.

Meisel, H. (1998) Overview on milk protein-derived peptides.International Dairy Journal, 8(5/6), 363–373.

Merrilees M.J., Smart E.J., Gilchrist N.L., Frampton C., Turner J.G., Hooke E., March R.L. & Maguire P. (2000) Effects of dairy food supplements on bone mineral density in teenage girls. Eur. J. Nutr. 39: 256-262.

Minervini F, Algaron F, Rizzello CG, Fox PF, Monnet V, Gobbetti M (2003) Angiotensin I-converting-enzyme-inibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase–idrolysed caseins of milk from six species. Appl. Environ. Microbiol., 69: 5297-5305.

Meyer J, Butikofer U, Walther B, Wechsler D, Sieber R (2009) Hot topic: changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different cheeses varieties. J. Dairy Sci., 92: 826-836.

Mikkänen H. M. and Wasserman R.H. (1980) Enhanced absorption of calcium by casein phosphopeptides in rachitis and normal chicks. Journal of Nutrition. 119: 2141-2148.

Miquel E., Gòmez J., Alegrìa A., Barberà R., Farrè R., Recio I. (2006) Identification of casein phosphopetides after dimulated gastrointestinal digestion by in tandem mass spectrometry. Eur Food Res Tecnol. 222: 48-53

Naito H., Kawakami A & Takano T.(1972) In vivo formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. Agriculture and Biological Chemistry. 36: 409-415.

Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T (1995) Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. J. Dairy Sci., 78: 777-783.

Noeson C.A., Green R.M., Hopper J.L. Sherwin A.J., Young D., Kaymakci B., Guest C.S., Smid M., Larkings R.G., & wark J.D. (1997) Aco-twin study of the effect of calcium supplementation on bone density during adolescence. Osteoporos. Int. 7 219-225.

Nurminen M.L., Sipola M., Kaarto H., Pihlanto-Leppala A.. (2000) •-lactorphin lower blood pressure via radiotelemetry in normotensive and spountaneously hypertensive rats. Life Sci. 66: 1535-1543.

Ondetti MA & Cushmann DW (1982) Enzymes of the rennin –angiotensin system and their inhibitors. Annual review of Bioche mistry, 51: 283-308.

Ono T., Ohotawa T. and Takagi Y. (1994) Complexes of casein phosphopeptide and calcium phosphate prepared from casein micelles by tryptic digestion. Bioscience, Biotechnology and Biochemistry. 58: 1376-1380.

Parrot S., Degraeve P., Curia C., Martial-Gros A. (2003) In vitro study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on Angiotensin I converting enzyme inhibitory peptides. Nahrung. 47 (2): 87-94.

Pennington, J.A. (1996) Intakes of minerals from diets and foods : Is there a need for concern? J. Nutr. 126: 2304S-2308S.

Perales, S., Barbera´ , R., Lagarda, M., & Farre´, R. (2005) Bioavailability of calcium from milk-based formulas and fruit juices containing milk and cereals estimated by in vitro methods (solubility, dialyzability, and uptake and transport by Caco-2 cells). Journal of Agricultural and Food Chemistry, 53, 3721–3726.

Peacock M. (1991) Calcium absorption efficiency and calcium requirements in children and adolescents. Am. J. Clin. Nutr., 54: 261S-265S.

Perez-Jarauuz M.D., Revilla M., Alvarez de los Heros J.I., Villa L.F.. & Rico H. (1996) Orophylaxis of osteoporosis with calcium, estrogens and/or eelcatonin: comparative longitudinal stydy of bone mass. Maturitas 23: 327-332.

Perich J., Kelly D.P. and Reynolds E.C. (1992) Efficent solution-phase synthesis of phosphopeptides related to casein and stratherin. International Journal of Peptide and Protein Research. 40: 81-88.

Prandini A., Sigolo S., Cerioli C., Piva G. (2009) Survey on conjgated linoleic acid (CLA) content and fatty acid composition of Grana Padano cheese produced in different season and areas. Italian J. Animal Science. vol 8:131-142.

Prentice A., Jarjou L.M., Cole T.J., Stirling D.M., Dibba B. & Fairweather- Tait S. (1995) Calcium requirements of lactating Gambian mothers: effects of a calcium supplement on breast-milk calcium concebtration, maternal bone mineral content, and urinary calcium excretion. Am. J. Clin. Nutr. 62: 58-67.

Prince R., Devine A., Dick I., Criddle A., Kerry D., Kent N., Price P. & Randell A. (1995) The effects of calcium supplementation (milk powder or tablets) and exercise on bone density in postmenopausal women. J. Bone Miner. Res. 10: 1068-1075.

Pringle, S. D., K. Giles, J.L. Wildgoose, J.P. Williams, S.E. Slade, K. Thalassinos, R.H. Bateman, M.T. Bowers, J.H. Scrivens. (2007) "An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument." International Journal of Mass Spectrometry 261(1): 1-12.

Recker R.R., Hinders S., Davies K.M., Heaney R.P., Stegman M.R., Lappe J.M. & Kimmel D.B. (1996) Correcting calcium nutritional deficiency prevents spine fractures in elderly women. J. Bone Miner. Res. 11: 1961-1966.

Revees R.E. and Latour N.G. (1958) Calcium phosphate sequestering phosphopetide from casein. Science. 128. 472.

Reeves P.G., Nielsen F. H., Fahey G.C. Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing Committee on the reformulation of the AIN-76 rodents diet. J. Nutr., 123: 1939-1951.

Reynold E.C. (1993) Phosphopetides for the treatment of dental calculus. World Patent WO 93/03707.

Reynold E.C. (1994) Anticariogenic Phosphopeptides. In Proceeding 24th International Dairy Congress, Melbourne, Australia. International Dairy Federation, Brussels, Belgium Record No. 7698-7796/10379.

Rossander L., Sandberg A.S., Sandsr• m B. (1992) The influnce of dietary fibre on mineral adsorption and utilization. In dietray Fibre-A component of Food, eds T.F. Schweizer and C.A. Edwards. Springer, London, pp. 197-216.

Ricci TY.A., Chowdhury H.A., Heymsfield S.B., Stahi T., Pierson R,N., Jr & Shapses S.A. (1998) Calcium supplementation suppresses bone turnover during weight reduction in postmenopausal women. J. Bone Miner. Res 13: 1045-1050.

Riggs B.L., O'Fallon W.M., Muhs J., O'Connor M.K., Kumar R. & Melton L.J., III (1998) Long-term effects of calcium supplementation on serum parathyroid level, bone turnover, and bone loss in elderly women. J. Bone Miner. Res. 13: 168-174.

Rohrbach M.S., Williams Jr E.B., Rolstad R.A. (1981) Purification and substrate specificity of bovine angiotensin-converting enzyme. J Biol Chem. 256: 225-230.

Sato R., Noguchi T. and Naito H. (1983) The necessity for the phosphate portion of casein molecole enhance Ca absorption for the small intestine. Agricultural and Biological Chemistry. 47: 2415-2417.

Sato R., Naguchi T., Naiti H. (1986) Casein Phosphopeptide (CPP) enhance calcium absorption from the ligated segment of rats small intestine. Journal of Nutrition Science and Vitaminology, 32, 67- 76.

Sato R., Shindo M., Gunshin H., Noguchi T and Naito H. (1991) Characterisation of phosphopeptides derived from b-casein: an inhibitor to intra-intestinal precipitation of calcium phosphate. Biochimica et Biophysica Acta. 1077:413-415.

Schaafsma G. (1997) Bioavailability of calcium. Dietary calcium and Health. Bulletin of the International Dairy Federation No. 322, pp 20-24.

Schlimme E., Meisel H. (1993) Bioactive peptides: structural, physiological and analytical aspects. Newsletter of the IDF, 139, October (Supplement), 57.

Schlimme E and Meisel H. (1995) Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. Die Narung. 39: 1-20.

Schwartz M.W. & Seely R.J. (1997) The new biology of body weight regulation. J. Am. Diet. Assoc. 97: 54-58.

Schoeller D.A. (1995) Limitations in the assessment of dietary energy intake by self-report. Metab. Clin. Exp. 44 (supp.2) : 18-22.

Seppo L., Jauhiainen T., Poussa T., Korpela R. (2003) A feremented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. Am. J. Clin. Nutrition. 77: 326-330.

Silva SV, Pihlanto A, Malcata FX (2006) Bio-active peptides in ovine and caprine cheeselike systems J. Dairy Sci., 89: 3336-3344.

Sforza S., Ferroni L., Galaverna G., Dossena A., Marchelli R. (2003) Extraction, Semi-Quantification, and Fast On-line Identification of Oligopeptides in Grana Padano Cheese by HPLC-MS. J. Agric. Food Chem. 51: 2130-2135.

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

Shi H., DiRienzo D. & Zemel M.B. (2002) Effects of dietary calcium on adipocyte lipid metabolism and body weight regulation in energy-restricted AP2-agouti transgenic mice. FASEB J. 15:291-293.

Spencer H., Kramer L. (1986) The calcium requirements and factors causing calcium loss. Fed. Proc., 45: 2758-2762.

Stein, J. L., & Imhof, K. (1978) Ullmanns Enz. Techn. Chem., 16, 689.

Storm D., Eslin R., Porter E.S., Musgrave K., Vereault D., Patton C., Kessenich C., Mohan S., Chen T., Holick M.F. & Rosen C.J. (1998) Calcium supplementation prevents seasonal bone loss and changes in biochemical markers of bone turnover in elderly New England women: a randomized placebo-controlled trial. J. Clin. Endocrinol. Metab. 83: 3817-3825.

Summer A., Fossa E., Sandri S., Pecorari M., Mariani P. (1997) Composizione chimica e proteolisi del Parmigiano Reggiano di 24 mesi in rapporto alla stagione di produzione. Scienza Tecnica Lattiero Casearia, 48: 135-145.

Terpstra A.H.M. (2004) Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overwiew of the literature. Am J. Clin. Nutr. 79: 352-361.

Van Dokkum K. (1998) Trace element intake in Europe: Safe and adequate? In Role of Trace Elements for Health Proportion and Disease Prevention (B. Sandstrom , and P. Walter , Eds.), pp 8-17.

Walsh D.J., BernardH., Murray B.A. (2004) In vitro generation and stability of lactokinin •-lactoglobulin fragment (142-148). J Dairy Sci. 87: 3845-3857.

Weaver C.M. (1994) Symposium: Required versus optimal intakes: A look at Calcium. Age related Calcium requirements due to changes in absorption and utilization. J. Nutrition. 124: 1418S-1425S.

Vermeirssen V., Van Camp J., Verstraete W. (2004) Bioavailability of Angiotensin I-converting enzyme inhibitory peptides. Brit. J. Nutr. 92: 357-366.

West D.W. (1990) Phosphopeptides and calcium absorption. In Dairying in a Changing World. Proceeding 23rd Intrenational Dairy Federation Congress, October, Montreal, Canada, International Dairy Federation, Brussels, Belgium, pp. 1208-1216.

Witteman JCM., Willett WC., Stampfer MJ., et al. (1989) A prospective study of nutritional factors and hypertension amoung US women. Circulation. 80: 1320-7.

Wolters M.G., Schreuder H.A.W, Van den Heuvel G., Van Lonkhuijsen H.J.J, Voragen A.G.J. (1993) A continuous in vitro methods for estimation of the bioavailability of minerals and trace elements in foods: Application to breads varying in phytic acid content. Br. J. Nutr., 69: 849-861.

World Health Organization (1996) Trace elements in Human Health and Nutrition. WHO. Geneva.

Xue B., Greenberg A.G., Kraemer F.B. & Zemel M.B. (2001) Mechanism of intracellular calcium ([Ca2+]i) inhibition of lipolysis in human adipocytes. FASEB J. 15: 2527-2529.

Yoshikawa M., Sasaki R., Chiba H. (1981) Effect of chimical phosphorylation of bovine casein components on the properties related to casein micelle formation. Agricultural and Biological Chemistry. 45: 909-914.

Zemel M.B., Shi H., Greer B., DiRienzo D & Zemel P.C. (2000) Regulation of adiposity by dietary calcium. FASEB J. 14: 1132-1138.