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FORTIFICATION OF LOQUAT JAM WITH CALCIUM EXTRACTED FROM EGGSHELL

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Abstract

The food industry, being a cornerstone of economies globally, confronts a significant challenge in managing its waste, particularly eggshells, which have garnered attention as major pollutants according to the Environmental Protection Agency. These eggshells, despite being rich in both organic and inorganic compounds, are often discarded in large quantities, leading to environmental degradation.

Calcium, a prominent component of eggshells, plays a vital role in human health, especially in maintaining bone density and preventing conditions like osteoporosis. This condition, affecting millions worldwide, underscores the importance of ensuring adequate calcium intake in diets.

Food fortification emerges as a cost-effective and evidence-based strategy to address nutritional deficiencies, including calcium. Over 140 countries have implemented food fortification programs, highlighting its global recognition and effectiveness in improving public health.

Additionally, the loquat fruit, with its abundance of nutrients, vitamins, and bioactive compounds, presents opportunities for both medicinal and food-related applications. Despite containing cyanogen glycosides in its seeds, which can be mildly toxic if ingested, the fruit itself is a rich source of essential nutrients such as vitamins A and B6, potassium, manganese, dietary fibers, and antioxidants.

Combining these insights, utilizing eggshells in fortified foods offers a novel and sustainable approach to enhance calcium intake, particularly beneficial for individuals with osteoporosis or those requiring increased calcium levels during

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critical growth phases, such as children. This innovative approach not only addresses environmental concerns related to waste but also contributes to improving public health outcomes.

Introduction

The food industry, as one of the largest industries around the world, is of primary importance to all national economies. The increase in the world population is leading to a sharp increase in the food production demand in the upcoming years. Under these circumstances, high volumes of food industry wastes attract increasing socioeconomic, political, and scientific attention. Eggshell waste has been ranked as the 15th major food industry pollution problem by the Environmental Protection Agency. They are considered as a major source of environmental pollution when not properly dumped off in specified locations, thus causing health hazards later due to fungal growth on these eggshells (Ajala, Eletta, Ajala, &Oyeniyi, 2018). A significant concern is the environmental impact of the production of such massive waste products. Even though eggshell waste, which is high in organic and inorganic compounds, could be used for different purposes, a large majority of these materials are thrown away, which leads toward the environmental issues. Vast amounts of chicken eggs are produced annually, with a significant proportion (30%) processed in food industries, lead to a massive accretion of eggshell waste (Ahmed et al., 2019).

The eggshell is composed of about 95.1% inorganic matter and 3.3% protein. The eggshell was reported to weigh on average 1.6% of the total chicken egg-based on wet weight (Adeyeye, 2009) with a thickness between 0.33 and 0.35 mm (Tadesse et al., 2015). About 91% of the weight of the hen's egg shell consists of inorganic matter, and about 98% of this is calcite (calcium carbonate crystals) (Burley and Vadehra, 1989) Around 37–39% of calcium can be isolated from chicken eggshell , the remaining 2% of inorganic material being composed of small amounts of magnesium carbonate and tricalcium phosphate. The remainder of the weight of the shell as a whole is made up of about 6% protein and some water. It has been reported that the amount of calcium absorption from chicken eggshells is higher when compared to that of dried milk solution as stated previously by Oguido et al. (1995).

Calcium deficiency can have significant consequences on various aspects of health. One of the most noticeable effects is weakened bone density, leading to conditions like osteopenia and osteoporosis.(Robinson, Greenway et al. 2025) Osteoporosis is a condition that reduces bone thickness, reduces specificity, and makes bones appear brittle is a major public health problem affecting both genders, chiefly women, Globally 200 million women suffer from osteoporosis. The average calcium intake in children in Pakistan was found to be 607.5 mg/day. According to the National Nutrition Survey Report in 2011, the daily calcium consumption in Punjab was 637 mg/day. In Pakistan, 51.1% of women suffer from hypocalcaemia, with 51.9% residing in urban areas and 50.8% in rural areas. In Punjab, the same survey reported that 52.3% of women have calcium deficiency. Encouragement of increased consumption of calcium-rich foods has the potential to be a cost-effective strategy for reducing fracture incidence later in life and for increasing patients' dietary quality and overall health. Food fortification stands out among public health interventions as one of the most effective methods of preventing nutritional deficiencies.

Fortification is an approach based on evidence by which micronutrient deficits can be averted and minimized. It is a proven, safe and cost-effective strategy for improving diets and for the prevention and control of micronutrient deficiencies (Ashraf 2025). Consensus ranked food fortification as one of the most cost-effective development priorities currently, over 140 countries globally have guidance or regulations in place for fortification programs, the majority of which are mandatory. Before describing calcium food fortification programs, we deemed it important to provide an overall description of current national micronutrient food fortification programs. Based on the WHO Global

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Nutrition Policy Review from 2018, 68% of a total of 155 countries surveyed have a national food fortification program (Bell, Rodrigues et al. 2024).

Loquat (Eriobotrya japonica) is an evergreen tree or shrub, widely cultivated for its edible fruit, and it belongs to genus Eriobotrya, family Rosaceae, and subfamily Maloideae. The color of the fruit may vary from pale yellow to deep orange depending on the cultivar (Lin, Lin et al. 2022). Because of the cyanogen glycosides they contain, the seeds of the loquat fruit have a mild poisonous effect. Ingestion of these may result in symptoms including vomiting and breathlessness (Lin, Lin et al. 2022).

For a very long time, people have used loquat as a medicinal herb to cure conditions like diabetes, cancer, inflammation, chronic bronchitis, and cough. Loquats are thought to be an excellent source of vital nutrients, including minerals, vitamins, and electrolytes, in their pulp, peel, seeds, and leaves. The plant's many components include bioactive substances and phytochemicals, including as flavonoids, carotenoids, and phenolic compounds, which exhibit strong antibacterial, free radical scavenging, and antioxidant properties (Nwozo, Effiong et al. 2023). Loquat has components that are good to health, which makes it a promising ingredient for pharmaceutical applications as well as the creation of food products with additional value.

The fruit is well-known for containing vitamins A and B6. It also contains higher levels of minerals like potassium and manganese as well as dietary fibers. The very sweet fruit has a high antioxidant content. Omega-3 and omega-6 fatty acids, which are low in carbohydrates and cholesterol, are among the monounsaturated acids found in the fruit (Nwozo, Effiong et al. 2023). The fruit has 50 kcal of energy, 0.4 g of proteins per 100 g of carbohydrates, 1.7 g of fiber per 100 g, and 16 and 14mgfolate per 100 g of fruit, respectively. Additionally, its vitamin C level is lower, at about 2 mg/100 g.

An effective way to increase calcium intake, one of which is through functional foods, has been studied in recent publications given the attractive characteristics of eggshells, food products containing this byproduct might be a useful alternative. Food products enriched with eggshells may be an attractive way to improve the diet of people with osteoporosis or to prevent the development of disorders and the diet of children during the intensive growth phase (Arnold, Rajagukguk et al. 2021).

Objectives

The major goal of this internship project is to investigate the viability of using egg shells as a source of calcium in different food items. The key aims of this study are as follows:

- Eggshell waste, which contains a lot of calcium, can be used to add calcium to food
- The aim of this study was to determine the nutritional contribution of each portion to the daily intake of calcium and to fortify classic and easily prepared loquat jam with eggshell powder
- To evaluate the sensory properties and consumer acceptance of food items enriched with calcium

Review of Literature

Eggshell waste is also a challenge as it adds up to costs and takes up landfills. Thus, the value addition of eggshells and their utilization should focus on the food processing industries. A considerable amount of work has already been done to utilize this waste and incorporate it into our daily diet as a source of calcium. Some of the products with the incorporation of eggshells are calcium-fortified pork sausage (Daengprok et al., 2002), nano-powdered eggshell (NPES) supplemented yogurt (Al Mijan et al., 2014), dietary calcium biscuits (Hassan, 2015), calcium-fortified roasted and ground coffee (de Paula et al., 2014), calcium-fortified chocolate cake (Ray et al., 2017) and other homemade food products like pizza and pasta (Brun et al., 2013).

Meeting the body's calcium requirements through dietary intake is crucial for maintaining overall health. Interestingly, researchers have discovered a potential solution within waste materials that contain valuable nutrients, particularly focusing on eggshells, bones, and seafood waste due to their high calcium content. However, eggshell waste is far from worthless. It contains a rich composition of

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both organic and inorganic compounds that can be repurposed and recycled into beneficial commodities. The composition of the eggshells is approximately 98.2, 0.9, 0.9% Calcium carbonate, Magnesium and Phosphorous (phosphate) respectively (Romanoff et al., 1949). Shell membranes comprise of 69.2% protein, 2.7% fat, 1.5% moisture and 27.2% ash (MacNeil, 1997). Shell membranes protein comprises of approximately 10% collagen (Froning, 1998). Eggshell and shell membranes are non-edible by-products with little saleable value but they may contain biologically active compounds (Nakano et al., 2003). Eggshells contain calcium and trace amounts of other micro elements, i.e. magnesium, boron, copper, iron, manganese, molybdenum, Sulphur, silicon and zinc (Bee, 2011). Eggshell calcium is probably the best natural source of calcium and it is about 90% absorbable (Bee, 2011).

The scientific community has been actively researching methods to transform this waste into valuable products, thus addressing both environmental concerns and nutritional needs. One innovative approach is the utilization of eggshell waste as a source of calcium supplements (Singh, Kelkar et al. 2021). By processing and purifying the calcium-rich compounds found in eggshells, researchers have developed calcium supplements that can be used to meet the body's daily calcium requirements.

This not only reduces waste but also provides a sustainable solution to combatting calcium deficiency in populations worldwide. Furthermore, eggshell waste has also found applications in agricultural practices. The calcium carbonate present in eggshells can be used as a soil amendment to improve soil quality and enhance plant growth. Calcium is an essential mineral needed for physiological functions, metabolic processes, and maintenance of bone and tooth tissue in the human body. Several health conditions related to calcium intake include calcium deficiency, osteoporosis. Stunting, and blood clotting (Afzal, 2020; Arnold et al., 2022; Sari et al., 2016). Recommendation Daily calcium requirements based on age and gender are 200 mg for ages 0-6 months. 250 mg for ages 7-11 months, 650 mg for ages 1-3 years, 1100 mg for teenagers and adults, 1300 mg for pregnant women and/or breastfeeding mothers (Food and Drug Supervisory Agency, 2016). Generally, calcium needs are obtained from consuming milk-based products, but for those who do not consume milk-based products, it is difficult to meet daily calcium needs. Therefore, other sources of calcium are needed apart from milk-based products, one of which is egg shells.

The use of egg shells as a source of calcium is done by first processing them into powder form through a process of soaking, boiling and drying. Fortification of egg shell powder has been carried out on various food products aimed at increasing calcium content, including soy milk jelly candy (Novelina et al., 2020), pudding (Pebrianti et al., 2023), snack bars (Handayani et al., 2020). 2022), tekwan (Telisa et al., 2022), chicken nuggets (Merta et al., 2020), jelly (Younas et al., 2021), muffins (Afzal, 2020), gingerbread (Arnold et al., 2022), biscuits (Shahnila et al, 2022), and ice cream (Hasan et al., 2023). The results of this research show that the addition of eggshell powder can significantly increase the calcium content compared to the control, so it can be concluded that eggshell powderhas health potential and is worth considering to be added to food processing.

Making eggshell powder can be done by using several methods which generally consist of cleaning, size reduction, flouring and filtering Extraction of calcium from biological resources involves multiple approaches such as fast and high intensity pulsed electric fields, microfluidic solvent extraction, and alkaline treatment, all collectively termed as biological methods (Bubel et al. 2016; Boskey and Posner 1976). Many methodologies and other associated downstream processes are involved in its purification. The production of calcium via chemical methods often utilizes procedures that include electrolytic production, distillation, or aluminothermic reduction (Jacob and Srikanth 1990; Wilhelm and Carlson 1964). High energy milling (HEM) processes are used in mineral processing industries and in powder metallurgy where a wide variety of materials are obtained from

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a pulverized metallic form. Large structures are broken down into smaller ones without allowing clumping. This method is used to create particles for a faster rate of reaction by increasing surface area. However, this process is inefficient and has a high energy requirement. The methodology is shown in Figure 8 (Waheed et al. 2019).

Materials and Method

Apparatus:

- Sterile test tubes or containers
- Sterile pipettes and tips
- Agar plates
- Incubator set to appropriate temperature (usually 30-35°C)
- Autoclave for sterilizing media and equipment
- Bunsen burner or alcohol lamp for sterilizing tools
- Sterile Petri dishes
- Sterile pipettes (1 mL, 0.1 mL)
- Sterile dilution bottles (e.g., 9 mL sterile water)
- Incubator set at appropriate temperature (e.g., 25°C)
- Microscope, slides and coverslips
- Microbial media (e.g., Sabouraud Dextrose Agar for yeast and Malt Extract Agarfor molds
- Sterile dilution bottles (160 ml)
- Sterile pipettes (1 ml, 10 ml)
- Sterile test tubes
- Incubator at 35°C
- Water bath at 45.5°C
- Mechanical blender
- Bunsen burner
- Autoclave for sterilization
- Microbiological media: Brilliant Green Bile Broth (BGB), Lauryl Tryptose (LT) broth, EC medium
- Levine's Eosin-Methylene Blue (L-EMB) agar, Tryptone broth, MR-VP medium, Koser citrate broth, Plate Count Agar (PCA)
- Pipette filler
- Weight Balance
- ELISA kit.

EQUIPMENTS USED

Laminar flow cabinet:

A laminar flow hood holds significant importance in laboratories for ensuring sterile and contamination-free environments. By directing HEPA-filtered air in a controlled, unidirectional flow, it safeguards sensitive samples from external contaminants, vital for accurate results in cell culture and microbiological work (BOUMAZA 2024). It allows researchers to manipulate samples while minimizing the risk of introducing contaminants. Laminar flow hoods are essential in industries like pharmaceuticals, biotechnology, and medical research, where maintaining aseptic conditions is paramount.

Incubator:

An incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains an optimal temperature, humidity, and other conditions such as the CO_2 and oxygen content of the atmosphere inside. Incubators are important for many experimental tasks in cell biology, microbiology, and molecular biology, and are used to culture both bacterial and eukaryotic cells. Louis Pasteur used the

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small opening under his stairs as an incubator. Hatchery equipment is also used as a replacement for chickens in poultry farming (Josephson 2020).

Autoclave machine:

The autoclave machine holds immense significance in laboratory settings due to its role in sterilizing equipment and materials. It employs high pressure and steam to eliminate microorganisms and their spores, ensuring aseptic conditions for experiments and procedures. The autoclave's capability to deactivate various pathogens makes it essential for preventing contamination and maintaining research integrity. Its application spans across medical, microbiological, and molecular research, as well as in industries like pharmaceuticals and biotechnology.

Colony counter:

A colony counter is a device used to count colonies of bacteria or other microorganisms growing on an agar plate. Biological measures are often based on precise counts of bacterial colonies and cells. Colony counters are used to estimate the density of microorganisms in a liquid culture by counting individual colonies on agar plates, slides, minigels, or Petri dishes (Sandle 2020).

Hot air oven:

Hot air ovens are electric devices that use dry heat to sterilize. They were originally developed by Pasteur. They usually use a thermostat to control the temperature. Their double wall insulation preserves heat and saves energy, the inner layer is a poor conductor and the outer metal. There is also an air-filled space between them to facilitate insulation. The circulation fan helps distribute the heat evenly. They are equipped with adjustable wire mesh covered carts or an aluminum base and can have an on/off button switch as well as temperature and hold time indicators and controls (OTEPOLA 2016).

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Magnetic stirring:

A magnetic stirrer is a device widely used in laboratories that consists of a rotating magnet or fixed electromagnet that produces a rotating magnetic field. This device is used to make a stir stick, dip a liquid, quickly centrifuge, or mix or mix a solution.

Most current magnetic stirrers rotate the magnets using an electric motor. This type of equipment is one of the simplest alloys. Magnetic stirrers are quiet and allow mixing of closed systems without isolation as with mechanical stirrers.(Zlokarnik 2008)

Analytical Weight Balance:

Analytical balances are highly sensitive laboratory instruments designed to accurately measure mass. Their readability ranges from 0.1 to 0.01 mg. Analytical balances have a draft guard or weighing chamber that prevents air currents from affecting very small samples (Mao 2024). They are designed to detect very fine impurities, so even the slightest vibration or wind can affect the results.

Vortex mixer:

A vortex mixer, or vortex, is a simple device often used in laboratories to mix small vials of liquid. It consists of an electric motor with a vertical drive shaft attached to a rubber casing mounted slightly off-center (Dickey and Fasano 2003). When the motor is running, the rubber part vibrates rapidly in a circular motion. If a test tube or other suitable container is pushed against the rubber cup (or touched to its edge), the movement is transferred to the liquid inside and a vortex is created (Mallanagoud and Anuradha).

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Stomacher:

A stomacher is a machine that places a sample in a sterile bag for microbiological work. It facilitates the thorough mixing of samples with dilution water, ensuring even distribution of microorganisms. This process is essential for accurate analysis of microbial populations and pathogens. The stomacher's gentle agitation prevents damage to delicate samples while maximizing extraction efficiency.

Dispensing Pipette:

Pipette (sometimes spelled pipette) is a commonly used laboratory tool in chemistry, biology, and medicine to transport a measured amount of liquid, often as an intermediate dispenser (Levron 2025). There are several different precision pipettes for different purposes, from single piece glass pipettes to more sophisticated adjustable or electronic pipettes.

Compound Microscope:

A compound microscope is an instrument, used to see magnified images of small objects on a slide. It can achieve higher magnification levels than stereomicroscopes or other low-power microscopes and reduce chromatic aberration. This is achieved by using two or more lenses in the objective and eyepiece. The lens or lenses in the nose have a short focal length and are located close to the object where it collects light and focuses the image of the object with the microscope. The second eye lens has a longer focal length and magnifies the image even more.

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Refrigerator:

Laboratory refrigerators must maintain a constant temperature to minimize bacterial contamination and the risk of explosion of volatile materials. To achieve high accuracy, the refrigerator needs air to circulate and a fan to maintain a constant temperature. The fan turns off when the door is open to prevent cold air from blowing out of the unit. Laboratory refrigerators have separate compartments to prevent cross-contamination and can store special medical supplies such as blood or vaccines.

Shaking Water Bath:

Shaking Water Baths are ideal for melting, heating, mixing, and shaking samples. Some applications include hybridization, bacterial culture, cell aeration, and molecular biological assays. Power can vary from vendor to vendor, from small desktop models to large industrial models. The inner part of the shaking water bath should be made of stainless steel for health and longevity. Most models use a magnetically coupled shock mechanism (Fan, Liu et al. 2025). The shake function can be linear or orbital with an adjustable shake speed. Some features to consider include temperature and uniformity, display, programmability, build quality, low-level indicator, and platforms available for customization.

pH meter:

The pH meter holds significant importance in laboratories due to its ability to measure pH levels accurately, aiding in various scientific disciplines. It is extensively used in Chemistry for reaction monitoring and endpoint determination (Nhivekar, Khandait et al. 2025). In biology, it helps understand enzyme activity and cellular processes. Industries rely on pH meters for quality control in food, pharmaceuticals, and water treatment. Environmental science benefits from pH meters for water quality assessment.

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Methods:

Extraction of calcium carbonate from eggshell:

Eggshell Collection and Preparation:

- Collect fresh eggshells from eggs used in cooking or baking.
- Rinse the eggshells under running water to remove any remaining egg residue.
- Allow the rinsed eggshells to air dry or pat them dry with a clean towel.
- Once dry, inspect the eggshells for any cracks or imperfections. Discard any damaged shell.

Fig: Washed Eggshells.

Fig: Ready To Dry in Oven

Separation of Eggshell Membranes:

- Manually separate the two membranes of the eggshell. The outer membrane is designated for calcium carbonate extraction, while the inner membrane is reserved for collagen extraction, if desired.
- Carefully peel away the membranes from the inner surface of the eggshell using tweezers or by gently scraping with a knife.

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Fig: Eggshell and inner membrane separation

Calcium Carbonate Extraction:

- Preheat an oven to 100°C (212°F),Spread the separated eggshells on a baking sheet in a single layer and place them in the preheated oven
- Allow the eggshells to dry in the oven for 10 to 15 minutes or until they are completely dry and brittle. Avoid overheating, as this can cause the eggshells to lose their calcium carbonate content.
- Once dried, remove the eggshells from the oven and let them cool to room temperature.
- Transfer the dried eggshells to a mortar and pestle or a food processor. Crush the eggshells into small pieces to increase the surface area for the extraction process, Place the crushed eggshells into a clean beaker or container.
- Prepare a solution of 10% acetic acid (CH3COOH) by mixing 10 parts of distilled water with 1 part of glacial acetic acid. Ensure that there is enough solution to completely cover the crushed eggshells.
- Carefully pour the prepared acetic acid solution over the crushed eggshells in the beaker or container until they are fully submerged as the acetic acid reacts with the calcium carbonate in the eggshells, bubbles and foam will appear, indicating the release of carbon dioxide gas.
- Close the beaker or container with a lid to prevent evaporation and contamination, Allow the mixture to stand at room temperature for a minimum of 24 hours, occasionally stirring the solution with a glass stirrer to facilitate the extraction process.

Fig: Acetic Acid

Fig: Acetic Acid poured into eggs

- After 24 hours, carefully remove the lid and check the progress of the extraction. The liquid should appear cloudy, indicating the presence of dissolved calcium carbonate, Transfer the cloudy liquid to a separate container, leaving behind any undissolved residue.
- Heat the extracted liquid on a hot plate or in a water bath to evaporate the solvent (acetic acid) and concentrate the calcium carbonate solution.Once the liquid has completely evaporated, a white powder consisting of calcium carbonate will remain at the bottom of the container.
- Carefully collect the calcium carbonate powder using a spatula or spoon and transfer it to a clean, dry container for storage or further analysis.

Fortification

An alarming proportion of the world's population suffers from 'hidden hunger', a phrase used to describe micronutrient deficiencies since the symptoms are frequently invisible or unfelt. Micronutrient deficiencies, which are little amounts of vitamins and minerals that the body need for physical and mental development,

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are ubiquitous, affecting more than one-third of the global population. Individuals and families suffer substantial effects in their absence, including learning difficulties, reduced labour capability, disease, and death. There are several strategies to boost micronutrient intake, including taking supplements on a regular basis or implementing dietary changes that encourage the consumption of micronutrient-rich foods and optimize their absorption in the diet. Numerous national governments acknowledge fortification as a crucial strategy for consistently and sustainably enhancing the health and nutrition of millions of people. Eggshell powder incorporated in numerous products such as jam, jelly.

Ingredients:

- 1 kg ripe loquats
- 500g granulated sugar
- Juice of 1 lemon and water
- 1 teaspoon finely ground eggshell powder

Procedure

- Firstly collect sound and fresh fruit free from any type of bruising and fruit must be fully ripened
- Thoroughly wash the loquats, remove the seeds, and chop them into small pieces. Then weight it on weighing balance.In a large pot, pour the chopped loquats, sugar, and lemon juice. Mix well and let

it sit for about 30 minutes to dissolve the sugar and blend the flavors.

- Add enough water to cover the loquats in the pot. Place the pot over medium heat and bring the mixture to a gentle boil, stirring occasionally.
- Once boiling, reduce the heat to low and let it simmer for 30-40 minutes or until the loquats are soft and the mixture thickens.
- While simmering, grinded clean and dried eggshells into a fine powder to make the eggshell powder.

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- When the jam reaches the desired consistency, stir in the finely ground eggshell powder thoroughly.Cook for an additional 5 minutes to allow the calcium from the eggshell powder to incorporate into the jam.
- Turn off the heat and let the jam cool slightly. Transfer the jam into sterilized jars, seal tightly, and store in a cool, dark place.

Analysis

Proximate Analysis:

1. Moisture Determination:

Introduction:

Moisture refers to the presence of liquid, particularly water, often in small quantities. It can be found in various forms, including atmospheric humidity, the water content of foods, and within commercial products. Moisture also encompasses the level of water vapor present in the air. The aim is to reduce moisture content in food to specific percentages, thereby enhancing shelf life and ensuring quality control.

Material and Method Requirements:

- Sample
- Oven
- Petri plate
- Desiccator
- Weighing balance

Oven drying method

- First of all we take the sample to determine the moisture content of that sample.
- Weight the Petri plate without sample and after weighing it, put the sample into Petri plate and weight it again.
- After this put the sample into oven having temperature 100° C for 3 to 4 hours.
- After the remove the sample and put into desiccators to cool down the sample in 10 to 15minutes.
- Than after cool down the sample again weight the sample.

Formula:

$$\text{Moisture \%} = \frac{\text{original weight} - \text{weight after drying}}{} \times 100$$

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Original weight

Fig: oven

1. Ash Determination

Principle

Ashing is incineration at high temperature (500-600° C) with muffle furnace, of organic part of food sample in the form of water and Co2 and produce minerals part. Ash content represents the total mineral content of food.

Material and Method

Requirements

- Measuring dish
- Dish holder
- Muffle furnace (dry oven)
- Crucible & Tongs
- Desiccator & Food sample

Fig: Desiccator

Procedure:

Fig: Muffle Furnace

Fig: Sample place

- Weigh the empty crucible and record its weight.
- Add 5 g of the sample to the crucible and record the weight.

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- Put the crucible into the oven for around 3 hours until the sample become white or grey.
- Put the crucible in the desiccators.
- Weigh the crucibles after it become cold and calculate the ash percentage according the following equation

Formula:

$$\text{Ash \%} = \frac{\text{weight of sample with crucible before ashing} - \text{weight of crucible}}{\text{Weight of ash}} \times 100$$

3. Crude Fat:

Introduction:

Crude fat is the term used to refer to the crude mixture of fat-soluble material present in a sample. Crude fat also known as the ether extract or the free lipid content is the traditional measure of fat in food products.

The lipid materials may include triglycerides, diglycerides, monoglycerides, phospholipids,steroids, free fatty acids, fat soluble vitamins, carotene pigments, chlorophylls, etc.

The common approach for total crude fat determination is based on the solubility of lipids in non-polar organic solvents such as hexanes, petroleum ether, or supercritical liquid carbondioxide with or without a solvent modifier.

Principle.

Extraction of a test portion, in a suitable apparatus, with hexane and petroleum which is not soluble in fat use to extract hexane. Elimination of the solvent and weighing of the extract obtained.

Fig: Soxhlet Apparatus

Materials and methods:

Requirements:

- Sample
- Thimble paper
- Flask
- Weighing balance
- Oven
- Filter paper

Reagents:

- Diethyl ether
- n-hexane (we use n-hexane)

Procedure:

- Weigh accurately 2g of sample (coriander) into a paper thimble.

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- Dry in an oven or preferably in a circulating air oven set at 98°C for 3 to 4 hrs
- Take the sample out the oven , cool and connect to soxhlet apparatus
- Weight the empty flask; add n- hexane in the volumetric flask up to 100ml.
- Put the sample into siphon tube and turn on the heating , n-hexane have low boiling point than fat so it start boil at 60 -65° C , go into siphon tube and dissolve fat from the sample.
- When siphon tube fill it again fell down into receiving flask, repeat 5-6 cycle and then turn off the heat.
- During all this process water should be run all the time, which is use for condensation of vapor and cooling purpose.
- From all this process n-hexane is recovered that can be use for next experiment.
- Remove the flask and put into oven that the remaining hexane should evaporate , and weight the flask because the remaining material is only fat that we extract from sample.

Formula:

$$\text{Fat \%} = \frac{\text{weight of beaker with fat} - \text{weight of empty beaker}}{\text{Weight of original sample}} \times 100$$

4. Crude Fiber

Introduction:

Fiber is a substance found in plants. Dietary fiber, which is the type of fiber you can eat, is found in fruits, vegetables, and grains. It is an important part of a healthy diet.

Materials and Methods:

Requirement:

- Sample
- Beaker
- Volumetric flask
- Filter paper
- Crucible
- Hot plate
- Furnace

Reagents:

- Sulphuric acid 1.25g/100ml
- Sodium hydroxide 1.25g/100ml
- Asbestose
- Petroleum ether
- Ethyl alcohol

Procedure:

- Take 5g of fat free sample after grinding the sample put it into beaker and add 200ml of H2SO4 solution.
- Boil it on the hot plate for 30 minutes.
- Filter the sample with muslin cloth.
- Take the retentate (upper portion after filtration) of the sample.
- Put this sample into the beaker and add 200ml of the NAOH solution.
- Again boil the sample for 30 mints on hot plate.

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- Filter the boil solution, and put into the crucible. Put into the oven for drying the sample.
- Weight after drying the sample. Then cheering of the sample or flame, and put into furnace at 550°C for 3-4 hours.
- After ashing remove the sample from furnace and weight the sample for fiber determination

Formula

$$\text{Fiber \%} = \frac{w1 - w2}{\text{Weight of sample}} \times 100$$

Fig: Hot plates

Microbial Analysis:

1. Total Plate Count Aerobic Count; Procedure:

Step 1: Preparation of Diluent and Media:

Prepare 500ml of butter field phosphate buffer by dissolving 34g of potassium dihydrogen arthophosphate anhydrous in 500ml of distilled water (dH2O) and adjust the pH to 7.2. Plate count agar is prepared by adding 7g of agar in 300ml of dH2O and autoclaved.

Step 2: Sample Preparation:

Weigh 1g of the calcium powder using a weight balance and combine it with 225ml of butter field phosphate buffer in a stomacher bag. Homogenize the mixture on a stomacher machine for 2 minutes.

Step 3: Serial Dilutions:

The homogenized sample serves as the 1st dilution. Transfer 1ml from the 1st dilution to a 2nd test tube containing 9ml of butter field phosphate buffer. Vortex the solution and transfer 1ml from the 2nd test tube to the 3rd one, and continue this process for 5 dilutions.

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Step 4: Plate Pouring & Incubation

All the dilutions are plated in duplicates, 1ml from each by pour plate method on empty Sterile plates. Molten media allowed to solidify at room temperature and then incubated at 37°C

Fig: Pouring Media into plates Plates

Fig: Incubating the plates

Step 5: COLONY COUNTING BY CFU:

All the plates were counted on colony counter.The plates having colony count between 25-250 is selected for CFU per g with the formula:

$$CFU/g = \frac{\text{No of Colonies} \times \text{Dilution Factor}}{\text{Volume of dilution poured on plate}}$$

Total Yeast and Mold Count (TYMC);

Procedure for Total Yeast and Mold Count:

Step 1: Diluent and Media Preparation

- 500ml 0.1% buffered peptone water is prepared by dissolving
- 0.5g peptone water in 500ml dH2O
- The pH is adjusted to 7.2
- Potato dextrose agar is prepared for ten plates and autoclaved
- After autoclave antibiotic chloramphenicol in conc. of 0.1 g is added to hot media

Step 2: Sample Preparation

10g of food sample i-e oats is weigh on weight balance and combined with 100ml of buffered peptone water in a stomacher bag. And homogenized on a stomacher machine
For 2 minutes.

Step 3: Serial Dilutions

Homogenized sample acted as 1st dilution. From 1st dilution 100µl was transferred to 2nd test tube containing 9ml of butter field phosphate buffer. Vortexed and again 1ml of mixture is taken from 2nd test tube to 3rd one and so on till 5 dilutions.

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Step 4: Plate Pouring and Incubation

All the dilutions are spread on media plates in duplicates using sterile glass spreader, 100µl from each and then incubated at 37°C for 6 days.

Step 5; Colony Countiong by CFU;

All the plates were counted on colony counter and the plates 3rd having colony count between 50-150 is selected for cfu per g with the formula;

$$\text{Cfu/g} = \frac{\text{no. of colonies X dilution}}{\text{factor volume of dilution spread on plate}}$$

Determination of Total Coliforms and E.COLI; Procedure for Determination of Total Coliforms and E.COLI;

Step 1; Media Preparation

0.9% saline solution 9ml for two test tubes, Laurayl Tryptose Soy broth and Brilliant green bile BGB broth 9ml for each of 10 tubes;also add inverted durhum tubes, Eosine methylene blue agar media has been prepared and autoclaved for 15mints at 15psi.

Step 2; Serial Dilution

Tap water 1ml is transferred to 1st dilution tube and 1ml from this tube to 2nd test tube.

i. **Presumptive Test:**
Three sets of LST tubes containing 3 tubes each is inoculated with the sample. First set is inoculated with undiluted 1ml water sample. Second set is inoculated with 1st dilution and third set is inoculated with 2nd dilution. One test tube was used as control. These tubes are then incubated at 37°C for 24-48 hrs.

ii. **Confirmatory Test:**
Tubes producing gas in durham tubes and turbidity are further tested to confirm the presence if coliforms. Tubes of BGB broth with inverted durham tubes are inoculated with a loopful of the positive LT tubes. Incubate at 35°C for 48 hours. Observe for gas production.

iii. **Completed Test:**
To check for the presence of faecal contamination, duplicate plates of EMB media are inoculated with 1ml of positive BGB tubes. One plate is incubated at 45.5°C for 48 hours and second plate at 37°C for 24-48 hrs.

Detection of Salmonella Species

Reagents

- Buffer peptone water (Primary Dillution)
- Rappaport vassilliadis Broth (RSV) & Mueller Kauffman Tetrathionate
- Xylose-Lysina Deoxycholate Agar (XLDA) (sticking of colonies)
- Nutrient agar Medium (Correct Sticking of Colonies)
- Brilliant Green phenol red Lactose agar (BGPRA) (Sticking)

Procedure

- Preparation of enrichment media, Mention Name on bottle's of related media
- Take 5 gram of Buffered pepton water, Add it into 225mL warm water, sterilise the media by using autoclave , RSV and MKTT medium are required as secondary Dilution or Enrichment

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- Sample pour in Bpw, Dissolve and homogenise the medium, place it at 37C° in incubator For 24 hour's
- Next day pour 1ml sample from Bpw into RSV Broth and MKTT Broth, we have to place RSv Broth into incubator for 24 hours at 41.5C°, MKTN for 24 hours at 37C°
- Takeout both From incubator , Streaking of MKTT done on XLDA and place it in incubator for 24 hours at 37C°, it show colonies of salmonella in black color

While Streaking of of BGPRA is done by RSV and it shows pinkish colonies of salmonella after incubation at 37C° for 2hour

Results and discussions:

Result of extraction:

During the study period, we were able to collect 900g of eggshells from 2 different hostels, we used just 300g of the external membrane to extract calcium carbonate and the result was high yield of pure material:
From 300g of external membrane of eggshells we managed to get 160g of calcium carbonate.

Fig: Calcium acetate powder

Fig: Calcium carbonate powder

1. Results of Proximate Analysis of eggshell powder

a) Moisture determination

Formula:

$$\frac{\text{Initial Weight} - \text{Final Weight}}{\text{Sample Wt}} \times 100$$

Calculation:

Sample name	Moisture dish Wt(g)	Moisture dish + Sample Wt(g)	Sample Wt (g)	Final Wt (g)	Moisture %
Calcium powder	11.2769	13.3221	2.0452	13.0228	14.634%

$$= \frac{13.3221 - 13.0228}{2.0452} \times 100$$

= **14.634%**

b) Ash Determination

Formula:

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$$\%ash = \frac{\text{weight of sample with crucible after ashing} - \text{weight of crucible}}{\text{Weight of ash}} \times 100$$

Calculations:

Sample Calcium powder	Wt of crucible(g)	Wt of sample(g)	After drying(g)	Ash %
1.	21.3117	3.0466	23.0522	57.1292%
2.	19.5890	3.0029	21.3154	57.4910%

1. $\%ash = \frac{23.0522 - 21.3117}{3.0466} \times 100$
   = 57.1292%

2. $\%ash = \frac{21.3154 - 19.5890}{3.0029} \times 100$
   = 57.4910%

c) Fiber Datermination

Formula:

$$\text{Fiber } \% = \frac{\text{Weight before ashing} - \text{Weight after ashing}}{\text{Sample Weight}} \times 100$$

Calculations:

Sample name	Sample Wt(g)	Wt before ashing (g)	Wt after ashing (g)	Fiber %
Calcium powder	2.0479	26.3636	26.3454	0.889%

$$\text{Fiber } \% = \frac{26.3636 - 26.3454}{2.0479} \times 100$$

= 0.889%

3. Results of Microbial Analysis:

a) Total plate count (TPC)

Dilutions	TOTAL PLATE COUNT	
	Plate 1	Media control
10-1	105	0
10-2	34	0
10-3	1	0
10-4	1	0
10-5	6	0

$$= \frac{73.5}{10^{-5} \times 1}$$

$$= \mathbf{14.3 \times 10^{6}\ CFU/g}$$

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4. Result of Yeast and mold

Dilutions	TOTAL PLATE COUNT	
	Plate 1	Media control
10-1	0	0
10-2	2	0
10-3	0	0
10-4	0	0
10-5	0	0

$$= \frac{1}{10^{-5} \times 1}$$

$$= 2.0 \times 10^{4} \text{ CFU/g}$$

5. Result of Spectroscopy

For the determination of calcium ion in the extracted sample the sample was sent to **National institute of health** (NIH)

Nutrition Division
National Institute of Health, Islamabad 45500
Phone +92-51-9255116, 9255079, Email:chiefnutritiondivision@nih.org.pk
WHO Focal Point for Food Safety / Federal Public Analyst Lab for Food Quality
Total Diet Study Laboratory
Analysis Report

Reception No.	7983	Receiving Date	21-03-2024
Laboratory No.	TDS-242	Reporting Date	27-03-2024
Name / Address of Client	Mr. Amer Mumtaz Hashmi, (PSO,NARC) Islamabad.		
Labelling / Packing	Product Name / Product Detail	Egg Shell Powder	
	Brand / Manufacturer	–	
	Packing	Composite Sheet bag	
	Batch/Lot No.	–	Expiry Date –
	Manufacturing Date.	–	
	Importer Address	–	

Physical Parameters

Parameters	Results	Parameters	Results
Color		Appearance	
Odor		Organoleptic	

Chemical Parameters

Parameters	Results	Reference Values
Calcium (Ca^{++})	739 mg/100gm	–

nclusion: Tests performed as per request.
marks:
ence: Pakistan Pure Food Rules / PPFR.

Officer Incharge (Laboratory) | Chief Nutrition Divis

Note:
- Authenticity of the sample rests with the Applicant
- Report Not Valid for Court Purpose

Discussion:

The aim of the research was to isolate calcium citrate from eggshells (ES), with a focus on determining the calcium citrate content in ES. The primary goal was to repurpose animal waste, making it useful and beneficial, while simultaneously reducing environmental pollution to safeguard human health. By extracting calcium citrate from eggshells, this study aimed to contribute to sustainable practices and promote resourcefulness in waste management.

Initially, eggshells were gathered and meticulously cleaned to ensure optimal hygienic conditions for the extraction process. The shells were sterilized,

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and their membranes were manually removed in preparation for further procedures. Subsequently, the eggshells were dried in an oven at a specific temperature for a defined duration and crushed into smaller fragments. These fragments were then treated with acetic acid and left to interact at room temperature under constant agitation until dissolution began. The resulting mixture underwent filtration and evaporation, leading to the formation of calcium acetate in the form of a white powder, yielding a specific quantity of 73.5g.

In order to determine the calcium ion content in the extracted powder, a spectroscopy technique was employed, revealing the presence of 739 mg of calcium ion per 100 g of the powder. Through these meticulous steps, the study successfully demonstrated a sustainable approach to reutilizing eggshell waste and deriving valuable calcium carbonate for potential use.

Analysis/ Experiments	Result
Moisture content %	14.634%
Ash content%	57.4910%
Fiber content%	0.889%
Total plate count	14.3×10^{-6} CFU/g
Yeast and mold	2.0×10^{-6} CFU/g
Spectroscopy	739 mg/100g of powder

Conclusion:

Eggshell is considered a polluter to nature because its decomposition produces compounds such as ammonia, hydrogen sulfide, and amine that release high concentrations of contaminants and odors. Additionally, eggshells can carry pathogens such as E. coli and salmonella that can be harmful to human health. The use and conversion of these bio-waste products as a primary source of calcium carbonate and calcium collagen is a major benefit to the nation's economy, especially since the use of calcium carbonate in the food, pharmaceutical, and other industries is imported from overseas.

The versatility of calcium carbonate (CaCO3) is truly remarkable! This mineral is utilized in various industries for a multitude of purposes. In the food industry, calcium carbonate powder serves as a natural food supplement, providing essential calcium to individuals.. In the pharmaceutical sector, CaCO3 is a key ingredient in antacids, calcium supplements, and antibiotics, showcasing its importance in promoting overall health and well- being. Moreover, in the realm of personal care products, calcium carbonate is utilized in toothpastes as a thickener and mild abrasive, contributing to oral hygiene and dental health.

Furthermore, the applications of calcium carbonate extend to the beauty industry, where it is utilized in the production of face powders, baby powders, eye shadows, and cosmetic foundations. Its whitening effect and ability to promote regular cell renewal make it a sought-after ingredient in skincare and cosmetic formulations. Additionally, mixtures containing calcium carbonate are utilized as feed supplements for animals, providing essential nutrients for their growth and development. The diverse uses of calcium carbonate underscore its significance across various sectors, making it a valuable resource in enhancing human health, beauty, and agricultural practices.

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