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Metabolomic approach study about goat cheese “Bouchon de chèvre” type after prolonged ripening

A dissertation presented by

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Dedication

To Eric, Néstor and Fátima. This work was elaborated only by thinking of you.
To my parents. This is your gift, just a little late.

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Abstract

Bouchon de chévre cheese is a surface-mold cheese made from goat's milk with a cylindrical shape and which is generally marketed after 45 days of maturation. It is produced by using starter and yeast cultures such as *Penicillium camemberti, Geotrichum candidum,* and *Kluyveromyces lactis*. At an international level, metabolomic analyzes of cheeses have been widely used to determine the metabolites produced after cheese elaboration to determine the final conditions of the cheese taking into account its chemical conditions, a situation that has not been seen in cheeses made in our country. That is why the objective of this work was to carry out an analysis of all the metabolites produced in a cheese made in a tropical area of the state of Veracruz, Mexico. Bouchon de chèvre type cheeses were ripened for 90 days under controlled conditions. to evaluate the impact of ripening time over physicochemical and texture parameters. Changes in hardness, cohesiveness, springiness, chewiness, and gumminess were evaluated by texture profile analysis (TPA). Moisture was evaluated with official methods, whereas pH (center and rind) was measured directly. The lactic acid determination was carried out by high-performance liquid chromatography. Proteolysis was measured using reverse-phase high-performance liquid chromatography. While the antioxidant activity was determined using a 2,2-diphenyl-1-picrylhydrazyl solution. Lipolysis was analyzed using gas chromatography. Analysis of variance (ANOVA) showed significant differences between all parameters except center pH. Correlation analysis exposed significant correlations between all texture parameters evaluated and lactic acid concentration. There was no significant correlation between moisture and other parameters. Analyzed cheeses showed peptides fraction in the retention time of 2.05, 18.36, and 50.11 min for acid-soluble fraction and non-protein protein nitrogen, and showed antioxidant activity from the first day of ripening to 73% discoloration in the DPPH solution at 55 ripening days. Obtained results suggested that ripened Mexican goat cheese had a DPPH radical scavenging activity related to peptides present originally in the milk or released by starter culture action during cheese ripening. The cheeses analyzed showed a few variations in lipid components during the entire ripening process. The ANOVA showed a total of 7 statistically significant compounds. PCA and GDA showed that petroselinic and vaccenic acid and the unidentified compound 31 are the ones that explain the behavior of the lipid fraction to a greater extent.

Keywords: **Surface mold-cheese; bioactive peptides; proteolysis; lipolysis; texture analysis; principal component principal analysis; general discriminant analysis.**

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Chapter 1

Introduction

Cheese is one of the dairy products that is most produced and consumed internationally, due to its flavor and variety, in addition to the cultural influence it represents. In Mexico, goat's milk represents 25.8% of total milk production and is the second consumed after cow's milk. On the other hand, the consumption of goat cheese represents about 40% of the total milk production of this mammal, and its distribution represents an important income for small and medium producers (Carver, 2003; FAO, 2017).

To obtain this goat cheese, the whole milk is precipitated by casein hydrolysis, which are the most important proteins found in milk. This process can be promoted by using commercial enzyme complexes, such as renin, or by using acid complexes for protein denaturation. Once the precipitation process finishes, the cheese is elaborated after the whey is separated from the precipitate and adding microorganisms that promote the ripening, which is the process that leads to important biochemical changes in the cheese matrix, modifying its composition, structure, appearance, and color, promoting the variation of the organoleptic characteristics depending on the ripening conditions. The main causes of ripening are the enzymes found intrinsically in the milk, the enzymes that promote the casein coagulation, and the microbial enzymes produced by the type of microflora found in each cheese. Bouchon de chèvre cheese is a surface-mold cheese produced with goat's milk and is subjected to 3 ripening months using fungi and proteolytic yeasts that promote the appearance of a crust on the external area of the product (Eck & Lagarriga, 1989; Spinnler, 2017).

On the other hand, metabolomics is a multidisciplinary omic science that aims to study the metabolites produced in a complex biochemical process. These are divided into peptidomics, the branch of metabolomics that studies the function of peptides in the biological systems, and lipidomics, which studies the behavior of lipids in the biological systems. The knowledge of the relationship between the production of metabolites during the cheese ripening is the key to understanding and predicting the product's final characteristics (Robles & Batista, 2012; Yanes, 2015).

Proteolysis is the process by which the proteins lose their quaternary, tertiary and secondary structure, remaining only as a chain of amino acids that, after hydrolysis, become free peptides, chains of between 5 and 20 amino acids. These peptides appear after an intense proteolysis process after the appearance of enzymes. The production of these peptides has been related to certain biological activities found both in milk and dairy products. In the case of cheese, the variation in the ripening process promotes the appearance of biological activities directly related to protein hydrolysis. The most studied are antihypertensive, antioxidant, immunomodulatory, antimicrobial, and opiate effects, among others. These conditions make cheese a functional food (Choi et al., 2012).

Lipolysis is another process that occurs thanks to lipolytic enzymes produced by certain microorganisms within the cheese matrix, which promote the release of fatty acids and glycerol molecules from triglycerides found in whole milk. The fatty acids found can vary in length from 4 carbons to 22 carbons, and it is possible to find saturated and unsaturated types. These molecules give the cheese its final characteristics, such as smell, taste, and texture. The knowledge of the variation of these metabolites during each ripening step gives the guideline to specify the final qualities of the cheese even before it is produced (Alonso et al., 1999; Fontecha et al., 2006).

The present study researches the knowledge of the complete metabolomic process present in bouchón de chèvre cheese subjected to a prolonged and controlled ripening, elaborated in a tropical region in Mexico. The proteolysis process, the presence of biological activities in the peptides produced, and the lipid profile is investigated, providing extensive knowledge on the metabolomics of Mexican cheeses.

1.1 Motivation

Mexico is a country with a great cheese tradition, which has contributed to the world a large number of local cheeses or which have converted international products to local conditions, with variations in the conditions of the animal develop, giving milk with different qualities. With this milk, the dairy products obtained, which have been made with the same processes, present different attributes, leading to different final conditions to products from other parts of the world.

In international cheeses, the metabolomic analysis has been widely studied, knowing in depth each f the metabolites produced, considering the production conditions, type of microorganism, and ripening times. Cheeses such as Edam, Camembert, and Gouda have been widely studied, and their metabolomic system is well known: their proteolytic qualities, their bioactive peptides, the fatty acids that are produced, and the final organoleptic conditions obtained from each one are known. However, and despite the significant amount of cheeses produced in Mexico, whether local or imitation, the metabolic modification of the compounds obtained after the ripening of Bouchon de chèvre type cheeses has been a few studied; external variations can lead to the appearance of different metabolites than those found in European cheeses.

1.2 Problem Statement and Context

The metabolomic qualities of Mexican cheeses have been few studied. Bouchon de chèvre goat cheese produced in a Mexican tropical zone can present different metabolomic qualities that make it different from cheeses made in other parts of the world. Likewise, it can produce a variety of peptides with biological activities, with different properties to those reported in other cheeses or the same type of cheese made in other regions. Other metabolites responsible for the organoleptic conditions of the final product may vary depending on the external conditions. The lack of knowledge delays the local production conditions and reduces the product's competitiveness with international cheeses.

1.3 Research Question

How many and what kind of metabolites are produced in Bouchon de chèvre cheeses made with an artisanal method in a Mexican tropical zone?

1.4 Solution overview

To fully understand the metabolites produced after the maturation of Bouchon de chèvre cheeses, it is necessary to use metabolomic analysis methodologies. The metabolomic study of the Mexican Bouchon de chèvre cheese made in a Mexican tropical zone could help to understand the similarities and differences with cheeses made in other countries of the world, taking into account the conditions in which it is produced. In addition, this

type of study opens the door to the elaboration of more in-depth studies in local cheeses so that their qualities and properties are known.

1.5 Hypothesis

If the goat cheese made in Veracruz type Bouchon de chèvre is an important source of metabolites after the ripening process, then these can be studied using metabolomic methodologies

Chapter 0

Theoretical Framework

2.1 Surface mold-ripened cheese

In the types of cheeses varieties, there are surface mold-ripened cheeses characterized by the presence of a white micellar crust produced thanks to the surface growth of yeasts such as *Penicillium camemberti*. This crust gives this type of cheese a characteristic appearance, taste, and smell because its ripening process is complex compared to other cheeses with simpler microbial flora. The typical example of this type of cheese is Camembert, which has a smooth consistency and a cylindrical shape of approximately 11 cm in diameter and an approximate weight of 250 g. Other cheeses of this type are Brie, Coulommier, Carré del´est and Bouchôn de chevre. The traditional shape of cheese can be observed in figure 2.1 (Leclercq-Perlat et al., 2015; Spinnler, 2017).

Figure 2.1 The traditional shape of surface mold-ripened cheese.

Traditional Camembert cheese is elaborate from raw cow's milk with added mesophilic starter cultures. The initial pH should be around 6.4, and the clotting time should be between 30 and 45 minutes. Once the curd is produced, it is molded using either manual or automatic methods, and the whey drains during the first hours at a temperature of 26 to 28 °C. When the curd temperature reaches 20 °C and the drainage is finished, the curd with a pH between 4.6 and 4.7 is cut into cubes, and after approximately 50 minutes, it is dried and salted. Once the classic cylindrical shape of the cheese is given, it is ripened for a total of 21 days at an average temperature between 11 and 13 °C with a relative humidity of 95% (O'Sullivan et al., 2005; Spinnler, 2017).

In this type of cheese, the microbial flora is the one that promotes variations in their organoleptic characteristics. Initially, the use of lactic acid bacteria, or LAB, produces an initial environment for the establishment of ripening microorganisms because they contribute to the peptidases released during initial milk hydrolysis, added to plasmin, and rennet activity modified the initial pH. Thanks to starter cultures, the flora is diverse in this type of cheese and it is difficult to prevent its development, although its presence promotes variations in pH levels and consequently creates the ideal environment for the growth of microorganisms during ripening. This mechanism can be observed in figure 2.2. If used milk is pasteurized, adventitious flora is added to the milk as a starter culture, and these microorganisms are those that promote the formation of the compounds that lead to changes in texture, taste, color, microbial activity, and specific sensory properties (Cotter & Beresford, 2017; Parente & Cogan, 2004; Spinnler, 2017).

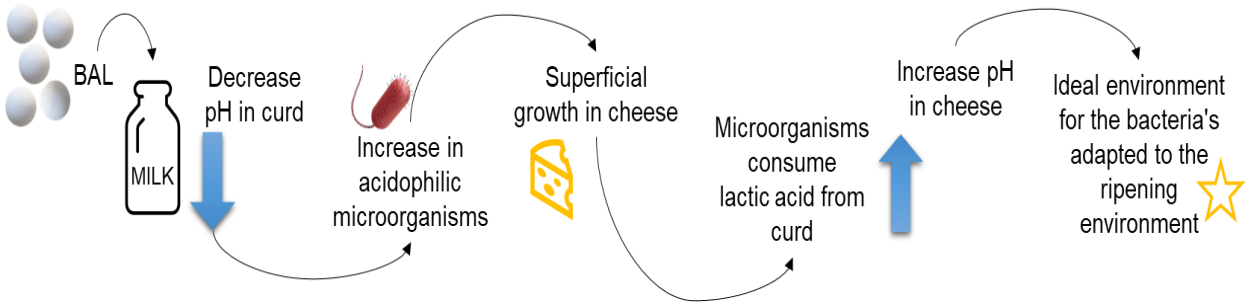

Figure 2.2 Environmental development mechanism for the establishment of microorganisms before ripening (Spinnler, 2017).

The cheese surface is highly complex. After the cheese production, the development of the yeasts begins, which produces a dense and whitish external crust of approximately 200 µm thick. The crust formation is due to the presence of *P. camemberti,* a yeast widely used in this type of cheese. This yeast develops in the surface area and with a few activities in the center of the cheese. Its growth begins from day 2 or 3 of ripening time. During ripening, the maximum growth peak is after 6 to 7 days and with a peak of enzyme production between 15 and 21 days. As the ripening time increases, the enzymatic capacity decreases. Its enzymatic capacity produces a large number of free amino acids thanks to the production of exopeptidases, which vary depending on the pH: if it is around 3.5 an acid carboxypeptidase is present, and if it is close to 8.0, an alkaline aminopeptidase is produced, causing variations in the sensory properties of the final product (Leclercq-Perlat et al., 2015; Sousa et al., 2001; Spinnler, 2017).

Another yeast that is used to produce surface-mold cheese is *Geotrichum candidum*. This yeast can produce a large number of proteinases with an optimum pH of 6.0. Generally, it is a yeast that does not have an important enzymatic activity, but when developed with *P. camemberti,* it has an intense proteolytic activity during the first five ripening days. On the other hand, if it is developed with *Kluyveromyces lactis, Debaryomyces hansenii*, and *Yarrowia lipolytica*, they cause an intense activity that catabolizes peptides and promotes the production of the characteristic flavor of cheese,

eliminating its bitterness. (Engel et al., 2001; Fox et al., 2017; Leclercq-Peralt et al., 1999; Mounier et al., 2005).

Once the yeasts have established themselves, and there have been significant changes in pH, de-acidification of the cheese, and other metabolic processes, aerophilic bacteria sensitive to acids, such as actinobacteria and proteobacteria, appears on the surface, such as *Brevibacterium linens, Arthrobacter, Micrococcus, Corynebacterium*, and *Brachybacterium*, which play an important role in the flavor and final appearance of the cheese (Fox et al., 2017; Mounier et al., 2017; Mounier et al., 2005; Spinnler, 2017).

Although *P. camemberti* gives the final product the fundamental characteristics, the secondary flora has an important complementary role in developing sensory properties. A large number of studies show that the combination of yeast, fungi, and bacteria promotes the appearance of a bacterial ecosystem in the cheese. Diverse authors have determined the biochemical qualities and the products obtained from the metabolism of each microorganism separately. However, when the cheese is understood as an ecosystem, these characteristics are modified taking into account the development conditions; for this reason, it is necessary to understand the importance of studying the product as a whole, which completes a life cycle due to its unique characteristics (Spinnler, 2017).

2.2 Glycolysis in surface-mold cheese ripening

The most important carbohydrate found in milk is lactose (4-O-β-D-galactopyranosyl-D-glucopyranose). This disaccharide, which can be observed in figure 2.3, is produced by the link between a D-galactose molecule and a D-glucose molecule. This union presents in its structure an anomeric carbon, promoting reduced characteristics. Of the carbohydrates found in food, lactose presents a few sweetening power and is less soluble than sucrose. Other oligosaccharides are also present in milk, which in general presents 3 to 10 carbons in its structure, which can be considered an important source of soluble fiber. These are capable of promoting the growth of lactic acid bacteria. In general, small ruminants milk contain these carbohydrates, but many authors have determined that in the case of goat milk, there is an important diversity of these molecules, with the existence of approximately 25 oligosaccharide fractions (Badui Dergal, 2016; Raynal-Ljutovac et al., 2008).

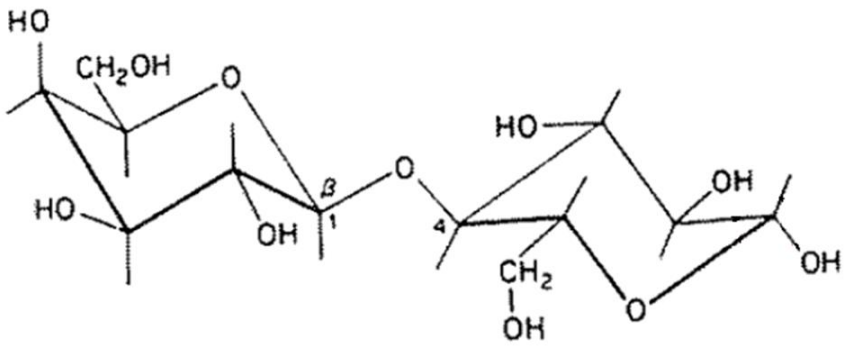

Figure 2.3 4-O-β-D-galactopyranosyl-D-glucopyranose molecule (Badui Dergal, 2016)

Milk generally has a pH between 6.3 and 6.4 which varies thanks to rennet addition. The addition of enzymes or precipitating substances promotes matrix acidification. After the whey elimination, the microorganisms begin the production of lactic acid in a protonated form, decreasing the pH at 4.6-4.8. The production of lactic acid and lactate promotes bacterial growth that gives the appropriate flavor conditions depending on the cheese type (Legg et al., 2017; Spinnler, 2017).

In surface-mold cheese, glycolysis is the first process that occurs after the establishment of microorganisms. In the case of surface mold cheeses, the first microorganism to consume lactose is *K. lactis*. Once this microorganism is established, it is displaced by *D. hansenii*, which consumes lactose and lactate produced by the first microorganisms. After 6 or 7 ripening days, and once a certain amount of lactate is produced in the cheese matrix from the consumption of lactose, the growth of some yeast such as *G. candidum* and *P. camemberti* is observed, which consume lactate for their growth as *D. hansenii*. This process promotes a decrease in pH since a large amount of lactic acid is produced due to the decrease in lactate consumed, which is also important in the final flavor of the product (Spinnler, 2017).

Once bacterial growth has started, an increase in external pH is observed thanks to the migration of lactate molecules and minerals such as calcium and phosphorus that go from the center to the surface, generating the formation of calcium phosphate, which helps the formation of the external crust. This entire process causes an increase in pH until reaching 7.0 after up to 21 days after the ripening process has started, where the enzymatic activities are developed, mainly those of the proteinic type. In the center of the cheese, the 6.0 pH can be observed thanks to the proteolysis produced and the lack of salts. All these variations lead to the rheological properties of the cheese, producing a softer center and the formation of the external crust characteristic of surface mold cheese. These biochemical changes only represent a part of all the machinery presented within the cheese, which will be seen in more detail in the next sections (Legg et al., 2017; Spinnler, 2017).

2.3 Proteolysis in surface-mold cheese ripening

2.3.1 Proteolysis

Cheese is a product that, by its nature, contains a large amount of protein. The most important and well-known are caseins, globular proteins that contain three fractions: α-casein, β-casein, and κ-casein, arranged in a spherical structure called micelle and which is balanced by the presence of diverse inorganic compounds. Its structure model can be observed in figure 2.4. The micelle is present in all kinds of milk regardless of the mammal from which it is obtained. This structure loses stability to external variations, such as the decrease in pH, changes in temperature, or the use of peptidases, which cause the denaturation of the κ-casein, promoting the precipitation of α and βcasein, which becomes narrow thanks to structures joined by the calcium content in milk (Akuzawa et al., 2009; Vázquez García, 2015).

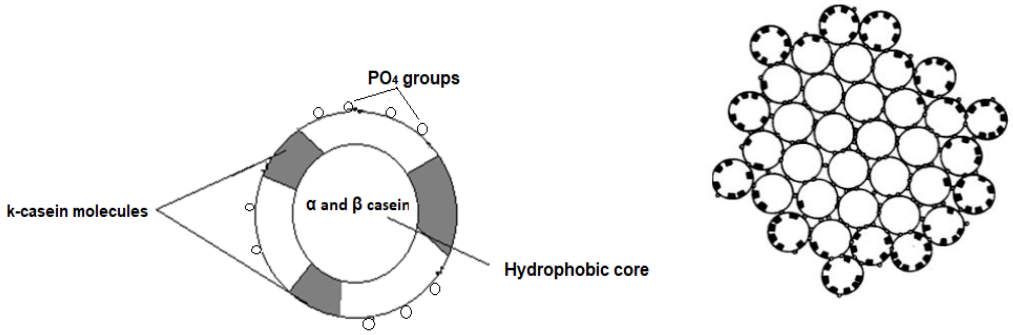

Figure 2.4 Micellar casein structure, proposed by Schmidt in 1982. Schematic representation of (a) Sub-micelle and (b) Caseine micelle composed by sub-micelles. Source: Vázquez García (2015) and Phadungath (2005)

The complete casein hydrolysis depends on each cheese variety. However, some mechanisms are present in all cheese varieties. Intact caseins in milk are hydrolyzed by coagulating enzymes added during the precipitation process that can destabilize κ-casein. Though, there are also enzymes contained in milk that promote the hydrolysis process, such as plasmin. Once the hydrolysis process has begun, a change in the pH of the milk is observed, and depending on the type of cheese, milk, and manufacturing characteristics, the caseins present different hydrolysis processes. Once this process occurs, added microorganisms can produce proteases that hydrolyze caseins to short peptides and free amino acids. The final cheese´s characteristics are affected by several process conditions like the type of milk, manufacturing requirements, pH, coagulant activity, moisture content, and the amount and type of microflora. For example, some cheeses are initially affected in α-casein, as in the Emmental cheese case. In contrast, there are cheeses such as Parmigiano, where β-casein is first hydrolyzed, and in the case of Mozarella cheese, the first one that is affected is the κ-casein (Ardö et al., 2017; Summer et al., 2017; Vázquez García, 2015).

As mentioned before, milk contains proteolytic enzymes that can destroy caseins, such as chymosin, the main protease used in the traditional precipitation of casein in cheese production. It can hydrolyze κ-casein, promoting destabilization of the micelle, and it is generally discarded after whey removal, although it is known that it remains in the clot on many occasions, causing more hydrolysis. Pepsin, which is widely related to chymosin in the coagulating agents used during casein precipitation, is very sensitive to changes in pH and is not closely related to the ripening process. Another important dairy enzyme within milk is plasmin, which is a proteinase that is directly related to the ripening of cheese, and whose main function is the hydrolysis of caseins without being greatly affected by changes in pH and by temperature variations, directly influencing the quality of the cheese (Ardö et al., 2017; Sanjuán et al., 2002; Uniacke-Lowe & Fox, 2017)

The casein hydrolysis also leads to the development of microorganisms since they take it as precursors of various metabolites, such as enzymes and other metabolic pathways. Ripened cheeses contain adventitious flora that also can produce peptidases in a similar way to starter microorganisms. Many of these microorganisms, like lactic acid bacterias, contain important and potent proteases, depending on the type of cheese and its ripening processes. LAB are microorganisms that require a significant amount of free amino acids for their growth. Milk does not contain amino acids; however, its proteolytic system causes caseins to become small peptides and free amino acids, which also contribute to the flavor of the cheese. The most used are *Lactococcus lactis* and *Lactobacillus*. The release of amino acids from peptides is catalyzed by a significant amount of intracellular peptidases released into the cheese matrix in cellular excretion. Species of the genus *Arthrobacter* grow on the surface with molds and yeasts help, this effect can be observed in cheeses such as Brie and Camembert. An example of these enzymes is lactocepin found in the internal part of the LAB cell released from the cell wall with the help of free calcium ions, taking advantage of the precipitation process as release initial. It is produced to hydrolyze caseins to oligopeptides (Liu et al., 2010; McAuliffe, 2017; O'Sullivan & Cotter, 2017).

Another agent that promotes the ripening process in cheese is the presence of proteolytic enzymes produced by molds and yeasts. Some cheeses are ripened from the surface with the help of molds and yeasts such as Camembert and Roquefort cheese. *Penicillium spp* is one of the major components of the microbiota and its enzymes are very important in the ripening process in cheeses such as those used in this project. *P. camemberti* and *P. roqueforti* are considered two widely used microorganisms for their ability to destroy proteins. Both synthesize an aspartyl proteinase, capable of hydrolyzing αS1-casein much faster than other caseins, has specificity for peptides containing hydrophobic or aromatic amino acids, and has an optimum pH of 3.4. Another enzyme they produce is the acidic proteinases that promote hydrolysis on β-caseins, with a preference for some bonds such as Lys-Val, Lys-Glu, or Lys-Ile. It is known that there is a close relationship between the presence of this enzyme and the release of amino acids that produce bitter flavors in cheese (Ardö et al., 2017; Batty et al., 2019; McSweeney, 2004; Sousa et al., 2001).

Another important yeast in cheese technology is *G. candidum*, which can synthesize both extracellular and intracellular proteinases. However, its contribution is less than other microorganisms. This yeast can be found on the surface of surface mold-ripened cheeses (Batty et al., 2019; Cotter & Beresford, 2017). In the case of surface mold cheeses, proteolysis is one of the most studied processes in ripening. In general, the most intense hydrolysis occurs in αS1-casein in all cheese areas. The β-casein is highly degraded in the external areas of the cheese, without degradation in the internal areas. Generally, the nitrogen contained in the caseins that are released during the proteolysis process represents about 35% of the total nitrogen and is mainly contained in small peptides. Like other cheeses, enzymatic proteolysis is promoted by the presence of rennet, plasmin contained in milk, and proteases produced by microorganisms. In Camembert cheese has been observed that about 15% of the rennet is retained in the curd, with intense proteolysis. The behavior of pH throughout the proteolysis process, which is also closely related to the glycolysis process, promotes the appearance of different types of enzymes, as shown in figure 2.5 (Bansal et al., 2009; Spinnler, 2017).

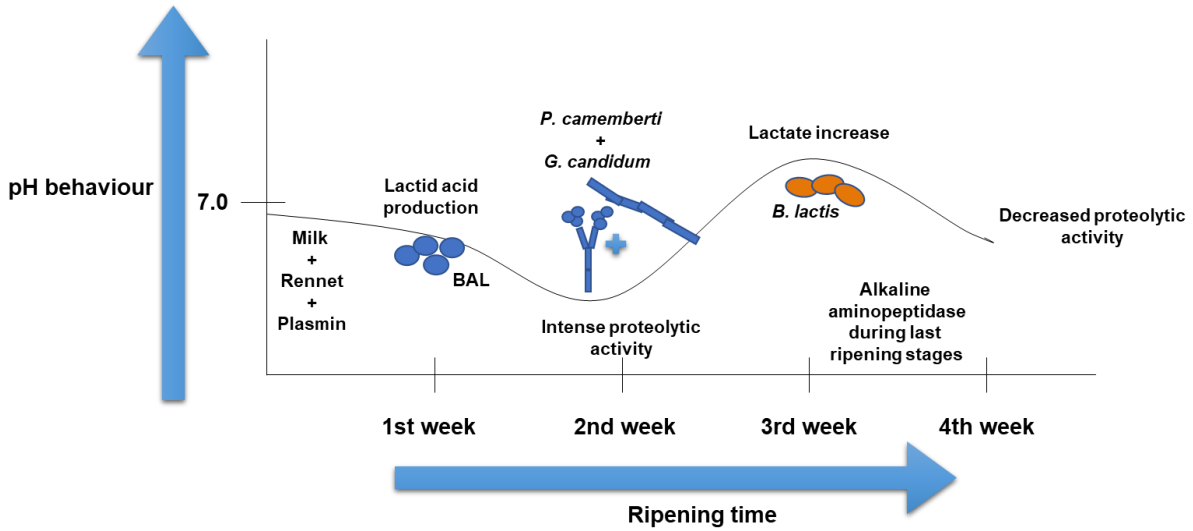

Figure 2.5 A scheme that represents the behavior of pH throughout the ripening process, the microorganisms involved, and produced substances.

2.3.2 Bioactive peptides in cheese

As already previously mentioned, cheeses represent an important source of peptides that are produced from the casein decomposition after cheese production and ripening thanks to the proteases and peptidases obtained from milk, the precipitation process, and the presence of microorganisms in cheese and the human digestive tract, which promote the production of free peptides. There is much evidence that shows that these peptides have certain biological activities that vary depending on the type of milk, type of cheese, ripening time, and the amount and type of microorganisms present, as the use of BAL as *L. helviticus, L. delbrueckii,* and *L. casei*, and the presence of yeast with high proteolytic activity (Abdel-Hamid et al., 2017; Choi et al., 2012; Dimitrov et al., 2015).

Some authors have demonstrated the presence of hypertensive effects in cheese, where it has been observed that there is inhibition of the angiotensin-converting enzyme, an enzyme that increases blood pressure affecting the kidney, heart, and blood vessels. Cow, goat, sheep, buffalo, and camel milk have been shown this activity, and the cheeses obtained from them have also shown an increase in biological effect after the ripening process, mainly in medium to extended ripening cheeses effect such as Crescenza, Gorgonzola, Mozzarella, Edam, Gouda, Cheddar, Roqueforti, Emmental, Parmesano, and Feta. This is the case of Parmigiano Reggiano cheese with 12 ripening months, Grana Padano cheese, Bulgarian white brined cheese, white cow cheese with up to 120 ripening days, Gouda cheese after 8 ripening weeks, Gamalost cheese, and Norvegia cheese (Barać et al., 2017; Basiricò et al., 2015; Bernabucci et al., 2014; Dimitrov et al., 2015; Nielsen et al., 2017; Paul & Van Hekken, 2011; Qureshi et al., 2013).

Another effect widely studied in dairy products is the antioxidant activity, where it has been observed that the peptides produced can prevent the oxidation of some reagents *in vitro*, such as ABTS and DPPH. It has been determined that cow, goat, buffalo, camel, and sheep milk show the activity and the cheeses derived from these such as white cow cheese, Parmigiano Reggiano cheese which presents an increase in activity after the ripening process, Cheddar cheese, Feta cheese, Pecorino cheese, and Roquefort cheese in different ripening times (Barać et al., 2016; Bottesini et al., 2013; De Gobba et al., 2014; Gupta et al., 2009; Huma et al., 2018; Meira et al., 2012).

Another effect that has been widely studied is the antimicrobial capacity of some peptides found in milk. This activity has been reported in buffalo, goat, and sheep milk against microorganisms such as *E. coli* and *B. cereus* using *in vitro* models. Activity has also been observed in dairy products such as cheeses and yogurt. These peptides have been used in the food industry as a preservative additive for processed products. In the case of ripened cheeses, activity against *E. coli* in Canastra cheeses after 30 ripening days have been observed (Fialho et al., 2018). Also, the activity against *L. innocua* and *L. monocytogenes* in Asiago d'Allevo cow cheeses after 18 ripening months (Lignitto et al., 2012) and in 12-month-old Cheddar cheeses, inhibitory activity against *E. coli, B. cereus*, and *S. aureus* was observed (Pritchard et al., 2010)Vivar Quintana et al., 2009. Another example of the effect is the inhibition of *P. aeruginosa* and *S. aureus* strains was found in camel whey and cheese (Abdel-Hamid et al., 2020).

Some studies have demonstrated the presence of other biological activities related to casein hydrolysis; although more studies are required to isolate the peptides responsible, such is the case of the anti-inflammatory activity, opiate activity, immunomodulatory activity, antithrombotic and potential effects on hormones and growth factors. The study of these activities is important to determine the effects of the final product on humans and the uses that can be made as a functional food (Abdel-Hamid et al., 2017; Choi et al., 2012).

In the case of surface mold cheeses, such as Camembert cheese, some authors have established the presence of peptides with anti-hypertensive activity, containing peptides that are inhibitors of the angiotensin-converting enzyme. There is evidence that this type of cheese presents immunomodulatory and opioid activity, particularly after 6 to 8 ripening weeks. Surface mold cheese produced with goat milk has been shown the same biological effects after 2 ripening weeks, and antioxidant effects were observed included in raw goat milk and fresh cheese (Bütikofer et al., 2007; Choi et al., 2012; Korhonen & Pihlanto, 2003; Saito et al., 2000).

2.4 Lipolysis in surface-mold cheese ripening

Contained fats are macronutrients that present the most important variations. Their variations depend on lipolysis that promotes the liberation of free fatty acids from triglycerides, diglycerides, and monoglycerides during all cheese elaboration and occurs when the triacylglycerides in whole milk are separated to produce free fatty acids. This effect is related to the origin of the milk, the type of cheese, the production process, and the ripening time to which the cheese is subjected, promoting the modification of nutritional and sensorial conditions in the final product, and are related to different circumstances, including the presence of lipolytic microorganisms, manufacturing temperature, storage time, oxygen concentration, moisture content and the use of antioxidant (Alichanidis & Polychroniadou, 2008; Barłowska et al., 2018; Lawlor et al., 2002; Martínez-Cuesta et al., 2011; Park, 2001; Sanchez-Macias et al., 2011)

Like milk, in the case of cheeses, variations in production, flavor, and characteristics depend on the climatic conditions where the milk is produced. It has been shown that variations can occur in the same regions that promote subtle changes in flavor (Alichanidis & Polychroniadou, 2008). It was observed that different stages in lactation time affect meltability, color, and sliceability in some cheeses obtained from goat milk, like cheddar and Colby cheeses (Olson et al., 2007). This effect is produced by different concentrations of fatty substances such as carotenoids and other lipid-type compounds obtained from the animal diet. These concentrations are directly affected by variations in the external conditions of the animal from which the milk is obtained; therefore, climatic conditions, the presence of rain, changes in humidity, and other environmental factors due to rainfall variations depending on whether it is a mountainous or plateau area. Also, it has been determined that the presence of greater or lesser amounts of cholesterol content is directly related to the feeding of the animal (Barłowska et al., 2018).

Another factor that shows variations in the effect of lipolysis during cheese production is the manufacturing process. Castillo et al. (2007) observed that the use of sanitized rennet without starter cultures promoted an increase in the concentrations of mono and diglycerides and short-chain fatty acids in ripened goat cheeses. The use of paste rennet has shown an increase in the lipolysis of goat cheeses with 15 days ripening, unlike the traditional procedure, where the cheese is ripened during 45 days with commercial rennet, improving times and organoleptic characteristics (Fontecha et al., 2006).

The presence of starter cultures is one of the factors that most directly affect the release of fatty acids, particularly by the production of acetic acid (2:0 Δ0), which appears in greater quantity when there are starter cultures, this thanks to the conversion of lactose by other metabolic pathways, modifying the flavor conditions in the final product (Kaminarides et al., 2007; Martínez-Cuesta et al., 2011; Voigt et al., 2010). This effect has been observed in the works elaborated by Kondyli et al. (2002), Zlatanos et al. (2002), and Manolaki et al. (2006) observed variations in Feta cheese with starter cultures that included *Lactococcus Lactis subsp. lactis* and *Lactococcus Lactis subsp. cremoris*, where the presence of free fatty acids and organic acids as acetic, butyric, and propionic acids was higher in cheeses made with whole milk as opposed to skim milk, both with positive effects in sensory properties. In this same way, Thierry (2004) observed that Propionibacterium freudenreichii in Emmental cheese promoted the liberation of 57 compounds, where short-chain fatty acids, esters can be found, and ketones that are related to lipolysis and aroma characteristics in the final product.

Pasteurization is a technological process that affects the number of fatty acids, as reported by M. Buffa et al. (2001), where cheeses made with raw and pasteurized milk were compared, observing that there is no significant difference between lipolysis with and without treatment after 60 ripening days. The use of preservation agents also modifies the lipolysis process, as is the case with brine. As observed by Kaminarides et al. (2007), using a 10% NaCl solution promoted the delay in lipolysis in Halloumi cheese, which after 45 days of ripening only presented some free fatty acids such as palmitic, caproic, and oleic acids. This same effect was observed by Pavia et al. (2000), who determined in brine vacuum impregnated Manchego cheese with the presence of less lipolysis in external areas of the cheese, which are closer to the saline solution after a month of ripening, with no difference detected by a sensory panel. Prieto, Urdiales, et al. (2000) observed a delay in lipolysis due to the amount of salt used during the manufacturing of Quesuco de Liébana cheese.

Ripening time is also a factor that promotes variations in lipid fractions. Nájera et al. (1998) observed that the ripening time promotes the appearance of different compounds. During the first two ripening months of Idiazabal cheese, the appearance of short-chain free fatty acids was observed, followed by the appearance of unsaturated fatty acids after three months of ripening, as well as a decrease in saturated fatty acids in the same period. Castillo et al. (2007) demonstrated the presence of up to 28 compounds belonging to families of fatty acids in goat cheeses made in different processes. The ripening process promotes lipolysis, either by the presence of starter cultures, by the pregastric esterases found in rennet, or by the presence of natural milk microflora. Sanchez-Macias et al. (2011) observed the same effect in goat cheese elaborated in the Canarian Islands after 28 ripening days with a lipolysis increase in full-fat cheese, promoting off-flavor in low-fat cheese.

These same results were observed by Franco et al. (2003) in a goat ripened cheese elaborated with raw milk that raised free fatty acid 18:1 Δ9, 16:0 Δ0, and 10:0 Δ0 concentration during the ripening process. Mallatou et al. (2003) observed the same effect in Teleme cheese elaborated with ewe, goat, and cow milk, finding a rise in

lipolysis process during 180 ripening with a high amount in free fatty acids production. El Galiou et al. (2013) showed that the concentrations of free fatty acids increased with the ripening of cheeses made with starter cultures, increasing the concentration of free fatty acids from 4:0 Δ0 to 18:2 Δ9,12. Toschi (2003) observed the increase of acetone after 90 days, and acetic acid, diacetyl, and acetoin after 180 ripening days in low-fat Kefalograviera cheese produced with starter cultures. Güler (2005) and Güler and Uraz (2004) note the presence of oleic, palmitic, myristic, and stearic acids in Kasar and Turkish white cheese after 3 ripening months. The same results were observed by Prieto, Franco, et al. (2000) and Prieto et al. (1999) in Picon Bejes-Tresvisoblue cheese after 120 ripening days. The changes in the release of fatty acids gave the cheese different sensory conditions that were observed by a panel concluding that the product's final characteristics have a highly significant correlation with its flavor.

The use of fortifying agents also has some effects on the lipolysis of ripened cheeses. The use of prebiotics such as inulin promotes the development of LABs that lead to the production of polyunsaturated fatty acids such as linolenic and linoleic acids. The use of oils such as fish and canola promotes the presence of medium and long-chain unsaturated free fatty acids that modify the sensory characteristics of the final product (DePeters et al., 2001; Güler, 2005; Kiss et al., 2019). In this respect, Mele et al. (2011) showed a Pecorino cheese elaborated with ewe milk fortified with conjugated linoleic acid during animal feeding that increased the concentration of cis-9, trans-11 linolenic acid, trans-11 C18:1, and alpha-linolenic acid.

In the case of surface mold cheeses, molds and yeasts also cause lipolysis since they produce high amounts of lipases. As in other types of cheese, the lipolysis process is fundamental in the formation of the final texture of the cheese since it is a solvent for flavor molecules, which are necessary for the perception of the food, giving it softness due to emulsification promoted by salivary lipase. It has been determined that lipolysis is much more intense under the crust, explaining the softness in this area of the product *P. camemberti* produces an extracellular alkaline lipase that is active at pH 6.0 and ripening temperatures. This enzyme is most active in triacylglycerides that contain low molecular weight fatty acids. *G. candidum* produces a lipase that releases oleic acid and other unsaturated fatty acids from triacylglycerides. On the other hand, *K. lactis* can accumulate short and medium-sized free fatty acid chains without much effect on fatty acids such as caprylic, caproic, capric, and lauric. This yeast is considered one of the most lipolytic presents in this type of cheese (Spinnler, 2017).

Chapter 3

Materials and Methods

To carry out the experimental phase of the present work, the methodology followed was separated into four parts: a) Initially, the first section was the controlled ripening process, followed by texture and physicochemical analysis, b) the second section was the proteolytic analysis and biological activity determination, c) the third section covered the lipid analysis section, and d) thee the fourth section that included the multivariate statistical analysis, elaborated with all obtained results during the three previous experimental phases. A general scheme of the entire experimental work can be observed in figure 3.1.

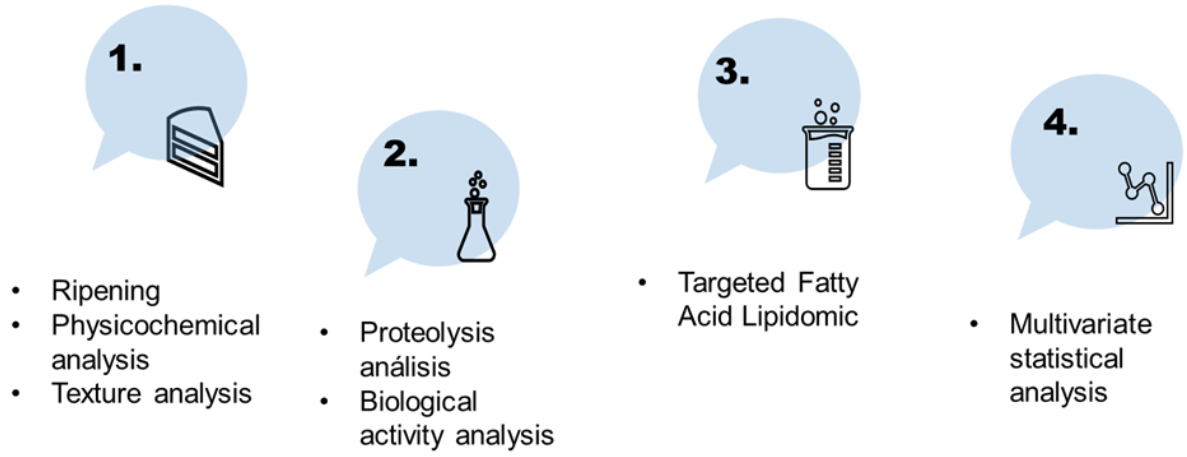

Figure 3.1 Methodology general scheme.

3.1 Cheese manufacture

The manufacture of the cheese used in the experimental phase of this work is described below. The cheese was produced using grazing feeding (grass, shrubs, shoots, and leaves of local plants such as *Brugmansia candida* and *Brugmansia sua veolens*) primiparous goat milk obtained in Pacho Viejo, Coatepec, México (at 1199 m above sea level). Goat milk was obtained by manual milking between April, May, and June to avoid climate and goat alimentation variation and reduce season variability using milk of the same animals. Then, milk was pasteurized for 30 minutes at 65 °C.

An artisanal method was carried out for cheese production, and when the temperature after pasteurization reached 30 °C, the milk was inoculated with *Staphylococcus lactis* and *Staphylococcus cremoris,* and *Penicillium candidum*. Milk coagulation was produced by adding bovine renin rennet at 1% (Coagulmex Co, Veracruz, Mexico). After rest for 8 hours, whey was eliminated, salt was added, and cheese was molded by hand, giving each piece a cylindrical shape with a height of 15 cm and a diameter of 5

cm, 200 g weight, and 24 hours of refrigeration. A total of three batches was elaborated following this procedure, every batch contained 27 cheese pieces, and a total of 81 pieces were used during all experimental phases. Finally, cheeses were transported under refrigeration at 4 °C to Tecnologico de Monterrey facilities on the same day of production.

3.2 Ripening process

All cheeses were placed in an electronic ripening chamber Membert HPP 260 (GmbH Co, Germany) as shown in figure 3.2, at a temperature between 13 and 14 °C, constant humidity (85%), and continued aeration for ammoniacal air elimination. All pieces were rotated once a day for 5 days, and then they were left to repose until 90 days were reached. Samples were obtained on the first (day 1), 5th (day 2) 8th (day 3), 12th (day 4), 19th (day 5), 29th (day 6), 40th (day 7), 55th (day 8), and 90th (day 9) ripening days.

Figure 3.2 Samples in the ripening chamber.

3.3 Physicochemical analysis

Each sample obtained from the ripening chamber was cut vertically at the center, exposing the cheese's central face as shown in figure 3.3. Then, the pH was valued directly on central and external faces by triplicate using an Orion 3-Star pH meter (Thermo Fisher Scientific) following the methodology by Guerra-Martínez et al. (2012). Moisture determination was elaborated by triplicate and following the oven drying method for moisture determination in milk and dairy products at 100 ± 2 °C (NOM-243-SSA1-2010, 2010).

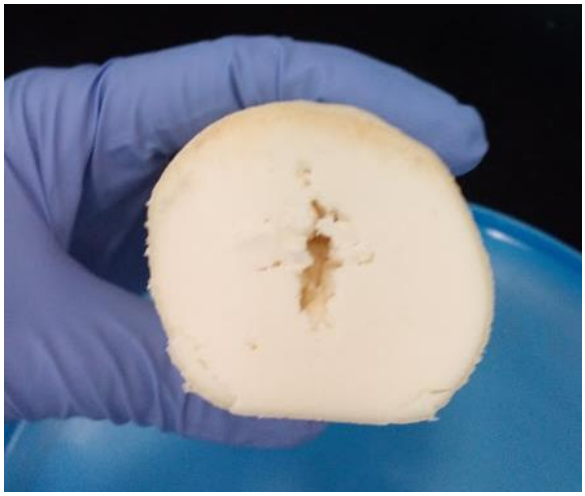

Figure 3.3 Cheese with central face exposed.

3.4 Instrumental texture profile analysis (TPA)

Each cheese was cut into 8 cm^3 cubes using a manual guillotine as is observed in figure 3.4, and of each cheese, there were obtained 8 pieces. Each cube was analyzed in a CT3 Texture Analyzer (Brookfield Engineering Laboratories, MA, USA), a device which can be observed in figure 3.5 ranging from 10 g to 50 kg of capacity. The TPA type manual test was elaborated using a 4.5 cm diameter cylindrical probe measuring cell model TA-AACC36 and height-adjustable rectangular base model TA-BT-KIT with a maximum of 50% of the height of the sample.

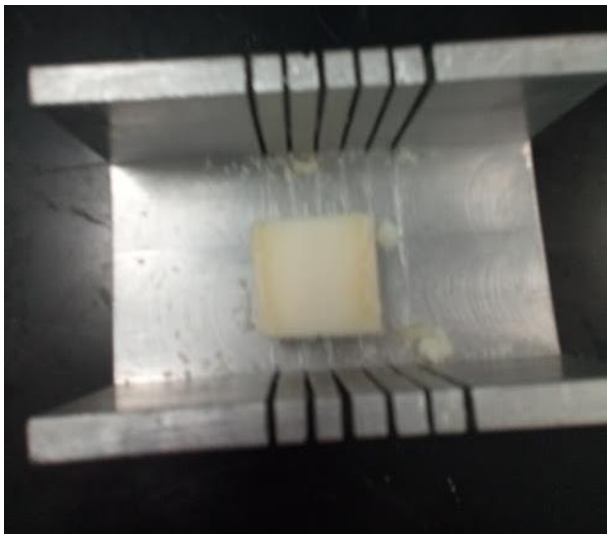

Figure 3.4 Cheese cube in a manual guillotine

Figure 3.5 Texture Analyzer (Brookfield Engineering Laboratories)

The conditions followed for the analyses were a trigger of 15%, deformation of 10 mm, and speed of 3.3 mm/s, and the analyzed parameters were hardness, cohesiveness, springiness, gumminess, and chewiness. All cheeses were analyzed by triplicate. (Guerra-Martínez et al., 2012).

3.5 Cheese homogenate

The cheese homogenate was and 3.5.1 section sample preparation was generated following method Martin-del-Campo et al. (2007). Once the texture analysis was carried out, every cheese sample was grounded until small fragments until uniform sample. 20 g of this sample were taken and diluted in 20 ml of deionized water (1:1 cheese/water). This mixture was dispersed using a homogenizer Ultra-Turrax T-18 basic (Ika Co, Germany) at 20,000 rpm for 5 minutes. Subsequently, the sample was stored in 50 ml tubes at -20 °C until later. These samples were labeled as CH (cheese homogenated).

3.5.1 Sample preparation for lipidomic analysis

5 g of the cheese homogenate taken and incubated at 40 °C in a water bath for one hour, followed by a second homogenization. Subsequently, the suspension was centrifuged for 30 minutes at 4 °C and 3000 g. The solidified fat layer was removed and stored at -20 °C until later use. The non-fat lower layer was homogenized again and stored at -20 °C .

3.5.2 Sample preparation for lactic acid analysis

Following the methodology developed by Leclercq-Peralt et al. (1999), 2.5 g of the homogenate of every non-fat lower layer were diluted in 2.5 ml of distilled water. This mixture was incubated at 50 °C for one hour in a water bath and then was dispersed using a homogenizer at 25,000 rpm for 2 minutes. After the samples were cooled to 25 °C, 2.5 ml of trichloroacetic acid (240 g/l) (Sigma-Aldrich, Steinheim, Germany) and 2.5 ml of distilled water were added to each suspension. This mixture was homogenized, incubated at 25 °C for one hour, and finally filtered with a Whatman filter no. 42

3.5.3 Sample preparation for nitrogen fraction analysis

3.5.3.1 No protein nitrogen (NPN)

The no protein nitrogen fraction was extracted as per Leclercq-Peralt et al. (1999). 5 g of the homogenate of every non-fat lower layer was diluted with 45 ml of NaCl solution (9 g/l) and homogenized at 25,000 rpm for 5 minutes. 20 ml of a trichloroacetic acid solution (240 g/l) were added to an aliquot of 20 ml of this suspension, and it was homogenized at 25,000 rpm for 2 minutes. After incubation at 25 °C for one hour in a water bath, it was centrifuged at 4,000 rpm at 7 °C for 10 minutes. The supernatant corresponded to the NPN fraction

3.5.3.2 Acid soluble nitrogen (ASN)

For acid-soluble nitrogen fraction was developed following the method proposed by Hernández-Galán et al. (2017); Huma et al. (2018). 10 g of the homogenate of every non-fat lower layer adjusted to a pH of 4.6 with HCl 2N. The mixture was incubated in bath water at 25 °C for 20 minutes, later was centrifugated at 6,000 rpm for 45 minutes at 7 °C. After centrifugation, every sample was filtered with a Whatman filter no. 42. The supernatant corresponded to the ASN fraction

3.5.3.3 Ethanol soluble and not soluble nitrogen (EtOH-SN and EtOh-NSN)

Finally, for ethanol soluble and not soluble nitrogen, the method developed by Hernández-Galán et al. (2017) was followed. The fraction is prepared by adding ethanol to 2 ml of the ASN solution until reaching a concentration of 700 ml / l. This mixture is incubated in a water bath for 24 hours at 25 °C. The soluble fraction is separated by centrifugation at 6,000 rpm for 20 minutes at 6 °C. The precipitate obtained corresponds to fraction EtOH-NSN, and the supernatant corresponds to EtOH-SN fraction. Subsequently, 2 ml of the EtOH-SN fraction were taken and concentrated until dryness in a speed-vac and solubilized in 1 ml of 70% ethanol. In the case of the precipitate corresponding to the EtOH-NSN fraction, it was solubilized in 0.5 ml of Tris HCl 50 µM and 0.5 ml of EDTA 1 µM. All samples were filtered on Whatman No. 42.

3.6 Lactic acid analysis

To carry out the lactic acid analysis, the technique proposed by Leclercq-Peralt et al. (1999) was followed. A high-performance liquid chromatograph (HPLC), Agilent Technologies, Model 1200 (Palo Alto, CA, USA) integrated with a diode array detector (DAD) was used. The device can be observed in Figure 3.6. The compounds separation was performed in a Carbohydrates column (5 µm, 4.6 nm I.d. x 150 mm, Agilent, USA) at 50 °C, with 0.005 M sulfuric acid solution at 0.6 ml/min flow. Quantification was developed using a standard solution of propionic acid (Sigma Aldrich) calibration curve (0.5 to 22.5 mg/l). DAD was set at 210 nm.

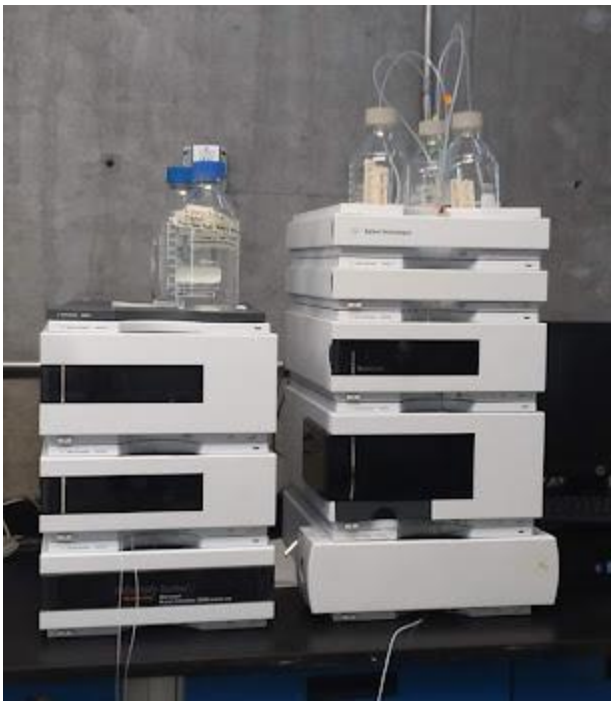

Figure 3.6 High-performance liquid chromatograph (HPLC) Model 1200

3.7 Nitrogen fractions determination

The nitrogen fractions determination was developed using the method proposed by Hernández-Galán et al. (2017). All fractions were filtered through a 0.45 µm Whatman filter before injection. A reverse phase HPLC technique was used, and an Agilent Technologies, Model 1200 (Palo Alto, CA, USA) integrated with a diode array detector (DAD) was used. The column was a Zorbax Eclipse XDB-C18 (5 µm, 4.6 µm i.d. x 150 mm, Agilent) at 0.75 ml/l flow in a biphasic solvent system. Phase A) was 100 ml/l of acetonitrile and 0.5 and 0.5 ml/l of TFA diluted in HPLC grade water. Phase B) was 600 ml/l of acetonitrile and 0.5 ml/l of TFA in HPLC grade water. Samples were initially eluted with 100% of phase A for 10 minutes, followed by a linear gradient from 0% to 49% of B for 98 minutes. Subsequently, B was increased from 50% to 80% up to 108 minutes, followed by a linear gradient from 80% to 100% of B up to 5 minutes, and it

was kept for 5 more minutes 100% of B. The injected volume was 10 µl and DAD was set at 215 nm

In each of the chromatograms obtained, the % area was obtained, and subsequently, the HO / HI ratio was determined following the De Llano et al. (1995) method, where it is established that all those peaks found between minutes 1 and 35 of the run are observed peptides with hydrophilic qualities, and from 35 minutes of running to 120 minutes, those with hydrophobic characteristics are retained.

3.8 Antioxidant activity determination on 2,2-diphenyl-1-picrylhydracyl (DPPH).

For the elaboration of this determination, the methodology proposed by Hernández-Galán et al. (2016) was followed, where the antioxidant activity is measured with the 2,2-diphenyl-1-picrylhydracyl reagent (DPPH). Initially, 0.02 ml of the ASN and NPN fractions were measured, and each of the samples was placed in a well of a 96-well flat-bottom plate. Subsequently, 0.22 ml of a DPPH solution (125 µM DPPH in 800 ml/l methanol in water) was added to each sample. The plate was covered with aluminum foil to promote darkness for 90 minutes. Subsequently, the plate was read at 520 nm in a UV-Vis spectrophotometer (X Mark Microplate Reader, Bio-Rad Laboratories, Inc Japan). Results were expressed as the percentage of discoloration, and reagent grade methanol (Karal, Leon, Gto. Mexico) was used as a blank.

3.9 Fatty acid profile determination

For this methodology, the method of Martin del Campo Barba (2006) was followed. To 100 mg of purified fat, 0.9 ml of chloroform was added. 0.1 ml of sodium methoxide diluted in methanol (0.5 N) is added to this mixture. The substance is added to a 3 ml vial, mixed, and allowed to stand for 20 minutes at room temperature for sedimentation. The analysis was followed on an Agilent Technologies 5957C insert MSD gas chromatograph (Palo Alto, CA, USA) with a triple-axis detector. A 112-88A7 HP-88 column was used for chiral compound length 100 m, diameter 0.250 mm, and film thickness 0.20 µm with temperature limits of 50 °C to 250 °C. The analysis conditions were an initial temperature of 70 °C by 2 minutes followed by a ramp to 20 minutes until 230 °C were reached and helium as carrier gas. The device can be observed in Figure 3.7. Famy of acids methyl esters was identified using the database found in the device interface (NIST). The identity of 33 compounds was confirmed using a calibration curve elaborated with 33 reference standards, which are shown in Table 1 (Sigma Aldrich).

Table 1. Reference substances used in the calibration curve for famy acids methyl esters identification.

Compound	Purity
Methyl butyrate	99.90
Methyl hexanoate	99.70
Methyl heptanoate	99.90
Methyl octanoate	99.90
Methyl nonanoate	99.90
Methyl decanoate	99.90
Methyl undecanoate	99.50
Methyl laurate	99.80
Methyl tridecanoic	99.90
Methyl tetradecanoate	99.70
Methyl myristoleic	99.90
Methyl pentadecanoate	99.60
Methyl palmitate	99.90
Methyl palmitoleate	99.90
Methyl heptadecanoate	99.40
Methyl octadecanoate	99.90
Cis-9-oleyc methyl ester	99.90
Methyl cis-6-petroselinic	99.90
cis-acido vacenico metil ester	99.00
Methyl linoleate	99.90
Methyl nonadecanoate	98.20
trans-9-eladic methyl ester	96.90
Methyl arachidonate	99.90
Methyl ester linoleic acid	99.90
Methyl eicosenoate	99.90
Methyl heneicosanoate	99.90
Methyl docosanoate	99.80
Methyl cis-5,8,11,14 Eicosatetraenoic	99.30
Methyl erucate	99.30
Methyl tricosanoate	99.80
Methyl lignocerate	99.80
Methyl nervonate	99.90
All cis-4,7,10,13,16,19 Docosahexaenoic Acid	99.90

Figure 3.7 Gas Chromatograph Agilent Technologies

3.10 Statistic analysis

For the first stage, the statistical analysis of physicochemical and texture parameters was developed using the statistical software Statistica v. 12.0 (Statsoft, Inc., Tulsa, OK, USA). A one-way analysis of variance (ANOVA) was elaborated to determine significant differences between the obtained texture variables: hardness, springiness, chewiness, cohesiveness, and gumminess; physicochemical variables: moisture, internal and external pH, and lactic acid. A statistical analysis of Fisher (LSD) was accomplished for each variable with showing significant differences with $\alpha = 0.05$. Correlation analysis was developed to determine the relationship between all parameters. A principal component analysis (PCA) was applied in response variables to evaluate the development of all parameters during all ripening times.

The same software was used to analyze the results related to proteolysis and antioxidant activity. All chromatographic data were separated in %Area of individual peaks of the NPN fraction, %Area of individual peaks in the ASN fraction, %Area of individual peaks in EtOH-NSN fraction, %Area of individual peaks in EtOH-SN fraction, Area ratio between hydrophilic and hydrophobic in all fractions, and Antioxidant Activity in the ASN and NPN fractions. ANOVA was applied to all data to evaluate significant statistical differences ($p < 0.05$) between days of ripening. Subsequently, a statistical analysis of LSD was carried out to find differences between groups. Correlation analyses were carried out to find correlations between peptides and antioxidant activity. Finally, a PCA was carried out to determine the behaviors throughout all ripening times of HI, HO, and the ratio HO/HI. For this analysis, DPPH was considered as supplementary data.

The same software was used to results related to the lipidic profile. All chromatographic data were separated in % corrected Area peaks and ANOVA was applied to evaluate significant statistical differences ($p<0.05$) between all ripening days. Subsequently, a

Fisher's least square determinant test was elaborated to determine differences between groups. To determine the behavior through the maturation process, a principal component analysis (PCA) was carried out, and finally, a general discriminant analysis (GDA) forward stepwise (P to enter 0.05; P to remove 0.05) was developed to determine the compounds that most affect the model.

Chapter 4

Results and discussion

4.1 Physicochemical and texture analysis

4.1.1 Analysis of variance (ANOVA)

In Table 2 can be observed the obtained results in ANOVA analysis, where all parameters were evaluated. All parameters showed highly significant differences during all ripening processes, except central pH. Hardness, moisture, and cohesiveness showed important variations in the last ripening stages. Superficial pH and lactic acid are parameters that showed low significance (p> 0.05) during the firsts ripening stages but showed important diminution after 2 or 3 weeks. This behavior can be explained thanks to the presence of microorganisms in the cheese matrix.

Table 2. Analysis of variance (ANOVA) of physicochemical and texture analysis (Vázquez-García et al., 2020).

		Storage time [b]								
Parameter	***p*[a]**	**Day 1**	**Day 5**	**Day 8**	**Day 12**	**Day 19**	**Day 29**	**Day 40**	**Day 55**	**Day 90**
pH Rind	0.002**	6.66^{AC}	7.62^{AB}	6.63^{AC}	7.34^{AB}	7.66^{AB}	7.86^{B}	7.36^{AB}	5.75^{C}	5.64^{C}
pH Centre	0.831	5.94^{A}	6.08^{A}	6.15^{A}	6.54^{A}	6.41^{A}	6.57^{A}	6.60^{A}	5.94^{A}	5.38^{A}
Moisture (%)	0.000 ***	49.8^{F}	35.02^{D}	31.3^{CD}	27.4^{C}	20.62^{B}	15.63^{AB}	14.46^{AB}	11.02^{A}	4.23^{E}
Hardness (g)	0.000 ***	3309.8^{C}	2616.5^{BC}	2096.1^{B}	5748.7^{A}	5179.5^{A}	5597.7^{A}	7271.5^{D}	7683.8^{D}	9507.5^{E}
Cohesiveness	0.000 ***	0.21^{D}	0.18^{C}	0.19^{C}	0.14^{B}	0.13^{AB}	0.13^{A}	0.14^{AB}	0.19^{CD}	0.27^{E}
Springiness	0.000 ***	3.919^{C}	3.03^{B}	2.75^{AB}	2.47^{A}	2.4^{A}	2.56^{AB}	2.93^{B}	4.5^{D}	5.99^{F}
Chewiness (g)	0.000 ***	4844.8^{C}	2301.4^{A}	1085.4^{B}	2277.6^{A}	1689.5^{AB}	1868.7^{AB}	2875.8^{A}	6573.1^{D}	16016.1^{F}
Gumminess	0.000 ***	860.4^{AC}	534.9^{BD}	390.9^{D}	861.7^{AC}	685.1^{AB}	741.0^{AB}	995.6^{C}	1381.7^{E}	2666.9^{F}
Lactic acid (mg/l)	0.000 ***	15.16^{AB}	16.31^{AB}	16.75^{AB}	24.39^{C}	28.11^{C}	18.89^{B}	15.9^{AB}	15.16^{BC}	13.74^{A}

Notes: [a] Significant at *** $p < 0.001$ and ** $p < 0.01$; [b] Means with different letters within the same row are significantly different $p < 0.05$

4.1.2 Moisture and pH analysis

As can be seen in Figure 4.1, it was observed a constant decrease in moisture content from the first day of ripening until reaching the 90th ripening day. The initial moisture value was 49.8% to end with a moisture value of 4.23%, completing a total of 91.5% moisture loss throughout all processes.

This effect can be compared with data obtained by diverse authors, where significant decreases are observed in the moisture of cheeses after different ripening times as

observed M. Buffa et al. (2001) in cheese after 60 ripening days, Bugaud et al. (2001) observed in Abondance cheese after 180 ripening days and Sanchez-Macias et al. (2011) in artisanal goat cheese after 28 ripening days. This effect is due to the loss of water due to internal physicochemical processes and the temperature to which the product is subjected.

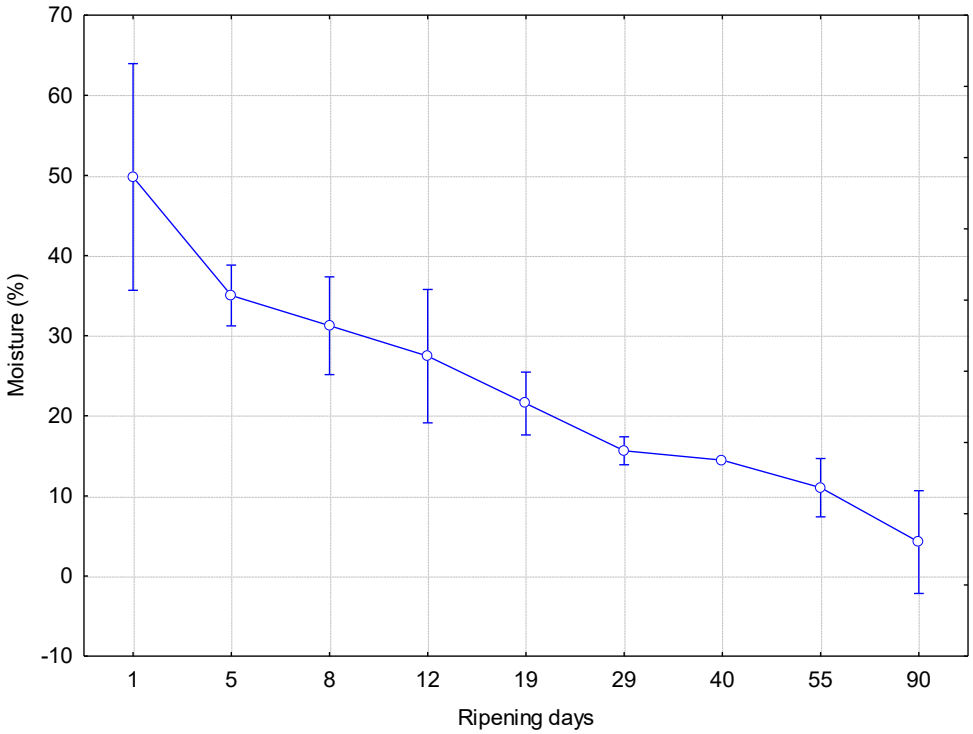

Figure 4.1 Moisture decrease during all ripening processes

On the other hand, Figure 4.2 shows the behavior of the pH in the center and the surface of the cheese during the whole ripening process. In both parameters, it can be observed that there are no significant changes in the pH values neither in the center nor on the surface, except for the last ripening day, where a slight decrease in pH is observed in both surfaces. This behavior has been reported by other authors, like Cholet et al. (2007) who observed a pH value diminution at the firsts ripening days induced by lactic acid bacteria and yeast as *Debaryomyces hanseni* and *Kluyveromyces lactis*. M. n. Buffa et al. (2001) did not observe a significant change in pH value in soft goat cheese after 60 ripening days. While Sahingil et al. (2014) observed an important pH decrease after 90 ripening days in white-brined cheese at different temperature stages.

The presence of lactic acid bacteria (LAB) and yeast, such as *Penicillium camemberti,* promotes the degradation of lactose to lactic acid during the first days of ripening, followed by a slight increase in the value of this parameter about 4 to 6 weeks caused by the release of casein due to the effect of plasmin produced by the same

microorganisms, to finally decrease again due to the presence of other compounds caused by proteolysis and lipolysis (Spinnler, 2017).

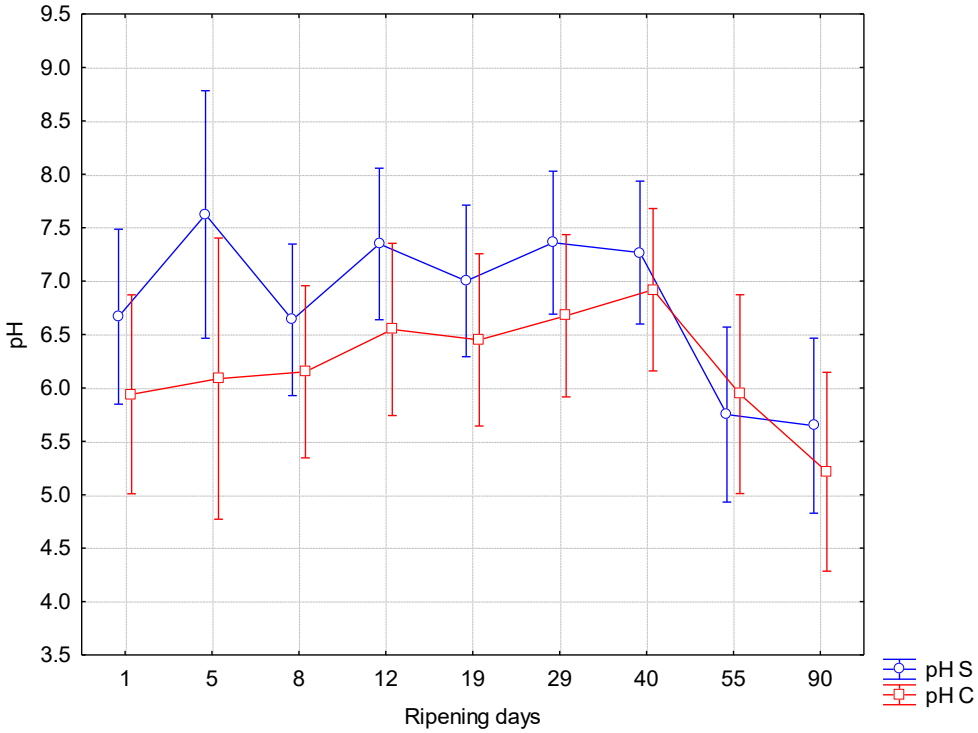

Figure 4.2 Centre and superficial pH during all ripening process

4.1.3 Texture analysis

In figure 4.3 can be observed the evolution in all texture parameters. All the parameters show the same tendency to decrease during the first 8 ripening days, followed by a fluctuating variation that remains until the 40th day of ripening, where the increase in all the texturometric properties of the product is observed until reaching the last day of ripening. It has been mentioned that all these characteristics are directly affected by the constant moisture lost in all ripening processes and the release of substances produced by the microorganisms found in the cheese matrix due to metabolic changes.

Ivanov et al. (2018) observed the same effect in hardness, gumminess, and chewiness, parameters that increased during 60 ripening days in Kashkaval cheese, and the variations were related to the proteolysis process. The same results were observed by Młynek et al. (2018) in Poland cheese after 30 ripening days, Martínez-Loperena et al. (2015) in artisanal Tepeque cheese after 165 ripening days, and Guerra-Martínez et al. (2012) in fresh panela cheese after 15 storage days, where there was observed a rise in the same parameters after-ripening. In all cases, variations are related to the proteolysis and lipolysis process, affecting the quality and sensory aspects of the final product directly.

On the other hand, some authors have mentioned that the variations present in the texture characteristics, mainly hardness case, are also related to the conditions to which the animal is exposed, such as climate, diet, and seasonal variations, modifying the rheological conditions of the cheese after the ripening process. This is the case of the observations elaborated by Tunick et al. (2007), Coulon et al. (2004), and Barłowska et al. (2018), where external variations that directly affect cheese production are mentioned, taking into account variables such as region and seasonally.

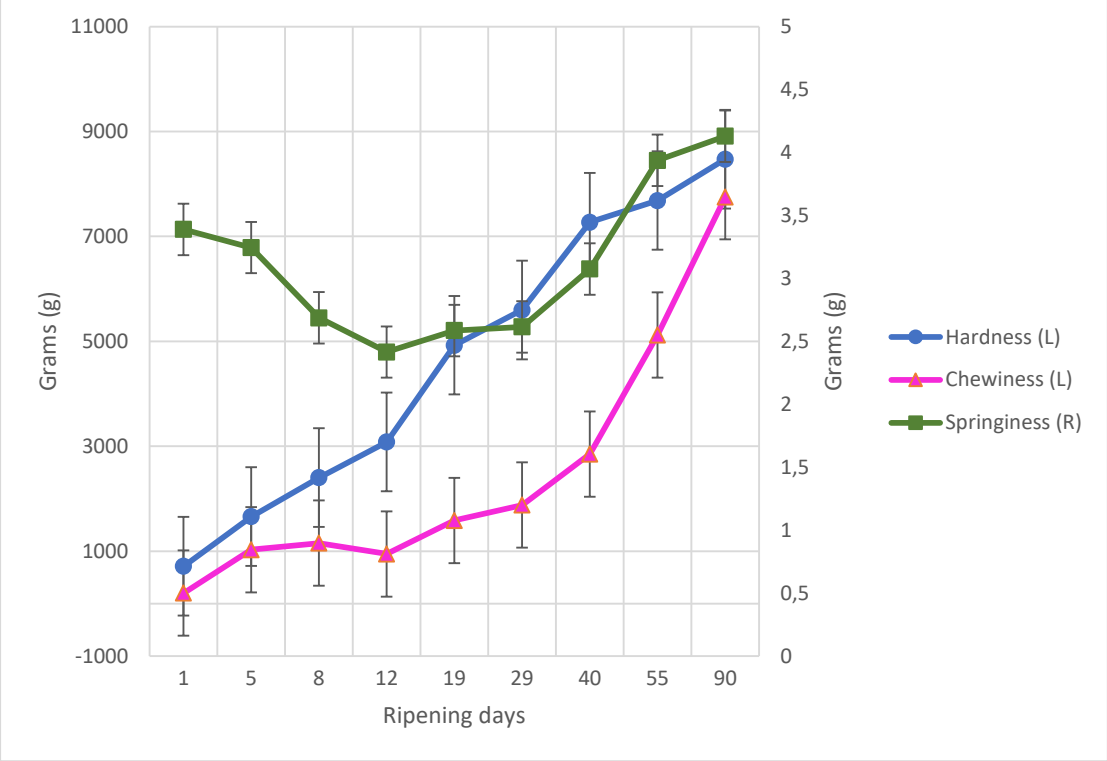

Figure 4.3 Texture parameters observed during all ripening process

4.1.4 Lactic acid analysis

Lactic acid behavior can be observed in Figure 4.4. There is a linear tendency during the first week of ripening followed by a significant increase in the concentration of the substance, which reaches a peak on 19 ripening days. From day 29, there is a decrease in concentration until the end of the ripening process, reaching a minimum value of 13.74 mg/l. This information is accordance with the studies carried out by Guerra-Martínez et al. (2012) in Panela cheese, and Güler (2005) in Kasar cheese.

As mentioned before, in surface-mold cheese as studied in this work, the presence of LAB and yeast promotes lactose consumption. Near the 21st ripening day, all lactose is consumed and converted to lactic acid, which migrates to the cheese surface and becomes lactate, promoting the pH decrease in surface cheese and modifying the texture characteristics (Spinnler, 2017).

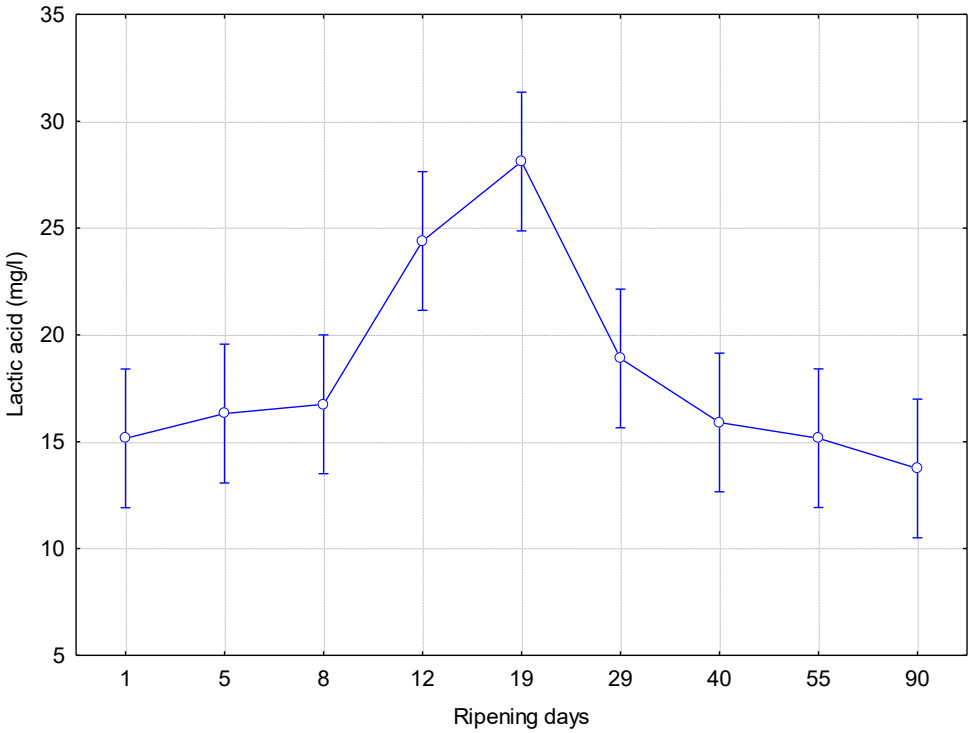

Figure 4.4 Lactic acid behavior during all ripening process

4.1.5 Correlation analysis

Table 3 can be observed the correlation analysis in all variables. Ripening time is significative correlated ($p < 0.05$) to almost all texture parameters in a positive way except cohesiveness, and is related to lactic acid, which, as seen previously, decrease during all ripening processes. On the other hand, it can be observed that the pH values are highly significavit correlated ($p < 0.01$) to the concentration of lactic acid, which has already been explained previously. Another data that can be observed is the highly significant negative relation ($p < 0.01$) between ripening and moisture, a parameter that did not present any relationship with any other variable studied. When hardness increase, moisture decrease, and this effect is related to the proteolysis produced in the cheese matrix, thanks to the production of ionized molecules of amino acid and carboxylic acid that promotes dehydration (Guerra-Martínez et al., 2012; Martínez-Loperena et al., 2015).

Table 3. Correlation coefficients[a] for the physicochemical and texture parameters were analyzed in goat surface mold cheese with an extended ripening process (Vázquez-García et al., 2020).

Variables	Ripening	Hardness	Cohesiveness	Springiness	Chewiness	Gumminess	pH Rind	pH Centre	Lactic acid
Hardness	0.75 ***								
Cohesiveness	0.37	0.52 **							
Springiness	0.45 **	0.73 ***	0.72 ***						
Chewiness	0.67 ***	0.90 ***	0.81 ***	0.85 ***					
Gumminess	0.71 ***	0.92 ***	0.80 ***	0.79 ***	0.99 ***				
pH Rind	-0.25	-0.43 *	-0.63 ***	-0.60 ***	-0.60 ***	-0.56 **			
pH Centre	-0.34	-0.45 **	-0.06	-0.38 *	-0.34	-0.32	0.53 **		
Lactic acid	-0.32 **	-0.12	-0.58 ***	-0.55 ***	-0.42 ***	-0.34 ***	0.32 **	0.41 ***	
Moisture	-0.84 ***	-0.39 *	0.03	-0.01	-0.24	-0.29	-0.06	0.18	0.06

Notes: [a]Correlation coeficients *Significant at $p < 0.1$; **Significant at $p < 0.05$; ***Significant at $p < 0.01$

4.1.6 PCA analysis

The principal component analysis (PCA) made it possible to carry out an analysis that can describe the behavior of the cheese during the entire ripening process, associating it with all the parameters analyzed in this experimental part. In the factorial map of scores and the eigenvectors, both shown in Figure 4.5 and Figure 4.6 we can see the separation of both all the parameters into two components called PC1 and PC2, which explain 90.6% of the total variance in the first case and 9.39% in the second case, adding a total of 99.99% concerning the total variation. In the vector map represented in the figure, the parameters' evolution can be observed depending on the ripening time. It can be seen that from day 1 to day 40 of ripening, it is related to PC1, and from day 40 to day 90 it can be seen that there is a combination of factors between PC1 and PC2.

The eigenvectors showed in Figure 4.6, hardness represented more importance in the first component, and chewiness presented more importance in the second component, followed by gumminess that also presented more importance on the second component, explaining in this way that there is a tendency to an increase in hardness and a decrease in chewiness during the first 40 ripening days, unlike that both increased from day 40 until the end of the process. These conditions are reflected in the results shown by the analysis of variance that indicates the behavior obtained in this analysis and that is explained with the sources mentioned before.

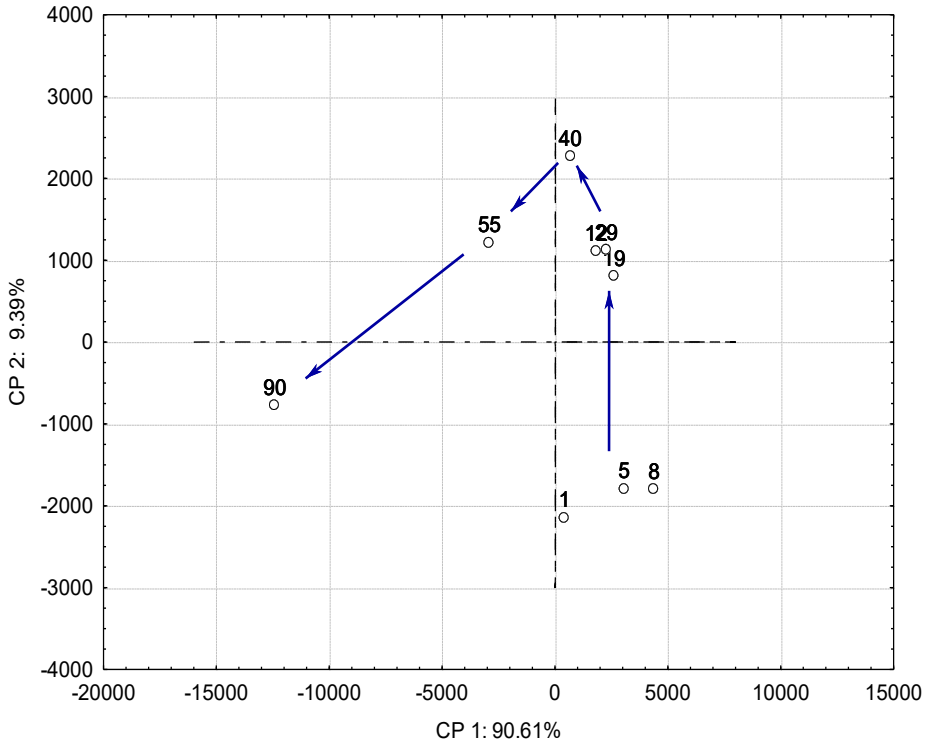

Figure 4.5 Factorial map of scores with all variables obtained by principal components analysis (PCA) of samples in different ripening days in a controlled system. Samples codes represent the ripening day.

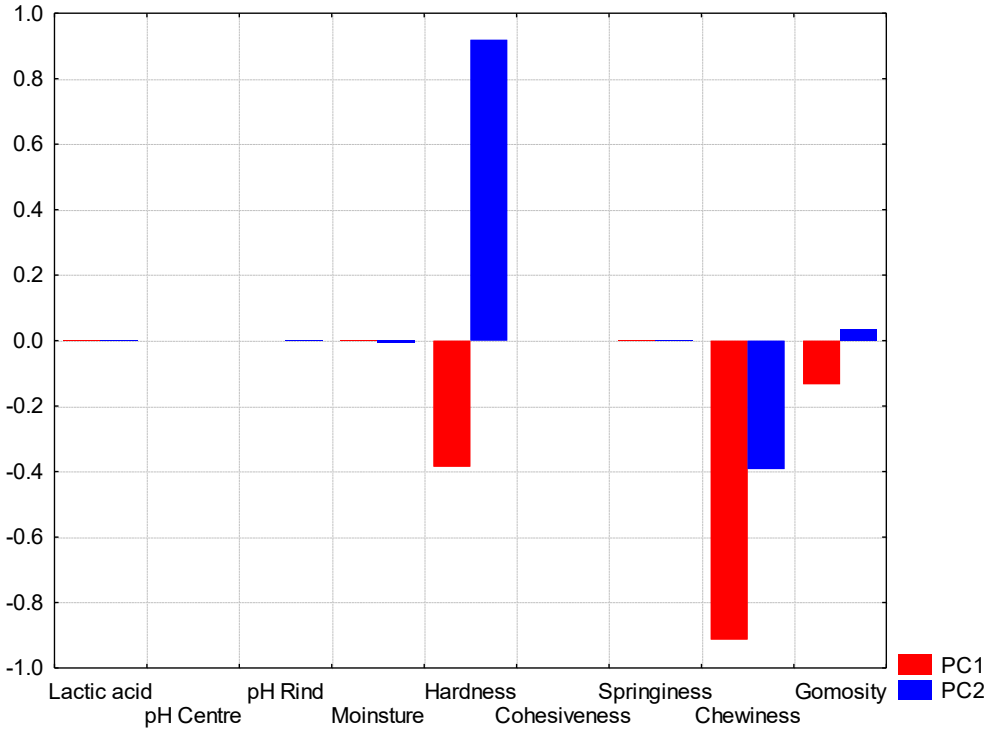

Figure 4.6 Eigenvectors of all parameters obtained during 90 ripening days

4.2 Nitrogen fraction and antioxidant activity determination

4.2.1 Analysis of peptide evolution

The data obtained followed a normal distribution, and the peaks found in the chromatograms were selected taking into account the permanence in at least 30 of the 90 ripening stages and that said peak presented more than 200 A.U. The data extracted from the integration reports were analyzed by area% using the ANOVA test with 95% significance. Figure 4.7 can be observed the evolution of the peptides found in the NPN fraction and were retained in 1.12±0.02, 2.15±0.02, 5.00±0.1, and 116.57±0.99 minutes. Their behaviors were varied; however, it was observed that the peptide in 2.10 and 116.57 minutes tended to increase throughout the entire process, reaching high levels towards the last ripening days. On the other hand, the peptide belonging to the retention time of 1.12 minutes showed a significant increase after the first 8 ripening days of followed by a gradual decrease. Finally, the peptide belonging to the retention time of 5.00 minutes showed a constant behavior throughout the entire ripening process.

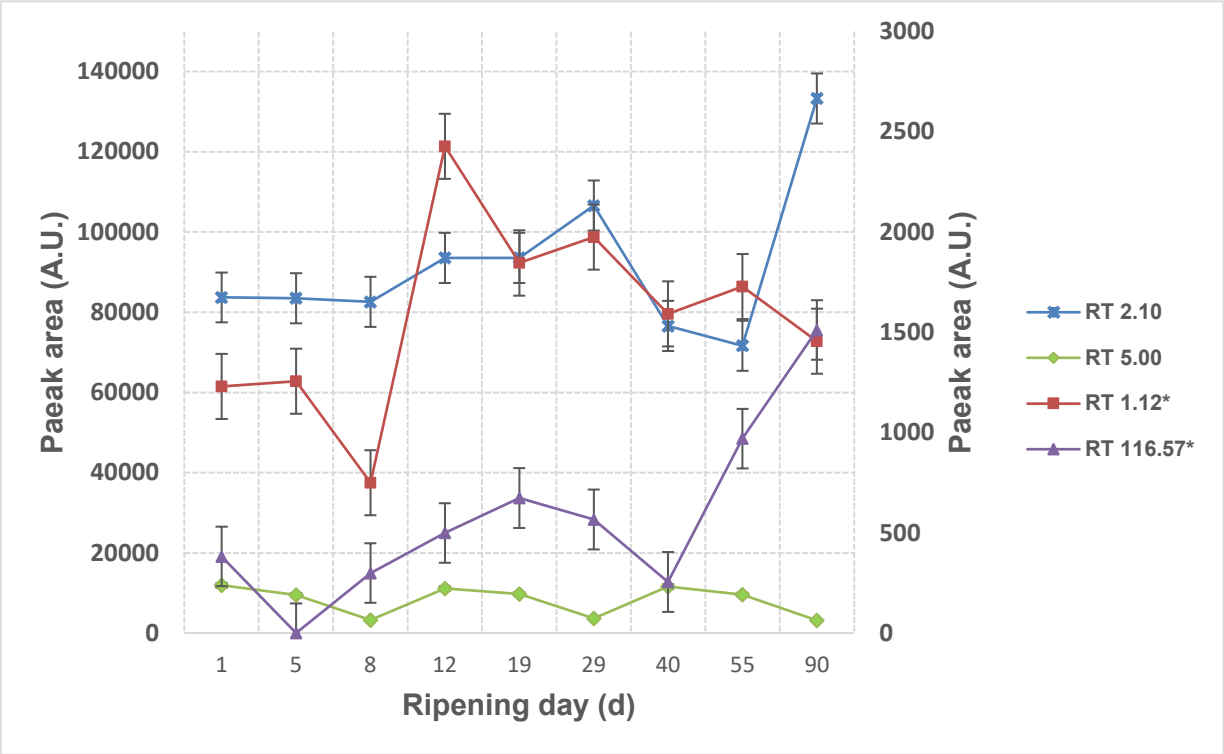

Figure 4.7 The evolution of peptides throughout the ripening process. NPN fraction where 4 main peaks are observed. RT: Retention time of the selected peak. * Values in the secondary y-axis.

Figure 4.8 shows the same information regarding the evolution of the peptides found in the fraction corresponding to the acid-soluble nitrogen, obtaining retention times at 1.18±0.02, 1.91±0.03, 2.43±0.03, and 106.41±0.07 minutes. Like NPN fraction, there is a tendency to increase the percentage in their concentration, particularly towards the last days of ripening. In the case of the retention time of 106.41 minutes, there is an

increase towards the 40th ripening day, followed by a decrease towards the 90th ripening day.

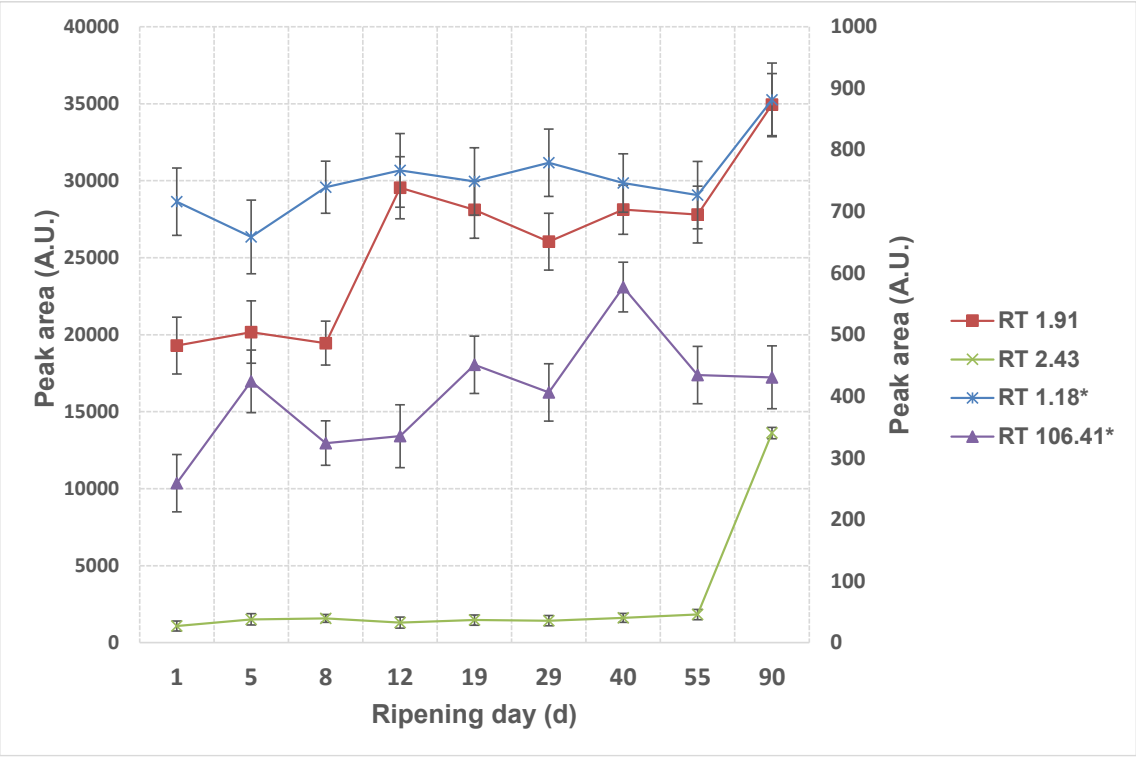

Figure 4.8 The evolution of peptides throughout the ripening process. ASN fraction where 4 main peaks are observed. RT: Retention time of the selected peak. * Values in the secondary y-axis.

For the case of the fraction belonging to the ethanol-soluble fraction shown in Figure 4.9, the presence of 4 peaks were observed: 1.097±0.07, 1.941±0.04, 2.454±0.03, and 117.23±0.34 minutes. Like the peaks observed in the previous fractions, the case of the nitrogen fraction soluble in ethanol presented high values in ripening times between 19th and 29th days, showing fluctuations before and after these points, to finally show a trend to increasing concentrations towards the end of the entire process.

In the case of the nitrogen fraction not soluble in ethanol, the last fraction studied that can be found in Figure 4.10, the trends are the same, unlike in this case only two peaks were observed throughout all the ripening process, the first found in 1.17±0.01 minutes, and the second found in 1.92±0.01 minutes. For this case, retention times were only found in the first sections of the chromatogram without finding data of peptides released towards higher retention times.

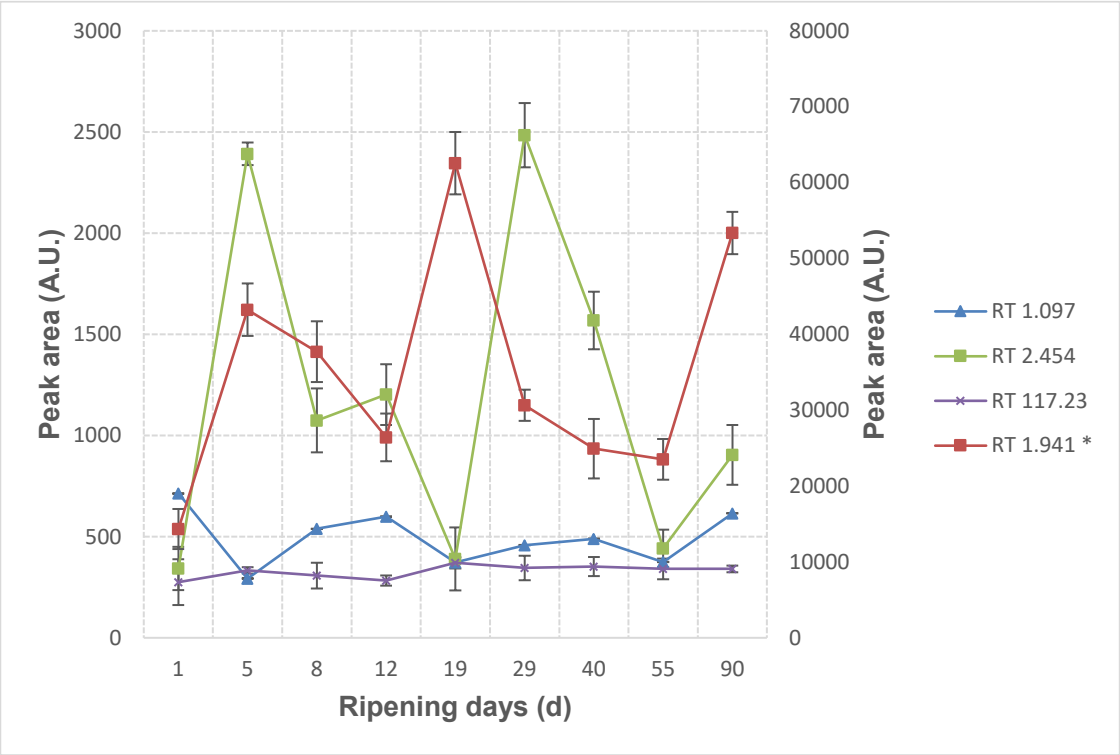

Figure 4.9 The evolution of peptides throughout the ripening process. EtOH-SN fraction where 4 main peaks are observed. RT: Retention time of the selected peak. *Values in the secondary y-axis.

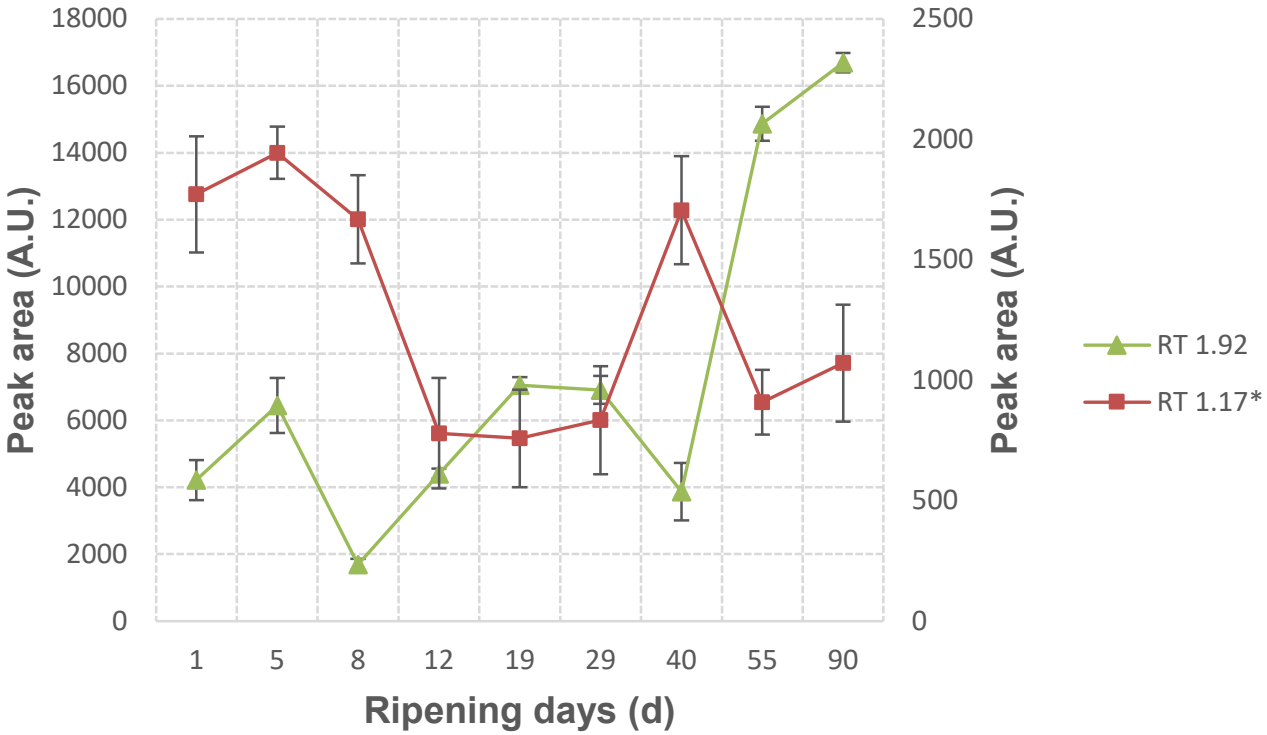

Figure 4.10 The evolution of peptides throughout the ripening process. EtOH-NSN fraction where 2 main peaks are observed. RT: Retention time of the selected peak. * Values in the secondary y-axis.

The fluctuating behavior observed in the four fractions is similar in almost all the peaks during the first days of ripening, in some cases during the first 8 days, in other cases close to day 19, 29, or 40, as already mentioned earlier. This evolution can be explained by the presence of proteolytic enzymes found in milk, such as plasmin or rennet. These enzymes work the first days after the cheese production process and break β-casein into short and medium-chain peptides. Then, the inactivation of the enzymes promotes a later decrease. Once this process has passed, a tendency to increase the peak concentrations is observed near the last ripening days, in some cases near 55^{th} or 90^{th} day. This behavior is related to the presence of peptidases released by microorganisms found in the cheese matrix and which develop further as the ripening process progresses, also releasing medium and short-chain peptides.

Diverse authors have reported these behaviors observed in cheeses subjected to prolonged ripening, such is the case of the data reported by Fox and Kelly (2006), Ardö et al. (2017), and McSweeney (2004) who determined that the presence of proteolytic enzymes from both milk and microorganisms promote the release of medium-chain, short-chain peptides and free amino acids from casein at different ripening times. Barać et al. (2016) observed this effect in water-soluble and insoluble peptide fractions obtained in white cow cheese during 50 ripening days, determining that the water-soluble fraction raised the peptide concentration from 1.88 g/100 mg in first ripening day to 14.46 g/100 mg at 30^{th} ripening day.

Gupta et al. (2009) observed the effect in water-soluble peptide fraction in cheddar cheese during 9 ripening months, with a rise of peptide concentration during the first ripening days with a decrease in these after the elimination of peptidases from milk and rennet, followed by an increase in their concentration. also observed the effect on the water-soluble nitrogen fraction obtained in buffalo and cow cheddar cheeses after 6 ripening months, and Basiricò et al. (2015) observed the same behavior in Parmigiano Reggiano after 12 ripening months, rising the peptide concentration from 8.46 to 21.55 mg/kg in water-soluble peptide fraction.

4.2.2 Analysis of HO/HI ratio

The relationship between hydrophilic and hydrophobic peptides can give us an indication of the relationship between them and the presence of biological activities. HO / HI ratio's evolution in each of the fractions and its behavior throughout the ripening process was determined. Table 4 shows the evolution of the HO/HI ratio throughout ripening.

Table 4. ANOVA and Fisher's LSD test (p<0.05) of %area of hydrophobic (HO), hydrophilic (HI) peptides, and the ratio (HO/HI) in the nitrogenous fractions throughout all ripening processes in Bouchon de chèvre type cheese (Vázquez-García et al., 2021).

Peptide fraction	Peptides proportion*	p-value	Storage time								
			Day 1	Day 5	Day 8	Day 12	Day 19	Day 29	Day 40	Day 55	Day 90
NPN	HI	0.375	32281.03^{ab}	31423.67^{ab}	28849.80^{ab}	35754.81^{a}	22247.37^{b}	37411.72^{a}	29912.20^{ab}	27655.34^{ab}	45992.09^{a}
	HO	0.000	382.03^{b}	0.000^{d}	299.51^{ab}	499.54^{c}	673.76^{e}	566.26^{c}	254.94^{a}	970.10^{f}	1511.05^{g}
	HO/HI	0.000	0.012^{ab}	0.000^{b}	0.010^{ab}	0.023^{ab}	0.030^{a}	0.031^{a}	0.009^{ab}	0.070^{c}	0.033^{ab}
ASN	HI	0.000	7029.73^{b}	7449.02^{b}	7258.29^{b}	10539.89^{a}	10110.30^{a}	9419.64^{a}	10160.42^{a}	10120.08^{a}	16480.31^{c}
	HO	0.000	259.08^{c}	424.26^{ab}	323.99^{ac}	335.30^{abc}	451.09^{b}	406.33^{ab}	577.35^{d}	434.63^{ab}	430.90^{ab}
	HO/HI	0.064	0.037^{ab}	0.057^{bc}	0.053^{bc}	0.032^{ac}	0.046^{abc}	0.043^{abc}	0.058^{c}	0.043^{abc}	0.026^{a}
EtOH-NSN	HI	0.000	2993.85^{a}	4195.68^{b}	1677.79^{c}	2592.69^{a}	3903.14^{b}	3872.45^{b}	2786.24^{a}	7887.87^{d}	8880.91^{e}
	HO	--	--	--	--	--	--	--	--	--	--
	HO/HI	--	--	--	--	--	--	--	--	--	--
EtOH-SN	HI	0.000	5132.72^{b}	15311.69^{e}	13108.84^{d}	9414.31^{a}	21112.98^{g}	11200.23^{c}	9000.98^{a}	8113.89^{a}	18296.92^{f}
	HO	0.161	275.40^{a}	333.54^{abc}	307.94^{abc}	283.03^{ab}	371.36^{c}	345.64^{abc}	353.22^{bc}	340.95^{abc}	340.89^{abc}
	HO/HI	0.000	0.055^{d}	0.021^{ab}	0.023^{ab}	0.030^{ac}	0.017^{b}	0.031^{ac}	0.039^{c}	0.042^{cd}	0.018^{ab}

*Different letters in values of the same fraction are significantly different (p<0.05).

The HO / HI ratio, which can relate the hydrophobic and hydrophobic peptides, is an indicator that can provide information about the quality of peptides released during all ripening processes and the proteolysis characteristics, particularly in the peptides that were released. This information is significantly related to the ripening process (Vivar Quintana et al., 2009). The presence of medium-sized peptides that are usually soluble in water can be observed in the HI fraction. These peptides are usually produced by the presence of peptidases that are obtained by starter and non-starter microorganisms. In the HO fraction case, long peptides are produced by added rennet and enzymes produced in the cheese matrix (Picon et al., 2007; Vivar Quintana et al., 2009).

For the case of the fraction corresponding to the peptides not soluble in ethanol (EtOH-NSN), no information was presented in the HO fraction, and therefore there was no HO / HI relationship because no peptide of the type hydrophobic was found in this fraction, expected result since the fraction contains a polar solvent. Fisher's posthoc analysis showed no differences between groups in almost all ripening processes; however, it can be observed that there is a much higher amount of hydrophilic peptides related to the presence of microorganisms that promote the proteolysis during the 90 ripening days. On the other hand, proteolysis's presence due to enzymes in the product matrix is observed to a lesser extent.

These results can also be observed in Figures 4.7, 4.8, 4.9, and 4.10 where it is evident that hydrophilic peptides are more common, which occur with retention times of less than 35 minutes, as opposed to hydrophobic peptides, which can be observed in only three fractions and a smaller proportion (3:1), ensuring that the analyzed peptides are

related to the proteolysis caused by both the microorganisms and the peptides found in the rennet and milk. This behavior was also reported by Tejada et al. (2008), who observed a trend towards a higher quantity of hydrophilic peptides in Murcia al Vino goat cheeses after 60 ripening days, observing a decrease in the HO / HI ratio throughout the ripening process although without significant differences between all stages.

On the other hand, the study elaborated by Vivar Quintana et al. (2009) showed the presence of a seasonal variation in the HO / HI ratio in matured cheeses from different mammals, observing that there is a higher proportion of hydrophobic peptides during winter, unlike cheeses produced in summer, and that after 6-month ripened trends towards hydrophobicity are observed in winter. On the other hand, the study elaborated by Hernández-Galán et al. (2017) observed a much higher HO / HI ratio in unripened goat cheeses.

4.2.3 Antioxidant activity determination

To carry out the determination of antioxidant activity, the DPPH discoloration percentage method was used in the fractions corresponding to non-protein nitrogen and acid-soluble nitrogen. Previously it has been determined that these fractions presented higher antioxidant activity than the fractions ethanol-treated (Hernández-Galán et al., 2017).
In Figure 4.11 the behavior of both fractions concerning the percentage of discoloration is observed. As can be seen, the NPN fraction showed a much higher percentage of discoloration than in the case of the ASN fraction. For both cases, the presence of the activity is observed throughout the ripening process without statistically significant changes. In the case of the NPN fraction, a statistically increase in antioxidant activity is observed after day 55 of maturation (0.02 p-value in ANOVA test and group differences in Fisher's LSD test, 0.95 confidence interval).

Tables 5 and 6 show the correlation values between the HO / HI values, antioxidant activity, and the retention times of each of the fractions. NSA fraction shows a highly significant positive correlation ($p < 0.05$) between DPPH and the peak at 2.43 minutes, which can be related to antioxidant activity. At the same time, a significant negative correlation ($p < 0.1$) is observed between the DPPH values and the retention time of 5.19 in the NPN fraction, values that indicate that the compounds which promote the antioxidant activity are present in the hydrophilic fraction. On the other hand, it is observed that there are close correlations between the HO / HI values and the retention times studied, demonstrating what was observed in the previous section.

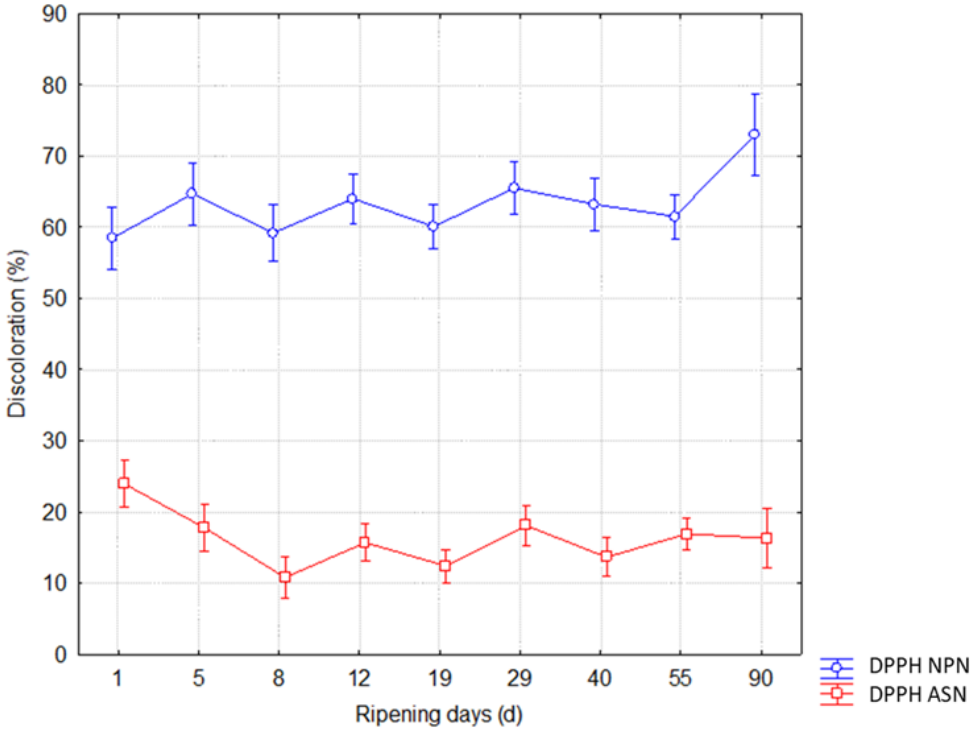

Figure 4.11 Evolution in discoloration percentage during all ripening processes in NPN and ASN fractions.

Table 5. Correlation values for retention time, peptides proportion, and antioxidant effect in ASN fraction in Bouchon de chèvre type cheese with prolonged ripening (Vázquez-García et al., 2021).

Variables	Ripening	RT 1.18	RT 1.91	RT 2.43	RT 106.41	HI	HO	HO/HI
RT 1.18	0.24*							
RT 1.91	0.65***	0.27**						
RT 2.43	0.52***	0.24*	0.46***					
RT 106.41	0.45***	0.07	0.36***	0.08				
HI	0.70***	0.32**	0.93***	0.75***	0.30**			
HO	0.45***	0.07	0.36***	0.08	0.99***	0.30**		
HO/HI	-0.10	-0.19	-0.54***	-0.28**	0.44***	-0.51***	0.44***	
DPPH		-0.15	0.16	0.31**	-0.05	0.25*	-0.05	-0.16

Notes: a Correlation values *Significant at $p < 0.1$; **Significant at $p < 0.05$; ***Significant at $p < 0.01$

Table 6. Correlation values for retention time, peptides proportion, and antioxidant effect in the NPN fraction in Mexican goat surface mold cheese with prolonged ripening (Vázquez-García et al., 2021).

Variables	Ripening	RT 1.12	RT 2.15	RT 5.19	RT 116.57	HI	HO	HO/HI
RT 1.12	0.25*							
RT 2.15	0.04	0.03						
RT 5.19	-0.11	0.30**	-0.08					
RT 116.57	0.69***	0.23*	0.11	-0.19				
HI	0.03	0.07	0.99***	0.01	0.10			
HO	0.69***	0.23*	0.11	-0.19	0.99***	0.10		
HO/HI	0.45***	0.19	-0.64***	-0.10	0.51***	-0.65***	0.51***	
DPPH	0.22*	0.11	0.013**	-0.10*	0.15	0.12	0.15	0.08

Notes: a Correlation values *Significant at $p < 0.1$; **Significant at $p < 0.05$; ***Significant at $p < 0.01$

Several authors have demonstrated the presence of antioxidant activity in milk and cheeses after-ripening process, particularly in water-soluble fractions (Abdel-Hamid et al., 2017; Ahmed et al., 2015; Barać et al., 2017). In the case of whole milk, there are reports of the presence of antioxidant activity in products obtained from cows, goats, sheep, and buffalo milk? (De Gobba et al., 2014). On the other hand, different types of cheeses have shown the presence of the activity even after different ripening times. In the work developed by Barać et al. (2016), the presence of constant antioxidant activity was observed in the water-soluble fraction with a 50% of antioxidant effect and at least 15% effect in the non-water-soluble fraction in white cow's milk cheeses in different ripening stages.

Also, in the case of the work developed by Bottesini et al. (2013), the presence of the same antioxidant activity in hydrophilic extracts in Parmigiano Reggiano cheeses was observed throughout 41 months ripening time without significant differences. In Cheddar cheese, there was observed the presence of the same activity in the works by Gupta et al. (2009) where an increase in antioxidant activity was observed after the first fifth ripening months and a significant decrease in it that reached the ninth ripening months. Huma et al. (2018) found in buffalo and cow cheddar cheeses ripened for 6 months in vitro antioxidant activity from day ripening 90, and that was constant until day 180, presenting a more pronounced activity in cheese from buffalo milk, and Meira et al. (2012) where Feta, Roquefort and Pecorino sheep cheeses from Brazil and Uruguay were analyzed. Roquefort cheese presented a higher antioxidant activity using the ABTS, TBARS, and DPPH methods as well as the presence of other biological activities such as antibacterial and antihypertensive.

The relationship between the proteolysis of cheese after the ripening process and the production of biological activities is widely documented. The production of certain antioxidant activity in the analyzed cheeses shows that the proteins and compounds in them present the same qualities as the cheeses produced and analyzed in other parts of the world.

4.2.4 PCA analysis

Principal component analysis of the NPN fraction demonstrates the relationship between the hydrophilic and hydrophobic fractions and the antioxidant activity analyzed by DPPH. In Figure 4.12 the factorial map shows that the behavior is explained 59.28% of total variance by the first principal component and 36.5% by the second principal component. The samples were separated into groups, observing a trend over the first 12 days of ripening representing young cheeses. A group was observed from days 19 to 55 of the ripening process and represents the cheeses in commercial ripening time. Finally, the third group represents the cheeses in the extended ripening stage after 90 days of ripening. In figure 4.13, the loading plot shows a very close relationship between PC1 and the HO / HI relationship, and individually it is observed that HO is related to PC1 and HI to PC2.

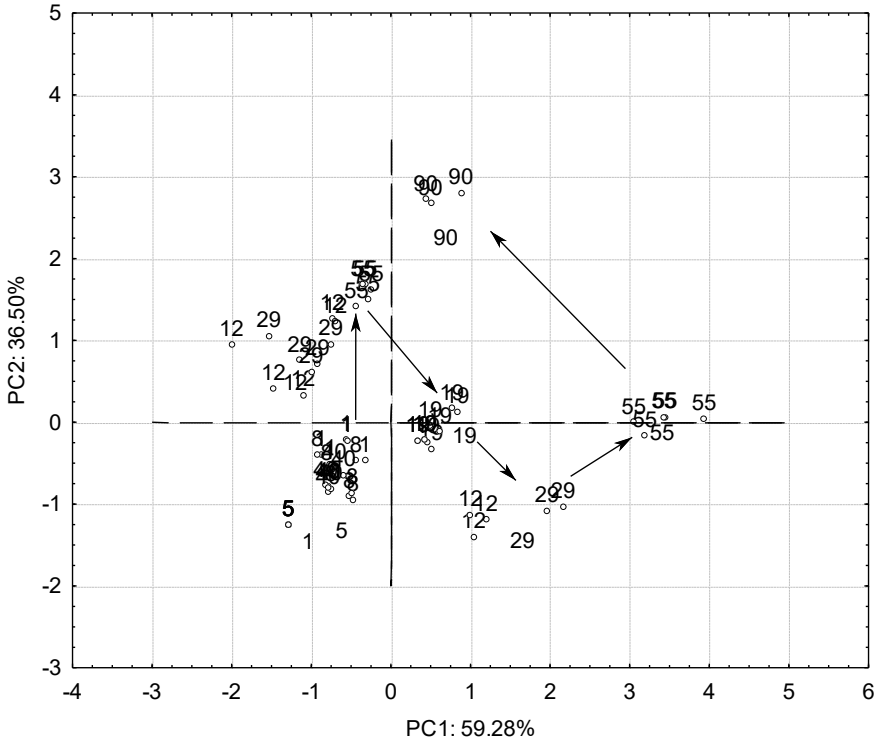

Figure 4.12 Factorial map obtained by principal components analyses (PCA) in NPN fraction. Arrows show the trend followed throughout the ripening process. Codes represent samples ripening day.

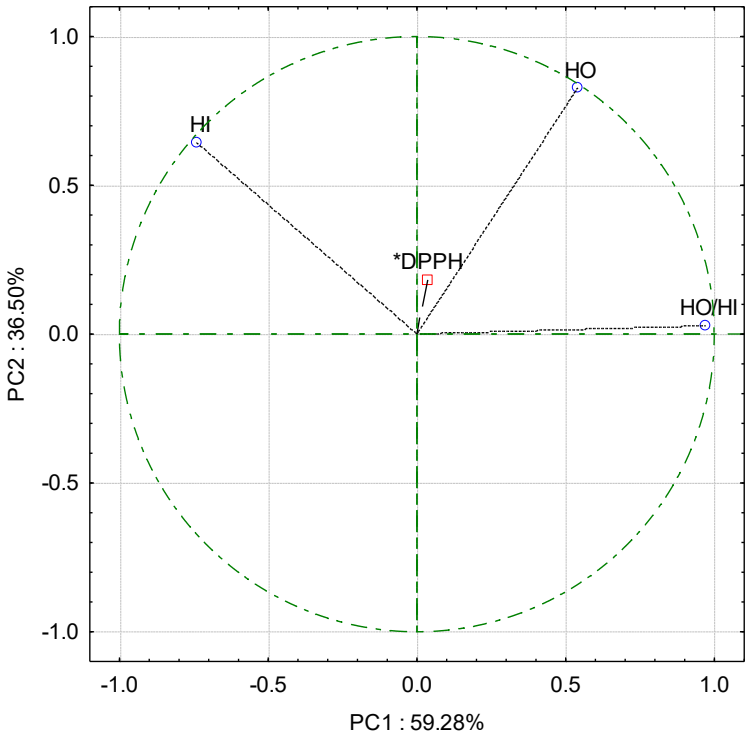

Figure 4.13 Factor loading plot in NPN fraction

On the other hand, the principal component analysis of the ASN shows similar behaviors over DPPH and HO and HI peptides. In Figure 4.14 the factorial map shows that the behavior is explained 51.61% by PC1 and 43.15% by PC2 of the total variance. As NPN fraction, the samples were separated into groups, observing a trend over the first 29 days of ripening representing young cheeses. A group was observed during the 40th ripening, representing the cheeses in commercial ripening time, and finally, the third group represents the cheeses in the extended ripening stage from 55 to 90th ripening days. In Figure 4.15, the loading plot shows a very close and negative relationship between PC1 and the HO / HI ratio, and, just like NPN fractions, individually, it is observed that HO and HI are related to both axes.

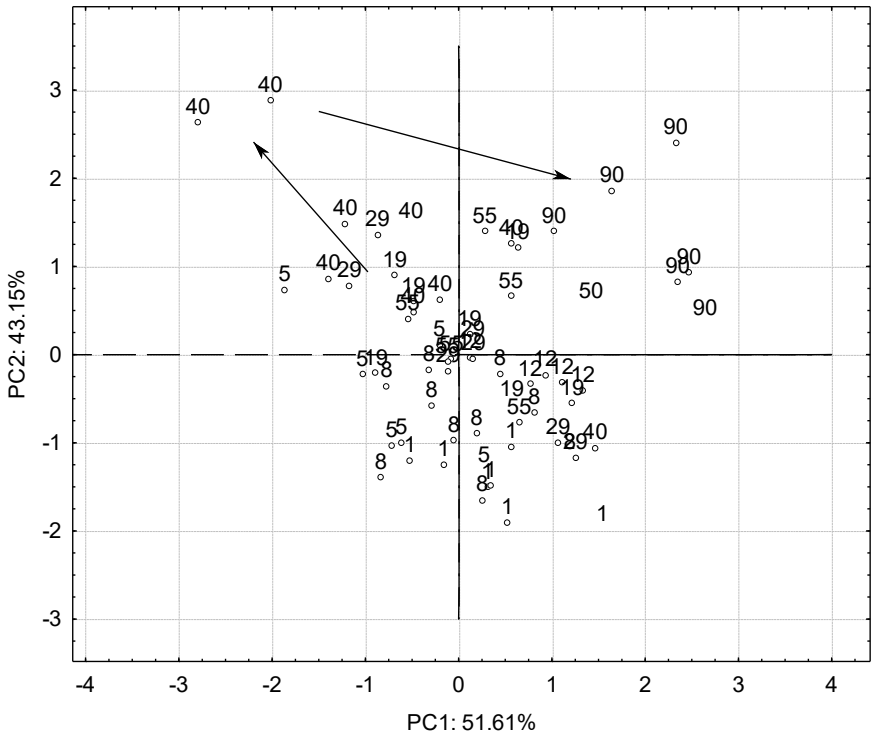

Figure 4.14 Factorial map obtained by principal components analyses (PCA) in ASN fraction. Arrows show the trend followed throughout the ripening process. Codes represent samples ripening day.

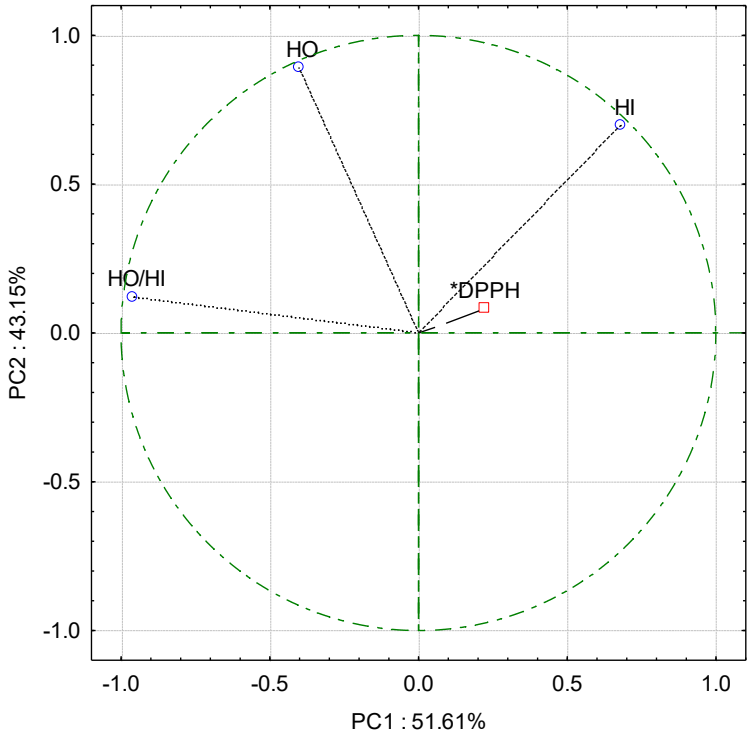

Figure 4.15 Factor loading plot in NPN fraction

4.3 Lipid profile determination

4.3.1 Lipid compound identification

In this work, A total of 80 compounds with lipid characteristics were located during the different samples through the complete maturation process. The identification of 33 of them was confirmed using reference standards. Table 7 shows all compounds found, identified or not. Of the total components, 64 were found during the entire ripening process, 13 stopped appearing during the last ripening days, some components as unidentified 7,8 and 17 from 40th ripening day, and others as petroselinic acid, unidentified 11 and 22 from 55th ripening day. Only 5 compounds appeared after the 8th ripening day.

During the entire ripening process, a few fluctuations in the production of lipid compounds were observed. Many unidentified compounds can be observed that disappear just before the last ripening days, except unidentified compounds 8, 9, 17, 23, and 38, wherein all cases stop appearing in the middle of the ripening process, corresponding to commercial ages (29 ripening days). In the case of the lipid compounds identified, such as butyric, hexanoic, heptanoic, and capric acid, there is no variation in their presence during the 90 ripening days that were studied. This behavior is related to the low amount of lipases produced by microorganisms.

Table 7. Lipidic compounds, identified and classified according to retention time and ripening time

Compound	#CAS[a]	Identification[b]	Retention time[c]	Ripening time (Days)								
				1	5	8	12	19	29	40	55	90
Butyric acid	107-92-6	MS, ST	11.726 min	√	√	√	√	√	√	√	√	√
Unidentified 7	--	MS, ST	12.685 min	√	√	√	√	√	√	√	√	√
Unidentified 8	--	MS, ST	13.967 min	√	√		√		√			
Unidentified 9	--	MS, ST	14.384 min	√	√	√	√	√	√			
Caproic acid	142-62-1	MS, ST	17.169 min	√	√	√	√	√	√	√	√	√
Unidentified 11	--	MS, ST	18.427 min	√	√	√	√	√	√	√	√	
Heptanoic acid	111-14-8	MS, ST	19.694 min	√	√	√	√	√	√	√	√	√
Caprilic acid	124-07-2	MS, ST	21.825 min	√	√	√	√	√	√	√	√	√
Unidentified 12	--	MS, ST	22.622 min	√	√	√	√	√	√	√	√	√
Unidentified 13	--	MS, ST	22.702 min	√	√	√	√	√	√	√	√	√
Nonanoic acid	112-05-0	MS, ST	23.578 min	√	√	√	√	√	√	√	√	√
Unidentified 14	--	MS, ST	24.171 min	√	√	√	√	√	√	√	√	√
Decanoic acid	334-48-5	MS, ST	25.221 min	√	√	√	√	√	√	√	√	√
Unidentified 15	--	MS, ST	25.755 min	√	√	√	√	√	√	√	√	√
Unidentified 16	--	MS, ST	26.351 min	√	√	√	√	√	√	√	√	√
Undecanoic acid	112-37-8	MS, ST	26.682 min	√	√	√	√	√	√	√	√	√

Unidentified 17	--	MS, ST	26.976 min	√	√	√	√	√	√			
Lauric acid	143-07-7	MS, ST	28.391 min	√	√	√	√	√	√	√	√	√
Unidentified 20	--	MS, ST	29.710 min	√	√	√	√	√	√	√	√	√
Unidentified 21	--	MS, ST	30.005 min	√	√	√	√	√	√	√		
Unidentified 22	--	MS, ST	29.295 min	√	√	√	√	√	√	√	√	
Unidentified 23	--	MS, ST	29.377 min	√	√	√	√	√	√			
Unidentified 24	--	MS, ST	29.710 min	√	√	√	√	√	√	√	√	√
Tridecanoic acid	638-53-9	MS, ST	30.395 min	√	√	√	√	√	√	√	√	√
Unidentified 25	--	MS, ST	31.704 min	√	√	√	√	√	√	√	√	√
Myristic acid	544-63-8	MS, ST	33.571 min	√	√	√	√	√	√	√	√	√
Unidentified 26	--	MS, ST	34.675 min	√	√	√	√	√	√	√	√	√
Unidentified 27	--	MS, ST	35.273 min	√	√	√	√	√	√	√	√	√
Myristoleic acid	544-64-9	MS, ST	36.170 min	√	√	√	√	√	√	√	√	√
Unidentified 28	--	MS, ST	36.773 min	√	√	√	√	√	√	√	√	
Pentadecanoic acid	1002-84-2	MS, ST	37.605 min	√	√	√	√	√	√	√	√	√
Unidentified 29	--	MS, ST	39.147 min	√	√	√	√	√	√	√		
Unidentified 30	--	MS, ST	40.388 min	√	√	√	√	√	√	√		
Unidentified 31	--	MS, ST	44.831 min	√	√	√	√	√	√	√	√	√
Unidentified 32	--	MS, ST	46.449 min			√	√	√	√	√		√
Unidentified 33	--	MS, ST	46.787 min	√	√	√	√	√	√	√	√	√
Unidentified 34	--	MS, ST	47.443 min	√	√	√	√	√	√	√	√	√
Unidentified 35	--	MS, ST	47.702 min	√	√	√	√	√	√	√	√	√
Palmitolec acid	373-49-9	MS, ST	48.329 min	√	√	√	√	√	√	√	√	√
Unidentified 36	--	MS, ST	49.624 min	√	√	√	√	√	√	√	√	√
Unidentified 37	--	MS, ST	50.615 min	√	√	√	√	√	√	√		
Heptadecanoic acid	506-12-7	MS, ST	52.699 min	√	√	√	√	√	√	√	√	√
Unidentified 38	--	MS, ST	54.109 min	√	√				√			
Unidentified 39	--	MS, ST	55.647 min	√	√	√	√	√	√	√	√	√
Stearic acid	57-11-4	MS, ST	57.833 min	√	√	√	√	√	√	√	√	√
Oleic acid	112-80-1	MS, ST	58.529 min			√	√	√	√	√	√	√
Petroselinic acid	593-39-5	MS, ST	58.900 min			√	√	√	√	√	√	
Vaccenic acid	693-72-1	MS, ST	59.134 min	√	√	√	√	√	√	√	√	√
Unidentified 41	--	MS, ST	59.288 min		√	√	√	√	√	√	√	√
Unidentified 42	--	MS, ST	59.304 min	√	√		√	√	√	√	√	√
Unidentified 43	--	MS, ST	59.453 min	√	√	√	√	√	√	√	√	√
Unidentified 44	--	MS, ST	59.654 min	√	√	√	√	√	√	√	√	√
Linoleic acid	60-33-3	MS, ST	59.781 min	√	√	√	√	√	√	√	√	√
Unidentified 45	--	MS, ST	60.009 min	√	√	√	√	√	√	√	√	√
Nodecylic acid	646-30-0	MS, ST	60.263 min	√	√	√	√	√	√	√	√	√
Unidentified 46	--	MS, ST	60.469 min		√	√	√	√	√	√	√	√

Elaidic acid	112-79-8	MS, ST	60.654 min	√	√	√	√	√	√	√	√	√
Unidentified 47	--	MS, ST	60.950 min	√	√	√	√	√	√	√	√	√
Unidentified 48	--	MS, ST	61.908 min	√	√	√	√	√	√	√	√	√
Arachidic acid	506-30-9	MS, ST	62.054 min	√	√	√		√	√	√	√	√
Alfa-linoleic acid	463-40-1	MS, ST	62.286 min	√	√	√	√	√	√	√	√	√
Gondoic acid	5561-99-9	MS, ST	62.635 min	√	√	√	√	√	√	√	√	√
Unidentified 49	--	MS, ST	62.955 min	√	√	√	√	√	√	√	√	√
Unidentified 50	--	MS, ST	63.173 min	√	√	√		√	√	√		
Heneicosilic acid	2363-71-5	MS, ST	63.342 min	√	√	√	√	√	√	√	√	√
Unidentified 51	--	MS, ST	63.825 min	√	√	√	√	√	√	√	√	√
Unidentified 52	--	MS, ST	64.063 min	√	√	√	√	√	√	√	√	√
Docosanoic acid	112-85-6	MS, ST	64.591 min	√	√	√	√	√	√	√	√	√
Unidentified 53	--	MS, ST	64.894 min	√	√	√	√	√	√	√	√	√
Arachidonic acid	506-32-1	MS, ST	65.079 min	√	√	√	√	√	√	√	√	√
Tricosylic acid	2433-96-7	MS, ST	65.708 min	√	√	√	√	√	√	√	√	√
Unidentified 54	--	MS, ST	66.299 min	√	√	√	√	√	√	√	√	√
Lignoceric acid	557-59-5	MS, ST	66.752 min	√	√	√	√	√	√	√	√	√
Nervoic acid	506-37-6	MS, ST	67.387 min	√	√	√	√	√	√	√	√	√
Unidentified 57	--	MS, ST	68.726 min	√	√	√	√	√	√	√	√	√
Docosahexanoic acid	2566-90-7	MS, ST	69.232 min	√	√	√	√	√	√	√	√	√
Unidentified 58	--	MS, ST	69.790 min	√	√	√	√	√	√	√	√	√
Unidentified 59	--	MS, ST	69.995 min	√	√	√	√	√	√		√	√
Unidentified 60	--	MS, ST	70.253 min	√	√	√	√	√	√	√	√	√
Unidentified 61	--	MS, ST	70.808 min	√	√	√	√	√	√	√	√	√

Notes: [a] CAS: Chemical Abstract Service registration number; [b] MS: Compounds identified based on the IE mass spectra in the Wiley library; [c] Retention time observed in the mass chromatogram.

To determine the presence of the compounds during the 90 ripening days, gas chromatograms were analyzed. The images of some of the analyzed chromatograms are in Figures 4.16, 4.17, and 4.18, where the presence of the compounds that presented the highest concentration can be observed, such as the case of butyric acid found at minute 11.726 of retention time, decanoic acid found at minute 25.221 of retention time, or petroselinic acid found at minute 58.900 of retention time. As can be observed in the three chromatograms that represent 3 different ripening stages, there are no variations in the presence and concordance of the compounds, information that is related to what is observed in table 7, where it is seen that the presence of the majority of the compounds is permanent throughout the entire ripening process.

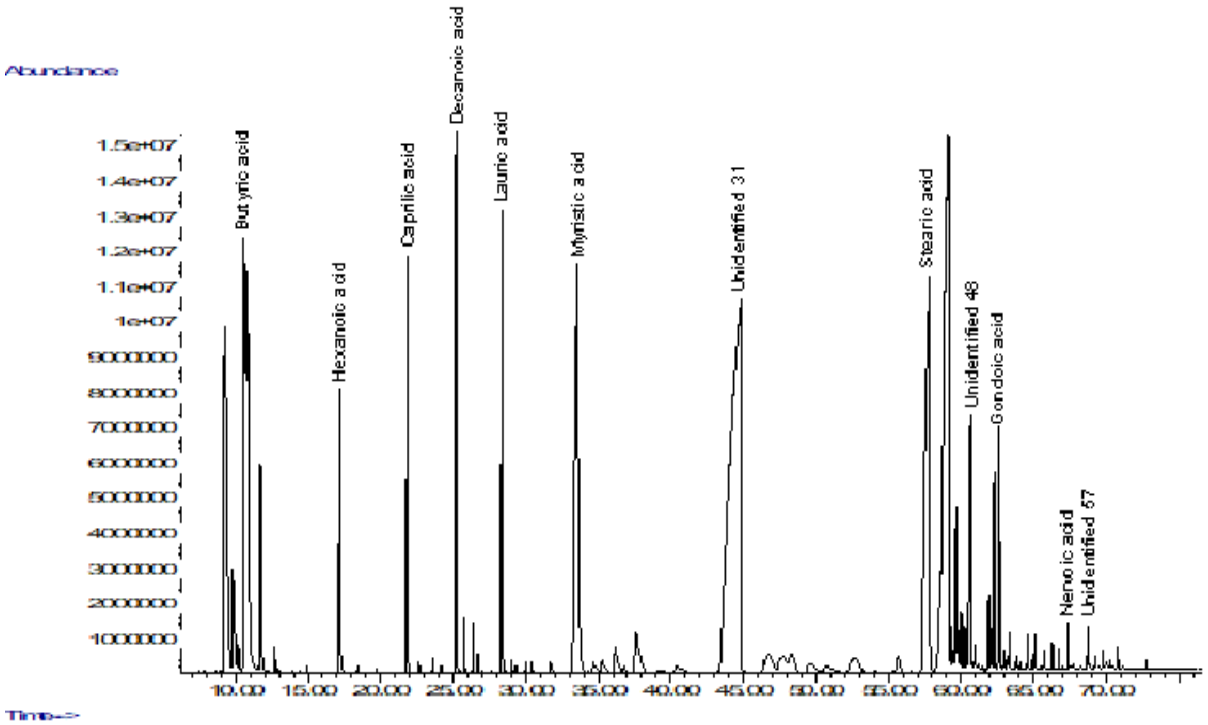

Figure 4.16 Chromatogram that represents lipid analysis during the first ripening day

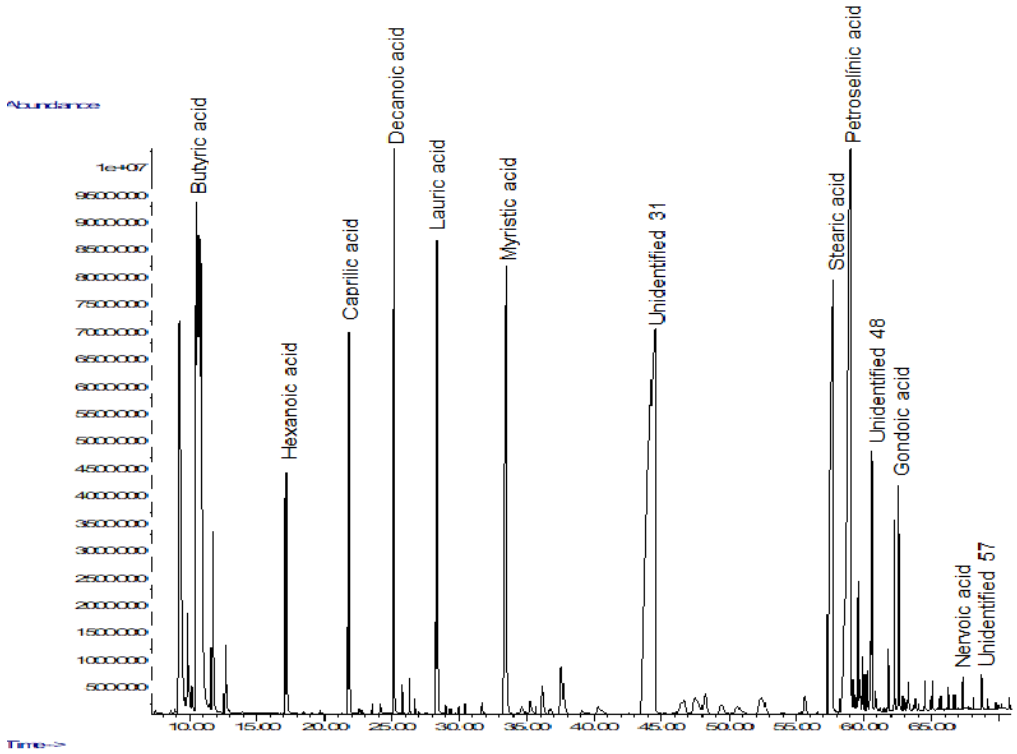

Figure 4.17 Chromatogram that represents lipid analysis during the 29th ripening day

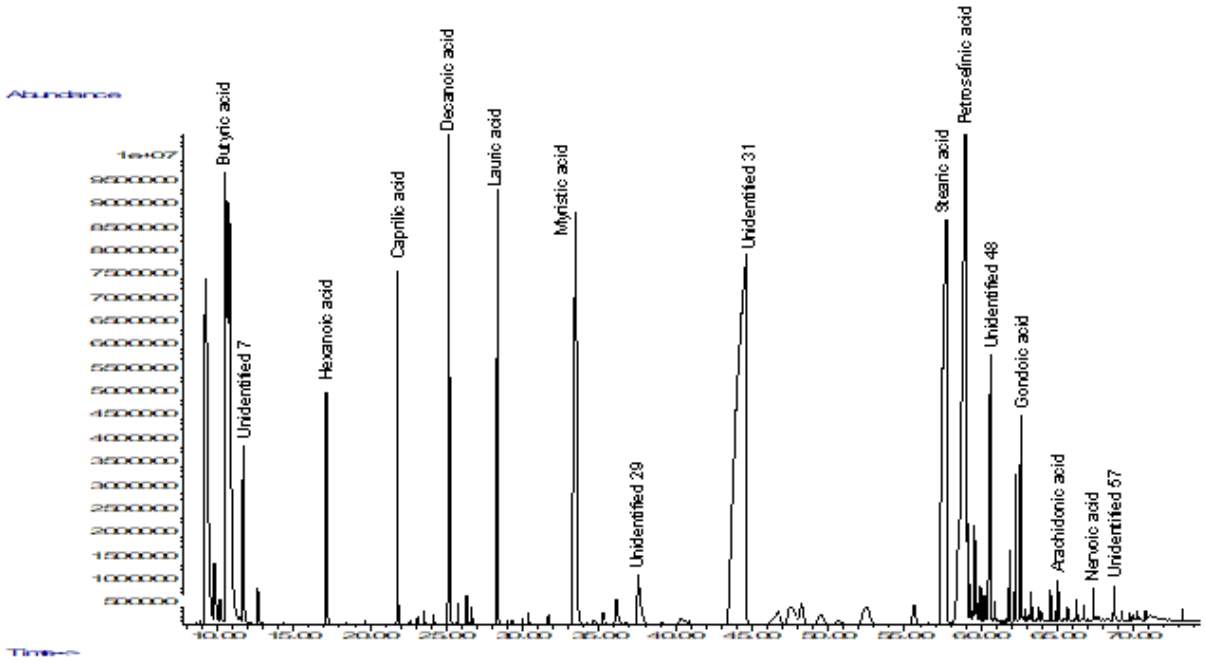

Figure 4.18 Chromatogram that represents lipid analysis during the 55th ripening day

This information can be related to was observed by {Alonso, 1999 #327} who determined the lipid composition of cheeses made with goat's milk where more than 20 fatty acids with more than 11 carbons were observed but did not present variations in the proportions of the compounds compared to cheeses. made with cow's milk from different herds, and in the studies developed by {Buffa, 2001 #402} where it was determined that there were no significant variations in the concentration of lipid compounds after the ripening of goat's milk cheeses produced with different treatments. Also, {Spinnler, 2017 #269} describes that the presence of short and medium fatty acids is due to the production of lipases from *P. camemberti*, *K. lacits* and the presence of other microorganisms; and the presence of unsaturated fatty acids is related to the presence of *G. candidum.*

Other authors have determined that there are no significant differences in the lipid profile of cheeses produced with goat milk that has been developed in the same climate and with the same diet, and although there are variations in the sensory conditions of the products obtained, no closely related to variations in the presence of lipid compounds, although there is an important relationship with their concentration {Barłowska, 2018 #471}. Finally, it has also been determined that the lipolysis promoted in surface-mold cheese is directly related to the amount and type of rennet used in its preparation, and to a lesser extent with the initiator bacteria; in this case, the use of

sanitized rennet and rennet paste directly affected the degree of lipolysis, an effect that was not observed when using commercial bovine rennet {Castillo, 2007 #404} {El Galiou, 2013 #376} {Fontecha, 2006 #450}.

4.3.2 Analysis of Variance (ANOVA)

Analysis of Variance (ANOVA) and Fisher´s least significant test (LSD) was developed to determine which compounds showed significant differences during all ripening processes. A data set of 33 quantified compounds was used for the identification. From all analyzed compounds. Only 8 compounds of all those identified presented statistical significance ($p < 0.05$) taking into account the ripening time. This information is reflected in Table 8.

Table 8. ANOVA and Fisher's LSD test ($p<0.05$) of significative lipidic compounds throughout all ripening processes in Bouchon de chèvre type cheese, media values of peak area were analyzed.

Compound	p-value	Ripening time (Days)								
		1	5	8	12	19	29	40	55	90
Unidentified 17	0.0126**	0.014^{bc}	0.018^{c}	0.003^{ab}	0.004^{ab}	0.004^{ab}	0.010^{bc}	0.000^{a}	0.000^{a}	0.000^{a}
Unidentified 21	0.0007***	0.021^{ab}	0.041^{b}	0.012^{a}	0.014^{a}	0.014^{a}	0.008^{a}	0.002^{a}	0.000^{a}	0.000^{a}
Unidentified 30	0.0142**	0.228^{a}	0.208^{a}	0.173^{a}	0.120^{ab}	0.177^{a}	0.161^{a}	0.018^{b}	0.000^{b}	0.000^{b}
Unidentified 37	0.0059**	0.088^{abcd}	0.196^{a}	0.146^{ad}	0.105^{abcd}	0.124^{acd}	0.149^{a}	0.037^{bcd}	0.000^{b}	0.000^{bc}
Unidentified 38	0.0000***	0.016^{a}	0.135^{b}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
Petroselinic acid	0.0001***	0.000^{a}	0.000^{a}	1.499^{a}	5.620^{ab}	1.309^{a}	5.347^{a}	12.333^{b}	5.441^{a}	0.000^{a}
Unidentified 50	0.0180**	0.032^{ab}	0.013^{a}	0.072^{b}	0.000^{a}	0.018^{a}	0.021^{a}	0.0145^{a}	0.000^{a}	0.000^{a}

Notes: [a] Significant at *** $p < 0.001$ and ** $p < 0.01$; [b] Means with different letters within the same row are significantly different $p < 0.05$

In the case of unidentified compound 21, an increase in its production was observed during the first 5 ripening days, to later decrease its presence gradually until reaching the 40th ripening day, to completely disappear from the product before the 55th ripening day. This behavior is different from the unidentified compound 38, which was only observed in the first 5 ripening days to later completely disappear from the product the rest of the process. Both compounds presented a highly significant level. Another compound that presented high significance was petroselinic acid, which was observed from day 8 of maturation with a fluctuating behavior until reaching a maximum production peak on 40th ripening day, where a high concentration of this compound is observed, for decrease again and disappear completely from the product for the last ripening weeks. The components with a low significance also present a fluctuating

behavior, as is the case of the unidentified compound 17 which disappears near the last ripening days, conditions that are repeated in the unidentified compound 30 and 37. In all these cases, the behavior of the compound is similar, observing a gradual decrease until it stops appearing. Concerning the other founded compounds, similar conditions of gradual reduction or general permanence can be observed throughout the controlled ripening. This information can be observed in Table 25 found in Appendix B.

Corroborating the information described above, the constant behavior of the amount and concentration of fatty acids has been previously reported. In the case of the studies developed by {Franco, 2003 #428}, it was determined that the release and variation of the concentration of fatty acids after the cheese ripening elaborated with raw goat's milk are not significant, observing that the fatty acids with the highest concentration were petroselinic acid followed by palmitoleic acid and decanoic acid. The same results were reported by {Fontecha, 2000 #483} where a greater amount of saturated medium chain compounds was observed and the presence of only 17% of the total unsaturated structures and 14% of the total polyunsaturated structures, and {Güler, 2005 #441} observed no significative differences in lipidic quantification in ripened Kasar cheese, with palmitic acid, caprylic acid, capric acid, and lauric acid as the predominant compounds.

Finally, it is estimated that the presence of unsaturated medium and long-chain compounds, such as oleic acid, vaccenic acid, linoleic acid, and nodecylic acid, among others, is due to the feeding of livestock. {Chilliard, 2001 #329} observed that the presence of this type of compound in whole milk and dairy products is closely related to mountain pasture feeding, unlike animals that consume silage, which tends to produce milk with saturated fatty acids. This behavior is observed in the cheese produced for the present study.

4.3.3 PCA and GDA analysis

To finalize the statistical analysis of this section, an analysis of main components and general discriminant analysis were carried out, the first to determine the behavior of the lipid compounds throughout the entire ripening process and the second to determine which are the compounds that mainly influence this evolution. The factorial map and the eigenvectors, both shown in Figures 4.19 and 4.20, the separation into two components called PC1 and PC2 can be observed, which only explain 42.52% of the behavior of the total variation. The factorial map represents the little relationship that exists between the components since there is no established pattern. The eigenvectors graph shows that the components that most cause variation in the lipid parameters of the cheese was the unidentified component 31 for factor 2, vaccenic acid for both factors, and petroselinic acid for factor 1. This information is consistent with the information provided by the analysis of variance, where it is specified that these compounds have a significant relationship with the ripening process and that they are related to the lipid profile.

This information is again confirmed by other authors who have observed the low presence of lipolysis during the ripening process in this type of cheese, and it is observed that there is the presence of unsaturated compounds, thanks to external conditions {Güler, 2005 #441} {Kaminarides, 2007 #452}. Finally GDA forward stepwise showed the same information, confirming that the only lipid components that are integrated into the general behavior are vaccenic acid (p = 0.000105), petroselinic acid (p = 0.001027), and the unidentified compound 31 (p = 0.000000).

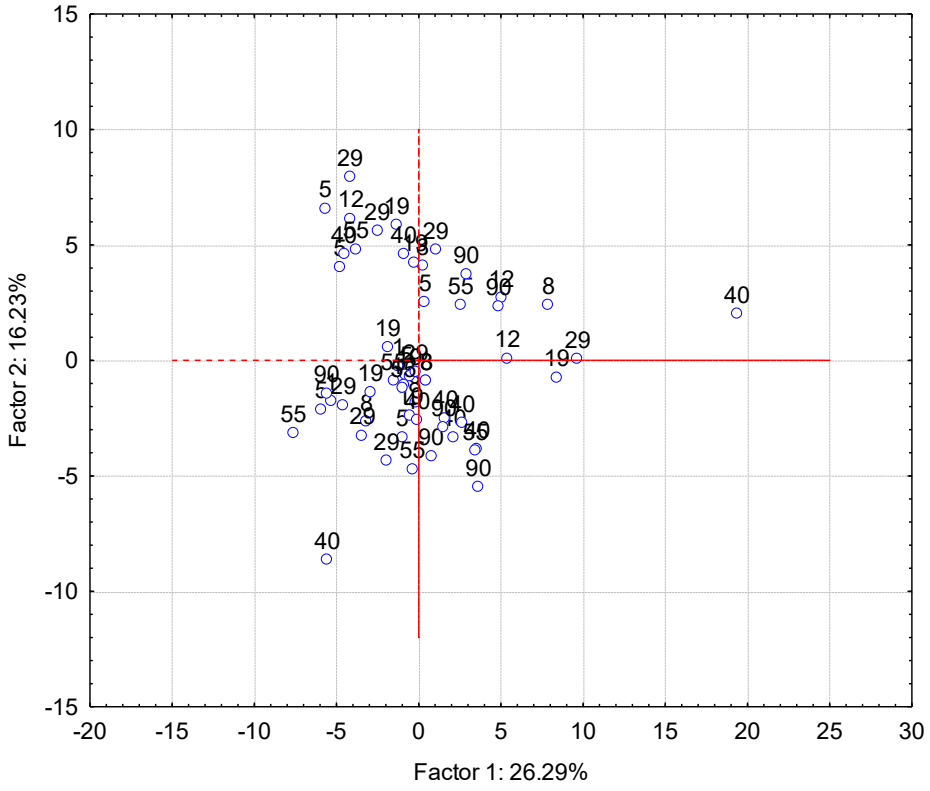

Figure 4.19 Factorial map of scores with all variables obtained by principal components analysis (PCA) of samples in different ripening days in a controlled system. Samples codes represent the ripening day.

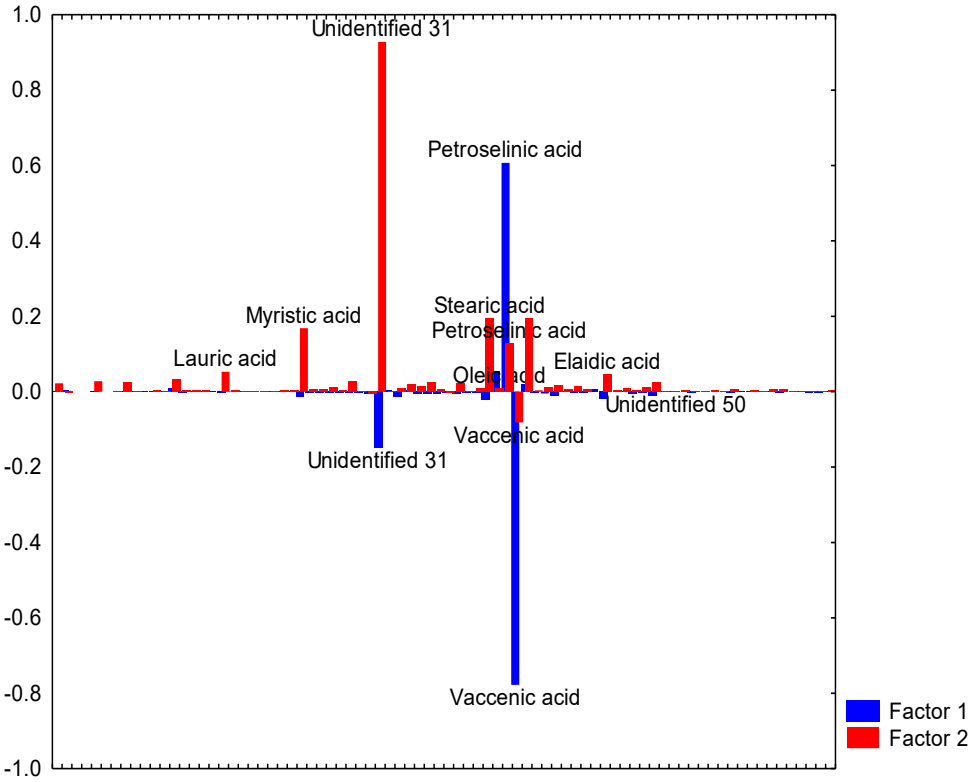

Figure 4.20 Eigenvectors all parameters obtained during 90 ripening days

Chapter 5

Conclusions

The Bouchòn de chevre cheese, made in a tropical area of Veracruz elaborated with a traditional method and with an extended ripening process, showed comparable characteristics to other extended ripened cheeses. In the case of the analysis of the rheological and physicochemical characteristics, the results obtained showed that the texture and the pH values, and the amount of lactic acid present significant changes during prolonged ripening, higher than the commercial age of the product. The central pH did not show significant variations and the PCA analysis made it possible to describe the evolution of ripening concerning these parameters. The results obtained showed a relationship between the cheese analyzed and similar cheeses made in other parts of the world, presented evidence of the behavior of the texture and physicochemical changes, without significant differences, ensuring that the cheese elaborated in the area presents the same quality compared to other products elaborated with similar procedures.

The determination of proteolysis in the cheeses analyzed showed that there is a production of peptides in the nitrogenous fractions analyzed, particularly in the non-protein nitrogen and acid-soluble nitrogen fractions. The peptides analyzed in these fractions showed strong evidence of producing some antioxidant activity using the DPPH radical scavenging activity method. In this case, it was observed that the activity is present throughout the ripening process with an increase in the average ripening days, ensuring that there is a relationship between proteolysis and the production of bioactive peptides. The PCA analysis showed the evolution of proteolysis during the prolonged ripening process in the analyzed fractions, observing significant changes. Despite the data obtained, there is no conclusive evidence to explain which are the peptides related to the activity, therefore it is suggested that there be more in-depth analyzes in this regard.

lipid analysis showed that few lipid compounds vary during the ripening process. It was observed that most of the compounds present in cheese are not modified during extended ripening, because the type of cheese does not present microorganisms that produce lipolytic enzymes. The presence of high concentrations of unsaturated fatty acids such as oleic, linoleic, and petroselinic acids were observed, mainly related to the feeding of the cattle at the time of making the cheese. Despite the data obtained, there is no conclusive evidence to explain all compounds unidentified, that were shown to be found in significant quantities and that they represent statistical significance in the lipid analysis of cheese ripening, therefore it is suggested that there be more in-depth analyzes in this regard

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Appendix A

Abbreviations and acronyms

Table A. 1 Abbreviations

	Description
LAB	Lactic acid bacteria
TPA	Texture profile analysis
NPN	No protein nitrogen
ASN	Acid soluble nitrogen
EtOH-NSN	Ethanol non-soluble nitrogen
EtOH-SN	Ethanol soluble nitrogen
HPLC	High-performance liquid chromatography
TFA	Trifluoracetic acid

Appendix B

Table 9. ANOVA table of texture parameters

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
Hardness	0.693642	0.481139	0.467962	1.559540E+09	8	194942519	1.681811E+09	315	5339081	36.51	0.00
Cohesiveness	0.665760	0.443236	0.429096	4.671986E-01	8	0	5.868640E-01	315	0	31.35	0.00
Elasticity	0.676365	0.457470	0.443691	2.929285E+02	8	37	3.473944E+02	315	1	33.20	0.00
Chewiness	0.793922	0.630312	0.620923	3.833284E+09	8	479160521	2.248279E+09	315	7137393	67.13	0.00
Gumminess	0.747242	0.558370	0.547154	8.454097E+07	8	10567621	6.686571E+07	315	212272	49.78	0.00

Table 10. ANOVA table of lactic acid parameter

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
Lactic acid	0.856572	0.733716	0.654817	745.360344	8	93.170043	270.510043	27	10.018890	9.29	0.000005

Table 11. ANOVA table of moisture parameter

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
Moisture	0.986414	0.973013	0.958620	4140.306013	8	517.538252	114.834250	15	7.655617	67.602425	0.000000

Table 12. ANOVA table of pH parameters

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
Superficial pH	0.557263	0.310542	0.208400	24.362168	8	3.045271	54.088321	54	1.001636	3.040298	0.006773
Centre pH	0.412332	0.170018	0.047058	14.307259	8	1.788407	69.844249	54	1.293412	1.382705	0.225090

Table 13. Factor-variable correlations (factor loadings), based on correlations

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8
Hardness	-0.885335	-0.297262	0.105098	-0.263808	0.183617	0.092225	0.069839	0.008835
Cohesiveness	-0.807517	0.421381	0.236398	0.257229	-0.048900	-0.208671	0.048214	0.006692
Elasticity	-0.880357	0.189625	-0.033340	-0.233644	-0.351225	0.099398	0.007463	-0.004295
Chewiness	-0.978321	0.009775	0.167632	-0.064130	0.051780	-0.012396	-0.086002	0.018639
Gumminess	-0.965163	-0.042958	0.208690	-0.044151	0.128756	-0.055965	-0.024747	-0.028151
Superficial pH	0.750502	-0.215314	0.424185	-0.409896	-0.071712	-0.193116	-0.001008	0.001183
Centre pH	0.474588	0.370434	0.774413	0.084373	0.021325	0.173936	0.001170	-0.000643
Moisture	0.228245	0.867160	-0.288596	-0.303620	0.142389	-0.013606	-0.002127	-0.000320

Table 14. Correlation table of texture, pH, and moisture parameters (Red values are significant in p < 0.01)

	Ripening days	Hardness	Cohesiveness	Elasticity	Chewiness	Gumminess	Superficial pH	Center pH	Moisture
Ripening days	1.00	0.75	0.37	0.45	0.67	0.71	-0.25	-0.34	-0.84
Hardness	0.75	1.00	0.52	0.73	0.90	0.92	-0.43	-0.45	-0.39
Cohesiveness	0.37	0.52	1.00	0.72	0.81	0.80	-0.63	-0.06	0.03
Elasticity	0.45	0.73	0.72	1.00	0.85	0.79	-0.60	-0.38	-0.01
Chewiness	0.67	0.90	0.81	0.85	1.00	0.99	-0.60	-0.34	-0.24
Gumminess	0.71	0.92	0.80	0.79	0.99	1.00	-0.56	-0.32	-0.29
Superficial pH	-0.25	-0.43	-0.63	-0.60	-0.60	-0.56	1.00	0.53	-0.06
Centre pH	-0.34	-0.45	-0.06	-0.38	-0.34	-0.32	0.53	1.00	0.18
Moisture	-0.84	-0.39	0.03	-0.01	-0.24	-0.29	-0.06	0.18	1.00

Table 15. Correlation table of texture, pH, and moisture parameters (Red values are significant in p < 0.05)

	Ripening days	Hardness	Cohesiveness	Elasticity	Chewiness	Gumminess	Superficial pH	Center pH	Moisture
Ripening days	1.00	0.75	0.37	0.45	0.67	0.71	-0.25	-0.34	-0.84
Hardness	0.75	1.00	0.52	0.73	0.90	0.92	-0.43	-0.45	-0.39
Cohesiveness	0.37	0.52	1.00	0.72	0.81	0.80	-0.63	-0.06	0.03
Elasticity	0.45	0.73	0.72	1.00	0.85	0.79	-0.60	-0.38	-0.01
Chewiness	0.67	0.90	0.81	0.85	1.00	0.99	-0.60	-0.34	-0.24
Gumminess	0.71	0.92	0.80	0.79	0.99	1.00	-0.56	-0.32	-0.29
Superficial pH	-0.25	-0.43	-0.63	-0.60	-0.60	-0.56	1.00	0.53	-0.06
Centre pH	-0.34	-0.45	-0.06	-0.38	-0.34	-0.32	0.53	1.00	0.18
Moinsture	-0.84	-0.39	0.03	-0.01	-0.24	-0.29	-0.06	0.18	1.00

Table 16. Correlation table of texture, pH, and moisture parameters (Red values are significant in p < 0.1)

	Ripening days	Hardness	Cohesiveness	Elasticity	Chewiness	Gumminess	Superficial pH	Center pH	Moisture
Ripening days	1.00	0.75	0.37	0.45	0.67	0.71	-0.25	-0.34	-0.84
Hardness	0.75	1.00	0.52	0.73	0.90	0.92	-0.43	-0.45	-0.39
Cohesiveness	0.37	0.52	1.00	0.72	0.81	0.80	-0.63	-0.06	0.03
Elasticity	0.45	0.73	0.72	1.00	0.85	0.79	-0.60	-0.38	-0.01
Chewiness	0.67	0.90	0.81	0.85	1.00	0.99	-0.60	-0.34	-0.24
Gumminess	0.71	0.92	0.80	0.79	0.99	1.00	-0.56	-0.32	-0.29
Superficial pH	-0.25	-0.43	-0.63	-0.60	-0.60	-0.56	1.00	0.53	-0.06
Centre pH	-0.34	-0.45	-0.06	-0.38	-0.34	-0.32	0.53	1.00	0.18
Moisture	-0.84	-0.39	0.03	-0.01	-0.24	-0.29	-0.06	0.18	1.00

Table 17. ANOVA table of NPN fraction

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
RT 1.12	0.880797	0.775803	0.741312	1.401944E+07	8	1.752430E+06	4.051425E+06	52	7.791202E+04	22.4924	0.000000
RT 5.19	0.400429	0.160344	0.031166	1.312600E+11	8	1.640750E+10	6.873564E+11	52	1.321839E+10	1.2413	0.294506
RT 2.15	0.841228	0.707665	0.662691	6.268174E+08	8	7.835218E+07	2.589367E+08	52	4.979552E+06	15.7348	0.000000
RT 116.57	0.976379	0.953317	0.946135	7.338016E+06	8	9.172520E+05	3.593365E+05	52	6.910318E+03	132.7366	0.000000
HI	0.381083	0.145224	0.013720	1.304166E+10	8	1.630207E+09	7.676200E+10	52	1.476192E+09	1.1043	0.375620
HO	0.976379	0.953317	0.946135	7.338016E+06	8	9.172520E+05	3.593365E+05	52	6.910318E+03	132.7366	0.000000
HO/HI	0.671372	0.450741	0.366239	2.768819E-02	8	3.461023E-03	3.374001E-02	52	6.488464E-04	5.3341	0.000066
DPPH	0.520085	0.270489	0.158256	6.551213E+02	8	8.189017E+01	1.766868E+03	52	3.397824E+01	2.4101	0.027069

Table 18. ANOVA table of ASN fraction

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
RT 1.18	0.380340	0.144659	0.002102	145355.63	8	18169.45	859463.44	48	17905.49	1.014742	0.437858
RT 1.91	0.766742	0.587894	0.519209	1396424927.51	8	174553115.94	978876713.19	48	20393264.86	8.559351	0.000000
RT 2.43	0.977221	0.954961	0.947455	672341402.00	8	84042675.25	31709720.92	48	660619.19	127.218036	0.000000
RT 106.41	0.659052	0.434350	0.340074	478507.71	8	59813.46	623157.43	48	12982.45	4.607257	0.000324
HI	0.873714	0.763375	0.723938	364596472.67	8	45574559.08	113014576.48	48	2354470.34	19.356608	0.000000
HO	0.659052	0.434350	0.340074	478507.71	8	59813.46	623157.43	48	12982.45	4.607257	0.000324
HO/HI	0.501252	0.251254	0.126463	0.006	8	0.001	0.017	48	0.0004	2.013397	0.064781
DPPH	0.588203	0.345983	0.236980	741.76	8	92.72	1402.15	48	29.21	3.174072	0.005735

Table 19. ANOVA table of EtOH-NSN fraction

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
RT 1.17	0.901287	0.812318	0.776570	10582025.09	8	1322753.14	2444916.83	42	58212.31	22.722913	0.000000
RT 1.92	0.974067	0.948807	0.939056	1013901229.09	8	126737653.64	54705553.45	42	1302513.18	97.302397	0.000000
HI	0.967120	0.935321	0.923002	233730158.59	8	29216269.82	16162720.58	42	384826.68	75.920593	0.000000

Table 20. ANOVA table of EtOH-SN fraction

Dependent variable	Multiple R	Multiple R^2	Adjusted R^2	SS Model	df Model	MS Model	SS Residual	df Residual	MS Residual	F	p
RT 1.097	0.728303	0.530425	0.426075	732401.35	8	91550.17	648380.08	36	18010.56	5.083139	0.000282
RT 1.941	0.980529	0.961436	0.952866	9623147203.90	8	1202893400.49	385990193.01	36	10721949.81	112.189800	0.000000
RT 2.454	0.957439	0.916689	0.898175	26362017.70	8	3295252.21	2395854.11	36	66551.50	49.514317	0.000000
RT 117.23	0.511242	0.261368	0.097228	41916.59	8	5239.57	118457.27	36	3290.48	1.592344	0.161628
HI	0.981419	0.963184	0.955003	1063349400.05	8	132918675.01	40644638.42	36	1129017.73	117.729484	0.000000
HO	0.511242	0.261368	0.097228	41916.59	8	5239.57	118457.27	36	3290.48	1.592344	0.161628
HO/HI	0.802967	0.644756	0.565812	0.01	8	0.0008	0.0035	36	0.0001	8.167333	0.000003

Table 21. Correlation table of NPN (Red values are significant in p < 0.01)

	RT 1.12	RT 5.19	RT 2.15	RT 116.57	HI	HO	HO/HI	DPPH
RT 1.12	1.00	0.03	0.30	0.23	0.07	0.23	0.19	0.11
RT 5.19	0.03	1.00	-0.08	0.11	1.00	0.11	-0.64	0.13
RT 2.15	0.30	-0.08	1.00	-0.19	0.01	-0.19	-0.10	-0.10
RT 116.57	0.23	0.11	-0.19	1.00	0.10	1.00	0.51	0.15
HI	0.07	1.00	0.01	0.10	1.00	0.10	-0.65	0.12
HO	0.23	0.11	-0.19	1.00	0.10	1.00	0.51	0.15
HO/HI	0.19	-0.64	-0.10	0.51	-0.65	0.51	1.00	0.08
DPPH	0.11	0.13	-0.10	0.15	0.12	0.15	0.08	1.00

Table 22. Correlation table of ASN (Red values are significant in p < 0.01)

	TR 1.18	TR 1.91	TR 2.43	TR 106.41	HI	HO	HO/HI	DPPH
TR 1.18	1.00	0.27	0.24	0.07	0.32	0.07	-0.19	-0.15
TR 1.91	0.27	1.00	0.46	0.36	0.93	0.36	-0.54	0.16
TR 2.43	0.24	0.46	1.00	0.08	0.75	0.08	-0.28	0.31
TR 106.41	0.07	0.36	0.08	1.00	0.30	1.00	0.44	-0.05
HI	0.32	0.93	0.75	0.30	1.00	0.30	-0.51	0.25
HO	0.07	0.36	0.08	1.00	0.30	1.00	0.44	-0.05
HO/HI	-0.19	-0.54	-0.28	0.44	-0.51	0.44	1.00	-0.16
DPPH	-0.15	0.16	0.31	-0.05	0.25	-0.05	-0.16	1.00

Table 23. Correlation table of EtOH-NSN (Red values are significant in p < 0.01)

	RT 1.17	RT 1.92	HI
RT 1.17	1.00	-0.35	-0.25
RT 1.92	-0.35	1.00	0.99
HI	-0.25	0.99	1.00

Table 24. Correlation table of EtOH-SN (Red values are significant in p < 0.01)

	RT 1.097	RT 1.941	RT 2.454	RT 117.23	HI	HO	HO/HI
RT 1.097	1.0000	-.2736	-.2123	-.2381	-.2743	-.2381	.2309
RT 1.941	-0.2736	1.0000	-0.0338	0.3134	0.9986	0.3134	-0.7454
RT 2.454	-0.2123	-0.0338	1.0000	0.148	0.0173	0.148	-0.1549
RT 117.23	-0.2381	0.3134	0.148	1.0000	0.3197	1.0000	0.2412
HI	-0.2743	0.9986	0.0173	0.3197	1.0000	0.3197	-0.7538
HO	-0.2381	0.3134	0.148	1	0.3197	1.0000	0.2412
HO/HI	0.2309	-0.7454	-0.1549	0.2412	-0.7538	0.2412	1.0000

Table 25. ANOVA table of lipidic compounds

Compound	p-value	Ripening time (Days)								
		1	5	8	12	19	29	40	55	90
Butyric acid	0.304226	1.324499^{A}	1.262450^{A}	1.046854^{A}	1.045961^{A}	1.159558^{A}	1.239609^{A}	1.260487^{A}	1.360092^{A}	1.238270^{A}
Unidentified 7	0.485363	0.255709^{A}	0.371214^{A}	0.369060^{A}	0.559963^{A}	0.401423^{A}	0.246592^{A}	0.300939^{A}	0.218015^{A}	0.214930^{A}
Unidentified 8	0.077900	0.004200^{AB}	0.000000^{A}	0.002202^{AB}	0.000000^{A}	0.001248^{A}	0.007971^{B}	0.000000^{A}	0.000000^{A}	0.000000^{A}
Unidentified 9	0.554286	0.010861^{A}	0.004155^{AB}	0.001557^{AB}	0.003839^{AB}	0.002753^{AB}	0.006660^{A}	0.000000^{B}	0.000000^{AB}	0.000000^{AB}
Hexanoic acid	0.337091	1.640544^{A}	1.547156^{A}	1.371155^{A}	1.366422^{A}	1.456029^{A}	1.507380^{A}	1.531690^{A}	1.647066^{A}	1.536808^{A}
Unidentified 11	0.146484	0.023769^{AB}	0.013125^{AB}	0.020802^{AB}	0.013220^{AB}	0.019074^{AB}	0.028190^{B}	0.019002^{AB}	0.014866^{AB}	0.000000^{A}
Heptanoic acid	0.287280	0.023848^{A}	0.037897^{A}	0.022950^{A}	0.028517^{A}	0.026677^{A}	0.030861^{A}	0.023570^{A}	0.027902^{A}	0.016983^{A}
Caprilic acid	0.346804	1.854610^{A}	1.768643^{A}	1.658080^{A}	1.649930^{A}	1.723247^{A}	1.736719^{A}	1.745627^{A}	1.835765^{A}	1.747544^{A}
Unidentified 12	0.554465	0.041517^{A}	0.027308^{A}	0.027538^{A}	0.022207^{A}	0.020768^{A}	0.032246^{A}	0.010774^{A}	0.023606^{A}	0.005571^{A}
Unidentified 13	0.116731	0.009228^{AB}	0.014841^{AB}	0.017689^{AB}	0.017271^{AB}	0.022269^{AB}	0.028305^{B}	0.008449^{AB}	0.013273^{AB}	0.005230^{A}
Nonanoic acid	0.367577	0.054932^{A}	0.078148^{A}	0.057922^{A}	0.069665^{A}	0.062302^{A}	0.068226^{A}	0.061667^{A}	0.068011^{A}	0.062772^{A}
Unidentified 14	0.640911	0.065796^{A}	0.059094^{A}	0.029331^{A}	0.055436^{A}	0.060506^{A}	0.047043^{A}	0.017764^{A}	0.036246^{A}	0.024527^{A}
Decanoic acid	0.312466	4.257912^{A}	4.156661^{A}	4.075497^{A}	4.120978^{A}	4.176984^{A}	4.224700^{A}	4.308064^{A}	4.383639^{A}	4.299185^{A}
Unidentified 15	0.386331	0.135923^{A}	0.121106^{A}	0.129186^{A}	0.105191^{A}	0.126186^{A}	0.134775^{A}	0.106139^{A}	0.155266^{A}	0.130795^{A}
Unidentified 16	0.357982	0.146665^{A}	0.152093^{A}	0.127384^{A}	0.124622^{A}	0.132367^{A}	0.142863^{A}	0.127323^{A}	0.149168^{A}	0.132863^{A}
Undecanoic acid	0.350297	0.074327^{A}	0.109919^{A}	0.081833^{A}	0.097813^{A}	0.086910^{A}	0.095436^{A}	0.085876^{A}	0.096918^{A}	0.096198^{A}
Unidentified 17	0.012600**	0.014709^{BC}	0.018345^{C}	0.003615^{AB}	0.004869^{AB}	0.004989^{AB}	0.010052^{BC}	0.000000^{A}	0.000000^{A}	0.000000^{A}
Lauric acid	0.315987	2.736395$^{\mathbf{A}}$	2.755597$^{\mathbf{A}}$	2.555864$^{\mathbf{A}}$	2.537520$^{\mathbf{A}}$	2.621120$^{\mathbf{A}}$	2.691053$^{\mathbf{A}}$	2.674621$^{\mathbf{A}}$	2.888180$^{\mathbf{A}}$	2.779196$^{\mathbf{A}}$
Unidentified 20	0.555044	0.061085^{A}	0.051551^{A}	0.053055^{A}	0.042814^{A}	0.052493^{A}	0.051536^{A}	0.049958^{A}	0.043571^{A}	0.024073^{A}
Unidentified 21	0.000700***	0.021002^{AB}	0.041583^{B}	0.012175^{A}	0.014683^{A}	0.014931^{A}	0.008465^{A}	0.002247^{A}	0.000000^{A}	0.000000^{A}
Unidentified 22	0.200748	0.028678^{AB}	0.004443^{AB}	0.012531^{AB}	0.017903^{AB}	0.022016^{A}	0.024750^{A}	0.007646^{AB}	0.006187^{AB}	0.000000^{B}
Unidentified 23	0.115632	0.003014^{AB}	0.002077^{AB}	0.000958^{AB}	0.001632^{AB}	0.003241^{AB}	0.004927^{B}	0.000000^{A}	0.000000^{A}	0.000000^{A}
Unidentified 24	0.272650	0.046902^{A}	0.054664^{A}	0.040811^{A}	0.040597^{A}	0.042874^{A}	0.047220^{A}	0.026472^{A}	0.024522^{A}	0.007903^{A}
Tridecanoic acid	0.329830	0.070474^{A}	0.089877^{A}	0.070038^{A}	0.078989^{A}	0.074737^{A}	0.078791^{A}	0.074948^{A}	0.084028^{A}	0.071112^{A}
Unidentified 25	0.389827	0.101433^{A}	0.091848^{A}	0.078777^{A}	0.073455^{A}	0.086277^{A}	0.081452^{A}	0.081797^{A}	0.096406^{A}	0.061245^{A}
Myristic acid	0.338562	7.329400^{A}	7.351028^{A}	6.614572^{A}	6.699015^{A}	6.792125^{A}	7.113614^{A}	6.890580^{A}	7.592989^{A}	7.367265^{A}
Unidentified 26	0.413210	0.141405^{A}	0.127940^{A}	0.113987^{A}	0.079425^{A}	0.125427^{A}	0.123080^{A}	0.092186^{A}	0.112000^{A}	0.025228^{A}
Unidentified 27	0.424683	0.209362^{A}	0.199345^{A}	0.169752^{A}	0.161903^{A}	0.186615^{A}	0.180350^{A}	0.160102^{A}	0.212339^{A}	0.191312^{A}
Myristoleic acid	0.329920	0.430485^{A}	0.456203^{A}	0.386528^{A}	0.379481^{A}	0.407511^{A}	0.422081^{A}	0.401655^{A}	0.455195^{A}	0.426226^{A}

Unidentified 28	0.633084	0.063483^{A}	0.046115^{A}	0.026815^{A}	0.033976^{A}	0.040351^{A}	0.043518^{A}	0.005580^{A}	0.182133^{A}	0.000000^{A}
Pentadecanoic acid	0.403157	0.974025^{A}	1.033290^{A}	0.885495^{A}	0.937780^{A}	0.926491^{A}	0.970203^{A}	0.919442^{A}	0.855942^{A}	1.029564^{A}
Unidentified 29	0.052898	0.088297^{C}	0.056859^{BC}	0.028316^{AB}	0.007265^{A}	0.017411^{A}	0.034764^{ABC}	0.005402^{A}	0.000000^{A}	0.000000^{A}
Unidentified 30	0.014203**	0.228163^{A}	0.208843^{A}	0.173079^{A}	0.120837^{AB}	0.177325^{A}	0.161014^{A}	0.018243^{B}	0.000000^{B}	0.000000^{B}
Unidentified 31	0.402285	21.44321^{A}	21.76034^{A}	19.67854^{A}	20.24093^{A}	20.34642^{A}	16.37806^{A}	20.33614^{A}	22.20228^{A}	22.21421^{A}
Unidentified 32	0.656063	0.000000^{A}	0.000000^{A}	0.014877^{A}	0.045001^{A}	0.025470^{A}	0.203429^{A}	0.106943^{A}	0.000000^{A}	0.061479^{A}
Unidentified 33	0.395582	0.614962^{A}	0.499529^{A}	0.542564^{A}	0.357305^{A}	0.533550^{A}	0.567033^{A}	0.296960^{A}	0.517326^{A}	0.216160^{A}
Unidentified 34	0.187751	0.369329^{A}	0.592752^{A}	0.581381^{A}	0.281566^{A}	0.318321^{A}	0.323520^{A}	0.582397^{A}	0.512481^{A}	0.488247^{A}
Unidentified 35	0.599362	0.320741^{A}	0.167968^{A}	0.043816^{A}	0.335837^{A}	0.354149^{A}	0.282388^{A}	0.134230^{A}	0.339697^{A}	0.278046^{A}
Palmitoleic acid	0.501608	0.569662^{A}	0.687486^{A}	0.493055^{A}	0.593561^{A}	0.533113^{A}	0.514647^{A}	0.490794^{A}	0.557451^{A}	0.516541^{A}
Unidentified 36	0.542260	0.306728^{A}	0.365850^{A}	0.309938^{A}	0.334902^{A}	0.290459^{A}	0.319782^{A}	0.183441^{A}	0.302496^{A}	0.314093^{A}
Unidentified 37	0.005945***	0.0882^{ABCD}	0.196251^{A}	0.146028^{AD}	0.105621^{ABCD}	0.124602^{ACD}	0.149374^{A}	0.037322^{BCD}	0.000000^{B}	0.000000^{BC}
Heptadecanoic acid	0.490229	0.744782^{A}	0.754406^{A}	0.656221^{A}	0.684337^{A}	0.621933^{A}	0.672252^{A}	0.670615^{A}	0.591375^{A}	0.694163^{A}
Unidentified 38	0.000011***	0.016472^{A}	0.135431^{B}	0.000000^{A}	0.000000^{A}	0.000000^{A}	0.000960^{A}	0.000000^{A}	0.000000^{A}	0.000000^{A}
Unidentified 39	0.629593	0.349096^{A}	0.364595^{A}	0.315424^{A}	0.348341^{A}	0.327439^{A}	0.296105^{A}	0.249174^{A}	0.284156^{A}	0.267236^{A}
Stearic acid	0.396136	9.805546^{A}	8.526793^{A}	8.283434^{A}	7.962287^{A}	8.476256^{A}	8.502100^{A}	8.478328^{A}	9.312894^{A}	9.231235^{A}
Oleic acid	0.169597	0.000000^{AB}	0.000000^{A}	0.312464^{AB}	1.228293^{AB}	0.313742^{AB}	0.257332^{AB}	1.209223^{B}	0.442708^{AB}	0.349578^{AB}
Petroselinic acid	0.000130***	0.000000^{A}	0.000000^{A}	1.49947^{A}	5.62090^{AB}	1.30947^{A}	5.34794^{A}	12.33468^{B}	5.44116^{A}	0.00000^{A}
Vaccenic acid	0.127882	15.08704^{AB}	14.72535^{A}	16.13742^{A}	3.85476^{AB}	13.03622^{A}	6.66809^{AB}	0.21785^{B}	6.50351^{AB}	12.57677^{A}
Unidentified 41	0.466636	0.000000^{AB}	0.085981^{A}	0.168093^{A}	4.082782^{B}	0.174891^{A}	0.072068^{A}	0.060378^{A}	2.045235^{AB}	1.348144^{AB}
Unidentified 42	0.140183	0.183131^{B}	0.170191^{B}	0.000000^{A}	0.064546^{AB}	0.027819^{A}	0.021662^{A}	0.009269^{A}	0.067684^{AB}	0.078041^{AB}
Unidentified 43	0.645610	0.094406^{A}	0.184749^{A}	0.127914^{A}	0.255051^{A}	0.223900^{A}	1.926648^{A}	0.173651^{A}	0.131527^{A}	0.448577^{A}
Unidentified 44	0.522479	0.757541^{A}	0.639215^{A}	0.498154^{A}	0.312740^{A}	0.492260^{A}	0.540121^{A}	0.499357^{A}	0.440375^{A}	0.231430^{A}
Linoleic acid	0.334324	0.213101^{A}	0.208763^{A}	0.248872^{A}	0.074524^{A}	0.169656^{A}	0.281917^{A}	0.227482^{A}	0.270970^{A}	0.130470^{A}
Unidentified 45	0.484606	0.416536^{A}	0.390606^{A}	0.344699^{A}	0.317606^{A}	0.324552^{A}	0.340799^{A}	0.332641^{A}	0.349668^{A}	0.341068^{A}
Nodecylic acid	0.399923	0.266324^{A}	0.255211^{A}	0.220073^{A}	0.202222^{A}	0.230900^{A}	0.238247^{A}	0.212991^{A}	0.238469^{A}	0.216257^{A}
Unidentified 46	0.114998	0.000000^{AB}	0.080134^{AB}	0.018267^{A}	0.103853^{AB}	0.099445^{AB}	0.024536^{A}	0.238957^{B}	0.066403^{AB}	0.102350^{AB}
Elaidic acid	0.485251	2.143181^{A}	2.136400^{A}	1.946295^{A}	1.959963^{A}	1.941971^{A}	1.993052^{A}	1.613461^{A}	1.920559^{A}	1.865478^{A}
Unidentified 47	0.323584	0.117438^{A}	0.121879^{A}	0.085749^{A}	0.097507^{A}	0.110200^{A}	0.115829^{A}	0.099211^{A}	0.108727^{A}	0.104524^{A}
Unidentified 48	0.145055	0.162810^{A}	0.269180^{A}	0.246387^{A}	0.232815^{A}	0.256552^{A}	0.245276^{A}	0.255399^{A}	0.285318^{A}	0.292257^{A}
Arachidic acid	0.020623**	0.268123^{AB}	0.347357^{B}	0.039240^{A}	0.000000^{A}	0.029851^{A}	0.062176^{A}	0.029449^{A}	0.107677^{A}	0.066955^{A}

Alfa-linoleic acid	0.629606	0.887224^{A}	0.635143^{A}	0.660452^{A}	0.522703^{A}	0.602434^{A}	0.617671^{A}	0.591030^{A}	0.637391^{A}	0.583733^{A}
Gondoic acid	0.463101	1.197900^{A}	1.188124^{A}	1.082317^{A}	1.078570^{A}	1.104021^{A}	1.059020^{A}	0.952338^{A}	1.022205^{A}	1.009285^{A}
Unidentified 49	0.539820	0.078312^{A}	0.090604^{A}	0.055454^{A}	0.037150^{A}	0.050781^{A}	0.059595^{A}	0.049165^{A}	0.058800^{A}	0.051724^{A}
Unidentified 50	0.018069**	0.032022^{AB}	0.013339^{A}	0.072303^{B}	0.000000^{A}	0.018605^{A}	0.021152^{A}	0.014595^{A}	0.000000^{A}	0.000000^{A}
Heneicosilic acid	0.455468	0.173498^{A}	0.134239^{A}	0.070762^{A}	0.089381^{A}	0.106123^{A}	0.112615^{A}	0.109089^{A}	0.154932^{A}	0.129501^{A}
Unidentified 51	0.933195	0.098819^{A}	0.071486^{A}	0.060002^{A}	0.051588^{A}	0.053136^{A}	0.051833^{A}	0.034603^{A}	0.046552^{A}	0.045602^{A}
Unidentified 52	0.533997	0.038124^{A}	0.048113^{A}	0.031455^{A}	0.049960^{A}	0.036564^{A}	0.032577^{A}	0.018145^{A}	0.034045^{A}	0.016053^{A}
Docosanoic acid	0.388873	0.129400^{A}	0.097489^{A}	0.100793^{A}	0.082353^{A}	0.101436^{A}	0.113310^{A}	0.101936^{A}	0.121003^{A}	0.116700^{A}
Unidentified 53	0.500513	0.037398^{A}	0.050492^{A}	0.012301^{A}	0.047969^{A}	0.027179^{A}	0.041803^{A}	0.030050^{A}	0.039970^{A}	0.027668^{A}
Arachidonic acid	0.358080	0.164943^{A}	0.205565^{A}	0.157008^{A}	0.176921^{A}	0.158219^{A}	0.174968^{A}	0.166272^{A}	0.190844^{A}	0.197178^{A}
Tricosylic acid	0.481770	0.068161^{A}	0.054789^{A}	0.056700^{A}	0.031598^{A}	0.050192^{A}	0.058708^{A}	0.046890^{A}	0.057094^{A}	0.049830^{A}
Unidentified 54	0.576200	0.105094^{A}	0.063127^{A}	0.089831^{A}	0.054949^{A}	0.062597^{A}	0.064152^{A}	0.074204^{A}	0.089704^{A}	0.069316^{A}
Lignoceric acid	0.537363	0.061045^{A}	0.049771^{A}	0.054266^{A}	0.032207^{A}	0.033333^{A}	0.043866^{A}	0.044714^{A}	0.052876^{A}	0.042042^{A}
Nervoic acid	0.515597	0.137162^{A}	0.145906^{A}	0.080007^{A}	0.170020^{A}	0.134537^{A}	0.122673^{A}	0.135676^{A}	0.174320^{A}	0.116990^{A}
Unidentified 57	0.502700	0.171701^{A}	0.139861^{A}	0.129051^{A}	0.106311^{A}	0.131163^{A}	0.127900^{A}	0.111748^{A}	0.163746^{A}	0.149231^{A}
Docosahexanoic acid	0.703112	0.046987^{A}	0.039616^{A}	0.039798^{A}	0.038528^{A}	0.032689^{A}	0.037473^{A}	0.035971^{A}	0.034184^{A}	0.032021^{A}
Unidentified 58	0.580758	0.044703^{A}	0.040176^{A}	0.034600^{A}	0.053749^{A}	0.041421^{A}	0.040551^{A}	0.035549^{A}	0.057188^{A}	0.017209^{A}
Unidentified 59	0.097157	0.01957^{ABC}	0.041487^{BC}	0.031792^{ABC}	0.065112^{C}	0.033372^{ABC}	0.033649^{ABC}	0.000000^{A}	0.012658^{AB}	0.009442^{AB}
Unidentified 60	0.253353	0.020994^{A}	0.050442^{A}	0.035565^{A}	0.043021^{A}	0.033905^{A}	0.033860^{A}	0.016327^{A}	0.035826^{A}	0.009276^{A}
Unidentified 61	0.449795	0.072499^{A}	0.081550^{A}	0.043357^{A}	0.100834^{A}	0.072268^{A}	0.078482^{A}	0.065900^{A}	0.085985^{A}	0.030786^{A}

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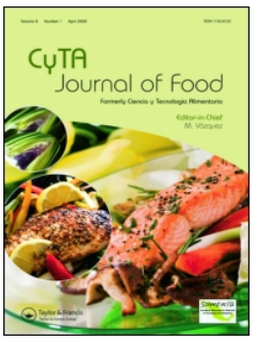

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Evolution of physicochemical and texture parameters throughout an extended ripening on a goat surface mold cheeses made in a tropical region in Mexico

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ABSTRACT

Mexican surface mold cheese manufactured with goat´s milk in a traditional process with milk produced in a tropical region and ripened for 90 days was analyzed to evaluate the impact of ripening time over physicochemical and texture parameters. Changes in hardness, cohesiveness, springiness, chewiness, and gomosity were evaluated by texture profile analysis (TPA). Moisture was evaluated with official methods, whereas pH (center and rind) was measured directly. The lactic acid determination was carried out by high-performance liquid chromatography. Analysis of variance (ANOVA) showed significant differences between all parameters except center pH. Correlation analysis exposed significant correlations between all texture parameters evaluated and lactic acid concentration. There was no significant correlation between moisture and other parameters. By principal component analysis (PCA), an evolution in two steps was observed depending on the ripening time; this evolution was determined principally by hardness and chewiness for both eigenvectors followed by gomosity.

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KEYWORDS
Surface mold cheese; Texture profile analysis; Lactic acid; Principal component analysis

PALABRAS CLAVE
Queso suave con hongos superficiales; Análisis de perfil de textura; Ácido láctico; Análisis de componentes principales

Evolución de parámetros fisicoquímicos y de textura tras maduración extendida de queso de cabra con hongos superficiales hechos en región tropical de México

RESUMEN

Se analizó queso con hongos superficiales mexicano fabricado con leche de cabra producido en una región tropical y madurado durante 90 días para evaluar el impacto del tiempo de maduración sobre los parámetros fisicoquímicos y de textura. Los cambios en dureza, cohesión, elasticidad, masticabilidad y gomosidad se evaluaron mediante análisis de perfil de textura (TPA). La humedad se evaluó con métodos oficiales; el pH se midió directamente. La determinación del ácido láctico se realizó por cromatografía líquida de alta resolución. El análisis de varianza (ANOVA) mostró diferencias significativas entre todos los parámetros, excepto el pH central. El análisis de correlación expuso correlaciones significativas entre todos los parámetros de textura y la concentración de ácido láctico. Por análisis de componentes principales (PCA), se observó una evolución en dos pasos dependiendo del tiempo de maduración; esta evolución fue determinada principalmente por dureza y masticabilidad de ambos vectores propios, seguida de la gomosidad.

1. Introduction

Surface mold-ripened cheeses type are characterized by the development of a white rind on the surface produced by the growth of some fungi microorganisms of *Penicillium* gender, particularly *Camemberti* and other microflora considered probiotic that can be produced by a different process of acidification, pressing, cut and ripening conditions (Ranadheera et al., 2019; Spinnler, 2017).

The sensorial and physicochemical characteristics are closely related to the biochemical process developed in the cheese matrix during ripening time and can be affected by some agents, like proteolytic enzymes associated with milk coagulation, the microbial development, and all ripening conditions, like temperature and humidity. The microbial growth promotes the sensorial characteristics by affecting the proteolytic process, providing specific characteristics to the final product including in products of different types of milk, such as the case of cheeses obtained from cow, goat, and sheep milk. (Ardö et al., 2017; Kiss et al., 2019; Sousa et al., 2001).

Mexican ripened cheeses have been little studied since its production gains interest in recent years due to globalization. The growth and feeding conditions of goats are different depending on the geographical area in which the country is located. Authors have shown that the presence of variations such as elevation above sea level, the botanical biodiversity with which the animals feed, and the climate, affect considerably the protein composition of milk and consequently the taste and general appearance in obtained cheese and therefore the ripening process and texture characteristics of the cheese (Barłowska et al., 2018; Vázquez García, 2015).

The present study aimed to evaluate the impact throughout an extended ripening time over physicochemical and texture parameters of goat surface mold cheeses made with milk from a tropical area in Mexico.

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2. Materials and methods

2.1. Cheese manufacture and ripening

Cheeses analyzed were manufactured with grazing goat milk obtained in Pacho Viejo, Coatepec, México (at 1199 m above sea level). Cheeses were made using an artisanal method. Goat milk was obtained by manual milking between April and May and then pasteurized (65°C for 30 minutes). When the temperature reached 30°C, it was inoculated with *Staphylococcus lactis, Staphylococcus cremoris*, and *Penicillium candidum*. Milk coagulation was done by the addition of bovine renin rennet at 1% (Coagulmex Co, Veracruz, Mexico). Every cheese was manually molded, giving a cylindric shape (about 15 cm height x 5 cm diameter), 200 g weight, and refrigerated by 24 hours. A total of two batches of 25 pieces of fresh cheese were obtained. Later, they were placed in an electronic ripening chamber Memmert HPP 260 (GmbH Co, Germany), at a constant temperature (13–14°C), the humidity of 85% and continued aeration for the ammoniacal air outlet. Cheeses were rotated once a day for 5 days and then let to promote the ripening process for a total time of 90 days. Cheeses were sampled after 1, 5, 8, 12, 19, 29, 40, 55, and 90 ripening days. To reduce variability among cheese batches, they were produced in the same season in the same month and using the milk of the same animals.

2.2. Physicochemical analysis

Each cheese was cut horizontally in two parts, exposing the central face. The pH value was determined directly on central face and rind by triplicate using an Orion 3-Star pH meter (Thermo Fisher Scientific, MA, USA), equipped with an Orion 9135APWP flat-surface pH electrode (Thermo Fisher Scientific) (Guerra-Martínez et al., 2012). For the determination of humidity, the AOAC oven drying method at 100 ± 2°C was followed (NOM-243-SSA1-2010, 2010, 2010). All the analysis was carried out by triplicate.

2.3. Instrumental texture profile analysis (TPA)

Each cheese was cut into 8 cm^3 cubes using a manual guillotine to obtain 8 cubes per sample. Each cube was analyzed in a CT3 Texture Analyzer (Brookfield Engineering Laboratories, MA, USA) with capacity range from 10 g to 50 kg following the TPA type manual test using a 4.5 cm diameter cylindrical probe measuring cell model TA-AACC36 and height-adjustable rectangular base model TA-BT-KIT with a maximum of 50% of the height of the sample, as marked by the method followed by Guerra-Martínez et al. (2012). The conditions followed for the analyses was a trigger of 15%, deformation of 10 mm and speed of 3.3 mm/s, and the analyzed parameters were hardness, cohesiveness, springiness, gomosity, and chewiness. All cheeses were analyzed by triplicate. Hardness is defined as the maximum strength required for the compression of the sample expressed in grams. The chewiness is defined as a secondary parameter obtained multiplying hardness X cohesiveness X elasticity, and it represents the times that the sample can be chew before it can be swallowed expressed in grams.

2.4. Lactic acid

For lactic acid determination, the samples were prepared by dispersion of 10 g of whole cheese and 10 ml of distilled water, homogenized during 5 min with an Ultra-Turrax T-18 basic (Ika Co, Germany) at 20,000 rpm. The mixture was incubated in bath water at 40°C for 1 h and centrifugated for 30 min at 3000 g and 4°C. The fat layer was removed, and the cheese suspension was resuspended and reincubated (50°C, 1 h). Then, samples were cooled down to 25°C, added with 10 ml water and 10 ml trichloroacetic acid (240 g/l) (Sigma-Aldrich, Steinheim, Germany). Subsequently, the samples were homogenized and incubated at 25°C for 60 min. Afterward, the samples were filtered through a Whatman no.42 filter paper (Leclercq-Peralt et al., 1999).

The high-performance liquid chromatography (HPLC) analyses were according to the method described by Picque et al. (1993) using an Agilent Technologies, Model 1200 (Palo Alto, CA, USA), integrated with diode array detector (DAD). The separation was performed by a Carbohydrate column (5 µm, 4.6 nm I.d. x 150 mm, Agilent, USA) at 50°C, with 0.005 M sulfuric acid at 0.6 ml/min flow. DAD was set at 210 nm. Quantification was done by comparison with propionic acid (Sigma-Aldrich) calibration curve (0.5 to 22.5 mg/l).

2.5. Statistical analysis

The statistical analysis was developed with the statistical software Statistica v. 13.0 (TIBCO Software Inc, Palo Alto, CA, USA). A one-way analysis of variance (ANOVA) was carried out to determine significant differences between the sampling days and the obtained dependent variables: humidity, internal and external pH, hardness, cohesiveness, springiness, gomosity, chewiness, and lactic acid. A statistical analysis of Fisher (LSD) was accomplished for each variable with the presence of significant differences with $\alpha = 0.05$. Correlation analysis was elaborated to determine the relationship between all parameters.

The principal component analysis (PCA) was applied using all the response variables and was carried out to evaluate the development of parameters throughout of cheese ripening. PCA makes it possible to obtain an overview of the data set information, showing the relationship between the new variables called principal components: PCs (PC1-Ripening days and PC2-Lactic acid), and the replacing of the original variables. The PCs contain almost all the information and are orthogonal among them.

3. Results and discussion

3.1. Analysis of variance (ANOVA)

Table 1 shows the results obtained from the analysis of variance (ANOVA) of all the evaluated parameters. Except for the results obtained in the central pH, all the parameters showed highly significant values. The hardness and the percentage of humidity were the parameters that showed more variations in the last ripening days.

3.2. pH and moisture

The pH rind showed a static fluctuation during almost all ripening until the 55^{th} day when the value decreased

Table 1. ANOVA results of physicochemical and texture parameters of goat surface mold cheese with an extended ripening process.
Tabla 1. Resultados del ANOVA de los valores fisicoquímicos y parámetros de textura para quesos de cabra blandos de hongos superficiales con una maduración extendida.

Parameter	p^a	Storage time[b]								
		Day 1	Day 5	Day 8	Day 12	Day 19	Day 29	Day 40	Day 55	Day 90
pH Rind	0.002**	6.66^{AC}	7.62^{AB}	6.63^{AC}	7.34^{AB}	7.66^{AB}	7.86^{B}	7.36^{AB}	5.75^{C}	5.64^{C}
pH Centre	0.831	5.94^{A}	6.08^{A}	6.15^{A}	6.54^{A}	6.41^{A}	6.57^{A}	6.60^{A}	5.94^{A}	5.38^{A}
Moisture (%)	0.000 ***	49.8^{F}	35.02^{D}	31.3^{CD}	27.4^{C}	20.62^{B}	15.63^{AB}	14.46^{AB}	11.02^{A}	4.23^{E}
Hardness (g)	0.000 ***	3309.8^{C}	2616.5^{BC}	2096.1^{B}	5748.7^{A}	5179.5^{A}	5597.7^{A}	7271.5^{D}	7683.8^{D}	9507.5^{E}
Cohesiveness	0.000 ***	0.21^{D}	0.18^{C}	0.19^{C}	0.14^{B}	0.13^{AB}	0.13^{A}	0.14^{AB}	0.19^{CD}	0.27^{E}
Springiness	0.000 ***	3.919^{C}	3.03^{B}	2.75^{AB}	2.47^{A}	2.4^{A}	2.56^{AB}	2.93^{B}	4.5^{D}	5.99^{F}
Chewiness (g)	0.000 ***	4844.8^{C}	2301.4^{A}	1085.4^{B}	2277.6^{A}	1689.5^{AB}	1868.7^{AB}	2875.8^{A}	6573.1^{D}	16016.1^{F}
Gomosity	0.000 ***	860.4^{AC}	534.9^{BD}	390.9^{D}	861.7^{AC}	685.1^{AB}	741.0^{AB}	995.6^{C}	1381.7^{E}	2666.9^{F}
Lactic acid (mg/l)	0.000 ***	15.16^{AB}	16.31^{AB}	16.75^{AB}	24.39^{C}	28.11^{C}	18.89^{B}	15.9^{AB}	15.16^{BC}	13.74^{A}

Notes: [a] Significant at *** $p < 0.001$ and ** $p < 0.01$; [b] Means with different letters within the same row are significantly different $p < 0.05$.
Notas: [a] Significativo a *** $p < 0.001$ y ** $p < 0.01$; Medias con letras diferentes dentro de la misma fila son significativamente diferentes $p < 0.05$.

significantly. pH center value showed no significant changes in all ripening time. In this regard, the results obtained by Tadjine et al. (2020) showed that the goat cheese obtained with pasteurized milk had a value of 4.56 in fresh cheese using the same microorganisms, a value lower than that obtained in the present study. The variation is estimated to be due to the growth qualities of the animals and the conditions in cheese making.

In the moisture case, the values obtained showed a significative decrease throughout the ripening, representing 91.5% of lost moisture (from 49.8% on day 1 to 4.23% on day 90) in analyzed cheeses. This behavior was also observed by Buffa et al. (2001) for soft goat cheeses during 60 days of ripening respectively under the same conditions of our study.

These results are similar to previous reports that observed an important diminution of pH value at the firsts ripening days induced by lactic acid bacteria and yeast as *Kluyveromyces lactis* and *Debaryomyces hansenii* (Cholet et al., 2007).On the other hand Buffa et al. (2001) did not observe significant pH variation between day 1 and day 60 for soft goat cheeses. In the last days of ripening, it has been observed the release of carbon dioxide; this could explain the decrease of pH observed in our study.

3.3. Texture profile

ANOVA showed that all texture parameters presented significant changes throughout ripening. All of them showed a significant decrease of values during the first 8 ripening days, followed by static fluctuation until days 45 or 55, finalizing with a significant increase.

The effects observed were similar to previous studies in different cheeses (Buffa et al., 2001; Guerra-Martínez et al., 2012; Martínez-Loperena et al., 2015).

3.4. Lactic acid

Lactic acid showed a static behavior with low values during the first 8 ripening days, followed by a significant increase between 12 to19 ripening days. Then, a significant constant decrease was observed until the last day.

These results are similar to other studies where the production of lactic acid is increased during the first ripening days, followed by a significant decrease for the last ripening days (Spinnler, 2017; Wolfe et al., 2014). This behavior is caused by lactose degradation by lactic acid bacteria, which produces lactic acid. Spinnler (2017) mentioned that for camembert cheeses, near the 21st ripening day, lactose has been consumed and lactic acid migrates to the cheese surface, becoming lactate and causing a decrease in rind pH and producing different effects on texture characteristics.

Some authors have concluded that the texture, flavor, smell, and other sensorial characteristics of the cheese is closely related to the external conditions of the animal, such as diet, climate, season, and geographical area (Coulon et al., 2004; Vázquez García, 2015). However, it has been shown in various studies that despite seasonal variations, there is little variability in texture and rheological properties that can be observed in matured cheeses. This is the case of cheeses made in other parts of Mexico, as well as few variations, are observed in cheeses made with milk produced in very different climatic zones, with few changes in the mentioned characteristics (Tunick et al., 2007). This gives the guideline to continue with the study of Mexican cheeses that may present characteristics very similar to European cheeses without taking into account the conditions of the cattle.

3.5. Correlation analysis

Table 2 shows the correlation coefficients obtained for the evaluated parameters.

Texture parameters showed significant correlations ($p < .05$) with ripening day, except for cohesiveness. From the physicochemical parameters, only rind and center pH did not show significant correlations with the ripening day. Additionally, significant negative correlations were observed between texture profile parameters and water content, except for cohesiveness, which showed a positive correlation. There was an important relationship among all texture parameters. The rind pH showed a significant correlation ($p < .01$) with cohesiveness, springiness, and chewiness. Lactic acid presented significant correlations with almost all texture parameters except hardness and all the physicochemical parameters, except moisture.

The hardness increases, while the moisture reduces, was observed by other authors, and is directly related to the proteolysis process in the cheese matrix that produces ionized molecules of carboxylic acid and amino groups and causes dehydration. In this way, the cheeses with more moisture are less hard (Guerra-Martínez et al., 2012; Martínez-Loperena et al., 2015).

Table 2. Correlation coefficients[a] for the physicochemical and texture parameters analyzed in goat surface mold cheese with an extended ripening process.
Tabla 2. Coeficientes de correlación[a] para los parámetros fisicoquímicos y de textura analizados en quesos de cabra blandos con hongos superficiales con una maduración extendida.

Variables	Ripening	Hardness	Cohesiveness	Springiness	Cheweness	Gomosity	pH Rind	pH Centre	Lactic acid
Hardness	0.75 ***								
Cohesiveness	0.37	0.52 **							
Springiness	0.45 **	0.73 ***	0.72 ***						
Cheweness	0.67 ***	0.90 ***	0.81 ***	0.85 ***					
Gomosity	0.71 ***	0.92 ***	0.80 ***	0.79 ***	0.99 ***				
pH Rind	−0.25	−0.43 *	−0.63 ***	−0.60 ***	−0.60 ***	−0.56 **			
pH Centre	−0.34	−0.45 **	−0.06	−0.38 *	−0.34	−0.32	0.53 **		
Lactic acid	−0.32 **	−0.12	−0.58 ***	−0.55 ***	−0.42 ***	−0.34 ***	0.32 **	0.41 ***	
Moisture	−0.84 ***	−0.39 *	0.03	−0.01	−0.24	−0.29	−0.06	0.18	0.06

[a]Correlation coeficients *Significant at $p < 0.1$; **Significant at $p < 0.05$; ***Significant at $p < 0.01$.
[a]Coeficientes de correlación *Significativo a $p < 0.1$; **Significativo a $p < 0.05$; ***Significativo a $p < 0.01$.

3.6. Principal component analysis (PCA)

Applying PCA analysis, it was possible to determine the information that can describe the behavior of cheese during ripening, and the association of time with the corresponding texture parameters and physicochemical changes, an analysis that was also elaborated by Vieira et al. (2020) where the behavior of the fermentation of an orange juice drink made from goat's milk whey was determined.

The factorial map and eigenvectors plot from PCA of texture and physicochemical parameters are shown in Figure 1(a and b, respectively). PC1 and PC2 explained 90.61% and 9.39% respectively of the total variance.

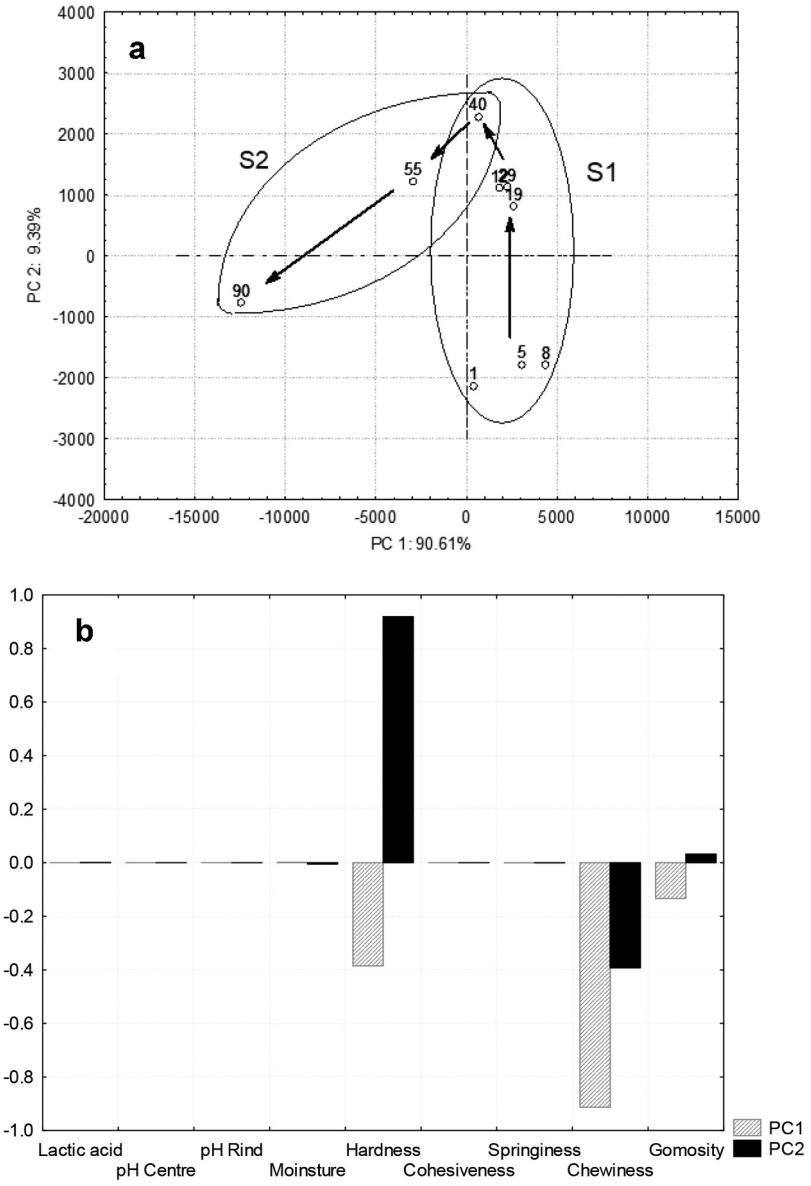

Figure 1. Plots of two principal components: PC1 and PC2: (a) Factorial map with all variables obtained by principal components analysis (PCA) of samples in different ripening days in a controlled system. Samples codes represent the ripening day. (b) Factor loadings of all parameters obtained during 90 ripening days of goat surface mold cheese produced in Veracruz, Mexico.
Figura 1. Gráficos de dos componentes principales: PC1 y PC2: (a) Mapa factorial con todas las variables obtenidas por análisis de componentes principales (PCA) de muestras en diferentes días de maduración en un sistema controlado. Los códigos de muestras representan el día de maduración. (b) Cargas factoriales de todos los parámetros obtenidos durante 90 días de maduración de quesos de cabra blandos con hongos superficiales producidos en Veracruz, México.

In the factorial map (Figure 1a) an evolution in two steps was observed depending on the ripening time. The first step (S1) from day 1 to day 40 principally in PC2, and the second step (S2) from day 40 to day 90 due to a combination of PC1 and PC2. The eigenvectors plot (Figure 1b) showed the most important parameters were hardness and chewiness for both eigenvectors followed by gomosity. Nevertheless, it was different depending on the eigenvector, for PC1 hardness was the most important while PC2 was chewiness. It seems that from day 1 to day 40 there was an increase of hardness and a decrease of chewiness while from day 40 to day 90, both parameters increased.

The observed behavior is related to ANOVA results, where hardness increase and chewiness decreases during the first 40 ripening days when the 3 parameters change significantly. At this point, the parameters increase from day 40 to 90 ripening days, presenting significant correlations between the evaluated parameters.

4. Conclusions

The obtained results showed that texture and physicochemical parameters present significant changes during an extended ripening time of surface mold cheeses. Center pH did not show significant changes. PCA analysis made it possible to describe the ripening evolution that is subject to texture parameters. Futures studies will be managed to evaluate some structural and chemical characteristics of the studied cheeses.

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Preliminary Study of Extended Ripening Effects on Peptides Evolution and DPPH Radical Scavenging Activity in Mexican Goat Cheese

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Abstract: Cheese ripening causes intense proteolysis, particularly when the cheese contains starter cultures. Several studies have shown the presence of bioactive peptides in goat's milk cheeses with antioxidant activity. Mexican goat cheeses' peptide fractions were evaluated at different ripening stages. Additionally, they were correlated with their antioxidant activity. Proteolysis was measured in the acid-soluble nitrogen and non-protein nitrogen fractions using reverse-phase high-performance liquid chromatography. While the antioxidant activity in both nitrogenous fractions was determined using a 2,2-diphenyl-1-picrylhydrazyl solution. Analyzed cheeses showed peptides fraction in the retention time of 2.05, 18.36, and 50.11 min for acid-soluble fraction and non-protein protein nitrogen, and showed antioxidant activity from the first day of ripening to 73% discoloration in the DPPH solution at 55 ripening days. Obtained results suggested that ripened Mexican goat cheese had a DPPH radical scavenging activity related to peptides present originally in the milk or released by starter culture action during cheese ripening.

Keywords: goat cheese; bioactive peptides; DPPH radical scavenging activity; principal component analysis

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1. Introduction

Certain foods can produce beneficial effects on the health of those who consume them. These effects are known as bioactivities and are present in vegetal or animal sources. Several authors have reported the presence of protein fractions with bioactivities such as antioxidant activity in the milk case. This effect is related to eliminating reactive oxygen species (ROS) and free radicals produced during oxidative metabolism. These can cause the appearance of degenerative diseases such as Parkinson's, Alzheimer's, and cancer [1,2]. Several authors have demonstrated the presence of bioactive peptides derived from hydrolyzed casein's milk that have antioxidant effects, such as cow's milk [3,4], buffalo milk [5,6], sheep's milk [7], and goat's milk [8,9]. Consequently, dairy products obtain the same effects. Different types of cheeses have been shown to have antioxidant activities regardless of their production. Such is the case of white cheese [10–12], Parmigiano Reggiano cheese [13], and Cheddar cheese [14,15]. Cheese is one of the most consumed dairy products due to its taste and nutritional qualities. Milk proteins undergo destruction processes during cheese production and ripening, either due to the presence of milk enzymes or microorganisms' effects. This process is known as proteolysis, and it occurs from the moment in which the enzymatic precipitation takes place until the end of the ripening [16,17]. Mexico has an important production of ripening goat cheeses in tropical areas such as Tabasco, Oaxaca, and Veracruz but the little knowledge and distribution contribute to a poor interest, also there are a few studies about their biochemical ripening

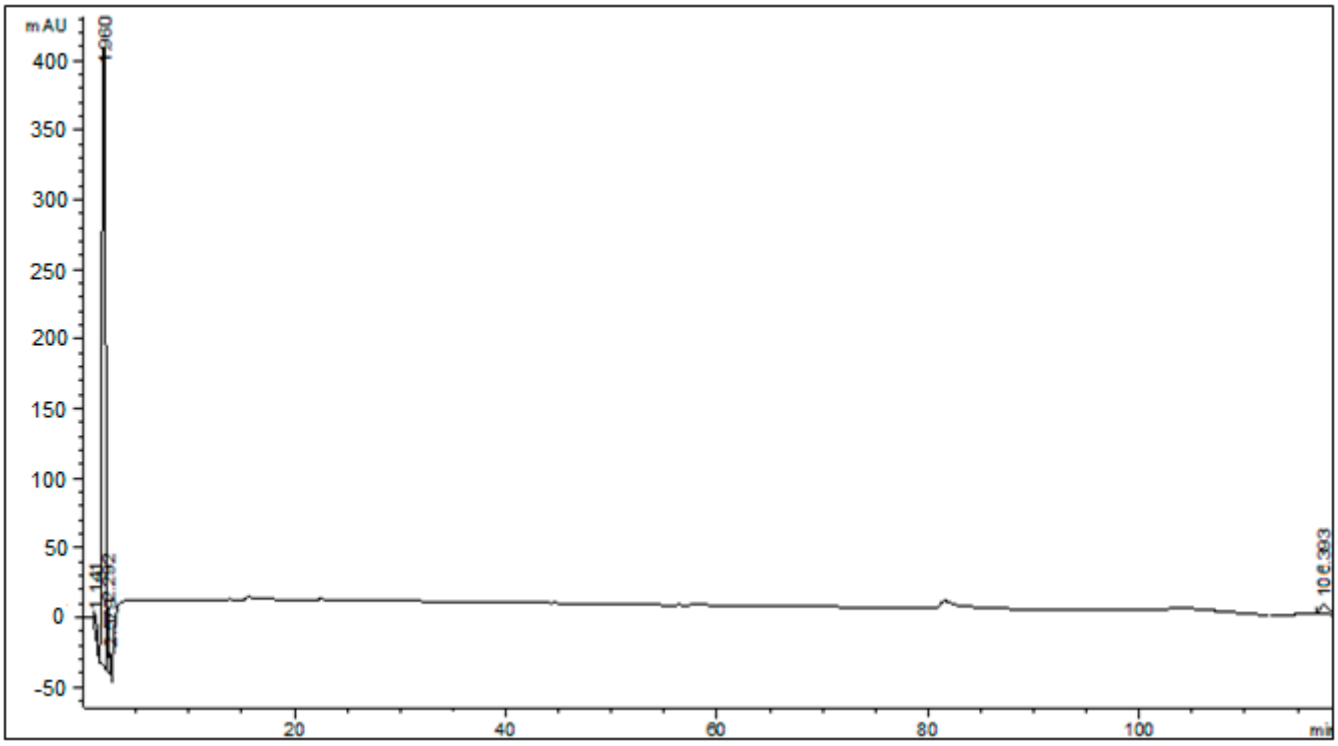
conditions [18]. Growth and feeding conditions in a different geographical area and environmental characteristics as climate, season, and elevation over the sea contribute to altering the protein composition of milk and consequently, the ripening process and bioactive peptides release during cheese making [19,20]. The importance of studying the bioactivities present in Mexican goat's milk and cheeses contributes to supporting producers and improving the quality of the products since they could be compared with other cheeses produced and studied in different geographical areas. It is considered that the study of the presence of bioactivities in goat's milk cheeses made in Mexico will give the product greater value by being able to compare it with other international products. This work aims to determine the protein profile and its relation to DPPH radical scavenging activity in cheeses with extended ripening of goat's milk made in Veracruz.

2. Results

2.1. Analysis of Peptide Evolution

Nitrogen fractions (NPN and ASN) were used to measure the proteolysis during prolonged ripening in Mexican goat cheese. Data were normally distributed. The peaks used for the analysis were selected, considering those presenting an area bigger than 200 A.U. and the presence in at least 30 of the 90 ripening days. Figure 1 shows the chromatograms in two of the ripening stages and the peaks observed in the NPN fraction.

The mean values of % area obtained by ANOVA test (95% of significance) of the frequent peaks in the chromatograms throughout the 90 days of ripening were obtained. For the statistical analysis, peaks appearing in all ripening stages were selected. Figure 2 could be observing the evolution of the mean peaks of ASN and its distribution. The ASN fraction retained the peaks at 1.13 ± 0.02, 1.91 ± 0.03, 2.43 ± 0.03, and 106.41 ± 0.07 min. For NPN, the retained peaks were at 1.92 ± 0.02, 2.15 ± 0.02, 5.19 ± 0.1, and 116.5 ± 0.99 min.



(**a**)

Figure 1. *Cont.*

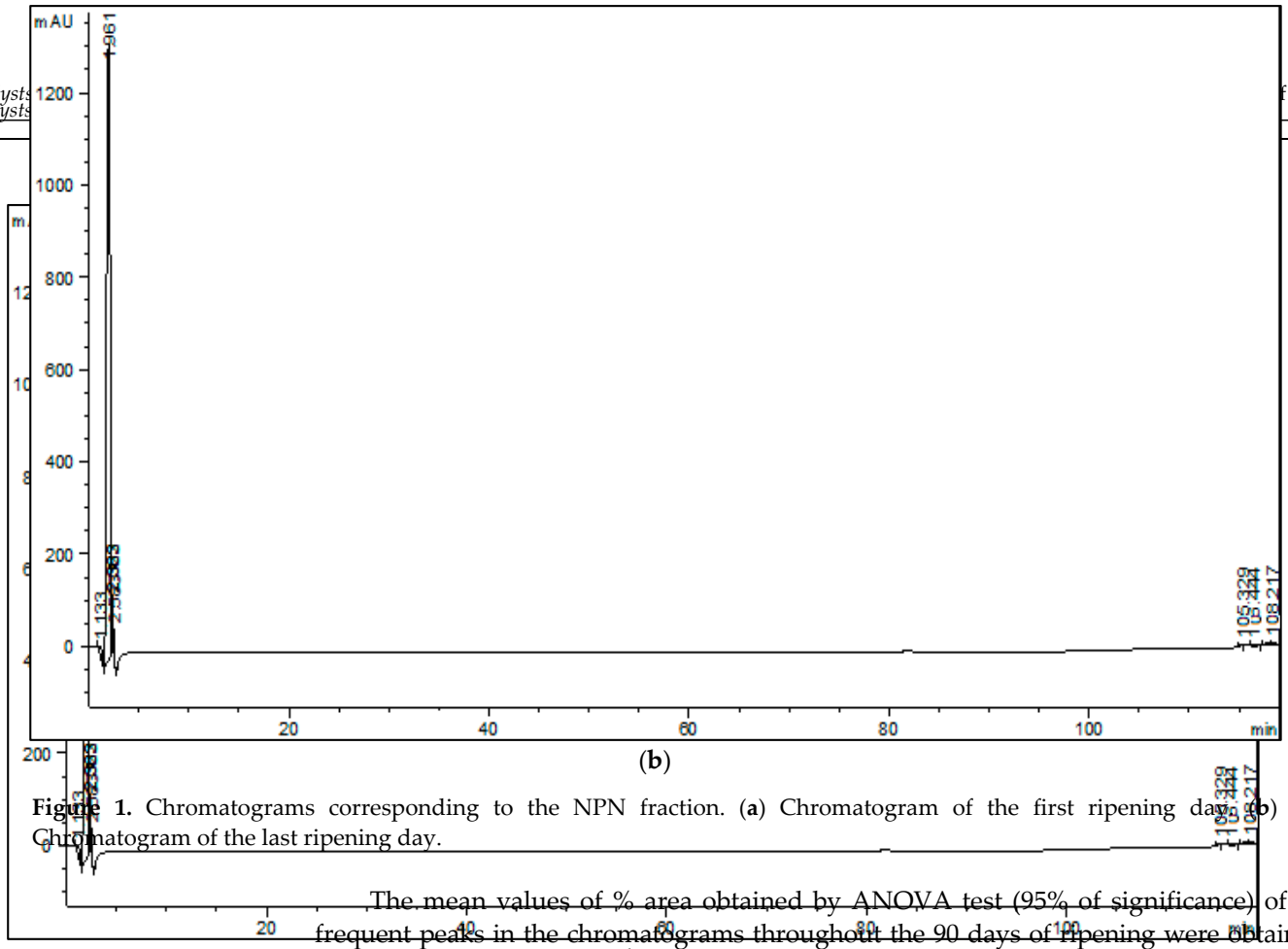


Figure 1. Chromatograms corresponding to the NPN fraction. (**a**) Chromatogram of the first ripening day. (**b**) Chromatogram of the last ripening day.

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The mean values of % area obtained by ANOVA test (95% of significance) of the frequent peaks in the chromatograms throughout the 90 days of ripening were obtained. For the statistical analysis, peaks appearing on all ripening stages were selected. In Figure 2 could be observed the evolution of the mean peaks of ASN, and in Figure 3 for NPN. For ASN were retained the peaks at 1.18 ± 0.02, 1.91 ± 0.03, 2.43 ± 0.03, and 106.41 ± 0.07 min. For NPN, the retained peaks were those at 1.12 ± 0.02, 2.15 ± 0.02, 5.19 ± 0.1, and 116.5 ± 0.99 min.

The mean values of % area obtained by ANOVA test (95% of significance) of the fr obtained. Fo n Figure 2 co NPN. For A min. For N 0.99 min.

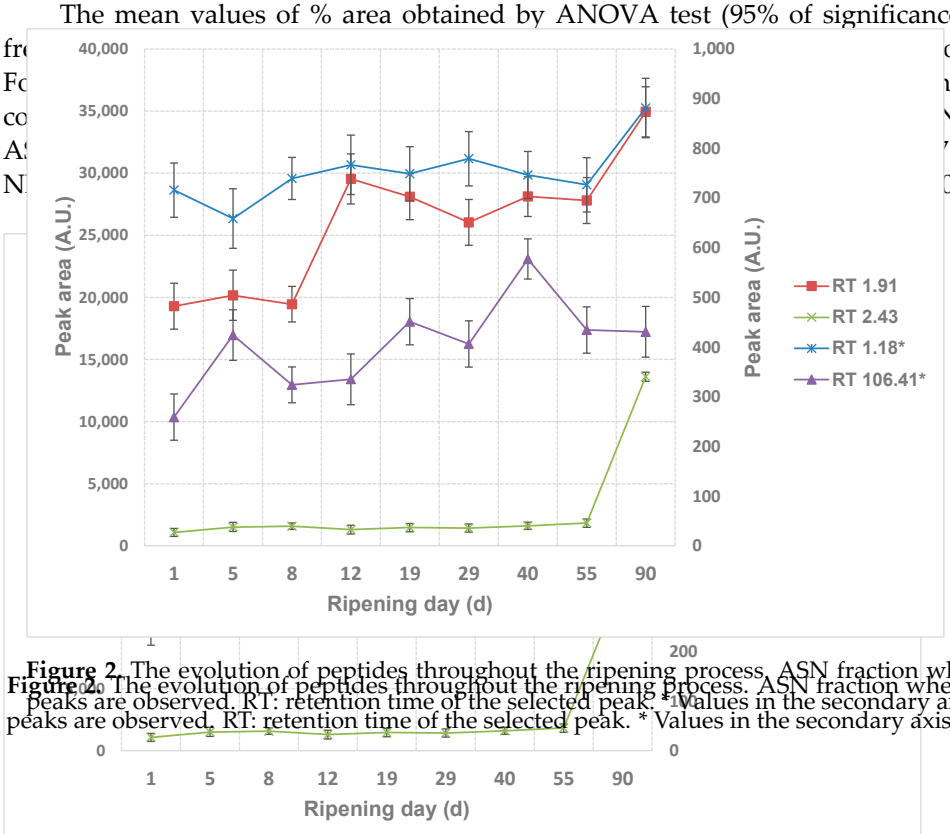

Figure 2. The evolution of peptides throughout the ripening process. ASN fraction where 4 main peaks are observed. RT: retention time of the selected peak. * Values in the secondary axis.

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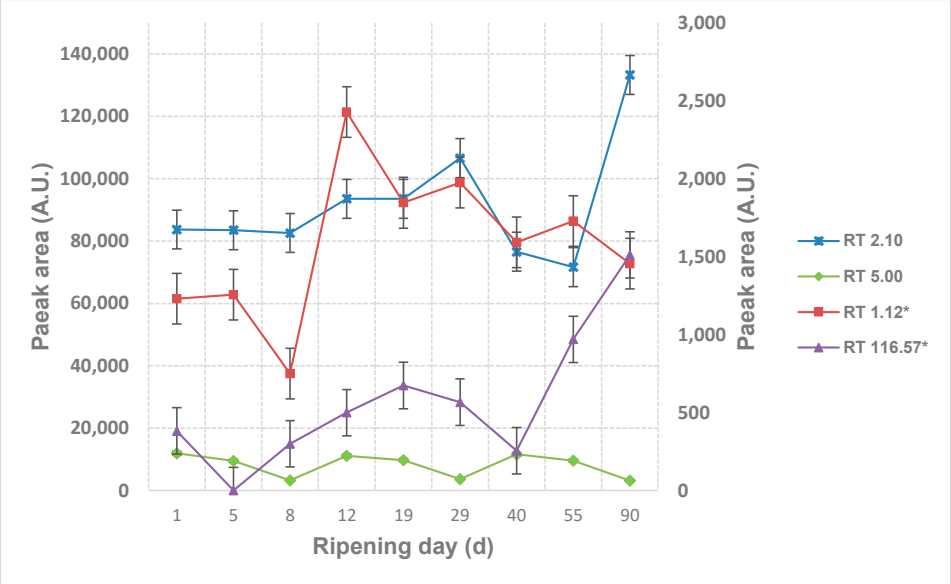

Figure 3. The evolution of peptides throughout the ripening process. NPN fraction where 4 main peaks are observed. RT: retention time of the selected peak. * Values in the secondary axis.

2.2. Analysis of HO/HI Ratio

The relationship that exists between hydrophilic and hydrophobic peptides can provide us an indication of the relationship that exists between them and the presence of biological activities. HO/HI evolution's evolution in the fractionations allows us to know how it behaves throughout the ripening process. Table 1 shows the evolution of the HO/HI ratio throughout ripening.

Table 1. ANOVA and Fisher's LSD test ($p < 0.05$) of % area of hydrophilic (HO), hydrophobic (HI) peptides, and the ratio (HO/HI) in the nitrogenous fractions throughout all ripening processes in Mexican goat cheese.

Peptide Fraction	Peptides Proportion *	*p*-Value	Storage Time								
			Day 1	Day 5	Day 8	Day 12	Day 19	Day 29	Day 40	Day 55	Day 90
NPN	HI	0.375 ns	32281.03 ^{ab}	31423.67 ^{ab}	28849.80 ^{ab}	35754.81 ^{ab}	22247.37 ^{b}	37411.72 ^{a}	29912.20 ^{ab}	27655.34 ^{ab}	45992.09 ^{a}
	HO	0.000	382.03 ^{b}	0.000 ^{b}	299.51 ^{ab}	499.54 ^{a}	673.76 ^{a}	566.26 ^{a}	254.94 ^{a}	970.10 ^{a}	1511.05 ^{a}
	HO/HI	0.000	0.012 ^{ab}	0.000 ^{b}	0.010 ^{ab}	0.023 ^{ab}	0.030 ^{a}	0.031 ^{a}	0.009 ^{ab}	0.070 ^{a}	0.033 ^{ab}
ASN	HI	0.000	7029.73 ^{b}	7449.02 ^{b}	7258.27 ^{b}	10539.89 ^{a}	10130.30 ^{a}	9419.61 ^{a}	10140.42 ^{a}	10120.68 ^{a}	16480.31 ^{a}
	HO	0.000	259.08 ^{c}	424.26 ^{ab}	323.99 ^{ac}	335.30 ^{abc}	451.09 ^{b}	406.33 ^{ab}	577.35 ^{d}	434.63 ^{ab}	430.90 ^{ab}
	HO/HI	0.064	0.037 ^{ab}	0.057 ^{bc}	0.053 ^{bc}	0.032 ^{ac}	0.046 ^{abc}	0.043 ^{abc}	0.058 ^{c}	0.043 ^{abc}	0.026 ^{a}

* Different letters in values of the same fraction are significantly different ($p < 0.05$). Each letter represents a different group obtained in Fisher's LSD test.

* Different letters in values of the same fraction are significantly different ($p < 0.05$). Each letter represents a different group obtained in Fisher's LSD test.

2.3. DPPH Discoloration Percentage Evolution

The % of DPPH discoloration shows the antioxidant effect in the evaluated fractions. The increase in the discoloration demonstrates higher antioxidant activity in the tested substance. Figure 4 shows the evolution of antioxidant activity during all ripening processes in both fractions.

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2.4. PCA Analysis NPN Fraction

Figure 5 shows the PCA analysis carried out on the NPN fraction. Figure 5a shows the factorial map of the peptides HO, HI, the relationship between these, and DPPH. They were defined as PC1 and PC2, and the behavior was explained in 59.28% and 36.50%, respectively. Samples were separated into three groups. The first one grouped cheeses from day 1 to day 12, corresponding to young cheeses. The second group grouped cheese from day 19 to day 55, corresponding with the commercial ripening time. At the same time, the third group corresponds to the extended ripened cheeses at 90 days. Figure 5b shows

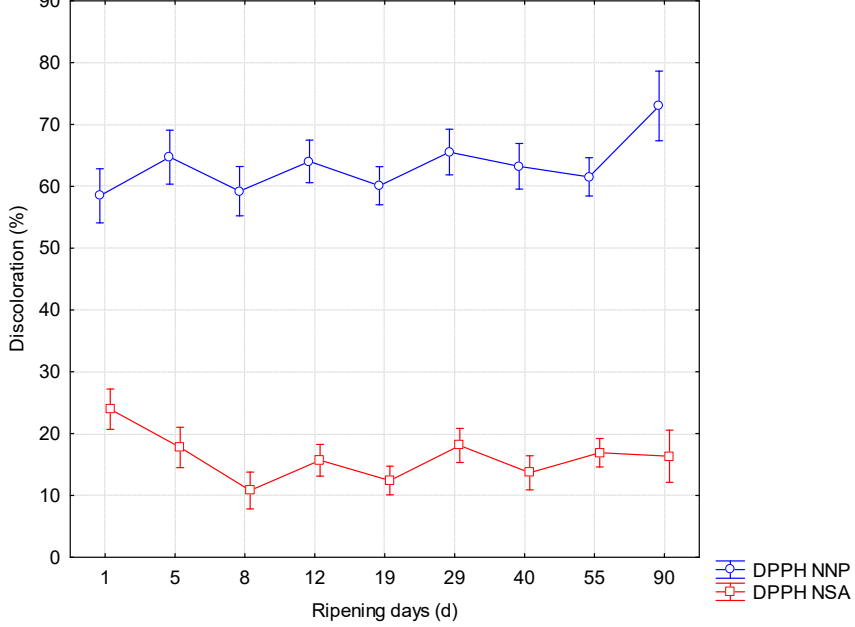

Figure 4. Evolution of DPPH discoloration percentage during the ripening process in both fractions.

2.4. PCA Analysis NPN Fraction

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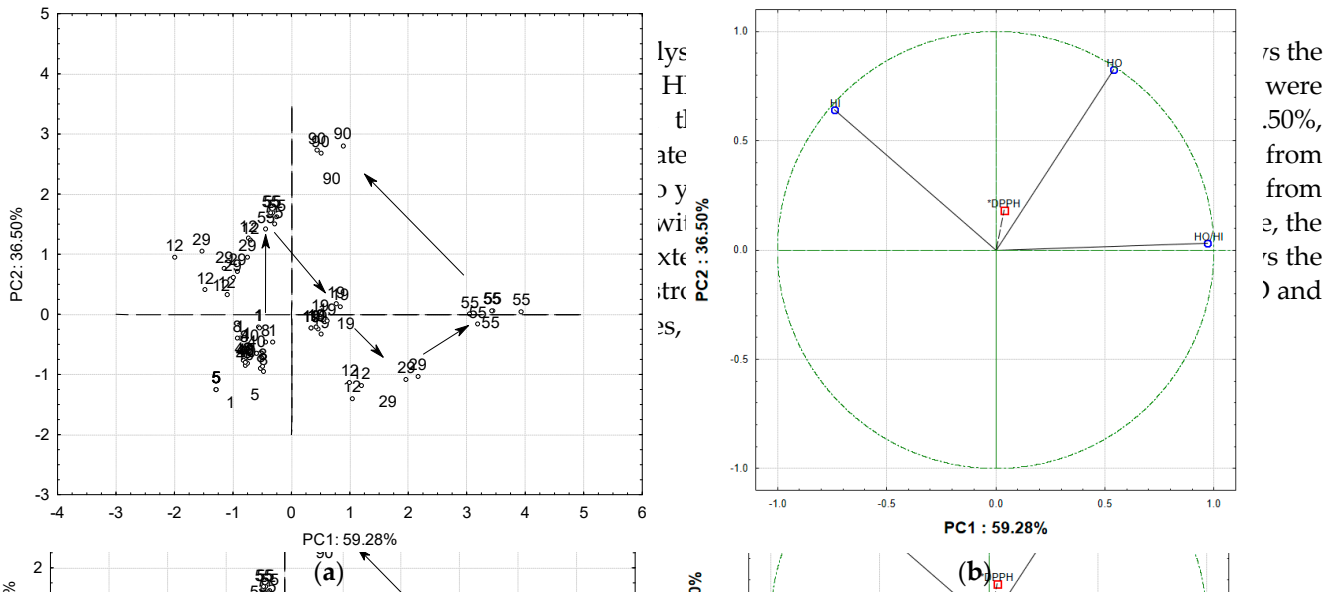


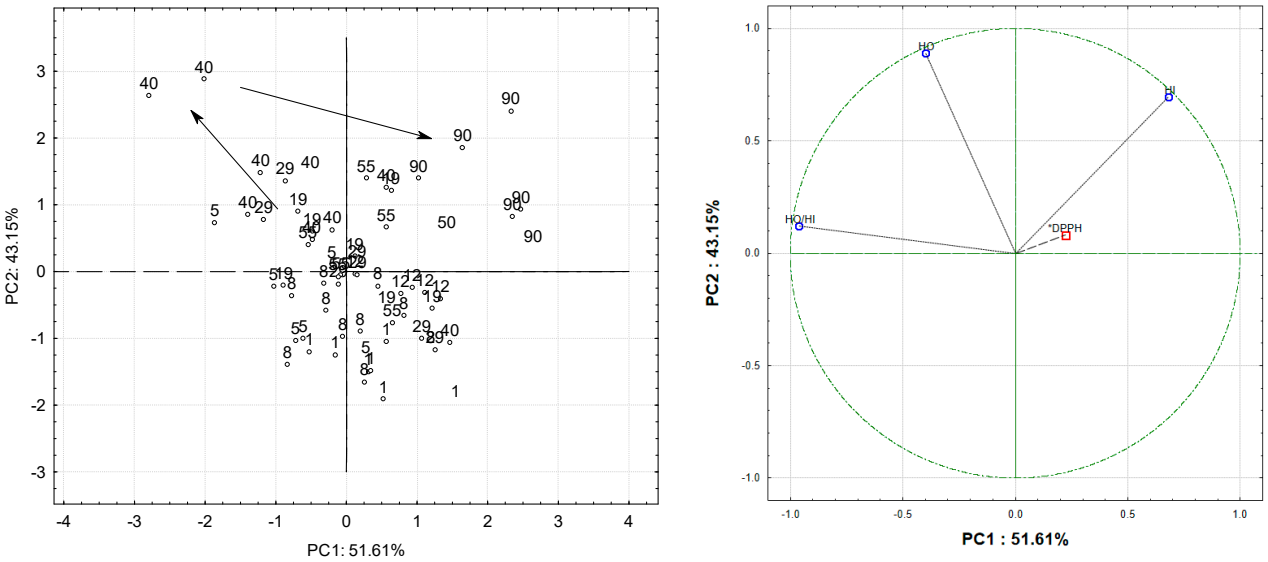
Figure 5. HI, HO, ratio HO/HI, and antioxidant activity in NPN profile. Two principal components (PC1 and PC2) are observed in PCA plots. (**a**) Factorial map obtained by principal components analyses (PCA) all in samples obtained by controlled ripening system. Arrows show the trend followed throughout the ripening process. (**b**) Factor loading plots (PC1 and PC2). Samples code represents the ripening day.

2.5. PCA Analysis ASN Fraction

Figure 6 shows the PCA analysis carried out on the ASN fraction. Figure 6a shows the factorial map of the peptides HO, HI, the relationship between these, and DPPH. They were defined by PC1 and PC2 that explained 51.61% and 43.15% of the total variance, respectively. In Figure 5a could be observed three main groups, one showing the first 29 days of ripening corresponding with young cheeses. Another is observed for the 40th

2.5. PCA Analysis ASN Fraction

Figure 6 shows the PCA analysis carried out on the ASN fraction. Figure 6a shows the factorial map of the peptides HO, HI, the relationship between these, and DPPH. They were defined by PC1 and PC2 that explained 51.61% and 43.15% of the total variance, respectively. In Figure 5a could be observed three main groups, one showing the first 29 days of ripening corresponding with young cheeses. Another is observed for the 40th ripening day corresponding to the commercial ripening time, and the last group, grouping cheeses from the 50th to 90th ripening day corresponding to the most ripened cheeses. Figure 6b shows the loadings plot. HO/HI ratio was strongly and negatively correlated with PC1. At the same time, HI and HO were correlated with both axes.



(**a**) (**b**)

Figure 6. HI, HO, ratio HO/HI, and antioxidant activity in ASN profile. Two principal components (PC1 and PC2) are observed in PCA plots. (**a**) Factorial map obtained by principal components analyses (PCA) all in samples obtained by controlled ripening system. Arrows show the trend followed throughout the ripening process. (**b**) Factor loading plots (PC1 and PC2). Samples code represents the ripening day.

2.6. Correlation Analysis

Table 2 shows the correlation between the individual peptides found in the ASN fraction, the HO and HI peptides, the HO/HI ratio, and the antioxidant effect found in this fraction. A highly significant correlation ($p < 0.05$) can be observed between antioxidant activity and the peak at 2.43 min. Table 3 shows the correlation between the peptides found in the NPN fraction, the HI and HO peptides, the HO/HI ratio, and the antioxidant effect found in this cheese fraction. Highly significant correlations were found between the relationships between retention times and the HO/HI ratio.

Table 2. Correlation values for retention time, peptides proportion, and antioxidant effect in ASN fraction in Mexican goat cheese with prolonged ripening.

Variables	RT 1.18	RT 1.91	RT 2.43	RT 106.41	HI	HO	HO/HI
RT 1.91	0.27 **						
RT 2.43	0.24 *	0.46 ***					
RT 106.41	0.24 *	0.46 ***	0.08				
HI	0.32 **	0.93 ***	0.75 ***	0.30 **			
HO	0.07	0.36 ***	0.08	0.99 ***	0.30 **		
HO/HI	−0.19	−0.54 ***	−0.28 **	−0.44 ***	−0.51 ***	0.44 ***	
DPPH	−0.15	0.16	0.31 **	−0.05	0.25 *	−0.05	−0.16

Notes: a Correlation values * significant at $p < 0.1$; ** significant at $p < 0.05$; *** significant at $p < 0.01$.

Table 3. Correlation values for retention time, peptides proportion, and antioxidant effect in the NNP fraction in Mexican goat surface mold cheese with prolonged ripening.

Variables	RT 1.12	RT 2.15	RT 5.19	RT 116.57	HI	HO	HO/HI
RT 2.15	0.03						
RT 5.19	0.30 **	−0.08					
RT 116.57	0.23 *	0.11	−0.19				
HI	0.07	0.99 ***	0.01	0.10			
HO	0.23 *	0.11	−0.19	0.99 ***	0.10		
HO/HI	0.19	−0.64 ***	−0.10	0.51 ***	−0.65 ***	0.51 ***	
DPPH	0.11	0.13	−0.10*	0.15	0.12	0.15	0.08

Notes: a Correlation values * significant at $p < 0.1$; ** significant at $p < 0.05$; *** significant at $p < 0.0.1$.

3. Discussion

The behavior of the two protein fractions' values shows the release of peptides throughout the ripening process. ASN and NPN fractions showed a significant increase in the peptides fraction mentioned above from day 1 to day 90, which can be observed in Figures 2 and 3. There is a fluctuating behavior in both fractions in the area of the peaks between days 8 and 40 of ripening. This can be explained by the presence of proteolytic enzymes found in milk as plasmin and rennet, which promote the release of medium and short-chain peptides from β-caseins. Subsequently, a tendency to a significant increase in the peaks' areas is observed between days 40 and 90 of ripening. This is related to the release of the same peptides thanks to peptidases produced by the microorganisms developed throughout the ripening process.

These results are related to the reported by Fox, P. et al. [21], Ardö, Y. et al. [22] and McSweeney, P. L. [23], who observed that intense proteolytic activity is not only due to the microorganisms during the ripening process, caseins are hydrolyzed thanks to the effect of digestive proteases contained in the food matrix. The proteins produced by these mechanisms are hydrolyzed to short peptides and free amino acids [22,24]. This effect is observed in long ripened cheeses as in the work elaborated by Barać, M. et al. [11] in white cow cheese where an increase in water-soluble fraction to 1.88 to 14.46 g/100 mg was observed; Gupta, A. et al. [14] in Cheddar cheeses at different stages of ripening; Huma, N. et al. [15] in Cheddar cheeses 6 months ripened, and Basiricó Basiricò, L. et al. [25] in Parmigiano Reggiano cheese with 12 ripening months.

The proportion of hydrophobic peptides with hydrophilic peptides (HO/HI) indicates a greater quantity of peptides released during all ripening stages and that there were increases in them near the 90th ripening day and is an important parameter that can indicate the proteolysis characteristics, particularly in the kind of peptide released and is significantly related to ripening process [26]. The formation of the HI fraction indicates the presence of medium-sized peptides that are usually soluble in water and frequently produced by the presence of peptidases produced by starter and non-starter microorganisms, unlike the HO fraction, which is represented by long peptides produced by the enzymes obtained by the rennet and enzymes found in the cheese matrix [26,27]. The obtained results show an increase in the parameters of hydrophilic peptides and hydrophobic peptides in both fractions. Fisher's posthoc analysis shows no differences between groups in almost all ripening processes; however, it can be observed that there is a much higher amount of hydrophilic peptides related to the presence of microorganisms that promote the proteolysis during the 90 ripening days. On the other hand, proteolysis's presence due to enzymes in the product matrix is observed to a lesser extent.

These results can also be observed in Figures 2 and 3, where hydrophilic peptides (less than 35 min of retention time) are found in a greater proportion compared to hydrophobic peptides (more than 35 min of retention time). Correlation analysis observed in Tables 2 and 3 shows a highly significant correlation between HI, HO, and retention times in both fractions. The results show the presence of proteolysis that is directly related

to the ripening process. Tejada, L. et al. [28] also reported this evolution. They found the presence of hydrophilic peptides in Murcia al Vino goat cheese with up to 60 days of ripening, observing a decrease in ratio HO/HI during all ripening processes, with fluctuations in values from 8.5 from the first ripening day to 5.5 to 60th ripening day without significative differences. On the other hand, there are the studies elaborated by Hernández-Galán, L. et al. [16] and Vivar Quintana, A. M. et al. [26], who observed a high value in the HO/HI ratio due to the presence of hydrophobic peptides after analyzing unripened goat, cow, and ewe cheeses.

The presence of antioxidant activity was observed from the first day of analysis. Figure 4 shows the evolution during all ripening processes in both fractions. Although both fractions showed antioxidant activity, the NPN fraction showed higher activity throughout ripening, significantly increasing after day 55, 0.02 *p*-value in ANOVA test and grouping differences in Fisher LSD test (0.95 confidence interval). The NSA fraction showed the same antioxidant activity during the entire ripening without significant changes. Correlation analysis observed in Table 2, where the ASN fraction is analyzed, shows a highly significant positive relationship between DPPH activity and retention time 2.43. The NNP fraction correlation analysis showed a significant positive relationship between DPPH activity and retention time 5.19, which indicates that the compounds that promote the activity are in compounds observed in the hydrophilic fraction.

Several authors have observed the presence of antioxidant activity in milk and cheese in water-soluble fractions and medium or short peptides [12,13]. On the other hand, it is known that whole milk, such as cow, goat, and buffalo milk, has antioxidant activity and that after the production process and cheese ripening, no significant variations in antioxidant activities [8,13]. This behavior has been observed in different types of cheeses that have exhibited activity, such as Barać, M. et al. [11], who observed the presence of constant antioxidant activity in white cheese with a percentage of antioxidant effect over 50% in water-soluble protein fraction and over 15% in non-water-soluble protein fraction after 50 ripening days; Bottesini, C. et al. [13] in hydrophilic fractions of Parmigiano Reggiano cheese with a no significative difference in antioxidant effect after 41 months in the ripening process, with a maximum of 13 µmols/mg of protein in TEAC during the first 24 ripening months. For Cheddar cheese, Gupta, A. et al. [14] and Huma, N. et al. [15] observed the antioxidant effect during 9 and 6 months, respectively, without significant variations in hydrophilic fractions. On the other hand, Meira, S. M. M. et al. [29] observed the effect using the DPPH test in a hydrophilic fraction on Roquefort cheese made from sheep's milk.

The principal component analysis made it possible to explain the 95.78% of total variability with the first two components. In the factor loading plots, an inversely proportional relationship between hydrophilic peptides and hydrophobic peptides indicates that the ripening process in this cheese promotes the main appearance of hydrophilic peptides, which can be compared with what is observed in the ANOVA and correlation tests mentioned above and that agree with what was found in the literature. On the other hand, it is observed that there is a relationship between the antioxidant activity with the principal component 1 in the tested fractions (ASN and NPN), which confirms that the fractions present close relationships with the bioactivity analyzed. This behavior has been observed in other cheeses of prolonged maturation, where retention times corresponding to hydrophilic peptides with the presence of antioxidant activity are observed [11–15,29].

Likewise, the principal component analysis factorial map shows the antioxidant activity's behavior that presents a trend from the first to the second component during the first days of ripening, followed by a trend toward the second component in the middle and last ripening days.

4. Materials and Methods

4.1. Cheese Production

For this study, 15 primiparous goats' free-grazing feeding (grass, shrubs, shoots, and leaves of local plants such as *Brugmansia candida* and *Brugmansia sua veolens*) were selected in Pacho Viejo, Coatepec, México (at 1199 m above sea level). Three batches of cheeses were produced in spring: April, May, and June to avoid climate and goat's alimentation variations. The 15 goats were milked for its batch, milk was mixed, and cheeses were produced the same day in the ranch. The milk was pasteurized at 65 °C for 30 min. The milk was allowed to cool, bovine renin rennet (Coagulmex Co., Veracruz, Mexico) was added in a concentration of 1%. It was left to rest for 8 h to remove the whey. The paste obtained was left scrubbed for 2 more hours, it was kneaded, and the strains of *Staphylococcus lactis*, *Staphylococcus cremoris*, and *Penicillium candidum* were added, taking into account the concentration set by the manufacturer. Once the paste was dry, the fresh cheeses were made in a cylindrical shape (about 5 cm diameter and 15 cm height) with a hand press. Each cheese weight was 150 g. Every batch gets 27 pieces of cheese, a total of 81 pieces of cheese for all experimental work. They were transferred to Tecnologico de Monterrey facilities on the same day of production under refrigeration at 4 °C.

4.2. Ripening Process

To carry out the ripening process, the cheeses were placed in an electronic ripening chamber brand Memmert HPP 260 (GmbH Co, Germany), programming constant temperature between 13 and 14 °C, constant humidity values of 85%, airspeed moderate, and ammonic gas output, conditions that no affect the inoculated microflora growth [18]. Sample withdrawal was carried out on the 1st, 8th, 12th, 19th, 29th, 40th, 55th, and 90th ripening days. On each sampling day, three pieces of cheese were removed and placed at −40 °C until analysis.

4.3. Sample Preparation

Each cheese was grated into small pieces. They were homogenized, and 10 g of the sample were taken and subsequently dispersed in 10 mL of deionized water in a 1:1 ratio. The dispersion was carried out using an Ultra-turrax homogenizer (IKA T18 basic instruments, Germany) for 5 min at 25,000 rpm. The obtained homogenate was incubated in a water bath at 40 °C for one hour, followed by a second homogenization of 5 min at 25,000 rpm. The suspension was centrifuged (Heraus X1, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 4 °C and 3000 rpm. From the product obtained, the solidified fat was separated by removing it with a spatula. The remaining sample was homogenized for 2 min at 25,000 rpm [30]. The cheese homogenate (CH) was stored at −20 °C until analysis.

4.4. Nitrogen Fractions

To evaluate the peptide fraction of the analyzed cheeses, a physicochemical fractionation was carried out. Before fractionation, the CH was diluted by adding 11.25 mL NaCl (9 g/L) to 1.25 g of CH. This solution was homogenized for 5 min at 25,000 rpm. The diluted cheese homogenate (DCH) was fractionated. Two nitrogen fractions were obtained: non-protein nitrogen (NPN) and acid-soluble nitrogen (ASN) at pH 4.6.

4.4.1. Non-Protein Nitrogen (NPN)

The method described by Leclercq-Perlat, M.-N. et al. [31] was used. A 10 mL aliquot of DCH was added with 10 mL of a trichloroacetic acid solution (240 g/L). Then, it was homogenized at 25,000 rpm for 2 min. This solution was incubated in a water bath for 1 h at 25 °C, then centrifuged at 4000 rpm at 7 °C for 10 min. The supernatant corresponding to the NPN fraction was then filtered using Whatman No. 42 paper and conserved at −18 °C until analysis.

4.4.2. Acid-Soluble Nitrogen (ASN)

To obtain the ASN fraction, the method described by Hernández-Galán, L. et al. [16] was applied. A 10 mL aliquot of CDH was adjusted to pH 4.6 with a 2 N hydrochloric acid solution and incubated in a water bath at 25 °C for 20 min. This solution was centrifuged for 45 min at 6000 rpm at a temperature of 7 °C. The supernatant corresponds to the ASN fraction. It was filtered using Whatman No. 42 paper and conserved at −18 °C until analysis.

4.5. Determination of Peptide Fraction

To determine the peptide fractions, the method described by Hernández-Galán, L. et al. [16] was used. A reverse-phase high-performance liquid chromatography (Agilent Technologies, Model 1200 Palo Alto, CA, USA) with a diode array detector (DAD) was used. The column was a Zorbax Eclipse XDB-C18 (5 µm, 4.6 µm i.d. × 150 mm, Agilent) at an elution rate of 0.75 mL/min in a two-solvent system. (Phase A) 100 mL/L acetonitrile and 0.5 mL/L TFA in HPLC grade water. (Phase B) 600 mL/L acetonitrile and 0.5 mL/L TFA in HPLC grade water.

Samples were initially eluted with 100% solvent A for 10 min. Subsequently, in a linear gradient, B was integrated from 0% to 49% by 98 min and 50% to 80% up to 108 min, followed by a linear gradient from 80% to 100% of B for 5 min. Once 100% was reached, B was maintained for 5 min. The wavelength used in the DAD detector was 215 nm. The injected sample volume was 10 µL, all samples were run in duplicate.

For each chromatogram, the % Area of each peak was obtained, and the compounds were coded with their retention time. The relationship between hydrophilic and hydrophobic peptides was determined following the method defined by De Llano, D. G. et al. [32], where it was established that the first 10 to 35 min of running are observed peptides with hydrophilic characteristics, and from 35 to 120 min, those peptides with hydrophobic tendencies are retained. These data were obtained using the peaks' areas and following the formula Hydrophobic peptides/Hydrophilic peptides.

4.6. Determination of Antioxidant Activity on 2,2-Diphenyl-1-picrylhydracyl (DPPH)

For this determination, the method described by Hernández-Galán, L. et al. [33] was applied. An aliquot of 0.02 mL of NPN and ASN fractions were taken and placed into a 96-well flat-bottom plate; they were added with 0.22 mL of DPPH solution (125 µM DPPH in 800 mL/L methanol). The plate was covered with aluminum foil to avoid light decomposition and kept at room temperature for 90 min. Once the time had elapsed, it was read at 520 nm in a UV-Vis spectrophotometer (X Mark Microplate Reader, Bio-Rad Laboratories, Inc., Japan). Results were expressed as the percentage of discoloration, and reagent grade methanol (Karal, Leon, Gto. Mexico) was used as a blank.

4.7. Statistical Analysis

The Statistica v 12 software (Statsoft, Inc., Tulsa, OK, USA) was used to analyze the results. All chromatographic data were separated in the following order: 1. %Area of individual peaks of the NPN fraction; 2. %Area of individual peaks in the ASN fraction; 3. Area ratio between hydrophilic and hydrophobic peptides; 4. Antioxidant activity in the ASN and NPN fractions. Analysis of variance (ANOVA) was applied to all data to evaluate significant statistical differences ($p < 0.05$) between days of ripening. If data were not normally distributed, a Kruska–Wallis test was applied. Subsequently, a Fisher's least square determinant test (LSD) was carried out to find differences between groups. Correlation analyses were carried out to find correlations between peptides and antioxidant activity. Finally, a principal component analysis (PCA) was carried out to determine the behaviors throughout all ripening times of HI, HO, and the ratio HO/HI. For this analysis, DPPH was considered as supplementary data.

5. Conclusions

The presence of peptides produced in the non-protein and acid-soluble nitrogenous fractions was determined after the prolonged ripening of Mexican goat cheese throughout extended ripening. These peptides showed evidence of producing antioxidant activity, which was observed in both fractions throughout the entire ripening of the cheese. However, it is required to perform a deep analysis to determine which peptide promotes de activity and other antioxidant tests. The study of bioactive peptides in Mexican cheeses gives rise to deep knowledge about the biochemical qualities of ripening in local cheeses. Further studies must be carried out to quantify and sequence the peptides as well as to evaluate the individual antioxidant activity after peptides fractionation.

Author Contributions: Conceptualization, R.V.-G., S.T.M.-d.-C. and S.T.M.-d.-C.; methodology, R.V.-G. and M.A.O.-L.; validation, A.C.-M. and S.T.M.-d.-C.; formal analysis, R.V.-G.; investigation, R.V.-G.; resources, S.T.M.-d.-C.; data curation, R.V.-G. and L.S.R.-H.; writing—original draft preparation, R.V.-G. and L.S.R.-H.; writing—review and editing, R.V.-G., A.C.-M. and S.T.M.-d.-C.; visualization, R.V.-G.; supervision, A.C.-M. and S.T.M.-d.-C.; project administration, A.C.-M. and S.T.M.-d.-C. All authors have read and agreed to the published version of the manuscript.

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Curriculum Vitae

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Dra. Rosa del Carmen Vázquez García

PhD Biotechnology

PROFESSIONAL PROFILE

Doctor in Biotechnology with specialty in dairy proteins and extensive experience with teaching at undergraduate level in several branches of chemical sciences.

WORK EXPERIENCE

Full Professor. Universidad Veracruzana/ Sep 2016-Aug 2017

- Full professor of Inorganic and Organic Chemistry course in the Chemical Engineering Degree.
- Development of academic programs and laboratory manuals

Full Professor. Centro de Estudios Superiores Hispano Anglo Francés (CESHAF)/ Sep 2014-Aug 2017

- Full professor of the subjects of Food Technology I and II, Food Chemistry, Development of New Products, Parasitology, General Chemistry, Biochemistry and Food Analysis I and II with the respective laboratory course in the Nutrition and Dietetics Degree.
- Development of academic programs and laboratory manuals.

Substitute teacher. Universidad Veracruzana/ Sep 2015-Aug 2016

- Substitute teacher of Sensorial Analysis in the Food Engineering Degree.

LABORATORY SKILLS

- Use of equipment for academic purposes such as HPLC, spectrophotometer, infrared spectrometer, gas and mass chromatography and glassware assembly for standard methodologies.
- Preparation of reagents for academic purposes.
- 572 TOEFL points

ACADEMIC TRAINING

PhD Biotechnology Degree/ Aug 2017-December 2021
Instituto Tecnológico de Estudios Superiores de Monterrey. Campus Querétaro.

Master in Food Science Degree/ Aug 2011-Jan 2015
Universidad Veracruzana. Campus Xalapa

Pharmacobiological chemist Degree/ Aug 2005-Jul 2010
Universidad Veracruzana. Campus Xalapa

LAST PUBLICATIONS

- Preliminary study of extended ripening effects on peptides evolution and DPPH Radical Scavening Activity in Mexican goat cheese; MDPI-Catalysts. 2021. DOI DOI 10.3390/catal11080967
- Milk and dairy products: New perspectives for ancient products. In Nova, editor. Super and Nutraceutical Foods; 2021.
- Antioxidant Activity and Fresh Goat Cheese. In: Springiner, editor. Plant Antioxidants and Health Reference Series in Phytochemistry: Springiner, Cham; 2020.
- Evolution of physicochemical and texture parameters throughout an extende ripening on a goat surface mold cheeses made in a tropical region in Mexico. CyTA-Journal of Food. 2020. DOI 10.1080/19476337.2020.1836028

SCIENCE POPULARIZATION

- ¿Es la leche un enemigo a vencer? In Transferencia Tec; June 2020
- Tecnología de la pasteurización. In Quimiofilia; August 2021