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Development and characterization of value-added cookies rich in vitamin D and calcium by incorporating eggshell powder and cod liver oil

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Abstract

Eggshell powder (ESP), because of its high bioavailability and low cost, can be added to the diet as an extra source of calcium. Similarly, cod liver oil (CLO), being a good source of vitamin D and omega 3 fatty acids, is a good choice to replace conventional shortenings in bakery products. Therefore, the goal of the current study was to valorize eggshell waste by adding ESP to the cookies that also contain CLO. Nutritional, physical, and sensory qualities of developed cookies were examined, with a focus on calcium and vitamin D contents. Comparative nutritional analysis of ESP and commercial straight grade flour (CSGF) revealed higher nutritional content in ESP, while CLO was determined to be a good source of polyunsaturated fatty acids and vitamin D. Nonsignificant results were reached regarding the weight, width, thickness, and spread factor of different treatments on cookies. The incorporation of ESP and CLO significantly ($p \leq 0.05$) increased the protein, fat, fiber, and ash contents of cookies. Further results showed that the calcium content of cookies was increased from 972 mg to 1732 mg with different treatments. The vitamin D content of cookies was also significantly ($p \leq 0.05$) improved from 588 IU to 1,684 IU with different treatments. Fourier transform infrared spectroscopy and X-ray diffraction analyses of the cookies confirmed the higher proportion of calcium and vitamin D in supplemented cookies. The sensory evaluation of cookies revealed that incorporation of up to 3 g of ESP and 9.6 mL of CLO as replacements of CSGF and shortening, respectively, was established as acceptable for the development of good-quality cookies, which although obtained lesser but very comparative sensory scores to the control, leading to consumer satisfaction. Thus, ESP and CLO could be used to develop nutritional bakery products having high contents of vitamin D and calcium.

Keywords: bakery formulation; functional oil; nutritional improvement; supplementation; waste valorization

Introduction

Utilization of food industry waste for the development of value-added food products has always been remained the topic of research. Egg's shell and membrane make up around 11% of the entire egg and is a significant waste product of the food industry. Owing to their high bioavailability and low cost, eggshells are added to the diet as an extra source of calcium (Nakilcioglu and Dadalı, 2024). If improperly disposed of, eggshells, an agricultural waste, pollute the environment. Eggshells, on the other hand, have a wide range of applications. Eggshells have become popular as adsorbents for the adsorption-based removal of colors and heavy metals. Supplements and dietary supplements are not the only applications of chicken eggs (Iftikhar *et al.*, 2024; Kalaycı *et al.*, 2025). In biomedical applications, eggshells are utilized as filler materials or implants (Kadhim *et al.*, 2022). Furthermore, research using hydroxyapatite demonstrate that eggshells are both biocompatible and biodegradable. Eggshells are used as ingredients in masks to tighten, brighten, and wash the face, and their membranes are encouraged as collagen in cosmetics (Baláž *et al.*, 2021; Hamidi *et al.*, 2017). Furthermore, eggshells are the natural and novel dietary supplements, which are a rich source of calcium, proteins, and other minerals, such as magnesium, strontium, fluorine, and selenium (Afzal *et al.*, 2020; Hasan *et al.*, 2023). Although there are prospects for eggshell waste valorization, the majority of the 8.3 million metric tons of eggshells generated annually worldwide, China, the United States, and India being the top producers, end up in landfills (Younas *et al.*, 2024).

Eggshell powder (ESP) is added to various food and non-food mixtures for technological and functional contributions (Ali and Badawy, 2017; Quddoos *et al.*, 2022; Waheed *et al.*, 2019). There is an increasing demand of calcium supplements in the people facing growth and development issues, although high demand is fulfilled by commercial calcium-containing salts, which may have some adverse effects (Prentice *et al.*, 2024). These commonly used calcium supplements contain calcium gluconate, calcium carbonate, and calcium citrate salts. Chicken eggshells, which are usually discarded as waste, are known to be best alternative source of calcium supplements, as calcium from eggshells is easily collected and extracted (Ajala *et al.*, 2018; Ray *et al.*, 2017). Calcium is present in both commercial calcium supplements and ESP, but ESP is readily absorbed. Commercial calcium supplements may not even be as effective as ESP at lowering the risk of osteoporosis (Singh *et al.*, 2021). Therefore, ESP-added bakery products are a good source of calcium.

Chicken ESP is an inexpensive and easily available source of calcium. It comprises 94% calcium carbonate, 1% magnesium carbonate, and 1% calcium phosphate. ESP with 39% elemental calcium is a potential and excellent source of dietary calcium. It has been noted that 1 g of ESP contains 370-mg calcium, 5-mg magnesium, 0.6-mg phosphorus, and appreciable quantities of other trace elements (Ali *et al.*, 2019; Gaonkar and Chakraborty, 2016; Hassan, 2015). ESP is utilized in industrial, medicinal and pharmaceutical products as it has nutritional contents, which may be useful for products and their intended users (Iftikhar *et al.*, 2024). To find the suitability of any material for pharmaceutical purposes, biological studies are conducted using different models. These *in vitro* and *in vivo* studies have revealed that ESP has a moderate immunostimulatory property, strong bone development capability, reduces osteoporosis, osteomalacia, and hypoparathyroidism, and has the ability to reduce neural degeneration (Baláž *et al.*, 2021; Bartter *et al.*, 2018; Opris *et al.*, 2020; Platon *et al.*, 2020). In clinical practice, eggshells are used in tissue engineering and wound dressings (Torres Gouveia *et al.*, 2025).

Synthetic calcium in the form of supplement has low bioavailability of calcium, while the prolonged use of synthetic supplements may have some adverse effects (Singh and Prasad, 2023). Eggshell's calcium has 60% more bioavailability, compared to synthetic calcium carbonate. Natural calcium in the form of eggshells may have positive health effects on bone mineral density, thus could help to prevent osteoporosis and bone loss in females (Faridi and Arabhosseini, 2018; Waheed *et al.*, 2020). ESP has an effective role in the development of bone mass and improves femoral neck bone density in humans. It contains matrix of proteins that promotes calcium transport across $CaCO_2$ cells in human intestines. ESP has a minute quantity of strontium, which has anabolic effect on bones (Bartter *et al.*, 2018; Iftikhar *et al.*, 2024; Torres Gouveia *et al.*, 2025). Eggshell membrane contains adequate amounts of bioactive components and has water retention property and biodegradability that make it a potential ingredient for clinical, nutraceutical, cosmetic, and nanotechnology applications (Kulshreshtha *et al.*, 2022; Milbradt *et al.*, 2015; Sakai *et al.*, 2017).

It is easy to extract eggshell calcium, which is acidic in nature and can be easily ionized (Dolińska *et al.*, 2016). It was observed that mice if fed with calcium in the form of ESP were discovered to have significant increase in the calcium content of the femur. Similar results in human studies discovered that intake of ESP had significant impact on bone mineral density (Alzaheb, 2018; Sakai *et al.*, 2017). Eggshells are a valuable product for human consumption and industrial utilization, thus their valorization may not only reduce the environmental waste but could also provide us value-added food products rich in nutritional contents.

The demand of oils rich in polyunsaturated fatty acids (PUFAs) is increasing, possibly due to increased chronic diseases. Therefore, consumers are nowadays more interested in food products containing fish oils rich in PUFAs and some vitamins (Li *et al.,* 2023; Liang *et al.,* 2023). Fish oils, especially cod liver oil (CLO), is a good source of vitamin D, vitamin A, and PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). CLO is used in many food products and pharmaceutical supplements. In the pharmaceutical industry, CLO is sold as a medicine or in the form of functional food oil as capsules or suspension. Functional food oils are the oils having potential benefits on human health. It has been estimated that 5 mL of CLO has 10-mg vitamin E, 10-µg vitamin D, 500-µg vitamin A, and 1.2-g PUFAs. Published data have reported that CLO supplements are dietary sources of DHA and EPA, which have clinical benefits in cardiovascular diseases and certain types of cancers (De Boer *et al.*, 2018; Ellulu *et al.*, 2015; Newell *et al.*, 2021). CLO is used for the fortification of foodstuff, such as bread, biscuits, baby foods, soups, and infant formulas. CLO is a good functional oil because of its ability to promote, treat, and prevent human health conditions. CLO is a good source of DHA and EPA used for the prevention of cardiovascular diseases and other health problems.

The PUFAs are known to have anti-inflammatory properties and suppress angiogenesis. Therefore, PUFA supplementation in cancer patients provides healthy fats with bioactive compounds that suppress tumor growth in the body (Aziz *et al.,* 2022; Marshall *et al.*, 2019; Newell *et al.*, 2021). CLO consumption may combat deficient serum vitamin D status. In a study, volunteers who took CLO daily, once a week, and six times a week had a serum vitamin D level of 60, 40, and 50 mmol/L, respectively (Kitagawa *et al.*, 2017). Adequate oral intake of vitamin D may help improve bone mass density, lower the incidence of osteoporotic fractures, and decrease bone loss, according to the results of several interventional studies conducted among older adults (Feng *et al.*, 2021; Polzonetti *et al.*, 2020). Compared to the subjects taking vitamin D only, another study discovered that intake of calcium supplements in addition to vitamin D increases bone mass density while lowering the risk of fractures (Hanai *et al.*, 2021). Thus, CLO, being rich in vitamin D and ESP having appreciable amount of calcium, could be added in food products to boost their nutritional contents.

ESP and CLO are widely utilized in cookies, snacks, baby foods, oils, and supplements. Researchers have developed some bakery products by adding ESP, and have explored that fortification with ESP in bakery products could increase nutritional and functional aspects (Ali *et al.*, 2019; Bradauskiene *et al.*, 2017; Nakilcioglu and Dadalı, 2024; Salem *et al.*, 2012; Zerek *et al.*, 2022). However, these studies had several limitations, as in some cases CLO and ESP were used separately, while in others, limited analyses were conducted. For example, no study conducted X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analyses of CLO- and ESP-added cookies. Therefore, there was a strong need to utilize both these ingredients in a unique combination to enhance both nutritional and bioactive contents of bakery products, which are commonly consumed in this modern era. Because cookies are commonly consumed by all age groups of people and are usually deficient in important nutritional contents, they were preferred for fortification. Thus, the main objectives of this work were the development and characterization of vitamin D and calcium-supplemented cookies through the incorporation of ESP and CLO. These formulated cookies were analyzed for their physicochemical and sensory parameters to assess increment in nutritional contents, especially vitamin D and calcium, while optimizing the sensory acceptance of products.

Materials and Methods

Ethical approval and consent to participate

The Biosafety and Ethical Review Committee (BERC) of the University of Sargodha, Sargodha, Pakistan, with a clearance number of UOS/IFSN/2023/07, provided ethical approval for this work. The informed consent was obtained from the participants who took part in sensory evaluation and were informed in detail about the purpose of this study.

Procurement of raw materials and reagents

Fresh eggshells, CLO, eggs, butter, sugar, wheat grains, for the development of commercial straight grade flour (CSGF), and baking powder were purchased locally from native markets of Rawalpindi and Islamabad, Pakistan. It was assured that same trade/brand ingredients were used in each trial to avoid variation in results. All the chemicals and reagents used in this study were of analytical grade and procured from Aladdin Chemicals, Shanghai, China.

Pretreatment of eggshells

By following the procedure adopted by Hassan (2015), the eggshells were collected and membrane removed, followed by washing under running water; then scrubbing was done with a sponge. After scrubbing, eggshells were immersed in a solution of sodium hypochlorite for disinfection, followed by rinsing and drying. In a post-drying procedure, eggshells were crunched to a fine powder

using pestle and mortar. Sieving of powdered eggshells was done through 0.18-mm sieve. Obtained powder was then dried in an oven (UNB 100, Memmert, Germany) at 80°C for 15 min. In order to remove all microorganisms from ESP, its sterilization was performed at 136°C for 18 min in an autoclave (MLS-3750, Labo Autoclave, Sanyo, Japan) using the protocols adopted by Kadhim *et al.* (2022). Packaging of ESP was done in low-grade polyethylene bags for safe storage. A flowchart for the pretreatment of ESP is presented in Figure 1.

Preparation and proximate analysis of commercial straight grade flour

For the preparation of flour, tempering of procured wheat grains was done at a moisture level of 15.5% after thorough cleaning. Tempered grains were stored in plastic bins at room temperature for 24 h in order to balance the water content of grains. The post-tempering moisture content was determined by method No. 26-95 of American Association of Cereal Chemists (AACC, 2000). The milling of tempered wheat grains was performed in Brabender Quadrumat Senior mill. By following the AACC (2000) technique No. 26-21A, the obtained milled fractions, such as break flour, reduction flour, bran, and shorts, were weighed and their proportions were determined using the total samples recovered. CSGF was yielded by blending of break roll flour and reduction roll flour ingredients. The fat, fiber, ash, protein, and nitrogen-free extract (NFE) of CSGF were determined using their respective methods provided in AACC (2000).

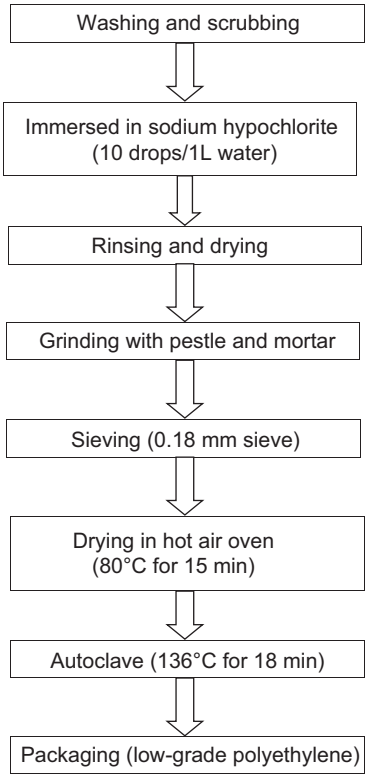

Figure 1. Pretreatment of eggshell powder.

Mineral analysis of eggshell powder

The mineral contents, such as iron, magnesium, phosphorus, calcium, potassium, and sodium, present in ESP were determined by the wet digestion method using atomic absorption spectrophotometer (5100PC, Norwalk, CT, USA) as described in Association of Official Analytical Chemists (AOAC, 2012), and further elaborated by Hussain *et al.* (2021). This method is commonly adopted for determination of wide range of macro and micro minerals because of its accuracy, cost effectiveness, and ease of use. A 0.5-g sample of ESP was taken and digestion was performed in a 100-mL conical flask on hot plate with 10-mL nitric acid solution. The sample was then heated for 20 min at 70°C. The same sample was then digested in 5 mL of 60% chloric acid at 190°C. The digestion of the sample was performed until contents in the flask turned clear. The sample volume of 100 mL was achieved by adding deionized water. In order to get standard curves, the samples were run with known strength of standards to determine the contents of iron, magnesium, calcium, and phosphorus. The sodium and potassium contents present in ESP were determined through flame photometer (Sherwood Scientific, UK) by following the method described in AOAC (2012).

Analysis of moisture content in eggshell powder

Moisture content in ESP was determined by following the method No. 44-15 of AACC (2000). Briefly, 2-g sample was collected in a petri dish that was previously weighed (W_1) and was heated to 105°C for 24 h. After that, the sample was placed for some time in a desiccator. The dried material was weighed again after cooling (W_2). Then the moisture percentage in ESP was estimated by using the following formula:

$$\text{Moisture } (\%) = \frac{W_1 - W_2}{W_1} \times 100,$$

where W_1 = pre-weighed weight, W_2 = weight after cooling of sample.

Analysis of protein content in eggshell powder

The protein content in ESP was determined using Kjeltech apparatus following the Kjeldahl's method

No. 46-30 of AOAC (2000). In the presence of digestion mixture, concentrated H_2SO_4 was used to break down 5 g of ESP. The mixture after digestion was run till a bright green hue was achieved. Then 250 mL of distilled water was used to dilute digestion mixture. Thereafter, 10 mL of diluted sample and 10 mL of 40% NaOH solution were combined for distillation in a distillation equipment. Then 4% boric acid solution was combined with ammonia, and until methyl red indication was attained, the sample was forced-running. The material was then titrated against 0.1-N H_2SO_4 solution. Finally, the following formula was used to estimate nitrogen:

$$\text{Nitrogen (\%)} = \frac{\text{Amount of } 0.1-\text{N } H_2SO_4 \times 0.0014 \times 250}{\text{Weight of sample} \times \text{volume of diluted sample}} \times 100.$$

The percentage of protein was calculated by the following expression:

$$\text{Protein (\%)} = \text{Nitrogen (\%)} \times 6.25.$$

Analysis of cod liver oil

High-performance liquid chromatography (HPLC) as reported by Feng *et al.* (2020) was used to analyze vitamins D and A contents, PUFAs, monounsaturated fatty acids (MUFAs), and other fatty acid-related parameters in CLO. This method was preferred due to high selectivity, rapid separation, simple sample preparation, and accurate quantification. First, 0.5-mL sample of fresh CLO was taken. Then a solution was prepared by mixing 25 mL of methanol with 700 mg of extremely pure potassium hydroxide and potassium methoxide. After that, ascorbic acid solution of 1% weight was prepared by mixing 165 mg of ascorbic acid in 10 mL of methanol. Then 500 mg of CLO was mixed with 2-mL ascorbic acid, followed by 5 mL of MeOK mixture in a glass flask. After that, the mixture was vortexed for 30 s. The flask was then placed in a silicone bath for 30 min at 80°C with reflux. The sample was cooled in ice water before being combined with 5 mL of n-hexane. The solid saponifiable sample was removed from hexane layer and dried under N_2 flux. The dried material was dissolved in 600 mL of MeOH. Standard vitamin D_3 fractions were produced in methanol and kept at 4°C in glass bottles to avoid exposure to sunlight.

The amount of vitamin D_3 was calculated by the following formula:

$$D_3(\text{ug/g}) = \frac{a_1 \times CF \times i}{a_2 \times W},$$

where

a_1 = peak area of vitamin D_3,
a_2 = peak area of vitamin D_2,
CF = correction factor

(The quotient of the peak areas of vitamins D_3 and D_2, was measured at identical concentrations of vitamin forms),

i = amount of internal standard added (g), and
W = weight of sample (g).

Preparation of supplemented cookies

Control and supplemented cookies were prepared by following the guidelines provided by Hussain *et al.* (2023b), using the recipe given in Table 1. Further, the treatment plan is provided in Table 2, where T_0 was control cookies, which had no source of calcium and vitamin D whereas treatments (T_1, T_2, T_3, and T_4) were prepared keeping in mind the recommended daily allowance (RDA) for calcium and vitamin D. Explaining in detail, first the raw materials were weighed with accuracy. After weighing, eggs, butter, and CLO were mixed, followed by beating. Dry ingredients, such as ESP, CSGF, baking powder, and sugar, were added during the beating process. The dough was kneaded with soft hands, then flattened and cut with a cookie cutter. The cookies were placed on greased baking trays and baked for 25 min at 175°C in a preheated oven. After cooling to room temperature, the prepared cookies were packed in bio-oriented polypropylene (BOPP) bags and kept at room temperature in laboratory for further investigations. Flow chart for preparing cookies is presented in Figure 2.

Characterization techniques used on supplemented cookies

Fourier Transform Infrared spectroscopy analysis

The presences of dietary calcium in ESP were analyzed using FTIR by following the guidelines provided by Gorsi *et al.* (2024). The ESP was analyzed by

Table 1. Recipe plan of cookies (g/100 g).

Ingredients	Weight (g)
CSGF	50
Shortening	30
Sugar	10
Eggs	10
Baking powder	0.2

CSGF: commercial straight grade flour.

Table 2. Different treatment plans for supplemented cookies (g/100 g).

Treatments	CSGF (g)	Butter (g)	ESP (g)	CLO (mL)	Egg (g)	Sugar (g)
T_0	50	30	-	-	10	10
T_1	47.5	24	2.5	6	10	10
T_2	47	20.4	3	9.6	10	10
T_3	46.5	16.8	3.5	13.2	10	10
T_4	45.5	12	4.5	18	10	10

CSGF: commercial straight grade flour; ESP: eggshell powder; CLO: cod liver oil.

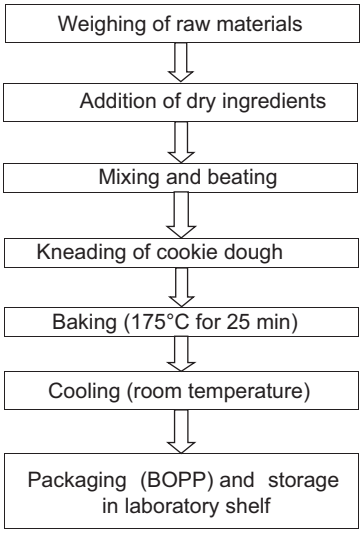

Figure 2. Flowchart for preparation of cookies.

the KBr pellet technique using FTIR system (Model 3000 Hyperion Microscope, Make-Bruker, Ettlingen, Germany). Before samples (0.5 g of ESP, control, and supplemented cookies) were mounted on FTIR instrument, the background spectra of the instrument were collected, and at 16 runs per scan, the records of the spectra were considered with characteristics peaks in wave numbers ranging from 400 to 4,000 cm^{-1}. The data were collected in the form of figures, which were presented and analyzed by identifying functional groups.

X-ray diffraction (XRD) analysis

Different fractions of ESP, control, and supplemented cookies were studied using XRD analysis as guided by Hamidi et al (2017). First, 5 g of samples were placed in an XRD sample holder and compressed with a stainless steel weight. An X-ray diffractometer (Model: X'Pert Pro and Make, PAN analytical, Almelo, the Netherlands) equipped with a divergence slit was used to analyze samples' crystalline properties and X-ray diffraction. The diffractometer was operated at 40 kV and 40 mA with a scan speed of 10/min, and the scanning angle was adjusted between 15° and 80° at a step size of 0.02°. Results in the form of microstructures were obtained as images, which were analyzed and discussed.

Physiochemical analysis

Physical analysis of cookies

Prepared cookies were evaluated for physical parameters, such as thickness (mm), weight (g), diameter (mm), and spread ratio (diameter/thickness) by following the guidelines provided by Hussain *et al.* (2023a). The prepared cookies were weighed on a weighing balance with triplicate measurements, and the mean value was considered. The cookies' diameter was also measured in triplicate with a calibrated ruler. The thickness was measured by placing three cookies upon each other and using the above-used ruler, and the spread ratio was calculated by dividing the diameter of cookies with thickness. Three rows of five cookies of the same size were placed and the height was estimated. The value of spread ratio was obtained by using the following formula:

$$\text{Spread ratio} = \frac{\text{Diameter (mm)}}{\text{Height (mm)}}.$$

Proximate analysis of cookies

Proximate analysis of cookies determined crude moisture, crude fat, crude protein, crude fiber, ash content, calorie value, and nitrogen-free extract (NFE) by using their respective methods mentioned in AACC (2000). The moisture content of cookies was determined by method No. 44-15 of AACC (2000). Crude protein in cookies was analyzed in Kjeltech apparatus using Kjeldahl's method No. 46-30 (AACC, 2000). Crude fat in cookies was analyzed according to method No. 30-25 (AACC, 2000) by using Soxhlet apparatus**.** Crude fiber in cookies was calculated by using method No. 32-10 (AACC, 2000). Crude ash in cookies was analyzed by using method No. 08-01 (AACC, 2000) by using muffle furnace at 550°C till grayish white residue was achieved. Cookies sample's NFE was calculated by subtracting the total of moisture, protein, ash, fiber, and fat from 100. The below-mentioned formula was used to compute the percentage of NFE (Afzal *et al.*, 2020):

$$\text{NFE (\%)} = 100 - (\text{protein [\%]} + \text{fat [\%]} + \text{fiber [\%]} + \text{ash [\%]}).$$

To determine calorific value, the protocols adopted by Salem *et al.* (2023) was followed: 1-g sample of cookies was placed in a device, lit by an electric spark to burn the

extra oxygen inside the bomb. Galvanometer system and thermocouple were used to analyze the maximum temperature of bomb calorimeter. Initial readings were taken in triplicate to calculate calorific value before the final reading.

Analysis of calcium content in cookies

The Calcium content of cookies was determined by following the same procedure mentioned in Section 2.6. The filtered digested sample solution of cookies was operated by an atomic absorption spectrophotometer as explained in earlier section 2.6.

Analysis of vitamin D content in cookies

Vitamin D content in ESP-supplemented cookies was estimated by HPLC method using the protocols provided in Section 2.9.

Sensory evaluation of cookies

In the current study, the control and supplemented cookies were exposed to sensory evaluation, which includes texture, color, flavor, mouth feel, and overall acceptability. The sensory evaluation was carried out in a laboratory by a panel of 40 semi-trained judges of both genders aged between 25 and 35 years, using a 9-point hedonic scale as guided by Rafique *et al.* (2023). The panel was briefed about the procedure and coded samples were provided along with evaluation proforma, and their informed consent was obtained. Proforma used for sensory evaluation of cookies is presented in Annexure 1.

Statistical analysis

This study considered possible variables that were classified in Statistical Package for Social Sciences (SPSS version 16) and Minitab (version 19). The effects of treatments of indicative parameters were examined using both one-way and two-way analyses of variance (ANOVA) with *post hoc* Tukey test, and between-group comparisons were carried out using the general linear model (GLM). For all statistical outcomes, a $p \leq 0.05$ was deemed statistically significant (Hipsey *et al.*, 2019).

Results and Discussion

Proximate analysis of commercial straight grade flour

Proximate analysis of CSGF showed the following: calcium = 18.5±0.51 mg/100 g, phosphorus = 107.25±1.70 mg/100 g, moisture content = 12.63±0.01%, and protein content = 10.51±0.021% (Table 3). The ash content recorded in CSGF was 0.94±0.01% and crude fiber was recorded as 0.37±0.021 whereas the iron content was 2.1±0.129 mg/100 g. Previous research findings by Ficco *et al.* (2009) and Mepba *et al.* (2007) were also in agreement with the results of the present study, as the authors also reported CSGF to be a poor source of calcium, iron, ash, and fiber. Some other research findings reported that the moisture content in CSGF was 13.33±0.60%, protein 11.01±0.7%, crude fat 0.84±0.25%, crude fiber 0.84±0.27%, and the ash content was 1.51±0.51% (Hussain *et al.*, 2023b). Mineral contents in CSGF, such as magnesium 5.51±0.37 mg/100 g, potassium 4.76±0.28 mg/100 g, calcium 18.61±0.66 mg/100 g, and iron 0.700±0.52 mg/100 g were reported by Khan *et al.* (2017). These findings confirmed the present results with strong agreements.

The proximate and mineral analysis of CSGF demonstrated that while preparing cookies, the incorporation of ESP might be useful in increasing ash, fiber, and mineral contents of the product, as CSGF has comparatively lower contents of these factors.

Proximate and mineral analysis of eggshell powder

Results after statistical analysis of proximate and mineral parameters of ESP are shown in Table 4. The presented results revealed that ESP contains calcium 38.5±0.17 mg/100 g, potassium 41.64±0.02 mg/100 g, phosphorus 99.34±0.03 mg/100 g, magnesium 375.52±0.98 mg/100 g, and sodium 87.06±0.025 mg/100 g. On the other hand, ESP contained protein 2.12±0.02%, ash 96.6±0.23%, and moisture 0.53±0.017%. These results were strongly supported by the findings provided by Waheed *et al.* (2019), as they reported that ESP contained calcium 38 mg/100 g, moisture 0.52%, and protein 2.13%, while phosphorus in ESP was reported as 99.36 mg/100 g. Similar findings were also observed in a study conducted by Murakami *et al.* (2007), who analyzed ESP and proposed that it is composed of 94% calcium carbonate, 1% of calcium

Table 3. Proximate (%) and mineral (mg/100 g) analysis of commercial straight grade flour.

Parameters	Quantity
Calcium (mg/100 g)	18.5 ± 0.51
Iron (mg/100 g)	2.1 ± 0.13
Phosphorus (mg/100 g)	107.25 ± 1.70
Moisture (%)	12.63 ± 0.01
Protein (%)	10.51 ± 0.02
Ash (%)	0.94 ± 0.01
Fat (%)	0.95 ± 0.02
Fiber (%)	0.37 ± 0.02
Nitrogen-free extract (%)	74.84 ± 0.03

Table 4. Proximate and mineral analysis of eggshell powder.

Parameters	Quantity
Calcium (mg/100 g)	38.5 ± 0.17
Potassium (mg/100 g)	41.64 ± 0.02
Sodium (mg/100 g)	87.06 ± 0.025
Phosphorus (mg/100 g)	99.34 ± 0.026
Magnesium (mg/100 g)	375.52 ± 0.98
Moisture (%)	0.53 ± 0.017
Protein (%)	2.12 ± 0.022
Ash (%)	96.6 ± 0.23

Table 5. Chemical analysis of cod liver oil.

Parameters	Quantity
Omega 3 fatty acids (g)	1.32 ± 0.22
Eicosapentaenoic acid (EPA) (mg)	741.25 ± 2.98
Docosahexaenoic acid (DHA) (mg)	461.25 ± 2.98
Other omega 3 fatty acids (mg)	199 ± 2.44
Vitamin D_3 (*μg*)	250.25 ± 1.70
Vitamin A (*μg*)	30002 ± 1.27
Total MUFA (g)	46.6 ± 0.25
Total PUFA (g)	22.475 ± 0.25
Total saturated fats (g)	22.57 ± 0.17
Cholesterol (mg)	570.25 ± 1.70

MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

phosphate, and 1% magnesium carbonate. ESP consists of 39% elemental calcium; therefore, it is a promising and effective source of dietary calcium.

The present results of ESP were also in accordance with the results achieved by Bartter *et al.* (2018), who analyzed calcium content of eggshell in three different studies and discovered it ranging from 360 mg/g to 400 mg/g of eggshell. The present study was also in accordance with the findings of Brun *et al.* (2013), who reported that eggshell mainly comprises calcium as 38 mg/g, potassium as 1.4 mg/g, sodium as 5.1 mg/g, and phosphorus as 4.4 mg/g. The present results of ESP were also in accordance with some previous findings, which estimated that ESP contained moisture as 0.46%, ash 94.6%, and protein as 3.92%. A high amount of calcium of around 40%, as calcium carbonate 98.43% and calcium phosphate 0.75%, along with magnesium 0.5%, and phosphorus 0.1%, have also been reported in ESP (Afzal *et al.*, 2020; Ali and Badawy, 2017; Ray *et al.* 2017). The present results and their relevant literature showed ESP to be a good source of ash, fiber, and important minerals, such as calcium and phosphorus.

Chemical analysis of cod liver oil

Analysis of CLO was conducted by HPLC, and as presented in Table 5, the following components were discovered in CLO: omega 3 fatty acids, 1.32±0.22 mg; eicosapentaenoic acid (EPA), 741.25±2.98 mg; dexosahexaenoic acid (DHA), 461.25±2.98 mg; other omega 3 fatty acids, 199±2.44 mg; vitamin D_3, 250.25±1.70 *μg;* vitamin A, 30,002±1.27 *μg;* total MUFA, 46.6±0.25 g; total PUFA, 22.475±0.25 g; total saturated fats, 22.57±0.17 g; and cholesterol, 570.25±1.70 mg. These findings showed CLO to be a good source of vitamins A and D, PUFAs, and MUFAs. The chemical composition of CLO presented by Higashi *et al.* (2014) demonstrated that CLO contained omega 3 fatty acids, 1.4 g; EPA, 740 mg; DHA, 460 mg; vitamin D_3, 250 *μg;* and other omega 3 fatty acids, 1.4 g per 5 mL. Similar findings were also demonstrated by Hubicka *et al.* (2020), who estimated that CLO contained high quantity of highly unsaturated fatty acids (HUFAs): DHA, 22:6, and EPA, 20:5. Similar composition was also reported by Almarri *et al.* (2017), who evaluated that CLO is a rich source of omega 3 fatty acids, HUFAs, and vitamin D_3 (252 *μg).*

Analysis of fortified cookies

Physical analysis of cookies

The physical parameters of different treatments of cookies are presented in Figure 3, which showed that the weight of cookies remained nonsignificant ($p \leq 0.05$) within the treatments, where ESP was replaced with CSGF and CLO with the shortening. The weight of control cookie (T_0) was reported as 15.41 g, followed by T_1 as 15.43 g whereas T_4 had a weight of 15.44 g. Results obtained in the present study demonstrated that the addition of core ingredients in cookies caused nonsignificant ($p \leq 0.05$) effect on their diameter (Figure 3). Control cookies had a diameter of 46.4 mm, followed by T_1, 46.40 mm and T_2, 46.40 mm, whereas T_4 showed the maximum diameter of 46.42 mm (Figure 3).

The results pertaining to thickness described that the thickness also varied nonsignificantly ($p \leq 0.05$) among different treatments. All cookie samples had a thickness ranging from 6.51 mm to 6.39 mm. It was observed that there was a nonsignificant ($p \leq 0.05$) increase in the thickness of cookies with the addition of ESP and CLO. Similarly, the effect of treatments showed that control cookie (T_0) had a spread ratio as 8.1, followed by T_1 8.1 and T_2 8.1, while the maximum spread ratio (8.3) was

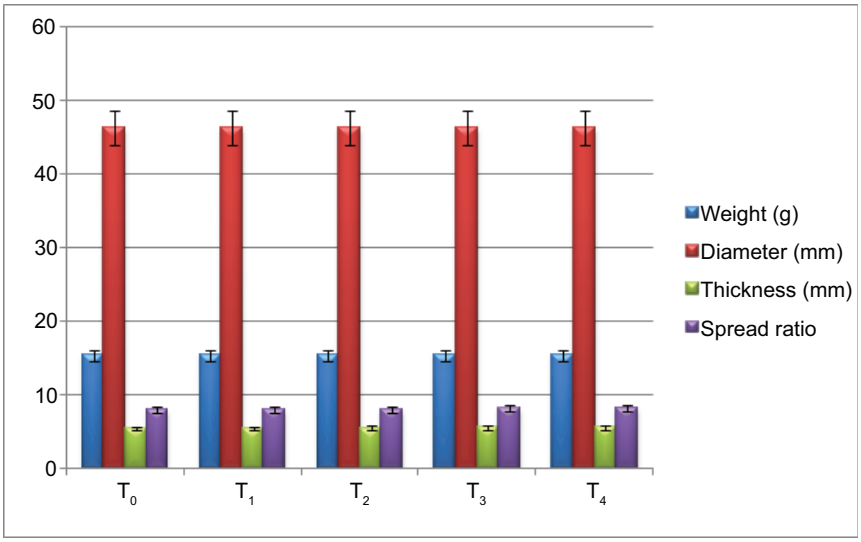

Figure 3. Effect of treatments on the physical parameters of cookies.

discovered in T_4 (Figure 3). An earlier study conducted by Salem *et al.* (2012) evaluated the physical properties of control biscuit and supplemented biscuit with 3%, 6%, and 9% ESP. Diameter, thickness, and spread ratio in T_0 were 46.0 mm, 5.93 mm, and 7.76 mm, respectively. Diameter and thickness increased but spread ratio decreased with the increasing level of ESP. The researchers observed that with the addition of 3–9% ESP, the diameter ranged from 45.8 to 45.0 mm, thickness from 5.97 to 6.01 mm, and spread ratio from 7.67 to 7.49 mm.

The present results were also in accordance with Hussain *et al.* (2023a), who reported that spread factor is dependent on fat content, because fat increased with increase in spread ratio. Previous study conducted by Brun *et al.* (2013) also observed similar results, reporting that spread ratio decreased with the increasing level of ESP. The present findings were also in accordance with those reported by Chilek *et al.* (2018), who observed that physical properties of ESP-supplemented bread were not much affected by up to 10% addition of ESP; however, a further increase in this level caused irregularities in physical properties. A recent study conducted by Nakilcioglu and Dadalı (2024) also supported the present results and discovered that physical features of a cake were not much affected at lower levels of ESP supplementation. The present results are also coherent with the findings reported by Salem *et al.* (2012), who proved that firmness or texture of cake improved with the addition of ESP during cake treatment. Findings of Quddoos *et al.* (2022) also proved that the incorporation of ESP in cookies had no negative impact on their physical parameters.

Proximate analysis of cookies

Table 6 presents the results of proximate analysis of ESP- and CLO-supplemented cookies, where the moisture content in T_0 was 4.74% followed by T_1 4.73%, and T_4 showed a minimum moisture content of 4.72%. The least fat content was detected in T_0, 31.05%, and the maximum fat of 31.45% was observed in T_4. Effect of treatments on crude fiber content of cookies was nonsignificant ($p \leq 0.05$) within the treatments. Crude fiber was maximum (0.18%) in T_0, followed by T_1 (0.17%), and was 0.165% in T_3, while the minimum value of 0.164% was observed in T_4. Effect of treatments on crude protein varied significantly ($p \leq 0.05$) within the treatments, as the crude protein presented in T_0 was 3.43%, followed by T_1 6.35% and T_2 6.31%, and the maximum crude protein of 6.18% was noted in T_4. Similarly, the ash content in cookies varied significantly ($p \leq 0.05$) among different treatments, as a minimum ash content of 2.42% was present in T_0 and the maximum 4.78% was discovered in T_4 treatment. Variation in NFE (%) depends on variations in other proximate analysis, because most of proximate analysis showed nonsignificant variations. The maximum NFE (%) was observed in T_0, followed by T_1 and T_2, while minimum NFE (%) was observed in T_4. Effect of treatments on caloric values of cookies showed that it significantly ($p \leq 0.05$) reduced from T_0 (549.7 kcal/g) to 536.22 kcal/g in T_4 (see Table 6).

Similar results were presented by Platon *et al.* (2020), who noted nonsignificant changes among treatments in bread prepared with ESP, which possibly was due to the smaller levels of incorporation of ESP. A similar earlier study conducted by Bradauskiene *et al.* (2017) also concluded that moisture content of bread showed a decrease in unleavened and leavened bread varieties, with increased concentration of ESP. Furthermore, protein content was determined to be varying between 9.79% and 9.89%, within different treatments of ESP-fortified flour. The decrease in moisture and increase in

Table 6. Effect of treatments on the chemical composition of cookies (mean values ± SD).

Treatment	Moisture	Protein	Fat	Fiber	Ash	Nitrogen-free extract	Calorie value (kcal/g)
T_0	4.74 ± 0.005^{a}	3.43 ± 0.02^{d}	31.048 ± 0.04^{d}	0.18 ± 0.028^{a}	2.42 ± 0.02^{de}	62.98 ± 0.012^{a}	549.7 ± 0.0082^{a}
T^1	4.73 ± 0.005^{a}	6.35 ± 0.02^{a}	31.43 ± 0.0002^{a}	0.17 ± 0.006^{a}	2.87 ± 0.002^{d}	59.18 ± 0.01^{b}	542.22 ± 0.004^{b}
T_2	4.73 ± 0.008^{a}	6.31 ± 0.002^{a}	31.43 ± 0.002^{a}	0.17 ± 0.014^{a}	3.34 ± 0.002^{c}	58.73 ± 0.027^{b}	540.72 ± 0.0018^{b}
T_3	4.73 ± 0.007^{a}	6.27 ± 0.002^{bc}	31.44 ± 0.002^{a}	0.17 ± 0.006^{a}	3.82 ± 0.002^{bc}	58.31 ± 0.013^{b}	539.22 ± 0.0017^{b}
T_4	4.72 ± 0.005^{a}	6.18 ± 0.002^{c}	31.45 ± 0.002^{a}	0.17 ± 0.003^{a}	4.78 ± 0.02^{a}	57.43 ± 0.114^{c}	536.22 ± 0.0026^{c}

Values having the same superscripted alphabets present nonsignificant results whereas those not having the same superscripted alphabets present significant results ($p \leq 0.05$).

protein were due to differences in the proximate composition of ESP and wheat flour. On the other hand, a study conducted by Ali *et al.* (2019) observed a significant reduction in protein content from 9.89% to 9.79% with the addition of ESP, because ESP is not a good source of protein and replacement of CSGF with ESP at relatively higher levels may decrease protein contents of cookies. The literature suggests that ESP is not a good source of fiber and contains similar amount of fiber as present in CSGF; therefore, effect of treatments was nonsignificant ($p \leq 0.05$) on the fiber content of breads (Uddin et al., 2025; Ali and Badawy, 2017).

The present results of crude ash were similar to the findings reported by Ray *et al.* (2017), who reported that ESP constituted 94.6% ash, and caused a significant ($p \leq 0.05$) increase in the ash contents of cakes. A study conducted by Chilek *et al.* (2018) also observed that addition of ESP increased the ash content of breads without effecting its protein and fat contents. The authors further reported that caloric value of ESP-supplemented cookies declined whereas addition of CLO in cookies caused increased caloric value. This increase in caloric value of food products having CLO could be attributed to the high caloric values of CLO, possibly because of high fat and protein contents. Research findings by Ajala *et al.* (2018) reported that ESP could be a good source of ash to be added to bakery product for the increment of mineral contents. The present results were also in accordance with the findings of Bradauskiene *et al.* (2017), who reported a significant decrease in NFE among different bread treatments enriched with ESP. As ESP, compared to CSGF, has a low amount of NFE, the NFE of cookies decreased because of increasing the level of ESP in CSGF.

Sensory analysis of cookies

Results of sensory analysis of control and ESP- and CLO-supplemented cookies are shown in Figure 4, which shows a significant ($p \leq 0.05$) difference among the scores of different treatments. Increasing the level of incorporation of ESP caused a decreased sensory acceptance of cookies. It is observed in Figure 4 that T_0 had maximum and T_4 had minimum color scores. The result further showed that maximum number of judges liked T_0, which received the maximum taste score of 7.2, followed by T_1 (7.1 ± 0.) whereas T_4 received minimum taste score (6.8). The effect of treatments showed that T_0 had the maximum mouth feel score of 7.4 whereas T_4 had a minimum score of 6.8. The results showed that T_0 had the best texture score of 7.16, followed by 7.1 for T_1, 7.0 for T_2, and T_4 having a minimum texture score of 6.8. The effect of treatments on the overall acceptability of cookies depicted that T_0 got the maximum score of 7.1, followed by T_1 (7.0), and T_4 had a minimum overall acceptability score of 6.6.

The sensory evaluation scores for different treatments of cookies were in accordance with the findings of Salem *et al.* (2012), who observed that ESP supplementation of up to 10% had a nonsignificant ($p \leq 0.05$) effect on the overall acceptability of cake. On the other hand, Hassan (2015) reported that ESP supplementation of up to 6% did not have any negative effect on the overall acceptability of biscuits. Similarly, Quddoos *et al.* (2022) also observed the consumer acceptance of cookies having ESP at an optimum level, just as in the present findings, the sensory scores of the treatments having smaller amounts of ESP were near to that of the control. The present findings regarding the sensory evaluation of supplemented cookies were in accordance to the results provided by Hasan *et al.* (2023), who revealed that supplementation of ESP in ice cream did not affect the color of the product, as ESP also had whitish color just like CSGF. The present results were in agreement with the findings of Brun *et al.* (2013), who discovered that ESP could be a suitable choice to be added in homemade food products, as its smaller levels does not affect their sensory features. However, a study conducted by Chilek *et al.* (2018) observed that addition of up to 20% ESP might cause a rise of sandy texture of bread; however, the bread with 12% ESP could be acceptable. Thus, as in the current work, smaller levels of ESP were introduced for cookies

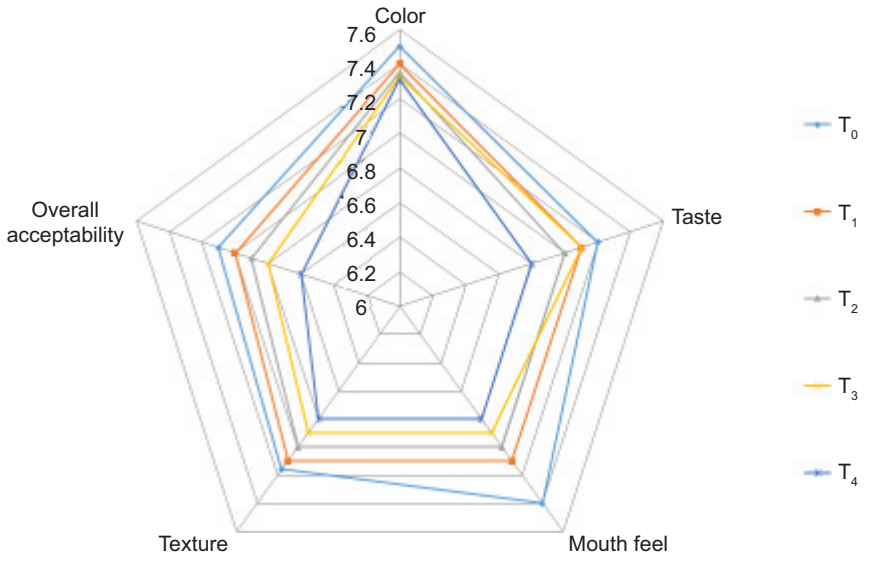

Figure 4. Effect of treatments on the sensory parameters of cookies.

baking process, and the sensory scores for all treatments were acceptable, with T_1 and T_2 having higher scores, compared to T_3 and T_4.

Calcium and vitamin D contents of cookies

Calcium and vitamin D have important biological functions in living bodies, as they help in strengthening bones and teeth. Vitamin D helps the body to absorb calcium. The recommended daily intake of calcium and vitamin D in adults is 1,000 mg and 600 IU, respectively (Prentice *et al.*, 2024; Singh *et al.*, 2021). Therefore, production and consumption of bakery products having high amounts of calcium and vitamin D could be advantageous for improving the overall health. Different treatments of cookies showed varied calcium content (Table 7), with a significant increase in calcium content of cookies having high levels of ESP. Calcium was 22.85 mg/100 g in T_0, followed by 972 mg/100 g in T_1, and T_4 was found with the maximum calcium content of 1,732 mg/100 g. Various treatments significantly affected the content of vitamin D ($p \leq 0.05$), as it increased from 39.8 IU in T_0 to 588 IU in T_1, and T_4 showed the maximum content as described in Table 7. This increase in the calcium content of cookies was due to high calcium content of ESP (Nakilcioglu and Dadalı, 2024). These results regarding increment in the calcium content of cookies were in accordance with the findings of Ali *et al.* (2019) and Bradauskiene *et al.* (2017), who discovered a significant increase in the calcium content of bread upon addition of ESP.

Table 7. Calcium and vitamin D contents of cookies.

Treatment	Calcium (mg/100 g)	Vitamin D (IU)
T_0	22.85 ± 0.019^e	39.8 ± 0.031^e
T_1	972 ± 0.0016^d	588 ± 0.011^d
T_2	1162 ± 0.017^c	916 ± 0.036^c
T_3	1352 ± 0.0015^b	1246 ± 0.027^b
T_4	1732 ± 0.016^a	1684 ± 0.053^a

Another study conducted by Khan *et al.* (2017) reported a significant improvement in the calcium content of breads fortified with chicken ESP, calcium carbonate, and bone extracts. The results regarding the increment of vitamin D content of cookies were in accordance with the findings of Bajaj and Singhal (2021), who also observed a significant increase in vitamin D levels in the fortified baked products than in the control, as they concluded that dough-based food products could be fortified by vitamin D. Just as in the present work, CLO, as a good source of vitamin D, was used to replace butter, and it caused a significant increase in vitamin D content of supplemented cookies. The present results were in line with the findings of Quddoos *et al.* (2022), who used ESP and bone powder in cookies and observed a significant increase in their calcium and vitamin D contents.

FTIR and XRD analysis of cookies

In chemical analysis, FTIR is used to assess a compound's functional group. Infrared spectroscopy is frequently employed in the food industry to check the quality of products, and food samples are identified and verified using it (Indrianingsih *et al.*, 2024). The FTIR spectrum of control cookies is shown in Figure 5A and that of ESP- and CLO-supplemented cookies is shown in Figure 5B. The absorbance bands of control cookies show at 2,920.4 cm^{-1}, 1,742.5 cm^{-1}, and 989.6 cm^{-1}, whereas

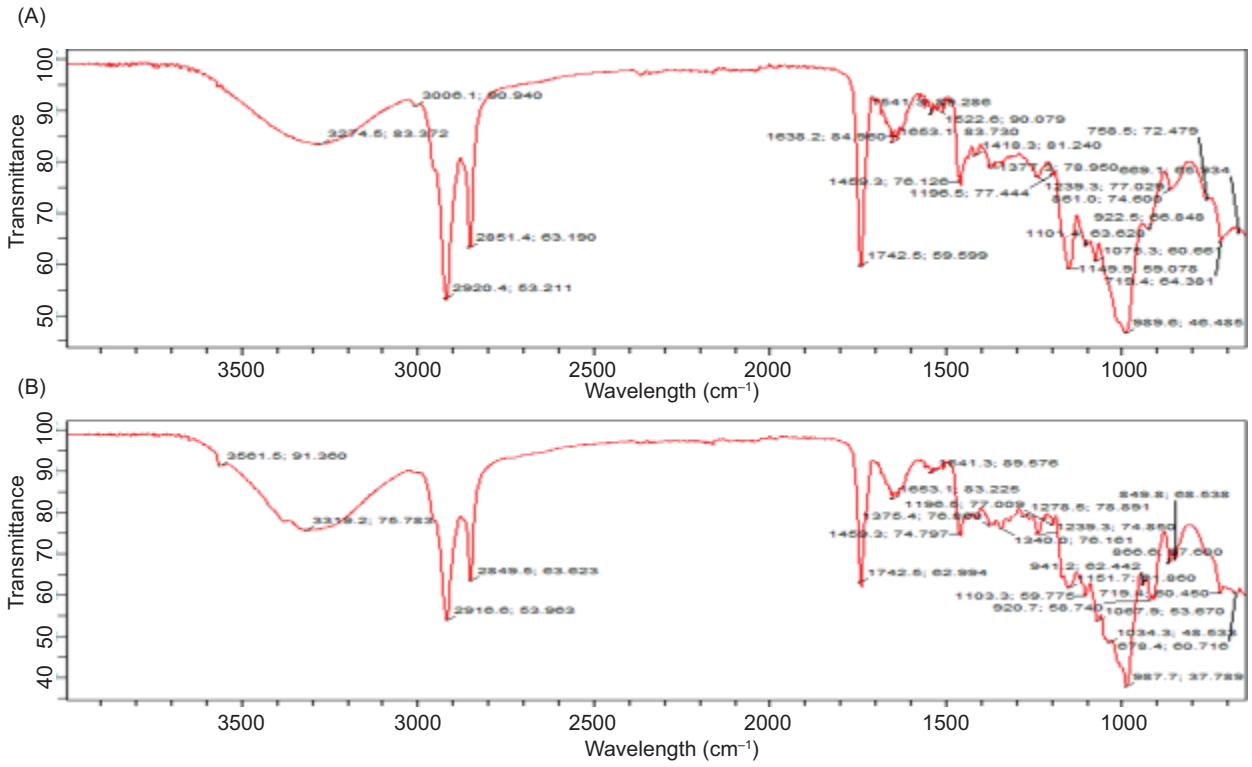

Figure 5. FTIR of control as well as calcium- and vitamin D-supplemented cookies. (A) FTIR of control cookie. (B) FTIR of calcium and vitamin D supplemented cookie Note. FTIR: Fourier Transform Infrared spectroscopy.

the sharp peaks of supplemented cookies show at 2916.6 cm^{-1}, 1742.5 cm^{-1}, and 987.7 cm^{-1}. The absorption of $^{-}$OH hydroxyl group, which occurred from carbohydrates or other substances such carboxylic aldehydes and ketones, was linked to vibration at roughly 2,900–3,000 cm^{-1}. This is attributed to vitamin D molecules, potential hydration in calcium carbonate, or content of moisture. In contrast, the control sample exhibits a weaker or less prominent peak, suggesting a lower concentration of hydroxyl group, possibly due to the absence of vitamin D supplementation. A strong vibration at a wave number of around 1,742.5 cm^{-1} came from the stretching vibration of C=O (carbonyl groups) discovered in vitamin D molecules. This peak is either absent or has lower intensity in the control sample, confirming that vitamin D ester groups are introduced only in the supplemented sample. Similarly, vibrations at 987.7 cm^{-1} showed the presence of fat and calcium carbonate in cookies, as CLO and ESP were introduced as replacements of butter and CSGF, and the fat from CLO and calcium carbonate from ESP had high contents of vitamin D and calcium, respectively. These peak intensities suggest increased calcium and vitamin D contents. In contrast, these peaks are weaker in the control sample, indicating a lower concentration of calcium and vitamin D or possibly differences in crystallinity. This FTIR comparison validates the incorporation of calcium and vitamin D, making the supplemented sample nutritionally enhanced compared to the control. The presence of Vitamin D functional groups and stronger calcium carbonate peaks suggests improved bioavailability of essential nutrients.

XRD plots of the control and supplemented cookies are shown in Figures 6A,B. The peaks are sharp and highly resolved that depicted the crystalline structure of ESP-fortified cookies. The ESP's crystallographic lattice of calcium carbonate was confirmed by diffractogram, which shows a single crystalline phase (calcite) with a peak at 29.4° in accordance with X-ray diffraction pattern. These bands also showed that the crystallinity of calcium in supplemented cookies was not affected by baking (Therdthai *et al.*, 2023). When Kalaycı *et al.* (2025) stated that the calcium carbonate peak in the XRD results of ESP was $2\Theta = 29.58°$ for various eggshells, it clearly confirmed the current findings about the existence of high calcium contents in supplemented cookies. The lack of calcium carbonate could be the cause of this peak's non-observance in the control cookies of this study. The present results are in strong agreement with the findings of that of Hamidi *et al.* (2017), who studied the crystalline structure of ESP and concluded that ESP could be used as an excellent source of dietary calcium. Cree and Rutter (2015) asserted that biowaste chicken eggshells had a high calcite or calcium carbonate content. The FTIR spectra and

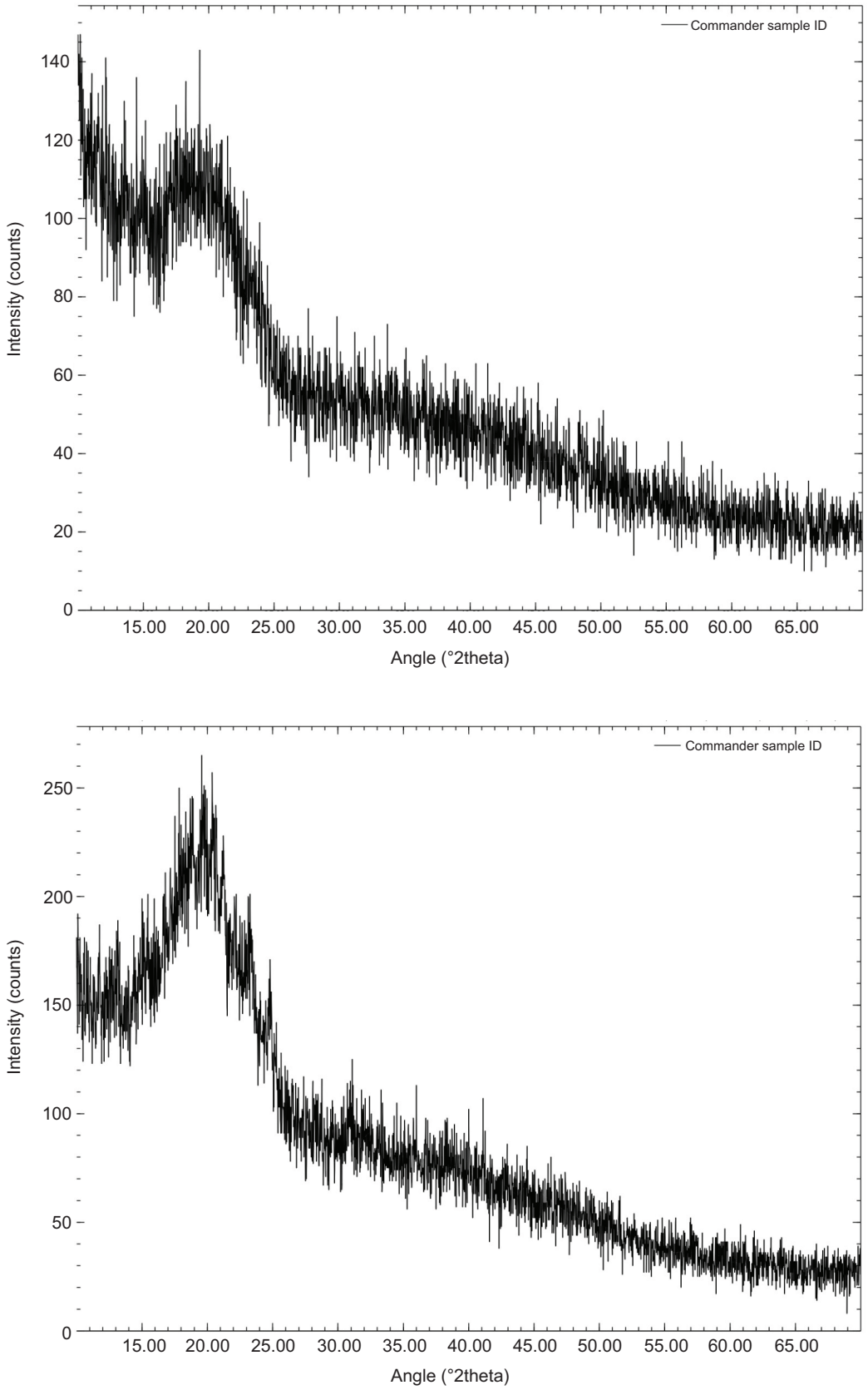

Figure 6. (A) XRD pattern of control cookies; (B) XRD pattern of supplemented cookie. XRD: X-ray diffraction.

XRD patterns of milled ESP were determined to be comparable with the current findings when ESP was used in cookies. Waste eggshells produced by processing industries could be utilized as a calcium fortification agent in various bakery products. The FTIR analysis of raw and boiled chicken ESP showed a similar absorption profile, with peaks at 1,394 cm^{-1}, 873 cm^{-1}, and 712 cm^{-1}, which supported the current findings. In a study conducted by Awogbemi *et al.* (2020), the XRD characterization of ESP showed that the proportion of calcium carbonate in raw and boiled samples was 79.3% and 99.2%, respectively, while these findings also supported the current results.

Conclusion

This study provided important information regarding the use of ESP and CLO as potential sources of calcium and vitamin D in cookies. ESP and CLO were discovered to be good sources of nutritional contents, especially calcium and vitamin D, respectively. The ESP- and CLO-fortified cookies were found to be good in terms of fat, protein, ash, fiber, and important minerals. The calcium analysis of developed cookies in the present study affirmed the significant increase in the calcium contents from 22.85 mg/100 g to 1,732 mg/100 g, whereas vitamin D content in the cookies were also significantly improved from 39.8 IU to 1,684 IU, which was due to the addition of ESP and CLO. The FTIR and XRD comparison of control and supplemented cookies validated the incorporation of calcium and vitamin D, making the supplemented sample nutritionally enhanced compared to the control. The presence of vitamin D functional groups and stronger calcium carbonate peaks suggested the improved bioavailability of these essential nutrients. The sensory evaluation of cookies presented nonsignificant results at lower levels of incorporation of ESP whereas at higher levels, a decrease in sensory scores was noted. The results suggested that the cookies developed with the replacement of CSGF and shortening, with ESP and CLO up to 3% and 9.6%, respectively, were acceptable for consumption, as their sensory scores were comparable to the control cookies developed from CSGF and butter as a shortening. Supplementation with ESP in cookies could be a great source of dietary calcium to combat calcium deficiency. CLO was used in mercantile food products as a vehicle to improve dietary vitamin D intake. More natural sources of calcium and vitamin D must be explored in the future and incorporated in daily diets as a part of nutritional supplementation.

Availability of Data and Materials

All the data generated in this research work has been included in the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

References

Afzal, F., Mueen-ud-Din, G., Nadeem, M., Murtaza, M.A. and Mahmood, S. 2020. Effect of eggshell powder fortification on the physicochemical and organoleptic characteristics of muffins. Pure Appl Biol. 9(2):1488–1496. http://doi.org/10.19045/bspab.2020.90154

Ajala, E.O., Eletta, O.A.A., Ajala, M.A. and Oyeniyi, S.K. 2018. Characterization and evaluation of chicken eggshell for use as a bio-resource. Arid Zone J Eng Technol Environ. 14(1):26–40. https://www.azojete.com.ng/index.php/Azojete/article/view/95

Ali, M. and Badawy, W.Z. 2017. Utilization of eggshells by-product as a mineral source for fortification of bread strips. J Food Dairy Sci. 8(11):455–459. https://doi.org/10.21608/jfds.2017.38960

Ali, R.S., Hasan, S.T. and Alssiraj, M.A. 2019. Effect of chicken eggshell powder fortification on the chemical physical and rheological characteristics of the bread. Biochem Cell Arch. 19(1):543–547. https://doi.org/10.35124/bca.2019.19.1.543

Almarri, F., Haq, N., Alanazi, F.K., Mohsin, K., Alsarra, I.A., Aleanizy, F.S. and Shakeel, F. 2017. An environmentally benign HPLC-UV method for thermodynamic solubility measurement of vitamin D3 in various (Transcutol + water) mixtures. J Mol Liquids. 242:798–806. https://doi.org/10.1016/j.molliq.2017.07.011

Alzaheb, R.A. 2018. The prevalence of hypovitaminosis D and its associated risk factors among women of reproductive age in Saudi Arabia: a systematic review and meta-analysis. Clin Med

Insights Women's Health 11:1179562X18767884. https://doi.org/10.1177/1179562X18767884

Association of Official Analytical Chemists (AOAC). 2000. Official Methods of Analysis, 17th Ed. AOAC, Washington, DC.

Association of Official Analytical Chemists (AOAC). 2012. Official Methods of Analysis. AOAC, Washington, DC.

Awogbemi, O., Inambao, F. and Onuh, E.I. 2020. Modification and characterization of chicken eggshell for possible catalytic applications. Heliyon. 6(10):e05283. https://doi.org/10.1016/j.heliyon.2020.e05283

Aziz T, Sarwar A, Daudzai Z, Naseeb J, Ud Din J, Aftab U, Saidal A, Ghani M, Khan AA, Naz S, Shahazad M, Cui H, Lin L. (2022). Conjugated Fatty Acids (CFAs) Production via Various Bacterial Strains and their Applications. A Review. Journal of the Chilean Chemical Society. 67 (1), https://doi.org/10.4067/S0717-97072022000105445

Bajaj, S.R. and Singhal, R.S. 2021. Fortification of wheat flour and oil with vitamins B12 and D3: effect of processing and storage. J Food Comp Anal. 96:103703. https://doi.org/10.1016/j.jfca.2020.103703

Baláž, M., Boldyreva, E.V., Rybin, D., Pavlović, S., Rodríguez-Padrón, D., Mudrinić, T. and Luque, R. 2021. State-of-the-art of eggshell waste in materials science: recent advances in catalysis, pharmaceutical applications, and mechanochemistry. Front Bioeng Biotechnol. 8:612567. https://doi.org/10.3389/fbioe.2020.612567

Bartter, J., Diffey, H., Yeung, Y.H., O'Leary, F., Häsler, B., Maulaga, W. and Alders, R. 2018. Use of chicken eggshell to improve dietary calcium intake in rural sub-Saharan Africa. Matern Child Nutr. 14:e12649. https://doi.org/10.1111/mcn.12649

Bradauskiene, V., Montrimaite, K. and Moscenkova, E. 2017. Facilities of bread enrichment with calcium by using eggshell powder. JAMA. 29:340–344.

Brun, L.R., Lupo, M., Delorenzi, D.A., Di Loreto, V.E. and Rigalli, A. 2013. Chicken eggshell as suitable calcium source at home. Int J Food Sci Nutr. 64(6):740–743. https://doi.org/10.3109/09637486.2013.787399

Chilek, T.Z.T., Kairuaman, N.A., Ahmad, F.I.S.A.L., Wahab, R.A., Zamri, A.I. and Mahmood, A.Z.I.Z.A.H. 2018. Development of white bread fortified with calcium derived from eggshell powder. Malays Appl Biol. 47(6):29–39.

Cree, D. and Rutter, A. 2015. Sustainable bio-inspired limestone eggshell powder for potential industrialized applications. ACS Sustain Chem Eng. 3(5):941–949. https://doi.org/10.1021/acssuschemeng.5b00035

De Boer, I.H., Zelnick, L.R., Lin, J., Schaumberg, D., Wang, L., Ruzinski, J., Friedenberg, G., Duszlak, J., Bubes, V.Y., Hoofnagle, A.N., Thadhani, R., Glynn, R.J., Buring, J.I., Sesso, H.D. and Manson, J.E. 2018. Vitamin D and omega-3 trial to prevent and treat diabetic kidney disease: rationale, design, and baseline characteristics. Contem Clin Trials. 74:11–17. https://doi.org/10.1016/j.cct.2018.09.014

Dolińska, B., Jelińska, M., Szulc-Musioł, B. and Ryszka, F. 2016. Use of eggshells as a raw material for production of calcium preparations. Czech J Food Sci. 34(4):256.

Ellulu, M.S., Rahmat, A., Patimah, I., Khaza'ai, H. and Abed, Y. 2015. Effect of vitamin C on inflammation and metabolic markers in hypertensive and/or diabetic obese adults: a randomized controlled trial. Drug Des Devel Ther. 9:3405–3412. https://doi.org/10.2147/DDDT.S83144. eCollection 2015

Faridi, H. and Arabhosseini, A. 2018. Application of eggshell wastes as valuable and utilizable products: a review. Res Agr Eng. 64(2):104–114. https://doi.org/10.17221/6/2017-RAE

Feng, F., Shi, G., Chen, H., Jia, P., Bao, L., Xu, F., Sun, Q.C. and Tang, H. 2021. Comprehensive interventions including vitamin D effectively reduce the risk of falls in elderly osteoporotic patients. Ortho Surg. 13(4):1262–1268. https://doi.org/10.1111/os.13009

Feng, L., Wu, L., Guo, Y., Hamada, N., Hashi, Y., Li, X. and Cao, L. 2020. Determination of vitamin D3 in daily oily supplements by a two-dimensional supercritical fluid chromatography-liquid chromatography-mass spectrometry system. J Chrom A. 1629:461510. https://doi.org/10.1016/j.chroma.2020.461510

Ficco, D.B.M., Riefolo, C., Nicastro, G., De Simone, V., Di Gesù, A.M., Beleggia, R., Platani, C., Cattivelli, L. and De Vita, P. 2009. Phytate and mineral elements concentration in a collection of Italian durum wheat cultivars. Field Crops Res. 111(3):235–242. https://doi.org/10.1016/j.fcr.2008.12.010

Gaonkar, M. and Chakraborty, A.P. 2016. Application of eggshell as fertilizer and calcium supplement tablet. Int J Innov Res Sci Eng Tech. 5(3):3520–3525.

Gorsi, F.I., Hussain, A., Kausar, T., Nisar, R., Siddique, T., Yaqub, S., Sidrah, Batool, S.A. and Akram, S. 2024. Structural and thermal interaction studies of aloe vera (aloe Barbadensis miller) gel powder and developed food bars. J Therm Anal Calorim. (JTAC), 149(10):4543–4559. https://link.springer.com/article/10.1007/s10973-024-13007-9

Hamidi, A.A., Salimi, M.N. and Yusoff, A.H.M. 2017. Synthesis and characterization of eggshell-derived hydroxyapatite via mechanochemical method: a comparative study. In AIP Conference Proceedings, Vol. 1835(1). AIP Publishing, Melville, NY. https://doi.org/10.1063/1.4981867.

Hanai, N., Shimizu, Y., Kariya, S., Yasumatsu, R., Yokota, T., Fujii, T., Tsukahara, K., Yoshida, M., Hanyu, K., Ueda, T. and Hirakawa, H. 2021. Effectiveness and safety of nivolumab in patients with head and neck cancer in Japanese real-world clinical practice: a multicenter retrospective clinical study. Int J Clin Oncol. 26(3):494–506. https://doi.org/10.1007/s10147-020-01829-0

Hasan, A., Bano, Y., Usman, S.K.M., Ahmad, T., Shukat, R., Abbas, S.Q. and Ali, M. 2023. Development and characterization of Ice cream using eggshell powder to curtail calcium deficiency. Neuro Quantol. 21(6):258.

Hassan, N.M. 2015. Chicken eggshell powder as dietary calcium source in biscuits. World J Dairy Food Sci. 10(2):199–206. https://doi.org/10.5829/idosi.wjdfs.2015.10.2.1152

Higashi, T., Goto, A., Morohashi, M., Ogawa, S., Komatsu, K., Sugiura, T., Fukuoka, T. and Mitamura, K. 2014. Development and validation of a method for determination of plasma 25-hydroxyvitamin D3 3-sulfate using liquid chromatography/tandem mass spectrometry. J Chrom B. 969:230–234. https://doi.org/10.1016/j.jchromb.2014.08.027

Hipsey, M.R., Bruce, L.C., Boon, C., Busch, B., Carey, C.C., Hamilton, D.P., Hanson, P.C., Read, J.S., De Sousa, E., Weber, M.

and Winslow, L.A. 2019. A General Lake Model (GLM 3.0) for linking with high-frequency sensor data from the Global Lake Ecological Observatory Network (GLEON). Geosci Model Develop. 12(1):473–523. https://doi.org/10.5194/gmd-12-473-2019

Hubicka, U., Padiasek, A., Żuromska-Witek, B. and Szlósarczyk, M. 2020. Determination of vitamins K1, K2 MK-4, MK-7, MK-9 and D3 in pharmaceutical products and dietary supplements by TLC-densitometry. Processes. 8(7):870. https://doi.org/10.3390/pr8070870

Hussain, A., Kausar, T., Aslam, J., Quddoos, M.Y., Ali, A., Kauser, S., Zerlasht, M., Rafique, A., Noreen, S., Iftikhar, K. and Waheed Iqbal, M. 2023a. Physical and rheological studies of biscuits developed with different replacement levels of pumpkin (*Cucurbita maxima*) peel, flesh, and seed powders. J Food Qual. 2023(1):4362094. https://doi.org/10.1155/2023/4362094

Hussain, A., Kausar, T., Din, A., Murtaza, M.A., Jamil, M.A., Noreen, S., Rehman, H.U., Shabbir, H. and Ramzan, M.A. 2021. Determination of total phenolic, flavonoid, carotenoid, and mineral contents in peel, flesh, and seeds of pumpkin (*Cucurbita maxima*). J Food Process Preserv. 45(6):e15542. https://doi.org/10.1111/jfpp.15542

Hussain, A., Laaraj, S., Kausar, T., Tikent, A., Azzouzi, H., Kauser, S., An, Q.U., Iqbal, A., Akram, S., Nisar, R. and Najam, A. 2023b. Food application of orange seed powder through incorporation in wheat flour to boost vitamin and mineral profiles of formulated biscuits. Int J Food Sci. 2023(1):6654250. https://doi.org/10.1155/2023/6654250

Iftikhar, L., Ahmad, I., Saleem, M., Rasheed, A. and Waseem, A. 2024. Exploring the chemistry of waste eggshells and its diverse applications. Waste Manag. 189:348–363. https://doi.org/10.1016/j.wasman.2024.08.024

Indrianingsih, A.W., Rosyida, V.T., Darsih, C., Apriyana, W., Iwansyah, A.C., Khasanah, Y., Kusumaningrum, A., Windarsih, A., Herawati, E.R.N., Muzdalifah, D. and Sulistyowaty, M.I. 2024. Physicochemical properties, antioxidant activities, β-carotene content, and sensory properties of cookies from pumpkin (*Cucurbita moschata*) and modified cassava flour (*Manihot esculenta*). Bioact Carbohydr Diet Fibre. 31:100398. https://doi.org/10.1016/j.bcdf.2023.100398

Kadhim, D.R., Hamad, T.I. and Fatalla, A.A. 2022. Use of eggshells as bone grafts around commercially pure titanium implant screws coated with nano calcium sulfate. Int J Biomater. 2022(1):8722283. https://doi.org/10.1155/2022/8722283

Kalaycı, T., Altuğ, D.T., Kınaytürk, N.K. and Tunalı, B. 2025. Characterization and potential usage of selected eggshell species. Sci Reports, 15(1):6241. https://www.nature.com/articles/s41598-025-87786-y

Khan, M.R., Wahab, S.A.I.D., Qazi, I.M., Ayub, M., Muhammad, A., Uddin, Z.A.H.O.O.R., Faiq, M., Tareen, A.K., Fahad, S.H.A.H. and Noor, M. 2017. Effect of calcium fortification on whole wheat flour based leavened and unleavened breads by utilizing food industrial wastes. Asian J Chem., 29(2), 423-430. https://doi.org/10.14233/ajchem.2017.20231

Kitagawa, Y., Uno, T., Oyama, T., Kato, K., Kato, H., Kawakubo, H., Kawamura, O., Kusano, M., Kuwano, H., Takeuchi, H. and Toh, Y. 2022. Esophageal cancer practice guidelines 2017 edited by the Japan Esophageal Society: part 1. Esophagus. 16:1–24. https://doi.org/10.1007/s10388-022-00935-4

Kulshreshtha, G., Diep, T., Hudson, H.A. and Hincke, M.T. 2022. High-value applications and current commercial market for eggshell membranes and derived bioactives. Food Chem. 382:132270. https://doi.org/10.1016/j.foodchem.2022.132270

Li, T., Guo, Q., Liang, M., Qu, Y., Zhang, Y, Wang, Q. (2023). Impact of additives on the formation of thermally induced trans linoleic acid in peanut oil. International Journal of Food Science & Technology, 58(5), 2498-2504. doi: https://doi.org/10.1111/ijfs.16391

Liang, M., Li, T., Qu, Y., Qin, J., Li, Z., Huang, X., Wang, Q. (2023). Mitigation mechanism of resveratrol on thermally induced trans-α-linolenic acid of trilinolenin. LWT, 189, 115508. https://doi.org/10.1016/j.lwt.2023.115508

Marshall, K.M., Loeliger, J., Nolte, L., Kelaart, A. and Kiss, N.K. 2019. Prevalence of malnutrition and impact on clinical outcomes in cancer services: a comparison of two time points. Clin Nutr. 38(2):644–651. https://doi.org/10.1016/j.clnu.2018.04.007

Mepba, H.D., Eboh, L. and Nwaojigwa, S.U. 2007. Chemical composition, functional and baking properties of wheat-plantain composite flours. Afr J Food Agri Nutr Devel. 7(1):1–22. https://www.ajol.info/index.php/ajfand/article/view/136129

Milbradt, B.G., Muller, A.L.H., da Silva, J.S., Lunardi, J.R., Milani, L.I.G., de Moraes Flores, E.M., Callegaro, M.D.G.K. and Emanuelli, T. 2015. Eggshell as calcium source for humans: mineral composition and microbiological analysis (Casca de ovocomofonte de calcio para humanos: composicao mineral e analisemicrobiologica). Ciencia Rural. 45(3):560–567.

Murakami, F.S., Rodrigues, P.O., Campos, C.M.T.D. and Silva, M.A.S. 2007. Physicochemical study of $CaCO_3$ from egg shells. Food Sci Tech. 27:658–662. https://doi.org/10.1590/S0101-20612007000300035

Nakilcioglu, E. and Dadalı, C. 2024. Optimization of gluten-free cake formulation by replacement of rice flour and baking powder with different levels of eggshell powder using response surface methodology. J Food Meas Charact. 18:5541–5551. https://doi.org/10.1007/s11694-024-02586-8

Newell, M., Mazurak, V., Postovit, L.M. and Field, C.J. 2021. N-3 long-chain polyunsaturated fatty acids, eicosapentaenoic and docosahexaenoic acid, and the role of supplementation during cancer treatment: a scoping review of current clinical evidence. Cancers. 13(6):1206. https://doi.org/10.3390/cancers13061206

Opris, H., Dinu, C., Baciut, M., Baciut, G., Mitre, I., Crisan, B., Armencea, G., Prodan, D.A. and Bran, S. 2020. The influence of eggshell on bone regeneration in preclinical in vivo studies. Biology. 9(12):476. https://doi.org/10.3390/biology9120476

Platon, N.I.C.O.L.E.T.A., Arus, V.A., Georgescu, A.M., Nistor, I.D. and Barsan, N.A.R.C.I.S. 2020. White bread fortified with calcium from eggshell powder. Rev. Chim, 71(7):299–306. https://doi.org/10.37358/RC.20.7.8248

Polzonetti, V., Pucciarelli, S., Vincenzetti, S. and Polidori, P. 2020. Dietary intake of vitamin D from dairy products reduces the risk of osteoporosis. Nutrients. 12(6):1743. https://doi.org/10.3390/nu12061743

Prentice, A., Jarjou, L.M., Goldberg, G.R., Schoenbuchner, S.M., Moore, S.E., Ward, K.A. and Cole, T.J. 2024. Effects of maternal calcium supplementation on offspring blood pressure and growth in childhood and adolescence in a population with a low-calcium intake: follow-up study of a randomized controlled trial. Am J Clin Nutr. 119(6):1443–1454. https://doi.org/10.1016/j.ajcnut.2024.02.025

Quddoos, M.Y., Mahmood, S., Yaqoob, M., Murtaza, M.A., Zahra, S.M., ud Din, G.M., Hussain, A., Rafique, A., Iftikhar, K. and Mustafa, S. 2022. The effects of natural and synthetic calcium utilization on quality parameters of cookies. Appl Food Res. 2(1):100093. https://doi.org/10.1016/j.afres.2022.100093

Rafique, H.S., Hussain, A., Nadeem, M., Rehman, A., Siddique, T., Najam, A., Haroon, H., Arif, M.R., Yaqub, S., Fatima, H. and Laaraj, S. 2023. Impact of different proportions of sweet potato (Ipomoea batatas L.) flour on physical, chemical and sensory parameters of straight grade flour-based cake rusk. Food and Humanity. 1:1282–1296. https://doi.org/10.1016/j.foohum.2023.09.024

Ray, S., Barman, A.K., Roy, P.K. and Singh, B.K. 2017. Chicken eggshell powder as dietary calcium source in chocolate cakes. Pharma Innov. 6(9, Part A):1.

Sakai, S., Hien, V.T.T., Tuyen, L.D., Duc, H.A., Masuda, Y. and Yamamoto, S. 2017. Effects of eggshell calcium supplementation on bone mass in postmenopausal Vietnamese women. J Nutr Sci Vitaminol. 63(2):120–124. https://doi.org/10.3177/jnsv.63.120

Salem, A.M., Ali, M.S. and Abdul Rahim, R.A. 2023. Evaluation of compressive strength and setting time of some types of calcium silicate based materials modified with chicken egg shell powder. Al-Azhar J Dental Sci. 26(4):477–483. https://doi.org/10.21608/ajdsm.2022.113951.1285

Salem, I.-S., Ammar, A.-S.-M. and Habiba, R.A. 2012. Effect of eggshell powder addition as a source of calcium fortification on butter cake quality. J Agri Veter Sci. 5(2):109–118.

Singh, A., Kelkar, N., Natarajan, K. and Selvaraj, S. 2021. Review on the extraction of calcium supplements from eggshells to combat waste generation and chronic calcium deficiency. Environ Sci Pollut Res. 28:46985–46998. https://doi.org/10.1007/s11356-021-15158-w

Singh, P. and Prasad, S. 2023. A review on iron, zinc and calcium biological significance and factors affecting their absorption and bioavailability. J Food Comp Anal. 123(2022):105529. https://doi.org/10.1016/j.jfca.2023.105529

Therdthai, N., Soontrunnarudrungsri, A. and Khotchai, W. 2023. Modified eggshell powder using thermal treatment and its application in Ca-fortified dog biscuits. Heliyon. 9(2):320. https://doi.org/10.1016/j.heliyon.2023.e13093

Torres Gouveia, M.E., Milhans, C., Gezek, M. and Camci-Unal, G. 2025. Eggshell-based unconventional biomaterials for medical applications. Adv NanoBiomed Res. 5(2):2400120. https://doi.org/10.1002/anbr.202400120

Ud Din , G. M. ., Usmani, W., Hussain, A., Bilal, M., Najam, A., Zia, M., Raza, M. S. ., Aziz, T., Zahra, N., Alshammari, J. M., Alharbi, M. H., Al-Asmari, F. (2025). Effect of multigrain flour mixes on the physicochemical, nutritional, textural, and sensory quality of cake rusks. Italian Journal of Food Science, 37(2), 261-275. https://doi.org/10.15586/ijfs.v37i2.2921

Waheed, M., Butt, M.S., Shehzad, A., Adzahan, N.M., Shabbir, M.A., Suleria, H.A.R. and Aadil, R.M. 2019. Eggshell calcium: a cheap alternative to expensive supplements. Trends Food Sci Technol. 91:219–230. https://doi.org/10.1016/j.tifs.2019.07.021

Waheed, M., Yousaf, M., Shehzad, A., Inam-Ur-Raheem, M., Khan, M.K.I., Khan, M.R., Ahmad, N. and Aadil, R.M. 2020. Channelling eggshell waste to valuable and utilizable products: a comprehensive review. Trends Food Sci Technol. 106:78–90. https://doi.org/10.1016/j.tifs.2020.10.009

Younas, K., Afzaal, M., Saeed, F., Shankar, A., Kumar Bishoyi, A., Khare, N., Imran, A., Mahmood, K., Amhed, A., Asghar, A. and Samar, N. 2024. A mini-review on egg waste valorization. J Sci Food Agric. 105(5):2748–2754. https://doi.org/10.1002/jsfa.13953

Zerek, E., Ersoy, N. and Ersoy, G. 2022. Determination of some nutritional and quality properties of eggshell powder added cookies: properties of eggshell powder added cookies. J Food Meas Charact. 16(5):3315–3320. https://doi.org/10.1007/s11694-022-01419-w

Annexure 1

(A)

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	2	3	4	5	6	7	8	9

(B)