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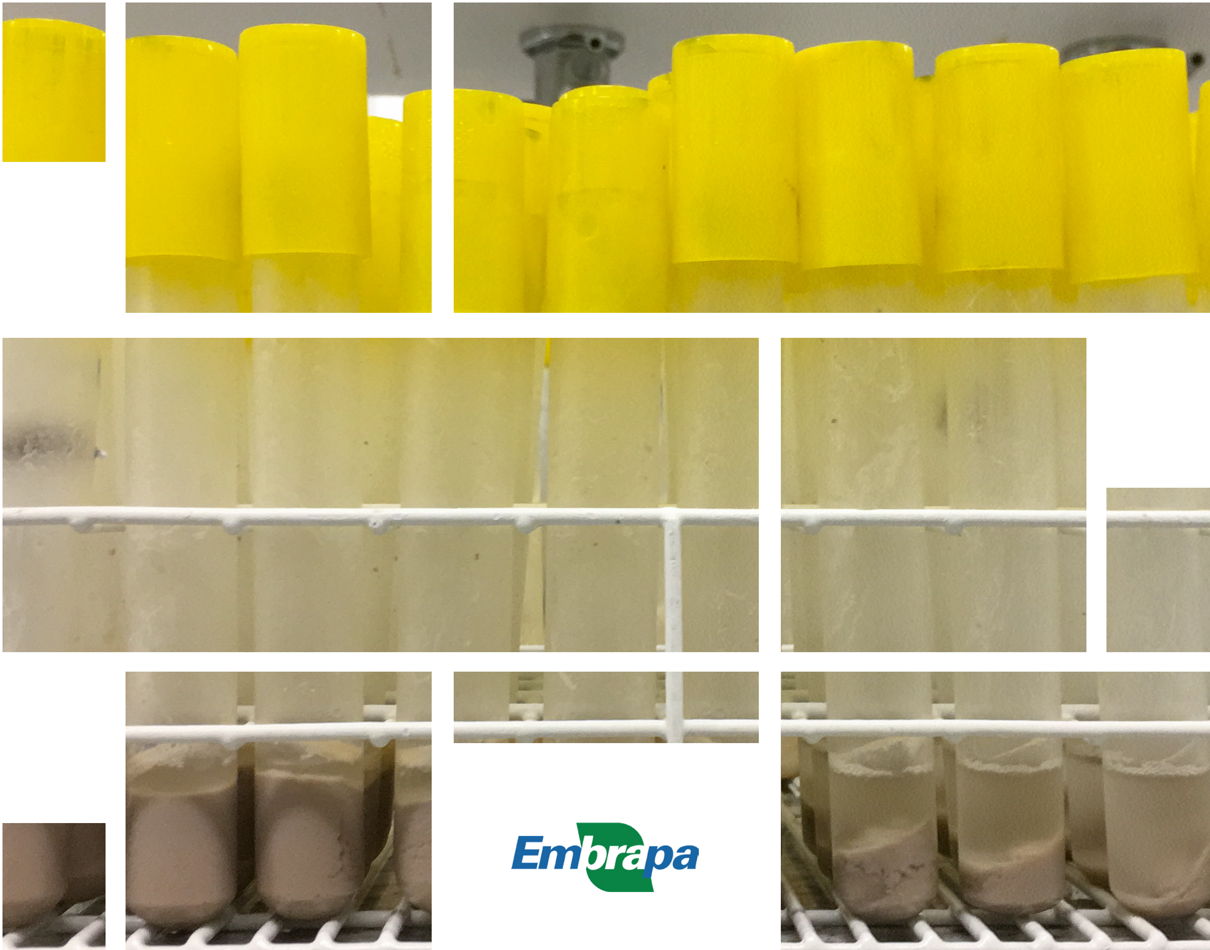
Guide for Technological-Functional Characterization of Protein Ingredients for the Plant-Based Market



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Embrapa Food Technology
Ministry of Agriculture, Livestock and Food Supply

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Rio de Janeiro, RJ
2022

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Foreword

Plant-based food analogues of animal-based products are mainly targeted to vegetarian, vegan, and flexitarian consumers and have shown exponential market growth in recent years. Due to this new demand, several vegetable protein ingredients have been developed for the food industry to be used as emulsifying, stabilizing, foaming, gelling, and dispersing agents in different product categories.

The technological applicability and performance of these ingredients are related to their technological-functional properties. However, there is still no national or international standardization of methodologies to determine these properties in plant-based protein ingredients. The lack of standardization impairs the comparison of the results obtained by different organizations.

Therefore, this guide was developed through selection and critical evaluation of the methods available in the literature, followed by laboratory testing. It presents a set of adapted and/or improved methodologies for five determinations in plant ingredients: (1) emulsifying activity and emulsion stability, (2) foaming capacity and foaming stability, (3) water and oil holding capacities, (4) water solubility, and (5) gelling capacity.

We hope that this guide will assist in obtaining representative and comparable results for each of the analyzed methods, thus helping to direct the application of plant protein ingredients in food products. This publication is intended for analytical laboratories, industries and researchers, and may become a document of reference for professionals in the field.

Edna Maria Morais Oliveira

General-Head of Embrapa Food Technology

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Introduction

Plant protein concentrates and isolates are being increasingly used as ingredients in the production of plant-based food analogues of animal-based products. However, there is still no standardization of methodologies to determine the technological-functional characterization of these ingredients that guide their use in foods. As a result, comparing results obtained for different ingredients, by different organizations, remains a significant challenge for the plant-based market.

The technical and scientific literature presents methods with a wide range of parameters, including differences in the sample preparation stage, analytical principles, variables of the methodological process, and forms of presenting the results. Thus, through the selection and critical evaluation of a compilation of methods available in the literature, followed by laboratory testing, this guide was prepared with a set of methodologies that were adapted and improved to facilitate the comparison of the properties of different protein ingredients and better direct the application of plant protein ingredients in food products.

Standard operating procedures (SOPs) were generated as a way of organizing and systematizing the data obtained for each evaluated methodology. The SOPs became part of the analysis portfolio of the Biochemistry Laboratory of Embrapa Food Technology. In addition to the corporate relevance represented by the increase in the laboratory's analytical capacity, the availability of these methodologies in the form of a guide shall facilitate the work of other groups, inside and outside Embrapa, to obtain comparable results of plant protein ingredients, either obtained by research or analyzed by demand from the private sector.

Steps Taken to Define the Methods Applied in the Technological-Functional Characterization of Plant Protein Ingredients

Selection of the technological-functional properties of interest

The selection of properties of interest was carried out by consulting the specialized literature on ingredients and protein foods, such as textbooks, scientific articles, and data sheets of related ingredients available in the national and international markets.

After consulting the technical and scientific documents, the selection was based on the abundance and interest of the properties found, as well as the technical complementarity of information that each property generates. Therefore, the selected properties were: emulsifying activity and emulsion stability, foaming capacity and foaming stability, water and oil holding capacities, water solubility, and gelling capacity.

Selection of ingredients to be tested according to the methodologies

Given the ingredients being developed in Embrapa's research projects, as well as the most abundant commercial plant-based ones on the market, the following ingredients were selected for the search of methodologies, as well as for application and laboratory testing of the methods: soy protein concentrate, soy protein isolate, pea protein concentrate, pea protein isolate, faba bean flour, faba bean protein concentrate (commercially acquired), common bean flour and common bean protein concentrate (produced at Embrapa).

Selection of methods available in the literature

The search for methods available in the literature was carried out containing the following inclusion criteria: internationally recognized methods and publications made by research groups with recognized competence in the area. The date of publication was not taken into account, since classical methods are typically found in older publications. In total, 27 scientific articles were selected for analysis.

Identification of the variables observed for each selected method

The selected literature was evaluated regarding the variables that made up each of the five methods analyzed. There were considered the variables regarding the sample preparation methods (sample concentration, dilution solvent, pH conditions, addition of salts and/or buffers, etc.), the fundamentals and analytical course of each method (variations regarding the analysis times, revolutions per minute and time in the centrifugation processes, the use or not of Ultra-Turrax type dispersers, etc.) and the units for expressing the results (%, g/g of sample, etc.).

Selection of parameters and laboratory testing

The variables selected in the previous section were critically analyzed. For some of them, a theoretical decision was made based on considerations of the ingredients of interest and the usefulness of the method for analyzing plant protein ingredients. For other variables, when there was technical doubt as to the best procedure to be adopted, laboratory testing was performed with the variables indicated by the literature. In these cases, these variables were tested with the eight ingredients previously mentioned, in order to select the most reproducible or most appropriate procedure for the expected responses. Finally, the selected procedures were used to evaluate the eight ingredients, in at least three replicates, and the results for each of the properties analyzed presented a coefficient of variation lower than 10%.

Methods for Technological-Functional Evaluation of Plant Protein Ingredients

It is suggested that all evaluations should be performed at least in three replicates, for each one of the methods described below.

Emulsifying capacity and emulsion stability

The emulsion of substances can be defined as a mixture composed of two immiscible liquids, where one component is defined as the dispersing or continuous phase and the other component as the dispersed or emulsified phase, as it is found in the form of small droplets.

Proteins are considered good emulsifying agents, as they have hydrophilic and hydrophobic regions in the same molecule, which reduces the surface tension between phases, allowing the formation of the emulsion. However, most proteins show a reduction or loss of emulsifying activity in a pH range close to their isoelectric point, where the net charge and solubility are reduced. Other factors that impair the emulsifying capacity of proteins are the presence of salts and exposure to heating, which can lead to protein denaturation.

The high emulsifying capacity of a protein ingredient directs its use for the production of solid or semi-solid foods in which proteins and fats, plus other hydrophilic and lipophilic ingredients, need to be emulsified, such as, for example, hamburgers, sausages, sauces, and desserts.

Procedure:

- In a 100 mL beaker, weigh 300 mg of the protein sample and add 60 mL of distilled water for each replicate. Homogenize and write down the exact concentration in g/mL;
- Measure the pH and adjust it to pH 7.0 using NaOH 0.1 mol/L or HCl 0.1 mol/L;
- Then, add 20 mL of commercial soybean oil to the solution and emulsify immediately with an Ultra-Turrax disperser (probe S 25 KV-18 G) at 9500 rpm for one minute at room temperature. The Ultra-Turrax disperser blade should be positioned at a height of 5 mm to 7 mm from the bottom of the beaker (Figure 1);
- Take an aliquot of 50 μL between the middle and the bottom of the beaker, immediately after homogenization;
- Add 50 μL aliquot to a test tube containing 5 mL of 0.1% (w/v) SDS (sodium dodecyl sulfate) aqueous solution and homogenize for 30 s in a vortex mixer;
- Zero the spectrophotometer with the 0.1% SDS solution;
- Measure the absorbance reading of the sample at a wavelength of 500 nm. This sample will be considered time zero (A_0);
- After 10 minutes, pipette 50 μL between the medium and the bottom of the beaker containing the emulsion, add in 5 mL of 0.1% SDS and homogenize for 30 seconds in a vortex mixer;
- Read in the spectrophotometer (A_{10});
- Calculate the emulsifying activity index (EAI) in m^2/g, using the following equation:

$$EAI\ (m^2/\,g)\ =\ \frac{2\ \times 2.303\ \times\ 100\ \times\ A_0}{c\ \times\ 0.25\ \times\ 10000}$$

where:

A_0 is the absorbance of the emulsion at time zero, that is, right after the end of homogenization.

c is the concentration of the protein sample (g/mL), that is, the weight of the sample divided by 60 mL.

NOTE: The numbers 2 and 2.303 are fixed values, as proposed by Pearce and Kinsella (1978). The number 100 corresponds to the dilution factor, 0.25 is the volume fraction of the oil added to the emulsion and 10000 is used to correct the unit for expressing the result.

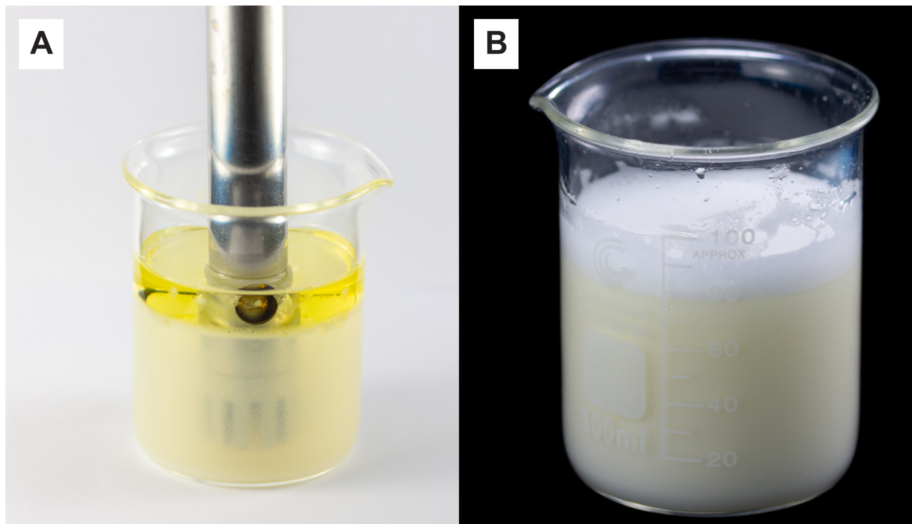
• Calculate the emulsion stability index (ESI) in minutes, using the following equation:

$$ESI\ (min) = \frac{A_0}{A_0 - A_{10}} x\ 10$$

where:

A_0 is the absorbance of the emulsion at time zero, that is, right after the end of homogenization.

A_{10} is the absorbance of the emulsion after 10 minutes.



Photos: Sidney Pacheco

Figure 1. Steps of emulsifying capacity analysis. (A) Disperser blade position to make the emulsion and (B) Resulting emulsified solution.

Emulsifying capacity and emulsion stability can be represented graphically or in tables. Figure 2 is an example of a graphical representation.

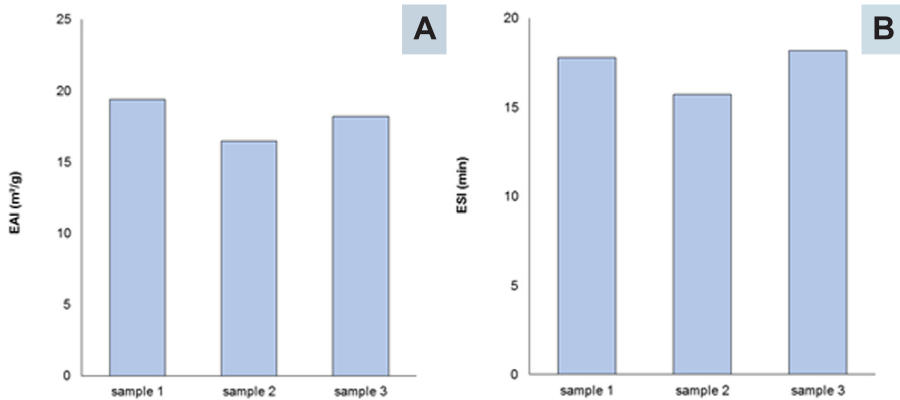


Figure 2. Graphical representative models of (A) emulsifying capacity – EAI and (B) emulsion stability – ESI.

The proposed methods were based on the information described by Pearce and Kinsella (1978), Naczk et al. (1985), Kaur and Singh (2005), Du et al. (2014), De La Rosa-Millán et al. (2018), and Gundogan and Karaca (2020).

Foaming capacity and foaming stability

The foams formed and present in food matrices consist of air droplets dispersed and enveloped in a liquid that contains a surfactant. The surfactant reduces the liquid-air interfacial tension and has the ability to form a film around the droplets, preventing their coalescence. The ability to form stable foam in the presence of air is an important functional property of protein ingredients, capable of influencing and modifying characteristics of various food products such as mousses, toppings, and bakery goods.

Procedure:

- Weigh 1.5 g of the protein sample in a 100 mL beaker;
- Solubilize the sample in 60 mL of distilled water using a glass rod and then, on a magnetic stirrer to maintain homogenization, carry out pH correction and aliquot removal (this volume of solution is sufficient for the experiment to be carried out in three replicates);
- Adjust the pH of the dispersion to 7.0 using 0.1 mol/L NaOH or 0.1 mol/L HCl;

- Remove 15 mL of the solution and add it to a 100 mL beaker (from here on, the procedure is described for each replicate);
- Homogenize using Ultra-Turrax for 2 min (probe S 25 KV-18 G), following without interruption the rotation/time ramp of 6500 rpm/30 s, 9500 rpm/30 s, and 13500 rpm/60 s. Note that the Ultra-Turrax disperser blade must be submerged 0.5 cm in the sample for good foam formation;
- Transfer the entire content of the beaker to a 50 mL measuring cylinder with the aid of a spatula adjusting the edges so the foam is leveled (it is recommended that the person responsible for the analysis fixes a maximum time to transfer the solution and foam to the measuring cylinder, minimizing the error between samples) (Figure 3);
- Measure and write down the foam volume observed in the measuring cylinder at 0 minutes, 10 minutes, 30 minutes, and 60 minutes;
- Calculate the foaming capacity (FC) and the foaming stability (FS) using the following equations:

$$FC\ (\%) = \frac{V_1 - V_0}{V_0}\ x\ 100$$

$$FS\ (\%) = \frac{V_2}{V_1}\ x\ 100$$

where:

V_0 is the initial volume of the protein sample solution.

V_1 is the volume after homogenization (solution + foam).

V_2 is the volume that remained (solution + foam) after standing for 10 minutes, 30 minutes, or 60 minutes at room temperature.

Photo: Sidney Pacheco

Figure 3. Foaming capacity.

Foam stability is usually graphically plotted as a function of time (Figure 4).

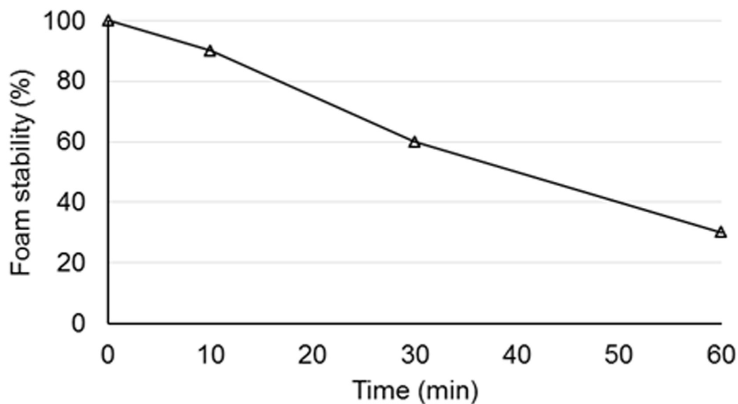


Figure 4. Graphical representative model for foam stability of protein ingredients.

The proposed methods were based on the information described by Poole et al. (1984), Kaur and Singh (2005), Zheng et al. (2008), Aydemir and Yemenicioĝlu (2013), Du et al. (2014), Shevkani et al. (2015), Lafarga et al. (2018), Gundogan and Karaca (2020), and Saricaoglu (2020).

Water Solubility

Solubility is a critical parameter for the functionality of a protein ingredient, being one of the most important technological properties for its use in food products. The solubility of the ingredients depends on their composition, mainly on the proportion and distribution of polar (hydrophilic) and nonpolar (hydrophobic) groups of the amino acids that constitute the protein structure, as well as on the pH of the medium since the solubility is closely related to the conformation of proteins in solution. The high solubility of a protein ingredient directs its use for the production of liquid foods, such as plant-based beverages that simulate the properties of milk.

Procedure:

- Weigh 300 mg of the protein sample in a 50 mL Falcon tube for each pH to be tested in each replicate;
- Add 30 mL of distilled water and homogenize;

- In a pH meter adjust the pH to the test values (3, 4, 5, 6, 7, 8, and 9) using 0.1 mol/L HCl or 0.1 mol/L NaOH. If there is a large change in volume, use more concentrated acidic or alkaline solutions;
- Shake the tubes for 30 min on an orbital shaker at room temperature (Figure 5);
- Take a sample of 2 mL and place it in an Eppendorf microtube;
- Centrifuge at 8000 rpm for 15 min in a microtube centrifuge;
- Remove the supernatant, transfer it to another microtube and determine the concentration of soluble protein by using the spectrophotometric method for the quantification of soluble proteins (Bradford, 1976);
- In parallel, for the preparation of control sample, prepare a solution of 1% of the protein sample in 0.1 M NaOH (it is considered that in this solution all the protein will be soluble in the supernatant) and determine the total concentration of protein in the sample using Bradford's method as previously mentioned;
- Calculate solubility using the formula:

$$Solubility\ (\%) = \frac{C}{C_T}\ x\ 100$$

where:

C is the protein concentration in the supernatant at each pH tested.

C_T is the total concentration of protein present in the control sample.

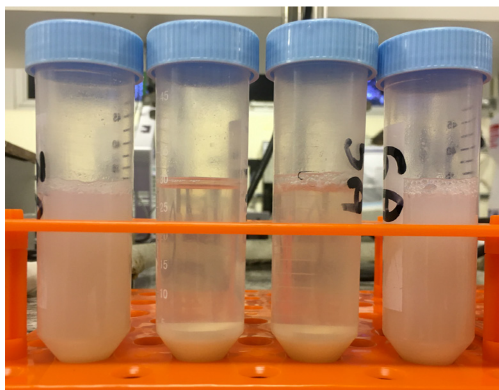


Photo: Sidney Pacheco

Figure 5. Tubes with protein samples solubilized at different pH levels (from left to the right, pHs 3, 4, 5, and 6).

Protein solubility in water is usually shown plotted versus pH (Figure 6). Solubility assessments can also be performed in relation to other parameters of interest, such as keeping the pH fixed and changing the temperature.

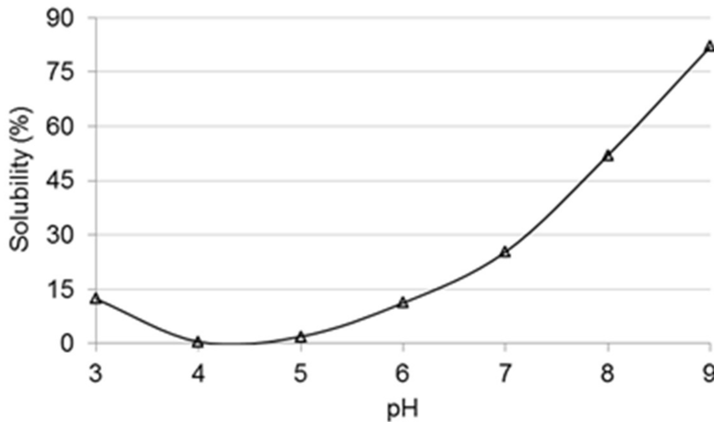


Figure 6. Graphical representative model for the solubility of protein ingredients

The proposed method was based on the information described by Boye et al. (2010), Joshi et al. (2012), Jarpa-Parra et al. (2014), Ladjal-Ettoumi and Chibane (2015), Ladjal-Ettoumi et al. (2016), Jarpa-Parra (2018), Alzuwaid et al. (2020), and Ibrahim et al. (2021).

Water and oil holding capacities

The water holding capacity of a protein ingredient is defined as the amount of water in grams that can be absorbed per gram of ingredient sample. Likewise, oil holding capacity is the amount of oil in grams that can be absorbed per gram of ingredient sample.

Water holding capacity is an important property for protein ingredients, as water retention is a considerable factor in the production of viscous foods, such as soups and in the baking process of some doughs. On the other hand, the oil retention of ingredients is especially important in the process of making processed meat-like products. For example, proteins with good oil holding capacity can help form emulsions in plant-based sausage manufacturing by joining water and fat within the product.

Procedure:

- Weigh and write down the mass of an Eppendorf-type microtube for each replicate (M_t);
- Weigh approximately 100 mg of the protein ingredient in this microtube and write down the value of the sample mass, discounting the microtube mass (M_0);
- Add 1 mL of distilled water (for water absorption capacity) or 1 mL of soybean oil (for oil absorption capacity) to the microtube;
- Close the tube and homogenize manually until the sample appears homogeneous;
- Agitate in a vortex mixer for 1 minute;
- Leave to rest for 30 min at room temperature;
- Centrifuge in a microtube centrifuge at room temperature at 12000 rpm for 20 min (Figure 7A);
- Pour and discard all the supernatant;
- Then touch the edge of the microtube on absorbent paper to drain residual supernatant (Figure 7B);
- Weigh the Eppendorf tube with the precipitate and write down the mass (M_1);
- Calculate the water holding capacity (WHC), or the oil holding capacity (OHC) using the following equation:

$$WHC\ or\ OHC\ (g/g\ sample) = \frac{M_1 - M_t - M_0}{M_0}$$

where:

M_1 is the mass (g) of the tube containing the wet sample after discarding the residual water or oil supernatant.

M_0 is the initial mass (g) of the sample.

M_t is the mass (g) of the Eppendorf microtube.

Photos: Sidney Pacheco

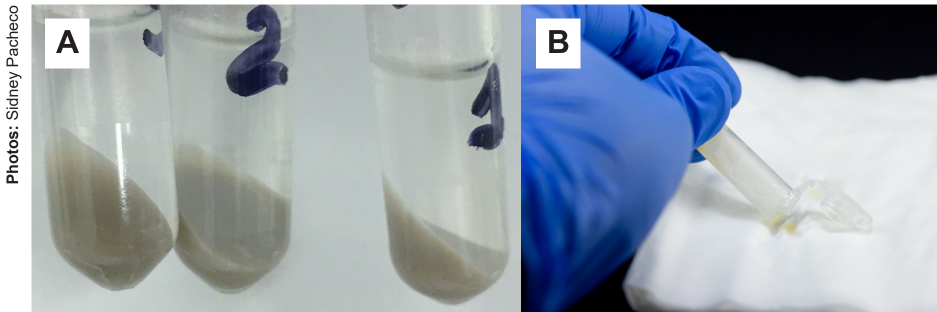


Figure 7. Analysis of water (or oil) holding capacity. (A) microtubes, (B) removal of residual supernatant from the microtube on absorbent paper.

Water and oil holding capacities can be represented graphically or in tables. An example of a graphic representation is shown in Figure 8.

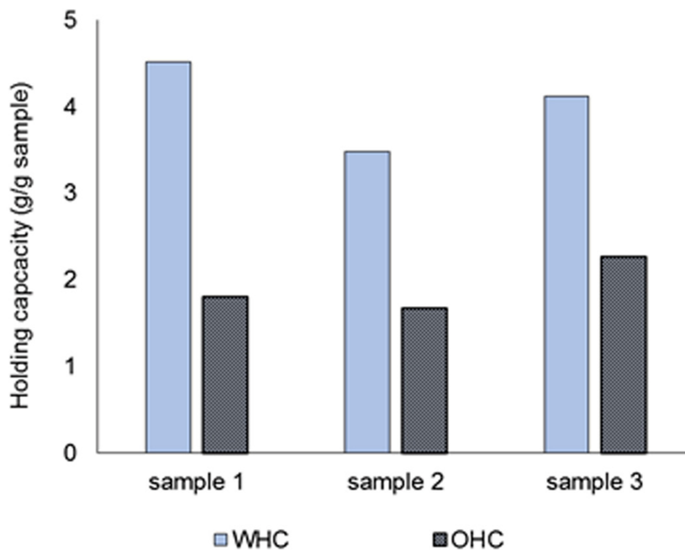


Figure 8. Graphical representative model of water holding capacity (WHC) and oil holding capacity (OHC).

The proposed method was based on the information described by Rodsamran and Sothornvit (2018), Čakarevic et al. (2019), Gundogan and Karaca (2020), Bozkurt et al. (2021), Marchini et al. (2021), and Omura et al. (2021).

Gelling capacity

Gelling capacity is generally expressed by the least gelling concentration, which can be defined as the minimum concentration of a substance necessary for the formation of a self-sufficient gel. Protein gelation is observed when proteins form a three-dimensional network after heating to a temperature higher than the protein's denaturation temperature, followed by subsequent cooling. The lower the least gelling concentration is, the better the gelling capacity. Protein gel formation is often favored by hydrophobic interactions and, in some cases, covalent disulfide bridges can also contribute to network development. This property is particularly important for making jellies or dairy-type desserts, such as puddings and flans.

Procedure:

- In 10 test tubes of 30 mL, with plastic caps without screws, weigh the following quantities of sample: 0.1 g, 0.2 g, 0.3 g, 0.4 g, 0.5 g, 0.6 g, 0.7 g, 0.8 g, 0.9 g, and 1.0 g;
- Add 5 ml of distilled water to each tube;
- Homogenize in a vortex mixer for 1 minute;
- Heat in a water bath at 100ºC (boiling water) for 60 minutes;
- Cool immediately in an ice bath;
- Place the tubes in the refrigerator at 4ºC;
- Keep refrigerated for 2 h;
- Remove from the refrigerator (Figure 9) and pour the tubes slowly, in increasing order of concentration, checking for flow;
- The test answer will be:

  (-) no gel, when the solution is liquid;

  (±) weak gel, when the solution flows, but more viscous;

  (+) gel, when the tube is inverted and the solution does not flow.
- The lowest concentration (g/mL) at which the sample does not flow, forming a firm gel, will be called the least gelling concentration (LGC) (Table 1) and the lower this concentration is, the better the gelling capacity will be.

Photo: Sidney Pacheco

Figure 9. Gelling capacity test with tubes in decreasing order of concentration. Tubes from left to right refer to concentrations of 0.20, 0.18, 0.16, 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02 g/mL.

Table 1. Model used to determine the least gelling concentration (LGC) of a protein ingredient.

Replicate	Concentration (g/mL)									
	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18	0.20
1	-	-	-	±	+	+	+	+	+	+
2	-	-	-	±	+	+	+	+	+	+
3	-	-	-	+	+	+	+	+	+	+

(-) no gel, (±) weak gel, (+) gel. In the example, the concentration at which the three replicates formed a gel, ie 0.10 g/mL, is considered as LGC.

The proposed method was based on the information described by Sathe and Salunkhe (1981), Ghribi et al. (2015), Jarpa-Parra (2018), and Ibrahim et al. (2021).

Final Remarks

As proposed, from methods described in the scientific literature and after review, improvement, and/or adaptation, analytical protocols were compiled to generate a standardization regarding the analysis of the main parameters that technologically and functionally characterize protein ingredients of plant origin.

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