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Use of chicken pepsin extract in the coagulation of cow, goat and camel milks for the manufacture of fresh cheese

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" وَأَن لَّيْسَ لِلإِنسَانِ إِلَّا مَا سَعَى وَأَنَّ سَعْيَهُ سَوْفَ يُرَى"

الحمدلله حباً وشكراً وامتنان على البدء والختام

الحمدُ والشكر لله الذي ما وصلت دون فضله فله المحامد كلّها على البلاغ والتمام. اهدي هذا النجاح لنفسي اولا ثم الى كل من سعى معي لإتمام هذه المسيرة دمتم لي سندا لا عمر له

الى من كلل العرق جبينه وعلمني أن النجاح لايأتي الا بالصبر والأصرار،الى النور الذي انار دربي والنور الذي لاينطفي بقلبي ابداً، من بذل الغالي واستمديت منه قوتي واعتزازي بذاتي.
(والدي حبيبي)

الى من علمتني الاخلاق قبل الحروف الى الجسر الصاعد بي الى الجنة الى الداعمة الاولى في حياتي الى الانسانه العظيمه التي طالما تمنت ان تُقر عيناها برؤيتي في يوم كهذا.
(والدتي غاليتي)

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ABSTRACT

The objective of this work is to evaluate chicken pepsin extract (CP) in liquid and freeze-dried form, and its use in the coagulation of different types of milk in order to establish a chicken pepsin production unit in Algeria. To achieve this objective, we extracted chicken pepsin from the proventriculus. We analyzed the physical and chemical properties of CP in parallel with the cheeses obtained. The clarified CP is essentially characterized by a coagulating activity (4.95 ± 0.49) RU / ml and a coagulating strength (1/2040.81) SU. The FTIR spectra of CP show OH, CH, C = O and C-O bonds. For diffraction, we recorded the presence of AL_2CaO_4. The results of the effect of storage on CP indicated that the use of liquid CP extract should not exceed one week at +4°C and one month at -20°C for milk coagulation; and the cooling and freezing of CP extract mainly affect the milk coagulation time. According to the coagulating strength, the recommended volumes of CP for milk coagulation were 0.5mL (for Cow Milk) 'CM', 1mL (for Goat Milk) 'GM' and 3mL (for Camel Milk) 'DM'. The best cheese yield with CM was 18.9% and coagulated with Freeze Dried Chicken Pepsin (FDCP). The physicochemical analyses of the six cheeses showed the highest DM and ash which were 53.3±1.2% and 8.75±0.4% respectively. The FTIR spectra of the cheeses showed the bonds (OH, CH, C=O and C-O and N-H). For diffraction, all the cheeses had an amorphous structure. The microstructure of cheeses is related to the type of milk and to LCP or FDCP. The casein network was more cohesive and more compact with FDCP. Finally, we can deduce that the use of chicken pepsin in freeze-dried form is recommended for milk coagulation and in order cow milk, goat milk and then camel milk.

Key Words: Coagulation, cow milk ,goat milk, camel milk ,freeze dried chicken pepsin

RÉSUMÉ

L'objectif de ce travail est d'évaluer l'extrait de pepsine de poulet sous forme liquide et lyophilisée, et son utilisation dans la coagulation de différents types de lait afin d'implanter une unité de production de pepsine de poulet en Algérie. Pour atteindre cet objectif, nous avons procédé à l'extraction de la pepsine de poulet à partir du proventricule. Nous avons analysé les propriétés physiques et chimiques de la CP en parallèle avec les fromages obtenus. L'extrait clarifié de CP se caractérise essentiellement par une activité coagulante (4,95 ± 0,49) UP /ml et une force coagulante (1/2040,81) US. Les spectres FTIR du CP apparaissent les liaisons OH, CH, C=O et C-O. Pour la diffraction, nous avons enregistré la présence de AL2CaO4. Les résultats de l'effet de la conservation sur CP ont indiqué que l'utilisation d'extrait liquide de CP ne doit pas dépasser une semaine à +4°C et un mois à -20°C pour la coagulation du lait ; et le refroidissement et la congélation de l'extrait de CP affectent principalement le temps de coagulation du lait. Selon la force coagulante, les volumes recommandé de CP pour la coagulation du lait étaient 0.5ml (pour CM), 1ml (pour GM) et 3mL (pour DM). Le meilleur rendement fromager avec CM était 18.9% et coagulé avec FDCP. Les analyses physicochimiques des six fromages ont montré respectivement MS et cendres les plus élevés qui étaient 53,3±1,2% et 8,75±0,4%. Les spectres FTIR des fromages montrent les liaisons (OH, CH, C=O et C-O et N-H). Pour la diffraction, tous les fromages avaient une structure amorphe. La microstructure des fromages est liée au type de lait et à LCP ou FDCP. Le réseau de caséine était plus cohésif et plus compact avec FDCP. Enfin, nous pouvons déduire que l'utilisation de la pepsine de poulet sous forme lyophilisé est recommandé pour la coagulation du lait et par ordre le lait de vache, le lait de chèvre et ensuite le lait camelin.

Mots clés : Coagulation, lait de vache, lait de chèvre, lait camelin, pepsine de poulet lyophilisé

ملخص

الهدف من هذا العمل هو تقييم مستخلص بيبسين الدجاج في صورة سائلة ومجففة بالتجميد، واستخدامه في تخثر أنواع مختلفة من الحليب من أجل إنشاء وحدة إنتاج بيبسين الدجاج في الجزائر. ولتحقيق هذا الهدف، قمنا باستخلاص البيبسين من الدجاج من البروفنتريكولوس. قمنا بتحليل الخواص الفيزيائية والكيميائية لـ CP بالتوازي مع الجبن الذي تم الحصول عليه. يتميز المستخلص الموضح لـ CP بشكل أساسي بنشاط تخثر (4.95 ± 0.49) UP /ml وقوة تخثر (2040.81/1) US. يُظهر أطياف FTIR لـ CP روابط OH وCH وC=O وCO. بالنسبة للحيود، سجلنا وجود AL2CaO4. أشارت نتائج تأثير الحفظ على CP إلى أن استخدام المستخلص السائل CP يجب ألا يتجاوز أسبوع واحد عند +4 درجة مئوية وشهر واحد عند −20 درجة مئوية لتخثر الحليب؛ ويؤثر تبريد وتجميد مستخلص CP بشكل رئيسي على وقت تخثر الحليب. اعتمادًا على قوة التخثر، كانت الكميات الموصى بها من CP لتخثر الحليب هي 0.5 مل (لـ CM)، 1 مل (لـ GM) و3 مل (لـ DM). وكان أفضل إنتاج للجبن مع CM هو 18.9% ومتخثر بـ FDCP. أظهرت التحاليل الفيزيائية والكيميائية للأجبان الستة على التوالي أعلى نسبة MS ورماد بلغت 53.3±1.2% و8.75±0.4%. يُظهر أطياف الجبن FTIR الروابط (OH، CH، C=O وCO−O وN−H). بالنسبة للحيود، كان لجميع أنواع الجبن بنية غير متبلورة. ترتبط البنية المجهرية للجبن بنوع الحليب وLCP أو FDCP. كانت شبكة الكازين أكثر تماسكًا وتماسكًا مع FDCP. وأخيرا، يمكننا أن نستنتج أن استخدام بيبسين الدجاج في شكله المجفف بالتجميد ينصح به لتخثر الحليب ومن أجل حليب البقر، وحليب الماعز، ثم حليب الإبل.

الكلمات الدالة: تخثر,حليب البقر ,حليب الماعز, حليب الابل, بيبسين الدجاج المجفف بالتجميد.

LIST OF ABBREVIATION

AL2CaO4: Calcium oxide gamma-alumina
αs1-CN: αs1 casein
αs2-CN: αs2 casein
β-CN: β-casein
κ- CN: K- casein
CM: Cow milk
CMP: Caseinomacropeptide
CP: Chicken pepsin
DM: Camel milk
ESEM: Environmental scanning electron microscope
FAO: Food and Agriculture Organization
FDCC: Cow cheese made with Freeze dried chicken pepsin
FDCP: Freeze dried chicken pepsin
FDDC: Camel cheese made with Freeze dried chicken pepsin
FDGC: Goat cheese made with Freeze dried chicken pepsin
FPC: Chymosin produced by fermentation
FTIR: Fourier-transform infrared spectroscopy
GM: Goat milk
GMO: Genetically modified organisms
LCP: Liquid chicken pepsin
LPCC: Cow cheese made with liquid chicken pepsin
LPDC: Camel cheese made with liquid chicken pepsin
LPGC: Goat cheese made with liquid chicken pepsin
MCA: Milk clotting activity
MFFB: Water content in defatted cheese
RU: Rennet unit
SEM: Scanning electron microscope
SU: Soxhlet units
TC: Clotting time
TS: Total solid
XRD: Diffraction X-Ray

LIST OF FIGURES

LIST OF TABLES

Summary

Summary

Summary

Introduction

Dairy products are an important source of daily nutrients for human consumption and are highly recommended as part of a healthy and balanced diet. Cheese in particular is among the typical dairy products produced by processing milk. The transformation of milk into cheese occurs under the influence of several physical, biochemical and microbiological processes **(Lepilkina and Grigorieva, 2023)**.

It has long been known that humans have used several coagulation methods, in which the coagulating enzyme is of plant or animal origin only (such as rennet), with rennet covering only 30% of world cheese production because the availability of calves' stomachs has become limited **(FAO, 2016)**. For animal-derived coagulating agents, there are studies that prove the effectiveness of camel rennet **(Boudjenah-Haroun *et al.*, 2011; Boudjenah-Haroun *et al.*, 2013s; Hattem *et al.*, 2017)**, goat rennet **(Zhang and Wang, 2007)** and chicken pepsin **(Benyahia-Krid *et al.*, 2016)** in the coagulation of cow milk. However, very limited studies have been conducted on the ability of chicken pepsin to coagulate goat and camel milk.

Pepsin is a protein of animal origin, and pigs, sheep, cows, poultry and aquatic animals are its main sources. It is also used as a biochemical agent and finds various applications in different fields (medicine, food, environment, etc.), and its demand is increasing day by day.

It has also been tested for its potential use in the cheese industry as a substitute for rennet. In 1974, more than 50% of cheese production was prepared from chicken pepsin **(Cuvellier 1993).** Chicken pepsin has been known in southern Algeria since ancient times and is used in the manufacture of traditional cheese known as "Takamrit ".

Poultry farming has developed to become one of the most productive industrial farms in Algeria **(Laouni et al., 2024)**. Poultry farming increased from 220,000 tons in 2008 to 300,000 tons in 2011, according to the Food and Agriculture Organization. At the local level, in the Oued region, the average number of chickens marketed in the last five years was 2,751,141 heads. Among the under-utilized parts of the chicken is the proventriculus.

The proventriculus and the duct are the first important site of enzyme activity. The main function of the proventriculus is to produce gastric juice and to push juice and food into the crop, which is the main site of gastric protein degradation **(Colin, 2015)**.

According to the National Office of Statistics, Algerian cheese factories imported about 2.25 tons of rennet and/or substitutes in 2015, for about $ 200,000. According to the same source, about 50 thousand tons of cheese were sold in the Algerian market, i.e. a consumption rate of 1.2 kg/person/year in 2015 compared to 0.7 kg in 2011.

The increase in the production and consumption of cheese in the world, on the one hand, and the impossibility of simultaneously increasing the production of rennet on the other, have caused a global supply shortage of this coagulant. These problems are aggravated especially in Muslim countries, for religious reasons, due to the rituals of slaughter. Algeria is no exception. The pepsin present in chicken seems to be a suitable substitute for rennet. There are many studies targeting chicken pepsin such as: **Benyahia-Krid *et al.*, 2016, Amimour 2019 , Bouras 2023, Bentiba 2023**.

This work aims to evaluate the chicken pepsin extract in liquid and specially in freeze-dried form, and its use in the coagulation of different types of milk such as cow , goat and even camel milks in order to establish a chicken pepsin production unit in Algeria, through :

- ✓ Extraction of chicken pepsin from proventriculus and characterization of the enzymatic extract obtained .
- ✓ Study of the effect of preservation on chicken pepsin extract
- Production of fresh cheese using liquid and Freeze dried chicken pepsin and its study in terms of :
- Physicochemical analysis
- Infrared spectroscopy and X-ray diffraction
- Microstructure

To report the results of our research work, the document mainly deals with 3 chapters:

- ✓ The first part is a bibliographical synthesis on milk coagulation enzymes and the milk coagulation process
- ✓ The second part describes the material and methods used
- ✓ The third part is dedicated to the results and discussion. It includes the results obtained on the characterization of the biological material (cow , goat and camel milks and chicken pepsin extract) and the detailed results for the characterization of fresh cheeses.

Part 1

BIBLIOGRAPHIC STUDY

Chapter 1

Coagulating enzymes of milk

In the world of biology, enzymes are considered vital agents that play a central role in accelerating biochemical processes. These catalytic proteins are indispensable in various living systems, from minute bacteria to the most complex organisms. In this chapter, we will review the general characteristics of enzymes and delve into their diverse applications in different industries such as cheese making.

We will particularly focus on the category of proteases, which are protein-breaking enzymes, presenting their definition and classification. We will also explore the sources of proteases, starting with animal sources such as rennet, chymosin, and pepsin, with an emphasis on different origins of pepsin such as chicken, bovine, fish, and porcine pepsin. We will also discuss the specific origin of chicken pepsin extracted from the proventriculus, and plant and microbial sources, leading up to recombinant enzymes.

1.Generalities about enzymes

Enzymes are high molecular mass protein macromolecules (from 10,000 to 100,000 daltons), which catalyze chemical reactions by increasing their rates by at least 106 times, compared to the reaction in their absence **(Machehalek and Yakhlef, 2015)**. Enzymes are precise in their action, and every enzyme is intended to initiate a particular reaction with a specific outcome **(Khan and Selamoglu, 2020)**. Also, enzymes have many environmental advantages: biodegradability, specific action, low toxicity and formation of byproducts compatible with the environment. Because of their many interesting properties, enzymes are revolutionizing the biotech industry. In several industrial processes, enzymes are favored because they overcome the disadvantages of chemicals and improve the cost-effectiveness relationships of the processes **(Boulakroune and Debbah, 2019).**

The global market for industrial enzymes reached approximately $5.6 billion in 2018, with a predicted increase of 4.9% at a compound annual growth rate, reaching an estimated $7.0 billion in 2023. Peptidases or protases are one of the most important classes of enzymes for use as catalysts, accounting for more than 65% of the total industrial enzyme market **(Nathiele et al., 2019)**.

2.Application of enzymes

2.1.Cheese making

Dairy products are among the classic examples of fermentation-derived foodstuffs, and their history of development goes back several millennia. Economical interest and scientific progress have led to the development of dairy food science as a special branch within food science, and it is the basis of control in manufacturing a huge variety of dairy products.

Emphasis on understanding and controlling taste and texture development has led to a still growing assortment of cheeses and desserts. Enzymes originating from raw materials, microbial starter cultures, or other sources are prime tools in improving existing and creating novel dairy products **(Aehle, 2007) .**

2.2.Fruit juice production and fruit processing

The first application of enzymes in the fruit juice industry was the use of pectinases for apple juice clarification in the 1930s [500]. The fast clarification of juice after breakdown of pectin by pectinases and the decrease in juice viscosity resulted in a shorter process and greatly improved the quality of industrial apple juice. Later, pectinases were applied to depectinization of red-berry juice **(Aehle, 2007).**

2.3.Baking

Wheat flour is the main component in the bread making industry. It contains insoluble proteins called gluten which determines the properties of bread. Endos and exopeptidases from *Aspergillusoryzae* are used to proteolytically modify the gluten in wheat flour **(Boulakroune and Debbah, 2019).**

2.4.Medical and pharmaceutical applications

Other uses have been described, such as a cysteine protease from the *Zingibermontanum rhizome* and its antioxidant activity with potential for the pharmaceutical and the enzyme from *Cissusquadrangularis L*, which has antibacterial properties. Here it is possible to describe the proteases used in medical and pharmacological sectors. The success of diseases treatments is possible due to enzyme specificity toward bio−molecules with anti-cancer drug development and anti-inflammatory activities. proteases is one of the most important classes to treat illnesses, being interestingly with cathepsins and calpains molecules **(Nathiele *et al.*, 2019).**

3. Proteases

3.1.Definition of proteases

Proteases (EC 3.4.21.62) are a class of hydrolases that hydrolyze large proteins into smaller peptides by cleaving peptide bonds. They are commonly found in a wide and diverse range including animals and plants, but microbes are considered preferred sources for protease production due to their technical and economic advantages. The use of proteases in the dairy industry is well recognised; It acts as a coagulant for the vital protective enzyme and enhances the shelf life and safety of dairy products. Proteases account for 60% of the total industrial enzymes on the market **(Kuddus, 2018).**

3.2.Origin of proteases

Coagulants from plant and animal sources have been used for centuries. With developments in biotechnology and microbiology coagulants obtained from bacteria and fungi have been used in recent years also recombinant enzymes as well as those produced by fermentation **(O'Connor, 1993)** look at the (Table 03) **(Barry and Tammim , 2010)**.

3.2.1. Anemal origin

3.2.1.1. Rennet

Rennet is a complex enzyme with chymosin as its main component (is the active part of the enzyme used in cheese making). A crude rennet extract can be obtained from the fourth stomach (abomasums of ruminants). including cows, goats and camels **(O'Connor, 1993; Merheb-Dini *et al*., 2011; Vejayan, 2019)**.

For centuries, It has been used as a milk coagulant in the production of all varieties of cheese. Rennet is used in medicinal products as well as for the manufacture of lactose. In Egypt and in the rest of the Arabic region the majority of rennet used is from animal sources. Commercial calf rennet consists mainly of two enzymes chymosin and pepsin. The relative proportion of the two enzymes varies with the age of the animal.

The major, milk-clotting component of standard rennet is chymosin (88 to 94%), although mature animal rennet may contain up to 90 to 94% of pepsin and only 6 to 10% of chymosin **(Hattem *et al.*, 2017)**.

3.2.1.2. Chymosin

Chymosin (E.C. 3.4.23.4) is an aspartyl proteinase of gastric origin, secreted by the young of a number of mammalian species. It is known as the most suitable coagulant which has a high milk clotting/proteolytic activity ratio. It plays a major role in the initial proteolysis of casein in many cheese varieties. The primary chymosin cleavage site in the milk protein system is the Phe 105-Met 106 bonds in κ-casein which is many times more susceptible to chymosin than any other bond in milk proteins; its hydrolysis leads to coagulation of the milk.

Chymosin is found in three allelic forms, A, B and C, and the main differences of these forms are shown in (Table 01).

Chymosin is weakly proteolytic indeed, limited proteolysis is one of the characteristics to be considered in the selection of proteinases for use as rennet substitutes **(Andrews and Varley, 2005)**.

Table 01: Chymosin A, B and C, main differences and identities **(Barry and Tammim, 2010)**

Chymosin A	Chymosin B	Chymosin C
Aspartic acid as amino acid no. 244	Glycine as amino acid no. 244	Several amino acid differences to chymosins A and B
Less abundant in animal rennet	Dominant in animal rennet	Minor component in animal rennet
Less stable, easily degraded, autocatalytic to chymosin A2 at lower pH values	More stable, is not as easily degraded at pH as chymosin A is	More stable, is not as easily degraded at low pH as chymosin A is
About 30% higher specific activity than for B; ∼290 IMCUa mg−1	Lowest specific activity; ∼223 IMCU mg−1	About 65% higher specific activity than for B; ∼368 IMCU mg−1
All known cheese making properties are the same as for chymosins B and C	All known cheese making properties are the same as for chymosins A and C	All known cheese making properties are the same as for chymosins A and B

3.2.1.3. Pepsine

Pepsin (EC 3.4.23.1) is a natural aspartic proteinase secreted in the stomach of mammals and also found in various birds, reptiles, amphibians and fishes.

Both chymosin and pepsin belong to the pepsin-like family of aspartic peptidases and their sequences are 55% identical. Pepsin and chymosin display the same overall structure. Both enzymes are gastric acid proteinases, derive from zymogens, pepsinogen and prochymosin, respectively, have about the same molecular weights, 35.0 and 35.6 kDa, respectively, act similarly on the peptide bond of κ-casein. However, they differ in their pH and temperature sensitivities, and their milk-clotting activity (MCA)/proteolytic activity ratio. Pepsin is less specific and hydrolyses any peptide bond with phenylalanine, tyrosine, leucine, or valine residues. These properties are of great importance for the cheese making industry due to their effect on cheese yield and quality.

Pepsin is characterized by a high content of acidic, β-hydroxylated amino acids and the presence of a small number of basic residues. It has a bilobed shape with an N-terminal domain and a C-terminal domain separated by a perpendicular cleft of the largest diameter (Figure 01). This slot contains the 2 residues aspartyls involved in the catalytic mechanism and constitutes a

large substrate binding site, capable of receiving approximately 6 residues **(OdileRolet-Repecaud et al., 2016) (Jesperandal , 2013)**.

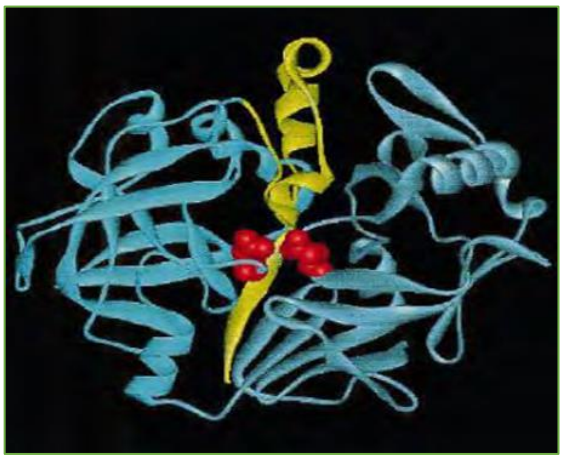


Figure 01: Tertiary structure of pig pepsinogen A The active enzyme moiety is shown in blue, the prosegment in yellow and the two active-site aspartates (Asp-32 and Asp-215) in red **(Richter *et al*., 1998).**

a. Different origins of pepsin

a.1.Chicken pepsin

Chicken pepsin (EC 3.4.23.1) is a protease capable of coagulating milk. It is extracted from the proventriculus of chickens, an organ discarded with the viscera during slaughter **(Benyahia-Krid *et al*., 2015)**. Chicken pepsin possesses a marked milk-clotting activity at pH 5.0 - 6.5 **(Bohak, 1970)**. An inactive precursor (pepsinogen) is secreted from the cells known as the fundus glands. Pepsinogen is a zymogen present in the gastric mucosa, the secretion opened in the acidic stomach, it quickly transforms into pepsin; although pepsinogen as well as similar zymogens are found in most vertebrate species **(Aouissi and Brinet, 2016)**. Chicken pepsinogen and chicken pepsin were partly purified by Herriott, Bartz, and Northrop in 1938. At temperatures above 55°C, pepsinogen is inactive. And Chicken pepsin was obtained by the activation of the pure zymogen at 25°C and pH between 1.8 and 2. It was observed by these investigators that chicken pepsin differed from swine and bovine pepsins in its stability in neutral and mildly alkaline solutions **(Bohak, 1969).** chicken pepsin was stable at pH 8 to 8.5. Inactivation at pH 6.5 to 7 is a characteristic feature of the pepsins of most vertebrate species so far Investigated. Besides chicken pepsin the only pepsin reported to be stable at pH 7 to 8 is pepsin B, a minor component of the gastric juice of swine **(Bohak, 1970).** It was found by amino acid analyses in conjunction with molecular weight determinations that chicken pepsinogen is composed of 387 amino acid residues with a molecular weight of 43000 Da and chicken pepsin of 308 amino acid residues and a molecular weight of 35,000 Da **(Bohak, 1969)**.

Also it is the only one of the stomach acid proteinases so far investigated that appears easily accessible to X-ray-crystallographic analysis. This is because a single thiol group is present in both the chicken pepsin and pepsinogen described by Bohak (1969) and in at least one of the three chicken pepsinogens, and the derived pepsin, isolated by Donta & Van Vunakis (1970). Bohak(1969) observed that the thiol group in pepsin reacted with p-chloromercuribenzoate, thus showing that a heavy atom could be fixed at a specific location in the molecule. The isolation of a pure chicken pepsinogen fraction and its conversion into a single pepsin. Some of the properties of both the pepsin and its zymogen are described. The presence of a single thiol group, which could be made to react with p-chloromercuribenzoate, has been established in both **(Green and Llewellin, 1973).** Chicken pepsin has also been used as a rennet substitute, mainly for religious reasons **(Andres, 2011).**

The amino acid composition of the pepsinogen fractions A, D, and C and their active pepsin forms (Table 02) is obtained after sedimentation applied at high speed followed by electrophoretic separation according to molecular weight by SDS-PAGE. The still heterogeneous pepsinogen B is obtained at very minute levels.

Table 02: Amino acid composition of pepsinogens and chicken pepsins **(Donta and Vanvunakis, 1970)**

Pepsinogens				**Pepsins**		
Amino acids	**A**	D	C	A	D	C
Lys	17	15	5	11	10	3
His	7	7	3	5	5	1
Arg	7	7	3	6	6	2
Asp	**44**	**44**	**41**	**44**	**45**	**35**
Thr	28	28	33	31	32	32
Ser	39	40	40	44	46	39
Glu	32	29	48	31	29	43
Pro	19	20	22	19	19	17
Gly	33	34	40	35	34	39
Ala	21	20	22	2	18	19
Half Cys	6	6	6	6	6	6
Val	27	28	22	27	28	21
Met	10	10	9	10	10	10
Ile	24	24	24	25	26	23
Leu	30	29	30	29	29	28
Tyr	24	27	24	24	24	20
Phe	25	22	27	23	22	24
Molecular weight	**42000**	**42000**	**42000**	**42000**		**38500**

a.2.Bovine pepsin

Bovine pepsin, enzymatically classified as a pepsin A (EC 3.4.23.1). It is one of the normal minor constituents of rennet after weaning. The pepsin becomes the major enzyme in gastric juices of adult ruminants and its molecular weight is 33400 Da.

The coagulant activity of bovine pepsin is not as pH dependent as that of porcine pepsin, and can coagulate milk at pHs above 6.9, its proteolytic activity is close to that of rennet. It is used in cheese making in a 50:50 mixture with rennet **(Boughellout, 2007; Barry and Tammim, 2010).**

a.3.Cod pepsin

Enzymatic extracts based on pepsin extracted from the internal wall of the cod stomach coagulate milk at 15°C, this is the best temperature for activity compared to calf chymosin **(Agrouche and Sehaki, 2016).**

a.4.Porcine pepsin

Porcine pepsin, enzymatically classified as a pepsin B (EC3.4.23.2), It is extracted from the stomach of pigs in inactive form, then activated by acidification to pH 2, its molecular weight is 34500 Da. This enzyme has milk-clotting activity, but its unspecific proteolytic activity hydrolyses bonds with Phe, Tyr, Leu or Val residues [8, 9], favouring the formation of undesirable peptides. Therefore, the application of this enzyme as a calf rennet substitute is rare **(Júnior *et al*, 2015; Saidi and Touahria, 2021)**.

b. Origin of chicken pepsin (proventriculus)

The proventriculus is the glandular compartment, which is functionally equivalent to the mammalian stomach. The proventriculus is a fusiform organ that varies in size and shape among avian species, being relatively small in granivorous species and relatively large in carnivorous and piscivorous species. In all birds except carnivore and piscivore species, the mucosal surface of the proventriculus lacks the longitudinal folds characteristic of the mucosal surface of the esophagus and is lined by mucous secreting cells. The orifices of the gastric glands are visible to the naked eye at the extremity of the papillae that project into the lumen of the proventriculus look at (Figure 02**).** The alveoli of these glands are lined with highly specialized cells secreting both hydrochloric acid and a proteolytic proenzyme: pepsinogen the system of collecting ducts, opening onto small papillae, brings gastric juice into the lumen of the proventriculus. The chyme, coming from the crop, stays in the proventriculus for a relatively short time (a few minutes to an hour) before passing into the gizzard. The gastric juice comes into action with the

food in the gizzard, in the first portion of the duodenum and eventually in the crop where it arrives by returning movements. The volume of gastric juice, which varies from 5 to 20 ml/h during a fasting period, reaches 40 ml after histamine stimulation **(Saidi and Touahria, 2021; Adoui, 2017).**

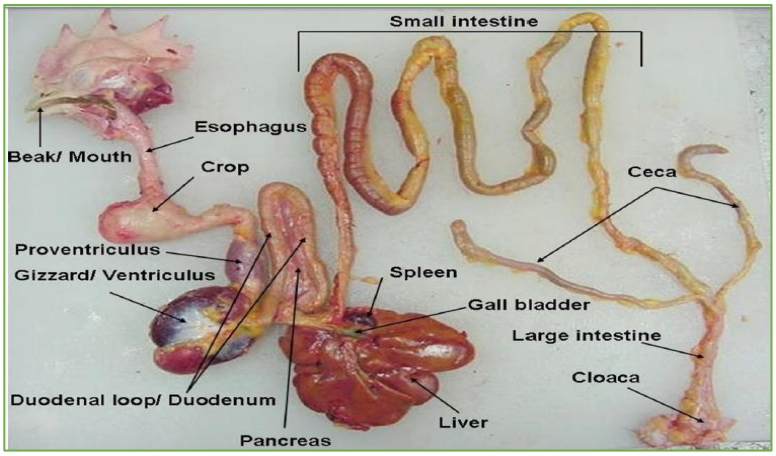


Figure 02: Parts of the digestive tract of a chicken

3.2.2.Vegetal origin

Plant extracts have been used as milk coagulants in cheese making since ancient times. Cheeses made with vegetable coagulant can be found mainly in Mediterranean, West African, and southern European countries **(Manzoor *et al*,2013).** several proteases obtained from vegetal sources such as fruits (e.g., kiwi, melon and papaya), roots (e.g., ginger rhizome), latex (e.g., Papaya fruit Carica p. and Sodom apple Calotropisprocera) and flowers (e.g., Cynaracardunculus and Centaureacalcitrapa) **(Mazorra-Manzano *et al.*, 2013).**However, the quality of the final products can impacted by bitter taste due to high proteolytic activity, which can be influenced by variations in plant sources, including their origin environment and cultivation **(Zannierah Mohsin *et al.*, 2024).**

3.2.3.Microbial origin

All the well-known microbial coagulants used for cheese making are of fungal origin. Most of the bacterial proteases described as milk-clotting enzymes have been found to be unsuitable, mainly because they have too high a proteolytic activity. Microbial coagulants are simple to manufacture by fermentation; they can be produced in large quantities at a lower cost than animal rennet. In addition, there is no risk of disease transmission from ruminants, and lacto-vegetarians agree with their use. the most important microbial derived coagulants that have been introduced into the commercial dairy sector are from three fungal species, namely from Rhizomucormiehei *R. miehei*, *Rhizomucorpusillus* (formerly *Mucorpusillus*), and

Cryphonectriaparasitica (formerly *Endothiaparasitica*) **(Barry and Tammim, 2010; Ait El Alia *et al*, 2023).**

3.2.4.Recombinant enzymes

During the 1980s, recombinant DNA technology was used to develop microorganisms capable of producing chymosin (chymosin produced by fermentation of a GMO is FPC), using the DNA sequence of chymosin from a calf abomasum cell. Today, there are two preparations on the market that are produced by a fermentation process involving either Aspergillusniger or Kluyveromyceslactis (both produce chymosin B). The third one, produced by Escherichia coli (chymosin A), which was allowed for use by the US Food and Drug Administration at the beginning of 1990, is no longer on the market after the patent was bought by a competitor manufacturing FPC producer **(Anders Andrén, 2011)**, that contain chymosin identical to the animal source, meaning that they have the same amino acid sequence as chymosin from the corresponding animal stomach, but it is just produced by more efficient means.

The main FPC, which contains bovine chymosin B, is today considered to be the ideal milk-clotting enzyme against which all other milk-clotting enzymes are measured. The production and application of bovine-type FPC has been reviewed by several authors **(Di Pierro; 2013)**. Recently, a new generation of FPC, identical to camel chymosin, has been developed. FPC (camelus) has been found to be an even more efficient coagulant for bovine milk than FPC (bovine), and is among others characterised by its very high specificity against casein, which leads to high cheese yields without creating any bitterness **(Andrews and Varley, 2005)**.

Nevertheless, the use of genetically modified organisms (GMOs) in their production raises ethical concerns related to consumer acceptance, labelling requirements, and potential long-term effects on human health and the environment.

These ethical considerations have sparked debates among consumers, regulatory bodies, and industry stakeholders. Even though, recombinant chymosin is functionally equivalent to animal rennet and offers advantages in terms of cost and consistency, its acceptance and market adoption depends on addressing these ethical concerns and ensuring transparent communication with consumers. Currently, strict regulations in certain countries, such as Germany, Netherlands, and France, regarding genetically modified foods pose challenges to recombinant chymosin **(Zannierah Mohsin *et al*., 2024)**.

Table 03: The most commonly used rennet and coagulants and their enzymes **(Barry and Tammim, 2010)**

Group	Source	Examples of rennet and coagulants	Active enzyme components
Animal	Bovine stomachs	Calf rennet, adult bovine rennet Rennetpaste	Bovine chymosin A, B and C, pepsin A and gastriscin The same as above, plus lipase
	Ovine stomachs	Lamb rennet, ovine rennet	Ovine chymosin and pepsin
	Caprine stomachs	Kid-caprine rennet, caprine rennet	Caprinechymosin and pepsin
Microbial	*Rhizomucormiehei* *Cryphonectria Parasitica*	Miehei coagulant type L, TL, XL and XLG/XP Parasitica coagulant	*Rhizomucormieheiaspartic* proteinase *Cryphonectriaparasiticaaspartic* proteinase
FPC[a]	*Aspergillus niger*	CHY-MAX™ CHY-MAX™ M	Bovine chymosin B Camelus chymosin
	Kluyveromyces marxianus var. lactis	Maxiren®	Bovine chymosin B
Vegetable	*Cynaracardunculus*	Cardoon	Cyprosin 1, 2 and 3 and/or cardosin A and B

Chapter 2

coagulation of milk

Milk clotting is a key pillar of dairy science, paving the way for the production of a rich variety of food products, especially cheese. This chapter highlights the coagulation process and the role of casein and its micelles in this process, reviewing the different types of caseins such as αs1 casein, αs 2 casein, β-casein, and κ- casein, and how they contribute to determining the characteristics of milk and its clotting susceptibility.

We deal in detail with the various types of coagulation, two divisions between acid and enzymatic coagulation, the latter consisting of three main stages: the primary enzymatic phase, the secondary stage, and the third stage. We also explore mixed coagulation and its effect on product diversity. In addition, the chapter presents the clotting abilities of different types of milk such as cow, goat and camel milks giving a comprehensive view of the factors affecting the clotting process.

1.Coagulation of milk

The transformation of milk into cheese occurs under the influence of many physicochemical, biochemical and microbiological processes. Milk coagulation is an important step and a complex process that plays a decisive role in the production of various dairy products, such as cheese and yogurt, and in the digestion of milk products **(Yang *et al.*, 2024; Lepilkina and Grigorieva., 2023; Dobozi *et al.*, 2023)**. This involves converting liquid milk into a gelatinous substance, also called cogolum or curd. There are two main types of coagulation: acid coagulation and enzymatic coagulation. However, in cheesemaking, milk curdling often results from the combined action of enzyme and acidification; Only the relative importance of their coagulation action differs. The mechanisms of action of these two coagulant agents at the micelle level are very different. Although both lead to coagulation formation, the rheological properties of the latter remain characteristic of the coagulation method **(Isselnane, 2014).**

1.1.Casein micelles

The vast majority of caseins in milk are packaged as large spherical macromolecular structures referred to as casein micelles (Figure 03). The size of the micelles ranges between 100 and 500 nanometers, with an average diameter of about 180 nm, and varies mainly according to the type of animal, season, and stage of lactation. The casein micelle can be thought of as a tangled spherical mass of thousands of individual casein molecules that are bonded together in part by calcium phosphate nanocrystals through ionic linkages with phosphoserine residues on adjacent casein molecules. Casein micelles consist of 92% protein and 8% minerals ,(calcium, magnesium, phosphate and citrate) .The four main proteins contained in the micelles are the

caseins αs1, αs2 , β and κ-casein. There is also γ δ -casein which is formed by the hydrolysis of β-casein by plasmin **(Vignola, 2002; Fox and Mc Sweeney, 1998; Kindstedt, 2013).**

1.2.Casein

Casein acts as a primary component of milk, and its relative average molecular weight ranges from 75,000 to 350,000 (Da). It forms about 80 g/100 g/L of milk protein in the form of casein micelles, which are insoluble in water. Interestingly, casein does not exist isolated within milk but forms a semi-spherical complex with calcium phosphate, which exists as a stable colloidal dispersion. Casein is the major nitrogenous fraction in all types of milk (i.e. cow, sheep, goat and buffalo) and is typically found at a level of 2.5%. There are four types of casein: αs1-, αs2-, β- and κ-casein. The composition of the casein micelle varies in the center and periphery. αs and β-caseins are more present in the center of the micelle and form a hydrophobic core, while the outer part of the micelle is composed of αs1-, αs2-, β-, and κ-caseins, and this part is more hydrophilic **(Vinola, 2002; Barry and Tammim, 2010; Pentsis and Papadimas, 2018; Yang et al., 2024)**.

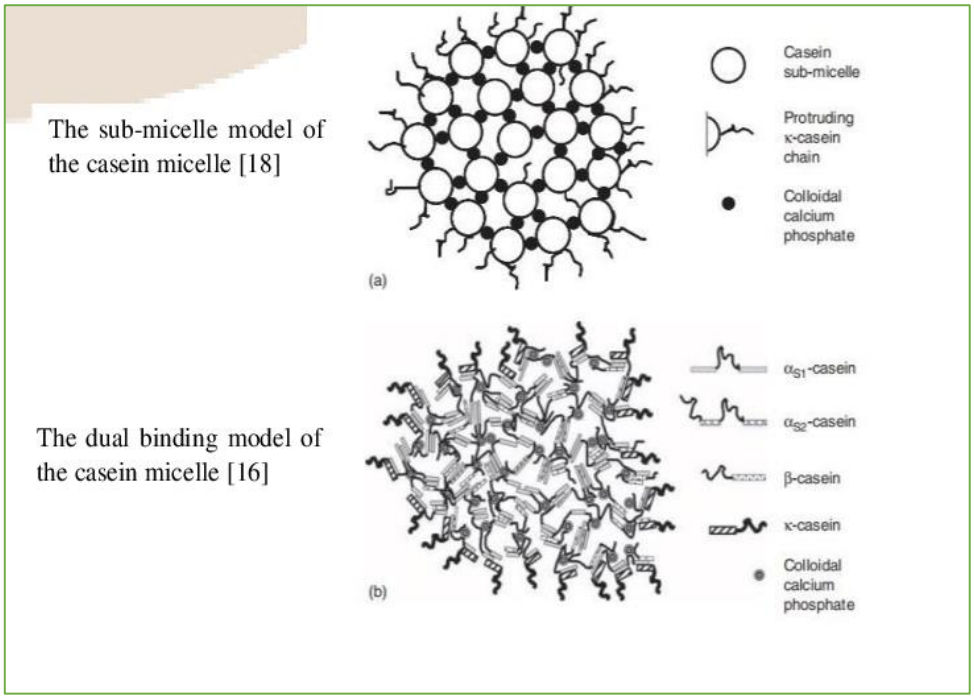


Figure 03: The structure of casein micelle **(Patel, 2020)**

1.3.Type of casein

Barana Hewa Nadugala et al, in their article (The effect of casein genetic variants, glycosylation and phosphorylation on bovine milk protein structure, technological properties, nutrition and product manufacture) in 2022, touched on the types of caseins (Table 04)

αs1 casein

αs1-casein is a phosphorylated single chain protein It is the most abundant protein in milk since it represents approximately 38 % of caseins, The B variant contains eight phosphate groups (αs1-CN B-8P) and is the most predominant genetic variant, and consists of 199 amino acid residues with a molecular mass of 23.6 KDa Its structure secondary has α-helices, β-sheets and β-bends

αs2 casein

αs2-casein constitutes approximately 10% of casein,and contains more phosphorylated residues than other caseins . The most predominant A variant contains 207 amino acid residues with 11 phosphorylated serine residues. As it contains two cysteine residues, purified αs2-CN can exist as a dimer because of intermolecular disulphide bonding . Five genetic variants (A, B, C,D and E) have been identified

β-casein

It is a protein which constitutes approximately 35% of casein and is found in a similar ratio to casein αs1-CN. There are up to 17 genetic variants, the most common of which is the A2 reference variant. It contains 209 amino acid residues with five phosphorylated serine residues and several proline residues, which greatly influence the structure of this protein due to the bends provided by this amino acid. It is the most hydrophobic of all the caseins, which places it more in the middle of the submicelles. Its secondary structure includes alpha helices and beta sheets. Its elliptical-shaped tertiary structure presents specific hydrophobic regions which may explain the high proportion of this type of casein cross-linked.

Κ- casein

Κ- casein is the smallest casein, It only represents around 12% of caseins, it is nevertheless the most common protein studied. There are two most common genetic variants are κ- CN A and B. Its primary structure has 169 acid residues amino acids and shows the presence of a glycomacropeptide portion which gives this protein its hydrophytic character. On the other hand, the rest of the protein chain is very hydrophobic in nature, which explains the loss of solubility of casein micelles during hydrolysis by chymosin

Table 04: Comparison of individual casein characteristics **(Nadugala *et al.*, 2022)**

Characteristic	**αs1-CN**	**αs2-CN**	**β-CN**	**κ- CN**
Native conformation	**unfolded structure**	**unfolded structure**	**unfolded structure**	**unfolded structure**
Quantity in milk (%)	**1.2_1.5**	0.3_0.4	0.9_1.1	**0.3_0.4**
No. of amino acid residues	199	207	209	**169**
Molecular weight	**~ 23,000**	**~25,000**	**~24,000**	**~19,000**
Location in milk at ambient temperature	**Micelle interior**	**Micelle interior**	**Micelle interior**	**Micelle surface**
No. of proline residues	17	10	33_35	**20**
No. of phosphate groups	7_9	9_15	4_5	**0_3**
No. of cysteine residues	0	2	0	**2**
Number of glycans	0	0	0	**0_6**
No. of S-S groups	0	1	0	**1**
No. of S-H groups	0	0	0	**0**
Number of genetic variants	10	5	17*	**14**

2.Types of coagulation

2.1.Acid coagulation

Acid coagulation precipitates caseins at their isoelectric point. It may result from biological acidification using enzymes that progressively convert lactose into lactic acid, or from chemical acidification (injection of carbon dioxide, addition of glucono-δ-lactone, etc.). Whatever the process, the acidification results in a drop in the degree of dissociation of the acidic groups of calcium phosphocaseinate. The H+ ions released by acidification gradually neutralize the electronegative charges: electrostatic repulsion decreases with H+ environmental enrichment and

then disappears. At room temperature, micelles begin to aggregate at pH 5.2. When the isoelectric pH of casein is reached (pH 4.6), there is total flocculation (Figure 04) **(Troch *et al.*, 2017)**

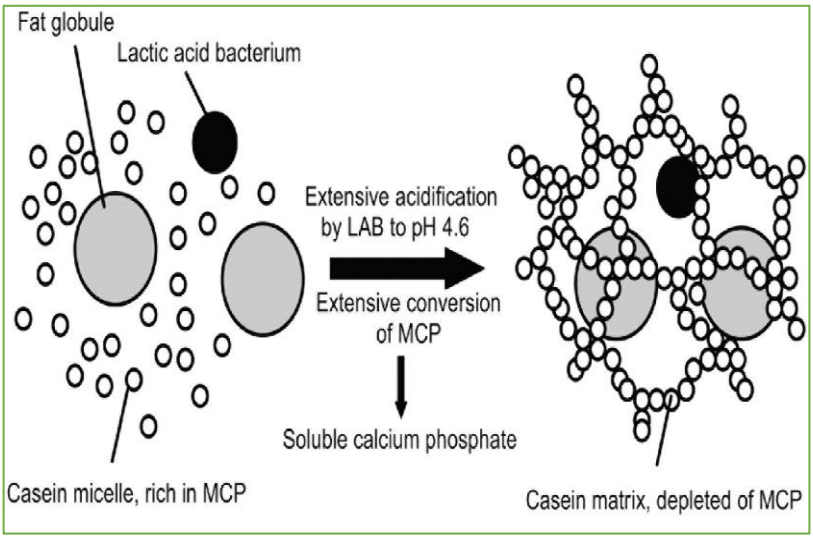


Figure 04: Diagrammatic representation of the process of acid coagulation **(Kindstedt, 2013).**

2.2.Enzymatic coagulation

Enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation Chymosin-induced coagulation of milk may be described by three phases **(Hallén 2008)** look at (Figure 05).

2.2.1.Primary Enzymatic Phase

The κ-casein molecules provide a steric stabilizing layer with their hydrophilic C-terminal peptides protruding into the aqueous phase. Gel formation is initiated by the proteolysis of the κ-casein molecules, which is accompanied by the release of a hydrophilic peptide, termed the caseinomacropeptide (CMP), into the serum (whey) phase. The remaining N-terminal region of the κ-casein, termed the para- κ-casein remains bound in the casein network. Gradual loss of the the CMP is accompanied by a decrease in the micellar zeta potential, which results in destabilization of the micelle and aggregation, ultimately into a gel **(Horne and Lucey, 2017).**

2.2.2.Secondary phase

When milk coagulates under normal conditions of pH and protein content, the viscosity does not increase until the enzymatic phase is at least 87% and > 60% complete. enzymatic coagulation time (visual). Thus, there is some overlap between the hydrolysis and aggregation stages; the extent of overlap depends on experimental conditions such as pH, temperature, and protein content. The high degree of the hydrolysis of κ-casein necessary for aggregation may be

due to the presence of other caseins on the micellar surface which also contribute to the repulsive barrier which opposes aggregation. When the repulsive barrier is sufficiently lowered by the removal of κ-casein hairs, aggregation can occur **(Bouras, 2023).**

2.2.3.Tertiary phase

The tertiary phase is what we call the crosslinking phase of the gel. This becomes more and more organized and structured. At the microscopic level, we observe an increase in bonds between the modified micelles, mainly hydrophobic and electrostatic interactions, as well as the formation of phosphocalcic bridges. It corresponds at the macroscopic level to the hardening of the gel **(Ronez, 2012).**

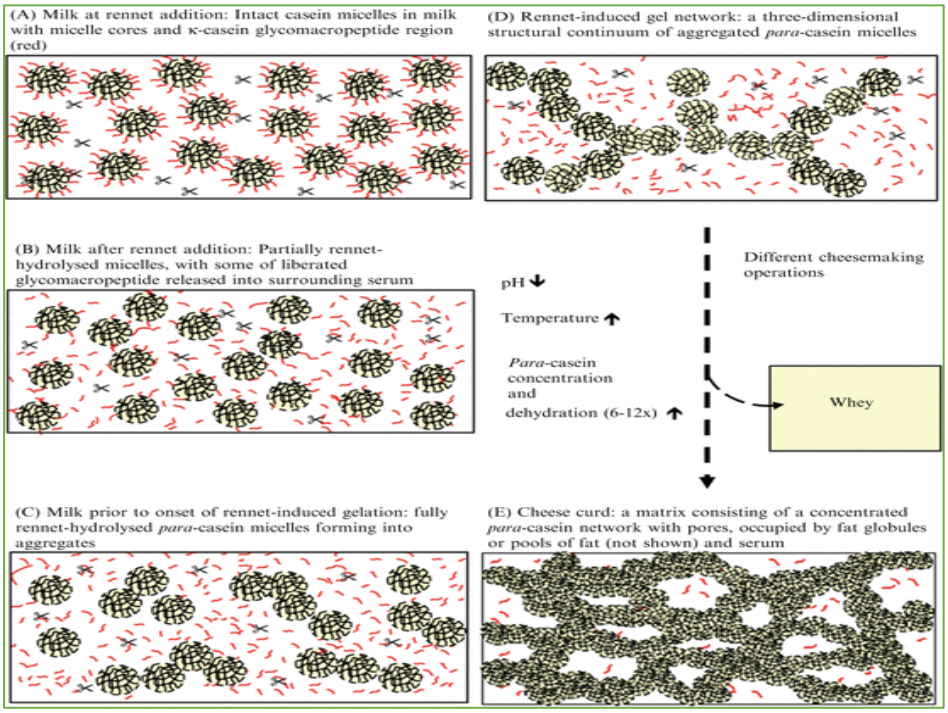


Figure 05: Schematic drawing of the various processes occurring during the enzymatic coagulation of milk **(Fox,2017)**

2.3.Mixed coagulation

Mixed coagulation is carried out by acidification of milk and addition of coagulating enzymes. In practice this method is used for the manufacture of fresh cheeses or soft cheeses. The coagulum obtained has intermediate characteristics between those of lactic gel and rennet. It is characterized by less flexibility and elasticity, greater firmness and friability than that of rennet gel **(Djaballah and Henka, 2022).**

3.Milk coagulation ability

The ability of milk to coagulate varies from one type to another depending on the proportions of its various components

3.1.Coagulation of cow milk

The coagulation ability of cow milk compared to goat and camel milks varies due to differences in milk composition, particularly the protein profile. Cow milk is known for its good coagulation properties, which are essential for cheese production **(Viraj *et al.*, 2022)**

3.2.Coagulation of goat milk

Milk protein and indigenous enzymes impact directly on the ability of milk to be processed and on the quality of dairy products. The characteristics of casein in goat milk are of particular interest due to the high number of polymorphism that are related to cheese making properties of milk**.**

Goat and cow milk have similar percentages of κ-casein and αs2-casein; however, goat milk contains lower αs1-casein content than cow milk .αs1-casein is the major casein fraction in cow milk . Whereas β-casein is the major casein fraction found in goat milk**.**

The coagulation ability of goat milk is strongly dependent on the αs1-casein content. If the content of this protein in the milk is higher, it is associated with a higher total protein content, a lower pH, a shorter coagulation time and a higher curd firmness **(Albenzio and Santillo, 2011; Seifu, 2023; Ben bouziane *et al.*,2023).**

3.3.Coagulation of camel milk

The processing of camel milk into cheese is difficult or even impossible ,There have been a number of attempts to produce cheese from camel milk , but most of these trials were unsuccessful and yielded contradictory results because of its properties that make it difficult to coagulate, especially with the use of classic rennet .Although it has more limited technological capabilities, this milk has been successfully tested in the manufacture of several derived products (cheese, fermented milks, butter, ice cream, etc.) Tests conducted to valorize this product by converting it showed a reduced ability to coagulate with rennet. This characteristic of the quantitative and qualitative composition of this milk results in two to three times longer flocculation and coagulation times compared to cow milk, which is attributed to differences in the size of casein molecules that are mainly related to the availability of κ-casein**,** only 3% verses 13% in cow milk **(Ben bouziane *et al.*,2023; Mati *et al.*,2005; Al haj O *et al.*, 2010)**

4.Factors affecting milk coagulation

Many factors are likely to modify the coagulation of milk and the physical characteristics of the coagulums. These factors are mainly related to temperature, pH, calcium content , micelle size and enzyme concentration.

4.1.Effect of temperature

The temperature also has an effect on milk clotting . Below 10 °C the enzyme is still active but gelation does not occur. Between 10 and 20 °C, coagulation is slow. Between 30 and 42 °C, clotting is gradual. Above 42 °C, coagulation begins to decrease and it stops completely at 55 °C. The effect of temperature on the enzymatic phase is relatively slight and is connected to the coefficient diffusion of the enzyme to its site of action **(Walstra *et al.*, 2006)**. Moreover, the cooling of the milk for a few days before its transformation also affects gelation. This is because at low temperatures, the colloidal calcium phosphate dissolves, causing disruption of the micelles. These effects are partially reversible **(Troch *et al.*, 2017).**

4.2.Effect of milk Ph

The pH of milk is an important environmental factor in the cheese formation stage. During the production of most cheeses in Western countries, it is common to add lactic acid bacteria to the milk, commonly known as “starters,” to develop acidity and promote coagulation. The pH of milk affects enzymatic and aggregation reactions. By lowering the pH, there is a decrease in the colloidal stability of the milk. The pH of milk directly affects the enzymatic activity of the coagulant **(Esteves *et al.*, 2003)**. By moving from pH 6.7 to 5.6, the speed of coagulation increases. This results from increased hydrolysis rate and thus increased gel stability rate. The hardness is much greater from pH 6.6 to pH 6.0 due to the greater availability of ionized calcium. Below pH 6.0, the casein is demineralized and disintegration of the micellar structure is accentuated until it is complete at pH 5.2. This leads to a weakening of the network **(Benyahia-Krid,2013)**

4.3.Effect of calcium ion content (CaCl2)

The effect of calcium content has been recognized for a long time; It is manifested in the coagulation time and gel hardness. Thus, it was demonstrated that the CaCl2 ratio varies significantly in “slow” milk and “normal” or “fast” milk, and the speed of coagulation increases when the calcium/phosphate + citrate ratio of the aqueous phase increases. Furthermore, a fairly close relationship between gel strength and calcium content was observed. Adding calcium chloride to milk is a common practice in cheese making. It has the effect of reducing coagulation

time and increasing gel hardness. This effect results in increased ionic calcium content, lowered pH and increased micellar calcium content, which is a limiting factor in the ability of milk to coagulate with rennet **(Remeuf *et al.*, 2020) .**

4.4.Size of the micelles

Thus, with regard to the dimensions of the micelles, it has long been accepted that the coagulation time was longer and the firmness of the gel lower when the average diameter of the micelles was small. Recent studies tend to question this relationship and it seems, on the contrary, that small micelles are the cause of a shorter coagulation time and the formation of a firmer gel than large ones **(Remeuf *et al.*, 2020) .**

4.5.Enzyme concentration

The clotting time is inversely proportional to the quantity of enzyme used The following formula allows you to obtain this coagulation time **(Saidi and Touahria, 2021)** :

$$TC = K/ E+Ta$$

TC : Clotting time

K : Inverse of the rate constant.

E : Enzyme concentration.

Ta: Time elapsed between the end of the enzymatic reaction and the moment of coagulation.

Part 2

Practical Part

Chapter 1

Material and methods

This work was carried out at different laboratoriesfor 2 month :

- Educational laboratory of the Faculty of Nature and Life Sciences, University of El Oued;
- Educational laboratory of the university business incubator of El Oued;
- Educational laboratory of chemistry and physics of the exact sciences faculty, University of El Oued;
- Laboratory Center for Research and Physio-Chemical Analysis (CRAPC) inBousmail.

The objective of this work is:

• Valorization of chicken pepsin extract in liquid and freeze-dried form.

• Use of chicken pepsin extract in the coagulation of different types of milk (cow milk, goat milk and even camel milk).

In the following part, we will explain the different methods of the parts announced in the experimental diagram of the study.

First part: Study of the raw material

- Physico-chemical characterization of cow, goat and camel milk
- Characterization of chicken pepsin extract
- Infrared Spectroscopy and X-Ray diffraction analysis
- Scanning electron microscope (SEM) analysis

Second part: Preservation's impact on chicken pepsin extract

A. On coagulant activity
    - Positive temperature (+4 C°)
    - Negative temperature (-20 C°)

B. On coagulant strength

Third part: Manufacture of fresh cheeses and cheese yield

- Manufacture of fresh cheese with liquid chicken pepsin extract
- Manufacture of fresh cheese with freeze-dried chicken pepsin

Fourth part :Characterization of fresh cheese

- Physico-chemical analysis
- Infrared Spectroscopy and X-Ray diffraction analysis
- Environmental scanning electron microscopy (ESEM) analysis

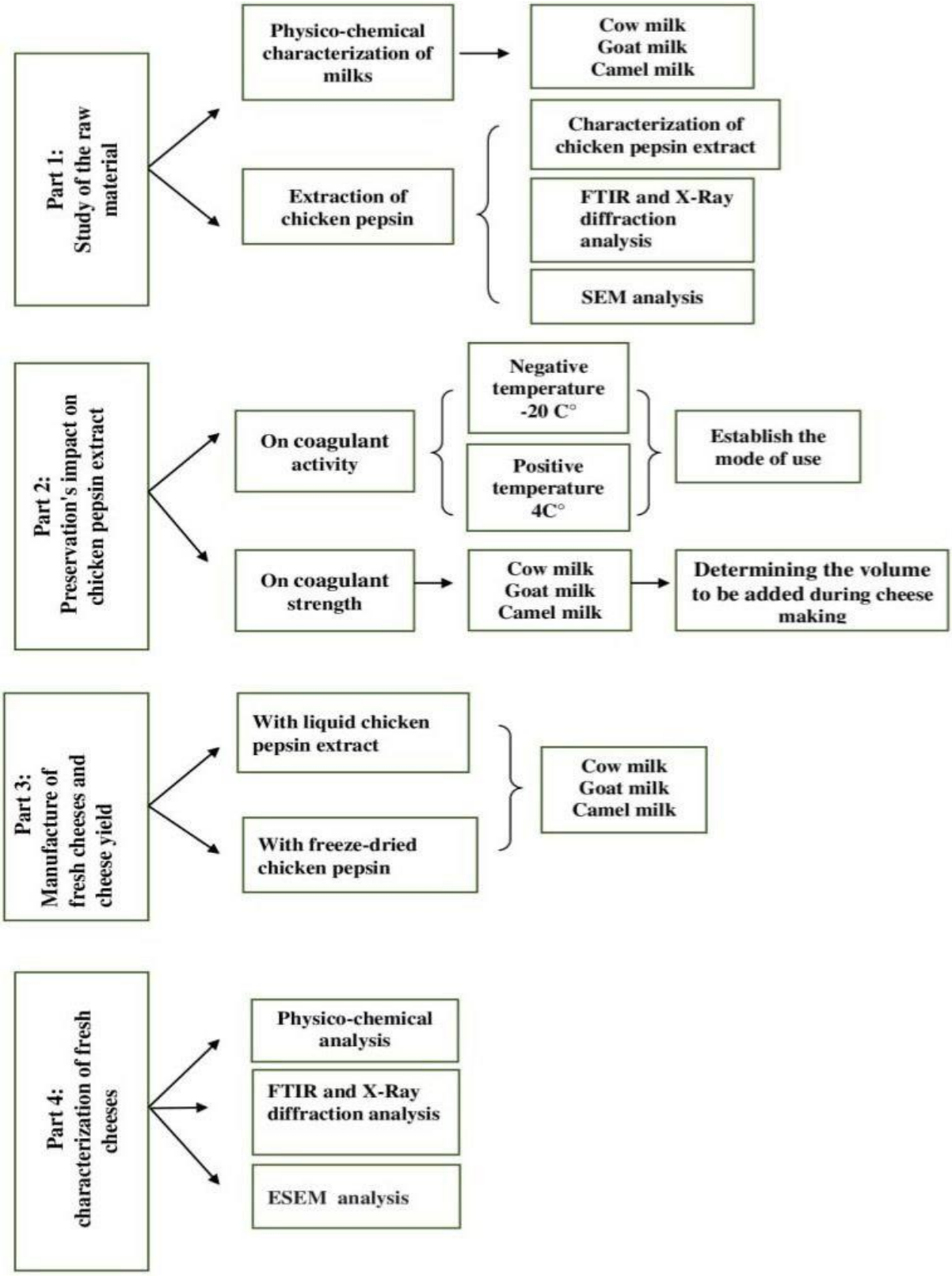


Figure 06: Experimental diagram of the study

1.Material

1.1.Machines

The different machines used during our study are as follows: precision Balance, Balance (KERN) , Milko Scan (Master Pro Touch), pH meter (Crison, C3010), Freezer at -20°, Refrigerator at + 4°C, Muffle furnace,Stirrer (Lab Tech), Centrifuge (SiGmA) ,Meat grinder, ESEM ,SEM ,IR, Incubationoven ,laboratory freeze dryer (Christ Alpha 1-4 LSCbasic), Kjeldah machine and X-ray diffraction.

1.2.Small equipment

A number of suitable small materials were also used in this study. it Consists of: Beakers, erlenmeyer flask, magnetic bars, spatula, gloves, filter cloth,sterileblades,petridish,funnel, sampling boxes, capsule, compresses Sterile, graduated cylindrical test tube without spout , volumetric flask, micropipette (1000 µL), tips micropipette, graduated pipette, Pasteur pipette, spatula, test tubes sterile, test tube, plate,paper tape, test tube rack, sterile bottles, wash bottle.

1.3.Chemicals and reagents

Solvents: Hydrochloric acid (HCL), Glutaraldehyde solution, Ethanol ,Distilled water, Lactic acid

Salts: Sodium bicarbonate (NaHCO3), Sodium chloride (NaCl) , Calcium chloride (CaCl2), Sodium hydroxide (NaOH), Sodium benzoate (E211).

1.4.Biological material

1.4.1.Cow milk

Cow milk was collected from the extensive farms of breeders from different regions of El-Oued (Al-Bayadha and Al-Rabah) under appropriate hygienic conditions.

1.4.2.Goat milk

Goat milk was collected from the Arabia race in extensive breeding from different regions of El-Oued (Al-Rabah, Al-Souihla, Al- Bayadha and Tiksebt) under suitable conditions.

1.4.3.Camel milk

Camel milk was collected from large-scale desert populations. Breeders in the area of Hassani Abdel Karim, Ben Gacha, Ghamra , Al-Bayadha and Guemar (in March and April) in El-Oued, in healthy conditions.

1.4.4.Berridge substrate

Skimmed milk (0% fat) powder (by lovely Milk), it is used as standard substrate in order to measure the flocculation time and the coagulating force according to the BERRIDGE substrate which is chosen for its good cheese-making capacity. This substrate is composed of 12g of

skimmed milk powder dissolved in 100ml of distilled water with 0.147g (0.01M) of CaCl2 in pH 6.4 with gentle stirring for 20 min. Next, the milk is kept for 24 hours at 4°C,to ensure physical and chemical balance .On the other hand, stabilization and rehydration of micelles and avoiding their microbial development **(Benyahia-Krid,2013).**

1.4.5.Preparation of the proventriculus

The proventriculus are recovered from an Al-hajj Al oudini poultry slaughterhouse located in the "HASSANI ABDELKERIM" region of el-oued. After slaughter, the proventriculus are recovered from the digestive tracts from chickens aged between 40 and 50 day , freed of the fat surrounding it then washed. Chicken proventriculus weighing between 8 and 14 g are opened using a knife and rinsed in tap water to remove adhering food particles then drained (Figure 7). They are then refrigerated and transported to the laboratory where they are immediately divided into 100g portions (10 to 13 pieces), then frozen at -20°C until use.

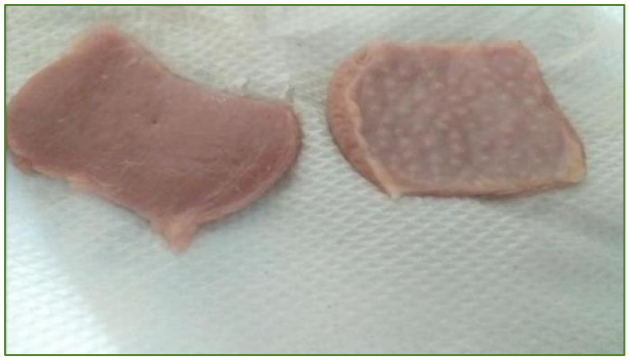


Figure 07 : Chicken proventriculus (internal and external face).

2.Method

In the following part, we will explained the different methods of the parts announced in the experimental diagram of the study.

Part I: Study of the raw material

I.1.Milk collection

The milk is drawn from cows, goats and camels in good health. It is collected neatly in new, clean plastic bottles. These were immediately placed in a cooler containing blocks of ice. Then frozen at -18°C until further use.

I.2.Physico-chemical parameters of milk

Physico-chemical analyzes carried out on the three milk samples (cow,goat,camel).The measured physico-chemical parameters are: pH, titratable acidity, density, total dry matter or total dry extract and protein content. All measurements have been carried out in 3 tests. In order

to determine the physic-chemical parameter of milk, we used a quick analysis by means of a Milkoscan device, the latter being an automatic milk analysis device by spectrometer infrared means, it displays the results on its digital screen.

I.3.Extraction of coagulating enzyme (chicken pepsin)

The extraction of chicken pepsin (CP) is carried out following the extraction protocol proposed by **(Bohak, 1970)**which consists of the steps shown on the diagram presented in (Figure 08).For each extraction, a 100 g portion is thawed at room temperature (20 to 25°C) for 30 min, then chopped in a meat grinder rotating at 5000 rpm for 10 seconds. The minced tissue is left for maceration in a solution of 3% NaCl and 0.7% (w/v) NaHCO3 (w/v) at 25°C for 3 hours with continuous stirring. After maceration, the mixture is filtered through double-layer gauze. The activation of pepsinogen into pepsin is obtained by acidification (Hcl 3N) of the filtrate at pH 2 for 15 min, followed by centrifugation at 3200 rpm for 30 min allowing the elimination of the Mucilage. The extract obtained is adjusted to pH 6.4 with (NaOH 3N), and stored in the refrigerator at 4°C until use.

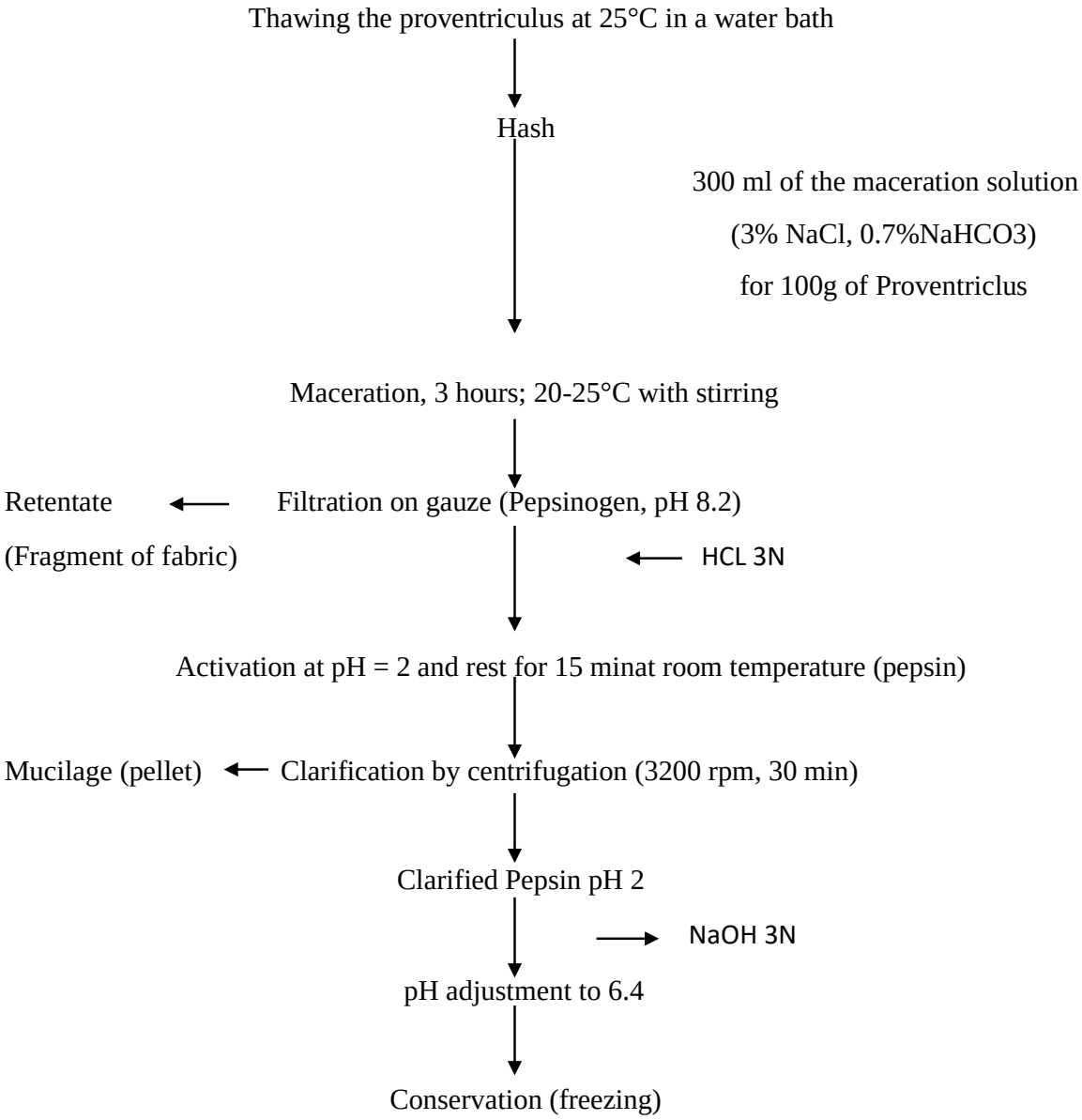


Figure 08: Chicken pepsin extraction diagram according to **(Bohak, 1970).**

(A) **(B)**

Figure 09: Clarified chicken pepsin extract in liquid state **(A)** and in freeze-dried state **(B)**

I.4.Characterization of chicken pepsin extract

I.4.1.Coagulant activity (R.U)

The coagulant activity, determined by the flocculation time, was carried out on skimmed cow's milk, and was measured according to the method of **BERRIDGE (1945)**. The technique consists of adding to milk heated to 30°C, 1mL of the enzymatic extract followed by homogenization (3 inversions), then noting the time which passes until visible casein flakes form on the walls. of the test tube.The coagulant activity unit (U.A.C) or the rennet unit (RU) is defined by the quantity of enzyme contained in 1mL of the enzyme solution which can coagulate 10mL of BERRIDGE substrate (100mL of milk skimmed containing 0.01M CaCl2) in 200 sec at 30° C **(Alais1974; Ramet, 1997; Mahaut *et al*., 2003).**

$$RU = 10.V/T.\ V'$$

RU: Rennet unit

V: Volume of milk

V' Volume of the enzymatic extract

T : Flocculation time

I.4.2.Coagulation time

The setting time is the point at which the first drops of whey appear on the surface of the gel, and the coagulant becomes rigid and does not flow down the walls of the tube **(Alais, 1974).**

Pour a volume of 10 ml of milk into a test tube kept at 30 °C in a water bath, then add it with 1 ml of enzyme solution, and leave the tube until the gel hardens and the first signs appear. Drops of serum on the surface of the gel. The elapsed time represents the setup time.Thus, for a coagulation time of 12 to 15 minutes, the setting time is between 25 and 30 minutes **(FAO, 1990).**

I.4.3.Coagulant strength

The coagulatingstrength (CS), is expressed in Soxhlet units (SU), it represents the number of volumes of fresh milk coagulated by a volume of rennet, in 40 min at 35°C **(Nouani *et al.*, 2009).**

$$CS = (2400 \times V) / (t \times v)$$

CS: Coagulation strength

V: Volume of adjusted milk (ml)

v: Volume of the enzymatic solution (ml)

T: Coagulation time in seconds (s).

I.4.4.Protein content

The Kjeldahl method is the reference method for dosage of total nitrogen for the food domain. It consists in performing a complete mineralization of molecules organic, transforming nitrogen present into ammonia which can be dosed by different techniques **(Guillou *et al.*, 1976).**

Protein content = Total content × coefficient

I.4.5.Dry matter and ash

Dry matter was calculated by drying 3 ml of chicken pepsin extract at 105°C for 24 h, and ash was determined by total incineration

DM%= Sample weight after drying (g) /sample weight before drying (g)*100

Ash%= (ώ - ῳ /ω)*100

ώ = Weight of crucible with ash (g)

ῳ = Weight of crucible (g)

ω = Weight of sample (g)

I.4.6.Specific activity

The specific activity is expressed by the ratio between the coagulant activity of the extract enzymatic and the protein level of this enzymatic extract and expressed in RU/ mg **(Nouani *et al.*, 2009).**

I.5.Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction analysis

1g of FDCP and 1 ml of LCP was used for sample

a.FTIR spectroscopy analysis

FTIR spectra of chicken pepsin were obtained using FT-IR spectrim (Agilent Technologies, Carry 630, USA). chicken pepsin was placed directly on top of the crystal and either pressed down onto or measured without pressing. Spectra were recorded at 20C° from

400 to 450 cm $^{-1}$ at a normal resolution of 8 cm $^{-1}$ with an accumulation of 4 scans for each spectrum. The background spectrum, which contains the absorptions of molecules present in the air, was scanned at the beginning of measurement by pouring the ATR cell with distilled water.

The same procedure was used for scanning blank spectra, which contains the absorption of distilled water. After each measurement, the ATR crystal was thoroughly washed with ethanol and distilled water and then dried. The data detected in the transmission mode. For each experiment, three replicates were rerformed**(Felfoul *et al.*, 2022; Razi *et al.*, 2024).**

b.Diffraction X-ray

The XRD profiles of chicken pepsin were investigated using powder X-Ray Diffractometer (PROTO AXRD, USA). The theta angle was adjusted, ranging from 5° to 50°,while the scanning rate remainde constant at 1 °/s. Operating voltage was 40KV **(Razi *et al.*, 2024).**

I.6.Microstructural analysis (SEM)

The purpose of this analysis was to visualize the microstructure of chicken pepsin enzyme powder by observation under a Phenom ProX electron microscope, operating at 15 kV, magnifier (X14000) for a scale of 158 µm.

Part II: Preservation's impact on chicken pepsin extract

II.1.On coagulant activity

The studies of the effect of the positive and negative temperature of conservation on the coagulant activity aim to establish the technical sheet or the mode of use of our product.

II.1.1.Positive temperature (4 C°)

The pepsin extract was maintained at a positive temperature, and the coagulation efficacy was assessed by adding 1 ml of CPL to 10 ml of Berridge substrate solution. This procedure was continued for one week, after which the coagulation time was measured.

II.1.2Negative temperature (-20 C°)

The pepsin extract was preserved at a negative temperature, and the coagulation efficacy was evaluated by adding 1 ml of CPL to 10 ml of the Berridge substrate solution. This process was carried out for a duration of one month, after which the coagulation time was measured.

II.2.On coagulant strength

Calculating the coagulant strength with different types of milk aims to determine the volume that should be added during cheese making.

1 ml of LCP from was added to a volume of 500 ml of cow, goat and camel milk, and the time required for clotting was calculated according to the above law ($CS = (2400 \times V) / (t \times v)$), at a temperature of 30 °C and (pH = 6.1).

Part III: Manufacture of fresh cheeses and cheese yield

III.1.Manufacture diagram of fresh cheese with enzymatic coagulation

In this part we followed the diagram for manufacturing fresh cheese from CP under the mentioned ideal condition Below:

Table 05: Optimal coagulation conditions of different milks with CP (liquid or freeze-dried)

Type of milk	Optimal coagulation conditions		References
	pH	Temperature	
Cow milk	5.82	42°C	**(Bouras *et al*., 2022)**
Goat milk	6.01	42°C	**(Bouras, 2023)**
Camel milk	5.00	42°C	

An experimental diagram summarizing the procedure for making fresh cheese from cow milk, goat milk, or camel milk, coagulated with CP and either liquid (LCP) or freeze-dried (FDCP), is shown in (Figure 10).

The freshness of the milk was confirmed by measuring the pH of each milk.

Next, the optimum pH was adjusted using lactic acid according to (Table 05), then CaCl2 (0.01%-0.015%) was added and each milk was allowed to settle for 30 minutes. The mixed milk was heated to the optimum coagulation temperature (see Table 05). Next, salting out (6 g/L) was performed. At these optimal parameters (temperature, pH), and based on the coagulation strength of (CP), the appropriate volume of it was added to each type of milk. After coagulation, which takes a maximum of 45 minutes, the cheese is automatically dried and then formed.

All cheeses made with chicken pepsin (LCP) or (FDCP) are made in clean food trays It is stored in the refrigerator at a temperature of 4°C.

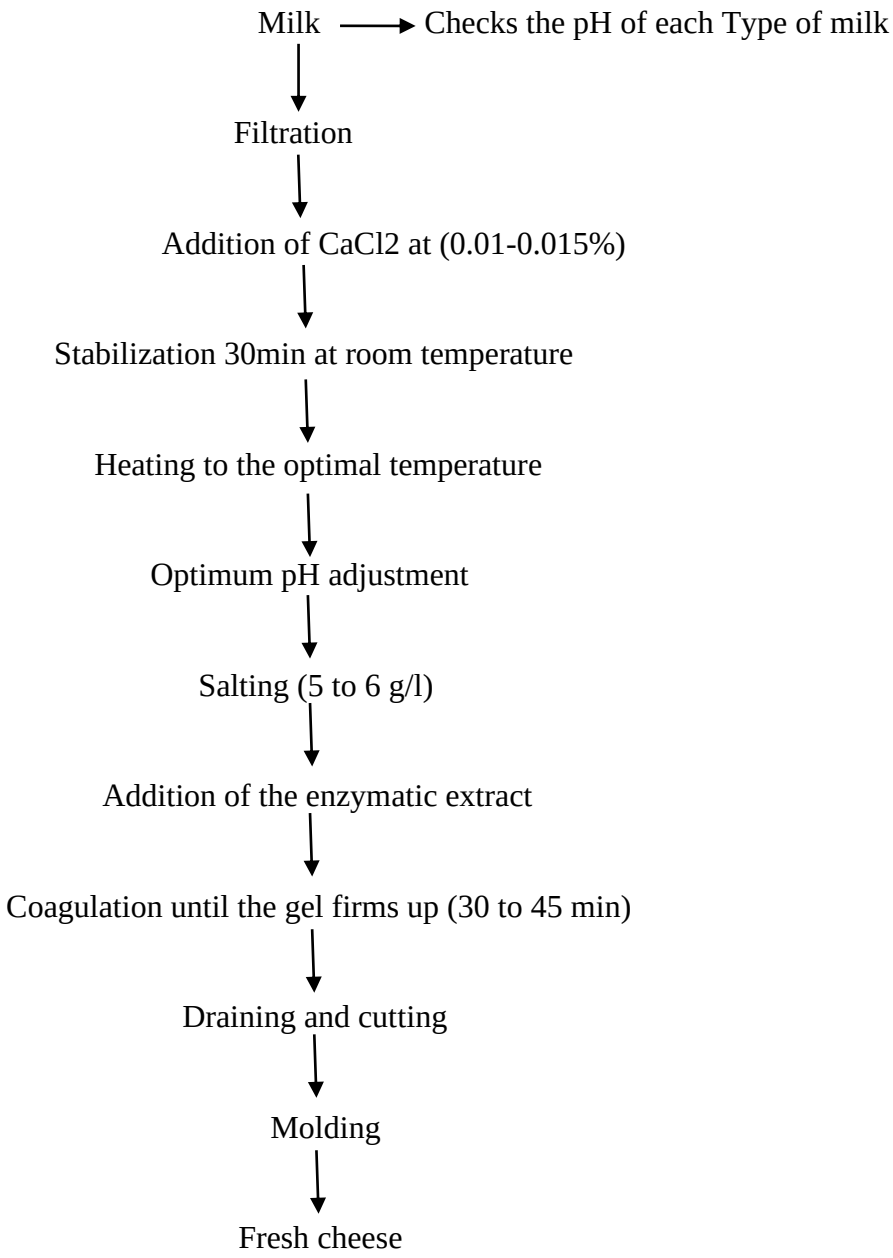


Figure 10: Diagram adopted for fresh cheese-making

III.2.Study of cheese yield according to the nature of pepsin extract (liquid and freeze-dried)

Cheese yield is an important parameter in cheese manufacturing which gives an idea of the value of the coagulating enzyme and the profitability of production.

Cheese yield is calculated by the following rule:

Yield = weight of cheese (g) / volume of milk (ml)

Part IV: Characterization of fresh cheese

The fresh cheeses are characterized based on raw composition, FTIR and X-ray diffraction measurement, microstructural analysis under ESEM.

IV.1.Raw composition

The physicochemical composition (protein, dry matter, fat and ash) and pH of cheese stored at 4°C for a maximum of 24 h were determined. Methodological criteria for analysis were followed as described by **(Bradley *et al.,* 1993).** The pH of the cheese was measured by a digital pH meter and total nitrogen was determined by **the Kjeldahl method**. Dry matter was calculated by drying 2 grams of fresh cheese at 103°C ± 2°C for 3 hours, fat was measured by **the van Gulik method** and ash was determined by total incineration. The mineral composition of goat and camel cheese can be measured using flame emission. Spectrometer, optical emission spectrometer and atomic absorption spectrometer. These methods measure the concentration of various minerals in cheese such as calcium, magnesium, potassium, sodium, iron, zinc and copper**.**

IV.2.FTIR spectroscopy analysis

FTIR spectra of fresh cheeses were obtained using FT-IR spectrim (Agilent Technologies, Carry 630, USA). A slice of each type of cheese was placed directly on top of the crystal and either pressed down onto or measured without pressing. Spectra were recorded at 20C° from 400 to 450 cm $^{-1}$ at a normal resolution of 8 cm $^{-1}$ with an accumulation of 4 scans for each spectrum. The background spectrum, which contains the absorptions of molecules present in the air, was scanned at the beginning of measurement by pouring the ATR cell with distilled water.

The same procedure was used for scanning blank spectra, which contains the absorption of distilled water. After each measurement, the ATR crystal was thoroughly washed with ethanol and distilled water and then dried. The data detected in the transmission mode. For each experiment, three replicates were rerformed **(Felfoul *et al.*, 2022; Razi *et al.*, 2024).**

IV.3.X-Ray Diffraction analysis

The XRD profiles of fresh cheeses were investigated using powder X-Ray Diffractometer (PROTO AXRD, USA). The theta angle was adjusted ranging from 5° to 50°, while the scanning rate remained constant at 1°/s. Operating voltage was 40 KV **(Razi *et al.*, 2024)**

IV.4.Environmental scanning electron microscope (ESEM) analysis

The purpose of this analysis was to visualize the microstructure of enzymatic gels based on cow milk, milk goat and camel milk by observing under an environmental scanning electron

microscope (ESEM – FEI QUANTA 250) operating under a large coil detector (LFD) and low vacuum. With an acceleration voltage of 10.00 kV.

First, a small piece of each fresh cheese, freshly curdled, is cut into fine pieces (0.5 cm long and 0.5 cm wide) and air dried for 4 to 5 hours. Then dried in an atmosphere saturated with glutaraldehyde overnight (12 hours). Each piece of cheese was then fixed with a series of ethyl alcohol from 10° to 100° for 5 minutes with the solution. Next, the cheese pieces were dried in the open air for a few hours before proceeding to observation **(Attia *et al.*, 1991).**

Chapter 2

Results and discussion

The objective of this work is to valorizethe chicken pepsin extract in liquid and freeze-dried form, and use it in the coagulation of different types of milk (cow milk, goat milk, and even camel milk).In the next part, we will discuss the results obtained after various applied studies of the parts announced in the experimental plan of the study.

Part I: Study of the raw material

I.1.Physicochemical characteristics of different type of milk

Table 06 presents the different physicochemical parameters of milks.

Table 06: Physicochemical characteristics of milks

Parameters	CM	GM	DM
pH	6.90±0.15^{a}	6.50±0.3^{a}	6.40±0.2^{a}
Specific gravity at 20°C (g/cm^3)	1.035±0.00^{a}	1.028±0.00^{a}	1.030±0.00^{a}
Total solids (TS)(g/L)	191±0.00^{a}	139±0.00^{b}	115.4±0.00^{c}
Proteins (g/L)	37±0.00^{a}	34±0.00^{b}	34±0.00^{c}
Fat (g/L)	17±0.00^{a}	46±0.00^{b}	23±0.00^{c}
Lactose (g/L)	56±0.00^{a}	51±0.00^{b}	51±0.00^{c}
Ashes (g/L)	8±0.00^{a}	8±0.00^{a}	8±0.00^{a}

a, b and c : Lettres indiquent la différence significative des moyennes dans la même ligne (P <0.05) (test ANOVA :test de Tukey).**CM:** Cow milk.**GM:** Goat milk**. DM:**Camel milk.

Statistical analysis shows that there is no significant difference in pH, specific gravity and ashes between the milks. Regarding dry matter and their components like proteins, fat and lactose; there is a significant difference between cow milk, goat milk and camel milk.

First, we recorded that the pH values of CM, GM and DM were overall lower than 7. Compared to the bibliography, the pH value of GM was within the recommended range of **FAO (1990)** that is between 6.45 and 6.70. For DM, our result was slightly lower than that reported by **Boura*et al.*, (2023)**and **Hadef*et al.*, (2021)**which was respectively 6.56 and 6.50**.**

In addition, for specific gravity, we did not report any dilution effect of the milks and the results were within the norm. For GM, **FAO (1990)** recommended a range between 1.027 and 1.035. Further more, our result was in agreement with **Al haj *et al.*, (2010)** who reported values between 1.026 and 1.035. The specific gravity depends directly on dry matter content and is strongly linked to irrigation frequency and animal's diet **Saidi and Touahria, (2021).**

Regarding TS, the values of CM, GM and DM were different with the higher value recorded for GM with 139±0.00 (g/L). In the literature, **Ismaili *et al.*, (2019)** reported a value of

104.2 g/L for DM and **FAO (1990)** recommended a range between 136 and 140 g/L for GM.Total solids contentvaries in milk according to the stage of lactation**Debouz*et al.*,(2014).**

For protein content, the highest value was recorded for CM with 37±0.00 g/L.Our results are in agreement with the results **of Attia*et al.*,(2000)** and **Konusbaeva*et al.*, (2009)**, which vary from 35 to 45 g/Lfor DM. However, our result was relatively higher than that reported by **Debouz*et al.*,(2014)** (28.1 g/L) and close to that reported by **FAO (1995)** (37 g/L) for GM. The proteins content varies depending on the season, the stage of lactation and the number of births **Debouz*etal.*,(2014).**

As for the fat content, the value recorded for GM (46±0.00 g/L) was similar to those given by **Bouras (2023)** and was significantly higher than for CM (17±0.00 g/L), where the fat content of milk was lower compared to the Algerian standard which should be 34 g/L**Meskiniet *al.*,(2023)**. For DM, our value was lower than that obtained by **Hadef*et al.*,(2021)**which was 30.95 g/L. The low-fat content observed in the present study can be attributed to the camel feeding in the study area. The fatty acid composition of DMis influenced by environmental factors such as: nutrition and physiological factors, including: lactation stage as well as differences in animal breed **Konosbayeva *et al.*,(2011)**

In terms of lactose content, CM presented the highest value with 56±0.00 g/L. For comparison, the lactose content of GM was higher than that reported by**Dhartiben*et al.*, (2016)** and FAO (2013) whose values were respectively41.6 g/L and 44 g/L. DM alsocontained a high value of lactosecompared to that presented by **Young *et al.*,(2017)**whichwas 45.6 g/L. In the literature, the lactose content of DMappears to depend not only on the breed but also on the stage of lactation and hydration status. It is low during the first hours after birth and undergoes an increase of 36% of the initial content, after 24 hours. A 37% reduction of the initial content was observed in dry camel conditions **Chethouna(2011).** These changes in lactose content are the origin of differences in the flavor of DM, and are responsible for the sweet and sometimes bitter taste of DM**Senoussi(2011).**

Our results showed a similarity in ash content inCM, GMand DM, with8±0.00 g/L; which was higher to those reported by**Houssou*et al.*,(2023)** for GM.while for DMour resultwas close to those reported by**Medjour*et al.*,(2023);**which was 8.05±0.07 g/L. It is worth mentioning that ash content, which represents the mineral content of DM, can be affected by factors such as water shortage **Yagil (1984)**. It also varies according to the lactation stage**Siboukeur(2007).**

I.2. Characterization of chicken pepsin extract (CP)

Table 07 presents the values of the different characteristic's parameters of chicken pepsin extract.

Table 07: Different characteristics parameters of chicken pepsin extract

Parameters		CP
Coagulant activity (RU.mL^{-1})		4.95±0.49
Coagulant strength (SU)		1/ 2040.81
Protein content (mg/mL)		18±0.1
Specific activity (RU.mg)		0.26±0.03
Dry matter (%)		15± 0.01
Ash (%)		3.4± 0.01
pH		6.4±0.00
Coagulation time(s)	**CM**	31.33±3.51
	GM	35.7±3.05
	DM	79.7±3.05

Chicken pepsin, extracted according to the protocol of **Bohak (1970)**, (100 g of chicken proventriculus soaked in 300 ml of saline solution (30 g/L NaCl and 7 g/L NaHCO3)). It is a yellowish liquid with a pH of 6.4.

The results of coagulation activity were recorded and expressed in the number of rennet units (RU), as shown in table 7, which is 4.95±0.49 RU.mL^{-1}; This is less than what was mentioned by **Aïssaoui-Zitoun*et al.*, (2017)** which is 10.2 ± 1.10 RU.mL^{-1}, and higher than that reported by **Bouras (2023)** (1.98 ± 0.02 RU.mL-1).

The coagulating strength of chicken pepsin extract is 1/2040.81(SU). Our result is lower than that estimated by **Saidi and Touahria (2021)**, **Bouras *et al.*, (2022)** and **Chelbi *et al.*, (2022)**; these authors reported the following values respectively (1/2192.60SU, 1/6153.85 SU, 1/3000 SU). The coagulant strength differs depending on the age of the chicken, the type of food, other soaking solutions and extraction conditions (**Amimour, 2019)**.

The protein content is 18±0.1 mg/mL; which is in agreement with **Saidi and Touahria (2021)** (18±0.00 mg/mL); it is higher than those reported by **Adoui (2007)** and **Aïssaoui-Zitoun*et al.*, (2017)** (8.77±0.41 mg/mL, 3.20±0.60 mg/mL respectively) and lower than that reported by **Bouras (2023)** (20±0.00 mg/ml).

Dry matter of chicken pepsin extract is 15 ± 0.01, and ash is 3.4 ± 0.01.

The specific activity is 0.26 RU/mg as shown in table 7. Our value is close to those estimated by **Saidi and Touahria (2021)**, which amount to 0.196 RU/mg. The specific activity is much higher than **Bouras (2023)** and **Chelbi *et al.*, (2022)** which reported 0.1RU/mg.

Finally, regarding the coagulation time, it is important to point out that the chicken pepsin extract reacts better and respectively with cow milk, goat milk and camel milk. So, we can say that the chicken pepsin extract coagulates cow milk faster in a time of 31.33±3.51s compared to other milks.

I.3.FTIR spectroscopy analysis of CP

Figure 11 presents the FTIR spectra of LCP and FDCP

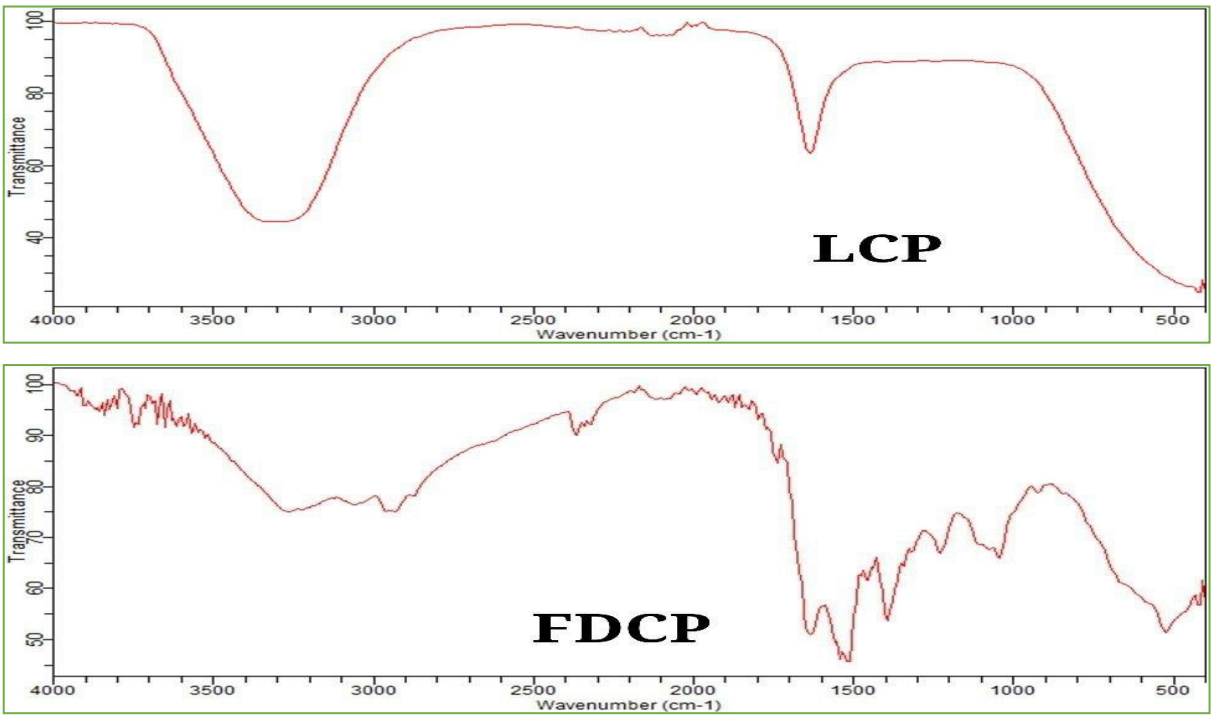


Figure 11: FTIR spectra of LCP and FDCP

FTIR analysis gave us an idea about the general structure of CP. In LCP, a very broad wave representing OH can be observed at 3200 to 3600 cm-1, and at 1600 to 1800 cm-1, the letter O=C appears. In FDCP, a broad wave representing OH can be observed at 3200 to 3600 cm-1 and an intermediate wave representing C=O at 1500 to 1800 cm-1. We can also detect an intermediate wave extending from 1300 to 1450 cm-1 representing the aliphatic bending group CH. In detail, the spectrum derived from LCP and FDCP shows distinct bands as follows:

At 3200 to 3600 cm-1, which represent the O-H stretching in hydroxyl groups. The different broad bands can be attributed to the presence of water in LCP. Where it absorbs water in the region of 3000 to 3600 cm-1 and with higher strength than 1650 cm-1. Moisture affected the N-H multiple bonds (amide I and amide II) in the regions of 2500 to 3500 cm-1 due to free water. At 1600 to 1800 cm-1, C=O for acids and esters is shown. In FDCP, at 1390 to 1700 cm-1, for amide I (C-O) and at 1700 to 1750 cm-1, for amide II (N-H) and thus binds to proteins. Concerning the shelf life of CP, we can say that the large OH bond is observed in LCP, which means that it has a shorter shelf life compared to FDCP.

I.4. X-ray diffraction analysis of FDCP

This technique allows both to demonstrate the crystalline or amorphous nature of a solid or apowder, as well as to determine the different crystalline phases.

Figure 12 presents the X-ray diffraction spectra of FDCP.

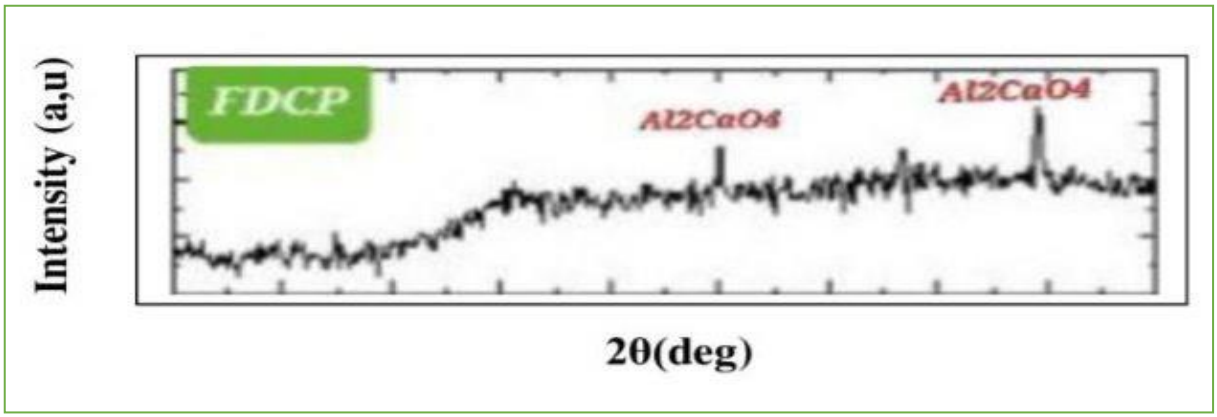
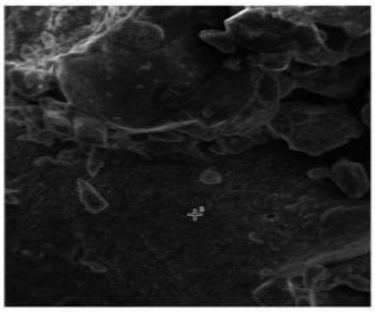


Figure 12: X-ray diffraction spectra of FDCP

Figure 12 shows the typical XRD pattern of FDCP diffraction peaks. A small peak appears at 2θ=30°, 38° and at 2θ=47°, 44.60°. The position of the diffraction peak is consistent with the standard card (JCPDS card No. 98--017-1762) Compound name: Calcium oxide gamma-alumina Chemical formula: AL2CaO4 (See the scientific card in the appendix).

I.5. SEM analysis of FDCP

Figure 13 presents the microstructure of FDCP



Spot 1

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	41.17	31.12
8	O	Oxygen	23.65	23.81
7	N	Nitrogen	22.70	20.01
11	Na	Sodium	7.33	10.61
17	Cl	Chlorine	4.45	9.92
45	Rh	Rhodium	0.70	4.52

Spot 2

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	42.00	34.57
8	O	Oxygen	16.48	18.08
7	N	Nitrogen	16.96	16.28
5	B	Boron	15.30	11.34
11	Na	Sodium	5.54	8.72
17	Cl	Chlorine	3.30	8.02
45	Rh	Rhodium	0.42	2.99

Spot 3

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	41.17	31.12
8	O	Oxygen	23.65	23.81
7	N	Nitrogen	22.70	20.01
11	Na	Sodium	7.33	10.61
17	Cl	Chlorine	4.45	9.92
45	Rh	Rhodium	0.70	4.52

Figure13: Micrograph of FDCP.

Figure 13 shows the different chemical elements that correspond to the micrograph presented. A part from the presence of carbon and oxygen, it is important to point out the presence of nitrogen; this element is related to chicken proteins or pepsin. In addition, sodium and chlorine were used as solutions to regulate the acidity of the extract. Finally, Boron and Rhodium were probably derived from the chicken proventriculus.

Part II: Preservation's impact on chicken pepsin extract

II.1. On coagulant activity

Figure 14 presents curve of the coagulant activity (CA) of chicken pepsin extract and preserved at positive (+4°C) and negative temperature (-20°C).

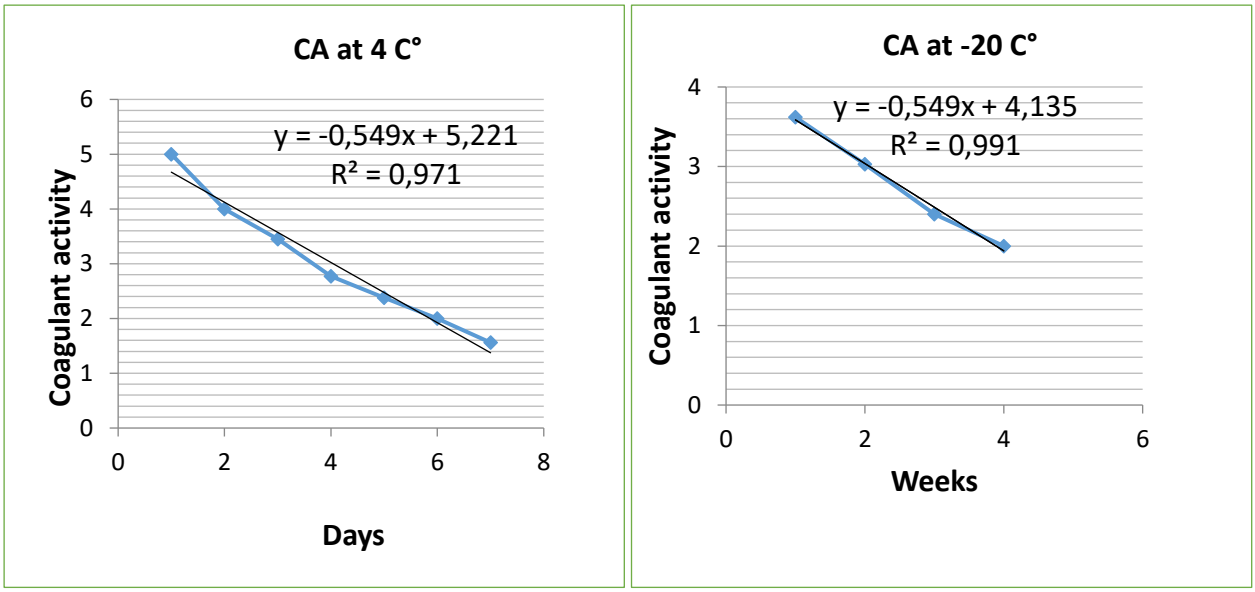


Figure14: Curve of the coagulant activity (CA) of chicken pepsin extract and preserved at positive **(A)**and negative temperature**(B)**

According to the coagulant activity test conducted on CP after storing it in the refrigerator for a week at 4° C and in the freezer for a month at -20°C, we noticed that:

At positive temperature, on the first day the coagulation activity value was 5 ($RU.mL^{-1}$) then gradually decreased over the days until it reached 1.56 ($RU.mL^{-1}$) on the seventh day.

At negative temperature, we noticed that its value at the end of the first week after dissolving the CP extract reached 3.62 ($RU.\ mL^{-1}$) then its value decreased until it reached 2 ($RU.\ mL^{-1}$) at the end of the fourth week, i.e. the last day of the month after extracting the CP.

Based on these results, we can conclude the following:

- ✓ The use of liquid chicken pepsin extract should not exceed one week at +4°C temperature for milk coagulation;
- ✓ The use of frozen chicken pepsin extract at -20°C should not exceed one month for milk coagulation;
- ✓ Refrigeration and freezing of chicken pepsin extract mainly affects the time of milk coagulation.

II.2.On coagulant strength

The coagulant strength was studied with chicken pepsin extract stored only at +4°C.

Table 08 presents the values of the coagulant strength of CP with different type of milk.

Table 08: The values of the coagulant strength of CP with different type of milk

Type of milk	Coagulant strength (SU)
CM	1/ 1966.56
GM	1/ 1307.19
DM	1/ 594.001

CM: Cow milk.**GM:** Goat milk. **DM:**Camel milk.

The coagulant strength gave us an idea about the recommended volume of chicken pepsin extract for milk coagulation. In addition, it is important to note that the value of the coagulant strength can vary with the quality of the milk.

- ✓ The coagulant strength of LCP with cow milk was 1/1966.56 (SU), which indicates that the volume of LCP required to be added to coagulate 1 liter of milk is 51 µl. ;
- ✓ The coagulant strength of LCP with goat milk was1/1307.19 (SU), which indicates that the volume of LCP is 77 µl to coagulate 1 liter of milk ;
- ✓ The coagulant strength of LCP with camel milk1/594.001 (SU), which indicates that the volume of LCP is169.5 µl to coagulate 1 liter of milk.

From these results, we can conclude that the recommended volume for milk coagulation is closely related with the type of milk. In this sense, camel milk needs the highest volume of chicken pepsin extract, followed by goat milk and cow milk.

Part III: Manufacture of fresh cheeses and cheese yield

Table 09 presents the volume of LCP and the amount of FDCP added to each type of milk, and their cheese yield.

Table 09: Volume of LCP and amountof FDCP added to each type of milk, and their Chees

Type of milk	LCP(mL)	Cheese yield(%)	FDCP(g)	Cheese yield(%)
CM	0.5	16.5	0.1	18.9
GM	1	12	0.2	17.12
DM	3	9.2	0.3	16.8

CM: Cow milk.**GM:** Goatmilk. **DM:**Camel milk.

Based on table 09, it is important to note that in practice, the coagulation of milks required additional volumes of LCP compared to those mentioned in the study of coagulant strength:

-For cow milk: 0.5mL of LCP instead of 51 µl;

-For goat milk: 1 mL of LCP instead of 77 µl;

-For camel milk: 3 mL of LCP instead of 169.5 µl.

Based on these results, we can conclude that the use of LCP extract may pose a dilution problem during the coagulation process and that the absence of preservatives may also affect the activity of LCP over time. For this reason, we recommend the use of CP under freeze-drying conditions, especially on an industrial scale.

Regarding the cheese yields obtained, we recorded the highest value (18.9%) with CM coagulated with FDCP. Moreover, the cheese yields of milk coagulated with FDCP were higher compared to LCP. According to **BojanicRasovic *et al.* (2013)**, fat and protein of milk are the main components of cheese, and their percentage in milk is of particular importance for the cheese yield and quality of the cheese.**Fox *et al.* (2017)** reported that cheese production is affected by many factors. The most important of these factors are the composition and quality of raw milk, milk handling and storage practices, and primary milk treatments (e.g., standardization of protein to fat ratio and protein content, homogenization, and pasteurization temperature).

Figure 15 shows the appearance of fresh cheeses made from different types of milk and coagulated with LCP and FDCP.

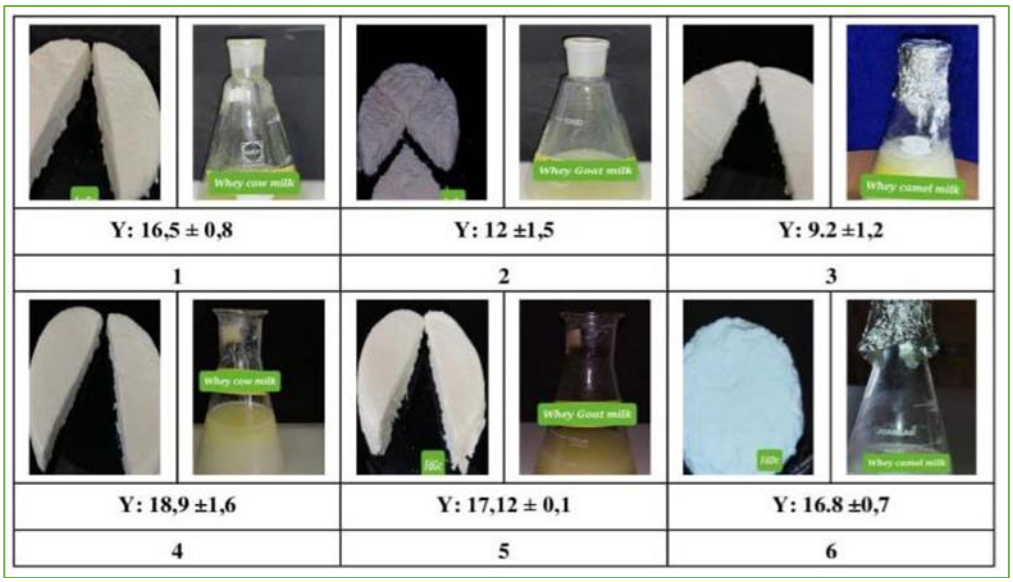


Figure15: Fresh cheeses coagulated with LCP andFDCP

Part IV: Characterization of fresh cheeses

Figure 16 shows the final appearance of fresh cheeses made from different types of milk.

A: LPCC (Cow cheese made with liquid pepsin), B: FDCC(Cow cheese made with freeze-dried),C: LPGC (Goat cheese made with liquid pepsin), D: FDGC (Goat cheese made with freeze-dried),E: LPDC (Camel cheese made with liquid pepsin), F: FDDC (Camel cheese made with freeze-dried).

Figure 16: Finished fresh cheeses with different types of milk

IV.1.Raw composition

Table 10 and 11 present respectively the values of the different physicochemical characteristics of fresh cheeses coagulated with LCP and FDCP

Table 10: Raw composition of finished fresh cheese coagulated with LCP (per 100g)

Parametres	LPCC	LPGC	LPDC
pH	5,75±0.1[a]	6.07± 0.01[b]	5.68± 0.03[c]
Total solids (TS%)	44.4±0.8[a]	50±1.1[a]	41,8± 0.5[c]
Pr/Ts (%)	63.06±1.5[a]	62±0.63[a]	62.8±2.6[a]
Fat/TS (%)	27.2±0.37[a]	32.3±1.0[b]	30.1±0.93[c]
MFFB (%)	63.15	57.64	65.11
Ash (%)	3.75±0.4[a]	5.93±0.02[b]	4.67±0.1[c]

a, b and c : Letters indicate significant difference of means within the same row (P<0.05) (ANOVA test: Tukey test), MFFB:moisture content on a fat free basis.

Statistical analysis showed a significant difference (P<0.05) between pH, dry matter, fat content and ash of the different fresh cheeses presented.

Regarding the pH, the results showed that the pH of the fresh cheeses vary between 5.68 and 6.07, where the lowest pH was recorded in camel cheese. For total solids, all the cheeses produced seem to have a considerable TS content; the minimum value is recorded with camel cheese, which is 41.4±0.5%. On the other hand, the highest value for Pr/TS ratio was in LPCC, which was 63.06±1.5%, and the lowest Fat/TS ratio was 27.2±0.37%. However, based on MFFB and fat/TS recommended by *Codex alimentarius* (1978), we can classify LPCC, LPGC and LPDC as firm and semi-fat cheeses. The ash content varies significantly depending on the fresh cheese. The maximum mineral value is recorded for LPGC with 5.93±0.02%.

In terms of literature, the coagulation of GM using chicken pepsin was studied by **Ait Amer Meziane (2008)**. The evaluation of this enzyme in CM was low. As for the mixture of GM and CM , **Aissaoui *et al.*, (2018), Saidi *et al.*, (2021), Chelbi *et al.*, (2022)** and **Bouras (2023)** reported that chicken pepsin seems encouraging in the transformation or coagulation of CM products giving fairly typical cheeses**.**

From these results, we can conclude that LCP reacts best with cow milk, goat milk and camel milk last with good retention of total solids and protein content.

Table 11: Raw composition of finished fresh cheeses coagulated with FDCP (per 100g)

Parametres	FDCC	FDGC	FDDC
pH	5.86± 0.1[a]	6,06± 0.1[a,b]	5.83± 0.03[b]
Total solids (TS%)	48,3± 1.0[a]	53,3± 1.2[b]	46± 0.5[c]
Pr/TS(%)	60.4±0.9[a]	63.78±0.5[a]	65.21±1.6[a]
Fat/TS (%)	28.98±0.8[a]	23.76±0.3[b]	22.56±1.9[b]
MFFB (%)	60.11	51.88	59.34
Ash (%)	4.06± 0.01[a]	8.75± 0.4[b]	7.85± 0.2[c]

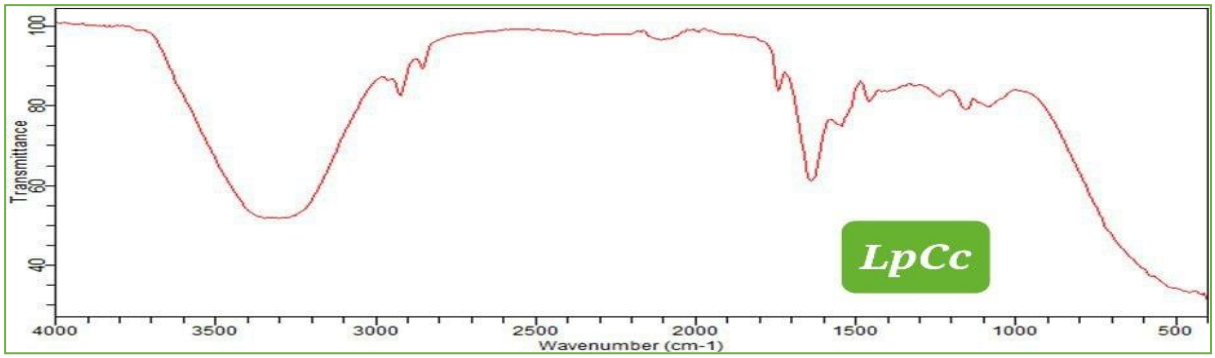
a, b and c : Letters indicate significant difference of means within the same row (P<0.05) (ANOVA test: Tukey test), MFFB:moisture content on a fat free basis.

According to table 11, statistical analysis showed a significant difference (P<0.05) between pH, dry matter, fat content and ash of the different fresh cheeses presented.

Regarding pH, the results showed that the pH of fresh cheeses made with FDCP ranged between [5.83 and 6.06], with the lowest pH recorded in FDDC cheese. As for total solids, the TS of fresh cheeses samples ranged between 46±0.5 % to 53.3±1.2% and the highest value was recorded in FDGC which also contained the highest Pr/Ts ratio value of 63.78±0.5%.Based on MFFB, we can classify FDCC and FDDC as firm cheeses and FDGC as hard cheese. For fat/TS, FDGC and FDDC are classified as partially skimmed cheeses and FDCC as semi-fat cheese. Regarding ash content, there seems to be a significant difference between fresh cheeses made with FDCP with the lowest FDCC value of 4.06%.

IV.2.FTIR spectroscopy analysis

Figure 17 presents the FTIR spectra of fresh cheeses made with different types of milk, coagulated with LCP, and freeze-dried FDCP.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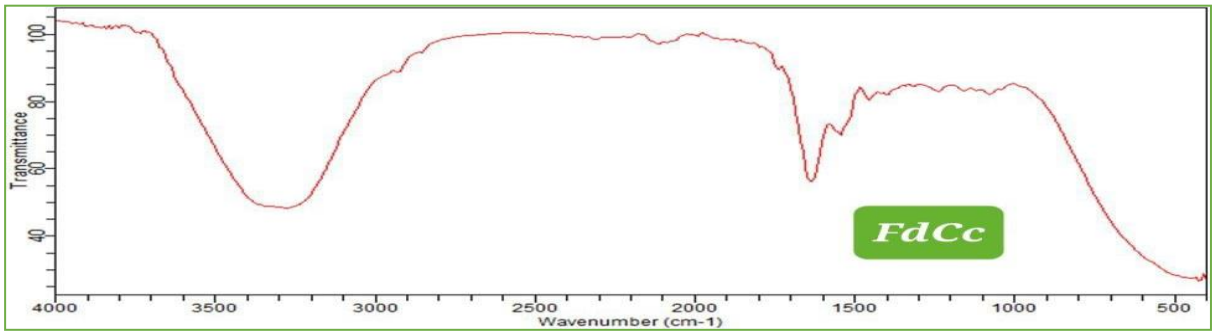
LpCc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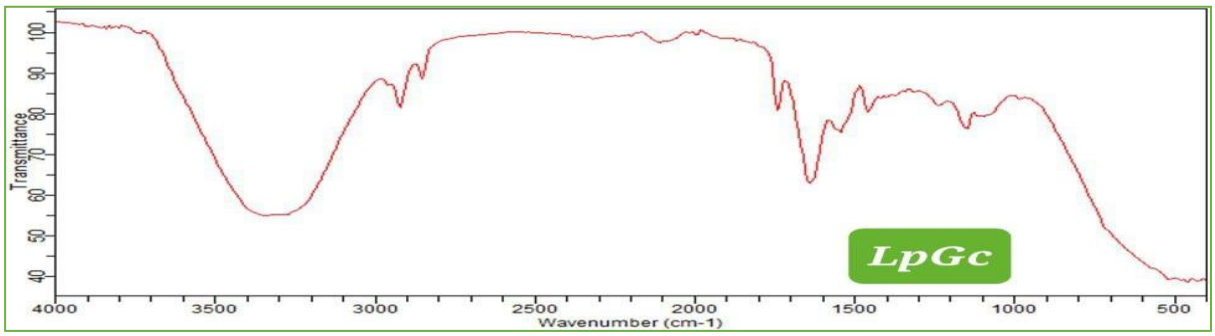
Wavenumber (cm-1)
FdCc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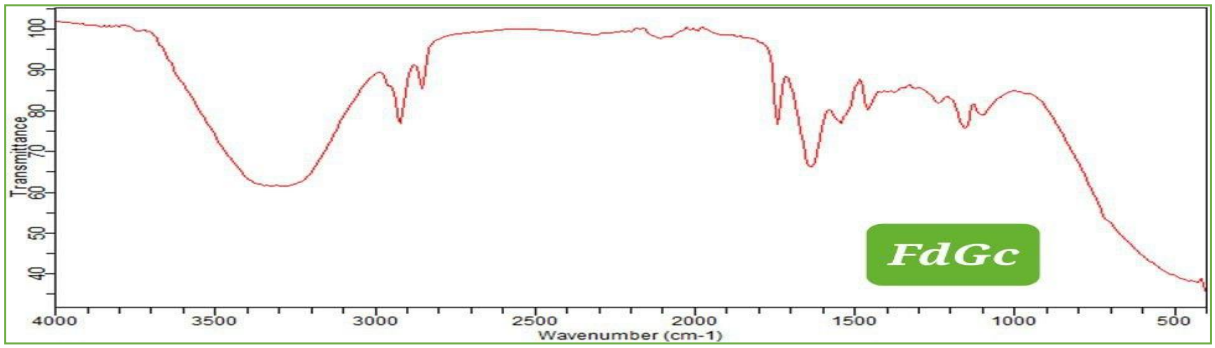
Wavenumber (cm-1)
LpGc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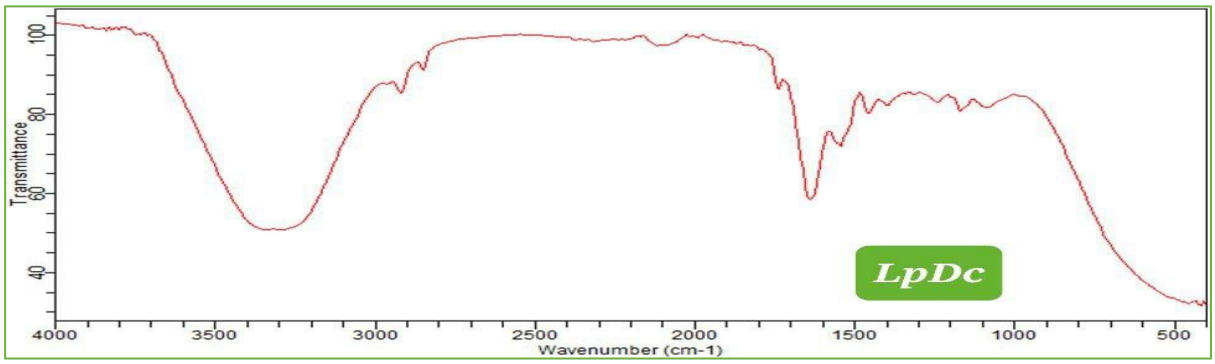
Wavenumber (cm-1)
FdGc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Wavenumber (cm-1)
LpDc

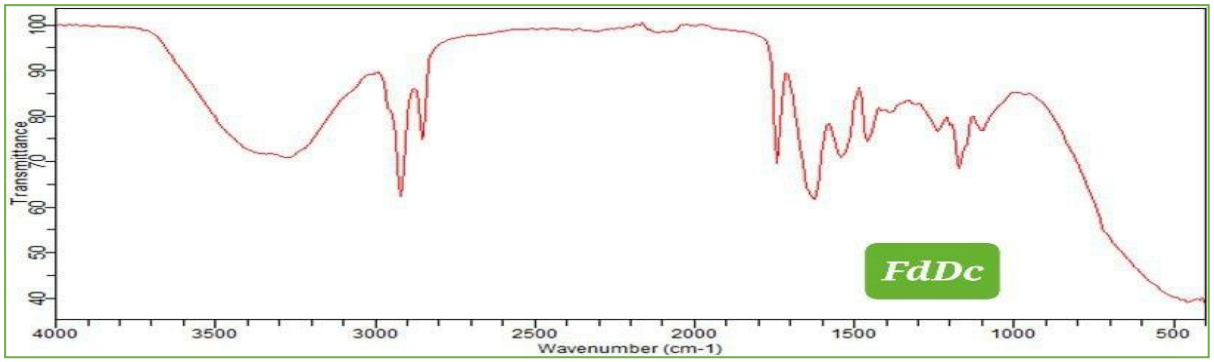


Figure 17: FTIR spectra of finished fresh cheeses

In fresh cheeses made from liquid chicken pepsin (LCP) and freeze-dried chicken pepsin (FDCP), OH bending can be observed in the region from 3000 to 3700cm-¹. At 2800 to 3100 cm-¹, two waves appear, one strong expressing CH. At 1700 to 1800 cm-¹, a wave expressing O = C appears. At 1100 to 1300 cm-¹, an intermediate C-O wave appears.

In LPCC and FDCC, at 3100 to 3700 cm-¹ we observed a broad wave expressing OH. At 1600 to 1700 cm-¹, the appearance of an intermediate wave expressing C=O could be detected.

In LPGC and FDGC, at 3000 to 3700 cm-¹ a strong broad wave expressing OH appears. At 2900 to 3100 cm-¹ a weak wave expressing CH is observed. At 1600 to 1700 cm-¹ a wave expressing C=O can be detected.

In LPDC and FDCC, at 3200 to 3700 cm-¹ a broad wave representing OH appears. At 2900 to 3100 cm-¹ a strong wave representing CH is observed. At 1600 to 1800 cm-¹ an intermediate wave representing C=O can be detected. At 1100 to 1300 cm-¹ an intermediate wave representing C-O appears.

In detail, the spectrum derived from cheeses made with LCP and FDCP shows characteristic bands as follows:

3700–3200 cm-1, indicating the -OH and -NH stretching in proteins; 3100–2800 $cm^{-1,}$ corresponding to the -CH olefin stretching due to unsaturated fatty raw materials; 1750–1650 cm-1, reflecting -C=O in fatty acids and esters; and 1650–1450 cm-1, for amide I (C-O) and amide II (N-H) and thus associated with proteins; and 1000–1200 cm-1, indicating the -C-O-C of polysaccharides; at 1022 and 1092 cm-1, corresponding to lactose

According to **Spina *et al.* (2024),** the specific absorption regions in the mid-infrared spectrum provide us with information about the composition of milk as reported by **Tarapoulouzi *et al.*(2024)** that the vibrations result from different compositions within the

peptide bonds and secondary structure of casein protein, and these results agree favorably with our results.

According to FTIR spectra, the cheeses prepared from chicken pepsin had a high water content, and the spectra of camel cheese were characterized by a strong C-H bond that indicates unsaturated fatty raw materials, because the amount of fat was less compared to other cheese.

In terms of references, FTIR analysis is another powerful analytical technique used to evaluate the authenticity and quality of cheese and provide valuable information for both research and industrial applications **(Tarapoulouzi *et al.*, 2024).**

Finally, FTIR analysis supported chemical analysis of cheeses made with LCP and FDCP.

IV.3.X-ray diffraction analysis

This technique allows both to demonstrate the crystalline or amorphous nature of a solid or a powder, as well as to determine the different crystalline phases.

Figure 18 presents the X-ray diffraction spectra of fresh cheeses made with different types of milk, coagulated with LCP, and freeze-dried FDCP.

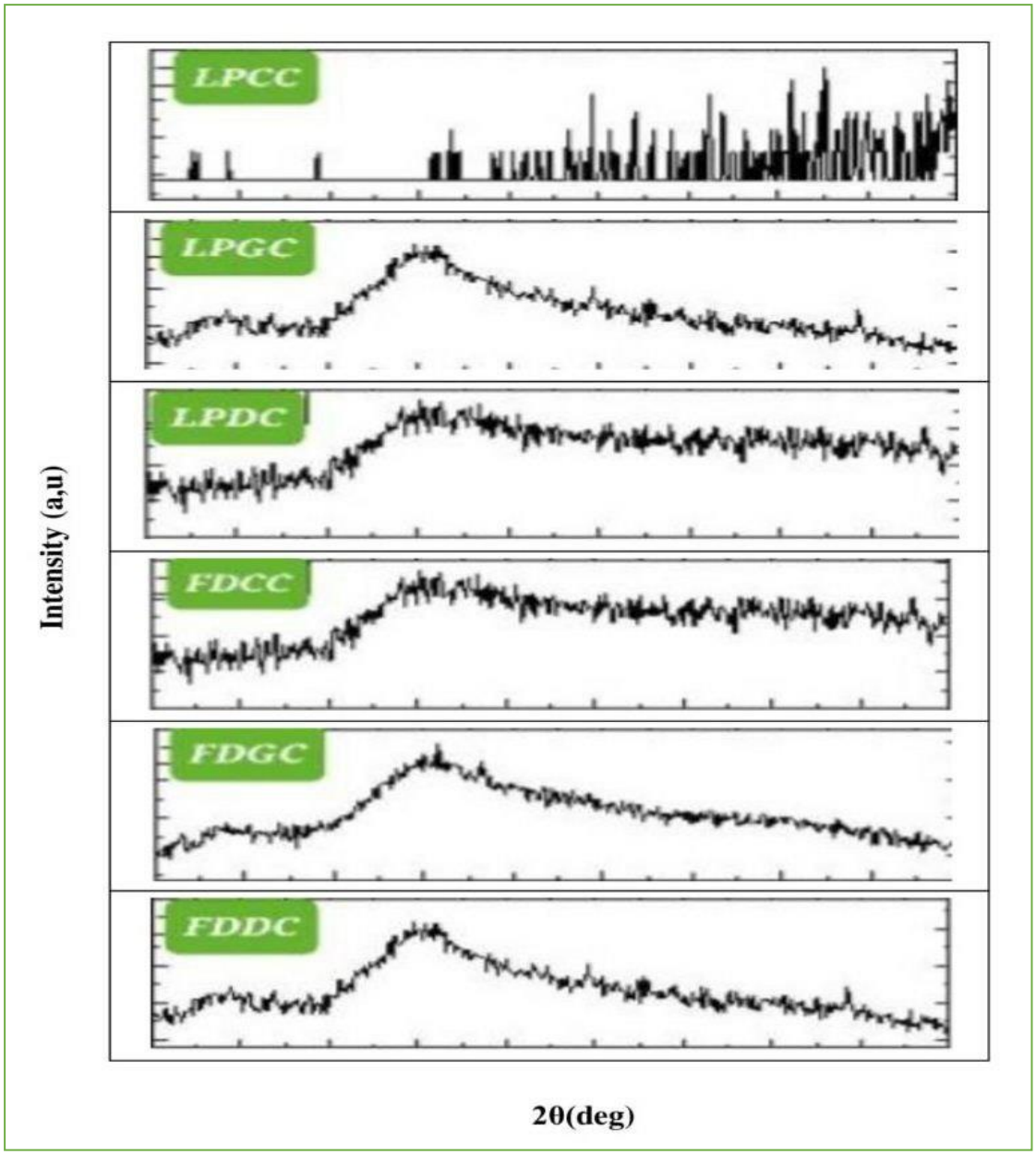
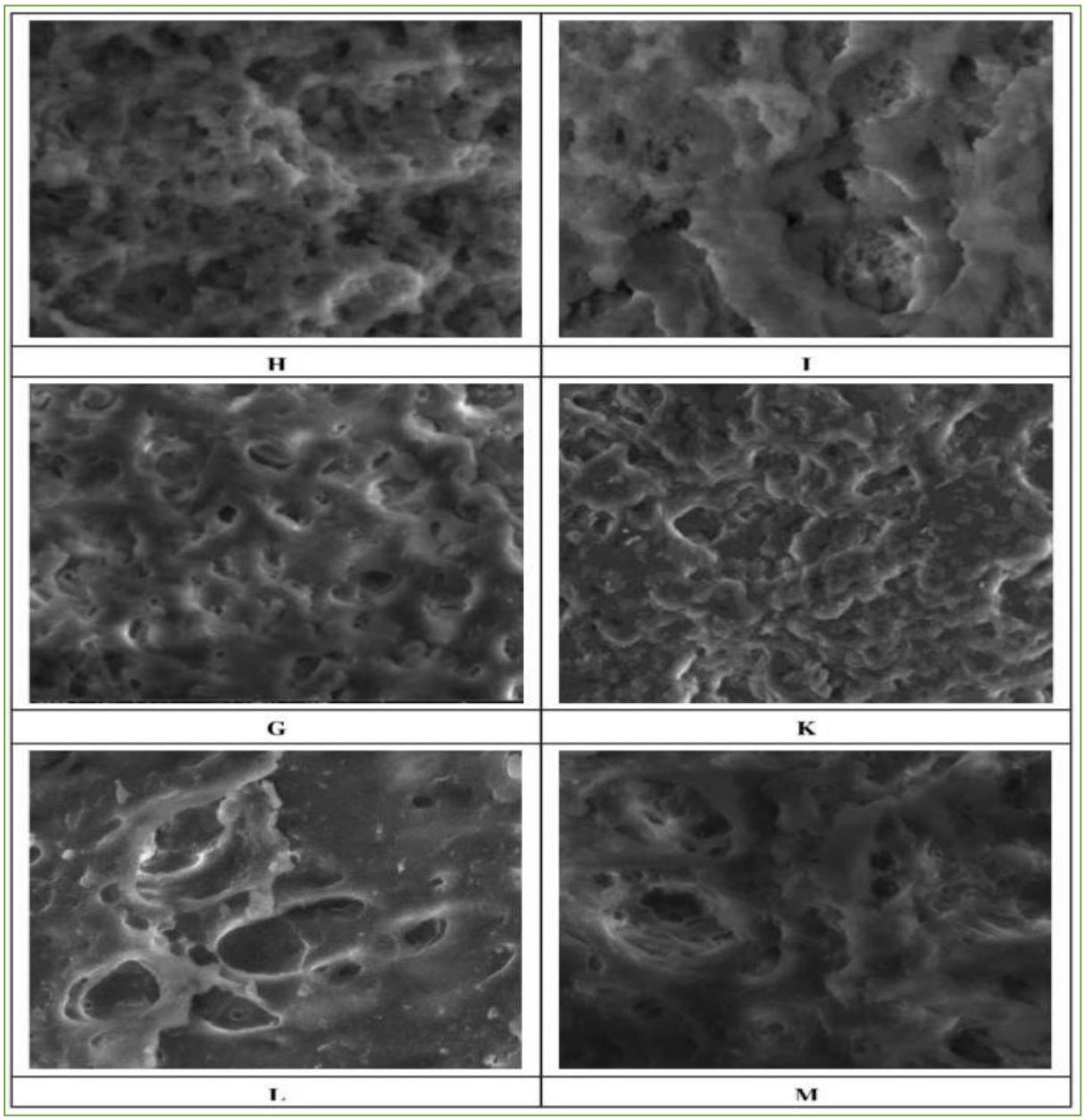


Figure 18: X-ray diffraction spectra of finished fresh cheeses

Figure 18 showed the X-ray diffraction spectra of cheeses made with LCP and FDCP . The difference between a crystalline and amorphous phase is an ordered structure for one and disordered for the other. The X-ray diffraction pattern of cheeses (LPCC,LPGC,LPDC, FDCC,FDGC,FDDC) was amorphous. It was made up of a very broad and diffuse line, indicating the absence of a structural unit which would repeat itself identical to itself at periodic intervals in the three dimensions.

IV.4.Microstructure

Figure 19 presents the environmental scanning electron micrographs (ESEM)of fresh cheeses made with different types of milk, coagulated with LCP, and freeze-dried FDCP.



H: LPCC (Cow cheese made with liquid pepsin), I: FDCC (Cow cheese made with freeze-dried),G: LPGC (Goat cheese made with liquid pepsin), K: FDGC (Goat cheese made with freeze-dried),L: LPDC (Camel cheese made with liquid pepsin), M: FDDC (Camel cheese made with freeze-dried).

Figure 19: Environmental scanning electron micrographs of finished fresh cheeses at a scale of 10 µm

The micrographs of fresh cheeses appear to have a connected and cohesive protein network (casein), which is justified by the production of cheese with enzymatic coagulation (chicken pepsin)

Regarding cheese made with LCP, it appears to have a less compact and cohesive casein network compared to FDCP cheese. In addition, it was observed that cheese made with camel milk had the largest pores compared to other types of milk, and we can conclude that the microstructure of cheese was closely related to the type of milk, and the nature and amount of coagulant used.

In terms of references. **Fox *et al.*(2000)** indicated that the presence of visible voids in cheese is related to the specificity of the coagulant. In addition, **Bouras *et al*. (2023)** studied the microstructure of fresh cheeses made with camel and goat milk mixture. These authors reported that in fresh cheeses with a high camel milk content, their micrographs appeared with fewer and more open pores (wide diameter).

Finally, according to the illustrated micrographs, we can deduce first that the liquid or freeze-dried nature of the chicken pepsin extract seems to have an effect on the microstructure of fresh cheeses. Secondly, it is important to point out that apart from the nature of the coagulant; the type of milk plays an essential role in the appearance of the micrographs of which the aptitude for coagulation of milk controls the enzymatic coagulation.

General Conclusion

The objective of this work is to evaluate the chicken pepsin extract in liquid and freeze-dried form, and its use in the coagulation of different types of milk such as cow , goat and even camel milks in order to establish a chicken pepsin production unit in Algeria.

We first studied the physical and chemical properties of CM, GM and DM: pH (6.90±0.15, 6.50±0.3, 6.40±0.2 respectively), specific gravity (1.035±0.00, 1.028±0.00, 1.030±0.00) g/cm3, total solids (191±0.00, 139±0.00, 115.4±0.00) g/l, protein content (37±0.00, 34±0.00, 34±0.00) g/l, fat (17±0.00, 46±0.00, 23±0.00) g/l, and lactose (56±0.00, 51±0.00, 51±0.00). g/l, ash (8±0.00 for each type of milk) g/l.

In this study we extracted chicken pepsin according to the Bohak protocol which made it possible to produce 300 ml of enzymatic extract from 100 g of proventriculus, giving a coagulation activity (4.95±0.49) U.A.C /ml , coagulation strength (1/2040.81) SU, protein content (18±0.1) mg/ml, specific activity (0.26±0.03) UP/mg, total solids (15±0.01) g/l, ash (3.4±0.01) g/l, pH (6.4±0.00), coagulation time(s): (on CM 31.33±3.51), (on GM 35.7±3.05), (on DM 79.7±3.05).

For FTIR, we noticed the difference in vibration between LCP and FDCP, especially the OH bond. For X-Ray diffraction, we identified the presence of a mineral component which is AL2CaO4.

In the second part, regarding the study of the effect of preservation on chicken pepsin extract, we reported that:

-The use of liquid chicken pepsin extract should not exceed one week at +4°C for milk coagulation ;

-The use of frozen chicken pepsin extract at -20°C should not exceed one month for milk coagulation;

-The cooling and freezing of chicken pepsin extract mainly affect the milk coagulation time.

We also reported that:

- The coagulation strength of LCP with CM was 1/1966.56 (SU), indicating that the volume of LCP required to be added to coagulate 1 liter of milk is 51 µL.

- The coagulation strength of LCP with GM was 1/1307.19 (SU), indicating that the volume of LCP is 77 µL to coagulate 1 liter of milk.

-The coagulant strength of LCP with DM was 1/594.001 (SU), which indicates that the volume of LCP is169.5 µl to coagulate 1 liter of milk.

In the third part, as for the cheese yield obtained, we recorded the highest value (18.9%) with CM coagulated with FDCP. Moreover, cheese yields from milk coagulated with FDCP were higher compared to LCP.

The physical and chemical properties of fresh cheeses made from liquid chicken pepsin (LPCC, LPGC and LPDC) were studied, where we reported the following results: Regarding pH (5.75±0.1, 0.01±6.07, 0.03±5.68 respectively),dry matter (44.4±0.8, 50±1.1, 0.5±41.8) %, Pr/TS ratio (63.06±1.5, 62±0.63, 62.8±2.6) %, Fat/TS ratio (27.2±0.37, 32.3±1.0, 30.1±0.93) ,ash (3.75±0.4, 5.93±0.02, 4.67±0.1) %. %

As for fresh cheeses made from chicken pepsin Freeze dried (FDCC, FDGC and FDDM), we reported the following results: pH (5.86±0.1, 6.06±0.1, 5.83±0.03 respectively), dry matter (50±1.0, 53.3±1.2, 46±0.5) %, Pr/TS ratio (60.4±0.9, 63.78±0.5, 65.21±1.6) %, Fat/TS ratio (28.98±0.8, 23.76±0.3, 22.56±1.9) %, ash (4.06±0.01, 8.75±0.4, 7.85±0.2) %, We also classified LPCC, LPGC and LPDC as hard and semi-fat cheeses, FDGC and FDDC as hard and partially skimmed cheeses and FDCC as hard and semi-fat cheese.

For FTIR, we detected the C=O, OH (water) and C-H (unsaturated fatty acids) vibrations of all cheeses, which supported the chemical analysis of cheese made from LCP and FDCP.

At the microstructural scale, the micrographs obtained by scanning electron microscopy showed that the LCP-coagulated cheeses had a less compact and cohesive casein network compared to FDCP cheese. Also, the camel milk cheese had the largest pores compared to other types of milk. We can conclude that the microstructure of the cheese was closely related to the type of milk and the nature and amount of the coagulant used.

As a perspective, it is important to complete this study with:

-Study of the characteristics of chicken pepsin with the incorporation of the preservative;

-Study of the optimization of the volume and the quantity to add chicken pepsin to the different types of milk in relation to the cheese yield.

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REFERENCES

A

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APPENDX

APPENDX

Appendix N°01: Physicochemical analysis of milk

Appendix N°02: Characterization of chicken pepsin extract

1. Coagulation enzyme extraction

Preparation of solution

Enzyme extraction solution (3% NaCl and 0.7% NaHCO3 solution):

Dissolve 3g of NaCl and 0.7g of NaHCO3 in 300 ml of distilled water for 100g of proventreculus.

Hcl 3N solution: V1=C2.V2/C1.

V1: Volume of concentrated Hcl.

C1: Concentration of Hcl.

C2: Concentration of diluted Hcl.

V2: Volume of diluted Hcl.

APPENDX

To prepare 10 ml of 3N HCl solution, 1.76 ml of concentrated HCl 17 mol/l are taken and diluted with distilled water to 10 ml.

NaOH 3N solution m=C.V.M

M: Mass of NaOH

C: Concentration of solution. V: volume of solution

M: Molar

To prepare 10 ml of 3N NaOH, dissolve 1.2 g of NaOH with a molar mass of 40 g/l in 10 ml of distilled water.

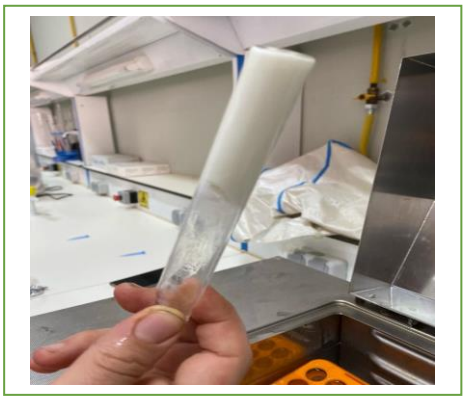
The steps of enzyme extraction

Enzymatic extract after drying

2.2. Preparation of BERRIDGE substrate

To prepare 300 ml of BERRIDGE substrate, dissolve 36 g of skimmed milk powder (0% fat) and 0.4 g of CaCl2 and complete the quantity with distilled water up to 300 ml. store at 4 ° C First, pour a small amount of solution over the entire powder so as to obtain a slurry by manual stirring, the rest of the calcium chloride solution is then added to this slurry, then stirred.

2.3. Clotting time



2.4.Coagulation strength

2.5. FTIR spectra

2.6.SEM

2.7. Diffraction X-ray

Appendix N°03:Method of Kjeldahl

- Mineralization

Mineralization is carried out using an excess of concentrated sulfuric acid, hot and in the presence of a mixture of catalyst (potassium sulfate, copper and selenium sulfate). In the presence of concentrated and hot sulfuric acid, carbon, oxygen, hydrogen and nitrogen of organic compounds are found in the form of CO2, H2O and NH3. Sulfuric acid being in excess, we have:

2NH3 + H2SO4 ---------- > 2NH4 + $SO4^{-2}$

Total nitrogen is therefore obtained in the mineral form NH4+ (ammonium ion). During mineralization, sulfuric acid is partially decomposed and reduced to SO2 and SO3 which form irritating and toxic white fumes .

- Distillation

To transform the ammonium ions of the mineralizate into ammonia (NH4+ into NH3), the mineralizate must be made alkaline with a large excess of a strong base which is 32% Na OH soda.

NH4+ OH ---------- > NH3 + H2O

The ammonia is recovered by distillation: the alkalized mineralisate is heated, the NH3 is released in the form of vapors which are condensed, which are captured by 4% boric acid and which are collected for the assay.

BO3H3 + 3NH3 ----- > H2BO3 (NH4)3

- Titration

The fixed ammonia is titrated with hydrochloric acid (0.1N) in the presence of a Tashiro or RB color indicator (mixture of methyl red and methylene blue).

NH3 + H+ --------- > NH4+

When the ammonia arrives in the boric acid, it alkalizes the medium which turns green. the calibrated solution of strong acid is then poured in to bring the indicator back to its sensitive hue.

Expression of results: **Total nitrogen = 1,40 x N x (Vi-V0) / P**

Appendix N°04:Fat

Using a micropipette, 10 ml of sulfuric acid (H2SO4) are taken and introduced into the butyrometer, 11 ml of milk or 3 g of cheese are introduced delicately without wetting the neck of

the butyrometer and avoiding premature mixing of the milk with the acid, 1 ml or 2 ml of isoamyl alcohol is then poured onto the surface of the milk, avoiding mixing the liquids.

Homogenization by inversion is carried out fifteen to twenty times, followed by centrifugation.

Remove the butyrometer from the water bath, with the stopper still pointing downwards, and carefully adjust it to bring the lower end of the fat column with the minimum movement of this column in front of the nearest mark, preferably a line- main mark.

- ✓ Expression of results

The value is read directly on the butyrometer, the fat content is expressed in% and each graduation of the butyrometer corresponds to 1% of fat.

Butyrometer

Appendix N°05: Preparation of ethyl solutions (Gay-Lussac table)

Table pour la dilution de l'alcool (Table de Gay-Lussac) appelée aussi Table de mouillage de l'alcool

		Concentration initiale													
		100	99	98	97	96	95	90	85	80	75	70	65	60	50
Concentration finale	95	6,5	5,15	3,83	2,53	1,25									
	90	13,25	11,83	10,43	9,07	7,73	6,41								
	85	20,54	19,05	17,58	16,15	14,73	13,33	6,56							
	80	28,59	27,01	25,47	23,95	22,45	20,95	13,79	6,83						
	75	37,58	35,9	34,28	32,67	31,08	29,52	21,89	14,48	7,2					
	70	47,75	45,98	44,25	42,54	40,85	39,18	31,05	23,14	15,35	7,64				
	65	59,37	57,49	55,63	53,81	52	50,22	41,53	33,03	24,66	16,37	8,15			
	60	72,82	70,80	68,8	65,85	64,92	63	53,65	44,48	35,44	26,47	17,58	8,76		
	55	88,6	86,42	84,28	82,16	80,06	77,99	67,87	57,9	48,07	38,32	28,63	19,02	9,47	
	50	107,44	105,08	102,75	100,44	98,15	95,89	84,71	73,90	63,04	52,43	41,73	31,25	20,47	
	45	130,26	127,67	125,11	122,57	120,06	117,57	105,34	93,30	81,38	69,54	57,78	46,09	34,46	11,41
	40	158,56	155,68	152,84	150,02	147,22	144,46	130,8	117,34	104,01	90,76	77,58	64,48	51,43	25,55
	35	194,63	191,39	188,19	185,01	181,85	178,71	163,28	148,01	132,88	117,82	102,84	87,93	73,08	43,59
	30	242,38	238,67	234,99	231,33	227,70	224,08	206,22	188,57	171,05	153,61	136,04	118,94	101,71	67,45
	25	308,9	304,52	300,18	295,86	291,56	287,28	266,12	245,15	224,3	203,61	182,83	162,21	141,65	100,73
	20	408,5	403,13	397,79	392,47	387,17	381,9	355,8	329,84	304,01	278,26	252,58	226,98	201,43	150,55
	15	574,75	567,43	560,53	553,55	546,59	539,66	505,27	471	436,85	402,81	368,83	334,91	301,07	233,64
	10	907,09	896,73	886,4	876,1	865,15	855,15	804,5	753,65	702,89	652,21	601,6	551,06	500,50	399,85

Les chiffres en noir indiquent la quantité d'eau en mL à ajouter à 100mL d'alcool de concentration initiale x **(en bleu)** pour obtenir la conc désirée.

Exemple : la table indique qu'il faut ajouter 105,34 ml d'eau à 100 mL d'alcool à 90° pour obtenir de l'alcool à 45°.
Attention : Le volume final est inférieur à la somme des volumes mis en jeu ! C'est le phénomène dit de « contraction de volume », variat fonction du titre de l'alcool initial.

Gay-Lussac

Appendix N°06: Scientific card

Name and formula

Reference code: 98-018-0997

Mineral name: Krotite
Compound name: Krotite
Common name: Krotite

Chemical formula: $Al_2Ca_1O_4$

Crystallographic parameters

Crystal system: Monoclinic
Space group: P 1 21/c 1
Space group number: 14

a (Å): 8,7000
b (Å): 8,0990
c (Å): 17,5037
Alpha (°): 90,0000
Beta (°): 119,6160
Gamma (°): 90,0000

Calculated density (g/cm^3): 2,94

Volume of cell (10^6 pm^3): 1072,21
Z: 12,00

RIR: 0,59

Subfiles and quality

Subfiles: User Inorganic
User Mineral
Quality: User From Structure (=)

Comments

Creation Date: 01/02/2012
Modification Date: 30/12/1899
Original ICSD space group: P121/N1. X-ray diffraction from single crystal
Structure type: NaBePO4. Temperature factors available
Temperature in Kelvin: 298
Compound with mineral name: Krotite
Structure type: NaBePO4
Recording date: 2/1/2012
Mineral origin: North West Africa 1934 meteorite
ANX formula: AB2X4
Z: 12
Calculated density: 2.94
R value: 0.0161
Pearson code: mP84
Wyckoff code: e21
Structure TIDY: TRANS -a,-b,a+c origin 0 0 1/2
Structure TIDY: REMARK Transformed from setting P 1 21/n 1.
Publication title: Krotite, Ca Al2 O4, a new refractory mineral from the NWA 1934 meteorite
ICSD collection code: 180997
Structure: NaBePO4
Chemical Name: Calcium Dialuminate
Second Chemical Formula: Ca (Al2 O4)

References

Structure: Schrader, D.L.;Sweeney Smith, S.A.;Rossman, G.R.;Beckett, J.R.;Connolly, H.C.jr.;Kampf, A.R.;Ma Chi, *American Mineralogist*, **96**, 709 - 715, (2011)

Peak list

No.	h	k	l	d [A]	2Theta[deg]	I [%]
1	0	0	2	7,60848	11,621	0,0
2	1	0	0	7,56341	11,691	1,3
3	1	0	-2	7,54205	11,724	0,2
4	0	1	-1	7,14944	12,370	0,3
5	1	1	-1	5,92793	14,933	3,8
6	0	1	-2	5,54531	15,970	2,0
7	1	1	0	5,52779	16,021	4,9
8	1	1	-2	5,51943	16,045	5,3
9	1	1	1	4,68126	18,942	24,7
10	1	1	-3	4,67112	18,984	28,7

11	1	0	2	4,38822	20,220	2,1
12	1	0	-4	4,37570	20,278	1,5
13	2	0	-2	4,34998	20,400	1,0
14	0	1	-3	4,29883	20,645	0,0
15	0	2	0	4,04950	21,931	19,1
16	0	2	-1	3,91330	22,705	1,7
17	1	1	2	3,85827	23,033	1,4
18	1	1	-4	3,84976	23,085	0,4
19	2	1	-2	3,83220	23,192	1,6
20	0	0	4	3,80424	23,365	0,5
21	2	0	0	3,78170	23,506	1,4
22	2	0	-4	3,77102	23,573	1,4
23	2	1	-1	3,71872	23,910	9,0
24	2	1	-3	3,71363	23,943	20,7
25	1	2	-1	3,67128	24,223	0,7
26	0	2	2	3,57472	24,888	0,8
27	1	2	0	3,57001	24,921	0,5
28	1	2	-2	3,56776	24,937	0,5
29	0	1	-4	3,44330	25,854	1,3
30	2	1	0	3,42656	25,983	3,7
31	2	1	-4	3,41861	26,044	2,9
32	1	2	1	3,30827	26,929	8,1
33	1	2	-3	3,30469	26,958	7,0
34	1	1	3	3,20492	27,814	4,1
35	1	1	-5	3,19841	27,872	16,6
36	0	2	3	3,16467	28,175	4,4
37	2	1	1	3,06191	29,141	13,0
38	2	1	-5	3,05341	29,224	1,4
39	1	2	2	2,97598	30,002	97,8
40	1	2	-4	2,97207	30,043	100,0
41	2	2	-2	2,96397	30,127	94,0
42	2	2	-1	2,91051	30,694	11,5
43	2	2	-3	2,90807	30,720	2,9
44	1	0	4	2,87564	31,075	9,0
45	1	0	-6	2,86976	31,140	3,1
46	2	0	2	2,86830	31,157	1,4
47	2	0	-6	2,85899	31,261	8,4
48	3	0	-2	2,85044	31,357	11,7
49	0	1	5	2,84889	31,374	0,9
50	3	0	-4	2,84700	31,396	2,9
51	0	2	4	2,77266	32,260	0,5
52	2	2	0	2,76390	32,365	1,9
53	2	2	-4	2,75972	32,416	2,2
54	3	1	-3	2,73024	32,776	1,0
55	1	1	4	2,70989	33,029	0,3
56	1	1	-6	2,70497	33,090	0,4
57	2	1	2	2,70375	33,106	0,3
58	2	1	-6	2,69594	33,204	0,9
59	3	1	-2	2,68877	33,296	0,1
60	3	1	-4	2,68588	33,332	0,4
61	0	3	1	2,65816	33,690	0,1
62	1	2	3	2,64357	33,882	1,8
63	1	2	-5	2,63992	33,930	0,0
64	1	3	-1	2,57838	34,765	3,2
65	3	1	-1	2,57232	34,850	0,5
66	3	1	-5	2,56727	34,921	0,5
67	2	2	1	2,56158	35,001	0,1
68	2	2	-5	2,55660	35,071	0,0
69	0	3	2	2,54425	35,247	0,0
70	1	3	0	2,54255	35,271	0,2

71	1	3	-2	2,54174	35,283	0,1
72	0	0	6	2,53616	35,363	51,5
73	3	0	0	2,52114	35,581	41,9
74	3	0	-6	2,51402	35,685	38,1
75	1	3	1	2,44269	36,764	1,0
76	1	3	-3	2,44125	36,786	8,7
77	0	2	5	2,43291	36,917	0,0
78	0	1	6	2,42027	37,117	12,0
79	3	1	0	2,40720	37,326	22,4
80	3	1	-6	2,40100	37,426	25,5
81	2	1	3	2,38662	37,659	9,0
82	0	3	-3	2,38314	37,717	1,1
83	2	1	-7	2,37991	37,770	0,4
84	3	2	-3	2,35775	38,138	0,3
85	1	2	4	2,34461	38,360	1,9
86	1	2	-6	2,34142	38,415	1,2
87	2	2	2	2,34063	38,428	4,7
88	2	2	-6	2,33556	38,515	1,8
89	1	1	5	2,33361	38,548	0,4
90	3	2	-2	2,33089	38,595	3,0
91	1	1	-7	2,32984	38,613	9,7
92	3	2	-4	2,32901	38,628	1,7
93	1	3	2	2,29937	39,146	2,9
94	1	3	-4	2,29757	39,178	2,9
95	2	3	-2	2,29382	39,244	4,7
96	2	3	-1	2,26877	39,696	12,8
97	2	3	-3	2,26762	39,717	0,8
98	3	2	-1	2,25380	39,971	0,6
99	3	2	-5	2,25040	40,033	0,0
100	3	1	1	2,22137	40,580	1,7
101	3	1	-7	2,21488	40,704	0,1
102	0	3	-4	2,20163	40,960	4,5
103	2	3	0	2,19723	41,045	6,9
104	2	3	-4	2,19513	41,086	7,2
105	2	0	4	2,19411	41,106	3,5
106	2	0	-8	2,18785	41,229	4,0
107	4	0	-4	2,17499	41,484	4,5
108	0	2	-6	2,14941	42,001	1,3
109	3	2	0	2,14024	42,190	1,8
110	3	2	-6	2,13588	42,280	4,7
111	1	3	3	2,13531	42,292	9,5
112	1	3	-5	2,13338	42,332	2,4
113	2	2	3	2,12574	42,491	0,4
114	2	2	-7	2,12100	42,591	0,0
115	2	1	4	2,11777	42,659	0,0
116	2	1	-8	2,11214	42,778	0,1
117	1	0	6	2,11064	42,810	3,0
118	1	0	-8	2,10739	42,880	0,9
119	3	0	2	2,10292	42,975	2,6
120	4	1	-4	2,10056	43,026	0,0
121	0	1	7	2,09954	43,048	0,6
122	3	0	-8	2,09603	43,123	1,7
123	4	0	-2	2,09304	43,188	1,4
124	2	3	1	2,09140	43,224	0,2
125	4	0	-6	2,08941	43,267	1,5
126	2	3	-5	2,08869	43,283	1,6
127	1	2	5	2,08803	43,297	0,0
128	1	2	-7	2,08532	43,356	0,0
129	4	1	-3	2,08172	43,435	0,0
130	4	1	-5	2,07994	43,474	5,4

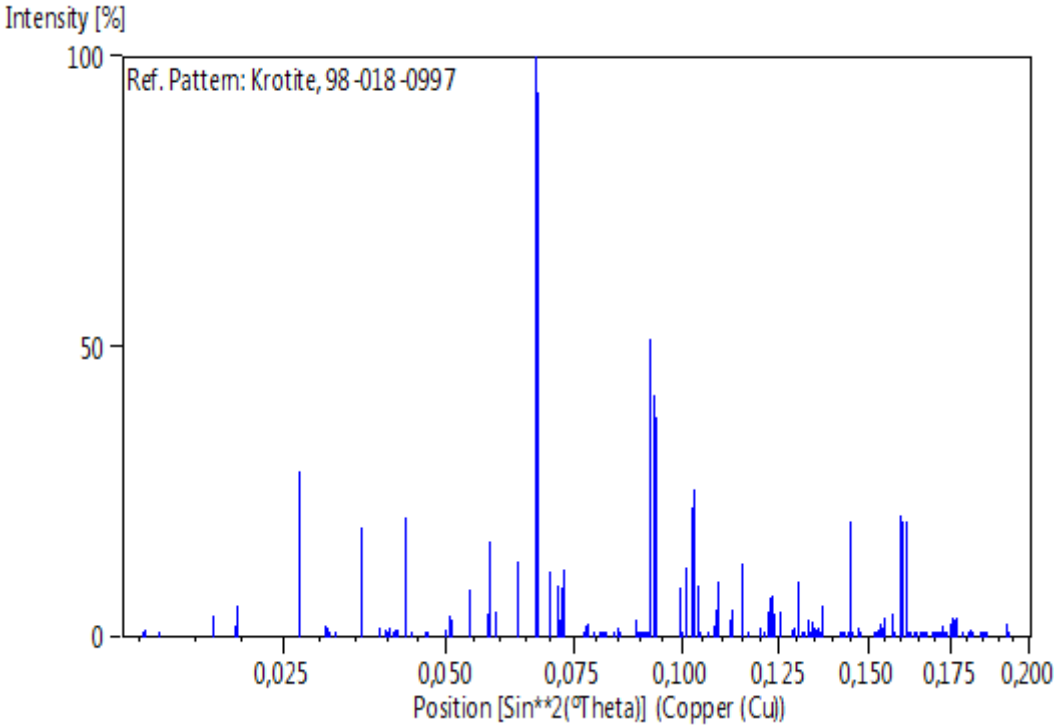
131	1	1	6	2,04242	44,314	0,0
132	1	1	-8	2,03947	44,382	0,1
133	3	1	2	2,03542	44,475	0,2
134	3	1	-8	2,02918	44,619	0,0
135	4	1	-2	2,02646	44,682	0,0
136	0	4	0	2,02475	44,722	20,1
137	4	1	-6	2,02317	44,759	0,1
138	0	3	-5	2,01959	44,843	0,1
139	0	4	1	2,00706	45,138	1,8
140	3	2	1	2,00647	45,152	0,1
141	3	2	-7	2,00168	45,266	0,0
142	3	3	-3	1,97598	45,888	0,8
143	1	4	-1	1,97205	45,985	0,3
144	1	3	4	1,96822	46,079	1,4
145	1	3	-6	1,96634	46,126	1,1
146	2	3	2	1,96587	46,138	2,4
147	2	3	-6	1,96286	46,213	1,8
148	3	3	-2	1,96009	46,282	1,8
149	3	3	-4	1,95897	46,310	3,4
150	0	4	2	1,95665	46,368	0,1
151	1	4	0	1,95588	46,387	0,2
152	1	4	-2	1,95551	46,396	0,0
153	4	1	-1	1,94291	46,715	4,0
154	4	1	-7	1,93856	46,826	1,1
155	2	2	4	1,92914	47,069	21,0
156	2	2	-8	1,92488	47,179	19,9
157	4	2	-4	1,91610	47,408	20,0
158	0	2	7	1,91532	47,429	0,0
159	3	3	-1	1,91357	47,475	0,1
160	3	3	-5	1,91149	47,530	0,2
161	1	4	1	1,90931	47,587	0,1
162	1	4	-3	1,90862	47,606	0,0
163	0	0	8	1,90212	47,778	0,6
164	4	2	-3	1,90177	47,788	0,7
165	4	2	-5	1,90041	47,824	0,2
166	2	1	5	1,89317	48,018	0,1
167	4	0	0	1,89085	48,081	0,0
168	2	1	-9	1,88848	48,145	0,2
169	4	0	-8	1,88551	48,226	0,4
170	0	4	3	1,88047	48,363	0,0
171	1	2	6	1,87167	48,606	0,3
172	1	2	-8	1,86940	48,668	0,0
173	3	2	2	1,86627	48,755	0,1
174	3	2	-8	1,86146	48,889	0,9
175	3	1	3	1,86103	48,901	0,2
176	4	2	-2	1,85936	48,948	1,0
177	4	2	-6	1,85681	49,020	0,1
178	3	1	-9	1,85531	49,062	1,9
179	0	1	-8	1,85174	49,163	0,1
180	0	3	-6	1,84844	49,257	0,8
181	3	3	0	1,84260	49,423	2,2
182	4	1	0	1,84133	49,459	0,2
183	3	3	-6	1,83981	49,503	1,6
184	1	4	2	1,83848	49,541	3,5
185	1	4	-4	1,83756	49,568	2,4
186	4	1	-8	1,83640	49,601	0,2
187	2	4	-2	1,83564	49,623	3,2
188	2	3	3	1,83332	49,690	1,6
189	2	3	-7	1,83027	49,779	3,3
190	2	4	-1	1,82273	49,999	0,0

191	2	4	-3	1,82213	50,016	0,0
192	1	1	7	1,81234	50,305	0,1
193	1	1	-9	1,80999	50,375	0,2
194	1	3	5	1,80896	50,406	1,3
195	1	3	-7	1,80720	50,458	0,2
196	4	2	-1	1,79419	50,850	0,0
197	4	2	-7	1,79076	50,954	0,2
198	0	4	-4	1,78736	51,058	0,5
199	2	4	0	1,78500	51,131	0,3
200	2	4	-4	1,78388	51,165	0,3
201	3	3	1	1,75515	52,065	0,8
202	2	2	5	1,75480	52,076	2,3
203	3	3	-7	1,75194	52,167	0,4

Structure

No.	Name	Elem.	X	Y	Z	Biso	sof	Wyck.
1	O1	O	0,09500	0,36430	0,14910	1,1922	1,0000	4e
2	CA1	Ca	0,19790	0,47015	0,42914	1,0896	1,0000	4e
3	CA2	Ca	0,50735	0,02887	0,24701	1,4212	1,0000	4e
4	AL1	Al	0,18540	0,32738	0,26168	0,8922	1,0000	4e
5	AL2	Al	0,18647	0,72376	0,26687	0,8843	1,0000	4e
6	AL3	Al	0,47929	0,32885	0,07786	0,8843	1,0000	4e
7	AL4	Al	0,53123	0,21941	0,41929	0,8843	1,0000	4e
8	AL5	Al	0,15614	0,22697	0,09133	0,8764	1,0000	4e
9	AL6	Al	0,15286	0,83446	0,09220	0,8843	1,0000	4e
10	CA3	Ca	0,15005	0,03429	0,41074	0,9949	1,0000	4e
11	O2	O	0,09455	0,03230	0,11035	1,2080	1,0000	4e
12	O3	O	0,27567	0,74540	0,19657	1,2475	1,0000	4e
13	O4	O	0,04492	0,24510	0,29712	1,1686	1,0000	4e
14	O5	O	0,40921	0,25900	0,47321	1,1607	1,0000	4e
15	O6	O	0,70928	0,35320	0,14078	1,1607	1,0000	4e
16	O7	O	0,24575	0,52420	0,31135	1,2791	1,0000	4e
17	O8	O	0,38885	0,22630	0,30455	1,2001	1,0000	4e
18	O9	O	0,37673	0,52260	0,05493	1,3028	1,0000	4e
19	O10	O	0,29109	0,64380	0,54659	1,2238	1,0000	4e
20	O11	O	0,38676	0,22580	0,13366	1,3028	1,0000	4e
21	O12	O	0,05276	0,23980	0,47636	1,1528	1,0000	4e

Stick Pattern



Appendix N°07: Incubator Supplement