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A.H.A. Allawi, F. Salehi, A. Daraei Garmakhany

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Address: College of Agriculture, Ferdowsi University of Mashhad, Iran

P.O.BOX: 91775- 1163

Fax: (98)051-38787430

E-Mail: ifstrj@um.ac.ir

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Research Article
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The Impact of Non-thermal Technologies on the Printability and Functional Properties of Quinoa Protein-Based Food Ink

M. Sarraf¹, S. Naji-Tabasi^{1*}, B. Ghorani^{1*}, B. Emadzadeh², O.S. Toker³

1- Department of Food Nanotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran

2- Department of Food Physics, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran

3- Chemical and Metallurgical Faculty Food Engineering, Yildiz Technical University, Istanbul, Turkey

(* - Corresponding Authors Email: s.najitabasi@rifst.ac.ir, b.ghorani@rifst.ac.ir)

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Abstract

In the past decade, the food industry has seen increased focus on diverse and practical designs. Accordingly, this research aims to use additive manufacturing to create gel structures from a new perspective. In this study, quinoa protein (QP), either untreated (B) or treated with cold plasma (CP) or pulsed electric fields (PEF), at two concentrations (9 and 12% w/w), was combined with polysaccharides (alginate and agar) to prepare gels. The printability and physicochemical (water activity, dry matter, water holding capacity, and color analyses), textural, and rheological properties of gels were then compared. Evaluation of the textural properties revealed that the highest hardness was observed 530.27, 416.52, and 334.82 g for the 12% B, 12% CP, and 9% PEF samples, respectively. However, in examining the results of the frequency sweep, 9% and 12% CP had the lowest storage modulus among the other gels. The 3D printing results of gels indicated that they are printable and showed high stability over 24 hr. In addition, phase separation did not occur in all treatments when gels were exposed to severe stress. Therefore, optimizing quinoa protein gels for 3D printing requires coordinating processing parameters with the composition of the formulation. This could advance the development of novel food designs with enhanced texture and stability, aligning with the food industry's focus on innovative and practical solutions.

Keywords: 3D food printing, Cold-Plasma, Hydrocolloids, Quinoa protein, Pulsed electric field

Introduction

“Three-dimensional printing” is a rapid prototyping technology that utilizes digital model files. This technique constructs physical components or structures through the layer-by-layer deposition of materials (Godoi, Prakash, & Bhandari, 2016; Shi, Zhang, & Bhandari, 2021). In the past decade, there has been a significant increase in the demand for specialized food products tailored to specific consumer groups. This trend is especially

prominent among individuals with specific nutritional requirements and personalized dietary preferences, leading to a growing demand for innovative food processing technologies that preserve desirable product properties (Godoi *et al.*, 2016; Shi *et al.*, 2021). Three D printing is a technology widely used in food, affecting not only the shape, taste, structure, and color of various foods but also offering potential improvements in precision, customization, and shelf life (Guo, Zhang, &



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Bhandari, 2019; Shi *et al.*, 2021; Xiao *et al.*, 2025). These materials possess a melty or mushy consistency at room temperature, enabling the creation of intricate and refined shapes through the use of Computer-Aided Design (CAD).

The most important features of the ink used in 3D printing are its ability to flow uniformly through the nozzle and to possess sufficient mechanical properties to maintain structural stability (Guo, Zhang, & Devahastin, 2020; Shi *et al.*, 2021; Sun, Zhang, & Chen, 2020). Applications of this printing technology within the food industry are diverse, as demonstrated in research on chocolate (You, Huang, & Lu, 2023), Flour products (Lim, Lim, Kim, & Park, 2023; Mallesham, Parveen, Pandiselvam, Rajkumar, & Naik, 2024), Meat productions (Dong, Wang, Yang, & Xu, 2023), and gels (Shi *et al.*, 2021; Yang, Zhang, Bhandari, & Liu, 2018).

In addition to the diversity of tastes, people's preferences have shifted towards healthy, nutritious and dietary foods. Therefore, the replacement of animal protein with plant proteins has also increased in consumer societies (Xiao, Zou, Hu, Zhu, & Wei, 2023; Xu *et al.*, 2024).

Quinoa is regarded as one of the most exceptional plant-based resources for protein supplementation. Classified as a pseudo-cereal, it possesses protein content comparable to that of legumes. Its popularity can be attributed to its distinctive amino acid profile and its significantly higher protein content in comparison to conventional cereals (Felix, Camacho-Ocaña, López-Castejón, & Ruiz-Domínguez, 2021; Manohar, Rehman, & Sivakumaran, 2021; Pereira *et al.*, 2019). Quinoa is primarily composed of globulins (37%) and albumins (35%), with a lesser proportion of prolamins (0.5–7.0%). This pseudo-cereal is noteworthy for its abundance of essential amino acids, including lysine, histidine, and methionine, which are typically limited in conventional cereal proteins (Xu *et al.*, 2024). These attributes contribute

significantly to its role in supporting growth and maintaining metabolic activity. Furthermore, quinoa's gluten-free nature, high antioxidant properties, substantial globulin content, and ability to form disulfide bonds underscore its potential as a valuable food ingredient in various nutritional applications (Galante, De Flaviis, Boeris, & Spelzini, 2020).

Recent studies have systematically investigated various modification approaches to enhance QP gelation properties (Farahmand *et al.*, 2026). Ma *et al.* 2025 developed hybrid protein gels with complementary amino acid profiles by blending ultrasound-pretreated (100–400 W) lentil and quinoa proteins (Ma, Xiong, & Jiang, 2025). Ultrasound treatment significantly enhanced protein solubility (32% and 22% for lentil and quinoa, respectively) and gelling capacity ($p < 0.05$), yielding 1.9-fold higher gel hardness. Improved rheological properties and network density were attributed to enhanced hydrophobic and polar interactions. The resulting binary gels exhibited superior digestibility, demonstrating potential for dysphagia foods, meat analogues, and other gel-based formulations. Xu *et al.* (2024) reported that gels made from quinoa protein treated with ultrasound showed improvements for use as egg substitutes in bread (Xu *et al.*, 2024). In the study by Cao *et al.* (2023), the gel strength of quinoa protein gel increased markedly by 94.12% with 600 W ultrasonic treatment, and the water-holding capacity increased from 56.6% to 68.33%. The gel solubility was reduced and free amino content led to increase the apparent viscosity and the consistency index (Cao *et al.*, 2023).

Among proteins extracted from various food sources, some exhibit desirable functional properties in the food industry, while others, having undesirable functional properties, are lost as waste or byproducts. To ensure a stable supply of food protein and manage associated costs, it is essential to modify protein structures (Yang *et al.*, 2025). Such modifications will enhance functional properties and significantly expand industrial applications (Nasrabadi, Doost, & Mezzenga, 2021). External factors,

for instance, oxidation effects, temperature, and pH, limit the functional properties of native proteins and restrict their application in the food industry (Wang *et al.*, 2023). Modifying the physicochemical properties of proteins by altering their structure and controlling their functional properties can be beneficial for utilizing proteins extracted from various food sources in different applications at an industrial level (Farahmand *et al.*, 2026; Wang *et al.*, 2024). Zare, Mollakhalili-Meybodi, Fallahzadeh, & Arab, (2022) systematically investigated atmospheric pressure cold plasma treatment of quinoa flour, demonstrating that treatment duration (5-20 min) and voltage (15-25 kV) significantly modified protein secondary structure. At optimal conditions (15 kV, 15 min), surface hydrophobicity increased by 47%, while disulfide bond formation enhanced by 32%. However, prolong treatment (>20 min) induced protein aggregation and decreased solubility by 28%, establishing critical processing thresholds. Ahmadian, Sohbatzadeh, Alashti, & Kenari, (2025) extended this work to protein-polysaccharide complexes, showing that surface dielectric barrier discharge plasma modified protein-polysaccharide interactions through controlled oxidation. Recently, Farahmand *et al.* (2026) provided foundational characterization of PEF-treated QP, demonstrating that 5000 V cm⁻¹ with 80 pulses induced partial unfolding (α -helix content reduced from 28% to 19%) while maintaining solubility (>85%). Surface hydrophobicity increased by 62%, and free sulfhydryl groups increased by 41%, indicating controlled denaturation favorable for subsequent gelation.

From another perspective, plant proteins are often not well-suited for 3D printing, which requires modifications to make them behave like ink. This issue can be attributed to poor solubility and aggregation, weak gelation, and particularly inadequate rheological properties. Three D printing demands specific rheological characteristics. The ink must exhibit a solid-like consistency when at rest while demonstrating liquid-like flow when subjected to force, a

phenomenon referred to as shear-thinning. Typically, native protein solutions lack this characteristic; therefore, modifications are essential to optimize the behavior of the proteins and achieve the necessary "extrude-and-hold" quality. Furthermore, these modifications facilitate the unfolding of the proteins, allowing them to establish a robust, elastic, self-supporting gel network that effectively retains the printed shape (Xu *et al.*, 2025).

One of these methods of modifying proteins is cold plasma (CP), which boosts the functional properties of proteins. CP is a novel, mainly untested non-thermal technology with tremendous promise for many food processing applications (Heydari *et al.*, 2023).

The pulsed electric field (PEF) technique is another non-thermal processing method that applies short bursts of high voltage to food products to electroporate cell membranes for various processing purposes. The benefits of PEF are cost-effective compared to the traditional method, less time, energy, and water consumption, and retaining the quantitative nutritional properties (Sarraf, Naji-Tabasi, & Beig-babaei, 2021).

The integration of proteins with polysaccharides facilitates the formation of thick, gel-like layers that exhibit charge characteristics (Sarraf *et al.*, 2023). Polysaccharides, like sodium alginate (SA), have properties such as biosafety, biocompatibility, and renewability (Gao *et al.*, 2020). It is a natural polysaccharide extracted from kelp or sargassum of brown algae and composed of β -D-mannuronic acid and α -L-guluronic acid (Guo, Qin, Chang, & Lee, 2023). It is challenging to use SA alone to cope with the complex water environment, as it encounters difficulty due to its poor mechanical strength, low structural stability, and low heat resistance (Ates, Koytepe, Ulu, Gurses, & Thakur, 2020).

Agar is another complex polysaccharide that is present in the cellular wall of red algae. It has a high gelling power in an aqueous solution; no need to add reagents to produce gelation and

used to improve printability. Agar also boasts remarkable reversibility when melted, and it possesses the highest natural gel strength of any gelling agent (Kim, 2014).

Until now, research has shown that QP emulsion gels can be 3D printed using various polysaccharides to deliver curcumin. One effective method involves fucoidan (FU) to improve printability and stabilize curcumin encapsulation (Zhao, Hao, Guo, Chang, & Shen, 2024, 2025). Additionally, plant-based 3D-printed dysphagia diets have been developed using pea protein isolate (PPI) with QP to enhance the essential amino acid profile, along with hydrocolloids to adjust the texture (Gu *et al.*, 2025). Enhancements in printability through electrostatic complexation with polysaccharides have led to better performance of QP. Notably, high internal phase emulsions (HIPEs) stabilized by QP and carrageenan exhibit optimal geometry, smooth texture, and stability. However, the effects of non-thermal technologies on the printability and functional properties of QP-based food inks remain unexplored (Zhong, Wang, & Shen, 2024). This paper investigates the combined use of CP and PEF to modify QP as a functional ink for 3D food printing. While both plant-based proteins and 3D printing are emerging fields, this study uniquely targets QP modification and systematically compares how these non-thermal techniques influence its gelation behavior with polysaccharides. In other words, despite these advances, no previous study has systematically compared cold plasma and pulsed electric field modifications of quinoa protein for gelation and 3D printing applications. The present study addresses this gap by investigating how CP and PEF treatments at two protein concentrations (9% and 12% w/w) influence the formation of composite gels with alginate and agar, evaluating their rheological, textural, microstructural, and printability characteristics. This work uniquely contributes to understanding concentration-dependent effects of non-thermal modifications and provides a foundation for developing optimized QP-based

food inks for additive manufacturing applications.

This approach offers a greener and more sustainable alternative to traditional thermal or chemical modifications for enhancing protein functionality in additive manufacturing. The paper adopts a hands-on approach, connecting 3D printing performance—such as printability and stability—with underlying microstructural, textural, and rheological properties. It highlights how controlled processes, CP and PEF, alter protein structures, affecting network formation with alginate and agar. This research offers essential insights for designing plant-based food inks, facilitating the creation of customized, nutritious printed food products.

Methods and Materials

Materials

Quinoa protein (QP) was extracted according to the procedure described elsewhere (Abugoch, Romero, Tapia, Silva, & Rivera, 2008) from Saponin-free quinoa flour (Protein content based on dry weight: 16.63%), which was purchased from Zarrin Daneh Meimeh company (Iran). Sodium alginate (SA) (Fufeng, China) and Agar (Ag) (Sigma Aldrich, Germany) were used for the preparation of gels. n-hexane and ethanol were prepared from Pars Shimi (Iran) and Taghtire-Khorasan (Iran), respectively. NaOH and HCl were purchased from Merck (Germany). All chemical products were of analytical grades. Purified deionized water was used for the solution preparation.

Extraction of Protein Quinoa (QP) from Quinoa Flour

Defatted quinoa flour was prepared by mixing n-hexane and quinoa flour (3:1) under a fume hood (24 h). The mixture was then centrifuged at 7000× g for 5 min to remove the solvent. It was repeated twice to ensure complete fat removal. Next, the collected defatted quinoa flour precipitate was dried. The extraction of quinoa protein powder was performed by solubilization at pH~9 and precipitation at pH~5. The defatted flour was suspended in water (10% (w/v), and pH~ 9 was

adjusted by NaOH (2N). The suspension was stirred at room temperature for 30 min, followed by centrifugation at $9000\times g$ for 15 min. The collected supernatant was adjusted on pH~ 5, and then it was centrifuged at $9000\times g$ for 20 min at 4 °C. The precipitate was suspended in water, neutralized with 2 N NaOH, and homogenized. The extract contained approximately 15% (w/v) solids. The extract was lyophilized by freeze-drier and the powder was stored in a cool, dry place (Abugoch *et al.*, 2008). The protein and moisture content were determined by the Kjeldahl method (AACC, 2000) and (AOAC, 2005), respectively.

Gels Preparation

The gels were prepared using a QP:SA:Ag in a ratio of 1:1:1 (based on pretreatment experiments). Initially, the QP mixtures, consisting of either 9 or 12 w/w%, were combined with 3% SA and subjected to heating in a circulator (Julabo, EH 19) at 85 °C for a duration of 30 min before being rapidly cooled. Subsequently, hot Ag (3 w/w %) was incorporated into the mixtures and thoroughly mixed. The six formulations performed include the following coding:

- (9, 12%) B-QP: 3% SA: 3% Ag;
- (9, 12%) CP-QP: 3% SA: 3% Ag;
- (9, 12%) PEF-QP: 3% SA: 3% Ag.

For the preparation of gels (QP and SA), biopolymers were dispersed in deionized water for 2 h and then were left overnight for complete hydration in the refrigerator (≈ 5 °C).

The pulse and plasma treatment conditions were selected according to our prior optimization work that provided the best gelation properties (Farahmand *et al.*, 2026). Accordingly, quinoa protein (QP) was treated using cold plasma (CP; 40 W, 15 min) and pulsed electric field (PEF; 5000 V cm^{-1} , 80 pulses), and compared with an untreated sample (blank).

3D-Printing

The gels, used as an ink, were 3D-printed by a 3D bioprinter (VafaToos Co, Mashhad, Iran)

to evaluate their potential in creating structured constructs. Printing patterns included a 20 mm x 20 mm cube with 12 layers and a 2 mm diameter nozzle. The printing speed was set to 15 mm.s^{-1} , 100% infill density, and the optimum printing temperature was 30 ± 3 °C.

Rheological Analysis

The evaluation of the strain and frequency sweeps of the gel inks were conducted using a rheometer (Anton Paar, Germany) featuring parallel plate geometry, with a diameter of 35 mm and a gap of 1 mm. Initially, the linear viscosity region (LVR) was determined, and an appropriate shear strain within this LVE region was selected. Strain sweep tests were conducted at a constant frequency of 1 Hz, and strain of 0.1-100% at 25 °C. The conditions used for frequency sweep included frequency of 0.1-100 rad.s^{-1} , temperature of 25 °C, and constant strain of 0.1%. The storage modulus (G'), loss modulus (G''), loss tangent ($\tan \delta$), and complex viscosity (η^*) were obtained (Rahman *et al.*, 2020).

Microstructure

Microstructure of the selected samples were studied at 25 °C using a Field Emission Scanning Electron Microscope (FESEM; GeminiSEM 300, Germany). The freeze-dried samples received a thin coating of gold to facilitate analysis. The fractures and surfaces of the gels were assessed at magnifications of $50\times$ and $200\times$ (Ates *et al.*, 2020).

Physicochemical Analysis

Evaluation of Water Activity (a_w)

The water activity of the gels was checked by a_w meter (LabMaster/Novasina, Swiss). The gels had to be placed in conditions without exchanging moisture with the environment. The sample was pipetted into a specialized plastic container and subsequently placed within the apparatus at a controlled temperature of 25 °C. The vapor pressure of the sample was recorded on the display once it stabilized at a constant level (Shahidi, Niazmand, Sarraf, & Shahidi, 2020).

Stability of Gel by Centrifugation Test

Centrifugal stability of gels was evaluated through centrifugation (Heidoph, Germany) at 3000 rpm for 30 min (Sarraf *et al.*, 2023). The data were analyzed using the following Eq. [1]:

$$\text{Stability (\%)} = \left(\frac{V_c}{V_t} \right) \times 100 \quad (1)$$

where V_c is the gel volume after centrifugation and V_t is the total volume of the gel (Sarraf *et al.*, 2023).

Colorimetric Analysis

The images of treatments were captured in a wooden box (45 cm above the sample and at an angle of 45° with the sample plane to give a uniform light) using a plate and a digital camera (Canon EOS 1000D, Taiwan) with a lens focal length of 35 mm. The color of gel images was evaluated using a computer (Opplé, 8 W, model: MX396-Y82; 60 cm in length). Parameters L^* , a^* and b^* of images (resolution of 300 dpi) were evaluated by the Image J software (Sarraf, Beigbabaei, & Naji-Tabasi, 2024).

Determination of FT-IR on the Optimal Gel

The functional groups of optimal gel were evaluated in powder form by a Fourier Transform Infrared Spectrometer (Thermo Nicolet AVATAR 370, USA) between wavelengths of 400 and 4,000 cm^{-1} (Ates *et al.*, 2020).

Printability Assessment

The printability of the gels printed was evaluated according to the method of Ouyang, Yao, Zhao, & Sun, (2016). To this end, the parameter associated with the gelation degree was analyzed through the measurement of the perimeter (A) and area (L) of the gels during the initial hour and after a period of one day. Additionally, the printability of the samples (Pr) was assessed in accordance with Eq. 2.

$$Pr = \frac{L^2}{16 A} \quad (2)$$

If the Pr is equal to 1, the gelation conditions are ideal; also, if $Pr < 1$, the printing of under-gelled samples (those that are not fully gelled)

becomes feasible. $Pr > 1$, indicates irregular filaments with fractured morphology (Aina *et al.*, 2024; Ouyang *et al.*, 2016).

Also, Shape retention that indicated the ability of filaments to retain their extruded shape upon deposition without significant deformation under gravity-induced stress. It is measured based on Eq. [3] (In, Jeong, Song, & Min, 2021).

$$\text{Shape retention (\%)} = \frac{h_{24}}{h_0} \times 100 \quad (3)$$

h_{24} is height after 24hr and h_0 is initial height.

Shape fidelity (%) was determined as the ratio of the printed gel height to the designed height (20 mm) (Eq. [4]) (Gao *et al.*, 2020).

$$\text{Shape fidelity (\%)} = (\text{Measured Dimension} / \text{Target Dimension}) \times 100\% \quad (4)$$

Dimensional Measurement

After printing each treatment, the height of the printed gels was measured twice: at the 1st h and 24th h of production. Changes were evaluated within a day at ambient temperature and a fixed place with appropriate lighting, using a ruler to observe the treatment height changes over time (In *et al.*, 2021, with modifications).

Texture Analysis

The textural characteristics of the gels printed were studied using a texture analyzer (Stable Micro System, TA-XT Plus). The parameters of hardness, adhesiveness, apparent elastic modulus, springiness, gumminess, cohesiveness, chewiness, and consistency were determined by the TPA method at 25°C. Based on the sample characteristics and dimensions of the prepared 3D gels, a probe P/75 (England) with a diameter of 40 mm was used at a rate of 1 mm.s^{-1} , with a Trigger force of 3 g and 30% strain. The test conditions used were based on the type of sample (Baydin, Arntsen, Hattrem, & Draget, 2022, with modifications).

Statistical Analysis

The results were analyzed as means $\pm$ SD by a completely randomized factorial design and data were analyzed using SPSS (version 11.0)

according to ANOVA. Also, the significant difference was obtained by Duncan's multiple range test ($p \leq 0.05$). The curves were plotted using Excel software 2016. Three replications were used for technical measurements.

Results and Discussion

Rheological Properties

Study on frequency sweeps evaluation, three types of systems are defined: gels, dilute solutions, and concentrated systems (Kutz, 2019). As shown in the frequency sweep curves in Fig. 1, the storage modulus (G') values were greater than the loss modulus (G'') within the specified frequency range. Additionally, the graphs for G' and G'' exhibited partial frequency dependence and did not intersect. These fluids are referred to as "weak gels or structural fluids." This indicates that the structure forms an intertwined network, which remains stable even at high frequencies.

As shown in Fig. 1, the G' and G'' of the treatments increased with increasing frequency. On the other hand, G' was higher than G'' in all samples, illustrating that the elastic property dominates the viscous behavior of the gels. The lowest value was associated with 9CP and then 12CP, while the highest G' was observed in PEF and B samples. The highest G'' was contributed by 12PEF. In a comparison between the B and PEF samples in different concentrations of QP, it was determined that non-thermal technologies had a positive effect on the gel properties of the QP-AG-SA gels, whereas CP weaken the biopolymer structure of QP.

The differences in rheological properties can be explained by the distinct mechanisms of the non-thermal treatments on protein structure. PEF treatment likely induced partial unfolding of quinoa proteins, exposing hydrophobic and charged groups and promoting protein–protein and protein–polysaccharide interactions. This promoted the formation of a denser three-dimensional network within the alginate–agar matrix, which increased the storage modulus

(G') and produced a stronger elastic gel (Farahmand *et al.*, 2026). In contrast, cold plasma causes mild oxidation and rearrangement of non-covalent interactions (Zare *et al.*, 2022). Therefore, this process produced a more flexible network with lower rigidity, explaining the reduced G' but improved flow behavior during extrusion. Therefore, CP formed softer but more extrudable gels, while PEF produced stiffer gels with higher structural strength. These structural differences are consistent with the printing behavior, where PEF samples exhibited greater resistance to flow and CP samples showed smoother extrusion. Also, Heydarian, (2024) reported that different conditions play a main role in creating cross-linking or depolymerization phenomena to modify quinoa. Zare *et al.* (2022) reported similar findings regarding the reduction in viscoelastic properties of quinoa flour after applying the CP process. They discovered that the elastic moduli of the control quinoa flour were higher than those of the quinoa flour treated with CP (Zare *et al.*, 2022).

As shown in Table 1, a comparison among parameters includes the storage modulus (G'), loss modulus (G''), loss tangent ($\tan \delta$), and complex viscosity (η^*) of gels was made at 1 Hz. Gels of B and PEF exhibited higher G' values than CP. Among the samples, G' was highest for the 12-B and 12-PEF gels. In the CP-treated gels, the G' of the 12% sample (1570.22 Pa) was approximately twice than the 9% sample (714.82 Pa).

A similar pattern was observed in the G'' modulus at a frequency of 1 Hz. The viscous modulus B for PEF gels at both concentrations of QP exceeded 400 Pa, whereas it remained below 300 Pa for the 9% and 12% CP samples. It is evident that the concentration of QP significantly influenced the increase in the G'' modulus; notably, the G'' modulus for the 12-QP concentration was consistently higher than that for the 9% concentration across all experimental treatments.

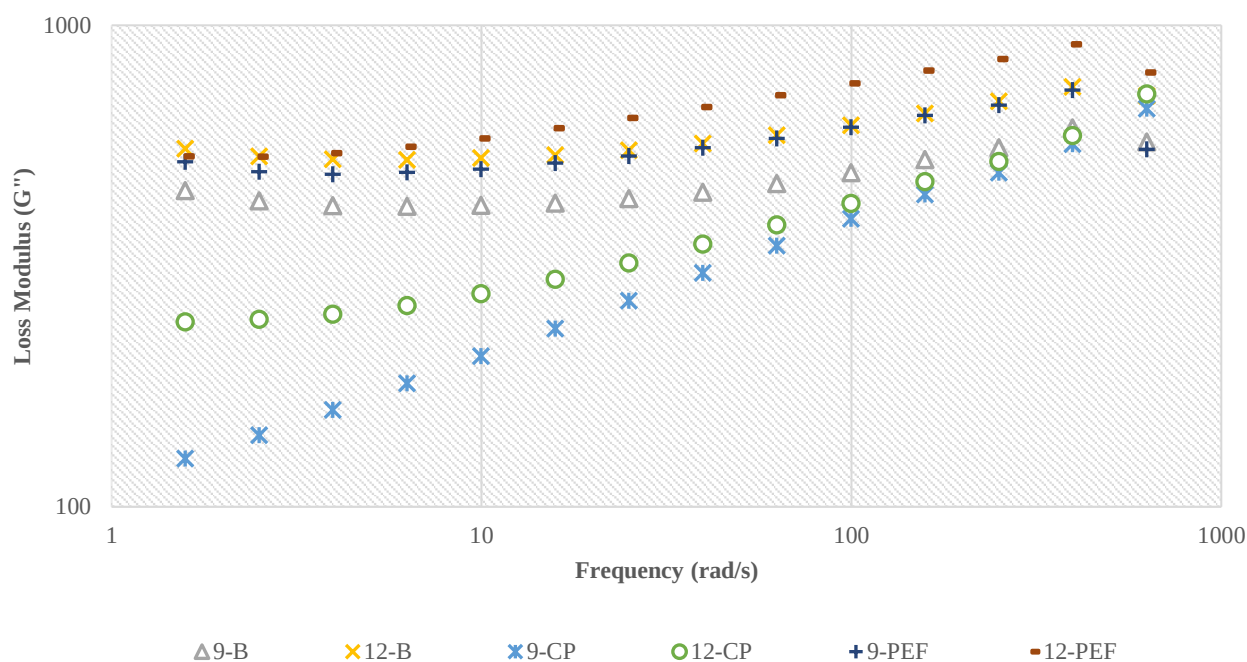


Fig. 1. Frequency sweep (i) G' and (ii) G'' of gels prepared with QP (B/CP/PEF) and Ag-SA at 25°C

Table 1- Dynamic rheological parameters of gels prepared with QP (B/CP/PEF) and Ag-SA by frequency sweep test at $f=1$ Hz and 25°C

Code	G' (Pa)	G'' (Pa)	η^* (Pa.s)	Tan δ
9-B	3054.90	420.39	491.03	0.14
12-B	3743.30	525.44	602.36	0.14
9-PEF	3419.4	494.84	550.16	0.14
12-PEF	3213.5	559.12	519.39	0.17
9-CP	714.82	180.46	117.39	0.25
12-CP	1570.22	261.78	253.49	0.17

The complex viscosity value (η^*) of gels containing 12% B and CP showed a higher value compared to gels containing lower concentrations (9%), which indicates an increase in gel strength with the help of QP concentration, except for PEF treatments, which showed the opposite trend and increasing QP concentration caused a decrease in η^* , so that, it was 550.16 and 519.39 Pa.s in 9PEF and 12PEF, respectively. The comparison of η^* treatments revealed that 12-B and then PEF (9 and 12% w/w) gels exhibited stronger structures, whereas the weakest were associated with CP (Table 1).

An additional parameter for the evaluation of gels is tan δ , which quantifies the ratio of energy lost to energy stored per cycle. The tan δ value exceeding 0.1 indicates that the material may not be classified as a true gel; instead, it suggests that the structure is situated between a highly concentrated biopolymer and an actual gel (Sarraf *et al.*, 2023). Steffe's theory suggests that a tan δ value between 0.24 and 0.33 indicates a weak elastic structure in biopolymer gels, leading to the formation of amorphous polymers (Steffe, 1996). The results obtained for the treatments were in the range of 0.14-0.17, except for the 9-CP (0.25).

The rheological behavior of quinoa protein gels in this study ($G' = 714$ -3743 Pa, tan $\delta =$

0.14-0.25) falls within the optimal ranges established for 3D food printing across various plant protein systems. Rahman *et al.* (2020) established that successful food inks require G' between 1,000-5,000 Pa and $\tan \delta$ between 0.15-0.25—criteria met by most QP formulations except 9-CP. Carranza *et al.* (2024) developed soy protein isolate (SPI)-alginate inks achieving optimal printability at G' values of 1,500-3,500 Pa, closely matching our 12-B (3743 Pa) and 12-PEF (3213 Pa) samples (Carranza, Tejo-Otero, Bengoechea, Guerrero, & de la Caba, 2024). The shear-thinning behavior observed across all QP gels aligns with requirements for extrusion-based printing, as documented for SPI (Carranza *et al.* 2024), pea protein (Jiang *et al.*, 2023), and sorghum protein (Barekat & Ubeyitogullari, 2025) systems.

Microstructural Features

The microstructural morphology of the gels prepared from three types of QP and polysaccharides was investigated using FESEM. As shown in Fig. 2, gels show a heterogeneous microstructure composed of protein-polysaccharide particles forming aggregates. According to Wang *et al.* (2024), proper gelation can only be achieved with suitable polysaccharide concentrations, whereas insufficient concentrations are a potential cause for failure in achieving acceptable 3D printability (Wang *et al.*, 2024). Based on Fig. 2 there are porous structures that enhance the movement of small molecules within the crystals. This phenomenon is caused by the contraction occurring through the lyophilization process (Sarraf *et al.*, 2024).

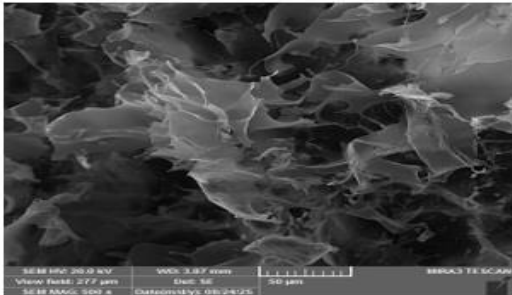
Feyzmanesh, Halvaei, & Baheiraei, (2022) prepared hydrogels by alginate and according to SEM images reported that porous structures with interconnected porosities were generated in the hydrogel structures.

The application of both treated and untreated QPs significantly impacts the structural characteristics of the gels ($p \leq 0.05$). Due to their hydrophilic nature and abundance of carboxyl and hydroxyl groups presence in SA, non-covalent bonds are generated and make promote hydrophobic interactions and hydrogen bonding (Wei *et al.*, 2023; Wang *et al.*, 2024).

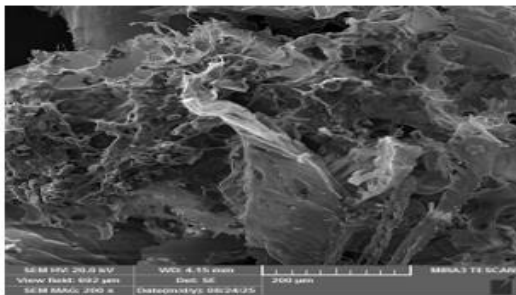
As demonstrated in Fig. 2 (magnifications: 50 $\times$ and 200 $\times$), sample 9-B presents a rough surface that lacks a coherent network; it contains large particles, loose aggregates, and exhibits reduced compaction. A notable distinction exists between the structural appearances of samples 9-B and 12-B.

The gel with 12% QP (12-B) contained a higher density of protein aggregates embedded in the Ag-SA matrix compared to the gel with 9% QP (9-B). The network appears more crowded in 12-B. The pores in 12-B sound to be smaller but are more irregular due to the filler effect of the additional protein (Fig. 2). This aligns with the lowest hardness values observed for sample 9-B (53.10 g) and a significant increase ($p \leq 0.05$) for sample 12-B (350.27 g). This suggests that merely adding more native protein serves as a filler, enhancing resistance without necessarily forming an elegant, cohesive network, as indicated by the low cohesiveness values ranging from 0.17 to 0.22 (Table 4).

9-B

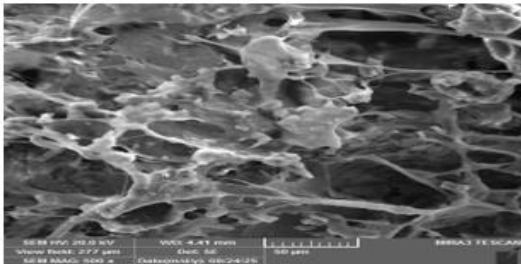


50 μm

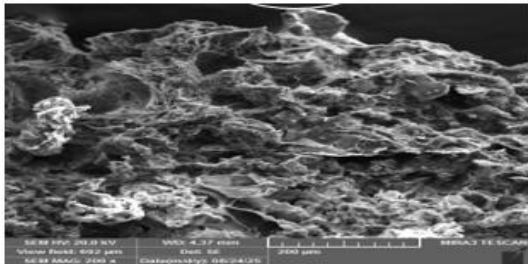


200 μm

12-B

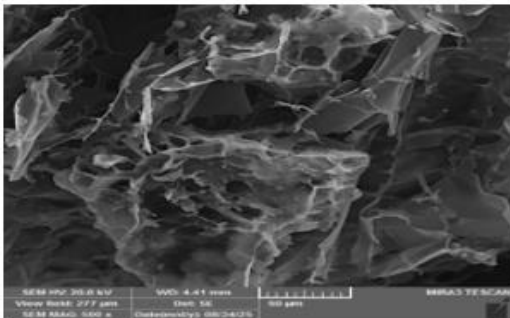


50 μm

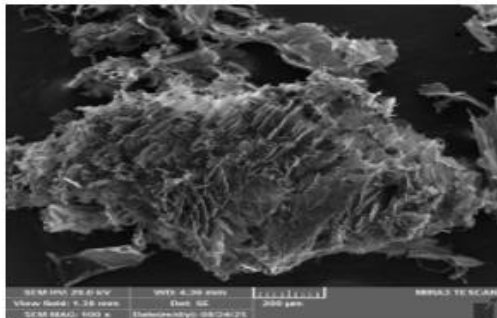


200 μm

9-PEF

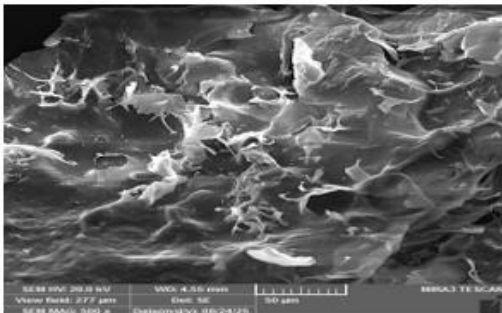


50 μm

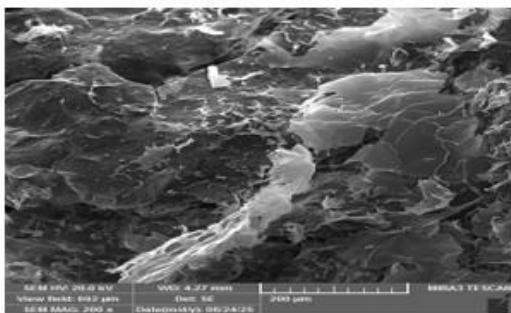


200 μm

12-PEF



50 μm



200 μm

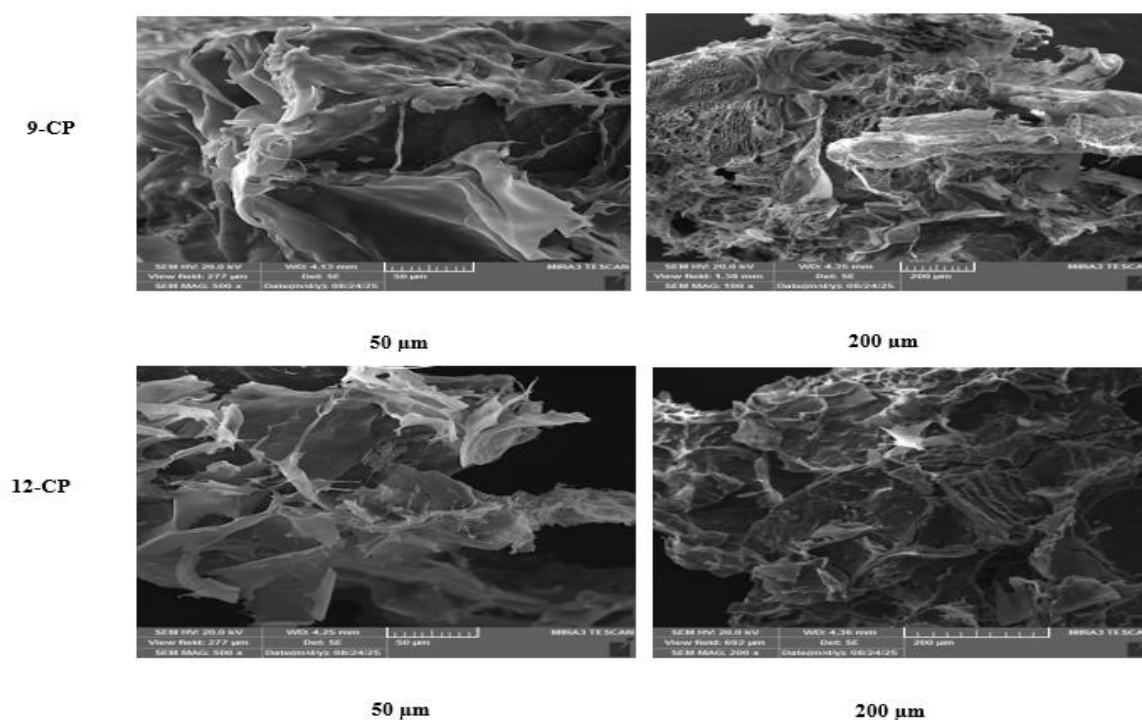


Fig. 2. The microstructural morphology (FESEM) of the gels prepared with QP (B/CP/PEF) and Ag- SA at 25 °C (50 and 100 μm)

In the 12-PEF and 9-CP images, the structure is completely heterogeneous, and roughness is observed at both magnifications. CP induces oxidation and cross-linking, creating a strong covalent protein network that integrates with Ag and SA. In light of this information, the structures of both the 9-CP and 12-CP samples exhibited a continuous and robust nature, demonstrating effective integration between the cross-linked QP and Ag-SA. This integration accounts for the elevated hardness and consistency values observed, such as the 12-CP sample, which displays a hardness of 416.52 g and a consistency of 1578.98 g·sec (Table 4). The significant cohesiveness of the 9-CP sample, with a value of 0.54, suggests that its network is not only strong but also notably balanced and resilient, resembling a highly uniform and interconnected web ($p \leq 0.05$). Conversely, the lower elasticity, indicated by G' values ranging from 714 to 1570 Pa in comparison to B/PEF gels, suggests a more rigid and less elastic microstructure (Table 1).

Similarly, PEF causes protein unfolding and electrostatic alignment without significant aggregation, promoting fine physical interactions within the matrix. Notably, the image of 9-PEF shows a somewhat uniform structure, with much less porosity and more compaction. This microstructure helps explain the high elastic modulus (G') values observed, with 9-PEF measuring 3419.4 Pa, making it the highest among all samples (Table 1). The fine, continuous network contributes to excellent elastic recovery, enabling it to store energy rather than dissipate it. The good hardness values for 9-PEF, recorded at 334.82 g, confirm that the structure is robust (Table 4). However, the lower cohesiveness compared to 9-CP indicates it may be less resistant to a second compression, likely due to the physical (non-covalent) nature of the bonds. To put it simply, the morphology of the protein-polysaccharide gels showed a more compact structure with minimal porosity compared to the protein images alone. These observations highlight the effect of non-thermal processing on protein-polysaccharide complexes, and the key role of

electrostatic interactions in determining their microstructural properties. For example, some studies reported that PEF promotes the formation of soluble protein aggregates by facilitating hydrophobic interactions among unfolded proteins (Taha *et al.*, 2023; Zhao & Yang, 2012). Also, Ahmadian *et al.* (2025) reported that cold plasma strengthens the interaction within the physicochemical properties of protein-polysaccharide complex that results as improve performance and increased suitability as a delivery wall material (Ahmadian *et al.*, 2025).

Physicochemical Analysis of Gels

Table 2 shows the results of water activity (a_w), dry matter (DM), water holding capacity (WHC), and color analyses of treatments. As can be seen, although a_w decreased with increasing QP percentage, no significant difference was observed among the gels. This may be attributed to the limited range of protein concentrations used and the predominance of

bound water in the gel network, which buffers the effect of increased QP on the water activity.

The results of the WHC tests on the gels indicated that the samples exhibited high strength against the stress generated by the centrifugal force during centrifugation. No phase separation or water separation was observed, which suggests a strong interaction between the biopolymers (Table 2). Zare *et al.* (2022) also subjected quinoa flour to the cold plasma technique and reported that by applying the process to quinoa flour, the WHC decreased at different times and voltages (Zare *et al.*, 2022). However, the results of this study indicate that neither the CP process nor the PEF process has an adverse effect on the WHC of the gel's structure. It is worth noting that the use of QP alone has been said to create a weak structure prone to syneresis (water leakage) and poor 3D printing capability, and the addition of polysaccharides can significantly improve this structure (Zhao *et al.*, 2024). In this study, the simultaneous use of QP and polysaccharides also created a high WHC (Table 2).

Table 2- Physicochemical properties (solid materials, a_w , holding capacity of water, and color parameters) of gels prepared with QP (B/CP/PEF) and Ag-SA

Code	Water Activity	Water Holding Capacity (%)	L*	a*	b*
9-B	0.86 ^a ±0.00	100.00±0.00	52.90 ^a ±0.67	0.98 ^e ±0.01	5.39 ^e ±0.01
12-B	0.85 ^{ab} ± 0.01	100.00±0.00	51.96 ^a ±0.23	1.38 ^d ±0.02	6.14 ^d ±0.00
9-PEF	0.85 ^{ab} ±0.00	100.00±0.00	45.34 ^c ±1.12	1.53 ^c ±0.02	6.61 ^d ±0.02
12-PEF	0.84 ^b ±0.00	100.00±0.00	48.46 ^b ±0.76	2.61 ^b ±0.01	9.18 ^c ±0.02
9-CP	0.85 ^{ab} ±0.01	100.00±0.00	41.00 ^d ±0.10	4.48 ^a ±0.01	11.75 ^b ±0.00
12-CP	0.84 ^b ± 0.01	100.00±0.00	44.53 ^c ±0.46	4.50 ^a ±0.00	12.40 ^a ±0.85

Regarding the color factor in gels, the brightness level has decreased in samples with a higher percentage of QP (12 %). On the other hand, the CP and the PEF increased the opacity of the produced protein, which in the same proportion reduced the brightness factor in the treated samples, so that the L* factor in 12% of B, PEF, and CP samples was 51.96, 48.46, and 44.53, respectively (Table 2).

Additionally, a* decreased in brightness, but expanded with protein percentage and pre-processing processes. Consequently, 12-CP exhibited the highest red spectrum and the lowest brightness among the treatments, which

was also predictable from the appearance of the QP powder. Factor b* showed a trend similar to that of factor a*, with the concentration of QP and pre-treatment positively influencing this factor. Pedrali *et al.* (2023) noted that the factors L*, a*, and b* were observed in samples when examining various types of QP. Color pigments are predominantly located in the pericarp. Upon grinding, these pigments integrate with the lighter particles of the endosperm (Pedrali, Giupponi, De la Peña-Armada, Villanueva-Suárez, & Mateos-Aparicio, 2023).

FTIR Analysis

FTIR spectra (4000–400 cm^{-1}) of QP (untreated, CP-treated, PEF-treated) and their

complexes with Ag/SA at 9% and 12% (w/w) were measured to ensure the interaction of the complex, as reported in Fig. 3.

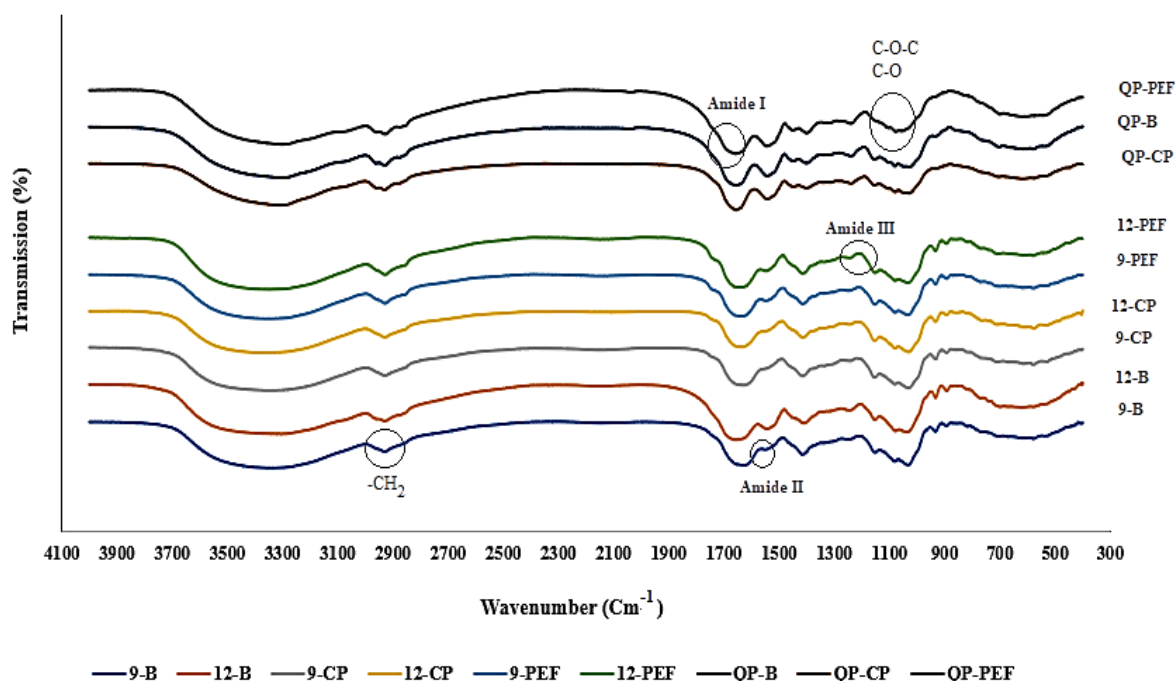


Fig. 3. FTIR spectra of dried gels prepared with QP (B/CP/PEF) and Ag-SA at 25°C

The absorption at wavenumbers of QP bands included amide I ($\approx 1650 \text{ cm}^{-1}$, mainly C=O stretch), amide II ($\approx 1540 \text{ cm}^{-1}$, N-H bend/C-N stretch), amide III ($\approx 1240 \text{ cm}^{-1}$), and amide A ($\approx 3300 \text{ cm}^{-1}$, N-H stretch). Alginate contributed symmetric/asymmetric $-\text{COO}^-$ stretches ($\approx 1600/1400 \text{ cm}^{-1}$) and $-\text{CH}_2$ vibration ($\approx 2900 \text{ cm}^{-1}$). (Derkach, Voron'ko, Sokolan, Kolotova, & Kuchina, 2020; Sarraf, Beig-Babaei, & Naji-Tabasi, 2021). On the other hand, the presence of the main polysaccharide structures was confirmed by a C-O-C stretching peak at $1020\text{--}1080 \text{ cm}^{-1}$ (Derkach *et al.*, 2020).

CP and PEF samples had intensity increase and slight shifts in the wavenumbers of $1020\text{--}1080 \text{ cm}^{-1}$ region in versus controls (9-B, 12-B). This alteration confirmed formation of hybrid QP/Ag-SA networks. The amide A band ($3300\text{--}3500 \text{ cm}^{-1}$) broadened and shifted to lower wavenumbers in treated samples (CP > PEF > control), reflecting stronger hydrogen bonding. CP-induced oxidation enhanced cross-linking, which is reflected in the texture

data, showing high hardness (Table 3) (Manzanilla-Valdez, Boesch, Martinez-Villaluenga, Montano, & Hernández-Álvarez, 2024). While PEF promoted protein unfolding, exposing $-\text{OH}/-\text{NH}_2$ groups for elastic, homogeneous networks.

The red shift and broadening of amide I after treatment indicated a partial unfolding (α -helix $\rightarrow$ random coil), and PEF induced a slight structural disorder relative to CP. Despite the pretreatment, the main interaction peaks were consistently formed in the samples, although the intensity variations were significant. Overall, the results illustrated that hydrogen bonding are essential for the formation and stability of protein-polysaccharide complexes. Another point is about the effect of pretreatment on the interaction changes between QP and polysaccharides.

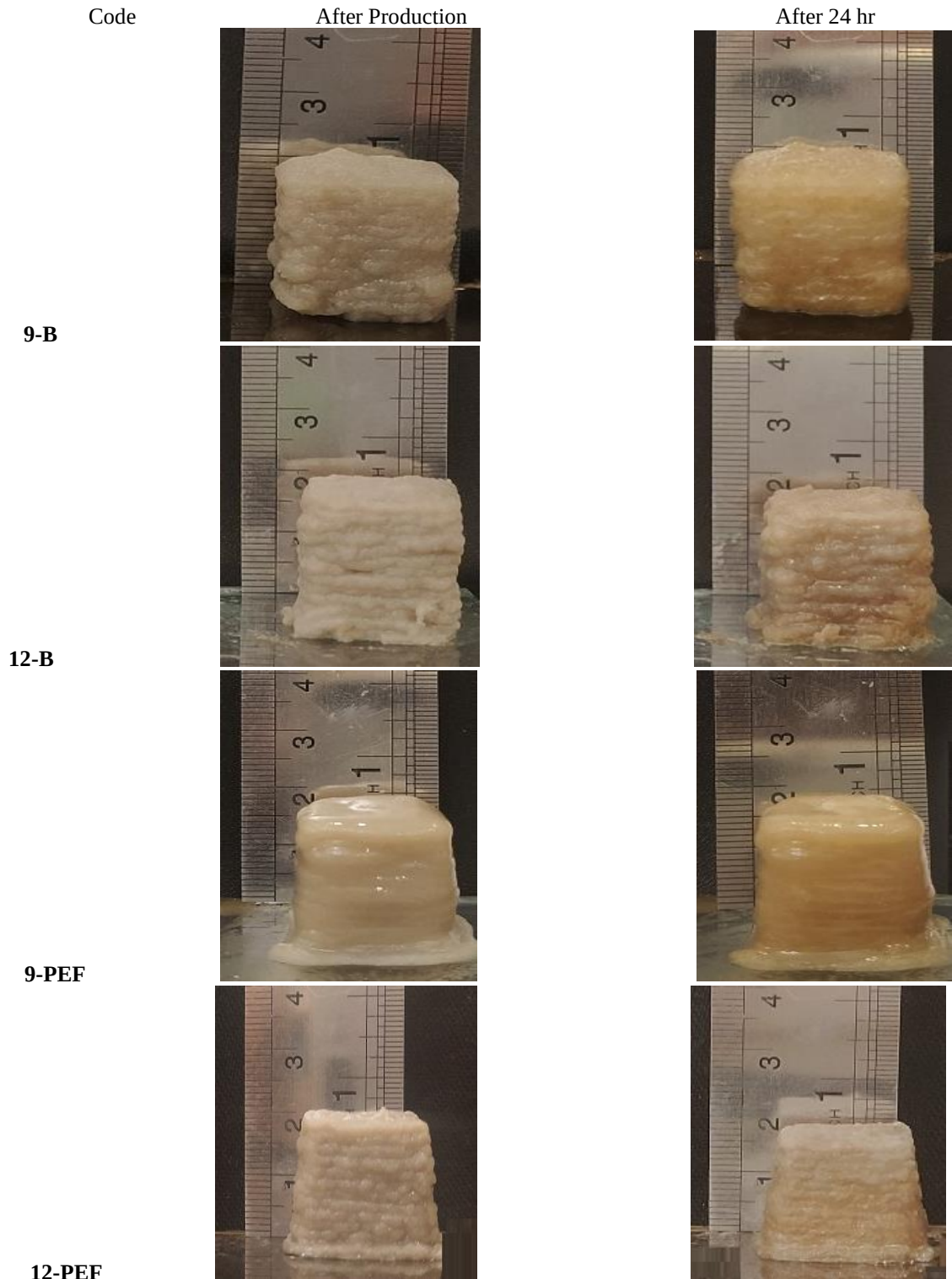
3D Printing Evaluation

Mechanism of 3D Printing

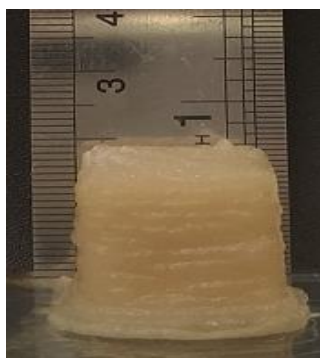
Evaluation of the 3D printability of the gels showed that all QP-polysaccharide composite

gels achieved (i) high-precision printing (ability to match the design model), (ii) high strength during printing (ability to maintain the printed form without collapsing), and (iii) strong

structural stability over time. However treated samples showed better and more stable structures, specially pulsed treated samples.



9-CP



12-CP

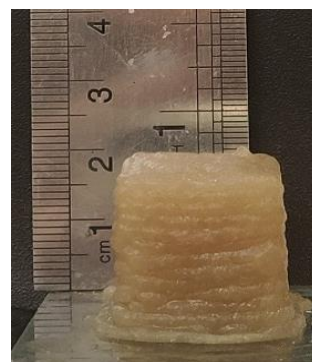


Fig. 4. 3D printability evaluation (cubic) of 3D printed gels prepared with QP (B/CP/PEF) and Ag-SA at 25 °C

On the other hand, the analysis of the rheological properties associated with the treatments reveals that all gels demonstrated shear-thinning characteristics (data not shown). This behavior indicates that, upon the application of force by the printer piston, the viscosity of the gel decreases, thus facilitating the extrusion process. It is essential to emphasize that a rapid recovery occurs during the process. Furthermore, upon completion of the process and the material's exit from the

nozzle, the gel structure promptly restores its high viscose and elastic modulus (high G' and G'') (Table 1). This characteristic ensures the stability of the printed layer, allowing it to bear the weight of subsequent layers effectively (Zhao *et al.*, 2024). The results were consistent across all treatments, and an evaluation of their height stability over a 24-h period showed that the productions possessed appropriate rheological properties (Fig. 4, Fig. 5).

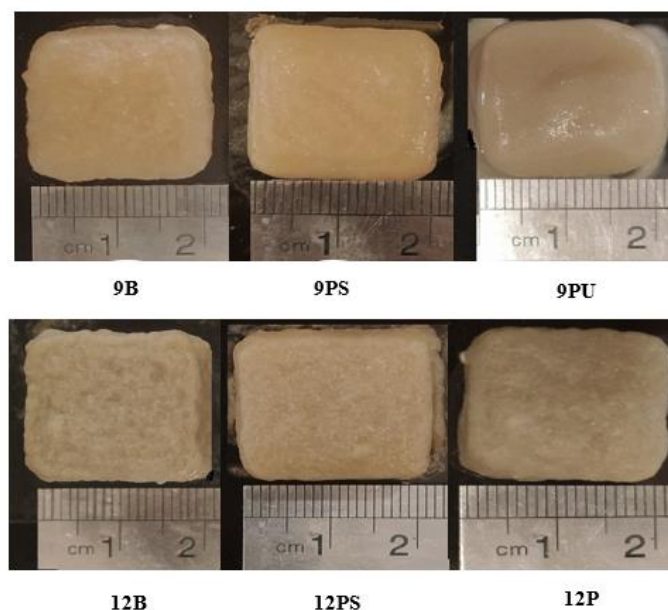


Fig. 5. Cross-section images of 3D printed gels prepared with QP (B/CP/PEF) and Ag-SA at 25 °C

Printability of 3D Printing Gels during 24 hr

The 3D printing process of the inks, as well as their dimensional measurements and the quality of appearance in additive manufacturing, were thoroughly evaluated at both 1 h and 24 h post-production (Fig. 4, Table 3).

After examining the printed gels that were stored at ambient temperature for 24 h, we observed no significant changes compared to their condition right after production ($p \leq 0.05$). This suggests that the gels have high stability and a long shelf-life. The height of the samples measured between 19.8 mm and 20 mm immediately after production, and maintained this height after 24 h. The gels exhibited strong viscoelastic behavior, and the storage modulus was high enough to maintain the ink's structure after extrusion (Zhao *et al.*, 2024).

The mechanism of this process is related to creation of cross-linking between proteins and polysaccharides, so that polysaccharides can bind to the QP (cationic) and form a dense, cross-linked network that traps water more effectively (Chen *et al.*, 2025; Chang, Gupta, Timilsena, & Adhikari, 2016). Moreover, polysaccharides can form extensive hydrogen

bonds with water and proteins, while their long polymer chains intertwine with the protein network, strengthening the entire gel structure.

The printability behavior was mostly related to the structural and rheological properties of the gel inks. The samples exhibited G' values higher than G'' , which showed elastic gel behavior capable of maintaining the printed shape after extrusion. The PEF treated samples showed a more compact and continuous microstructure, which increased the storage modulus and structural strength. As a result, these samples exhibited good shape fidelity but relatively more difficult extrusion due to higher resistance to flow. Conversely, the CP treatment promoted a balanced network structure with good cohesiveness, allowing smoother extrusion while still preserving the geometry of the printed construct. The negligible change in height after 24 h confirms that the viscoelastic network was sufficiently strong to resist gravitational deformation. Therefore, printability was governed by the balance between extrusion ability (flowability) and post-printing stability (shape retention), both of which were modulated differently by CP and PEF treatments.

Table 3- Printability values and height changes (after 24h) of gels prepared with QP (B/CP/PEF) and Ag-SA at 25°C

Code	3D Printing					
	Height of the gel after formation (mm)	Height of the gel after 24 h (mm)	Printability of gel	Weight of printed gel	Shape retention (%)	Shape fidelity %
9-B	20.00 ^a ±0.00	20.00 ^a ±0.00	1.50	6.31 ^c ±0.08	100.00	100.00
12-B	19.80 ^c ±0.01	19.70 ^c ±0.00	1.44	7.14 ^{ab} ±0.05	99.49	99.00
9-PEF	19.80 ^c ±0.01	19.80 ^b ±0.01	1.44	7.25 ^{ab} ±0.15	100.00	99.00
12-PEF	20.00 ^a ±0.00	19.80 ^b ±0.00	1.50	6.40 ^c ±0.5	99.00	100.00
9-CP	19.90 ^b ±0.00	19.80 ^b ±0.01	1.47	6.87 ^b ±0.06	99.50	99.50
12-CP	19.80 ^c ±0.01	19.80 ^b ±0.00	1.47	7.57 ^a ±0.25	100.00	99.00

By analyzing the printability of the gels, it can be stated that the range obtained was $Pr > 1$, as mentioned in section 3.8.1, which indicates excessive gelation that produces irregular filaments.

On the one hand, considering the stability over time and the retention of water under tension, and on the other hand, the high printability of these gels, the data are acceptable in response to the gels produced (Fig. 4).

The 24-h shape retention of treated/untreated QP gels (>99% for all samples) exceeds values reported for pea (85-95%) (Jiang *et al.*, 2023), and soy (90-97%) (Carranza *et al.*, 2024) protein systems, demonstrating exceptional structural stability of QP-Ag-SA formulations. The shape fidelity of the QP gels exceeded 99%, indicating that the printed structures closely matched the designed geometry (20 mm height) immediately after printing.

Appearance of 3D Printed Cube during Ink Extrusion

The extrusion conditions for the inks were broadly similar, except for the 12-PEF. While it demonstrated sufficient strength both during printing and over time, it felt heterogeneous to the touch, and the extrusion process was challenging. Additionally, the 9-PEF inks had a different texture and appearance compared to the 12-PEF inks (Fig. 4, Fig. 5).

The inks containing CP gels exhibited sufficient cohesion, allowing the printing

process to be completed faster than the other treatments. The 9 and 12% control samples (9-B, 12-B) also showed an acceptable extrusion process and did not present any challenges. In addition, no broken filaments during printing was observed, which might be related to the smoothness effect provided by polysaccharides. In a study of Liu *et al.* (2018), the objects printed using xanthan- mashed potatoes and *k*-carrageenan - xanthan- mashed potatoes and the results displayed a smooth surface texture and suitable appearance due to the enhancement of soft structure and coating properties of the gum (Liu, Zhang, & Bhandari, 2018).

Texture Analysis of Gels

There are two main effective parameters on the textural properties of the gels, namely protein concentration and the type of pretreatment used on QP. The hardness parameter indicated strength of the gel structure under compression. The hardness parameter was observed to increase with QP concentration in B and CP gels. Accordingly, the highest hardness values and stiffness parameters were obtained for 12-CP and 12-B, which were 416.52 and 350.27 g, respectively. This indicated the formation of a stronger network and improved water retention compared to the gel with a lower concentration of QP. In contrast, samples of PEF exhibited reverse results, with 9-PEF showing greater hardness (Table 4).

Table 4- Textural properties of gels prepared with QP (B/CP/PEF) and Ag-SA at 25°C

Code	Hardness(g)	Adhesiveness (g.sec)	Consistency (g.sec)	Apparent elastic modulus (g.sec ⁻¹)	Springiness (mm)	Gumminess (g)	Chewiness (g.mm)	Cohesiveness
9-B	53.10 ^c	-10.12 ^b	322.60 ^d	6.20 ^d	8.54 ^b	11.68 ^f	99.75 ^d	0.22 ^c
12-B	350.27 ^a	-30.79 ^a	1369.72 ^b	54.11 ^b	10.12 ^a	59.55 ^d	602.65 ^c	0.17 ^d
9-PEF	334.82 ^a	-20.48 ^{ab}	1298.10 ^b	66.08 ^b	8.18 ^b	97.10 ^c	794.28 ^b	0.29 ^c
12-PEF	111.10 ^c	-16.77 ^{ab}	607.13 ^c	18.26 ^c	8.54 ^b	14.44 ^e	123.32 ^d	0.13 ^d
9-CP	238.50 ^{ab}	-35.59 ^a	1216.29 ^b	56.22 ^b	8.09 ^b	128.79 ^b	1041.91 ^a	0.54 ^a
12-CP	416.52 ^a	-26.73 ^{ab}	1578.98 ^a	94.15 ^a	7.86 ^b	154.11 ^a	1211.30 ^a	0.37 ^b

Another important factor to be considered is adhesiveness, which measures the forces that keep a product attached to the surface of another material. The results indicate that in gels containing treated QP, adhesiveness decreased as the concentration of QP increased—conversely, the level of adhesiveness increased in gels that contain untreated QP. The consistency is another parameter and indicates the internal strength of the bonds. This characteristic showed the same results as the hardness parameter, which was predictable. The highest consistency was related to 12-B, 12-CP, and 9-PEF, which showed 1369.79, 1578.98 and 1298.10 g, respectively.

The apparent elastic modulus indicates the stiffness of the gels. Notably, the highest levels of this parameter were recorded for the samples designated as 12-B, 12-CP, and 9-PEF. Therefore, increasing the QP to 12% can have a significant effect on the hardness, stiffness and consistency of the texture in B and CP ($p \leq 0.05$). In contrast, the protein concentration of PEF poses a limitation, and the effects of this pretreatment on the network's strengthening or lack thereof should be considered.

Other parameters, such as springiness, gumminess, and chewiness, also showed an upward trend with increasing the quantities of QP. It is important to note that the texture properties of the PEF sample yielded different

results; specifically, the gumminess and chewiness of the 9-PEF sample were significantly higher ($p \leq 0.05$). Chewiness in the QP pulsed samples exhibited a weak response as the concentration of QP increased; for instance, the chewiness of the 9-PEF sample measured 794.28 g.mm, while it decreased to 123.32 g.mm when the concentration of QP pulsed increased from 9% to 12%. However, in other treatments, chewiness improved with rising QP levels.

The gumminess results are completely similar to that of chewiness, and the parameter increased in B and PEF compared to CP gels, indicating a direct relationship with the chewiness characteristic.

Furthermore, the concentration of protein increased the cohesiveness of the gels, with the highest cohesiveness observed at a concentration of 9-CP. This finding indicates that while higher concentrations of QP can enhance hardness and stiffness, they negatively impact the cohesion of the texture. The results suggest an inverse relationship between the concentration of QP and cohesiveness; as the concentration increases, cohesiveness significantly decreases ($p \leq 0.05$). Therefore, high concentrations of QP did not positively influence cohesiveness and consistency.

Textural properties of QP gels (hardness: 53-416 g) span ranges comparable to optimized plant protein systems.

Textural properties of QP gels (hardness: 53-416 g) span ranges comparable to optimized plant protein systems. Xu *et al.* (2024) reported that QP gels for eggless bread achieved hardness of 425 g at 12% concentration, matching our 12-CP sample (416 g). Zhao *et al.* (2025) developed QP-fucoidan emulsion gels with hardness of 280-450 g and printability values ($Pr = 1.42-1.52$) almost identical to our QP-Ag-SA systems ($Pr = 1.44-1.50$), suggesting that Pr values of 1.4-1.5 represent a universal optimum for protein-polysaccharide composite inks.

Conclusion

This study was conducted to examine the printability, physicochemical properties, and mechanical characteristics of gels derived from QP (both treated and untreated) at concentrations of 9% and 12%, in conjunction with polysaccharides. The analysis of their rheological, textural, and printability properties revealed that one of the significant conditions to obtain optimal performance was the dependence of gel characteristics on protein concentration. In addition, the application of the non-thermal technique helped to enhance the functional properties of the gels; however, there was no statistically significant difference observed when compared to the untreated samples. Overall, optimal results were achieved by 12-B, 12-CP, and 9-PEF. This indicates that while high concentration (12%) is generally beneficial for creating firm gels, the effectiveness of novel non-thermal techniques like cold plasma and PEF is particular to the protein concentration, with PEF showing a distinct advantage at the lower 9% concentration. In future research, the use of alternative polysaccharide types, different conjugation methods, or varying process parameters could be useful to achieve the desired improvements in printability.

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Author Contribution

Mozhdeh Sarraf: Original draft, Software, Investigation, Formal analysis, Data curation. **Sara Naji-Tabasi:** Conceptualization; Project administration; Supervision; Methodology; Validation; Review & editing. **Behrouz Ghorani:** Conceptualization; Project administration; Supervision; Methodology; Validation; Review & editing. **Bahareh Emadzadeh:** Methodology; Validation; Review & editing. **Omer Saeid Toker:** Validation; Review & editing.

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Availability of data and materials

Data are available on request from the authors.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for Publication

All authors read and approved the final manuscript.

Competing Interests

The authors declare they have no competing interest.

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مقاله پژوهشی

جلد ۲۲، شماره ۵، آذر - دی ۱۴۰۵، ص. ۴۴۹-۴۷۲

تأثیر روش‌های غیرحرارتی بر قابلیت چاپ و خواص عملکردی جوهر غذایی مبتنی بر پروتئین کینوا

مژده صراف^۱ - سارا ناجی طبسی^۱ ID* - بهروز قرآنی^۱ ID* - بهاره عمادزاده^۲ ID* - عمر سعید توکر^۳

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چکیده

در دهه گذشته، صنعت غذا شاهد تمرکز فزاینده‌ای بر روی طرح‌های متنوع و کاربردی بوده است. بر همین اساس، این تحقیق با هدف استفاده از تولید افزایشی برای ایجاد ساختارهای ژل از منظری جدید انجام شده است. در این مطالعه، پروتئین کینوا (QP)، چه بدون تیمار (B) و چه تحت تیمار با پلاسمای سرد (CP) یا میدان‌های الکتریکی پالس (PEF)، در دو غلظت (۹ و ۱۲ درصد وزنی/وزنی)، با پلی‌ساکاریدها (آلژینات و آگار) ترکیب شد تا ژل‌ها تهیه شوند. سپس قابلیت چاپ و خواص فیزیکوشیمیایی (فعالیت آبی، ماده خشک، ظرفیت نگهداری آب و آنالیز رنگ)، خواص بافتی و رئولوژیکی ژل‌ها مورد بررسی قرار گرفت. ارزیابی خصوصیات بافتی نشان داد که بالاترین میزان سختی مربوط به نمونه‌های ۱۲ درصد B (۵۳۰/۲۷ g)، ۱۲ درصد CP (۴۱۶/۵۲ g) و ۹ درصد PEF (۳۳۴/۸۲ g) می‌باشد. با این حال، در بررسی نتایج فرکانس متغیر، نمونه ۹ و ۱۲ درصد CP کمترین مدول ذخیره را در بین سایر ژل‌ها نشان داد. نتایج چاپ سه‌بعدی ژل‌ها، قابلیت چاپ و پایداری بالا در طول ۲۴ ساعت در دمای محیط را نشان داد. علاوه بر این، جداسازی فازی در معرض تنش شدید در هیچ یک از تیمارها رخ نداد. بنابراین، بهینه‌سازی ژل‌های پروتئین کینوا برای فرآیند چاپ سه‌بعدی نیاز به هماهنگی پارامترهای پردازش با ترکیب فرمولاسیون دارد. این امر می‌تواند طراحی‌های غذایی نوین همراه با ارتقای بافت و پایداری را توسعه داده و با تمرکز صنعت غذا بر راه حل‌های نوآورانه و عملی همسو باشد.

واژه‌های کلیدی: پروتئین کینوا، پلاسمای سرد، چاپ سه بعدی محصولات غذایی، میدان الکتریکی پالس، هیدروکلوئید

۱- گروه نانوفناوری مواد غذایی، مؤسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران

(*)- نویسندگان مسئول: Email: s.najitabasi@rifst.ac.ir; Email: b.ghorani@rifst.ac.ir

۲- گروه فیزیک مواد غذایی، مؤسسه پژوهشی علوم و صنایع غذایی، مشهد، ایران

۳- دانشکده شیمی و متالورژی، مهندسی مواد غذایی، دانشگاه فنی بیلدیز، استانبول، ترکیه

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Inhibition of Enzymatic Browning in Mango Juice Using Antioxidant-Rich Eggplant Peel Extracts: A Kinetic and Preservation Study

M. Aldabbagh¹, O. AL-Jammaas², R. Altaee¹, O. AL-abbasy^{1*}

1- Department of Chemistry, College of Education for Pure Science, University of Mosul, Iraq

(*- Corresponding Author Email: chem.omar1978@uomosul.edu.iq)

2- Department of Food Science, College of Agriculture and Forestry, University of Mosul, Iraq

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Abstract

Enzymatic browning processes negatively influence many food products, such as fresh juices, particularly their sensory properties and nutritional value, ultimately leading consumers to reject such products. Natural antioxidants have been used to reduce this phenomenon, preserving these products and preventing quality deterioration. This research investigates the antioxidant and enzyme inhibitory activities of eggplant peel ethanolic (EEPE) and aqueous (AEPE) extracts, by scavenging DPPH radicals method in mango juice (MJ) during cold storage. EEPE had greater DPPH radical scavenging capacity (0.66 mg/g) than AEPE (0.52 mg/g), although both extracts were weaker than vitamin C (0.73 mg/g). Enzymatic browning assays revealed that EEPE suppressed PPO and POD activities in mango juice (MJ), contributing to decreased browning intensity and better preservation of phenolic content after 15 days storage at 4°C, more than AEPE. Purified mango pulp PPO was of approximately 54 kDa molecular weight and was effectively extracted and purified using ammonium sulfate precipitation, dialysis, and CM-Cellulose ion exchange chromatography with a purification fold value of 15.07. Kinetic studies revealed that EEPE acted like a competitive inhibitor of PPO, while AEPE exhibited non-competitive inhibition. This finding reflects the potential of eggplant peel, as an agricultural waste, is a potent natural substance to be used in food preservation due to its antioxidant and anti-browning activities.

Keywords: Antioxidant activity, Eggplant peel extract, Enzymatic browning, Food preservation, Mango juice, Peroxidase, Polyphenol oxidase

Introduction

Fruit juice (FJ) is considered as nutrient-dense beverage, containing micronutrients, minerals, and antioxidants (Buvé, Pham, Hendrickx, Grauwet, & Van Loey, 2021). It shares the composition of fruits and is made from whole fruit (edible parts), with natural sugars, making it low-in-energy (Walton & Kehoe, 2025). It's convenient to consume and has a longer shelf life without chemical

preservatives. The global juice market, was valued at USD 145.2 billion in 2022. The market is projecting to grow due to health-conscious consumers seeking natural, preservative-free beverages (Konar, Gunes, Palabiyik, & Toker, 2022). The market is expected to expand at a CAGR of 5.60% by 2030. The variety of juice products has increased efficiency in quality control and maintenance, facilitated by minimal processing



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(Mieszczakowska-Frąć, Celejewska, & Płocharski, 2021).

Mango (*Mangifera indica* L.) belonging to the family Anacardiaceae is one of the most important fruits consumed in fresh or processed form globally (Singh, Singh, Sane, & Nath, 2013). It is extensively cultivated in tropical and subtropical climates worldwide because of its high nutritional value, flavor, and aroma (Dhanyasree, Rafeekher, & Premachandran, 2025). This fruit is a good source of minerals, dietary fiber, vitamins, and antioxidants like polyphenols, which have been connected to several health advantages (Wu *et al.*, 2018). Like many fruits, mango juice turns brown when exposed to air, which changes the color and taste, thus spoiling the product and making it undesirable to drink.

Browning in food products like FJ occurs during processing and storage, resulting in color changes due to two key chemical reactions: non-enzymatic browning (NEB) and enzymatic browning (EB). Nonenzymatic chemical reactions like caramelization, ascorbic acid degradation, and the Maillard reaction are common quality issues in concentrated FJ, leading to nutrient loss and undesirable compounds (Abdul Kareem & Ali Al-Jammaas, 2024). On the other hand, EB is mainly caused by polyphenol oxidase (EC 1.14.18.1, PPO) (Al-abbasy, Alsarraf, Mahdi, & Al-barwari, 2025). This enzyme oxidizes phenolic compounds to quinones that spontaneously polymerize with other compounds (phenolics, sugars, soluble amino proteins), to generate brown pigments (Bobo-García, Arroqui, Merino, & Vírveda, 2020). Several studies have focused on preventing or inhibiting PPO activity in food products. Numerous methods and mechanisms have been suggested over the years for controlling this enzyme activity. Analysis revealed that most chemical or synthetic inhibitors were hazardous compounds that might react with cellular constituents to produce toxicity. Hence, the most important and safest method is using natural sources in products that are minimally processed (AL-BURGUS, Ali, & AL-

ABBASY, 2024; Weerawardana, Thiripuranathar, & Paranagama, 2020). For example, studies have demonstrated that, aloe vera (Alberio, Muratore, Licciardello, Giardina, & Spagna, 2015), whey protein (Yi & Ding, 2014), and rice bran extracts (Sukhonthara, Kaewka, & Theerakulkait, 2016) inhibit browning and maintain quality.

Eggplant (*Solanum melongena* L.) is a widespread vegetable crop in temperate and tropical regions (Mat Sulaiman *et al.*, 2020). Eggplant fruit is included in the basic diets of many local communities (Davidar *et al.*, 2015). It contains many active compounds compared to other crops of the same family. The largest part of these active compounds is particularly abundant in the peel (approximately 30-60%), and is represented by polyphenols, flavonoids, and phenolic acids (Niño-Medina, Urías-Orona, Muy-Rangel, & Heredia, 2017). Anthocyanins and chlorogenic acid are the main and most important compounds in the peel (Yarmohammadi, Rahbardar, & Hosseinzadeh, 2021). These compounds have a high antioxidant capacity.

The main objective of this research was to develop PPO inhibition in mango juice using eggplant peel extracts as natural inhibitors. Thus, the project aimed to reduce browning and increase the shelf life of mango juice.

Materials and Methods

Chemicals such as Folin-Ciocalteu reagent, bovine serum albumin, Triton X-100, ammonium sulfate, and ninhydrin were purchased from Merck Co. Additionally, catechol, polyethylene glycol, polyvinylpyrrolidone, gallic acid, guaiacol, petroleum ether, ethanol, and carboxymethyl (CM) cellulose were obtained from Sigma-Aldrich Co.

Preparation of Soxhlet Extraction

Eggplant fruit was purchased freshly from a local market in Mosul City, Iraq, in October 2024 (peak season). The plant was washed with a tap water and then manually peeled and the peel was chopped into small pieces. The pieces

were dried at 40°C for 72 h until a constant weight was achieved, using a digital forced-air convection oven (5-200 °C, Model UN55, Memmert GmbH Co. KG, Schabach, Germany). The dried pieces were ground to obtain fine homogeneous powder in a high-speed analytical laboratory grinder fitted with 180W motor drive (Model A11 Basic, IKA-WERKE GmbH & Co. KG, Staufen, Germany). For extraction, 25g powder was subjected to Soxhlet extraction with 250 ml petroleum ether for 8 hours at 60-80°C for defatting. The extract was evaporated using a vacuum rotavapor. The defatted peel was macerated in ethanol (70%) for 24 h., extracted by Soxhlet for 6 h., evaporated at 50°C, and kept at -20°C (Al-Abbasy, Ali, & Younis, 2020). This extract is defined as ethanolic eggplant peel extract (EEPE).

Preparation of Aqueous Eggplant Peel Extract

Twenty-five grams of eggplant peel powder were mixed with 250 mL of distilled water and the mixture was incubated on a rotating shaker at 1,500 rpm at 35°C for 2 days. The extract was then filtered through a cheesecloth and centrifuged at 10,000×*g* for 15 min. The supernatant was evaporated on a vacuum rotavapor at 70°C (Taqi, Al-Shahery, & Al-Abbasy, 2025). This extract is defined as aqueous eggplant peel extract (AEPE) and kept at -20°C.

Preparation of Mango Juice

Mango was acquired freshly from a local market in Mosul City, Iraq, in May 2025 to ensure maximum enzymatic activity. After washing and peeling, the mango pulp was homogenized with DW at a ratio of 1:10 (W/V), using blender powered with a 370-W motor driver (Model 8011G, Waring Commercial, Torrington, CT, USA). The crushed mango was filtered through many layers of muslin cloth. The produced juice was centrifuged at 3,000×*g* for 10 min. This was defined as mango juice (MJ). The MJ was divided into two groups treated with 5% of each EEPE and AEPE separately. For comparison, the control was MJ

without any extract (Hasan Ali AL-Jammaas, 2023).

Antioxidant Activity Measurement by DPPH Radical Scavenging

According to Matthäus (Matthäus, 2002), two milliliters of EEPE were mixed with three milliliters of DPPH solution (0.5 mg/mL), and twenty-five milliliters of methanol. After thorough shaking, the mixture was kept in the dark for forty-five minutes. The decrease in absorbance was measured at 515 nm. Vitamin C (1%) was used as an antioxidant control. The following formula is used to calculate the percentage:

$$\text{Inhibitory \%} = [(A_c - A_s) \div A_c] \times 100$$

When: A_c : Control reaction absorbance, A_s : Testing specimen absorbance.

Intermediate Compounds and Browning Intensity Monitoring

By using spectrophotometry, a colorless intermediate at A_{294} nm was recorded. The brown intensity of a hue was measured at A_{420} nm (Tsai, Yu, Chen, Liu, & Sun, 2009).

PPO Assay

Spectrophotometrically, PPO activity was monitored. One milliliter of 0.01M catechol (substrate), 0.9 ml buffer (pH 7.2), and finally 0.1 ml of enzyme were mixed. The absorbance was measured at 420 nm with a ten-second interval for five minutes (Oktay, Küfreliolu, Kocaçalışkan, & Şaklrolu, 1995).

Peroxidase Assay

To assess POD activity, a 100 µL aliquot of the extracted enzyme was added to the reaction mixture along with 1.5 mL of 0.1 M phosphate buffer (pH 7.0), 0.5 mL of guaiacol (1.68%), and 0.5 mL of H₂O₂ (1.8%). The total volume was then adjusted to 3 mL with distilled water. All components of the reaction mixture, except the enzymatic extract, were replaced with water to create blanks. The absorbance was measured at 470 nm at 30 °C (Lagrimini, Gingas, Finger, Rothstein, & Liu, 1997).

Content of Total Phenols

To a 25 ml volumetric flask, one ml of the sample, one ml of Na_2CO_3 (20%), and 0.75 ml of Folin-Ciocalteu reagent were added. Distilled water was then added to make the total amount of 25 ml. Using spectrophotometer (PU 8800 UV/VIS, Shimadzu, Double Beam), the absorbance at 765 nm was measured following a 30-minute incubation period in a 55 °C water bath. A standard curve for gallic acid (GA) was utilized to quantify the total phenolic content. The result was reported as GA equivalents per gram of fresh weight (mg/g) (Girgžde, Samsone, & Gailis, 2019).

Determination of Protein Concentration

The protein concentration was assessed using bovine serum albumin as a stock solution. The absorbance was monitored at 650 nm (Scharcterle & Pollack, 1973).

Enzyme Extraction

To purify PPO, a procedure described by (Rashan & Al-Abbasy, 2021) with some modifications was used. After washing and peeling, 50 grams of fresh mango fruit were homogenized with 150 ml of cold phosphate buffer (0.1 M, pH 7.2), including (10 mM ascorbic acid, 3% polyvinylpyrrolidone (PVPP), 2% PEG, and 1% Triton X-100), using a blender for 2 minutes. The homogenate was frozen using liquid nitrogen to ensure disruption of the cell wall. After that, the extract was filtered through cheesecloth in an ice bath and then centrifuged at $10,000 \times g$ for 15 minutes at 4 °C. The supernatant was collected and considered as crude enzyme, then kept at 4°C for further analysis.

Precipitation and Dialysis

The crude enzyme solution was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to give a saturation of 30-80% and left overnight. The centrifugation for 30 min at $3000 \times g$ was achieved. The precipitated fractions were resolubilized in a minimum volume of phosphate buffer (0.1M, pH 7.2) . The dialysis procedure via a 14 kDa cut-off membrane for 24 h. was done against the same buffer at 4°C with six buffer changes.

The centrifugation for 5 minutes to obtain a clear supernatant (Mahdi, Younus, Al-Burgus, & Al-Abbasy, 2025).

Ion Exchange Chromatography

On a column containing CM-cellulose resin, the dialyzed solution was loaded. A protein was eluted using phosphate buffer at a flow rate of 0.8 ml/min (three milliliters/fraction) and monitored at 280 nm. Following detection, pooling, and lyophilization, the fractions with PPO activity were employed in the subsequent studies (Alrushdi *et al.*, 2025).

Gel Electrophoresis

SDS-PAGE was carried out in a mini gel equipment (Bio-Rad Mini-PROTEIN Tetra Cell, Bio-Rad) as mentioned by Laemmli (Laemmli, 1970). Protein solutions were denatured by heating for 5 minutes at 99 °C (Eppendorf Thermomixer Comfort). The samples were then put in 5% stacking and 9% resolving polyacrylamide gels before being run at 120 V. The gel was stained using Coomassie Brilliant Blue G-250 (Al-Taie, Alaboo, Al-Abbasy, & Taqi, 2026). The protein markers were used to calculate the molecular weight of the purified PPO.

Inhibition Mode of PPO

The inhibition of purified mango PPO was examined in the presence of EEPE and AEPE as inhibitors. The activity was monitored at 420 nm using (0.25-3 mM) of substrate after incubation of 0.2 ml of enzyme with 0.1 ml of inhibitor for 30 minutes (Alabbas, Salih, & Al-Abbasy, 2022).

Statistical Analysis

All acquired data were represented by mean $\pm$ S.D. The data were processed using the SPSS Statistics programme for one-way ANOVA analysis followed by Duncan's Multiple Range test. The comparisons between various groups were carried out, and a p-value of <0.05 indicated the statistically significant differences.

Results and Discussion

Table 1 shows the scavenging impact of eggplant peel extracts on DPPH radicals. Both extracts were found to have antioxidant properties. EEPE showed higher scavenging properties ($0.66 \text{ mg} \cdot \text{g}^{-1}$) than AEPE ($0.52 \text{ mg} \cdot \text{g}^{-1}$) compared to vitamin C ($0.73 \text{ mg} \cdot \text{g}^{-1}$).

Table 1- The scavenging effect of eggplant peel extracts ($\text{mg} \cdot \text{g}^{-1}$) for quenching DPPH radicals

Solution	$\text{mg} \cdot \text{g}^{-1}$
Vitamin C	0.73 ± 0.015
EEPE	0.66 ± 0.021
AEPE	0.52 ± 0.035

The DPPH free radical scavenging activity of the ethanolic extract of eggplant pulp powder was evaluated using the common antioxidant ascorbic acid as a standard. The antioxidant activity was observed to be concentration-dependent and to be significantly greater than that of ascorbic acid (Nasraldeen Majeed, 2024; Salih & Sadoon, 2026). The antioxidant activity of ethanolic and water extracts from different parts of eggplant was evaluated. The extracts of peel and calyx parts exhibited a higher DPPH radical scavenging activity in both extracts (Jung, Bae, Jo, Jo, & Lee, 2011). The biological process underlying the antioxidant property is linked to the scavenging of free radicals, specifically hydroxyl radicals, and metal chelation (Akanitapichat, Phraibung, Nuchklang, & Prompitakkul, 2010). Eggplant peel is frequently discarded as biological waste, resulting in significant losses of organic matter that typically contains high levels of bioactive compounds (Agnello *et al.*, 2020).

Several studies have revealed that plant extracts are accomplished of inhibiting PPO activity in foods. The ability of extracts to inhibit PPO varies depending on the phytochemicals they contain and the antioxidant potential of the compounds. Further, Fig. 1 shows an evaluation of the

inhibitory effects of EEPE and AEPE extracts compared to vitamin C on PPO.

Compared to the untreated juice sample, EEPE showed an inhibitory effect of 45.87%, which is better than AEPE, which inhibited the enzyme by 16.84%. However, both extracts were less effective than vitamin C, considered a potent antioxidant, with a 56.79% inhibitory effect. The ability of these extracts to inhibit oxidation processes may rely on several mechanisms, involving reduction activity, radical scavenging, and chelating of metal ions (Billaud *et al.*, 2005).

Effect of Extracts on Browning Intensity and Intermediate Compounds in MJ during Storage

According to Ali *et al.* (Ali, Al-Burgus, Al-Abbasy, & Haddad, 2026) absorbance at 294nm and 420nm indicates the existence of colorless intermediate particles and browning compounds. The browning intensity and intermediate compound values of MJ after treatment with 5% of EEPE and AEPE and storing for 15 days at 4°C are shown in Fig. 2 and Fig. 3. The values of MJ-treated AEPE were higher than those of EEPE-treated at all storage times. These results suggest that EEPE could diminish the increase in absorbance at 420 and 294 nm in MJ during storage more than AEPE. The EEPE might contain phenolic compounds capable of inhibiting the PPO isozyme in MJ, with each phenolic compound potentially having different specific inhibitory activities on various PPO isozymes. These compounds have structures similar to the substrate and might inhibit browning (Ajila, Rao, & Rao, 2010). Regardless of the type of natural anti-browning chemical utilized, the findings correspond with those documented by others (Jirasuteeruk & Theerakulkait, 2020; Shomodder, Hossain, Das, Ranganathan, & Mazumder, 2021).

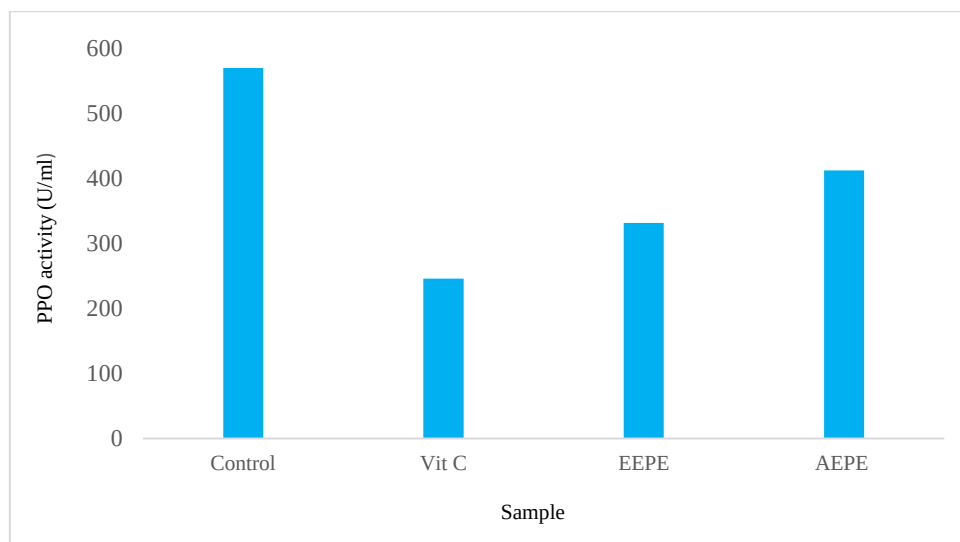


Fig. 1. Comparison the effects of eggplant peel extracts and vitamin C on purified PPO from Mango pulp

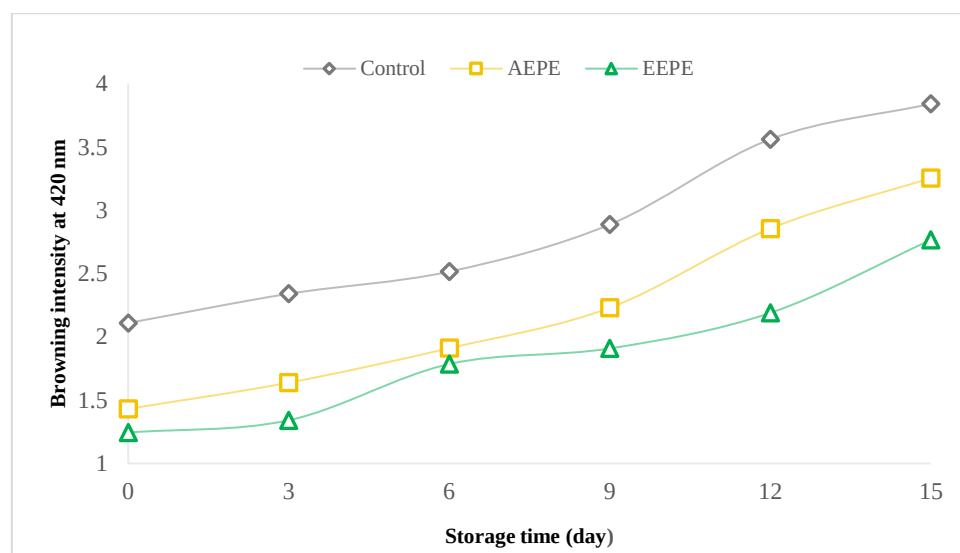


Fig. 2. Extracts effect on browning intensity in MJ during storage

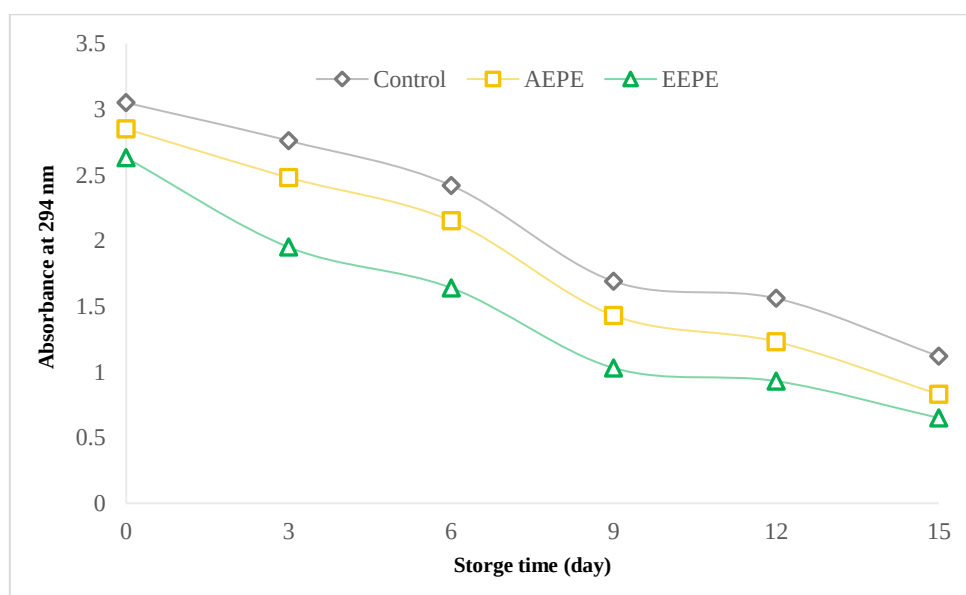


Fig. 3. Extracts effect on intermediate compounds in MJ during storage

Effect of EEPE and AEPE Addition on the PPO Activity during Storage

The impact of EEPE and AEPE on mango juice PPO enzymatic activity during storage for 15 days at 4°C is presented in Fig. 4. The results

showed that both extracts could inhibit PPO compared to the control group for all storage times. However, the percentage of enzyme inhibition in the presence of EEPE was stronger than that of AEPE in the treated juice samples.

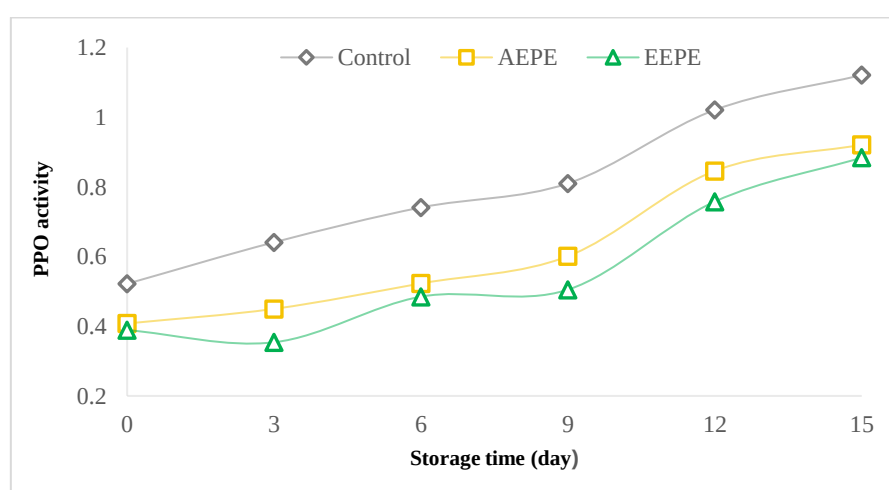


Fig. 4. Inhibitory effect of extracts on PPO in MJ during storage

Several studies have attempted to inhibit PPO activity by utilizing extracts from sources rich in antioxidant compounds. For example, it has been found that aqueous extracts of mango peels of several cultivars inhibited the activity of PPO in potato puree to varying degrees (AL-abbasy, M Mahdi, Younus, Sheej Ahmad, & Al-Azzawi, 2025; Jirasuteeruk & Theerakulkait, 2020). Previous results

indicated that the oil extract of cinnamon bark showed 40% inhibition of PPO activity in mushrooms (Marongiu *et al.*, 2007). It was also observed that the aqueous extract of ginger has an anti-tanning effect by reducing the enzymatic activity of PPO of *Annona muricata* and *Musa acuminata* (Weerawardana *et al.*, 2020). Different concentrations of garlic and onion extracts presented a higher inhibitory

effect on the yam PPO activity (Yapi, Gnangui, Dabonné, & Kouamé, 2015). Such inhibition may be due to the high total phenolic content in these extracts.

Effect of EEPE and AEPE Addition on POD Activity during Storage

The effect of eggplant peel extracts on POD activity was evident Fig. 5. A gradual increase

in POD activity was observed during storage at 4°C for untreated juice, reaching its maximum on the 15th day. When juice samples were treated with EEPE and AEPE extracts (5%), POD activity decreased significantly compared to untreated juice samples. The results demonstrated that EEPE was more effective than AEPE in inhibiting POD.

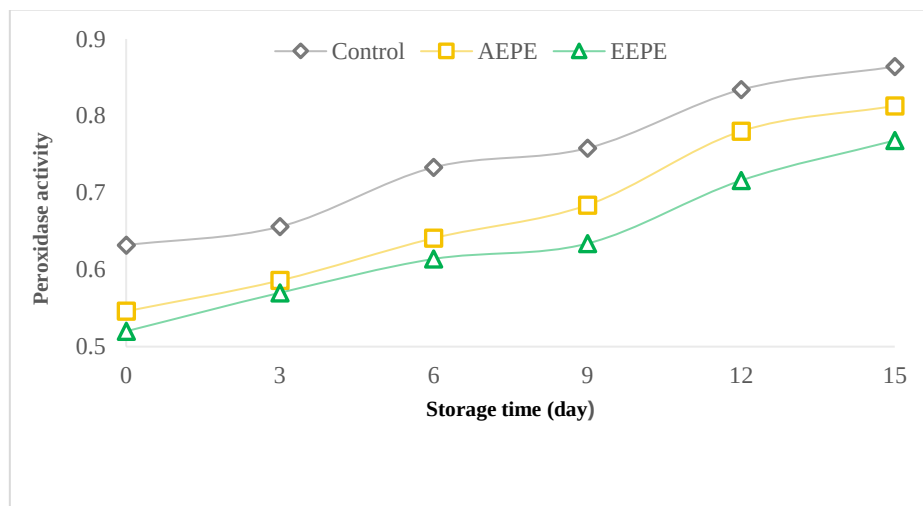


Fig. 5. Inhibitory effect of extracts on POD in MJ during storage

POD is an enzyme that catalyzes the oxidation of phenols by peroxide, which is the major cause of enzymatic browning in fruits and vegetables (Oms-Oliu *et al.*, 2010). During litchi fruit storage, POD activity in the pericarp steadily rose in tandem with the skin browning index (Zhang, Pang, Xuewu, Ji, & Jiang, 2005). POD activity increased steadily at 4°C throughout the storage period, with the highest activity level recorded in untreated mandarin fruit. In contrast, enzyme activity at 8°C rose during the first two weeks, followed by a sharp decline for the remainder of the storage period. (Tao, Wang, Luo, & Yan, 2018). Each vegetable substrate under study had a unique reaction of POD activity to the antioxidant treatment, which was contingent upon storage conditions. Freshly cut veggies are reported to be best preserved when stored in a refrigerator (Xu *et al.*, 2012). The use of antioxidant solutions, such as tea tree essential oil and propolis extract, at 5°C under optimal storage

conditions for 14 days inhibited POD activity in butternut squash throughout the storage period. Furthermore, the use of propolis delayed browning and preserved the product's visual quality for a longer period compared to untreated sample (Alvarez, Moreira, & Ponce, 2015).

Effect of Extracts on Phenolic Content in MJ during Storage

Fig. 6 depicts the total phenolic content (mg/100 ml) in MJ treated with EEPE and AEPE throughout storage. The amount of phenolic content significantly declined during storage at 4°C, reaching its minimum on the 15th day. Treatment of MJ with 5% of EEPE and AEPE showed a significant increase compared to the untreated juice at 0 and 15th day of storage. It is clear from the diagram that the alcoholic extract had a better effect in maintaining the level of phenolic compound content than the aqueous extract. This may be due to high levels of active phenolic

compounds in these extracts, which can reduce PPO activity and thus prevent the oxidation of

the phenolic compounds initially present in MJ and preserve them during the storage period.

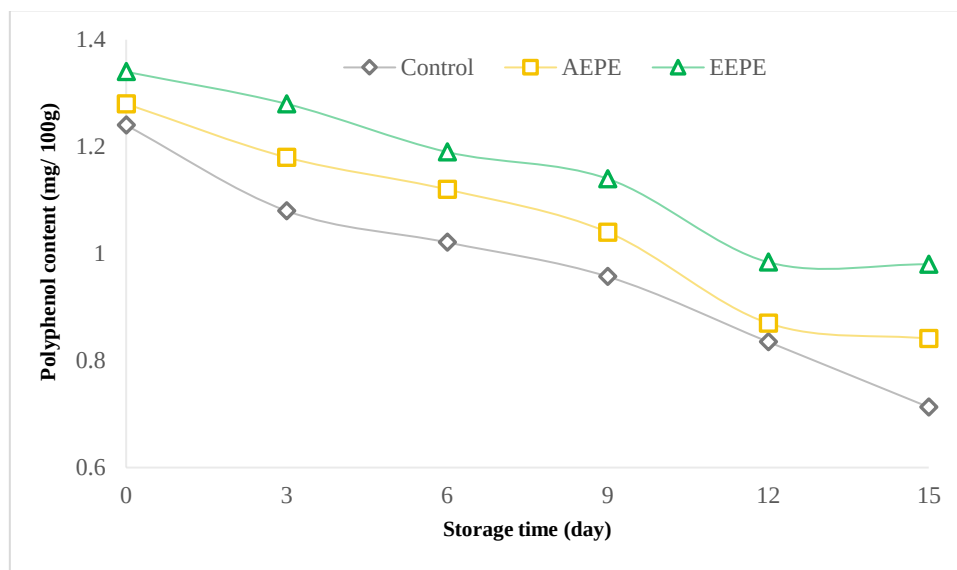


Fig. 6. Extracts effect on polyphenol content in MJ during storage

Similarly, Altunkaya *et al.* (2013), reported a significant increase in the phenolic content of apple juice treated with pomegranate peel extracts (Altunkaya *et al.*, 2013). An apparent increase in the concentration of phenolics was also observed, directly proportional to the concentration of the added natural extracts, as in apple juice (Phaiphan, Panichakool, Jinawan, & Penjumras, 2019) and quince juice (Rashan & Al-Abbasy, 2021) during storage.

Enzyme Purification

Precipitation of the enzyme-containing protein with 30-80% ammonium sulfate increased the specific activity of PPO to 2449.11 units /mg protein, with a purification

fold of 5.61 compared to the crude. After dialysis to remove salts, peptides, and low-molecular-weight compounds, the results indicated that PPO was more purified by 7.00-fold. The protein concentration decreased after this step, and the specific activity value increased to 3055.9 units /mg protein (Table 2). To obtain almost completely pure enzyme, the CM-cellulose ion exchange column chromatography was used, and the elution profile showed a single band (Fig. 7) with a specific activity of 6573.42 compared to the crude enzyme, and the purification fold was raised to 15.07.

Table 2- Purification steps of PPO from mango pulp

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Sp. Ac. (U/mg protein)	Yield %	Purification Fold
Crude extract	66.5	55.86	24361.61	436.12	100	1.0
Ammonium sulfate	12	6.48	15870.24	2449.11	65.14	5.61
Dialysis	13.2	4.818	14723.28	3055.9	60.43	7.00
CM-Cellulose	24.2	1.76	18301.39	10398.51	75.12	23.84

* The unit is defined as the amount of enzyme that oxidizes 1 micromole of catechol per minute at 25 °C.

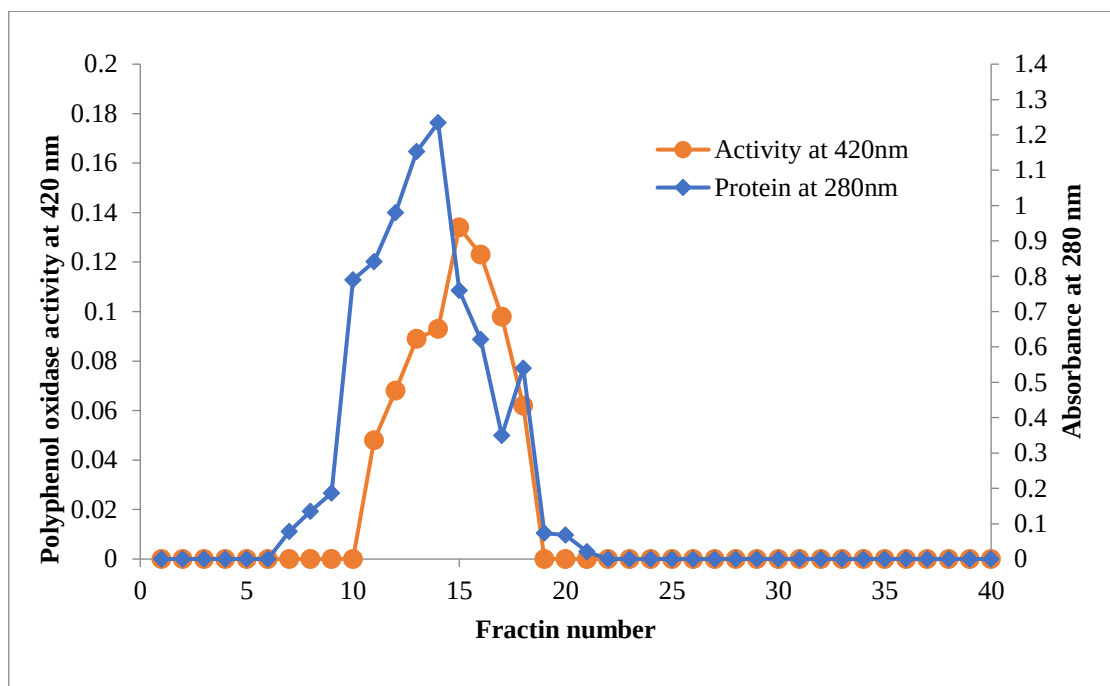


Fig. 7. Elution profile of mango pulp PPO by ion exchange chromatography

Numerous studies have used ion exchange chromatography to purify PPO from various plant tissues. This technique relies on the positive or negative charges present on the exchanger used for separation. The PPO was purified from *Prunus domestica* L. 36-fold using DEAE-cellulose chromatography (Siddiq, Sinha, Cash, & Hanum, 1996). One peak of PPO was obtained from the Mirabelle plum with a purification fold of 26.31 after passing the enzyme extract on a column containing the cationic exchanger, CM-cellulose (Younus, Mahdi, Al-Abbasy, & Sheej Ahmad, 2025). DEAE Sephadex A-50 and DEAE-Sepharose columns were used to purify PPO from the seeds of Melon (Adeseko & Fatoki, 2021) and the leaves of *Cleome gynandra* L. (Gao, Liu, & Xiao, 2011), respectively.

Molecular Weight Determination Using SDS-PAGE

This technique has been used to document the molecular weights of PPO, with masses ranging from 25 kDa for pineapple (Das, Bhat, & Gowda, 1997) to 120 kDa for beans (Paul & Gowda, 2000). In our analysis, the purified PPO

obtained after ion exchange chromatography performed as a single protein band on a gel with a molecular weight of 54 kDa (Fig. 8). This result is very close to the PPO enzyme purified from African bush mango peel, which was observed to have a molecular weight of 53 kDa (Adeseko *et al.*, 2021). It is slightly less than that purified from Mexican Golden Delicious apples (58 kDa) (Macheix, Fleuriet, & Billot, 1990), Turkish Alyanak apricots (57 kDa) (Ünal & Şener, 2016), and potato (50 kDa) (Aksoy, 2020). On the other hand, the molecular weight of several mango parts was reported and found to be significantly lower than what we obtained (Adeseko, Sanni, & Lawal, 2022; Palma-Orozco, Marrufo-Hernández, Sampedro, & Nájera, 2014).

Inhibition Type of PPO

The inhibition type of the purified mango PPO activity was examined in the presence of EEPE and AEPE extracts. It was verified by drawing a Lineweaver-Burk plot using various concentrations of a substrate. The results demonstrated a competitive inhibition type with EEPE (Fig. 9). The value of V_{max} was approximately 0.208 units /ml/ min. The K_m

value increased from 1.020 to 2.127 mM. When using the AEPE as an inhibitor, Fig. 10 shows that the inhibition type was non-competitive. The V_{max} value decreased from 0.208 to 0.116

units/ml/min. The K_m value was stable at 1.020 mM.

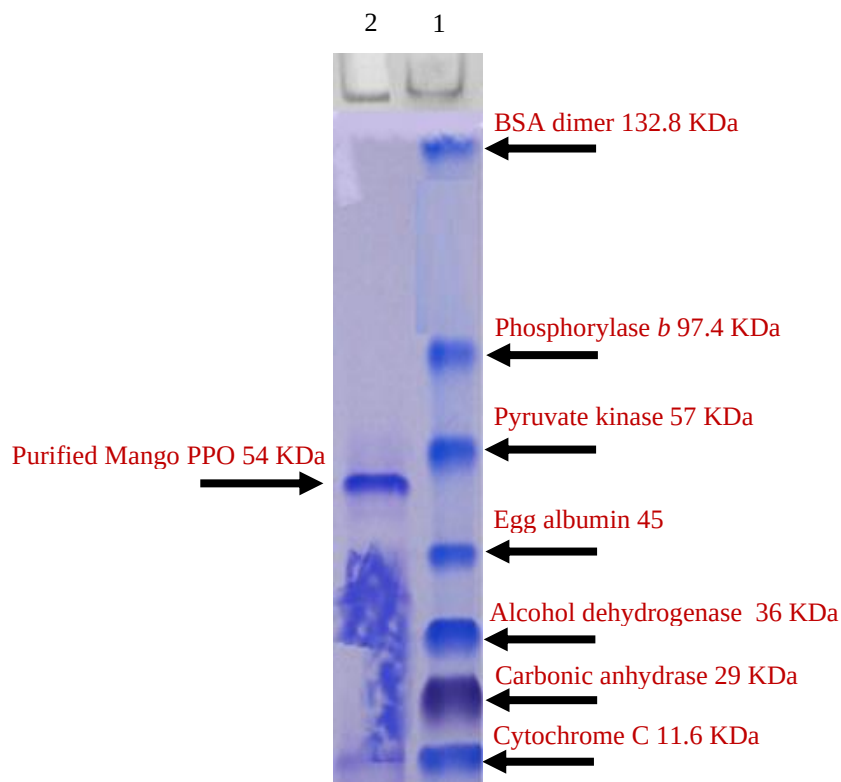


Fig. 8. Photograph of SDS-PAGE electrophoresis. Lane 1: Marker protein bands. Lane 2: Band of purified PPO from the mango of molecular weight approximately 54 kDa

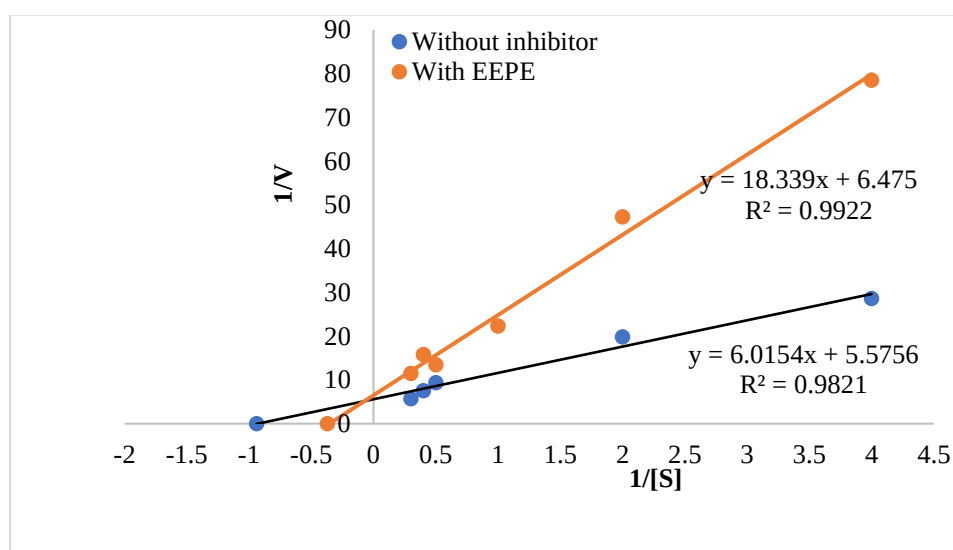


Fig. 9. Inhibition mode of PPO by ethanolic eggplant peel extract

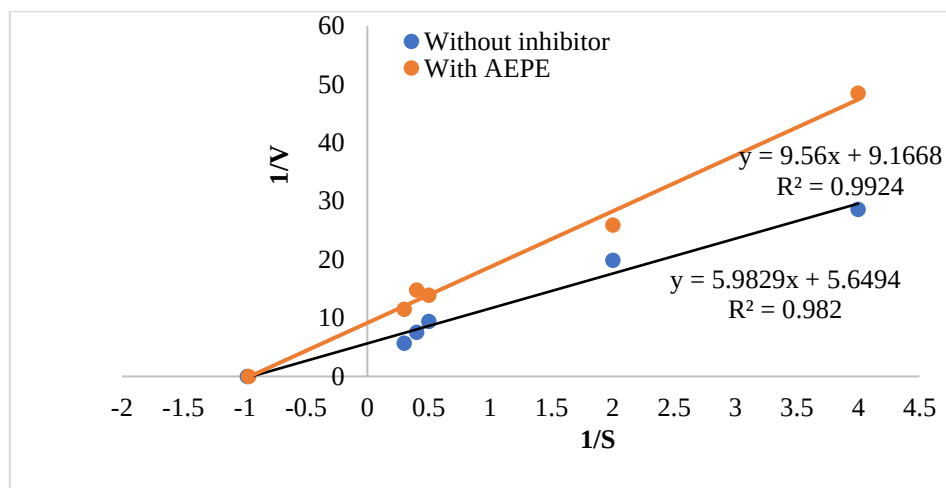


Fig. 10. Inhibition mode of PPO by aqueous eggplant peel extract

Competitive inhibition may be attributed to the presence of high levels of phenolic compounds in these extracts, as they are structurally similar to the substrate and are able to bind to the active site of the enzyme to hinder the process of enzyme reaction (Sadoon & Abbasy, 2026; Zheng, Shi, & Pan, 2012). Noncompetitive inhibition, on the other hand, may be due to chelation of the metal copper ions present in the enzyme (Al-Barwari & Abbas, 2026; Sun *et al.*, 2012). Many studies have indicated the inhibition of polyphenol oxidase purified from plant sources using extracts such as *Rhus coriaria* L. (Salim, Al, & Sadoon, 2026), *Rosa roxburghii* (Yu *et al.*, 2021), chili pepper (Lim & Wong, 2018), onion (Yapi *et al.*, 2015), oregano, and lovage extracts (Sierocka & Świeca, 2024).

Conclusion

The findings of this study indicated that eggplant peel extracts, particularly the ethanolic extract (EEPE), exhibit strong antioxidant and anti-browning activities. EEPE showed higher DPPH free-radical-scavenging activity and more effective inhibition of PPO and POD enzymes than the aqueous extract (AEPE). This reduced browning intensity and improved the preservation of phenolic compounds in mango juice during storage. Kinetic analysis confirmed that EEPE is a competitive inhibitor of PPO, whereas AEPE is a non-competitive

inhibitor. The successful purification and characterization of mango PPO further confirmed its enzymatic nature. Overall, utilizing eggplant peel, a relatively underutilized agro-industrial by-product, presents an environmentally friendly and efficient approach to preventing EB and oxidative spoilage in fruit juices. These findings justify further research into incorporating such extracts into food preservation programs, aligning with global efforts toward waste valorization and cleaner-label foods.

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Authors Contribution

M.G.A: Methodology, Resources, Software, Validation, Software, Visualization. **O.H.A:** Conceptualization, Data curation, Formal analysis, Investigation, Writing—original draft. **R.T.A:** Methodology, Project administration, Supervision, Resources, Software, Validation, , Writing—review and editing. **O.Y.A:** Conceptualization, Data curation, Writing—original draft, Investigation.

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Abbreviation

Fruit juice (FJ); Eggplant Peel Ethanolic Extract (EEPE); Eggplant Peel Aqueous

extract (AEPE); Mango Juice (MJ); Enzymatic browning (EB); Polyphenol oxidase (PPO); Peroxidase (POD).

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مقاله پژوهشی

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مهار قهوه‌ای شدن آنزیمی در آب انبه با استفاده از عصاره‌های پوست بادمجان غنی از آنتی‌اکسیدان: یک مطالعه سینتیکی و نگهداری

می جی آمین الدباغ^۱ ID - اودی اچ علی الجماس^۲ ID - رعنا تی ابراهیم التایی^۱ ID - عمر وای العباسی^۱ ID*

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چکیده

فرآیندهای قهوه‌ای شدن آنزیمی تأثیر منفی بر بسیاری از محصولات غذایی، مانند آبمیوه‌های تازه، به‌ویژه خواص حسی و ارزش غذایی آنها دارند و در نهایت منجر به رد محصولات آسیب‌دیده توسط مصرف‌کنندگان می‌شوند. از آنتی‌اکسیدان‌های طبیعی برای مقابله با این پدیده، حفظ این محصولات و جلوگیری از افت کیفیت آنها استفاده شده است. این تحقیق به بررسی فعالیت‌های آنتی‌اکسیدانی و مهار آنزیمی عصاره‌های اتانولی پوست بادمجان (EEPE) و آبی (AEPE) و همچنین ظرفیت آنها در مهار رادیکال‌های DPPH و مهار آنزیم‌های قهوه‌ای شدن در آب انبه (MJ) در طول نگهداری در یخچال می‌پردازد. EEPE ظرفیت مهار رادیکال DPPH بیشتری (۰/۶۶ میلی گرم بر گرم) نسبت به AEPE (۰/۵۲ میلی گرم بر گرم) داشت، اگرچه هر دو ترکیب از ویتامین C (۰/۷۳ میلی گرم بر گرم) ضعیف‌تر بودند. سنجش‌های قهوه‌ای شدن آنزیمی نشان داد که EEPE فعالیت‌های PPO و POD را در آب انبه (MJ) سرکوب می‌کند و به کاهش شدت قهوه‌ای شدن و حفظ بهتر محتوای فنلی پس از ۱۵ روز در دمای ۴ درجه سانتی‌گراد، بیشتر از AEPE، کمک می‌کند. PPO پالپ انبه خالص شده تقریباً ۵۴ کیلو دالتون وزن مولکولی داشت و با استفاده از رسوب سولفات آمونیوم، دیالیز و کروماتوگرافی تبادل یونی CM- سلولز با مقدار خالص‌سازی ۱۵/۰۷ به‌طور مؤثر استخراج شد. مطالعات سینتیکی نشان داد که EEPE مانند یک مهارکننده رقابتی PPO عمل می‌کند، در حالی که AEPE مهار غیررقابتی از خود نشان می‌دهد. این پاسخ، پتانسیل پوست بادمجان، ضایعات کشاورزی، را به‌عنوان یک ماده طبیعی برای نگهداری مواد غذایی به‌دلیل فعالیت‌های آنتی‌اکسیدانی و ضد قهوه‌ای شدن آن نشان می‌دهد.

واژه‌های کلیدی: آب انبه، پراکسیداز، پلی فنل اکسیداز، عصاره پوست بادمجان، فعالیت آنتی‌اکسیدانی، قهوه‌ای شدن آنزیمی، نگهداری مواد غذایی

۱- گروه شیمی، دانشکده علوم تربیتی (علوم پایه)، دانشگاه موصل، عراق

(*)- نویسنده مسئول: Email: chem.omar1978@uomosul.edu.iq

۲- گروه علوم و صنایع غذایی، دانشکده کشاورزی و جنگلداری، دانشگاه موصل، عراق

Research Article

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Effects of Indian and Semi-industrial Extraction Methods on the Quality and Acceptability of Isinglass Produced from Swim Bladders of Cultured Beluga (*Huso huso*), Persian Sturgeon (*Acipenser persicus*), and Stellate Sturgeon (*Acipenser stellatus*)

M. Seifzadeh^{1*}, A. Koochakian Sabour¹

1- National Fish Processing Research Center, Inland Water Aquaculture Research Institute, Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization, Anzali, Iran

(*- Corresponding Author Email: M.seifzadeh@areeo.ac.ir)

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Abstract

The protein with a gel like structure, extracted from collagen, is named isinglass, which is derived from the swim bladder of various fish, but is mostly extracted from sturgeon. Isinglass extracted from three types of sturgeon fish, which are commonly reared in Iran, namely Beluga, Persian, and Stellate sturgeon fish, using two different methods, namely the Indian method and the semi-industrial method. In this study, the extracted isinglass was tested for quality in terms of physical, chemical, microbiological, and organoleptic properties for 7 months at room temperature. The percentage of extraction as well as the extraction time were found to be higher in the semi industrial method (63-67% and 3-7 days) than in the Indian method (<24% and 8-24 days). ($P < 0.05$). The protein content in semi-industrial method was 74-75%, while in the Indian method, it was about 64%. Moisture, TVB-N, and peroxide were better in the semi-industrial method compared to the Indian samples after 7 months. The semi-industrial samples had the lowest bacterial count, and their sensory evaluation showed higher acceptability scores compared to the Indian samples (above 4 after 7 months). However, the Indian samples were not acceptable in terms of sensory evaluation. The Indian samples had higher nutritional values and lower energy consumption, the semi-industrial method had higher yield, better chemical and microbial quality, and good sensory quality. Thus, it is concluded that semi-industrial method is a better choice for isinglass production on a large scale.

Keywords: Isinglass, Indian method, Physicochemical properties, Semi-industrial method, Sturgeon

Introduction

Isinglass, which is a transparent material that is rich in collagen extracted from the swim bladder of fishes, especially those of the sturgeon family. Isinglass, while being processed and extracted from the swim bladder, the collagen in the fish swim bladder gets transformed into clear and jellylike matter, and has been employed as an effective clarification

and adhesion substances since the long history. Nowadays, isinglass, as a fining material applied in wine, beer, fruit juice, vinegar, and others, for clarity and adhesive abilities (Zhao, Sang, & Han, 2022; Sousa *et al.*, 2020; Lallemand Brewing, 2023).

The isinglass raw material is considered the highest quality when it comes from fish that have a large swim bladder with an abundance



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of collagen. In this regard, the most appropriate source is believed to be the native species in the southern Caspian Sea, particularly beluga, Persian sturgeon, and stellate sturgeon, owing to their anatomical and Biochemical properties (Dong & Dai, 2022; Carvalho *et al.*, 2024). As reported by the Iranian Office of Planning and Budget (2025), over the last 5 years, the production of farmed sturgeon in Iran has surpassed 4000 tons per year, and approximately half of these productions come from three aforementioned species. In commercial utilization of sturgeons, caviar constitutes 10–15% of their total body weight and 50–60% edible flesh; however, the remaining 30–40% body weight like skin, bones, viscera, and swim bladder are usually disposed as industrial waste or converted to low value products, such as fishmeal or animal feed (Akter, Rahman, Bhowmik, Haidar, & Alam, 2016; Zhang, Adachi, Ura, & Takagi, 2019). Even though the contribution of the swim bladder as a by-product in the total processing is low, due to its high protein content, especially collagen, it constitutes an economically valuable product and can make a significant value-added to the waste product (Hussain, Reddy, Chari, & Kumar, 2024a).

Proper valorization of fish processing by-products has gained significant attention in promoting sustainable fisheries and circular bio economy concepts. The extraction of collagen from swim bladder opens new avenues to valorize abundant unused biological materials to value-added commercial products, optimizing the use of resources, decreasing pollution from the processing industry and creating additional value to fish processing industries (Cruz-Lopez *et al.*, 2021; Li *et al.*, 2023; Kumar, Nidheesh, Govindaraju, Jyoti, & Suresh, 2017; Yuan *et al.*, 2024).

Different methods for the extraction of isinglass have been developed, and a factor that affects the efficiency of extraction of isinglass is the processing conditions applied. The generally obtained yield is between 15 - 25% (dry wt) of the swim bladder (Sui *et al.*, 2025). Two major systems for the preparation of

isinglass are the traditional method and the semi-industrial method. The traditional Indian method is being followed in most parts of India and Southeast Asian countries. This process involves many simple operations such as steeping, drying, boiling, and filtration. This method is characterized by low cost and requires only simple equipment. However, this method suffers from quality inconsistency, long processing time, and unhygienic process conditions due to high dependence on the prevailing climatic conditions. But the semi-industrial method is associated with controlled drying and extraction parameters such as temperature, pH, and processing time, and in general, this process gives the highest yields and uniform and hygienically safer products (Sarkar *et al.*, 2023).

So far, most published research on collagenous fish by-products has dealt with their utilization for collagen isolation, production of gelatin, or studies on the physicochemical and functional properties of collagen. Production of isinglass from cultured sturgeon species under semi-industrial scale has not been extensively studied, and only little knowledge exists on the topic. No earlier report has focused on a comprehensive comparison between the Indian traditional and semi-industrial production processes of commercial isinglass from farmed Iranian sturgeon with simultaneous characterization of extraction yield, physicochemical attributes, nutritional value, microbial load, sensory parameters, shelf-life, and industrial relevance.

Because the industry of cultivating Iranian sturgeon is growing, the lack of proper production procedures and commercial requirements on isinglass has been regarded as a vital obstacle in further development. Development of a process is needed to increase production and usage of swim bladder, augment the added value of byproducts of sturgeon processing, and provide additional space for commercial production of high-value added-collagen products. Hence, the purpose of this study is a comparison of a traditional Indian procedure and a semi-industrial procedure to

produce isinglass from the swimming bladders of cultured Beluga, Persian sturgeon, and stellate sturgeon.

Materials and Methods

Collection and Initial Preparation

In the experiment, the cultured sturgeons were conveyed to the Aquatic Products Processing Laboratory within 15 min following the fish harvest. The fresh sturgeons were handled right upon arrival at the laboratory (10 minutes). The fish were washed, eviscerated, and the swim bladder was picked off from both ends with a stainless-steel knife in hand, making efforts to avoid being damaged manually. Then they were repeatedly rinsed with tap water to detach blood, connective tissue, and adipose tissue. After the cleaning and trimming operation of the detached tissues, the swim bladders were immediately placed for the extraction process. It took about 30-45 minutes to process one fish to obtain a clean swim bladder, and the duration varied

depending on the size of the fish and the technician skillful (Carvalho *et al.*, 2024).

Isinglass Production

Indian Method

Exposed directly to the atmosphere and the sun, the swim bladders were dried as completely as possible so that their moisture content would be as low as possible prior to the flattening procedure (Rodrigues *et al.*, 2021). If the swim bladders are still moist (i.e., soft) at this point, the swim bladders are placed on clean board made of wood or metal so they would not wrinkle during the flattening process. A simple means of manually flattening or smoothing out the surface of the swim bladders, is the application of rolling pin made of wood or equivalent to create a uniform thickness and remove any trapped air or remaining fluid from the inside of the swim bladders. The swim bladders are then cut into sheets and left out in direct sunlight for seven days to thoroughly dry out (Fig. 1) (Koochekian, Zareh, & Yousefi Jourdehi, 2006).



Fig. 1. Preparation of isinglass of sturgeon swim bladders by Indian method

Semi Industrial Method

Thermal driers are used for drying cleaned swim bladders to provide uniform and controlled drying conditions. The dryer temperature was set at 60 °C to allow gradual evaporation of internal moisture while maintaining the collagen structure of the swim bladder. Depending on sample thickness, this required between 8 and 24 hours. Upon partial drying of the samples, the sample was then passed between two stainless steel (or Teflon)

plates in an evenly heated flat press. Each sample would be controlled to apply a uniform pressure, further heating required to create a smooth, uniform surface, thus improving the appearance of the final product through the application of both controls in this stage of processing. At the end of this process, each bladder becomes isinglass and will be molded into a flat sheet of isinglass (Fig. 2) (Koochekian *et al.*, 2006).



Fig. 2. Preparation of isinglass from sturgeon swim bladders by semi industrial method

Packaging and Storage

The isinglass sheets were enclosed in polyethylene bags and stored for a period of 7 months at room temperature under laboratory conditions at an ambient temperature of nearly 25°C and relative humidity of 70% according to local climate situations. During this time, the quality of the isinglass was evaluated using a number of tests, including physical, chemical, microbiological and sensory analysis.

Nutritional Analyses

Moisture Determination

After drying, the test container was weighed and then used to measure the sample's moisture using the oven-drying method. Sample size: approximately 10 g of isinglass was placed, and the weight of both the container and the sample was recorded. The sample was transferred to an oven (105°C) for 24 h. After cooling in a desiccator and ambient temperature, the weight of the sample was recorded. The amount of moisture present in the sample was calculated by determining the loss in weight of the material after drying (AOAC, 2005).

Protein Determination (Kjeldahl Method)

A Kjeldahl flask was used to dissolve 2 g of Isinglass using concentrated sulphuric acid with the aid of a catalyst until a clear solution was obtained. The digestion was cooled and alkalinized before distilling for the release of ammonia gas to capture it in a solution of boric acid. The captured ammonia was then titrated using Normal sulphuric acid in order to determine the nitrogen content. The total

protein content was then calculated by multiplying the nitrogen percentage by 6.25 (AOAC, 2005).

Fat Determination (Soxhlet Method)

Five grams of dried isinglass were weighed, placed into an extraction pouch or filter thimble, and placed into the Soxhlet extractor and extracted for 6 to 8 h using hexane as the extraction solvent. After evaporation of the solvent, residue that was obtained in the sample flask, it was reweighed, and fat content was computed on the basis of the original weight (AOAC, 2005).

Ash Determination

The gravimetric method was used to determine ash content. In other words, to determine ash content, a clean porcelain crucible is placed in an electric muffle furnace and then removed to cool in a desiccator. After weighing the crucible, a 5g sample of isinglass is placed in the crucible and is ashed until the smoke stops. The crucible is then placed back into the furnace and heated for 4 hours to 550 °C until white or light grey ash is formed. After another period of cooling in the desiccator, the crucible is weighed, and the ash content is calculated as a percentage of the original weight of the sample (AOAC, 2005).

Chemical Analyses

Peroxide Value Determination

The peroxide value was measured by using a colorimetric method. An approximate amount of 5 g of the defatted sample, in a solution of potassium iodide, was added to a solution of

acetic acid and chloroform. This solution was kept away from light for 1 min. The mixture was then diluted with distilled water and titrated using sodium thiosulfate to remove all the yellow color. Once the yellow color was removed, some starch indicators were added, and the titration continued until complete discoloration occurred. The peroxide value was then calculated; results were expressed as the meq /kg of fat (FAO, 1986).

Thiobarbituric Acid (TBARS) Assay

Using a colorimetric method, a thiobarbituric acid assay was performed by dissolving about 200 mg of isinglass (Whole sample without lipid extraction) in trichloroacetic acid. The resulting clarified solution was mixed with thiobarbituric acid indicator and placed into the water bath at 95 °C for 30 minutes. After cooling down, the solution's absorbance at 532 nm was determined with a spectrophotometer, allowing for the calculation of malondialdehyde levels (lipid oxidation values) (FAO, 1986).

Total Volatile Nitrogenous Bases (TVB-N)

The amount of total volatile nitrogenous bases was quantified using the distillation process. A 10g of the sample was used with 2 g of magnesium oxide and 300 mL of distilled water and a few boiling stones in a Kjeldahl flask. After boiling the sample for a few minutes, the solution was allowed to distill for 25 minutes, and the vapors of the distillate was collected in an Erlenmeyer flask which was filled with 25 ml 2% boric acid solution with few drops of methyl red indicator and titrated with 0.1N sulfuric acid (FAO, 1986).

Microbiological Analyses

Total bacterial Count

To perform the test for total bacterial count, a 25-gram sample of isinglass was aseptically mixed with a sterile diluent (commonly 0.1% peptonized water). Several serial dilutions (dilution factors of 10^{-2} or 10^{-3} , or more if necessary) were then performed, and 1 mL from each dilution of isinglass was pour-plated onto

sterile Plate Count Agar (PCA) and subsequently incubated for 48 hours at 37 °C. Once the incubation time was completed, the colonies formed during that time on the PCA were then counted to give a total count of all viable bacteria as logarithmic colony-forming units per gram of sample (log CFU/g) tested (Maturin & Peeler, 2001; Andrews & Hammack, 2003; Feldsine, Abeyta, & Andrews, 2002).

Yeast and Mold Count

For determining yeast and mold counts, the sample dilution was completed as specified above. 1 mL of the proper dilution was surface plated onto Sabouraud dextrose agar. The plated samples were incubated at 25 °C for 7 days. After incubation period, the fungal colonies formed were counted and then expressed in terms of log colony-forming unit/gram sample (Tournas, Stack, Mislivec, Koch, & Bandler, 2001; Kovács *et al.*, 2025).

Pathogenic Bacteria Identification

The possible presence of pathogenic microorganisms such as *Salmonella*, *Staphylococcus* spp., *Escherichia coli* and coliforms were assessed. To detect *Salmonella*, 25 g of the sample was incubated for 18–24 h at 37 °C in buffered peptone water, then transferred to selective media, such as Xylose Lysine Deoxycholate agar and *Salmonella*-*Shigella* agar (Andrews *et al.*, 2024), and incubated for an additional 24 hours. Potential *Salmonella* colonies were then further processed to confirm their identity with biochemical testing (Catalase, coagulase, and indole testing). *Staphylococcus aureus* (25 g) (Bennet & Lancette, 2001) was detected on Bird Parker Agar, and *E.coli* (50 g) (Feng, Weagant, Grant, & Burkhardt, 2020) was identified on Eosin Methylene Blue Agar.

Sensory Evaluation

Trained panelists assessed sensory characteristics (color, odor, appearance, texture (brittleness) on a scale of 1 (dislike extremely) to 5 (like extremely) for seven months at

ambient (room) temperature as part of an ongoing study to evaluate potential changes in visual quality and overall acceptability of isinglass over the storage period. During sensory analysis, each of the isinglass was identified with a code number (Gilbert, 2013).

The sensory evaluation was carried out by thirty well trained panelists male and female (Master's and PhD in seafood processing and food industry), aged around 40, and they had past experience in the sensory evaluation of fish and fishery products. In addition, training was given to panelists before the experiment, where they learned the objectives of the study, the sensory attributes evaluated (appearance, color, odor, texture (brittle), and overall acceptance), the procedures in sensory evaluation, and were provided with reference samples with different quality levels. Reference samples were given in order to ensure uniformity in the interpretation of the criteria to be evaluated and to enhance similarity in panelists' scoring. All evaluations were carried out under similar environmental conditions with consistent protocols for the storage period.

Color was observed for the changes shown in transparency, yellow, darkness, and cloudiness, and then given a score. The odor was identified based on whether it was natural, rancid, or offensive, and a numerical score was given to it. Product's Appearance was observed on the basis of whether the original shape of the product is still intact, whether there were any defects on the product (cracks and deformation), as well as whether all the products have a similar shape. Isinglass texture was checked through touches and moderate pressures based on the brittleness, flexibility, as well as excessiveness of softness of the products, and eventually, all of the above attributes are also considered, thus resulting in an acceptable overall rating for each product after the respective scores were given (Gilbert, 2013).

Physical Analyses

The physical characteristics of isinglass were evaluated over seven months of storage at ambient temperature. Assessments of dry weight, texture (or flexibility and brittle nature), color, and pH were conducted to determine how the sample reacted to the environment (FAO, 1986).

Texture Analysis

Texture was determined by visual inspection or objectively by a texture analyzer that measures the force required to break (or bend) the sample under a particular pressure. By determining how much effort was needed to break the sample determine how strong the sample was and how much it had deteriorated during storage. A sudden decrease in the amount of force required to break, or increased brittleness, would indicate the deterioration of the product during storage.

pH Measurement

The potentiometric technique was used to measure the pH levels. To do this, approximately 1 g of isinglass was added to 100 mL of distilled water, and the solution was placed at room temperature. When the pH electrode was inserted into the solution, the pH value was read directly from the pH meter (FAO, 1986).

Color Measurement

Color values of all samples were determined with the Hunter Lab color flex (USA) color-measurement instrument, recorded in Hunter color values of lightness (L), Red-Green (a), and Yellow-Blue (b).

Mineral Composition

Mineral concentrations, including calcium, phosphorus, magnesium, sodium, iron, zinc, and copper, were analyzed by AAS. Samples were placed in the electric furnace, and after being calcified to a white powder, they were dissolved in 1ml concentrated nitric acid. The contents of each crucible were poured into 250ml volumetric flasks. They were made to a final volume of 250ml using deionized water,

shaken up thoroughly, and sequentially diluted if necessary. Then, lanthanum trichloride of 0.1 (1/10 of the total volume) was added to each flask, filled with sufficient deionized water, and mixed well. Analyses were performed with blank solutions and with an air acetylene flame atomic absorption burner within the wavelength range of 400-800nm. The standards were prepared by dissolving 1000 mg of each pure mineral element in 1L deionized water to get stock solutions containing 1000mg/L. Each 100ml volumetric flask was pipetted 0ml, 1ml, 2ml, 3ml, 4ml, 5ml of standard solution, added 10ml lanthanum trichloride, and added deionized water up to 100ml, then mixed thoroughly (AOAC, 2005).

Amino Acid Profile

After the sample cooled to 24°C, it was rotated at 4000 RPM (3756×g RCF) for five minutes to precipitate any undissolved material. Then, a 10 µl of the supernatant mixed with 1 mL of distilled water in a new vial. For most amino acids, used stable isotope labeled mixtures as both calibration and internal standards. Isoleucine and histidine got a slightly different treatment; leucine was the internal standard and 3-methylhistidine included. Then, 50 µl of the sample was blended by 50 µl of the labeled standard and 700 µl of indicator. After mixing for five seconds, it was injected into a C18 HPLC column. All of them were conducted using mobile phases A and B, running a gradient elution at 0.7 mL/min. Detection occurred at 254 nm (Weber, 2022; Peace & Gilani, 2005).

Yield Determination

To calculate yield, used the formula of $r=P/C$ where: P - Product (Final), C - Raw Material (Starting). Therefore, used 500 g to compute yield. Then computed yield by dividing the difference between the amount of isinglass produced from each treatment divided by the total weight of raw material (500 g) multiplied by 100 (Demerjian, 2018).

Water Activity

Measurements of water activity of the isinglass were performed with a calibrated water activity meter (AquaLab 4TE, Meter Group Inc., Pullman, WA, USA). Before analysis, the instrument was calibrated with standard saturated salt solutions following the instructions of the manufacturer. A finely cut portion of 2-3 g of isinglass was loaded into the clean, dry sample cup to cover the bottom surface, and the sample was not placed above the edge of the cup. Then the cup was loaded into the measuring chamber of the instrument, and the a_w value was taken after it reached the steady state at 25 °C. Measurements of a_w were made under laboratory conditions, just after the processing and handling of the samples to avoid water with surroundings air and water loss or absorption.

Sampling

Once per month, over a period of seven months (at room temperature), a range of tests were performed on the samples stored for varying times. These tests included physical, chemical, sensory, and nutritional analyses. The amino acid profiles and mineral compositions of products were tested once during the study.

Statistical Analysis

SPSS Statistical Package version 25 was used for comparing experimental samples based on microbiological, chemical, mineral, amino acid profiling, and physical analyses. The experimental design of the current study utilized three independent variables: fish species, method of extraction, and storage duration. Based on this experimental design, the data analysis addressed the main effects of all variables as well as any potential interactions among variables. For factorial experiments, analysis of variance (ANOVA) are more suitable for statistical analyses than using simple pairwise comparison tests. It can test for species, method, and time effects simultaneously, and also it can test for interaction effects (Species- Method, Species- Time, Method- Time, and Species- Method

Time interactions). Interactions among the variables are crucial.

This study uses factorial ANOVA and post hoc analyses like Tukey's HSD or repeated measures over time to analyze the isinglass quality characteristics. Statistical differences at $p < 0.05$ were performed using a 95% confidence interval. In order to evaluate differences in sensory evaluation data, the Kruskal Wallis test was used for non parametric analysis, and the Mann Whitney test was performed if necessary. The effect of storage time on microbial, chemical and sensory characteristics within each treatment and comparison of treatment means over time were measured using a two-way ANOVA. All data are presented as mean $\pm$ standard deviation. Fish from each sturgeon healthy stocks of cultured populations have been randomly selected for this study. The experimental procedures' treatments were 6 resulting from the crossing of 3 species and 2 extraction methods, which were Indian traditional method and semi industrial method. The two procedures were carried out on each treatment by 3 biological independent replicas, were in the same parameters of conditions. Thus each replica not only consisted of fishes of another source origin, but also formed an independent batch of samples, instead of an analytic repeat from a specific product batch. All biological replica samples were stored in the same environmental conditions after extraction, then examined at specific dates: month zero and subsequent 7 months. In each specific time of examinations 3 sets of analyses have been carried out per replicate (24 analyses for each treatment: 3 replicates $\times$ 8 time points) giving 72 samples/per method of extraction; totally 144 samples examined for 2 methods of extraction.

Independent analysis of species, extraction, and their interactions on all determined parameters was performed using the factors identified. Biological samples used to generate each individual quality measurement of each treatment combination were biological replicates to remove variability from the batches and to enable separation between the

differences among the sturgeons and differences derived from the extraction method.

Sample Size Justification

The sample size was determined based on the factorial experimental design and the availability of biological materials while ensuring independent biological replication for each treatment combination. Three independent biological replicates were included for each combination of fish species, extraction method, and storage time, providing a balanced experimental design suitable for three-way analysis of variance. The observed treatment effects across the primary response variables indicated that the selected sample size provided sufficient sensitivity to detect biologically meaningful differences under the experimental conditions of the present study.

To further evaluate the adequacy of the experimental design, a post hoc power analysis was performed. The analysis indicated that the selected sample size provided sufficient statistical power (power > 0.80) to detect the observed differences among treatments, supporting the reliability of the statistical comparisons

Results

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable differences among sampling times ($P < 0.05$), whereas the same lowercase letters show no main difference ($P > 0.05$). Dissimilar uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

From [Table 1](#), it can be seen that there was a decline in the protein content of items produced using the Indian method after seven months of storage ($P < 0.05$). Simultaneously, the moisture content increased significantly during this time ($P < 0.05$).

Table 1- The results from aproximate analysis of isinglass treatments made by means of industrial and Indian methods for seven months at room temperature (%)

Indian method												
Index	Ash			Moisture			Fat			Protein		
Storage period (Month)	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon
Month 1	1.45±1.12 ^{aA}	1.98±1.11 ^{aA}	2.67±1.66 ^{aA}	28.98±1.95 ^{bA}	28.17±1.99 ^{bA}	28.91±1.18 ^{Ab}	0.73±0.51 ^{Aa}	0.95±0.24 ^{Aa}	0.84±1.28 ^{Aa}	68.84±1.19 ^{aB}	68.90±1.24 ^{aB}	67.58±1.23 ^{aB}
Month 7	0.98±0.76 ^{aA}	1.35±0.95 ^{aA}	1.95±1.59 ^{aA}	33.05±1.84 ^{aA}	32.14±1.93 ^{Aa}	33.18±1.39 ^{aA}	0.61±0.47 ^{Aa}	0.81±0.31 ^{Aa}	0.73±1.36 ^{Aa}	65.36±1.27 ^{bC}	65.70±1.98 ^{bC}	64.14±1.14 ^{bC}
Industrial method												
Month 1	0.72±0.37 ^{aA}	0.99±0.54 ^{aA}	0.84±0.25 ^{aA}	23.87±1.54 ^{aB}	23.65±1.42 ^{Ba}	23.85±1.25 ^{Ba}	0.14±0.10 ^{aA}	0.18±0.12 ^{aA}	0.17±0.11 ^{aA}	75.27±1.43 ^{aA}	75.18±1.17 ^{aA}	75.14±1.15 ^{aA}
Month 7	0.70±0.39 ^{Aa}	0.85±0.62 ^{Aa}	0.61±0.41 ^{Aa}	24.52±1.19 ^{Ba}	24.18±1.75 ^{Ba}	25.16±1.36 ^{Ba}	0.11±0.08 ^{aA}	0.14±0.13 ^{Aa}	0.12±0.09 ^{aA}	74.67±1.76 ^{aA}	74.83±1.56 ^{aA}	74.11±1.90 ^{aA}

Table 3– Comparison of yield and production indices of isinglass treatments from sturgeon swim bladders prepared by industrial and Indian methods

Treatment	Industrial			Indian		
Fish species	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon
Index						
Yield on Dry Matter Basis (%)	63 ^b	67 ^a	65 ^{ab}	22 ^a	24 ^a	23 ^a
Production time	8-24 hours	8-24 hours	8-24 hours	3-7 day	3-7 day	3-7 day
Labor (hours per kilogram, h/kg)	3-5 ^a	3-5 ^a	3-5 ^a	0.5-1.5 ^b	0.5-1.5 ^b	0.5-1.5 ^b
Energy (kwh/kg)	1-2 ^b	1-2 ^b	1-2 ^b	4-8 ^a	4-8 ^a	4-8 ^a
Water (l/kg)	120-250	120-250	120-250	80 -150	80 -150	80 -150
Batch-to-Batch Quality Consistency	High	High	High	Medium	Medium	Medium
Waste/Rejection	3 ^b	3 ^b	3 ^b	6 ^a	6 ^a	6 ^a

Fresh swim bladders from Beluga sturgeon (mean body weight: 12–15 kg) had a mean weight of about 300–400 g and were obtained about 120–160 g after drying. Swim bladders from Persian sturgeon (mean body weight: 8–12 kg) were in the range of 200–300 g, and 80–

120 g dried isinglass; whereas swim bladders from stellate sturgeon (mean body weight: 3–5 kg) were smaller (approximately 80–120 g) and 35–55 g dried isinglass. On average, 8 Beluga, 11 Persian sturgeon and 21 stellate sturgeon were used for each treatment.

Table 4– Major micronutrients in sturgeon isinglass prepared by Indian and industrial methods (mg/100 g)

Processing method	Industrial			Indian		
Fish species	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon
Mineral components						
Calcium	26±1.17 ^b	29±1.53 ^a	28±1.58 ^{ab}	55±2.17 ^b	58±1.99 ^a	57±2.13 ^{ab}
Phosphorus	14±1.83 ^b	19±1.76 ^a	18±1.27 ^a	36±1.24 ^b	39±2.16 ^a	38±2.26 ^{ab}
Magnesium	2±1.26 ^b	5±1.19 ^a	4±1.12 ^{ab}	5±1.56 ^b	10±1.34 ^a	9±1.16 ^a
Sodium	53±2.65 ^b	58±2.19 ^a	57±1.92 ^a	74±1.95 ^b	78±2.15 ^a	77±2.34 ^a
Iron	0.1±0.01 ^a	0.3±0.05 ^a	0.2±0.07 ^a	0.3±0.2 ^a	0.5±0.3 ^a	0.4±0.13 ^a
Zink	0.1±0.03 ^a	0.3±0.04 ^a	0.2±0.06 ^a	0.2±0.01 ^a	0.4±0.2 ^a	0.3±0.21 ^a
Copper	0.01±0.02 ^a	0.02±0.01 ^a	0.01±0.03 ^a	0.01±0.02 ^a	0.03±0.01 ^a	0.02±0.11 ^a

Results are exhibited as mean ± standard deviation (n = 3). Unlike lowercase letters within the same column, present remarkable

differences among sampling times (P<0.05), whereas the same lowercase letters show no main difference (P> 0.05). Dissimilar

uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

The calcium, phosphorus, sodium, and magnesium, in the isinglass processed by Indian method is higher compared to the semi industrial made isinglass (Table 4). However, the difference in the concentrations of copper, iron and zinc were non-significant at a 5% level between the two methods ($p > 0.05$).

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable differences among sampling times ($P < 0.05$), whereas the same lowercase letters show no main difference ($P > 0.05$). Dissimilar uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

Statistically there were no differences in total microbial counts for all samples during months 1 and 2 ($P > 0.05$), but from month 3 and thereafter the comparison of both methodologies resulted in significant

differences ($P < 0.05$). No molds or yeasts were detectable for either samples until month 7 at which point mold and yeast were observed on all Indian methodology prepared isinglass materials. In addition to this, all pathogenic microorganisms (*Salmonella*, coliform, *S. aureus*, *E. coli*) were not detected in any of the materials prepared using either method. Based on this data, it was determined that the industrial method maintained good microbiological quality of isinglass for the entire storage period (7 months) compared to the Indian method which had acceptable microbiological quality at the end of 6 months (Table 5).

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable differences among sampling times ($P < 0.05$), whereas the same lowercase letters show no main difference ($P > 0.05$). Dissimilar uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

Table 5– Microbiological characteristics of isinglass treatments prepared from sturgeon swim bladders by industrial and Indian methods during seven months of storage at room temperature (log CFU/g)

Processing method		Indian method				
Index		Total bacterial counts			Molds and yeasts	
Treatment	Persian sturgeon	Beluga	stellate sturgeon	Persian sturgeon	Beluga	stellate sturgeon
Day 1	3.19 $\pm$ 0.39 ^{eA}	3.69 $\pm$ 0.38 ^{dA}	3.75 $\pm$ 0.15 ^{bA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 1	3.25 $\pm$ 0.28 ^{deA}	3.88 $\pm$ 0.27 ^{cdA}	3.78 $\pm$ 0.17 ^{bA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 2	3.41 $\pm$ 0.89 ^{dA}	3.97 $\pm$ 0.19 ^{cA}	3.87 $\pm$ 0.26 ^{abA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 3	3.68 $\pm$ 0.58 ^{cdA}	4.16 $\pm$ 0.33 ^{bcA}	3.91 $\pm$ 0.45 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)

Month 4	3.94±0.53 ^{bcA}	4.25±0.49 ^{bA}	4.01±0.32 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 5	4.21±0.64 ^{abA}	4.37±0.67 ^{bA}	4.14±0.29 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 6	4.57±0.66 ^{aA}	4.51±0.54 ^{bA}	4.28±0.35 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 7	5.47±0.69 ^{aA}	5.17±0.79 ^{aA}	5.36±0.84 ^{aA}	2.60+1.37 ^a	2.60+1.45 ^a	2.74+1.56 ^a
Industrial method						
Day 1	1.59±0.11 ^{aA}	1.82±0.19 ^{bA}	1.72±0.35 ^{cA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 1	1.66±0.18 ^{aA}	1.85±0.15 ^{bA}	1.91±0.24 ^{cA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 2	1.70±0.29 ^{aA}	1.90±0.23 ^{bA}	2±0.17 ^{bcA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 3	1.78±0.68 ^{aA}	1.94±0.43 ^{bA}	2.19±0.38 ^{bA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 4	1.85±0.65 ^{aA}	2.04±0.39 ^{abA}	2.28±0.46 ^{bA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 5	1.94±0.69 ^{aA}	2.17±0.27 ^{aA}	2.40±0.64 ^{abA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 6	2.04±0.63 ^{ab}	2.31±0.31 ^{aA}	2.54±0.51 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)
Month 7	2.28±0.88 ^{aB}	2.56±0.73 ^{aA}	2.78±0.52 ^{aA}	CFU/g10> (detection limit)	CFU/g10> (detection limit)	CFU/g10> (detection limit)

Total volatile nitrogen bases and peroxide values increased significantly more in the Indian method than the industrial method ($P<0.05$). By the second month, the differences between the two methods were statistically significant ($P<0.05$), but the respective levels of chemical stability of the industrial method were superior (Table 6). Over the 7-month period peroxide values increased gradually in all treatments; though it rose significantly in Indian method prepared samples than semi-industrial preparation ($P<0.05$) without significant differences among the three sturgeon species ($P>0.05$).

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable

differences among sampling times ($P<0.05$), whereas the same lowercase letters show no main difference ($P> 0.05$). Dissimilar uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P<0.05$), whereas identical uppercase letters display no notable difference ($P> 0.05$).

Table 6– Evaluation of the chemical properties of isinglass treatments prepared from sturgeon swim bladders by industrial and Indian methods during seven months of storage at room temperature

Index		TBARS (mg MDA/kg)					Peroxide (meq/kg lipid)					TVB-N (mg/100g)						
Treatment		Industrial		Indian			Industrial		Indian			Industrial		Indian				
Storage period	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon
Day 1	0.68±0.53 ^{aA}	0.46±0.32 ^{aA}	0.54±0.42 ^{aA}	0.54±0.31 ^{aA}	0.28±0.18 ^{aA}	0.24±0.13 ^{aA}	1.44±1.36 ^{bA}	1.34±1.27 ^{cA}	1.42±1.49 ^{bA}	1.76±1.20 ^{eA}	1.64±1.27	1.82±1.14 ^{dA}	8.32±1.8 ^{hA}	8.12±1.19 ^{hA}	7.84±1.12 ^{hA}	11.64±1.67 ^{hA}	11.46±1.17 ^{hA}	11.26±1.26 ^{hA}
Month 1	1.26±0.59 ^{aA}	0.68±0.52 ^{aA}	0.78±0.55 ^{aA}	0.68±0.17 ^{aA}	0.46±0.37 ^{aA}	0.32±0.22 ^{aA}	1.86±1.58 ^{abA}	1.78±1.69 ^{abA}	1.66±1.50 ^{bA}	2.38±1.23 ^{dA}	2.16±1.93	2.44±1.22 ^{aA}	8.98±1.26 ^{gA}	8.96±1.46 ^{gA}	8.46±1.15 ^{gA}	12.68±1.89 ^{gA}	12.84±1.31 ^{gA}	12.68±1.13 ^{gA}
Month 2	1.68±0.78 ^{aA}	1.56±0.93 ^{aA}	1.64±0.64 ^{aA}	0.95±0.39 ^{aA}	0.74±0.54 ^{aA}	0.68±0.57 ^{aA}	2.24±1.19 ^{aA}	2.16±1.35 ^{aA}	2.05±2.12 ^{aA}	4.62±1.36 ^{aA}	4.54±1.57	4.68±1.31 ^{aA}	9.64±1.35 ^{fA}	9.58±1.54 ^{fA}	9.42±1.23 ^{fA}	14.58±1.91 ^{fA}	14.24±1.42 ^{fA}	13.42±2.11 ^{fA}
Month 3	1.50±0.99 ^{aA}	1.34±0.91 ^{aA}	1.32±0.65 ^{aA}	0.84±0.58 ^{aA}	0.62±0.52 ^{aA}	0.54±0.34 ^{aA}	1.98±1.34 ^{aA}	1.88±1.77 ^{aA}	1.84±1.16 ^{abA}	4.38±1.39 ^{abA}	4.32±1.78	4.46±1.39 ^{abA}	10.54±1.49 ^{eA}	10.46±1.59 ^{eA}	10.12±1.32 ^{eA}	15.84±1.88 ^{eA}	15.35±1.45 ^{eA}	14.80±1.97 ^{eA}
Month 4	1.42±0.28 ^{aA}	1.20±0.99 ^{aA}	1.28±0.71 ^{aA}	0.75±0.38 ^{aA}	0.54±0.45 ^{aA}	0.52±0.40 ^{aA}	1.66±1.52 ^{bA}	1.54±1.17 ^{bcA}	1.68±1.14 ^{bcA}	4.02±1.40 ^{bA}	3.88±1.75	4.00±1.42 ^{bA}	11.34±1.85 ^{dA}	11.14±1.47 ^{dA}	11.16±1.47 ^{dA}	17.14±2.14 ^{dA}	16.98±1.53 ^{dA}	15.46±2.43 ^{dA}
Month 5	1.26±0.39 ^{aA}	1.08±0.95 ^{aA}	1.12±0.82 ^{aA}	0.61±0.21 ^{aA}	0.45±0.12 ^{aA}	0.48±0.31 ^{aA}	1.38±1.17 ^{bcA}	1.32±1.23 ^{cA}	1.24±1.25 ^{cdA}	3.54±1.90 ^{bcA}	3.42±1.71	3.46±1.38 ^{cA}	11.98±1.86 ^{cA}	11.96±2.25 ^{cA}	12.46±1.52 ^{cA}	18.68±2.29 ^{cA}	18.46±2.25 ^{cA}	17.28±2.18 ^{cA}
Month 6	0.94±0.61 ^{aA}	0.76±0.60 ^{aA}	0.85±0.51 ^{aA}	0.49±0.17 ^{aA}	0.38±0.29 ^{aA}	0.42±0.25 ^{aA}	1.16±1.13 ^{cA}	1.12±1.03 ^{cdA}	1.18±1.13 ^{dA}	3.20±1.72 ^{cA}	3.12±1.80	3.24±1.13 ^{cA}	13.18±1.97 ^{baA}	12.98±2.16 ^{baA}	13.12±1.59 ^{baA}	20.44±2.24 ^{baA}	20.56±2.43 ^{baA}	20.82±2.24 ^{baA}
Month 7	0.90±0.78 ^{aA}	0.72±0.59 ^{aA}	0.80±0.76 ^{aA}	0.42±0.13 ^{aA}	0.34±0.26 ^{aA}	0.39±0.28 ^{aA}	1.07±1.05 ^{cA}	1.05±1.01 ^{dA}	1.11±1.09 ^{dA}	3.11±1.79 ^{cA}	2.99±1.81	3.10±1.50 ^{cA}	14.48±1.59 ^{aA}	14.12±1.99 ^{aA}	14.98±1.63 ^{aA}	22.95±2.30 ^{aA}	23.17±1.90 ^{aA}	22.14±2.36 ^{aA}

Table 7– Evaluation of the sensory attributes of isinglass treatments prepared from sturgeon swim bladders by industrial and Indian methods during seven months of storage at room temperature

Index	Indian method											
	Overall acceptance			Texture			Odor			Color		
Treatment Storage period	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon	stellate sturgeon	Beluga	Persian sturgeon
Day 1	3.54±1.73 ^{aA}	3.64±1.22 ^{aA}	3.59±1.58 ^{aA}	3.26±1.21 ^{aA}	3.35±1.19 ^{aA}	3.29±1.24 ^{aA}	3.40±1.98 ^{aA}	3.53±1.23 ^{aA}	3.46±1.12 ^{aA}	3.70±1.27 ^{aA}	3.95±1.36 ^{aA}	3.75±1.15 ^{aA}
Month 1	3.51±1.67 ^{aA}	3.62±1.24 ^{aA}	3.54±1.49 ^{aA}	3.22±1.36 ^{aA}	3.31±1.25 ^{aA}	3.25±1.32 ^{aA}	3.39±1.92 ^{aA}	3.41±1.20 ^{aA}	3.42±1.21 ^{aA}	3.65±1.24 ^{aA}	3.86±1.53 ^{aA}	3.64±1.8 ^{aA}
Month 2	3.49±1.50 ^{aA}	3.58±1.43 ^{aA}	3.52±1.38 ^{aA}	3.18±1.54 ^{aA}	3.29±1.14 ^{aA}	3.22±1.48 ^{aA}	3.35±1.90 ^{aA}	3.35±1.59 ^{aA}	3.39±1.32 ^{aA}	3.63±1.43 ^{aA}	3.53±1.62 ^{abA}	3.50±1.25 ^{aA}
Month 3	3.42±1.59 ^{aA}	3.45±1.49 ^{aA}	3.49±1.33 ^{aA}	3.11±1.86 ^{aA}	3.27±1.37 ^{aA}	3.15±1.53 ^{aA}	3.29±1.88 ^{aA}	3.29±1.66 ^{aA}	3.30±1.39 ^{aA}	3.55±1.39 ^{aA}	3.34±1.95 ^{bA}	3.39±1.19 ^{aA}
Month 4	3.35±1.39 ^{aA}	3.34±1.45 ^{aA}	3.38±1.35 ^{aA}	3.08±1.13 ^{aA}	3.22±1.42 ^{aA}	3.13±1.54 ^{aA}	3.28±1.82 ^{aA}	3.15±1.58 ^{aA}	3.27±1.40 ^{aA}	3.51±1.38 ^{aA}	3.29±1.84 ^{bA}	3.32±1.27 ^{aA}
Month 5	3.17±1.35 ^{aA}	3.21±1.51 ^{abA}	3.26±1.44 ^{aA}	2.95±1.44 ^{aA}	3.15±1.18 ^{aA}	3.05±1.61 ^{aA}	3.12±1.73 ^{aA}	3.08±1.33 ^{abA}	3.15±1.43 ^{aA}	3.24±1.27 ^{abA}	3.16±1.75 ^{bA}	3.20±1.12 ^{abA}
Month 6	3.11±1.23 ^{abA}	3.14±1.56 ^{bA}	3.15±1.47 ^{abA}	2.83±1.49 ^{abA}	3.08±1.49 ^{aA}	2.95±1.72 ^{aA}	3.05±1.69 ^{aA}	3.01±1.29 ^{bA}	3.09±1.45 ^{abA}	3.18±1.39 ^{bA}	3.11±1.76 ^{bcA}	3.12±1.16 ^{bA}
Month 7	2.98±1.19 ^{bA}	3.04±1.63 ^{bA}	3.05±1.50 ^{bA}	2.74±1.39 ^{bA}	2.97±1.53 ^{aA}	2.91±1.83 ^{aA}	2.96±1.60 ^{aA}	2.99±1.11 ^{bA}	2.95±1.19 ^{bA}	2.98±1.41 ^{bA}	3.00±1.65 ^{cA}	3.02±1.29 ^{bA}
Industrial method												
Day 1	4.55±1.21 ^{aA}	4.65±1.98 ^{aA}	4.61±1.27 ^{aA}	4.42±1.56 ^{aA}	4.52±1.36 ^{aA}	4.45±1.33 ^{aA}	4.55±1.28 ^{aA}	4.68±1.33 ^{aA}	4.59±1.73 ^{aA}	4.62±1.16 ^{aA}	4.85±1.39 ^{aA}	4.76±1.52 ^{aA}
Month 1	4.43±1.19 ^{aA}	4.65±1.86 ^{aA}	4.52±1.63 ^{aA}	4.38±1.54 ^{aA}	4.50±1.53 ^{aA}	4.41±1.28 ^{aA}	4.51±1.31 ^{aA}	4.65±1.55 ^{aA}	4.54±1.79 ^{aA}	4.55±1.50 ^{aA}	4.80±1.36 ^{aA}	4.73±1.73 ^{aA}
Month 2	4.40±1.67 ^{aA}	4.60±1.38 ^{aA}	4.46±1.12 ^{aA}	4.32±1.23 ^{aA}	4.45±1.93 ^{aA}	4.39±1.42 ^{aA}	4.43±1.20 ^{aA}	4.60±1.56 ^{aA}	4.53±1.80 ^{aA}	4.53±1.60 ^{aA}	4.78±1.80 ^{aA}	4.65±1.72 ^{aA}
Month 3	4.39±1.69 ^{aA}	4.58±1.14 ^{aA}	4.43±1.29 ^{aA}	4.30±1.90 ^{aA}	4.41±1.98 ^{aA}	4.35±1.40 ^{aA}	4.40±1.22 ^{aA}	4.58±1.63 ^{aA}	4.49±1.89 ^{aA}	4.48±1.34 ^{aA}	4.65±1.82 ^{aA}	4.60±1.67 ^{aA}
Month 4	4.35±1.37 ^{aA}	4.57±1.19 ^{aA}	4.39±1.43 ^{aA}	4.23±1.92 ^{aA}	4.35±1.56 ^{aA}	4.30±1.39 ^{aA}	4.36±1.37 ^{aA}	4.52±1.66 ^{aA}	4.42±1.85 ^{aA}	4.40±1.52 ^{aA}	4.61±1.87 ^{aA}	4.58±1.69 ^{aA}
Month 5	4.30±1.90 ^{aA}	4.51±1.67 ^{aA}	4.31±1.49 ^{aA}	4.20±1.95 ^{aA}	4.32±1.43 ^{aA}	4.26±1.71 ^{aA}	4.30±1.55 ^{aA}	4.50±1.90 ^{aA}	4.40±1.74 ^{aA}	4.38±1.91 ^{aA}	4.55±1.83 ^{aA}	4.50±1.51 ^{aA}
Month 6	4.28±1.43 ^{aA}	4.50±1.50 ^{aA}	4.30±1.52 ^{aA}	4.15±1.66 ^{aA}	4.30±1.24 ^{aA}	4.23±1.70 ^{aA}	4.28±1.98 ^{aA}	4.49±1.59 ^{aA}	4.35±1.68 ^{aA}	4.31±1.99 ^{aA}	4.43±1.49 ^{aA}	4.41±1.70 ^{aA}
Month 7	4.24±1.53 ^{aA}	4.47±1.48 ^{aA}	4.25±1.22 ^{aA}	4.13±1.50 ^{aA}	4.26±1.27 ^{aA}	4.19±1.65 ^{aA}	4.20±1.83 ^{aA}	4.45±1.79 ^{aA}	4.31±1.66 ^{aA}	4.30±1.93 ^{aA}	4.41±1.54 ^{aA}	4.38±1.79 ^{aA}

Table 8– Evaluation of the physical properties of isinglass treatments prepared from sturgeon swim bladders by industrial and Indian methods during seven months of storage at room temperature

Indian method															
Index	pH		Texture analysis				Color analysis								
Treat ment	stellat e sturge on	Beluga	Persia n sturge on	stellate sturgeo n	Beluga	Persian sturgeon	stellate sturgeon			Beluga			Persian sturgeon		
Index							L (Lightnes s)	A Red–) (Green	B Blue–) (Yellow	L (Lightne ss)	A Red–) (Green	B Blue–) (Yellow	L (Lightne ss)	A Red–) (Green	B Blue–) (Yellow
Day 1	6.51± 1.38 ^{aA}	6.50± 1.80 ^{aA}	6.45± 1.15 ^{aA}	74.16± 1.65 ^{aA}	74.84±1. 12 ^{aA}	74.56±1. 33 ^{aA}	85.93±1. 14 ^{aA}	2.12±1. 24 ^{eA}	6.51±1. 55 ^{dA}	85.16±1. 13 ^{aA}	2.18±1. 26 ^{eA}	6.96±1. 23 ^{cA}	85.28±1. 76 ^{aA}	2.23±1. 93 ^{gA}	6.67±1. 13 ^{dA}
Month 1	6.49± 1.54 ^{aA}	6.48± 1.49 ^{aA}	6.43± 1.38 ^{aA}	73.58± 1.37	74.17±1. 23 ^{bA}	74.13±1. 43 ^{aA}	85.17±1. 99 ^{bA}	2.26±1. 25 ^{eA}	6.60±1. 51 ^{dA}	84.73±1. 19 ^{aA}	2.34±1. 24 ^{deA}	7.12±1. 20 ^{bcA}	84.79±1. 79 ^{bA}	2.45±1. 54 ^{fgA}	6.85±1. 34 ^{dA}
Month 2	6.42± 1.18 ^{aA}	6.40± 1.72 ^{aA}	6.37± 1.89 ^{aA}	72.97± 1.17	73.67±1. 39 ^{cA}	73.56±1. 56 ^{bA}	84.53±1. 95 ^{cA}	2.59±1. 16 ^{eA}	6.75±1. 92 ^{cdA}	84.12±1. 35 ^{bA}	2.69±1. 34 ^{cdA}	7.20±1. 60 ^{bA}	83.12±1. 46 ^{bA}	2.93±1. 45 ^{efA}	6.99±1. 73 ^{cdA}
Month 3	6.37± 1.27 ^{aA}	6.47± 1.79 ^{aA}	6.35± 1.90 ^{aA}	72.15± 1.88	73.15±1. 40 ^{dA}	73.12±1. 45 ^{bA}	83.67±1. 94 ^{dA}	3.11±1. 15 ^{dA}	6.80±1. 99 ^{cA}	83.67±1. 36 ^{cA}	3.12±1. 38 ^{cA}	7.34±1. 54 ^{bA}	82.59±1. 47 ^{cA}	3.17±1. 44 ^{eA}	7.18±1. 75 ^{cA}
Month 4	6.34± 1.98 ^{aA}	6.41± 1.32 ^{aA}	6.26± 1.50 ^{aA}	71.84± 1.69	72.54±1. 51 ^{eA}	72.64±1. 44 ^{bA}	82.91±1. 99 ^{eA}	3.56±1. 32 ^{cdA}	6.92±1. 98 ^{cA}	82.95±1. 33 ^{cA}	3.85±1. 39 ^{bA}	7.45±1. 53 ^{abA}	81.95±1. 64 ^{dA}	3.84±1. 40 ^{dA}	7.24±1. 80 ^{cA}
Month 5	6.25± 1.87 ^{aA}	6.24± 1.43	6.13± 1.29 ^{aA}	71.24± 1.60	71.99±1. 62 ^{fA}	71.85±1. 77 ^{cA}	81.53±1. 90 ^{fA}	3.78±1. 39 ^{bcA}	7.13±1. 75 ^{bcA}	81.34±1. 39 ^{dA}	4.12±1. 40 ^{bA}	7.51±1. 78 ^{aA}	81.16±1. 62 ^{eA}	3.15±1. 28 ^{cA}	7.45±1. 82 ^{bcA}
Month 6	6.14± 1.66 ^{aA}	6.15± 1.56 ^{aA}	6.08± 1.54 ^{aA}	70.98± 1.55	71.52±1. 59 ^{fgA}	71.14±1. 84 ^{dA}	80.47±1. 88 ^{gA}	4.18±1. 30 ^{abA}	7.47±1. 17 ^{bA}	80.27±1. 41 ^{eA}	4.56±1. 42 ^{abA}	7.68±1. 77 ^{aA}	80.39±1. 60 ^{fA}	4.37±1. 31 ^{bA}	7.59±1. 89 ^{abA}
Month 7	6.07± 1.57 ^{aA}	6.05± 1.67 ^{aA}	6.02± 1.59 ^{aA}	70.43± 1.49	71.08±1. 77 ^{gA}	70.82±1. 89 ^{dA}	79.13±1. 85 ^{hA}	4.56±1. 28 ^{aA}	7.83±1. 23 ^{aA}	79.16±1. 77 ^{fA}	4.85±1. 24 ^{aA}	7.83±1. 28 ^{aA}	79.28±1. 68 ^{gA}	4.93±1. 33 ^{aA}	7.99±1. 92 ^{aA}
Industrial method															
Day 1	6.30± 1.12 ^{aA}	6.37± 1.27 ^{aA}	6.39± 1.22 ^{aA}	88.73± 1.59 ^{aA}	88.97±1. 50 ^{aA}	88.85±1. 27 ^{aA}	88.28±1. 56 ^{aA}	1.17±1. 12 ^{bA}	4.21±1. 19 ^{bA}	88.14±1. 25 ^{aA}	1.45±1. 54 ^{bA}	4.51±1. 40 ^{bA}	88.39±1. 39 ^{aA}	1.12±1. 67 ^{bA}	4.87±1. 17 ^{bA}
Month 1	6.28± 1.15 ^{aA}	6.35± 1.29 ^{aA}	6.35± 1.23 ^{aA}	88.65± 1.50 ^{aA}	88.90±1. 56 ^{aA}	88.80±1. 89 ^{aA}	88.34±1. 63 ^{aA}	1.20±1. 35 ^{bA}	4.27±1. 36 ^{bA}	88.11±1. 13 ^{aA}	1.52±1. 94 ^{bA}	4.55±1. 98 ^{bA}	88.30±1. 47 ^{aA}	1.18±1. 45 ^{bA}	4.89±1. 24 ^{bA}
Month 2	6.24± 1.89 ^{aA}	6.30± 1.48 ^{aA}	6.32± 1.34 ^{aA}	88.60± 1.57 ^{aA}	88.86±1. 45 ^{aA}	88.78±1. 17 ^{aA}	88.43±± 1.53 ^{aA}	1.28±1. 15 ^{bA}	4.31±1. 29 ^{bA}	88.03±1. 14 ^{aA}	1.63±1. 66 ^{bA}	4.63±1. 44 ^{bA}	88.25±1. 82 ^{aA}	1.29±1. 77 ^{bA}	4.95±1. 22 ^{bA}
Month 3	6.18± 1.18 ^{aA}	6.27± 1.40 ^{aA}	6.30± 1.56 ^{aA}	88.51± 1.55 ^{aA}	88.80±1. 34 ^{aA}	88.70±1. 59 ^{aA}	88.50±1. 32 ^{aA}	1.35±1. 22 ^{abA}	4.40±1. 57 ^{abA}	87.96±1. 28 ^{aA}	1.68±1. 78 ^{bA}	4.70±1. 60 ^{abA}	88.15±1. 85 ^{aA}	1.35±1. 12 ^{bA}	5.11±1. 82 ^{abA}
Month 4	6.15± 2.21 ^{aA}	6.20± 1.35 ^{aA}	6.25± 1.67 ^{aA}	88.40± 1.39 ^{aA}	88.72±1. 39 ^{aA}	88.61±1. 77 ^{aA}	88.61±1. 73 ^{aA}	1.45±1. 31	4.45±1. 72 ^{aA}	87.85±1. 43 ^{aA}	1.75±1. 27 ^{abA}	4.75±1. 92 ^{aA}	88.09±1. 18 ^{aA}	1.49±1. 79 ^{abA}	5.15±1. 19 ^{aA}
Month 5	6.13± 1.35 ^{aA}	6.15± 1.17 ^{aA}	6.21± 1.36 ^{aA}	88.37± 1.76 ^{aA}	88.60±1. 42 ^{aA}	88.55±1. 76 ^{aA}	88.69±1. 71 ^{abA}	1.50±1. 30 ^{aA}	4.56±1. 80 ^{aA}	87.80±1. 88 ^{aA}	1.80±1. 35 ^{aA}	4.82±1. 24 ^{aA}	88.97±1. 27 ^{aA}	1.53±1. 13 ^{aA}	5.23±1. 31 ^{aA}
Month 6	6.10± 1.28 ^{aA}	6.12± 1.24 ^{aA}	6.18± 1.52 ^{aA}	88.29± 1.88 ^{aA}	88.52±1. 54 ^{abA}	88.43±1. 78 ^{aA}	87.75±1. 21 ^{bcA}	1.53±1. 85 ^{aA}	4.68±1. 18 ^{aA}	87.79±1. 19 ^{abA}	1.83±1. 58 ^{aA}	4.93±1. 73 ^{aA}	87.91±1. 46 ^{abA}	1.56±1. 16 ^{aA}	5.29±1. 12 ^{aA}
Month 7	6.05± 1.16 ^{aA}	6.07± 1.68 ^{aA}	6.12± 1.48 ^{aA}	88.27± 1.46 ^{aA}	88.40±1. 93 ^{bA}	88.40±1. 89 ^{aA}	87.41±1. 13 ^{cA}	1.89±1. 86 ^{aA}	4.99±1. 91 ^{aA}	87.60±1. 26 ^{bA}	2.11±1. 99 ^{aA}	5.14±1. 84 ^{aA}	87.83±1. 38 ^{bA}	1.95±1. 80 ^{aA}	5.56±1. 56 ^{aA}

Data presented in Table 7 show that the industrial method maintained scores above 4.0 at all-time points, with an insignificant reduction in overall acceptability ($P > 0.05$). The industrial method also received higher sensory scores than the Indian method across color, smell, texture, and overall acceptability at every time point during the study ($P < 0.05$). The decline in quality of the Indian method became significant after 3 months and by 7 months ($P < 0.05$), many attributes were unfavorable whereas with respect to the industrial method largest number of attributes maintained acceptable ranges.

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable differences among sampling times ($P < 0.05$), whereas the same lowercase letters show no main difference ($P > 0.05$). Dissimilar uppercase letters within the same row illustrate

meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

The data in Table 8 give physical property changes based on colorimetric testing (L^* , a^* , b^* values) that show higher brightness and uniformity ($p < 0.05$) from the industrial method and better retention of that brightness over time. Texture results from the industrial method had greater tissue cohesiveness and resistance ($p < 0.05$) than tissue from isinglass prepared using the Indian method (brittle, variable). The pH values decreased slightly over the two methods but showed greater stability with the industrial method, and pH values decreased more quickly for the Indian method compared to the industrially processed product, indicating overall physical stability and desirability of the product processed through an industrial manner.

Table 9– Evaluation of the water activity of isinglass treatments prepared from sturgeon swim bladders by industrial and Indian methods during seven months of storage at room temperature

Index	Water activity					
	Industrial method			Indian method		
Sample Sampling time	Beluga	Persian sturgeon	<i>Stellate sturgeon</i>	Beluga	Persian sturgeon	<i>Stellate sturgeon</i>
0 month	0.48 $\pm$ 0.25 ^{aA}	0.49 $\pm$ 0.29 ^{aA}	0.50 $\pm$ 0.18 ^{aA}	0.53 $\pm$ 0.28 ^{aA}	0.54 $\pm$ 0.32 ^{aA}	0.55 $\pm$ 0.41 ^{aA}
7 month	0.53 $\pm$ 0.21 ^{aA}	0.54 $\pm$ 0.14 ^{aA}	0.55 $\pm$ 0.23 ^{aA}	0.58 $\pm$ 0.24 ^{aA}	0.59 $\pm$ 0.37 ^{aA}	0.60 $\pm$ 0.35 ^{aA}

Results are exhibited as mean $\pm$ standard deviation ($n = 3$). Unlike lowercase letters within the same column, present remarkable differences among sampling times ($P < 0.05$), whereas the same lowercase letters show no main difference ($P > 0.05$). Dissimilar uppercase letters within the same row illustrate meaningful differences between treatments at the same keeping time ($P < 0.05$), whereas identical uppercase letters display no notable difference ($P > 0.05$).

Table 9 shows the changes in water activity of isinglass produced by the traditional Indian and semi-industrial methods during seven months of storage at room temperature. A gradual increase in a_w was observed in both treatments over the storage period.

Nevertheless, the semi-industrial method maintained consistently lower water activity values than the traditional Indian method throughout storage.

Discussion

The rate of moisture loss during drying, as well as retention of non-collagenous components during extraction and drying processes can determine the nutritional composition of isinglass (Chen *et al.*, 2024). In the experimental isinglass, protein, moisture, fat and ash content showed significant difference between traditional Indian and semi-industrial processes (Table 1). Protein content of semi-industrial isinglass ranged between 74.11-74.63% while it was 64.14-65.70% for

the traditional Indian isinglass. It was evident from the results that higher protein recovery was achieved in semi-industrial process with better preservation of protein.

Strict control over washing process, quick dehydration and hot air drying were supposed to have contributed to the minimization of protein loss and the higher collagen recovery (Bao, Ertbjerg, Estévez, Yuan, & Gao, 2021). Traditional Indian method that involves drying under atmospheric condition for a long time, resulted in some degradation of protein and decreased collagen recovery (Yu *et al.*, 2023). There were considerable differences in the moisture content. Semi-industrial isinglass showed a moisture content ranging between 24.18 and 25.16%, while the values for traditional Indian isinglass were between 32.14 and 33.19% during the period of storage. Lower moisture content is of paramount importance as it reduces the possibility of microbial spoilage, enzymatic activity and oxidative reactions during the shelf life of food product.

Lower microbial count, TVB-N, peroxide value and TBARS value with acceptable sensory scores found in semi-industrial treated samples were attributed to their lower moisture content. Fat content in semi-industrial product (0.11-0.14%) was also much lower than that of traditional method (0.61-0.81%), implying that the amount of readily oxidizable substrates is low in the former and therefore less oxidation occurs during storage. In addition, the low ash content (0.61-0.85%) in the semi-industrial isinglass signified removal of more non-collagenous impurities and less mineral salts, yielding higher purified collagen (Rodrigues *et al.*, 2021).

Asty, Yasman, & Wardhana, (2017) prepared semisolid isinglass from cultured catfish swim bladder. They were determined moisture, protein, fat, and ash contents 98.97%-99.08%, 0.49%-0.53%, 0.20%-0.21%, and 0%, respectively, in isinglass samples treated with acetic acid. In our study, moisture, protein, fat, and ash contents were 33.05-33.18%, 64.14 – 65.36%, 0.61-0.73 and 0.98-1.95%, respectively, in the Indian method.

Moisture, protein, fat, and ash contents were 24.52-25.16%, 74.11-74.67%, 0.11-0.12%, and 0.61-0.70%, respectively, in samples processed by the semi-industrial method. In detail, the production conditions were different in these studies. Their isinglass was prepared by extraction with acetic acid and dried at low temperature (39–40 °C). The dried isinglass samples in the current study were extracted by other methods, such as Indian and semi-industrial processes, without the addition of acid. However, this comparison was made to show the effect of the processing method on the quality of isinglass.

Nevertheless, the comparison serves the purpose to prove that the extraction method plays an important role on the nutritional properties of the isinglass. Therefore, the selection of a proper extraction and drying procedure not only enhances the nutritional value and market acceptability but also ensures the shelf-life stability of commercial isinglass and thus provides a useful guide to the process development for the production of high quality dried isinglass from cultured sturgeon swim bladders (Chen, Liu, & Wang, 2022).

Isinglass amino acid profile provides an insight into collagen structure and nutritional value. As shown in Table 2, glycine (26–28 residues/1000 residues), proline (12 - 14residues/1000 residues) and hydroxyproline (12-14residues/1000 residues) were identified as major amino acids in all the samples, confirming the collagen rich nature of the extracted isinglass. These amino acids represent major constituent elements of the collagen matrix; with glycine maintaining the triple helix integrity and proline and hydroxyproline contributing towards the collagen thermal stability and tensile strength due to hydrogen bonding at an inter-chain level (Yu *et al.*, 2023).

No significant variation was noted in the amino acid profile of samples obtained through the traditional Indian and semi-industrial extraction methods. This implies that neither extraction process led to any significant changes in the primary structure of the collagen

molecules. Small variations were observed for certain amino acids in their proportions among the samples. It is very probable that these numerical differences may simply reflect inherent biological variation among samples rather than any processing related degradation. Moderate drying temperature utilized in the semi-industrial method along with absence of chemical agents during processing likely contributed in maintaining the original composition of amino acids (Zhang *et al.*, 2022).

The proportions of glycine and proline obtained in the present work are similar to those reported by Cruz-López *et al.* (2023) who found glycine and proline 29.19 and 12.10 residues/1000 residues as principal amino acids of the collagen from the swim bladders of farmed *Totoaba macdonaldi*. Asty *et al.* (2017) reported the proline and glycine as 462.82 - 605.88 and 1069.35 - 1163.08 ppm in semisolid isinglass prepared of cultured catfish swim bladder. Our data showed that glycine varied between 26 – 28 and 25-27 residues/1000 residues in Indian and semi-industrial treatments, respectively. Proline content was 12-14 residues/1000 residues in the semi-industrial and Indian methods. Minor discrepancies were, as expected, attributed to the different fish species, maturity, extraction procedure, and analytical methods employed in each study.

In addition, similar amino acid profiles in both traditional and semi-industrial products were consistent with better protein content and better physicochemical stability of the semi-industrial isinglass samples. The semi-industrial method allowed for higher protein yields and improved water, oxidation and microbial stability, but without affecting the fundamental composition of the amino acids of the collagen molecule. This indicated that the advantages provided by the semi-industrial method are chiefly related to improved collagen quality preservation rather than collagen structural alteration (Zheng *et al.*, 2024).

As it is apparent from Table 3, the processing method had significant effect on

process efficiency, product yield, processing time, resource consumption and manufacturing performance. In contrast to the traditional Indian method yielding 22-24% of dry isinglass recovery, the semi-industrial process showed much higher dry isinglass yield in the range of 63-67%. The high recovery from semi-industrial method more likely due to the controlled washing, trimming, drying (hot air drying) which minimize the collagen loss during the processing and hence improve the recovery of the swim bladder matrix. The prolonged exposure to processing environment and unskilled workers likely caused increased mechanical losses in the traditional process leading to much lower collagen recovery. Although the efficiency of the processing system was markedly improved in the semi-industrial method, the present comparison study evaluated two whole processing systems and not individual processing parameters and so improvements obtained should be interpreted as resultant effect of the overall process parameters (Wei *et al.*, 2025).

Similarly, processing time for both processing systems was also highly different. The semi-industrial process resulted in total processing time of 8-24 hours while it takes 3-7 days for traditional Indian process to complete the isinglass processing. The reduction in processing time also enhance the manufacturing capacity of processing units and provides reduced duration of exposure to environmental contamination and microbial degradation. It is in consonance with observed significantly low microbial counts, lower TVB-N values and good oxidation and sensory quality in semi-industrial isinglass product during storage. It is thus confirmed that enhancement in process efficiency leads to enhancement of quality and shelf-life of the isinglass.

Though operator handling in semi-industrial processing was higher (3-5 h/kg) than traditional process (0.5-1.5 h/kg) due to well-controlled washing, trimming, quality checks and monitoring drying conditions, it also results in better reproducibility, uniform product and

consistent quality. Higher labor was for better quality control than processing disadvantages.

In terms of resources consumption, the semi-industrial method consumed less electricity (1-2 kWh/kg) than traditional Indian method (4-8 kWh/kg) even with shorter processing time and higher product recovery. This could be attributed to quick moisture removal in hot air drying thereby minimizing energy loss. However, water consumption for semi-industrial method was higher (120-250 l/kg) than traditional process (80-150 l/kg) owing to the increased washing steps required to improve sanitary quality and to remove non-collagenous substances prior to drying. Though high water consumption adds to processing costs, whereas additional washing steps likely account for lower microbial population, ash content and higher protein content.

Process consistency was another major advantage for the semi-industrial processing. More consistent temperature, airflow and drying conditions for the processing of samples produced a more consistent product with less than 3% processing loss compared to about 6% for the traditional Indian process. A lower loss due to processing rejection is an economic benefit and promotes resource sustainability through optimum utilization of raw materials. More consistent semi-industrial process resulted in low variability in the physicochemical, microbiological and sensory parameters during the entire period of storage, proving that improved process control is correlated with increased process efficiency.

To the best of our knowledge, no such research has been carried out on evaluation of traditional Indian and semi-industrial processing procedures for commercializing cultured sturgeon swim bladder isinglass with simultaneous investigation of process efficiency, yield, resource utilization, quality attributes and shelf life. Hence, the present work is one of the first systematic integrated assessment of both technology and quality consequences of both these production processes. Overall results strongly advocate that semi-industrial processing strategy

provided better economic and technological advantage for production of a shelf stable, uniform, higher quality and safe commercial isinglass through improved collagen yield, shortened process time, efficient use of energy and minimized loss during processing.

Although the traditional Indian extraction methodology is a simple technique and does not require sophisticated infrastructure, but efficiency of the process may be significantly improved through systematic experimental optimization of different processing steps via soaking pH and duration, temperature and duration of drying, initial moisture level prior to pressing, and addition of different processing aids. This may further lead to improve extraction yield, physico-chemical quality, stability and overall product performance. Thus, further work should be performed for identifying optimal processing conditions (Li, Yang, Wu, Chen, & Lin, 2022).

Minerals are generally resistant to degradation at typical drying temperatures. The concentration of the minerals in isinglass therefore mainly reflects the washing, purification, and the extent of removal of non-collagenous materials from tissues and surfaces throughout processing and the loss of some residual matter from washing step. Minerals were detected lower in the semi-industrial processed sample than in the traditionally processed sample (For example: calcium was 26 -28 and 55-58 mg/100 g in semi- industrial and Indian methods, respectively. Phosphorus was 14 – 19 and 36 -39 mg/100 g in semi-industrial and Indian methods, respectively. Sodium was 53 -58 and 74 -78 mg/100 g in semi- industrial and Indian methods, respectively. Magnesium was 2 -5 and 5 -10 mg/100 g in semi- industrial and Indian methods, respectively) (Table 4). This indicates that the semi-industrial process has more removal efficiently for remaining tissues, blood, and other residues clinging to the swim bladder during washing and purification, and the reduction in minerals was a benefit resulting in higher purity. Any reduced mineral levels observed should therefore be regarded not as an

indicator of a nutritional loss, but of greater purity achieved during processing. More of residual connective tissue, other cellular materials and their contained inorganic substances remain in the fish collagen as a result of inadequate washing and handling during the traditional process of producing this material. This resulted in increased contents of mineral materials than with further cleansing, rinsing and other standardizing procedures in semi-industrial processing. Therefore, these differences in mineral content mainly depend upon processing but not due to the loss from the drying stage itself. However, minor differences found between processing methods of isinglass would be unlikely to make a substantial difference to any significant contribution from isinglass to mineral intake and it serves well as a functional collagenous product, not as a source of mineral nutrition (Shi, 2025).

Instead, such enhancement in isinglass purity achieved in semi-industrial processing could further improve the protein purity, ash, physicochemical properties, and microbial stability of product throughout storage. Overall, for the first time, comparison between minerals of isinglass from farmed sturgeon swim bladders with semi-industrial process and traditional Indian process was specifically addressed. However, this is generally in line with studies on the extraction of fish collagen in general that indicate a loss of remaining mineral and the further increase of purity due to further extensive purification during fish collagen processing which is also without detrimental effects on the quality of collagen globular form (Wei *et al.*, 2025). The present study suggests that the use of a semi-industrial process while preserving collagen mineral characteristics. Together with better nutritional content and reduced microbial activity as documented in storage evaluation studies, this provides further support for its commercial viability.

Microbiological quality is one of the principal criteria used to evaluate the safety, hygienic status, and storage stability of food products. In the present study, total bacterial

counts gradually increased throughout the seven-month storage period in all treatments. At the end of storage, total bacterial counts ranged from 5.17 to 5.47 log CFU/g in isinglass produced by the traditional Indian method and from 2.28 to 2.78 log CFU/g in samples produced by the semi-industrial method (Table 5), indicating that the semi-industrial process provided substantially greater microbiological quality during storage. The gradual increase in bacterial counts suggests that a limited residual microbial population survived processing and was capable of slow multiplication under ambient conditions. Nevertheless, the relatively low growth rate indicates that the low moisture content and reduced water activity of dried isinglass considerably restricted microbial proliferation, resulting in much slow microbial development (Pan, Li, Tao, & Guo, 2018).

A clear difference was observed between the two extraction methods. Samples produced by the traditional Indian method consistently exhibited higher microbial counts than those produced by the semi-industrial method. The variability can probably be the result of extended drying times and continuous presence of environmental contamination, i.e., dust, microorganisms, airborne materials, oxygen and so forth in sun dried products during traditional processing method. Whereas, drying at 60 °C for controlled hot-air drying under the semi-industrial processing conditions accelerated the process, effectively lowering the moisture content and at the same time denaturing the many endogenous microorganisms and enzymes present. Furthermore, washing, controlled handling, and more uniform dehydration during semi-industrial processing likely reduced the initial microbial load and minimized subsequent microbial growth throughout storage. These findings clearly demonstrate that processing conditions exert a major influence on the microbiological quality of dried isinglass (Li *et al.*, 2022).

Yeasts and molds were detected only at low levels during storage, with their occurrence

being more evident in samples prepared using the traditional Indian method after prolonged storage. This may relate to a slow take-up of ambient moisture and that an environment becomes more conducive to the fungal process, though this does not become extensive throughout the period of storage and thus must be interpreted relating to the low activity of water in dried isinglass and hence the absence of widespread spoilage organisms. Hence the microbiological changes suggest slow quality loss, rather than significant spoilage (Kovács *et al.*, 2025). To the best of our knowledge, no previous studies have evaluated the microbiological quality of isinglass produced from cultured sturgeon swim bladders using traditional Indian and semi-industrial extraction methods, making direct comparison with previous investigations unavailable.

Samples with lower bacterial counts exhibited lower TVB-N, peroxide value, and TBARS value, along with better scores in sensory evaluation during the storage period. The bacterial proliferation promoted development of volatile nitrogen compounds, accelerated oxidation deterioration and induced off odor, appearance and acceptability. Since microbial load gradually increased during storage, the proliferation rate of microorganisms seemed slow to cause sensory failure in early stage. Thus, storage quality deteriorations are attributed to cumulative effects from mild microbial contamination, protein degradation, and oxidative reactions rather than by gross microbial spoilage (Pan *et al.*, 2018).

TVB-N is widely used as an indicator of protein degradation in seafood represents the formation of volatile nitrogen compounds through the enzymatic and microbial activities (Bao *et al.*, 2021). In the current investigation, there was a progressive increase in the TVB-N in all treatments during 7 months' storage indicating gradual degradation of protein. This increase was throughout and not marked in either of the extraction method indicating slow protein degradation under the condition

employed for the storage. TVB-N remained at comparatively low level with the final TVB-N between 11.26 and 23.17 mg/100 g (traditional Indian method) and between 7.84 and 14.12 mg/100 g (semi-industrial method) respectively (Table 6). Though TVB-N was the widely used parameter for the spoilage of fishery products, it has been explored very little to characterize dried swim bladders products, and no TVB-N data were reported for sturgeon isinglass produced by traditional and semi-industrial method. Hence TVB-N values of the dried products can only be considered as relative indicator of the extent of protein deterioration and shelf life. These findings demonstrated that the semi-industrial method had much better efficiency in minimizing the development of volatile nitrogenous compounds. This reduction in the TVB-N in semi-industrial method could be ascribed to a more stringent process control of washing, dehydrating and hot air drying (60 °C), rapidly brought down the moisture content, repressed bacterial proliferation and suppressed the activity of endogenous enzymes. On the other hand, the extended duration and ambient condition of drying of the samples under traditional Indian method had allowed more exposure to the air and prolonged drying that favor the proliferation of microorganisms and the degradation of protein to develop higher TVB-N (Koochakian *et al.*, 2006).

As observed earlier for other parameters the TVB-N also followed a similar trend showing strong correlations with microbial and sensory scores which means that protein spoilage, microbial trend and sensory quality change in same directions during storage of the dried isinglass. Similar progressive increase in the PV also indicated simultaneous occurrences of protein degradation and primary lipid oxidation during storage. The fact that the TVB-N values in the semi-industrial products were always lower demonstrates clearly better preservation of chemical quality and greater storage stability in this method (Zhang *et al.*, 2022).

In spite of observed changes, the steady progression in TVB-N and PV values would

probably make it possible to describe their change during storage by kinetic models; an experiment with 8 points over 7 months should be feasible for future kinetic modeling. With the sampling time points and measurements provided, the degradation rate constants of these parameters can be estimated, which then can be used for modeling such as first-order kinetics, pseudo-first-order reaction, etc. for predicting shelf-life and storage characteristics.

PV and TBARS are considered as an important parameters for the evaluation of primary and secondary lipid oxidation, respectively, and these parameters when combined offer a holistic view of oxidative changes that occur during storage. In this study, both PV and TBARS demonstrated gradual increase throughout the seven months of storage for all treatments, suggesting that lipid oxidation was continually occurring (Table 6). However, the levels remained low throughout the entire period, suggesting that generally, the oxidative stability of the isinglass was satisfactory under the storage conditions encountered. The steady increase in PV reflected the accumulation of lipid hydroperoxides as primary oxidation products. Though isinglass is a collagen-rich material with little lipid, trace quantities of phospholipids and membrane lipids remain from the swim bladder and these are prone to oxidation upon extended storage. The low rates of PV increase suggested that low moisture and reduced water activity in dried isinglass likely hindered the oxidative process (Zhao *et al.*, 2018).

The PV in samples processed using the traditional Indian method (1.64–4.68 meq/kg oil) was significantly higher compared to the samples processed using the semi-industrial method (1.05–2.24 meq/kg oil), which indicates that the oxidative stability was poorer when using the traditional Indian process. Similarly, the levels of TBARS rose from 0.46 to 1.98 mg MDA/kg for the Indian method, but only rose moderately from 0.24 to 0.95 mg MDA/kg for the semi-industrial method, suggesting that controlled drying methods have

improved the oxidative stability of the product throughout its shelf life. Prolonged air drying combined with the exposure of product to ambient air and direct sunlight in the Indian traditional process potentially accelerated the oxidation of lipid, whereas in the semi-industrial method hot-air drying (60 °C) reduced the moisture content of the swim bladders very quickly thereby inhibiting microbial activity, minimizing subsequent reactions and thereby reducing oxidation. Improved washing and greater removal of residual impurities during semi-industrial processing likely further reduce the substrate availability of oxidation, contributing to the lowered PV and TBARS values observed (Li *et al.*, 2023; Chen *et al.*, 2019; Zhang, Chen, & Hu, 2025).

The continuing increase of TBARS suggested gradual decomposition of lipid hydroperoxides to secondary products of oxidation such as malondialdehyde, a volatile aldehyde is the main contributor to the unpleasant odor and off-flavor in oxidizing lipids. The PV and TBARS changes mirrored each other, suggesting a parallel progress of primary and secondary lipid oxidation throughout the period of storage (Ahmed, Verma, & Patel, 2020). However, the consistently lower TBARS values in samples prepared with the semi-industrial process indicated that controlled drying conditions were also successful in limiting the secondary products of oxidation, and not only their primary counterparts.

These observed oxidative changes were consistent with the gradual changes in the sensory attributes of isinglass during storage. As shown, products with lower PV and TBARS generally scored better in terms of odor, color and overall acceptability. The decline in both odor and color was likely the result of progressive lipid oxidation, with subsequent development of flavors and colors and degradation of natural chromospheres. Moreover, oxidative reactions may cause slight modifications in collagen matrix leading to a gradual decrease in lightness (L value) and a

gradual increase in the yellowness (b value) of the product which explained changes in Instrumental Color Parameters during storage (Zhao *et al.*, 2018).

The oxidative changes obtained in this study, indicated that TVB-N and microbial count, changes in instrumental color and sensory scores, are related to lipid oxidation, protein degradation, and microbial growth proceeded simultaneously during storage, and oxidative reaction could be one of major mechanism causing the quality degradation of dried isinglass (Bekhit, Holman, Giteru, & Hopkins, 2021). PV and TBARS value were highly related to sensory evaluation, therefore could be potentially utilized to predict the sensory quality and oxidative stability during storage of isinglass (Huang, Song, & Yuan, 2026). As far as the authors know, no data on the change of peroxide value and thiobarbituric acid reactive substances in the isinglass prepared from farmed sturgeon swim bladders through both traditional Indian and semi-industrial extraction methods have been reported.

Sensory score of isinglass made by the traditional Indian and semi-industrial processes stored for seven months at ambient temperature (room temperature) presented in Table 7. As seen, for both methods, the sensory scores tend to drop over storage time; however, the degradation is more intense in the samples obtained by the traditional Indian method. All sensory scores decreased during storage for the traditional method from about 3.7 at the start to about 3.0 for color, from about 3.5 to below 3.0 for odor and from 3.3 to below 3.0 for texture and overall quality. Color is characterized by progressive darkening and loss of transparency, attributed likely to the exposure to sunlight and the atmosphere during the traditional drying, resulting in oxidation of the surface collagen structure. Similarly, a gradual increase of TVB-N in these samples resulted in an increased production of volatile nitrogenous compounds causing changes in the odor profile. Increased brittleness and reduced firmness were reflected in lower texture scores attributed to further moisture losses and protein and collagen

structure degradation due to longer storage time under uncontrolled environmental conditions (Huang *et al.*, 2026). All this translated to a decline of overall acceptability from approximately 3.6 to 2.9 by month 7, falling below the minimum consumer acceptance level. In contrast, the isinglass made by the semi-industrial method retained high sensory quality throughout storage time. The color scores dropped from about 4.62 -4.85 to 4.30 -4.41, while the other sensory attributes much less affected during storage (Odor: from 4.55-4.66 to 4.20 – 4.45, Texture: From 4.42-4.52 to 4.13-4.26 and overall acceptability: from 4.55-4.65 to 4.24 -4.47), as compared to the traditional product, suggesting effective retention of appearance, odor and texture, under the conditions of semi-industrial process, involving drying at 60 °C. The latter condition resulted in a limited growth of microorganisms and an effective prevention of the degradation of collagen (Seriš *et al.*, 2024). Overall acceptability ranged between good and very good throughout storage, clearly demonstrating that controlled semi-industrial processing is much superior for the production of high quality product.

These differences are supported by the differences in the chemical indices of the product stored at room temperature for 7 months. TVB-N, peroxide value, TBARS values and total microbial counts were consistently lower for the semi-industrially produced isinglass as compared to the traditional one. The parallel trend observed in the two storage methods between these physicochemical indices and the sensory evaluation suggests that deterioration of sensory properties is likely due to the concomitant protein degradation (high TVB-N), oxidation of lipids (high peroxide value and TBARS) and microbial activity, the combined effects are negatively impacted sensory attributes such as appearance, odor, texture and overall acceptability. These findings indicate that TVB-N, peroxide value and TBARS could be utilized as practical predictive indexes for sensory quality evaluation and monitoring of

the storage stability of commercial isinglass (Wei *et al.*, 2025).

pH is a physicochemical attribute that is considered as a main indicator of the general deterioration during storage period (Table 8). This index was 6.45 – 7 in Indian method and 6.30 – 6.12 in semi-industrial method. Since dried isinglass has low moisture content (32.14 – 33.18% in Indian method and 24.16 – 24.52% in semi-industrial method at the end of storage period) (Table 1) and correspondingly low water activity (0.53 – 0.55 in semi-industrial method and 0.58 – 0.60 in Indian method after the 7 months' storage) (Table 9), this would have provided limitation for microbial and enzyme activity thereby minimizing the buildup of acidic or alkaline components. A significant difference was obtained between the two extraction methods, where by semi-industrial production method results in a relatively stable pH compared with the traditional Indian method (Table 8), meaning that the chemical stability of the collagen matrix was maintained to a great extent in samples produced by semi industrial methods due to effective washing, quicker dehydration, reduction of initial microbial load and drying by hot air under control temperature (60 °C). However, drying under normal ambient temperature in open places during traditional Indian method exposes to a lot of microbes and oxygen of the atmosphere thereby gradually facilitating some kind of biochemical change leading to a gradual change in pH during storage time (Bekhit *et al.*, 2021). Low pH values correlated with lower TVB-N, PV, TBARS and bacterial counts and with higher sensory evaluation scores (Tables 5-7). The present results indicate that pH is not an independent criterion of quality evaluation, but it should be viewed as an index of deterioration representing all three major process such as protein breakdown, oxidative degradation and microbial contamination. Hence, pH should not be used alone as quality index, but as a supplemental test in conjunction with the other indices (Tang, Ying, & Shi, 2020). Asty *et al.* (2017) determined pH 2.41-2.69 in semisolid

isinglass of cultured catfish swim bladder. In our study, pH was 6.02-6.07 and 6.05-6.12 in samples processed by Indian and semi-industrial methods, respectively. There are significant differences between our study and their study. The different results obtained in our study compared to the aforementioned study can be attributed to the different conditions used to produce isinglass.

What about color aspects, isinglass of Indian origin has significant decrease in lightness L and significant changes in color indices a, b after storage. Originally, the L was around 85-86 quite light and transparent color, but at the end of 7th month it was around 79 -80 (Table 8). During this period, index a (2.12-4.93) and b (6.51-7.99) increased. So isinglass became darker and more yellowish during storage. This process was driven by oxygen, light, humidity, created by the outdated drying technology, and also by oxidative reactions of surface organic compounds. While for the isinglass from the semi industrial production L remained high and stable (around 87-88) and changes of a (1.12-2.11), b (4.21-5.56) were insignificant. The product remained transparency and stability of color due to controlled temperature (60°C), no direct contact with sunlight and less oxidative reactions, therefore, it was lighter and more color stable than Indian style product. In both methods after filtration, all samples were pressed to get uniform thickness through a mechanical pressing which provided better transparency and homogeneity (Koochakian *et al.*, 2006).

Visual appearance is among the most important factors affecting acceptance by consumers of collagen dried products. Minor reductions in L and some variations of b over storage may have contributed to reduce samples' visual appeal. Such changes related to the extent of oxidative processes and minor changes to the collagen structure during storage, thereby accounting for the drop in overall appearance scores recorded by the panelists (Seifzadeh, 2023).

Color indices (L, a, and b) can provide information on not only the appearance but also

on any changes in the structure within collagen during storage. In the context of material science, the interaction of light with a protein-based structure (i.e., absorption, reflection, scattering) affects the optical properties of collagenous matrices. Therefore, if the structure of the collagen matrix changes during storage, the values of color parameters can be expected to change. In the current study, decreasing values of L indicate progressive loss of brightness of the isinglass samples during storage, possibly as a result of subtle changes in the organization of the collagen network, cross-linking and aggregation of protein chains that reduce scattering and increase light absorption by protein. The samples thus become progressively darker (Poojary & Lund, 2022).

Increasing values of a and particularly of b values reflect structural and chemical modification of the collagen network. Increase of yellowness may result from accumulation of lipid oxidation secondary products and also minor browning reactions during prolong storage, while oxidation products of lipids as chromophores alter optical reflection properties. Similarly change in a index leads to formation of protein oxidation secondary products. So both surface and deep color change of isinglass samples reflect oxidation and structural changes in the collagen network (Poojary & Lund, 2022).

It is clear that two preparation methods result in two products differing in appearance properties. The semi-industrial produced sample possessed a more stable appearance and color over storage, compared to the traditionally Indian prepared samples, which exhibited lower L and greater increases of b over storage time. The superior quality of the semi industrial samples was a result of adequate washing and the use of hot air drying at elevated temperature, with no light exposure. This prevented significant oxidation and degradation of structural elements of the collagen.

Changes in instrumental color of isinglass during storage highly correlated with lipid oxidation, expressed by PV and TBARS

analysis, as increasing amount of secondary oxidation products caused changes in reflection and scattering of light and consequently, affect the L and b indices of the samples. In addition, significant correlation between changes in color and the increasing values of TVB-N, indicated that oxidation processes and protein degradation ran concurrently (Yu *et al.*, 2023).

Results on sensory assessment were also well correlated with instrumental color data. Higher L values and lower changes of b in samples, resulting from their preparation by the semi-industrial method, corresponded to highest scores for appearance and overall acceptability of the samples. These results supported that deterioration of the appearance of isinglass product was not related to just surface discoloration but also to an internal degradation and modification of collagen structure, therefore instrumental color measures can provide useful information for evaluating structural integrity and storage stability of collagen based products and for practical application to industrial quality control of collagen-based food and drug products (Gustami, Nurilmala, & Jacob, 2025). The agreement of sensory evaluation with Instrumental color, PV, TBARS, TVB-N, and bacterial counts indicates that during storage the isinglass deteriorates via a multifactorial process involving protein degradation, oxidation and microbial proliferation rather than by the decline of a particular quality characteristic.

Texture is considered one of the most significant commercial quality attribute for isinglass because it depends directly upon the integrity of the collagen network, as well as upon the ease with which it may be handled, processed and the extent to which it can be sold to commercial buyers. The semi-industrial drying method resulted in significantly better texture (88.40-88.73) throughout storage when compared with Indian method (71.08-74.16), as it can be seen from Table 8, the semi-industrial method of extraction maintained higher hardness and flexibility, a uniform and a more cohesive texture throughout the storage when

compared to traditional Indian method which tended to be softer and brittle. The degradation in texture for traditional Indian dried samples may be associated with increasing the moisture absorption and slow and progressive physicochemical alteration occurring in collagen matrix with additional influence of limited oxidative degradation. It is plausible that, under relatively variable climatic conditions encountered during traditional drying, there is more probability of developing more inhomogeneous structure within collagen, hence becoming easily weak over the time. Moreover, semi-industrial controlled hot-air drying method facilitates uniform removal of moisture content thereby developing homogenous collagen network which gives enhanced physical strength to the end product and consequently better textural property storage (Zhou *et al.*, 2024). This improved textural property is well corroborated by physiochemical attributes that the samples showing low moisture content, TVB-N, Peroxide value and TBARS and lower microbial counts sustained much better texture throughout.

This fact points to that the maintenance of the collagen network not only rests upon the drying process, but also on the capability of preventing both bacterial activity, protein degradation and oxidation to occurring throughout the storage period. In this view, texture deterioration seems to be a composite process of physical events and chemical alterations. Moreover, physical dimension and consistency produced in the semi industrial process of sturgeon isinglass contributed significantly to improve the physical quality, thereby enabling even and controlled dehydration of the product which led to reduction of garble, cracked texture, Brittleness etc, during the storage. The traditional Indian drying method, by nature of environmental factors has inherent capability to render heterogeneity to the final product structure, which was detectable from better physical uniformity produced in semi industrial drying (Seriš *et al.*, 2024). In the entire of this study, it

is established from the physical tests, conducted that the semi -industrial drying method is better suited over the traditional Indian process with respect to enhancing product structure stability, texture, physical appearance and quality throughout the storage of sturgeon isinglass which are well corroborated by the physicochemical, microbiological and sensorial analyses performed.

One of the most crucial parameters that determine the microbiological stability and shelf-life of dried collagen-based products is water activity. During the 7 months of storage under ambient condition, there were only minimal increases in water activity for both extraction methods, indicating low moisture uptake of the product during the storage (Table 9). Samples produced by the semi-industrial process, with a_w values between 0.48 and 0.55, were characterized by lower values than those produced by the traditional Indian process (0.53–0.60), reflecting better dehydration performance and enhanced storage stability. Lower water activity is a major prerequisite to achieving higher stability during storage even relatively low differences in the a_w may have significant consequences for shelf-life, as it might directly contribute to higher proliferation rates of microorganisms or acceleration of chemical reactions (Hussain, Reddy, Chari, & Kumar, 2024b).

These differences in a_w between the products from semi-industrial process and Indian process might related to more controlled hot air drying under standard temperature and more efficient removal of water due to the homogenous nature of the produced collagen. However, extended exposure to fluctuate temperature and humidity throughout open air sun drying (Indian process) probably allowed the samples to be exposed to non-uniform conditions and thus increased moisture content. Similar findings were demonstrated for other dried products, such as fish collagen based materials (Zhu, Sun, & Dong, 2022), in which control over the drying process was shown to yield lower a_w values and enhanced stability compared to open-air drying processes. The low a_w observed in both

products, in the order of 0.48–0.60, is responsible for the low bacterial and fungal counts recorded throughout the entire storage time as water activity of ≤ 0.60 can seriously limit the growth of the most spoilage bacteria, yeast, and mold, reduce protein degradation and the rate of oxidation processes in food during storage (Kumar & Suhag, 2024).

These results were also in good agreement with the results obtained from the microbiological count, TVB-N, PV, TBARS and sensory measurement where semi-industrial process resulted in better quality attributes. In addition to all mentioned quality indicators, water activity should be considered as important contributing factor of physical, chemical and microbiological attributes, and sensory qualities of dried isinglass product. As far as our knowledge, there is no existing report that investigated water activity of commercial isinglass from cultivated sturgeon swim bladder using both traditional Indian and semi-industrial extraction processes.

The use of a factorial experimental design permits to evaluate independently the influence of species and extraction method. Indeed, the data revealed that the extraction method shows a higher significance level than the species effect for the vast majority of the parameters (physicochemical, microbiologic, and sensorial), whereas differences attributed to the species are usually very low or null. This confirms that the benefits are due more to the processing method than to intrinsic properties between the sturgeon species.

From an industrial viewpoint, choice of extraction technique cannot be based solely on one operating parameter; a composite techno-economic evaluation based on capital investment, equipment maintenance and labor costs, cost of utilities, throughput, yield of isinglass, cost of disposal of waste streams and value of product is needed. The study was not, of course, structured to offer a full cost-benefit analysis; however, the production figures and quality results presented here provide the necessary technical information to support economic modelling studies. Such an

integration of these technical factors with economic parameters will permit estimation of production costs per kg of isinglass, overall profitability and return on capital invested thus facilitating the rational comparison of the relative industrial viability of the two extraction methods.

Study Limitations

In absence of established, internationally accepted quality limits for dried collagen based products, like isinglass the parameters like physicochemical and microbiological parameters analyzed in this study served primarily as indicators for relative differences in product quality and shelf life rather than definitive acceptance values. The results were analyzed, consequently used as comparative differences between conventional Indian and semi-industrial extraction systems. The obtained results were also supplemented with literature data for similar products based on fish collagen. Even though the semi-industrial process resulted in consistently better yield and overall improved product physicochemical, microbial quality and acceptability, but due to the fact that entire extraction system was compared, isolated processing steps like temperature of drying, duration of drying, method of pressing and environmental controls cannot be separated and attributed definite individual impacts, likewise many species inherent characteristics (swim bladder thickness, amount of collagen, microstructure of swim bladder tissue, age of the fish, biochemistry of swim bladder) may have contributed for difference in the extraction efficiencies and product quality, but their isolated impact was not analyzed as such in this experimental approach. Hence the conclusion can be generalized for relative advantage of complete process rather than impact of each processing units or species specific characterization.

Additionally, no commercial isinglass reference standards were prepared, since typical commercial isinglass products derive from species other than sturgeon and are

produced by patented industrial processes and cannot be readily compared. Thus, in this study, we only set up a comparison between the two extracted products by using common experimental setup and raw materials. However, in the future research, prepare of external quality references in form of isinglass standards commercial products can support comparison with standards for the purpose of overall quality valuation.

Future Perspectives

Even though the traditional Indian method is uncomplicated and inexpensive to utilize facilities it needed however, quite a lot of performance which could be enhanced through optimization. This could be done to the main operating factors. The main processing elements are soaking time, drying, dry duration and temperature, Initial moisture and also pressing requirements. Additional factors should also be taken in to account, because the optimization associated with such variables could help to increase proteins hydration, proteins swelling, moisture elimination, conservation associated with collagen mesh work, extraction yield, and good quality for instance bodily plus chemical. Additionally, it will increase microbiological security, sensory properties plus shelf-life associated with isinglass. A Design associated with Experiments (DoE) is recommended for long term scientific research, as the factorial testing framework plus Reply Area Strategy (RSM) is often made use of for pinpointing optimal set associated with variables together with evaluating person and together influences associated with these parameters (Box, Hunter, & Hunter, 2005; Shadish, Cook, & Campbell, 2002; Montgomery, 2019; Myers, Montgomery, & Anderson-Cook, 2016). Variables this sort such as extraction yield, proteins, peroxide value, TVB-N, color characteristics, microbial good quality, TBARS and also sensorial approval could then be assessed to determine optimum mixture regarding processing factors which may be utilized within a standardized, replicable

process suitable for commercial production of large quality isinglass. As stated earlier, although the Indian process has a lower start investment cost as well as lower facilities requirement, its considerably lower extraction yield may result in an increased cost per product produced. Additionally, it demands a far more significant processing time as well as can outcome in much higher product losses. Higher microbiological security, greater proteins retrieval and excellent final product are likely result in a more economical manufacturing of isinglass, even though greater gear and running costs, increased vitality consumption and pressing devices, are necessary for the semi-industrial process.

Future scientific studies ought to be centered not merely about process optimization and also techno-economic investigation, but should also be devoted to creating predictive models associated with top quality and degradation through storage. The obtained observations from samples of dry isinglass, over storage and analyzed for TVB-N, peroxide value, TBARS, aerobic plate counts and total yeasts and molds, recommend that the final products degrade considerably during time. These factors might thus be integrated in to future predictive shelf life designs for isinglass. Although this kind of research was designed mostly to finding possible process variations it could still generate useful data for the future establishment of forecasting quality models. The data obtained would make suitable bases for kinetic models if future research employ appropriate kinetic designs. The actual kinetic design could be put to use in assessing destruction rate and future predictions associated with shelf-life under varying circumstances associated with storing (Kutner, Nachtsheim, Neter, & Li, 2005).

Conclusion

Our results proved that the production method exerted significant effect on production yield, physicochemical and microbial qualities, physical parameters, oxidative stability and sensory acceptability of isinglass made from

sturgeon swim bladders during seven-month ambient storage. Compared with the Indian traditional extraction method, semi-industrial method consistently was offered significantly high production yield and protein content, lower moisture content, better microbial quality, reduced TVB-N and peroxide values, improved color stability, stronger texture and better sensory score throughout the entire storage. Hence, semi industrial method appeared to be more successful to maintain the quality integrity of isinglass during storage. These quality indices implied the semi industrial process was exhibited good storage stability for isinglass production with good product quality. This information could provide valuable scientific references for the

development and commercialization of sturgeon swim bladder based isinglass production.

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Author Contributions

Dr. Koochakian Sabour was responsible for the conceptualization and project management. **Dr. Seifzadeh** collaborated on project implementation, conducting experiments, and writing the article.

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مقاله پژوهشی

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تأثیر روش‌های استخراج هندی و نیمه‌صنعتی بر کیفیت و مقبولیت آیزین گلاس تولید شده از کیسه شنای فیل ماهی (*Huso huso*)، تاس ماهی ایرانی (*Acipenser persicus*) و ازون‌برون (*Acipenser stellatus*) پرورشی

مینا سیف زاده^{۱*} - انوشه کوچکیان صبور^۱

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چکیده

آیزین گلاس پروتئینی با ساختار ژل مانند است که از کلاژن استخراج می‌شود. این پروتئین از کیسه شنای ماهی‌های مختلف گرفته می‌شود، اما بیشتر از ماهیان خاویاری استخراج می‌شود. آیزینگلاس از سه نوع ماهی خاویاری که معمولاً در ایران پرورش داده می‌شوند. آیزین گلاس از ماهی‌های فیل ماهی، تاس ماهی ایرانی و ازون‌برون با استفاده از دو روش مختلف شامل هندی و نیمه‌صنعتی، استخراج شد. در این مطالعه، آیزینگلاس استخراج شده به مدت ۷ ماه در دمای اتاق نگهداری شد و از نظر ویژگی‌های فیزیکی، شیمیایی، میکروبیولوژیکی و ارگانولپتیکی مورد آزمایش قرار گرفت. درصد استخراج در روش نیمه صنعتی (۶۳-۶۷٪) بیشتر از روش هندی (>۲۴٪) بود. زمان استخراج نیز در این نمونه‌ها کوتاه‌تر بود (۳-۷ روز در مقابل ۸-۲۴ روز) ($P < 0.05$). روش‌های نیمه صنعتی حاوی ۷۴-۷۵٪ پروتئین بودند، در حالی که در روش هندی حدود ۶۴٪ بود. رطوبت، TVB-N و پراکسید در روش نیمه‌صنعتی در مقایسه با نمونه‌های هندی پس از ۷ ماه بهتر بودند. نمونه‌های نیمه‌صنعتی کمترین تعداد باکتری را داشتند و ارزیابی حسی آنها امتیاز پذیرش بالاتری را در مقایسه با نمونه‌های هندی نشان داد (بالای ۴ پس از ۷ ماه). با این حال، نمونه‌های هندی از نظر ارزیابی حسی قابل قبول نبودند. در حالی که نمونه‌های هندی ارزش غذایی بالاتر و مصرف انرژی کمتری داشتند، روش نیمه‌صنعتی بازده بالاتر، کیفیت شیمیایی و میکروبی بهتر و کیفیت حسی مطلوبی داشت. بنابراین، روش‌های نیمه‌صنعتی انتخاب بهتری برای تولید آیزینگلاس در مقیاس تجاری هستند.

واژه‌های کلیدی: آیزین گلاس، خواص فیزیکی شیمیایی، ماهیان خاویاری، روش هندی، روش نیمه‌صنعتی

۱- مرکز ملی تحقیقات فرآوری آبزیان، پژوهشکده آبی پروری آب‌های داخلی، مؤسسه تحقیقات علوم شیلاتی کشور، سازمان تحقیقات آموزش و ترویج کشاورزی، انزلی، ایران
(*- نویسنده مسئول: Email: M.seifzadeh@areeo.ac.ir)

Research Article

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Valorization of Dairy Sludge as a Low-Cost Substrate for Enhanced Biomass and Exopolysaccharide Production by *Enterococcus durans* K48

N. Taheri Nasab¹, M.R. Edalatian Dovom^{1*}, M. Moeenfar¹, L. Roozbeh Nasiraei²

1- Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

(*- Corresponding Author Email: edalatian@um.ac.ir)

2- Department of Food Science and Technology, No.C., Islamic Azad University, Nour, Iran

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Abstract

Dairy industry waste, particularly organic-rich dairy sludge, represents a promising and sustainable resource for biotechnological applications. This study evaluated the potential of dairy sludge as a cost-effective medium for the co-production of biomass and exopolysaccharides (EPS) by selected *Enterococcus* strains. The physicochemical parameters of the dairy sludge were characterized, revealing a composition of 32.70% fat, 29.32% protein, 9.56% ash, and 30.17% carbohydrates (which adjusted to 28.50% after spray-drying). Five strains of *Enterococcus* were screened through a quantitative culture-dependent approach to evaluate their growth and EPS production. Among the tested isolates, *Enterococcus durans* K48 exhibited the highest production potential and was selected for further study. Response Surface Methodology (RSM) was employed to optimize the concentrations of key medium components: dairy sludge, sucrose, and yeast extract. Under the optimized conditions (15% dairy sludge, 5% sucrose, and 2.5% yeast extract), a maximum biomass yield of 8,440 mg/L and an EPS yield of 394.2 mg/L were achieved. These findings demonstrate that dairy sludge can serve as a valuable source and sustainable substrate for *Enterococcus* cultivation, facilitating efficient biomass and EPS production while contributing to the valorization of dairy processing waste.

Keywords: Biomass, Dairy sludge, Lactic acid bacteria, Medium optimization, Valorization

Introduction

Dairy industries are among the main producers of organic waste within the food processing sector. The sludge generated from their wastewater treatment systems possesses considerable biotechnological potential due to its high organic load and its content of lactose, proteins, and micronutrients (Shete & Shinkar, 2013; Ryan & Walsh, 2016). One of the primary components of these effluents is dairy sludge, originating from processes such as separation and bacto-fugation (McCarthy, 2011). Effective management and value-added

utilization of this waste stream could not only reduce the environmental impacts associated with disposal, but also facilitate the recovery of high-value biological compounds (Shete & Shinkar, 2013). In recent years, the use of dairy sludge as a fermentation medium for producing bioactive compounds has gained growing interest, however, its efficiency as a targeted substrate for exopolysaccharide (EPS) production remains unexplored.

EPS are high-molecular-weight polymers secreted by microorganisms into their surrounding environment (Wang *et al.*, 2023).



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Owing to their rheological, functional, and bioactive properties, EPS hold significant relevance for food, pharmaceutical, and biotechnological industries (Naykodi, Doriya, & Thorat, 2025). Although conventional EPS production commonly depends on costly culture media, recent strategies focus on utilizing low-cost raw materials and food wastes—such as cheese whey, molasses, and agricultural by-products—to improve economic feasibility (Sakr, Massoud, & Ragaei, 2021). Lactic acid bacteria (LAB) are widely recognized for their EPS-producing potential (Surayot *et al.*, 2014). In particular, the genus *Enterococcus*, which naturally inhabits dairy environments, exhibits promising EPS synthesis capabilities with diverse structural and functional characteristics (Gaur & Annapure, 2025).

Despite these advantages, limited information is available regarding the performance of *Enterococcus* strains in unconventional, nutrient-rich substrates such as dairy sludge. Moreover, the relationship between sludge composition, strain metabolism, and EPS productivity remains poorly understood. This gap highlights the need for systematic investigation into the suitability of dairy sludge as a fermentation medium for EPS-producing *Enterococcus* strains.

To Address this research gap, the present study aimed to investigate the feasibility of utilizing dairy sludge as a sustainable and low-cost substrate for microbial biomass and exopolysaccharide (EPS) production. Five *Enterococcus* strains isolated from traditional dairy products were screened for their biomass-forming ability and EPS production, and the most promising strain (*Enterococcus durans* K48) was selected for further investigation. Biomass production was subsequently optimized using Response Surface Methodology (RSM). The novelty of this study lies in demonstrating the potential of dairy sludge, an underutilized dairy by-product, as an alternative medium for microbial cultivation, thereby contributing to waste valorization, reducing production costs, and promoting a

more sustainable bioprocess without relying on conventional culture media.

Material and Methods

Bacterial Strains and Activation

Five *Enterococcus* strains (*Enterococcus durans* K48, *Enterococcus faecium* R114, *Enterococcus faecium* T52, *Enterococcus faecium* NB4, and *Enterococcus durans* ND1) previously isolated from traditional Iranian dairy products, were used in this study. Taxonomic identification had been confirmed by molecular assays and 16S rRNA gene sequencing. Stock cultures were maintained in MRS broth (MIRMEDIA, Iran) supplemented with 30% (v/v) glycerol and stored at -80°C . Prior to experiments, each strain was activated in fresh MRS broth at 37°C for 24 h to obtain metabolically active cultures (Oleksy-Sobczak, Klewicka, & Piekarska-Radzik, 2020; Rahnama Vosough, Edalatian Dovom, Habibi Najafi, Javadmanesh, & Mayo, 2022).

Screening of EPS-Producing Strains

The five strains were screened for biomass formation and EPS production. Biomass formation was determined by measuring the dry cell weight (DCW). Following cultivation, the fermentation broth was centrifuged at 4,000 rpm for 10 min. The supernatant was discarded, and the cell pellet was washed twice with distilled water to remove residual medium components. The washed biomass was dried at 80°C until a constant weight was obtained. Biomass production was expressed as dry cell weight (mg/L) (Choi, Lee, & Paik, 2021). EPS extraction was performed as described by Kavutake *et al.* (2024) with slight modifications. Briefly, the strains were incubated in MRS broth at 37°C for 48 h, and the cell biomass was harvested by centrifugation at 4,000 rpm for 10 min. To precipitate proteins, the cell-free supernatant was treated with trichloroacetic acid (TCA) to a final concentration of 10% (w/v) and incubated at 4°C for 12 h. The precipitated proteins were removed by centrifugation (4,000 rpm, 15 min, 4°C), and the EPS in the supernatant was subsequently

precipitated by adding three volumes of ice-cold 96% (v/v) ethanol, followed by incubation at 4 °C for 24 h. The EPS precipitate was collected by centrifugation (4,000 rpm, 15 min, 4 °C), dried to a constant weight in a desiccator, and weighed to determine the EPS yield (Hu *et al.*, 2021; Kavitake *et al.*, 2024).

Dairy Sludge Source and Characterization

Dairy separator sludge was obtained from a renowned dairy processing plant in Khorasan Razavi, Iran. The raw sludge had an initial pH of 5.5 and a total solids content of 14% (w/v). Chemical characterization was conducted to assess its suitability as a nutrient-rich substrate for microbial cultivation.

Chemical Composition Analysis

Crude protein

Protein content was determined using the Kjeldahl method according to the Iranian National Standard No. 9188-1. Samples were digested with concentrated sulfuric acid in the presence of a catalyst to convert organic nitrogen to ammonium ions. The released ammonia was distilled, absorbed in boric acid, and titrated against standardized hydrochloric acid. A nitrogen-to-protein conversion factor of 6.38 was applied (Iranian National Standardization Organization, 2015).

Fat Content

Fat content was measured using the butyrometric (Gerber) method following Iranian National Standard No. 23626. Samples were mixed with sulfuric acid and amyl alcohol in a butyrometer, centrifuged, and incubated at 65 °C. The fat percentage was determined by reading the height of the fat column on the calibrated scale (Iranian National Standardization Organization, 2024).

Ash Content

The Ash content was determined by incineration. Samples were dried at 105 °C to constant weight and then combusted in a muffle furnace at 550 °C for 4–6 h. After cooling, ash percentage was calculated according to Eq. (1) (Quirino, Palma, Franco, & Detmann, 2023).

$$\text{Ash Content} = \frac{\text{Weight of Ash}}{\text{Original Dry Sample Weight}} \times 100 \quad (1)$$

Total Carbohydrates

Total carbohydrate concentration was determined using the phenol–sulfuric acid method. The samples were reacted with phenol and sulfuric acid, heated in a boiling water bath, cooled, and the absorbance was measured at 490 nm using a UV–Vis spectrophotometer (Unico, Iran). The concentration was calculated against a glucose standard curve (Gao *et al.*, 2022).

Preparation of Dairy Sludge Fermentation Medium

Dairy sludge (pH 5.5, 14% total solids) was used as the basal substrate for bacterial growth. To prepare the dairy sludge as growth medium, two different approaches were employed depending on the experimental purpose. For the RSM experiments, spray-dried dairy sludge was used to ensure substrate homogeneity, facilitate the accurate preparation of defined concentrations, and improve the reproducibility required for statistical optimization. The sludge was pasteurized (72 °C, 30 min) and spray-dried (Buchi, Switzerland) to obtain a powder (Falah, Mortazavi, Danesh, Yazdi, & Ramezani, 2024). The resulting powder was reconstituted in distilled water at 5%, 10%, and 15% (w/v). The medium was supplemented with sucrose, yeast extract, 0.02% (w/v) MnSO₄, and 0.2% (w/v) KH₂PO₄, and sterilized at 121 °C for 15 min (Esmaeilnejad-Moghadam, Mokarram, Hejazi, Khiabani, & Keivaninahr, 2019).

In contrast, liquid dairy sludge was used for the scale-up experiment to better simulate industrial processing conditions while avoiding the additional energy consumption and cost associated with spray drying. For scale-up experiments, the pH was adjusted to 6.4 using 1 N HCl. Caseins were removed via heat treatment (95 °C, 15 min) followed by centrifugation and filtration (Che *et al.*, 2025). The clarified supernatant was supplemented with calcium carbonate (CaCO₃) to enhance

buffering capacity during fermentation and then sterilized (Fusi, Primicerio, & Monti, 2015).

Growth Assessment of *E. durans* K48

The growth kinetics of the selected strain were evaluated in both MRS broth and the formulated dairy sludge medium supplemented with sucrose and yeast extract. Cultures were incubated at 37 °C for 24 h in a shaker incubator at 250 rpm. To monitor the growth curve, samples were collected at hourly intervals, and the optical density (OD 600) was measured using a UV–Vis spectrophotometer. To account for the inherent turbidity of the dairy sludge medium, samples were appropriately diluted with distilled water prior to spectrophotometric analysis to ensure the absorbance remained within the linear range (Esmaeilnejad-Moghadam *et al.*, 2019).

Optimization of Biomass Production

Response Surface Methodology (RSM) was employed to optimize biomass production using Design-Expert software (version 13). A Central Composite Design (CCD) was

employed to evaluate the effects of three independent variables, including (A) dairy sludge concentration, (B) sucrose concentration, and (C) yeast extract concentration. Each variable was evaluated at three distinct levels, as shown in Table 1. Six center-point replicates were included to estimate experimental error. The experimental data were fitted to a second-order polynomial model, expressed as Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j \quad (2)$$

where Y represents biomass production, β_0 is the intercept, β_i and β_{ii} are linear and quadratic coefficients, respectively, and β_{ij} represents interaction coefficients (Ahmed *et al.*, 2023).

To validate the predictive capability of the developed RSM model, an independent confirmation experiment was performed under the predicted optimal conditions. Three independent experiments were conducted under these optimized conditions, and the mean experimental biomass yield was compared with the value predicted by the model.

Table 1- Levels of independent process variables and their values

Independent variable	Symbol	Unit (w/v)	High Level (+1)	Medium Level (0)	Low Level (-1)
Dairy sludge concentration	A	%	15	10	5
Sucrose concentration	B	%	5	3	1
Yeast extract concentration	C	%	2.5	1.5	0.5

EPS Production, Extraction, and Purification

EPS production was carried out in the optimized dairy sludge medium using a modified procedure adapted from Kavitate *et al.* (2024). The medium was inoculated with a 3% (v/v) active culture of *E. durans* K48 (adjusted to an optical density of OD₆₀₀=1.0) and incubated at 37°C for 48 h. Post-incubation, the bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min at 4°C.

To precipitate the proteins, trichloroacetic acid (TCA) was added to the cell-free supernatant to achieve a final concentration of 10% (w/v), and the mixture was incubated at 4°C for 12 h. The precipitated proteins were

removed by centrifugation at 4,000 rpm for 20 min at 4 °C. Subsequently, the crude EPS was precipitated from the clarified supernatant by adding three volumes of ice-cold 96% (v/v) ethanol, followed by incubation at 4 °C for 24 h.

The resulting EPS precipitate was collected by centrifugation (4,000 rpm, 20 min, 4°C), dissolved in deionized water, washed twice with ethanol, and dialyzed against deionized water for 48 h at 4°C using a 10 kDa molecular weight cut-off (MWCO) dialysis membrane (with regular water changes every 12 h). Finally, the dialyzed EPS solution was pre-frozen at –80 °C and lyophilized using a freeze-

dryer for 48 h. The purified EPS was weighed to determine the yield and stored at -20°C for further analysis (Kavitake *et al.*, 2024; Wu, Wu, Pan, Luo, & Zhong, 2024).

Statistical Analysis

Experimental design, model fitting, and response surface plots were conducted using Design-Expert software (version 13.0.1.0). Analysis of variance (ANOVA) was performed to evaluate the statistical significance of the fitted regression model and the linear, quadratic, and interaction effects of the independent variables on biomass production. Statistical significance was considered at $p \leq 0.05$, with highly significant effects indicated at $p \leq 0.001$. Additional statistical analyses were performed using SPSS software (version 27). All experiments were performed in triplicate, and the mean values were used for statistical analysis.

Results and Discussion

Screening of EPS-Producing Strains and Selection of the Superior Strain

Simultaneous assessment of biomass formation and EPS production enabled clear discrimination among the screened *Enterococcus* strains. Biomass measurements revealed statistically significant variation ($p \leq 0.001$), allowing classification of the strains into distinct homogeneous groups. Among the isolates, strain K48 exhibited the highest biomass concentration (approximately 2.1 g/L), followed by R114 (1.7 g/L), NB4 (1.5 g/L), ND1 (0.9 g/L), and T52 (0.7 g/L). The superior growth of K48 indicates a greater physiological tolerance to the fermentation environment and

implies more efficient substrate uptake and energy metabolism, which are commonly associated with enhanced polysaccharide biosynthesis (Netrusov *et al.*, 2023). As illustrated in Table 2, EPS production followed a growth-associated pattern, consistent with the biomass trends. EPS yields among the tested strains ranged from 0.12 to 0.40 g/L, with K48 achieving the maximum titer of 0.40 g/L (denoted by the letter ‘a’ in Table 2). This elevated productivity may be attributed to inherent genetic variations in the regulation of glycosyltransferases and precursor-sugar biosynthetic pathways (Cui, Jiang, Hao, Qu, & Hu, 2017). Similar EPS production levels (approximately 0.40–0.50 g/L) have been reported for *Enterococcus* strains cultivated in carbohydrate-rich media (Kowsalya *et al.*, 2023), supporting the reliability of the present results. The lower performance of strains such as T52 and ND1 may be attributed to reduced expression of EPS operons or less efficient carbon-source metabolism. Such strain-specific metabolic limitations have been previously reported in LAB, where energy allocation between growth and exopolysaccharide formation varies among species and isolates (Liang, Wang, Li, & Liu, 2024). From a bioprocessing perspective, selecting a strain capable of achieving both high biomass and EPS productivity is crucial for cost-effective fermentation, particularly when low-value substrates such as dairy sludge are used. The markedly superior performance of *E. durans* K48 supports its selection as the most promising candidate for subsequent optimization studies and scale-up development.

Table 2- Comparison of biomass concentration and EPS production among the screened *Enterococcus* strains (R114, T52, K48, ND1, and NB4)

Bacterial strains	EPS production (g/L)	Biomass content
R114	0.226 ± 0.028^c	1.702 ± 0.050^b
T52	0.112 ± 0.006^e	0.773 ± 0.018^e
K48	0.401 ± 0.017^a	2.108 ± 0.016^a
ND1	0.158 ± 0.021^d	0.978 ± 0.010^d
NB4	0.298 ± 0.005^b	1.501 ± 0.004^c

Different letters above columns with the same color denote statistically significant differences among strains ($p < 0.05$).

Chemical Properties of Dairy Sludge

The chemical profile of dairy sludge—comprising protein, ash, fat, and total carbohydrates—is presented in Table 3. The sludge contained $29.32 \pm 0.42\%$ protein (dry basis), indicating a substantial concentration of nitrogenous compounds derived from residual milk proteins such as casein and whey (Prazeres, Carvalho, & Rivas, 2012). The ash content was $9.56 \pm 0.73\%$, reflecting a rich mineral profile (including calcium, phosphorus, and magnesium) essential for supporting microbial metabolism (Kwapinska, Pisano, & Leahy, 2023).

Notably, the fat content reached $32.73 \pm 0.52\%$, likely due to incomplete fat separation during industrial processing and the subsequent accumulation of fat globules in the sludge (Ganta, Bashir, & Das, 2022). Regarding the carbon source, total carbohydrate content exhibited a slight but statistically significant decrease ($p < 0.05$) from $30.17 \pm 0.93\%$ in the raw sludge to $28.29 \pm 0.50\%$

after spray-drying. This reduction is potentially attributable to the partial thermal degradation or Maillard reactions involving lactose and other reducing sugars during the drying process (Ziaee, O'Connor, Murphy, & O'Reilly, 2023). Nevertheless, the high residual carbohydrate levels confirm the suitability of dried dairy sludge as a valuable carbon source for *Enterococcus* cultivation.

These results are generally consistent with existing literature, although some variations exist. For instance, McCarthy (2011) reported that dairy sludge (dry basis) typically contains 40–53% nitrogenous compounds and approximately 31.3% lactose, but significantly lower fat levels (1.7–2.3%). The observed discrepancies in fat and protein concentrations compared to previous studies may be attributed to differences in milk origin, specific dairy processing techniques (e.g., cheese vs. yogurt production), and seasonal variations in sludge sampling.

Table 3- Chemical composition of the dairy sludge

Sample	Protein (%)	Ash (%)	Fat (%)	Total carbohydrate (%) before spray drying	Total carbohydrate (%) after spray drying
Dairy sludge	29.32 ± 0.42	9.56 ± 0.73	32.7 ± 0.52	30.17 ± 0.93	28.50 ± 0.50

Comparative Growth of *E. durans* K48 in Dairy Sludge and MRS Media

The growth profiles of *E. durans* K48 cultivated in MRS broth and dairy sludge medium over a 24 h fermentation period are shown in Fig. 1. Both media supported a characteristic sigmoidal growth pattern; however, distinct differences were observed in their kinetic parameters.

A prolonged lag phase was observed in the dairy sludge medium, with exponential growth initiated after approximately 6–7 h. In contrast, cells grown in MRS broth adapted more rapidly, entering the exponential phase by hour 3. This initial delay in dairy sludge can be attributed to the time required for the bacterial

cells to synthesize extracellular enzymes needed to metabolize complex substrates (such as residual casein and lactose) compared to the readily bioavailable nutrients in MRS (Esmaeilnejad-Moghadam *et al.*, 2019).

Interestingly, following the adaptation period, *E. durans* K48 exhibited a rapid acceleration in cell density within the dairy sludge medium (between 8 and 14 h), reaching a plateau ($OD_{600} \approx 1.60$ – 1.65) by hour 14. Conversely, growth in MRS broth progressed at a steady rate, attaining a higher maximum cell density ($OD_{600} \approx 1.90$) at hour 24. The earlier onset of the stationary phase in dairy sludge may result from the depletion of specific micronutrients or the accumulation of organic

acids during fermentation. Nevertheless, achieving nearly 85% of the total biomass produced in refined MRS broth confirms that

dairy sludge is a highly effective, low-cost nutrient substrate for *E. durans* cultivation and subsequent EPS production.

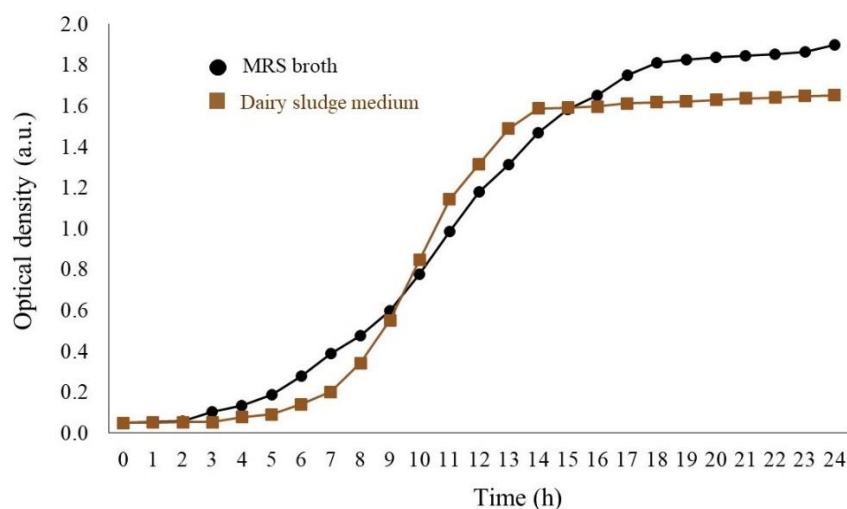


Fig. 1. Comparative growth curves of *E. durans* K48 in MRS broth and dairy sludge medium during 24 h of incubation

Optimization of Biomass Production Using Response Surface Methodology

A central composite design (CCD) comprising 21 experimental runs was applied to evaluate the effects of (A) dairy sludge concentration, (B) sucrose concentration, and (C) yeast extract concentration, on biomass production by *E. durans* K48. The experimental design matrix, including the actual concentrations of the independent variables and the corresponding experimental and predicted biomass yields for all 21 runs, is presented in Table 4. The experimental responses were fitted to a second-order polynomial model (Eq. 3), which is commonly used to describe nonlinear interactions among process variables:

$$Y = 7713.43 - 18.99A + 53.58B + 504.95C + 49.63AB + 72.70AC + 74.99BC + 396.43A^2 + 22.71B^2 - 391.87C^2 \quad (3)$$

Analysis of variance (ANOVA) indicated that the regression model was highly significant ($F = 39.61$, $p < 0.0001$), demonstrating a strong relationship between the tested variables and biomass production. ANOVA showed that the model was highly significant, and several linear, interaction, and quadratic terms

significantly affected biomass production ($p \leq 0.0059$), indicating that variations in dairy sludge, sucrose, and yeast extract concentrations markedly influenced cell growth. The model exhibited a high coefficient of determination ($R^2 = 0.9701$), with an adjusted R^2 of 0.9456, indicating a good fit between the model and the experimental data. The coefficient of variation (CV) was low (1.48%), suggesting good precision and reliability of the experimental results. The predicted R^2 was 0.3142, while the adequate precision was 21.1384, exceeding the desirable value of 4 and indicating an adequate signal-to-noise ratio for the model. The positive coefficients for sucrose and yeast extract indicate that biomass formation was enhanced by increasing the availability of easily metabolizable carbon and nitrogen sources. Although the interaction coefficients (AB, AC, and BC) were positive, their corresponding effects were not statistically significant ($p > 0.05$); therefore, no significant synergistic interaction among the tested variables could be established. The lack-of-fit test was not significant ($p = 0.0864$), confirming that the

developed model adequately represented the experimental data within the studied range.

Table 4- Central composite design matrix for biomass production by *E. durans* K48, including actual and predicted values

Run	A: Dairy sludge concentration (%)	B: Sucrose concentration (%)	C: Yeast extract concentration (%)	Bio Mass (mg/L)	Predicted Value (mg/L)
1	10	3	1.5	7590	7713.43
2	10	3	1.5	7660	7713.43
3	5	5	2.5	8290	8270.87
4	15	1	2.5	8100	8121.17
5	10	3	1.5	7580	7713.43
6	15	5	2.5	8440	8477.57
7	15	1	0.5	7150	7115.83
8	5	5	0.5	7240	7256.40
9	15	5	0.5	7110	7172.28
10	10	3	1.5	7780	7713.43
11	5	3	1.5	8230	8128.85
12	10	3	2.5	7970	7826.51
13	10	3	1.5	7610	7713.43
14	15	5	0.5	7210	7172.28
15	5	1	2.5	8100	8113.01
16	10	3	0.5	6730	6816.60
17	10	5	1.5	7940	7789.72
18	10	3	0.5	6910	6816.60
19	5	5	2.5	8180	8270.87
20	15	3	1.5	8140	8090.87
21	10	3	1.5	7760	7713.43

Main and Interactive Effects of Process Variables on Biomass Yield

Fig. 2 presents the effects of dairy sludge concentration (A), sucrose concentration (B), and yeast extract concentration (C) on biomass production by *E. durans* K48, as illustrated by three-dimensional response surface plots. The plots reveal that biomass production was influenced not only by the individual levels of the variables but also by their combined effects.

As shown in Fig. 2a, the combined effect of dairy sludge and yeast extract markedly influenced biomass formation. Increasing yeast extract concentration enhanced biomass production up to an optimum level, confirming the critical role of nitrogen availability in supporting bacterial growth. However, further increases in yeast extract resulted in a slight decline in the response, which is consistent with

the negative quadratic coefficient of C^2 in the fitted model and suggests the presence of an optimum concentration range.

Fig. 2b illustrates the interaction between dairy sludge and sucrose concentrations. Biomass production increased when both variables were maintained at moderate-to-high levels, indicating a favorable combined effect between the complex nutrients supplied by dairy sludge and the readily metabolizable carbon source provided by sucrose (Ashekuzzaman, Forrestal, Richards, & Fenton, 2019; Kwapinska *et al.*, 2023). However, increasing sucrose concentration beyond the optimal range did not result in a substantial additional improvement in biomass yield.

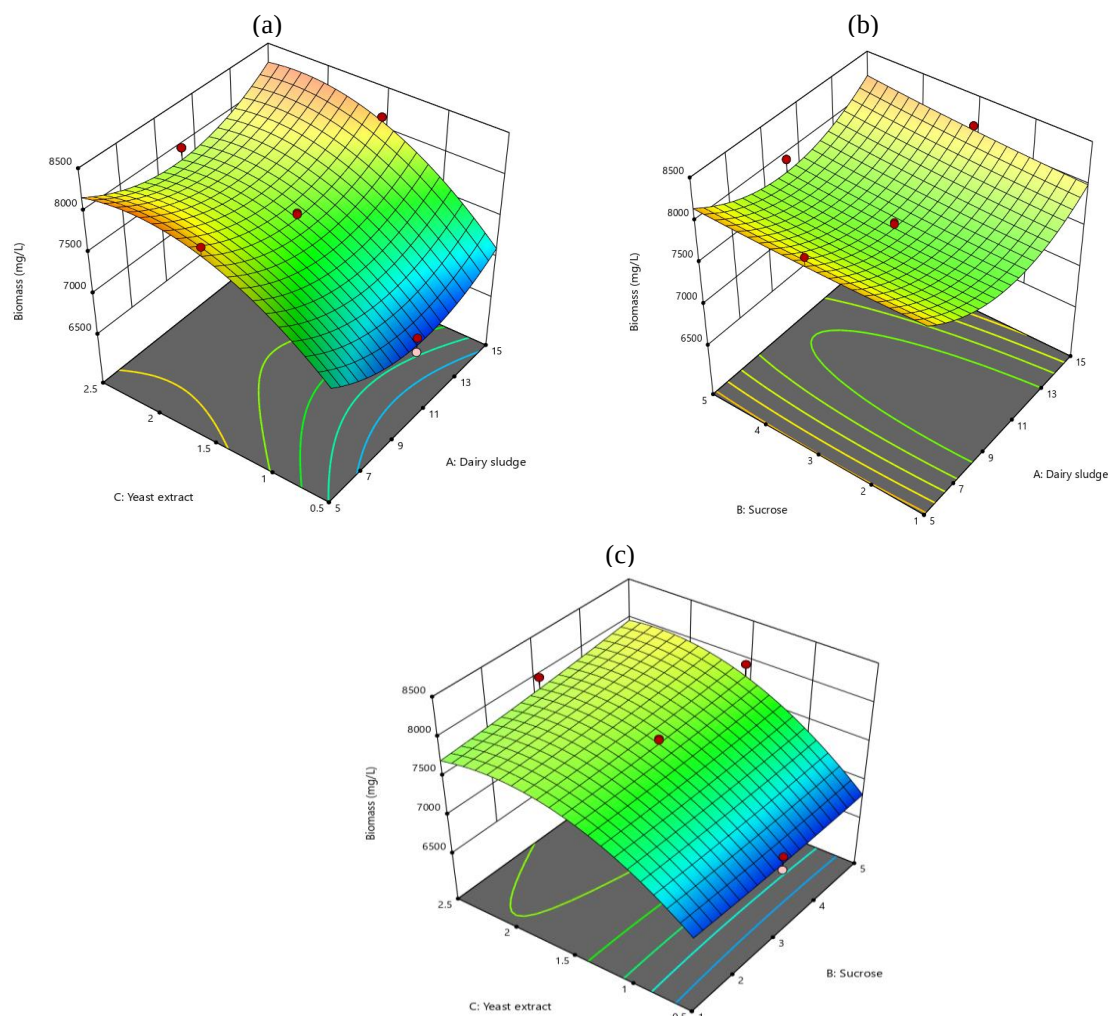


Fig. 2. Three-dimensional response surface plots showing the interactive effects of (a) dairy sludge concentration and yeast extract concentration, (b) dairy sludge concentration and sucrose concentration, and (c) sucrose concentration and yeast extract concentration on biomass production by *E. durans* K48

As presented in Fig. 2c, biomass production was maximized when sucrose and yeast extract concentrations were properly balanced, emphasizing the importance of coordinated carbon and nitrogen supplementation. Deviations toward either insufficient or excessive levels of these components adversely affected cell growth (Esmailnejad-Moghadam *et al.*, 2019). Overall, the curved response surfaces and the elliptical nature of the contour plots suggest the presence of interaction effects among the investigated variables. These

findings indicate that biomass production is governed by the combined influence of medium components, and therefore, optimization should be performed simultaneously rather than by varying one factor at a time.

The stimulatory effect of dairy sludge on biomass production can be attributed to its complex nutritional composition. Dairy sludge contains residual lactose, proteins, peptides, lipids, and minerals derived from dairy processing effluents (McCarthy, 2011). These constituents may serve as carbon and nitrogen

sources as well as growth-promoting factors for lactic acid bacteria. Fermentable carbohydrates such as lactose provide energy for metabolism, whereas proteins and peptides contribute amino acids required for biosynthesis. In addition, minerals and trace elements may enhance enzymatic activity and overall metabolic efficiency. Therefore, incorporation of dairy sludge into the culture medium creates a nutritionally enriched environment that supports microbial proliferation. Similar observations have been reported for other dairy by-products, such as whey, which have been successfully used as low-cost medium for microbial biomass production (Esmaeilnejad-Moghadam *et al.*, 2019). Collectively, these results highlight the potential of dairy sludge as an economical medium and support its valorization as a sustainable resource in microbial bioprocessing.

Determination of Optimal Conditions and Model Validation

Biomass yield varied from 6730 to 8440 mg/L across the CCD experiments. The maximum biomass concentration (8440 mg/L) was obtained at A = 15%, B = 5%, and C = 2.5%. This combination likely provided a favorable balance of carbon, nitrogen, and complex nutrients from dairy sludge, and create favorable conditions for efficient cell proliferation. To verify the accuracy of the developed quadratic model, predicted values were compared with experimental data. The scatter plot presented in Fig. 3 shows that most data points were closely distributed along the

line of equality, indicating a strong agreement between the model-predicted and experimentally observed biomass yields. The absence of major deviations or outliers further confirms the robustness, reliability, and predictive capability of the RSM model for optimizing biomass production in dairy-sludge-based media.

The predicted optimal conditions were experimentally validated through three independent confirmation experiments. The experimental results were in close agreement with the model predictions, confirming the predictive capability and reliability of the developed RSM model.

Exopolysaccharide Production, Extraction, and Purification

Following optimization of the fermentation parameters, EPS production was evaluated under the identified optimal conditions (dairy sludge 15%, sucrose 5%, and yeast extract 2.5%). Under these conditions, *E. durans* K48 yielded approximately 394.2 mg/L of EPS. After extraction and lyophilization, the recovered EPS appeared as a fine white powder with a fibrous, cotton-like texture, which is typical characteristic of highly polymerized microbial polysaccharides. The EPS yield obtained in this study was comparable to several previously reported values. For instance, Jia *et al.* (2019) reported an EPS production of 130 mg/L using *E. faecium* WEFA23, while Zanzan *et al.* (2023) achieved 382.27 mg/L by *E. mundtii* A2, under their optimized fermentation conditions.

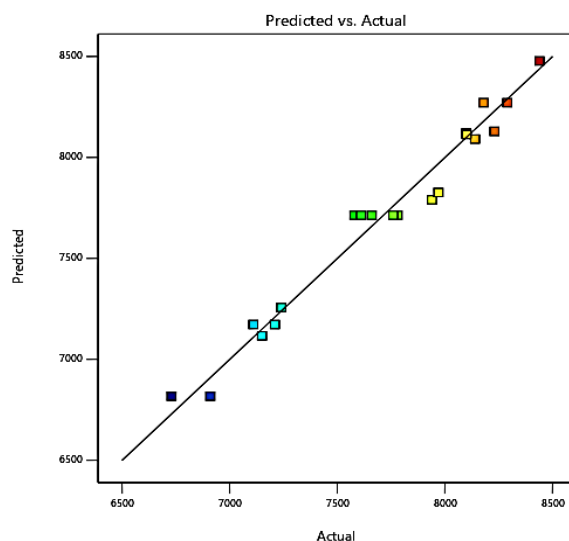


Fig. 3. Comparison between predicted and experimental values of biomass production based on the developed regression model

The relatively high yield observed in the present work may be attributed to the combined effect of the selected strain and the optimized nutritional composition of the culture medium determined, which was established using response surface methodology. These findings indicate that *E. durans* K48 is a promising candidate for efficient EPS production. Furthermore, the use of dairy sludge as a fermentation substrate highlights the potential of this process as a cost-effective and sustainable approach for microbial polysaccharide production with possible industrial applications.

While the obtained EPS yield and the use of dairy sludge as a low-cost substrate are promising, further investigations are required to fully assess the potential of the produced EPS and the strain itself for future applications. Comprehensive structural and functional characterization of the EPS, including analyses of monosaccharide composition, molecular weight distribution, rheological behavior, and other physicochemical and functional properties, was beyond the scope of the present study and is being addressed in a separate follow-up investigation focused on EPS characterization. In parallel, the potential application of the strain itself requires further consideration. The primary objective of the

present study was to evaluate the feasibility of dairy sludge as a sustainable and low-cost fermentation substrate for microbial biomass and EPS production, rather than to assess the suitability of *E. durans* K48 for direct food or probiotic applications. Therefore, further safety assessments, including genomic analysis for the detection of potential virulence factors and antibiotic resistance determinants, are needed before considering this strain for such applications (Farkhondeh, Yavarmansh, Tabatabaei Yazdi, & Edalatiane, 2023; Zanzan *et al.*, 2023).

Conclusion

This study demonstrated the feasibility of utilizing dairy sludge as a sustainable, nutrient-rich medium for microbial biomass and exopolysaccharide (EPS) production. Among the screened isolates, *E. durans* K48 exhibited the highest growth performance and EPS-producing capacity in the dairy-sludge-based medium. Response surface methodology (RSM) identified the optimal cultivation conditions as 15% dairy sludge, 5% sucrose, and 2.5% yeast extract, under which the maximum biomass and EPS yields reached 8440 mg/L and 394.2 mg/L, respectively. These results highlight dairy sludge as a valuable, low-cost resource capable of supporting

efficient microbial metabolism, thereby demonstrating the potential of this optimized bioprocess for the cost-effective valorization of dairy industry waste. The robust performance of *E. durans* K48 further underscores its applicability in the food, pharmaceutical, and biotechnology-related sectors. To facilitate industrial implementation, future research should focus on process scale-up, detailed structural and functional characterization of the recovered EPS, and a comprehensive techno-economic assessment of the integrated bioprocess.

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Author Contributions

Nazanin Taheri Nasab: Conceptualization, Methodology, Software, Investigation, Formal analysis, Data Curation, Writing–Original Draft, Visualization. **Mohammad Reza Edalatian Dovom:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing–Review & Editing. **Marzieh Moeenfar:** Conceptualization, Validation, Supervision, Writing–Review & Editing. **Leila Rouzbeh Nasiraei:** Conceptualization, Validation, Supervision, Writing–Review & Editing.

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ارزش آفرینی از لجن لبنی به عنوان بستری کم هزینه برای افزایش تولید زیست توده و اگزوپلی ساکارید توسط *انتروکوکوس دورانس* K48

نازنین طاهری نسب^۱ - محمد رضا عدالتیان دوم^{۱*} - مرضیه معین فرد^۱ - لیلا روزبه نصیرایی^۲

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چکیده

پساب صنایع لبنی، به ویژه لجن لبنی غنی از مواد آلی، یک منبع امیدبخش و پایدار برای کاربردهای بیوتکنولوژیک به شمار می رود. این پژوهش، پتانسیل لجن لبنی را به عنوان یک محیط کشت مقرون به صرفه برای تولید همزمان زیست توده و اگزوپلی ساکاریدها (EPS) توسط سویه های منتخب *انتروکوکوس* ارزیابی کرد. ابتدا پارامترهای فیزیکوشیمیایی لجن لبنی مشخص شد که ترکیبی از ۲۲/۷۰٪ چربی، ۲۹/۳۲٪ پروتئین، ۹/۵۶٪ خاکستر و ۳۰/۱۷٪ کربوهیدرات (که پس از فرآیند خشک کردن به ۲۸/۵۰٪ تنظیم شد) را نشان داد. پنج سویه از طریق یک رویکرد کمی وابسته به کشت غربالگری شدند تا رشد و تولید EPS آنها ارزیابی شود. در میان جدایه های آزمایش شده، *انتروکوکوس دورانس* K48 بالاترین پتانسیل تولید را نشان داد و برای مطالعه بیشتر انتخاب شد. از روش سطح پاسخ (RSM) برای بهینه سازی غلظت اجزای کلیدی محیط کشت: لجن لبنی، ساکارز و عصاره مخمر استفاده شد. تحت شرایط بهینه (۱۵٪ لجن لبنی، ۵٪ ساکارز و ۲/۵٪ عصاره مخمر)، حداکثر بازده زیست توده برابر با ۸۴۴۰ میلی گرم بر لیتر و بازده EPS برابر با ۳۹۴/۲ میلی گرم بر لیتر به دست آمد. این یافته ها نشان می دهند که لجن لبنی می تواند به عنوان بستری مناسب و پایدار برای کشت *انتروکوکوس* مورد استفاده قرار گیرد و ضمن فراهم کردن امکان تولید کارآمد زیست توده و EPS، به ارزش آفرینی از پسماندهای حاصل از فراوری صنایع لبنی نیز کمک کند.

واژه های کلیدی: ارزش آفرینی، باکتری های اسیدلاکتیک، بهینه سازی محیط کشت، زیست توده، لجن لبنی

۱- گروه علوم و مهندسی صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران
(*)- نویسنده مسدول: edalatian@um.ac.ir (Email:)

۲- گروه علوم و صنایع غذایی، دانشگاه آزاد اسلامی واحد نور، نور، ایران

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Isolation, Purification and Characterization of Proteins and Peptides from Calabash (*Crescentia cujete* L.) Fruit Flesh with Antihypertensive and Lipid-Lowering Properties

J. C. Villanueva^{1*}, A. C. Sajise², A. C. Laurena³, M. O. Torio⁴, V. P. Migo⁵

1- Philippine Genome Center Program for Agriculture, Livestock, Fisheries and Forestry, Office of the Vice Chancellor for Research and Extension, University of the Philippines Los Baños, College, Laguna, Philippines

(*- Corresponding Author Email: jcvillanueva3@up.edu.ph)

2- T. T. Chang Genetic Resources Center, International Rice Research Institute, Los Baños, Laguna, Philippines

3- Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, Philippines

4- Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna, Philippines

5- Department of Chemical Engineering, College of Engineering and Agro-Industrial Technology, University of the Philippines Los Baños, College, Laguna, Philippines

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Abstract

Calabash (*Crescentia cujete* L.) fruit has increasingly been studied in recent years for its significant contribution to human health and well-being. The reported bioactivities are linked to the presence of phytochemicals such as polyphenols, but little is known about the role of their proteins. This represents a significant gap, as fruits are generally overlooked as sources of proteins compared to legumes and other proteinaceous products, despite their potential functional and therapeutic applications. This study aimed to isolate, purify, and characterize proteins and peptides from calabash fruits, and evaluate their potential antihypertensive and lipid-lowering activities. Proteins were extracted, purified, and characterized through a combination of biochemical techniques such as sodium phosphate buffer extraction, ammonium sulfate precipitation, gel-filtration chromatography, and *in vitro* biological assays namely pancreatic lipase inhibition, cholesterol micellar solubility inhibition, and angiotensin-converting enzyme (ACE) inhibition assays. Crude protein isolate (CPI) had a protein concentration of 688 µg/mL with observed protein bands at 12.1 kDa to 46.7 kDa. After simulated gastrointestinal digestion, CPI showed average inhibition activities of 76.5% for ACE, 22.3% for pancreatic lipase, and 84.0% for cholesterol micellar solubility, while the 2-h digest of the purified protein fraction showed 73.4%, 44.9%, and 82.2% inhibition, respectively. Proteins and peptides exhibited strong bioactivity, with inhibition responses across all assays approaching those of the assay controls under the tested conditions. This is the first study to demonstrate the bioactive potential of proteins and peptides extracted from *C. cujete* fruit, providing promising insights into their possible functional properties associated with the preventive management of obesity, cholesterol levels, and hypertension. Further peptide fractionation and sequence identification are needed to confirm the most active components.

Keywords: Antihypertensive, Bioactive peptides, Calabash, Cholesterol micellar, Functional food



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Introduction

Functional foods contain bioactive compounds that provide physiological and health benefits beyond nutrition, and the emergence of these foods has led people around the world to gradually transition to a diet rich in proteins, essential oils, vitamins, polyphenols, and carotenoids (Angeles *et al.*, 2021). Among the major bioactive constituents of functional foods, proteins and peptides have attracted considerable attention because of their diverse physiological activities. Proteins play a fundamental role in providing the nutrients for human diet derived from foods. Proteins derived from functional foods may exhibit various health benefits, including regulation of blood pressure, cholesterol levels, and blood sugar.

This growing interest in functional foods is largely associated with the increasing prevalence of chronic non-communicable diseases, particularly cardiovascular diseases (CVD). Two major modifiable risk factors of CVD are hypertension and dyslipidemia. Hypertension affected around 1.3 billion adults in 2023, while dyslipidemia accounts for approximately 4 million CVD-related deaths worldwide (Zhao, 2021; World Health Organization, 2023; Abera, Worede, Hirigo, Alemayehu, & Ambachew, 2024). Increased consumption of plant proteins has been associated with reduced CVD risk. Due to this emerging need of the populace, the development of nutritionally enhanced and functional foods to address health concerns is rapidly increasing.

The physiological effects exhibited by proteins are usually present in the peptide sequences encrypted in the parent protein, which become active when released as peptide fragments or free amino acids. Bioactive peptides found in foods have been widely investigated for their potential role in the prevention of conditions such as hypertension, cancer, dyslipidemia, microbial infections, and cardiovascular diseases (Cicero, Floor & Albertoni, 2017). Protein hydrolysates and

peptides from cereals, legumes, and animal products have been widely investigated for their ACE-inhibitory and lipid-lowering activities, yet fruits are generally overlooked as sources of proteinaceous compounds due to their relatively low protein content. This gap underscores the need to explore unconventional fruit sources using targeted workflows that combine protein extraction, characterization, digestion, and *in vitro* evaluation aligned with antihypertensive and lipid-related outcomes.

Calabash (*Crescentia cujete* L.) is of particular interest because its fruit has long been used in traditional medicine and has recently gained considerable attraction in the Philippines due to anecdotal claims of treating hypertension, diabetes, dyslipidemia and cancer. Belonging to the family *Bignoniaceae*, calabash is a seasonal fruit that is widely propagated usually in home gardens in Central and South America, Africa and Asia. Calabash fruit has been incorporated into herbal mixtures in many traditional medicine systems for its anti-inflammatory, and anti-bacterial effects and its use in managing cough and colds (Shastry, Bhalodia Maulik & Aswathanarayana, 2012). Although the biological activities of calabash are commonly attributed to phytochemicals such as apigenin, quercetin, anthraquinones, carotenoids, and β -sitosterol, the contribution of its proteinaceous components remains poorly understood.

In this context, the present study aims to isolate, purify and characterize the proteins and peptides released after simulated gastrointestinal digestion of calabash fruit flesh, thereby providing baseline information on its protein content. This study also aims to investigate the potential effects of proteins and peptides in managing elevated blood pressure and high cholesterol levels, providing scientific validation for anecdotal claims.

Materials and Methods

Sample Preparation and Crude Protein Isolation

Calabash (*C. cujete*) fruits were collected from the province of Davao, Philippines. Three randomly selected trees were used as biological

sources, and three mature fruits were harvested from each tree yielding a total of nine fruits for subsequent analysis. Fruit maturity was determined based on fruit size, hard outer rind and external color, which are used for morphological identification of mature calabash fruits. The fruit flesh was lyophilized using a -85°C laboratory floor-mode freeze dryer (IlShin Biobase, Korea). Lyophilized fruit flesh from each fruit was extracted individually for generate nine independent samples, and downstream assays were performed in triplicate for each extract. Two grams of lyophilized calabash flesh were homogenized with 10 mL of 0.1 M sodium phosphate buffer pH 7.0 containing 0.05 M Tris-HCl and 0.4 M NaCl. The mixture was incubated overnight at 4°C with shaking, and then filtered through cheesecloth and centrifuged at 11,872 x *g* at 4°C for 10 min. The resulting filtrate was labeled as crude protein isolate (CPI).

Purification of Proteins

Ammonium Sulfate Precipitation

Five mL of CPI was subjected to ammonium sulfate precipitation to achieve approximately 40% saturation by slow addition of 1.21 grams of powdered ammonium sulfate under constant stirring. This saturation level was selected, based on preliminary experiments, to recover a protein-enriched fraction. The samples were centrifuged at 11,872 x *g* for 10 min at 4°C using Beckman Coulter X-30R (USA). The supernatant was discarded, and the pellet was reconstituted using 0.1 M sodium phosphate buffer pH 7.0.

Dialysis

The reconstituted proteins were dialyzed overnight at 4°C against distilled water using a cellulose dialysis tubing with molecular weight cut-off of 3.5 kDa. The distilled water was changed every 3 h.

Gel Filtration Chromatography

Dialyzed protein samples were purified using Sephadex G-100 (Sigma-Aldrich, USA)

pre-swollen for 3 days and equilibrated using extraction buffer. Three milliliters of dialyzed protein samples were loaded into the column and eluted with cold extraction buffer at a flow rate of 1.0 ml/min. Fractions of 1 mL each were collected, and absorbance of eluates was monitored at 210 nm using a UV-Vis spectrophotometer (Spectroquant Pharo 300, Merck Millipore, Germany). Pooled fractions were labelled as purified proteins (PP).

Protein Quantification Using Bradford Assay

Protein quantification was performed using Quick Start Bradford Assay (Bio-Rad, USA). A standard calibration curve was prepared using different concentrations of bovine serum albumin (BSA). In a 96-well microtiter plate, 2.5 µL of sample and 125 µL 1x Bradford reagent were thoroughly mixed and incubated for 10 min. The absorbance was monitored at 595 nm using a microplate reader (BioTek Epoch, Agilent, USA).

Sodium Dodecyl Sulfate–polyacrylamide Gel Electrophoresis (SDS-PAGE)

Twelve percent (12%) discontinuous denaturing gels were prepared and run at 110V for approximately 100 minutes using a Mini-PROTEAN Tetra Cell (Bio-Rad, USA). Gel bands were visualized using 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad, USA). A protein reference ladder (NEB, MA, USA) was used to estimate the molecular weights of the protein subunits.

Enzymatic Hydrolysis of Proteins

PP were subjected to digestion in a 12:1 protein-to-enzyme ratio. Solutions of pepsin, trypsin and chymotrypsin (1 mg/mL) were prepared in distilled water. These enzymes were chosen to simulate the main stages of human gastrointestinal protein digestion and to generate a broad spectrum of peptide fragments with different sequence specificities under physiologically relevant pH and temperature conditions. Sequential digestion was performed, wherein pepsin was added first and allowed to digest for 1 h at pH 2.0. The

remaining enzymes were then added simultaneously after adjusting the pH to 7.0 using 1.0 M NaOH. The mixture was incubated for 1, 2, 3, 4, 6 and 24 h at room temperature. The hydrolysis was terminated by immersing the samples in a boiling water bath for 5 min. Progression of hydrolysis was assessed via SDS-PAGE.

Normalization of Protein Activity

The normalized protein activity of CPI was obtained by dividing the percent inhibition by the mass of protein loaded per assay. For CPI, the protein mass was calculated from the Bradford-derived concentration (688 µg/mL) and the assay loading volume, and the values are expressed as apparent normalized inhibition per mg protein. Activities were therefore expressed as apparent normalized percent inhibition per mg protein loaded. Because the purified fractions fell below the Bradford detection limit, normalization of PP digest activity should be considered approximate unless verified using a more sensitive protein assay.

In vitro Assays for Antihypertensive, Anti-obesity and Hypcholesterolemic Activities

The sample preparation and laboratory analysis were all done at the Biochemistry Laboratory of the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. All chemicals and reagents used were of analytical grade and purchased from reputable suppliers.

ACE Inhibition Assay

ACE inhibition activity was determined using the method of [Cushman & Cheung \(1971\)](#) with modifications. An assay mix of 25 µL 100 mM sodium phosphate buffer pH 8.3, 25 µL 300 mM NaCl, 50 µL 5 mM hippuryl—histidyl-leucine (HHL), 25 µL of sample, and 25 µL crude ACE extract (which was added last) from pig lungs was incubated for 30 min at 37°C. After incubation, 125 µL of 1 N HCl was added to stop the reaction. A blank control was prepared by adding 25 µL of phosphate buffer

to the assay mix instead of the sample. Captopril at a concentration of 1 mg/mL was used as a positive control and added to the assay mix. Negative control was also prepared wherein the stop solution (1 N HCl) was initially added to the assay mix without ACE. Addition of 750 µL ethyl acetate into the mix was then performed to extract hippuric acid. Five hundred microliters of the ethyl acetate layer were collected and allowed to evaporate to dryness. The extracted hippuric acid was dissolved in 500 µL distilled water and the response was monitored at absorbance of 228 nm. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank} - \text{Absorbance of negative control}} \times 100\% \quad (1)$$

Pancreatic Lipase Inhibition Assay

The pancreatic lipase inhibition activity was determined using the method of [Chheda, Kaikini, Bagle, & Seervi, \(2016\)](#). Freshly prepared 0.1 M potassium phosphate buffer pH 7.2 with 150 mM NaCl and 0.5% (v/v) Triton X-100 was used to dissolve 6 mg of lipase enzyme from porcine pancreas (≥125 units/mg protein) and diluted to 10 mL with phosphate buffer. The substrate of the assay was prepared using 8.45 µL *p*-nitrophenyl butyrate (PNPB) and diluted to 10 mL with acetonitrile. Inhibitory activity was determined by mixing 25 µL of the sample, 100 µL phosphate buffer, 50 µL lipase enzyme, and 25 µL PNPB solution. The mixture was incubated at 37°C for 30 min and the absorbance was monitored at 400 nm using a microplate reader to estimate the amount of *p*-nitrophenol released from the reaction. One mg/mL Orlistat dissolved in dimethyl sulfoxide was used as a positive control. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\% \quad (2)$$

Cholesterol Micellar Solubility Inhibition Assay

The cholesterol micellar solubility inhibition activity was determined using the method of [Bangoura, Wenshui, & Jiali, \(2009\)](#).

Cholesterol micellar solution was initially prepared by mixing 10 mM sodium deoxycholate, 0.4 mM cholesterol, 132 mM NaCl, 1 mM oleic acid, and 15 mM potassium phosphate buffer (pH 7.4). The mixture was sonicated before use.

A 1:1 mixture of micellar solution and sample was incubated at 37°C for 24 h and centrifuged at 11,872 x *g* for 10 min. Using a 96-well titer plate, 60 µL of supernatant was mixed with 75 µL of acetic acid followed by the slow addition of 90 µL Zak's reagent (0.1% FeCl₃ in concentrated H₂SO₄). The reaction mixture was incubated at room temperature for 30 min prior to absorbance determination at 560 nm using a microplate reader. One mg/mL cholestyramine dissolved in phosphate buffer was used as positive control. The blank sample was prepared by substituting the protein isolates with the micellar solution. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\% \quad (3)$$

Statistical Analysis

The data collected were expressed in mean ± standard deviation and analyzed using R Studio (Posit Software, Boston, Massachusetts). The number of samples, *n*, refers to independently prepared fruit extracts, whereas assay measurements from each extract were treated as technical replicates. The bioactivities were analyzed using Kruskal–Wallis test to determine the significant differences among the given groups. A pairwise post hoc test using Dunn's test with Benjamini-Hochberg (BH) correction was also performed to identify which specific groups differ compared to the activity of positive control.

Results and Discussion

Protein Isolation and Characterization

This study extracted soluble protein-containing fractions from *C. cujete* fruit flesh to evaluate their functional applications and potential health benefits through *in vitro* bioactivity assays. The extracted protein has an

average concentration of 688 µg/mL in terms of extraction buffer or 0.344 g/100 g dry weight of fruit flesh. The median and interquartile range (IQR) values were recorded at 671 and 449 µg/mL, respectively. The quality of CPI was assessed using SDS-PAGE as shown in Fig. 1a. Five distinct bands were observed with estimated molecular weights of 12.1 kDa, 23.2 kDa, 25.7 kDa, 30.1 kDa, and 46.7 kDa. This suggests that CPI contains relatively low-molecular-weight proteins and peptides. Many classical structural and enzymatic proteins in cellular compartments have higher molecular masses (usually 40–60 kDa), but numerous functional proteins and bioactive peptides are substantially smaller. Classic examples include insulin, a 5.8 kDa hormone, and the 4-kDa hormone-like legume peptide that interacts with a 43-kDa receptor-like kinase in soybean. Plant-derived proteolytic enzymes like fruit bromelain (25–32 kDa) from pineapple, widely used for its protease activity and reported anti-inflammatory property further demonstrate that functional proteins can span a broad molecular-weight range. Reviews on plant- and food-derived bioactive peptides also report many active sequences with molecular weights below 10 kDa, supporting the view that the relatively small size of the CPI proteins and peptides observed in this study is compatible with functional and bioactive roles (Hanada & Hirano, 2004; Hikiş & Bernasinska-Slomczewska, 2021; Mayer, Zhang, & DiMarchi, 2007; Zhu *et al.*, 2023).

Crude proteins were isolated using sodium phosphate buffer containing Tris-HCl and NaCl. Phosphate buffers are highly effective in maintaining pH stability, thereby preventing extreme changes that could result in protein denaturation or aggregation. Maintaining a neutral pH (7.0) is crucial for preserving the native conformation of proteins, ensuring their functionality and bioactivity remain intact. Furthermore, sodium phosphate buffer is a preferred choice in protein extraction protocols due to its minimal reactivity with proteins, which reduces the likelihood of undesirable chemical interactions (Pavani, Kumar, Rani,

Venkatesu, & Lee, 2021). CPI contains less than 1% protein, consistent with the typically low protein content of most fruits and vegetables, which still offer dietary benefits (Ford *et al.*, 2023).

The CPI was precipitated using ammonium sulfate at 40% saturation as a partial enrichment step and subsequently dialyzed to remove excess salts. This precipitation indicates selective recovery of a subset of soluble proteins rather than confirmed precipitation of the total protein complement. Classical protein fractionation shows that proteins precipitate over a broad range of saturations (e.g., 60-80%) to recover more complete protein fractions (Tola & Missihoun, 2023; Wingfield, 1998). Future work will combine stepwise fractionation with trichloroacetic acid or total nitrogen measurements to validate total protein recovery. The partially purified proteins were evaluated using SDS-PAGE which showed three remaining distinct bands with improved resolution (Fig. 1b) which correspond to molecular weights of 12.1 kDa, 23.2 kDa, and 25.7 kDa, as estimated in CPI in Fig. 1a. Different molecular weight protein markers were used for Fig. 1a and 1b due to reagent availability during separate experimental runs; however, band size estimations were independently performed using the corresponding protein ladder for each gel. The partially purified proteins were then subjected to size-exclusion chromatography using Sephadex resin and 25 fractions of 1 mL each were collected. The elution profile showed two distinct peaks: one between 5-9 mL and another broad peak from 11-22 mL with observed peak shouldering from 15 mL to 18 mL. The presence of shouldering suggests the presence of multiple species due to close molecular weight or the existence of multiple states such as oligomer or protein aggregates. After conducting several electrophoretic runs, the

remaining visible band corresponded to a protein of approximately 12.1 kDa, matching the last broad peak observed in the elution profile as illustrated in Fig. 2. Bradford assay could not determine the remaining protein concentration in PP because of the possibility of having few low molecular weight proteins and peptides. Another possibility is that the protein concentration of PP, estimated to be less than 20 µg/mL, is already below the detection limit of the Bradford assay, thus resulting in no detectable response. Other protein quantification methods with higher sensitivity and detection limit such as Lowry and Bicinchoninic Acid (BCA) assays can be utilized in the future to verify the protein content of calabash fruit flesh.

PP was used to simulate gastrointestinal digestion. Enzymatic digestion releases peptides and amino acids that were initially inactive within the native protein structure. These peptides are known to exhibit protein-protein inhibition due to their variability in size and specificity (Daliri, Oh & Lee, 2017). After 1 h of digestion, protein bands were no longer visible in the polyacrylamide gels, indicating successful protein hydrolysis resulting in low molecular weight proteins or peptide fragments. This observation suggests the efficiency of the hydrolysis process and highlights its potential to support biochemical reactions analogous to natural digestive processes. In addition, purified protein hydrolysates were not detectable by Bradford assay, likely due to low concentration or reduced dye binding of low-molecular-weight species, hence, the extent of total protein recovery could not be fully verified in the present study.

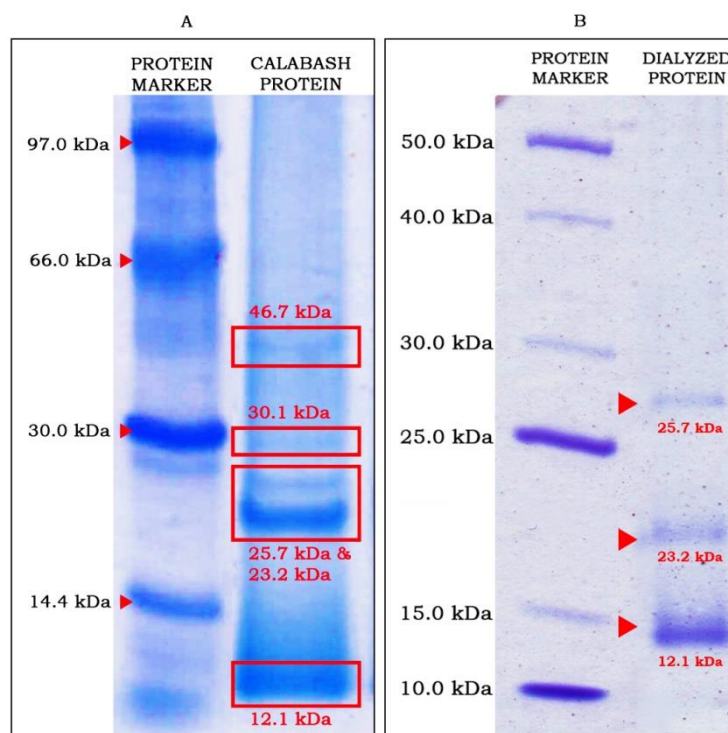


Fig. 1. Electrophoretograms of (a) CPI highlighting five major protein bands; and (b) partially purified proteins from ammonium sulfate fractions

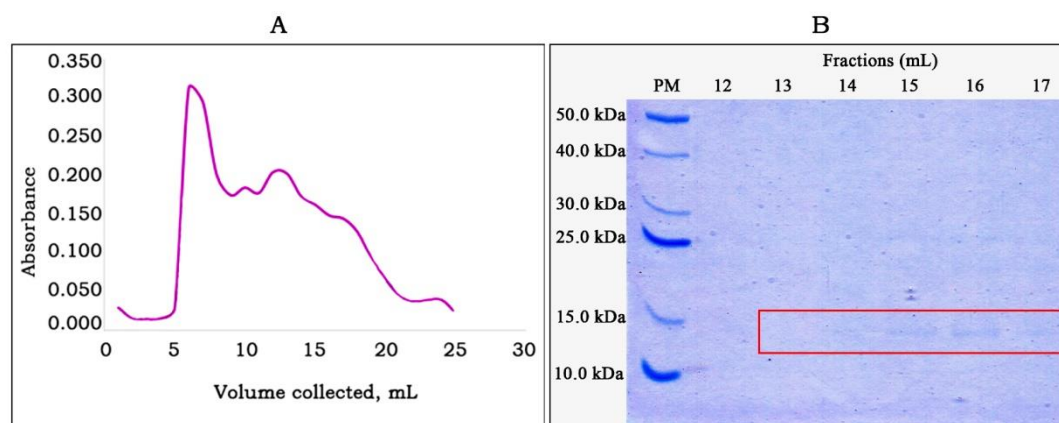


Fig. 2. (a) Elution profile of the dialyzed 40% ammonium sulfate-recovered protein fractions, and (b) SDS-PAGE profile of PP with visible bands with approximate molecular weight of 12.1 kDa

The apparent normalized inhibition values reported for CPI (Table 1) represent protein-normalized endpoint responses rather than classical enzyme specific activities, which are typically expressed in units per mg based on kinetic measurements. Because the purified protein fraction and its hydrolysates could not be reliably quantified by Bradford, equivalent normalization for these samples was not

performed, and their activities are presented as percent inhibition per reaction as presented in Table 2.

ACE Inhibition Activity

The *in vitro* antihypertensive and lipid-lowering properties of CPI and PP hydrolysates were determined spectrophotometrically. For antihypertensive activity, CPI exhibited high

ACE inhibition of 76.5%, which showed no significant difference from Captopril, a known ACE inhibitor drug, with 78.9% inhibition ($p > 0.05$). CPI contributed approximately 0.0172 mg protein per reaction, corresponding to an apparent normalized inhibition of about 4.45×10^3 % inhibition per mg protein. This value provides a protein-normalized estimate of the ACE inhibitory response of CPI and supports the observation that the crude isolates contain proteinaceous components with inhibitory potential. However, this apparent normalized inhibition should be interpreted only as an internal comparative metric because the assay was performed as an endpoint inhibition assay rather than a kinetic assay. In

addition, inhibition values of the calabash CPI approached those of captopril under the assay conditions, such comparisons should be interpreted cautiously because the tested materials differed markedly in composition, purity, and active mass. In addition, results showed that ACE inhibition of protein hydrolysates had comparable values from the 1st to 4th h of digestion but showed a significant decrease at the 6th h of digestion. However, an increase in inhibition activity was later observed for the 24-h digests. For visualization, the effect of digestion on the purified samples over time using ACE inhibitory activity is shown in Fig. 3.

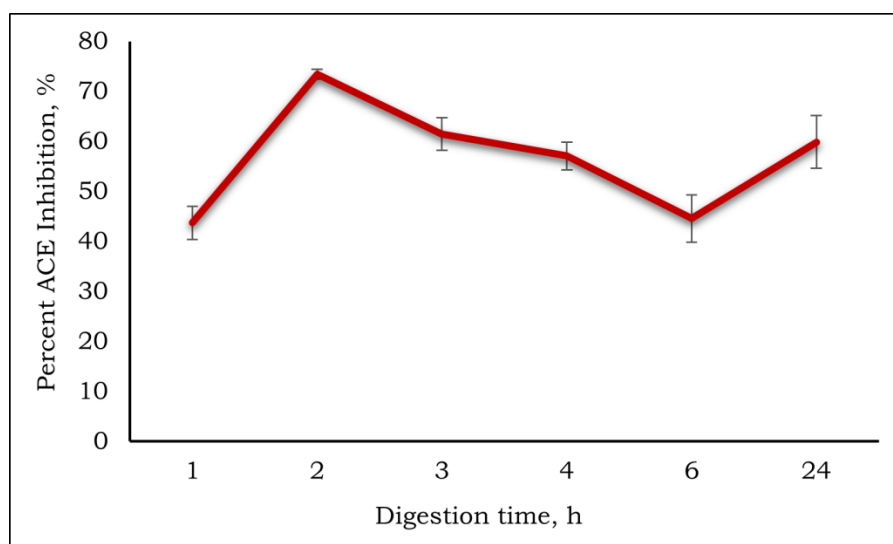


Fig. 3. Effect of digestion time on ACE inhibition of *C. cujete* fruit flesh purified protein hydrolysates

Table 1- Comparison of the apparent normalized inhibitory activities of CPI

	Apparent % inhibition per milligram protein		
	ACE inhibition	Lipase inhibition	Cholesterol micellar inhibition
Crude Protein Isolate (CPI)	4.45×10^3	1.30×10^3 %	2.03×10^3 %

Table 2- Summary of inhibitory activities of CPI and two (2)-hour PP digests

	ACE inhibition, %		Lipase inhibition, %		Cholesterol micellar inhibition, %	
	CPI	PP digest	CPI	PP digest	CPI	PP digest
Positive control	Captopril 78.9 ± 5.94 ^a		Orlistat 72.6 ± 6.36 ^a		Cholestyramine 91.5 ± 0.16 ^a	
Calabash	76.5 ± 3.54 ^{a,b}	73.4 ± 1.00 ^{a,b}	22.3 ± 5.76 ^{b,c}	44.9 ± 0.680 ^{a,c}	84.0 ± 1.20 ^{a,b}	82.2 ± 8.17 ^{a,b}

*Values are expressed as mean ± SD (n = 3). In the same column, values followed by the same superscript letters are not significantly different at the 5% level of significance based on the Kruskal-Wallis test followed by Dunn's test. Letters before the comma indicate column-wise comparisons; letters after the comma indicate row-wise comparisons per bioassay.

For digested PP, it was observed to have high ACE inhibition after 2 h digestion time with 73.4% inhibition compared to other digestion times. The 2-h digests were selected to use in the succeeding bioassays after the ACE inhibition assay exhibited the highest bioactivity across digestion time.

Crude extracts may contain various proteins in their native conformation including the presence of other bioactive compounds such as polyphenols, which could contribute to the observed increased bioactivity. Previous studies have shown that certain phenolics and flavonoids exhibit ACE inhibitory activity (Balasuriya & Rupasinghe, 2011; Simaratanamongkol, Umehara, Noguchi, & Panichayupakaranant, 2014). The results obtained from this study were comparable to the ACE inhibitory activity of mango flesh and 'Señorita' banana cultivar flesh crude proteins with 71%-74% and 85.2% inhibition, respectively (Ferrerias, Bercansil-Clemencia, Hizon-Fradejas, Uy, & Torio, 2021; Rojas *et al.*, 2021). The highest ACE inhibition was observed in 2 h digested PP, followed by a significant decrease at the 6th h of digestion. Although the 2-hour digest exhibited highest ACE inhibition, the present study did not fractionate the hydrolysate into peptide subfractions. The initial increase in activity likely reflects the release of ACE-inhibitory peptides during early digestion time whereas the subsequent decrease may be attributed to the further hydrolysis of proteins into smaller fragments with reduced inhibitory response. ACE inhibitory peptides are typically short sequences (2–12 amino acids), because smaller peptides can more easily access the ACE active site. Their activity is commonly associated with hydrophobic or aromatic residues, especially at the C-terminal end. These features are inferred to enhance binding through hydrophobic interactions within ACE's substrate-binding pockets leading to an improved inhibitory response (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozelik, 2017; Hu *et al.*, 2025). ACE inhibition activity can also be increased by using higher enzyme

concentration and longer hydrolysis time which may generate shorter peptides with potential bioactivity (Rojas *et al.*, 2021; Shalaby, Zakora, & Otte, 2006). Similar studies also reported higher ACE inhibitory activity when exposed to longer hydrolysis time (Abuine, Rathnayake & Byun, 2019). In addition, a minimal contribution can be linked to trace amounts of other compounds, such as polyphenols because PP underwent purification and dialysis utilizing a molecular weight cut-off of 3 kDa which effectively removes compounds smaller than this threshold. Any residual effect would therefore be minor compared to peptide-mediated inhibition.

Pancreatic Lipase Inhibition Activity

For pancreatic lipase inhibition, CPI exhibited 22.3% inhibition and showed significantly lower inhibition than Orlistat, a known lipase inhibitor, with an inhibition value of 72.6 %. The crude protein isolate corresponded to an apparent normalized inhibition of approximately 1.30×10^3 % inhibition per mg protein. This lower normalized response relative to ACE and cholesterol micellar solubility inhibition suggests that, within CPI, lipase-inhibitory activity was weaker or less concentrated than the other measured bioactivities. On the other hand, 2-h PP digest showed higher lipase inhibition at 44.9% compared to CPI and was not statistically significant from Orlistat ($p > 0.05$). The values obtained in this study were lower compared to Orlistat but higher compared to the reported lipase inhibitory activity of sea buckthorn seed protein hydrolysates with 35% inhibition (Xiang *et al.*, 2020). Studies also reported peptides isolated from millet grains, rice bran, milk, and some fish species that showed moderate to strong inhibitory activity (Jakubczyk, Szymanowska, Karaś, & Złotek, 2019; Ketprayoon *et al.*, 2021). In physiologic conditions, pancreatic lipase interacts at the lipid interface of emulsion droplets, where it hydrolyzes triglycerides to release free fatty acids. One possible mechanism for pancreatic lipase inhibition by

proteins involves the ability to reduce the interfacial tension at the lipid-water interface. This reduction allows the proteins to penetrate the monomolecular film of phospholipids and glycerides, even under high surface pressure. The disruption hampers the ability of pancreatic lipase to hydrolyze its substrate efficiently (Gargouri, Julien, Pieroni, Verger, & Sarda, 1984). Another possible mechanism includes the formation of protein aggregates or peptide complexes that limit its accessibility to lipid substrates, as demonstrated by lipase activity with flavonoids under intestinal conditions (Bustos, Hakansson, Linares-Pasten, Peñarrieta, & Nilsson, 2020). However, because of the observed low-molecular weight nature of proteins and peptide fractions, peptide-mediated inhibition is more plausible than the aggregate-related effects. Low molecular weight peptides tend to diffuse more efficiently to the lipid-water interface, adsorb at the emulsion surface, and interact directly with the substrate-binding regions of lipase, thereby interfering with substrate access that reduce enzymatic turnover (Ketprayoon *et al.*, 2021; Wang *et al.*, 2022).

Cholesterol Micellar Solubility Inhibition Activity

For cholesterol micellar solubility inhibition, CPI and the 2-hour PP digest showed high inhibitory responses of 84.0% and 82.2%, respectively. CPI corresponded to an apparent normalized inhibition of approximately 2.03×10^3 % inhibition per mg protein. This suggests that CPI may contain components capable of reducing cholesterol incorporation into mixed micelles under the simplified *in vitro* conditions of the assay. Cholestyramine showed a comparable inhibition of 91.5%, with no significant difference ($p > 0.05$) from CPI. Both CPI and protein hydrolysates likely bound cholesterol thereby reducing its solubility within micelles. Proteins and peptides may interact with bile components, bind free cholesterol through hydrophobic interactions or disrupt the structural conformation of micelles (Udenigwe & Rouvinen-Watt, 2015). Such interactions could potentially limit the

bioaccessibility of cholesterol during intestinal digestion. From a physiological perspective, non-solubilized cholesterol tends to segregate into an oil phase within the intestinal lumen, thereby becoming inaccessible for absorption and can be excreted through the hepatobiliary pathway or transintestinal cholesterol excretion (Estribillo *et al.*, 2022; Jesch & Carr, 2017). Similar cholesterol micellar solubility inhibition activities have been reported for peptides generated from royal jelly and fermented seabass by-product which reported suppression of cholesterol solubility greater than 80% (Chen, Lin, Huang, & Lin, 2021; Kashima *et al.*, 2014). This simplified *in vitro* assay provides an indicator of reduced cholesterol bioaccessibility potential rather than a direct measure of *in vivo* cholesterol-lowering efficacy. Overall, these findings highlight the promising potential of CPI and PP digests to contribute to the preventive management of elevated blood cholesterol level and obesity, and when combined with their ACE inhibitory activity, to support blood pressure control.

This study represents an initial screening effort on the biological activity evaluation of calabash proteins; the most active peptide fraction and individual peptide sequences were not identified. In addition, all bioactivities were evaluated using *in vitro* assays, which provide useful screening evidence but do not demonstrate *in vivo* antihypertensive or lipid-lowering efficacy. These constraints highlight the need for further work involving improved protein quantification methods, more detailed peptide fractionation and identification (e.g. LC-MS/MS), and *in vivo* validation.

Conclusion

Protein- and peptide-enriched fractions from *C. cujete* fruit flesh exhibited promising *in vitro* ACE inhibitory, pancreatic lipase inhibitory, and cholesterol micellar solubility inhibitory activities. The observed biological activities may be associated with the release of sufficient bioactive peptides during hydrolysis in a time-dependent manner. The 2 h hydrolysate of the

PP digest showed the most consistent *in vitro* antihypertensive potential, with high ACE inhibitory activity and notable inhibitory effects on pancreatic lipase and cholesterol micellar solubility, under the conditions used in this study. To the best of our knowledge, this is the first study to report on the isolation and characterization of bioactive peptides with ACE and lipase-inhibiting properties from *Crescentia cujete* fruit flesh. However, the present work represents an initial screening study; total protein recovery at the ammonium sulfate step was not validated as total precipitation, the most active peptide fraction was not isolated, and peptide sequences were not identified. Future work may involve validation using complementary protein precipitation techniques, peptide identification and sequencing using LC-MS/MS, and other approaches to confirm target interactions. Animal bioassays can also be conducted to further support the therapeutic potential of calabash fruit-derived proteins and peptides.

Authors Contributions

JC Villanueva conceptualized, designed, acquired funding, analyzed data, and wrote the manuscript for this study; **AC Sajise** helped in

acquiring funding, sample collection and reviewing the manuscript; **AC Laurena** assisted in reviewing and revising the manuscript; **MO Torio** provided laboratory resources and assisted in reviewing the manuscript; **VP Migo** helped in designing the study and assisted in reviewing the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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مقاله پژوهشی

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جداسازی، خالص سازی و شناسایی پروتئین ها و پپتیدهای موجود در گوشت میوه کدویی (*Crescentia cujete* L.) با ویژگی های ضد فشارخون و کاهنده چربی خون

جریس ویلانوا^{۱*} - اندرس گادوین ساجیسی^۲ - آنتونیو لائورنا^۳ - ماری آن توریو^۴ - ورونیکا میگو^۵

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چکیده

میوه کدویی (*Crescentia cujete* L.) در سال های اخیر به دلیل نقش قابل توجه آن در سلامت و تغذیه انسان، به طور فزاینده ای مورد مطالعه قرار گرفته است. فعالیت های زیستی گزارش شده برای این میوه با حضور ترکیبات فیتوشیمیایی مانند پلی فنول ها مرتبط دانسته شده اند، اما درباره نقش پروتئین های آن اطلاعات اندکی وجود دارد. این موضوع یک خلأ مهم پژوهشی محسوب می شود، زیرا میوه ها در مقایسه با حبوبات و سایر فرآورده های غنی از پروتئین، معمولاً به عنوان منابع پروتئینی نادیده گرفته می شوند؛ با وجود آنکه می توانند کاربردهای بالقوه عملکردی و درمانی داشته باشند. هدف این پژوهش، جداسازی، خالص سازی و شناسایی پروتئین ها و پپتیدهای موجود در میوه کدویی و ارزیابی توان بالقوه آن ها در کاهش فشار خون و چربی خون بود. پروتئین ها با استفاده از مجموعه ای از روش های بیوشیمیایی، از جمله استخراج با بافر فسفات سدیم، رسوب دهی با سولفات آمونیوم، کروماتوگرافی ژل فیلتراسیون و آزمون های زیستی درون کشتگاهی (*in vitro*) استخراج، خالص سازی و شناسایی شدند. آزمون های زیستی شامل مهار لیپاز پانکراس، مهار حلالیت میسلی کلسترول و مهار آنزیم مبدل آنژیوتانسین (ACE) بودند. ایزوله پروتئینی خام (CPI) دارای غلظت پروتئینی ۶۸۸ میکروگرم بر میلی لیتر بود و نوارهای پروتئینی مشاهده شده در محدوده ۱۲/۱ تا ۴۶/۷ کیلودالتون قرار داشتند. پس از شبیه سازی هضم دستگاه گوارش، CPI به ترتیب میانگین فعالیت مهار ۷۶/۵٪ برای ACE، ۲۲/۳٪ برای لیپاز پانکراس و ۸۴/۰٪ برای حلالیت میسلی کلسترول نشان داد. در حالی که هضم دوساعته فراکسیون پروتئینی خالص شده، به ترتیب ۷۳/۴٪، ۴۴/۹٪ و ۸۲/۲٪ مهار را در این آزمون ها نشان داد. پروتئین ها و پپتیدها فعالیت زیستی قابل توجهی از خود نشان دادند و میزان مهار آن ها در تمامی آزمون ها، در شرایط آزمایشی مورد بررسی، به مقادیر مشاهده شده در نمونه های کنترل آزمون نزدیک بود. این پژوهش نخستین مطالعه ای است که توان زیست فعال پروتئین ها و پپتیدهای استخراج شده از میوه *C. cujete* را نشان می دهد و دیدگاه های امیدوارکننده ای درباره ویژگی های عملکردی احتمالی آن ها در مدیریت پیشگیرانه چاقی، سطح کلسترول و فشار خون بالا ارائه می کند. با این حال، برای تأیید فعال ترین ترکیبات، انجام فراکسیون بندی بیشتر پپتیدها و شناسایی توالی آن ها ضروری است.

واژه های کلیدی: پپتیدهای زیست فعال، ضد فشارخون، غذای عملکردی، کدویی، میسل کلسترول

۱- برنامه مرکز ژنوم فیلیپین در حوزه کشاورزی، دامپروری، شیلات و جنگلداری، دفتر معاونت پژوهش و ترویج، دانشگاه فیلیپین در لوس بانوس، کالج، لاگونا، فیلیپین

(*)- نویسنده مسئول: (Email: jcvillanueva3@up.edu.ph)

۲- مرکز منابع ژنتیکی تی. تی. چانگ، مؤسسه بین المللی تحقیقات برنج، لوس بانوس، لاگونا، فیلیپین

۳- مؤسسه علوم زراعی، دانشکده کشاورزی و علوم غذایی، دانشگاه فیلیپین در لوس بانوس، کالج، لاگونا، فیلیپین

۴- مؤسسه شیمی، دانشکده هنر و علوم، دانشگاه فیلیپین در لوس بانوس، کالج، لاگونا، فیلیپین

۵- گروه مهندسی شیمی، دانشکده مهندسی و فناوری صنایع کشاورزی، دانشگاه فیلیپین در لوس بانوس، کالج، لاگونا، فیلیپین

Research Article

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Impact of Sprouted Oat Powder as a Rice Flour Substitute on Physicochemical, Textural, and Sensory Characteristics of Gluten-free Sponge Cake

A.H.A. Allawi¹, F. Salehi^{1*}, A. Daraei Garmakhany²

1- Department of Food Science and Technology, Faculty of Food Industry, Bu-Ali Sina University, Hamedan, Iran

2- Department of Food Science and Technology, Faculty of Engineering and Natural Resources, Bu-Ali Sina University, Hamedan, Iran

(* - Corresponding Author Email: F.Salehi@basu.ac.ir)

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Abstract

The present study examined the impact of replacing rice flour with sprouted oat powder (SOP) at various levels (0–100%) on the physicochemical, textural, and sensory attributes of gluten-free rice cake. Rheological analysis revealed pseudoplastic behavior, with apparent viscosity (AV) decreasing as shear rate increased, while higher levels of SOP resulted in increased batter viscosity. The inclusion of SOP led to reduced lightness index (L^*) in both batter and cake, with the lowest lightness recorded in the 100% sprouted oat sample. Batter yellowness index (b^*) increased significantly with substitution, while redness index (a^*) showed no consistent trend. Sprouted oat substitution significantly increased cake weight and reduced baking loss. However, it had no statistically significant effect on cake volume or density. Cakes formulated with higher levels of SOP exhibited increased moisture (from 17.93% to 21.76%) and ash content (from 1.08% to 2.08%), reflecting the nutritional richness of the sprouted flour. The maximum acidity (0.83%) and minimum pH (6.73) were recorded in the sample prepared with 100% SOP. Increasing the concentration of SOP led to a systematic elevation in both the antioxidant potential and the total phenolic levels within the gluten-free cakes. The protein content of the final cakes also increased proportionally (up to 4.49% in the 100% oat sample), while the fat content remained relatively stable across formulations. Increased replacement ratios elevated cake crust hardness (from 0.04 N to 0.55 N), while texture profile analysis (TPA) results indicated no significant changes in crumb firmness or cohesiveness. Sensory evaluation revealed that up to 75% substitution was acceptable without negative impacts on aroma, flavor, texture, or overall acceptance, whereas higher replacement ratios negatively affected crust and crumb color.

Keywords: Batter, Fat, Protein, Total phenolic content, Viscosity

Introduction

Sponge cake is a popular bakery product known for its soft, airy texture, typically made from wheat flour, eggs, sugar, milk, and oil. Its porous structure results from leavening agents like baking soda that release gas during baking. The stability of this structure relies on the gluten network and coagulated egg proteins,

which help retain gas cells and support the cake's volume and texture (Ataei Nukabadi, Nehchiri, Keramat, & Momeni Shahraki, 2022; Karami, Zarringhalami, & Bimakr, 2025; Salehi, 2019, 2020).

There is a growing consumer demand for cereal-based food products that offer added health benefits, reflecting a broader trend



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toward functional and health-promoting foods (Salehi, 2019). Among various cereal grains, oats (*Avena sativa* L.) have recently garnered significant attention from both researchers and the food industry due to their unique nutritional profile and associated health benefits. This heightened interest has led to the increased incorporation of oats as a primary ingredient in a wide array of food products, including breakfast cereals, breads, cookies, infant formulas, and functional beverages (Cao *et al.*, 2022; Figueroa-Pérez, Reynoso-Camacho, Ramos-Gómez, Mendoza-Sánchez, & Pérez-Ramírez, 2024; Solmaz, Levent, & Şahin, 2025). The versatility and health-promoting properties of oats make them a valuable component in the improvement of novel food formulations aimed at improving public health outcomes (Samary, Salehi, Aliverdi, & Daraei Garmakhany, 2025b; Xu *et al.*, 2025). Numerous studies have verified that oats are a rich source of phenolic compounds and antioxidants, contributing to a range of beneficial biological effects, including the reduction of blood lipid levels in humans. In addition to these bioactive components, oats are widely recognized for their dense nutritional profile and health-promoting characteristics, which are primarily attributed to their high dietary fiber content, particularly β -glucan, a soluble fiber known for its cholesterol-lowering effects (Coria-Hernández, Rueda-Enríquez, Soto-Bautista, & Meléndez-Pérez, 2025; Gramza-Michalowska *et al.*, 2018; Samary *et al.*, 2025b). Furthermore, oats are unique among cereals in their content of avenanthramides, a class of polyphenolic alkaloids with well-documented antioxidant and anti-inflammatory properties. These compounds, along with a distinct group of N-cinnamoyl anthranilic acid derivatives found exclusively in oats, play a key role in their functional food potential (Figueroa-Pérez *et al.*, 2024; Gramza-Michalowska *et al.*, 2018; Solmaz *et al.*, 2025). According to recent findings by Xu *et al.* (2025), the incorporation of oat flour offers significant potential for enhancing the quality and stability of gluten

free cereal matrices. The study highlights that substituting traditional ingredients with oat flour optimizes functional attributes such as water absorption, starch structural integrity, and viscoelastic behavior, while simultaneously exerting a retarding effect on starch gelatinization and the progression of short-term retrogradation.

Seed sprouting and seedling emergence represent the initial and most critical phases in the establishment and development of plants (Yılmaz Tuncel *et al.*, 2025). In cereals, the sprouting process is accompanied by a range of biochemical and physiological transformations that considerably improves the nutritional profile of the grain (Aparicio-García, Martínez-Villaluenga, Frias, & Peñas, 2021; Schendel, 2019). Notably, sprouting leads to an increase in antioxidant compounds, an improvement in both the content and quality of proteins, and greater bioavailability of essential minerals (Ahmed, Ragab, Gadallah, Alhomaid, & Almujaýdil, 2024; Singh, Rehal, Kaur, & Jyot, 2015). Simultaneously, there is a reduction in the levels of antinutritional factors and a lowering of the glycemic index, making the sprouted product more suitable for health-conscious consumers. Additionally, the sprouting process stimulates the biosynthesis of various bioactive compounds, including polyphenols, which contribute to the enhanced functional and nutritional properties of the final manufactured goods (Singh *et al.*, 2015; Yılmaz Tuncel *et al.*, 2025). There has been growing scientific interest in exploring the properties of sprouts derived from various cereal grains. This attention is largely due to the significant enhancement in bioactive compounds that occurs during sprouting, which can lead to improved health-promoting properties compared to unsprouted seeds (Cao *et al.*, 2022). These improvements have positioned cereal sprouts as promising functional ingredients, prompting extensive research within the food scientists to better understand their nutritional potential and application in health-oriented food products (Benincasa, Falcinelli, Lutts, Stagnari, &

Galieni, 2019). For instance, oats are rich in a wide array of phytochemicals, including quinones, benzoic and cinnamic acids. Remarkably, the concentrations of these bioactive compounds have been reported to increase three- to four-fold during the sprouting process. Furthermore, the levels of avenanthramides (polyphenolic compounds) also rise significantly during sprouting (Schendel, 2019).

The development and enhancement of gluten-free foods for individuals with celiac disease represents a significant area of research within the food industry (Jnawali, Kumar, & Tanwar, 2016; Karami *et al.*, 2025). People affected by this autoimmune disorder must adhere to a strict gluten-free diet, relying on alternative protein sources such as rice and oats (Cao *et al.*, 2022; Davtalab, Naji-Tabasi, & Shahidi, 2025). Sprouting has been shown to improve the nutritional profile of cereals, increasing their functional and health-promoting properties. To the best of our knowledge, limited studies have systematically investigated the application of SOP as a complete or partial substitute for rice flour in gluten-free sponge cake and its comprehensive impact on physicochemical, textural, and sensory attributes. To address the demand for high-quality gluten free alternatives, this study examines the functional and sensory implications of replacing rice flour with SOP in sponge cake formulations. The resulting data are intended to facilitate the creation of nutritionally superior products, catering to the growing market of health-conscious consumers and those who must adhere to a gluten-free regimen.

Materials and Methods

Materials

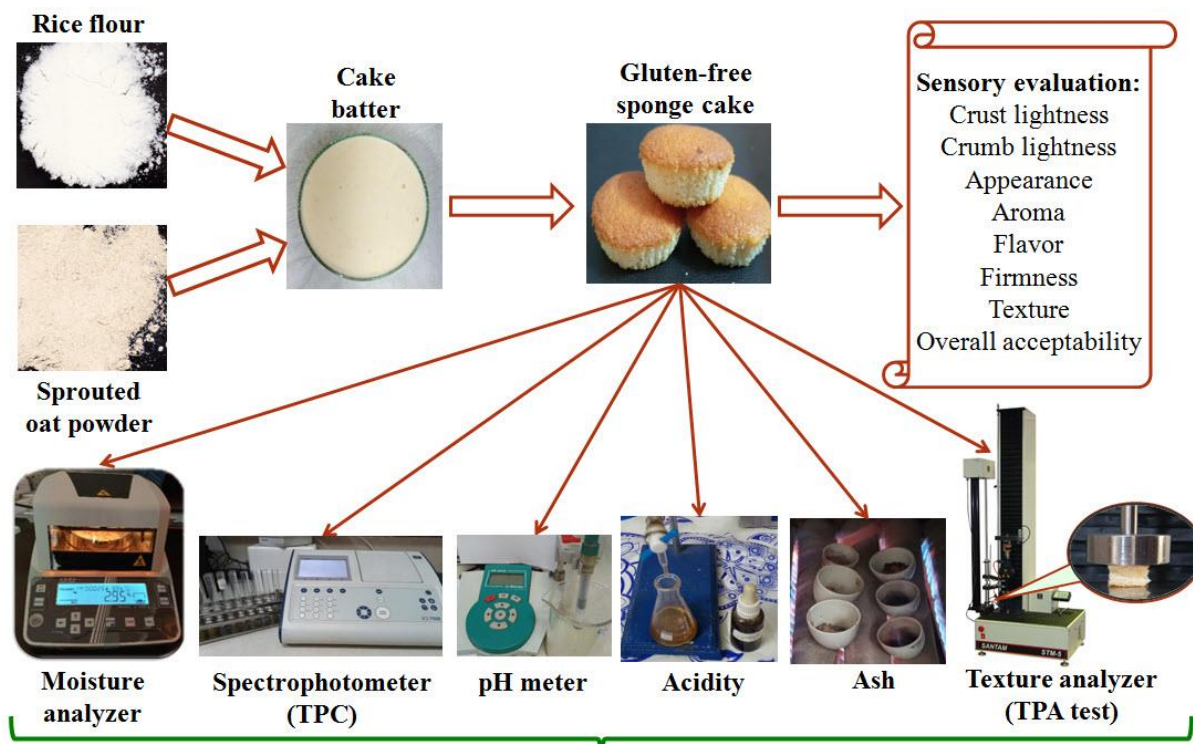
Rice flour (Tarkhineh, Iran), sugar (Hamedan Sugar Co., Iran), fresh eggs, oil

(Oila, Iran), milk powder (Kalleh, Iran), baking powder (Golestan, Iran), and vanilla (Bartar, Iran) required for the preparation of the cakes were obtained from regional markets located in Hamedan, Iran. Commercial SOP was obtained from Food Industries Group 111, located in Mazandaran, Iran.

Cake Preparation

Following a review of the relevant literature and preliminary trial-and-error testing, an appropriate cake formulation was established, consisting of 100 g of rice flour, 0.5 g of vanilla, 2 g of baking powder, 57 g of oil, 72 g of sugar, 6 g of milk powder, 102 g of egg, and 30 g of water. In the initial stage of cake preparation, egg whites were separated from the yolks. The egg whites were then whipped using an electric mixer for 5 min until stable white foam was formed. Sugar was then added, and the mixing process was continued for a further minute. In a separate bowl, the egg yolks were blended with oil, vanilla, water, powdered milk, and baking powder. Following this, flour was incorporated gradually, with varying replacement ratios of SOP at 0% (100% rice flour), 25%, 50%, 75%, and 100% (0% rice flour) (Fig. 1).

In the final step, the previously whipped egg whites were gently folded into the yolk-flour mixture along with the remaining sugar, using a circular folding motion to maintain the aerated structure. The resulting batter was poured into round paper cake pans. Cakes were baked in a Leopard fan-type oven (model L4215, Iran) at 170 ± 2 °C for 15 min. Upon completion of baking, the cakes were taken out from the oven and permitted to cool down to ambient temperature (approximately 25 °C). After cooling, the cakes were placed in polyethylene containers with moisture- and oxygen-barrier properties to maintain product quality.



❖ Sprouted oat powder enhances nutrition without compromising sensory quality

❖ Up to 75% substitution: Acceptable flavour and texture

Fig. 1. Evaluation of sprouted oat powder addition on the physicochemical and sensory quality of gluten-free rice cakes

Evaluation of Cake Batter Viscosity and Color

A rotational viscometer (Brookfield DV2T, RV model, USA) with an RV-04 spindle was employed to measure the AV of the cake batter. The determination involved recording viscosity at 0-120 seconds intervals across spindle speeds of 5, 10, and 15 rpm, all under a constant temperature of $25 \pm 1^\circ\text{C}$.

Color parameters of the cake batter were determined by image processing technique. Digital images of the batter were captured with a 12-megapixel camera (LG K52, China) under standardized illumination. The captured images were then processed using ImageJ software (version 1.42e, USA) with the appropriate color analysis plugin. The color space of the photos was converted from RGB to the CIELAB system, providing values for L^* (lightness), a^* (green to red spectrum), and b^* (blue to yellow spectrum) indices (Samary, Salehi, Aliverdi, & Daraei Garmakhany, 2025a).

Baking Loss Determination

Baking loss was calculated following [Vejdanivahid & Salehi report \(2025b\)](#). It was determined by recording the mass of the cake batter prior to baking and the corresponding weight of the baked sponge cake. The percentage of baking loss was calculated based on the reduction in mass during baking relative to the initial batter weight and expressed as a percentage.

Volume and Density Determination

Cake volume was determined using the canola seed displacement method. A container larger than the cake sample was first filled with canola seeds of known density until leveled at the top. The seeds were then transferred into a second, identical container. The cake sample was placed in the first container, and the canola seeds from the second container were poured over it until the container was once again level, with excess seeds removed using a ruler. The

mass of the removed seeds, along with the remaining seeds in the second container, was recorded. Based on the known density of the canola seeds, the displaced seed volume (equivalent to the cake's volume) was calculated (Salehi & Vejdaniwahid, 2025). Cake density was calculated by measuring the volume displaced using the canola seed displacement method and dividing the cake's mass by this volume.

Moisture and Ash Contents Determination

Moisture content for rice flour, SOP, and cake samples was measured by a Kern DBS60-3 digital moisture analyzer (Germany). Samples were analyzed by weight under controlled conditions, with the device automatically calculating and reporting results as a percentage based on heat-induced weight loss (Salehi & Vejdaniwahid, 2026).

Ash content was determined by weighing 3 g of each sample, followed by pre-ashing over a flame to remove organic matter. The samples were then incinerated in a muffle furnace (Pars-Azma-Co., Iran) at 600 °C until constant weight was reached (Hosseini, 2006).

pH and Acidity Determination

The pH of rice flour, SOP, and cake samples was determined using a Metrohm 827 pH Lab (Switzerland). Samples (10 g) were homogenized with 100 mL of distilled water in a laboratory blender and allowed to stand for 20 min at 25°C. The calibrated pH meter with pH 4.0 and 7.0 buffer solutions was used.

Acidity of rice flour, SOP, and cake samples was determined following Samary *et al.* (2025a). Samples (10 g) were mixed with 50 mL of 67% ethanol, stirred, and filtered. A 25 mL aliquot of the filtrate was titrated with sodium hydroxide (0.1 N) using phenolphthalein indicator (3%). Acidity was calculated and expressed based on the NaOH volume required for a persistent pink endpoint (Pourghasemian, Pourfarzad, & Babakhani, 2025).

Total Phenolic Content (TPC) and Antioxidant Capacity (AC) Determination

The TPC of rice flour, SOP, and cake samples was determined following the technique described by Samary *et al.* (2025a). Results were reported as µg GAE/g of sample, calculated from a gallic acid standard calibration curve.

AC of samples was determined using the DPPH radical-scavenging assay according to Salehi, Ghazvineh, & Inanloodoghous, (2023).

Color Parameters Determination

Color parameters of the cake crust and crumb were analyzed using image processing. Photographs of cake samples were captured with a 12-megapixel LG K52 camera (China), while rice flour and SOP images were obtained via an HP Scanjet-300 scanner. ImageJ software (v1.42e, USA) with a dedicated plugin was employed to convert RGB values to L*, a*, and b* color space parameters (Samary *et al.*, 2025b).

Protein and Fat Determination

The protein content of the cakes was determined using the Kjeldahl technique (Bordbar Lomer & Ghannadiasl, 2025). To determine the protein content, 1 g of the sample was placed into a digestion flask. A catalytic tablet consisting of 10 g potassium sulfate, 0.3 g copper(II) sulfate pentahydrate, and 0.3 g titanium dioxide was then added. Subsequently, 20 mL of concentrated sulfuric acid was added to each flask. The flasks were then positioned in a digestion unit and heated to 420 ± 10 °C. After a minimum of 2 hours of incubation, the samples were cooled and 50 mL of distilled water was then added. In a 125 mL Erlenmeyer flask, 50 mL of 2% boric acid solution was prepared, to which 10 drops of methyl red indicator were added. For the neutralization process, more than 5 mL of 40% sodium hydroxide solution was added in a Kjeldahl distillation apparatus. The distillate was then titrated with 0.1 N sulfuric acid until the endpoint, indicated by a color change to pink. The volume of sulfuric acid used was recorded

and applied to the nitrogen content calculation formula. Lastly, the samples' total protein content was ascertained through the multiplication of their nitrogen content by the standard protein conversion factor, 6.25 (Hosseini, 2006).

The fat content of the cakes was determined using a Soxhlet extraction apparatus. Initially, 5 grams of each sample were placed into an extraction thimble. Subsequently, 80 mL of n-hexane (Extra Pure, Arman Sina Co., Iran) was added as the solvent. The extraction was carried out using a laboratory-scale Soxhlet apparatus (Behr E4, Germany) for duration of 7 hours at a constant temperature of $70 \pm 2^\circ\text{C}$ (Hosseini, 2006).

Puncture and TPA Tests

Crust hardness was determined using a puncture test with a Santam STM-5 texture analyzer (Santam, Iran) equipped with a 5 mm diameter cylindrical probe. The probe penetrated the cake crust to a depth of 10 mm at a constant speed of 1 mm/s. The maximum force (N) recorded during penetration was considered as crust hardness. Measurements were performed at room temperature (approximately $25 \pm 2^\circ\text{C}$), and each sample was analyzed in triplicate (Samary *et al.*, 2025b).

Texture Profile Analysis (TPA) was performed using a Santam STM-5 texture analyzer (Santam, Iran) equipped with a 50 mm cylindrical probe. Cake samples ($2 \times 2 \times 2 \text{ cm}^3$) were subjected to a double compression cycle to 50% of their original height at a constant crosshead speed of 1 mm/s, with a fixed interval between compressions. The parameters of firmness, cohesiveness, springiness, and chewiness were calculated from the force–time curves according to standard TPA definitions. All measurements were conducted at room temperature ($25 \pm 2^\circ\text{C}$), and each formulation was analyzed in triplicate (Goharpour, Salehi, & Daraei Garmakhany, 2024).

Sensory Evaluation of Cake

The sensory evaluation was conducted in the Laboratory of New Technologies at Bu-Ali Sina University by 20 trained panelists representing different age groups (8 men and 12 women, aged 20-50 years). The study received ethical approval from the Biomedical Research Ethics Committee of Bu-Ali Sina University, Iran (Approval No. 5667). Before the evaluation, panelists received basic training to familiarize them with the sensory attributes and scoring procedure, thereby improving the consistency of their assessments. Cake samples were coded with random digit numbers and presented in a randomized order under identical testing conditions. Panelists evaluated crust lightness, crumb lightness, appearance, aroma, flavor, firmness, texture, and overall acceptability using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely). Drinking water was provided for palate cleansing between samples (Samary *et al.*, 2025b).

Statistical Analysis

All experiments were performed using three independent replicates, and each measurement was conducted in triplicate (analytical replicates). Results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) in SPSS software (version 21.0, IBM, USA). Differences among means were determined using Duncan's multiple range test at a significance level of $p < 0.05$.

Results and Discussion

Viscosity of Cake Batter

Fig. 2 illustrates the effect of spindle rotation speed and time on the AV of rice cake batter formulated with 50% SOP. At higher spindle speeds, the AV progressively decreased, indicating a shear-thinning behavior of the batter. This rheological trend remained consistent across all tested concentrations of SOP, indicating that its inclusion does not fundamentally change the batter's flow characteristics.

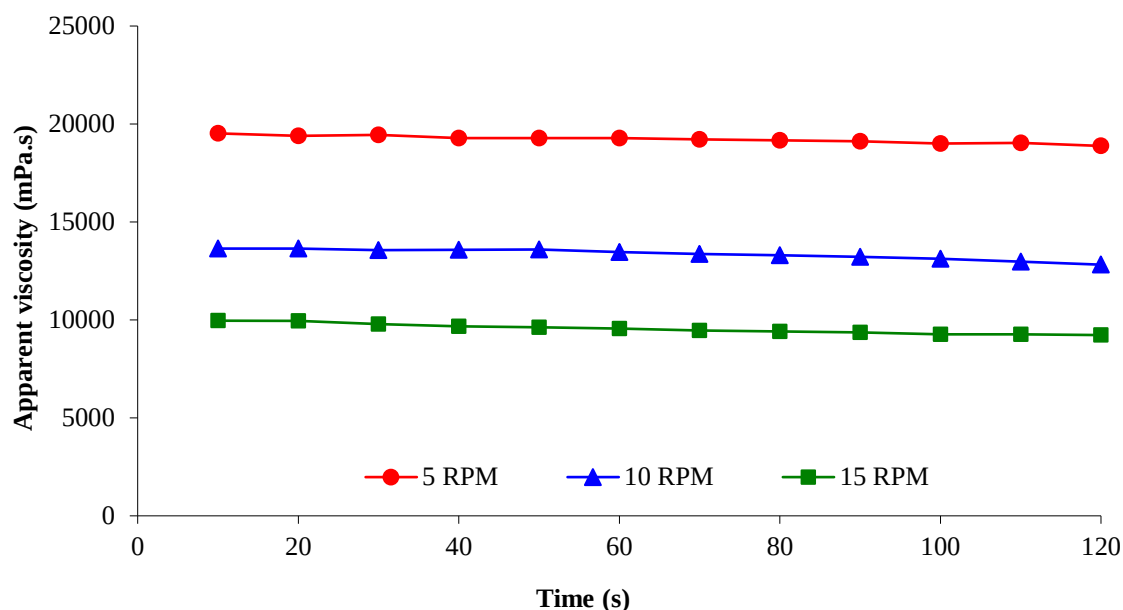


Fig. 2. Impact of spindle rotation speed (5–15 RPM) and time (s) on the apparent viscosity of rice cake batter containing 50% sprouted oat powder

During spindle rotation, the batter's viscosity remained largely stable over time, exhibiting minimal reduction. This indicates a lack of significant time-dependent rheological behavior, such as thixotropy, under prolonged shear. The batter's structural stability under shear is therefore maintained, which is important for consistent processing and handling in rice cake production.

The relationship between AV and spindle rotation speed for rice cake batter, as influenced by different levels of SOP substitution, is presented in Fig. 3. Higher levels of SOP in the formulation led to increased batter AV. This implies that the inclusion of SOP results in a thicker batter consistency. This thickening is likely attributable to the powder's elevated fiber and soluble solids content, which can improve water-binding capabilities and promote the formation of a more robust network structure within the batter. The increase in AV with higher levels of SOP can be attributed mainly to the presence of oat β -glucan, a soluble dietary fiber with strong water-binding and viscosity-enhancing properties. Previous studies have shown that β -glucan increases dough stiffness and reduces extensibility by limiting molecular mobility and promoting a

more structured system (Londono, Gilissen, Visser, Smulders, & Hamer, 2015). Therefore, the higher viscosity observed in this study is likely related to the functional role of β -glucan in increasing water immobilization and strengthening the batter structure in gluten-free systems.

In addition, viscosity decreased as spindle speed increased at all replacement ratios, indicating pseudoplastic behavior of the batter. Such shear-thinning behavior is commonly observed in starch- and hydrocolloid-based systems, in which molecular alignment under shear reduces resistance to flow. While the incorporation of SOP leads to an enhancement in the AV of the batter, the non-Newtonian characteristics of the rice cake batter remains unaffected, as indicated by these results.

Color Indices of Cake Batter

Fig. 4 illustrates the effect of SOP replacement ratios on the color parameters (lightness (L^*), redness (a^*), and yellowness (b^*)) of rice cake batter. As the proportion of SOP increased in the formulation, a remarkable darkening of the batter was observed, as reflected by a decrease in the lightness (L^*) index. The lowest lightness value was recorded

in the batter prepared with 100% SOP, indicating a significant reduction in brightness compared to formulations containing higher levels of rice flour.

The redness index (a^*) values of the batters ranged from -1.5 to 0.8, showing minimal variation across replacement ratios. However, a clear trend was observed in the yellowness index (b^*): as the percentage of SOP increased, the yellowness of the batter also increased. The formulation containing 100% rice flour exhibited the lowest b^* value, whereas the highest b^* value was recorded for the batter with 100% SOP. This increase in yellowness is likely attributable to the natural pigments or Maillard reaction products present in sprouted oats. The difference in yellowness between the two extremes (0% and 100% substitution) was statistically significant ($p < 0.05$), highlighting the substantial impact of SOP on the color profile of the batter.

Baking Loss

Fig. 5 presents the influence of SOP replacement levels on the weight and baking loss of rice cakes. As the proportion of SOP increased, the final weight of the baked cakes also increased. Consequently, a corresponding decrease in baking loss percentage was observed. Cake sample made with 100% rice flour exhibited the minimum post-baking weight and the maximum baking loss, while the sample formulated with 100% SOP showed the maximum weight and the minimum baking loss. The differences between these two formulations were considerable ($p < 0.05$) for both weight and baking loss parameters. The observed increase in cake weight and reduction in baking loss with higher levels of SOP may be attributed to the superior water absorption and retention capacity of sprouted oats, likely due to their higher fiber and protein content.

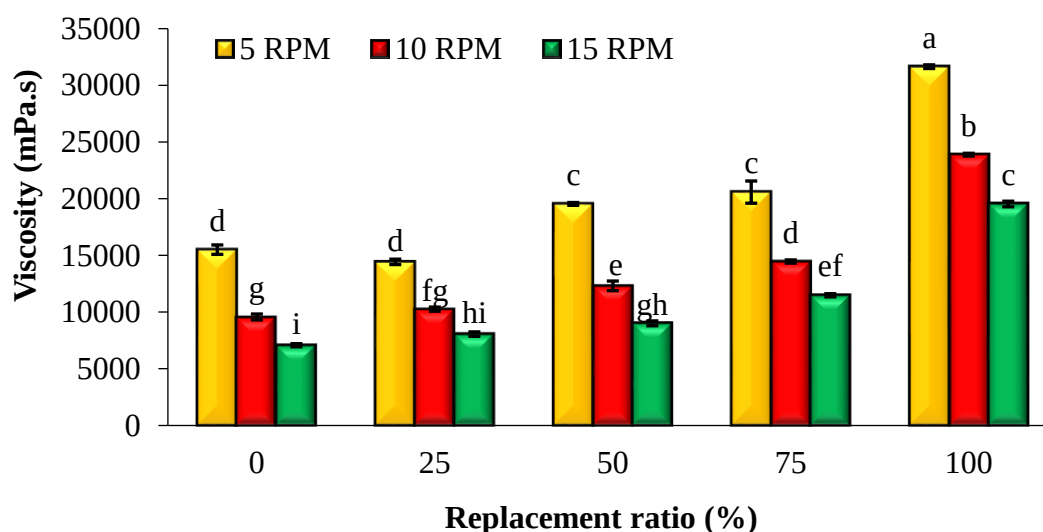


Fig. 3. Apparent viscosity of rice cake batter: influence of sprouted oat powder content and spindle rotation speed

Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

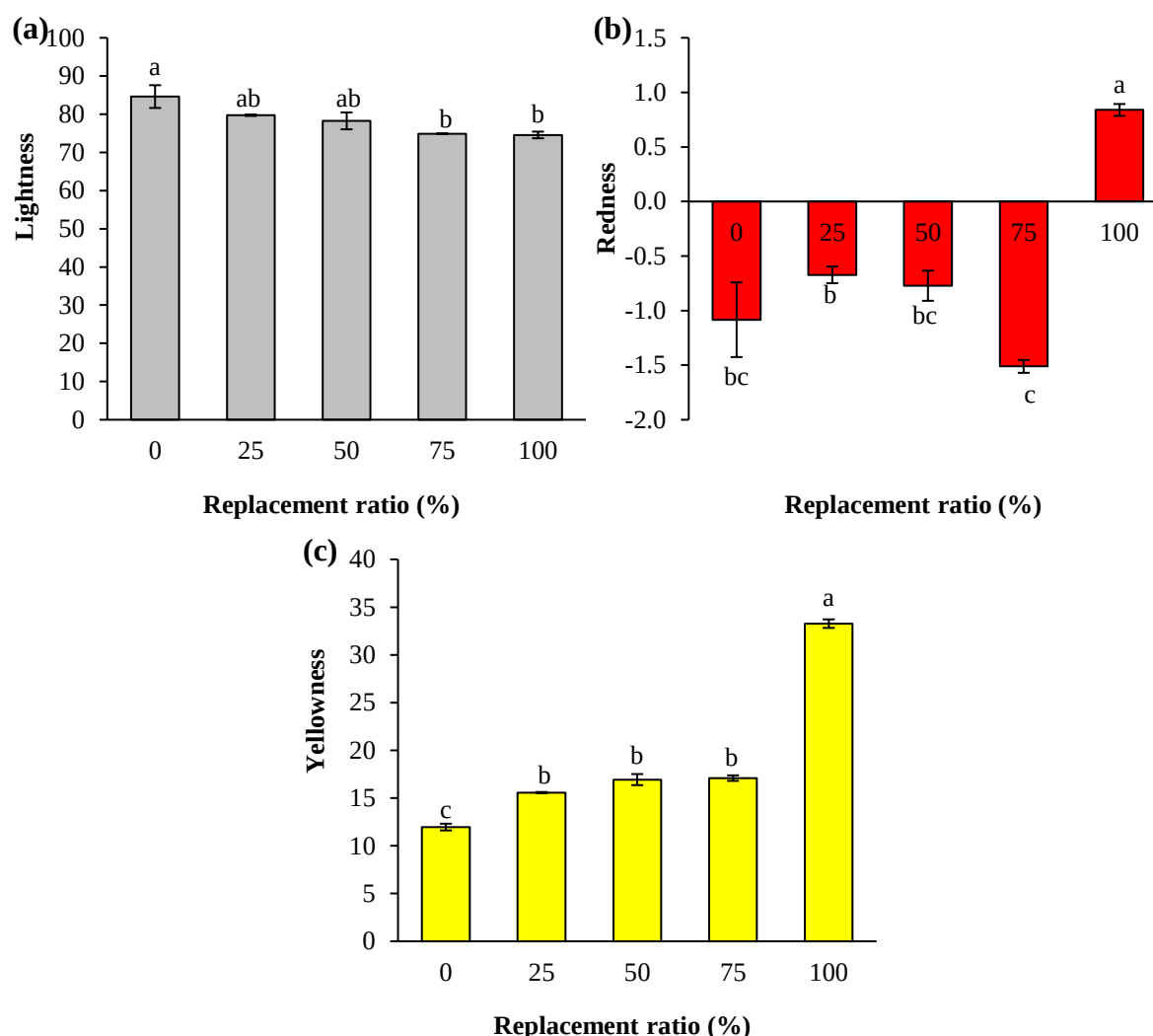


Fig. 4. Color properties of sponge cake batter as affected by sprouted oat powder incorporation
Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

These components may enhance the water-holding capacity of the batter during baking, thereby minimizing moisture evaporation and structural shrinkage. The reduced baking loss is a desirable trait, as it contributes to improved yield and product consistency in baked goods. Nazari & Gharekhani (2021) reported that increasing the level of sprouted mung bean flour in bread dough up to 15% resulted in a non-significant reduction in baking loss, indicating that moderate replacement ratios may slightly improve moisture retention during baking without significantly altering water loss dynamics.

Volume and Density

Cake volume is a key quality parameter, often used to evaluate the extent of leavening and the batter's ability to retain gas during baking. Fig. 6 displays the effect of different replacement ratios of SOP on the volume and density of rice cakes. The results show that there were no statistically significant differences in either volume or density among the various formulations ($p > 0.05$).

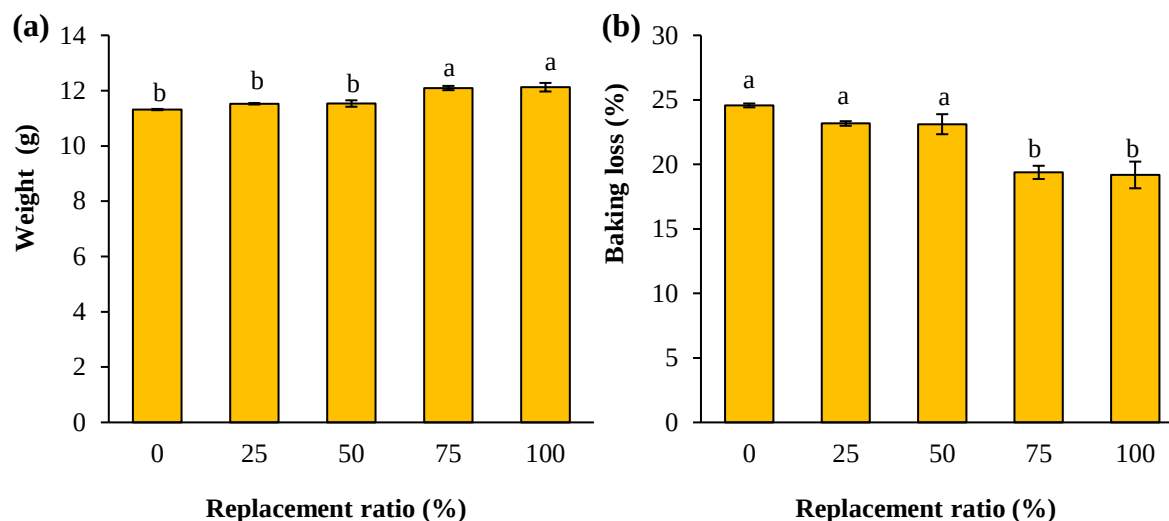


Fig. 5. Influence of sprouted oat powder addition on cake weight (a) and baking loss (b)
Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

In the present study, cake volume varied between 9.3 and 9.6 cm³, while density values fell within the range of 1202.5–1311.7 kg/m³. These findings suggest that replacing rice flour with SOP, even at high levels, did not adversely affect the cake's ability to expand or maintain

structural integrity during baking. This indicates that SOP can be used as a functional ingredient in rice cake formulations without compromising volumetric quality or increasing product compactness.

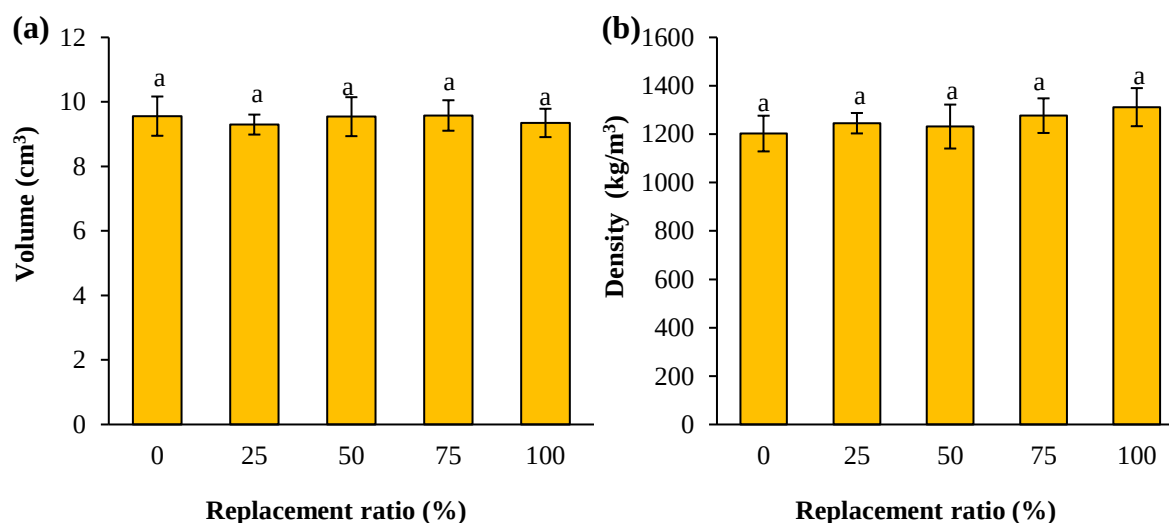


Fig. 6. Effect of sprouted oat powder substitution on the volume (a) and density (b) of rice sponge cakes
Same letters above the columns denote statistically no significant differences ($p > 0.05$).

Moisture and Ash Contents

Rice flour and SOP exhibited moisture contents of 8.17% and 6.72%, respectively. Fig. 7a illustrates the impact of SOP replacement ratio on the moisture content of cakes. Despite the slightly lower initial moisture content of

SOP, its incorporation into rice cake formulations had a notable impact on the final moisture content of the baked products. As the level of SOP substitution increased, the moisture content of the resulting sponge cakes increased. Cake sample made entirely with rice

flour (0% substitution) exhibited the minimum moisture content, while the highest value was recorded for the cake made with 100% SOP. The difference between these two formulations was statistically considerable ($p < 0.05$). The observed rise in moisture retention can likely be ascribed to the elevated fiber and protein levels in SOP, which potentially augment the water-holding capacity within the batter. These results suggest that SOP not only contributes nutritional value but also improves the functional moisture-retention properties of gluten-free sponge cakes during baking. Consistent with our findings, Balighi, Alami, & Sadeghi, (2024) investigated the impact of replacing rice flour with raw and sprouted mung bean powder (MBP) on the physicochemical attributes of gluten-free batter and cake. Their research corroborated that the inclusion of sprouted MBP can favorably affect the quality parameters of cakes. Specifically, they observed the maximum cake moisture content at a 20% replacement ratio of sprouted mung bean flour, highlighting its capacity to improve moisture retention in gluten-free baked goods.

Ash content is an indicator of the total mineral content in food products and can reflect the nutritional enhancement introduced by

ingredient substitution. Aparicio-García *et al.* (2021) observed that oat seed powder is characterized by substantial levels of both macro- and micro-minerals, with potassium (K) being the predominant mineral, followed by phosphorus (P), magnesium (Mg), and calcium (Ca). Additionally, the micro-minerals iron (Fe), zinc (Zn), and manganese (Mn) were present in notable quantities, whereas sodium (Na) and copper (Cu) were detected at lower levels. The average ash content of the rice flour and SOP used in this study was 0.7% and 2.7%, respectively. Fig. 7b illustrates the impact of SOP replacement level on the ash content of gluten-free sponge cakes. The substitution of rice flour with increasing levels of SOP led to a proportional increase in the ash content of the formulation. Cake sample made with 100% rice flour exhibited the lowest ash content, while the highest ash content was recorded for the cake containing 100% SOP. This finding is consistent with the higher intrinsic mineral content of SOP compared to rice flour. The progressive increase in ash content with higher replacement ratios indicates that incorporating SOP can effectively enhance the mineral profile of gluten-free sponge cakes, offering potential nutritional benefits without compromising product integrity.

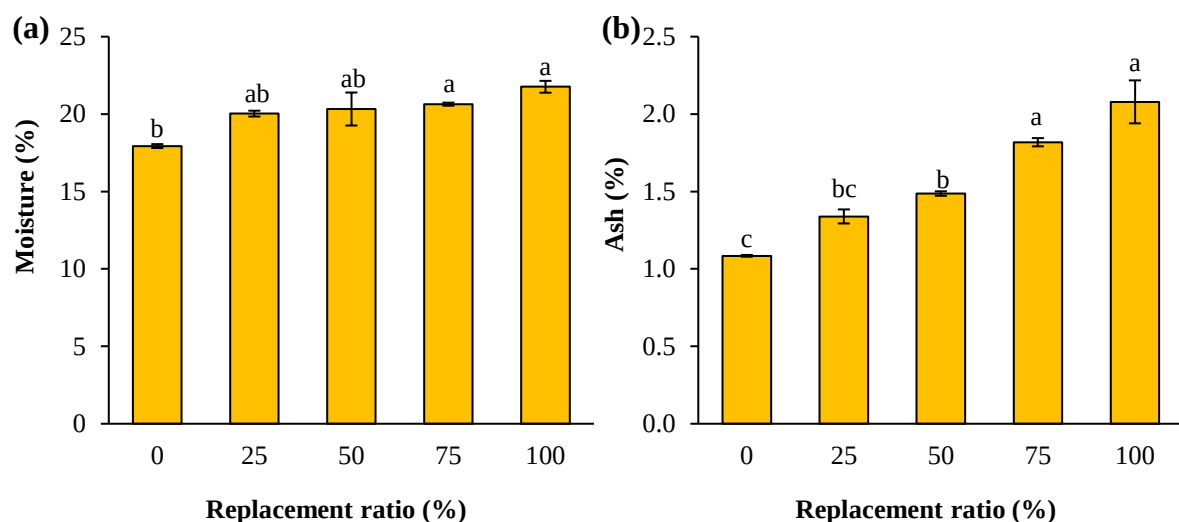


Fig. 7. Changes in moisture (a) and ash (b) contents of rice cake as influenced by different levels of sprouted oat powder incorporation

Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

pH and Acidity of Cake

The sprouting process is known to reduce the pH and increase the titratable acidity of sprouted cereal flour due to the formation of organic acids during sprouting (Fateh Nikoo, Naghipour, & Faraji, 2019). As a result, bakery products formulated with sprouted cereal flour typically exhibit lower pH and higher acidity compared to those made with non-sprouted flours (Samary *et al.*, 2025b; Vejdaniyahid & Salehi, 2025b). The average pH values of the rice flour and SOP used in this study were 6.83 and 5.65, respectively, while their corresponding average titratable acidity values were 0.25% and 1.55%. These differences reflect the biochemical changes that occur during the sprouting process, including the production of organic acids that lower pH and increase acidity.

The influence of SOP replacement on the pH and acidity of rice cakes is presented in Fig. 8. A clear trend emerged: as the concentration of SOP increased, the titratable acidity of the baked cakes increased, while the pH values

correspondingly decreased. The lowest acidity (0.29%) and highest pH (7.25) were observed in the sample containing 100% rice flour. In contrast, the highest acidity (0.83%) and lowest pH (6.73) were recorded in the sample made with 100% SOP. These differences were statistically significant ($p < 0.05$). The observed trends can be attributed to the acidic nature of SOP, which introduces a greater concentration of organic acids into the batter. This increase in acidity and decrease in pH may influence not only the flavor profile but also the shelf life and microbial status of the final product. Such changes are important considerations in the formulation of functional baked goods. Vermelho *et al.* (2024) and Vejdaniyahid & Salehi (2025b) reported that, in the baking industry, acidity resulting from organic acids plays a crucial role in inhibiting microbial growth, thereby enhancing the shelf life of bread and other baked goods, while simultaneously contributing to flavor development.

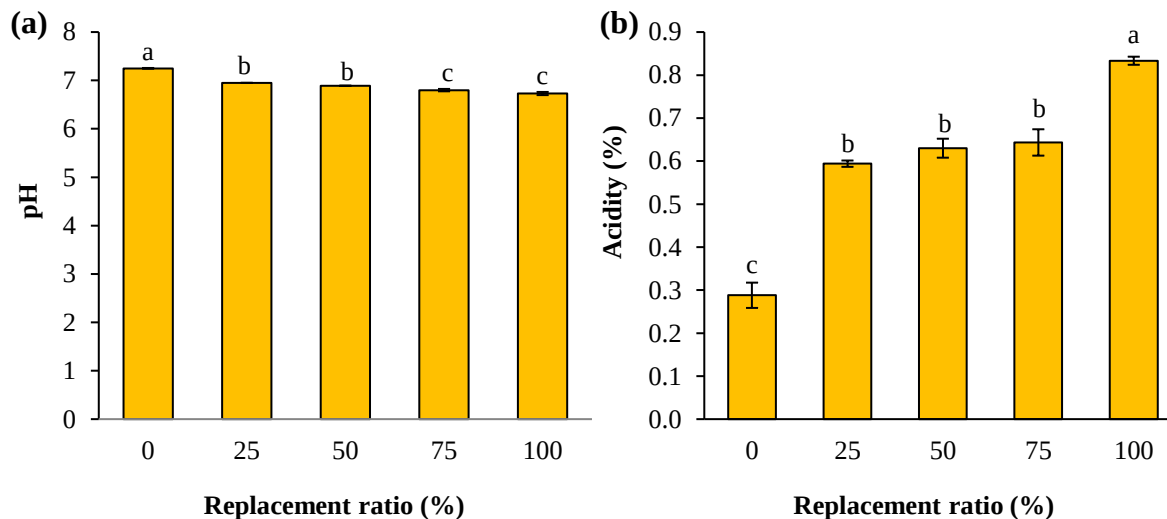


Fig. 8. Impact of sprouted oat powder incorporation level on the pH (a) and acidity (b) of rice cake
Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

TPC and AC

Sprouting has been widely reported to improve the nutritional profile of cereal grains by increasing the concentration of antioxidant, anti-inflammatory, and other bioactive compounds associated with improved

regulation of blood lipid and glucose levels (Schendel, 2019; Singh *et al.*, 2015). In the current study, the TPC and AC of the rice flour and SOP were 390.7 $\mu\text{g GAE/g}$ and 1580.2 $\mu\text{g GAE/g}$, and 17.1% and 93.6%, respectively. These values highlight the significantly higher

functional potential of SOP compared to rice flour.

The influence of SOP replacement on the TPC and AC of gluten-free rice cakes is shown in Fig. 9. A consistent trend of increasing TPC and AC was observed with higher proportions of SOP. The 100% rice flour control sample yielded the minimum TPC and AC, in contrast to the 100% SOP sample, which demonstrated the maximum values. These extreme values were statistically significant ($p < 0.05$). These findings highlight the promising role of sprouted grains, particularly SOP, as functional ingredients for developing more nutritious, antioxidant-rich, and health-promoting gluten-free bakery products.

Cao *et al.* (2022), investigated the substitution of wheat starch with SOP in bread

and reported increased polyphenol and butyric acid content, suggesting that sprouting enhances both digestibility and nutritional value. Similarly, Ahmed *et al.* (2024), evaluated the effect of sprouted wheat grain on the AC of cupcakes and found that sprouting for up to three days significantly improved the nutritional composition and AC of the final product, resulting in a healthier baked good. Moreover, Aparicio-García *et al.* (2021), evaluated sprouted oat as a promising gluten-free ingredient with improved nutritional and bioactive characteristics and reported that sprouting increased the levels of free phenolic compounds, γ -aminobutyric acid (GABA), and AC in oat powder.

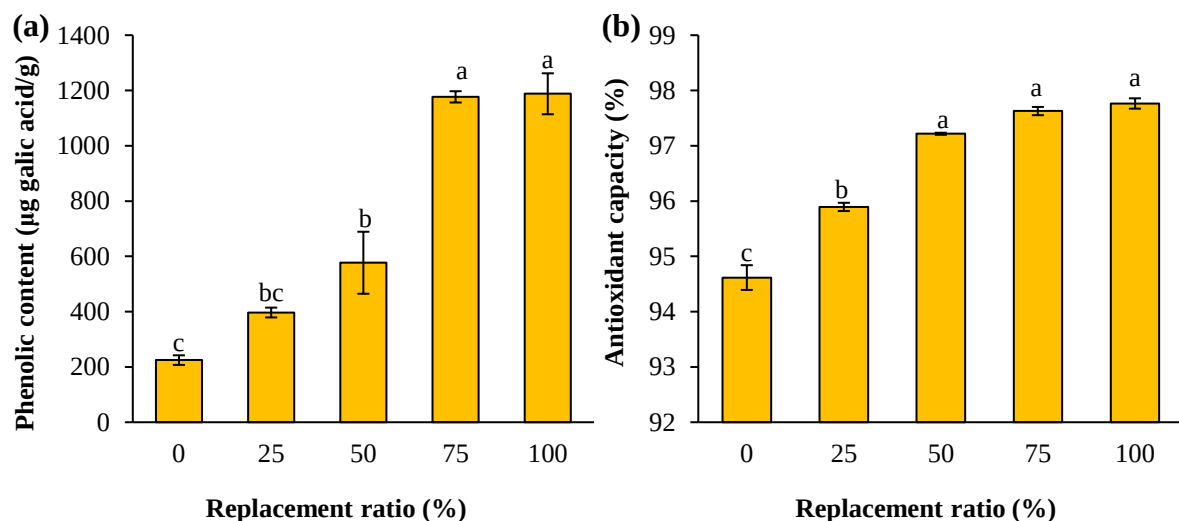


Fig. 9. Impact of sprouted oat powder incorporation level on the total phenolic content (a) and antioxidant capacity (b) of rice cake

Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

Crust and Crumb Color Indexes of Cake

The sprouting process enhances enzymatic activity, which promotes both enzymatic and non-enzymatic browning reactions, ultimately leading to a reduction in product lightness (Ahmed *et al.*, 2024; Javaheripour, Shahsoni Mojarad, Mahdikhani, & Inanloo, 2022; Vejdaniyahid & Salehi, 2025a). In this study, the color parameters of the rice flour and SOP used in the formulations were notably different. The rice flour exhibited a lightness (L^*) value

of 97.31, a redness (a^*) value of 0.01, and a yellowness (b^*) value of 3.54, indicating its bright and neutral color profile. In contrast, the SOP showed a lower lightness value of 91.31, along with higher redness ($a^* = 2.32$) and yellowness ($b^* = 11.12$) values, reflecting its darker and more intensely pigmented appearance. These intrinsic color differences between the two flours help explain the observed reductions in cake lightness and the

increase in crumb redness and yellowness as the level of SOP substitution increased.

Fig. 10 presents the impact of SOP incorporation level on the crust and crumb color parameters of gluten-free rice cakes. As the proportion of SOP increased, both crust and crumb lightness values decreased significantly. The control sample made with 100% rice flour exhibited the highest lightness values, while the sample containing 100% SOP showed the lowest. In this study, crust lightness values ranged from 45.2 to 65.8, and crumb lightness ranged from 61.9 to 89.0. Similar observations were made by [Javaheripour et al. \(2022\)](#), who reported decreased lightness and darkening of sponge cakes prepared with sprouted wheat flour, attributing the changes to elevated enzymatic activity and the accumulation of reducing sugars during sprouting. Likewise, [Ahmed et al. \(2024\)](#), found that sprouting wheat grains led to significant reductions in cupcake lightness, from 51.79 in the control to 43.17 and 32.73 after 1 and 5 days of sprouting, respectively. [Nazari & Gharekhani \(2021\)](#), reported that the L^* index of gluten-free bread decreased as the proportion of sprouted mung bean flour replacing rice flour increased. This decline in lightness was attributed to the darker color of sprouted mung bean flour, as well as the enhanced browning reactions associated with sprouting.

In terms of the redness index (a^*) of the cake crust, no consistent trend was observed across different replacement ratios. However, a considerable increase ($p < 0.05$) in crumb redness values was detected with higher levels of SOP substitution. This suggests that while surface browning may be influenced by complex interactions during baking, the internal color development of the crumb is more directly affected by the increasing concentration of pigments and reducing sugars associated with SOP. The redness values of the cake crust were notably higher than those of the crumb, likely due to non-enzymatic browning

reactions that predominantly occur on the surface during baking. In the present study, the redness index of the crust ranged from 20.3 to 23.2, while the crumb ranged from -2.8 to 9.3.

Regarding the yellowness index (b^*), increasing the proportion of SOP led to a rise in crumb yellowness, whereas the yellowness of the crust showed a decreasing trend. These contrasting patterns may result from differences in moisture loss, pigment degradation, and thermal reaction intensity between the crust and crumb during baking. In the present study, the yellowness index of the crust ranged from 25.6 to 52.8, while the crumb ranged from 25.0 to 47.5.

Protein and Fat Contents

The protein content of SOP was notably high, reaching 10.7%, highlighting its potential as a nutrient-rich ingredient for gluten-free formulations ([Aparicio-García et al., 2021](#)). The protein content of the rice flour and SOP used in this study was 9.8% and 13.0%, respectively. This difference reflects the naturally higher protein content of oats, which can be further enhanced through the sprouting process due to increased enzymatic activity and nutrient bioavailability. Fig. 11a illustrates the impact of SOP replacement ratio on the protein content of gluten-free rice cakes. Protein content increased progressively as the level of SOP incorporated into the formulation increased. The maximum protein content was recorded in the cake made with 100% SOP. This trend demonstrates that SOP can serve as an effective protein enhancer in gluten-free baked products. The increase in protein content is particularly relevant for improving the nutritional quality of gluten-free formulations, which often lack sufficient protein. Incorporating SOP may therefore contribute to the development of more balanced and nutritionally adequate gluten-free products.

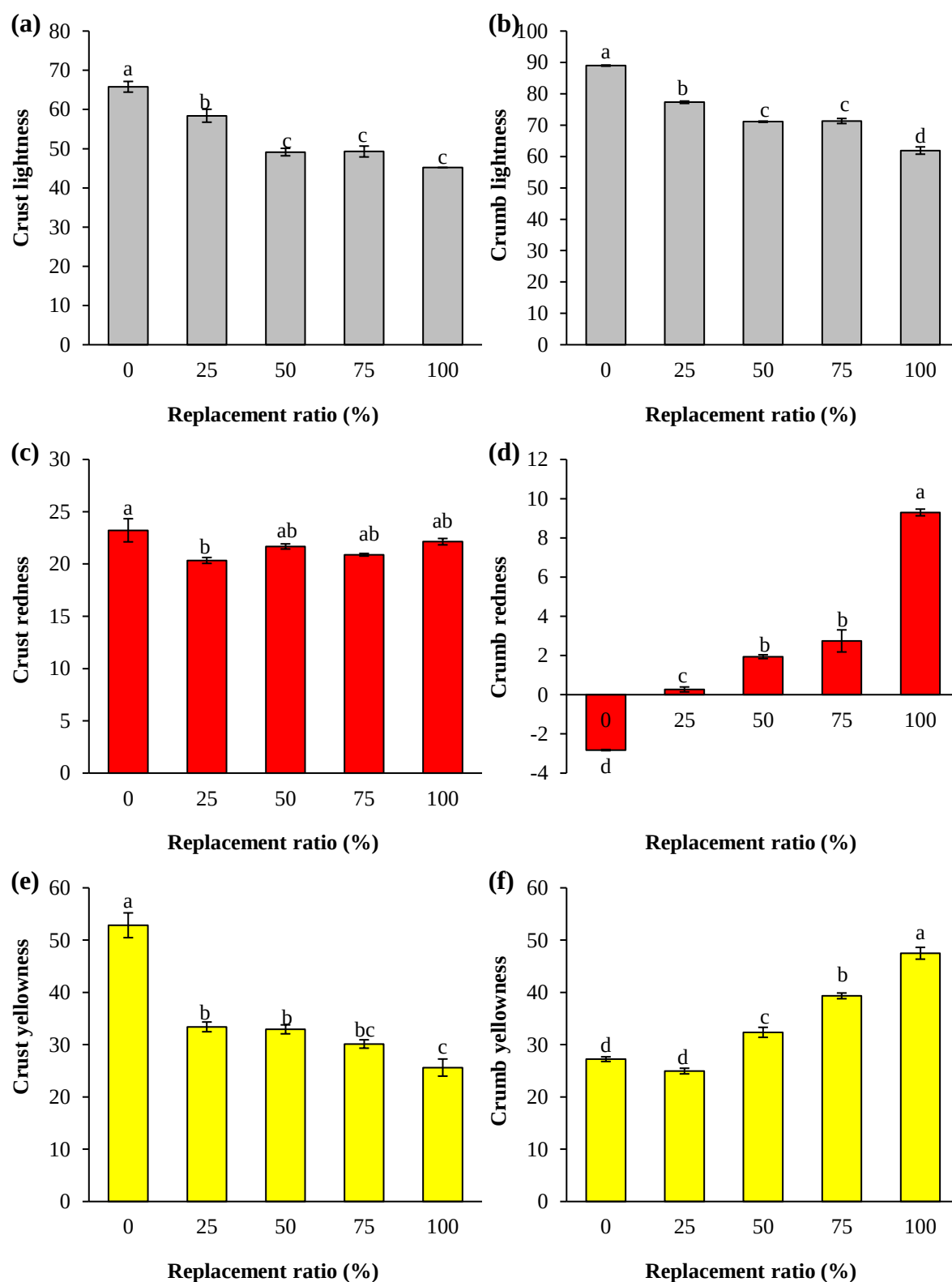


Fig. 10. Impact of sprouted oat powder incorporation level on the lightness (crust (a) and crumb (b)), redness (crust (c) and crumb (d)), and yellowness (crust (e) and crumb (f)) of rice cake
Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

Aparicio-García *et al.* (2021), reported the fatty acid composition of oat powder, with linoleic, oleic, and palmitic acids representing the majority (>94%) of total fatty acids. Also, SOP exhibited a similar overall profile; however, sprouting significantly increased the levels of palmitoleic, linoleic, α -linolenic, and cis-11-eicosenoic acids, while oleic acid decreased by approximately 3%, indicating that sprouting can enhance the nutritional quality of oat lipids. The average fat content of the rice flour and SOP used in this study was 1.3% and 2.5%, respectively. These values reflect the naturally higher lipid content of oats compared to rice, which could potentially influence the fat content of the final product when used as a substitute. Fig. 11b demonstrates the effect of SOP replacement ratio on the fat content of gluten-free rice cakes. Unlike the trends

observed for ash and moisture content, no consistent or statistically significant pattern was evident in the fat content across the different replacement ratios. The fat content of the cakes ranged from 22.3% to 24.1%, regardless of the proportion of SOP used. This lack of a clear trend in fat content may be attributed to several factors, including interactions between ingredients during baking, changes in the moisture content of the product, and variations in the amount of oil absorbed by the paper cake pans. Additionally, differences in fat retention and the influence of other formulation components could have contributed to the observed variability. Nonetheless, the overall fat content remained within a relatively narrow range, suggesting that substitution with SOP does not considerably alter the lipid level of the final product.

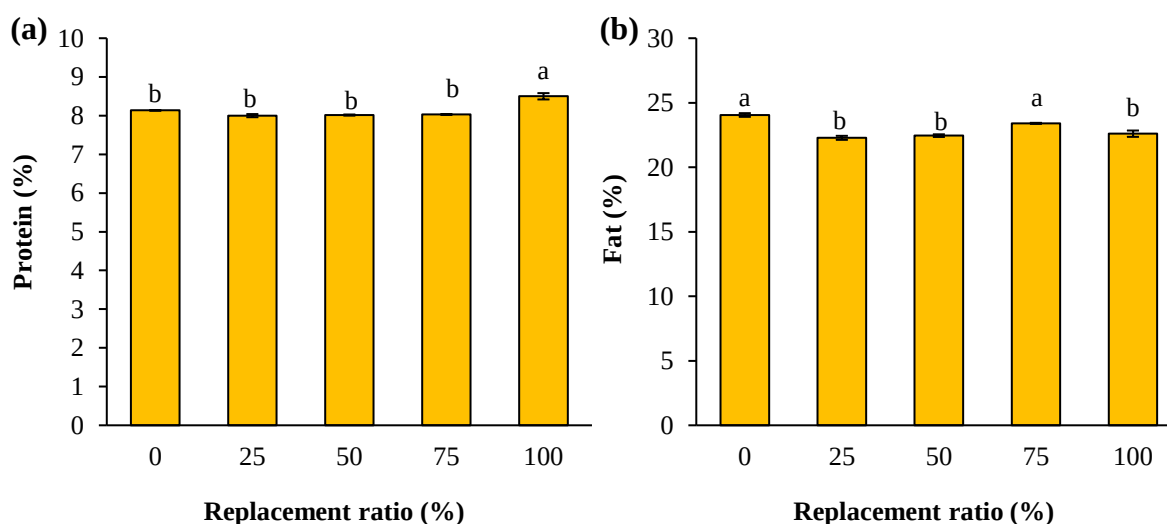


Fig. 11. Changes in protein (a) and fat (b) contents of rice cakes as affected by the incorporation level of sprouted oat powder

Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

Puncture and TPA Tests Results

Among the paramount quality indicators for food products, texture stands out as particularly crucial, especially in bakery goods like cake, assume a pivotal role in the food industry and consumer perception and acceptance. Among the various textural parameters, crust hardness is especially important as it influences the initial mouthfeel and overall eating experience. The influence of SOP substitution on crust

hardness in gluten-free rice cakes is shown in Fig. 12. As the level of SOP increased, crust hardness increased correspondingly. The control sample prepared with 100% rice flour recorded the minimum hardness (0.04 N), while the sample formulated entirely with SOP showed the maximum hardness (0.55 N), with significant differences between them ($p < 0.05$). Our results align with the observations of Javaheripour *et al.* (2022), who documented a

notable enhancement in the textural firmness of sponge cakes when sprouted wheat flour was integrated into their formulations.

In alignment with our results, [Nazari & Gharekhani \(2021\)](#), examined the effects of replacing rice flour with both raw and sprouted MBP on the TPC and physicochemical attributes of gluten-free bread. They reported

that the inclusion of sprouted MBP, particularly up to a 5% substitution rate, enhanced bread volume in rice flour-based formulations. Furthermore, their study showed that higher levels of MBP in the formulation resulted in a significant increase in TPC and greater sample hardness.

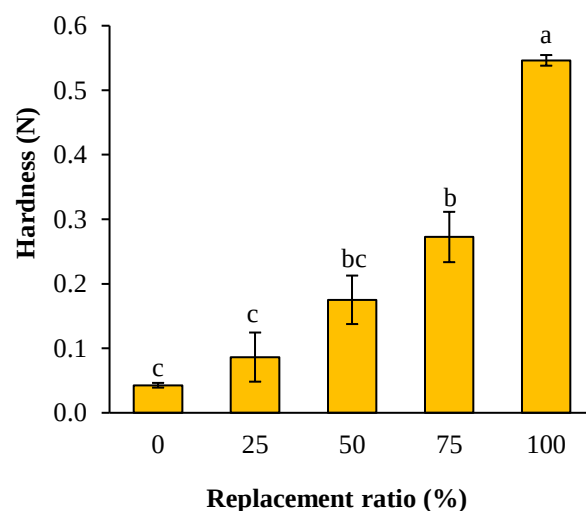


Fig. 12. Impact of sprouted oat powder incorporation level on the crust hardness (puncture test) of rice cake
Distinct letters above the columns denote statistically significant differences at the $p < 0.05$ level.

[Table 1](#) presents the effect of SOP replacement ratio on the textural properties of gluten-free rice cakes, as measured by the texture profile analysis (TPA) test. The results indicate that there were no statistically significant differences ($p > 0.05$) in either firmness or cohesiveness among the various formulations. Firmness values ranged from 2.08 N to 3.80 N, while cohesiveness values varied from 0.33 to 0.63. These findings suggest that the replacement of rice flour with SOP, even at high replacement ratios, did not negatively affect the crumb structure or internal firmness of the cakes, thereby maintaining overall textural integrity during baking. In terms of springiness and chewiness, no consistent trends were observed across the samples. The differences between treatments were generally not significant, further supporting the stability of the cake's internal texture. Springiness values ranged from 0.22 to

0.50, while chewiness values ranged from 0.16 N to 1.07 N. Overall, the TPA results suggest that SOP can be effectively incorporated into gluten-free rice cake formulations without compromising key textural attributes, making it a suitable functional ingredient for improving the nutritional value of baked goods while preserving desirable sensory qualities.

Sensory Evaluation

The impact of SOP replacement level on the sensory attributes of rice cakes is presented in [Table 2](#). Due to the naturally darker color of SOP compared to rice flour, its incorporation into the cake formulation resulted in a darker crust and crumb. As the replacement ratio increased, the visual darkness of the cakes intensified, leading to lower sensory scores for crust lightness, crumb lightness, and overall appearance. The highest scores for these parameters were observed in the control sample containing 100% rice flour, which showed a

statistically significant difference ($p < 0.05$) compared to the sample made with 100% SOP.

Table 1- Impact of sprouted oat powder incorporation level on the textural properties (texture profile analysis test) of sponge cake

Replacement ratio	Firmness (N)	Cohesiveness	Springiness	Chewiness (N)
0%	3.80±1.37 ^a	0.57±0.04 ^a	0.50±0.08 ^a	1.07±0.41 ^a
25%	2.08±0.07 ^a	0.63±0.27 ^a	0.44±0.12 ^a	0.64±0.43 ^{ab}
50%	2.69±0.70 ^a	0.45±0.04 ^a	0.34±0.12 ^{ab}	0.46±0.25 ^{ab}
75%	2.14±0.42 ^a	0.33±0.03 ^a	0.22±0.02 ^b	0.16±0.05 ^b
100%	2.77±0.05 ^a	0.45±0.07 ^a	0.31±0.04 ^{ab}	0.39±0.04 ^{ab}

Data are shown as mean ± standard deviation (N = 3). Distinct letters within the columns denote statistically significant differences at the $p < 0.05$ level.

In terms of aroma, a considerable variation was only observed in the complete replacement cake (100% SOP), which received the lowest score among all formulations. A similar trend was noted for flavor, texture, and overall acceptance, with the 100% SOP sample consistently receiving the lowest ratings. However, consistent with the instrumental TPA results, no significant differences were detected in perceived texture firmness among the samples, suggesting that the mechanical and

sensory evaluations of this parameter are in agreement.

Overall, the sensory evaluation results indicate that up to 75% of rice flour can be successfully substituted with SOP in gluten-free rice cake formulations without negatively impacting key sensory attributes such as aroma, flavor, texture, or overall acceptance. This finding supports the use of SOP as a functional ingredient capable of enhancing the nutritional profile of gluten-free products while maintaining consumer acceptability.

Table 2- Changes in sensory attributes of sponge cake with different levels of sprouted oat powder

Replacement ratio	Crust lightness	Crumb lightness	Appearance acceptance	Aroma acceptance	Flavor acceptance	Firmness	Texture acceptance	Overall acceptance
0%	8.2±1.2 ^a	8.3±1.3 ^a	7.9±1.2 ^a	6.6±2.0 ^a	7.2±1.8 ^a	5.6±1.9 ^a	6.9±1.8 ^a	7.5±1.6 ^a
25%	7.2±1.5 ^{ab}	7.2±1.6 ^{ab}	7.4±1.4 ^a	6.5±1.8 ^a	6.7±1.7 ^a	6.3±1.8 ^a	6.8±1.4 ^{ab}	7.2±1.3 ^a
50%	6.8±1.4 ^{bc}	6.8±1.3 ^{bc}	7.1±1.2 ^{ab}	7.1±1.3 ^a	6.8±1.1 ^a	6.1±1.4 ^a	6.4±1.1 ^{ab}	7.2±0.6 ^a
75%	6.0±1.7 ^{cd}	6.0±1.5 ^{cd}	6.2±1.2 ^{bc}	6.6±1.2 ^a	6.1±1.3 ^{ab}	6.1±1.5 ^a	6.4±1.3 ^{ab}	6.7±1.0 ^a
100%	5.4±1.7 ^d	5.4±1.7 ^d	5.3±1.4 ^c	5.3±1.8 ^b	5.5±1.3 ^b	5.8±2.0 ^a	5.7±1.1 ^b	5.1±1.6 ^b

Data are shown as mean ± standard deviation (N = 20). Distinct letters within the columns denote statistically significant differences at the $p < 0.05$ level.

Conclusion

Considering the growing interest in nutrient-enriched bakery products, the present work examined the application of SOP as a partial substitute for rice flour in the formulation of gluten-free sponge cake. The present study comprehensively examined the impact of replacing rice flour with SOP on the quality attributes of rice cakes. The findings indicate

that such substitution significantly modified and, in some aspects, enhanced the nutritional properties of the final product, particularly by increasing weight, moisture, ash, acidity, TPC, AC, and protein. These enhancements are primarily attributed to the compositional richness of SOP, which contains higher levels of ash (2.7%), TPC (1580.2 µg GAE/g), AC

(93.6%), and protein (13.0%) compared to rice flour.

Despite these nutritional improvements, the substitution also influenced the quality of the cakes. The lightness (L^*) of both batter and cake decreased significantly with increasing sprouted oat content due to intensified Maillard and enzymatic browning reactions during baking. Texture analysis revealed that crust hardness increased with higher levels of substitution, yet the internal crumb firmness, cohesiveness, springiness, and chewiness were largely unaffected. This suggests that the structural integrity of the cakes remained stable even at elevated levels of substitution. The greatest sensory differences among the cake samples were associated with their color. The 100% sprouted oat sample received the lowest scores in crust/crumb color, aroma, and overall acceptability. However, cakes containing up to 75% SOP substitution were well-accepted by the panelists in terms of taste, texture, and general appeal, showing no significant differences from the control. In conclusion, SOP presents a promising functional ingredient

for use in gluten-free baking products. Its incorporation enhances the nutritional profile of rice cakes without compromising key quality attributes, particularly when replacement ratio does not exceed 75%.

Founding Sources

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Author Contributions

Alaa Hussein Allawi Allawi: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Fakhreddin Salehi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing–original draft, Writing–review and editing. **Amir Daraei Garmakhany:** Conceptualization, Methodology, Supervision, Writing–original draft.

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مقاله پژوهشی

جلد ۲۲، شماره ۵، آذر-دی ۱۴۰۵، ص. ۵۵۷-۵۷۸

تأثیر پودر جودوسر جوانه زده به عنوان جایگزین آرد برنج بر ویژگی های فیزیکوشیمیایی، بافتی و حسی کیک اسفنجی بدون گلوتن

علاء حسین علاوی^۱ - فخرالدین صالحی^{۱*} - امیر دارایی گرمه خانی^۲

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چکیده

این مطالعه تأثیر جایگزینی آرد برنج با پودر جودوسر جوانه زده را در سطوح مختلف (۰ تا ۱۰۰ درصد) بر ویژگی های فیزیکوشیمیایی، بافتی و حسی کیک برنجی بدون گلوتن ارزیابی کرد. آنالیز رئولوژیکی، رفتار سودوپلاستیک را نشان داد؛ به طوری که با افزایش سرعت برشی، ویسکوزیته ظاهری کاهش یافت، در حالی که مقادیر بالاتر پودر جودوسر جوانه زده منجر به افزایش ویسکوزیته خمیر شد. افزودن پودر جودوسر جوانه زده منجر به کاهش شاخص روشنایی در خمیر و کیک شد که کمترین میزان روشنایی در نمونه ۱۰۰ درصد جودوسر ثبت گردید. شاخص زردی خمیر با افزایش جایگزینی به طور معنی داری افزایش یافت، در حالی که شاخص قرمزی روند ثابتی نشان نداد. جایگزینی با جودوسر به طور معنی داری وزن کیک را افزایش و افت پخت را کاهش داد. با این حال، تأثیر آماری معنی داری بر حجم یا دانسیته کیک نداشت. کیک های تهیه شده با سطوح بالاتر پودر جودوسر جوانه زده، مقدار رطوبت (از ۹۳/۱۷ به ۷۶/۲۱ درصد) و خاکستر (از ۰/۸/۱ به ۰/۸/۲ درصد) بیشتری نشان دادند که بازتاب دهنده غنای تغذیه ای آرد جوانه زده است. بیشترین اسیدیته (از ۰/۸۳ درصد) و کمترین pH (۶/۷۳) در نمونه تهیه شده با ۱۰۰ درصد پودر جودوسر جوانه زده ثبت شد. با افزایش سطح جایگزینی پودر جودوسر جوانه زده، محتوای فنل کل و ظرفیت آنتی اکسیدانی کیک ها نیز به تناسب افزایش یافت. محتوای پروتئین کیک های نهایی نیز به طور متناسب افزایش یافت (تا ۴۹/۴ درصد در نمونه ۱۰۰ درصد جودوسر)، در حالی که محتوای چربی در تمامی فرمولاسیون ها نسبتاً ثابت باقی ماند. افزایش سطوح جایگزینی باعث افزایش سختی پوسته کیک شد (از ۰/۴/۰ نیوتن به ۵۵/۰ نیوتن)، در حالی که نتایج تحلیل پروفیل بافت (TPA) تغییر معنی داری را در سفتی یا انسجام مغز کیک نشان نداد. ارزیابی حسی نشان داد که جایگزینی تا ۷۵ درصد بدون تأثیر منفی بر عطر، طعم، بافت یا پذیرش کلی قابل قبول است، اگرچه سطوح بالاتر جایگزینی، تأثیر منفی بر رنگ پوسته و مغز کیک داشت.

واژه های کلیدی: پروتئین، چربی، خمیر، محتوای فنلی کل، ویسکوزیته

۱- گروه علوم و صنایع غذایی، دانشکده صنایع غذایی، دانشگاه بوعلی سینا، همدان، ایران

*- نویسنده مسئول: (Email: F.Salehi@basu.ac.ir)

۲- گروه مهندسی علوم و صنایع غذایی، دانشکده فنی و منابع طبیعی تویسرکان، دانشگاه بوعلی سینا، همدان، ایران

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

مندرجات

مقالات پژوهشی

- ۴۷۲ تأثیر روش‌های غیر حرارتی بر قابلیت چاپ و خواص عملکردی جوهر غذایی مبتنی بر پروتئین کینوا
مژده صراف - سارا ناجی طبسی - بهروز قرآنی - بهاره عمادزاده - عمر سعید توکر
- ۴۹۰ مهار قهوه‌ای شدن آنزیمی در آب انبه با استفاده از عصاره‌های پوست بادمجان غنی از آنتی اکسیدان: یک مطالعه سینتیکی و نگهداری
می جی آمین الدباغ - اودی اچ علی الجماس - رعنا تی ابراهیم التایی - عمر وای العباسی
- ۵۲۵ تأثیر روش‌های استخراج هندی و نیمه‌صنعتی بر کیفیت و مقبولیت آیزین گلاس تولید شده از کیسه شنای فیل ماهی (*Huso huso*)،
تاس ماهی ایرانی (*Acipenser persicus*) و ازون‌برون (*Acipenser stellatus*) پرورشی
مینا سیف زاده - انوشه کوچکیان صبور
- ۵۴۱ ارزش آفرینی از لجن لبنی به‌عنوان بستر کم‌هزینه برای افزایش تولید زیست‌توده و اگزوپلی ساکارید توسط *انتروکوکوس دورانس*
K48
نازنین طاهری نسب - محمد رضا عدالتیان دوم - مرضیه معین فرد - لیلا روزبه نصیرایی
- ۵۵۶ جداسازی، خالص‌سازی و شناسایی پروتئین‌ها و پپتیدهای موجود در گوشت میوه کدویی (*Crescentia cujete* L.) با ویژگی‌های
ضد فشارخون و کاهنده چربی خون
جریس ویلانوا - اندرس گادوین ساجیسی - آنتونیو لائورنا - ماری آن توریو - ورونیکا میگو
- ۵۷۸ تأثیر پودر جودوسر جوانه‌زده به‌عنوان جایگزین آرد برنج بر ویژگی‌های فیزیکوشیمیایی، بافتی و حسی کیک اسفنجی بدون گلوتن
علاء حسین علاوی - فخرالدین صالحی - امیر دارایی گرمه‌خانی

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با شماره پروانه ۱۲۴/۸۴۷ و درجه علمی-پژوهشی شماره ۳/۱۱/۸۱۰ از وزارت علوم، تحقیقات و فناوری
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آذر - دی ۱۴۰۵

شماره ۵

جلد ۲۲

صاحب امتیاز: دانشگاه فردوسی مشهد

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سر دبیر: دکتر مسعود پاورمنش

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دکتر مرتضی خمیری

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دکتر کلیفورد نکمناسو اوبی

دکتر ساموئل ایوفمی اولالکان آدیبه

میکروبیولوژی و بیوتکنولوژی، دانشگاه فردوسی مشهد

میکروبیولوژی مواد غذایی، دانشگاه فردوسی مشهد

میکروبیولوژی، دانشگاه فردوسی مشهد

میکروبیولوژی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان

مهندسی و خواص بیوفیزیک مواد غذایی، دانشگاه فردوسی مشهد

شیمی مواد غذایی، دانشگاه فردوسی مشهد

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مهندسی مواد غذایی، دانشگاه علوم کشاورزی و منابع طبیعی گرگان

تکنولوژی مواد غذایی، دانشگاه فردوسی مشهد

مهندسی مواد غذایی، دانشگاه فردوسی مشهد

مهندسی مواد غذایی، دانشگاه تبریز

بیوتکنولوژی مواد غذایی، دانشگاه صنعتی شریف

نانو فناوری مواد غذایی، مؤسسه پژوهشی علوم و صنایع غذایی، مشهد

زیست مولکولی، بیمارستان زنان و بزرگام، ایالت متحده آمریکا

میکروبیولوژی غذایی، دانشگاه متروپولیتن لندن

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مهندسی عمران و محیط زیست، دانشگاه آریزونا

مهندسی تولید مواد غذایی، دانشگاه فنی دانمارک

بیوتکنولوژی مواد غذایی، دانشگاه صنعتی شریف

گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه ارومیه

گروه علوم و صنایع غذایی، دانشگاه صنعتی اصفهان

گروه علوم و صنایع غذایی، دانشگاه تربیت مدرس

گروه علوم تغذیه و مواد غذایی، دانشگاه پورتو، پرتغال

گروه علوم مولکولی، دانشکده منابع طبیعی و علوم کشاورزی، دانشگاه سوئد

گروه میکروبیولوژی، دانشگاه کشاورزی مایکل اوکپارا، ایالت ابیا، نیجریه

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جلد ۲۲ شماره ۵
سال ۱۴۰۵

شاپا: ۴۱۶۱-۱۷۳۵

شماره پیاپی ۱۰۱

عنوان مقالات

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- تأثیر روش های غیرحرارتی بر قابلیت چاپ و خواص عملکردی جوهر غذایی مبتنی بر پروتئین کینوا ۴۷۲
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- مهار قهوه ای شدن آنزیمی در آب انبه با استفاده از عصاره های پوست بادمجان غنی از آنتی اکسیدان:
یک مطالعه سینتیک و نگهداری ۴۹۰
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- تأثیر روش های استخراج هندی و نیمه صنعتی بر کیفیت و مقبولیت آیزین گلاس تولید شده از کیسه شای فیل ماهی
(*Huso huso*)، تاس ماهی ایرانی (*Acipenser persicus*) و ازون برون (*Acipenser stellatus*) پرورشی ۵۲۵
مینا سیف زاده- انوشه کوچکیان صبور
- ارزش آفرینی از لجن لبنی به عنوان بستری کم هزینه برای افزایش تولید زیست توده و اگزوپلی ساکارید
توسط *انتروکوکوس دورانس* K48 ۵۴۱
نازنین طاهری نسب- محمد رضا عدالتیان دوم- مرضیه معین فرد- لیلا روزبه نصیرایی
- جداسازی، خالص سازی و شناسایی پروتئین ها و پپتیدهای موجود در گوشت میوه کدویی
(*Crescentia cujete* L.) با ویژگی های ضد فشارخون و کاهنده چربی خون ۵۵۶
جریس ویلانوا- اندرس گادوین ساجیسی- آنتونیو لائورنا- ماری آن توریو- ورونیکا میگو
- تأثیر پودر جودوسر جوانه زده به عنوان جایگزین آرد برنج بر ویژگی های فیزیکوشیمیایی، بافتی و حسی
کیک اسفنجی بدون گلوتن ۵۷۸
علاء حسین علاوی علاوی- فخرالدین صالحی- امیر دارایی گرمه خانی