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PRODUCTION OF BREAD WITH EGGSHELL POWDER AND THE INCREASE OF CALCIUM CONTENT IN THE BODY

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ABSTRACT

Calcium is the most abundant mineral in the body. An inadequate intake of this mineral can cause problems in several vital processes. Eggshell powder is a rich source of calcium that has been shown to have positive effects on bone metabolism. However, the eggshell is classified as a waste material by the food industry, therefore it is not used despite having different properties, its calcium content being one of the most important. The aim of the present investigation was to produce bread using eggshell powder as the raw material in order to develop a bread product rich in calcium. Also, to determine the increase of the calcium content in the body by means of biological analysis. The investigation started with the production of eggshell powder (EP) and the determination of its calcium content by atomic absorption. Then it was replaced in 3 different percentages (10%, 15% and 20%) in addition to a blank sample (0%) to compare the results. The methodology included the conduct of the biological study, where the substitution at 10% obtained higher percentage of assimilation (9.19%) compared to 15% and 20% which were 6.87% and 4.35% respectively. Proximal, mineral (Ca) and microbiological evaluations (molds) of the bread were performed after obtaining the optimal substitution. The results were obtained: humidity (23.8%), ash (5.24%), fat (3.83%), proteins (9.25%), fiber (2.10%), carbohydrates (55.78%), kilocalories (295.79 KJ) and calcium (29.47 mg/g). Finally, the microbiological analysis showed harmlessness in bread.

1. Introduction

Calcium (Ca) is the most abundant divalent cation in the human body. It constitutes between 1.5 – 2.0% of the body's total weight. More than 99% of the body's calcium is found in the skeleton. It occupies a central place in biological systems and is responsible, together with other elements, for structural functions affecting the skeleton and soft tissues, and for regulatory functions such as neuromuscular transmission of chemical and electrical stimuli, the secretion of cells, blood clotting, oxygen transport, and enzymatic activity (Valdés, 2009).

Additionally, a hen's eggshell is an excellent natural source of calcium, consisting of 96% calcium carbonate (Alais *et al.*, 1990). However, the eggshell is commonly considered a waste. On the other hand, it is commonly consumed by people with scarce resources, those who, in some cases, say they prepare the powdered shell in different homemade solutions such as milk, orange juice or lemon, as well as in vinegar (5% acetic acid) or in soup (hot water). Therefore, a study that supports the use of the common eggshell as a source of calcium and as the use of a waste would be very useful and applicable (Gómez, 2011).

Roverský *et al.* (2003) also note that eggshell powder is a natural source of calcium with positive effects on bone metabolism. In addition, different studies have shown that eggshell powder has positive antirachitic effects in rats and humans.

To provide consumers with more opportunities to increase their calcium intake without resorting to supplementation, manufacturers in the United States are recommended to fortify foods and beverages with calcium if certain criteria are met: the food should be commonly consumed. The additional ingredient compatible with the product must be added, and the technology to strengthen the food must be simple, safe and cost effective (Nollet and Toldrá, 2012). In addition, fortification with calcium has been shown to be an economical way to obtain additional calcium (Keller *et al.*, 2002). Moreover, the human body cannot produce adequate amounts of calcium without external support. Additionally, calcium is daily lost through hair, skin, nails, sweat, urine, and feces. This loss of calcium must be replaced, or the body will take calcium from the bones to perform other functions (Kessenich, 2008).

It is important to highlight that bread is one of the staple foods in many countries of the world. Currently, the consumption of bread and bakery products made from refined wheat flour is often the usual trend. However, this flour is characterized by a limited nutritional value (Isserliyska *et al.*, 2001).

In the present, different food products for children are being developed, which are mainly characterized as fortified and nutritious products. Within them we can find bakery products made by replacing wheat flour with flour of tubers, of cereals or of native grains, which increases their nutritional value (Obrego *et al.*, 2013).

Therefore, the present study aimed to investigate the increase of calcium content in the body through biological tests with mice.

2. Materials and methods

2.1. Obtaining eggshell powder

Eggshells were collected from different sources such as restaurants, dealerships, bakeries, food trucks , etc. They were washed and disinfected with a 100 ppm sodium hypochlorite concentration for 5 min, according to tests carried out for validation by a HACCP system in a Food Dealer audited by SMC Slovensko (The Mediterranean Certification Society) in 2018. Next they were dried at 120°C for 60 min based by preliminary tests. Then the shells were ground and passed through a mesh sieve for 10 min with an opening of 106 μm based by preliminary tests. The calcium content was then determined by atomic absorption.

2.2. Bread making

The method used for the production of the product is the direct method. The loaves were prepared according to the standardized formulation with a slight modification of Mesas and Alegre (2002). The wheat flour was replaced by eggshell powder (EP) in 3 different percentages which were 10% (F1), 15% (F2), and 20% (F3), these were compared with a blank sample with 0% (F0) EP substitution. The kneading lasted 5 min. and 50 gr were weighed for each bread. The bread was baked at 180°C for 15 minutes. The loaves were then removed, cooled and stored for further study.

2.3. The Biological Analysis

2.3.1. Animals

Fifteen (15) albino male mice (Mus musculus) of Balb/c/CNPB strain of experimentation were selected for the investigation at an age of 35 to 38 days old, with a weight in the range of 19 to 22 g. They were divided into five groups (samples in triplicate) distributed as follows:

One standard group (GP)

One white group (GB)

Three experimental groups (GE10, GE15 and GE20)

After two weeks of adaptation, in which the mice were fed a standard maintenance feed for rodents (1152.09 mg Ca/100g), the standard group was analyzed to know the initial conditions of calcium in their bodies. It should be noted that each rodent was in a cage with its own drinking trough and feeder, thus avoiding aggression and injuries between them.

2.3.2. Diet

The white group (GB) and the three experimental groups (GE10, GE15 and GE20) received food orally "ad libitum" for 28 days, the difference was the calcium content in the food supplied. The white group's food was bread with a conventional formulation (F0) containing 0.32mg Ca/g of bread, while the different formulations: Fp1, Fp2, Fp3 contained 29.94 mg, 44.75 mg and 59.55 mg of calcium per gram of bread respectively.

2.3.3. Environmental conditions

The environmental conditions in which the animals were kept throughout the experiment were constant, 12 light hours and 12 dark hours in a temperature range between 20°C to 25°C (Fuentes *et al*., 2008).

2.3.4. Cleaning up

The cages were cleaned and disinfected 3 times a week. The cages were washed using detergent and a brush, then they were disinfected with 0.5% sodium hypochlorite. In the same way the plates containing rodent feces were cleaned. They were left to breathe for 5 min so that the chemical used was volatilized.

2.3.5 Collection of data and samples

During the time of the study the amount of food ingested was weighed daily and the variation in the animals' body weight was measured weekly. A precision scale was used for these measures.

2.3.6. The slaughter

The experimental protocol to which the animals were subjected was designed according to the guidelines proposed in the "Guide for the management and care of laboratory animals: Mouse", which exists in Peru approved by the Resolution N°309-2008-J-OPE/INS.

After 28 days of experimentation, rodents were killed by inhalation of chloroform. The animals were placed in a glass bell containing the chemical, with the lid closed. After 30 to 50 seconds the animals were immobilized. To ensure the animal's death you should wait at least 30 seconds after its last breath. (Servicio de Experimentación Animal, 2005)**.**

2.3.7. Determination of Calcium

Calcium was determined by the atomic absorption method.

2.4. Optimal product analysis

2.4.1. Proximate composition

The proximal composition of the optimal product was determined. Humidity by the NTP 209.085 method, ash by the 2.173 method of AOAC , fat by the 209.093 method, proteins by the 2.057 method of AOAC, fiber by the NTP 209.074 method and carbohydrates by the 31.043 method of the AOAC.

2.4.2. Microbiological analysis

Mould count: ICMSF Volume I, 2nd. Edition, Part II, Method I, p. 166-167.

2.4.3. Calcium content of the optimal product

Calcium content was determined by atomic absorption.

2.5. The Statistical Analysis

The collected data were statistically evaluated by a variance analysis (ANOVA) and the Tukey test with a reliability level of 95%, using the statistical software Minitab 18.

3.Results and discussions

3.1. Calcium content in eggshell powder

The calcium content in the eggshell powder turned out to be 394.58 mg/g, this high calcium content was due to the calcium carbonate content present in the composition of the flour studied.

Brun *et al.* (2013) found that a hen's eggshell has a high calcium content of 381±89 mg Ca/g of eggshell, content similar to that written by Bartter *et al.* (2018) who indicate that the eggshell contains 380 mg Ca/g of calcium. Likewise, Schaafsma *et al.* (2000) found 385 to 401 mg/g Ca in the eggshell, depending on its origin. These values are similar to those obtained in this work.

3.2. Biological Analysis

3.2.1. Food intake

The food intake for the white group (GB) and the experimental groups (GE10, GE15 and GE20) was 44.98 ± 2.48 g / week.

The intake was constant and equivalent throughout the duration of the treatment, as shown in table 1.

Table 1. Data on food intake during the treatment period

Groups	Weeks				Average (g)
	1	2	3	4	
GB	35.45	40.22	49.89	44.42	44.49
GE10	42.77	48.77	48.46	37.96	47.46
GE15	42.07	48.13	50.70	48.93	43.04
GE20	37.61	43.39	50.17	40.99	42.50

Note: GB = White group, GE10 = Experimental group of 10% substitution, GE15 = Experimental group of 15% substitution, GE20 = Experimental group of 20% substitution.

According to Fuentes *et al.* (2008), laboratory mice had a daily consumption of 3 to 6 grams of food, which means that they had a consumption between 21 and 42 g / week.

3.2.2. Total Body Weight

The variation of the animals' body weight for each experimental group and during the 4 weeks of treatment is shown in table 2.

Table 2. Variation of the body weight of the mice during the experimental time

Groups	Weeks (g)				
	0	1	2	3	4
GB	26.07	29.53	31.13	33.23	33.40
GE10	30.85	30.85	32.23	33.50	31.83
GE15	27.70	28.80	31.67	32.50	31.14
GE20	24.03	24.03	24.67	26.80	26.90

The white group (GB) has a greater increase in body weight. In addition, a slight weight gain is observed in the experimental groups with calcium-rich diets (GE10, GE15 and GE20). However, these values are not significantly different ($p > 0.05$).

In the same way as indicated by different authors, such as Gunther *et al.* (2005), after subjecting three groups of subjects to a treatment with different calcium content (800, 1000, and 1300 mg Ca / day respectively), no significant differences in the body weight of the individuals were found. Lappe *et al.* (2004), after administering to groups of people a diet with different calcium content (900 vs 1500 mg Ca / day), did not notice any difference in the weight of the subjects neither. Snijder *et al.* (2007), after reviewing the data from the HOORM´S study, also found no association between body weight and calcium intake.

However, other publications show inconsistent results. Zemel *et al*. (2000), after conducting a study in rats, concluded that high calcium diets reduced weight gain. Metz *et al*. (1988) pointed out that, after subjecting Wistar Kyoto rats to a normocaloric treatment with high calcium content (4 g / kg feed) for eleven weeks, they also found an association between the high level of calcium in the diet and the reduction in the animals' body weight. González *et al.* (2013) worked with adults from 20 to 59 years of age, where they concluded that there is an inverse association between dietary calcium intake and high body mass index.

3.2.3. Calcium content in rodents

According to the results obtained from the variance analysis regarding the calcium content, there was a significant difference ($p < 0.05$). This shows that not all the averages of the set out groups are equal.

When there was a significant difference, the respective Tukey significance tests for treatments were performed, which indicate us the similarities and differences and help us select the best treatment statistically.

Table 3. Calcium content of experimental groups

Experimental group	Calcium content (g/100 g)
GB	1119.02[b]
GE10	1251.53[a]
GE15	1224.95[a]
GE 20	1195.99[ab]

Values are average of triplicate analysis. The different letters in the same row indicate significant differences P <0.05 between the variants

The best results are shown by GE10 with a diet of 29.94 mg Ca / g of the F1 product (bread with 10% replacement). It shows a significantly higher calcium content compared to the white group (GB) that had a diet based on a bakery product with traditional formulation (F0).

3.2.4. Calcium Assimilation

After 2 weeks of adaptation and before starting the experiment, the analysis of calcium content in the standard group (GP) was performed giving an average value of 1146.15 mg Ca / 100 g. This value shows us the initial conditions of the experimental units, that way we can know the mineral assimilation for each group.

The assimilation of calcium from the different formulations for making the bread with partial replacement of wheat flour with eggshell powder (F1, F2 and F3) and a traditional formulation (F0), was determined according to the content of initial calcium (GP).

Table 4. Calcium Assimilation

Groups	Calcium content (mg/100g)		Assimilation %
	Initial	Final	
GB	1146.15	1119.02	- 2.37
GE10	1146.15	1251.53	9.19
GE15	1146.15	1224.95	6.87
GE20	1146.15	1195.99	4.35

In table 4 it is shown that the experimental groups that had a diet based on bread with eggshell powder increased the calcium content, obtaining an assimilation of 9.19%, 6.87% and 4.35% for groups GE10, GE15 and GE20 respectively. However, the opposite is true regarding the white group (GB) where the assimilation was negative (-2.37%).

The white group (GB) presents a loss of calcium with a decrease of 2.37%. This is due to the change of diet of 11.52 mg Ca / g of the feed to a diet based on bread with traditional formulation with a calcium content of 0.33 mg Ca / 100 g, causing bone resorption.

In addition, GE10 has a greater assimilation of calcium after having changed from a feed diet to a diet with 29.94 mg Ca / g of bread with partial substitution of wheat flour with eggshell powder (10%). Nevertheless, GE15 and GE20 have lower assimilations despite having a higher calcium content in the diets provided.

Malm (1958), Heaney *et al*. (1975), Oguido and De Angelis (1995) showed that the fraction of calcium absorbed by the diet is inversely proportional to the amount ingested. This fact could be due to a delay in the disintegration of the mineral during intestinal transit (Milbradt *et al.* 2017).

Bao *et al*. (1998) studied the possibility of rats absorbing and using calcium from the fortified diet with different sources of calcium, within which eggshell powder was mentioned and then was revealed to have a very high absorption and utilization capacity.

In addition, Dutch and Japanese researchers reported that eggshell calcium has positive effects (Ali and Badawy, 2017).

In the same way, Schaafsma *et al.* (2002); Rovenský *et al.* (2003); Brun *et al.* (2013) and Fina *et al.* (2016) showed in humans that a calcium supplementation from eggshell powder has a positive effect with an adequate intake of this mineral.

3.3. Optimal product analysis

3.3.1 Proximate Composition

In table 5, bread with partial substitution of wheat flour with eggshell powder in 10% (F1) has 23.80% moisture. This value is within the parameters indicated in the Sanitary Standard for Manufacture, Processing and Expenditure of Bakery, Biscuit and Pastry Products RM No. 1020-2010 / MINSA, which indicates that common or tilled bread must contain a moisture of 23% (min.) - 35% (max.).

On the other hand, the ash content is 5.24%. This is due to the amount of minerals that eggshell powder contains. Ray *et al.* (2017) made chocolate cake using eggshell powder, in which they found that a substitution of 9% contained 5.5% of ashes.

Table 5. Proximate Composition of bread with 10% substitution

Analysis	Unity	Results
Moisture	%	23.80
Ash	%	5.24
Fat	%	3.83
Protein	%	9.25
Fiber	%	2.10
Carbohydrates	%	55.78
Energy	KJ	295.79

According to the National Institute of Health (INS, 2009), tilled bread contains 9.60% proteins, 0.3% total fat, 71.8% total carbohydrates, 1.2% crude fiber and 328 KJ energy. According to the results shown in table 5, the Proximate Composition of the product is greater regarding the fat and fiber content. However, it is lower regarding the protein, carbohydrate and energy content.

3.3.2. Microbiological analysis

Table 6 shows that the mold count is less than 10 in CFU / g. This parameter is permissible according to the Sanitary Standard for the Manufacture, Processing and Expenditure of Bakery, Biscuit and Pastry Products R.M. N ° 1020-2010 / MINSA.

Table 6. Microbiological analysis of bread with 10% substitution

Determination	Results	Units
Molds	<10	UFC/g

Note: UFC = Colony forming units

3.3.3. Calcium content in bread

Table 7 shows the calcium content in the optimal product, resulting in 29.47 mg / g. This result is in line with Ali and Badawy (2017), who mentioned that increasing the content of eggshell powder in bread strips, increases the calcium content in comparison to a control sample. In the substitution at 10% they found a calcium content of 30.78 mg / g of bread.

Table 7. Calcium content of bread with 10% substitution

Analysis	Results	Unity
Calcium	29.47	mg/g

Each bread weighs around 40 grams. Therefore the calcium content is 1178.95 mg / unit of bread.

However, INS (2009), in the Peruvian Food Composition Tables, indicate that the calcium content of tilled bread is 0.4 mg / g of the food. The content of this mineral in the final product has a high value compared to that written by the National Institute of Health, this is because of the high ash content in eggshell powder which mostly comes from calcium carbonate ($CaCO_3$).

Piscoya C. (2002) mentions that calcium carbonate is a food additive that has greater advantages over others, such as citrate and acetate, since carbonate has a higher percentage of elemental calcium (40.04%), has low cost and Food Codex indicates its conditions of use.

Most studies agree on the need to maintain a high calcium intake in adolescence, as a dairy diet, fortified foods or medicated supplements, because the only modifiable determinants of bone mass peak are exogenous (Sánchez *et al.*, 2003).

4. Conclusions

According to the results, the formulation of bread with partial substitution of wheat flour (HT) with eggshell powder (HCH) at 10% (F1) has the highest calcium assimilation compared to bread with conventional formulation (F0). It is worth mentioning that this product rich in calcium and generated from an organic residue would contribute to solve calcium deficit in the body.

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