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Optimization Of Solvent Concentration And Extraction Time Of Chicken Eggshell Protein For Gel Peel-Off Face Mask Application Using Response Surface Methods

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ABSTRACT

Chicken eggshells, commonly discarded as household and industrial waste, present a valuable opportunity for sustainable use due to their abundant quantity. This study explores the potential of utilizing chicken eggshells as a natural protein source to create eco-friendly skincare products, particularly peel-off gel masks, which can combat free radicals and enhance skin elasticity and moisture. The research focused on determining the optimal conditions for protein extraction from chicken eggshells using acetic acid solvent and varying extraction times. Response Surface Methodology (RSM) was employed to optimize these conditions. The protein extraction process involved testing the extracted protein for biuret reaction using spectrophotometry, determining protein content with Ultra Performance Liquid Chromatography (UPLC), and evaluating the physical properties of the resulting peel-off gel mask. The results indicated that both solvent concentration and extraction time significantly impact the yield of protein. The optimal conditions were found to be an acetic acid concentration of 0.5 M and an extraction time of 24 hours, which produced a yield of 5.064%. The RSM model validation showed a p-value < 0.05, a desirability index of 0.887, and a predicted yield of 3.080%, with a deviation of 1.98% from the actual yield. These findings suggest that the model is accurate and reliable for further development of the peel-off gel mask. This approach not only provides a sustainable use for eggshell waste but also contributes to the development of high-quality, natural skincare products.

Introduction

Indonesia ranks second as the country with the most food waste in the world. Each resident can produce 300 kg of food waste per year. One type of waste produced is eggshells, which correlates with the level of egg consumption. The production of poultry eggs in Indonesia increases annually. In 2022, poultry egg production in Indonesia reached 5.6 million tons, making eggshells a type of waste that has the potential to cause pollution due to microbial activity in the environment [1].

Although often considered as waste, chicken eggshells have interesting characteristics and chemical properties, and contain various useful components. Some studies abroad have utilized the benefits of chicken eggshells [2].

Eggshells are the outer part of the egg, known to be rich in protein and calcium. The composition of eggshells consists of water (2%) and dry matter (98%). The dry matter contains mineral elements (92%) and protein (6%). Based on the mineral composition, eggshells are composed of $CaCO_3$ crystals (98.43%), $MgCO_3$ (0.84%), and $Ca_3(PO_4)_2$ (0.75%) [3].

Chicken eggshell protein consists of various types of proteins (about 3.5%), including glycoproteins and proteoglycans, as well as water content. One of the proteins found in it is ovocleidin-17 (OC-17), which is the first result from the purification of chicken eggshells. Proteomic analysis conducted by

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researchers shows that OC-17 is present in very abundant amounts in the chicken eggshell matrix. The OC-17 protein plays a significant role as a catalyst in accelerating eggshell formation. Scientists from the University of Sheffield and Warwick used the HEC-ToR supercomputer to reveal that OC-17 plays a crucial role in initiating the crystallization process, which is the initial stage of eggshell formation. The OC-17 protein has the ability to transform calcium carbonate into calcite crystals that form the eggshell structure. Although calcite crystals are also found in various bones and skeletons of other birds, their formation process in chickens occurs more rapidly. Chickens can produce about 6 g of eggshell every 24 hours [4].

To obtain protein content from chicken eggshells, an extraction process is required. Protein extraction from natural sources is based on the type of solvent used, such as water, alkali, organic solvents, and acids. Although it depends on the characteristics of the protein being extracted, the process conditions also affect the final yield of the obtained protein. Extraction methods are often used because proteins have high solubility and good stability when isolated in water. Additionally, this extraction process is easy to operate and has low costs [5].

Gel-based peel-off masks enriched with antioxidants are considered an excellent choice. This is because the use of gel on the skin can enhance the absorption of polyphenols into the skin. Using peel-off gel masks is quite practical and offers several benefits, such as helping to tighten and refresh the skin. The antioxidant effects can achieve optimal results when the peel-off gel mask can be evenly spread on the skin's surface. Additionally, the main requirement for an acceptable peel-off gel mask is a drying time between 15-30 minutes. Ingredients like HPMC and PVA are the main components that influence the gel's characteristics, including the spreadability and drying time of the gel product [6].

The use of natural ingredients in cosmetics is preferred over synthetic ones because synthetic ingredients can cause side effects and even damage the skin's natural structure. Peel-off gel face masks are usually formulated using a polyvinyl alcohol (PVA) base, which, when applied and dried, forms a layer that tightly covers the face. Active substances are added to the formulation to enhance the sealing and tightening effects. This formulation includes softeners, moisturizers, preservatives, surfactants, fragrances, and active ingredients. PVA plays a crucial role in providing the peel-off effect due to its adhesive properties, which allow it to form a film layer that can be easily peeled off once dried [7].

RSM is a combination of statistical and mathematical techniques used to determine the optimal conditions of an independent variable that affects a response and to optimize the response. The advantage of RSM is the reduced number of experimental units needed to obtain statistically acceptable results, thereby saving time and costs. This method is used to develop, improve, and optimize the process of determining the optimal formulation. Its application is particularly important in the fields of design, development, and formulation of new products, as well as in the improvement of existing product designs [8]. This research will optimize the protein extraction process using the RSM method on the main raw material, namely chicken eggshells, to achieve the desired physical and chemical properties. The research outcome, in the form of a gel peel-off face mask, will contribute to the development of environmentally friendly cosmetic products.

Methods

Chicken eggshells were collected, cleaned with distilled water, dried at 40°C for 60 minutes, and ground into powder. The proximate analysis of the powder included tests for ash, moisture, fat, and protein content using standard methods. For protein extraction, 50 g of eggshell powder was mixed with 200 ml of 0.1N NaOH, soaked at 10°C for 48 hours, and then rinsed until neutral pH. The mixture was hydrolyzed with 500 ml of acetic acid at varying concentrations (0.5M, 1M, 1.5M, 2M, and 2.5M) and extraction times (24, 48, and 72 hours). The hydrolyzed solution was stored at 10°C for 48 hours, washed with water at 45°C, and then dried to obtain protein powder. The Biuret test was used to measure protein content, and the optimum protein extraction was analyzed by UPLC. RSM was employed to determine the optimal conditions for extraction and peel-off gel formation, including acetic acid concentration and extraction time, using Design Expert-13. The gel peel-off mask was prepared by dissolving PVA, glycerin, methyl paraben, and propyl paraben, combined with HPMC and the optimized eggshell extract, and allowed to gel. The physical characteristics of the gel peel-off mask, including pH, spreadability, adhesion, drying time, and antioxidant activity, were evaluated.

Results and Discussions

Proximate Analysis of Chicken Eggshells

Chicken eggshells are the outermost part of the egg, serving to protect the inner components of the egg from physical, chemical, and microbiological

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damage. So far, eggshell waste has not been optimally utilized for cosmetic purposes and is only used for crafts and animal feed. The mineral content within eggshells plays an important role in maintaining bodily functions at the cellular, tissue, organ, and overall body levels. To assess the nutritional content of chicken eggshells, proximate analysis has been conducted, with the results presented in **Table 1.**

Table 1. Proximate Analysis of Chicken Eggshells

Sampel	Parameter	Result (%)	Standard of AOAC 2005 (%)
Chicken Eggshell	Ash Content	44.66	90
	Water Content	1.25	1.50
	Fat Content	1.27	2.70
	Protein Content	6.50	7.05

The proximate analysis of chicken eggshells reveals significant deviations from AOAC 2005 standards. The ash content (44.66%) is notably lower than the 90% standard, likely due to residual membrane components or differences in raw materials. This low ash content indicates reduced mineral presence, which is crucial for skin health benefits. The moisture content (1.25%) is slightly below the standard, suggesting better product stability and reduced microbial growth, beneficial for peel-off gel masks. The fat content (1.27%) is lower than the AOAC standard (2.70%), which is advantageous for non-comedogenic formulations. The protein content (6.50%) is close to standard levels and supports skin health by enhancing skin structure, elasticity, and active ingredient penetration [9].

The Effect of Acetic Acid and Extraction Time on Chicken Eggshell Protein Extract

The protein extraction process from chicken eggshells involves several stages to maximize yield. Initially, eggshell samples are dissolved in a 0.1N NaOH solution and soaked for 48 hours at 10°C to separate the protein from the solid matrix [10]. The mixture is then extracted using acetic acid at varying concentrations 0.5M; 1M; 1.5M; 2M; 2.5M and extraction times 24 hours; 48 hours; 72 hours. Following extraction, the solution is stored at a cold temperature to maintain sample stability. The acetic acid hydrolysis solution is washed with distilled water at 45°C to remove residual solvents and impurities. Finally, the wet protein is dried using a freeze dryer to produce a stable, dry protein suitable for long-term storage.

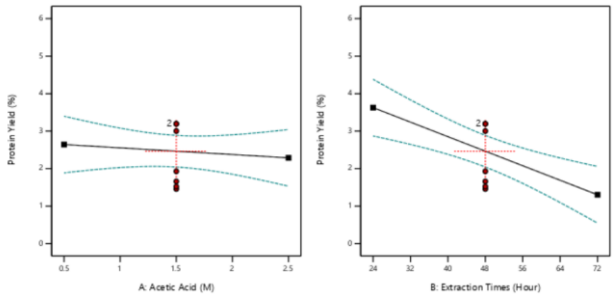

Figure 1. Effect of Acetic Acid and Extraction Time on Protein Content

The results of the eggshell extraction showed that the protein content in the sample reached 5.281% with a yield of 5.064% at an acetic acid concentration of 0.5M and an extraction time of 24 hours **(Figure 1)**. This indicates that low concentrations of acetic acid and shorter extraction times are more optimal, as higher concentrations can denature proteins or damage their structure, and extended extraction times can lead to protein degradation or excessive washing, reducing the extractable protein content. Previous research on collagen extraction from chicken feet and buffalo skin supports these findings, showing optimal values for similar concentration and extraction time parameters [11]. Excessive acid concentration can cause over-hydrolysis, converting collagen chains into gelatin. Additionally, the duration of acid extraction should be precise, as longer extraction times increase water absorption by the skin, making collagen fibers easier to separate and extract [12].

Protein Content Analysis

The protein content of chicken eggshell extract was analyzed using the Biuret method with a UV-Vis spectrophotometer, known for its specificity to proteins and its ability to exclude non-protein nitrogen components. This method is favored for its rapid execution compared to other techniques [13]. Proteins are composed of long peptide chains, and in the Biuret test, the intensity of the purple color formed correlates directly with the length of the peptide chains or the concentration of protein in the sample; longer peptide chains or higher protein concentrations yield a more intense purple color [14].

In this study, a Bovine Serum Albumin (BSA) standard solution was prepared in varying concentrations, as shown in **Figure 2**. The chicken eggshell protein extract was reacted with Folin Phenol (FP) reagent, resulting in a color change to purple. The Biuret reaction is specific to peptide bonds within proteins, where a higher protein concentration leads to a more intense purple color.

This color change is due to the formation of a complex between Cu^{2+} ions in the Biuret reagent and N-terminal amino groups in the albumin solution under alkaline conditions, producing a purple-colored complex [15].

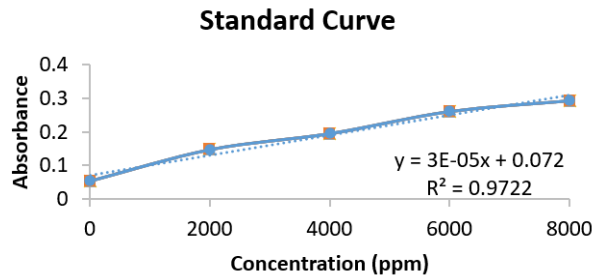

Figure 2. Calibration Curve of Standard Protein Solution (BSA)

The absorbance of protein sample solutions was measured using a spectrophotometer at a wavelength of 540 nm. The measured absorbance was used to construct a calibration curve that relates absorbance to BSA concentration. This calibration curve assists in determining the protein concentration in samples based on the absorbance measured at the same wavelength, ensuring accurate and consistent results. The linear regression analysis of the calibration curve yielded the regression equation Y = 3e-05x + 0.072 with a correlation coefficient (r) of 0.9722. This correlation coefficient indicates a good fit, meeting the acceptance criteria of $0.99 < r < 1$, which ensures that the method is reliable for accurate analysis [16].

Table 2 shows the protein concentrations for different acetic acid concentrations and extraction times. Higher concentrations of acetic acid and longer extraction times lead to decreased protein concentrations. The highest protein concentration was found in the first variation, with an acetic acid concentration of 0.5 M and an extraction time of 24 hours, resulting in 5.264%.

Table 2. Extraction Time with Variations in Acetic Acid Concentration

Extraction Time (Hour)	Acetic Acid (M)	Protein Content (%)
24	0.5	5.264
	1	2.379
	1.5	3.738
	2	2.524
	2.5	2.352
48	0.5	2.788
	1	4.827
	1.5	3.242
	2	3.182
	2.5	1.180
72	0.5	2.404
	1	2.640
	1.5	1.204
	2	1.088
	2.5	1.095

Further protein analysis was conducted by introducing the biuret-tested protein samples into an Ultra Performance Liquid Chromatography (UPLC) system. In UPLC, the protein samples pass through a chromatography column, interacting with both the stationary phase and the mobile phase. The protein molecules exiting the column are detected and separated by the UPLC detector.

Table 3. UPLC Amino Acid Analysis of Chicken Eggshell

Amino Acid	Chicken eggshell powder (mg/kg)	*Limit of Detection*
L-Arginin	1323.5	-
Glisin	180.04	-
L-Asam Aspartat	Not detected	57.17
L-Alanin	Not detected	25.39
L-Asam Glutamat	Not detected	45.73
L- Histidin	Not detected	88.53
L-Isoleusin	Not detected	51.30
L-Leusin	Not detected	50.19
L-Lisin	Not detected	100.82
L-Valin	Not detected	38.63
L-Fenilalanin	Not detected	142.82
L-Prolin	Not detected	38.51
L-Serin	Not detected	44.92
L-Treonin	Not detected	48.93
L-Tirosin	Not detected	182.40

The UPLC data reveal the amino acid composition in chicken eggshell samples **(Table 3)**. L-Arginine was found at 1325.35 mg/kg, while Glycine was present at 181.06 mg/kg. L-Arginine, a non-essential amino acid, plays a crucial role in human metabolism, enhancing skin hydration, repairing epidermal damage, and boosting collagen and elastin production, which helps maintain skin elasticity and prevent wrinkles. Glycine, the simplest non-essential amino acid, offers benefits such as mild cleansing and detoxification, aiding in pore purification and cosmetic residue removal, and possesses anti-irritant properties that soothe and prevent skin irritation. Other amino acids were not detected by UPLC, possibly due to their concentrations being below the instrument's detection limit.

Optimum Analysis Using RSM (Response Surface Methodology)

RSM is employed to understand the complex relationship between independent variables (X1 and X2) and the response (protein extraction yield) through mathematical modeling. The RSM analysis provides data on the minimum and maximum values for acetic acid concentration (X1) and extraction time (X2), as detailed in **Table 4**.

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Table 4. Independent Variables

Independent Variabel	Symbol	Min	Max
CH_3COOH Concentration (M)	X1	0.5	2.5
Ekstraction Time (hour)	X2	24	72

The values of the independent variables were input into the Design Expert-13 program using the Response Surface Methodology (RSM) Central Composite Design to illustrate the influence of the independent variables on the response. The resulting graph shows the interaction between two independent variables and their effect on the optimal yield of protein content.

Table 5. Model Selection Based on Summary Statistics

Source	Std Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	Ket.
Linear	**0.7518**	**0.5820**	**0.5123**	**0.3273**	**10.91**	**Suggested**
2FI	0.7224	0.6462	0.5497	0.1893	13.15	
Quadratic	0.7360	0.6995	0.5326	0.2569	12.06	
Cubic	0.8086	0.7179	0.4358	-2.1727	51.48	Aliased

The model recommended by Design Expert-13 is the linear model **(Table 5)**. This model has a standard deviation of 0.7518. While the cubic model boasts the highest R^2 value at 0.7179, the linear model has the lowest R^2 value at 0.5820. According to Anderson, adding more complexity to a model can improve statistical data [17]. However, Table 3 shows that the cubic model does not demonstrate significance and is not supported by the Central Composite Design, as it is marked as Aliased. This indicates that some parameters in the cubic model cannot be specifically estimated [18].

With an R^2 value of 0.5820, the linear model suggests that the factors of extraction time and acetic acid concentration contribute to 58.20% of the response variation, while the remaining 41.8% is influenced by other factors not studied in this research. The linear model has an Adjusted R^2 of 0.5123 and a Predicted R^2 of 0.3273, indicating its ability to predict data that was not used. Additionally, Table 3 shows the PRESS (Predicted Error Sum of Squares) value, with the linear model having the lowest PRESS value of 10.91, which measures the model's prediction error. These statistics imply that while the linear model may not capture all the complexities of the data, it still provides a reasonable fit for the variables under consideration, suggesting a balance between model simplicity and explanatory power.

Table 6. ANOVA Results for Linear Response of Protein Yield

Source	Sum of Squares	Df	Mean Square	F-value	p-value	Ket.
Model	9.44	2	4.72	8.35	< 0.0001	*Significant*
A-Asam Asetat	0.2153	1	0.2153	0.3810	0.0548	
B-Waktu Ekstraksi	9.23	1	9.23	16.32	0.0016	
Residual	6.78	12	0.5652			
Lack of Fit	2.83	6	0.4710	0.7143	< 0.6533	*Not significant*
Pure Error	3.96	6	0.6594			
Cor Total	16.23	14				

To determine if protein yield is influenced by extraction time and acetic acid, a linear model analysis was conducted using Analysis of Variance (ANOVA)**.** For the model to be considered significant, the p-value must be less than 0.05. According to **Table 6**, the model has a p-value < 0.0001, indicating a noise probability of only 0.01%, making the model significant. Meanwhile, the p-value for factor A (acetic acid) is 0.0548, and for factor B (extraction time) it is 0.0016, suggesting that the combination of these two factors affects the response. The non-significant Lack of Fit (p-value < 0.6533) indicates that the model fits the data well or there is no significant evidence that the model does not fit the data.

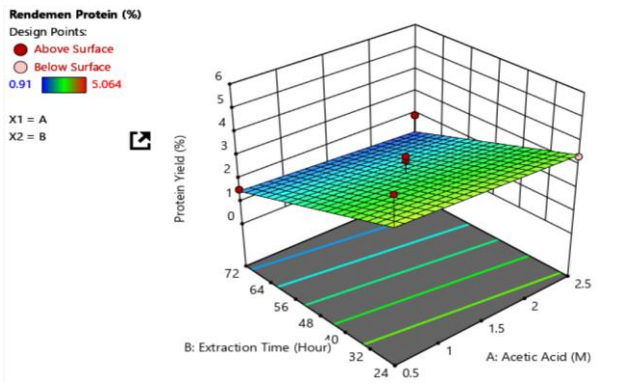

Figure 3. The Effect of Acetic Acid Concentration and Extraction Time on Protein Yield

Figure 3 presents a three-dimensional response graph showing that protein yield decreases with higher acetic acid concentration and longer extraction time. The peak of the graph indicates the optimal extraction conditions, which are at an acetic acid concentration of 0.5 M and an extraction time of 24 hours, yielding 5.064%. This supports the theory that acetic acid is a commonly used organic solvent for protein extraction, as it can alter pH, affecting protein charge density and modifying electrostatic interactions and protein structure [19].

Extraction is also influenced by time, as molecular movement during diffusion depends on the duration [20]. Previous research corroborates these findings [11], showing that an acetic acid concentration of 0.7 mol/L and an extraction time of 12 hours resulted in the highest protein yield of 0.73 g protein/100 g, validating the proposed model ($p<0.05$). This evidence confirms that both acetic acid concentration and extraction time significantly affect the protein yield.

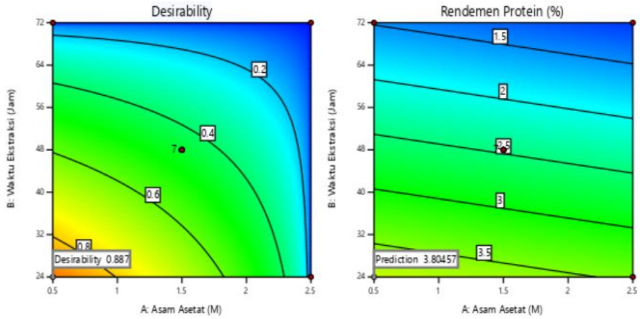

Figure 4. Desirability and Protein Yield vs. Prediction

In **Figure 4**, the desirability value obtained is 0.887. According to Anderson [17], a desirability value close to 1 indicates that the target or goal has been achieved. The accuracy of the model generated by Design Expert was then tested by comparing the predicted results with actual research findings. The program's predicted yield was 3.080%, with a deviation of 1.98% from the actual yield. The prediction and actual result difference of less than 5% indicates that the model is sufficiently accurate for processing [21].

Testing the Physical Properties of Peel-Off Gel Face Masks

The process of creating a peel-off gel face mask begins with preparing the main ingredients. Polyvinyl alcohol (PVA) is mixed with water and heated in a water bath at 80°C until fully dissolved. PVA acts as a film-forming agent that imparts the peel-off property to the mask. At high temperatures, PVA undergoes solubilization, breaking hydrogen bonds between PVA molecules and forming new bonds with water molecules. Next, glycerin, methyl paraben, and propyl paraben are mixed with hot water in a porcelain dish. Glycerin serves as a humectant to maintain skin moisture, while the parabens act as preservatives. These ingredients are then combined with the dissolved PVA solution to form Mixture A, which is stirred until homogeneous. In Mixture B, hydroxypropyl methylcellulose (HPMC) is mixed with cold water to create a gel structure. After optimal protein extract from chicken eggs is added to Mixture B, it is combined with Mixture A and further diluted with water. This solution is left to gel, forming a solid peel-off mask through the interaction of HPMC and water molecules, as well as the protein which enhances gel strength. The resulting peel-off gel mask has the right consistency for application and easy removal. Once formed, the mask is stored in an airtight container to prevent microbial contamination and maintain product stability.

Table 7. Physical Characteristics of Peel-Off Gel Face Masks

Parameters	Requirements	Result
pH	6.4 – 7.1	6.9
Spreadability	5-7 cm	5.1 cm
Adhesion	> 4 seconds	29.83 seconds
Drying Time	15-30 minutes	20 minutes

The physical characteristics of the peel-off gel face mask were evaluated through tests for pH, spreadability, adhesion, and drying time **(Table 7)**. The pH test, conducted with a pH meter AS218, showed a value of 6.97, indicating a near-neutral pH suitable for facial application, as the optimal range is 6.4-7.1 (SNI 16-4399-1996). Spreadability, which measures how widely the gel spreads on the skin, initially showed 2.1 cm and increased to 5.1 cm with an additional 125 g load, meeting the acceptable range of 5-7 cm. Adhesion testing, which determines how well the mask sticks to the skin, showed a separation time of 29.84 seconds, exceeding the required 4 seconds [22]. Finally, the drying time test indicated that the mask dries in 20 minutes, within the acceptable range of 15-30 minutes as per SNI standards.

Table 8. Antioxidant Testing of Peel-Off Gel Face Mask

Sample	Absorbance	Inhibition (%)
Peel-Off Gel Mask	0.785	15.470
	0.787	15.255
	0.787	15.255

Antioxidant testing was conducted to measure the mask's ability to inhibit free radicals using the DPPH method. The gel sample's absorbance was measured using UV-Vis spectrophotometry at a wavelength of 520 nm. The results showed absorbance values of 0.785 with an inhibition percentage of 15.470%, and 0.787 with an inhibition

percentage of 15.255% **(Tabel 8)**. The antioxidants in the mask function to protect the skin from oxidative damage caused by free radicals.

Conclusions

Based on the research findings, it can be concluded that the optimal protein content from chicken eggshells is achieved with an extraction time of 24 hours and an acetic acid solvent concentration of 0.5M, resulting in a yield of 5.064%. Higher solvent concentrations and longer extraction times reduce protein content. The optimal protein yield condition, with a p-value < 0.05, a desirability of 0.887, and a predicted yield of 3.080% with a 1.98% deviation, indicates that the model is sufficiently accurate for further processing. Additionally, the physical characteristics of the peel-off gel mask, including pH, spreadability, adhesion, drying time, and antioxidant properties, meet the SNI 16-4399-1996 standards.

Acknowledgment

Further research and development are necessary to explore methods for utilizing chicken eggshells, with the aim of creating patented cosmetic or beauty products. By advancing the techniques for processing and incorporating eggshells into cosmetic formulations, it is possible to enhance their efficacy and innovation in the beauty industry. This could lead to the development of unique products that leverage the natural benefits of eggshells, such as their protein content and potential antioxidant properties, thereby providing new opportunities for sustainable and effective skincare solutions.

Author Contributions

The authors' contributions to the paper are as follows: study conception, design, analysis, and interpretation of results: EA, ASR, B; data collection: EA; draft manuscript preparation: EA. All authors have reviewed the results and approved the final version of the manuscript.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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