Trop J Nat Prod Res, January 2025; 9(1): 321 - 328 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Enriched Facial Skin Serum Formulation Using Nanogold and Oligochitosan Particles: Optimization by Box Behnken Design

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ARTICLE INFO

Article history:
Received 04 September 2024
Revised 20 September 2024
Accepted 20 December 2024
Published online 01 February 2025

ABSTRACT

Facial skin continuously exposed to oxidative stress can accelerate the skin aging process, and the effects of aging can be prevented using cosmetic products, such as facial serums containing antioxidants. Consequently, nanogold is widely used as a modern cosmetic ingredient because of it antioxidant properties. The addition of oligochitosan minimizes the use of synthetic antibacterials in serum preparations. Therefore, this study aimed to formulate, optimize, and characterize an anti-aging serum with a mixture of nanogold and oligochitosan. The serum formulation design was carried out using the Box Behnken (BBD) surface response methodology (RSM) with Design Expert 13.0.5.0. The fixed variables were methylparaben, propylene glycol, ascorbic acid, glycerin, nanogold, collagen, Tween 80, and double distilled water. The independent variables were oligochitosan, hyaluronic acid, and citric acid. The optimum formula was determined based on the physical properties (pH and viscosity), whereas the characterization included stability tests, antioxidant activity using the DPPH radical scavenging assay, and particle size analysis using transmission electron microscopy (TEM). The optimum concentrations of oligochitosan and hyaluronic acid in the serum formulation were 2.5% and 1%, respectively, with a serum pH value of 5.02, a viscosity value of 1320 cP, and a particle size in the range of 57-123 nm. This serum formulation exhibited low antioxidant activity.

Keywords: Antioxidant, Box Behnken design, Face serum, Nanogold, Oligochitosan.

Introduction

Facial skin aging is a complex process causing changes in the biological and biochemical properties of the skin, as well as secondary structural changes, underlying muscles, subcutaneous fat tissue, and bone structure, which results in impaired biological function and various diseases.[1] Many factors, such as extrinsic and intrinsic factors can cause facial skin aging. Facial skin continuously exposed to oxidative stress, such as pollutants, UV radiation, chemical oxidants, and others can accelerate the skin aging process. Oxidative stress usually results from reactive oxygen species (ROS), which are unstable, very reactive, and accelerate skin aging.[2,3] Antioxidants can play an essential role in neutralizing ROS and protecting cells from oxidative damage. Furthermore, it can neutralize free radicals by accepting or donating electrons, effectively halting radical reactions. Antioxidant molecules directly react with reactive radicals, to form new, and less active species.[4] Cosmetics enriched with antioxidants are highly effective in slowing down or preventing the signs of aging.[5] One type of cosmetics that is currently gaining a lot of attention is serum, which is a concentrated product containing ten times more organic ingredients than cream.

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Citation: Salsabila A, Widikdo W, Sugita P, Arifin B, Restu WR. Enriched Facial Skin Serum Formulation Using Nanogold and Oligochitosan Particles: Optimization by Box Behnken Design Trop J Nat Prod Res. 2025; 9(1): 321 – 328 https://doi.org/10.26538/tjnpr/v9i1.41

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

A facial serum is a highly concentrated emulsion available in water-based and oil-based forms,[6] which is faster and more effective in overcoming skin problems, such as aging.[7] Anti-aging serum stimulates the regeneration of physiological processes in the body, thereby reducing and protecting the skin from aging. This serum generally contains ingredients that function as humectants to maintain moisture, antioxidants to protect the skin from oxidation caused by ROS,[8] and antibacterial substances to prevent/delay cosmetic changes and protect them from bacterial contamination. Nanogold is one of the cosmetic ingredients, which is inert and non-toxic with high antioxidant activity,[9] and particle size ranging from 1–100 nm,[10] having a large surface area, and the ability to be easily absorb into the skin.[11-13] Gold nanoparticles (AuNPs) are an excellent choice for biomedical applications due to their ease of synthesis, stability, and functionalization, low toxicity, and ease of detection.[14] Collagen formation by the skin cells can be assisted by nanogold. Regular collagen regeneration makes the skin tissue dense, making the skin firmer, younger and attractive.[9]

Chitosan has excellent potential as an anti-aging cosmetic ingredient because it contains some good characteristics such as being non-toxic, biocompatible,[15] and has antibacterial and antioxidant activities.[8,16] However, chitosan has disadvantages, such as being insoluble in water or high pH medium, and only soluble in acidic medium.[17] The solubility of chitosan can be increased through modification in order to enable more comprehensive uses.[18] Subsequently, oligochitosan is a chitosan derivative produced from the depolymerization of chitosan,[19] consisting of 2-10 β-1,4-linked D-glucosamine units. It is amorphous in nature,[20] soluble in water or neutral solvents, non-toxic, low molecular weight, and has antioxidant and antibacterial activities.[21] Oligochitosan is added to cosmetic products to reduce the use of synthetic antibacterials.

However, the optimization of the production process of anti-aging serum using nanogold and oligochitosan as ingredients has not performed, it is therefore necessary to formulate and optimize anti-aging serum to obtain the best formulation. Therefore, this study aimed to formulate, optimize, and characterize an anti-aging serum with a mixture of nanogold and oligochitosan as well as examine the antioxidant capacity of the serum formulation.

Materials and Methods

Study design
The anti-aging serum was formulated using the Box Behnken (BBD) surface response methodology (RSM) with Design Expert 13.0.5.0. The fixed variables included methylparaben, propylene glycol, ascorbic acid, glycerin, nanogold, collagen, Tween 80, and double distilled water. The independent variables were oligochitosan, hyaluronic acid, and citric acid. The minimum and maximum limits of the independent variables are shown in Table 1. The range between the minimum and maximum values set was inputted into BBD to perform random combinations in order to obtain 15 serum formulation designs. The responses measured by BBD were pH and viscosity with one experimental replicate performed.

Table 1: Variables for BBD

Factor	Variable	Minimum (%)	Maximum (%)
A	Oligochitosan	0	5
B	Hyaluronic acid	0.2	1
C	Citric acid	0	4

Preparation of serum formulation
Oligochitosan was dissolved in double distilled water. Methylparaben was dissolved in propylene glycol and added to the oligochitosan solution. Ascorbic acid solution was then added, followed by citric acid solution in double distilled water, and collagen solution in propylene glycol. Furthermore, Tween 80 and hyaluronic acid were dissolved in the solution, and left to stand followed by the addition of glycerin and double distilled water. Finally, nanogold was added and stirred until homogeneous. The facial serum preparation was then evaluated by testing for pH, viscosity, homogeneity, and stability.

pH test
The pH test was performed using a pH meter (Hanna HI 2211 bench top), calibrated with a buffer solution. The pH meter electrode was dipped into the serum preparation until a stable value was obtained.

Viscosity test
Viscosity was measured with a spindle viscometer (Brookfield TV-10) placed at the appropriate depth in the serum preparation. The spindle and speed used were adjusted to the highest torque value obtained in each serum, and then the viscosity value was recorded.

Homogeneity test
The similarity of color and texture was observed visually. Homogeneity was indicated by the same color and the absence of coarse grains in the serum.

Stability test
The stability test of the serum preparations was carried out for 35 days at room temperature (24 ± 2°C). The pH, viscosity, odour, colour, and homogeneity were measured on days 0, 7, 14, 21, 28, and 35.

Evaluation of antioxidant activity
Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Waksman De Torres *et al.* (2011)[22] with some modifications. Briefly, 1600 µL of sample solutions (500, 250, 125, 62.5, 31.25, 15.6, and 7.8 µg/mL) were added separately to 400 µL of DPPH solution, the solution was mixed thoroughly, and incubated at room temperature in the dark for 30 minutes. The absorbance of the resulting solution was measured at 517 nm using a UV-Vis spectrophotometer6 (SPECTRO star Nano). The percentage inhibition of DPPH radical was calculated using the formula as shown in equation 1. The half maximal inhibitory concentration (IC_{50}) was determined from a linear regression equation ($y = ax + b$) (equation 2) of the calibration curve made by plotting % inhibition (y) vs concentration (x).

$$\% \text{ inhibition} = \frac{(\text{blank absorbance} - \text{sample absorbance})}{\text{blank absorbance}} \times 100\% \quad \ldots(1)$$

$$IC_{50} = \frac{(50-b)}{a} \quad \ldots.(2)$$

Where; a is the slope and b is the constant of the linear regression equation.

Transmission electron microscopy (TEM) of serum formulation
The morphology and size distribution of facial serum particles were evaluated using transmission electron microscopy (Tecnai G2 20S-Twin) operating at an accelerating voltage of 200 kV. The size distribution was determined using ImageJ and Origin imaging software.

Results and Discussion

Optimized serum formulation
The BBD method was used to determine the effect of independent variables on the response to obtain a relationship model between independent variables, and selected conditions that produced the best response (pH and viscosity) from a combination of oligochitosan, hyaluronic acid, and citric acid concentrations.[23] This method was used for optimizing the facial serum ingredients. The advantages of the BBD method are that it reduces the number of trials, and predicts the potential interaction between variables. The result was then analyzed using multivariate analysis using linear, 2-factor interaction, and quadratic models. The pH values of the serum formulations ranged from 2.39–5.02 (Table 2). The desired pH range of skin care products is 4.5–6.5, which the standard pH range of the skin.[24] Serum preparations should have a pH that is neither excessively acidic nor alkaline. The viscosity values of the serum formulations as presented in Table 2 ranges from 15–1.22×10^6 cP, whereas the viscosity of serum products in the market ranges from 2×10^2–2×10^4 cP. Surini *et al.*[25] reported that serum preparations with low viscosity have better skin penetration than those with high viscosity. The lower the viscosity value, the higher the spread ability, hench the active substances in the serum preparation will spread more widely on the skin.[26]

The quadratic model is best suited for pH optimization based on R^2 values and the lowest standard deviation values and p-values. From the ANOVA results, the pH response has an R^2 value of 0.9971, with the predicted R^2 value of 0.9546, and adjusted R^2 value of 0.9920 (Table 3). R^2 value close to 1.0, indicates a good correlation.[27] Results from the ANOVA shows a p-value < 0.05, meaning the model is significant.[28] The viscosity response was also analyzed using quadratic models, and the model shows an R^2 value of 0.7898 and a p-value of 0.1118 ($p > 0.050$), meaning that the model is not significant. This can be caused by the nature of the serum, and a non-Newtonian fluid. Non-Newtonian fluids have non-constant viscosity because the relationship between shear rate and shear stress is not constant, thereby the measured fluid viscosity is called "apparent viscosity". Serum is a non-Newtonian fluid with pseudoplastic flow, which shows a decrease in viscosity with increasing flow rate.[29] Based on this, the actual viscosity value is far from the results predicted by BBD, due to the apparent viscosity of serum. The viscosity of non-Newtonian fluids is measured with a rheometer.

The ANOVA results showed a lack of fit in the viscosity response with an R^2 value of 0.0610 ($p>0.05$), meaning that the lack of fit is not

significant. An insignificant lack of fit value is a requirement for a good model because it shows that the response data obtained is in accordance with model.[30] Meanwhile, the lack of fit in the pH response has a value of $p < 0.05$ (0.0182), indicating a significant lack of fit.

Table 2: pH and viscosity of the serum formulations

Run order	Chitosan (%)	Hyaluronic acid (%)	Citric acid (%)	pH	Viscosity
1	2.5	1	4	2.6	1.22×10^6
2	2.5	1	0	5.02	1.32×10^3
3	5	0.2	2	2.77	45
4	5	0.6	4	2.52	9.8×10^4
5	0	1	2	2.94	1.5×10^5
6	0	0.6	4	2.52	7×10^4
7	2.5	0.2	4	2.39	20
8	0	0,2	2	2.73	20
9	5	0.6	0	4.64	1.5×10^2
10	5	1	2	2.94	6×10^4
11	2.5	0.6	2	2.85	8×10^4
12	2.5	0.6	2	2.86	6×10^4
13	0	0.6	0	4.78	1.4×10^2
14	2.5	0.2	0	4.87	15
15	2.5	0.6	2	2.88	2×10^5

This is because the midpoint variance (standards 13, 14, and 15) is not wide enough, causing the lack of fit to be significant. The pH value in facial serum formulation needs to be considered because if the skin's pH value changes, the skin's normal flora, and natural functions will be disrupted. Subsequently, pH changes on the skin can cause problems such as acne, peeling skin, and excessive sebum secretion.[31] Formulating a facial serum with an appropriate pH range can help improve the skin's protective function. The RSM equation for optimizing the serum formula against pH response is presented in equation (3).

$$\text{pH} = 2.86 - 0.0125\text{A} - 0{,}0925\text{B} - 1.16\text{C} - 0.0100\text{AB} + 0.0350\text{AC} + 0.0150\text{BC} - 0.0617\text{A}^2 + 0.0433\text{B}^2 + 0.8133\text{C}^2 \quad \ldots(3)$$

Where; A is oligochitosan; B is hyaluronic acid; and C is citric acid.

Equation (3) shows that the pH response increases in direct proportion to the increase in hyaluronic acid concentration, the interaction between oligochitosan and citric acid, the interaction between hyaluronic acid and citric acid, the interaction between hyaluronic acid, and the interaction between citric acid, indicated by a positive constant value. Conversely, the pH response decreases with the increase in oligochitosan, citric acid, and the interaction between oligochitosan, and this is indicated by a negative constant value. The three-dimensional (3D) graph in Figure 1 is a surface form of the interaction between the components of the facial serum composition and the pH response. It was observed that the increase and decrease in pH are significantly influenced only by the concentration of citric acid. This is because citric acid in cosmetics functions as a pH regulator.[32]

As shown in Equation (4) below, the viscosity increases in direct proportion to the increase in the concentration of hyaluronic acid and citric acid, the interaction between oligochitosan and citric acid, and the interaction between hyaluronic acid and citric acid. Conversely, the viscosity decreases with the increase in the concentration of oligochitosan and the interaction between oligochitosan and hyaluronic acid. Figure 2 shows that the concentration of citric acid and hyaluronic acid significantly influenced the viscosity. According to Šmejkalová *et al.*, (2015),[33] hyaluronic acid added to cosmetic formulas increases the viscosity of the formulation. Similarly, the addition of citric acid increases viscosity and decreases pH.

$$\text{Viscosity} = 1.293\times10^5 - 7745.62\text{A} + 1.789\times10^5\text{B} + 1.733\times10^5\text{C} - 22506.25\text{AB} + 6997.50\text{AC} + 3.047\times10^5\text{BC} \quad \ldots(4)$$

Where; A is oligochitosan; B is hyaluronic acid; and C is citric acid.

Hyaluronic acid improves the moisture levels in the skin, yielding multiple benefits such as reducing the visibility of wrinkles and facilitating wound healing, among others. Furthermore, hyaluronic acid is an important element of the extracellular matrix, with depletion commencing at the age of 25 years. Because of its superior hygroscopic, rheological, and viscoelastic qualities, hyaluronic acid is found in many cosmetic formulations. Additionally, some investigations emphasized the advantages of hyaluronic acid applied topically in dermatological practice. According to numerous clinical trials, hyaluronic acid is well-tolerated and effective as a support treatment for post-surgical care and facial rejuvenation procedures.[34] Citric acid as a pH adjuster,[35] chelating agent,[32] antioxidant,[36] and preservative,[37] in cosmetic products.

The best serum formula was selected based on the pH and viscosity responses that met the facial serum standards. Therefore, formula two was selected because it has a pH value of 5.02, which falls within the standard skin pH range of 4.5–6.5,[24] and the viscosity value was 1320 cP. Furthermore, the selected formula was tested for stability for five weeks.

Stability of facial serum preparations

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The stability of the serum preparation was tested to determine the durability of the preparation during storage. Stability test was carried out by storing the serum preparation at room temperature for five weeks or 35 days. The results of the stability of formula 2 facial serum preparation for 35 days are shown in Table 4.

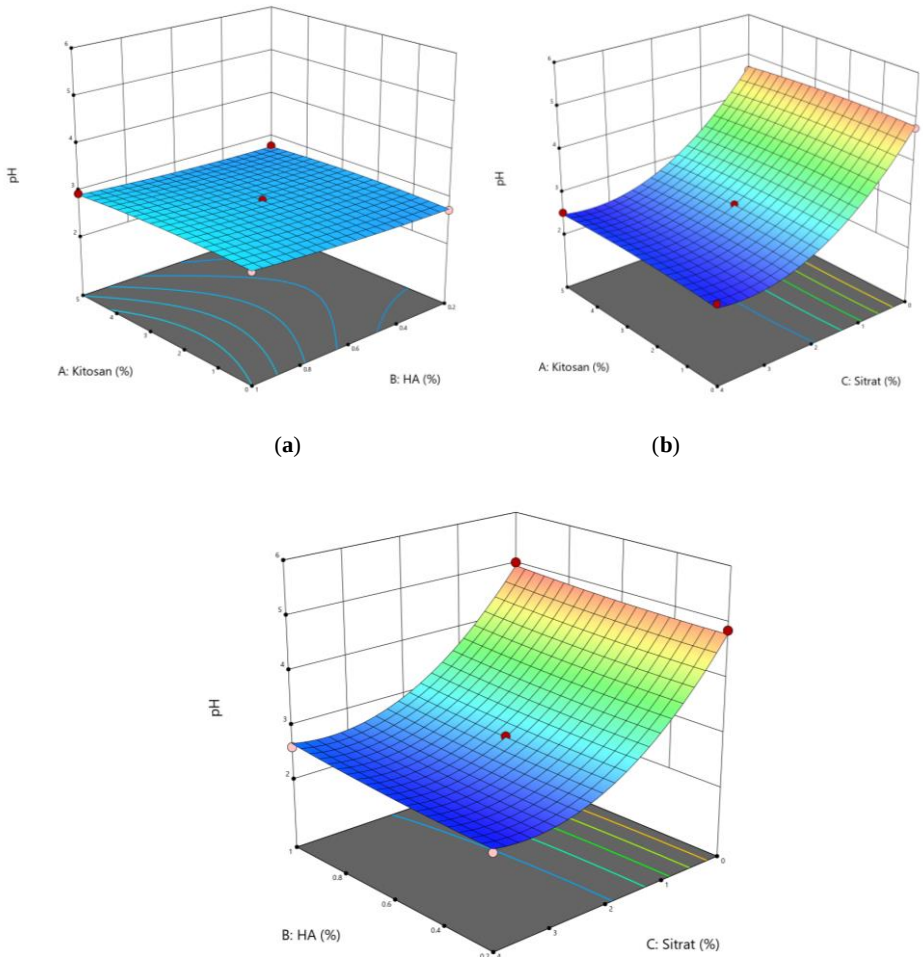


FIgure 1: 3-D graph of pH response of **(a)** citric acid vs chitosan:HA; **(b)** hyaluronic acid vs chitosan:citric acid; **(c)** chitosan vs hyaluronic acid :citric acid

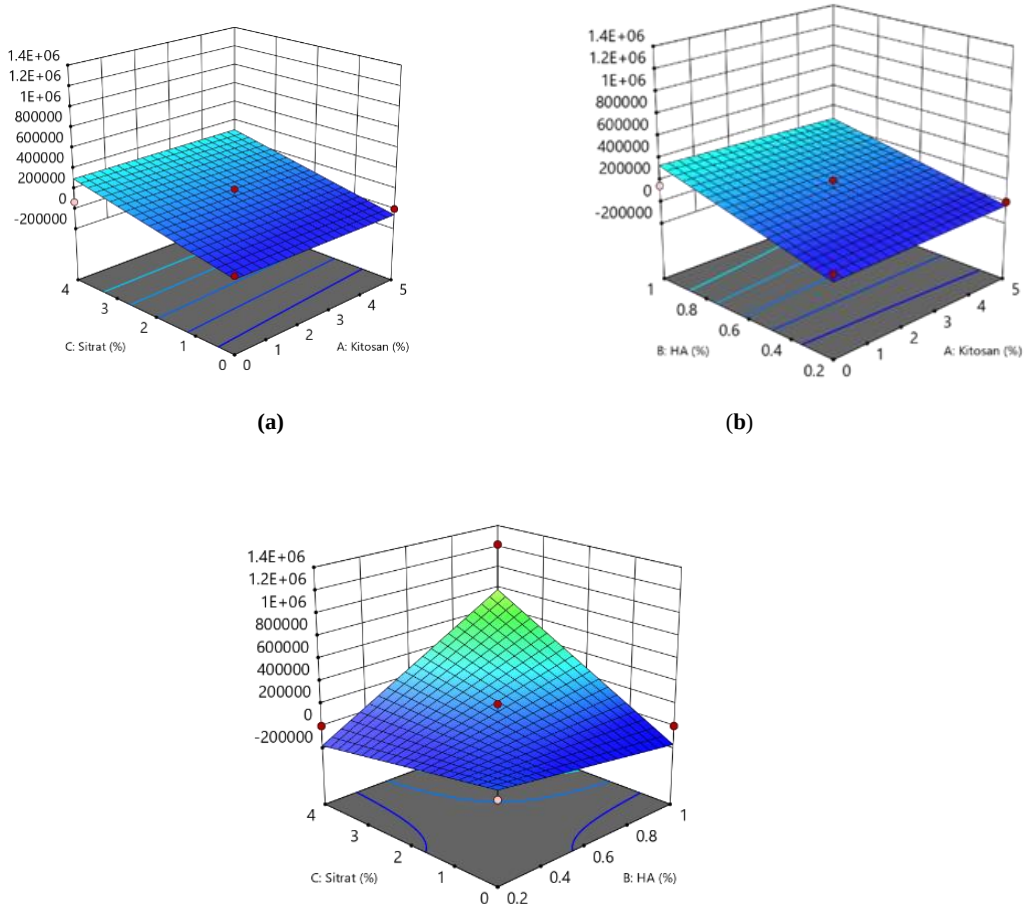
Formula 2 serum preparation during 35 days of storage at room temperature remained stable with a distinctive odour of oligochitosan. During storage, formula two serum preparation showed a colour change from clear yellow in weeks 0–4 to dark yellow in week 5. This colour change occurs due to the oxidation of the ascorbic acid component in the facial serum.[38] Ascorbic acid is a material with a high level of instability and reactivity to temperature and light.[39] In addition, ascorbic acid is reversibly oxidized when exposed to light, heat, transition metal ions, and pH (alkaline conditions) to dehydroascorbic acid (DHA) which is a form of ascorbic acid with low stability (Figure 3). The oxidation of ascorbic acid changes the colour of the serum sample to a darker colour.[38] Ascorbic acid (Vitamin C) acts as an antioxidant in topical cosmetics, which is essential for the development and maintenance of connective tissues, significantly contributing to bone formation, wound healing, and the production as well as preservation of collagen.[40,41] Daily topical application of ascorbic acid is predominantly safe for extended durations and can be effectively utilized alongside other common topical anti-aging medicines, including sunscreens, serum, tretinoin, other antioxidants, and alpha-hydroxy acids, such as glycolic acid.[42]

Oxidation occurs because high concentrations of ascorbic acid have a lower degradation rate constant.[39] Therefore, this study used a low concentration of ascorbic acid (0.1%), so that the degradation rate could be higher. In addition, the addition of an ascorbic acid stabilizer such as citric acid to the preparation is necessary to effectively inhibits the degradation of ascorbic acid in both light and dark conditions.[43] However, in this study, the use of citric acid resulted in a reduction in the pH of the serum preparation, rendering it unsuitable for facial serum application and preventing its use. The serum preparation had a pH in the range of 4.5–5.5, whereas, the stability of ascorbic acid is best at pH 3.4.[44] In acidic pH conditions, ionization of ascorbic acid occurs which makes it less susceptible to degradation. Decreasing pH can increase the proportion of undissociated ascorbic acid to maintain its stability.[43]

Table 3: Optimization parameters of serum formulation using the BBD method

Response	Significant (p <0.05)	Lack of fit (p >0,05)	R^2	R-adj	R-pred
pH	<0.0001	0.0182	0.9971	0.9920	0.9546
Viscosity	0.2161	0.0610	0.7898	0.4116	-2.2438

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)



(a) **(b)** **(c)**

Figure 2: 3-D graph of viscosity response of **(a)** citric acid vs chitosan:HA; **(b)** HA vs chitosan:citric acid; **(c)** chitosan vs HA:citric acid

The results of the pH determination of the facial serum preparations showed no significant changes during 35 days of storage at room temperature. Formula 2 had a pH value of 5.08 on day 35 (Table 4). This pH is within the normal skin pH range of 4.5–6.5.[45] The skin surface has a fairly acidic pH, which is necessary condition to maintain stratum corneum homeostasis and layer permeability. The most important function of acidic pH appears to be related to the process of keratinocyte differentiation, the formation and function of epidermal lipids and corneocyte lipid sheaths, and the maintenance of the skin microbiome.[46] Serum preparations with too-acidic pH can cause skin irritation and acne while a pH that is too alkaline can cause dry and scaly skin.[47] The use of facial care products with a pH different from the physiological pH of the skin can damage the skin's acid mantle. The slightly acidic, protective layer of moisture on the skin is called the acid mantle. The acidity is caused by a combination of secretions from the sebaceous glands and sweat glands.[48] The acid mantle prevents the skin from becoming dry and infected by bacteria and fungi because it comprises amino acids and fatty acids from skin sebum. Homogeneity is evaluated in order to know the effectiveness of mixing of the ingredients used in the serum preparation.[49] A homogeneous serum preparation will mix well when applied, therefore, the active ingredient content is assumed to be always the same for each use. Inhomogeneous serum preparations can cause the substances in the preparation not to spread evenly on the skin surface. The homogeneity examination during a storage period of 35 days showed that the serum preparation had good homogeneity, indicated by the even distribution of serum preparation particles without coarse, inhomogeneous granules. Good homogeneity of serum preparations is indicated by the absence of granules or materials that have not been mixed in the preparation.[48] The results of the examination of the homogeneity of the serum preparation of formula 2 are presented in Figure 4.

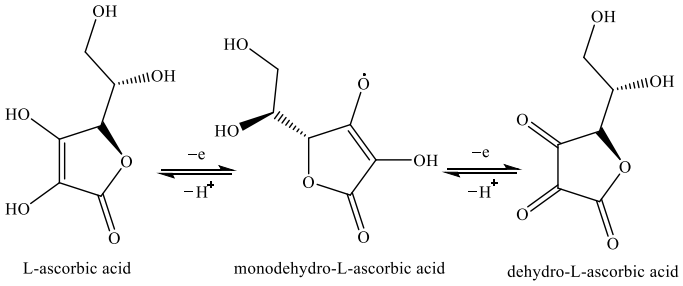


Figure 3: Degradation of L-ascorbic acid to dehydroascrobic acid.[40]

Viscosity is a measure of resistance of fluids to deformation due to shear,[50] which describes a fluid's internal resistance to flow and can be thought of as a measure of fluid friction. A viscosity test was conducted to determine the particle flow rate in the serum. The viscosity value of the facial serum preparation formula 2 on day 35 was 64.2 cP (Table 4). The difference in viscosity is influenced by the ingredients, for example, commercial serum preparations containing thickeners, such as Carbopol and xanthan gum, have a higher viscosity. Meanwhile, the serum preparation in this study did not use thickeners, therefore the

viscosity was lower. However, the low viscosity was not too problematic because it was related to its penetration ability.

Table 4: Stability of serum formula 2 at room temperature

Evaluation parameter	Observation (per week)				
	0	**1**	**3**	**4**	**5**
pH	5.02	5.25	5.22	5.13	5.08
Viscosity	1320 cP	68.2 cP	64 cP	47.7 cP	64.2 cP
Smell	Distinctive	Distinctive	Distinctive	Distinctive	Distinctive
Colour	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow
Homogeneity	Good	Good	Good	Good	Good

It has been reported that serum preparations with low viscosity penetrate better than those with high viscosity.[25] The lower the viscosity value, the higher the spread ability, hence, the active ingredients in the serum preparation will spread more widely on the skin.[26] The viscosity value of facial serum preparations tends to decrease during 35 days of storage. This is because the longer the storage time, the lower the viscosity of the preparation. The longer the storage time, the more the effect of environmental factors, such as moisture, which causes the serum preparation to absorb water vapour, leading to increased volume of water in the serum.

Figure 4: Visualization of serum preparation (formula 2)

Antioxidant activity of the serum preparations

The antioxidant activity of the serum preparations was measured using the DPPH radical scavenging assay. IC_{50} value is used to indicate the antioxidant ability of a compound, the smaller the IC_{50} value, the more active a compound is as a DPPH radical scavenger. On the basis of the IC_{50} value, antioxidant activity can be classified into three categories, namely; the strong category if the IC_{50} value is 10–50 µg/mL or 0.001–0.005%, the moderate category if the IC_{50} value is 50–100 µg/mL or 0.005–0.01%, and the weak category if the IC_{50} value is >100 µg/mL or >0.01%.[51] The contents of serum preparations known to have antioxidant activity are ascorbic acid, and nanogold.[52] The IC_{50} value of formula 2 was 6.11%, which indicates a weak antioxidant activity (IC_{50} >0.01%). This can happen because the serum may have undergone oxidation due to the ascorbic acid content which is very sensitive to changes in pH and light. Therefore, it is necessary to add ingredients that can reduce the rate of ascorbic acid oxidation in the serum.

Serum characterization results with TEM

Nanogold and oligochitosan serum preparations were analysed using TEM. The TEM micrographs of formula 2 showed spheroidal particles in the particle size range of 57-123 nm (Figure 5). Some of the nanogold particles in formula 2 were larger than 100 nm, but most were within the desired range, around 80-100 nm (Figure 5). This difference in size can occur due to agglomeration, due to the high surface energy of nanoparticles, which tend to agglomerate to neutralize this energy. Aggregation is caused by nanoparticles forming chemical bonds with other nanoparticles.[27]

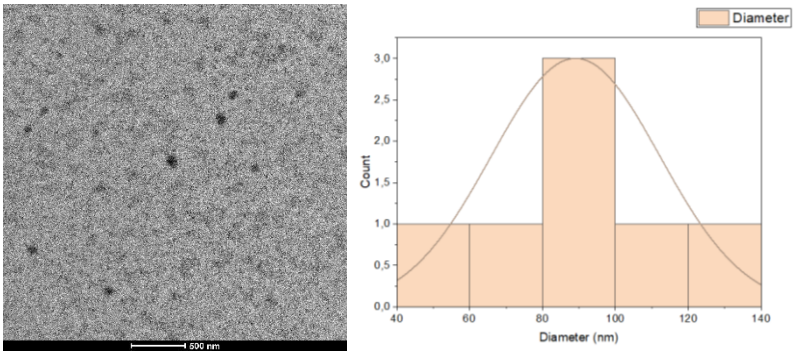


Figure 5: TEM micrograph of serum preparation formula 2 (magnification at 500 nm)

Conclusion

In conclusion, the best formula of anti-aging serum preparation based on nanogold and oligochitosan that met the criteria of skin pH range and serum viscosity, was produced from the addition of oligochitosan and hyaluronic acid without the addition of citric acid. The best combination of oligochitosan and hyaluronic acid concentrations were 2.5% and 1%, respectively. This formula has a pH of 5.02 and a viscosity of 1320 cP. However, the antioxidant activity was weak.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors express profound gratitude to Research and Technology, Ministry of Education, Culture, Research and Technology Indonesia, for financial support through the *Riset Kolaborasi Indonesia (RKI) 16 PTNBH Tahun Anggaran 2022* scheme under IPB University, Project No.3340/IT3.L1/PT.01.03/P.B/2022. The authors are also grateful to the National Research and Innovation Agency which provided TEM facilities.

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