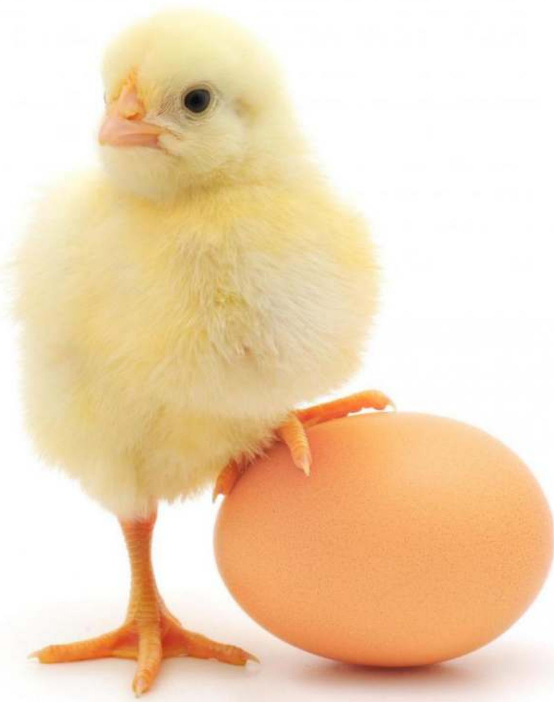
ISSN 1682-8356
ansinet.org/ijps



INTERNATIONAL JOURNAL OF POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

OPEN ACCESS International Journal of Poultry Science
ISSN 1682-8356
DOI: 10.3923/ijps.2017.451.456

Research Article
Influence of Separation Techniques with Acid Solutions on the Composition of Eggshell Membrane

[1]A.C. Torres-Mansilla and [2]E. Delgado-Mejía

[1]Adriana Torres-Mansilla, Faculty of Dentistry, Universidad Nacional de Colombia, Bogota, Colombia
[2]Department of Chemistry, Faculty of Science, Universidad Nacional de Colombia, Bogota, Colombia

Abstract
Background and Objective: The eggshell membrane is a biomaterial widely used in multiple fields of science. In order to use this biomaterial, the membrane needs to be separated from the shell. This procedure can be easily done manually but it could be inefficient and time consuming. Opposite to that, the acid dissolution of the shell represents an easy and efficient technique to obtain eggshell membrane. Some of the common acids used in this procedure are: acetic acid, EDTA and HCl. However, there is not enough information on the effect that these acids could cause in the organic eggshell membrane, neither on how these methods could affect its properties as a biomaterial. The objective of this study was to compare the influence of separation techniques with acid solutions, using Fourier Transform Infrared Spectroscopy (FTIR). **Methodology:** Pieces of ~0.5 g eggshell were immersed into acid solutions, with an acid mass equivalent to four times the stoichiometrically calculated to dissolve the shell mineral. Manually obtained eggshell membranes were used as control. Afterwards, all the membranes were removed, washed with deionized water dried at 37°C overnigth. The samples of dried membranes were analyzed with FTIR-KBr (potassium bromide) and ATR (Attenuated Total Reflection) sample preparation techniques. **Results:** The FTIR-KBr and ATR techniques revealed that the absorption bands, related to the organic structure of the eggshell membrane, were modified by the HCl and EDTA-Na_2 solutions, while the CH_3COOH produced minimal changes. **Conclusion:** The eggshell membrane separation technique with CH_3COOH solution preserves the organic structure of the eggshell membrane. Among the other acid solutions, the CH_3COOH solution might be suitable to separate the eggshell membrane more efficiently than manual separation, without producing changes in the chemical composition of its structure.

Key words: Eggshell membrane, fourier transform infrared spectroscopy, biomaterial, eggshell

Received: May 27, 2017 Accepted: July 08, 2017 Published: October 15, 2017

Citation: A.C Torres-Mansilla and E. Delgado-Mejía, 2017. Influence of separation techniques with acid solutions on the composition of eggshell membrane. Int. J. Poult. Sci., 16: 451-456.

Corresponding Author: Edgar Delgado-Mejía, Department of Chemistry, Faculty of Science, Universidad Nacional de Colombia, Bogota, Colombia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The eggshell membrane constitutes a biomaterial source for many fields of science, whose properties were previously underestimated as it was only considered as waste[1]. The acquisition of this biomaterial could be optimized using acid solutions to dissolve the calcium carbonate mineral but it is not well known if the acid solutions could also affect the organic structure of the eggshell membrane[1].

The eggshell membrane has a fibrous structure composed of three sub-membranes: An external in intimate contact with the eggshell mineral, an internal and a limiting membrane in contact with the egg albumen[1].

In both, the external and internal membranes, the eggshell membrane fibers are randomly organized and are composed by proteins and glycoproteins with cross-linked lysine rich bonds. Collagen (type I, V and X) represent around of the 10% of the total protein content[1].

The eggshell membrane structure contributes to a wide range of functions. Among others, it provides mechanical support to the eggshell, acts as an antimicrobial barrier and controls the nucleation and growth of calcite crystals[1]. Besides these physiological tasks, the eggshell membrane is also multifunctional as a biomaterial. This membrane, as revised by Balaz[1], contributes to the synthesis of nanoparticles, works as an adsorbent of metallic ions (as of organic dyes and other contaminant agents in water) and supplies food nutritional values[1].

In order to work with this biomaterial, it is necessary to remove the membrane from the mineral shell. This could be achieved not only by manual separation, but also, with acid solutions[1,2-6]. As the external membrane of the eggshell membrane is tightly bonded to the mineral shell, in most of the cases, the structure of the eggshell membrane couldn't be obtained in large pieces[1].

Larger pieces of eggshell membrane can be recovered with the use of acid solutions[1], but these solutions could cause a change in the chemical structure and therefore, possibly also have an effect on its properties as a biomaterial.

The studies that use the eggshell membrane as a biomaterial[2-6], commonly use Ethylene Diamine Tetraacetic Acid Disodium (EDTA-Na_2), Hydrochloric acid (HCl) and Acetic Acid (CH_3COOH) to remove the eggshell mineral. These studies, however, use heterogeneous times and concentrations and do not analyze the possible alteration in the organic membrane caused by these solutions.

In contrast to the reviewed literature, the present study compared the effect of 4 separation methods in the structure of the eggshell membrane: EDTA-Na_2, HCl, CH_3COOH and manual separation, with the use of Fourier Transform Infrared Spectroscopy (FTIR) using KBr pellet sample preparation and Attenuated Total Reflectance (ATR) accessory to determine which of these methods would cause minimal changes in the structure and the chemical composition of the eggshell membrane.

MATERIALS AND METHODS

Materials: Commercial red hen eggs (average weight ~60 g). Analytic solutions: Ethylene Diamine Tetraacetic Acid Disodium (EDTA-Na_2), Hydrochloric acid (HCl) and Acetic Acid (CH_3COOH).

Methods: Pieces of ~0.5 g of eggshell were immersed into 25 mL of the following acid solutions: Ethylene Diamine Tetraacetic Acid Disodium Salt (EDTA-Na_2), Hydrochloric acid (HCl) and Acetic Acid (CH_3COOH). The stoichiometrically quantity of solution needed to dissolve the calcium carbonate of the eggshell was calculated for each acid reagent and the solutions were prepared with a concentration four times higher.

To set the control group, a sample of eggshell membrane was manually detached from the eggshell mineral in order to later compare its chemical composition with those membranes which would be immersed into the different acid solutions. After 24 h, the fragments which had been immersed into acid solutions were removed from those solutions, washed with deionized water and desiccated at 37°C in an ESCO oven overnight. A replicate was made for each method.

Fourier Transform Infrared spectroscopy (FTIR): Each of the desiccated membranes was subsequently cut in half, one half was milled in agate mortar and a KBr pellet was prepared, the other half, in turn, was mounted in the Attenuated Total Reflection (ATR) accessory. The spectra of each membrane were obtained with Nicolet iS10 (Thermo Scientific) in a 400-4000 cm^{-1} range and 16 scans of each sample were taken.

RESULTS

Fourier Transform Infrared spectroscopy, KBr pellet sample preparation (FTIR-KBr): The FTIR-KBr spectra of the manually separated eggshell membranes showed the characteristic absorption bands/peaks related to the organic structure of the membrane and a peak related to the eggshell mineral as shown in (Fig. 1).

The protein/amide characteristic bands appeared as 3 highly intense bands (Fig. 1): A broad band in 3400 cm^{-1}

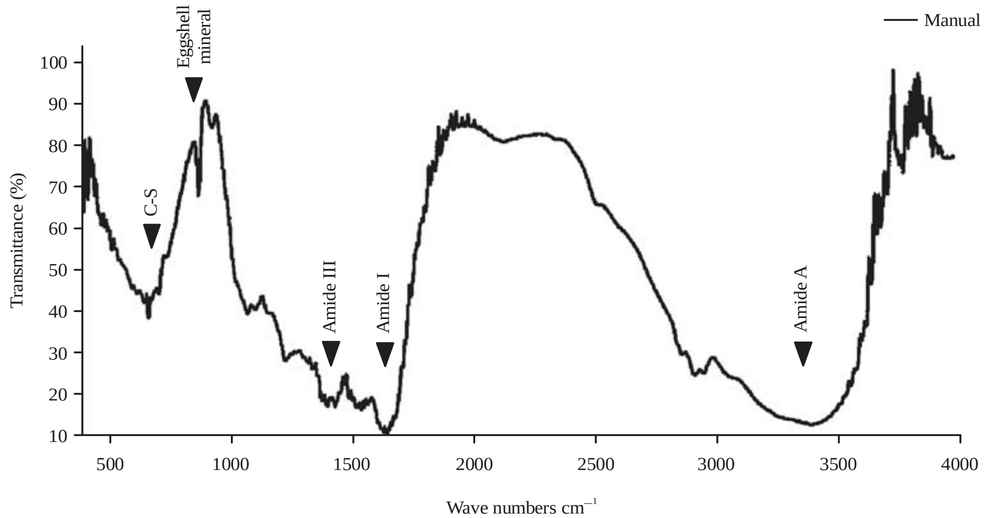


Fig. 1: Characteristic absorption bands of eggshell membrane. Spectrum (FTIR-KBr) of manually separated eggshell membrane

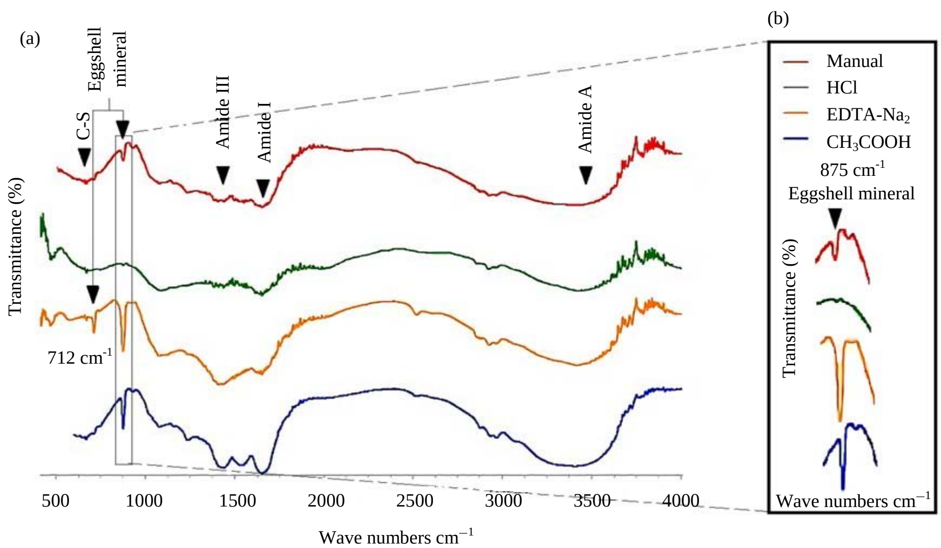


Fig. 2(a-b): (a) FTIR-KBr spectra for the eggshell membrane obtained by manual separation and acid dissolution of the shell mineral (HCl, EDTA-Na$_2$ and CH_3COOH) and (b) Zoom in of absorption band originated in the eggshell mineral

(amide A: NH stretching vibration) and 2 sharper bands in 1650 cm^{-1} (amide I: C = O stretching vibrations) and 1440 cm^{-1} (amide II: CN stretching and NH in plane bending)[7]. An absorption band, also related to the eggshell membrane structure, appeared in the position of 670 cm^{-1}, corresponding to the stretching vibration of C-S bonds associated with cysteine-rich proteins of the membrane fibers[8]. A sharp absorption band related to the eggshell mineral was also present: Present in the 875 cm^{-1} position of the spectrum (bending vibration out of the plane of CO_3^{2-})[6]. In every spectra, the bands related to the organic membrane displayed lower percentages of transmittance that the bands related to the eggshell mineral.

The spectra of the eggshell membranes separated manually and with acid dissolution of the eggshell mineral showed differences in the presence of the absorption bands as well as in their shape and intensity (Fig. 2).

The spectra of the membranes obtained with HCl eggshell mineral dissolution didn't have the absorption band amide III and the band related to the eggshell mineral (875 cm^{-1}). The amide I peak was present but less defined in comparison with the spectra of the membranes

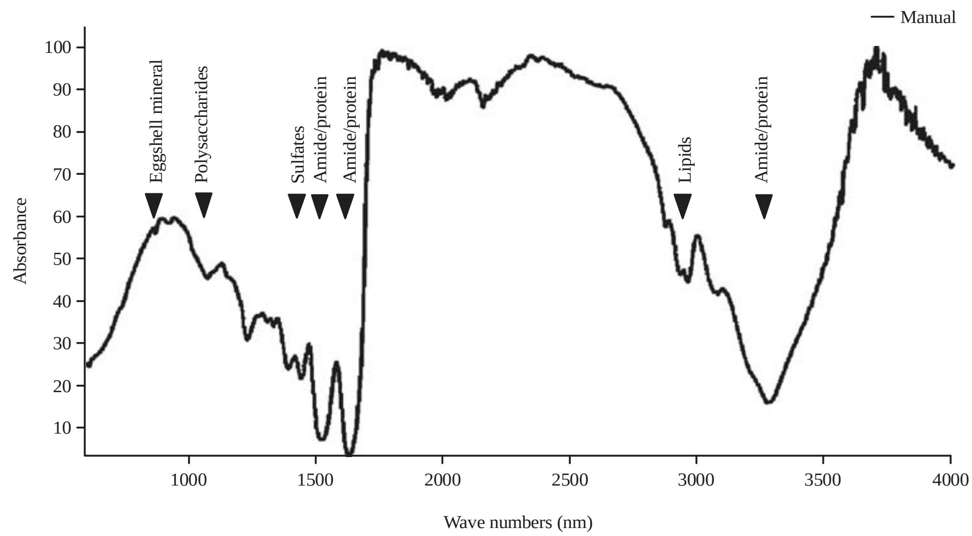


Fig. 3: Characteristic absorption bands of eggshell membrane. Spectrum (FTIR-ATR) of manually separated eggshell membrane

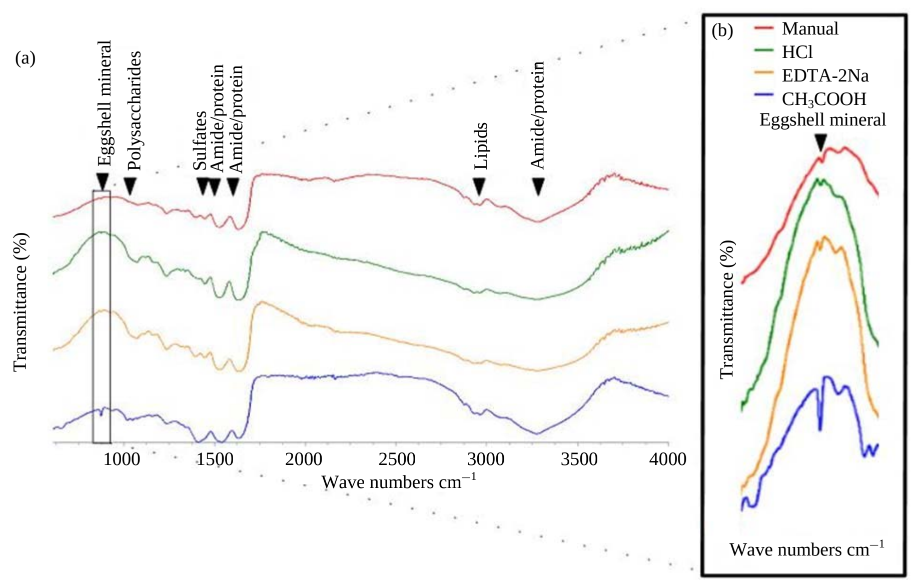


Fig. 4(a-b): (a) FTIR-ATR spectra for the eggshell membrane obtained by manual separation and acid dissolution of the shell mineral (HCl, EDTA-Na_2 and CH_3COOH) and (b) Zoom in of absorption band originated in the eggshell mineral

separated with the other treatments. On the other hand, the spectra of the membranes, separated with EDTA-Na_2 and CH_3COOH, displayed the characteristic absorption bands related to the organic constituents of the eggshell membrane similar to the spectrum from the manually separated membrane. Nevertheless, in the spectrum of the membrane separated with EDTA-Na_2, a sharp, but not very intense peak in the position 712 cm^{-1} appeared, related to the eggshell mineral (O-C-O bending vibration in the plane)[9].

Fourier Transform Infrared Spectroscopy-Attenuated Total Reflection (FTIR-ATR): The FTIR-ATR spectrum of the manually obtained membrane, displayed the characteristic absorption bands[9] Amide/Proteins 3280, 1640 and 1520 cm^{-1}, polysaccharides at 1065 cm^{-1}, sulfates at 1435 cm^{-1} and lipids -CH bonds- at 2850, as well as a band related to eggshell mineral (Fig. 3).

The FTIR-ATR spectra from the membranes obtained with manual separation and acid dissolution of the eggshell with the use of EDTA-Na_2, HCl and CH_3COOH preserved the protein, polysaccharides, sulphates and lipids absorption bands (Fig. 4). However, the spectra of the HCl and EDTA-Na_2 treated membranes presented less defined lipid absorption peaks. The band related to eggshell mineral could be seen in the FTIR-ATR of all samples.

DISCUSSION

The FTIR spectroscopy provides information of vibrational modes of the atoms in a molecule. In turn, as these vibrations depend on features like the degrees of freedom and symmetry of the molecules, this procedure provides information about the functional chemical groups and therefore when these vibrations change their frequency, it also indicates chemical changes, represented in the position of the spectrum of the absorption bands in the abscissa axis, plotted against the intensity of the absorption (transmittance%) of these bands[8].

The acid dissolution of the eggshell mineral is a commonly used procedure by many of the studies that use the eggshell membrane as a biomaterial, as well as the manual separation[2-6]. There are FTIR-KBr and ATR characterizations of the manually detached membrane, used as a reference to compare the other eggshell membrane separation treatments in this study[1,9], as the characterization for membranes obtained by dissolution of the calcite with acid solutions is limited. This study compared membranes obtained by acid dissolution of the mineral with EDTA-Na_2, HCl and CH_3COOH with the use of FTIR-KBR and ATR sample technique preparation. With the KBr pellet preparation is possible to obtain sharper and defined absorption bands. In contrast, with the ATR accessory, it is possible to compare our findings with previous and relevant reference spectrum of the eggshell membrane, specially the one referenced by Rodriguez-Navarro *et al.*[9], who analyzed the membranes during different stages of eggshell mineralization.

In the present study, the FTIR-KBr and the FTIR-ATR spectra from the eggshell membranes obtained with different separation methods showed differences, not only regarding the characteristic absorption bands of the organic constituents but also in absorption peaks related to the eggshell mineral (Fig. 2, 4).

It seems that the HCl treatment did affect the secondary protein backbone structure of the organic membrane because the amide III peak was not possible to identify and the amide I peak diminished in intensity in the FTIR-KBr spectrum of the eggshell membrane obtained with this method (Fig. 2). This treatment as well as the EDTA-Na_2 separation treatment may also affect the lipids band; in the FTIR-ATR spectra of the eggshell membranes separated with these treatments (Fig. 4), the lipids bands appeared broader and less intense compared to the other methods.

We can infer, however, that the separation with CH_3COOH acid solution didn't change the chemical structure of the eggshell membranes, as the spectra of the membranes obtained with this method had the characteristic organic absorption bands similar to the manually obtained eggshell membranes.

Another characteristic to highlight is that all the eggshell membrane spectra, regardless the method used to obtain it, presented peaks of absorption related to the eggshell mineral. In the position of 875 cm^{-1} of all FITR-KBr eggshell membrane spectra and also in 712 cm^{-1} of the FTIR EDTA-Na_2 spectrum.

This finding can be explained by looking into the organic-inorganic relation in the eggshell biomineralization. The eggshell membrane has some active sites which induce the nucleation of the mineral, in consequence it has an active role in the calcite crystal growth[9]. These active sites and the calcite crystals are tightly bonded together and even though the membrane was subjected to immersion in acid solution, some of the mineral might remain deep inserted in the fibers of the eggshell membrane. Further investigation about the presence of calcite mineral absorption band in eggshell membranes by acid dissolution of the eggshell mineral should be considered.

With the growing expansion of the use of eggshell membrane biomaterial, there's a need to find a separation technique that preserves the eggshell structure and could also be used in a large industrial scale[1]. The eggshell mineral separation techniques with acid solutions, are currently being widely used and so, it is necessary to deepen our knowledge on the incidence that those acids could have on the chemical structure of the membrane. There is even a publication of a patent made of a machine designed for this purpose[10]. This machine uses cavitation and acetic acid to dissolve the eggshell mineral.

The comparison made in this study, with three of the most common acid solutions used for eggshell membrane separation, demonstrates that the dissolution of the eggshell mineral with CH_3COOH was the only method that preserved the most of the eggshell membrane structure. This was demonstrated by means of the FTIR-KBr and ATR analysis. The HCl and EDTA-Na_2, on the other hand, modified the absorption bands related to the organic structure of the eggshell membrane, thereby causing a possible alteration of its structure.

Lastly, there's another finding worth to be explored: The presence of the absorption bands associated with the eggshell mineral (calcium carbonate) in all the membranes mineral in all the membranes separated, regardless the acid solution used in the dissolution of the eggshell mineral.

CONCLUSION AND RECOMMENDATIONS

The use of HCl and EDTA-Na_2 acid solutions to dissolve the eggshell mineral and obtain the eggshell membrane produce changes in the chemical structure of the eggshell membranes as demonstrated by the FTIR-KBr an FTI-ATR analysis. The use of the acid solution CH_3COOH produce minimal chemical changes, compared to the other eggshell membrane separation techniques evaluated in the present study.

SIGNIFICANCE STATEMENT

The dissolution of the eggshell mineral with acetic acid solution (CH_3COOH) to separate and obtain the eggshell membrane could represent a conservative procedure more efficient than manual separation, with possible applications in biomaterial research or in an industrial scale.

ACKNOWLEDGMENTS

Authors would like to acknowledge and thank the Chemistry Department of the National University of Colombia for providing the technical requirements that made this study possible and Karen Torres for her valuable work in the translation issues of this article.

REFERENCES

1. Balaz, M., 2014. Eggshell membrane biomaterial as a platform for applications in materials science. Acta Biomater., 10: 3827-3843.
2. Hsieh, S., H.H. Chou, C.W. Hsieh, D.C. Wu, C.H. Kuo and F.H. Lin, 2013. Hydrogen peroxide treatment of eggshell membrane to control porosity. Food Chem., 141: 2117-2121.
3. Wu, T.M., J.P. Rodriguez, D.J. Fink, D.A. Carrino, J. Blackwell, A.I. Capalan and A.H. Heuer, 1995. Crystallization studies on avian eggshell membranes: Implications for the molecular factors controlling eggshell formation. Matrix Biol., 14: 507-513.
4. Tsai, W.T., J.M. Yang, C.W. Lai, Y.H. Cheng, C.C. Lin and C.W. Yeh, 2006. Characterization and adsorption properties of eggshells and eggshell membrane. Bioresour. Technol., 97: 488-493.
5. Armitage, O.E., D.G.T. Strange and M.L. Oyen, 2012. Biomimetic calcium carbonate-gelatin composites as a model system for eggshell mineralization. J. Mater. Res., 27: 3157-3164.
6. Balaz, M., A. Zorkovska, M. Fabian, V. Girman and J. Briancin, 2015. Eggshell biomaterial: Characterization of nanophase and polymorphs after mechanical activation. Adv. Powder Technol., 26: 1597-1608.
7. Barth, A., 2007. Infrared spectroscopy of proteins. Biochim. Biophys. Acta (BBA)-Bioenerg., 1767: 1073-1101.
8. Larkin, P., 2011. Infrared and Raman Spectroscopy: Principles and Spectral Interpretation. Elsevier, Boston, MA., ISBN: 9780123870186, Pages: 230.
9. Rodriguez-Navarro, A.B., P. Marie, Y. Nys, M.T. Hincke and J. Gautron, 2015. Amorphous calcium carbonate controls avian eggshell mineralization: A new paradigm for understanding rapid eggshell calcification. J. Struct. Biol., 190: 291-303.
10. Vlad, V., 2011. Eggshell membrane separation method. Publication No. US8056844 B2, November 15, 2011. https://www.google.com/patents/US8056844