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ANALYSIS OF THE CHANGES IN ELEMENTAL COMPOSITION OF THE CHICKEN EGGSHELL DURING THE INCUBATION PERIOD

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ABSTRACT

The egg can be considered as a primary "capsule of life", in which all ingredients are included in precise amounts necessary to sustain the development of an embryo and give rise to birth of a healthy chick. Thus, the chicken eggshell (CE) contains ions and compounds that are necessary to generate new life, serving as a building material of the new organism. Therefore, it is not surprising that CE of the fertilized egg changes its composition during the process of incubation, which in chicken lasts 21 days. Embryonic development in birds is associated with elution and subsequent absorption of some elements from the shell. The changes in elemental composition of the chicken eggshell during the incubation period were studied using the inductively coupled plasma optical emission spectrometry (ICP-OES). The samples of eggshells were collected on the 1st, 14th, 18th and 21st day of incubation. The statistical analysis (ANOVA) revealed significant differences between the concentrations of five studied elements. The levels of barium, phosphorus and strontium were found to increase whereas those of calcium and magnesium - to decrease during the incubation period. The levels of the other studied elements: boron, iron and zinc remained unchanged during that process. The changes in elemental concentrations that occur during the incubation process can be associated with the request of the growing embryo for certain elements and the solubilities of alkaline earth carbonates.

Keywords: chicken eggshell, incubation, ICP-OES, elements.

INTRODUCTION

The chicken eggshell (CE), which serves to protect and provide nutrients to the enclosed embryo is sometimes described as a "natural composite bioceramic", containing organic (50g kg^{-1}) and inorganic (95 g kg^{-1}) phases (Arias *et al.* 1993). The organic matrix is composed of 1.0 g kg^{-1} (w/w) proteins (Mine *et al.* 2003), whereas the fatty acid content in the insoluble eggshell layers (after decalcification) is in the range of 2–4 g kg^{-1} (Miksik *et al.* 2003). The inorganic part of the **CE** consists mostly of calcium carbonate in the polymorphic form of calcite. However, other inorganic salts are also present in this interesting biomaterial.

The egg can be considered as a primary "capsule of life", in which all ingredients are included in precise amounts necessary to sustain the development of an embryo (Ishaq *et al.* 2014) and give rise to birth of a healthy chick (Iqbal *et al.* 2012). Thus, the **CE** contains ions and compounds that are necessary to generate new life, serving as a building material of the new organism (Hincke *et al.* 2012). Therefore, it is not surprising that **CE** of the fertilized egg changes its composition during the process of incubation, which in chicken lasts 21 days (Yalçin and Siegel 2003). For skeletal mineralization, the avian embryo mobilizes calcium from its calcitic eggshell. This occurs through dissolution of specific interior regions of the shell in a process that also weakens the shell to allow hatching. Changes in the calcium reserve body - an essential sub-compartment of mammillae - consistent with it being an early, primary source of calcium essential for embryonic skeletal growth. (Chien *et al.* 2009). Although these changes have not been fully recognized yet, it was proven by the authors (Pisklak *et al.* 2012) that the mechanism of calcium liberation from the insoluble calcium carbonate is based on the formation of calcium bicarbonate $Ca(HCO_3)_2$, according to the reaction:

$$2H^+ + 2CaCO_3 \rightarrow Ca(HCO_3)_2 + Ca^{2+}$$

Although the mineral composition of the **CE** was studied previously (Schaafsma *et al.* 2000), there is no information concerning the changes in the concentration of elements during the incubation period, except for the calcium (Glaser and Piehler 1934, Gabrielli and Accili 2010) which is the main component of the **CE**. According to the previous reports, the composition of the **CE** remains unchanged during the first two weeks of incubation. Rapid changes begin on ca. 14th day of this process, which is a direct consequence of the embryo skeletal system development (Crooks and Simkiss 1974). The chorioallantoic membrane (CAM) is the tissue responsible for translocating eggshell calcium into the circulation of the developing chick embryo. Calcium transport by the CAM is highly specific and is expressed as a function of growth (Tuan 1987). It was proposed three ways of CAM calcium transport pathway: a calcium-binding protein (CaBP), a Ca2+-ATPase, and carbonic anhydrase (Gabrielli and Accili 2010).

The aim of this study was to analyze the changes that occur in the **CE** during the process of incubation, in particular in its mineral composition. This subject is not only interesting from the scientific point of view but also important due to the increasing role of the *in-ovo* injections in the poultry immunoprophylaxis (Wiliams 2007) whose efficiency also depends on the strength and structure of CE.

To the best of our knowledge, this is the first report showing the changes in elemental composition of the chicken eggshell during the incubation period using the inductively coupled plasma optical emission spectrometry.

The inspiration for this work was the publication of Ogawa *et al.* (2004) in which authors measured the amount of calcium, magnesium and phosphorus in the CE at various times during the stay in the uterus. We wanted to continue this subject by measuring the concentrations of those and other elements during incubation. Some similar experiments have been performed recently to evaluate the changes in the loggerhead turtle (*Caretta caretta*) eggshell mineral composition, which shows that this is an interesting problem not only in the case of avian species (Al-Bahry *et al.* 2011).

MATERIALS AND METHODS

Materials preparations: Sections (9 cm^2) from the mid-region of 24 eggshells from a commercial strain (Ross 308) of chicken broiler breeders (*Gallus gallus domesticus)* at the age of 34 weeks, were collected on the 1st, 14th, 18th, and 21st day of incubation process at a hatchery localized in Mazovian Province, Poland. The samples were washed with water, then the membranes were removed and finally the eggshells were dried in air and powdered. Next 500 mg of each sample was weighted and placed in a digestion PTFE vessel, 6 mL of 65% nitric acid (Suprapur; Merck, Darmstad, Germany) was added and the samples were placed in the microwave sample preparation system MULTIWAVE made by Anton Paar (Perkin Elmer, Waltham, MA). The sample digestion was performed in accordance with the program given in Table 1.

After the digestion, the samples were quantitatively transferred into 10 mL flasks (class A, Brand®) and filled up to the mark with deionized water (Milli Q, Billerica, MA). Because of the expected high concentrations of calcium all the previously prepared samples were diluted 1:100 with deionized water (Milli Q, Billerica, MA) to enable the analysis of Ca concentration.

Instrumentation

An inductively coupled plasma-optical emission spectrometer (Optima 3100XL - Perkin Elmer) was used for the measurements of the following elements: B, Ba, Ca, Fe, Mg, P, Sr, Zn. The setup parameters of the spectrometer are presented in Table 2.

Calibration and Accuracy of the Method: The calibration of the spectrometer for each element was performed using aqueous calibration standards. Standard solutions were prepared from 1000 mg⁄ L stock solution (Merck) by dilution in 5% (v/v) HNO_3. For each element seven standard solutions of different concentrations were prepared. The ranges of calibration curves were as follows: 0–10 mg ⁄L for Ba, Fe, Sr, and Zn; 0–50 mg⁄L for B; 0–100 mg⁄L for P; 0–200 mg ⁄L for Mg and 0–500 mg⁄L for Ca. Beryllium and yttrium were chosen to be internal standards. The quality control of the method was performed using the standard reference materials NIST 1486 (bone meal), NCS ZC73012 (cabbage). The measured values were within 95–105% of the certified values.

Statistical analysis: The results were expressed as a mean with corresponding standard deviation (SD). Differences between samples from different days of incubation were estimated by analysis of variance (ANOVA) followed by Tukey’s “Honest Significant Difference” test. Normality and homoscedasticity of the data were evaluated for all parameters, using the Shapiro−Wilk Test and Levene’s Test, respectively. The statistical significance level was set to 0.05. The results were statistically analyzed in Statistica- version 6.0 (StatSoft Ltd., UK).

RESULTS AND DISCUSSION

The results of the elemental analysis are presented in Table 3 and Table 4. Letters following the values in each column (a, b, c) show the classification of the concentrations to different groups based on significant differences ($p<0.05$) determined using the ANOVA. They are compared with the literature data (Schaafsma *et al.* 2000) for the eggshells that did not undergo the process of incubation.

Boron: The boron concentration does not change during the incubation period and remains at the level of 200 µg/g. What is worth mentioning, our results are different from the only available literature data (Schaafsma *et al.* 2000), exceeding the reported ones by more than 4,000 times. Those differences may be the result of different fodder composition or, less likely, the analytical methods used for the analysis of boron concentrations (ICP-OES versus ICP-MS). It was proven that dietary boric acid inclusion improved feed efficiency of broiler chickens (Yildiz *et al.* 2013). However, the effect of the boron on the growing embryo and the development of its skeletal system has not been studied so far.

Calcium: We proved that there is a statistically significant difference between the concentration of calcium in the **CE** before and after the incubation process (Tab. 3). The rapid changes in this element extraction from the **CE** to the embryo occur between the 14th and 18th day of incubation. This is consistent with the previous reports of Glaser and Piehler (1934), Crooks and Simkiss (1974) as well as our earlier results (Pisklak *et al.* 2012) and confirms that the mobilization of calcium, being the result of the reaction between the hydrogen ions and calcium carbonate, intensifies during the last week of incubation. Our results obtained for the **CE** from the 1st day of incubation are comparable with the literature data (Schaafsma *et al.* 2000) for the eggshell powder which was prepared from the eggs that did not undergo the process of incubation.

Magnesium: Although the results obtained for the **CE** from all the studied days are in the range of the data presented in the literature (Schaafsma *et al.* 2000), we proved that the magnesium concentration in the **CE** decreases constantly during the incubation period and that after the hatch it is ca. 25 % lower than on the first day of incubation (Tab. 3).

Strontium: The concentration of strontium increases slightly during the last few days of incubation (Tab. 4). Once again, our results were consistent with the ones obtained (Schaafsma *et al.* 2000), but the range of our results was much narrower than of the reported ones (98-110 versus 50-693 [$\mu g\ g^{-1}$]). Strontium supplementation was proved to induce large positive effects on bone density, volume, and microarchitecture in laying hens (Shahnazari *et al.* 2006). Surprisingly, it seems that the majority of the strontium from the **CE** is not absorbed during the incubation.

Phosphorus: We proved that the concentration of phosphorus increases during the incubation period. Similarly as in the case of calcium, our results for the **CE** from the first day of incubation do not differ from the literature data (Schaafsma *et al.* 2000) for the eggshell powder which was prepared from the fresh eggs.

Barium: To the knowledge of the authors the amount of barium in the **CE** has not been determined so far. However, our results are similar to the data obtained for the passerine birds eggshells (Mora 2003). The physiological role of barium is still unclear. Barium compounds mostly impact the environment as a result of industrial waste water. Probably the presence of barium in the **CE** is just the direct result of water or fodder contamination with this element. That would explain the statistically significant higher concentrations of barium in the **CE** from the 21st day of incubation, the barium is not eluted to the albumen and due to the total eggshell weight loss its concentration increases (Tab. 4).

Iron and zinc: We were not able to prove any significant differences in the levels of iron and zinc in the **CE** collected from various days of incubation. Our results were similar to the literature data (Schaafsma *et al.* 2000).

According to Glaser and Piehler (1934) total **CE** weight decreases during the incubation process. Therefore, if some elements are not eluted during this period (Ba, P), their concentrations will increase. The elution of calcium from the $CaCO_3$ (%Ca=40) which is built into the embryo skeleton and formation of calcium bicarbonate ($Ca(HCO_3)_2$) (%Ca=25) (Crook and Simkiss 1974) is responsible for the decrease of the calcium concentration. Another characteristic feature is that the changes in the levels of the alkaline earth metals can be correlated with the K_{sp} of their carbonates (De Visscher *et al.* 2012) (5.60×10^{-10} for $SrCO_3$, 2.58×10^{-9} for $BaCO_3$, 5.00×10^{-9} for $CaCO_3$ and 6.82×10^{-6} for $MgCO_3$ respectively). This suggests that the differences in the elution of those elements from the **CE**, besides the efficiency of their active transport mechanism, can be at least partially explained by different solubilities of their carbonates. Moreover, it is not surprising that the decrease of the concentrations occurs for the metals which have the highest percentage in chicken body composition (Ca and Mg) as one of the roles of eggshells is to provide building material for the growing embryo.

Table 1. Digestion program of eggshell samples

Step	Initial power, W	End power, W	Ramp time, min
1	100	300	5
2	0	0	1
3	300	600	5
4	0	0	1
5	600	800	5
6	0	0	1
7	800	1000	5
8	0	0	1
9	1000	1000	10
10	0	0	15 (cooling)

Table 2. Operating parameters of ICP OES

Parameter	Value
Plasma gas flow (L/min)	15
Auxiliary gas flow (L/min)	0.5
Gas flow through atomizer (L/min)	0.5
Plasma power (W)	1450
Plasma observation height (mm)	15
Sample flow (mL/min)	0.65
Delay time (sec)	45

Table 3. Concentrations of the elements in the CE from different days of incubation, presented as a mean ± standard deviation (SD) and compared with the literature data (Schaafsma *et al.* 2000).

Day of incubation	Ca [mg g^{-1}]	Mg [mg g^{-1}]	P [mg g^{-1}]	Fe [µg g^{-1}]
1st	390 ± 6 *a*	5.1 ± 0.30 *a*	1.08 ± 0.14 *a*	28.8 ± 1.3 *a*
14th	370 ± 12 *a*	4.84 ± 0.16 *a*	1.15 ± 0.11 *a*	31.6 ± 2.3 a
18th	345 ± 11 *b*	4.55 ± 0.14 *b*	1.49 ± 0.05 *b*	32.7 ± 3.6 a
21st	332 ± 16 *b*	4.06 ± 0.15 *c*	1.7 ± 0.20 *c*	29.7 ± 5.6 *a*
Lit.	386-415	3.5-5.5	0.6-1.4	20-25

**a, b, c* groups based on significant differences ($p<0.05$)

Table 4. Concentrations of the elements in the CE from different days of incubation, presented as a mean ± standard deviation (SD) and compared with the literature data (Schaafsma *et al.* 2000).

Day of incubation	Zn [µg g^{-1}]	B [µg g^{-1}]	Sr [µg g^{-1}]	Ba [µg g^{-1}]
1st	5.7 ± 0.5 *a**	200 ± 20 *a*	98 ± 2 *a*	1.4 ± 0.2 *a*
14th	5.4 ± 0.2 *a*	201 ± 13 *a*	106 ± 4 *a*	1.7 ± 0.1 *a*
18th	5.8 ± 0.4 *a*	197 ± 3 *a*	105 ± 8 *a*	2.2 ± 0.2 *b*
21st	5.9 ± 0.6 *a*	196 ± 6 *a*	110 ± 5 *b*	2.5 ± 0.3 *b*
Lit.	4-6	≤ 0.5	50-693	ND

**a, b, c* groups based on significant differences ($p<0.05$)

Conclusions: The levels of barium, phosphorus and strontium were found to increase whereas those of calcium and magnesium - to decrease during the incubation period, the levels of the boron, iron and zinc remained unchanged during that process. The changes in elemental concentrations that occur during the incubation process can be associated with the request of the growing embryo for certain elements and the solubilities of alkaline earth carbonates. Comparison of elemental concentrations of the **CE** before and after the incubation revealed that both types of eggshell are a good source of natural calcium, whereas the amounts of other than calcium valuable macro- and microelements are neglible when compared with their RDA values (Szeleszczuk *et al.* 2015). On the other hand, attention should be paid to strontium which is present in both types of the eggshell, because of its beneficial role in the prevention of osteoporosis. The amount of strontium in the **CE** is much lower than the therapeutic range, which guarantees the safety of the eggshell if used as a dietary supplement. Another valuable element present in the **CE** is boron which reduces the urinary excretion of calcium. The synergistic effect between Ca, Sr and B which are the components of the **CE** can be used in the treatment of osteoporosis and other diseases caused by calcium deficiency.

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