OPTIMIZATION AND CHARACTERIZATION OF EXTRACTED CALCIUM CHLORIDE FROM EGGSHELL

A THESIS

BY

MANOBENDRO SARKER

Student No: 1305179
Session: 2013-2014
Semester: July-December, 2014

MASTER OF SCIENCE (MS)
IN
FOOD PROCESSING AND PRESERVATION

DEPARTMENT OF FOOD PROCESSING AND PRESERVATION

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

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DEPARTMENT OF FOOD PROCESSING AND PRESERVATION

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

DECEMBER, 2014

Affectionately

Dedicated To

My Parents

ABSTRACT

Considering the public health, an attempt was taken to optimize the extraction technique of Calcium as $CaCl_2$ from eggshell and characterize chemically for possible use of human welfare. For extraction different concentration of HCl, variation in time and temperature were applied. Characterization of extracted calcium salt was performed by X-ray diffraction. Among the treatments, 0.9N HCl at 70^oC for 3 hours were selected as optimum conditions. Highest yield of calcium chloride (95.08%) contains calcium (635.57mg/g), magnesium (11.63mg/g) and zinc (9.63mg/g) usually at pH 7.45 and showed high solubility. Heavy metal constituents in terms of arsenic (0.16ppm) and lead (1.46ppm) were found, which are within safety limits for human consumption. The extracted calcium salt could be stored in laminated foil at ambient condition for more than six months to make a potential use as dietary calcium source.

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The Author

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LIST OF ABBRIVIATION

AOAC = Association of Analytical Chemists
BCSIR = Bangladesh Council of Scientific and Industrial Research
BMD =Bone Mineral Density
BR = Bradford Reagent
BSA = Bovine Serum Albumin
$CaCl_2$ = Calcium Chloride
et.al. = and others
FAO = Food and Agriculture Organization
FAOSTAT = The Statistics Division of FAO
gm = Gram
HCl =Hydrochloric Acid
HDPE = High Density Polyethylene
hr = Hour
LA = Laminated foil
mg = Milligram
MIC = Micron
min = Minute
ml = Milliliter
MoFL = Ministry of Fisheries and Livestock-Government of Republic Bangladesh
N = Normality
^oC = Degree centigrade
ppm = Parts per Million
SAS = Statistical Analysis System
SCI = Specular Component Included
SD = Standard deviation
sp. = Species
XRD = X-ray Diffraction
μm = Micrometer

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INTRODUCTION

CHAPTER I

INTRODUCTION

The eggshell is the outermost calcified layer of egg, which protects the embryo acting as strong physical barrier for microorganism invasion, infection and water loss from egg. Eggshell has another important function to help in gaseous changes and it is an indigenous calcium source for the formation of embryo skeleton. An egg weighing 60 g consists of approximately 4-6 g shell, wherein 96 g inorganic and only 4 g organic material per 100 g solid matrix. Typically, eggshell is a three layered structure, specifically, cuticle layer, a calcareous layer and shell membrane from outside to inside (Stadelman, 2000). Matrix of mammillary and spongy layers consists of protein fibers attached to calcite or calcium carbonate crystal. The chemical compositions of shell membrane includes protein (69.2%), fat (2.7%), ash (27.2%) and moisture (1.5%) (King'ori, 2011). The inorganic chemical compositions of eggshell are calcium carbonate (96%), magnesium carbonate (1%) and calcium phosphate (1%) (Tsai *et al.,* 2006).

All over the world even in Bangladesh, eggshell is regarded as waste disposed from different domestic sources like food industry, restaurant, homes and poultry farms. Various challenges including cost, disposal site availability, odor, and abrasiveness are associated with disposing of eggshell waste, which causes environmental pollution (Phil and Zhihong, 2009). Generally, eggshell wastes are used as fertilizer (26.6%), as animal feed (21.1%), in municipal dumps (26.3%) and in different ways (15.8%).

Moreover, eggshell powder is an excellent calcium source for making low cost fish feed and feed for piglets. Eggshells have been also used in layer diets as a rich calcium source that has no adverse effect on feed intake, live performance, egg production and shell quality. Adverse effect of sterile and ground eggshell as calcium source on health, egg production and hatchability has not been found till now (Gongruttananun, 2011). Recently, some research works have been carried out to find out possible uses of eggshell in human nutrition. Eggshell calcium is an excellent dietary calcium source, which is also substitute of crustacean shell (Sugoro *et al.*, 2000). One of the possible uses of eggshell powder is calcium supplement in human food but the elimination of pathogenic organisms is required before using. Dairy-based

supplement enriched with eggshell powder is effective diet, which increases bone mineral density (BMD) and delays bone demineralization (Schaafsma and Pakan, 1999). Meanwhile, it is reported that eggshell is good source of various calcium salts like calcium chloride, calcium lactate and calcium citrate. Moreover, these calcium salts have been commonly used as firming agent, flavoring agent, flavor enhancer, leavening agent, stabilizer, thickener and nutrient supplement (Whipple and Koohmaraie, 1993).

Calcium lactate produced from eggshell is used in fermented pork sausage as a dietary calcium source (Daengprok *et al.*, 2002). Eggshell calcium chloride is being used as a stabilizer in fruit and vegetable products and as a thickener in dairy products. Eggshell calcium chloride increases water holding capacity and keeps horse meat tenderized (Pe'rez-Chabela *et al*., 2003). In general, calcium chloride solution can be injected or marinated to increase the tenderization of beef steak. Considering the consumer health point of view, heavy metal constituents in calcium chloride should be under safety limits (Garnjanagoonchorn and Changpuak, 2007).

Unfortunately, any research has not been yet conducted on extraction of calcium salt from eggshell in Bangladesh, which could be used as dietary calcium source to recover the calcium loss and prevent calcium deficiency diseases. Considering the medicinal value of eggshell calcium salt, it is highly appreciated to use extracted calcium salt in value added food products. In such a way, the utilization of eggshell calcium chloride would also be an important issue for product market strategy. If it is possible to optimize the extraction technique of eggshell calcium chloride, it would be great achievement in Bangladesh. Indeed, the extraction of eggshell calcium chloride could be an excellent opportunity for developing calcium enriched products. Considering the above views and ideas, the present research work was carried out with following specific objectives.

a) to explore the effects of solvent concentration, extraction time and extraction temperature on the extraction of calcium chloride from eggshell
b) to optimize the extraction conditions for calcium chloride from eggshell
c) to characterize the extracted calcium chloride chemically


REVIEW OF
LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 Preamble

Review of related literature in any research is necessary because it provides a scope for reviewing the stock of knowledge, primary concept and relevant information to the proposed research. These knowledge, concept and information give a guideline in designing and conducting the research successfully. It is essential for reviewing that gives a proper instruction in designing a future research problems and validating the new findings.

Egg is one of the excellent sources of high quality protein. As a major nutrient contributor to the nation's food chain, large amount of consumed egg come from food processing plants manufacturing bakery products such as cakes, biscuit, pasta etc. Ultimately huge amount of eggshell is produced every year, which is discarded as serious waste in the food processing area. But eggshell can be used to make valuable commodity. Though a good numbers of researches have been completed on poultry production, broiler production, layer production and egg production, but the study on the extraction of eggshell calcium chloride is rare one in Bangladesh. Studies on the extraction of eggshell calcium salt are of recent origin in abroad. In present part, the most common and fruitful relevant studies about eggshell structure, composition and uses, extraction of eggshell calcium salt with uses are given, which were conducted in home and abroad in the recent past.

2.2 Egg and eggshell waste production

According to report of FAOSTAT (2009), the total egg production in Bangladesh was 219700 tons wherein hen egg (in shell) was 154000 tons.

Hossain and Hassan (2013) studied on the domestic production of egg in Bangladesh. The production of egg in the past year of 2011-2012 was 7303 million in number.

In the last years of 2012-2013, the total egg production in Bangladesh was 7617.38 million in number (MoFL, 2013). An egg weighs 55-60g and consists of 10% shell and membrane

(Stadelman, 2000), so total eggshell waste production was 0.42 to 0.46 million tons in 2012-2013.

2.3 Eggshell structure and its composition

The eggshell is the outer crust of an avian egg, which consists of porous bioceramic material and has been studied greatly since 1964. Because of scanning electron microscopy and micro-focus X-ray scattering techniques, the structure of eggshell and membrane is now properly understood (Lammie *et al.,* 2005).

Tsai *et al*. (2006) stated that eggshell was made up of calcium carbonate calcium carbonate (96%), magnesium carbonate (1%), and calcium phosphate (1%) as inorganic matter. Nys and Gautron (2007) stated that eggshell consisted of calcium carbonate (95%) and minor amount of organic matter (3.5%) and could be divided into six different layers.

Different soluble and insoluble protein, minerals are deposited to form eggshell and later it is used up by developing embryo within eggshell. The insoluble proteins have been observed to act as structural framework and the soluble proteins become embedded in the calcified layers. The deposited calcium is necessary for the formation and development of embryo's skeleton (Lammie *et al*., 2005).

From inside to outside, inner shell membrane is 20 μm thick, which is in direct contact with the egg albumen. The outer membrane lies just above the inner shell membrane is approximately 50 μm thick. Both of the inner and outer membrane consists of protein fibers and provide structural support to the eggshell as a whole (Lammie *et al*., 2005; Nys and Gautron, 2007). Eggshell strength greatly depends on both of two shell membranes, which also prevent microorganism penetration.

Stadelman (2000) stated that the calcified portion (calcium carbonate crystals) of the eggshell could be divided into three layers; the mammillary layer, palisade layer and the vertical crystal layer.

The palisade layer is 200 μm thick, which lies just above the mammillary layer and forms the major portion of calcium carbonate crystal of eggshell. In this layer, the calcite crystals grow

perpendicularly to the shell membranes. It has been reported that small portion (2-5%) of organic matrix incorporated in the palisade layer. Pores formed in the palisade layer help in the exchange of gases. The pores (gap between crystals) in eggshell take place when the crystals fail to join completely each other with their side surfaces (Lammie *et al*., 2005; Stadelman, 2000; Nys and Gautron, 2007).

Lammie *et al.* (2005) and Nys and Gautron (2007) studied on the vertical crystal layer and they reported that vertical layer was 8 µm thick, which was the upper most layer of calcite crystals.

The most outer layer of eggshell is cuticle layer (10-30 µm), which acts as water insoluble layer (Lammie *et al*., 2005; Nys and Gautron 2007). Lammie *et al.* (2005) stated that cuticle layer made of 90% protein with a high amount of cystine, glycine, glutamic acid, lysine and tyrosine. Fucose, galactose, glucose, hexosamines, mannose and sialic acid have been reported as constituents of polysaccharides (Stadelman, 2000).

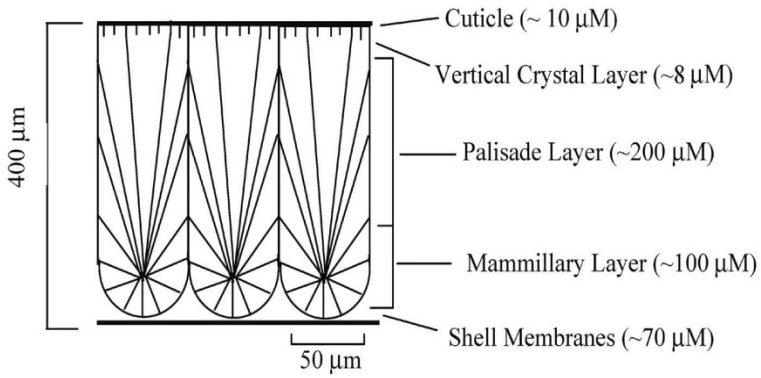

Figure 2.1: Schematic diagram of the structure and different layers of eggshell
(Source: Lammie *et al*., 2005)

2.3.1 Polypeptides and Polysaccharides of the Eggshell Membrane

To indentify and characterize the eggshell protein components and the organic membrane, numerous examinations have been carried out since 1990. Various biochemical and molecular biological techniques have been applied to identify proteins matrix. Gautron and Nys (2007) used the functional genome tools and the sequence of the chicken genome to the identification and characterization of eggshell matrix components.

Gautron and Nys (2007) observed that, the eggshell matrix made of a complex mixture of interwoven polysaccharides and protein fibers where at least 70% of the matrix being proteins. They also reported that, 11% of the matrix was polysaccharide including chondroitin sulphate A and B, hyaluronic acids, dermatan sulphate, keratan sulphate and uronic acids.

It has been observed that little amount of ovalbumin (egg white protein) is localized in mammillary layer of the eggshell. The lysozyme and ovatransferrin two other major egg white proteins, are identified as constituent of eggshell layer (eggshell membranes, mammillae) (Gautron and Nys, 2007). Various glycoproteins named osteopontin and clusterin have been reported as constituent in the different layers of the eggshell (Gautron and Nys, 2007).

Gautron and Nys (2007) stated that high amount of arginine, glutamic acid, methionine, histidine, cystine, hydroxyproline, hydroxylysine, and desmosine were found as amino acid of the eggshell membrane.

2.4 Use of eggshell

Considering the high disposal cost of eggshell, shortage of landfill and increasing environmental concerns, it is necessary to find an alternative method that would transform the waste eggshells into a valuable item; giving financial benefits to the competitive egg processing industry. Apart from giving manufacturers a new profit stream, it would help overcome the high disposal costs and environmental concerns (MacNeil, 2006).

There are many uses of separated eggshell and membrane (MacNeil, 2001) but not many when they are attached. The following section gives a brief review of various potential uses of separated eggshell.

2.4.1 Source of calcium carbonate in Agriculture

Calcium carbonate is the major constituent of shell accounting to 94% of solid mass. Separated or processed shell can be used as source of calcium carbonate or calcium in producing fertilizer or animal feed, bio-plastics and coating on paper (Anton *et al*., 2006).

Abdullah (2000) reported that membrane free eggshell could be used as calcium supplement or lime substitute in agricultural field. He also suggested that protein-rich shell membrane attracted rats, insects and others.

John and Paul (2006) observed that eggshell was environmental friendly and effective liming source for the growth of plants. They used eggshell and agricultural lime as liming sources in the field of corn and soybean. They observed that soil pH increases rapidly with traditional agricultural lime within six months following application. But eggshell found to be effective liming source after six months to fifty four months following application.

Holmes *et al.* (2011) demonstrated the usefulness of eggshell as a liming agent. Aglime (agricultural lime) and ground eggshell were used as liming material for the production of corn and soybean. Increasing of soil pH was higher with ground eggshell application than aglime, which resulted high yield of both cprn and soybean. Based on soil pH response and yield of corn and soybean, ground eggshell was proved effective liming material compared to agricultural lime.

2.4.2 Source of calcium in feed

Many studies have been studied to make use of eggshell as source of calcium in making feed for fish, poultry and others.

Scheideler (1998) examined the effect of eggshell calcium on egg production, egg quality and Ca digestibility in laying hens. First or third cycle laying hens were fed with various diets containing different amount of eggshell and/or oyster shell. Eggshell product was found to be same to fine limestone as a source of calcium. Eggshell products were suggested to combine with another calcium source to make optimum calcium mix for better both of egg and shell quality.

Gongruttananum (2011) examined the egg and eggshell quality, productive performance, bone mineralization, plasma Ca concentration, and gonadal characteristics of laying hens on feed with sterilized and ground eggshell as calcium source. He divided hens into three groups and first group was feed 100% fine local limestone, whereas 50% and 100% ground eggshell were supplied to second and third group with local lime stone respectively for 10 wks. Desired

parameters of three hens group were not significantly ($P > 0.05$) different. It was concluded that ground eggshell could be used as Ca source in chicken feed without any detrimental effects on egg and eggshell quality, productive traits, bone mineralization, plasma Ca balance, and gonadal performance.

Gongruttananum (2011) conducted a research to examine the effect of sterile and ground eggshell in diet on live performance, plasma calcium concentration, bone mineralization semen quality, gonadal and visceral organ characteristics of Rhode Island Red breeder males. He divided Rhode Island Red breeder males into three groups, and first group was feed 100% fine local limestone, whereas 50% and 100% ground eggshell were supplied to second and third group with local lime stone respectively for 10 wks. Desired parameters of three hens group were not significantly ($P > 0.05$) different. It was concluded that ground eggshell could be fully used as Ca source in breeder male feed without any detrimental effects on live performance, plasma calcium concentration, bone mineralization semen quality, gonadal and visceral organ characteristics.

Gongruttananum (2011) observed the effect of sterile and ground eggshell as calcium source in diet on the egg and shell quality. To find out the effect of eggshell feeding on egg production, egg and eggshell quality, eggshell ultrastructure and hatching characteristics, laying hens were divided into three groups. First group was fed 100% fine local limestone, whereas second and third group were fed 50% and 100% ground eggshell with local lime stone respectively for 10 wks. Desired parameters of three hens group were not significantly ($P > 0.05$) different. Results indicated that ground eggshell could be fully used as calcium source in layer diets without any detrimental effects on egg production, egg and eggshell quality, eggshell ultrastructure and hatching characteristics.

Chakrabarty *et al.* (2010) conducted a research to observe the effect of various feeds on gonad weight in juvenile fish (*Colisa fasciata)* and biomass conversion rate. Three types of feed including local fish feed, laboratory prepared feed (LPF) were fed, whereas LPF was prepared with eggshell powder, dried earthworm powder and rhizome (*Commelina* sp.). LEF was proved to be best economical feed among tested feeds. Eggshell constituent of LEF was proved to be rich source of albumin and calcium.

Bag *et al.* (2012) conducted a research to evaluate the growth performance of Mozambique tilapia (*Oreochromis mossambicus*) feeding low lost feed. Three groups of tilapia were fed with three types of feed hydrated poultry feather meal (PFM), earthworm meal (EWM) and slaughter house offal meal (SOM). EWM feed was found to be best lost cost fish feed, which made with dry earthworm dust, mustard oil cake, eggshell powder and rice bran.

Schaafsma and Beelen (1999) conducted a comparative study of piglet diets with $CaCO_3$ or eggshell powder. Piglets were placed on casein based diet with $CaCO_3$ or eggshell powder at first and then on soy protein isolate based diet with $CaCO_3$ or eggshell powder. In both diet, digestibility coefficient for calcium from eggshell was high than purified $CaCO_3$. Adverse effect of eggshell power in diet was not observed in both of studies.

2.4.3 Source of dietary calcium in human nutrition

Schaafsma and Pakam (1999) observed the short-term effects of dairy-based product enriched with eggshell powder on bone mineral density in osteoporosis or osteopenia patient. In this study, bone mineral density (BMD) of lumbar spine and hip of nine women and one man (mean age±SD, 63.9±8.1 years) osteoporosis patients were measured. One intervention group was placed on diet twice daily with dairy-based supplement for 4-8 months, which resulted in daily intake of 3 g of egg shell powder. Result showed the significant ($p < 0.05$) increasing in total proximal femur and BMD of lumbar spine. Reduction in pain and improvement in well-being were resulted after four months of intervention. Six women of the intervention group were again allowed continuing dairy-based diet only once daily for 24 months, whereas double dose was allowed for last three months. Second study didn't show any change in BMDs from baseline. Eggshell powder was concluded as a rich source of bioavailable calcium, which increased BMD and delayed bone demineralization for longer time.

Schaafsma *et al.* (2000) conducted a research on the potential use of mineral, amino acid and hormonal constituent of hen eggshell powder in human nutrition. Chicken eggshell powder was recorded as attractive calcium source for human nutrition. Level of toxic component such as lead, aluminum, cadmium and mercury in eggshell powder was recorded low, whereas oyster shell supplement proved to have high level of these toxics.

Daengprok *et al.* (2003) reported that chicken eggshell was attractive source of dietary calcium for human health. Eggshell was proved to be effective to increase bone mineral density (BMD) and reduce pain of elder population with osteoporosis. Eggshell matrix protein (1%) attached with calcium carbonate ($CaCO_3$) enhanced calcium transportation in body through intestinal epithelial cell Caco-2.

Hirasawa *et al.* (2001) studied on the effect of combination of 1alpha-hydroxyvitamin D3 and eggshell calcium on bone metabolism in osteopororotic rat. Bone mineral density (BMD) of intervention group of rat was increased significantly compared to control group. Eggshell calcium was found to be similar to $CaCO_3$ on bone metabolism. The result suggested that eggshell could be used as effective Ca source for osteoporosis people with vitamin D3.

Murakami *et al.* (2007) conducted a physicochemical study of eggshell $CaCO_3$. Physicochemical and thermal properties of eggshell calcium carbonate were evaluated in this study. They observed that eggshell could be used for making dispersible tablets and calcium supplement for human. Eggshell was found to be better than oyster shell because of its toxic components including lead, aluminum, mercury and cadmium.

Than *et al.* (2012) examined the eggshell powder as excipient to make fats and sustained release acetaminophen tablets. Four types of eggshell powder including untreated, water treated, chloroform treated and ethanol treated powder were used to make acetaminophen as a model drug. Results of this study showed that eggshell powder was suitable as excipient to control drug releasing from any tablet like acetaminophen.

2.5 Extraction of eggshell calcium salt

Daengprok *et al.* (2002) studied on the process of eggshell calcium lactate preparation. Eggshell powder, lactic acid and water with a ratio of 1:2:28 (w/v/v) were kept at 4ºC overnight, and then centrifuged at high speed (14000 rpm) for 10 minutes and subjected to heating at 75ºC for 5 minutes. Then the supernatant was filtered and freeze-dried to get eggshell calcium lactate. They also fortified Nhams (sausage) with eggshell calcium lactate and found that calcium lactate increased the pH and decreased the texture without affecting total acceptance. They suggested that calcium supplementation should not be more than 150 mg/100 g that is equivalent to 18.75% of the RDA for adults.

Garnjanagoonchorn and Changpuak (2007) conducted a research on the extraction of calcium chloride from eggshell. They considered two factors namely hydrochloric acid and ratio of eggshell powder to acid solution in the extraction of calcium chloride. Hydrochloric acid concentration at 3%, 4% and 5% (w/v) and ratio of shell powder to acid solution at levels 1:5, 1:10 and 1:15 (w/v) were studied in this research. Highest yield of calcium chloride (87.38%) was recorded for an extraction period of three hours using 4% (w/v) hydrochloric acid solution with the ratio of 1:15 (w/v). Finally, X-ray diffraction indicated that extracted eggshell calcium chloride was mainly composed of $CaCl.2H_2O$.

2.6 Insights from the review of literature

It is clear from the review of literature that a lot of researches have been carried out on eggshell, and a few researches have been conducted on eggshell calcium salt in aboard. The main efforts of most of the relevant researches were directed on eggshell structure analysis, comparative analysis on shell structure of different eggs, use of shell membrane and eggshell in feed. From the review of literature it is proved that eggshell calcium salt can be used as important source of dietary calcium for human being. Indeed an appropriate extraction technique of eggshell calcium chloride helps to reduce the eggshell waste in any country. But no study was carried out on the extraction of eggshell calcium chloride in Bangladesh still now. Therefore, the present research work was conducted to find the optimum conditions for the eggshell calcium chloride extraction, which is indeed a new research in Bangladesh. This research is very important in terms of producing dietary calcium salt utilizing eggshell waste.


MATERIALS AND
METHODS

CHAPTER III

MATERIALS AND METHODS

This chapter deals with the materials and methods used to carry out the present research work. The materials and methods used to extract and characterize the eggshell calcium chloride ($CaCl_2$) are described under the following different sub-titles.

3.1 Experimental site

The research work was carried out in the laboratories under the Faculty of Engineering, Hajee Mohammad Danesh Science and Technology University. Partial characterization of eggshell calcium chloride was accomplished in the laboratories under the Institute of Food Science and Technology (IFST) and the Institute of Glass and Ceramic Research and Testing (IGCRT), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka.

3.2 Sample collection

The experimental material of this research work was “Brown eggshell”, which was collected from local restaurant at Basherhat where eggshells are seemed and discarded as waste material (Figure 3.1).

Figure 3.1: Eggshell discarded as waste

3.3. Extraction process of Calcium Chloride ($CaCl_2$) from eggshell

3.3.1 Preparation of membrane free eggshell powder

Brown eggshells were sorted out from collected samples to make unique brown eggshell sample for research. Then, eggshells were washed thoroughly with de-ionized water to remove adhered dirt with shell. After removing dirt, shell membranes were separated from eggshell manually. Following the method of Than *et al.* (2012), membrane free shells were then boiled in water at 130^{o}C for 20 minutes to kill the pathogenic microorganism.

Sterile eggshells were crushed to small piece approximately 0.25cm and dried in cabinet dryer at 60^{o}C for 6 hours. Dried eggshells were ground and sieved through a mesh (Mic-200) and obtained 0.2mm of particle size (Figure 3.2). Ground eggshell powder was packed in high density polyethylene bag and stored at ambient condition to use as raw material for the research work.

Figure 3.2: Membrane free eggshell powder

3.3.2 Extraction of calcium chloride from eggshell

Eggshell powder was then mixed with different HCl solution (0.5, 0.7 and 0.9N) with a ratio of powder to HCl solution of 1:20 (w/v). Mixtures were transferred on hot plate with magnetic

stirrer to heat with continuous stirring at 50°C and 70°C for 1hr, 2hr and 3hr. The following reaction between HCl and $CaCO_3$ of eggshell produced calcium chloride (Garnjanagoonchorn and Changpuak, 2007):

$$CaCO_3 + 2HCl \xrightarrow{\text{Heating}} CaCl_2 + CO_2 + H_2O$$

The mixtures were then cooled to room temperature and filtered through Whatman No. 40 filter paper using vacuum filter. The filtrate was subjected to hot plate to dry at 200°C for six hours to make it nearly anhydrous drying. Finally, whitish eggshell calcium chloride was packed in laminated foil and high density polyethylene (HDPE) for further analyses.

3.4. Parameter studied

The following parameters were studied in the research work.

3.4.1 Phase analysis

Phase analysis by X-ray diffraction pattern

3.4.2 Physico-chemical analysis

Proximate composition (moisture, ash, protein and fat content), color (L, a, b), pH, calcium, magnesium, and phosphorous content and heavy metal content (arsenic, lead and zinc)

3.4.3 Methods

3.4.3.1 Phase analysis by XRD

Phase analysis was carried out by X-Ray diffraction according to modified method of Farooque *et al.* (2009).

Working procedure

The phase composition of the eggshell calcium chloride was determined by the X-Ray diffraction (XRD). For XRD analysis, an automated powder diffractometer designed with a Cu-Kα radiation source (Cu-Kα = 1.5406Å) operated at 40 KV-30mA was used. Af first, 1g

of sample was taken in primary beam, and selected radiation was passed through the sample to get X-ray diffraction data. A nickel filter was placed before the sample to reduce CuKβ radiation and finally CuKα radiation was only used as the primary beam. The selected scan range was 5-75^o with a step size of 0.020^o and counting time of 0.30s. All the X-ray diffraction data of the sample were analyzed using computer to get d values and peak intensities. The d-spacing was calculated using Bragg relation where λ is the wavelength of the incident radiation and for CuKα = 1.5406Å. The d-values and their intensity ratios were interpreted by auto matching with the data available in 'X'Pert High Score Plus' software of the XRD system. Finally, the unknown compound was identified from the observed data.

3.4.3.2 Determination of Moisture content

Moisture content was determined by oven drying method (AOAC, 2004) as described below.

Working Procedure

First of all, weight of empty previously dried crucible (at 100^{o}C for 1hr) with cover was recorded, and 5g of sample was taken in it. Then the crucible was placed in an air oven (thermostatically control) and dried at a temperature of 105^{o}C for 24hr. After drying, the crucible with sample was kept in a desiccator to cool. It was then weighed with covered glass.

Calculation

Moisture content was determined as follows:

$$\text{Moisture content}\,(\%) = \frac{M_1 - M_2}{M_1 - M_0} \times 100$$

Where,

M_0 = Mass of the crucible

M_1 = Mass of the crucible and test sample before drying

M_2 = Mass of crucible and test sample after drying

3.4.3.3 Determination of Ash content

AOAC method (2000) was used to determine the total ash content in sample as described below.

Working procedure

The sample (5g) was taken in a previously cleaned, dried and weighed porcelain crucible. At first, the crucible containing sample was placed in oven at 105^{o}C for 4hr to remove moisture. The moisture free sample was completely charred followed by heating in a muffle furnace for 6hr at 550^{o}C and ignited until light gray ash resulted (or to constant weight). It was cooled in desiccators and weighed. To ensure the completion of ashing, the crucible was again transferred in muffle furnace for half an hour and then cooled in desiccator and weighed again. This process was repeated until a constant weight was obtained and the ash became almost white and grayish in color.

Calculation

Total ash content was calculated using the following formula:

$$\text{Ash Content (\%)} = \frac{M_1 - M_2}{M_0 - M_2} \times 100$$

Here,

M_1 = Mass of the crucible and residue after ashing

M_2 = Mass of the crucible

M_0 = Mass of crucible and test sample before ashing

3.4.3.4 Determination of Protein content

Bradford method was used to determine the crude protein content in sample with quite modification as described by Kruger (1997).

Working Procedure

5ml of BR solution was taken in a test tube. Then 100µl of sample solution was added to BR solution and mixed using vortex mixer. It was allowed to incubate for five minutes. Then absorbance was measured at 595nm against sodium chloride blank. A standard curve was prepared using BSA solution.

Calculation

The protein content of the sample was calculated using prepared standard curve.

3.4.3.5 Determination of Fat content

Fat content of the sample was determined according to the method (AOAC, 2004) as described below.

Working procedure

The dried sample remaining after moisture determination was taken in tracing paper and transferred to thimble. Top of thimble was then tightly plugged with fat free cotton. The thimble was dropped into the extraction tube attached to a Soxhlet flask. Roughly, 90-120ml of anhydrous petroleum ether was poured into the Soxhlet flask. The top of the fat extraction tube was attached to the condenser. The sample was extracted for 16hr on heater at 70-80^{o}C. At the end of fat extraction, the thimble was removed from extraction tube, and most of the ether was distilled off by allowing or collected in soxhlet tube. The ether was poured off when the extraction tube was nearly full. When the ether reached a small volume, it was poured in a previously dried and weighed beaker.

The flask was rinsed and filtered thoroughly, using ether. The ether was evaporated on heater at low heat, and it was then dried at 100^{o}C for 1hour, cooled and weighed. The difference in the weights gave the ether soluble material present in the sample.

Calculation

Fat content was calculated by the following formula:

$$\text{Fat Content (\%)} = \frac{\text{Weight of ether extract}}{\text{Sample weight}} \times 100$$

3.4.3.6 Color measurement

Color measurement was carried out by Minolta colorimeter following the modified method developed by HunterLab (1995).

Working procedure

At first, colorimeter was calibrated where zero calibration was performed first and then white calibration. After that, color values were measured in terms of L, a, b (SCI) where L, a, b value indicates lightness, redness and yellowness respectively.

3.4.3.7 Determination of pH

The pH of the sample was determined according to modified method of Garnjanagoonchorn and Changpuak (2007).

Working procedure

At first, 1g of sample was mixed with distilled water at a ratio of 1:20 (w/v) to make sample solution for pH determination. The electrode assembled with pH meter was dipped into the standard buffer solution of pH 7.0 and pH 4.0 respectively. After adjusting in both buffer solutions, the electrode was washed with distilled water. After that, electrode was dipped into the sample solution and pH was readout directly, which was recorded. Before measuring pH of another sample, electrode was washed with distilled water again. The pH of all samples was determined by this way.

3.4.3.8 Determination of Calcium content

Calcium content of the sample was determined following the method developed by AOAC (1995).

Working procedure

Sample digestion

1g sample was taken in a 50 ml conical flask. 5ml of di-acid mixture was added to it. The flask was then placed on hot plate and digested at 195^{o}C for 1hr when the solution was clear. Distilled water was added to the solution and mixed thoroughly. The solution was then filtered through Whatman no. 42 filter paper and the volume was made up to 100ml with distilled water. The solution was then ready for the estimation of calcium.

Estimation of Calcium

5ml digestion mixture was taken in a 250ml conical flask and then 50ml distilled water was added. 5ml of 10% NaOH solution was added to the flask. Masking reagent (10 drops potassium ferocyanide, 10 drops hydroxylamine hydrochloride, 10 drops triethanolamine) was added to the flask and 6 drops calcon indicator was added. The flask was shaken vigorously and titrated against 0.01M of Na_2EDTA. The solution turned into blue. A blank was run following the same procedure as describe above. The data were recorded and the amount of calcium present in the sample was calculated.

Calculation

The percent of calcium was calculated according to the formula:

1ml of 0.01M EDTA solution = 0.2004mg of Ca

$$\text{Calcium content (\%)} = \frac{\text{mg of calcium obtained}}{\text{weight of sample}} \times 100$$

3.4.3.9 Determination of Magnesium content

Magnesium content of the sample was determined following the method described by AOAC (1995).

Working procedure

Sample digestion

1g sample was taken in a 50ml conical flask. 5ml of diacid mixture was added to it. The flask was then placed on hot plate and digested at 195^{o}C for 1hr when the solution was clear. Distilled water was added to the solution and shaked thoroughly. The solution was then filtered through Whatman no. 42 filter paper and the volume was made up to 100ml with distilled water. The solution was then ready for the estimation of magnesium.

Estimation of magnesium

5ml digestion mixture was taken in a 250ml conical flask and then 50ml distilled water was added. 5ml NH_3-NH_4 buffer solution was added to the flask. Masking reagent (10 drops

potassium ferocyanide, 10 drops hydroxylamine hydrochloride, 10 drops triethanol amine and 10 drops sodium tunstate solution) was added to the flask and 6 drops EBT indicator was added. The flask was shaken vigorously and titrated against 0.01M of Na_2EDTA. The solution turned into blue. A blank was run following the same procedure as describe above. The data were recorded and the amount of calcium present in the sample was calculated.

Calculation

The percent of calcium was calculated according to the formula:

1ml of 0.01M EDTA solution = 0.2432mg of Mg

$$\text{Magnesium}\,(\%) = \frac{\text{mg of magnesium obtained}}{\text{weight of sample}} \times 100$$

3.4.3.10 Determination of Arsenic content

Arsenic content of the sample was determined following spectrophotometric method according to Erisbie *et al.* (2005).

Working procedure

Sample preparation

At first, 5g of sample was taken in arsine generator (Erlenmeyer flask) and mixed with 0.35ml of KI (50%). Then, 0.35ml of $SnCl_2.2H_2O$ (40%) was mixed. Finally, 35ml of volume was made with distilled water. It was subjected to heat for 1min to reduce As (v) to As (III).

Scrubber preparation

At first, 0.17g of glass wool was placed onto a filter paper. Then, 10 drops of Lead (ii) acetate trihydrate solution (10%) were placed to glass wool. The glass wool was squeezed to remove excess solution. The glass wool was fluffed and placed in the scrubber.

Absorber preparation

Firstly, 2.5ml of I_2/KI solution was taken in a test tube (20ml). Then, 0.5ml of sodium bi-carbonate (1M) solution was delivered to test tube and mixed properly. Finally, mixture was poured into the absorber and a cap was tightly affixed to the absorber.

Arsine generation and color development

At first, 2ml of sulfuric acid (conc.) was added to the treated sample in Erlenmeyer flask and mixed. 10ml of concentrated HCl was mixed to solution in Erlenmeyer flask. Then 5g of zinc was added to mixture in Erlenmeyer flask. The scrubber and absorber were connected to the Erlenmeyer flask immediately. It was allowed to heat gently and stand for 30min for complete generation of AsH_3 from Erlenmeyer flask to absorber. The absorber liquid was poured to test tube. Again, absorber was rinsed with 1ml of distilled water to pour residual liquid to test tube. 1ml of sulfuric acid-ammonium molybdate tetrahydrate solution was added to test tube and mixed. Then 0.5ml of Sodium metabisulfite solution (6%) was added to test tube and deep reddish-brown color changed to faint yellow. Finally, 0.5ml of Tin (II) chloride dehydrate solution (0.2%) was added to test tube and allowed to stand for 30min for developing bluish-green arsenomolybdate color.

Spectrophotometry

A small amount of mixture was taken in cuvette and placed in spectrophotometer. Finally, absorbance was read at 835nm. A standard curve was prepared using standard arsenic solution.

Calculation

The Arsenic content of the sample was calculated from prepared standard curve.

3.4.3.11 Determination of Lead content

Graphite Furnace Spectrophotometry was used to determine lead content of the sample as developed by GB (2010).

Working procedure

At first, 1g of sample was weighed and taken into a crucible. Then it was heated on the electric heating plate until no smoke and transferred into muffle furnace at 550^{o}C for 6hr. After ashing, crusible was cooled to room temperature, and the sample was dissolved in nitric acid (0.5mol/l). The sample digestion solution was washed carefully and transferred into a 25ml volumetric flask. A small amount of distilled water was used to wash the crucible and poured into the volumetric flask. Finally, the volume was made up to the mark and mixed properly.

Spectrophotometry

10µL of sample was pipetted and injected into the graphite furnace to measure absorbance. Then the concentration was calculated according to the formula obtained from standard curve where standard curve was prepared standard lead solution.

Calculation

The lead content of the sample was calculated using prepared standard curve.

3.4.3.12 Determination of Zinc content

Zinc content of the sample was determined following spectrophotometry according to Korn *et al.* (1999) with a slight modification.

Working procedure

Sample preparation

At first, 2g of sample was taken in porcelain crucible and then transferred to muffle furnace at 550^{o}C for 6hr. After ashing, it was cooled to room temperature and 5ml of conc. Hydrochloric acid was added to porcelain crucible. Solution was transferred in a 500ml volumetric flask, and volume was made with distilled water.

Spectrophotometry

Firstly, 5ml of prepared sample solution was taken in 25ml volumetric flask. Then 1ml of p-NIAZOXS solution and 3ml of borax buffer solution were added to sample solution. Volume

was made with distilled water and mixed properly. Then the absorbance was measured at 520nm against distilled water. A standard curve was also prepared using standard Zn solution.

Calculation

The zinc content of the sample was calculated from currently prepared standard curve.

3.5 Statistical analysis

In this experiment, data obtained from various treatments were statistically analyzed using SAS 9.1 software. Multi-way analysis of variance (ANOVA) was used to determine the significance of difference between the means of data obtained from various extraction techniques. Least significant difference (LSD) with the level of significance at 5% and Duncan Multiple Range Test (DMRT) were used to compare the significance of difference between pair of means.

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

This research was carried out to find out the potential use of eggshell waste as extracted calcium chloride. The effect of different treatments in relation to extraction of calcium chloride from eggshell, and characterization of extracted calcium salt were studied in this research. The results obtained from the research are presented and discussed in this chapter under the following headings.

4.1 Proximate composition of eggshell powder

Proximate compositions of sterile eggshell powder before using as raw sample are showed in Table 4.1.

Table 4.1: Proximate composition of eggshell powder (*)

Composition (%)	Content
Moisture	0.64 ±0.03
Ash	95.74±0.04
Protein	2.54±0.03
Fat	0.27±0.02

*Values are mean of triplicate analysis with standard error mean

4.1.1 Moisture content

Moisture content of ground eggshell powder is shown in Table 4.1. The moisture content (0.64%) was found in eggshell powder, which indicated that drying temperature and duration were suitable to produce low moisture content powder. Because of non-hygroscopic and low moisture content, eggshell powder would be stable in high density polyethylene package for

several months at room temperature. The moisture content in eggshell powder of this study was less than previous values 0.98% and 0.8% reported by Garnjanagoonchorn and Changpuak (2007) and Gongruttananum (2011) respectively.

4.1.2 Ash content

Result shown in Table 4.1 revealed that the major component in eggshell powder was ash content (95.74%). One of possible reasons of high ash content in eggshell powder is 96% inorganic material in eggshell (Tasai *et al.*, 2006). This result of the present study was supported by Garnjanagoonchorn and Changpuak (2007) who reported ash content (95.74%) in membrane free eggshell powder, but differed considerably from result reported by Hemung (2013) who observed ash content (75.83%) in Tilapia fish bone powder.

4.1.3 Protein content

The protein content in membrane free eggshell powder was found 2.54% (Table 4.1). This result was in agreement with the finding of Garnjanagoonchorn and Changpuak (2007) who observed protein (3.22%) in membrane free eggshell powder. In contrast, higher protein content (5.35%) in ground eggshell powder was resulted by Gongruttananum (2011). Difference in protein content of eggshell powder may be resulted due to separation technique and use of different pretreatment.

4.1.4 Fat content

It has been reported that a little amount of lipid (3%) is contained in cuticle layer of brown eggshell (Rose-Martel *et al*., 2012). Except cuticle, there is no additional source of fat in eggshell, which would supply fat in membrane free eggshell powder.

The fat content in eggshell powder of this study was higher than the result carried out by Garnjanagoonchorn and Changpuak (2007) who reported 0.03% fat in membrane free eggshell powder. They also stated that membrane free eggshell was supposed to be less auto-oxidized because of very little amount of fat content.

4.2 Color of eggshell powder

Color of eggshell powder before using as raw sample was measured in terms of L, a, b value (SCI) and shown in table 4.2.

Table 4.2: Color of eggshell powder (*)

Color	SCI Value
L	70.38±3.40
a	2.71±0.16
b	8.43±0.20

* Values are mean of triplicate analysis with standard error mean

The color of eggshell powder in terms of 'L', 'a', and 'b' value were measured where 'L', 'a' and 'b' value indicated lightness, redness and yellowness of sample respectively. The visible color of eggshell powder was found to be whitish. The 'L' value for eggshell powder was recorded 70.38 in this study, which indicates less lightness property of powder and the presence of organic component in powder. This result was much lower than the value of 98.08 reported by Hemung (2013) for Tilapia fish bone powder, but similar to the result carried out by Bragadottir *et al.* (2007) who observed 'L' value 73.75 for Saithe fish bone powder.

The 'a' value for eggshell powder was observed 2.71, which was indication of slight redness or brown of powder. Generally, redness of eggshell powder is related to protoporphyrin-IX pigment contained in brown shell of egg. In earlier study, Bragadottir *et al.* (2007) observed 'a' value (0.91) of Saithe fish bone powder stored at 30^{o}C. On the other hand, Hemung (2013) found 'a' value (16.79) for Tilapia fish bone powder. This value indicates the reddish color of Tilapia fish bone powder.

The 'b' value for eggshell powder was found 8.43 (Table 4.2). Higher 'b' value means the yellowness of sample. The 'b' value (8.43) of present study indicates light yellowness of powder. In contrast, higher 'b' value 17.19 for Saithe fish bone powder and 79.48 for Tilapia

fish bone powder were reported by Bragadottir *et al.* (2007) and Hemung (2013) respectively. Both of 'b' values indicated more yellowness of fish bone powder.

4.3 Extraction of calcium chloride from eggshell

4.3.1 Effect of solvent concentration on calcium chloride extraction

Different concentrations of hydrochloric acid were used to extract calcium chloride from eggshell in order to measure the effect of hydrochloric acid concentration on the yield. It was found that hydrochloric acid concentration significantly ($p<0.001$) influenced the yield of calcium chloride (Table 4.3; supplementary Figure 4.1). Yield of calcium chloride was increased with the increasing of solvent normality (Figure 4.1).

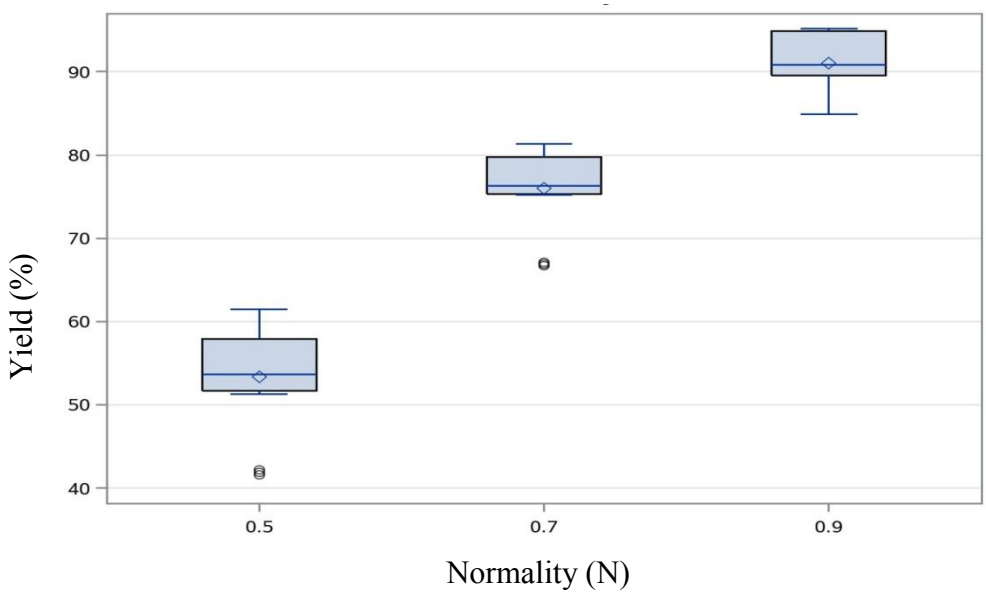

Figure 4.1: Distribution of calcium chloride yield depending on concentration

However, the average yield of calcium chloride 53.32%, 75.97% and 91.04% were resulted for 0.5N, 0.7N and 0.9N HCl solution respectively. This finding is in good agreement with Garnjanagoonchorn and Changpuak (2007) who found highest yield of calcium chloride for 5% of hydrochloride acid solution among three HCl concentrations of 3%, 4% and 5% (w/v).

Table 4.3: Yield of calcium chloride extracted from eggshell (*)

Treatment	Concentration of HCl (N)	Temperature (°C)	Time (hr)	Yield (%)
1	0.5	50	1	41.88±0.17^{p}
2	0.5	50	2	52.09±0.26^{o}
3	0.5	50	3	55.15±0.18^{n}
4	0.5	70	1	51.65±0.21^{m}
5	0.5	70	2	57.89±0.14^{l}
6	0.5	70	3	61.27±0.15^{k}
7	0.7	50	1	66.89±0.09^{j}
8	0.7	50	2	75.31±0.05^{i}
9	0.7	50	3	76.17±0.08^{h}
10	0.7	70	1	76.49±0.09^{h}
11	0.7	70	2	79.85±0.08^{g}
12	0.7	70	3	81.11±0.13^{f}
13	0.9	50	1	85.06±0.08^{e}
14	0.9	50	2	88.86±0.11^{d}
15	0.9	50	3	89.66±0.06^{c}
16	0.9	70	1	92.02±0.05^{b}
17	0.9	70	2	94.92±0.07^{a}
18	0.9	70	3	95.08±0.08^{a}

* Values are mean of triplicate analysis with standard error mean; Means in the same column with different letters are significantly different ($p<0.001$).

4.3.2 Effect of temperature on calcium chloride extraction

Experiment of calcium chloride extraction from eggshell at different temperature was performed to know the effect of temperature on yield. Both temperatures were very effective in increasing yield of calcium chloride (Table 4.3; supplementary Figure 4.2).

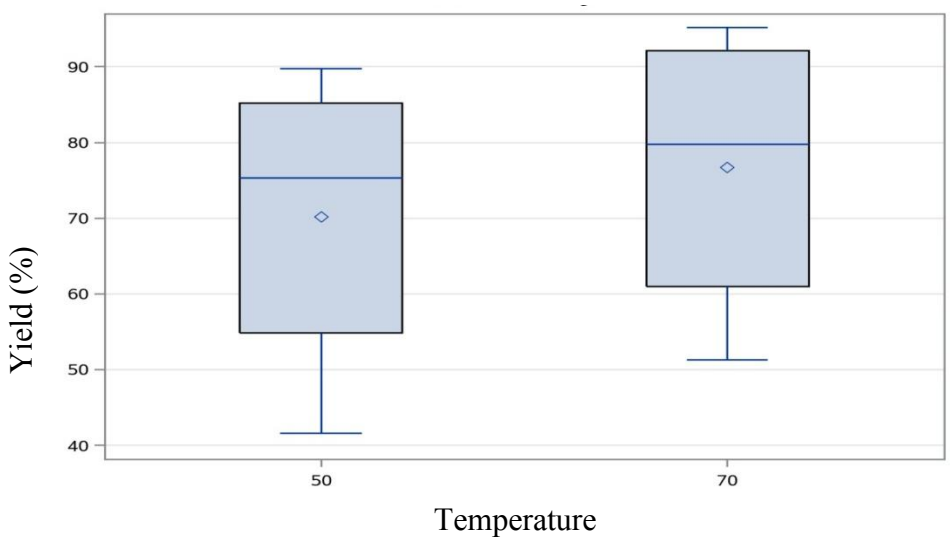

Figure 4.2: Distribution of calcium chloride yield depending on temperature

The average yield of calcium chloride 70.19% and 76.70% were scored at temperature 50^{o}C and 70^{o}C respectively. Based on these results, it was suggested that increasing extraction temperature from 50^{o}C to 70^{o}C could enhance the extraction rate of calcium chloride.

4.3.3 Combined effect of concentration and temperature on calcium chloride extraction

Combination of hydrochloric acid concentration and extraction time significantly ($p<0.001$) affected the yield of calcium chloride (Table 4.3). The yield of calcium chloride was increased with increase in both concentration and extraction temperature (Figure 4.3). Results also indicated that the yield of calcium chloride was significantly increased with the increase of extraction temperature of same concentration of HCl solution. Highest average yield of calcium salt (94.01%) was resulted for combination of 0.9N and 70^{o}C, and lowest one (49.70%) resulted for combination of 0.5N and 50^{o}C.

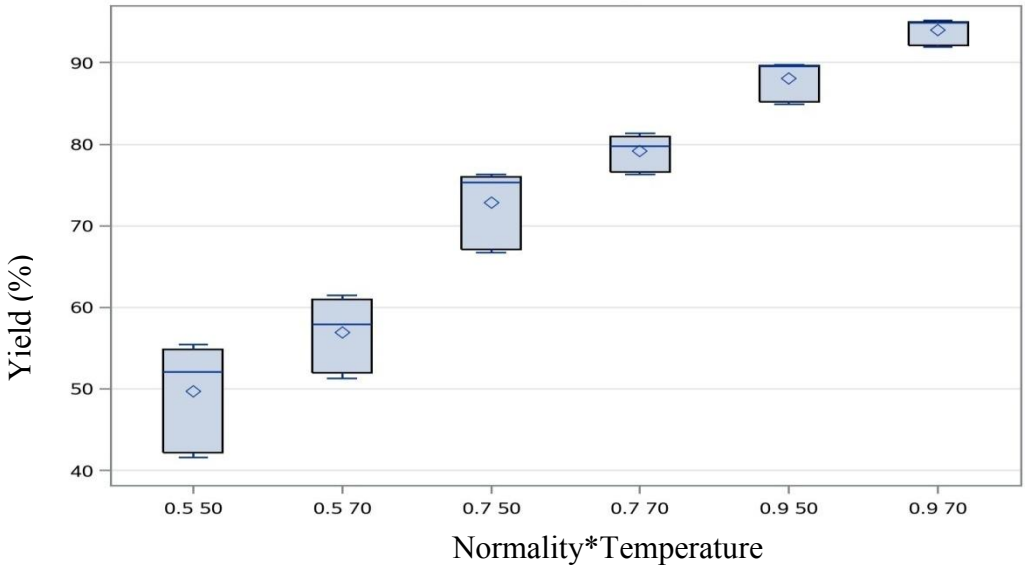

Figure 4.3: Distribution of calcium chloride yield depending on combination of concentration and temperature

4.3.4 Effect of extraction time on the yield of calcium chloride

Extraction period significantly ($p<0.001$) influenced the extraction of calcium chloride from eggshell (Table 4.3). There was a rapid increase in the yield of calcium chloride in first two hours followed by a slow increase in last hour of extraction (Figure 4.4).

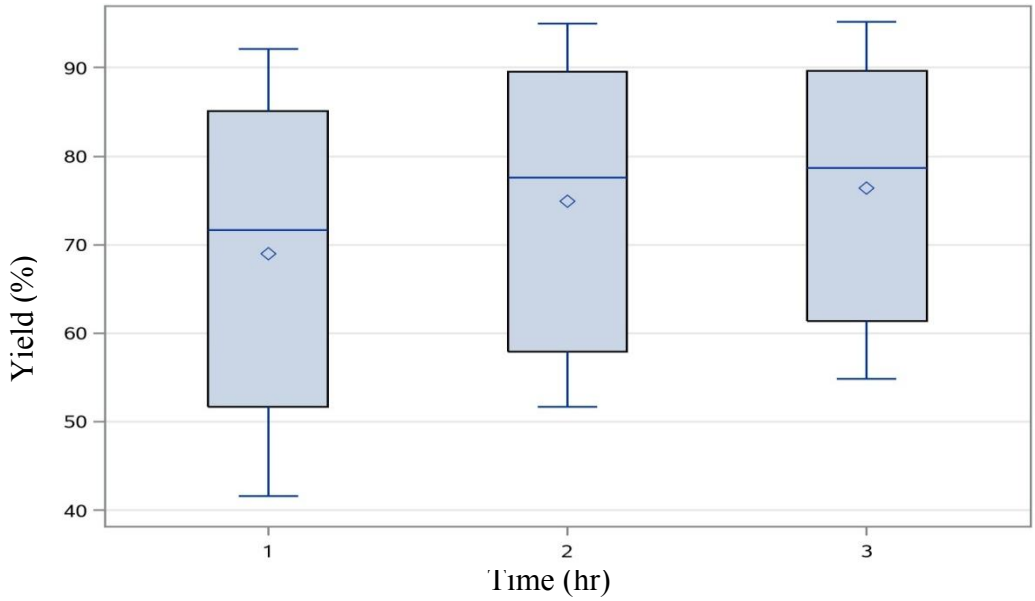

Figure 4.4: Distribution of calcium chloride yield depending on time

Average yield of calcium chloride 69.00% and 76.407% found for 1hr and 3hr extraction period respectively, whereas 74.93% calcium chloride was found for 2hr extraction period. Slow increase in the yield of calcium chloride in last hour may be due to slow reaction rate between HCl and $CaCO_3$.

4.3.5 Combined effect of concentration and time on calcium chloride extraction

Combination of concentration and time had a significant ($p<0.001$) effect on the yield in respect of calcium chloride (Table 4.3). Figure 4.5 shows that yield of calcium chloride increased with the increase of both concentration and extraction time. The highest average yield (58.21%) and lowest average yield (46.76%) were observed for 3hr and 2hr of extraction period at 0.5N.

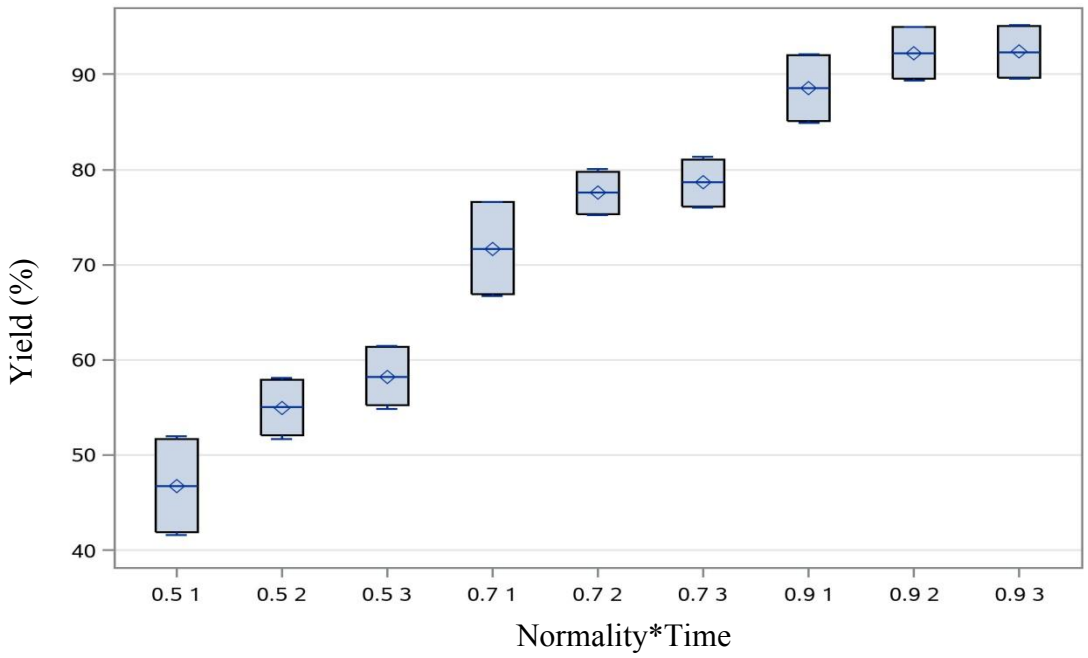

Figure 4.5: Distribution of calcium chloride yield depending on combination of concentration and time

A rapid increase in the yield of calcium chloride was resulted in first two hours and followed by a slow increase in yield at last hour in case of 0.7N and 0.9N. In case of 0.7N, highest average yield (78.64%) and lowest average yield (71.69%) were found for 3hr and 2hr of extraction period respectively. Using solvent concentration of 0.9N, 92.37% and 88.54%

average yields were resulted for 3hr and 2hr extraction period. A contradictory study was carried out by Hemung and Sriuttha (2014) who extracted calcium from Tilapia fish bone powder. It was reported that acetic acid (0.25M) was used at a ratio of 1:50 (w/v) with continuous stirring for 48hr at room temperature.

4.3.6 Combined effect of time and temperature on calcium chloride extraction

Yield of calcium chloride was found to be increased significantly ($p<0.001$) as combined effect of normality and temperature (Table 4.3; supplementary Figure 4.6). At 50°C, lowest mean value of calcium chloride (64.61%) and highest mean of calcium chloride (73.66%) were observed for 0.5N and 0.9N respectively. At 70°C, lowest average yield (73.39%) and highest average yield (79.15%) were observed for 0.5N and 0.9N respectively, whereas second highest average yield (77.55%) resulted for combination of 0.7N and 70°C. Daengprok *et al.* (2002) also observed that combination of heat treatment (75°C) and extraction time (5min) positively affected the extraction of calcium lactate from eggshell powder.

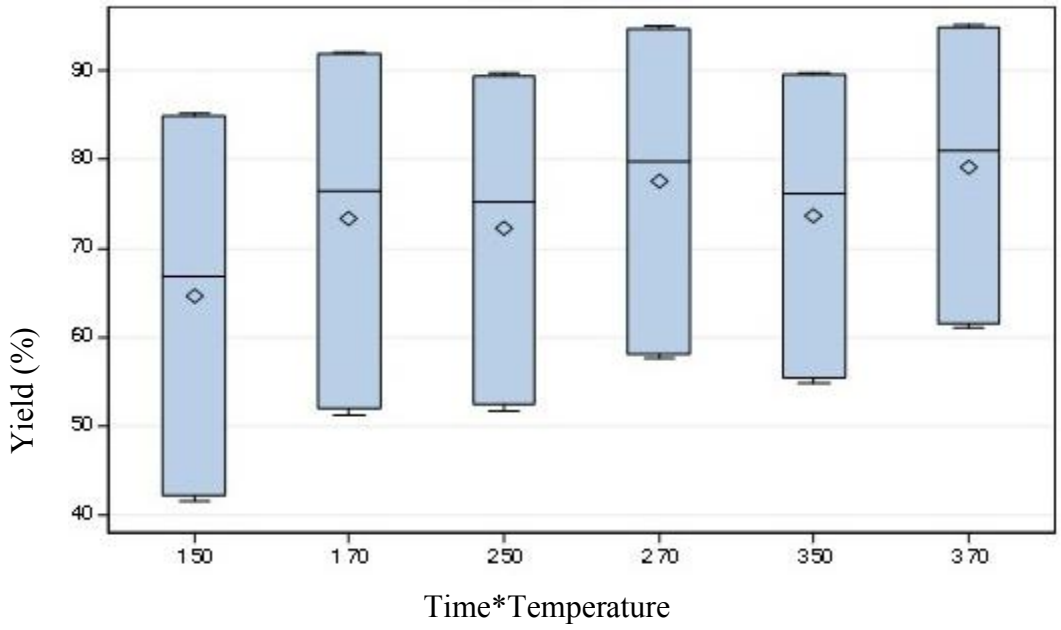

Figure 4.6: Distribution of calcium chloride yield depending on combination of time and temperature

4.3.7 Combined effect of normality, time and temperature on calcium chloride extraction

Highly significant ($p<0.001$) effect was revealed on yield of calcium chloride among the combined interaction of concentration, time and temperature (Table 4.3; supplementary Figure 4.7).

The treatment interaction of 0.5N and extraction temperature 50°C resulted average yield (55.15%) after 3hr extraction period and average yield (41.88%) after 1hr extraction period. At normality 0.5 and extraction temperature 70°C, average yield (61.27%) was obtained after 3hr extraction period, whereas average yield (51.65%) was obtained after 1hr extraction period.

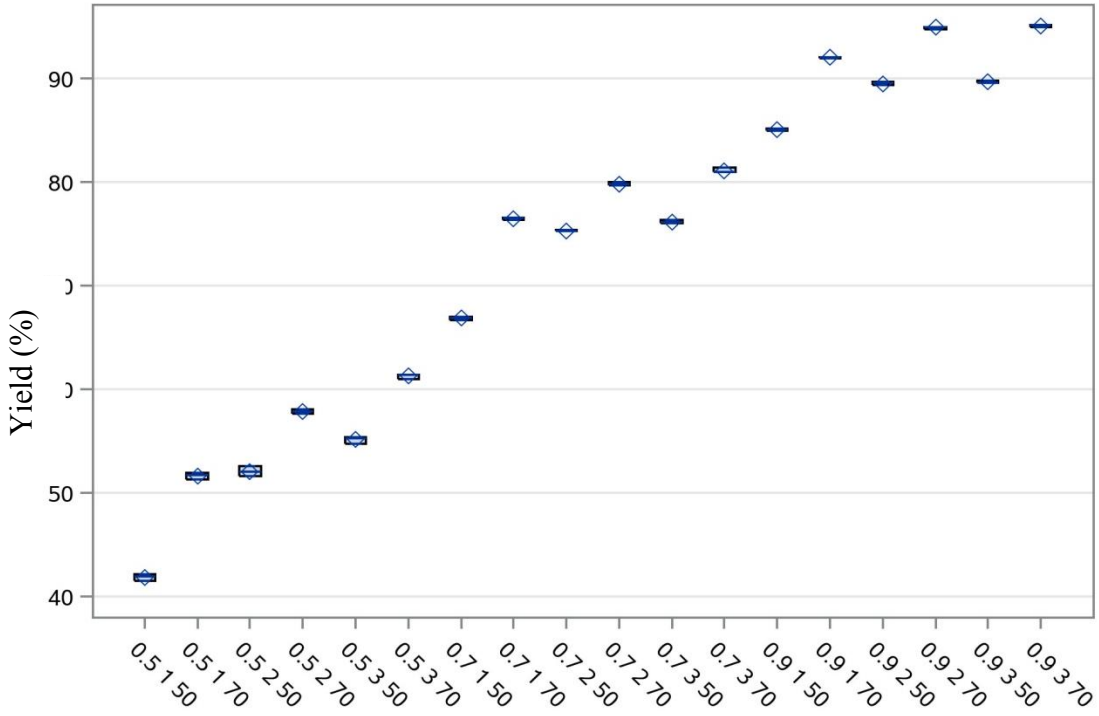

Figure 4.7: Distribution of calcium chloride yield depending on combination of concentration, time and temperature

As combined effect of 0.7N and extraction temperature 50°C, average yield (76.17%) was found after 3hr extraction period. On the other hand, average yield (81.11%) was resulted after 3hr extraction period at 70°C using 0.7N HCl.

The highly significant ($p<0.001$) difference in the yield of calcium chloride was resulted as combined effect of 0.9N, temperature of 50°C and time of 1hr, 2hr and 3hr (Table 4.3). Average yield of 89.66% was resulted after 3hr extraction period at 50°C using 0.9N HCl.

But a significant ($p<0.05$) difference in the yield of calcium chloride was not observed at 70°C using 0.9N HCl for 2hr and 3hr extraction period (Table 4.3). However, highest average yield of calcium chloride 94.92% and 95.08% were resulted after 2hr and 3hr extraction period respectively. These results were comparatively higher than the yield of calcium chloride (90.80%) carried out by Garnjanagoonchorn and Changpuak (2007).

4.4 Phase analysis of Extracted Calcium Chloride

The X-ray diffraction (XRD) patterns of extracted sample are shown in Figure 4.8. The highest peak of XRD spectra was resulted at 2-Theta 32° (Figure 4.8).

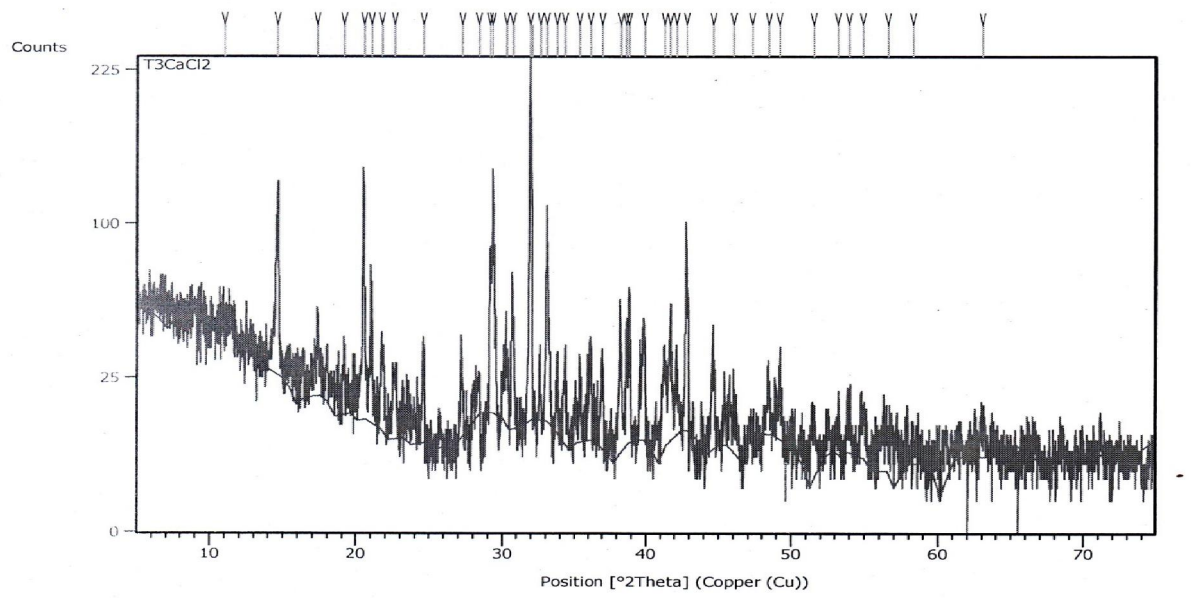

Figure 4.8: X-Ray Diffraction (XRD) patterns of Extracted Calcium Chloride

The XRD patterns for the respective sample indicated that the extracted sample was crystal calcium chloride ($CaCl_2$). The finding of this study was in agreement with the result of Garnjanagoonchorn and Changpuak (2007), who observed highest peak at 2-Theta 32°. They also reported that extracted eggshell calcium chloride was $CaCl_2.2H_2O$ based on XRD patterns.

In contrast, the X-ray diffraction patterns of commercial calcium chloride indicated the existing of $CaSO_4$ as a component of $CaCl_2.4H_2O$ (Garnjanagoonchorn and Changpuak, 2007).

4.5 Physico-chemical analysis of extracted calcium chloride

Proximate compositions of extracted calcium chloride from eggshell are showed in Table 4.4. Ash content in sample T_2 was significantly ($p<0.05$) higher than that of T_3 samples. Ash content 94.73% and 94.37% were observed in sample T_2 and T_3 respectively (Table 4.4). A similar result for ash content (94.37%) in extracted calcium chloride from eggshell was observed by Garnjanagoonchorn and Changpuak (2007).

Significant ($p<0.05$) difference was not found in fat content for increasing extraction period. Moreover, negligible amount of fat content was observed in both extracted samples.T_2 and T_3 samples were scored with 0.09% and 0.07% fat content respectively (Table 4.4). On the other hand, Garnjanagoonchorn and Changpuak (2007) observed no lipid in calcium chloride extracted from eggshell.

Table 4.4: Proximate composition of extracted calcium chloride (*)

Composition	T_2	T_3
Ash (%)	94.73 ± 0.18^a	94.37 ± 0.09^b
Protein (%)	1.08 ± 0.02^a	1.08 ± 0.02^a
Fat (%)	0.09 ± 0.02^a	0.07 ± 0.01^a
pH	8.27 ± 0.03^a	7.45 ± 0.19^b
Calcium (mg/g)	799.45 ± 1.49^a	635.57 ± 2.08^b

* Values are mean of triplicate analysis with standard error mean

T_2 - Calcium Chloride after 2hr extraction (0.9N and 70^{o}C)

T_3 - Calcium Chloride after 3hr extraction (0.9N and 70^{o}C)

The pH of extracted calcium chloride was decreased significantly ($p<0.05$) with the increase of extraction period but both of the samples were scored with higher pH value than neutral

condition (pH 7.0). The pH value 8.27 was recorded for sample T_2, whereas pH 7.45 was found in T_3 sample (Table 4.4). These findings were higher than that of Garnjanagoonchorn and Changpuak (2007) who found pH 5.25 in the extracted eggshell calcium chloride. This variation might be occurred because of using different concentrations of hydrochloric acid solvent.

Table 4.4 shows that calcium content in extracted sample was significantly ($p<0.01$) decreased with the increase of extraction period. In the sample T_2, highest calcium content (799.45mg/g) was recorded, whereas T_3 was scored with lowest value (635.57mg/g). Possible reason of decreasing calcium content in extracted calcium chloride with respect to extraction period is the increasing of unwanted mineral.

4.6 Metal constituents in extracted calcium chloride

Metal constituents in calcium chloride extracted from eggshell are presented in Table 4.5. It was observed that magnesium content was increased significantly ($p<0.01$) in sample T_3 compared to sample T_2. Highest magnesium content (11.63mg/g) was resulted in sample T_3, whereas T_2 was scored with lowest magnesium content (9.79mg/g).

Table 4.5: Metal constituent in extracted calcium chloride (*)

Composition	T_2	T_3
Magnesium (mg/g)	9.79 ± 0.11^b	11.63 ± 0.13^a
Zinc (mg/g)	9.63 ± 0.01^b	10.09 ± 0.02^a
Arsenic (ppm)	0.37 ± 0.01^a	0.16 ± 0.01^b
Lead (ppm)	1.51 ± 0.02^a	1.46 ± 0.03^a

* Values are mean of triplicate analysis with standard error mean

T_2 - Calcium Chloride after 2hr extraction (0.9N and 70°C)
T_3 - Calcium Chloride after 3hr extraction (0.9N and 70°C)

Table 4.5 shows that the level of zinc content was increased significantly ($p<0.01$) in sample T_3 than sample T_2. Highest zinc content (10.09mg/g) was investigated in sample T_3, and lowest value (9.63mg/g) was recorded in sample T_2 (Table 4.5).

Significant ($p<0.01$) effect of extraction period on arsenic content in extracted calcium chloride was found. Arsenic content 0.37ppm and 0.16ppm were found in sample T_3 and sample T_2 respectively (Table 4.5). Arsenic content decreased significantly with the increase of extraction period at same temperature. This finding was in agreement with the finding of Dahl *et al.* (2010) who found significant ($p<0.05$) decrease in arsenic concentration in boiled sea fish samples than raw samples. The same trend (43% to 50% reduction in arsenic by boiling) was investigated by Laparra *et al.* (2004) in edible seaweed.

Significant ($p<0.05$) difference in lead content of extracted calcium chloride samples was not found in this study. But a little decrease in lead content was observed in sample T_3 (1.46ppm) compared to T_2 sample (1.51ppm) (Table 4.5). Several studies have resulted that processing (boiling, heating and frying) reduces heavy metal constituent (lead) in perishable food products. Musaiger (2008) observed that cooking process resulted considerable reduction in heavy metal constituents (mercury, lead and cadmium) in rice. Considerable reduction in lead content by microware cooking and baking in fish was recorded by Ersoy (2011).

4.7 Storage of extracted calcium chloride

Effects of packaging materials on extracted calcium chloride during storage are shown in Figure 4.9 and Figure 4.10.

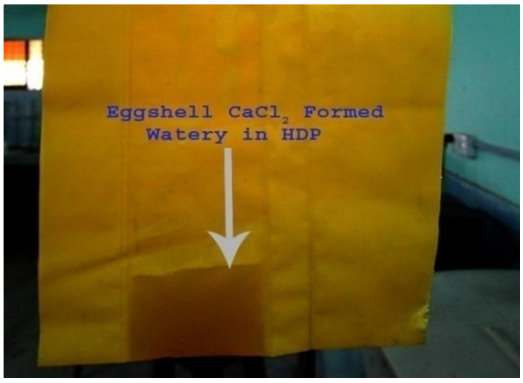

Figure 4.9: Calcium chloride in HDPE

Figure 4.10: Calcium chloride in LF

A watery solution of calcium chloride was observed in high density polyethylene (HDPE) package after second month of storage study (Figure 4.9). Watery solution in high density polyethylene package was resulted because of poor barrier property of HDPE package to water vapor transmission and high hygroscopic property of salt. On the other hand, extracted calcium chloride in laminated foil (LF) was found dry like commercial calcium chloride after six months of storage (Figure 4.10). Laminated foil is highly gas barrier and prevents water vapor transmission. Generally, Teflon bottle is used to store commercial calcium chloride to keep it anhydrous. This finding revealed that extracted calcium salt could be stored in laminated foil as appropriate packaging material instead of using Teflon bottle.


SUMMARY AND
CONCLUSION

CHAPTER IV

SUMMARY AND CONCLUSION

The findings of this study revealed that calcium salt could be extracted from eggshell by hydrolysis of membrane free eggshell powder with different concentrations of hydrochloric acid. In this study, calcium was extracted as calcium chloride that was identified by X-ray diffraction. This study clearly demonstrated the effect of hydrochloric acid concentration, extraction temperature and time on yield. HCl concentration (0.9N), extraction temperature (70^oC) and extraction period (2hr and 3hr) were identified as optimum conditions to extract calcium chloride form eggshell. Significant ($p<0.05$) difference was not found between maximum yields of 94.98% and 95.08%. Extracted calcium chloride was found to be highly soluble and alkaline. Eggshell calcium chloride was found to contain heavy metals in terms of arsenic and lead, which would be health issue. Arsenic (0.16ppm) and lead (1.46ppm) were resulted in calcium chloride (after 3hr extraction period) where safety limit of arsenic is 0.01-0.5ppm, and 0.01-2ppm for lead suggested by World Health Organization. But further studies need to be performed in order to purify the extracted calcium chloride. Regarding the potential health benefits of calcium salt, extraction and purification of eggshell calcium chloride would be possible use of eggshell, which is generally discarded as waste.

In addition, eggshell calcium chloride contains high amount of dietary calcium. Therefore, it is concluded that extracted calcium chloride could be used as fortificant in food product to prevent calcium deficiency and meet the daily calcium requirement as well.

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APPENDICES

Appendix I: Analysis of variance (ANOVA) for yield of calcium chloride from eggshell

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Normality	2	12980.92456	6490.46228	132343	<.0001
Temperature	1	571.41547	571.41547	11651.4	<.0001
Normality*temperature	2	3.98100	1.99050	40.59	<.0001
Time	2	553.37325	276.68662	5641.76	<.0001
Normality*time	4	89.30785	22.32696	455.26	<.0001
Time*temperature	2	34.97823	17.48911	356.61	<.0001
Normality*time*temperature	4	5.77576	1.44394	29.44	<.0001

Appendix II: Duncan's Multiple Range Test (DMRT) for yield of calcium chloride from eggshell

Treatment	Concentration of HCl (N)	Temperature (°C)	Time (hr)	Yield (%)
1	0.5	50	1	41.88^{p}
2	0.5	50	2	52.09^{o}
3	0.5	50	3	55.15^{n}
4	0.5	70	1	51.65^{m}
5	0.5	70	2	57.89^{l}
6	0.5	70	3	61.27^{k}
7	0.7	50	1	66.89^{j}
8	0.7	50	2	75.31^{i}
9	0.7	50	3	76.17^{h}
10	0.7	70	1	76.49^{h}
11	0.7	70	2	79.85^{g}
12	0.7	70	3	81.11^{f}
13	0.9	50	1	85.06^{e}
14	0.9	50	2	88.86^{d}
15	0.9	50	3	89.66^{c}
16	0.9	70	1	92.02^{b}
17	0.9	70	2	94.92^{a}
18	0.9	70	3	95.08^{a}

LSD = 0.15; $p<0.05$

Mean with same superscript within a column are not significant at $p<0.05$

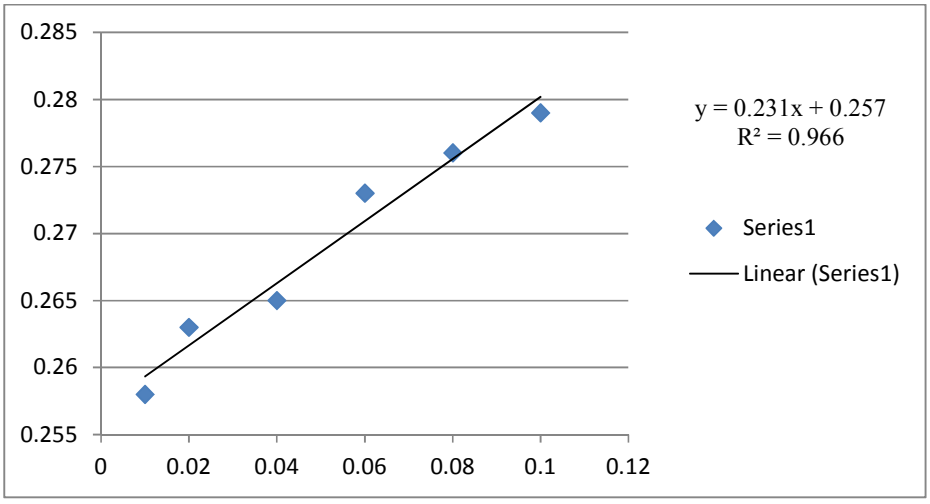
Appendix III: T-test for physico-chemical compositions of extracted calcium chloride

		Levene's Test for Equality of variances		t-test for equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Ash	Equal variances assumed	1.561	.280	3.072	4	.037	.35667	.11609	.03434	.67899
	Equal variances are not assumed			3.072	2.981	.055	.35667	.11609	-.01410	.72743
Protein	Equal variances assumed	.000	1.000	.535	4	.621	.00667	.01247	-.02796	.04130
	Equal variances are not assumed			.535	4.000	.621	.00667	.01247	-.02796	.04130
Fat	Equal variances assumed	15.876	.016	-.911	4	.414	-.19767	.21700	-.80016	.40483
	Equal variances are not assumed			-.911	2.000	.459	-.19767	.21700	-1.13132	.73599
pH	Equal variances assumed	.571	..492	25.107	4	.000	.82000	.03266	.72932	.91068
	Equal variances are not assumed			25.107	3.039	.000	.82000	.03266	.71680	.92320
Calcium	Equal variances assumed	1.914	.239	51.605	4	.000	163.88000	3.17568	155.06290	172.69710
	Equal variances are not assumed			51.605	3.051	.000	163.88000	3.17568	153.86878	173.89122

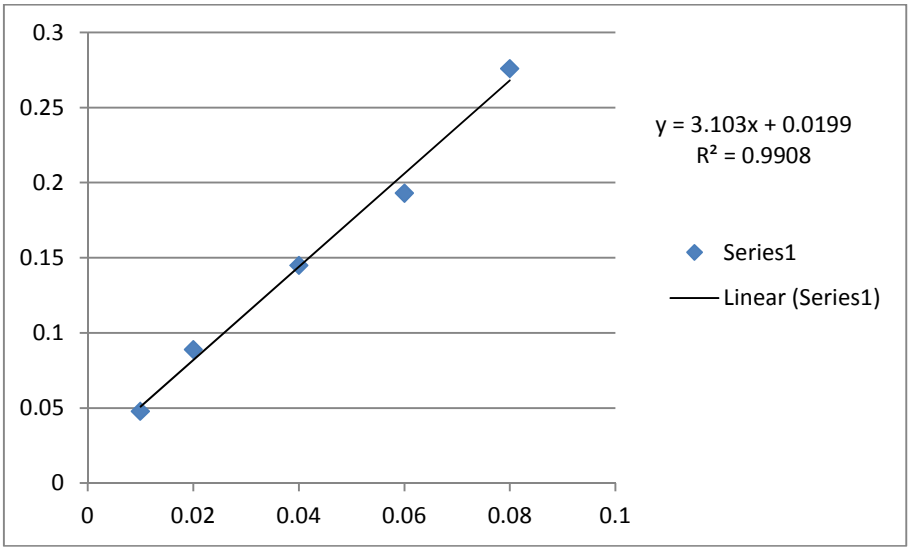
Appendix IV: T-test for metal constituents in calcium chloride extracted from eggshell

		Levene's Test for Equality of variances		t-test for equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Magnesium	Equal variances assumed	.058	.822	-10.387	4	.000	-1.83333	.17651	-2.32340	-1.34326
	Equal variances are not assumed			-10.387	3.892	.001	-1.83333	.17651	-2.32883	-1.33783
Zinc	Equal variances assumed	1.313	.316	-8.625	4	.001	-.45000	.05217	-.59486	-.30514
	Equal variances are not assumed			-8.625	2.941	.004	-.45000	.05217	-.61794	-.28206
Arsenic	Equal variances assumed	.727	.442	20.239	4	.000	.21333	.01054	.18407	.24260
	Equal variances are not assumed			20.239	3.448	.000	.21333	.01054	.18212	.24454
Lead	Equal variances assumed	.472	.530	1.329	4	.255	.05333	.04014	-.05811	.16478
	Equal variances are not assumed			1.329	3.226	.270	.05333	.04014	-.06948	.17615

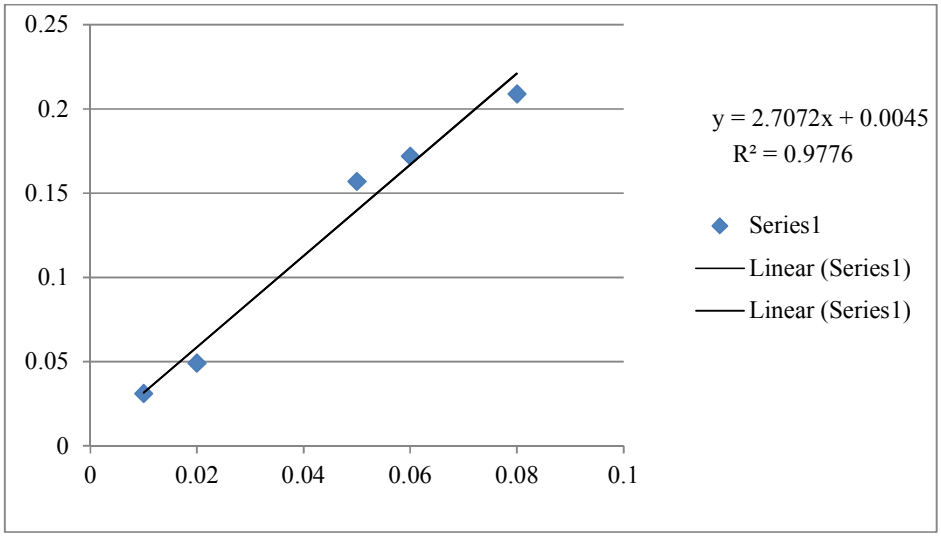
Appendix V: Standard curve of protein



Appendix VI: Standard curve of Arsenic



Appendix VII: Standard curve of Lead



Appendix VIII: Standard curve of Zinc