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Theme

Extraction of calcium carbonate and collagen from egg shells

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ABSTRACT

Around the world, large quantities of eggs are consumed in a variety of ways, producing enormous numbers of eggshells. These eggshells may wind up in landfills, rivers, or coastal waters, which may have an adverse effect on people's health, contaminate water supplies, or otherwise harm the environment. Additionally, in recent years, particular attention has been paid to industrial sectors that pollute the environment.

Eggshells have some organic membranes and contain a lot of CaCO3, although they are typically regarded as garbage. ES comprise roughly 95% calcium carbonate, and the ES membrane's fibrils are primarily made of proteins, around 10% of which are collagen (types I, V, and X).

Due to the versatility of both calcium carbonate and collagen in many fieldssuch as medicine, pharmaceutic, alimentation and cosmetic. In this study, we used waste eggshell as raw material to extract calcium carbonate and collagen as bio and natural products, and in this study we gave an attempt to reduce waste eggshell, including environmental pollution, in order to preserve our health and achieve the zero waste rule.

1 kg of eggshell collected from 2 different restaurants, we used just 100g of the extern membrane to extract $CaCO_3$ and 50g from the inner membrane to extract collagen. The eggshells were cleaned and sterilized, both membranes were separated manually. The outer membrane was ground and mixed with 10% of acetic acid in order to extract $CaCO_3$. The inner membrane went through two stages, the first one is the pre-treatment to remove impurity such as soluble non-collagenous compound, lipids, pigments and aftertaste. The second one is the extraction step of collagen by 0.5M of citric or acetic acid.

In this study, the presence of calcium carbonate content in ES was determined by back titration.

The result of this study was high yield of pure material of $CaCO_3$ and collagen, the rest of the details and information you will get through the upcoming papers.

Keywords: Eggshell, calcium carbonate, collagen, extraction.

Résumé

Dans le monde entier, de grandes quantités d'œufs sont consommées de diverses manières, produisant d'énormes quantités de coquilles d'œufs. Ces coquilles peuvent se retrouver dans les décharges, les rivières ou les eaux côtières, ce qui peut avoir un effet néfaste sur la santé des personnes, contaminer les réserves d'eau ou nuire à l'environnement de quelque manière que ce soit. En outre, ces dernières années, une attention particulière a été accordée aux secteurs industriels qui polluent l'environnement.

Les coquilles d'œuf ont des membranes organiques et contiennent beaucoup de $CaCO_3$, bien qu'elles soient généralement considérées comme des déchets. Les coquilles d'œufs sont constitués d'environ 95 % de carbonate de calcium et les fibrilles de la membrane des coquilles d'œufs sont principalement composées de protéines, dont environ 10 % de collagène (types I, V et X).

En raison de la polyvalence du carbonate de calcium et du collagène dans de nombreux domaines tels que la médecine, la pharmacie, l'alimentation et la cosmétique. Dans cette étude, nous avons utilisé des coquilles d'œuf usagées comme matière première pour extraire le carbonate de calcium et le collagène en tant que produits bio et naturels, et nous avons tenté de réduire les déchets de coquilles d'œuf, y compris la pollution de l'environnement, afin de préserver notre santé et d'atteindre la règle "zéro déchet".

1 kg de coquilles d'œufs collectées dans 2 restaurants différents, nous avons utilisé seulement 100g de la membrane externe pour extraire le $CaCO_3$ et 50g de la membrane interne pour extraire le collagène. Les coquilles d'œufs ont été nettoyées et stérilisées, les deux membranes ont été séparées manuellement. La membrane externe a été broyée et mélangée à 10% d'acide acétique afin d'extraire le $CaCO_3$. La membrane interne a subi deux étapes : la première est le prétraitement pour éliminer les impuretés telles que les composés solubles non-collagéniques, les lipides, les pigments et l'arrière-goût. La seconde est l'étape d'extraction du collagène par l'acide citrique ou acétique 0,5M.

Dans cette étude, la présence de carbonate de calcium dans les coquilles d'œufs a été déterminée par titrage en retour.

Le résultat de cette étude est un rendement élevé de la matière pure de $CaCO_3$ et de collagène, le reste des détails et des informations vous seront fournis dans les prochains documents.

Mots-clés : Coquille d'œuf, carbonate de calcium, collagène, extraction.

الملخص

في جميع أنحاء العالم، يتم استهلاك كميات كبيرة من البيض بطرق متنوعة، مما ينتج عنه أعداد هائلة من قشر البيض. قد ينتهي الأمر بقشر البيض في مدافن النفايات أو الأنهار أو المياه الساحلية، مما قد يكون له تأثير سلبي على صحة الناس، أو يلوث إمدادات المياه، أو يضر بالبيئة. بالإضافة إلى ذلك ، في السنوات الأخيرة ، تم إيلاء اهتمام خاص للقطاعات الصناعية التي تلوث البيئة.

تحتوي قشور البيض على بعض الأغشية العضوية وتحتوي على الكثير من كربونات الكالسيوم ، على الرغم من أنها تعتبر عادةً قمامة. تشتمل قشور البيض على ما يقرب من 95٪ من كربونات الكالسيوم ، وتتكون ألياف غشاء قشر البيض بشكل أساسي من البروتينات ، حوالي 10٪ منها عبارة عن كولاجين (أنواع I و V و X).

نظرًا لتعدد استخدامات كل من كربونات الكالسيوم والكولاجين في العديد من المجالات مثل الطب والتغذية ومستحضرات التجميل. في هذه الدراسة ، استخدمنا نفايات قشر البيض كمادة خام لاستخراج كربونات الكالسيوم والكولاجين كمنتجات حيوية وطبيعية ، وفي هذه الدراسة قمنا بمحاولة للحد من نفايات قشر البيض بما في ذلك التلوث البيئي ، وذلك للحفاظ على صحتنا وتحقيق قاعدة صفر نفايات.

تم جمع 1 كجم من قشر البيض من مطعمين مختلفين ، استخدمنا 100 جرام فقط من الغشاء الخارجي لاستخراج كربونات الكالسيوم و 50 جرامًا من الغشاء الداخلي لاستخراج الكولاجين. تم تنظيف وتعقيم قشر البيض ، وتم فصل الغشاءين يدويًا. تم طحن الغشاء الخارجي وخلطه مع 10٪ من حمض الأسيتيك لاستخراج كربونات الكالسيوم. مر الغشاء الداخلي بمرحلتين ، الأولى هي المعالجة المسبقة لإزالة الشوائب مثل المركب غير الكولاجيني القابل للذوبان ، والدهون ، والأصباغ ، والمذاق اللاحق. الخطوة الثانية هي خطوة استخلاص الكولاجين بمقدار 0.5 مول من حمض الستريك أو حمض الخليك.

في هذه الدراسة ، تم تحديد وجود محتوى كربونات الكالسيوم في قشر البيض عن طريق المعايرة الخلفية.

كانت نتيجة هذه الدراسة عائد مرتفع من المواد النقية من كربونات الكالسيوم والكولاجين وبقية التفاصيل والمعلومات سوف تتحصلون عليها من خلال الأوراق القادمة.

الكلمات المفتاحية: قشر البيض ، كربونات الكالسيوم ، الكولاجين ، الاستخلاص

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First of all, we would like to thank ***ALLAH*** *for giving us the will, health and patience to complete these long years of study.*

We would like to express our gratitude and thanks to our thesis supervisor ***Pr.Rouabhi Rachid****, who was always available throughout the realization of this work, he guided us and helped us to find solutions to move forward, thank you for being very patient with us.*

We dedicate this work and our deepest gratitude to those who gave us life, love and courage; to you our dear and sweet mothers and our dearest fathers all our joys and our love, and our gratitude for the education they gave us. By all means and at all costs, the sacrifices they made for us and for their trust and for the sense of duty they gave us, taught us since our childhood and all that they have done for us; Thanks to their moral and economic support. May Allah protect you and protect you for us. Insha'Allah we will make you very proud.

We would also like to thank the members of the jury for accepting to judge this thesis.

DEDICATION

I would like to dedicate this work as a sign of respect and love to my dearest parents ***Djamel*** *and my paradise* ***Nadia.***

Thank you so much for everything! Words can hardly describe my thanks and appreciation to you. You have been my source of inspiration, support, and guidance .You have taught me to be unique, determined, to believe in myself, and to always perservere. I am truly thankful and honored to have you as my parents.

To take a quote from Albert Schweitzer, "At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us". You, mom and dad, have been that spark for when my blew out. Thank you for your unwavering love and support al. I love you both always and forever.

May ***Allah*** *keep You always in good health..*

And to my dear brother ***Oussama.***

To all my friends without exception whether they are close or far.

To my partner ***Anfel*** *and all her lovely family.*

To all those who are dear to me.

NIHEL

I dedicate this work.

To my dear parents ***Abdulrahman*** *and* ***Warda****, for all their sacrifices, their love, their tenderness, their support and their prayers throughout my studies.*

May Allah keep my mom in good health and protect her for me. She's the only person who makes me happy in this dunia.

To my dear brothers, sisters and my cute nieces and nephews.

To all my friends without exception whether they are close or far.

To my partner ***Nihel*** *and all her lovely family.*

To all those who are dear to me.

Please, I wish everyone who passed through here to pray for ***Rahma*** *for my beloved father, that great man who did a lot for me. I love you very much* ***PAPA****. I hope you are proud of me.*

ANFEL

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ABBREVIATION LIST

ES: Eggshells

$CaCO_3$: Calcium carbonate

Ca_2^+ : Calcium

CO_3^{2-}: Carbonate

HCl: Hydrochloric Acid

NaOH: Sodium hydroxide

CH_3COOH: Acetic Acid

NaCl: Sodium Chloride

$H_2\ SO_4$: Sulphuric Acid

$C_6\ H_3\ O_7$: Citric Acid

H_2O_2: Hydrogen peroxide

$KHC_8H_4O_4$: Potassium hydrogen phthalate

H_2O : Water

$CaCl_2$: Calcium Chloride

CO_2: Carbon dioxide

ANNEXES LISTE

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Introduction

Globally, hundreds of thousands of tones of eggshells which the EU considers to be hazardous waste are thrown. This egg manufacturing waste is a useful source material that can be applied in a variety of industries **(King'Ori, 2011)**. ES can be used for a number of things that reduce the environmental harm they cause. ES are perhaps the greatest natural source of calcium since they contain trace levels of other minerals and present healthy, balanced calcium. Collagen is a part of the eggshell membrane, similar to other mammalian collagens, eggshell membrane collagen has a very low incidence of autoimmune and allergic

Calcium carbonate is a white or translucent mineral that takes the shape of crystals is identified by the chemical formula $CaCO_3$. A calcium salt of carbonic acid makes up calcium carbonate, often known as calcite. Solid dosage forms frequently use calcium carbonate, a pharmaceutical excipient, as a diluent. Additionally, it serves as a basis for pharmaceutical and dental preparations, a food additive, a calcium supplement, and a buffering and dissolve aid for dispersible tablets. Mineral salts are abundant in egg shells, especially calcium carbonate, which makes up around 94% of the shell. Large volumes of shells are produced by layer farms, and their final disposal presents environmental challenges **(Murakami et *al*., 2007)**.

Collagen, a fibrous macromolecule of a glycoprotein origin, is found widely in the animal kingdom and makes up about one-third of the proteins human body. The production of glue, one of humanity's oldest industries, is dependent on the conversion of collagen into gelatin. The tanning or leather business is another ancestor of collagen, most recently has been utilized in both medicine and surgery. Catgut is the collagen found in mammalian intestinal submucosa, which is also used to make strings for musical instruments. Additionally, collagen-based cosmetic items, such as collagen lotion, and dressings (for burns, for example), have been developed **(Robert, 2023)**.

In terms of egg production, he said that Algeria produces eight billion units annually, while consumption does not exceed six billion at a rate of 160 eggs per citizen. This is what results tons of eggshells polluting the environment.

In this context, the objective of our study is the extraction of calcium carbonate and collagen from eggshells as bio natural products and trying to reduce environmental pollution by recycling waste.

Our manuscript will be divided into three chapters. The first chapter, a bibliographical synthesis, will present eggshells, calcium carbonate and collagen. In the second chapter, we will describe our practical work approach collecting of eggshells till the extraction of $CaCO_3$ and collagen. Finally, in the third chapter, we will analyse and discuss the results obtained.

Chapter 01:

Bibliographic synthesis

1. Eggshell

An eggshell is the hard, outer covering of an egg. It consists mostly of calcium carbonate, a common form of calcium. The rest is made up of protein and other minerals **(Daengprok et *al*., 2003)**

1.1.Chemical composition of eggshell

Did you know? Your eggshells are worth gold in your trash! A very underrated food waste, eggshells have superpowers.

Eggshells from hens typically weigh 5.5 g and range in thickness from 280 to 400 µm. Minerals make up 95.1% of the eggshell's contents, followed by proteins (3.3%), and water (1.6%). Calcium makes up the majority of the mineral weight (37.3%) and is the most prevalent type in crystalline form, followed by calcium triphosphate (0.8%) and magnesium carbonate **(Neunzehn et *al*., 2015).**

Chemical tests reveal that eggshells comprise roughly 95% calcium carbonate **(Yoshinori and Jennifer, 2004).**The average eggshell has traces of sodium, potassium, zinc, manganese, iron, and copper as well as roughly 3% phosphorus and 3% magnesium **(Miles et *al*., 2019)**. Chemically, the eggshell membrane's fibrils are primarily made of proteins, around 10% of which are collagen (types I, V, and X), and between 70 and 75 percent of which are other proteins and glycoproteins with lysine-derived crosslinks **(Kheirabadi et *al*., 2018).** Additionally, the inner and outer eggshell membranes have slightly different morphologies as well as chemical makes-ups. Type I and V collagen make up the majority of the core proteins of the inner eggshell membrane, whereas type I makes up the majority of the outer eggshell membrane **(Carrino et *al*., 1997).** Type X collagen was found in both membranes **(Arias et *al*., 1997).**

1.2.Egg structure

The eggshell develops in several sections of the hen's oviduct and is made up of multiple layers arranged in a well-organized structure**(Yamamoto et *al*., 1996).**

While the eggshell is forming, a variety of proteins both soluble and insoluble minerals, and fibers are deposited; these materials are later consumed by the developing embryo.

According to **Figure 01**, the eggshell is primarily composed of three components: the shell, albumen, and yolk. Albumen layers encircle the inner portion of the yolk, which is protected by the egg's tough shell. A thin film layer termed the cuticle, a layer of limestone, and the inner and outer shell membranes make up the outer eggshell layer **(Golakiya et*al*., 2020).**

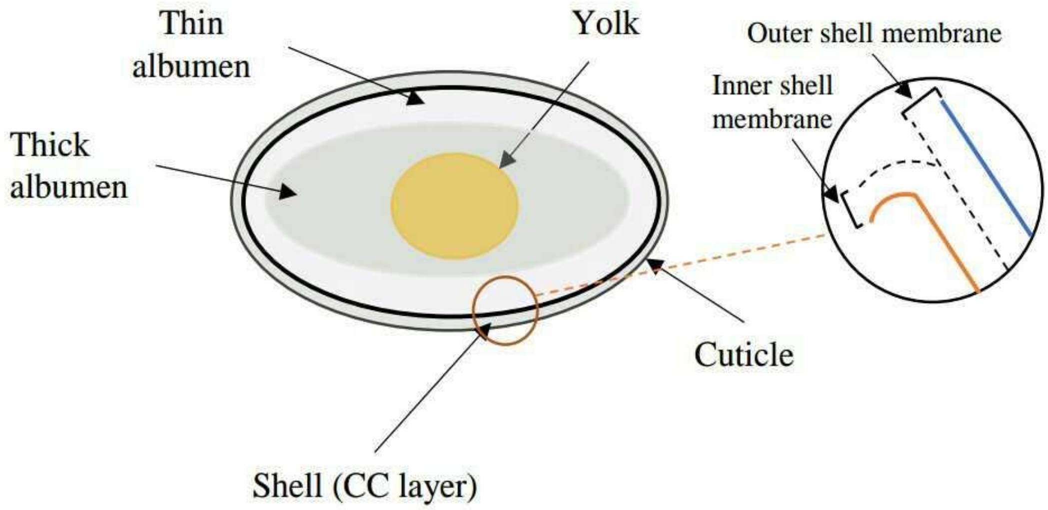

Figure 01: Egg structure and its different components**(Golakiya et *al*., 2020).**

The cuticle, which is the outermost layer and is between 10 and 20µm thick, limits the passage of bacteria and moisture to some extent (**Bain et *al*., 2013) (Chen et *al*., 2019).**The eggshell membranes are made up of inner and outer membranes that are, respectively, 50 µm and 15µmthick **(Yamamoto et *al*., 1996).** Different kinds of collagen proteins, amino acids, and carboxylic acids were discovered in both the membrane's inner and outer layers by (**Maxwell et *al*., 2012).** The yolk is covered with an albumen layer that is both thin and thick, making up the egg albumen. The yolk is mostly composed of lipids and proteins, with yellow yolk making up about 98 % and white yolk about 2 %.

1.3.Global egg production

In every country in the globe, chicken eggs are a staple of the daily diet and are a good source of affordable, wholesome food **(Ahmed et *al*., 2019a).** Over 76.7 million metric tons of eggs were produced worldwide in 2018. **(1)** China, the United States, Indonesia, India, and Mexico are the top five egg-producing nations, and they collectively produced around 63% of the world's 99 million tonnes (or 1,652 billion eggs) of eggs in 2019. **(2)**

Over 86.3 million metric tons of eggs were produced globally in 2021, down from about 87.07 million metric tons in 2020. The volume of eggs produced globally has increased by more than 100% since 1990. **(1)**

1.4.Applications of egg shells

The literature indicates that industrial eggshell uses can be divided into two categories: raw material and operating supplies. ES waste is rich in bioactive chemicals. Options in the first scenario include biomaterial composite, pure calcium carbonate, soil amendment, food additive, and soil amendment. ES can be utilized as a catalyst and sorbent as an operating supply **(Quina et *al*., 2017).**

Since eggshell organic membranes contain vital substances including collagen, hyaluronic acid, glucosamine, and chondroitin sulphate, they have been utilised in the medical, pharmaceutical and cosmetic industries. These elements are scarce in nature or it may be challenging to attain the desired purity, therefore when they are recovered from other natural resources, processing costs might be high.

Eggshells have a wide range of uses in many different industries, including food, art, building, fertilizer, and medicine. It is also believed that they are a better source of calcium than limestone. It has been suggested that eggshells can be used as an alternate source of soil stabilizing agent. The eggshell waste can be utilized in the manufacturing of biodiesel as a solid base catalyst used to reduce biodiesel pollutants, lowering the cost of producing biodiesel and making the process entirely ecological and environmentally friendly; as a severe environmental concern in the ecosystem, heavy metals from wastewater can be used as an absorbent; as a biomaterial to replace bone tissues due to an increase in patients; and as a fertilizer and calcium supplement in food for people, animals, plants, etc. **(3)**

1.5.Membrane removal methods

There are a lot of egg shells as a result of the technical methods used in the egg industry, which causes environmental issues and adds to the cost of managing them. It is required to remove eggshell membranes from eggshells for a superior valorization to produce active principles with high biological and commercial value. As a result, environmentally friendly and effective methods have been created. The value of the finished products is increased by the thorough separation of membranes and shells. The shell membrane can be obtained using a variety of techniques **(Devi et *al*., 2012).**

1.5.1. Chemical treatments to remove membranes from eggshell

Information was evaluated regarding the potential use of egg shell minerals and protein membranes in technology. There are many potential uses, ranging from low-cost ones like fertilizer and animal feed to high-cost ones like converting into food for humans, absorbing heavy metals, treating paper, biodiesel production catalysts, producing hydrolyzed or concentrated protein, making bone, and creating dental implants. The most recent applications require significant investments, but they can yield more beneficial outcomes. In order to add value by utilising these materials in ecologically safe ways, industrial plants that can utilise egg shell leftovers can be developed. Process alternatives and their anticipated environmental implications are taken into consideration as a preliminary study. Overall, all of the offered procedures offer significant economic and environmental advantages (**Oliveira et *al*., 2013)**

(Cusack and Fraser, 2002) suggested that membranes might be successfully removed from eggshell using treatments with sodium hypochlorite (such as bleach), hydrochloric acid (HCl) or by plasma ashing (etching). With the intention of subsequently extracting intra- and intermineral proteins, these three approaches are contrasted. Plasma ashing is appropriate for subsequent SEM of the eggshell's inner surface but is completely inappropriate for subsequent protein extraction. Both acid (HCl) treatment and the use of sodium hypochlorite are effective ways to remove the inner membrane. Since the protein production is maximum here and, unlike acid treatment, it does not partially eliminate the calcite fraction, sodium hypochlorite treatment is the favored approach.

1.5.2. Mechanical processes to remove membranes from eggshell

Eggshells and membranes, which are typically discarded as waste, contain a wealth of valuable bioactive components that can be recovered by effective separation of eggshell and membrane **(Hussain and Abid, 2023).**

In order to solve the environmental and financial problems connected with the disposal of used egg shells, **(MacNeil and Joseph, 2003)**devised a method for passively dissociating eggshell particles in a tank of liquid (ideally water) to separate the membranes from the eggshell particles. By using a reduction device, where the cutting operation reduced the size of the shell particles to between 0.5 mm and 4.0 mm, the abrasion was accomplished. The eggshell fragments were then added to a sizable water tank, where they were allowed to sink to the bottom as depicted in **Figure 02**. The membranes and shell fragments totally separated as a result of the water's turbulence when the particles landed. Gallon-sized quantities of water were needed for the invention's industrial setup, which raised the cost of production. Additionally, this strategy only works in batches.

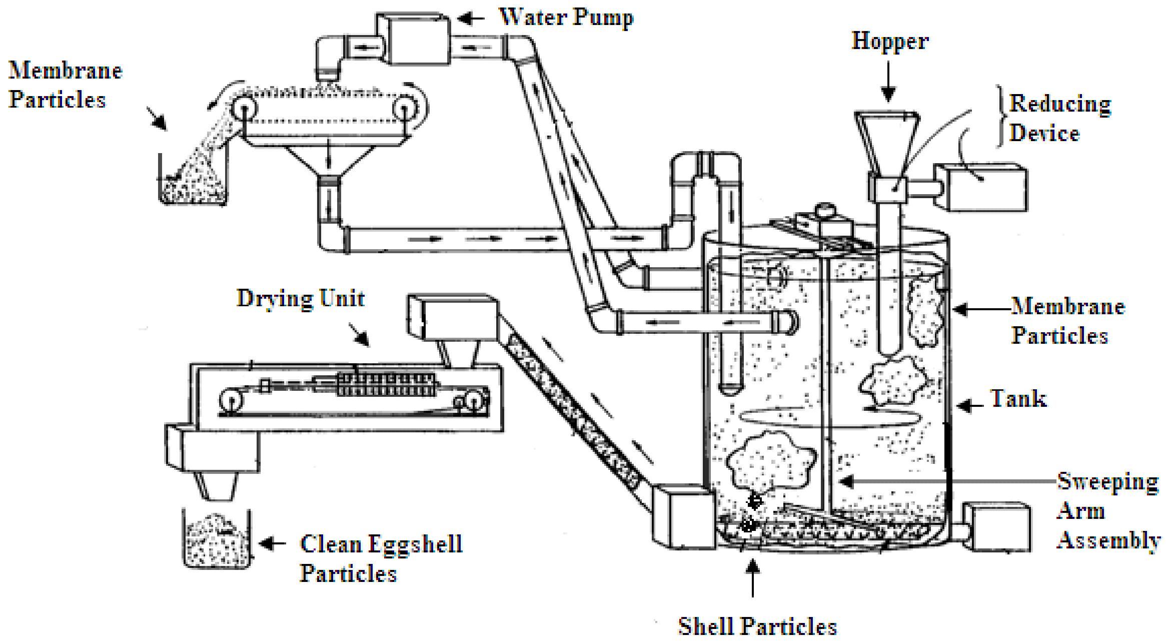

Figure 02: Apparatus for separation of eggshell and membrane **(MacNeil, 2001)**

2. **Calcium carbonate**

With the chemical formula ($CaCO_3$), calcium carbonate is the carbonic salt of calcium made up of calcium (Ca_2^+) and carbonate(CO_3^{2-}) ions. Calcium carbonate can be found as colorless crystals or as a white, odorless powder. Water practically insoluble. **(4)**It is a typical substance that is present in rocks all over the world (most prominently as limestone), and it is the primary constituent of the shells of marine animals, snails, coal balls, pearls, and eggshells. $CaCO_3$ comes in a variety of polymorphs, each of which has a unique stability that depends on a wide range of factors **(Al Omari et *al*., 2016).**

$Ca^{2+} + CO_3^{2-} \rightleftharpoons CaCO_3$

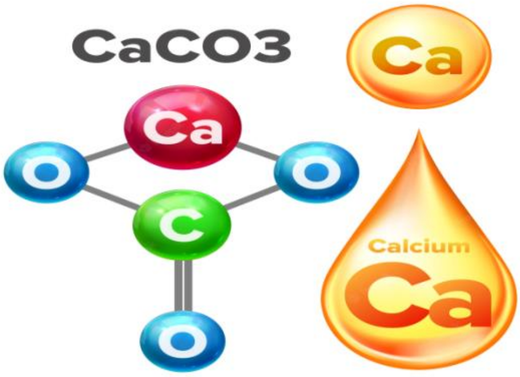

Figure 3: Molecular structure of calcium carbonate.

Table (1): Physico-chemical data of calcium carbonate.

Formula	**Mass Molar**	**Mass fractions**	**Melting Temperature**	**Solubility**	**PKa**	**Molar entropy**	**Molar heat capacity**
$CaCO_3$	100,0869 ± 0,006 g/mol	Carbon 12 % Calcium 40,04 % Oxygen 47,96 %	825 °C	0.013g/L (at 25°C)	9	93 J/mol.K	81.9 J/mol.K

2.1. Nomenclature

2.1.1. SystematicChemicalNames

- Calcium carbonate.
- Carbonic acid calcium salt.

2.1.2. Non proprietary Names

- ✓ **Recommended international non proprietary name:** Calcium carbonate.
- ✓ **Synonyms:** E 170, calcite, aragonite, vaterite, chalk, CI pigment white 18, drop chalk, prepared chalk, whiting, English white, Paris white **(Al Omari et *al*., 2016).**

2.1.3. Proprietary Names

Calcium Carbonate is available under the following different brand names:

Maalox, Mylanta, Rolaids, Tums, Alka-Mints, Calel-D, Caltrate 600, Chooz, Os-Cal 500. **(5)**

Tums, Tums Chewy Delights, Tums Extra, Tums Freshers, Tums Kids, Tums Regular, Tums Smoothies, and Tums Ultra or Children's Pepto**. (6)**

2.2. Calcium carbonate applications

Among the carbonates, calcium carbonate, with a worldwide market share of approximately 70%, plays the dominant role. How is calcium carbonate used in everyday life? Applications of calcium carbonate in industries?

Calcium carbonate ($CaCO_3$) is a substance that is widely used for many different things. For instance, it is used as a filler and pigment material in paper, plastics, rubbers, paints, and inks as well as in pharmaceutics, cosmetics, construction materials, asphalts, and as a nutritional supplement in animal foods....

- ➢ **Health and food uses**

In the field of medicine, calcium carbonate is used as a food additive for its nutritional benefits, as a calcium supplement to treat low serum calcium conditions, as an antacid for stomachaches, as a phosphate binder for chronic kidney disease, as a tableting excipient for other pharmaceutical agents, and as a tableting excipient for foods.

For low serum calcium conditions like osteoporosis, osteomalacia, hypothyroidism, hypoparathyroidism, pseudohypoparathyroidism, DiGeorge syndrome, kidney dysfunction, pancreatitis, rheumatoid arthritis, Fanconi syndrome, pregnancy, nursing mothers, post-menopausal women, and when taking specific medications, calcium carbonate is recommended **(Salisbury and Terrell, 2022; Karatzas et *al*., 2017; Sunyecz, 2008; Bilezikian et *al*., 2016; Jorde et *al*., 2002; Nakamura et *al*., 2017; Scott, 1981).**

The drugs that necessitate taking calcium supplements concurrently are:

- Rifampin (antibiotic)
- Loop-diuretics
- Anti-seizuremedications (phenytoin and phenobarbital)
- Bisphosphonates (alendronate, ibandronate, zoledronic acid, and risedronate)
- Calcitonin
- Chloroquine
- Corticosteroids
- Plicamycin
- Citrate use during apheresis (anticoagulant)**(Lim et *al*., 2009; Lee and Arepally, 2012; Buckley et *al*., 1996).**

NSAID upper gastrointestinal mucosal damage, duodenal and gastric ulcers, biliary reflux, stress gastritis, exocrine pancreatic insufficiency, bile acid-mediated diarrhea, non-ulcer dyspepsia, and urinary alkalization are all conditions for which calcium carbonate is prescribed as an antacid **(Al Omari et *al*., 2016; Salisbury and Terrell, 2022; Matonand Burton, 1999; Dağlıand Kalkan, 2017).**Calcium carbonate improves intestinal motility and has applications for treating constipation, just like aluminum and magnesium salts **(Matonand Burton, 1999; Matzkies et *al*., 1984).**

- **Cosmetics Uses**

Because calcium carbonate can be produced in a relatively quick, cheap, and efficient reaction, $CaCO_3$ is also employed in the cosmetics sector. The dry, white powder is used to make cosmetic foundation, baby powder, eye shadow, and face powder. Calcium carbonate, which whitens teeth, is a common component in toothpastes.**(7)**

- **Agriculture and animal husbandry uses**

A calcium fertilizer improves deficient soil, and mixes containing calcium carbonate are ideal animal food supplements. Carbonate is frequently used in agricultural and animal husbandry. The amount of calcium carbonate ($CaCO_3$) in the soil is a good predictor of soil quality; the more $CaCO_3$, the less stable and productive the soil is. Calcium bicarbonate is used by dairy cattle producers to increase lactation in cows and strengthen the gastrointestinal system in animals. Similar to calcium carbonate used for hens, calcium carbonate used for cattle improves livestock health by reducing infections, weakness, and acidity.**(8)**

3. Collagen

The term collagen means "glue producer" (the name comes from the Greek word kolla meaning "glue"). Its properties were used by the Egyptians 2,000 years before. Native Americans used it in the 5th century. The oldest known glue is made from collagen, and dates back 6,000 years. **(9)**

A class of protein is collagen. In actuality, it is the structural protein that is most prevalent in mammals. A structural protein is one that provides your cells and tissues with their structure or framework **(Nezwek and Varacallo, 2022).**

There are 28 varieties of collagen that are now recognized, with type I collagen making up 90% of the collagen in the human body **(Ricard, 2011).**

The four most prevalent kinds are:

- **Type I:** All connective tissue, especially scar tissue, tendons, ligaments, bone, cornea, skin, and dentin, contain type I collagen, which is also the most prevalent form. Type I collagen is strong, flexible, and resistant to force, tension, and strain.
- **Type II:** the articular and hyaline cartilage of joints, as well as the intervertebral discs, give resistance to pressure.
- **Type III:** provides a flexible meshwork for cellular support. Reticulum fibers are frequently seen in organs like blood vessels and skin. Additionally prevalent in the early phases of wound healing and contributes to the production of granulation tissue **(Ehrlich and Krummel, 1996).**
- **Type IV:** The kidney, inner ear, and lens of the eye all include basement membrane, which is made up of a meshwork that supports and attaches to the extracellular matrix beneath.

Fibroblasts, macrophages, adipocytes, leukocytes, and mast cells are common connective tissue cells **(Mescher, 2017).**

Glycine, proline, and hydroxyproline are the three amino acids that make up the majority of collagen. The triple-helix structure of collagen is made up of these amino acids, which are organized into three strands **(Saghaleini et *al*., 2018; Szulc, 2018).**

Collagen is found in connective tissue, skin, tendons, bones, and cartilage. It supports tissues structurally and is crucial for several biological functions, including **((10); Zhao et *al*., 2021; Izu et *al*., 2020; Yamada and Sixt, 2019) :**

- ✓ tissue repair
- ✓ immune response
- ✓ cellular communication
- ✓ cellular migration, a process necessary for tissue maintenance

Collagen is created and maintained by fibroblasts, which are connective tissue cells. Collagen fragmentation, reduced fibroblast activity, and slowed collagen production are all effects of aging. **(10)(11)**.Age-related symptoms like drooping skin and wrinkles are brought on by these changes as well as the loss of elastin, another important structural protein.

3.1.Collagen Synthesis and Structure

In the rough endoplasmic reticulum of intracellular space, DNA translation of a polypeptide chain initiates the manufacture of collagen. Preprocollagen is a polypeptide chain made up of alpha chains with repeated glycine-X-Y sequences (where X and Y are either proline or lysine). **(Pinnell, 1982)**

The vitamin C dependent hydroxylases hydroxylate the proline and lysine residues of the alpha chain, which aids in the formation of a stable structure. Scurvy, a disorder caused by a lack of vitamin C, is characterized by deficient preprocollagen hydroxylation and inefficient collagen production **(Lykkesfeldt et *al*., 2014).**

The alpha chain's hydroxylated lysine then goes through glycosylation by being given a carbohydrate. Following glycosylation, a triple helix is created when disulfide and hydrogen connections form between three distinct alpha chains. Procollagen is the final structure.

Osteogenesisimperfecta, which is characterized by poor bone matrix production, is brought on by defective triple helix formation **(Marini et *al*., 2017).**

Next, exocytosis is then used to transport procollagen to the extracellular environment. Procollagen is split at the C- and N-termini once it reaches the extracellular space, creating a substance that is now known as tropocollagen. The cleavage results in the insoluble nature of tropocollagen in water.

By covalently attaching hydroxylated lysine residues to tropocollagen, multiple nearby tropocollagen molecules are then strengthened, forming collagen fibrils. A copper-dependent lysyl oxidase enzyme enables the cross-linking of hydroxylysine residues.

Finally, a number of these collagen fibrils combine to form a substantial bundle that is known as collagen fibers**(Last and Reiser, 1984).**

3.2.Applications of collagen

Collagen is the primary raw material used to create gelatin from an industrial and economic standpoint. Tropocollagen breaks up into its three strands when it is partially hydrolyzed. Thus, gelatin is created. Additionally, it has been applied to cosmetics, biomedical and the pharmaceutical industry...

- **Cosmetic applications**

Due to its moisturizing, renewing, and film-forming qualities, collagen is one of the major ingredients in cosmetic compositions. The skin's proper water content is maintained throughout the day thanks to its excellent capacity to bind water. The skin is hydrated and become softer. In addition to being a natural humectant, collagen also has film-forming abilities that lessen. Damage to the skin and hair brought on by mechanical deficiencies is avoided by peptide occlusion. Occlusion also improves skin's radiance, illumination, and smoothness**(Helfrich et *al*., 2008; Chung et *al*., 200).**Collagen according to research, expedites wound healing and aids in tissue regeneration **(Secchi, 2008; Peng et *al.*, 2004; Nagelschmidt and Struck, 1974; Li et *al*., 2005; Pachence et *al*., 1987; Jridi et *al*., 2015).** As a result, collagen is frequently employed in the cosmetics industry. It can be utilized for both hair and skin care. **Figure 4** illustrates collagen's potential for cosmetic use.

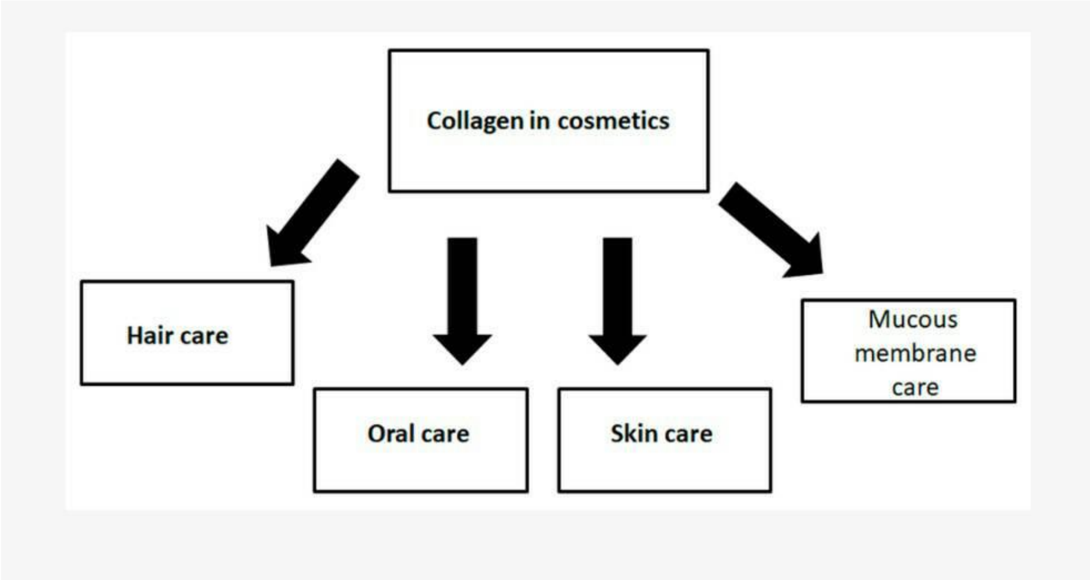

Figure 04: The application of collagen in cosmetics.

In cosmetic medicine, collagen fillers are further employed. By mending its dermatological flaws, the subcutaneous injection of soluble collagen enhances the quality and density of the skin (**Pachence et *al*., 1987).** Age-related indications of aging are removed with collagen fillers. The extender must to be biocompatible, non-allergic, simple to take out, and eventually biodegradable. Tests for allergies must be performed prior to the surgery. The fact that collagen fillers are safe, natural, biodegradable, and have repeatable results makes them quite popular **(Newman, 2009; Requena et *al*., 2011; Buck et *al*., 2009; DeVore et al., 1996).**

Many hydrogels that can be used as a "beauty mask" contain collagen as their primary ingredient. This kind of cosmetic ought to improve skin elasticity and anti-aging results **(Mitura et *al*., 2020)**. In addition, a variety of polymers with natural origin can be used as thickening agents in cosmetic compositions. Although collagen can be employed as a thickening agent on its own, the exorbitant cost of natural collagen precludes such a decision. The preferred thickening ingredient is typically gelatin, a far less expensive, denatured version of collagen **(Sionkowska et *al*., 2016).**

- **Medical applications**

Since more than 50 years ago, collagen and products made from collagen have been utilized successfully in medicine. Recent years have seen a sharp rise in the number of academic papers discussing collagen's use in creating scaffolds for tissue engineering. Collagen has been used in medicine for a long time. Current uses include decellularized collagen matrices, soluble collagen injections, and solid structures created from solutions. Collagen material is effective in treating chronic wounds, burns, venous and diabetic ulcers, plastic and reconstructive surgery, urology, proctology, gynecology, ophthalmology, otolaryngology, neurosurgery, dentistry, cardiovascular, bone, and cartilage surgery, as well as cosmetology, according to analysis of published data **(Shekhter et *al*., 2019).**

Chapter 02:

Material and methods

1. Study framework

Our work was carried out in the laboratory of Toxicology, department of applied biology, Larbi Tébessi University, Tébessa, during the period from 22/02/2023 to 15/05/2023.

2. Objective

Eggshells are considered a pollutant to nature because their decomposition produces compounds like ammonia, hydrogen sulfide and amine, releasing high levels of contaminants and odors. Eggshells can also carry pathogens like E. coli or salmonella which is harmful to human health. The goal of this study is to extract calcium carbonate and collagen as bio natural products, and trying to reduce environmental pollution by recycling waste.

3. Material

Eggshells were obtained from two different restaurants in Tebessa, about1 kg in one day.

Figure 05: Collected eggshells.

3.1.Reagents required for calcium carbonate extraction

- 10% Acetic acid (CH_3COOH) or Hydrochloric acid (HCl)

3.2.Reagents required for collagen extraction

- 0.45 M Sodium chloride (NaCl)
- 0.2% Sodium hydroxide (NaOH)
- 0.2% Sulphuric acid (H_2SO_4)
- 0.7% Citric acid ($C_6H_8O_7$)

- 10% Sodium chloride (NaCl)
- 1% Hydrogen peroxide (H_2O_2)
- 0.01M Sodium hydroxide (NaOH)
- 0.5 M Acetic acid or 0.5 M Citric acid.

3.3.Equipment and glassware

The equipment, instruments and glassware used are listed in Annexe (1).

4. Methods

4.1.Eggshell preparation

The eggshells are first collected, then they are washed well with water and sterilized.

Eggshell membranes are separated manually, chemically or mechanically. In our work, we separated the two membranes manually.

The outer membrane is for the extraction of calcium carbonate and the inner membrane for collagen.

4.2.Calcium carbonate extraction

After separation the eggshells are dried in an oven at 200° F (93° C) for 10 to 15 minutes or until they are completely dry and brittle. Then they are crushed into small pieces using a mortar and pestle or a food processor

Figure 06: Eggshells crushing process in the mortar.

The crushed eggshells are transferred to a beaker or container with a lid. Then enough 10% acetic acid (CH_3COOH) is poured over the eggshells to completely cover them. The ratio used is (1:1 w v-1) i.e. g/ml. 100g of eggshells with 100ml of acetic acid solution.When acetic acid is poured on crushed egg shells, bubbles and foam appear as shown in **Figure 07.**

Figure 07: Bubbles and foam appear after pouring the (CH_3COOH)

The beaker or container is closed with the lid and left to stand at room temperature for at least 24 hours with the mixture each time with the stirrer as shown in **figure 08**, when we notice that it has started to dissolve, we increase the volume of the solution and we continue to stir until the eggshells are completely dissolved.

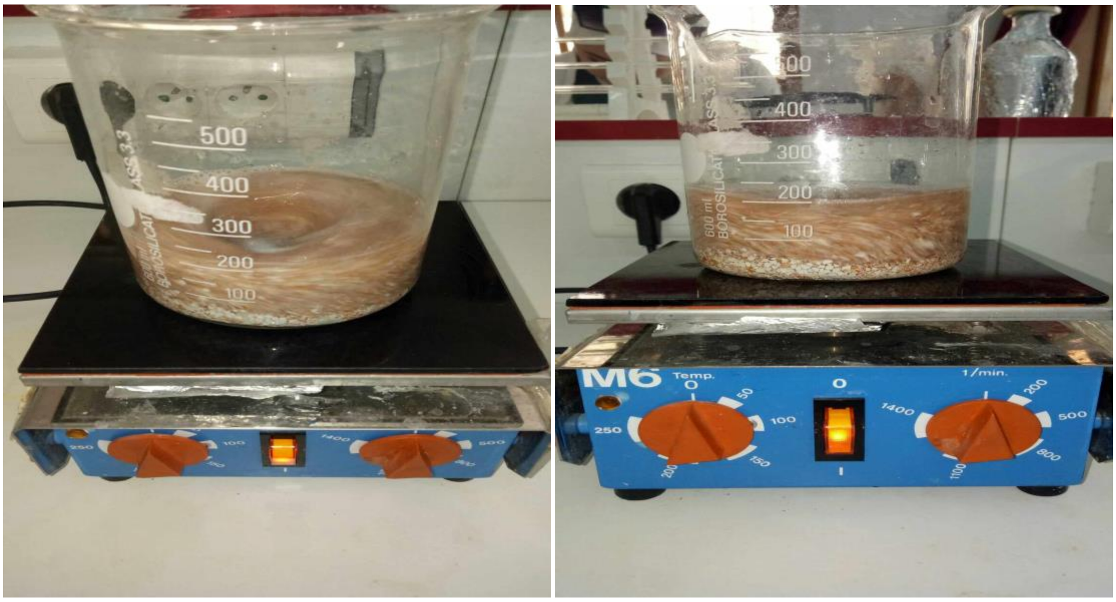

Figure 08: Stirring the mixture by magnetic stirrer.

Then, the mixture is filtered through a coffee filter or cheesecloth to remove any remaining shells or impurities.

Figure 09: Mixture filtration process.

The filtered liquid is transferred to a clean beaker and allowed to evaporate in a warm, dry place (Shaker water bath in 80°C).

Figure 10: Evaporation process.

Once the liquid has completely evaporated, we obtain a white powder, which is calcium carbonate.

Figure 11: Calcium carbonate powder.

4.3.Collagen extraction

➢ **Pre-treatment of eggshell membrane**

The pretreatment process is to remove impurity such as soluble non-collagenous compound, lipids, pigments and aftertaste.

In the first step, soluble non-collagenous compounds were removed by cold water.

In our work, we used the ratio (1:6 w v-1). 50g of eggshell inner membrane with 300 ml of acid solution.

The eggshell membrane was mixed with cold water (1:6 w v-1) using a blender at 4 °C for 3 min and filtered.

The membrane was then mixed with 0.45 M NaCl (1:6 w v-1) using shaker for 3 min. The retentate was homogenised with 0.45 M NaCl (1:6 w v-1) at 6 000 rpm for 4 min as shown in **figure 12**. Then the retained membrane was washed with distilled water(1:6 w v-1) and centrifuged at 2 000 g, 4 °C for 30 min**(figure 13)**.

Figure 12: Retentate homogenisation.

Figure 13: Centrifugation of the membrane.

Finally, the precipitate was collected to remove lipids, pigments and off-flavour by stirring in the following chemicals: 0.2% (w v-1) NaOH, 0.2% (w v-1) H_2SO_4 and 0.7% (w v-1) citric acid, respectively, at the ratio of precipitate to each solution 1:7 (w v -1), for 4 min, washed with water to pH 7 and filtered **(Figure 14, 15)**. After that, the retentate was soaked in 10% NaCl (w v-1) for 24 h at room temperature, filtered, bleached with hydrogen peroxide (1% v v-1) H_2O_2 in 0.01 M NaOH) at the ratio of 1 to 6(w v-1) for 24 h at room temperature, neutralised and washed with distilled water.

Figure 14: pH regulation.

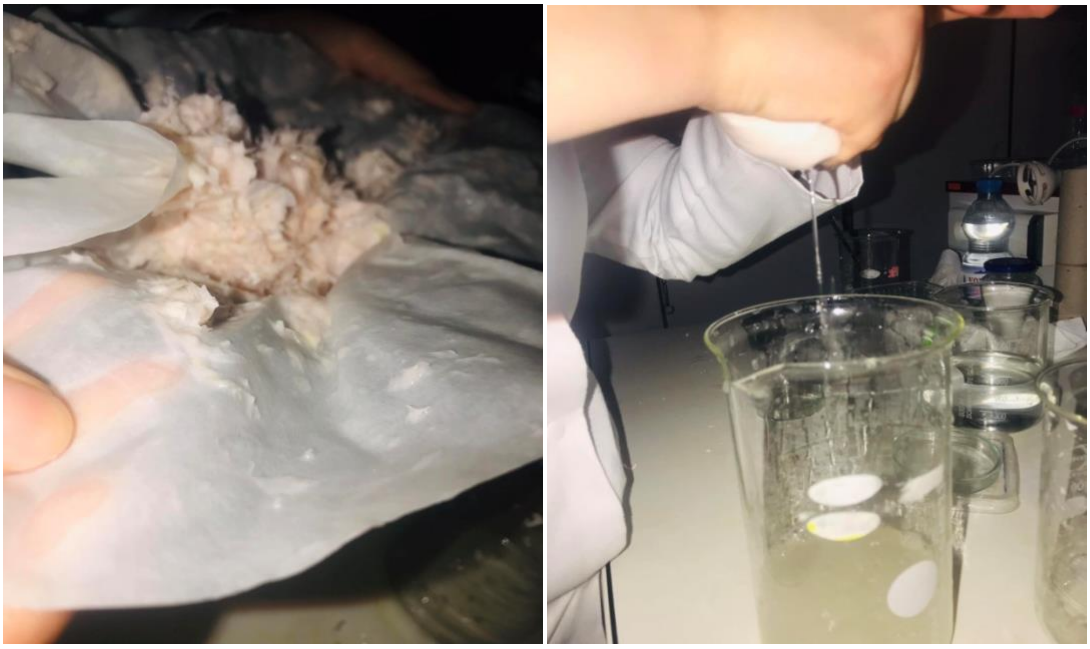

Figure 15: Filtration by filter paper.

- **Extraction process**

Collagen was extracted from the membranes, obtained from the pre-treatment process, with either 0.5 M acetic acid or 0.5 M citric acid. In our work, we used acetic acid. Membranes were thoroughly mixed with either acid always in the ratio (1:6 w v-1), in a water bath at 4 °C for 2 h. The mixtures were then removed, centrifuged for 4 min at 6.000 rpm.

Mixed again in the water bath (4 °C) for 24 h, homogenised for 2 min (6.000 rpm, 4 °C) and finally centrifuged at 10.000 g for 20 min. The precipitate was extracted three times. The final result is soluble collagen **(Figure 16)**.

Figure 16: Soluble collagen.

5. Determination of calcium carbonate content in eggshell by back titration

Back titration is done when an excess amount of standard solution is required for a complete reaction with the analyte. Thus, the analyte cannot be titrated directly. Instead, an excess amount of standard solution is added to the analyte. The amount of excess reagent is determined by titration (back titration).

In this experiment, the calcium carbonate content in eggshell is to be determined by back titration. The amount of calcium carbonate can be determined by measuring the amount of standard hydrochloric acid required to react completely with it. The reaction of HCl with $CaCO_3$ is as follows:

$$CaCO_3\,(s) + 2\,HCl(aq) \rightarrow CaCl_2(aq) + H_2O(l) + CO_2(g)$$

Since $CaCO_3$ is insoluble in water then an excess amount of HCl should be added for a complete reaction. The amount of the HCl should be exactly known. After the reaction has been completed there will be some excess HCl. The number of mole of the excess HCl can be determined by titration with a standard NaOH solution. Then, the number of mole of HCl that reacted with $CaCO_3$ can be calculated. From this, the amount of $CaCO_3$ in the sample can be determined.

5.1.Objective

To determine the calcium carbonate content in eggshell.

5.2.Chemicals and materials

- 0.25 M Potassium hydrogen phthalate ($KHC_8H_4O_4$)
- Sodium hydroxide solution
- Hydrochloric acid solution
- Phenolphthalein indicator
- Methyl orange indicator
- Eggshell

5.3. Procedure

- **Standardization of the NaOH solution**

1. The burette filled with the NaOH solution as shown in **figure 17**.

Figure 17: Burette full of NaOH solution

2. Pipette 20 mL of the standard potassium hydrogen phthalate, $KHC_8H_4O_4$ solution into a beaker then we add 2 drops of phenolphthalein indicator **(figure 18)**.

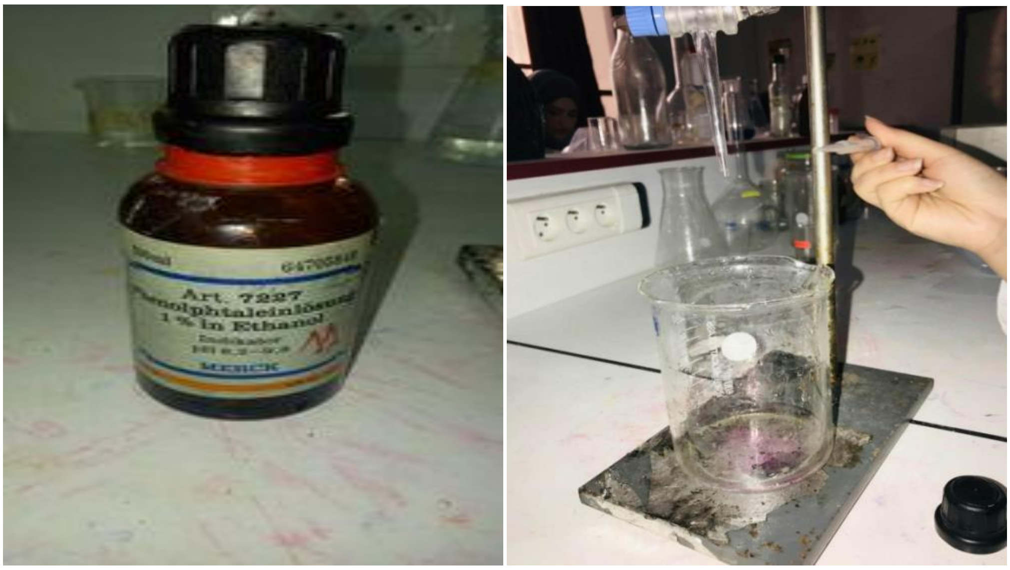

Figure 18: Adding of phenolphthalein indicator

3. The $KHC_8H_4O_4$ titrated with the NaOH solution.

Figure 19: Appearance of purple colour after titration

4. Repetition of the titration two more times.

5. Then we calculate the concentration of the NaOH solution.

- **Standardization of the HCl solution**

1. Pipette 10 mL of the HCl solution into a beaker, then we add 2 drops of phenolphthalein indicator.

2.HCltitratedwith the NaOH solution from the burette the color change as the **figure 19**.

3. The titration repeated once.

4. Then we calculate the concentration of the HCl solution.

- **Analysis of the eggshell**

1. Some dried eggshells have been obtained with the inner membrane removed. The eggshell was ground in a mortar into small particles. (about the size of fine sand).

Figure 20: Grind eggshell.

2. About 0.5 grams of eggshell was weighed into a beaker.

3. Pipette 25 mL of the standard HCl solution into the Beaker that contains the eggshell. Wait for about 2-3 minutes for the reaction to complete.

4. The reaction mixture was heated until it boiled. It was cooled and two drops of methyl orange indicator were added to it.

Figure 21: Adding of methyle orange indicator.

5. The mixture titrated with the NaOH until the colour changed to yellow.

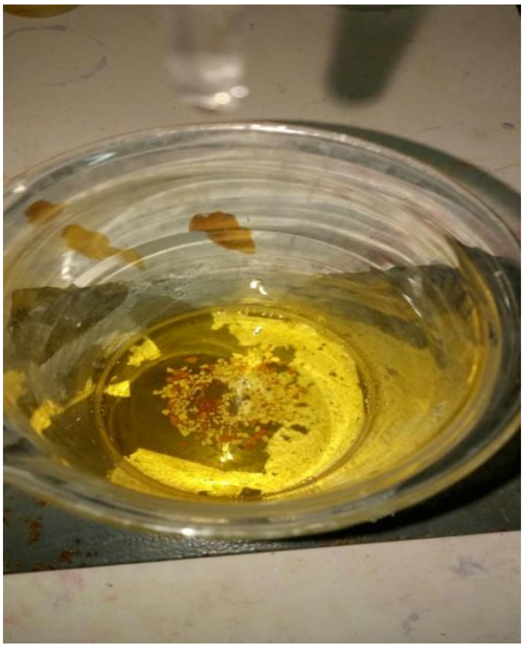

Figure 22: The colour of solution changed to yellow after titration

6. Steps 2 to 5 repeated.

7. The percentage of $CaCO_3$ in the eggshell is calculated in each iteration.

8. The mean percentage of $CaCO_3$ is calculated in the eggshell.

Chapter 03:

Results and discussion

1. Result of the extraction

During the study period, we were able to collect 1 kg of eggshells from 2 different restaurants, we used just 100g of the extern membrane to extract calcium carbonate and 50g of the inner membrane to extract collagen and the result was high yield of pure material:

From 100g of extern membrane of eggshells we managed to get 70,33 g of calcium carbonate. **(Figure 23)**

Figure 23: Result of $CaCO_3$ extraction.

From 50g of inner membrane of eggshells we managed to get 55ml of soluble collagen.

Figure 24: Result of collagen extraction.

2. Result of the determination of calcium carbonate content in eggshell by back titration

a) Standardization of the NaOH solution

Table (2): Standarization of the NaOH solution

	Replicate 1	**Replicate 2**	**Replicate 3**
Initial burette reading (mL)	0	0	0
Final burette reading(mL)	34.4	35.6	35.5
Volume of NaOH used (mL)	34.4	35.6	35.5

b) Standarization of HCl solution

Table (3): Standarization of HCl solution

	Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate 4**
Initial burette reading (mL)	0	0	0	0
Final burette reading(mL)	25.6	25.7	25.8	25.9
Volume of NaOH used (mL)	25.6	25.7	25.8	25.9

c) <u>Analysis of the eggshell</u>

Weight of the eggshell = 0.5 g

Table(4): Analysis of eggshell.

	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Initial burette reading (mL)	0	0	0	0
Final burette reading(mL)	28.7	28.9	28.8	28.9
Volume of NaOH used (mL)	28.7	28.9	28.8	28.9

- **Calculation of the concentration of NaOH solution for the standardization of the NaOH solution**

Equation: $KHC_8H_4O_4$+ NaOH - > $NaKC_8H_4O_4$+ H_2O

Molarity of $KHC_8H_4O_4$ = 0.25M

Volume of KHC_8H_4O = 0.02L

Molarity = n / v

0.25 = n / 0.02 L

= 0.005 mol

Ratio : 1 mole of NaOH : 1 mole of $KHC_8H_4O_4$, this means that NaOH is 0.005 mol . Average volume of NaOH used 35.17 mL .

Molarity NaOH = n / v

= 0.005 mol / 0.03517 mL

= 0.142 M

- **Calculation of the concentration of HCl solution**

Equation:**NaOH + HCl - >NaCl + H_2O**

Average Volume of NaOH used = 25.75 mL

Molarity of NaOH = 0.142M

Molarity NaOH = n / v

0.142 n / 0.02575 L

n = **0.00366 mol**

Ratio : 1 mole of NaOH : 1 mole of HCL , this means that HCI is 0.00366 mol .

Average volume of HCI used = 0.01 L

Molarity of HCI = 0.00366 mol / 0.01 L

= 0.366 M

- **Calculation of the number of moles excess HCl reacted with the eggshell by using the help of NaOH**

Equation:**NaOH + HCl - >NaCl + H_2O**

Average volume of NaOH used in analysis of eggshell = 0.02825 L

Number of mol of NaOH = Molarity of NaOH x volume of NaOH used

= 0.142 M x 0.02825 L

= 4.0115X 10^{-3} mol

Ratio : 1 mole of NaOH : 1 mole of HCL . Thus,

mole of HCL = 4.0115X 10^{-3} mol

Initial mol of HCI = Molarity of HCI x volume of HCI used

= 0.366 M x 0.025 L

= 0.00915 mol

Number of mol excess HCl = 0.00915 mol 4.0115X 10^{-3} mol

= 5.1385x 10^{-3} mol

- **Calculation of the mass of $CaCO_3$ in eggshell**

  Equation:**CaCO3 + 2HCl - >$CaCl_2$ + H_2O + CO_2**

Ratio: 1 mole of $CaCO_3$: 2 mole of HCl, thus 5.1385x 10^{-3} mol must be divided into 2 5.1385x 10^{-3} / 2 = 2.56925x 10^{-3}mol of CaCO3

Mass of CaCO3 = number of moles x molar mass

= 2.56925x 10^{-3} mol x 100.1 $gmol^{-1}$

= 0.257 g

- **Calculation of the percentage of $CaCO_3$ in the eggshell in each replicate and its mean percentage.**

  Replicate 1:

  Equation: **NaOH + HCl - >NaCl + H_2O**

NaOH = 28.7 mL

Molarity of NaOH = 0.142M

HCl = 25 mL

Initial mole of HCI = 0.00915 mol

$M_1V_1 = M_2V_2$

M_{HCl} =(0.142M X 0.0287L) / 0.025L

= 0.163M

No of mole HCI used = 0.163M X 0.025L

= 4.075 x 10^{-3} mol

Moles of HCI reacted = 0.00915 mol - 4.075 x 10^{-3} mol

= 5.075 x 10^{-3} mol .

$CaCO3 + 2HCl$ - >$CaCl_2 + H_2O + CO_2$

Ratio : 1 mole of $CaCO_3$: 2 mole of HCL

CaCO3 = 5.075 x 10^{-3} mol / 2

= 2.5375 x 10^{-3} mol

Mass of $CaCO_3$ = number of moles x molar mass

= 2.5375 x 10^{-3} mol x 100.1 $gmol^{-1}$

= 0.254g

Percentage = (mass of $CaCO_3$ / mass of eggshell) x 100 %

= 0.254g / 0.5g x 100 %

= 50.8 %

Replicate 2:

NaOH = 28.9

Molarity of NaOH = 0.142M

HCI = 25 mL

Initial mole of HCI = 0.00915 mol

$M_1V_1 = M_2V_2$

M_{HCl}=(0.142M x 0.0289L) / 0.025L

= 0.164M

No of mole HCI used = 0.164M X 0.025L

= 4.104 x 10^{-3} mol

No of mole HCI reacted = 0.00915 mol - 4.104 x 10^3 mol

= 5.046 x 10^3 mol

$CaCO3 + 2HCl$ - >$CaCl_2 + H_2O + CO_2$

Ratio : 1 mole of CaCO3 : 2 mole of HCl

CaCO3 = 5.046 x 10^{-3} mol / 2

= 2.523 x 10^{-3} mol

Mass of CaCO3 = number of moles x molar mass

= 2.523 x 10^{-3} mol x 100.1 $gmol^1$

Percentage = 0.253g

= (mass of $CaCO_3$ / mass of eggshell) × 100 %

= 0.253g / 0.5g x 100 %

= 50.6 %

Replicate 3:

NaOH = 28.8

Molarity of NaOH = 0.142M

HCI = 25 mL

Initial mole of HCl = 0.00915 mol

$M_1 V_1 = M_2V_2$

M_{HCl}= (0.142M x 0.0288L) / 0.025L

= 0.163M

No of mole HCI used = 0.163M x 0.025L

= 4.089×10^{-3} mol

No of mole reacted Percentage= 0.00915 mol - 4.089×10^{-3} mol

= 5.061×10^{-3} mol

$CaCO3 + 2HCl \text{ - >} CaCl_2 + H_2O + CO_2$

Ratio : 1 mole of CaCO3 : 2 mole of HCL

$CaCO_3$ = 5.061×10^{-3} mol / 2

= 2.531×10^{-3} mol

Mass of $CaCO_3$ = number of moles x molar mass

= 2.531×10^{-3} mol x 100.1 $gmol^{-1}$

= 0.253g

Percentage = (mass of $CaCO_3$ / mass of eggshell) x 100 %

= 0.253q / 0.5g x 100 %

= 50.6%

Replicate 4:

NaOH = 28.9

Molarity of NaOH = 0.142M

HCI = 25 mL

Initial mole of HCl = 0.00915 mol

$M_2V_1 = M_2V_2$

$M_{HCl=}$(0.142M x 0.0289L) / 0.025L

= 0.164M

No of mole HCI used = 0.164M X 0.025L

= 4.104 x 10 - ^{3} mol

No of mole HCI reacted Percentage = 0.00915 mol - 4.104 x 10^{-3} mol

= 5.046 x 10^{3} mol

$CaCO_3 + 2HCl$ - >$CaCl_2 + H_2O + CO_2$

Ratio : 1 mole of $CaCO_3$: 2 mole of HCL

CaCO3 = 5.046 x 10^{-3}mol / 2

= 2.523 x 10^{-3}mol

Mass of CaCO3 = number of moles x molar mass

= 2.523 x 10^{-3} mol x 100.1 $gmol^{-1}$

= 0.253g

Percentage = (mass of CaCO3 / mass of eggshell) x 100 %

= 0.253g / 0.5g x 100 %

= 50.6 %

3. Discussion:

The objective of the study was to extract calcium carbonate and collagen from ES and it was supported by the determination of $CaCO_3$ content in ES. In order to recover the animal waste and make it beneficial and usable, and from it trying to limit environmental pollution to be able to preserve human health.

There are four parts performed to achieve the goal. First, the collect of eggshells which were cleaned, sterilized, and their membranes separated manually in preparation for the extraction process.

Second, The ES was dried in an oven at 200° F (93°C) for 10 to 15 minutes and crushed by a mortar into small pieces, a quantity of 100g was used wish covered by 10% of acetic acid and left to stand at room temperature for 24h with constant mixing by magnetic stirrer until it has started to dissolve. Then, the mixture filtred and evaporated in a water shaker bath at 80° until a white powder obtained which is calcium carbonate. The quantity we get from this process is 70.33gof $CaCO_3$.

Third, the membrane obtained from the separation treated with a pretreatment process to remove impurity such as soluble non-collagenous compound, lipids, pigments and aftertaste. The ratio used in this processes is (1:6 wv^{-1}), in the first step the membrane mixed with cold water by a blender, after that with 0.45M of NaCl in shaker, the mixture homogenized at 6000 rpm for 4 min. The retained membrane washed with distilled water and centrifuged at 2000g for 30 min. The precipitate was collected to remove lipids, pigments and off-flavour by stirring in the following chemicals: 0.2% (w v-1) NaOH, 0.2% (w v-1) H_2SO_4 and 0.7% (w v-1) citric acid, respectively, at the ratio of precipitate to each solution 1:7 (w v -1), for 4 min, washed with water to pH 7 and filtered. After that, the retentate was soaked in 10% NaCl (w v-1) for 24 h at room temperature, filtered, bleached with hydrogen peroxide (1% v v-1) H2O2 in 0.01 M NaOH) at the ratio of 1 to 6 (w v-1) for 24 h at room temperature, neutralised and washed with distilled water.

Collagen was extracted from the membranes obtained from the pretreatment with 0.5M of acetic acid. Membranes were thoroughly mixed with the acid always in the ratio (1:6 w v-1), in a water bath at 4 °C for 2 h. The mixtures were then removed, centrifuged for 4 min at 6.000 rpm. Mixed again in the water bath (4 °C) for 24 h, homogenised for 2 min(6.000 rpm, 4 °C) and finally centrifuged at 10.000 g for 20 min. The precipitate was extracted three times.

The final result is 55 ml (27.5%) of soluble collagen. The results obtained in our study differ from those reported in other studies carried out in the same context**(Kheirabadi et *al*., 2018)** noted that the highest amount of extraction was theoretically predicted to be 8.55%, under the optimal by using 0.1–0.9 mol/L of acetic acid and this was done to find the optimum combination of acetic acid concentration, the result was calculated to be 8.35%, which confirms the predicted value and which confirmed that the concentration used in our study is correct but our rate of yield was greater. In another study **(Kuwahara and Junko, 2021)**, the collagen has been extracted from another source tilapia scales with a yield of 1.58% by the aeration of ultrafine bubbles of carbon dioxide gas in a 0.1 M acetic acid.

The 4th part is the determination of $CaCO_3$ content in ES which in turn is divided into 3 parts: a) standardization of the NaOH solution, part b) standardization of the HCI solution and part c) analysis of the eggshell. The experiment started with part a) where Sodium Hydroxide, NaOH solution was titrate with standard potassium hydrogen phthalate, $KHC_8H_4O_4$ solution to standardize the NaOH solution . An indicator was used as the end point of the titration. The endpoint of the reaction was observed when titration change colour to purple. The average volume of NaOH used in titration were 35.7 mL which then used to determine the molarity of NaOH solution. The reaction between two substance is NaOH + $KHC_8H_4O_4$ + $KNaC_8H_4O_4$ + H_2O. From the equation 1 mole of NaOH is equal to one mole of $KHC_8H_4O_4$. The mole of the NaOH is 0.005 mol. So, the molarity of the NaOH was determined as 0.142 M.

Next, part b) was performed by titrating Hydrochloric acid, HCI with NaOH to standardize the HCI solution. Phenolphthalein indicator too was used in the experiment. The average volume of HCI used were 25.75 ml. The reaction between two substances is HCI + NaOH→NaCl + H_2O. From the equation, I mole of HCL is equal to I mole of NaOH. So, the mole of HCL is 0.00366 mol and the molarity determined as 0.366 M. Part c) was performed to analysis the eggshell. The determination was achieved by adding an excess of HCI to dissolve all the calcium carbonate and titrate it with NaOH to calculate the percentage of calcium carbonate in the eggshell. Weight of the grinded eggshell recorded as 0.5 g in a beaker. Then, 25 mL of the standard HCl solution into the Beaker that contains the eggshell and left for 3 minutes. The reaction mixture was boiled, cooled and added 2 drops of methyl orange indicator. Mixture was titrated with NaOH until the colour changes to yellow and whole procedure repeated. The average of HCI used were 28.825 mL. To find the percentage of CaCO3 in the eggshell, Percentage = (mass of $CaCO_3$ / mass of eggshell) x 100 % . The reaction between two substance is $CaCO_2 + 2HCl \rightarrow CaCl_2 + H_2O + CO_2$ From the equation 1 mole of CaCO3 is equal to 2 mole of HCL. So, the percentage of the eggshell determined as 41.03 %. The back titration formula was used to find the number mole HCL excess. Mole excess = Initial mole-mole reacted. The initial mole of HCL found as 0.00915mol and the reacted mole of HCL was $4.0115X\ 10^3$ mol. So, the mole excess of HCL was determined as $5.1385\ x\ 10^3$mol. As recommendations, the titration should be done once the phenolphthalein indicator change colour to get an accurate endpoint reading. Observation on the colour changes is crucial. Next, meniscus level must be considered for every measurement of solute to avoid systematic error. Avoid direct contact with NaOH and HCI because it is corrosive.

Conclusion

Conclusion

Eggshells are considered a pollutant to nature because their decomposition produces compounds like ammonia, hydrogen sulphide and amine, releasing high levels of contaminants and odours. Eggshells can also carry pathogens like E. coli or salmonella which is harmful to human health.

Therefore, the use and transformation of these biological wastes as a basic source for calcium carbonate and collagen is a very important gain for the national economy, especially since the calcium carbonate used in the food industry, pharmaceutical and collagen are imported from foreign countries.

The number of possible uses of $CaCO_3$ is truly impressive! Calcium carbonate powder is used as a natural food supplement (calcium supplement), and as an effective leavening agent and pH regulator in baking powder for bakery products. And also added in pharmaceutical products (for example: antacids, calcium supplement, antibiotics), $CaCO_3$ is mainly used in toothpastes as a thickener and mild abrasive. Calcium carbonate is obtained by a relatively simple, fast and economical reaction, which is why $CaCO_3$ is also used in the beauty industry (used in the manufacture of face powders, baby powders, eye shadows or cosmetic foundations) because of its whitening effect and in order to maintain regular cell renewal.

Calcium carbonate is commonly used in agriculture and animal husbandry a calcium fertilizer enriches depleted soils, while mixtures containing calcium carbonate are perfect feed supplements for animals.

Collagen is a protein present in all structures of the body: skin, cartilage, tendons, ligaments and connective tissues. It represents 30% to 35% of the body's total proteins and ensures the cohesion, elasticity and regeneration of all these tissues.

Collagen is an active ingredient in anti-aging creams, anti-wrinkle cosmetic treatments, masks for mature skin or concealer serums.

With this in mind, our work aimed to extract $CaCO_3$ and collagen from eggshells. The objective of extraction and determination of $CaCO_3$ in eggshells was achieved. As a result of this work, we obtained a high quantity of both products 70.33 g of $CaCO_3$ from 100g of ES and

55ml of collagen from 50g of ES membrane and the mass of percent of $CaCO_3$ in the ES is determined as 50.65%

Acetic acid could significantly extracted calcium carbonate and collagen from eggshells.

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Annexes

Annexe01: Apparatus and glassware used.

Apparatus	Instruments and consumables	Glassware

- Balance -Magnetic Stirrer -Blender -Homogenizer -Centrifuge -Shaker water bath -Oven.	-Spatula - Micropipette - Tips	-Beaker -Erlenmyer flask -Graduated cylinder -Funnel -Burette -Volumetric flask -Droppers -Petri dishes