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DEVELOPMENT AND APPLICATION OF PLANT-BASED PROTEINS FOR APPLE JUICE CLARIFICATION

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Abstract

Fruit juice clarification is important for ensuring product quality. However, conventional fining agents, such as gelatine, are derived from animal sources. This raises ethical and environmental concerns, as well as conflicting with vegan diets. This research addresses the demand for sustainable alternatives by utilizing legume proteins, specifically those derived from green pea pods and red beans.

The materials used in this study were plant-based proteins derived from green pea and red bean by-products, which were applied at low, medium, and high concentrations. The proteins were obtained using a traditional alkaline extraction (AE) method. A comparative analysis was conducted to determine the efficacy of the aforementioned agents when used in combination with mineral agents, such as GranuBent Pore-Tec (bentonite) and KlarSol 30 (silicic acid). The commercial fining agents included in the study were ErbiGel (gelatine) and LittoFresh Liquid (plant-protein-based). The data were analysed using the following methods: turbidity (NTU), transmittance (T%), antioxidant capacity (FRAP), and total polyphenol content (TPC). Statistical evaluation was conducted using a one-way analysis of variance (ANOVA).

The results of this research demonstrate that plant-based legume proteins effectively contribute to the reduction of turbidity, though higher protein concentrations do not necessarily result in greater clarification efficiency. The investigated proteins exhibited notable advantages in transmittance and colour stability. Notably, applying red bean protein at a medium concentration alongside bentonite emerged as the optimal treatment, delivering promising clarification efficacy while successfully preserving bioactive compounds such as FRAP and TPC.

Given the summary of results, this study demonstrates that novel proteins derived from legume by-products possess significant potential as vegan-friendly clarification agents. In particular, the optimal pairing of medium-concentration red bean protein with bentonite yields a proven dual benefit: achieving clarity while simultaneously maintaining the product's valuable bioactive profile.

***Keywords**: Plant-based proteins, Traditional alkaline extraction, Juice clarification, Green pea, Red bean.*

1. Introduction

The clarification of fruit juices is a fundamental step in modern food processing technology, essential for ensuring product purity, visual appeal, and shelf stability. Freshly pressed juices, such as apple juice, contain a significant amount of suspended solids, pectins, starches, and plant cell wall materials (Berutu *et al.*, [1]). These substances create colloidal systems that cause high turbidity and undesirable increases in viscosity, necessitating targeted fining processes to achieve physical and chemical stabilization (Uzuner [2]). Furthermore, haze formation in fruit juices is heavily influenced by complex interactions between naturally occurring proteins and polyphenolic compounds, which bind together to form insoluble aggregates over time (Granato *et al.*, [3]).

Historically, the beverage industry has relied heavily on animal-derived proteins, specifically gelatin, as the primary fining agent. Gelatine, often combined with silicic acid or bentonite, carries a positive charge in low-pH environments, allowing it to react effectively with negatively charged phenolic compounds to rapidly remove suspended matter. However, given that gelatine is derived from animal connective tissues, its extensive application gives rise to considerable religious, ethical, and environmental concerns. The replacement of animal-based gelatine is increasingly driven by vegetarian, halal, and kosher market demands, pushing the industry toward sustainable, "green" hydrocolloids (Russo Spena *et al.*, [4]). While alternative sources like fish-derived gelatine meet some religious dietary laws, they fail to satisfy the rapidly growing consumer demand for strictly vegan products.

Consequently, the development of sustainable, plant-based alternatives has become an industry necessity. Fining agents derived from plant proteins, such as cereals, potatoes, and legumes, have emerged as highly viable substitutes due to their excellent functional properties and lack of allergenic risks (Marangon *et al.*, [5]). For example, soy and pea protein isolates have demonstrated clarification efficiencies comparable to traditional animal-protein agents, effectively reducing turbidity without the need for strict allergen labelling required for animal proteins like casein or egg albumin (Ficagna *et al.*, [6]). These plant-derived proteins are not only effective at flocculation but also interact selectively with phenolic compounds to modify astringency and colour characteristics, mimicking the desirable effects of traditional gelatine (Versari *et al.*, [7]). Recent processing studies confirm that enzymatic treatments (e.g., mannanase enzyme) can significantly enhance transmittance while preserving vital sensory characteristics (Nadaroglu *et al.*, [8]).

While there are commercially available vegan fining agents, immense untapped potential lies in utilizing agro-industrial by-products as novel protein sources within a circular economy framework. The modern food sector is rapidly transitioning from a linear model to a circular bioeconomy, focusing on waste prevention and the value-added recycling of by-products. Legumes, including green pea pods and red beans, demonstrate favourable solubility and aggregation properties, making them highly suitable for use in colloidal beverages. Furthermore, utilizing these specific by-products not only achieves clarification goals but also maximizes nutritional retention, specifically the preservation of total polyphenol content (TPC) and antioxidant capacity (FRAP), which are often unintentionally stripped during traditional fining.

This study is pioneering in its approach to the extraction and application of these lesser-used plant proteins for the clarification of apple juice. It employs a circular economic approach that seeks to minimize waste while maximizing product quality, optical clarity, and bioactive compound retention.

2. Materials and Methods

2.1 Materials

The primary raw materials used in this study were plant-based proteins derived from agro-industrial by-products, specifically green pea pods and red beans. Apple juice from the Idared variety was selected as the standard medium for all clarification trials. To establish a foundation for the study, commercial fining agents were employed as controls, including ErbiGel (gelatine) and LittoFresh Liquid (pea protein). In addition, mineral agents such as bentonite (Seporit Pore-Tec) and silicic acid (KlarSol 30) were utilized in combination with the proteins. The materials needed for the experiment were obtained from Erbslöh Austria GmbH.

2.2 Protein extraction

Before processing the agro-industrial by-products, an evaluation of some protein extraction technologies was conducted to determine the optimal balance between yield, structural preservation, and sustainability (Pojić *et al.*, [9]). Advanced methodologies, such as ultrafiltration and diafiltration membranes, provide a gentle extraction process that effectively preserves the native structure of proteins. However, their industrial application remains limited by high capital equipment costs and the persistent challenge of membrane fouling when processing complex plant matrices (Tanudjaja *et al.*, [10]). Emerging green technologies, such as deep eutectic solvents (DES), offer a highly sustainable and biodegradable alternative; nevertheless, they present significant technical challenges regarding low extraction selectivity and high dynamic viscosity, which severely impede mass transfer during scale-up (Sahin *et al.*, [11]; Xu *et al.*, [12]).

Ultimately, traditional alkaline extraction (AE) combined with isoelectric precipitation was selected as the most viable method for this research. While this conventional method possesses the environmental drawback of producing wastewater that requires pH neutralization, it was chosen primarily due to its reliable efficacy, excellent scalability, and low operational costs (Nehmé *et al.*, [13]). Furthermore, AE is highly effective at chemically disrupting the complex cellulosic network of legume by-products, promoting the dissociation of protein bodies and maximizing protein recovery yields (Stone *et al.*, [14]).

The dried plant by-products were solubilized in distilled

water at a 1:20 (w/v) solid-to-liquid ratio. This specific ratio was selected to optimize the concentration gradient for adequate mass transfer, as higher solvent volumes have been shown to cause excessive dilution that lowers extraction energy density and efficiency (Peng *et al.*, [15]). Protein solubilization was achieved by adjusting the dispersion to pH 9.5 using sodium hydroxide (NaOH). Because the primary native legume proteins (globulins and albumins) have an isoelectric point around pH 4.5, this highly alkaline environment imparts a net negative surface charge to the proteins. As described in recent literature (Tiruneh *et al.*, [16]), the resulting electrostatic repulsion disrupts hydrogen bonding and forces the proteins to dissociate from the insoluble plant matrix and dissolve into the aqueous phase. This mixture was then subjected to centrifugation (4500 rpm, 10 min) to effectively separate the liquid protein-rich extract from the insoluble residues.

Next, the targeted proteins were recovered from the clarified supernatant via isoelectric precipitation. The pH was lowered to 4.5 using citric acid, a food-grade, organic acid, chosen specifically to prevent the irreversible structural damage often caused by harsh mineral acids (Ratnaningsih and Songsermpong, [17]). A pH of 4.5 corresponds to the approximate isoelectric point (pI) of the majority of legume proteins. As documented in pulse protein extraction literature, at this pH range (4 - 5), the net electrostatic charge of the proteins approaches zero, leading to a loss of solubility, subsequent hydrophobic aggregation, and precipitation (Shevkani *et al.*, [18]). Finally, the samples were subjected to centrifugation to discard the remaining supernatant and collect the settled protein for further processing.

In the final step, the product was freeze-dried (lyophilized). This process yielded a highly stable, dry plant protein powder with optimal functional characteristics, which was subsequently utilized as the active fining agent in the apple juice clarification experiments.

2.3 Apple juice production

The raw material used for the juice production consisted exclusively of the Idared variety. The process started with washing to remove surface impurities, followed by a manual sorting process to ensure that any unsuitable, damaged, or inedible fruit parts were completely removed prior to processing. The cleaned apples were then mechanically crushed using an apple mill to achieve the optimal mash consistency for juice extraction.

To optimize the pressing efficiency and juice yield, the apple mash underwent an initial enzyme pre-treatment using the Pektinex Ultra AFP enzyme preparation, applied at an equivalent dosage of 200 mL/ton. The physical separation of the liquid phase was executed using a press. After pressing, the extracted raw juice was subjected to a secondary enzyme treatment with Pektinex Ultra AFP at a reduced dosage of 100 mL/ton to further break down soluble pectins before the clarification stage.

The clarification experiments were set up by filling 500 mL measuring cylinders with the enzyme-treated Idared apple juice. The fining process relied on the combination of negatively and positively charged agents. The negatively charged mineral agents employed in the study included silicic acid (KlarSol 30) and two variants of bentonite: GranuBent Pore-Tec (sodium bentonite) and Seporit Pore-Tec (calcium bentonite).

For the positively charged clarification agents, the custom-extracted plant proteins (green pea and red bean) were utilized to facilitate flocculation. Once the respective fining agents were added and homogenized, the juice samples were allowed a designated clarification time of 1 hour to permit the full formation and settling of the floccules before analytical measurements were taken.

The control sample was not treated with the clarification agents; however, it was treated with the pectinase enzyme.

2.4 Analytical methods

To evaluate the efficacy of the plant-based fining agents, the treated apple juice samples were subjected to physical, optical, and chemical analyses.

2.4.1 Physical and optical properties

The primary indicator of clarification, turbidity, was measured and expressed in Nephelometric Turbidity Units (NTU). The volume of the sediment-free phase was recorded as a percentage (%) to quantify the amount of sludge generated during the precipitation process, which is a critical factor for industrial processing efficiency. Additionally, the optical clarity of the juice was assessed by measuring light transmittance (T%) using a spectrophotometer set at a wavelength of 440 nm. To determine visual quality and stability, the colour difference (ΔE*) was calculated to assess any deviations from the desired product colour.

2.4.2 Chemical and bioactive properties

Key quality indicators, such as pH and water-soluble solids (expressed in °Brix), were measured to confirm that the clarification processes did not negatively affect the natural characteristics of the apple juice.

To evaluate the retention of its nutritional properties, the following analyses were performed:

- To determine the Total Polyphenol Content (TPC), a colorimetric method utilizing phosphomolybdic-phosphotungstic acid reagents was applied (Singleton and Rossi, [19]), with values expressed in milligrams of gallic acid equivalents per liter (mg GAE/L).
- Antioxidant capacity was subsequently assessed using the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, [20]), and the results were recorded as milligrams of ascorbic acid equivalents per liter (mg AAE/L).

2.4.3 Statistical analysis

All analytical measurements were performed in multiple replicates. Except for the colour stability evaluation, the collected data were subjected to analysis of variance (ANOVA) to establish the statistical significance of the results across the different protein types, varying concentrations, and accompanying mineral agents. To evaluate colour stability, the colour difference (ΔE*) was calculated using the 3D Pythagorean theorem. This method determined the Euclidean spatial distance between the two points of the CIELab coordinates representing the treated samples and the control.

3. Results and Discussion

Abbreviations for understanding the results are shown in Table 1.

3.1 Turbidity and clarified layer

The primary goal of clarification is the removal of suspended solids to achieve a physically stable juice. The results (Figure 1) demonstrate highly variable efficacy depending on the clarification agents used.

The traditional combination of silica sol and gelatin (S-G) was the most effective treatment for reducing overall turbidity, achieving the lowest value of 52.33 NTU. In contrast, pairing silica sol with alternative plant-based proteins largely failed to flocculate the suspended particles; in several cases, such as the silica sol + red bean combination (682 - 797 NTU), the turbidity significantly increased compared to the control. This indicates poor compatibility and insufficient electrostatic interaction between silica and these proteins.

Bentonite-based treatments performed moderately better than silica when paired with alternative proteins. Despite the wide variations in turbidity, physical separation was largely successful across all treatments. The clarified layer volume remained exceptionally high, exceeding 97% for nearly all alternative combinations, demonstrating that while the supernatant may remain cloudy, the sedimentation behavior is stable.

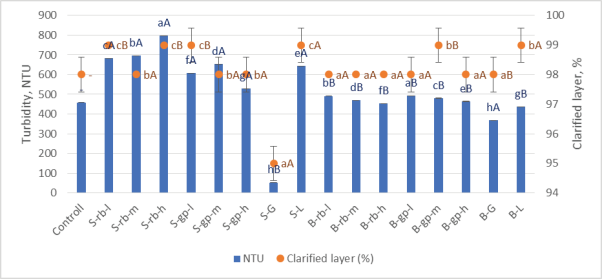

Figure 1. Turbidity and clarified layer values of apple juices

The presence of different letters indicates that the groups in question are significantly different.
Lower-case letter: comparison of proteins at fixed mineral clarification agents and concentrations.
Upper-case letter: comparison of mineral clarification agents at fixed protein and protein concentrations

Table 1. Abbreviations of samples

Sample name	Negative agent	Positive agent	Concentration of the positive agent
Controll	**No clarification agent added**		
S-rb-l	Silicic acid (S)	Red bean (rb)	low (l)
S-rb-m			medium (m)
S-rb-h			high (h)
S-gp-l		Green pea (gp)	low(l)
S-gp-m			medium(m)
S-gp-h			high(h)
S-G		Gelatine (G)	
S-L		Littofresh (L)	
B-rb-l	Bentonite (B)	Red bean (rb)	low(l)
B-rb-m			medium(m)
B-rb-h			high(h)
B-gp-l		Green pea (gp)	low(l)
B-gp-m			medium(m)
B-gp-h			high(h)
B-G		Gelatine (G)	
B-L		Littofresh (L)	

3.2 Transmittance

While turbidity measures scattered light due to suspended particles, transmittance evaluates the percentage of light passing directly through the juice, serving as a key indicator of visual clarity (Figure 2).

Interestingly, the transmittance data does not perfectly mirror the turbidity results. While the traditional S-G treatment improved transmittance to 57.33%, the highest transmittance values were achieved using bentonite paired with alternative (green pea) proteins. Specifically, the B-gp-m treatment achieved the highest clarity at 79.33%, followed by B-rb-h at 68%. This suggests that while bentonite and alternative protein combinations might leave some microscopic particulates in suspension (as seen in the NTU data), they are highly effective at removing the specific colloidal complexes that block light transmission, resulting in a visually clearer liquid.

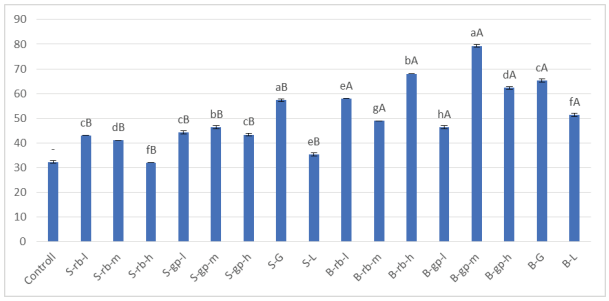

Figure 2. Transmittance of apple juices
The presence of different letters indicates that the groups in question are significantly different.
Lower-case letter: comparison of proteins at fixed mineral clarification agents and concentrations.
Upper-case letter: comparison of mineral clarification agents at fixed protein and protein concentrations

3.3 pH and refraction

Maintaining the fundamental physicochemical properties of apple juice during clarification is crucial for preserving its characteristic flavour and quality. The data indicate that the clarification treatments had low to no significant impact on the basic chemistry of the juice (Figure 3).

The unclarified control juice had a pH of 3.34 and a refraction value (Brix) of 11%. Across all clarification treatments, regardless of whether silica or bentonite was used, or the type and concentration of the protein, the pH remained stable between 3.32 and 3.40. Similarly, the soluble solids content fluctuated only marginally between 10.8% and 11.2%. This demonstrates that the applied fining agents successfully targeted suspended colloidal matter without stripping the juice of its intrinsic organic acids and soluble sugars.

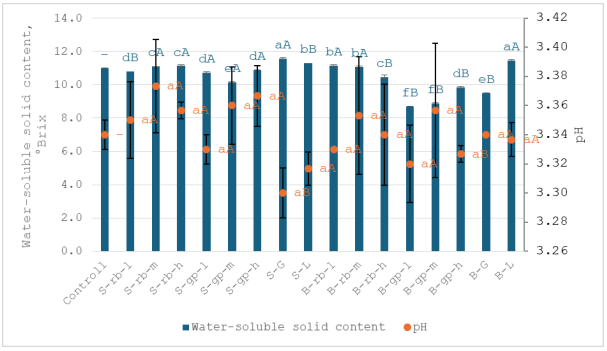

Figure 3. Water-soluble solid content and pH of apple juices
The presence of different letters indicates that the groups in question are significantly different.
Lower-case letter: comparison of proteins at fixed mineral clarification agents and concentrations.
Upper-case letter: comparison of mineral clarification agents at fixed protein and protein concentrations

3.4 Bioactive compound preservation

A major challenge in juice clarification is preventing the unintentional stripping of beneficial compounds, specifically polyphenols. The total phenolic content (TPC) and ferric reducing antioxidant power (FRAP) results (Figure 4) highlight the trade-offs between physical clarity and nutritional retention.

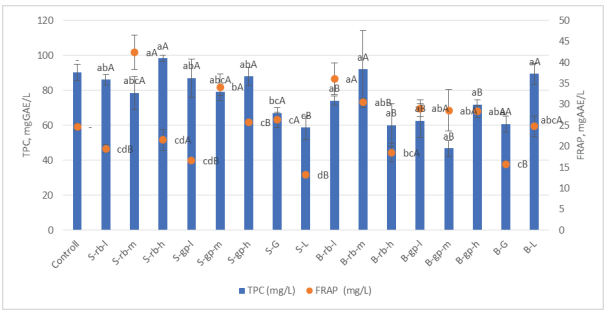

Figure 4. TPC and FRAP values of apple juices
The presence of different letters indicates that the groups in question are significantly different.
Lower-case letter: comparison of proteins at fixed mineral clarification agents and concentrations.
Upper-case letter: comparison of mineral clarification agents at fixed protein and protein concentrations

The control juice contained a TPC of 90.41 mg/L and a FRAP value of 24.67 mg/L. Several alternative treatments, such as silicic acid + red bean in high concentration, bentonite + Littofresh, and bentonite + red bean on medium concentration, successfully preserved the phenolic content at levels comparable to the unclarified control. Conversely, the treatments that were most effective at reducing turbidity, such as S-G, showed a notable reduction in TPC (67.16 mg/L), indicating that traditional gelatine-silica fining actively precipitates phenolic compounds alongside proteins and pectins.

Notably, the antioxidant capacity (FRAP) exhibited significant variability. Certain alternative protein treatments, particularly S-rb-m and B-rb-l, demonstrated an increase in antioxidant capacity compared to the control. This suggests that certain plant-based fining agents may either protect easily oxidizable compounds during processing, release bound antioxidants from the juice matrix, or contribute their own intrinsic antioxidant properties to the final product.

3.5 Colour stability

Colour stability is a crucial quality parameter for fruit juices, as treatment-induced colour shifts can negatively impact the visual appeal and marketability of the final product. To evaluate this, the colour difference (ΔE*) was calculated using the 3D Pythagorean theorem within the CIELab colour space, determining the spatial visual deviation of the plant-protein treated samples from their respective commercial gelatine controls (S-G for the silicic acid control, and B-G for the bentonite control) (Table 2). The analytical data revealed contrast in colour-preservation depending on the mineral agent utilized:

- Silicic Acid (KlarSol 30 / S) treatments: These combinations performed poorly regarding visual quality. Regardless of the legume protein type or concentration applied, all silicic acid treatments resulted in a "Very noticeable" degree of colour difference from the gelatine standard. The observed (ΔE*) values were exceptionally high, ranging from a minimum of 8.633 (S-gp-h) to a maximum of 14.634 (S-rb-h).
- Bentonite (GranuBent Pore-Tec / B) treatments: In contrast, bentonite pairings demonstrated highly effective colour preservation. The green pea combinations yielded only "Noticeable" visual differences (ranging from ΔE* 1.519 for B-gp-m to 2.034 for B-gp-l).

The most successful optical outcomes were observed with the red bean protein applied at low and medium concentrations alongside bentonite, both of which achieved a "Barely noticeable" degree of difference. Specifically, the medium-concentration red bean treatment (B-rb-m) yielded the lowest overall colour deviation with an ΔE* of just 0.838. Because an ΔE* below 1.0 is generally considered to be indistinguishable to the human eye, this result confirms that the B-rb-m treatment can perfectly replicate the desired visual profile of the industry-standard gelatine fining agent without causing unwanted colour degradation.

4. Conclusions

- The primary goal of clarification is the removal of suspended solids to achieve a physically stable juice. The results demonstrate highly variable efficacy depending on the clarification agents used. The traditional combination of silica sol and gelatine (S-G) was the most effective treatment for reducing overall turbidity, achieving the lowest value of 52.33 NTU. In contrast, pairing silica sol with alternative plant-based proteins largely failed to flocculate the suspended particles; in several cases, such as the silica sol and red bean combinations, the turbidity significantly increased compared to the traditional methods. Bentonite-based treatments performed moderately better than silica when paired with alternative proteins. Despite these variations in turbidity, physical separation was largely successful across all treatments, with the clarified layer volume remaining exceptionally high (exceeding 97%) for nearly all alternative combinations.
- Interestingly, the highest transmittance values were achieved using bentonite paired with alternative green pea proteins, rather than the traditional S-G treatment. Specifically, the bentonite and medium-concentration green pea (B-gp-m) treatment achieved the highest clarity at 79.33%, followed by the high-concentration red bean (B-rb-h) at 68%. This suggests that these specific alternative combinations are highly effective at removing the colloidal complexes that block light transmission on 440 nm.
- Maintaining the fundamental physicochemical properties of apple juice is also crucial for preserving its flavour and quality. The clarification treatments had low to no significant impact on the basic chemistry of the juice. Across all treatments, the pH remained stable between 3.32 and 3.40, and the soluble solids content fluctuated only marginally between 10.8% and 11.2%. This demonstrates that the applied fining agents successfully targeted suspended colloidal matter without stripping the juice of its organic acids and soluble sugars.

Table 2. ΔE* values

Parameters	S-G	Degree of difference	Parameters	B-G	Degree of difference
S-rb-l	14.183	Very noticeable	**B-rb-l**	1.353	Barely noticeable
S-rb-m	12.419	Very noticeable	**B-rb-m**	0.838	Barely noticeable
S-rb-h	14.634	Very noticeable	**B-rb-h**	3.064	Clearly visible
S-gp-l	10.930	Very noticeable	**B-gp-l**	2.034	Noticeable
S-gp-m	13.008	Very noticeable	**B-gp-m**	1.519	Noticeable
S-gp-h	8.633	Very noticeable	**B-gp-h**	1.669	Noticeable

- Several alternative treatments, including bentonite combined with medium-concentration red bean protein, successfully preserved the phenolic content at levels comparable to the unclarified control. Conversely, the treatments most effective at reducing turbidity, such as S-G, showed a notable reduction in TPC (67.16 mg/L), indicating that traditional gelatine-silica fining actively precipitates phenolic compounds. Furthermore, certain alternative plant-based treatments demonstrated an increase in antioxidant capacity compared to the control, suggesting they may protect easily oxidizable compounds or contribute their own antioxidant properties to the final product.
- Colour stability is another critical parameter, as treatment-induced colour shifts can negatively impact visual appeal. Silicic acid combinations performed poorly in this regard, resulting in a "Very noticeable" degree of colour difference from the gelatine standard across all legume types and concentrations. In contrast, bentonite pairings demonstrated highly effective colour preservation. The most successful optical outcomes were observed with the bentonite + medium-concentration red bean treatment (B-rb-m), which yielded a colour deviation (ΔE*) of just 0.838. Because an ΔE* below 1.0 is generally considered indistinguishable to the human eye, this confirms that the B-rb-m treatment can perfectly replicate the desired visual profile of the industry-standard gelatine fining agent.
- Ultimately, this study demonstrates that novel proteins derived from legume by-products possess significant potential as vegan-friendly clarification agents. Specifically, the optimal pairing of medium-concentration red bean protein with bentonite emerged as the best treatment, delivering promising clarification efficacy while successfully preserving valuable bioactive compounds and matching the visual profile of traditional methods.

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