DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

CHEESEMAKING WITH VEGETABLE RENNET (*O. tauricum*):

Evaluation of fatty acid composition and volatile profile.

TYPE OF DISSERTATION: research

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ACADEMIC YEAR 2020-2021

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ACRONYMS AND ABBREVIATIONS

MCA	Milk-clotting activity
AP	Aspartic protease
PA	Proteolytic activity
SP	Serine protease
AC	Coagulant activity
AR	Animal rennet
A_W	Water activity
GMP	Glycomacropeptide
CP	Cysteine protease
GMO	Genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
EC	Enzyme commission
MFGM	Milk fat globule membrane
LPL	Lipoprotein
FFA	Free fatty acid
SCFA	Short chain fatty acid
MCFA	Medium chain fatty acid
LCFA	Long chain fatty acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
FID	Flame ionization detector
FAME	Fatty acid methyl ester

CB	Control body
FAO	Food and agricultural organization
WHO	World health organization
GRASS	Generally recognized as safe
FDA	Food and drug administration
VOC	Volatile organic compound

INTRODUCTION AND AIM OF THE THESIS

Since centuries, plant proteases have been used either as crude extracts or in purified form in cheesemaking and plant rennets, due to their easy availability and simple purification processes, have gained progressively increasing interest in cheese industry. For several years, indeed, various types of artisanal cheeses, mainly produced in the Mediterranean countries, Southern Europe and West Africa, have been obtained using plant extracts. Additionally, the employment of plant proteases as alternative to calf rennets in cheese manufacturing has allowed a greater acceptability by vegetarians improving their nutritional intake. Actually, plant coagulants offer an alternative to the calf rennets conciliating religious factors, diet or ban on recombinant calf rennet in some countries and answering issues due to the scarce availability causing the price increase on the market. Therefore, plant tissues are sources of enzymes essential to the cheesemaking process that can be obtained from their natural source or through in vitro culture to guarantee a continuous stream of plant proteases. Although most of the enzymes used as milk coagulants belong to the aspartic proteases, enzymes from other groups such as cysteine and serine protease have been reported as possessing the ability to coagulate milk under suitable conditions. Lower yields of cheese, bitter flavors and consistency imperfections are however defects due to the excessive proteolytic nature of most vegetable coagulants, limiting their use in the production of cheeses; this pushes the search for new potential rennets for the coagulation of milk from plants to meet the growing world demand. Therefore, the growing global demand for cheese coupled with the reduced supply of calf rennet made necessary to select a suitable vegetable coagulant. The selection of an appropriate substitute for calf rennet requires a thorough study of the technological properties; this entails analyses relating to milk coagulation activity (MCA) and proteolytic activity (PA) which are considered crucial steps in the study since the activities' ratio impacts the rheological and sensorial properties of the produced cheeses. An excellent product with desirable firmness and no release of the typical bitter aromas of plant proteases is generally achieved when a high value of this ratio is obtained. Therefore, the study of the enzymatic and technological properties of vegetable rennet, previously analysed in the literature, provides a clear overview of the key elements for the choice of the appropriate vegetable rennet. [1]

Consistent with what has been said, the examination and evaluation of the diverse physio-chemical properties of cheeses obtained with plant proteases are the primary aim of this study. Types and sources of diverse plant proteases involved in milk coagulation are discussed and particular attention is given to plant extract of *Onopordum tauricum* whose chemical, technological and coagulant properties are analysed. *Onopordum tauricum* extract's functional properties and enzymatic role in cheese production are evaluated. The cheese under analysis is produced in the Marche foothills and is characterized by the use of local spontaneous flora of *Onopordum tauricum* used for the preparation of aqueous extracts used as milk coagulant. Specifically, fatty acids composition and volatile profile of cheeses obtained with *Onopordum tauricum* enzyme preparation are object of laboratory's studies carried out in the Department of the Università Politecnica delle Marche. In addition, effects of plant coagulants on rheological properties of gels and influencing factors are explored. Examples of cheeses available on market are reported. Lastly, a general analysis on the designation of origin that provide for the use of vegetable rennet in the European context is proposed.

Finally, considerations and conclusion are provided on the bases of the scientific literature available, and the laboratory's activities results with the intention of contributing to the promotion of this product and its production area.

Chapter 1
PREFACE

The main phase for the production of cheese consists in the coagulation of the milk by coagulating enzymes, which are preparations of proteolytic enzymes. Their use in cheese production seems to date back thousands of years, and to be even among the oldest known applications of enzymes as confirmed by cave paintings dating back to 5000 BC indicating cheese production. Historically, although most of the enzymatic preparations used for cheese production were extracted from the stomach of ruminants, especially that of calves, the use of coagulants from microbes and plants also began very early on.

The nomenclature of enzymes involved in milk coagulation has changed over time as the nature of enzymes was realized and understood thus leading to the growing knowledge of their identity and diversity. The first name for the milk coagulation enzyme was chymosin (from ancient Greek 'χυμός' – khymos, meaning gastric juice, given by Deschamps in 1840). In 1890 the name rennin, derived from the word rennet, was suggested for the same enzyme. Though, because of misunderstanding with the related proteolytic enzyme renin, the main milk clotting enzyme was again named chymosin (International Union of Biochemistry and Molecular Biology - IUBMB, 1992). [2] Chymosin (EC 3.4.23.4) is therefore the foremost enzyme component extensively used in cheesemaking and, being ruminant stomach the major source of rennet, they were largely exploited to supply the dairy industry. However, since 1960s due to the limited availability of ruminant stomachs, the rennet supply decreased; this caused the increase in the price of rennet that coupled with religious concerns (e.g., Islam and Judaism), diet (vegetarianism and veganism) or the prohibition of recombinant calf rennet (in France, Germany and The Netherlands), have paved the way for the search for alternative milk-clotting sources. The necessity to replace calf rennet in cheesemaking prompted the research to discover alternative milk-clotting enzymes, including microbial, recombinant, and plant-based enzymes which have been isolated and studied. Particularly, despite rennet substitutes produced by microorganisms and genetically engineered microorganisms were considered suitable replacements for animal rennet, vegetable coagulants, e.g., the milk-clotting enzymes extracted from plants, gained a rising relevance. [3]

Therefore, the origin of the milk coagulation ferments is diverse. The active enzymes that have been shown to be effective for cheese production are aspartic proteinases (IUBMB EC 3.4.23). Therefore, according to the International Diary Federation (IDF), the term “rennet” indicates an extract of ruminant abomasum while the term "coagulants" is intended for milk-clotting preparations of other origins and among these there are microbial and vegetable coagulants. Instead, chymosin produced by a genetically modified organism (GMO) is generally referred as “fermentation-produced chymosin” (FPC). [2]

According to Tamer and Mavituna (1997), almost all types of plant tissues possess these enzymes, and it seems to be a general rule that, under appropriate conditions, all proteolytic enzymes have the ability to coagulate milk. However, although almost all the enzymes used as milk coagulants belong to the aspartic proteases, enzymes from other groups such as cysteine and serine protease have also been used. As previously mentioned, the production of cheese also took place through the use of plant extracts used as milk coagulants since ancient times.[3] Surprisingly, historical finds dating back to the Roman period make references to the use of different types of rennet. Indeed, Lucius Junius Moderatus Columella, the Roman soldier and writer of agriculture, in his treatise *De Re Rustica* (c. 50 BC), considered one of the most meticulous ancient narratives of cheesemaking, describes the different uses of lamb or kid rennet and even reports that cheeses can also be produced with rennet of vegetable origin. Columella, in addition to seeds of *Carthamus tinctorius* (thistle’s similar flower), also mentions the use of wild thistle flowers (probably *Cynara cardunculus*) and fig sap to coagulate the milk of small ruminants.[4]

The Caciofiore di Columella cheese can be considered a sort of "ancestor" of the Pecorino Romano cheese, made with rennet based on wild thistle (*Cynara Cardunculus*). Columella in his treatise "*De Rustica*" reports:

> *“It should usually be curdled with rennet obtained from a lamb or a kid, though it can also be coagulated with the flower of the wild thistle or the seeds of the safflower, and equally well with the liquid which flows from a fig-tree if you make an incision in the bark while it is still green...”* (Conceição, 2018)

Today in the Roman countryside, the "motherland" of wild thistle and artichoke, some producers, taking up these writings, use the flower of thistle as rennet and propose raw milk pecorino cheese with an ancient flavor. The Romans also used *Carthamus tinctorius* seeds, also commonly known as "false saffron", green pine nuts and shredded thyme and vinegar to curdle the cheese.

Particularly, some countries of the Mediterranean, West Africa and Southern Europe, are still producing typical cheeses manufactured with vegetable coagulants; countries such as Spain and Portugal have the largest variety and production of vegetable rennet cheeses obtained from

Cynara sp. Portuguese cheeses such as Serpa and Serra, and Spanish cheeses such as La Serena, Los Pedroches and Torta del Casar (from milk of sheep) and Los Ibores cheese (from milk of goat) and Flor de Guía cheese (from a mixture of milk of sheep and cow) are examples of cheeses obtained with extracts of *Cynara spp*. The use of vegetable coagulant is also particularly widespread in the Iberian Peninsula which possess a large number of Protected Designation of Origin (PDO) cheeses. Crude extracts obtained from flowers or even leaves of thistles are characterized by thermostability and a high proteolytic activity due to the presence of aspartic proteases, known as cardonsins (commonly referred as cynarases and cyprosins), with a high specificity for caseins. However, the lower yield of the cheese and the defects in flavor and texture due to the excessive proteolytic nature of most of the vegetable coagulants has limited their use in the production of cheese. This pushes the search for new potential lactic ferments for the coagulation of milk from plants to proceed, so as to make them optimal to the industrial application aimed at diversified and high-quality cheese production. [3]

Chapter 2
CHEESEMAKING PROCESS: BASIC OPERATIONS OF CHEESEMAKING TECHNOLOGY

Two are the paramount goals in the technology of cheesemaking:

- establish the parameters making cheeses desirable (e.g., flavour, body, texture and stretch properties); and
- establish protocols of manufacturing and ripening reporting parameters to be routinely replicate every time cheese is made.

Despite involving complex chemical and physical phenomena, cheesemaking can be considered a simple process. Cheesemaking consists in a process relying on the coagulation of the major milk protein, casein and proceeding with manufacturing steps meant at controlling the chemistry of the casein molecules. The interactions between casein molecules will dictate and govern the cheese's physical and rheological characteristics.

Several factors (e.g., pH, proteolysis, temperature, cheese composition and others) influence the cheesemaking process and despite each of them can be considered independently, each must also be considered in relation to all the others.

2.1 **Basic cheese manufacture**

Cheese manufacture essentially involves the coagulation of casein micelles to form a gel that entraps the fat globules, if present; when the gel is cut or broken, the casein network contracts (syneresis), expelling whey. The resulting curds may be consumed fresh as mild-flavoured products or ripened for a period ranging from 2 weeks (e.g., for Mozzarella) to over 2 years (e.g., for Parmigiano-Reggiano).

The most important step in cheesemaking is the coagulation of micelles that will precipitate forming the curdle. Thus, the cheesemaking essentially involve micelles that interact each other forming a sort of gel which entraps fat globule.

There are several methods to induce the coagulation:

The first method, which is used for most ripened cheeses and accounts for approximately 75% of total cheese production, relies on the addition of coagulating enzyme. The addition of coagulating enzymes is responsible of the destabilization of the colloidal suspension of casein's micelles and makes them aggregate to form a gel network. Many are the sources from which coagulants derived: coagulants can derive from calf stomachs (rennets) of from plants and fungi. (More in-depth analysis on the types of rennets and coagulants is proposed in chapter 3)

The second method relies on the acidification to pH of approximately 4.6 at 30 – 36°C by in situ production of acid by fermentation of lactose to lactic acid by lactic acid bacteria or direct acidification by the addition of acids or acidogens (e.g., gluconic acid-δ-lactone). Is needed to remember that caseins are denatured very easily because they do not have the secondary structure. Most acid-coagulated cheeses are consumed fresh and represent about 25% of total cheese production. Major examples of acid-coagulated cheeses are cottage, quark, and cream cheeses.

The third method relies on the acidification of milk, whey, or mixtures thereof to a pH of approximately 5.2 and heating to approximately 90 °C. These cheeses are usually consumed fresh and common examples include Ricotta and Queso Blanco cheeses.

Independently from the method of casein precipitation, milk fat is surrounded by the casein as the coagulum forms and is trapped together with serum which contains water-soluble components, namely lactose, whey proteins and minerals. Based on how the casein is coagulated, further different processing steps are applied to remove the whey from the coagulum.

To facilitate the removal of whey from the coagulated casein, the coagulum is cut into small grains. The curd instantly starts shrinking and expelling whey. During syneresis the casein molecules reorganize themselves; this results in squeezing the whey from the casein gel network. Hereafter, depending on the type of cheese, the way the curd and whey mixture is handled varies.

The curd and whey mixture are stirred, heated and subsequently separated. Then, the curd can be textured, ground and salted and later placed in a mold generally referred as "hoop". Generally, the hopping is performed after or during dripping for the production of soft cheeses. It is also possible to apply pressure to the container; the amount and duration of pressing vary according to the type of cheese. Furthermore, the size and shape of the container depend on the type of cheese desired. Before putting the curd into the container, salt is added to the whey-free curd (direct salting) or once the pressed cheese block has been removed from the "hoop". Salting can be done directly to the surface of the cheese (dry salting) or by dipping the block of cheese in brine. When the cheese has been pressed and salted, it is seasoned.

Depending upon the type of cheese the ripening process varies. Changes to the body, texture and physical properties as well as development of flavour of the cheese will occur during ripening as result of the chemical and enzymatic reactions. Several factors affect these changes such as temperature during ripening, pH of the cheese, manufacturing protocol and the addition of specific enzymes and microorganisms. [21]

2.2 **Stages of cheesemaking**

Once arrived in the production plant, milk is subjected to microbiological, chemical and physical analysis before being introduced in the production process. Despite cow's milk is the most common milk used, other animal origin milk can be used as well such as sheep's, goat's and buffalo's milk. However, milk may have different characteristics resulting in different quality products.

2.2.1 Standardization of milk

To produce a cheese of consistent composition and to standardize the production to obtain a cheese that does not vary with time, is necessary to utilize milk of consistent composition. Indeed, milk composition can vary in dependence to the weather, animal-feeding practices, breed of animal and others, all factors over which the cheesemaker has no control. However, milk composition can be altered (standardized) by adding milk solids (condensed or milk powder) or by removing cream.

Cheese composition is determined by the ratio of casein to fat (C/F) in terms of the amount of fat in the total solids (TS) portion of cheese, e.g., fat-in-dry matter (FDM). The total amount of casein and fat, in part, determines the yield potential of milk. Standardization of milk is an optional phase carried out depending on the desired type of cheese to be produced. [21]

2.2.2 Heat treatment of milk

After receipt and standardization, milk is usually subjected to heat treatment. However, in many minor traditional cheese production plants, the milk is never heated; that because heat treatment of milk is not compulsory in EU countries. Nevertheless, it is mandatory to report on the label that raw milk is used.

A significant debate regarding whether or not to require a mandatory minimum milk's heat treatment that is to be processed into cheese manufacturing is still occurring. Since no pathogen

has been shown to survive in pasteurized milk (treated at 72 °C for 15s), pasteurization is suggested to avoid public health issues. On the other hand, cheese made from pasteurized milk does not taste the same as cheese made from raw or minimally heated milk, and those arguing the necessity of the heat treatment, consider that there might be as well an opportunity for post-pasteurization contamination of cheese with pathogens; hence, subjecting the milk to heat treatment is not essential. According to those, using pathogen-free milk and producing cheese in a pathogen-free environment, combined with appropriate milk handling, it is enough to obtain a cheese free of pathogens. [21]

2.2.3 Addition of the starter culture

In cheesemaking starter cultures are added to enrich the milk with particular microbial flora appropriate for the type of cheese to be produced. The starter is the acid-producing bacterial culture or any bacteria, intentionally added to the milk to influence the taste, aroma and texture of the cheese because of their production of specific flavour compounds or gas.

The metabolism of the starter cultures induces the reduction in pH resulting in the dissolution of colloidal calcium phosphate from the casein, an increase of the rate of coagulant's enzyme activity and of the syneresis' rate, and an inhibition of some bacteria's growth including some pathogens. In addition to the loss of calcium phosphate, the net charge repulsion between casein molecules increases initially but then decreases as the pH nears the isoelectric point of casein thus influencing the chemistry of the casein network, particularly the casein molecules' mobility and the aggregating micelles' final configuration. Therefore, the addition of starter significantly affects the physical properties of cheese such as the texture (firmness, smoothness of mouthfeel) as well as the colour of the cheese. This justifies the accurate choice of starter bacteria employed in cheesemaking which beyond the tradition, is based on the flavour desired, rate and extent of acid development preferred. The amount of starter to be added to milk for cheesemaking varies in dependence of factors comprising the rate and extent of acid development and conditions of culture propagation. To standardize the entire cheesemaking procedure, large-scale cheese manufacturers need the acid development's rate to be predictable every time cheese is made. Sensitivity to salt, temperature and pH are variable characteristics of starter strains exploited in cheesemaking. [21]

2.2.4 Heating

Depending on the type of cheese to be produced, milk is heated to temperatures between 15 °C and 35 °C in order to create the ideal conditions for the coagulant and proteolytic activity of the enzyme.

2.2.5 Addition of coagulating enzymes

The addition of coagulating enzymes results in the formation of the curd incline to contract and expel serum. The selection and application of the coagulants accomplishes the final characteristics of the finished product. A description of the major types of coagulant used for cheesemaking today is reported in chapter 3.

2.2.6 Coagulation and cutting

Coagulation determines the transformation of the milk into curd due to the gelatinization of the casein micelles. Subsequently, the curd tends to contract during a process called syneresis while the whey is expelled. Coagulation can be of two types: acidic or enzymatic.

Enzymatic coagulation consists of three steps. In the first step which is the only one to be fully known, that can occur under different conditions of pH (5.5 to 7) and temperature (4 to 45 °C), and in absence of calcium ions, chymosin selectively cleaves the casein's peptide bond between $Phenilalanin_{105}$–$Methionine_{106}$ of k-casein yielding a hydrophilic glycopeptide (caseinmacropeptide, CMP) that is released, and para-k-casein, a hydrophobic peptide that remains in the micelle. Once chymosin has removed from k-casein the hydrophilic/glycosylated part, all the micelles aggregate and precipitate. The glycomacropeptide is released in the serum and para-k-casein, being a hydrophobic peptide, remains so in the curdle.

The second step occurs at temperatures above 15 ° C to allow the formation of hydrogen bridges between the micelles in the presence of ionic calcium (Ca^{++}).

In the third steps, the clot shrinks expelling the whey resulting in an increasing consistency. A slow non-specific proteolytic activity, resulting from the release of some casein peptides αs1 and ß, takes place affecting the cheese's maturation. The gel's syneresis can be spontaneous and very slow or induced. During the production of cheeses, the latter generally occurs because it is faster.

The cheese's organoleptic characteristics are strongly influenced by the coagulation and syneresis phases.

During slow coagulation, a network of fine strands and small aggregates is formed from the casein micelles; the whey fills the small spaces between the strands and aggregates. There is the formation of what is referred as "fine coagulum". Nearby the fat globules, which are huge

compared to micelles and aggregates of micelles, a network develops. While coagulation proceeds, what is called a "coarse coagulum" is obtained where the filaments form larger, interconnected aggregates and the pores between the aggregates become bigger. Since the interaction between and amongst the casein micelles is of a lesser extent, the fine coagulum is softer than the coarse coagulum. Once the soft coagulum is cut, the curd particle begins to shrink rapidly expelling large amounts of whey. Due to the loss of fat and whey the curd develops a denser "skin" or layers of casein micelles which prevents further fat loss. Skin development is often referred to as the "healing" of the curd as it makes it more resistant to stress and less prone to breaking or tearing. However, if the curd is too weak when subjected to stirring or agitation or in general under sufficient stress it can break.

Basically, it is like cutting the curd into smaller curd particles called “fines”. When the whey and curd are subsequently separated, the curd particles, due to their small size, may not be incorporated into the curd mass resulting in a loss of dairy yield. Again, new skin develops, albeit more slowly. The same process of curd shrinkage and skin formation occurs in hard or coarse coagulum, but even more slowly resulting in a curd more prone to breakage when stirred. Therefore, shaking immediately after the clot is cut is more harmful to a hard cut coagulum than to a soft one. Therefore, cutting too immediately or too lately can result in increased fines and fat loss, especially if the subsequent stirring rate is too high for the resilience of the curd; this is because if the coagulum is cut and shaken too soon, even the curd can be subject to tearing and breaking. Traditionally, the cheesemaker measures the coagulum firmness. The optimal firmness will change for different cheeses. The curd size as well is chosen according to the desired type of cheese to be produced. The smaller the coagulum is cut, the greater is the surface area exposed and the more fat is lost: the curd is cut small and soft to produce low-moisture cheeses, while the curd is cut large and firm to produce high-moisture cheeses. [21]

2.2.7 Stirring, heating and syneresis

Once cut, the curd is stirred and heated. Stirring and heating combined with the acid development resulting from the activity of the starter, have a significant impact on moisture and calcium phosphate’s dissolution and consequently on the final characteristics of cheese. During syneresis casein molecules rearrange resulting in casein network’s tightening since moisture is expelled from the network. Temperature, pH drop after the curd’s cut and pressure are the most important factors influencing syneresis. After cutting the coagulum, the greater the drop in pH, the more moisture is removed from the curd; the higher the temperature used to heat the curd, the lower is the moisture in the curd. In addition to other factors, also the pressure applied on the curd

increase the rate of syneresis and the rate at which the ‘free serum’ is removed from the curd as whey. Indeed, when pressure is applied with high intensity and for long time, the amount of whey that is removed from the curd increase.

In addition, since most of water in cheese is mobile because just mechanically trapped within the casein network, it can freely move out of the cheese if sufficient ‘force’ is applied. Low pH (<4.95), low humidity or drying, pressed block of cheese’s, dry salting and proteolysis or the breakdown of the casein network, are forces enough to move water out of the casein network. [21]

2.2.8 Whey removal, hooping and salting

The texture of the cheese as well colour and flavour are influenced by the way whey and curd are separated. Basically, whey can be removed in three ways. Briefly, whey is drained from the holes of the moulds to produce soft cheese, while in the production of most hard and semi-hard cheese, whey is drained from the vat, the curd is held behind a sieve and a channel is made in the curd mat to allow the whey to flow. Salt is sprinkled onto the curd absorbing more moisture from it. With or without continuous stirring, the salty whey starts to drain. The curds are then put into moulds and pressed. [21]

2.2.9 Brining and/or dry surface salting

If before pressing salt is not added to the curd, cheese can be added with salt by soaking in a saturated saline solution (brine) or by rubbing salt on its surface during maturing. The brine is regenerated daily to prevent infections due to the presence of unwanted microorganisms and to restore the initial saline concentration which decreases due to salt absorption and serum release from the cheese. In addition, microorganisms causing turbidity must be eliminated.

Rubbing salt onto the cheese is a traditional method of maturing cheese and is typically performed in small farm. Cheesemakers can allow yeasts and moulds, or in general microorganisms possessing the ability to survive in dry, high-salt conditions to grow on the cheese. The inside of the cheese is protected by the rind; however, yeast and mould metabolites can move through the rind and result in a desirable or undesirable cheesy flavour depending on the taster's reference point. The cheese looks "traditional" and has a certain natural, earthy appeal. [21]

2.2.10 Pressing

During pressing the curd assumes the desired shape, the whey is expelled and, under pressure, the curd welds more quickly. The condition of the curd at the time of pressing and the decrease in pH (loss of colloidal calcium) during pressing are factors affecting the time, pressure and efficiency of pressing.

2.2.11 Ripening

The ripening of cheese implicates profound physical and chemical-physical modifications causing the conversion of the curd's components into substances characterizing the taste, aroma, colour, appearance, and the texture of the final product. [21]

Chapter 3
TYPES OR RENNETS AND COAGULANTS

Several types of rennet and coagulants have been employed for cheese's manufacturing. Several authors have analysed the types of rennet and coagulants and their characteristics. According to their sources, rennet and coagulants are generally categorized as described in Table 1 reporting the major types of coagulant used for cheesemaking today, together with their active enzyme components. [2]

3.1 **Animal rennet**

Animal rennet contains all the enzymes, in particular chymosin (or renin) and pepsin, which acting directly on the protein chains, are necessary for the digestion of milk. Animal rennet is obtained from the abomasum of non-weaned ruminants including calves, lambs, and little goats. Among the animal origin's product, calf rennet, due to its high content of chymosin is considered the ideal enzyme product for cheesemaking. Depending on the animal's age and the feeding regime, the proportion between the two enzymes, chymosin and pepsin, varies. Generally, chymosin production is stimulated in milk-feeding and young age ruminants. Extracts from young calves have high proportion of chymosin content, while adult bovine rennet is an extract from older animals and has a much higher content of pepsin; consequently, since animals are slaughtered at different ages and all kinds of mixtures of the extracts exist, currently a broad range of composition for commercial rennet exist. Adult bovine rennet, containing the same active enzymes as calf rennet, is the most widely used substitute. High sensitivity to pH and a higher general proteolytic activity is characteristic of the adult bovine rennet due to the high pepsin content. Lamb/ovine and kid-caprine/caprine rennets are some of the numerous niche products currently existing on the market; however, despite they are very similar to calf/adult bovine rennet, their application is appropriate for clotting milk of their own species. A mixture of animal rennet and lipases is used to provide a characteristic flavour typical of the South Italian cheeses whose production often involves their use. Such products are generally referred as rennet paste and are obtained by maceration and drying of stomachs from calves, lambs or kid-caprine, which

have recently been suckling, to have the stomachs filled with milk. This result in a rennet paste containing a mixture of rennet and lipase enzymes. [2,16]

3.1.1 Specific molecular aspects of the enzymes

Chymosin (EC 3.4.23.4) is an endopeptidase of animal origin that allows the formation of curdle by the hydrolysis of k-casein. Is the most specific milk-clotting enzyme characterized by high milk-clotting activity and its low general proteolytic activity. It belongs to the hydrolase class, necessary for the digestion of casein present in milk. Together with pepsinogen, it is produced by adelomorphic (or zymogenic) cells at the level of the gastric glands proper of the stomach mucosa. Its production is greater in the neonatal period. The specificity of the enzyme is very wide-ranging and overlays that of pepsin A. Milk's coagulation for the production of rennet coagulated cheese exploits the casein system's distinctive characteristic. The casein in milk exists as large colloidal particles, known as casein micelles. The micelles are stabilized by k-casein, which is the only casein to be glycosylated and is concentrated on the surface. Hence, k-casein keeps all micelle in suspension into milk avoiding the aggregation with other micelle. The micelles' stability is lost when the surface k-casein layer is damaged by heat, alcohol, or proteinases. Chymosin selectively hydrolyses the peptide bond between two specific amino acid residues in k-casein: phenilalanin$_{105}$ and metionin$_{106}$ yielding a hydrophilic glycopeptide (casein-macropeptide, CMP) that is released, and para-k-casein, a hydrophobic peptide that remains in the micelle. (Figure 1) Glycomacropeptide is hydrophilic, so it means that contains the sugar. Once chymosin has removed from k-casein the hydrophilic/glycosylated part, all the micelles aggregate and precipitate. The glycomacropeptide is released in the serum and para-k-casein remains in the micelle because is a hydrophobic peptide, remaining so in the curdle. Para-k-casein precipitates in the presence of Calcium. Thereby, k-casein loses its stabilizing effect: in the presence of a critical concentration of Ca^{++}, and at temperatures above 18 °C micelles aggregates causing the curdle formation. Chymosin of the different species varies in relation to the specific clotting activity of milk, as well as for all other milk coagulation enzymes. Because since the beginning the development and production of most cheese varieties has mainly employed calf rennet (80-90% chymosin), the use of other coagulants will result in different flavours than a preparation dominated by chymosin. [2,16] In addition to chymosin, rennet contains other enzymes including pepsin and a lipase.

Pepsin (EC 3.4.23.1) is the name given to several digestive enzymes secreted by glands that line the stomach; its etymology derives from Greek word πέψις -pepsis- meaning "digestion" and it was discovered in 1836 by the German physician and physiologist Theodor Schwann. Pepsin is

an aspartic protease possessing a pH optimum of between 1.5-2 and that uses a catalytic aspartate in its active site. At pH over 3.5 the proteolytic activity of pepsin is lost while at pH 5 gets irreparably denatured. Stomach parietal cells also secrete hydrochloric acid allowing the pepsinogen, the inactive precursor (or zymogen) to be converted to active pepsin by the enzymatic action of pepsin itself. In the stomach, pepsin hydrolyses proteins at peptide bonds on the amino-terminal side of the aromatic amino acid residues Phe, Trp, and Tyr, cleaving long polypeptide chains into a mixture of smaller peptides. [19] Pepsin possesses a greater proteolytic activity compared to chymosin, however, is less specific.

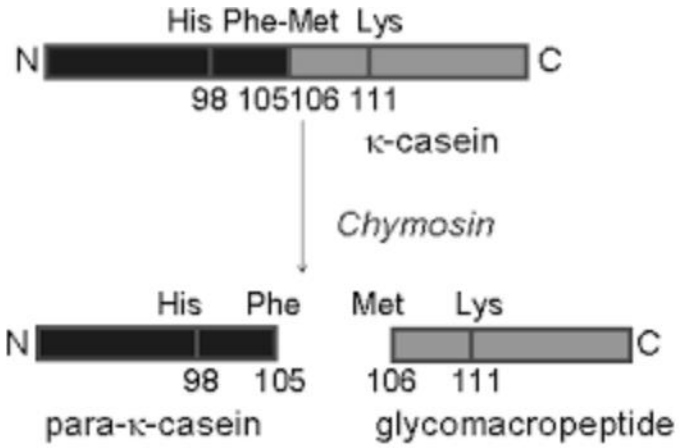

Figure 1: The aspartic proteases hydrolyse the Phe_{105}-Met_{106} bond of k-casein, dividing the protein into two: the hydrophobic para-k-casein and the hydrophilic macropeptide casein.

Lipases (EC 3.1.1.3) are a class of hydrolases, mainly accountable for the hydrolysis of acylglycerides. Differently from esterase, lipases have the capability to interact at the aqueous and non–aqueous interface. Particularly, lipases catalyse the hydrolysis of fatty acid ester bond in the triacylglycerol at the interface between an insoluble substrate phase and the aqueous phase releasing free fatty acids (FFAs) through a reversible reaction. Hence, in absence of water the esterification and formation of glycerides from fatty acids and glycerol occurs. [20] The hydrolysis of triacylglycerols by lipases into mono- and diacylglycerols and FFAs is commonly referred to as lipolysis. By releasing short chain fatty acids (SCFA), lipases provide new flavours and aromas affecting significantly the quality of food products including cheeses. In milk great part of lipolysis is carried out by the native lipoprotein enzyme (EC 3.1.1.3.4). However, lipoprotein enzyme (LPL) is almost completely inactivated during conventional pasteurization treatment (72 °C for 15 s) resulting hence in a limited contribution to lipolysis in milk or cheese, unless the milk fat globule in the raw milk is physically damaged, allowing access of the LPL to the milk fat triacylglycerols. Lipolysis must be controlled to avoid unpleasant consequences in taste and the

formation of undesired spicy flavours and aromas. Nevertheless, a high level of lipolysis is desired in rennet-curd cheeses (e.g., blue-type cheeses), where lipases or lipases from secondary starter cultures are added to access easily fats and resulting in the selective hydrolysis of the triacylglycerols and release of FFA that lead to the desired flavour. C16: 0 (palmitic), C18: 1 (oleic) and C14: 0 (myristic) are the principal fatty acids in milk fat on a total weight basis; instead shorter chain fatty acids (C4:0 to C12:0), occurring in lower quantities on a weight basis, are the main responsible for the piquant flavour of hard Italian cheeses, (e.g. Parmesan and Romano), and of the typical flavour of sharp goatee-like of soft-milk cheeses. Lipase enzymes, as a result of damage to the milk fat globule membrane (MFGM) during the processing and aging of the cheese, have access to these fatty acids that are hydrolysed. Added exogenous enzymes (e.g., added rennet paste, pregastric esterase), secondary flora (*Brevibacterium linens*, *Penicillium roqueforti*, *Geotrichiun candidum*) and lactic acid bacteria starter culture are the primary sources of lipases.[2]

Table 1: The most commonly used rennet and coagulants and their enzymes. [2]

Group	**Source**	**Example of rennet and coagulant**	**Active enzyme components**
Animal	Bovine stomach	Calf rennet, adult bovine rennet	Bovine chymosin A, B and C, pepsin A and gastriscin
		Rennet paste	The same as above, plus lipase
	Ovine stomachs	Lamb rennet, ovine rennet	Ovine chymosin and pepsin
	Caprine stomachs	Kid-caprine rennet, caprine rennet	Caprine chymosin and pepsin
Microbial	*Rhizomucor miehei*	Miehei coagulant type L, TL, XL and XLG/XP	*Rhizomucor miehei* aspartic proteinase
	Cryphonectria parasitica	Parasitica coagulant	*Cryphonectria parasitica* aspartic proteinase
Fermentation produced chymosin (FPC)	*Aspergillus niger*	CHY-MAX™ CHY-MAX™ M	Bovine chymosin B Camelus chymosin
	Kluyveromyces marxianus var. *lactis*	Maxiren®	Bovine chymosin B
Vegetable	*Cynara cardunculus*	Cardoon	Cyprosin 1, 2 and 3 and/or cardosin A and B

Fat in cheese is the most important component that influences, not only its physical properties, but mainly the development of flavour. Sanjuán et al. (2002) found that cheeses made with vegetable rennet (*C. cardunculus*) contained more fat than cheeses made with animal rennet, since

it seems that vegetable rennet has a greater capacity to capture fatty components in the curd. This higher fat content may explain the finer texture and higher unctuousness described for the cheeses produced with vegetable rennet when they were sensory evaluated. [22]

3.2 **Genetically modified rennet**

On the market since 1990, rDNA-products with the general name of "Fermentation-produced Chymosin" are one of the most important alternatives to calf rennet. FPC is chymosin produced by fermentation of a GMO (Table 1). FPG possessing the same amino acid sequence as chymosin from the corresponding animal stomach, contains chymosin equal to the animal source, however, it is just produced more efficiently. The main FPC, containing bovine chymosin B, is actually considered the ideal milk-clotting enzyme compared to all other milk-clotting enzymes. Research has also been carried out on recombinant chymosin identical to camel chymosin. FPC (camelus) was discovered to have very high specificity against caseins thus leading to high cheese yields without creating any bitterness. Due to such properties, FPC (camelus) is considered to be a more efficient coagulant for bovine milk than bovine FPC.

3.3 **Microbial coagulants**

The most broadly known microbial coagulants used for cheesemaking are of fungal origin and are produced using *Rhizomucor pusillus, Rhizomucor miehei* and *Cryphonectria parasitica.* (Table 1). Instead, due to their too high proteolytic activity, bacterial proteases have been found to be unsuitable as milk-clotting enzymes. *Rhizomucor miehei* is the predominant microbial coagulant dominating the market and it is available in four types, all significantly more proteolytic than chymosin. The *Cryphonectria parasitica* coagulant has a very high general proteolytic activity and a low pH dependency, however, being very heat labile, is inactivated at high temperature, thus *parasitica* coagulants does not influence the ripening process of cheeses. Due to these features, *parasitica* coagulants is merely used in the manufacturing of cheeses cooked at high temperatures. *Rhizomucor pusillus* coagulant is no longer produced commercially because it has no further advantage over *Rhizomucor miehei* to which it is very similar. Microbial coagulants possess the advantage of unlimited availability since they can be easily produced by fermentation. [2,16]

3.4 **Vegetable coagulants**

The last group of enzymes reported in Table 1 derives from plants. Although enzymes extracted from *Cynara cardunculus* cardoon are considered to be particularly suitable in cheesemaking, several enzymes from other plants have been found to coagulate milk and hence to be suitable as well. Mainly used in the past in the traditional dairy sector, recently vegetable coagulants have gained more attention and interest for reasons related to dietary and ethical choices. Indeed, vegetable coagulant are the subject of many studies. Plant coagulant's enzymatic activity is mainly related with the activity of aspartic proteases (APs) or those with serine and cysteine residues. [2,16] In cheese manufacturing, the degradation level of the protein matrix of milk is influenced by the use of different plant proteases, thus leading to differences in cheeses' sensory properties.[1]

Lower yields of cheese, bitter flavours and consistency imperfections are however defects occurring due to the excessive proteolytic nature of most vegetable coagulants, limiting their use in the production of cheeses. Consequently, the appropriate selection of suitable plant coagulant and control of different gelation parameter is of paramount importance to obtain high-quality final products. Despite the drawbacks, encourage the production of dairy products based on vegetable coagulants is important to solve and overcome issues related to the restrictions imposed by religions (e.g., Islam and Judaism), to support vegetarian diets and comply with legislative prohibitions not allowing the use animal rennet (e.g., Germany, the Netherlands and France). In addition, the use of vegetable coagulants in the artisanal field allows the production of niche cheeses characterized by a bitter note with a creamy consistency, acidic flavour and sometimes piquant. In addition, different studies suggest that the viscoelastic properties of soft-acid-curd cheeses is favoured since the mobility and water retention of individual caseins is increased as a consequence of the general hydrolysis carried out on casein micelles by plant-derived proteases. Therefore, during the manufacture of soft-acid-curd cheeses, the acid-induced gelation could be assisted by the use of milk plant- derived proteases. [18]

Chapter 4
MILK-CLOTTING ENZYMES. BIOCHEMICAL PROPERTIES OF PROTEASES AND PRODUCTION METHODS EMPLOYED.

To understand the similarities and differences between the products, the analysis of the molecular aspects of the milk-clotting enzymes present in rennet and coagulants is of paramount importance. [2] Based on the catalytic mechanism used during the hydrolytic process; plant proteases are classified into various groups. Aspartic, serine, and cysteine proteases are the main classes of milk-clotting proteases. [1] That of aspartic proteases is the family to which all enzymes primarily used for making cheese belong to and are characterized by having the same catalytic mechanism, with two aspartic acid residues in the catalytic site. Several diverse types of rennet and coagulants as well as their characteristics were reviewed by several authors and have been used for manufacturing of cheese. According to what reported in the literature, milk can be coagulated by the activity of any enzyme of plant origin, however, the extract from the thistle *Cynara cardunculus* seems to be particularly suitable. Since ancient times indeed, in many countries and especially in Portugal, *Cynara cardunculus* flowers have been used in the artisanal production of high-quality cheeses such as Serra and Serpa. [2]

4.1 **Types and sources of plant proteases involved in milk coagulation**

Plants require proteases in all aspects of their life cycle being involved in many phases including during seed germination for the mobilization of storage proteins to the initiation of cell death and senescence programs.

Based on the catalytic mechanism used during the hydrolytic process, proteases have been divided into groups: aspartate, serine, cysteine, and metalloproteases are the main catalytic types; however, only aspartate, serine, cysteine plant proteases are used as milk coagulants and none from metalloproteases (Table 2). Serine and cysteine proteases are very diverse for the catalytic point of view from aspartic and metalloproteases in that the catalytic site's nucleophile is part of an amino acid, whereas it is an activated water molecule in the other two groups. [3]

Plant rennet's enzymatic activity is mainly related to the action of APs or those with cysteine and serine residues. In fact, the degradation level of the protein matrix of milk is influenced by the use of different types of plant proteases in cheese technology, thus leading to differences in sensory properties of cheese. Most cheeses produced with plant coaguloant are characterized by bitter flavours due to excessive PA, this limits plant coagulant industrial use. Hence, appropriate plant coagulant's selection and the different gelation parameters' control are of great importance to obtain a better quality of final product. [1]

4.1.1 Aspartic proteases

Aspartic proteases take part in different mechanisms during the development process of plants; indeed, they are involved in the mechanisms of preservation and degradation of proteins and in the responses to stress, attacks by pathogens and in the plants' senescence. Two aspartic residues are responsible for the catalytic activity of these enzymes, whose specificity of action is preferred for the cleavage of peptide bonds between hydrophobic amino acid residues. Most of these enzymes are heterodimeric proteins with large subunit of 28–35 kDa and small subunit of 11–16 kDa, and only very small number of monomeric proteins with molecular mass of 36–65 kDa. [1]

Two aspartic residues are present in the catalytic site of the aspartic proteases and according to Domingos et al. (2000), aspartic proteases are most active at acidic pH and show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues responsible for the catalytic activity. [5]

Aspartic proteases with milk-clotting activity have been reported in rice kernels, milk thistle (*Silybum marianum L. Gaertn*.), artichoke (*Cynara scolymus L*.); in *Onopordum taurcicum* and *Centaurea calcitrapa*. Mediterranean regions traditionally use cardoon (*Cynara cardunculus*) flowers in cheesemaking. Cardosins and cyprosins are aspartic proteases produced by cardoon where accumulate in petals and pistils of mature flowers but not in leaves or seeds. [3] Cardosins possess a characteristic high milk-clotting activity, cleaving the peptide bond Phenilalanin$_{105}$ - Metionine$_{106}$ in bovine and ovine k-casein. Such vegetable coagulant has a marked proteolytic action in vitro, as well as a coagulating activity similar to that of chymosin. An extensive degradation of caseins in the dairy matrix is obtained by this coagulating activity producing cheeses characterized by the development of a typical aroma and a creamy and slightly spicy flavour and a soft and buttery texture. [9]

Table 2: example of milk-clotting proteases from plants: source, classification, and milk-clotting activity.

Plant	Organ	Type	Name	Number and class	MCA/total MCA/specific MCA	References
Cynara cardunculus	Flowers	Aspartic protease	Cardosin	8 Cardosin A Q9XFX3/AJ 132884 Cardosin B Q9XFX4/AJ 237674 Cardosin C Cardosin D Cardosin E P85136 Cardosin F P85137 Cardosin G P85138 Cardosin H P85139	Extract: 0.131 ± 0.025 UAC/mL (1 h of maceration) 0.164 ± 0.024 UAC/mL (24 h of maceration) Cardosin A: 1160 UACI/g Cardosin B: 7556 UACI/g	[23- 24]
Cynara scolymus	Flowers	Aspartic protease	Cynarase	3 Cynarase A Cynarase B Cynarase C	Extract: Between 60 and 70 CAU/mg 30 CAU/mg 100 CAU/mg Between 30 and 40 CAU/mg	[25-26]
Cynara humilis	Flowers	Aspartic protease	Cardosin A like	-	-	[27]
Silybum marianum	Flowers	Aspartic protease	Enzymatic extract	-	0.083 CAU/mL	[28]
Orytza sativa	Seeds	Aspartic protease	Oryzasin	1	-	[29]
Moringa oleifera	Flowers	Aspartic protease	Enzymatic extract	-	1.9 CAU	[30]
Onopordum acanthium	Flowers	Aspartic protease	Onopordosin	-	-	[31]
Cirisum vulgare	Flowers	Aspartic protease	Cirsin JN703462	-	-	[32]
Centaurea calcitrapa	Cell suspension	Aspartic protease	Enzymatic extract	-	2.023 U/mg	[33]

Albizia lebbeck	Seeds	Cysteine protease	Enzymatic extract	-	Crude extract: 156×10^{-3}U/mg Concentrated extract: 591×10^{-3} U/mg	[34]

4.1.2 Serine proteases

A serine residue is present in the active site of the serine proteases which possess and share a series of biochemical and physiological characteristics. Particularly abundant in fruits, they are widespread among the taxonomic groups in plants where they are present in almost all parts. In plants their main role is similar to that of APs, but with some additional features. Plant serine proteases have been found and extracted from, seeds, latex, stems, leaves, flowers and roots. [3]

4.1.3 Cysteine proteases

Cysteine proteases or thiol-proteases, rely their catalytic mechanism on a cysteine group in the active site. Thanks to their capability of being active over a broad range of temperature and pH, beside the food industry, cysteine proteases have great potential in biotechnology and pharmaceutical industries. Since, cysteine proteases occur naturally in different tissues, and in some cases in some cases in excessive amount, plants represent an attractive alternative to produce CPs. In example, ficin can be isolated from the latex of different *Ficus* species like *Ficus racemose;* and possessing specific ability to digest casein, is particularly adequate in cheesemaking for its milk-clotting property.[3]

4.2 **Production of plant proteases**

According to Gonzalez-Rabade et al. (2011), proteases used as milk coagulants have been identified and analysed from almost all plant part whether it may be seed, flower, or latex. These enzymes can be obtained from their natural source or through in vitro culture ensuring a continuous supply of plant proteases. [3, 6]

4.2.1 Production from natural sources

Generally, various vegetable organs such as flowers, seeds, roots and leaves are used as a natural source for the extraction of these enzymes by aqueous maceration. The aqueous extract of plant material can be obtained by different preparation methods. As described by Roseiro et al.

(2003), once the dried whole or crushed cardoon flowers are soaked in water at room temperature for a variable time period, the filtrate is collected, and this crude extract is used as coagulant. [7] Grinding the dried flowers with crude kitchen salt, is and alternative method of extraction; and after laying the paste on a cotton cloth, the enzymes are solubilized by percolation with warm milk. Depending upon the desired degree of purification, further purification of the crude extract can also be performed to obtain partially purified enzyme or pure enzyme. [3]

4.2.2 In-vitro production

In vitro techniques are an alternative well-established method to obtain milk-clotting proteases. In vitro techniques have numerous advantages and potentials like the capability to overcome the low enzyme yield and difficulties encounter during the extraction from the natural sources. In addition, in vitro techniques allow to solve problems due to climate and season conditions, and product's heterogeneity obtained from plant parts.[3] Numerous studies are reported in literature regarding the in vitro production of enzymes for coagulation of milk. They use different vegetable and technical materials. In example, Tamer and Mavituna (1997) used the culture of *Mirabilis jalapa* to produce proteases and found that the proteolytic yield was higher with proteases produced in vitro as compared to the proteases from the intact plant.[8]

Chapter 5
APPLICATION OF RENNET AND COAGULANTS: ANALYSIS OF COAGULANTS

Because of the increasing range of products and mixtures of products available on the market, since the 1970s, the demand to be able to analyse rennet and coagulants has increased.

Though belonging to the same group of aspartic proteinases and possessing similar milk-clotting properties, all enzymes used for cheesemaking reveal many slight but important differences for the application. Indeed, the difficulties faced when evaluating milk-clotting enzymes is due to the great similarity among analogous enzymes. However, since different rennet and coagulants have different values for cheesemaking, the analysis of the products from a financial and quality perspective is of paramount importance. Analytical methods are genenerally employed. The strength (enzyme activity), enzyme composition, identity and purity are the most relevant parameters of rennet and coagulants to be analysed. Many methods have been used to measure the strength; among these, Soxhlet and Berridge are the most employed. The German agricultural chemist Von Soxhlet (1877) defined the strength (the total milk-clotting activity) of a rennet as Soxhlet units. Soxhlet units are defined as the volume of milk, which one volume of enzyme preparation is able to clot in 40 min at 35 °C. The strength is expressed as ratios referring to the amount of rennet that is able to clot a certain amount of milk, e.g., 1:14 000 means that 1 mL of rennet is able to clot 14 000 mL of milk. Despite the Soxhlet unit is easy to be understood by cheesemakers, it is greatly affected by the pH and the milk's quality and the lack of reference standards to be used. This justifies the sporadic use of the Soxhlet unit to be used as a guideline for the approximate strength. To improve the exactness of milk-clotting activity tests, the English scientist Berridge (1952) instead of raw milk, introduced the use of a standardized milk powder reconstituted in 0.01 M $CaCl_2$. Afterward, the Berridge units (or rennin units - RU) have been extensively used. One RU is expressed as the activity which is able to clot 10 mL of standardized milk in 100 s at 30 °C. Nevertheless, since the Berridge milk's calcium content is high and the pH 6.3 of Berridge substrate is lower compared to the level of most cheesemaking (pH 6.4–6.6), even the Berridge method has drawback resulting in a misleading strength compared to how the products behave during most cheesemaking. Despite the drawbacks of both methods, the fact

remains that Soxhlet and Berridge' s idea influenced all the different national definitions of rennet strength and the following standard methods used. The International Organization for Standardization (ISO) and the International Dairy Federation (IDF) developed the international standard method employed nowadays for the analysis of the strength. Specifically, for the analysis of total milk-clotting activity of animal rennet and FPC, the IDF method (IDF, 2007) was developed, whereas the analyses of microbial coagulants employ the IDF (2002) method. The idea is that the clotting time is measured in milk at pH 6.5, for a sample relative to the international reference standards with the same enzyme composition as the sample. This method is very robust because the standards would react in the same way to any variations in the test conditions. The strength measured by the IDF methods is expressed in international milk clotting units (IMCU). [2]

Chapter 6
USE OF VEGETABLE COAGULANTS IN CHEESEMAKING

Beside the capacity to coagulate milk, numerous plant extracts have also proteolytic activity (Table 3). Proteases such as ficin from *Ficus sp*., papain from *Carica papaya* and cynarases or cyprosins, commonly referred as cardosins, from *Cynara sp*., are mainly components of plants' leaves or flowers latex, and sometimes of fruits, roots, seeds and/or sap.

Table 3: sources of vegetable coagulants

Scientific name	Common name	Reference
Albizia julibrissin	Silk tree	[35]
Ananas comosus	Pineapple	[36]
Calotropis procera	Sodom apple	[37, 38]
Carica papaya	Papaya	[39]
Centaurea calcitrapa	Red star thistle	[40]
Cirsium	Thistle	[41]
Cucurbita pepo	Pumpkin	[42]
Cynara cardunculus, C. humilis, C. scolymus	Cardoon and artichoke	[43]
Ficus carica, F. glomerata, F. religiosa	Fig tree	[39]
Lactuca sativa	Lettuce	[44]
Silybum marianum	Holy thistle	[45]
Streblus asper	Siora or Rusa	[39]
Taraxacum officinale	Dandelion	[35]
Withania coagulans	Withania berry	[39]

Animal and microbial coagulants possess the advantage of preventing the labor-intensive and expensive collection of plants, are cheaper and easier to use; in addition, they even provide more consistent products. Instead the plant coagulants, not possessing the same qualities, make the production of the cheese mainly linked to the artisanal scale, in farmhouse or small dairies. However, they provide an important socio-economical contribution to the dairy sector both at regional and local areas thus playing a significant part in the local agricultural economy. [7]

6.1 General use of vegetable coagulants: enzymatic role in milk coagulation and cheesemaking

When vegetable rennet is added, the two main steps of enzymatic coagulation of milk occur identically as coagulation with calf rennet (or calf chymosin) previously discussed in chapter 3. Beside the main role of plant proteases in the coagulation of milk, they have a significant role in the initiation of cheese maturation. Substrates essential for a certain bacterial microflora are produced following the hydrolysis of the caseins in the cheese by the residual coagulant, which results in the development of flavour during maturing. However, the type of vegetable coagulant, its dose and its enzymatic activities influence the intensity of these effects on the quality of the cheese. [1] Although, vegetable coagulants have been employed for many centuries, investigations revealed that vegetable coagulants have some characteristic downsides that limit their use. This is mainly due to low MCA/PA ratios and the presence of too proteolytic plant proteases responsible of the generation of abundant acidity, bitter flavours and also texture defects in cheese such as increased tendencies for shape's loss. Therefore, the estimation of enzymatic activities and their comparison with those of commercial rennet (chymosin) is an essential first step in selecting an appropriate vegetable coagulant. [1,7]

Typical Portuguese and Spanish ewe's milk cheeses are traditionally produced using *Cynara sp.* (cardoon) extract. In West African countries, such as Nigeria and the Republic of Benin, traditional cheesemaking employs *Calotropis procera* (Sodom apple) extract. [7]

Chapter 7
ONOPORDUM TAURICUM: CHEMICAL, TECHNOLOGICAL AND NUTRITIONAL TRAITS

Mediterranean Basin is considered to be one of the planet's biodiversity "hotspots", due to the region's high level of endemism. [11] *Onopordum tauricum* is a biennial thistle occurring in uncultivated and ruderal environments and possessing a peculiar etymology; indeed, "*Onopordum*" originates from the Greek ονος -onos- meaning "donkey" and from de Greek πορδη -pordè- meaning "fart", due to the presumed effects of intestinal turbulence that the plant provides to the donkeys who are greedy for it. The specific epithet from the Latin "*tauricum*" meaning of Tauria (Crimea), in reference to the predominantly Pontic distribution area that extends from the Balkans to Ukraine, Turkey and Syria. This species native to Eurasia mainly with a Pontian range, belong to the *Asteraceae* family and has been chosen as candidate wild plant for possible use as milk-clotting agent in research aimed at the production of traditional Mediterranean cheeses with non-animal rennet in order to meet the increasing demand of vegetarian and animal rights consumers. [12]

(a) (b)

Figure 2: inflorescence (flower head) of Onopordum tauricum (a) whole; (b) section.

The biennial herbaceous plant, 50-200 cm tall, has robust, green, not white-tomentose stems, but densely glandular, winged up to the apex under the flower head; with very wide wings, often interrupted, equipped with long patent thorns.

The basal leaves are oblong-lanceolate, grossly toothed with triangular lobes, strongly thorny, sprinkled with musky odor glands and with long decurrent caulins on the stem. The hemispherical flower heads, 4-6 cm in diameter, are terminal and mostly solitary, with imbricated, green or often purple, scales of the envelope, lesiniform and 3-5 mm wide, densely glandular on both faces and equipped with robust spines 3-7 mm; the lower scales, on the other hand, are generally reflected. Flowers are purple tubuloses, hermaphrodites, long 25-27 mm, glabrous or with rare glands. Stamens have hairless filaments and the receptacle is bare, without scales or hairs, with alveoli with denticulate edges. The fruit is a cypsela (achene) without a beak, with transverse furrows and with a pappus of simple, serrated bristles, ring-welded at the base. [13, 14, 15]

Nevertheless, Taurian thistle's data about the chemical and nutraceutical traits are scarce. Petkova and Mihaylova (2016), performed phytochemical studies aimed at revealing the presence of prebiotics (fructans, inulin) and antioxidants (polyphenols) in ethanol and water extracts obtained from flower heads of *O. tauricum* Willd grown in Bulgaria. For the first time, *O. tauricum* Willd. flower heads were discovered to be a rich source of prebiotics and total phenols revealing the potential of *O. tauricum* plant to be used as a source of prebiotics and soluble dietary fibers for the human nutrition. This suggests even other application of the valuable plant not only in the food sector but event in herbal cosmetics as a natural source of bioactive compounds and phytonutrients. [48] Bruno et al. (2011) described the occurrence of ten sesquiterpene lactones, including a new elamanolide and four new eudesmanolides, flavonoids (apigenin, acacetin, luteolin, hispidulin, nepetin, apigenin 7-O-glucoside, luteolin-7-glucoside), and derivatives of cinnamic acid (caffeic acid, chlorogenic acid) in the chloroform extract of *O. tauricum*'s leaves. [49] In addition, Targan et al. (2018) investigated the macro (Na, Mg, and Ca) and trace elements (Li, Fe, Zn, Mn, Se, Al, V, Cr, Ni, Cu, Pb, As, Co, Cd, and Hg) by inductively coupled plasma-mass spectrometry (ICP-MS), after microwave digestion of the aerial parts. [50] Finally, Erciyes et al. (1995) reported that linoleic acid is the most abundant component of *O. tauricum* seed oil. [51]

Chapter 8
CHEESEMAKING WITH WATER CRUDE EXTRACT OF FLOWERS OF ONOPORDUM TAURICUM

8.1 **Plant material and crude extract preparation**

Raw materials: Artichoke violet flowers (*Onopordum tauricum*) were collected in July 2020 in experimental farm of the Marche Polytechnic University in Agugliano, Italy (43°32' N, 13°22' E). The materials were prepared according to Mozzon et al. (2020). Immediately after harvesting, with the help of forceps and scalpels, the violet flowers were separated from receptacle and macerated in demineralized water (1:10 *w*/*v*) for 24 h at 4 °C. Through a muslin cloth, the liquid phase was recovered by filtration and then centrifuged (5000× *g*, 10 min). Finally, the resultant aqueous crude extract was freeze-dried (VirTis Advantage benchtop freeze dryer Steroglass S.r.l., Perugia, Italy) and stored at −20 °C until it was used. At the time of use, the dried extract was reconstituted in demineralized water 1:10 *w*/*v*. [10]

8.2 **Cheese manufacture**

The cheeses studied in this thesis were made raw ewe's milk and water crude extract of *Onopordum tauricum* as rennet.

Briefly, cheeses were manufactured from 5 l of raw ewe's milk which was heated at 37 °C. Milk coagulation occurred by the addition of an aqueous extract of *Onopordum tauricum* thistle flower, without the addition of any commercial starter culture. The technological optimization applied for the manufacturing process of cheese identified the optimum conditions to obtain the greatest quality cheese as being 3 ± 1 g of *O. tauricum* plant extract plus 10 mM of $CaCl_2$. The coagulation time was approximately 30-40 min, after which it was manually cut to rice-sized grains. Curd's portions of irregular shape and size were transferred to moulds to release whey. After whey drainage, cheeses were removed from the moulds and externally salted.

The ripening process, lasted 2 months, occurred in controlled environment at 12 °C, 70% of relative humidity (HR). In this phase, cheese loses humidity and allows for microbial growth

favourable for ripening. The main steps involved in the manufacture of cheeses are shown in Figure 3. The same methods have been adopted for the production of cheese with commercial rennet of *Cynara cardunculus;* about 1.4 mL in 5 l of raw ewe's milk.

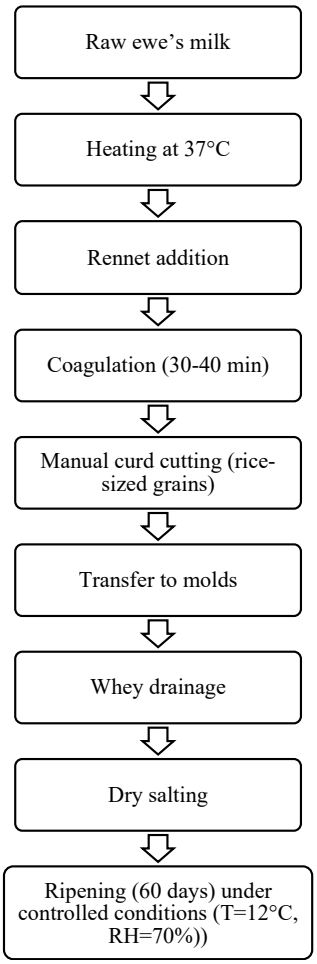

Figure 3: flow chart for cheese manufacture

8.3 **Experimental section**

The cheese samples were coded with a letter (O representing *Onopordum*, COM for ''Commerciale" and a number (1–3) representing the tree plant extract used in the cheese making. Cheese sample obtained with the plant extract were analysed in duplets and coded as "BIS". A total of nine samples were analysed.

Chapter 9
CHARACTERIZATION OF FATTY ACIDS IN CHEESES

9.1 **Characterization of the acidic composition**

The main components of the lipid fractions of animal origin are fatty acids. The term "fatty acids" groups a whole series of molecules with very different characteristics. Fatty acids differ from each other in chain length, degree of unsaturation (monounsaturated, polyunsaturated, conjugated etc.) as well as in geometric isomerism (cis, trans).

In order to identify the greatest number of fatty acids, complex analytical techniques using advanced chromatographic and spectroscopic methods are required.

The main instrumental analytical techniques used for the separation and subsequent identification and quantification of individual fatty acids in food and biological matrices are chromatographic techniques such as gas chromatography (GC) and HPLC (High Performance Liquide Chromatography).

The gas chromatography technique is certainly the most common and convenient one as it allows, with a single analytical run, the determination of a large number of fatty acids thanks to the use of a universal detector such as the FID (Flame Ionization Detector).

9.2 **Derivatization of fatty acids**

The identification and quantification of the fatty acids that make up the various lipid fractions through gas chromatography or HPLC techniques is preceded by their conversion by derivatization into apolar molecules with low molecular weight. This transformation, called methylation, produces methyl esters; and is fundamental as it allows the chromatographic analysis of compounds that would otherwise be difficult to analyse, also allowing accurate identification and quantification of the compounds themselves.

The formation of methyl esters (FAME, Fatty Acid Methyl Esters) normally occurs in the presence of a catalyst. To conduct the laboratory analyses, hydrochloric acid was employed.

9.3 **Identification of fatty acids by GC**

The most commonly used methods for determining the content of fatty acids present in matrices of natural origin are gas chromatography (GC) techniques.

9.4 **Material and methods**

9.4.1 Fat determination in cheese samples by Soxhlet extraction

To extract the lipid fraction from cheeses, FatExtractor-500 was employed. The dry matter was assessed by a gravimetric method. With the analytical balance, 9 aluminum cones were labelled that to correspond to each cheese sample and were weighed. Subsequently, 50 g of cheese were weighed in aluminum cones for all nine samples and introduced into the freeze dryer (VirTis Advantage benchtop freeze dryer, Steroglass S.r.l., Perugia, Italy). After extracting the cones from the freeze dryer, they were weighed again and dry matter was calculated. All data are reported in table 5 and 6. The samples were finely shredded in order to increase the exposed surface necessary for the crude fat content determination by solvent extraction with diethyl ether. Dry and clean backers were used for the Soxhlet extraction. Backers were previously dried at 102°C for at least 30 min; then they were let cooled down to ambient temperature in a desiccator for at least 1 h. The beakers were taken with a pincer and transferred from the desiccator inside the analytical balance. The exact weight prior to extraction was recorded. A white thimble to be introduced into the Soxhlet was associated with each sample. In each cellulose thimble 5 g of finely shredded cheese sample was introduced. In order to improve the accuracy of the quantitative analysis, the exact weights were recorded. The cellulose thimble containing the sample were placed into the extraction chamber and the level sensor to the sample's height was adjusted. Subsequently, 100 ml of petroleum ether (ApplieChem, Darmstadt, Germany) were introduced into each backer which was later placed in the corresponding heating plate. The safety shield was closed, and the rack lowered. The occupied positions were activated and the cooling water opened. The extraction started according to the parameter listed in the table 4.

Table 4: parameters for the Soxhlet extraction with the FatExtractor E-500

Parameter	Value	Heating level
Solvent	Petroleum ether	
Extraction	120, resp 360	6[5]
SOX valve open time	Mid	
Rinse	5 min	6[5]
SmartDrying	On[6]	-
Solvent volume	100	

The backers containing the extract were dried in a drying oven at 120 °C until a constant weight is reached. Once the Soxhlet extraction was completed, the solvent was removed from the sample by placing the backers in an oven at 60 °C. The beakers were cooled down to ambient temperature for at least 1 h in a desiccator and the weights were recorded.

Subsequentially, the extracted fat was weighed and the fat content calculated. The results were calculated as percentage of the fat according to equation 1.

$$\% Fat = \frac{(m_{Total} - m_{Beaker})}{m_{Sample}} x\ 100\% \quad (1)$$

%Fat: Percentage of fat in the sample

m_{Total}: Beaker + extract (g)

m_{Beaker}: Empty beaker weight (g)

m_{Sample}: Sample weight (g)

Once removed the solvent, the resulting oils were transferred in 17x18 mm diameter neutral glass test tubes and stored in refrigerators at - 40°C.

Subsequently, by using a pipette, for each sample, 1 cm of oil was transferred in a clean glass tube and added with 2 ml of hydrogen chloride solution. All 9 class tubes, each corresponding to the cheese sample, was introduced in oven at 60 °C for almost 2 hours. 45 minutes after placing the samples in the oven, the contents were gently shaken. Once removed the tubes from the oven; the double extraction with hexane proceeded. In each tube, 1 ml of water was added to stop the reaction, then 1 ml of hexane was introduced to induce the separation of fats. The content was gently agitated. With the help of a pipette, the fat was carefully removed and transferred into a clean tube. The operation was carried out again. To be sure that the water was completely removed from the fat, a small amount of granular sodium sulfate (Sigma-Aldrich, St. Luis, MO, USA) was added to the tubes. Samples were closed with a special cap and sealed with parafilm. Samples were stored at – 20 °C. Analysis of FAMEs was carried out on a CP-9001 gas chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a column CP-Sil 88, 50 m length × 0.25 mm i.d., 0.2 μm film thickness (Chrompack). Nitrogen was used as carrier gas at a linear velocity of 20 cm/s. The oven temperature was set at 160 °C for 1 min, then increased at a rate of 2 °C/min to the final temperature of 240 °C, which was kept for 5 min. Injector and flame ionization detector (FID) temperatures were set at 250 °C.A Supelco (Bellefonte, PA, USA) standard solution containing a mixture of 37 FAME and a Sigma-Aldrich (St. Louis, MO, USA) linoleic acid methyl ester, cis/trans-isomers mix, was used for the identification of

peaks. Fatty acid (FA) compositions (wt % of total FAs) were calculated by the peak area normalization method. [57]

Chapter 10
DETERMINATION OF VOLATILE AROMA PROFILE OF CHEESES.

The study of the VOCs of a food aims to provide the characterization of the aromatic profile, thus allowing to identify the most important compounds in defining the organoleptic characteristics of the product.

A volatile compound, to significantly contribute to the aroma of a food, must be present at a concentration higher than its threshold value. This value is defined as the minimum quantity of substance capable of arousing perception.

The main components of food (proteins, amino acids, carbohydrates, lipids and fatty acids) undergo degradation processes as a result of conservation processes (e.g., seasoning) or due to technological treatments (e.g., cooking), thus giving rise to a wide range of compounds such as hydrocarbons, esters, aldehydes, ketones, alcohols, nitrogen and sulphur compounds, which impart the characteristic aroma to the food.

The determination of volatile organic compounds in food plays a role of considerable importance: these substances are in fact responsible for the smell of the product, which can fall within the norms of normality and acceptability and even be a peculiar characteristic of the product or present anomalies due to the presence of substances which impart an unpleasant odour, the so-called “off-flavours”.

It is therefore important to identify and quantify the compounds normally present in the volatile fraction of food products, in order to characterize their aromatic profile and to study their variations according to geographical origin, origin (e.g., milk obtained from different types of animals), production technology, curing or interaction with packaging material.

It is also important to identify the compounds responsible for organoleptic alterations and determine their content, in order to be able to hypothesize their origin and remove the causes that led to their presence.

Great technological importance is conferred to the changing knowledge of cheese during ripening. Indeed, the accurate choice of ripening time can improve cheese quality, thus preventing sensory attributes’ degradation. Particularly, in the case of PDO cheeses, where quality is still a

challenge for cheesemakers, the accurate choice of ripening time is of paramount importance to meet the increasing demand for cheese characterization. Due to the importance of sensory analysis, research groups have studied the formation of volatiles and the connotation of volatiles for different cheese varieties; this have allowed the definition of the attributes required for characterizing traditional cheeses and for helping to prevent cheeses adulterations and imitations. [53, 54] Different sampling techniques can be adopted to extract volatile compounds; for the purpose of this study, solid-phase microextraction–gas chromatography/mass spectrometry (SPME-GC/MS) was used to characterize volatile profile of the cheeses under analysis. Despite, sampling of substances can be performed by exposure of the fiber both in the headspace above the matrix and by immersion, the first technique, suitable for volatile analytes, was adopted. To facilitate the extraction of the analytes, the sample was heated.

10.1 **Material and methods**

For each sampling, 1,5 gr of cheese was finely grounded with knife and introduced on 10-mL vials respectively and closed with a screw cap equipment with elastomeric septum. Vials were sealed with parafilm and stored in freezers at – 20 °C until the time of analysis. The sample vials were allowed to equilibrate in a heating bath at 40 °C for 5 min. Subsequently, the SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane, 1 cm, 50/30 µm) from Supelco/Sigma-Aldrich (Milan) stainless steel needle in which the fiber is housed was pushed through the vial septum; the fiber was exposed to the headspace of the sample for 10 min.

Once the fiber was pulled into the needle sheath, the SPME device was removed from the vial and introduced into the injection port of the GC system for thermal desorption. Thermal desorption of the compounds from the fiber took place in the GC injector at 220 °C for 15 min. The injection was performed in the splitless mode (splitless time 0.3 min) at 220 °C. The GC-MS runs were performed with a Varian 3900 gas chromatograph coupled to a Saturn 2100Tion trap mass spectrometer (Varian Analytical Instruments, Walnut Creek, CA, USA). The chromatographic separation was performed on a TG-5MS capillary column (Thermo Scientific, 30 m × 0.25 mm I.D., film thickness 0.25 µm). Volatile profile was analysed and identified according to Mozzon, Foligni, and Mannozzi (2020). [55] Briefly, the injector temperature was prepared at 250 °C; the oven temperature was increased from 40 °C to 220 °C at the rate of 6 °C/min and maintained for 5 min. Gas flow (He) was used in constant mode at 1.0 mL min; the ion trap and the transfer line were set at 200 °C and 220 °C, respectively. Full scan MS data were acquired in the mass range of 31–250 atomic mass units (amu). The identification of compounds was made according to Maoloni et al., 2021 by matching the mass spectral data with those

collected in the NIST/EPA/NIH Mass Spectral Library (National Institute of Standards and Technology, MD, USA) and the Kovats retention Indices (RIs) with those available in the public access database Pubchem. Chemical ionization (methanol) spectral data (parent and base peaks) were also used to confirm the molecular weight of volatile substances. [56]

Chapter 11
RESULTS AND DISCUSSION

11.1 **Fatty acids profile of cheeses**

The fatty acids profile is described by 31 fatty acids among which palmitic acids (C16:0), oleic acid (C18: 1 cis-9), capric acid (C10:0) and myristic acid (C14:0) were the most abundant fatty acids in the three cheese samples analysed. Precisely, in all cheese samples, saturated fatty acids (SFAs) were the most abundant accounting for approximately 75%, followed by monounsaturated fatty acids (MUFAs) accounting for approximately 20% and polyunsaturated fatty acids (PUFAs) accounting for approximately 5% of total fatty acids.

In the SFA class, the highest values determined were for palmitic acids (C16:0) representing almost 22% of total FA in both O1 and COM cheese samples while 20% in sample O2, myristic acid (C14:0) found in greater amount in in sample O2 (14%) compared to sample O1 (12,7%) and even more to sample COM (9%). Capric acid (C10:0), caproic acid (C6:0), caprylic acid (C8:0) and lauric acid (C12:0) were found in similar amount in all three cheese samples, while stearic acid (C18:0) and butyric acid (C4:0) were more abundant in cheese sample COM compared to O1 and O2. Undecylic acid (C11:0), tridecylic acid (C13:0), pentadecylic acid (C15:0), margaric acid (C17:0), arachidonic acid (C20:0) and lignoceric acid (C24:0) were the least represented and their quantity was negligible in all the samples analysed.

From the MUFAs class, the highest value was established for oleic acid (C18:1 cis-9) accounting for approximately 18% of total fatty acids and present in all analysed samples in almost similar amount.

In the PUFAs class, linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3) were the most representative among the nine PUFA detected. Both acids were more abundant in sample COM (4% and 2% respectively of total fatty acids) compared to samples O1 and O2. (Table 9).

Table 5: changes in composition of cheese obtained with vegetable coagulant (O. tauricum)

Sample	Weight (g)	Dry matter (g)
O1	50	44.6
O1BIS	50	45.2

O2	50	43.3
O2BIS	50	45.4
O3	50	42.0
O3BIS	50	44.9

Table 6: changes in composition of cheese obtained with commercial rennet

Sample	Weight (g)	Dry matter (g)
COM1	50	42.4
COM2	50	45.1
COM3	50	49.4

Table 7: crude fat content of cheeses obtained with plant coagulant determined with FatExtractor E-500

Sample	m_{backer} (g)	m_{sample} (g)	m_{total} (g)	% Fat	N° cycles
O1	109,2464	5,3689	111,4818	41,64	25
O1BIS	113,3318	5,3764	115,1946	34,65	27
O2	113,3420	5,1348	115,5174	42,37	25
O2BIS	109,9224	5,1565	111,7558	35,56	23
O3	109,9280	5,4390	112,2530	42,75	27
O3BIS	109,2134	5,2015	111,1444	37,12	27

Table 8: crude fat content of cheeses obtained with commercial rennet determined with FatExtractor E-500

Sample	m_{backer} (g)	m_{sample} (g)	m_{total} (g)	% Fat	N° cycles
COM1	109,5752	5,3633	112,0630	46,39	29
COM2	109,9760	3,3783	112,2422	67,08	30
COM3	113,0748	5,0665	115,1750	41,45	28

11.1.1 Discussion

For years, the attention of experts was attracted by the chemical composition of products of animal origin, and particularly the content of ingredients as fatty acids because of their impact on human health. Indeed, the cheese's fatty acid composition is an important marker for determining the cheese's characteristic properties.

Saturated fatty acids (SFA) have mainly an energetic significance and are present not only in animal fats, but even in tropical oils and cottonseed oil. Numerous epidemiological studies

indicate that diets with a high SFA content are associated with high levels of serum cholesterol (in particular LDL) and, therefore, with a high incidence of coronary heart disease. The short-chain SFAs (<C10) do not lead to an increase in blood cholesterol, while the lauric (C12: 0), myristic (C14: 0) and palmitic (C16: 0) SFAs are atherogenic as already observed by Keys et al. in 1965. [62] Many studies have then shown that medium-chain SFA, myristic (C14: 0), palmitic (C16: 0) are also thrombogenic.[63] Myristic acid is the main atherogenic acid since it has a hypercholesterolemic power four times higher than that of palmitic acid as already observed by Hegsted et at. in1965. [64] Stearic acid (C18:0) more abundant in cheese sample COM on the other hand, although saturated, is not very atherogenic as it is rapidly desaturated by the body to oleic acid. [65] The recommended daily intake of saturated fatty acids should be less than 10% (7-10%) in terms of total calories, and the optimal dietary SFA/UFA ratio should be 1: 2 which is far from what was examined in all three cheese samples. [66] SFAs including lauric acid (C12: 0) taken above energy needs, have hyperlipidaemic and hypercholesterolemic effects, but if the diet is balanced in terms of SFA and UFA, then lauric acid, as well as palmitic (C16:0) and stearic acid (C18:0), can mostly be oxidized and without a hyperlipidaemic effect. [67]

Among the monounsaturated trans fatty acids, vaccenic acid (C18:1) is present in scarce quantities in the analysed samples. This is not very positive since, vaccenic acid has important positive effects on the health of the consumer as a precursor of rumenic acid whose nutraceutical properties are known. Vaccenic acid is in fact desaturated to rumenic acid both in the mammary gland of ruminants and in some tissues of the human body (liver) by the enzyme Δ9 desaturase. Usually, MUFA consumption is related to a positive effect on human health. Consumption of MUFA is associated with a reduction of the LDL cholesterol (bad cholesterol), an increase of the HDL cholesterol (good cholesterol) and as result an overall general improvement of LDL-HDL ratio. The recommended daily intake of monounsaturated fatty acids in the diet should cover 20% of the total caloric requirement (WHO, 1990).

The composition of organic acids in cheese is of paramount importance since it contributes to cheese flavor which is one of the most important qualitative criteria for fresh and mature cheeses and thus in the characterization of cheese. Butyric, caproic and caprylic acid (C4:0, C6:0 and C8:0 respectively) are the main carriers of the cheese aroma, long-chain acids are present but do not affect smell, while medium-chain acids are responsible for the smell of sheep fats. A change and increase in the composition of organic acids occurs during the cheese' s ripening and the content of organic acids can be taken as the index of cheese ripening. From the MUFA group, the highest concentration was determined for oleic acid (C18:1 cis-9), and the dominant content from the PUFA group was established for linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3). [67]

Both, linoleic and alpha-linoleic acids are considered essential fatty acids (EFA) and they must be introduced with the diet. When these two precursors are introduced with the diet, the organism is able to synthetize other polyunsaturated fatty acids; indeed, they are called ω-3 and ω-6 precursors. However, only a minor part of this acid derives from food absorption, while great part of it derives from the body's reserves, partly compensating for oscillations in food intake. Cheese is an important source of C18: 2 n-6 and its isomers, which have a hypothetical role in reducing the risk of cancer and cardiovascular disease. The relationship between SFA, MUFA and PUFA of the analysed cheeses is inversely proportional to the needs of a balanced diet and the impact on human health. The Σ SFA in the samples of the examined cheese was higher than Σ MUFA and Σ PUFA, which appears to place the cheese samples in a bad position from a nutritionist's point of view. [67] (Table 10)

The conditions of breeding, feeding and production technology are probably responsible of the resulting fatty acid composition.

Table 9: Fatty acids profile of cheeses

Fatty Acids		**Samples**					
		O1		**O2**		**COM**	
		LIST OF SFA					
Butyric acid	C4:0	4,37	± 0,37	4,13	± 0,44	4,67	± 0,68
Caproic acid	C6:0	6,36	± 0,22	6,31	± 1,03	6,25	± 1,34
Caprilyc acid	C8:0	4,37	± 0,45	4,33	± 0,29	4,18	± 0,25
Capric acid	C10:0	10,15	± 0,08	11,43	± 0,72	10,85	± 0,21
Undecylic acid	C11:0	0,18	± 0,01	0,17	± 0,02	0,16	± 0,01
Lauric acid	C12:0	5,36	± 0,35	5,00	± 0,26	5,55	± 0,35
Tridecylic acid	C13:0	0,15	± 0,01	0,15	± 0,01	0,13	± 0,03
Myristic acid	C14:0	12,69	± 0,92	14,00	± 0,76	9,04	± 0,08
Pentadecylic acid	C15:0	1,18	± 0,02	1,12	± 0,02	1,45	± 0,21
Palmitic acid	C16:0	22,25	± 0,32	20,45	± 0,63	21,95	± 0,35
Margaric acid	C17:0	0,53	± 0,02	0,55	± 0,03	0,45	± 0,07
Stearic acid	C18:0	6,31	± 0,33	7,53	± 0,46	9,90	± 0,99
Arachidic acid	C20:0	0,05	± 0,02	0,05	± 0,00	0,07	± 0,01
Lignoceric acid	C24:0	0,07	± 0,02	0,03	± 0,02	0,06	± 0,01
		LIST OF MUFA					
Myristoleic acid	C14:1Δ9	0,57	± 0,03	0,78	± 0,01	0,65	± 0,07
Palmitoleic acid	C16:1	1,36	± 0,03	1,40	± 0,02	1,15	± 0,07
Oleic acid	C18:1Δ9c	18,38	± 0,25	17,12	± 0,66	16,75	± 0,64

Heptadecenoic acid	C17:1Δ10	0,27	± 0,02	0,25	± 0,02	0,40	± 0,14
Gadoleic acid	C20:1Δ11	0,05	± 0,01	0,03	± 0,00	0,06	± 0,02
Erucic acid	C22:1Δ13	0,01	± 0,00	0,01	± 0,00	0,02	± 0,01
Nervonic acid	C24:1Δ15	0,02	± 0,01	0,01	± 0,01	0,02	± 0,02
Vaccenic acid	VACC	0,44	± 0,13	0,47	± 0,05	0,50	± 0,00
		LIST OF PUFA					
Linoleic acid	C18:2 n-6	3,06	± 0,43	2,93	± 0,10	3,60	± 0,14
Alpha-linolenic acid	alpha-18:3	1,30	± 0,29	1,26	± 0,11	1,73	± 0,10
Eicosadienoic acid	C20:2Δ11,14	0,04	± 0,02	0,06	± 0,02	0,03	± 0,00
Eicosatrienoic acid	C20:3 n-6	0,03	± 0,01	0,02	± 0,01	0,01	± 0,00
Eicosatrienoic acid	C20:3 n-3	0,19	± 0,02	0,16	± 0,01	0,18	± 0,01
Arachinonic acid	C20:4 n-6	0,07	± 0,03	0,08	± 0,01	0,05	± 0,01
Eicosapentaenoic acid EPA	C20:5 n-3	0,08	± 0,01	0,07	± 0,01	0,07	± 0,00
Docosadienoic acid	C22:2Δ13,16	0,06	± 0,01	0,04	± 0,03	0,06	± 0,01
Docosahexaenoic acid DHA	C22:6 n-3	0,06	± 0,01	0,05	± 0,01	0,04	± 0,01

Results are expressed as mean ± SD

Table 10: The ratio of fatty acid in cheese samples

	O1	**O2**	**COM**
Σ SFA	74,00	75,25	74,70
Σ MUFA	21,11	20,06	19,54
Σ PUFA	4,89	4,68	5,76
Σ UFA	26,00	24,75	25,30
	Ratio of fatty acids		
SFA/MUFA	3,51	3,75	3,82
SFA/PUFA	15,13	16,07	12,97
MUFA/PUFA	4,32	4,28	3,39
SFA/UFA	2,85	3,04	2,95

11.2 **Volatile aroma profile: water crude extract of cheeses obtained with flower from *Onopordum tauricum***

The SPME-GC/MS allowed to identify 24 volatile compounds in the headspace of cheeses O1 and COM1: a total of 4 alcohols (n-propanol, 2-pentanol, 3-Methyl-1- butanol, and 2-heptanol), 7 acids (acetic acid, isobutyric acid, butanoic acid, 3-methylbutanoic acid, 2-methylbutanoic acid, 4-Methyl-2-oxovaleric acid, and hexanoic acid) , 7 esters (butyl acetate, ethyl butyrate, propyl butyrate, ethyl hexanoate, isopentyl isobutyrate, 2-nonanone, and ethyl octanoate), 3 ketones (2-butanone, 2-pentanone, and 2- heptanone), 2 aromatic hydrocarbons (benzene and toluene), 1 aldehyde (3-methyl-butanal) were identified. The main identified volatile compounds found in the cheeses obtained with *Onopordum tauricum* and commercial rennet are listed in Table 11 together with the chromatographic retention times. The volatile compounds identified in the cheeses sample O1 and COM 1 were numerically different (Figure 5). Some compounds have been identified in both samples (3-methyl- butanal, 2-pentanone 3-methyl-1-butanone, toluene, isobutyric acid, 2-heptanol, ethyl hexanoate, hexanoic acid, isopentyl isobutyrate and ethyl octanoate). Other compounds, however, were not found or found in negligible quantities in both of the cheese samples (benzene, 2-pentanol, butyl acetate, 3-methylbutanoic acid, 2-methylbutanoic acid, propyl butyrate and 4-Methyl-2-oxovaleric acid). Particularly, n-propanol, 2-pentanone, 3-Methyl-1-butanol, toluene, isobutyric acid, butanoic acid**,** isopentyl isobutyrate, ethyl octanoate were identified in higher concentration sample O1 while 2-butanone, acetic acid, 3-methyl-butanal, 2-heptanone, 2-heptanol, ethyl hexanoate, hexanoic acid and 2-nonanone had a higher concentration in sample COM1. As shown by the graph, 3-Methyl-1-butanol, isobutyric acid and hexanoic acid are particularly abundant in the O1 sample compared to the COM1 sample. Alcohols were the most abundant identified volatile compounds in both cheese samples analysed followed by acids which were identified in grater amount in COM1 compared to O1 cheese sample.

Table 11: The main identified volatile compounds found in cheeses sample O1 and COM1 obtained with Onopordum tauricum crude extract

RT [min]	Name	CAS Number	Category	Abundance	
				O1	COM 1
1,97	n-propanol	71-23-8	Alcohols	6,65E+04	2,54E+06
2,164	2-butanone	78-93-3	Ketones	0,00E+00	1,58E+06
2,341	acetic acid	64-19-7	Acids	0,00E+00	4,90E+05
2,519	3-methyl-butanal	590-86-3	Aldehydes	1,01E+06	4,64E+05
2,616	benzene	71-43-2	Aromatic hydrocarbons	0,00E+00	0,00E+00
2,808	2-pentanone	107-87-9	Ketones	3,97E+05	1,47E+04
2,938	2-pentanol	6032-29-7	Alcohols	0,00E+00	0,00E+00
3,377	3-Methyl-1-butanol	123-51-3	Alcohols	4,55E+06	1,59E+06
3,828	toluene	108-88-3	Aromatic hydrocarbons	8,43E+04	3,99E+04
3,698	butyl acetate	123-86-4	Esters	0,00E+00	0,00E+00
4,006	isobutyric acid	79-31-2	Acids	5,60E+06	1,08E+06
4,151	ethyl butyrate	105-54-4	Esters	0,00E+00	1,52E+06
5,255	butanoic acid	107-92-6	Acids	2,21E+05	0,00E+00
5,855	3-methylbutanoic acid	503-74-2	Acids	0,00E+00	0,00E+00
5,954	2-methylbutanoic acid	116-53-0	Acids	0,00E+00	0,00E+00
6,084	2-heptanone	110-43-0	Ketones	0,00E+00	1,02E+05
6,244	propyl butyrate	105-66-8	Esters	0,00E+00	0,00E+00
6,34	2-heptanol	543-49-7	Alcohols	1,08E+05	2,50E+04
7,316	4-Methyl-2-oxovaleric acid	816-66-0	Acids	0,00E+00	0,00E+00
8,623	ethyl hexanoate	123-66-0	Esters	1,06E+06	3,45E+05
8,816	hexanoic acid	142-62-1	Acids	2,73E+06	3,45E+05
10,016	isopentyl isobutyrate	2050-01-3	Esters	4,84E+04	2,47E+04
10,921	2-nonanone	821-55-6	Esters	0,00E+00	1,16E+05
13,461	ethyl octanoate	106-32-1	Esters	4,23E+05	2,08E+04

11.2.1 Discussion

The SPME- GC/MS technique allowed to detect the major and minor volatile compounds in cheeses obtained with crude extract of fresh flowers of *Onopordum tauricum* as a coagulating agent. As known, a combination of factors including milk enzymes, rennet and microbial enzymes influence cheese flavour. Specifically, the role of lactic acid bacteria, and particularly such of autochthonous species, coupled with moulds, yeasts and non-pathogenic adventitious microorganisms that naturally occur in the raw materials (vegetable rennet and raw milk), are fundamental in the development of cheese's volatile profile. Volatile organic compounds (VOCs)

are produced during the cheese's ripening by numerous enzymatic reactions, such as glycolysis, lipolysis, proteolysis, and amino acid degradation. The nature of milk as well as milk treatments, as pasteurization reducing the amount of volatile compounds, influence the cheese volatile profile thus explaining the stronger flavour and aroma of raw milk cheeses in respect with those produced using pasteurized milk. [60]

Cheeses object of analyses possesses a few unique properties that render them quite different from other industrially produced cheeses using pasteurized or heat-treated cow's milk. Foremost, the cheeses analysed were manufactured from raw sheep's milk possessing peculiar properties compared to cow's milk, e.g., ewe's milk has almost twice fat content compared to cow's milk (7.1 vs. 3.8%). Likewise, the total fatty acid amount in sheep's milk is greater compared to cow's milk. In addition, compared to cheeses manufactured from heat-treated milk, the microbial load in raw milk is quite high especially in terms of *Enterobacteriaceae* which are known to produce lipases that breakdown milk fat. Furthermore, differently from commercial rennets, which usually possess a strong lipolytic capacity, the coagulant used in cheese making is a crude protease mixture of plant origin. Finally, ripening does not favour lipolysis since it occurs at a relatively low temperature (12 °C).

A correlation between volatile compounds and sensory characteristics exists. So far, more than 600 VOCs have been identified in different types of cheeses available on market and many of them have been related to specific and peculiar flavour notes. [58] (Table 12)

Many of VOCs have been associated to precise odour and aroma notes and are responsible for cheese sensory characteristics. The volatile fraction of the analysed cheeses is very rich in alcohols, acids and aromatic hydrocarbons, and its odour and aroma have cheesy, fruity and sour notes. Consistently to what available in literature, ketones are typical VOCs commonly related with the aroma of surface-mould ripened and blue veined cheeses. [58] Among the four alcohols identified in cheeses, 3-methyl-1-butanol was the most abundant in sample O1. It usually originates from the reduction of its corresponding aldehyde, which is derived from leucine. Instead, primary alcohols such as 1-propanol, particularly abundant in sample COM1 can be produced in cheese by the reduction of their corresponding aldehyde and methyl ketone. The presence of 2-butanone has been identified in cheese sample COM1 and the sensory descriptors typically associate is of butter, cheese, chemical, chocolate, ethereal, and gaseous. Butanoic acid exclusively present in cheese sample O1 has already been identified in sheep's cheeses as Canestrato Pugliese PDO cheese, Fiore Sardo PDO cheese, Torta del Casar PDO cheese, Pecorino Romano PDO cheese and Terrincho PDO cheese and is typically associated with strong unpleasant odours. [60, 61] Finally, identified in higher concentration in

cheese sample O1 compared to COM1 is hexanoic acid which is commonly associated with waxy, soapy and goaty odours. [61] Interestingly, hexanoic acid is considered an active antimicrobial compounds resulting from the metabolic activity of lactic acid bacteria with antagonistic effect on fungi and mycotoxin production.

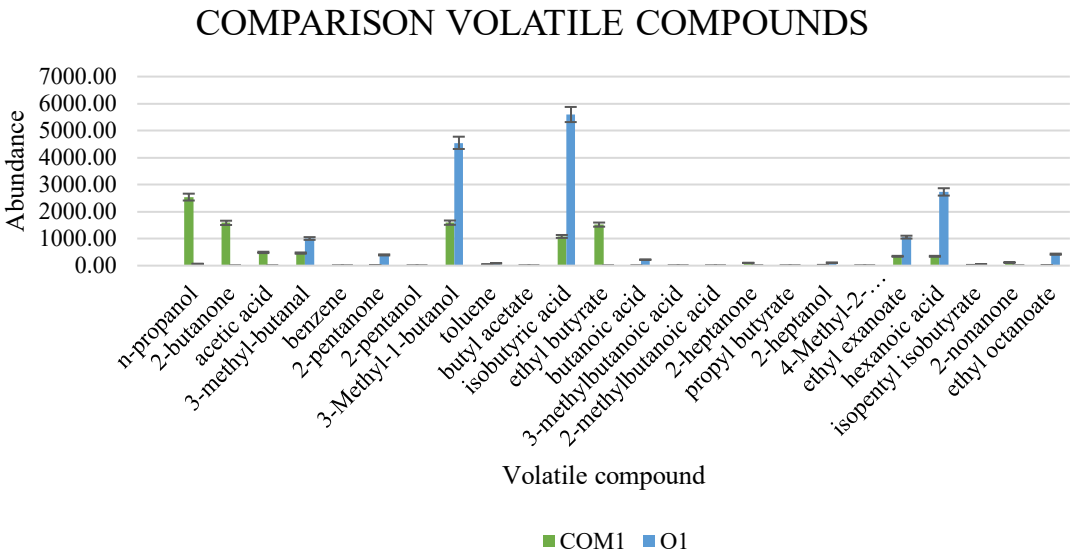

Figure 4: comparison volatile compounds O1 and COM1 cheese sample

Table 12: Odorous sensations associated with some volatile molecules found in cheese (Curioni and Bosset, 2002) [58]

Odorous sensation	Volatile compounds
Fruity	Ethyl acetate, ethyl butyrate, ethyl hexanoate, lactones
Butter	Diacetyl, acetoin
Blue cheese	2-heptanone, 2-nonanone, 8-nonen-2-one
Herbal, green	2-pentanol, 2-heptanol, hexanal, heptanal, 2-nonenal
Sour, acrid	acetic, butyric, hexanoic acids
Cheesy	butyric acid
Goat	hexanoic acid, nonanoic acid
Mushroom	1-octen-3-one
Mushroom, earthy	1-octen-3-olo
Garlic, onion, cabbage	methylsulfide, dimethylsulfide, dimethyldisulfide
Floral	phenylethanol, phenylacetaldehyde

Therefore, a direct contribution of the aqueous extract to the cheese flavour and aroma/fatty acid composition also emerged, thus suggesting a deep connection of the plant extract with the manufacturing process. During lipolysis free fatty acids (FFA) are released and short- and medium-chain FFA (C4:0–C8:0 and C10:0–C14:0, respectively) play an important role because they contribute, together with the volatile compounds and the proteolysis products, directly to

cheese flavour. Instead, long-chain FFA (>14 carbon atoms), due to their high perception thresholds, play a minor role in cheese flavour. In addition, fatty acids beside being aroma compounds by themselves, also act as precursor molecules for a series of catabolic reactions leading to the production of flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols. Furthermore, cheese fatty acids profile showed high levels of long-chain fatty acid (LCFA) such as palmitic (C16:0) and oleic (C18:1), as well considerable amounts of medium-chain fatty acid (MCFA) and short-chain fatty acid (SCFA) were detected. This is consistent with the results reported by Delgado et al. (2009) for Torta del Casar cheese where the higher LCFA amount could be due to palmitic and oleic acids, which are the predominant FFA in ewe milk. [68] Due to their considerably lower perception thresholds, SCFA and MCFA play an important role on the typical cheese flavour as each one provides peculiar flavour notes; [69] and in tourn, thanks to their higher volatility compared to MCFA, SCFA have a higher impact on cheese flavour. Among SCFA caproic acid (hexanoic acid), the major SCFA in this study as already mentioned is typically associated to a fatty, cheesy, waxy, and like that of goats odour. Despite acetic acid is not a product of lipolysis but mostly a product of other biochemical pathways, probably resulting from the fermentation of lactate or the metabolism of amino acids by bacteria, it is found in high content in COM cheese sample. This result agrees with Torta del Casar cheese volatile profile analysis, where acetic acid was the major volatile acid isolated (Delgado et al., 2009) greatly contributing to the final flavour of Torta del Casar cheese adding ‘‘pungent’’ flavour note.

Chapter 12
LEGISLATION AND APPROVALS

In accordance with the RD 2003/25:

"Cheese is the product obtained from the acid or enzymatic coagulation of whole or partially skimmed milk or from cream, also making use of enzymes and salt. The term "cheese" is reserved for cow's milk derivatives, while the use of milks different form cow's one, implies that the term "cheese" must be accompanied by the animal species from which the milk originates. "

The classification of cheeses is carried out on the basis of various characteristics such as origin, maturation time, the percentage of fat and water and the consistency. Further elements to be considered are the breed of the animal and the technology used for production and processing (e.g., the milk treatment applied: raw, pasteurized, whole, skimmed), as well as indications regarding the origin of the territory. Alternative classification can be made based on the type of rind: washed, flowery, brushed, etc. Considering the various types of classification, each cheese could belong to several reference groups: to overcome this problem, the fundamental characteristic of the cheese under analysis is generally used as the main reference.

Follow the types of cheese classification and the main categories.

Cheeses are classified based on the milk animal origin as:

- Sheep cheeses
- Cow cheeses
- Goat cheeses
- Buffalo cheeses

In any cheese produced from milk different from the cow's one, the indication of the species employed shall indicated in the label, e.g., "pecorino cheese" derives from sheep's milk. The cheeses that have obtained the PDO, PGI and TSG certification follow the specific provisions on the subject.

Based on the duration of ripening, cheeses are classified as:

- Fresh: cheeses not subjected to seasoning that must be consumed within few days from the production. (e.g., Mozzarella, Fiordilatte and Crescenza)
- Short ripening: cheeses whose ripening period does not exceed 30 days (e.g., Asiago and Caciotta di Urbino)
- Average-ripening: cheeses with a ripening period between 1-6 months (e.g., Fontina, Gorgonzola and Provolone)
- Slow ripening: cheeses with a ripening period longer than 6 months (e.g., Parmigiano Reggiano and Grana Padano).

This classification is only conventional as the curing or maturation times can be very variable.

Based on the temperature of the curd and technology applied, cheeses are classified as:

- Raw pasta cheese: the curd is not subjected to any heating above the coagulation temperature; the maximum temperature reached is around 40 °C (e.g., Crescenza, Gorgonzola and Taleggio).
- Semi-cooked pasta cheese: the maximum temperature reached is around 48 °C (e.g., Fontina, Asiago and Provolone)
- Cooked pasta cheese: obtained by heating the curd over 48-56 °C (e.g., Grana Padano, Parmigiano Reggiano and Emmenthal)
- "Pasta filata" cheese: the curd is placed in water at 80-90 °C (e.g., Mozzarella and Provolone)
- Blue paste cheese: selected molds are intentionally added so that they will develop within the cheese paste. Due to their specific enzymatic activities, molds will contribute to maturation. (e.g., Gorgonzola and Castelmagno)

This type of classification is preferred by many technicians and is based on the principle that the type of cheese processing is crucial for its final qualities.

Based on the fat contents expressed in dry substance cheeses are classified as:

- Fat cheeses: fat in the dry matter higher than 35% (e.g., Fontina, Robiola and Bitto.)
- Light cheeses: fat in the dry matter varies between 20 to 35%
- Lean cheeses: with a fat content of less than 20% of dry substance and generally prepared with skimmed milk.

This system, which measures the fat content of cheese, represents a rigorous classification, as the percentage of fat can be measured with great precision.

In the past, excessive skimming was considered a food sophistication, for this reason the fat content is regulated by specific laws.

In relation to the percentage of water contained, cheeses are classified on the bases of the consistency of the dough as:

- Hard cheeses: they are obtained by breaking the curd into very small fragments, which are cooked at 50-60 °C and stirred continuously; the dough is then compressed, salted and subjected to a seasoning that can last for months or years. This result in an amount of water that is less than 35%. This category includes cheeses such as Parmigiano Reggiano, Grana Padano and Pecorino.
- Semi-hard cheeses: they are produced by breaking the curd into small fragments, which are compressed and left to mature for a short time. The water content is between 35% and 45%. (e.g., Fontina and Asiago)
- Soft cheeses: they are produced by breaking the curd into large fragments, then squeezed and kneaded. The water content is high (greater than 45%) and the cheeses obtained with this type of processing must be eaten immediately or stored in the refrigerator. (e.g., Stracchino, Crescenza, Burrata, and Squacquerone).
- Fresh cheeses: they are not seasoned; they never have a rind or surface patina and should be consumed within a few days of production. They have a percentage of water higher than 60% and from this derives the soft structure. Among others, Mozzarella and Robiola belong to this category.

Thus, the classification based on consistency is based on the presence of water, which can be extremely variable. Although this type of classification is widely used, it is quite ambiguous and occasionally confusing as the hardness of the pasta can undergo many modifications depending on the curing times. However, this type of classification remains the most used.

In addition, when cheeses are produced using only milk, rennet, salt, enzymes or cultures of microorganisms, the indication of the ingredients is not required; instead, fresh cheeses and melted cheeses are exceptions, for which it is necessary to indicate on the label the presence of salt. If, on the other hand, other ingredients in addition to those mentioned above are used (e.g., herbs, olives, etc.), the list of ingredients must be reported, and if an ingredient is characterizing, the provisions of Annex VIII of EU Reg. 1169/ 2011 must be respected, according to the so-called QUID rule.

12.1 **Legislation that regulates products with protected designation (PDO, PIG, TSG)**

The regulations relating to the quality designations of agricultural and food products are contained, in *Regulation (EU) no. 1151/2012 on quality schemes for agricultural and food products*. This regulation comes into force on January 3, 2013 repealing the previous regulations (EC) no. 509/2006 and no. 510/2006. It aims to provide a common and coherent legislative framework regarding certification schemes and indications that add value to agricultural products. The regulations on the protection of the quality of products provided by Regulation (EU) no. 1151/2012 does not apply, by explicit provision of the same provision (article 2) to wines and wine products, for which the provisions of Regulation no. 1308/2013 and spirit drinks, for which the regulations on the protection of geographical indications contained in Regulation (EC) no. 110/2008.

Regulation (EU) no. 1151/2012 is part of the "Quality Package"; it was proposed by the European Commission at the end of 2010 in order to define a more coherent agricultural product quality policy and aimed at helping farmers to better communicate the added value of their products. In addition to the above-mentioned regulation, a proposal on marketing standards is also part of the "Quality Package", aimed at facilitating the Commission's changes to the current marketing and origin labelling standards, and a series of guidelines on good practices applicable to voluntary certification schemes and the labelling of products that use protected geographical indications as ingredients.

With regard to the previous regulations, Regulation (EU) no. 1151/2012 introduces significant changes including a further speeding up of the recognition procedures, a general strengthening of the role of producers, trade associations and protection Consortia, the provision of the new optional indications " *Mountain product* " and " *Product agriculture of the island* " and the inclusion of novel foods in the list of products authorized to obtain Community certification. With regard to the TSGs, the main change refers to the increase from 25 to 30 years of the minimum period of use on the market of the product to be certified.

Furthermore, in relation to the above-mentioned regulation, the Ministerial Decree of 14 October 2013 and the Implementing Regulation (EU) no. 668/2014 of the Commission were published in order to specify the methods of implementation and application. In particular, the Ministerial Decree reports in its articles the methods to benefit from the PDO and PGI certification, which are the subjects entitled to submit the application for registration and the information to be attached to the request; the procedures to be implemented in the event that multiple applications are submitted for the same product/denomination or for similar

products/denominations; the procedure for evaluating registration applications and any reasons for opposition. In addition, Commission Regulation (EC) No 1216/2007 of 18 October 2007 laying down detailed rules for the implementation of Council Regulation (EC) No 509/2006 on agricultural products and foodstuffs as traditional specialities guaranteed was enacted.

There are three quality systems in the European Union and these are widely used in the characterization of cheeses currently existing on the market. A general overview about the *Protected Geographical Indication* (PGI), *Protected Designation of Origin* (PDO) and *Traditional Specialty Guaranteed* (TSG) is provided. List of some of cheeses obtained with vegetable coagulant available in the European market is reported in Table 13.

12.1.1 Protected designation of origin (PDO)

It is a trademark of legal protection of the denomination that is attributed by the European Community (EC) to foods whose particular characteristics essentially or exclusively depend on the territory in which they are produced. The geographical environment includes both natural factors (climate, environmental characteristics), and human factors (production techniques handed down over time, expertise, knowhow) which, combined together, make it possible to obtain an inimitable product outside a specific place. In order for a product to be granted as a PDO the stages of production, transformation and processing must take place in a defined geographical area. With the PDO the link between the territory and the production is indissoluble: if even just one characteristic of the place of origin were to change, then even the final product would no longer be the same. Producers of PDO products must comply with the strict production rules established in the production specification.

The production specification is the law that defines the production and commercial requirements of a PDO, PGI or TSG product; it follows that any product bearing a protected denomination/ indication has a detailed production specification which is periodically revised for any updating or modification. As a law, the violation of the established indications and disciplinary rules constitutes an illegal act and therefore is punished with an administrative sanction according to the current legislation. Pursuant to Article 19 of Regulation (EU) No. 1151/2012, the production specification must contain at least: the name proposed for registration, the description of the product with its physical, chemical, microbiological or organoleptic characteristics, the description of the method of production and the key elements that establish the traditional character of the product. Compliance with these rules is guaranteed by a specific control body (CBs). The CBs that intend to carry out control and certification activities of

operators who produce products registered as PDO, PGI and TSG and want to be registered in the list pursuant to law 128/1998, must be accredited by ACCREDIA as certified bodies according to EN 45011 and also they must be authorized by *The Department of central inspectorate for fraud repression and quality protection of the agri-food products and foodstuffs* (ICQRF) to which all the CBs are required to present a control plan for their authorization.

12.1.2 Protected geographical indication (PGI)

Designates a mark of origin that is recognized by the European Community to those agricultural and food products for which a certain quality, reputation or other characteristic are affected by the geographical origin, and whose production, transformation and/or elaboration occurs in a geographic area. To be granted as PGI, therefore, at least one phase of the production process must occur in a specific area. Those producing PGI granted products must comply with the severe production rules established in the production specification. Compliance with these guidelines is guaranteed by a specific CB. The PGI trademark, like the PDO, denotes a connection with the territory of origin, but in a less stringent and binding way. Indeed, some stages of processing can also take place in an area other than the certified one.

12.1.3 Traditional specialty guaranteed (TSG)

It is a mark of origin aimed at protecting productions that are characterized by traditional compositions or production methods. This certification, unlike other quality system, such as PDO and PGI, is aimed at agricultural and food products that have a "specificity" linked to the production method or composition linked to the tradition of an area, but which are not necessarily produced only in that area. The TSG trademark, therefore, refers not so much to a specific place of production, as to a product obtained using traditional raw materials of a territory, or with the use of traditional production/processing techniques, linked to particular uses or customs.

Many Italian cheeses fall into this category, but not all. Actually, there are many that are excluded from the special register despite being produced according to ancient dairy traditions or with a strong territorial link.

12.2 **Sanctioning rules relating to violation in the field of production and commercialization**

It is useful to remember that pursuant to Article 13 of Regulation (EU) no. 1151/2012, registered PDO and PGI products are protected against:

- any improper commercial use of unregistered products whose placing on the market has the purpose of exploiting the reputation of the protected name as they have characteristics that could be misunderstood as similar.
- any usurpation, imitation or evocation, even if the true origin of the goods or services is indicated or if the protected name is a translation or is accompanied by expressions such as "method", "style", "type" or "similar", even when such products are used as an ingredient;
- any other false or misleading indication relating to the provenance, origin, nature or essential qualities of the product that is used on the packaging, advertising or on documents relating to the product in question, and the packaging of the product in containers that can mislead as to its origin; and any other practice that could mislead the public as to the true origin of the product.

On the other hand, with regard to the protection of TSG products pursuant to Article 24 of Regulation (EU) No. 1151/2012, the registered names are protected against any usurpation, imitation or evocation or against any other practice that could mislead the consumer. Member States shall guarantee that sales descriptions used at national level do not lead to misunderstanding with registered names.

On 15 December 2004 it was published in the Gazzetta Ufficiale della Repubblica Italiana, no. 293, the Legislative Decree 19 November 2004, n. 297, concerning "*Disciplinary provisions in application of Regulation (EEC) No 2081/92, concerning the protection of geographical indications and designations of origin for agricultural products and foodstuffs* ". This Legislative Decree consists of five chapters for a total of 12 articles which intend to organize a form of protection that guarantees the quality, the characteristics and conservation of specific products; moreover, aims at protecting product names against abuse and imitations and ultimately provide consumers with information on the specific characteristics of products. Although quality systems are voluntary, once a manufacturer joins, he is subject to control, and it becomes mandatory to follow the requirements of the control plans, including the payment of the control structure. The control system for the PDO, PGI and TSG is established in accordance with Regulation (EC) No 882/2004 which requires Member States to put in place the control system that best satisfy their needs, on the bases of a risk analysis.

Table 13: List of some of cheeses obtained with vegetable coagulant available in the European market.

Denomination	**Cat.**	**Milk origin**	**Vegetable material**	**Place**
Queijo Serra da Estrela	PDO	sheep	*Cynara cardunculus*	Portugal
Torta del Casar	PDO	sheep	*Cynara cardunculus*	Spain
Queso de Flor de Guía / Queso de Media Flor de Guía / Queso de Guía	PDO	sheep	*Cynara cardunculus*	Spain
Queso de La Serena	PDO	sheep	*Cynara cardunculus*	Spain
pecorino delle balze Volterrane	PDO	sheep	*Cynara cardunculus*	Tuscany, Italy
Pecorino Toscano	PDO	sheep	*calf or vegetable rennet*	Tuscany, Italy
Queijo de Azeitão	PDO	sheep	*Cynara cardunculus*	Portugal
Queijo de Castelo Branco	PDO	sheep	*Cynara cardunculus*	Portugal
Queijo de Evora	PDO	sheep	*Cynara cardunculus*	Portugal
Queijo de Nisa	PDO	sheep	*Cynara cardunculus*	Portugal
Queijo Serpa	PDO	sheep	*Cynara cardunculus*	Portugal
Gran Kinara	-	cow	*Cynara cardunculus*	Piemonte, Italy
Lou Bergier	-	cow	*Cynara cardunculus*	Piemonte, Italy
Lou Jaun	-	cow	*Cynara cardunculus and turmeric*	Piemonte, Italy
La Blancha	-	cow	*Cynara cardunculus*	Piemonte, Italy
Caciofiore Columella	PAT	sheep	*Cynara cardunculus or Cynara scolymus*	Lazio, Italy

Chapter 13
CONCLUSION

Historically, although most of the enzymatic preparations used for cheese production were extracted from the stomach of ruminants, especially that of calves, the use of coagulants from microbes and plants also began very early on. For centuries, plant proteases have been used as milk coagulants in cheesemaking either as crude extracts or in purified form. High price of rennet, religious factors, diet or ban on recombinant calf rennet in some countries make plant proteases an alternative to the calf rennet. Moreover, the market niche of vegetarians as well as the growth of the market for Kosher and Halal foods is in increasing expansion resulting in a need the search for rennet substitutes from plant sources, to make products adapted for these specific market segments. [4] In almost all kinds of plant tissues such enzymes can be found and obtained from their natural source however, since the extraction of milk-clotting proteases from intact plants parts is labour intensive, plant in vitro culture is a viable alternative to obtain clotting enzymes to ensure a continuous supply. The crude extracts can be further purified to obtain partially purified or pure enzyme depending upon the degree of purification. [3] Consumers and the market have recently focused their attention on thistle-curdled cheeses and despite most of these are still manufactured in small dairy plants; thanks to their unique sensory characteristics and traditionality, many of these cheeses are included in the Registry of the Protected Geographical Indication (PGI) and Designation of Origin (PDO) products. The present study, although carried out on a limited number of samples, has enabled first to evaluate the technological properties of raw extracts obtained from *Onopordum tauricum* collected from the area of Agugliano (Marche, Italy) by investigating its fatty acids profile and associated volatile metabolites as described in the previous chapter.

Several factors (e.g., pH, temperature, water activity, and microflora) influence the chemical features of cheese throughout ripening; and its sensory characteristics are a consequence of the dynamic interaction of chemical and microbiological parameters. As well documented by Buchin et al., 1998, due to the high levels of native lactic acid bacteria present in raw milk cheeses manufactured from raw milk has a more intense flavor than those produced from pasteurized or heat-treated milks. Consequently, variability is a main problem in cheeses manufactured from raw

milk. This is an issue not only related to the cheeses under analyses but is even typical of Serra da Estrela cheese, a Portuguese artisanal raw (ewe's) milk cheese, coagulated with plant rennet without deliberate addition of any starter culture. Strategies aimed at minimizing the aforementioned variability are necessary, that to improve microbiological safety and evn to guarantee a final product characterised by typical aroma attributes, on both artisanal and industrial levels. In example, adding native cultures may be a strategy to reduce the heterogeneous quality of cheese. The study on the molecules responsible for the unique organoleptic attributes of cheese could be useful to this aim. From a sensory viewpoint, odour descriptors associated with the cheeses under analyses are "acidic," "sweaty," and "sheepy-like" notes. These descriptors indicate that FFA play a key role in the aroma character of cheeses. consequently, the cheese's FFA profile could be used to establish the optimum ripening time described as that providing the aroma attributes qualitatively and quantitatively mostly appreciated by consumers. As already reported in literature, a wide collection of volatiles, for example, alcohols, esters, ketones, aldehydes, and FFA, are among the families of compounds reported to be present in cheeses manufactured from ewe's milk. Among those, short-chain FFA thus considered of paramount importance providing the aroma impact in the final product. [59]

However, particular concern is the lack of sanitary control of the flowers, since like any other agricultural product, they are exposed to dust, insects, animal waste, which is why they could have a high microbial load. These microorganisms become part of the microflora of the cheese, since the coagulants are not subjected to further technological processes aimed at guaranteeing their sanitary safety. This translates into the use by the cheesemakers of materials that nature offers them and which can be subject to any risk. For all these reasons, one of the possible solutions would be a correct cultivation of a type of *Onopordum* with the best characteristics of vegetable coagulant in a specific type of soil thus solving the further concern about the lack of traceability. To date, the lack of traceability is caused by the presence of other plants and species that grow spontaneously and which could be confused with *Onopordum* due to their similarity. The use of *Onopordum* with other similar species involves their mixture as a vegetable coagulant; this could be considered a fraud for designation of origin.

In conclusion, much work remains to be done to understand the development of texture and flavour during ripening of artisanal cheeses made from raw ewe's milk and coagulated with *Onopordum tauricum* extracts. Work has been started to characterize proteases from *O. tauricum* and the aspartic proteases with a specific activity in hydrolyzing the Phe_{105} –Met_{106} linkage of k-casein. The contribution to the health benefits of bioactive components from *O. tauricum* extracts

as source of prebiotic and antioxidant have already been studied and are available in the literature. The combination of limited production and rising demand, particularly for the PDO cheeses made by traditional procedures, provides the impetus to improve understanding of the role of *O. tauricum* extracts during maturation and hence to provide more consistent extracts to help to optimize the cheesemaking process. [7]

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