Original Article

Investigation Of Different Chicken Breeds Egg Shells Through High Throughput Chemical And Elemental Analysis.

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Abstract

An eggshell (ES) is an essential protective layer against pathogens and physical stress during the early development of an avian embryo and until hatching with adequate metabolic and nutritional supply. Nevertheless, calcium carbonate-enriched ES waste is now used for various industrial and agricultural applications. The present study analyzed the chemical composition of nine varieties (aseel, white leghorn, vanaraja, black rock, color cross, kadaknath, Rhode Island Red, red carnish and venzaguda) of chicken breeds ESs available at Central Poultry Development Organization (CPDO), Bhubaneswar, Odisha. Further, ES and eggshell membrane (ESM) morphology and chemical characteristics were analyzed through ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS), atomic absorption spectroscopy (AAS), X-ray diffraction (XRD), X-ray fluorescence (XRF), red, green, blue (RGB) analyzer and thermal gravimetric analysis (TGA). According to calcinations and XRF analysis, the percent of calcium (CaO) is 97.60-98.36, sulfur trioxide (SO3) is 1.097-1.49, and potassium oxide (K2O) is 0.198-0.28. Trace elements such as chlorine, sulfur, potassium, lutetium, iron, manganese and strontium, zirconium, and zirconium oxide were also recorded. The FTIR data for all ES and all ESM showed comparatively similar composition types, except for XRF data, which could be more helpful for preparing any ES-based composites and materials.

Keywords: Chicken eggshells, Chemical composition, Elementary analyses, Advanced spectroscopic analyses.

INTRODUCTION

Globally, chicken eggs are a common daily human diet with a rich source of high-quality nutritional value. In total, China, the United States, Indonesia, India, and Mexico produce more than half of the world's eggs (approximately 63 percent or 1652 billion eggs) (Shahbandeh, 2020; FAO, 2020). At the same time, approximately 2.3 million tons of eggshell (ES) waste are produced and need to be properly disposed of for environmental safety. As ES disposal costs are also high, research is now focused on converting ES waste into a value-added product for biotechnological, biomedical, and intraductal applications (Ahmed *et al.,* 2019; FAO, 2020).

From composition point of view, ES contains calcium carbonate (CaCO3) in mineral form as calcite (nearly 95%), organic matrix (nearly 3.5 %) (Athanasiadou *et al.,* 2018). Typically, organic matrices consist of proteins and proteoglycans, which contribute to the formation of specific microstructures and mechanical properties of the ES. Overall, the ES contain a multilayer resilient structure composed of eggshell membrane (ESM), mamillary cone, palisade and cuticle layers. Consequently, the calcium carbonate enriched ES utilized for value-added biomedical applications such as calcium lactate, calcium phosphate, health-promoting products, food processing, plant growth promoters, fertilizer biomass and collagen from ESM has used been health, biochemical, pharmaceutical, food and cosmetics industries (Vandeginste 2021; Zain *et al.,* 2021; Hsieh *et al.,* 2021; Bharti *et al.,* 2020). As indicated by the increase in patents filed on ES-based products, ES is not a waste and several up-scaling platforms have been developed to repurpose ES.

Currently, several new generation or hybrid eggs are being developed through different genetic engineering approaches to enhance egg quality, nutritional value, egg size, etc. As a result, there is a possibility of change in ES composition and functional characteristics. Therefore, the present hypothesis is to determine the chemical composition of nine breeds (aseel, white leghorn, vanaraja, black rock, color cross, kadaknath, Rhode Island red, red carnish and venzaguda) of chicken based on spectroscopic analyses. Briefly, ESs of 9 different breeds of chickens were collected from the Central Poultry Development Organization (CPDO), Bhubaneswar, Odisha and to examine the morphology and chemical composition of both ES and EPS were characterized through ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS), atomic absorption spectroscopy (AAS), X-ray diffraction (XRD), X-ray fluorescence (XRF), Red, Green, and Blue (RGB) analyzer, thermal gravimetric analysis (TGA).

METHODS

Sample collection and preparation

Nine different breeds of chicken ESs were collected from the Central Poultry Development Organization (CPDO), Bhubaneswar, Odisha. Firstly, decontaminate the shells by washing them several times in ultra-pure water, drying in an EcoTherm oven at 60°C for 60 mins and storage at room temperature for 24 hrs. For the membrane separation, we initially used 1M acetic acid and nitric acid separately to extract the shells (Liu et al., 2017). Moreover, about 17 to 60 minutes after incubation, membrane separation, and the solid shell of the egg were collected. Then, three groups of 20 g ESs were calcined (unseparated, dissolving components 17 minutes, and 60 minutes) before being deposited in a Carbolite® CWF-1100 muffle furnace for 30 minutes, 1 hour, 3 hours, and 5 hours at 300°C, 500°C, 700°C, and 900°C, respectively. Unseparated shells, washed shells at 17 min, and 60 min were referred to as UNS, Sep17, and Sep60, respectively (Rose et al., 2009; Liu et al., 2017). The leaching rate was mainly determined by degrading 20 g of ES in 13 sacrificial reactors with 500 mL/mol acetic acid. With the pH record individual reactors were filtered/ washed with distilled water and dried at 60°C. Further, to track $CaCO_3$ loss over time, the pH of a next reactor (mean 14th position) was monitored for 180 mins (Rose *et al.,* 2009; Liu *et al.,* 2017).

ES and ESM composition analyses

Primarily, measure the pH to assess the concentration at which CaCO3 and other ES elements were released into the acid medium. Then, record the changes in the chemical composition and specific functional group after leaching through FTIR (GX-FT-IR, Perkin Elmer, USA) within the spectral range 4000-400 cm^{-1} (Kınaytürk *et al.,* 2021). After functional groups, calcium concentration in leached liquid was measured, where CaCl2 was used for standard linear calibration curve generation with AAS (Perkin Elmer AANAlyst 400, Waltham, Massachusetts). Briefly, 1M KCl was applied to prevent Ca ionization in water, then samples were diluted with ultra-pure water to a Ca concentration of 5 mg/L for these analyses (Kobus-Cisowska *et al.,* 2020). Mainly, three different ES particle sizes were used with soaking times of 17 mins, 30 mins and 60 mins respectively for the experiment.

Thermogravimetric analyzer (TGA) (Perkin Elmer Pyris Diamond-11 using indium as a standard to measure calcilation (the degradation of CaCO3 to CaO at high temperatures). Thus, individual samples were scanned over the range of 30-300°C with scanning rate of 10°C/min in a nitrogenic enforcement (150 mL/min) (Awogbemi *et al.,* 2020). Then, using the Image J software (https://imagej.nih.gov/ij/), the 3D interactive surface and pixel vs. intensity assessment plots were generated and analyzed the temperature variation in colorimetric terms. Further, to examine the surface morphology of ESs, the advanced SEM technique was employed. Briefly, the ES powder sample was placed in an aluminum stub containing coating material. Afterward, ES coated stubs were placed in a SEM chamber and randomly scanned and photomicrographs were recorded at a voltage of 10 kV (Fecheyr-Lippens et al., 2015). Also, XRF (Shimadzu EDX-720) was used to determine oxide compositions and mainly the mineral components by XRD (Ultima-III, Rigaku, Japan) of prepared ES poweder followed the appropriate methods previously described (Brahimi *et al.,* 2020; Laohavisuti *et al.,* 2021). In addition, the collagen in the leachate ESM sample was determined using UPLC-Q-TOF-MS followed by the software MassLynx v.4.1 (Yamauchi *et al.,* 2013).

Statistical analyses

The overall statistical analysis consisted of mean and standard deviation values calculated from our triplicate data as well as one-way analysis of variance (ANOVA) and Bonferroni multiple comparisons. All analysis was done using the software, GraphPad® Prism v.5.0 (San Diego, USA).

RESULTS

Physical characteristics ES and ESM. As per the analyzed data, the percent of composition of the ES and ESM was calculated as 97.5% and 2.05%, respectively (**Table 1**). In addition, the densities of the unseparated and separated shell were 1.07 g/cm^3 and 1.73 g/cm^3, respectively (**Table 1**).

Table 1. Characteristics of egg shell and shell membrane.

Characteristics	Value
% Composition of the shell	97.5
% Composition of the membrane	2.05
Density of unseparated shell (g/cm^3)	1.07
Density of separated shell (g/cm^3)	1.73

Comparatively, the present findings contradict the results of previous studies (Tangboriboon *et al.,* 2012; Ketta and Tůmová, 2016). Technically, the removal of the highly porous membrane layer results in a lower density and that porosity helps to dye absorbent for value-added product development (Murcia-Salvador *et al.,* 2020; Tsai *et al.,* 2006).
Calcilation rates of ES and ESM in acidic medium

ES and ESM interaction was lost by applying acetic and nitric acids to leach ES calcium into the acid medium separately. Then the AAS analyzed the presence of calcium after 60 mins and 180 mins of incubation. The results indicated that, approximately 6 g (30%) and 15 g (75%), respectively of $CaCO_3$ were presented in acidic medium. Mainly, the rate of

calcium dissolved in acidic medium means mass loss of CaCO3 during the leaching process in two different time interval was calculated by below equation-I (Tangboriboon *et al.*, 2012).

$$[Ca^{2+}] = [CO_3^{2-}] + [HCO_3^{-}] + [H_2CO_3] \ldots\ldots\ldots\ldots \text{(I)}$$

Thermal analyses of ES and ESM components

At an increased temperature at specific time interval (30 minutes, 1 hr, 3 hrs, and 5 hrs at 300°C, 500°C, 700°C, and 900°C), the effects of on separated and unseparated ES and ESM were investigated (**Figure 1)**.

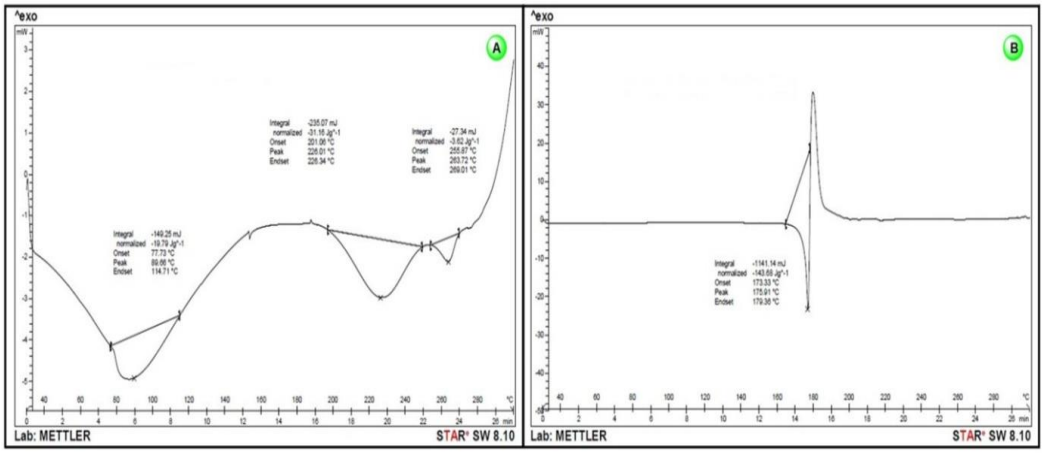

Figure 1. DSC thermograms: (A) leached sample (B) unseparated sample

The depicted figure indicated that, loss of mass was increased by increase in temperature. The thermograms showed a significant difference in both leached and unseparated samples. Mainly, the presence of ESM in the unseparated ES burns faster than the separated ES. Based on percent of mass loss, unseparated ES lost 47% of their initial mass, while separated ES lost 45% from TGA analyses.

RGB of ES and ESM components

The rate of calcilation of ES and ESM components was recorded by taking photographs at a constant distance and illumination in the tri-color (red, green, blue) frame. The data revealed that, the colour changed from brown to dark brown, grey at 300°C and appeared white at 900°C similar to previous studies (Murcia-Salvador et al., 2020). It was also revealed that when samples were leached for 60 minutes they did not show as much dark as raw samples due to lower carbon content in Sep60. Nevertheless, all samples showed a white spectrum after 3 hours of calcination. The lack of white colour at 700°C combined with XRD analysis data indicated that, calcination was only optimal at 900°C, while calcination was complete after 3 hrs. Therefore, Sep17 samples behaved similarly to Sep60 samples and leaching beyond 17 minutes to more than 3 hrs of calcination seems not to aid calcination.

Micro-structural analysis of ES and ESM

Based on recorded SEM image, the surface morphology of ES is porous, crystalline, and angular **(Figure 2)**.

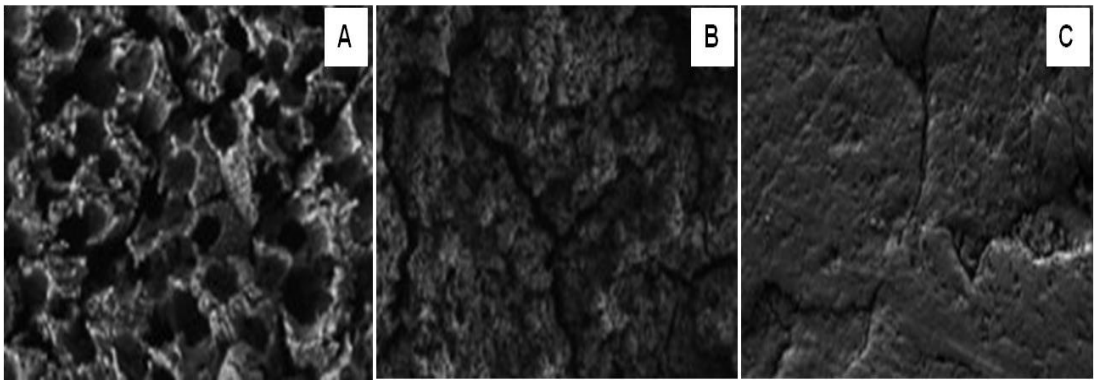

Figure 2. SEM microphotographs of shell at (A) 500x magnification, (B) 2000xmagnification, and (C) 7000xmagnification

In addition, an agglomeration of surface particles was also observed. As a result of the presence of Cao, honeycomb-like pore surfaces with rod-like particles were observed. Similarly, the SEM image of ESM indicated a large surface area with fiber-like pores. Technically, porous fibril structure in ESM acts as an excellent adsorbent as studies showed (Zulfikar *et al.*, 2013).

Oxide composition analyses of ES and ESM

The oxide composition of calcined ES at 900°C for 3 hrs was recorded by X-ray fluorescence (XRF) analysis (**Table 2**).

Table 2. XRF data analysis of different egg shells.

Elements name	AA	BR	CX	K	OR	RC	VG	VN	WL
Al_2O_3	Nil	Nil	Nil	0.463%	Nil	Nil	Nil	Nil	0.46%
SO_3	1.49%	1.29%	1.35%	1.097%	1.639%	1.669%	1.118%	1.171%	1.229%
Cl	0.24%	0.22%	0.16%	0.19%	0.27%	0.222%	0.197%	0.216%	0.191%
K_2O	0.28%	0.27%	0.20%	0.237%	0.276%	0.257%	0.221%	0.198%	0.225%
CaO	97.89%	98.01%	98.09%	97.78%	97.60%	97.75%	98.36%	98.30%	97.682%
SrO	Nil	0.14%	0.15%	0.142%	0.146%	Nil	Nil	Nil	0.148%
V_2O_5	Nil	Nil	51.4ppm	Nil	Nil	Nil	Nil	Nil	Nil
TiO_2	103.3ppm	85.4ppm	Nil	118.7ppm	132.1ppm	Nil	126.3ppm	90.8ppm	99.5ppm
MnO	74.5ppm	41.6ppm	31.2ppm	Nil	36.7ppm	34.8 ppm	57.7 ppm	Nil	28.6 ppm
Fe_2O_3	219.2ppm	177ppm	129.3ppm	283.7ppm	128.5ppm	305.3 ppm	155.4 ppm	111.3 ppm	178.3 ppm
ZrO_2	217ppm	8.3ppm	3.2 ppm	10.2 ppm	8.8 ppm	212 ppm	202 ppm	219.6 ppm	10.6 ppm
SnO_2	188.2 ppm	174 ppm	Nil	186.6 ppm	Nil	Nil	167.6 ppm	184 ppm	164.4 ppm
Co_3O_4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Nd_2O_3	Nil	95.1 ppm	114.1 ppm	Nil	Nil	90.2 ppm	Nil	105.6 ppm	Nil
CeO_2	71.7 ppm	Nil	Nil	Nil	83.3 ppm	80.6 ppm	Nil	89.5 ppm	81.7 ppm
Pr_2O_3	Nil	Nil	Nil	Nil	39.1 ppm	Nil	Nil	84.6ppm	Nil
Yb_2O_3	Nil	Nil	Nil	17.4 ppm	Nil	Nil	Nil	Nil	Nil
Sm_2O_3	Nil	Nil	20 ppm	30.5 ppm	29.5 ppm	Nil	54.1 ppm	9.9 ppm	Nil
Eu_2O_3	Nil	47.4 ppm	Nil	Nil	21.2 ppm	Nil	Nil	52 ppm	Nil
Er_2O_3	78.1 ppm	158.9 ppm	217 ppm	144.4 ppm	88.4 ppm	191.3 ppm	135.5 ppm	106.2 ppm	48.4 ppm
Lu_2O_3	46.9 ppm	36.3ppm	23.7ppm	23.9 ppm	36.7 ppm	40.5 ppm	44.6 ppm	32.6 ppm	37.7 ppm
CO_2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

*__AA:__ aseel, __BR:__ black rock, __CX:__ color cross, __K__: kadaknath, __OR:__ rhode island red, __RC:__ red carnish, __VG:__ venjaguda, __VN:__ vanaraja, __WL:__ white leghorn

Mainly, to determine the loss of ignition, the samples were roasted at 1000°C. Further, one gram of the roasted sample was placed in a Pt/Au pot with 6 g of Li2B4O7 and fused into a glass bead at 1050°C. The software searched for all elements in the periodic table between Na and U, but only those with atomic masses greater than the detection limits were reported. The results were normalized to include the sample's LOI, which indicates crystal water and/or oxidation state changes. With each batch of samples, blank and certified reference matter was analyzed.

Mineralogy profiles of ES and ESM

The mineralogy profiles of both ES and ESM after calcination were determined by XRD. The generated diffraction pattern reports were compared with the International Crystal Structure Database (ICSD). The depicted diffraction patterns in **Figure 3**

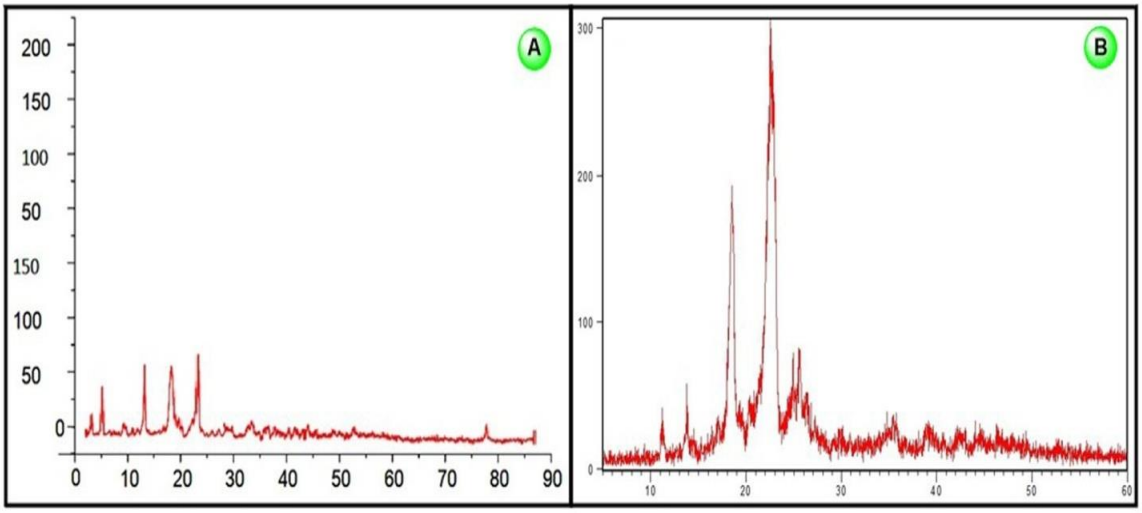

Figure 3. XRD diffractogram: (A) $CaCO_3$ in uncalcined shells (B) CaO after 3 hrs of calcinations.

Showed that, uncalcined $CaCO_3$ was transformed into CaO after 3 hrs of calcination. Thus, uncalcined ES had $CaCO_3$ peaks, but ESM (unseparated) had slightly higher peaks than ES (separated shells). In addition, the CaO peak was observed after calcination as hygroscopic and faint peaks [Ca (OH) 2]. However, soaking time had no effect on CaO formation in Sep17 and Sep60 samples.

Recovery of collagen and other compounds

Both negative and positive ion data are collected and the obtained precise masses are compared to known compounds' precise masses in the compound database (ChemSpider, PubChem, etc.). Majority of compounds were found in nitric acid-leached shells, where 6, 8, and 11 were not found in acetic acid-leached shells (**Table 3**).

Table 3. Identified compounds using UPLC-MS.

Sl. No.	Name of the components	Molecular formula	Molecular mass (g/mol)	Presence in Nitric acid leached effluent	Presence in Acetic acid leached effluent
1	Collagen	$C_8H_7FO_3$	170.1378	+	+
2	Collagen ointment	$C_{18}H_{28}N_2O$	288.4277	+	+
3	Collagen (type II fragment)	$C_{13}H_{24}N_4O_4$	300.3541	+	+
4	Lysozyme	$C_{36}H_{61}N_7O_{19}$	895.4022	+	+
5	Uronic acid	$C_6H_{11}NO_6$	193.0586	+	+
6	Uronic acid	$C_{12}H_{17}Cl_3O_9$	411.6170	+	-
7	Sialic acid	$C_{11}H_{19}NO_9$	309.2699	+	+
8	Sialic acid	$C_{20}H_{36}N_2O_{17}$	576.5030	+	-
9	Chondroitin sulphate A	$C_{14}H_{23}NO_{15}S$	477.3951	+	+
10	Dermatan sulphate	$C_{14}H_{21}NO_{15}S_{-2}$	475.3792	+	+
11	Dermatan sulphate	$C_{18}H_{31}NO_{14}S$	517.5020	+	-
12	Hyaluronic acid	$C_{33}H_{54}N_2O_{23}$	846.7815	+	+
13	Keratan sulphate	$C_{28}H_{48}N_2O_{32}S_4$	1052.9349	+	+

However, 5, 7 and 10 were discovered in acetic and nitric acid leached shells. Collagen was found in both positive and negative modes in Sep17 and Sep60 samples. Herein, three types of collagens were discovered and indicated ES collagen with other valuable compounds can be recovered before shell calcined form CacO3 to CaO.

Functional composition analyses of ES and ESM

Based on the recorded FTIR spectral data, the functional part of the ES and ESM were determined (**Figure 4**).

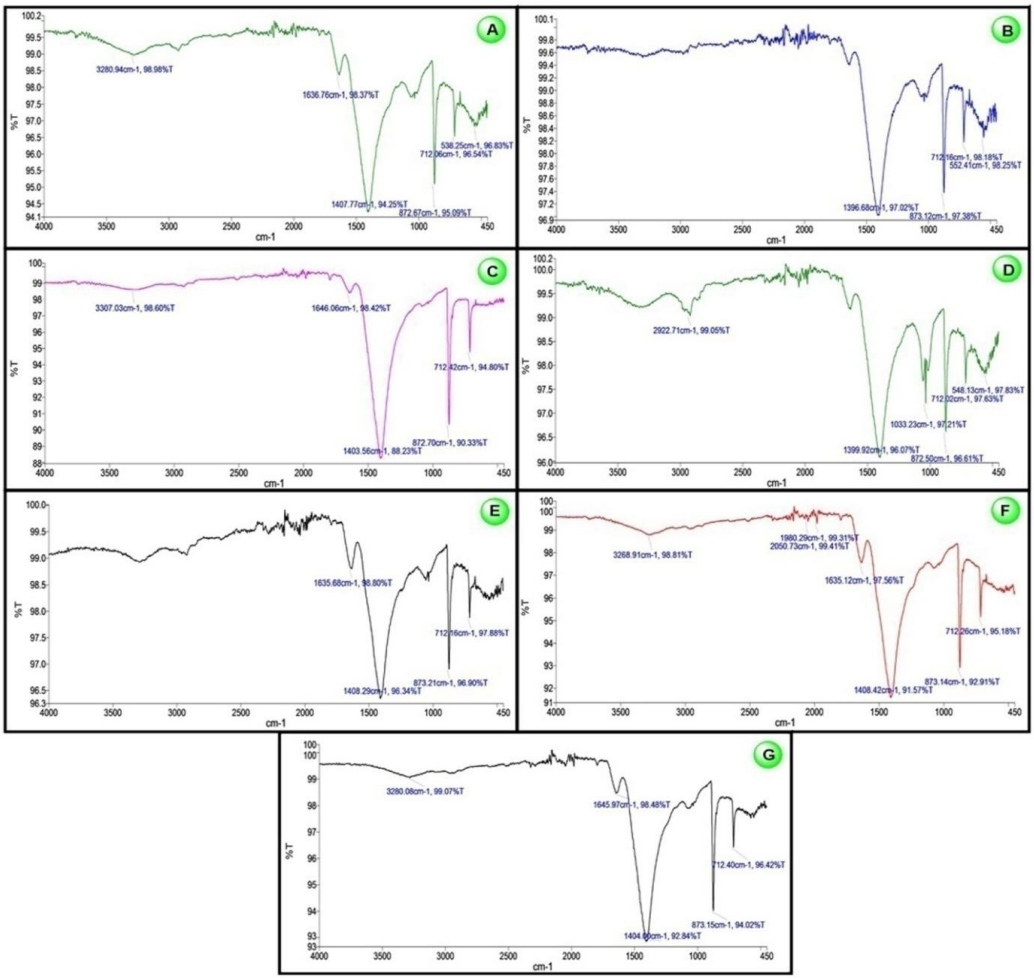

Figure 4. FT-IR spectrum for determining composition. (**A). BR:** black rock, (**B). CX:** color cross, (**C). K**: kadaknath, (**D). OR:** rhode island red, **(E). RC:** red carnish, **(F).VN:** Vanaraja, (**G). WL:** White Leghorn

All ESs exhibited the imperative stretching, O-H (hydroxyl), N-H (amide), alkanes (C=C=C), C=N (azomethine), S=O (sulfoxide) as well as bending like CH_2 (methyl component), halogens (C-F, C-Cl, and C-Br), O-H (hydroxyl), alkenes (C=C) and many more (**Table 4**).

Positions of the maxima of absorption spectra and assignment to the relevant vibrations as recorded in different egg shells.

Sample types	FT-IR	Type of vibrations
	Position of bands (cm^{-1})	
AA	2922.71	$N\text{-}H_{stretch}$
	1399.92	CH_{2bend}
	872.5	$C\text{-}F_{stretch}$
	712.02	$C\text{-}Cl_{stretch}$
	548.13	$C\text{-}Br_{stretch}$
WL	3280.08	$O\text{-}H_{stretch}$
	1645.97	$C\text{-}H_{bend}$
	1404	$C\text{-}F_{stretch}$
	873.15	$C{=}C_{bend}$
	712.4	$C\text{-}Cl_{bend}$
VN	3268.91	$O\text{-}H_{stretch}$
	2050.73	$C{=}C{=}C_{stretch}$
	1980.29	$C{=}C{=}C_{stretch}$
	1635.12	$C{=}C_{stretch}$
	1408.42	$S{=}O_{stretch}$
	873.14	$C{=}C_{bend}$
	712.26	$C\text{-}Cl_{bend}$
BR	3280.94	$O\text{-}H_{stretch}$
	1636.76	$C{=}N_{stretch}$
	1407.77	$C\text{-}F_{stretch}$
	872.67	$C{=}C_{bend}$
	712.06	$C\text{-}Cl_{bend}$
	538.25	$C\text{-}Br_{stretch}$
CX	1396.68	$O\text{-}H_{bend}$
	873.12	$C{=}C_{bend}$
	712.16	$C{=}C_{bend}$
	552.41	$C\text{-}Br_{stretch}$
K	3307.03	$N\text{-}H_{stretch}$
	1646.06	$N\text{-}H_{stretch}$
	1403.56	$C\text{-}F_{stretch}$
	872.7	$C{=}C_{bend}$
	712.42	$C\text{-}Cl_{bend}$
OR	2922.71	$N\text{-}H_{stretch}$
	1399.92	CH_{2bend}
	1033.23	$C\text{-}F_{stretch}$
	712.02	$C\text{-}Cl_{bend}$
	548.13	$C\text{-}Br_{stretch}$
RC	1635.68	$N\text{-}H_{bend}$
	1408.29	CH_{2bend}
	873.21	$C\text{-}F_{bend}$
	712.16	$C\text{-}Cl_{bend}$
VG	2923.25	$N\text{-}H_{stretch}$
	1408.55	CH_{2bend}
	873.27	$C{=}Cl_{bend}$
	712.53	$C\text{-}Br_{bend}$

***AA:** Aseel**, BR:** Black Rock, **CX:** Color Cross, **K**: Kadaknath, **OR:** Rhode Island Red, **RC:** Red Carnish, **VG:** Venjaguda, **VN:** Vanaraja, **WL:** White Leghorn

Based on this study, we concluded that ES contained several organic components as well as numerous inorganic substances; however, their concentration varied between 9 nine chicken breeds ESs.

DISCUSSION

Generally, eggs are composed of three main parts; yolk, albumen and shell. Mainly, their quality depends on their physical make up and chemical composition. Egg quality is the most significant factor contributing to the economics of the sale. Thus, there is a competition in the polarity field and a challenge to provide a constant supply of high-quality eggs to the consumer. Alternatively, genetic engineering and hybrid breeding methods are adopted to improve the egg quality. As a result, hybridization effects on ES composition and quantity of organic materials. In the present study, we looked at the chemical composition of ES and the characteristics of different breeds.

Prior to utilization as value added product rather disposal is of ES. A number of patent filing within 2015-2020 indicated that there is a lot of up scaling research is going on for utilization ES in different biomedical, pharmaceutical applications. Recently several similar types of data have been analyzed on ES with and our data also showed such similarity of profiles. For example, starting from plant growth promoter, food processing, as an active calcium source, catalytic reagent in biodiesel production, as natural component of plastic production, functionalized nanoparticle formation and environment

friendly for heavy metal, dyes, organics, sulfonates and fluoride (Siemiradzka *et al.*, 2018; Ahmed 2019; Apalangya *et al.*, 2019; Ahmed *et al.*, 2021), Thus, towards development of specific value-added products, primary composition is more essential which is supported by this research.

CONCLUSION

As comprehensive preliminary analyses on nine varieties of ES indicated that, the improvement of egg quality also impacts on the percent of chemical composition in ES. Herein, from the calcination and XRF-analysis indicated that, both ES and EMS are composed of calcium with a range of 97.60-98.36%with traceable elements such as chlorine, sulfur, potassium, lutetium, ethereum, iron, manganese, strontium, zirconium and zirconium oxide were present in minimum quantities. In addition, UPLC-MS analysis, biological components such as collagen, uronic acid, sialic acid, hyaluronic acid, keratan sulfate and lysozyme were also found in the collected sample towards more similar to recent studies. In conclusion, the preliminary biochemical, physical analyses help to use ES as a raw biomaterial for various applications with sustainable waste management.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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