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Evaluation of the physicochemical, bioavailability, nanoscale, and FTIR properties of eggshell, chicken, and cattle bones powders for muffin preparation

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ORIGINAL ARTICLE

Abstract

Minerals, especially Ca, P, and Mg, are undoubtedly essential nutrients associated with the maintenance of life. The proposed research work was designed with different treatments to fortify muffins using chicken eggshell powder (ESPck), chicken bone extract powder (T2 (BEPck)), and cattle bone extract powder (T3 (BEPct)) to augment mineral content and evaluate various quality parameters. According to the statistical outcomes of the research, the proximate composition of raw minerals showed a significant amount of ash in ESPck, fat and NFE (nitrogen-free extract) in T2 (BEPck), and moisture and protein in T3 (BEPct). Significant amounts of Ca and Na were present in ESPck, while P, Mg, K, and Fe were found in T2 (BEPck), and Zn was identified in T3 (BEPct). High levels of Ca and Mg were observed in nano T3 (BEPct). The minerals-fortified muffins (MFM) showed a significant effect only in moisture and ash content, while mineral composition showed a significant amount of Ca in C_{+ve} (control positive), and P and Mg in T3 (BEPct). High mineral bioavailability was observed for Ca in T2 (BEPck), and for P and Mg in T3 (BEPct). Although Ca bioavailability was greater in T2 (BEPck), bone powder did not yield highly preferred results in sensory and other parameters, whereas ESPck achieved the highest score in overall acceptability. It can be concluded that muffins fortified with chicken eggshell powder contain more calcium and received positive sensory scores.

Keywords: bone powder, Bioavailability, Eggshell, FTIR, mineral, muffins

Introduction

Nowadays, food scientists are increasingly concerned with food fortification, focusing on food safety, quality, security, diet-health linkages, and malnutrition (Olson *et al.*, 2021). Micronutrient deficiency is common in both developed and developing countries. Even a minor micronutrient shortage can have wide-ranging consequences for human body systems and health, contributing to a high prevalence of morbidity and mortality from various diseases (Lowe, 2021). In 2000–2002, 852 million people worldwide were categorized as undernourished, according to the Food and Agriculture Organization (FAO, 2003). Currently, more than 800 million people suffer from undernourishment globally. In 2011, 1.1 and 3.5 billion people suffered from Zn (zinc) and Ca (calcium) deficiencies, respectively, due to limited availability in the dietary supply (Kumssa *et al.*, 2015).

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Minerals are essential for vital bodily structures and functions. Macro-minerals are needed in quantities greater than 100 mg/dl, while micro-minerals are required in amounts less than 100 mg/dl (Lowe, 2021). Ca is a mineral required by the human body to maintain bone tissues and support various physiological functions. Ca, along with P (phosphorus), constitutes the mineral portion of teeth and bones. Ca plays a key role in treating and maintaining the demineralization of bones, blood coagulation, neuromuscular excitability, hormone secretion, reproductive functions, the release of cellular enzymes, the transfer of inorganic ions across membranes, and acts as a metabolic component. A decrease in phosphorus levels in serum is associated with rickets and hyperparathyroidism (Lecoq *et al.*, 2020; Quddoos *et al.*, 2020). Magnesium (Mg) deficiency symptoms often result from malabsorption, diarrhea, or alcoholism. Severe Mg deficiency can lead to vasodilation, with erythema and hyperemia appearing after a few days on a deficient diet. A proper balance of minerals is important for both humans and animals. Overdose, deficiency, or imbalance of inorganic nutrients can negatively affect health (Lecoq *et al.*, 2020; Quddoos *et al.*, 2020). Similar to eggshells' mineral, Proteins, minerals, enzymes, vitamins, saponins, and flavonoids are all abundant in garlic. Garlic has several health benefits and has been used as a remedy to prevent sickness (Zerlasht *et al.*, 2024).

The chicken eggshell is a waste material derived from various native sources such as homes, hatcheries, poultry farms, restaurants, and factories that produce egg-based products. It mainly comprises 94% $CaCO_3$, along with some calcium phosphate and magnesium carbonate deposited on an organic matrix. Eggshell Ca is an excellent dietary Ca source and serves as a suitable replacement for significant crustacean shells (Quddoos *et al.*, 2020). Chicken eggshell powder, containing approximately 38% Ca, is a little-known but promising source of Ca for human nutrition. Commercial chicken eggshells are richer in mineral content than those of other animals. Ca is the most abundant mineral in chicken eggshells, with a concentration of 2534.4±10.60 mg/100g. Other minerals found in chicken eggshells include Mg, Na, P, K, Fe, and Zn, in quantities of 247.7, 168.9, 139.8, 82.2, 7.64, and 0.93 mg/100g, respectively. These minerals can help reduce pain in osteoporosis patients and contribute to increased bone density.

Chicken bones, available from chicken slaughtering plants and by-products, are rich in Ca and other minerals. Chicken bone extract powder (T2 (BEPck)) is a source of Ca and minerals that is well-received when fortified into products such as chili paste and shrimp chips. T2 (BEPck) serves as a substitute and inexpensive source of Ca, as it contains a high Ca content (30 g per 100 g) and has a Ca:P ratio of approximately 2:1. Ca can be efficiently obtained from T2 (BEPck), making it suitable for use in bakery items like bread and cookies without affecting their texture, appearance, or taste (Sittikulwitit, 2004).

The consumption of poultry meat is increasing alongside the generation of by-products from poultry slaughter. Various secondary products are produced as a result of operations on any poultry farm (Mokrejš *et al.*, 2019).

Processing poultry secondary raw materials is a promising avenue. The increase in poultry meat production has made this issue increasingly relevant. It is customary to produce soup sets and feed products from secondary raw materials and waste generated during chicken slaughter and processing (Merenkova *et al.*, 2021). Various chemical, biotechnological, and mechanical techniques are used to extract nutrients—such as fats, proteins, and minerals—from poultry meat and bone residue for food applications. Finely grinding meat and bone residue to a pasty consistency is the simplest and most gentle processing method. To achieve a pasty consistency with a particle size of less than 100 μm, it is necessary to use fine grinding equipment, adhere to temperature guidelines to prevent overheating and protein denaturation, control moisture content, and ensure the desired consistency and rheological characteristics of the finely ground poultry meat and bone residue (Yessimbekov *et al.*, 2021; Bekeshova *et al.*, 2022).

When bones are finely ground in a mill or grinder, the friction between the particles and the rapidly spinning rotor knives generates a significant amount of heat. This heat can cause the temperature of the product to rise, potentially leading to thermal effects such as thermal expansion or even material damage (Li & Zhao, 2012; Sajid *et al.*, 2023).

Cattle bones have an average Ca content of 37.06%, which does not vary with age. The Ca concentration in the ash of bovine bone ranges from 36.3% to 36.9%. The Ca composition in cattle bone ash remains relatively constant. Cattle bones can be used to produce cattle bone extract powder (T3 (BEPct)) for utilization in food fortification. Bone mineral content (BMC) results from the balance between bone formation and resorption, and it is influenced by physiological state, diet, and age. Approximately 20% of the bone's wet weight is water, 35% is organic matter (OM), and 45% is ash in adult mammals. The ash content consists of 37% calcium, and phosphorus makes up 18.5%. The mineral content is 65% to 70%, while OM comprises 30% to 35% on a dry weight basis (Li *et al.*, 2021). Body composition can be measured through bio-electrical impedance-based scale BF-105 in whch following parameters can be measured and also showed bone composition, weight (kg), bone mass (%),

body fat (%), muscle mass (%), body water (%), basal metabolic rate (BMR), and active metabolic rate (AMR) Hameed *et al.*, (2024).

There is an increasing need to prevent the development of musculoskeletal system (MSS) disorders by implementing preventive measures, such as incorporating specialized products and functional foods with enhanced compositions into everyday diets. Calcium and phosphorus, in particular, are essential minerals that must be balanced as they enter the human body, as they play a key role in preventing and treating MSS diseases. Studies by foreign researchers have observed that when animals are fed foods with a high phosphorus content and low calcium level, or vice versa, bone tissue can decline by up to 56% in laboratory mice (Begot *et al.*, 2011).

Farm animal bones are currently processed primarily in the country's industry through a process that involves grinding, heat treatment, polishing, and drying. These technological processes only allow for the extraction of a portion of the bone mass and do not sufficiently purify the bone product, which is then used solely as meat and bone meal for farm animal feed (Grishaeva *et al.*, 2021; Saeed *et al.*, 2023).

Throughout the study, a technique for processing deer bone was developed that allows for the extraction of a bio-substance with a high mineral content. Using the following technological parameters, the fat extraction method removed 18.6% of the fat: two to three hours of exposure to infrared radiation at a temperature of 80 to 85°C. Ultrasound equipment and home enzymes were employed to remove the entire 25.8% protein fraction at a temperature of 75–80°C for over 4 to 5 h.

The process of softening bone raw materials to produce a mineral supplement, constituting 94.3% of the raw material volume, was carried out for three hours at 120°C and 1.5 atm of pressure. The data on changes in the blood chemistry of dogs following the use of the bone supplement clearly demonstrated good assimilation of the bone product. Specifically, the levels of calcium increased by 47.3%, phosphorus by 2.5 times, magnesium by 19.0%, protein by 44.2%, and hemoglobin by 66.7% (Grishaeva *et al.*, 2021).

The development of bone processing technology is therefore necessary to produce a highly purified product that can be used as food. In conclusion, waste-free deer bone processing can help processing companies better utilize production facilities, reduce labor and energy costs, and increase overall profitability in the deer breeding industry. As organic bio-waste, cow bone has been refined into various particle sizes, including macro, micro, and nanoparticles. According to Ikumapayi and Akinlabi (2019), the process of creating microparticles from cow bones involved first carbonizing the bones at 750°C in a heat-treatable furnace without oxygen, then sieving them through mesh sizes of 150, 300, and 600 μm. These particles were then added to the polymer as reinforcement to create polymatrix composites and have also been used as reinforcement nanoparticles during friction stir welding of metal composites.

In this investigation, cow bone was ground for one hour, then carbonized for two hours at 500°C in a muffle furnace, and finally allowed to cool to ambient temperature. It is well known that cow bones are waste products that contribute to environmental pollution. Despite this, researchers are working diligently to transform these agricultural wastes into more practical technical applications, particularly for reinforcement. According to Ahmad *et al.* (2014), cow bone, as a natural animal fiber, should have strong surface compatibility in addition to meeting the structural compatibility requirements for biomaterials.

The study by Ikumapayi *et al.* (2021) involved the collection of cow skulls (cow head bones) from a slaughterhouse located in Johannesburg, South Africa. After being scrubbed of meat and cleaned with deionized water, the skulls were left in the sun for nearly six weeks to ensure complete drying. After six weeks, they were cleaned again with deionized water to remove any potential contaminants and pollutants. The skulls were then dried in an electric oven set at 50°C for five hours each day over the course of seven days to ensure complete moisture removal. Following this, the skulls were cleaned with acetone before being crushed and ground. The material was then sieved to a size of 150 μm using the standard range of ASTM meshes with the King Test Sieve. Finally, it was further milled for sixty minutes using a digital vibratory disk milling machine (VDMM) before use.

Fortification of food is the process of adding one or more essential nutrients to it, whether or not they are typically present, in order to prevent or improve the occurrence of one or more nutrient deficiencies in a community or specific population group. Wahlqvist (2008) reports that this approach is cost-effective and widely accepted in industrialized countries as a means to increase nutrient consumption and improve nutritional status. The term "fortification" refers to the addition of necessary nutrients to address specific public health needs (Olson, 2021). Food fortification can take various forms, including bulk, market-driven, and tailored fortification. Gao *et al.* (2016) described muffins as "quick breads" because their preparation does not involve kneading, resting, or rising, as no yeast is used in them. They are also referred to as soft cupcakes because they are typically shaped like cups. Additional ingredients such as chocolate chips, fruits

(e.g., banana, orange, blueberries, raspberries, peach), nuts, cheese, spices, chopped meats, and herbs (for medicinal purposes) can also be added to muffin recipes. These ingredients make muffins excellent sources of major water-soluble vitamins, minerals, and carbohydrates.

The bioavailability of fortified trace micronutrients has been identified as a crucial factor for maintaining normal body health (Marze *et al.*, 2017). Diego Quintaes *et al.* (2017) explained that the widely accepted definition of bioavailability is the amount of trace elements fortified in a food that plays a role in the body's normal digestive and physiological functions. It was noted that the availability of minerals in the human body can vary between 1% and 90%, and it is the primary factor that determines the effectiveness of a food (Schönfeldt *et al.*, 2016).

The study aims to develop mineral-fortified muffins and investigate the influence of adding chicken eggshell powder (ESP_{ck}), chicken bone extract powder (T2 (BEPck)), and cattle bone extract powder (T3 (BEPct)) on the physicochemical and sensory properties, mineral content, and bioavailability, particularly the bioavailability of Ca, P, and Mg in ESP_{ck}, T2 (BEPck), and T3 (BEPct) fortified muffins.

Materials and Methods

Dietary interventions play a vital role in preventing micronutrient deficiencies, particularly minerals such as Ca.

Product development

Different muffin treatments were prepared using natural sources of minerals, as shown in Table 1 below.

Preparation of raw materials

Commercially available wheat flour (Maida), milk, salt, sugar, eggs, baking powder, and ghee were purchased from the local market in Sargodha. Chicken eggshells, chicken bones, and cattle bones were obtained from the local market as well.

Table 1. Different treatments used to prepare fortified muffins.

Treatment	ESP_{ck} (g)	T2 (BEPck) (g)	T3 (BEPct) (g)	$CaCO_3$ (g)
C_{-ve}	–	–	–	–
C_{+ve}	–	–	–	0.63
T1 (ESP_{ck})	0.66	–	–	–
T2 (BEPck)	–	0.83	–	–
T3 (BEPct)	–	–	1.14	–

T1 ESPck = Eggshell Powder of chicken; T2 BEP_{ck} = Bone extract powder of chicken; T3 (BEPct) = Bone extract powder of Cattle. $C_{-ve:}$ Unfortified muffins/Placebo, C_{+ve} = $CaCO_3$.

Preparation of powders

Chicken eggshell powder (T1 (ESPck)) and chicken bone extract powder (T2 (BEPck)) were prepared using the method described by Quddoos *et al.* (2020), while cattle bone extract powder (T3 (BEPct)) was extracted following the method outlined by Li *et al.* (2021).

Preparation of eggshell powder

Chicken eggshells were collected and washed twice with running tap water, and then processed according to the following steps. The eggshells were washed with water for approximately 25 minutes. Next, the eggshells were dried in a hot air oven (Model: ED 115, Binder, Germany) for 1 hour at 75°C. They were then sterilized for 15 minutes in an automatic sterilizer autoclave (Model JSAT-45 Autoclave, Vertical, JS Research INC, Gongju-City, Korea) at 134°C. Each eggshell treatment was ground into a fine powder using a household mill (Braun, Germany) and used to make the muffins, as described by Quddoos *et al.* (2020).

Preparation of chicken and cattle bone powders

Chicken bones were obtained from local chicken shops, and cattle bones were sourced from the local market. Bone extract powders (BEP) were prepared using an alkaline treatment method. In this method, 50 g of bones were dried in a hot air oven (Model: ED 115, Binder, Germany) for 2.5 hours at 65°C. A 3% NaOH solution (Hunan Mingrui Xiangsheng Trade Co., Ltd., China) was added to the dried bones in a ratio of dried bones to 3% NaOH = 1:3 w/v, and the mixture was boiled for 1.5 hours. The bones were treated thoroughly to eliminate microbes and all organic matter before being used as a calcium fortificant. They were then washed with 1% HCl (Shijiazhuang Xinlongwei Chemical Co., Ltd., Hebei, China) in a ratio of bones to 1% HCl = 1:1 w/v, and rinsed with deionized water until reaching a neutral pH. The neutralized bones were then dried in a hot air oven for 1.4 hours at 100°C and ground into a fine powder using a Chakki atta roller mill (Model No: MFFC with Cyclone 2101, Anyang Best Complete Machinery Engineering Co., Ltd., Henan, China) until the powder could pass through a 100-mesh sieve. This powder was then used for the next stage of the experiment (Quddoos *et al.*, 2020; Li *et al.*, 2021).

Determination of mineral content of fortificant's powders

The powders were analyzed for mineral profiles, including Ca, P, and Mg, using an Atomic Absorption Spectrophotometer (Model: AA-6300, Shimadzu Corporation, Japan) following the AACC (2000) method.

Digestion of powder

Powder samples were prepared using the wet digestion method for mineral estimation. For each treatment, 0.5 g

of sample was taken and digested on a hot plate (Model: AH-120E, China) in a 100 ml conical flask at 60–70°C for 20 minutes with 10 ml of HNO_3 (catalog #84378). This was followed by digestion at 190°C with 5 ml of $HClO_4$ (60% v/v, catalog #311413) until the contents in the flask turned clear. The volume of each sample was then made up to 100 ml using double-distilled and deionized water, transferred to 100 ml volumetric flasks, and filtered. The filtered samples were analyzed using an Atomic Absorption Spectrophotometer (Model: AA-6300, Shimadzu Corporation, Japan). Standard solutions of known concentrations were first analyzed to generate standard curves. The concentrations of calcium, phosphorus, and magnesium in the samples were then determined using these curves, following the AACC (2000) method and also referencing the PerkinElmer Atomic Absorption Spectrophotometer.

Estimation of mineral contents by atomic absorption spectrophotometer

An Atomic Absorption Spectrophotometer was used to analyze the filtered sample solutions. Standard curves for each mineral were prepared by running samples of known concentrations. The mineral content of each sample was then determined using the respective standard curves, following methods No. 40-70 and 40-71 of AACC (2006).

Preparation of nanoscale bone powder of T2 (BEPck) and T3 (BEPct)

The extraction procedure was followed to produce all of the bone powder. The dried bones were ground into powder using a grinder (Model No: MFFC with Cyclone 2101, Anyang Best Complete Machinery Engineering Co., Ltd., Henan, China), and the resulting bone meals were further degreased using the method outlined by Buckley *et al.* (2012). Lipid extraction was performed twice on the bone meals using 100% hexane at a 1:1 (w/v) ratio, with continuous mixing for 15 minutes during the first extraction and 2 hours during the second.

The supernatants from the lipid-extracted samples were discarded after centrifugation at 2,031 g. The residual sediments were then dried in a drying oven (Model: ED 115, Binder, Germany) at 55°C until a constant weight was achieved, following the removal of surface red plasma protein using a medical spoon. The bone meal particles were subsequently dried, defatted, and ground into micron-sized particles using a vibrating micro-grinder (Songyue Machinery Co., Ltd., Jinan, China) set to vibrate for 20 minutes at a 5 mm vibration amplitude. The final step involved ball milling the micron-scale bone powders (MBPs) in a planetary ball mill (XQM-0.4, Tianchuang Powder Technology, China) to produce N (BEPck) and N (BEPct). This procedure followed the method of Li *et al.* (2018), with the exception of the ball milling duration. N (BEPck) and N (BEPct), also referred to as NBPs, were produced through a five-hour milling process.

Preparation of minerals-fortified muffins

Muffins were prepared by incorporating different levels of fortificant powders (as shown in Table 1) along with ingredients such as maida, salt, sugar, eggs, milk, ghee, and baking powder, with slight modifications. The preparation was carried out in the canning hall of the Institute of Food Science and Nutrition, University of Sargodha, using the straight dough method described by Kim and Shin (2022), with minor adjustments. The formulation of the fortified muffins included flour (500 g), sugar (275 g), eggs (4), butter/ghee (275 g), milk (50 ml), salt (2 g), baking powder (15 g), and fortificant powders as per Table 1 (Kim and Shin, 2022).

Preparation of muffin

Fortified muffins were prepared using a method adapted from Kim and Shin (2022), with certain modifications. Ghee and icing sugar were creamed together first. Eggs were beaten separately and then mixed into the cream for 4 to 5 minutes. Flour and baking powder were sifted and gradually added to the cream mixture until a homogeneous batter was formed. Milk and fortificant powders were then incorporated into the mixture as specified in Table 2. The muffin tray was greased with oil, and the batter was poured into the pans and baked in an oven (Model No. YXY-12A) at 180°C for 35 minutes. After baking, the muffins were cooled at room temperature for 1 hour and then placed into butter paper cups for further analysis.

Physico-chemical analysis

To analyze the physical properties and chemical composition of the prepared muffins, they were subjected to various physicochemical tests.

Table 2. Formulation of Fortified Muffins.

Ingredients	Quantity
Flour	500g
Sugar	275g
Eggs	4 No
Butter/ghee	275g
Milk	50mL
Salt	2g
Baking powder	15g
Fortificant powders	As per Table 3.1

(Kim and Shin, 2022).

Chemical analysis of minerals fortified muffins

Proximate analysis of the fortified muffins—namely moisture, crude fat, crude protein, crude ash, crude nitrogen, and nitrogen-free extract (NFE)—was conducted at the beginning of the study and then fortnightly for up to 30 days (AACC, 2000; AOAC, 2000; Nishat *et al.*, 2024).

Determination of mineral content of fortified muffins

The mineral composition of the fortified muffins was analyzed using an Atomic Absorption Spectrophotometer, following methods No. 40-70 and 40-71 of AACC (2000).

Digestion of muffin

Muffin samples were prepared for mineral estimation using the wet digestion method. A 0.5g sample of each treatment was taken and digested on a hot plate in a 100ml conical flask at 60-70 °C for 20 minutes with 10ml of HNO_3. The digestion continued at 190 °C with 5ml of $HClO_4$ (60%) until the contents in the flask became clear. Each sample was then double-distilled and de-ionized to a final volume of 100 ml, after which it was transferred into 100 ml volumetric flasks. The samples were filtered before analysis. The filtered samples were analyzed using an Atomic Absorption Spectrophotometer (Model AA-6300). To create standard curves, samples of known concentration were run first. The concentrations of minerals such as calcium, phosphorus, and magnesium were then determined using these standard curves (AACC, 2000) with a PerkinElmer Atomic Absorption Spectrophotometer.

Estimation of mineral contents

Samples of known concentration were first run on the Atomic Absorption Spectrophotometer to obtain standard curves. Minerals such as calcium, phosphorus, and magnesium were then determined by using these individual standard curves. The mineral content of each sample was calculated by referencing the corresponding standard curve for each mineral, following methods No. 40-70 and 40-71 of the AACC (2006).

In-vitro bioavailability of minerals

In-vitro bioavailability was assessed using the method outlined by Sittikulwitit *et al.* (2004), with some modifications, to evaluate the bioavailability of the minerals.

The method consisted of three stages: first, peptic digestion; second, pH adjustment; and finally, pancreatic digestion with equilibrium dialysis. These steps simulate the digestion process in the stomach and intestine.

Peptic digestion

In the first step, 25 g of dry muffin samples were suspended in a 200 ml Milli-Q water bottle (Millipore Co., Etten-Leur, The Netherlands). After adjusting the pH to 2.1 using HCl, 7.5 ml of pepsin suspension was added. The pH was then carefully adjusted to 2.00 ± 0.03 using Milli-Q water, and the total sample weight was brought to 250 g. The mixture was placed in a shaking water bath and incubated for two hours at 37°C. The pH was checked and adjusted to 2.00 every 30 minutes.

pH adjustment for pancreatic digestion

Following gastric digestion, the suspension was divided into five sections, each weighing 20 g. The sections were then transferred into plastic bottles, and each bottle was filled to a volume of 25 ml with Milli-Q water. Dialysis tubing segments (28.6 mm in diameter, with a molecular weight cut-off of 12,000 to 14,000; Spectra/Por, Spectrum, Houston, TX, USA) were prepared, filled with a $NaHCO_3$ solution (60 g/l), which matched the titratable acidity. The dialysis tubing was placed inside the bottles, and the bottles were incubated in a shaking water bath at 37°C for 30 minutes. At this point, one of the bottles was labeled "T0" and had its incubation stopped.

Pancreatic digestion with equilibrium dialysis

The samples were then incubated at 37°C for intervals of 0.5, 1, 2.5, and 4 hours ($t_{0.5}$, t_1, $t_{2.5}$, and t_4). After 5 milliliters of the pancreatin-bile extract mixture were added to each of the four remaining bottles, they were placed in the shaking water bath. Following the addition of the pancreatin-bile extract, the final pH of the mixture ranged from 6.7 to 7.0, depending on the buffering capacity of the food sample and dialysis against $NaHCO_3$. After the pancreatic digestion process, the pH remained relatively stable and did not change significantly.

The concentrations of Ca, P, and Mg in the dialysates were measured. Any trace elements from the reagents and dialyzable minerals were corrected by running a blank for each experiment. The equilibrium formation of the semipermeable membrane served as the basis for the technique. Equilibrium was achieved after 2.5 hours. Therefore, the dialyzed amounts of Ca, P, and Mg were determined as the mean values from the $t_{2.5}$ and t_4 time points. Since the volumes on both sides of the semipermeable membrane were similar, the amounts of dialyzed Ca, P, and Mg represented only half of the total dialyzable amount. Consequently, the total dialyzable minerals were calculated by doubling the amount dialyzed. The bioavailability (or duplicability) of the minerals was expressed as the percentage of Ca, P, Mg, and Cu present in the food sample. Duplicability was calculated using the following equation:

20 WA dualizability (YO) = ~ x 100,

where,

D is the amount of mineral dialyzed, which is calculated as the mean of values at t 2.5 and t 4 (mg).

W is the dry weight of the food sample being used for pancreatic digestion (g).

A is the mineral concentration contained in a dry food sample (mg/g). Sitikulwitit *et al.* (2004).

Microbial analysis

Microbial analysis, mold/yeast count, and total plate count were performed according to method No. 42-11 of AACC (2000).

Total plate count

Total plate count (TPC) was conducted following the AACC (2000) method 42-11. A sterile spoon and blender jar were used to prepare a 1g sample of the muffin, which was then combined with 9 mL of buffered phosphate diluent. After dilution and shaking, the mixture was transferred onto petri dishes and incubated for 48 hours at 35°C. Following incubation, colonies were enumerated, and the total plate count per gram was determined by calculating the arithmetic average. Only plates with between 30 and 300 colonies were considered for counting. The dilution factor was applied, and the arithmetic average was used to determine the total number of plates per gram.

Mold/yeast count

Mold and yeast counts were performed according to method 42-11 of the AACC (2000). Potato dextrose agar (70139, Merck, Germany) was used to prepare the media. A 1g muffin sample was aseptically prepared and homogenized with 9 mL of buffered phosphate diluent. Serial dilutions (1:10) were prepared, shaken, and transferred to duplicate petri dishes. Potato dextrose agar was then added to the dishes, and the plates were incubated at 22-25°C. After incubation, colonies were enumerated, and the mold count per gram was calculated using the arithmetic average. Plates with fewer than 50 colonies were counted, and the colony count was multiplied by the dilution factor to determine the mold count per gram.

Sensory evaluation

All muffin samples were evaluated by a trained panel of 20 judges, aged between 25 and 60 years, selected from the Institute of Food Science and Nutrition. The panel assessed sensory attributes including color, flavor, texture, taste, and overall acceptability to identify the most suitable treatment for the muffins. Each muffin sample was placed on separate plates, labeled with random codes, and presented in a randomized order along with a glass of water for palate cleansing. Judges were instructed to taste each sample and score them using a 9-point hedonic scale (Wichchukit & Mahony, 2022; Nishat *et al.*, 2024), where scores ranged from 1 (dislike extremely) to 9 (like extremely).

Statistical analysis

Data collected were analyzed using the statistical software Minitab 16. The data were processed through an analysis of variance (ANOVA) technique to determine significant differences between treatments and groups. To further identify specific differences, the Least Significant Difference (LSD) Test was employed (Bertinetto *et al.,* 2020).

Results and Discussion

In the present study, the influence of fortification with chicken eggshell powder (T1 (ESPck)), chicken bone extract powder (T2 (BEPck)), and cattle bone extract powder (T3 (BEPct)) on the physicochemical, sensory properties, mineral content, and bioavailability of muffins was evaluated. Eggshell and bone powders were prepared using various treatment methods, and their proximate and mineral compositions were analyzed. Different muffin treatments, incorporating varying levels of fortificant powders from these natural mineral sources, were prepared. Sensory evaluations were conducted, followed by physicochemical, microbial, mineral, and bioavailability analyses. The muffin treatment demonstrating the highest mineral bioavailability was considered the most suitable among all the treatments. The results are discussed according to the following work plan.

Proximate composition of fortificant powder

The proximate composition of the source powders was measured, revealing significant variations in protein, ash, fat, moisture, and nitrogen-free extract (NFE) content. The mean values for each source treatment are presented in Table 3. The highest protein content (10.040 ± 0.31^{A}) was found in T3 (BEPct), while the lowest (0.9500 ± 0.03^{C}) was observed in T2 (BEPck). The ash content reached its maximum value (94.220 ± 0.53^{A}) in T1 (ESPck) and its minimum value (82.000 ± 0.72^{C}) in T3 (BEPct). The highest fat content (6.736 ± 0.06^{A}) was found in T2 (BEPck), with the lowest (0.3467 ± 0.03^{C}) in T1 (ESPck). Moisture content was highest (3.6067 ± 0.56^{A}) in T3 (BEPct) and lowest (0.4633 ± 0.02^{B}) in T1 (ESPck). The highest NFE content (6.0500 ± 2.08^{A}) was found in T2 (BEPck), while the lowest (1.0767 ± 0.57^{B}) was observed in T1 (ESPck)

The ash content of eggshell powder was found to be the highest, likely due to the high percentage of ash in the

Table 3. Effect of treatments on proximate composition (%) of raw material (Sources).

Treatments	Ash	Fat	Moisture	Protein	NFE
T1 (ESPck)	94.220±0.53^{A}	0.3467±0.03^{C}	0.4633±0.02^{B}	3.8933±0.05^{B}	1.0767±0.57^{B}
T2 (BEPck)	85.793±2.06^{B}	6.7367±0.06^{A}	0.4700±0.03^{B}	0.9500±0.03^{C}	6.0500±2.08^{A}
T3 (BEPct)	82.000±0.72^{C}	1.9200±0.05^{B}	3.6067±0.56^{A}	10.040±0.31^{A}	2.4333±0.61^{B}

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle).
Statistical differences by letter.

eggshell itself (Table 3). Our findings align with those of Arif *et al.* (2022), who reported that eggshell powder contained 0.46% moisture, 3.92% protein, 94.61% ash, and a minimal fat content of 0.35%. Similarly, Hassan *et al.* (2015) found that eggshell powder contained 5.40% crude protein, a negligible fat content of 0.02%, and high ash content (90.2%). These results are consistent with our study, which also found eggshell powder to have the highest ash content (94.69%) and the lowest crude fat content (0.04%). Ray *et al.* (2017) noted that ash is a major component of eggshells (94.6%), followed by protein (3.92%) and water (0.46%). Ali and Badawy (2017) also analyzed white eggshells and reported that the constituent levels of ash, protein, fat, and water were 96.70%, 3.17%, 0.06%, and 0.95%, respectively.

The protein content in T2 (BEPck) was found to be very low (Table 3), likely due to the minimal protein present in chicken bone extract powder. The proximate composition of chicken bone extract powder has been reported to include low moisture (0.47 g/100g) and protein (0.96 g/100g), with a higher fat content (6.71 g/100g) and a substantial ash content (86.74 g/100g). In contrast, T3 (BEPct) exhibited the highest protein content among the treatments, which could be attributed to the higher proportion of protein present in cattle bone powder.

According to Yessimbekov *et al.* (2023), chicken meat-bone paste possesses a high protein content. Specifically, meat-bone paste prepared from chicken breast bones, both with and without added water in a 1:0.25 ratio, exhibited the highest protein levels—22.58% and 22.78%, respectively. These findings suggest that the type of bone used and the amount of water added during processing significantly influence the final protein content of the meat-bone paste.

Our results are in line with the studies of Li *et al.* (2021), who investigated the proximate composition of bone powders of different animals. They demonstrated that the fat, moisture, protein, and ash content of cattle bones were 1.95%, 3.92%, 10.18%, and 84.0%, respectively.

Mineral composition of raw materials (sources)

The mineral composition was measured in the source powders, and it was observed that mineral content varied highly significantly in treatments. Mean values for the source treatments are accessible in Table 4. The maximum Calcium content (33137±118^{A}) was found in T1 (ESPck), while the minimum (20870±337.79^{C}) was found in T3 (BEPct). Phosphorus content showed a maximum value (14307±517.91^{A}) in T2 (BEPck), while a minimum (151.80±2.72^{C}) value was found in T1 (ESPck). The maximum Magnesium content (728.33±9.50^{A}) was found in T2 (BEPck), while the minimum (261.60±2.69^{C}) was found in T1 (ESPck). The Na (80±16.24^{A}) was found in T1 (ESPck), and the maximum amounts of K and Fe were found in T2 (BEPck), 159.46±0.36^{A} and 205.33±0.65^{A}, respectively. Zn maximum was found in T3 (BEPct) (86.42±1.02^{A}).

Hassan *et al.* (2015) reported results which matched our findings, that Calcium, Phosphorus, and Magnesium in eggshell powder are 35080, 150.2, and 262.0 mg/100g, respectively. The mineral composition of eggshell powder, as reported by Arif *et al.* (2022), includes Calcium, Phosphorus, and Magnesium in the range of 34.12%, 0.29%, and 0.04%, respectively. Ca is the highest mineral in chicken eggshells; 2534.4±10.60 mg/100g. Other minerals found in chicken eggshells are Mg, Na, P, K, Fe, and Zn in quantities of 247.7, 168.9, 139.8, 82.2, 7.64, and 0.93 mg/100g, respectively.

The mineral composition of our results is in line with the results of Suchy *et al.* (2009) who described that chicken bone extract powder (T2 (BEPck)) is a Ca as well as minerals source. T2 (BEPck) is a substitute, inexpensive Ca source as it is abundant in the Ca (30g per 100g), and it comprises a Ca: P ratio of about 2:1. Calcium and Phosphorus were 30.72 and 14.58 g/100g of the bone extract powder respectively. Chicken bones have Ca, P, and Mg in quantities 10.45, 6.31, and 1.46 g/kg respectively as reported by the findings of Suchy *et al.* (2009).

Venkatesan *et al.* (2015) revealed that Porcine and bovine bone had greater percentages of ash (>64%), which was in line with the calcium trend. Cow bones have greater

Table 4. Effect of treatments on mineral composition (mg/100g) of raw material (Sources).

Treatments	Ca	P	Mg	Na	K	Zn	Fe
T1 (ESPck)	33137±118^{A}	151.80±2.72^{C}	261.60±2.69^{C}	80±16.24^{A}	60.20±9.40^{B}	0.67±0.03^{C}	11.47±0.87^{B}
T2 (BEPck)	30673±830.02^{B}	14307±517.91^{A}	728.33±9.50^{A}	4.48±0.02^{B}	159.46±0.36^{A}	70.59±0.66^{B}	205.33±0.65^{A}
T3 (BEPct)	20870±337.79^{C}	10773±210.08^{B}	573.33±40.41^{B}	–	–	86.42±1.02^{A}	–

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle).
SD – standard deviation; means carrying different letters across the same row differ significantly ($P < 0.05$); a indicates the highest value group across the same row.

magnesium content. Ca^{2+}, Mg^{2+}, and other ions are adsorbed on the surface of calcium hydrogen phosphate ($CaHPO_4$) and hydroxyapatite (Ca10 (PO4) 6(OH)^{2}), which make up the majority of bone salts.

Chemical composition of nanoscale bone powders

Before they can be polished, raw animal bones must first be softened because they are extremely hard and tough to shatter directly. To soften the bones for this study, traditional high-pressure cooking was employed. Following three hours of cooking at 110°C, it was evident that the chicken bone had softened; nevertheless, the cow bone's hardness had increased due to overloading (the instrument's maximum load was 10 kg), making it the hardest bone ($p < 0.05$) after the chicken bone. According to Yin *et al.* (2015), the primary causes of bone hardness are hydroxyapatite crystals and the structure of the collagen network. The natural triple helix structure of bone collagen is broken down by high-temperature pretreatment, which reduces the stress associated with bone fracture failure. The variation in mineral salt thickness and leftover collagen may be connected to the difference in bone hardness. Cattle have high hardness due to their massive bones, thick sclerotin layers, and ability to retain more collagen under cooking conditions (as demonstrated by Table 5's Gly-Pro-Hyp content study results). Depending on the bone's hardness, the degree of difficulty in breaking and softening it can be deduced—chicken bones are easy to break, whereas cattle bones are harder to fracture.

The smallest average particle size was found in rabbit bone powder (269.87 ± 5.76 nm) by Li *et al.* (2021), followed by pig bone powder (327.50 ± 13.65 nm), bovine bone powder (432.43 ± 13.97 nm), and chicken bone powder (840.23 ± 32.32 nm) ($p < 0.05$). The greater number of protein agglomerates that attached to the chicken bone's surface may have contributed to its larger particle size (Figure 4). The size of the chicken bone, as well as that of the bovine and pig bones, also decreased as the milling duration was increased.

FT-IR results

There were three infrared bands (1,462, 1,419, and 876 cm^{-1}) and two Raman bands (1,431 and 889 cm^{-1}) in the region of carbonate ion vibrations. Raman and infrared spectra of NBPs revealed the presence of distinct phosphate group peaks, with the Raman spectrum displaying more detailed phosphate group representations. There was a dominant very strong peak at 969 cm/cm (v1), medium intensity peaks at 430 cm−1 (v2), 589 cm−1 (v4), 1,069 cm−1 (v3), and lower intensity peaks at 441 cm−1 (v2), 610 cm−1 (v4), and 1,045 cm−1 (v3), whose distributions resembled the structures of natural and synthetic hydroxyapatite (Heidari *et al.*, 2018; Nam *et al.*, 2019). A high degree of overlap in the waveforms of multiple NBPs suggests that their chemical compositions and structures are similar. Proteins like collagen and hydroxyapatite were evident from the distinctive peaks. The findings of the NPBs' chemical composition analysis are in line with this. Minerals like hydroxyapatite and leftover proteins like collagen make up the majority of NBPs. The slight variations in the NBPs spectra could be caused by the degree of protein and collagen breakdown found in various animal bones, as well as the replacement of carbonate ions for phosphate groups in the hydroxyapatite lattice (D'Elía *et al.*, 2013; Venkatesan *et al.*, 2015).

Table 5. Chemical composition of nanoscale bone powders.

Measure	Nano T3 (BEPct)	Nano T2 (BEPck)
Proximate		
Fat	1.95±0.22^{b}	2.02±0.11^{b}
Moisture	3.92±0.16^{a}	3.51±0.21^{a}
Protein	10.18±0.25^{b}	14.05±0.47^{a}
Ash	84.00±0.42^{a}	80.53±0.95^{b}
Minerals		
Ca (mg/g)	309.03±2.18^{c}	285.83±2.55^{d}
Mg (mg/g)	7.44±0.18^{a}	7.28±0.14^{a}
Zn (mg/kg)	94.13±2.55^{c}	241.55±2.83^{a}

T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle).
SD – standard deviation; means carrying different letters across the same row differ significantly ($P < 0.05$); a indicates the highest value group across the same row.

Li *et al.* (2021) discovered that the Raman spectra were dominated by a very strong peak at 963 cm/cm (v1), with medium intensity peaks appearing at 432 cm−1 (v2), 586 cm−1 (v4), and 1,073 cm−1 (v3), and lower intensity peaks appearing at 442 cm−1 (v2), 609 cm−1 (v4), and 1,042 cm−1 (v3). These distributions were comparable to the structures of both synthetic and natural hydroxyapatite (Heidari *et al.*, 2018; Nam *et al.*, 2019).

Chemical (proximate) analysis of mineral fortified muffins

Moisture content of mineral fortified muffins (powder)

A significant variation was observed in the moisture content of muffins due to treatments (Table 6). Mean values for muffins fortified with different levels of fortificants are presented in Table 6. The maximum moisture content (35.69±0.139%) was found in C_{-ve}, while the minimum (30.55±1.01%) was in T2 (BEPck). This was probably because, as the concentration of fortificant increased, the moisture content decreased, either due to the fortificants being in dry powder form or because of the hygroscopic nature of the fortificant. The results of our study showed the same pattern as Khan *et al.* (2017), who documented a decrease in the moisture content of leavened and unleavened breads because of the increased concentration of these calcium sources. Khan *et al.* (2017) reported similar results: the moisture content of the $CaCO_3$, eggshell, and bone extract powder-fortified breads decreased from 34.42% to 33.06%. The moisture of those breads decreased as fortification with $CaCO_3$, eggshell, and bone extract powder at different levels was carried out. Bread with 1.5% eggshell powder showed the highest reduction. A decrease in moisture content was observed with increasing levels of these fortificant powders. The decrease in moisture content might be due to the use of dry calcium sources. These results were supported by Fik *et al.* (2012), who stated a decrease in moisture content of fortified breads with calcium carbonate because of the increase in dry fortificant. Khan *et al.* (2020) also reported that the moisture content of enriched flour decreased significantly from 12.7% (T0) to 11.76% (T3), and the protein content decreased from 9.89% (T0) to 9.79% (T3), according to nutrient composition data.

Crude protein of mineral fortified muffins (powder)

Analysis of variance showed non-significant variation in the protein content of muffins due to treatments, as shown in Table 6. The mean values of the muffin treatments fortified with different mineral sources are accessible in Table 6. The maximum protein content (8.691±0.18%) was found in T3 (BEPct), while the minimum (8.543±0.07%) was in C_{-ve}. This might be due to the lack of protein source addition in the treatments, as these are mineral-enriched sources with very little protein, and since the quantities of fortificant powders added were very small, they contributed no significant change in the protein content of the muffins. Arif *et al.* (2022) reported lower protein content in eggshell ash and a protein content of 0.96 g/100g in chicken bone extract powder. Since the quantities of these powders were used at the lowest level, they contributed no significant change in the protein content of the muffins. Quddoos *et al.* (2020) reported non-significant differences in cookies fortified with natural sources like bone extract powder and eggshell powder in treatments and storage. The lack of differences in crude protein may be due to the absence of a protein source in the treatments.

Crude fat of mineral fortified muffins (powder)

The values of analysis of variance show the non-significant variation of treatments on the fat content of muffins (Table 6). Mean values of muffins fortified with natural mineral sources are accessible in Table 6. The maximum fat content (22.641±1.11%) was found in C_{-ve} (Table 6), while the minimum (22.43±1.18%) was in T3 (BEPct). The non-significant effect on fat content in muffins was probably due to the addition of these mineral-enriched

Table 6. Proximate analysis, Total plate count (TPC), and Mold count of mineral-fortified muffins (%).

Treatments	Moisture	Protein	Fat	Ash	Fiber	NFE	TPC (log10 cfu/g)	(MC) (cfu/g)
C_{-ve}	35.69±0.139^{a}	8.543±0.07^{a}	22.64±1.11^{a}	2.10±0.003^{e}	1.23±0.026^{a}	29.77±1.26^{e}	1.28±0.023^{a}	20.00±2.0^{a}
C_{+ve}	31.70±0.217^{cd}	8.569±0.19^{a}	22.53±1.11^{a}	2.14±0.002^{cd}	1.24±0.02^{a}	33.81±0.97^{b}	1.27±0.023^{a}	12.00±1.0^{d}
T1 (ESPck)	32.67±0.28^{bc}	8.560±0.07^{a}	22.63±1.18^{a}	2.35±0.015^{c}	1.28±0.044^{a}	32.49±0.99^{bc}	1.20±0.022^{a}	11.0±2.5^{de}
T2 (BEPck)	30.55±1.01^{d}	8.580±0.19^{a}	22.63±1.17^{a}	2.559±0.005^{b}	1.25±0.025^{a}	34.41±2.28^{a}	1.26±0.022^{a}	13.0±1.2^{c}
T3 (BEPct)	33.53±1.12^{b}	8.691±0.18^{a}	22.43±1.18^{a}	2.67±0.001^{a}	1.27±0.057^{a}	31.38±2.50^{d}	1.21±0.031^{a}	14.0±2.1^{b}

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle).
C_{-ve}: Unfortified muffins/Placebo, C_{+ve}= $CaCO_3$.
SD – standard deviation; means carrying different letters across the same row differ significantly ($P < 0.05$); a indicates the highest value group across the same row.

sources, which have very low amounts of fat content. Arif *et al.* (2022) reported a very low fat content of 0.35% in eggshell powder. Hassan *et al.* (2015) also concluded very little (0.02%) fat content in eggshell powder. Quddoos *et al.* (2020) reported non-significant differences in the fat content of cookies fortified with eggshell powder and chicken bone powder, both in treatments and during storage. These results are in line with Mahmood *et al.* (2008), who also stated non-significant changes in the fat content of cookies during storage.

Ash of mineral fortified muffins (powder)

The values show a significant effect of treatment on the ash content of muffins. Mean values of mineral-fortified muffins are mentioned in Table 6. The maximum ash content (2.67±0.001%) was found in T3 (BEPct), while the minimum (2.10±0.003%) was in C_{-ve}.

Significant variations in the ash content of muffin treatments might be due to a gradual variation in the mineral sources, which have greater and lesser ash contents. Ash content increased because of the addition of $CaCO_3$, eggshell powder, and bone extract powder, as reported by studies conducted by Khan *et al.* (2017). The maximum ash content was found in the bone extract powder-fortified breads at a rate of 1.5%, while the minimum ash content was observed in whole wheat flour-based breads, in which fortification with CaCO3 was done at a rate of 0.5%. Unleavened bread also showed higher ash content when bone extract powder (1.5%) fortification was done, and lower ash content was found in bread with CaCO3 at a rate of 0.5%. This is evident from the data that these fortificants had higher quantities of ash, so they significantly increased the ash content of the breads. Ash content increased due to the rise in calcium levels.

According to Niharika *et al.* (2020), the three muffin versions 1, 2, and 3 had greater calcium contents (915.6 mg, 990.4 mg, and 958.5 mg, respectively), particularly in light of the 95% calcium content of eggshells. Eggshells from chicken can also be used to boost the calcium content of brownie products.

Crude fiber of mineral fortified muffins (powder)

Table 6 shows a non-significant variation in the fiber content of muffins with respect to the treatments. The mean values of muffin treatments fortified with natural mineral sources are presented in Table 6. The maximum fiber content (1.28±0.044%) was found in T1 (ESPck), while the minimum (1.23±0.26%) was in C_{-ve}.

The non-significant effect of fiber content in muffins is due to the fact that these natural sources contain very little fiber, being rich in minerals. Our results are contradictory to those of Kaur *et al.* (2020), who reported an increase in the fiber content of muffins from 1.57 to 5.41 by increasing the levels of flaxseed, which has fiber content. Since the sources in our study were not fiber-rich and had no significant fiber content, no increase in fiber content was observed. Quddoos *et al.* (2020) reported non-significant differences in crude fiber for eggshell and chicken bone powder cookies, which might be due to the absence of any added fiber source in the cookies.

Nitrogen-free extract (NFE) of mineral fortified muffins (powder)

The treatments highly significantly affected the NFE content of the muffins, as shown in Table 6. The mean values of the fortified muffins, using natural mineral sources as fortificants, are presented in Table 6. The maximum NFE content (34.41±2.28%) was found in T2 (BEPck), while the minimum (29.77±1.26%) was observed in C_{-ve}.

The change in the NFE content is related to the change in other parameters, as it might compete with a significant change in the internal NFE content of the muffins. The results of Butt *et al.* (2008) also support this study, as they found a significant difference in the NFE content of cookies enriched with Vitamin A due to the specificity of Retinal acetate. Afzal *et al.* (2020) observed NFE with mean values for the control (T_0), T_1 with 8%, T_2 with 16% eggshell powder, T_3 with 8% calcium carbonate, and T_4 with 16% calcium carbonate, which changed as the concentration of calcium increased: 46.20±1.03%, 46.31±0.85%, 48.75±0.89%, 47.56±1.16%, and 48.32±0.96%, respectively. A change in nitrogen-free extract levels was observed with increased levels of mung bean and mash bean in wheat flour through fortification. Our results are also supported by the findings of Kenny *et al.* (2000), who reported significant differences.

Microbial analysis of minerals fortified muffins

Total plate count (tpc) (log10 cfu/g) of mineral fortified muffins (powder)

The table values show the non-significant variation in TPC (log10 cfu/mg) of mineral-fortified muffins across treatments (Table 6). The mean values of the fortified muffins indicate that the maximum microbial growth (1.28±0.023 cfu/g) was found in C_{-ve} (Table 6), which is outside the threshold limits (1.3979 to 2.477 cfu/mg) (Rossi *et al.*, 2012), while the minimum (1.20±0.022 cfu/g) was observed in T1 (ESPck).

The normal shelf life of muffins is one week to 10 days, so a storage study of up to 30 days results in high microbial levels. Akbar (2018) reported that cookies made from maize flour were microbiologically safe for up to 2 months of storage but caused health problems and

became unacceptable after 3 months of storage. The highest admissible TPC limits in baked products such as bread, cakes, muffins, and biscuits are 2.0x10^5 cfu g^-1 for coliform bacteria (<200 MPN g^-1), yeast and mold (<1.0x10^4 cfu g^-1), and the absence of E. coli, according to the WHO standard (1994). Quddoos *et al.* (2020) also reported TPC in the range of 1.51 to 1.52 log10 cfu/g. TPC values of 1.51 from T0 to T3 in calcium-fortified cookies were within the safe range (Rossi *et al.*, 2012).

Mold count (MC) of mineral fortified muffins (powder)

High-significant values of MC (cfu/mg) for the muffin treatments were observed due to treatments and storage days, showing changes in values (Table 6). The mean values of fortified muffins indicate that the maximum mold count (20.00±2.0 cfu/g) was found in C_{-ve} (Table 6), while the minimum (11.0±2.5 cfu/g) was observed in T1 (ESPck). These values are within the threshold limits, meaning they are not hazardous to health. Kaur *et al.* (2020) stated that the total plate count of flaxseed meals varied significantly during storage for both control and fortified muffins, showing that the total plate count increased during the storage period. According to the WHO standard (1994), the highest admissible limits in baked products such as bread, cakes, and muffins for yeast and mold is <1.0x10^4 cfu g^-1, and E. coli should be absent.

Mineral content (mg) of mineral-fortified muffins

Calcium (mg) of mineral-fortified muffins (powder)

Table 7 shows that the treatments highly significantly affected the calcium content of the muffins. The mean values of the muffin treatments are provided in Table 7. The maximum calcium content (282.59±10.22 mg/100g) was found in C_{+ve} (Table 7), while the minimum (31.26±0.99 mg) was observed in C_{-ve}. The increase in the calcium content of the muffins may be attributed to the addition of rich mineral sources. These results are well supported by the studies of Hussain (2006), who prepared chapattis by adding flaxseed, both partially defatted and in full-fat form. Significant effects on the calcium content of the chapattis were observed with the addition of different levels of fortificants, while storage showed no significant effects.

Table 7. Effect of treatments on mineral composition (mg/100g) of mineral-fortified muffins.

Treatments	Calcium	Phosphorus	Magnesium
C_{-ve}	31.26±0.98^{c}	40.130±0.99^{e}	8.366±0.15^{d}
C_{+ve}	282.59±10.22^{a}	42.537±0.88^{cd}	8.38±0.06^{d}
T1 (ESPck)	280.59±9.73^{a}	43.653±0.02^{c}	8.66±0.03^{c}
T2 (BEPck)	280.31±6.85^{a}	161.27±0.09^{ab}	10.36±0.11^{b}
T3 (BEPct)	171.04±5.02^{b}	163.36±2.30^{a}	14.64±0.04^{a}

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle). C_{-ve}: Unfortified muffins/Placebo, C_{+ve}= $CaCO_3$.
SD – standard deviation; means carrying different letters across the same row differ significantly (P < 0.05); a indicates the highest value group across the same row.

Arif *et al.* (2022) prepared chocolate cakes using eggshell powder as a dietary calcium source. The results indicated that the addition of eggshell powder led to a pronounced increase in calcium content in the supplemented cakes, with values of 504.5 mg/100g, 816.8 mg/100g, and 1364.5 mg/100g at the rates of 3%, 6%, and 9%, respectively. Hassan *et al.* (2015) prepared biscuits supplemented with T1 (ESPck) at ratios of 3%, 6%, and 9%. The results indicated that adding T1 (ESPck) led to a distinct increase in the mineral content, especially calcium, in the supplemented biscuits, with values of 607.33 mg/100g, 1378.11 mg/100g, and 2175.23 mg/100g at 3%, 6%, and 9%, respectively. Khan *et al.* (2017) fortified bread with $CaCO_3$, eggshell powder, and bone extract powder and found a significant increase in the calcium content of both leavened and unleavened bread. The maximum calcium content (1545.3 mg/100g) was observed in wheat flour in which eggshell powder (1.5%) was used for fortification in leavened bread, while the same calcium content was recorded in bone extract powder (1.5%) fortified bread. This is due to the fact that these are rich natural calcium sources.

The use of chicken eggshell powder in chocolate cake as a dietary calcium source at 3%, 6%, and 9% was also investigated by Ray *et al.* (2017). Based on their research, cakes supplemented with chicken eggshell powder had significantly higher calcium contents of 504.5 mg/100g (3%), 816.8 mg/100g (6%), and 1364.5 mg/100g (9%). It was determined that cake formulations can benefit from the use of chicken eggshell powder up to 6% to achieve the desired calcium content, texture, and sensory quality.

Phosphorus (mg) of mineral-fortified muffins (Powder)

The analysis shows a highly significant variation in the phosphorus content of muffins due to the treatments (Table 7). The mean values of the muffin treatments are presented in Table 7, showing the maximum phosphorus content (163.36±2.30 mg) in BEA_{ct}, while the minimum phosphorus content (40.130±0.99 mg) was observed in C_{-ve}.

Khan *et al.* (2017) concluded that the mineral content, especially calcium, also increased in the fortified bread samples (P≤0.05). However, breads fortified with eggshell powder and bone extract powder showed higher mineral content than those fortified with CaCO3. They suggested that further studies be conducted on the

bioavailability of calcium and minerals in both leavened and unleavened bread. The phosphorus content, as reported by Khan *et al.* (2017), was significantly higher in the fortified breads containing eggshell powder and bone extract powder. The maximum phosphorus content was found in bread fortified with bone extract powder, as chicken bones are known to contain good amounts of phosphorus, as revealed by Schaafsma *et al.* (2000). These results align with those of Sittikulwitit *et al.* (2004), who also stated that calcium and phosphorus are in a 2:1 ratio in bone extract powder. On the other hand, phosphorus content was significantly reduced in breads fortified with $CaCO_3$ compared to the control bread. The reduced phosphorus content in these breads may be due to the baking process, which likely reduced phytate levels.

Magnesium (mg) of mineral-fortified muffins (Powder)

A highly significant variation in the magnesium content of the mineral-fortified muffins was observed due to the treatments. The mean values of the muffin treatments fortified with mineral-rich natural sources are presented in Table 7. The maximum magnesium content (14.65±0.04 mg) was found in T3 (BEPct), while the minimum (8.36±0.15 mg) was observed in C_{-ve}. Afzal *et al.* (2020) conducted a study to address mineral deficiency by preparing eggshell powder-fortified muffins. Muffin treatment T1, which included eggshell powder at a rate of 8g, showed a significant increase in minerals, with calcium (2462.7±0.69 mg per 100g) and magnesium (5.65±0.28 mg per 100g) being the most notable.

Mean magnesium content values in fortified bread showed the highest magnesium content in bread made with whole wheat flour fortified with chicken eggshell powder at all levels. This may be due to the high magnesium content in eggshell powder. Schaafsma *et al.* (2000) reported that eggshells are composed of 96% calcium carbonate, 1% magnesium carbonate, 1% calcium phosphate, and smaller amounts of organic substances. A smaller increase in magnesium was observed in both leavened and unleavened bread fortified with CaCO3 and bone extract powder (1% and 1.5%), while a decrease in magnesium content was observed at a 0.5% fortificant level.

Bioavailability of minerals

The analysis showed that a highly significant variation was found in the calcium bioavailability of the muffins across the treatments (Table 8). The mean values for bioavailability indicated that the maximum calcium bioavailability (140.46±2.04 mg) was found in T2 (BEPck), while the minimum (4.37±0.19 mg) was observed in C_{-ve} (Table 8). The mean values for phosphorus bioavailability in the fortified muffins showed the maximum phosphorus bioavailability (114.62±1.09 mg) in T3 (BEPct), while the minimum (6.14±0.05 mg) was found in C_{-ve} (Table 8). The results indicated a highly significant variation in the magnesium bioavailability of the muffins across the treatments (Table 8). The mean values for magnesium bioavailability in the fortified muffins showed the maximum magnesium bioavailability (4.11±1.09 mg) in BEA_{ct}, while the minimum (1.66±0.05 mg) was observed in C_{-ve} (Table 8).

Sittikulwitit *et al.* (2004) conducted a study to determine the bioavailability of Ca in T2 (BEPck) and the fortified products of T2 (BEPck) using an in-vitro equilibrium dialysis method. The effects of phytate and dietary fiber from food products on the Ca bioavailability of T2 (BEPck) were evaluated. T2 (BEPck) exhibited excellent Ca bioavailability in the results. The Ca bioavailability (% dialyzable Ca) of BEP (52.07±1.2) was higher than that of CaCO3 (47.4±0.2) and milk (28.5±1.8). Although phytate and dietary fiber reduced the bioavailability of Ca, T2 (BEPck) showed the least effect among all sources of Ca.

Sittikulwitit *et al.* (2004) carried out Ca fortification using chicken powder, which was a high source of Ca (30 g/100 g), and P in a 2:1 Ca:P ratio. Ca bioavailability was analyzed by the in-vitro equilibrium dialysis process. The results of this method indicated that the bioavailability of Ca (% dialyzable Ca) in BEPck (52.07±1.2) was much higher than that in milk (28.5±1.8) and $CaCO_3$ (47.4±0.2) from the fortified products. Hassan *et al.* (2015) prepared biscuits supplemented with ESP at ratios of 3%, 6%, and 9%. The Ca bioavailability from the biscuits containing 3%, 6%, and 9% ESP powder was recorded as 26.0%, 35.4%, and 41.43%, respectively.

Eggshells are the most bioavailable, least expensive source of calcium in the dietary needs of humans. They

Table 8. Effect of treatments on minerals bioavailability (mg).

Treatments	Calcium mg	Phosphorus	Magnesium
C_{-ve}	4.37 ± 0.19^{H}	6.14 ± 0.05^{F}	1.66 ± 0.05^{F}
C_{+ve}	127.37 ± 1.15^{B}	0	0
T1 (ESPck)	113.09 ± 0.46^{C}	10.79 ± 1.09^{E}	2.14 ± 1.09^{C}
T2 (BEPck)	140.46 ± 2.04^{A}	54.65 ± 0.90^{C}	3.24 ± 0.90^{B}
T3 (BEPct)	34.60 ± 1.12^{G}	114.62 ± 1.09^{B}	4.11 ± 1.09^{B}

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle).
C_{-ve}: Unfortified muffins/Placebo, C_{+ve}= $CaCO_3$
SD – standard deviation; means carrying different letters across the same row differ significantly ($P < 0.05$); a indicates the highest value group across the same row.

recovered and utilized the byproducts of fish bone calcium. Fishbone powder-fortified white flour bread's reliability was found to range b/w 34.5–35.7%. This study depicted that powder obtained from fish bones is a rich source of bioavailable calcium and could improve the calcium intake status of the Vietnamese population and among common people. Kobus-Cisowska *et al.* (2020) assessed the effect of calcium fortification on the bio-availability, quality, and rheological attributes of Polish bread spread made from eggshells. In comparison to the control sample, enriching eggshell powder in bread spread boosted calcium levels by >2.5 times.

Dietary magnesium limitation has been found in epidemiologic research to induce osteoporosis, and intake of this element is closely correlated with bone density. According to the National Health and Nutrition Examination Survey, nearly half (48%) of US citizens did not get the recommended daily intake of magnesium (320 mg) from diet in 2005–2006. Moreover, the treatment did not affect the increased amounts of magnesium that the steers receiving supplements retained (1.6 vs. 0.9 grams/day). Mg retention was measured as a percentage of intake and absorbed magnesium, with average values of 8.8 and 25.2%, respectively, and was consistent across all regimens (Maizes *et al.*, (2009).

Sensory evaluation of mineral fortified muffins

Color (scores) of muffins fortified with minerals (powder)

The significant color variation (score) of the minerals-fortified muffins is shown in treatments (Table 9). The maximum color (score) (7.96±1) was found in T1 (ESPck), while the minimum (6.96±0.93) was in T3 (BEPct). In storage, color may change with time. The color of the muffins might be due to the fact that eggshell is white, and the color of the muffins became lighter rather than yellow because of the whitish eggshell powder. Our results are in line with the studies of Hassan *et al.* (2008) for color scores of eggshell and calcium carbonate-fortified muffins at different levels, which showed (7.8), (7.6), (7.4), (6.7), and (6.3) color scores in treatments, showing that chicken eggshell supplementation did not affect the color of the biscuits, as eggshells are whitish.

Muffins should have good, attractive, and appealing colors. Our results are aligned with the studies of Khan *et al.* (2017), who reported that the color of bread containing bone extract powder changed due to the dark color of the bone powder extract. Gomathi and Parameshwari (2022) reported that bread color was affected by the addition of buckwheat, which has a dark color. The color reduction might be due to a reaction between reducing sugar and amino acids (Dhingra and Jood, 2002).

Odor (scores) of muffins fortified with minerals

Table 9 shows a non-significant variation in odor (score) of the minerals-fortified muffins in treatments. Mean values of muffin treatments fortified with minerals are presented in Table 9. The maximum odor (score) (7.43±0.58) was found in T2 (BEPck), while the minimum (7.07±1.01) was in C_{+ve}. Fortification of calcium sources like eggshells might change the aroma of the muffins. Khan *et al.* (2020) reported a significant reduction in aroma score from 6.93 of control to 3.96 of T3 of bread fortified with different quantities of eggshell powder. The eggshell powder fortification potential at different levels for bread production. Sensory analysis of this study showed a significant decrease in odor as the amount of eggshell powder was increased in the bread samples. The control cookies have a sweet odor caused by mixing butter with eggs, which produces that sweaty odor of cookies.

Taste (scores) of mineral fortified muffins

Analysis shows a non-significant variation in taste (scores) of mineral-fortified muffins in treatments (Table 9). The maximum taste (score) (7.54±1.10) was found in T1 (ESPck), while the minimum (6.92±0.91)

Table 9. Sensory Evaluation (Scores) of Mineral Fortified Muffins

Treatments	Color	odor	Taste	texture	overall acceptability
C_{-ve}	7.92±1.091^{a}	7.13±1.05^{a}	7.49±0.56^{a}	7.30±1.0^{a}	7.33±1.15^{a}
C_{+ve}	7.89±1.02^{abc}	7.07±1.01^{ab}	7.35±0.58^{ab}	7.27±1.1^{a}	7.02±1.02^{ab}
T1 (ESPck)	7.96±1^{ab}	7.09±1.01^{ab}	7.54±1.10^{a}	7.43±1.35^{a}	7.26±0.59^{ab}
T2 (BEPck)	7.33±1.43^{abcd}	7.43±0.58^{a}	6.98±1.00^{abcd}	6.74±0.92^{ab}	6.99±1.1^{abc}
T3 (BEPct)	6.96±0.93^{bcd}	7.14±0.04^{a}	6.92±0.91^{abcd}	6.69±1.1^{abc}	6.96±1.11^{abc}

T1 (ESPck)= Egg Shell Powder (Chicken); T2 (BEPck) = Bone Extract Powder (Chicken); T3 (BEPct) = Bone Extract Powder (Cattle); SD – standard deviation; means carrying different letters across the same row differ significantly ($P < 0.05$); a indicates the highest value group across the same row.

was in T3 (BEPct). The eggshell powder fortification potential at different levels for bread production. Sensory parameters showed non-significant values in taste scores at the initial point but significance at different quantity ratios and during storage studies, due to the increased eggshell powder levels with different ratios. Afzal *et al.* (2020) found in studies that the highest taste score value was shown in control T0 (7.8), followed by T2 having eggshell powder at 16% (7.7); the lowest taste score was found in T4, with calcium carbonate at 16%. According to Niharika *et al.* (2020), variation-2's taste mean and SD value (8.2±0.72) were greater than those of variation-1 (6.6±1.48) and variation-3 (4.1±1.23).

Texture (scores) of mineral fortified muffins

Table 9 shows a non-significant variation in the texture (scores) of the mineral-fortified muffins across treatments (Table 9). The maximum texture score (7.30±1.0) was found in C_{-ve}, while the minimum (6.69±1.1) was observed in T3 (BEPct). The source of minerals might not influence the texture of the muffins, but during extended storage, the texture might decline due to moisture absorption from the atmosphere and the different fortificant sources added to the muffins. Sharif *et al.* (2005) reported a similar effect of moisture absorption on texture, which decreases the quality score. Our findings are in line with studies in which texture scores showed no significant differences in control, $CaCO_3$, and eggshell powder-fortified bread, while a reduction in texture scores was observed in the case of BEP fortified at levels of 1% and 1.5%, as reported by Khan *et al.* (2017). According to Niharika *et al.* (2020), the texture mean and SD value of variation-2 (7.9±0.92) was higher than that of variation-1 (6.9±1.34), while the lowest score was found in variation-3 (5.4±1.42).

Overall acceptability (scores) of mineral fortified muffins

Analysis shows a non-significant variation in overall acceptability (scores) of the minerals-fortified muffins across treatments (Table 9). The maximum overall acceptability score (7.33±1.15) was found in C_{-ve}, while the minimum (6.96±1.11) was observed in T3 (BEPct). The overall acceptability score of the muffins did not change due to other parameters like color, odor, taste, and texture, which showed no change or only slight variation due to the use of eggshell powder as compared to the control. Arif *et al.* (2022) also indicated that although fortified cake with a maximum level of eggshell powder (9%) was nutritionally beneficial, the overall acceptability analysis showed it was not satisfactory. Cake fortified with a lower quantity (3%) of eggshell powder chicken was found to be satisfactory compared to other treatments with higher levels of eggshell powder. Better color and overall acceptability were found in bread containing eggshell powder as compared to the control bread, although the odor and flavor became inferior or remained unchanged.

In order to assess the impact of calcium fortification on leavened and unleavened bread, Khan *et al.* (2017) added $CaCO_3$, chicken eggshell powder, and chicken bone extract to whole wheat flour at levels of 0.5, 1.0, and 1.5%. They found that the total acceptance value was significantly ($p<0.05$) impacted by bread fortified with bone extract, chicken eggshell powder, and $CaCO_3$ at varying doses. Overall, as the amount of all fortifiers increased—particularly with bone extract—the sensory quality decreased.

Conclusions

Eggshell powder and cattle bone powder are among the richest sources of minerals and hold potential as functional foods, reflecting the growing interest in health-promoting dietary sources. Therefore, the study aims to develop mineral-fortified muffins and examine the influence of adding chicken eggshell powder (T1 (ESPck)), chicken bone extract powder (T2 (BEPck)), and cattle bone extract powder (T3 (BEPct)) on physicochemical and sensory properties, mineral content, and bioavailability (particularly of Ca, P, and Mg in ESPck, T2 (BEPck), and T3 (BEPct) fortified muffins). The fortificants (T1, T2, T3) showed the highest values in proximate composition: ash in T1 (94.220), fat in T2 (6.736), moisture in T3 (3.606), protein in T3 (10.04), and NFE in T2 (6.05). Muffins developed using various mineral sources indicated that treatment with T1 (ESPck) at the 25% RDA level was considered best among all, showing better results in sensory, physicochemical, microbial, mineral, and bioavailability assessments. In sensory evaluation, the maximum overall acceptability score (7.26±0.59) was found in T1 (ESPck), while the minimum (6.96±1.11) was in T3 (BEPct). The Ca, P, and Mg bioavailability values for T2 (BEPck) (140.46±2.04), T3 (BEPct) (114.62±1.09), and (4.11±1.09), respectively, were higher; however, bone powders showed less favorable results in sensory and other parameters, and thus were not considered superior to T1 (ESPck). The maximum mineral contents—Ca, P, and Mg—were highest in T1 (ESPck) (280.59±9.73), T3 (BEPct) (163.36±2.30), and 14.64±0.04, respectively. The highest TPC and mold counts were found in T2 (BEPck) (1.26±0.022) and T3 (BEPct) (14.0±2.1). At the nanoscale, the best results were found in T3 for Ca and Mg, and in T2 for Zn. However, these natural mineral sources can be used in combination in various bakery and food items to combat mineral deficiencies, as they are rich and bioavailable sources of minerals that can help overcome calcium, phosphorus, and magnesium deficiencies.

Ethics Statement

The present study was carried out in accordance with the standardized ethical guidelines for sensory testing, as approved by the Departmental Ethics Committee. All participants provided written informed consent regarding their willingness to participate in the sensory evaluation. We confirm that the research complies with all national and institutional regulations concerning human subject research. Participants were fully informed about the product and the nature of the study and gave written consent prior to participation. The safety of the food products was thoroughly documented, and participants' well-being was prioritized throughout the study. Although there was no formal "ethics approval system" in place, we ensured that our research adhered to core ethical principles, including voluntary participation, safety, and transparency.

This study involved a sensory evaluation of muffins, conducted in accordance with ethical guidelines for human subject research. Prior to participation, all subjects were provided with detailed information about the study, including its purpose, procedures, and any potential risks. Informed consent was obtained from all participants before the sensory evaluation commenced.

The study followed the principles of the Declaration of Helsinki and respected the rights, dignity, and well-being of the participants. All participants were volunteers, and no minors or vulnerable populations were included without appropriate consent from legal guardians. The sensory evaluation was non-invasive, involving the tasting and assessment of muffin samples, and participants retained the right to withdraw from the study at any time without consequence.

Participants were informed of any possible allergens or dietary concerns related to the consumption of the muffin products. To minimize potential risks, the samples were prepared under strict hygiene and safety standards in a controlled environment, ensuring compliance with food safety regulations.

All data collected during the sensory evaluation were anonymized to protect the participants' identities and confidentiality. The results of the study were used solely for research purposes and will be published in aggregate form, with no personally identifiable information disclosed.

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Availability of Data and Materials

Collective data after the state is available for publishing but data cannot be provided by an individual author.

Ethics Approval

Not applicable because no human and animal study performed.

Authors Contributions

Conceptualization, Data curation, Formal analysis and Methodology: Z.; Project administration: S.M., M.N., M.Y.Q.; Journal format setting and uploading to journal: M.Y.Q.; Resources, Funding acquisition: D.M.A.N., I.M.; Supervision: S.M., M.N.; Writing original draft: Z.; Review & editing: S.M., M.Y.Q., K.A.

Conflict of Interest

The authors declare no conflict of interest.

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