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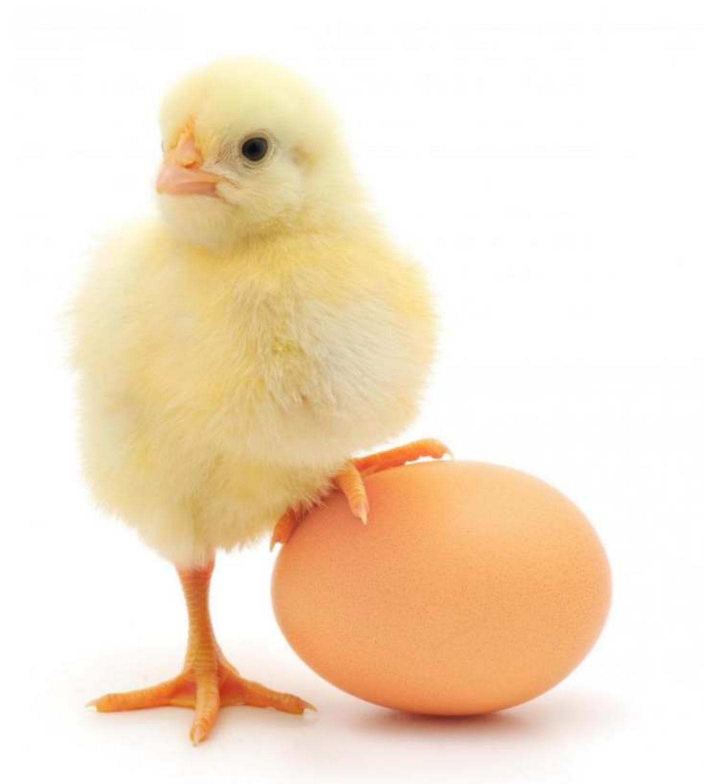

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Elemental and Ultrastructural Analysis of the Eggshell: Ca, Mg and Na Distribution During Embryonic Development via LIBS and SEM Techniques

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Abstract: The avian eggshell is composite structure of calcium carbonate in association with organic components. Calcium, magnesium, and sodium are major inorganic constituents of the avian eggshell. However, Ca distribution is not homogenous throughout the shell thickness, its relative concentration increases from inside to outside. This phenomenon is more pronounced after hatching indicating the consumption of the inner layer contents by the embryo during its development. On the other hand Mg and Na concentrations in the internal layers of the eggshell before hatching are higher than after hatching for the same reasoning. It has been suggested that an increase in magnesium content of the shell is directly related to an increase in shell hardness. In the present work Laser Induced Breakdown Spectroscopy (LIBS) technique has been used to study the avian eggshell elemental composition before and after hatching. Depth profiling of the shell is also carried out to follow-up different elements throughout the shell thickness using the same laser spectroscopic technique. The evaluation of the LIBS spectra of the examined samples provided us with quantitative information about the consumption of different elements during incubation. Scanning electron microscopy was also used to assess the relationship between layers of the eggshell before and after hatching. The thicknesses of the mammillary, palisade, vertical crystal, and cuticle layers relative to the total eggshell thickness were measured before and after hatching. The percent contribution of the layers was different in the thinnest and thickest eggshell after and before hatching respectively.

Key words: Avian eggshell, hatching eggs, laser light, LIBS, SEM

Introduction

It is known that the egg-shell consists of several mutual layers of $CaCO_3$. Major elements found in the eggshell are calcium, magnesium, sodium, and carbon. The elemental composition of eggshell has been reported to be about 98.2% calcium, 0.9% magnesium and 0.9% phosphorus (present in shell as phosphate) (Powrie, 1972). Phosphate was detected in the cone region, but most of the phosphate and magnesium have been found in the outer portion of the shell. The inclusion of magnesium in the shell is probably limited by the properties of calcite. In recent years laser methods of spectroscopy are widely used in basic biological research. Laser Induced Breakdown Spectroscopy (LIBS) can be used as an elemental analytical technique to investigate the elemental composition of the eggshell. Eggshell structure is well documented in the literature, the initiation, growth and termination of eggshell formation and the role of eggshell matrix proteins are increasingly well understood (Nys *et al.*, 1997; 2000). However, the precise mechanisms involved, including the inorganic components and their interactions with the organic components remain to be fully elucidated. Proceeding from the innermost layer, these are the mammillary knob layer, palisade, vertical crystal and cuticle (Solomon, 1991). Most Ultrastructural studies however have focused on the mammillary layer of the eggshell. The mammillary layer is the major source of calcium for the developing embryo; any variation in its thickness may affect the amount of calcium available. Approximately 80% of the calcium requirements of the chick by the time of hatch is derived from the eggshell (Simkiss, 1961). Scanning Electron Microscopy (SEM) has supplied much data on the shell structure of the normal avian egg (Simons, and Wiertz, 1970; Quintana and Sandoz, 1978), but to our knowledge no comprehensive ultrastructural study of thin –shelled and shell-less egg is available in the literature. The mammillary, palisade and vertical crystal layers were identified according to the descriptions of (Solomon, 1991; Mikhailov, 1987; Dennis *et al.*, 1996).That is, the mammillary layer was taken to be the multiple cone-shaped structures whereas the palisade layer consists of long vertical elongations that juxtaposed the mammillary layer. The vertical crystal layer was considered to be the numerous narrow vertically orientated columns immediately above the palisade layer. The cuticle was clearly visible as the outermost layer of the eggshell (Solomon, 1991; Ruiz and Lunam, 2000).

Cross-sectional measurements of the mammillary and palsied layers were made at x200 magnification whereas the vertical crystal and cuticle were measured at magnification of x2000 (Ruiz and Lunam, 2000). The cross-sectional length of the calcified region (mammillary, palisade and vertical crystal layers) of the eggshell was measured at x200 magnification. The cross-section of each layer was measured directly from the microscopic image by using scale bar within the field of view. To eliminate any error in the cross sectional length that may have resulted from variations in the angles of positioning and fracture of eggshell pieces, the relative proportion(%) of the total shell thickness made by the mammillary, palisade and vertical crystal layers and the cuticle was also determined.

The aim of the present study is to study in details:

1- The elemental composition of the eggshell to follow up the major elements before and after hatching via LIBS technique.
2- The depth profiling of the eggshell to explore the stratigraphy of calcium concentration throughout the shell thickness to confirm the LIBS results in this concern.
3- The ultrastructure of the avian eggshell to assess the relationship between different layers of the eggshell before and after hatching adopting SEM technique.

Materials and Methods

Eggshells from broiler breeder that has 36 to 40 weeks are used as samples throughout the present investigations. A typical LIBS setup (Sabsabi *et al*., 2003) has been exploited in the elemental analysis of the eggshell samples. Laser induced plasma is obtained using a Nd:YAG laser (Surlite I, Continuum, USA) delivering 7ns laser pulses at its fundamental wavelength (λ = 1064 nm), with adjustable repetition rate up to 10Hz (i.e. 10 pulses per second).The laser pulse energy was adjusted by suitable combination of beam splitters, as an external attenuator at constant operating high voltage (1.3kV) to ensure spatial beam profile stability. An energy meter (NOVA, Ophir Optronics Ltd. ,USA) was employed to monitor the shot-to-shot pulse energy. Plasma production on the eggshell surface was attained via a quartz plano –convex lens of 100 mm focal length. A 1m length fused silica optical fiber mounted on a micro xyz-translation stage is used to collect the emission light from the plasma plume and feed it to an echelle spectrometer (Mechell 7500, Multichannel instruments, Sweden).The echelle grating spectrometer ,designed for operation in high orders and high angles of incidence and diffraction, can provide high resolution in a more compact size and cover a much wider spectral range than conventional grating spectrometers. The Mechell 7500 provides a constant spectral resolution of 7500 corresponding to 4 pixels FWHM (Full Width at Half Maximum), over a wavelength range 200 - 1000nm displayable in a single spectrum. A gateable intensified CCD camera (DiCAM-Pro-PCO Computer Optics, Germany) coupled to the spectrometer was used for the time resolved detection of the dispersed light. The gating of the camera and the timing for spectral data accumulation were controlled via PC. The overall linear dispersion of the spectrometer-camera system ranges from 0.006 (at 200nm) to 0.033 nm/Pixel (at 1000 nm).To avoid electronic interference and jitters, the intensifier high voltage is triggered optically. The ICCD camera control was performed via special Multichannel Instruments software. Echelle spectra display, processing and analysis were done using 2- and 3-D Gram/32 software programs (National Instruments, USA). In addition to the atomic database used by the mentioned software, spectral lines identification was checked by the most up-to-date electronically published database. All measurements were performed in air under atmospheric pressure. Six single spectra have been accumulated for each eggshell, three at each of the two different parts of the eggshell (middle and lower). The average of the 6 spectra acquired represents the elemental spectrum of such eggshell. A total of 100 samples, 50 before and 50 after hatching have been analyzed by the system.

Eggshell preparation for SEM: In order to examine the layers of each shell, samples were selected at random of some egg shells before and some after hatching. Following manual removal of the inner shell membrane, a single 1 cm^2 section was cut from each shell using a dental drill with a rotary blade attached. The shell was fixed to aluminum stubs with silver paint then coated with 35 nm layer of palladium using a polaron E5200 automatic coater for 3 min to be ready for scanning electron microscopy examination in a JEOL SEM, model (JSM-T330A).

Results and Discussion

The concentration of magnesium is not constant throughout the eggshell thickness; however Mg concentration decreases strongly after hatching (much less than its original value before hatching). LIBS experiments have been performed to follow up the Mg concentrations in the shell before and after hatching, to quantify our results it is necessary to obtain a calibration curve for magnesium. This has been done by preparing samples of calcium carbonate with different known concentrations of magnesium. The prepared standard samples are measured via LIBS technique and the obtained spectra have been normalized to avoid experimental fluctuations. Calibration curve for the Mg line at 285.22 nm is obtained by plotting its intensity versus the concentration in each sample. Fig. 1 shows the calibration line of magnesium. From the analysis of all the obtained LIBS spectra of the 100 measured samples, it is found that Mg concentration is decreasing by about 60% in the average with respect to the values before hatching as shown in the Fig. 2. This result is reasonable in view of the consumption of Mg during the

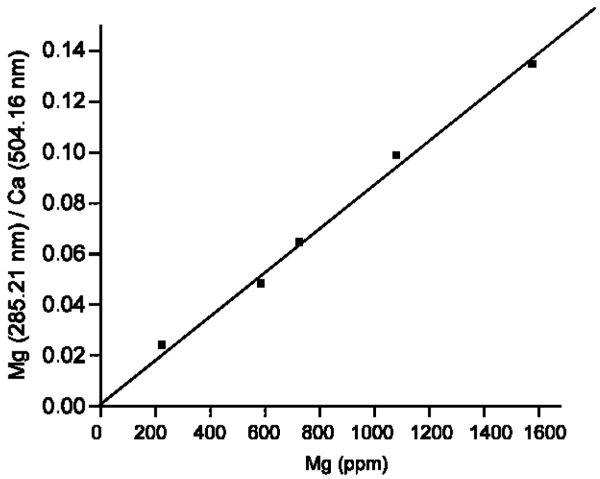

Fig. 1: Calibration curve of Mg

embryonic development stage. Similarly, the concentration of Na in the eggshell has been found to be lower after hatching than in the eggshell before hatching. This result is depicted in Fig. 3. For further quantitative analysis, the 588.9 nm line has been used. The calibration line of Na is shown in Fig. 4. Compared to magnesium the drop in the concentration of sodium after hatching is much less. In the average, the decrease in the Na concentration was about 30%. As we mentioned such decrease in Sodium concentration after hatching is less than that of magnesium. This is of course because the Sodium needs for the development of the embryo is much less than that of magnesium.

Calcium is the most important constituent of the eggshell. The results of the present study demonstrate that calcium distribution in the eggshell differs before and after hatching too.

The same samples investigated for magnesium and sodium are now considered concerning calcium concentration (qualitatively) via LIBS analysis. It was not possible to obtain quantitative results because of the difficulty of having standard calcium samples. However, relative concentration, before and after hatching was available. The spectral range extending from 370 up to 650 nm has been investigated to follow up the intensities of calcium spectral emission lines before and after hatching. The intensities of the calcium lines show a clear increase for the samples measured after hatching compared with the other samples before hatching as shown in Fig. 5. This result is opposite to what obtained in case of magnesium and sodium.

The results show a general increase in the calcium spectral lines intensity of more than 90% for the samples after hatching. Such increase in the calcium concentration can be interpreted in view of the depletion of the mammillary layer during the incubation period. The embryo consumes the elements and calcium in such inner layer for its development. Once the mammilary layer is consumed, after hatching, we are left with the inner most layer of the shell which is rich in calcium (in the form of calcium carbonate), and poor in other elements. To confirm such interpretation, a depth profiling LIBS measurements have been performed on a fresh egg shell (not hatched).

LIBS is an appealing technique for depth profiling purposes due to its capabilities for performing fast analysis in air at atmospheric pressure without limitations of sample size or nature. Thus, in the present work we exploited LIBS in investigating the depth profile of the egg shells in order to follow up its elemental composition in depth. In fact, this study was necessary and complimentary to confirm the previous results concerning the distribution of different elements throughout the shell thickness.

In order to perform such measurements, LIBS spectra of the same spot of the eggshell have been collected after increasing numbers of laser shots, 5, 10, 15, ... up to 35 at the same experimental conditions. An SEM micrograph of the laser induced crater on the egg shell after 20 shots as shown in Fig. 6. The cone shaped tips of the mammillary layer show up clearly in the micrograph. At fixed laser energy (100mJ pulse^{-1}) for the depth analysis a pronounced increase of the calcium spectral lines intensities takes place with depth, i.e. with increasing number of laser shots. In general the increase in the intensities, and consequently in the calcium concentration, after 35 shots relative to that after 5 shots is around 90% as shown in the spectra in Fig 7.

It is well known that approximately 80% of the calcium requirements of the chick by the time of hatch are derived from the eggshell (Simkiss, 1961). This leads to the consumption of a complete layer (the mammillary layer) which contains the required calcium and other elements too. That is why the eggshell looses much of its thickness after hatching. The left layer contains mostly calcium carbonate and therefore it is expected to find higher calcium concentration in the inner layer of the shell as confirmed by the LIBS depth profiling measurements. With higher number of laser shots we remove more material and reach deeper layers in the shell. The spectroscopic results show that the deeper we go (after 35 shots) the higher are the intensities of the spectral lines of calcium which means that the calcium concentration is increasing with depth.

It is worth to mention that the increase in the calcium spectral line intensities in some cases is relatively lower than the average. For example the intensity enhancement of the lines at 393.3 and 398.4 nm is only around 80%. This is primarily due to the fact that both lines are resonant lines that suffer strongly from the self absorption effect.

The relative thickness of the mammillary, palisade and vertical crystal layers did differ between eggshells after and before hatching. Recent findings (Fraser *et al*.,1998)

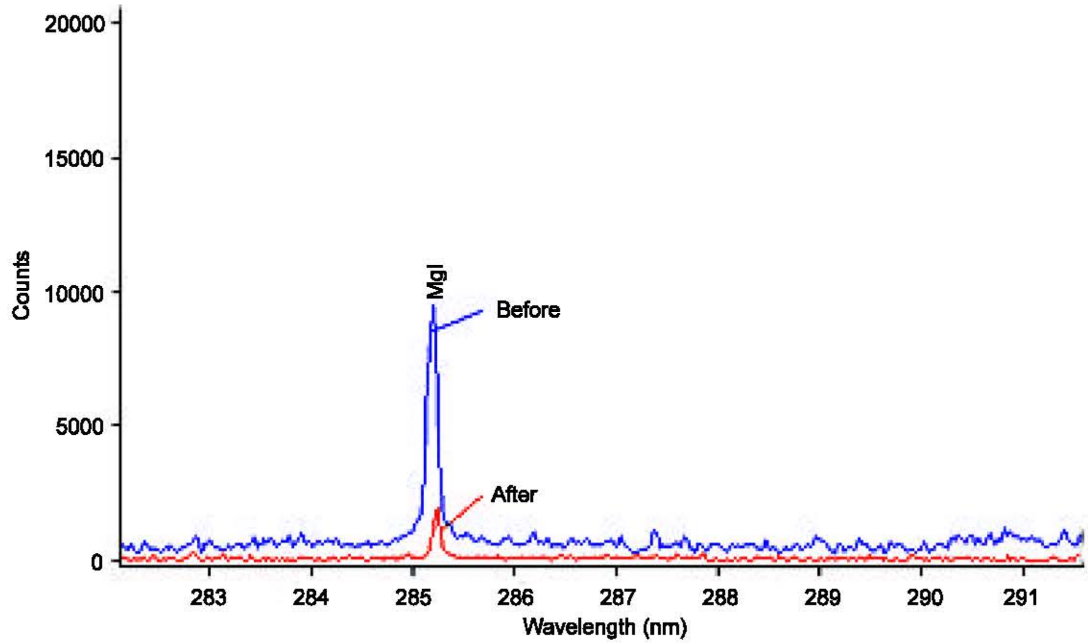

Fig. 2: Emission spectrum of the eggshell showing the Mg spectral line at 285.21nm

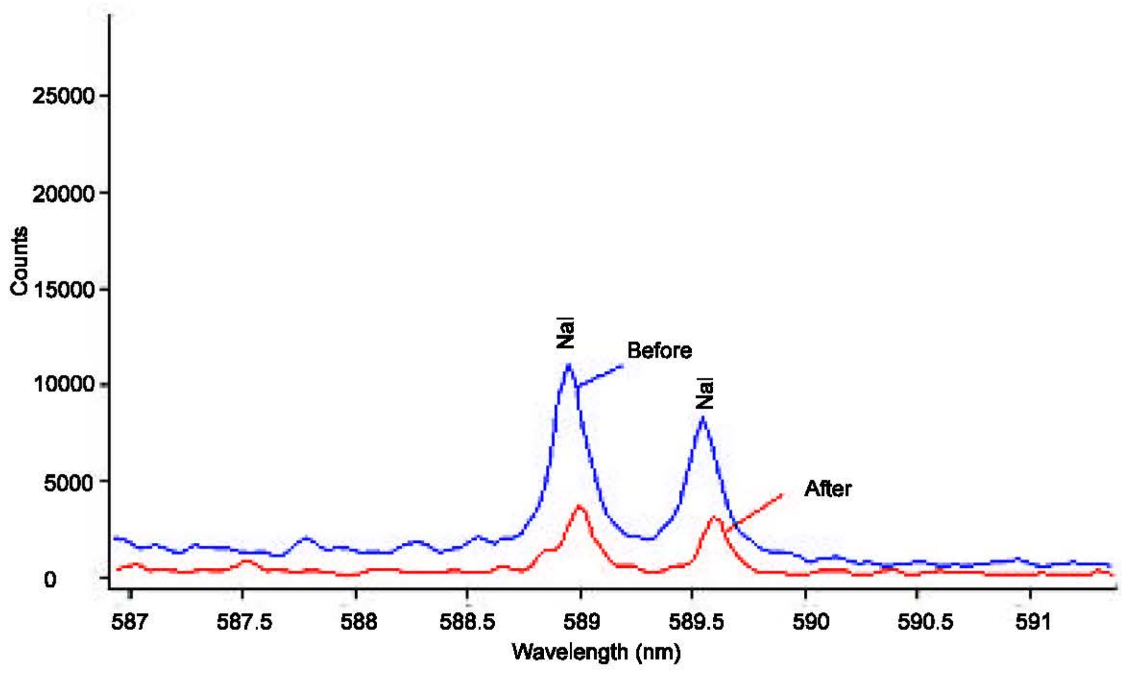

Fig. 3: LIBS spectrum of the eggshell showing the Na intensity before and after hatching.

suggest that the organic matrix of the eggshell could play a role in determining shell quality as the broiler hen ages. The quality of the mammillary layer ultrastructure is directly correlated with eggshell physical and material properties (Bain, 1992). In broiler breeder eggshells, the major changes in the main inorganic species after calcium carbonate; magnesium and phosphorus, occur in the outer region of the eggshell and therefore have no influence on the mammillary layer. In a separate study (Ruiz and Lunam, 2000.), the authors examined the material properties and ultrastructural characteristics of the broiler breeder eggs and noted that there was no significant decline in eggshell quality over the period of lay.

The scanning electron microscopy (SEM) is used to study the ultrastructure of eggshell. The SEM

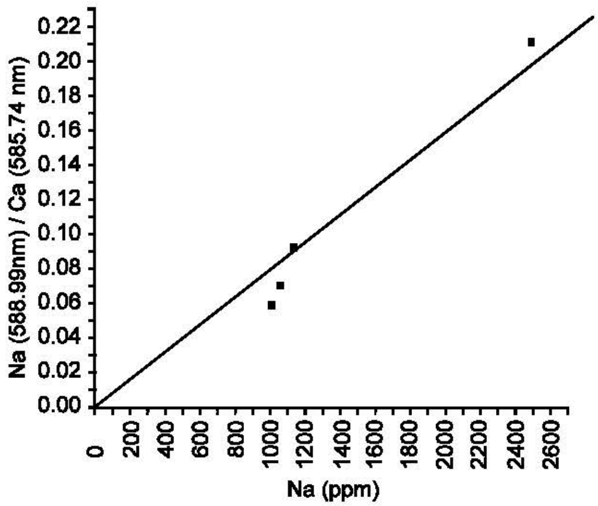

Fig. 4: Calibration curve of Na

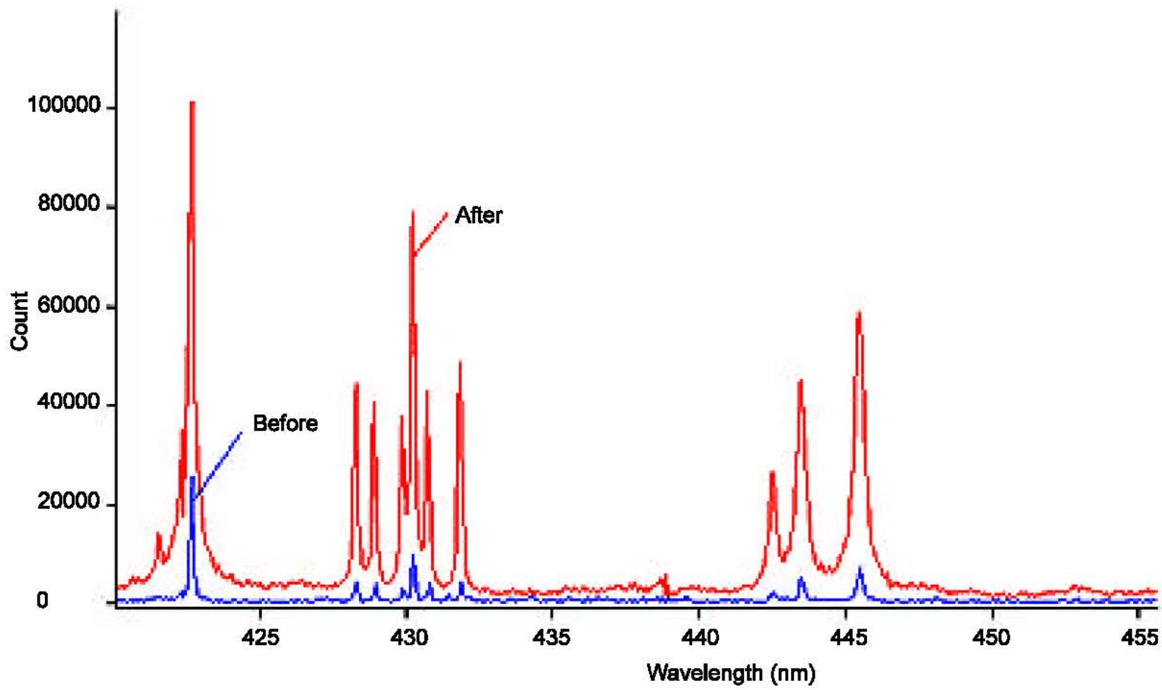

Fig. 5: LIBS spectrum of Ca in the eggshell before and after hatching.

micrographs displayed in Fig. 8, 9 show clearly that the thickness of the eggshell after hatching is much less than that before hatching. The mammillary layer looses about 50% of its original thickness after hatching as measured at three different positions. This loss in thickness is obviously due to the consumption of the inner mammillary layer by the embryo during incubation period.

The physical thickness of the eggshell has been measured via a micrometer too. A total of 100 samples (50 before and 50 after hatching) have been measured. Analysis of the measurements gives about 50% reduction in the thickness after hatching which is consistent with the SEM measurements.

Conclusion: In conclusion, using LIBS technique in the present study revealed that Magnesium concentration in the eggshell is decreasing after hatching by about 60% in the average with respect to its values before hatching. Sodium, on the other hand decreases by about 30% of the hatching. This is reasonable in view of the consumption of such elements, magnesium and sodium, during the embryonic development. On the contrary calcium concentration increased by more than

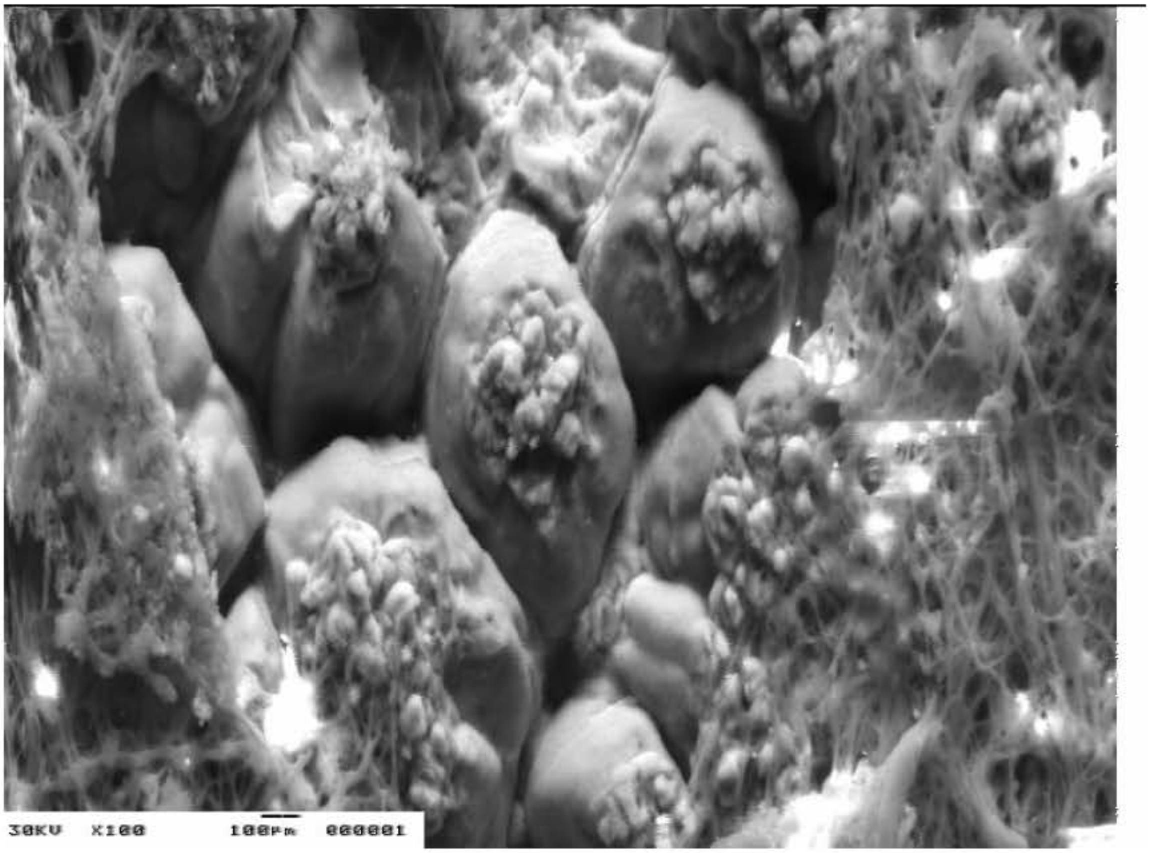

Fig. 6: Scanning electron microscopy (SEM) micrographs of a Crater produced by 20 laser shots. (100x)

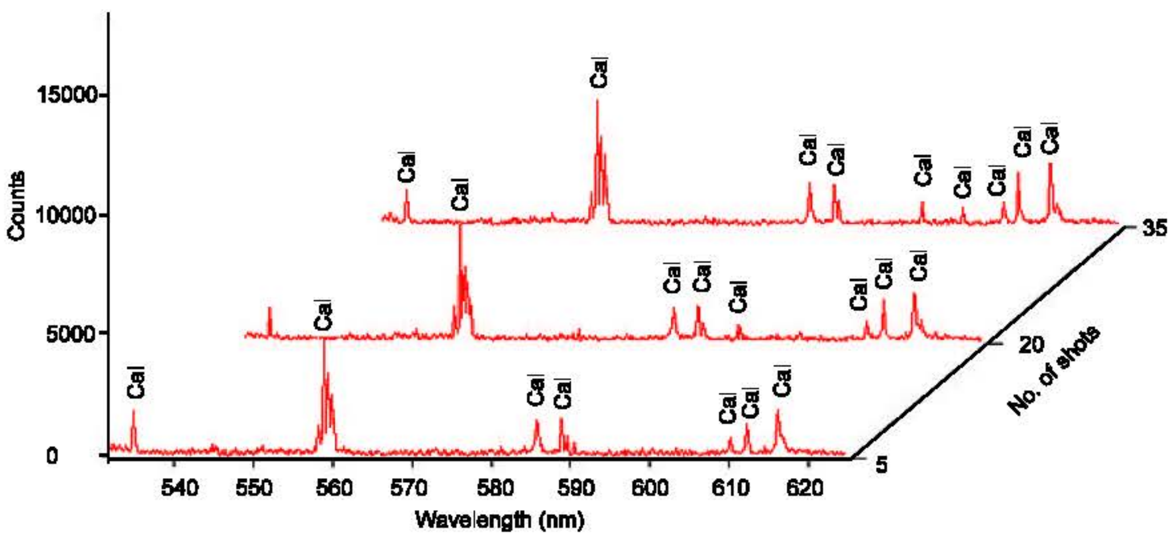

Fig. 7: 3-D spectra demonstrating the depth profile of the eggshell from 520 to 620nm.

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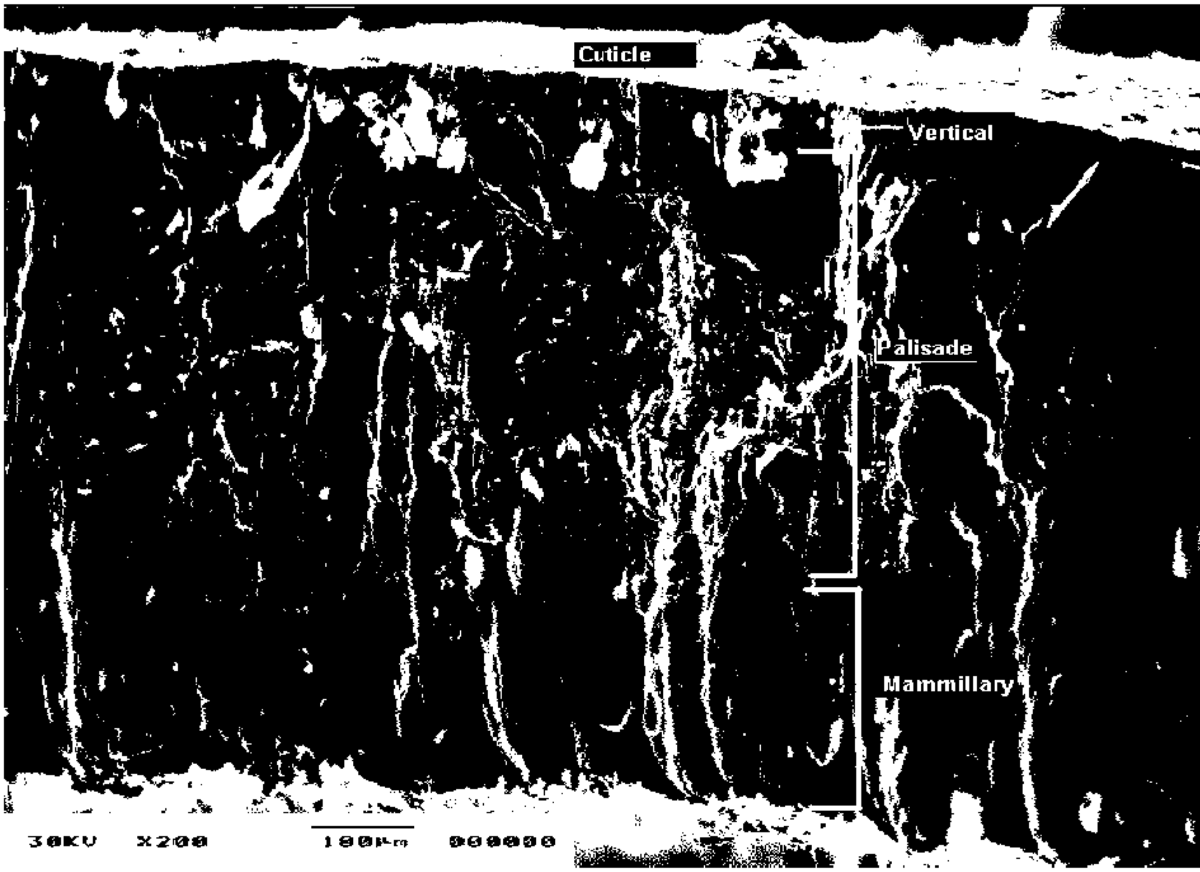

Fig. 8:Cross-sectional scanning electron micrograph through the eggshell of broiler breeder before hatching

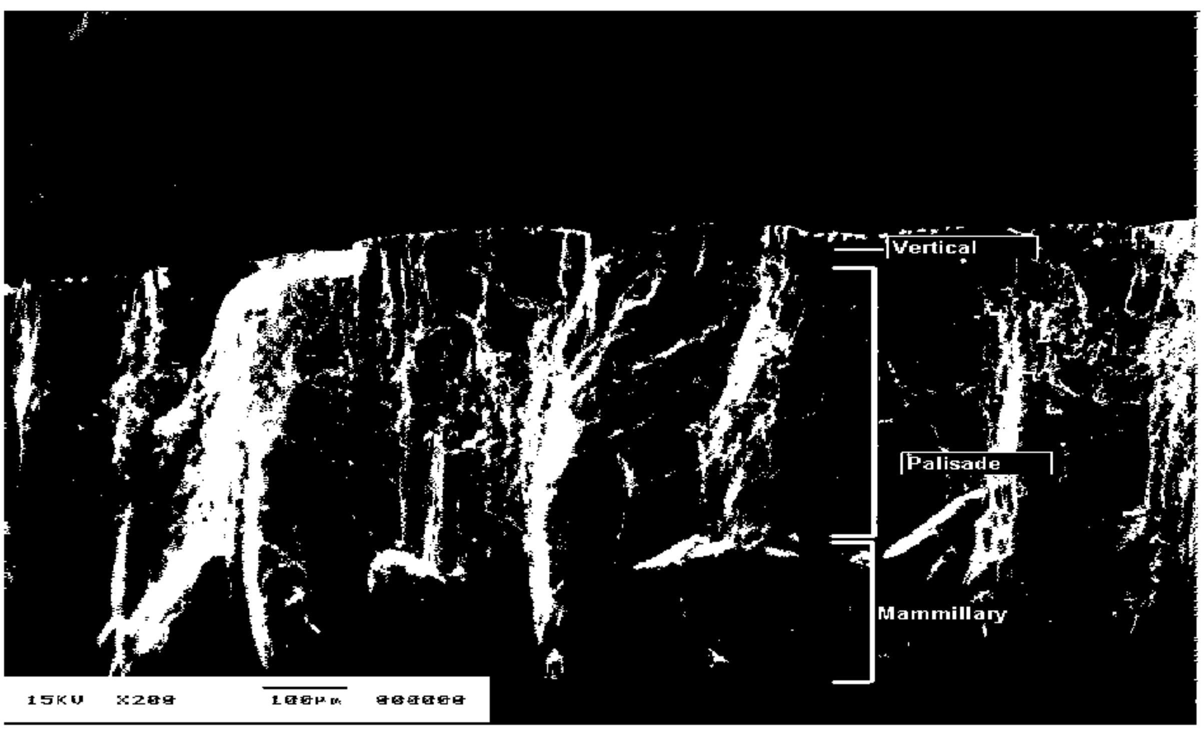

Fig. 9: Cross- sectional scanning electron micrograph through the eggshell of broiler breeder after hatching.

90% for the samples investigated after hatching via LIBS technique. The increase in the calcium concentration can be interpreted in view of the depletion of the mammillary layer during the incubation period. The embryo consumes the elements and calcium in such inner layer for its development. Depth analysis measurements showed that the calcium concentration, after 35 shots relative to that after 5 shots is higher by around 90% confirming the values obtained before and after hatching. The eggshell looses much of its thickness after hatching. The remaining layer contains mostly calcium carbonate and therefore it is expected to find higher calcium concentration in the outer layer of the shell as proven by the LIBS depth profiling measurements.

The present study shows the usefulness of using LIBS as an elemental analysis technique in poultry as a new applied field. In the same time and in view of the possibilities furnished by LIBS, in situ measurements (in farms and incubation centers) are feasible, that saves time and cost.

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