Research Article

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Transformation of eggshell waste to egg white protein solution, calcium chloride dihydrate, and eggshell membrane powder

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Abstract: The present study investigated the possibility of complete utilization of eggshell waste (ESW) transforming it to adherent egg white protein solution, calcium chloride dihydrate, and eggshell membranes (ESM). Adherent egg white protein solution was obtained by washing ESW three times with distilled water at 25°C, followed by filtration, and analyzed for the protein content and lysozyme activity. ESM and calcium chloride were obtained simultaneously by the exposure of washed eggshells to 5% hydrochloric acid treatment at 25°C for 3 h, followed by separation by filtration. The separated ESM were washed, dried, and milled to powder and analyzed for protein and lipid content. The calcium chloride solution was exposed to the neutralization of excess hydrochloric acid by calcium hydroxide, followed by evaporation to one-tenth of volume. Calcium chloride crystals were precipitated from the concentrated solution with acetone, separated by filtration, dried at 110°C, and analyzed for chemical composition and purity. The obtained results revealed that 100 g of ESW can be transformed to 1.61 ± 0.34 g of adherent white proteins containing 485,821 U of lysozyme activity, 2.84 ± 0.16 g of ESM powder, and 108.74 ± 3.62 g of calcium chloride dihydrate of high purity.

Keywords: eggshell waste utilization, adherent egg white proteins, eggshell membranes, calcium chloride, lysozyme

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1 Introduction

One of the greatest problems worldwide is the enormous accumulation of waste of all kinds, and among them are those from the agri-food industry whose accelerated development caused by exponential population growth and concomitant increased demand for food, results in the generation of large amounts of waste. However, agri-food industry waste is not just a waste that can be simply discarded, since it presents a source of high-value biocomponents that can be used as secondary raw materials for the production of various high-value-added products such as chemicals, fine chemicals, nutraceuticals, pharmaceuticals, enzymes, etc. [1–3]. However, the implementation of the proposed waste management models is still not efficient in terms of significantly reducing the amount of waste generated in the agri-food industry. The reason for this lies in the fact that different ways of waste utilization are still far from the "*zero waste*" model, which is the main challenge of sustainable waste management and the foundation of the circular bio-economy.

Among the various wastes produced in the agri-food industry, eggshell waste (ESW) is prominent. A total of 1,141 scientific papers related to the possibilities of using

ESW have been published in the WoS CC database from 1973 until today, with over 100 published papers recorded annually, starting from 2018.

Based on the annual worldwide production of hen eggs of 78,949,623 t and 7,770,000 t in the European Union in 2018 [4], and the fact that eggshells comprise about 10–11% of egg mass [5–7], it can be estimated that at least 7,894,962 t of ESW is generated annually worldwide of which 777,000 t is in the European Union. Dependent on its production origin and legal regulations, ESW can be classified into two basic groups: municipal waste and by-products of animal origin, category 3, which is not intended for human consumption. Regardless of whether it is a by-product of animal origin or municipal waste, the majority of ESW unfortunately end up in landfills, additionally burdening the environment. However, if one considers the data on the chemical composition of ESW [6,8–10], it is clear that it is a valuable raw material for the production of a whole range of different products, such as eggshell membranes (ESM), various calcium salts, collagen, hyaluronic acid, bioplastic, etc. This is supported by numerous studies and patents on its utilization [6–8,11–15]. Besides the numerous products that can be derived from ESW and used for various biotechnological applications (among them are the catalysts for biodiesel production or adsorbent for wastewater treatment [11]), the production of high-value-added products from ESW is more oriented toward "zero-waste" model is still of great interest.

The majority of the abovementioned patents and scientific articles dealing with the production of high-value-added products from ESW are oriented toward the production of a single product, thereby resulting in unused waste streams containing valuable components of ESW. Among them, the production of various calcium salts from ESW results in the discarding of adherent egg white proteins and ESM.

In this respect, we have investigated the possibility of complete utilization of ESW for the simultaneous production of adherent egg white proteins as a possible source for the production of lysozyme, ovalbumin, and ovotransferrin; ESM as potential food/pharmaceutical/medical supplement; and calcium chloride as potential food-grade additive. We carefully designed the transformation process oriented toward the "zero-waste" model, where each part of the ESW was used (*adherent egg white proteins, calcified matrix, eggshell membranes*) and each production stream during the transformation was examined on the selected parameters.

2 Materials and methods

2.1 Materials and chemicals

ESW was collected from households and local restaurants from the city of Osijek, Croatia, and kept frozen in hermetically sealed plastic containers at −20°C until further use.

Hydrochloric acid, sulfuric acid, and chloroform were purchased from Carlo Erba (France), while acetone, copper (II) sulfate pentahydrate, and sodium chloride from Gram-Mol d.o.o. (Croatia). Calcium hydroxide and calconcarboxylic acid were obtained for Acros Organics (Spain), sodium hydroxide and ammonium sulfate from Kemika (Croatia), and ethylenediaminetetraacetic acid disodium salt from Fischer Scientific (UK). Bradford reagent was purchased from Bio-Rad (Germany), sodium sulfate from Lach-Ner (Czech Republic), and egg white lysozyme and *Micrococcus lysodeikticus* from Sigma-Aldrich (USA). Glycine, glycerol, bromophenol blue, acrylamide, *N,N′*-methylenebisacrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine, sodium dodecyl sulfate, and Coomassie Brilliant Blue G-250 were purchased from Serva (Germany), and LMW-SDS protein standards were from Cytiva (USA).

2.2 Chemical analysis of ESW

The ESW collected from households and local restaurants, as well as those prepared by washing with distilled water 3 × 30 min, was examined for basic chemical composition, including the dry matter, protein, lipid, and calcium carbonate. The dry matter content was determined by drying the ESW at 100°C until a constant mass was obtained. The protein and the lipid contents were determined by the Kjeldahl method [16,17] and the Folch method [18], respectively; calcium carbonate was determined by complexometric titration using 25 mM EDTA-2Na as a titrant and calconcarboxylic acid as an indicator.

2.3 Production of adherent egg white protein solutions, ESM, and calcium chloride

The production of adherent egg white protein solutions, ESM, and eggshell-derived calcium chloride is shown in Figure 1.

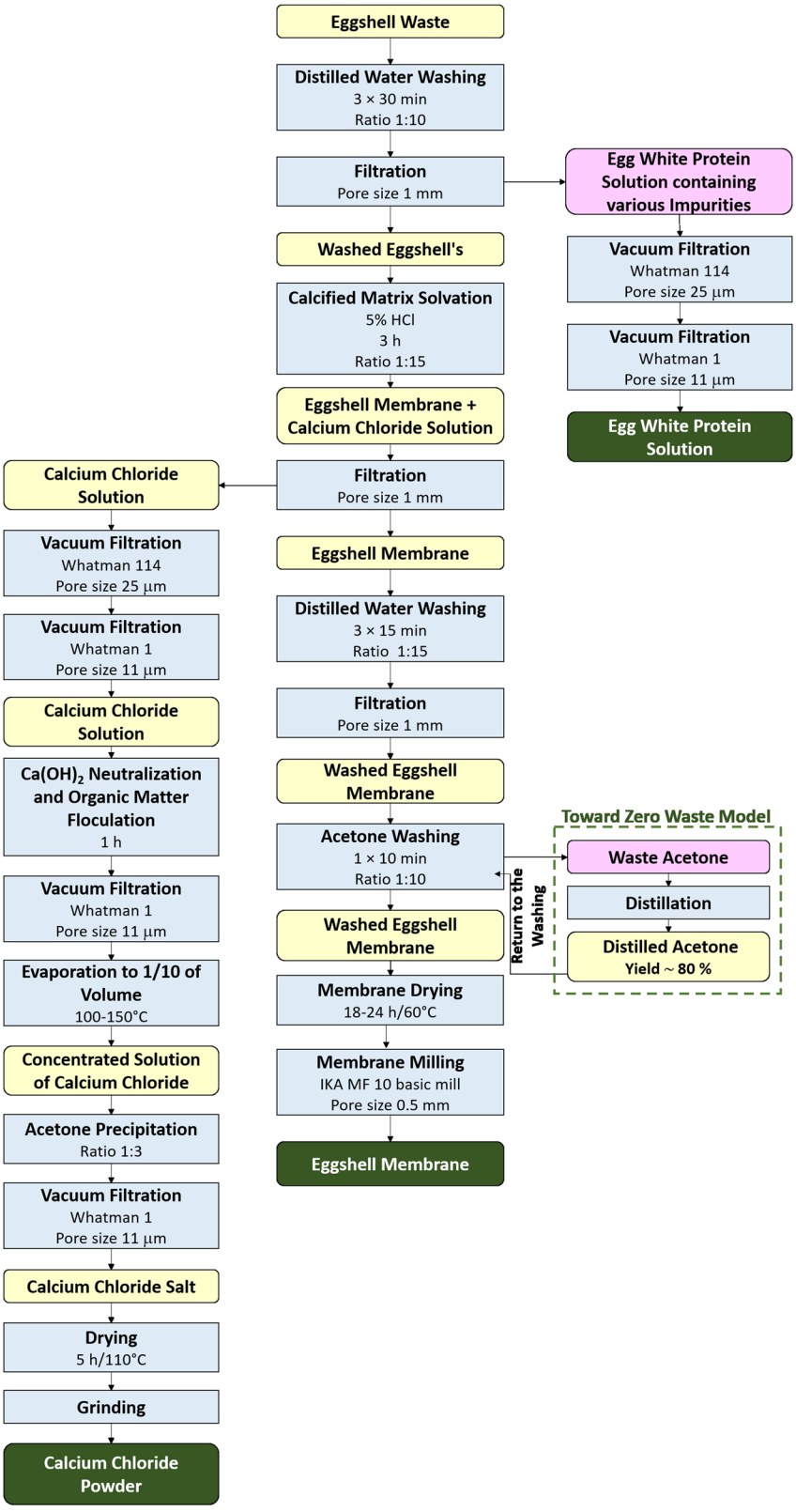

Figure 1: Flow diagram of the proposed procedure for complete utilization of ESW.

The preparation of adherent egg white protein solutions included 3 × 30 min washing of 90 g of ESW with 900 mL of distilled water on an orbital shaker IKA KS 260 basic (IKA, Germany) set at 250 rpm·min^{-1}. Each of the protein solutions obtained after washing was successively filtered through filter paper Whatman 114 followed by Whatman 1. The clear protein solution obtained was used for the analysis of protein content and lysozyme activity.

The washed ESW obtained after three steps of washing was mixed with 1,350 mL of 5% hydrochloric acid. Simultaneously, calcium chloride and ESM were mixed in a magnetic stirrer (LLG, Germany) set at 500 rpm·min^{-1} for 3 h at room temperature. The end of the production process was noted by the absence of foam on the surface of the solution and the absence of any eggshell particles in the solution. After the complete conversion of calcium carbonate to calcium chloride in the ESW, ESM were separated from the calcium chloride solution by filtration through a plastic mesh screen of 1 mm pore size.

The obtained ESM were washed with distilled water 3 × 15 min on an orbital shaker (IKA, Germany) set at 250 rpm·min^{-1}, followed by washing with acetone for 10 min under the same shaking conditions. After separation from acetone, the membranes were dried between 18 and 24 h at 60°C in a thermostatic incubator (Heraeus, Germany). Afterward, the dried ESM were milled using a laboratory mill IKA MF 10.1 (IKA, Germany), equipped with a 0.5 mm pore size sieve, at a speed rotation of 4,500 rpm. They were then kept in closed plastic containers until analysis. A small amount of raw egg-shell membranes was also prepared for comparison. In this case, ESM were separated manually by forceps from the washed ESW, dried between 18 and 24 h at 60°C, and milled with a laboratory mill IKA MF 10.1.

The eggshell-derived calcium chloride solution obtained after separation from the ESM was subjected to two successive steps of vacuum filtration through filter paper Whatman 114 followed by Whatman 1; a clear solution was thus obtained. Excess hydrochloric acid in the clarified calcium chloride solution was neutralized by the addition of calcium hydroxide. $Ca(OH)_2$ was added slowly with continuous mixing of the solution on a magnetic stirrer set at 500 rpm·min^{-1} until all calcium hydroxide was dissolved and the pH of the solution reached 7. Afterward, mixing was shut off and the solution was left to stand for 30 min for organic matter flocculation. The calcium chloride solution was separated from the flocculated matter by vacuum filtration through filter paper Whatman 1, followed by calcium chloride solution evaporation to 1/10th of the volume on a magnetic stirrer, equipped with a temperature probe for heating. The concentrated solution of the eggshell-derived calcium chloride was left to cool to room temperature. Then, three volumes of acetone were added to precipitate the calcium chloride from the saturated solution. The calcium chloride precipitate was separated from the rest of the liquid by vacuum filtration through filter paper Whatman 1 and dried at 110°C for 5 h in a heating oven (Memmert, Germany); white, hard deliquescent fragments were obtained. The fragments were ground in a mortar and pestle to obtain a white powder of calcium chloride, which was stored in sealed plastic containers until analysis.

The process of calcium chloride production was monitored by determining the protein and calcium content in the solution. The protein content was determined by the Bradford method [19], and calcium was determined by complexometric titration using 25 mM EDTA-2Na as a titrant and calconcarboxylic acid as an indicator.

2.4 Chemical and biochemical analysis of the adherent egg white protein solution

The protein content in the prepared adherent egg white protein solution was determined by the Bradford method [19], while lysozyme activity was determined by continuous turbidimetric assay [20].

SDS-PAGE in 15% T polyacrylamide gels was performed on a vertical Hoefer SE 600 Ruby electrophoresis unit, with effective cooling at 15°C maintained by a Thermostatic circulator (Cytiva, USA). Proteins in two parallel gels were separated for 5 h at a nominal voltage of 600 V and a current of 60 mA [21]. The protein bands were stained using the method of Neuhoff et al. [22].

2.5 Characterization of ESM

Chemical analysis of the produced ESM included determining the dry matter, total protein, and lipid content using the same methods as previously mentioned for the analysis of ESW.

FTIR-ATR analysis of the milled raw and HCl-derived ESM was performed on a Cary 630 FTIR ATR spectrometer (Agilent, USA) in the range 650–4,000 cm^{-1}. SEM analysis of the raw and HCl-derived ESM was performed on a Hitachi TM 3030 electron microscope (Hitachi, Japan).

2.6 Characterization of eggshell-derived calcium chloride

The dry matter content of the calcium chloride powder was determined by drying at 105°C until a constant mass,

while the calcium content was determined by complexometric titration using 25 mM EDTA-2Na as a titrant and calconcarboxylic acid as an indicator.

The soluble protein content prepared in a 10% calcium chloride solution was determined by the Bradford method [19]. The prepared 10% calcium chloride solution was also used for pH measurement. The solubility of eggshell-derived calcium chloride was determined according to Domrongpokkaphan and Khemkhao [23].

The free alkali in the produced calcium salts, as well as magnesium and alkali salts, was determined according to FAO [24], and the fluoride content was determined by the ion-selective electrode method [25]. As, Pb, and Hg contents in the eggshell-derived calcium chloride were determined by the ICP-MS technique, which was performed by an external accredited laboratory.

FTIR-ATR analysis of the commercially available calcium chloride anhydrous, calcium chloride dihydrate, and ESW-derived calcium chloride was performed on a Cary 630 FTIR ATR spectrometer (Agilent, USA) in the range 650–4,000 cm^{-1}.

XRD measurement of ESW-derived calcium chloride was performed using a powder X-ray diffractometer (Malvern Panalytical, Netherlands) with Cu-Kα radiation at a scan rate of $2^{o} \cdot min^{-1}$.

3 Results

The present study investigated the possibility of complete utilization of ESW for the production of adherent egg white proteins, ESM, and calcium chloride (ESW-$CaCl_2$) (Figure 1), with ESW-$CaCl_2$ as a potential food-grade additive. The first step in the process development was the analysis of the chemical composition of ESW. This was followed by a carefully designed transformation process oriented toward a "*zero-waste*" model, where each part of ESW was used (*adherent egg white proteins, calcified matrix, eggshell membranes*) and each production stream during the transformation process was examined on the selected parameters.

3.1 Chemical composition of ESW

The basic chemical compositions of originally collected ESW and ESW devoid of adherent egg white proteins (*washed eggshells*) are shown in Table 1. Calcium carbonate was found to be a major component of both the examined ESWs (>92%), followed by proteins, while the lipid content was found to be less than 0.5%. The originally collected ESW contained about 85% of dry matter. The greatest difference between the examined ESW samples was in the protein content; the washed eggshells contained almost half of the proteins less than the originally collected ESW. This was expected since the use of the liquid part of hen eggs for various food preparations results in waste eggshells that contains a sticky layer of adherent egg white proteins, which was removed during ESW washing with distilled water.

Table 1: Chemical composition of ESW[1]

Content	Eggshells	Washed eggshells[2]
Dry matter ($g \cdot 100\ g^{-1}$)	85.00 ± 2.31	99.50 ± 0.03
Proteins ($g \cdot 100\ g_{d.w.b.}^{-1}$)[3]	6.64 ± 0.41	3.87 ± 0.24
Lipids ($g \cdot 100\ g_{d.w.b.}^{-1}$)	0.35 ± 0.05	0.44 ± 0.06
$CaCO_3$ ($g \cdot 100\ g_{d.w.b.}^{-1}$)	92.88 ± 0.29	93.99 ± 0.74

[1]Resulta are presented as mean ± standard deviation of six independent determinations. [2]Washed eggshells were dried for 24 h at 60°C. [3]d.w.b., dry weight basis.

The obtained data on the calcium carbonate, protein, and lipid contents of the examined ESWs were well in accordance with the literature reports of Waheed et al. [8], Ray et al. [9], and Walton et al. [10].

3.2 Adherent egg white proteins of ESW

The washing of ESW with distilled water was the first step in ESW utilization (Figure 1). After two successive filtrations, clear solutions of egg white proteins obtained were examined for the protein content and lysozyme activity (Table 2).

Table 2: Protein content and lysozyme activity in water extracts obtained after ESW washing with distilled water

Washing step	Protein content ($mg \cdot g\ ESW^{-1}$)[1,2]	Lysozyme activity ($U \cdot g\ ESW^{-1}$)[1,2]
First washing	14.88 ± 3.22	3,324.34 ± 983.34
Second washing	1.06 ± 0.34	873.43 ± 318.79
Third washing	0.15 ± 0.14	387.15 ± 182.14
Total	**16.10 ± 3.44**	**4,585.21 ± 1,251.84**

[1]Results are presented as mean ± standard deviation of three independent determinations, each performed in triplicate. [2]ESW, eggshell waste; per 1 g of ESW.

The majority of proteins (92%) and lysozyme (72%) were removed from ESW during the first washing, while with the two next washing steps complete removal of adherent egg white proteins occurred. The total amount of adherent egg white proteins extracted from ESW during three washing steps was 16.1 mg per 1 g of ESW (~1.6%), containing 4,585 U of lysozyme activity per 1 g of ESW. This indicated that a small portion of egg white proteins was inevitably lost during egg breaking and subsequent ESW disposal.

When the data on the total amount of proteins extracted by washing (Table 2) are compared with the estimated amount of adherent egg white proteins calculated as the difference of the protein content in the originally collected ESW and washed ESW (Table 1), a somewhat lower amount of proteins was detected in protein solutions. This was expected since the Bradford method [19] detects proteins in solutions by the dye binding to basic and aromatic amino acid side chains of proteins, while the Kjeldahl method detects all amino-containing compounds within the sample, including amino sugars. It is well known that some of the egg white proteins are glycoproteins whose carbohydrate structure contains amino sugars [26]. Therefore, it is not surprising that a greater amount of proteins was detected by the Kjeldahl method.

While the data on the protein content and lysozyme activity in the protein solution after ESW washing indicated that the adherent egg white proteins has been removed from ESW, it was necessary to prove that it belongs to the egg white proteins. Therefore, SDS-PAGE analysis of proteins in solutions after ESW washing and of egg white proteins was performed (Figure 2).

SDS-PAGE analysis (Figure 2) revealed an identical pattern of proteins present in the water solution after ESW washing and proteins present in the egg white. The protein band intensities of the second washing step were much lower and can be attributed to the protein load, especially if one considers that the amount of proteins in solutions of the second ESW washing was about 14-fold lower than that in solutions of the first ESW washing (Table 2). Nevertheless, SDS-PAGE analysis confirmed that proteins in solution after ESW washing are of egg white protein origin.

Based on all the aforementioned results, it can be safely concluded that ESW contains a small portion of adherent egg white proteins whose presence should not

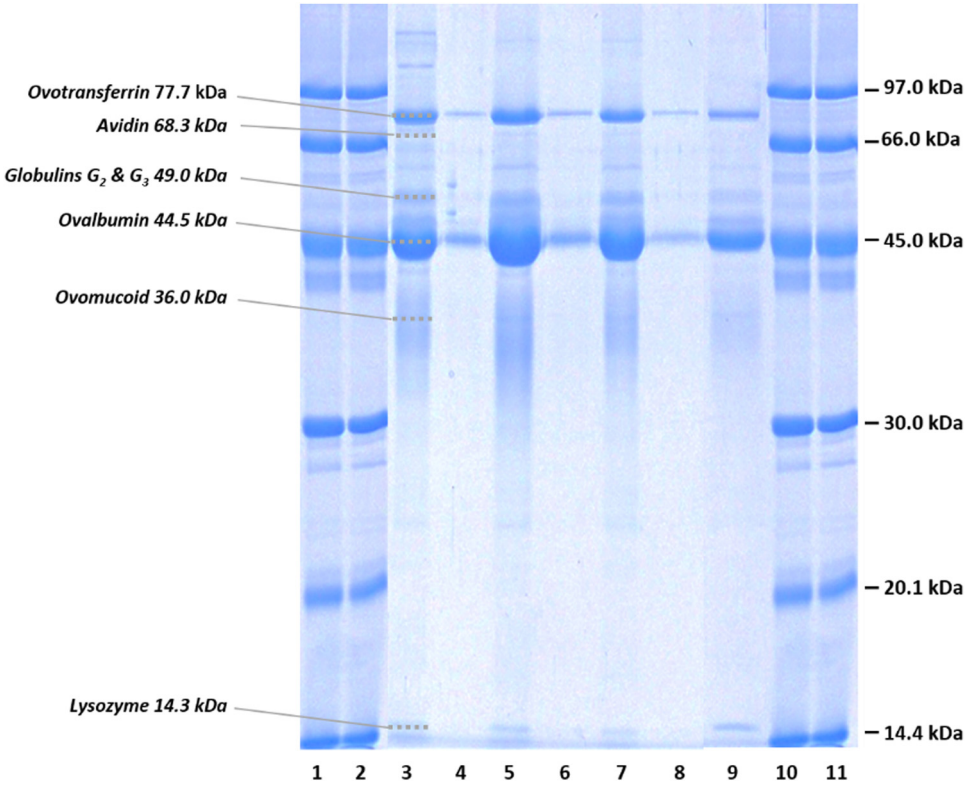

Figure 2: SDS-PAGE (15% T) of adherent egg white proteins extracted during washing of ESW.: lines 1, 2, 10, 11 – low molecular weight protein standards; lines 3, 5, 7 – adherent egg white proteins of three independent production processes (first washing); lines 4, 6, 8 – adherent egg white proteins of three independent production processes (second washing); line 9 – proteins of egg white.

be neglected, especially if one considers the possibility of the production of high-value-added purified proteins such as lysozyme, ovalbumin, and ovotransferrin.

3.3 Characterization of HCl-derived ESM

ESM produced by 5% hydrochloric acid treatment of the washed ESW were (after separation from the calcium chloride solution) washed with distilled water and acetone, and subsequently, dried at 60°C. The dry membranes (Figure 3) were then milled to granulation ≤0.5 mm and used for analysis.

Hydrochloric acid treatment of the washed ESW yielded 2.84 ± 0.16 g of ESM per 100 g of originally used ESW. To the best of our knowledge, this is the first report on the yield of ESM produced by the acid treatment of ESW, so far. However, it should be pointed out that the yield of ESM-HCl found in this research was much lower than the yield (6–10%) reported in the patents of Thoroski [27] and MacNeil [28], where ESM were produced without acid treatment.

Torres-Mansilla and Delgado-Mejía [29] indicated that exposure of ESW to 5% hydrochloric acid might lead to some chemical and physical changes in ESM; therefore, a small amount of raw ESM was prepared by peeling it off from washed ESW and was used for comparison after drying.

Table 3 shows the chemical composition of raw and 5% HCl-produced ESM. Both ESM contained a similar amount of dry matter and lipids but differed in their protein content. The HCl-derived ESM contained a greater amount of proteins than the raw ESM and be attributed to the acid treatment during membrane production. The higher amount of proteins found in HCl-derived ESM was probably caused by the extraction and/or partial hydrolysis of the non-proteinaceous organic matter.

Table 3: Chemical composition of ESM[1]

Content	ESM-Raw	ESM-HCl
Dry matter ($g \cdot 100\ g^{-1}$)	91.45 ± 0.36	92.17 ± 0.39
Proteins ($g \cdot 100\ g_{d.w.b.}^{-1}$)[2]	84.26 ± 0.77	97.03 ± 1.57
Lipids ($g \cdot 100\ g_{d.w.b.}^{-1}$)	0.57 ± 0.06	0.56 ± 0.11

[1]Results are presented as mean ± standard deviation of five independent determinations. [2]d.w.b., dry weight basis.

SEM analysis of raw and HCl-derived ESM (Figure 4) partially supported the abovementioned observation. The three-dimensional network of protein fibrils seemed less uniform, more loosened with thinner protein fibrils, and greater pore sizes in HCl-derived ESM than in raw ones, indicating the extraction of some organic matter during HCl treatment. On the micrographs presented in Figure 4a and b, it can be observed that the raw ESM have a relatively uniform tubular structure with a diameter ranging from 2 to 4 µm; however, in the case of HCl-derived ESM, this structure is eroded with HCl treatment and the morphology loses uniformity (Figure 4c and d). In addition, the presence of small white particles embedded within the surface of raw ESM indicates the presence of calcium carbonate particles, which were obviously stripped off from the eggshells during the manual separation of ESM.

In order to further elucidate the observed differences in the protein content between the raw and HCl-derived ESM, as well as to pinpoint the non-proteinaceous organic

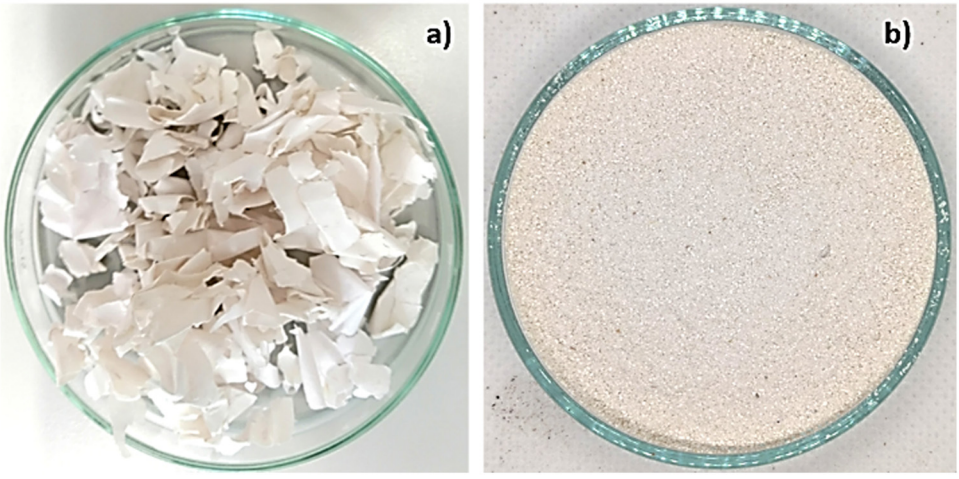

Figure 3: ESM produced after drying (a) and milling (b). (a) Pieces of dry ESM; (b) ESM milled to a size of 0.5 mm.

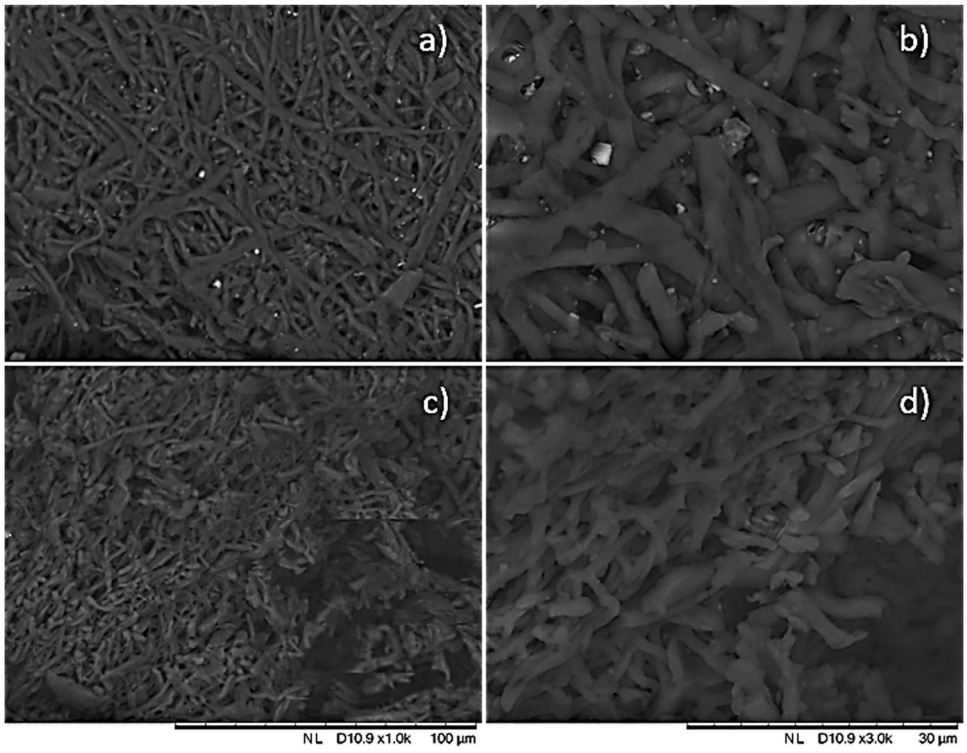

Figure 4: SEM micrographs of ESM. (a and b) Raw ESM. (c and d) ESM prepared by hydrochloric acid solvation of ESW.

matter that has been probably extracted during hydrochloric acid treatment, FTIR-ATR analysis of raw and HCl-derived ESM was performed. The FTIR-ATR spectra of raw and HCl-derived ESM are shown in Figure 5. Both ESM show an identical position of absorption bands attributable to the constitutive proteins, polysaccharides, glycosaminoglycans, and lipids. The band centered at 3,306 cm^{-1} could be assigned to N–H and O–H stretching vibrations in proteins and polysaccharides, the band at 2,911 cm^{-1} could be assigned to the C–H stretching vibration in lipids, and the bands at 1,640 cm^{-1} could be assigned not only to C═O and C–N stretching vibrations of the amide bonds present in proteins, including collagen fibers, but also to the amide bonds present in glycosaminoglycans containing *N*-acetyl-glucosamine and *N*-acetyl-galactosamine including hyaluronic acid; however, the band centered at 1,513 cm^{-1} was solely attributable to the N–H deformation and C–N stretching vibration of amide bonds present in the proteins. The band centered at 1,152 cm^{-1} could be assigned to the C–O stretching vibrations in proteins and carbohydrates, while the bands present in the region from 1,077 to 995 cm^{-1} could be assigned to the C–O stretching vibration in polysaccharides [30–33]. The majority of the observed bands were more intense in the FTIR-ATR spectra of raw ESM than those in the HCl-derived ESM, except the bands detected at 1,640 and 1,513 cm^{-1}. All these implied that polysaccharides were probably the major non-proteinaceous organic matter that was extracted from ESM during the hydrochloric acid treatment of ESW. On the other hand, increased intensities of bands detected at 1,640 and 1,513 cm^{-1} in HCl-derived ESM confirmed the higher amount of proteins detected in ESW-HCl (Table 3). Similar findings

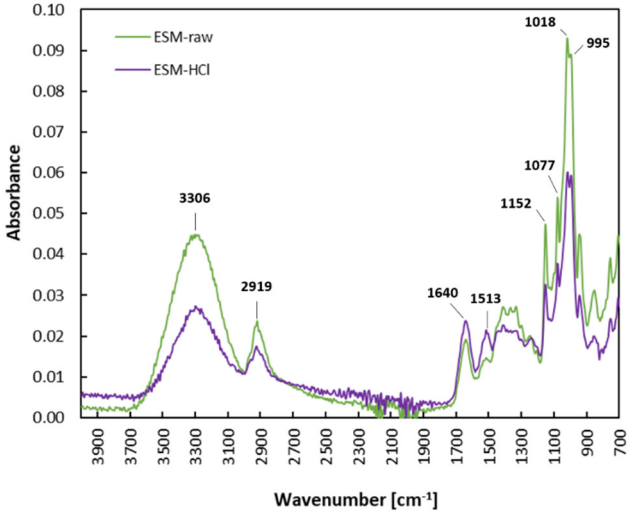

Figure 5: FTIR-ATR of eggshell membranes. ESM-raw – raw eggshell membranes; ESM-HCl – eggshell membranes prepared by hydrochloric acid solvation of ESW.

on the effect of hydrochloric acid on FTIR-ATR spectra of ESM were reported by Torres-Mansilla and Delgado-Mejía [29].

Hence, it can be concluded that about 2.8 g of ESM enriched in proteins can be produced from 100 g of ESW by the hydrochloric acid treatment of washed ESW. This is quite important, especially in considering the positive effect of ESM consumption on the alleviation of joint pains, as reported by Kiers and Bult [34].

3.4 Production and characterization of eggshell-derived calcium chloride

The production of calcium chloride from ESW (Figure 1) was started by mixing washed ESW and 5% hydrochloric acid in a ratio of 1:15, where the estimated molar ratio of ESW calcium carbonate/HCl was 1:2.55. It should be pointed out that HCl was present in excess in order to ensure the complete conversion of ESW calcium carbonate to calcium chloride. The process of complete conversion of ESW calcium carbonate to calcium chloride lasted approximately 3 h, and the end could be noticed by the absence of foam on the surface of the solution and by the lack of any eggshell particles in the solution. Following the separation from ESM and two successive filtrations, the solution was subjected to neutralization of excess HCl by the addition of calcium hydroxide and subsequent organic matter flocculation, which was removed from the solution by filtration. The prepared calcium chloride solution was evaporated to 1/10th of the volume, cooled to room temperature, and mixed with acetone in a volume ratio of 1:3 in order to precipitate calcium chloride. The precipitated calcium chloride was separated by filtration and dried at 110°C for 5 h, milled in mortar and pestle, and the white calcium chloride powder obtained was stored in tightly closed containers until analysis.

The production process was performed in three independent batches, and each batch was monitored by determining the calcium and protein concentrations in calcium chloride solutions.

Table 4 shows the changes in the calcium chloride concentration in solutions during the production process, as well as the $CaCl_2$ yield expressed per dry weight basis of ESW. After the complete conversion of eggshell calcium carbonate to calcium chloride, the $CaCl_2$ concentration in solution was 59.18 $g{\cdot}L^{-1}$, and it increased to 74.61 $g{\cdot}L^{-1}$ upon neutralization of excess HCl with $Ca(OH)_2$. This $CaCl_2$ increase was expected since the reaction between the excess HCl and $Ca(OH)_2$ would result in the formation of $CaCl_2$ as a product. Consequently, the $CaCl_2$ yield increased from 101.33% to 131.92%. The evaporation of the calcium chloride solution to 1/10th of the volume did not change the yield but the calcium chloride concentration was almost 10-fold higher than what was expected.

Table 4: Calcium chloride concentration and yield in solution during production[1]

Production step	γ (calcium chloride) ($g{\cdot}L^{-1}$)	Yield (%)[2]
5% HCl solvation	59.18 ± 1.71	101.33 ± 2.50
$Ca(OH)_2$ neutralization	74.61 ± 2.73	131.92 ± 2.63
Evaporation to 1/10th of the solution volume	730.89 ± 18.92	129.03 ± 4.95

[1]Results are presented as mean ± standard deviation of three independent production processes, each performed in triplicate. [2]Yield is expressed as per dry weight basis of ESW.

A small amount of proteins was found in the calcium chloride solution after the complete conversion of eggshell calcium carbonate to calcium chloride (Table 5). These proteins originated from the eggshell matrix proteins, which were released into the solution during eggshell solvation by 5% HCl but were removed in subsequent production steps. The neutralization of calcium hydroxide and subsequent evaporation resulted in the almost complete removal of proteins present in calcium chloride solutions. In the end, the concentration of proteins present in the concentrated solution of calcium chloride was around 0.03 $g{\cdot}L^{-1}$.

The production of powdered calcium chloride from ESW was found to be quite satisfying with an average yield of 108.74 ± 3.62 g of calcium chloride per 100 g of wet weight of ESW. Therefore, it was of interest to characterize the calcium chloride produced and to determine whether it meets the criteria as an additive prescribed by

Table 5: Changes in the protein concentration during the production of eggshell-derived calcium chloride[1]

Production step	γ (proteins) ($g{\cdot}L^{-1}$)	Protein removal (%)[2]
5% HCl solvation	0.35 ± 0.04	—
$Ca(OH)_2$ neutralization	0.11 ± 0.01	67.06 ± 4.10
Evaporation to 1/10 of solution volume	0.03 ± 0.01	98.99 ± 0.46

[1]Results are presented as mean ± standard deviation of three independent production processes, each performed in triplicate. [2]Expressed as the ratio of the protein content present in the solution and protein content in the solution after 5% HCl solvation.

FAO [24,25] and EU [35]. The characterization of eggshell-derived calcium chloride powder is shown in Table 6.

The obtained white powder of eggshell-derived calcium chloride contained about 28.37% of calcium, as determined by complexometric titration, which was slightly higher than the theoretically calculated amount of calcium (27.26%) in calcium chloride dihydrate, indicating that the calcium chloride formed might be in the dihydrate form. The eggshell-derived calcium chloride was quite soluble in water with a solubility of up to 0.8 g per 1 mL of water. The pH value of the 10% solution was about 9.34, while soluble proteins were only present in traces. The ESW-derived $CaCl_2$ fully met all prescribed criteria as an additive. Free alkali was less than 0.074%, which agreed with FAO criteria [24]; >0.15% was not allowed. The magnesium and alkali salt contents were about 5-fold and the fluoride content was about 4-fold lower than those allowed by FAO [24] and EU [35]. The As, Pb, and Hg contents in the produced eggshell-derived calcium carbonate were below the defined maximal values prescribed by EU: As (<3 $mg \cdot kg^{-1}$), Pb (<2 $mg \cdot kg^{-1}$), and Hg (<1 $mg \cdot kg^{-1}$) [35].

Since the data of complexometric titration indicated that the produced eggshell calcium chloride might be in the dihydrate form, the calcium chloride powder was further analyzed by more sophisticated methods: FTIR-ATR and X-ray diffraction. The FTIR-ATR spectra of the three production batches of eggshell-derived calcium chloride are shown in Figure 6. It can be seen that the produced calcium salts showed almost identical positions of absorption bands as those of commercially available calcium chloride anhydrous and calcium chloride dihydrate, all attributable to the symmetric or asymmetric O–H stretching in crystalline water (3,485, 3,345, 3,216 cm^{-1}) and H–O–H bending vibration frequency in crystalline water (1,628, 1,613 cm^{-1}) [36]. The absorption

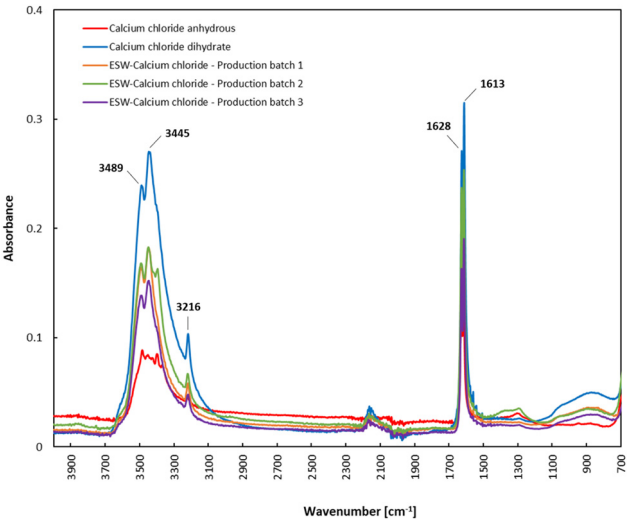

Figure 6: FTIR-ATR of eggshell-derived calcium chloride. Infrared spectra of eggshell-derived calcium chloride of the three production batches are compared with the spectra of commercial calcium chloride anhydrous and calcium chloride dihydrate.

band intensities of all three production batches of ESW-$CaCl_2$ were quite similar, indicating the uniformity of the production process. When FTIR-ATR spectra of the produced ESW-$CaCl_2$ were compared with the spectra of commercially available $CaCl_2$ and $CaCl_2 \cdot 2H_2O$, it could be seen that the ESW-$CaCl_2$ absorption band intensities were higher than those of $CaCl_2$ anhydrous, and a bit lower than those of calcium chloride dihydrate bands, indicating that ESW-derived calcium chloride is probably in the form of dihydrate.

The XRD analysis, shown in Figure 7, confirmed that the produced eggshell-derived calcium chloride is

Table 6: Characterization of eggshell-derived calcium chloride

Parameter	Value
Dry matter (%)	98.81 ± 0.41
Calcium ($\%_{d.w.b.}$)	28.37 ± 0.43
Solubility in water ($g \cdot mL^{-1}$)	≤0.8
pH of 10% solution	9.34 ± 0.15
Free alkali (%)	<0.074
Soluble proteins ($g \cdot 100\ g_{d.w.b.}^{-1}$)	0.02 ± 0.01
Magnesium and alkali salts ($mg \cdot g^{-1}$)	9.77 ± 1.99
Fluoride ($mg \cdot g^{-1}$)	<10
As ($mg \cdot g^{-1}$)	<1
Pb ($mg \cdot g^{-1}$)	<1
Hg ($mg \cdot g^{-1}$)	<0.01

d.w.b., dry weight basis.

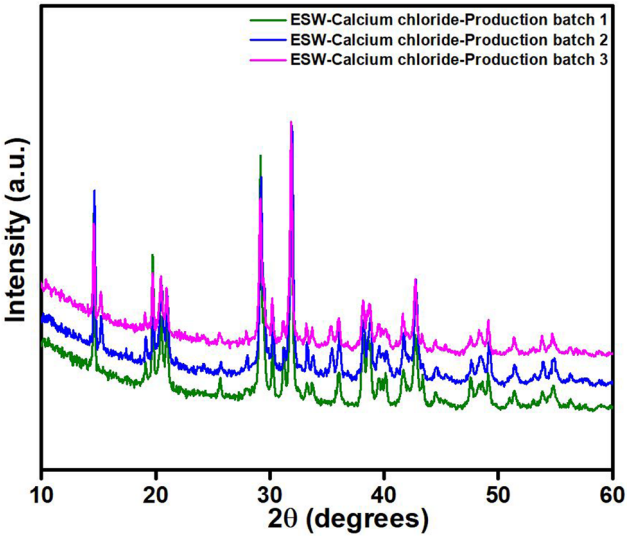

Figure 7: XRD analysis of ESW-derived calcium chloride.

predominantly of the dihydrate form. The position and intensity of the major peaks were identical to those reported by Garnjanagoonchorn and Changpuak [37], while the peak sharpness indicated the crystalline state of eggshell-derived calcium chloride. The identical position and similar peak intensities of calcium chloride of the three production batches confirmed the uniformity of the production process, while the lack of secondary phases indicated the high purity of the produced salts.

Based on all the abovementioned results, it can be safely concluded that about 109 g of calcium chloride dihydrate of high purity can be produced from 100 g of the wet weight of ESW by the hydrochloric acid treatment. Moreover, based on the fact that produced salts fully meet the criteria for calcium chloride as an additive, the production of calcium chloride dihydrate from ESW seems quite promising.

Although several reports on the production of calcium chloride from ESW have been already published [23,37–39], it should be pointed out that the authors did not use the originally collected ESW *per se* for the calcium chloride production but used the dried and milled one. Moreover, these reports were solely oriented toward calcium chloride production, without analyzing the solution after ESW washing and information on the concomitant ESM production. On the contrary, we used originally collected ESW fragments, and after the washing step, we immediately performed the simultaneous production of calcium chloride and ESM. Nevertheless, all these reports, including ours, showed the possibility of calcium chloride production from ESW by the hydrochloric acid treatment.

When the data on the production yield and characteristics of eggshell-derived calcium chloride dihydrate are compared with the abovementioned literature reports, it can be concluded that the calcium chloride produced in our work was of the highest purity and highest yield. For example, Garnjanagoonchorn and Changpuak [37] reported a yield of 90.80%, while Domrongpokkaphan and Khemkhao [23] reported a yield of 80.35%, which are lower than the yield of 108.74% obtained in our research. However, it should be pointed out that the increased yield of calcium chloride obtained in our work was obviously due to the neutralization of the excess hydrochloric acid with calcium hydroxide, which was not carried out by Garnjanagoonchorn and Changpuak [37] and Domrongpokkaphan and Khemkhao [23]. Organic matter flocculation following neutralization of the excess hydrochloric acid by calcium hydroxide obviously led to the production of calcium chloride of high purity, as can be seen in Table 6. In comparison with the data on the impurity content in calcium chloride in the work of Garnjanagoonchorn and Changpuak [37], the produced calcium chloride dihydrate in our work had lower amounts of magnesium and alkali salts, fluorides, and heavy metals (calculated as Pb).

4 Discussion

Keeping in mind the current state of ESW utilization/landfilling, as well as the need/wish for the production of high-value-added products, we have designed the ESW transformation process oriented toward "zero-waste" model, where each part of ESW was used (*adherent egg white proteins, calcified matrix, eggshell membranes*) and each production stream was examined on the selected parameters (Figure 1).

From 100 g of ESW collected from households and restaurants, containing 85% of dry matter (Table 1), we have successfully produced 1.61 g of egg white proteins present in filtered water solution (Tables 2), 2.84 g of ESM powder, and 108.74 g of powdered calcium chloride dihydrate of additive purity.

It is well known that egg-breaking in households and restaurants for food production and consumption, and egg-breaking in plants for the production of liquid and/or frozen eggs, results in ESW containing a small amount of egg white visible as a sticky layer associated with ESM. While the presence of adherent egg white proteins in ESW produced in egg-breaking plants has been reported by Walton et al. [10], to the best of our knowledge, there are no available data on their composition. This has been overcome by our research where we have proved by SDS-PAGE analysis (Figure 2) that adherent egg white proteins of ESW originate from egg white. Moreover, a significant activity of egg lysozyme has been detected in the clarified protein solution (Table 2). The use of egg white for the production of various purified proteins, including lysozyme as a high-value-added product, i.e., fine chemicals, has been well established [26,40]. Although the amount of proteins found in the water solution after washing ESW with distilled water was low (Table 2), it seems quite possible that the use of HiTrap ion-exchange columns used for protein purification [41] might overcome this problem, simply by concentrating the proteins of interest. Among proteins present in the clarified adherent egg white protein solutions, the most prominent are ovotransferrin, lysozyme, and ovalbumin, whose cost in the market of fine chemicals can reach up to 388 € per g of purified protein [6]. Thus, it seems quite possible that egg white proteins removed from ESW during washing might

be used for the production of purified high-value-added proteins. Nevertheless, further elaboration on the possibility of their purification from adherent egg white protein solution obtained after ESW washing is one of the future investigations of our research group.

The exposure of washed eggshells to 5% hydrochloric acid treatment at room temperature resulted in the subsequent production of calcium chloride and ESM.

Hydrochloric acid-derived ESM differed from the raw ones both by chemical composition (Table 3) and physical characteristics (Figures 4 and 5). HCl-derived ESM contained a greater amount of proteins (Table 3) and possessed thinner protein fibrils in the 3-D protein network compared to the raw ones (Figure 5). The differences between raw and HCl-ESM were caused by the hydrochloric acid treatment where complete conversion of eggshell calcium carbonate to calcium chloride was obtained, and concomitant extraction and/or hydrolysis of constitutive ESM polysaccharides by HCl occurred, which was observed by FTIR-ATR analysis (Figure 4). While the yield of HCl-derived ESM was lower than the reported yield of membranes produced without acid treatment [27,28], the positive effect of ESM on human health [12,34] and quite promising use of ESM as carriers for enzyme immobilization [7] justifies their production.

Calcium chloride dihydrate was the major product of the designed ESW utilization process. The white powder of eggshell-derived calcium chloride was predominantly in the dihydrate form (Figures 6 and 7) and fully met all prescribed criteria for calcium chloride as additive no. 509 [24,35].

5 Conclusions

The present study investigated the possibility of transformation of ESW to value-added products by using the designed transformation process, which was found successful in the complete utilization of ESW at the laboratory scale level, where adherent egg white protein solution, ESM, and calcium chloride dihydrate of additive purity were produced. However, for any possible implementation at the industrial level, scaling-up of the proposed process is necessary. This is one of the future investigations planned by our research group, where besides the process scaling-up, industrial ESW from egg-breaking plants is planned to be used. Moreover, the implementation of spray drying instead of acetone precipitation followed by drying will be examined since it might lead to the production of calcium chloride of potentially higher purity, or even the production of anhydrous calcium chloride. In addition, another part of our future investigation will be oriented toward the examination of the possibility of the production of purified lysozyme, ovalbumin, and ovotransferrin from the adherent egg white protein solution by ion-exchange chromatography. All this will further contribute to the development of the process of complete utilization of ESW approaching the "*zero waste*" model intended for the possible industrial application.

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Data availability statement: The datasets generated for this study are available on request to the corresponding author.

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