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Original Article

HPLC Quantification of Glucosamine Sulfate in Eggshell membranes from White and Brown Chicken Eggs

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ABSTRACT

Eggshell membrane (ESM), a thin membrane lining the eggshell of chicken eggs, is a natural byproduct attracting the attention of many researchers worldwide due to its valuable composition and beneficial biological activities. Its main active ingredients include collagen (types I, V, X), glucosamine sulfate, chondroitin/dermatan sulfate, and hyaluronic acid, so it possesses a beneficial value for maintaining healthy joints. Variation in eggshell color/breed of hens has been reported to affect the quality and composition of eggs and eggshells. So, the current study aimed to compare glucosamine sulfate content in ESM separated from white and brown eggs using the HPLC technique and UV detection at 195 nm. The peak area quantification method was used to determine the concentration of glucosamine in both samples. Results revealed that ESM separated from white eggs has a higher content of glucosamine sulfate (11.83% w/w) than ESM separated from brown eggs (10.68% w/w). We conclude that the use of ESM from white eggs may be more valuable than ESM from brown eggs in further biological studies to determine the effect of ESM in the prevention and management of osteoarthritis.

Keywords: *Eggshell membrane; glucosamine sulfate; HPLC; white eggs; brown eggs.*

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1. INTRODUCTION

Based on the global trend of recycling to convert waste products into valuable raw materials, eggshell membrane (ESM) is considered one of the most promising natural products in the pharmaceutical field instead of being an environmentally waste product. ESM is a thin, bi-layered proteinaceous membrane found between the albumen and calcified eggshell. Recently, many researchers have focused on its variable biomedical applications **[1]**. It has been reported for its therapeutic activity in the case of connective tissue disorders **[2]**, in wound healing

[1], and it can be used in vascular grafts **[3]**. ESM is rich in proteins, such as collagen (type I, X, V) and elastin, glucosamine sulfate, glycosaminoglycans (such as dermatan sulfate, chondroitin sulfate, and hyaluronic acid), and many other components, giving it a beneficial role in maintaining healthy joints **[4].**

Glucosamine ($C_6H_{13}NO_5$), a naturally occurring amino sugar, is found in almost all tissues of the human body and is present in the highest concentrations in cartilage and synovial fluid **[5]**. It stimulates the production of proteoglycans and glycosaminoglycans (GAGs), which are fundamental components of cartilage responsible for giving elasticity, flexibility, and strength to joints **[6]**. The biosynthetic pathway of many GAGs, such as dermatan sulfate, chondroitin sulfate, hyaluronic acid, heparin sulfate, and glycoproteins, depends on the presence of glucosamine **[7] (Fig. 1)**. Glucosamine also prevents the degradation of collagen via the inhibition of inflammatory cytokines **[8]**. With old age, the ability of the body to produce sufficient levels of glucosamine may be decreased, resulting in loss of joint flexibility and an increased risk factor for osteoarthritis **[9]**. So, it plays an important role in osteoarthritis prevention and management. It is considered to be the most commonly used dietary supplement for patients suffering from osteoarthritis **[10]**.

There is no food source for glucosamine. The majority of commercially produced glucosamine is from chitin, the exoskeleton of shellfish such as crabs, shrimp, and lobsters **[11]**. Glucosamine can be available in many forms including, glucosamine sulfate (the most common form), glucosamine hydrochloride, and *N*-acetyl glucosamine **(Fig. 2)**. It is thought that glucosamine sulfate is the best source of glucosamine due to its sulfur content as it is essential for matrix stabilization of connective tissue of cartilage, tendons, and ligaments. And also, stimulates the uptake of sulfate ions to enhance GAGs biosynthesis by chondrocytes **[9]**. Many human studies on the pharmacokinetics of orally administered glucosamine sulfate, including absorption, distribution, and elimination, showed a great absorption rate of as high as 98%, and once absorbed, it is primarily distributed into joint tissues **[12]**.

Identification of glucosamine sulfate combined with chondroitin/dermatan sulfate, hyaluronic acid, collagen, and other components such as desmosine/isodesmosine, sialic acid, ovotransferrin, lysozyme, and lysyl oxidase in ESM attracts many researchers to evaluate its biological efficacy, especially in osteoarthritis management **[13]**.

According to literature studies, it has been reported that variation in egg color and origin leads to quality variability in egg composition **[14]**. Brown chicken eggs have been found to possess a heavier weight and higher levels of albumen and vitamin E than white eggs. White eggs are lighter in weight and have higher levels of yolk, vitamin A, and total fat content (because of increased polyunsaturated and saturated fats in white eggs) than brown eggs **[15]**. The mineral composition and quality of eggshells have also varied according to egg color/origin, with brown eggshells having greater breaking strength and higher levels of calcium, magnesium, copper, aluminum, and zinc than white eggshells **[14]**. The effect of different egg colors/origins on quality as well as the activity of ESM has not been studied before. So, we compared ESM separated from white and brown chicken eggs according to glucosamine content using HPLC analysis.

2. MATERIALS AND METHODS

2.1. Materials and reagents

White eggshells were obtained as waste

products from Hathout, a pastry and sweets factory, Shibin ElKom, Menoufia, Egypt. The white and brown eggs were from different chicken breeds (Lohmann white and Isa brown), grown by El-Fath poultry company, Beheira, Egypt. EDTA powder was obtained from EL-Nasr Pharmaceutical Chemicals Co., Egypt. Sodium dihydrogen orthophosphate and HPLC-grade acetonitrile were obtained from Merck. EMSURE® ACS, ISO, Reag. Ph Eur, Germany. Standard glucosamine sulfate was obtained from AUG Pharm. And certified to contain 99.99(w/w). Ascorbic acid reference was obtained from Arabcomed factory for pharmaceutical products, Obour city, Egypt. Glucosamine compound tablets, Eva Pharma, Egypt.

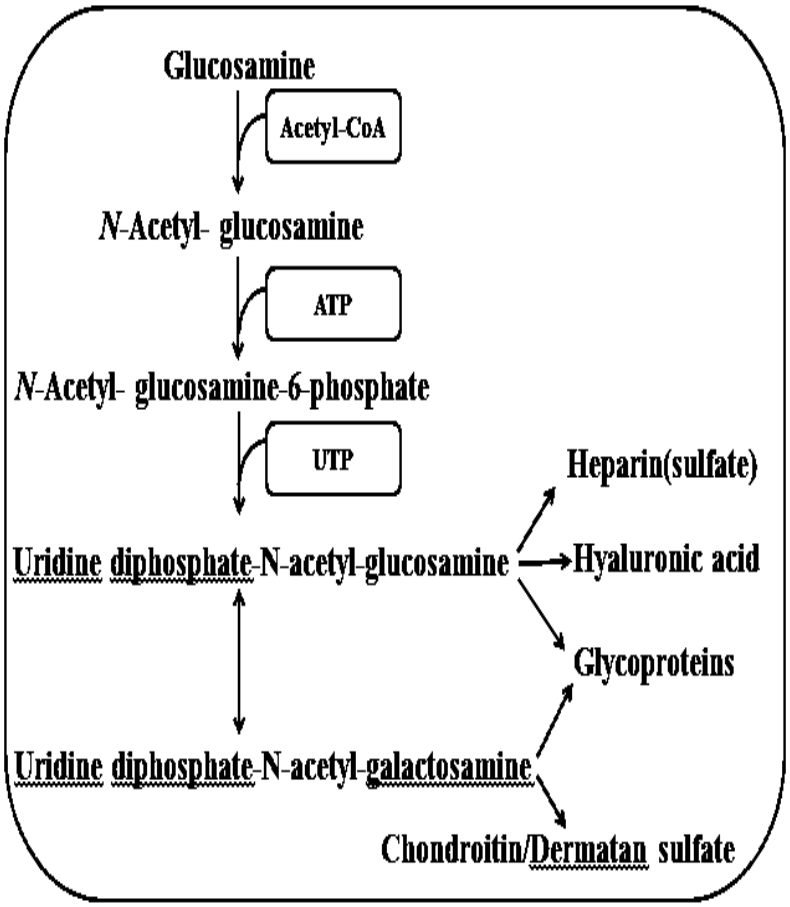
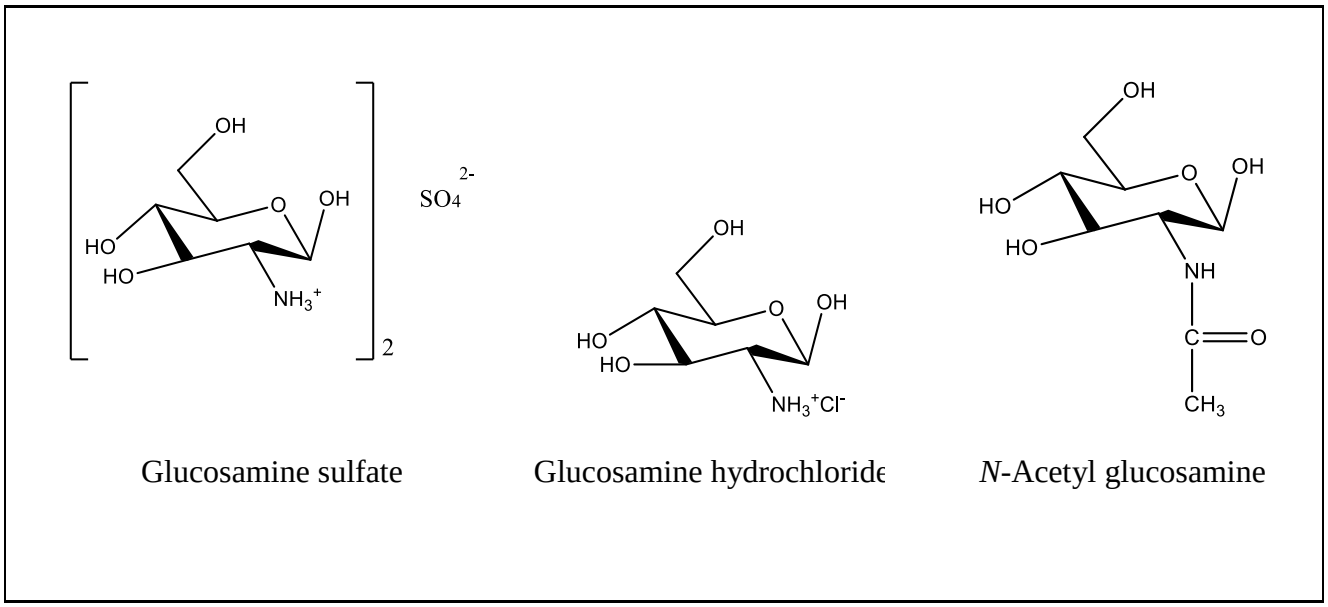


Fig. 1. Role of glucosamine in the biosynthesis of GAGs

Acetyl CoA, acetyl-coenzyme; ATP, adenosine triphosphate; UTP, uridine triphosphate



Glucosamine sulfate Glucosamine hydrochloride *N*-Acetyl glucosamine

Fig. 2. Chemical structures of available glucosamine forms

2.2. Methods

2.2.1. Preparation of eggshell membrane powder

White and brown eggshell waste products were washed with distilled water for 7-10 minutes and then left to dry at room temperature in a well-ventilated and dry place for 3-4 days. The dried shells were ground using a grinder mill (high-speed grinder, China) to obtain a fine powder of eggshell. Separation of the membrane from the calcified shell was done via stirring 100 gm of eggshell powder with 1 liter of 5% EDTA solution by using a magnetic stirrer for (20-30) minutes at a speed of 50 rpm **[16]**. The principle of separation depends on the chelating activity of EDTA as the light particles of the membrane float while the heavier particles of the calcified shell sink. After collecting the membrane through filtration and washing it with distilled water, it was dried in the electric oven (40 °C) taking into consideration avoiding high temperatures to prevent protein content from denaturation **[17]**. This method yielded ESM with a quantity up to 5-7% w/w.

2.2.2.Glucosamine assay in the eggshell membrane by HPLC

A high performance liquid chromatographic method was performed for the assay of glucosamine in ESM samples from white and brown chicken eggs and glucosamine standard (Glucosamine compound® Tablets, Eva pharma, Egypt. Each tablet contained 500 mg of glucosamine sulfate, 400 mg of chondroitin sulfate, and 100 mg of ascorbic acid) by using a C18 reversed-phase sphere orb column with dimensions of 250 mm X 4.6 mm, and a 10 µm particle size using isocratic elution mode with the mobile phase consisting of sodium dihydrogen orthophosphate and acetonitrile with the addition of 1 mL of orthophosphoric acid with a ratio 98:2. The flow rate of the mobile phase was 1 mL/min, the injection volume was 10 µL, and UV detection was at 195 nm **[18]**.

2.2.3. Glucosamine sulfate assay

The quantification of glucosamine sulfate in each sample was calculated through substitution

in the regression equation of the calibration curve.

2.2.4. Statistical analysis

Linear regression was used to calculate the concentration of glucosamine sulfate in samples. Microsoft EXCEL 2010 program was performed for data analysis and drawing the graph. Data were presented as mean ± SD. The level of significance was set at $p<0.0001$.

3. RESULTS

According to the data obtained from the HPLC chromatogram of standard glucosamine sulfate (**Fig. 3)**, the retention time of glucosamine sulfate was identified at 3.869 min. Based on this data, we identified the peak of glucosamine sulfate in glucosamine compound® tablets and made a calibration curve **(Fig. 4)** using different concentrations (100 mg/mL, 250 mg/mL, 375 mg/mL, 500 mg/mL, 625 mg/mL, 750 mg/mL) to be used in the glucosamine sulfate assay in ESM by substitution in the calibration curve equation. By analyzing ascorbic acid reference via HPLC, its retention time was 1.680 min **(Fig. 5)**.

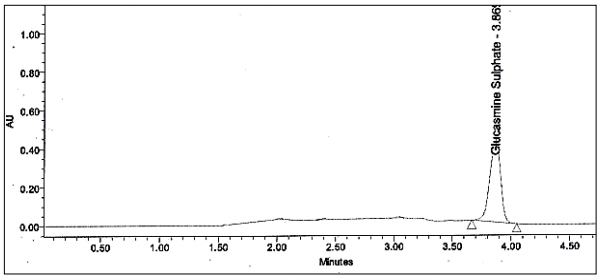


Fig. 3. HPLC chromatogram of Standard Glucosamine sulfate.

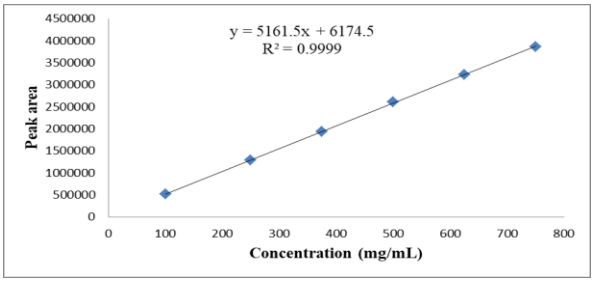


Fig. 4. Calibration curve of glucosamine sulfate

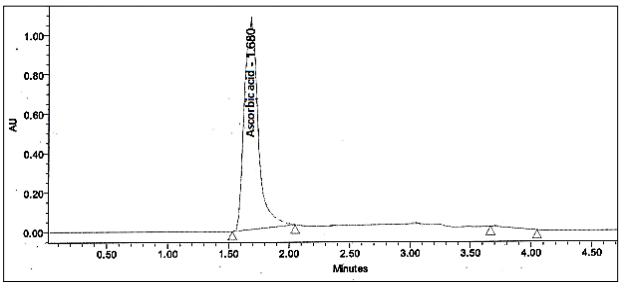


Fig. 5. HPLC chromatogram of Standard Ascorbic acid

The main ingredients of glucosamine compound® tablets are glucosamine sulfate, chondroitin sulfate, and ascorbic acid. After the identification of the peaks of both glucosamine sulfate and ascorbic acid, we could conclude that the third peak, which eluted at the retention time of 2.711, is chondroitin sulfate **(Fig. 6)**. Six replicated injections of the standard solution (glucosamine compound® tablets) show that the area of glucosamine sulfate achieves the system suitability with an RSD% of 0.47 (less than 2.0%) and three replicated injections of each test sample: (A) ESM from white eggs and (B) ESM from brown eggs show that the peak area of glucosamine sulfate achieves the system suitability with an RSD% of 0.025 and 0.033, respectively.

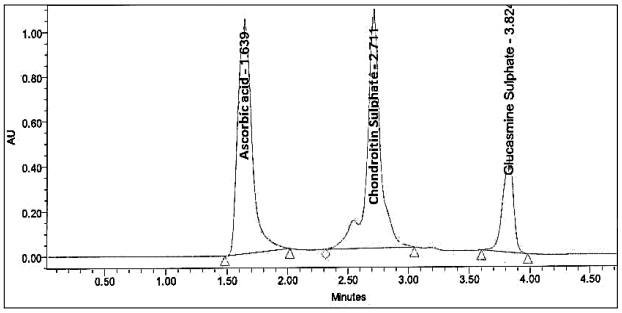


Fig. 6. HPLC chromatogram of Glucosamine compound® tablets

As shown in **Fig. 7**, glucosamine sulfate has been identified in both samples of ESM from white eggs **(Fig. 7A)** and brown eggs **(Fig. 7B)** at retention time 3.83, and chondroitin sulfate has been identified in both samples 2.71.

Mean values ± SD of three measurements of peak areas of glucosamine sulfate obtained from HPLC analyses for standard, ESM from white eggs, and ESM from brown eggs were 2568706 ± 12091.6, 616604 ± 156.476, and 557453 ± 181.165, respectively, where the concentration of ESM in each of the two test samples was 1000 mg/mL. By substitution in the calibration curve equation:

y = 5161.5x + 6174.5, we could calculate the concentration of glucosamine sulfate in each sample, was as follows:

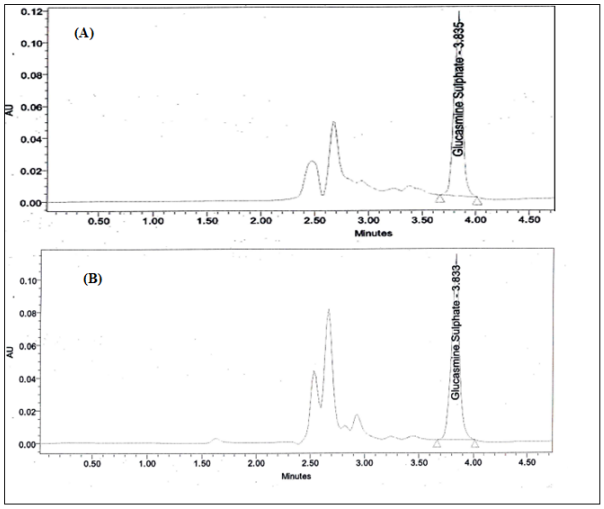


Fig. 6. HPLC chromatograms of test samples: (A) ESM from white eggs. (B) ESM from brown eggs

Concentration of glucosamine sulphate in ESM from white eggs = 118.27 ± 0.03 mg/mL

Concentration of glucosamine sulphate in ESM from brown eggs = 106.8 ± 0.035 mg/mL

So, the percentages of glucosamine sulfate in ESM from white and brown eggs were 11.83% ± 0.003, and 10.68% ± 0.0035, respectively.

4. DISCUSSION

For many years, dietary supplements containing glucosamine sulfate combined with chondroitin sulfate have been widely marketed to be used to manage osteoarthritis all over the world **[19]**. The occurrence of all these valuable ingredients in ESM confirms its ability to maintain healthy articular joints, synovium, and connective tissues and also to repair the harmful effects of osteoarthritis, including cartilage degradation, and inflammation **[20]**, pain, stiffness, and physical disability **[13]**.

In 2020, Marimuthu et al. reported that quantitative analysis of pure natural eggshell membrane separated from eggs of chicken strain BV-300 (white eggs) prepared without any chemical hydrolysis showed 96% total protein content, 17% collagen (type I, V, X), 28.3% chondroitin sulfate, 20.5% GAGs (consisting of repeating units of glucosamine or galactosamine), 27% elastin including desmosine and isodesmosine, and 5% hyaluronic acid **[21]**.

Due to the lack of data reported about the comparison of the glucosamine assay in ESM separated from different chicken breeds, the current study aimed to cover this point. HPLC analysis of ESM separated from Egyptian eggs (white/brown) has shown that glucosamine sulfate content in ESM from white eggs is significantly higher than that from brown eggs ($P<0.0001$).

Conclusion

Different origins of chicken eggs can lead to variations in the quantities of active components identified in ESM. Using HPLC analysis, the glucosamine sulfate content in ESM separated from white eggs was found to be higher than that separated from brown eggs. Further studies are recommended to evaluate the variation in biological activities of ESM from white and brown eggs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data generated or analyzed during this study were included in the main manuscript.

Competing interests

The authors declare that there are no competing interests.

Funding statement

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