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# Improving foam stability: Calcium addition, high hydrostatic pressure, and their combinations on soybean protein isolates

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# Innovative Food Science and Emerging Technologies

## Improving foam stability: calcium addition, high hydrostatic pressure, and their combinations on soybean protein isolates

--Manuscript Draft--

<b>Manuscript Number:</b>	IFSET-D-24-01047R1
<b>Article Type:</b>	Research Paper
<b>Keywords:</b>	Soy protein; calcium addition; foam formation; foam stability; High hydrostatic pressure
<b>Corresponding Author:</b>	Francisco Speroni Center for Research and Development in Food Cryotechnology La Plata, ARGENTINA
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<b>Order of Authors:</b>	Francisco Speroni Marie de Lamballerie Marc Anton
<b>Abstract:</b>	Various combinations of high hydrostatic pressure treatment (HHPT, 0.1 or 600 MPa), calcium addition (0, 0.50, or 0.75 mmol CaCl <sub>2</sub> /g protein), and protein content ([Prot.], 1, 5, or 10 g/L) were applied to soybean protein isolate (SPI) dispersions to analyze their effects on the formation and stability of foams. The significant interactions between these factors led to several combinations that improved foam stability via different mechanisms that involved different solubilities, aggregate sizes, and molecular structures of proteins. Thirty 30 min after foam formation, calcium combined with HHPT improved volume of foam (VF30) and volume of liquid retained (VLr30), by the formation of strong interfacial films owing to the increased cross-linking ability of soluble and intermediate-sized aggregates. Calcium without HHPT had a greater effect on VLr30 than on VF30, which could be owing to the insoluble and large aggregates that blocked Plateau borders. HHPT without calcium led to small denatured aggregates that were soluble and improved the VLr30 when [Prot.] was lower.
<b>Suggested Reviewers:</b>	Laura Laguna laura.laguna@iata.csic.es  Pascale Moll p.moll@uni-hohenheim.de  Ai Sato asato.kyoto@gmail.com
<b>Response to Reviewers:</b>	The respond to Reviewers was attached as a file

La Plata, June 19<sup>th</sup>, 2024

Editor in Chief  
Innovative Food Science & Emerging Technologies  
Dietrich Knorr

We are sending the revised version of the manuscript **Improving foam stability: calcium addition, high hydrostatic pressure, and their combinations on soybean protein isolates**, by Francisco Speroni, Marie de Lamballerie, and Marc Anton to be considered for publication in Innovative Food Science & Emerging Technologies.

The study deals with combinations of treatments oriented to improve the technofunctional properties of soybean proteins added with calcium in order to contribute in the change towards a greater consumption of plant proteins. The effect of high hydrostatic pressures on the protein structure is exploited for this purpose. In the context of plant proteins, our group has extensive experience in the study of the technofunctional properties of soybean proteins added with calcium such as gelation, emulsification and colloidal stability. This work is the first to study the combination of high hydrostatic pressures, calcium and soybean proteins in the formation and stability of foams.

The manuscript presents data that are useful for a potential application in the food industry while trying to explain the physicochemical aspects that ruled the phenomena.

The manuscript was improved thanks to the reviewers' questions.

Sincerely yours

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The authors thank both reviewers for carefully reading the manuscript. The authors believe that after modifying the text, based on the questions, the study has improved considerably. The changes appear in red font.

**Reviewer #1:**

Title: " Improving foam stability of soybean protein isolates by combining high hydrostatic pressure with calcium addition"

General comments

1. Revis the title of the study. See Title and Materials and methods section.

The title was modified according to the reviewer's suggestion. The Materials and Methods section was modified according to the reviewer's suggestion.

2. Revise English language in the whole manuscript.

The whole manuscript was modified according to the reviewer's suggestion. We have used the Language Editing Services from Elsevier.



Abstract

3. Revise English language in the whole section.

The section was modified according to the reviewer's suggestion.

4. Revise the entire abstract.

The abstract was modified according to the reviewer's suggestion.

5. Lines 12-13. Protein of what source? (the words "plant protein" make the information confusing, author should be careful with that).

The source of protein appears at the end of the sentence. Line 14.

6. Lines 12-13. Revise. Include within parentheses the amounts of pressure, Ca and proteins content.

The text was modified according to the reviewer's suggestion. Lines 12-13.

7. Lines 16-17. Which combination?

Various combinations improved foam stability (in terms of VF30 and VLR30) when compared the combination vs. the sample without calcium addition and without HHPT. The journal guidelines impose a maximum word count of 150 for the abstract; consequently, it is not possible to include such detail in the abstract.

8. Lines 17-18. After 30 min of what?

After 30 min of foam formation. The text was modified according to the reviewer's suggestion. Lines 17 and 18.

9. The whole abstract should be revised for making it understandable.

The abstract was revised according to the reviewer's suggestion

#### Keywords

1. Protein of which plant? (the words "plant protein" make the information confusing, author should be careful with that).

"plant protein" was removed from key words.

2. Include "foam formation".

"foam formation" was included in key words.

#### Industrial relevance

1. Revise English language in the whole section.

The section was modified according to the reviewer's suggestion

#### Introduction

1. Revise English language very carefully in the whole section.

The section was modified according to the reviewer's suggestion

2. Revise that all the technical-scientific content be correct.

The technical-scientific content is correct.

3. Lines 55-58. ??? Unclear paragraph. Revise all paragraphs in the whole section.

The text was modified according to the reviewer's suggestion. Lines 68-71.

4. Line 69. Revise the way for reporting citations. Revise this in the entire manuscript and in the references section.

According to the journal guidelines, citations in the text follow the referencing style used by the American Psychological Association (Publication Manual of the American Psychological Association, Seventh Edition, 2019). Reporting citations were checked according to the reviewer's suggestion.

5. Line 73. "... has to be supplemented ... "? Why?

The text was modified according to the reviewer's suggestion. Lines 91-92.

6. Authors should continue revising the whole section according to the above suggestions.

The whole section was revised according to the reviewer's suggestion.

7. Line 102. Delete "our grout". Include instead the citations found below.

The text was modified according to the reviewer's suggestion. Lines 121-122.

8. Lines 111-112. Revise objectives to make them clearer.

The text was modified according to the reviewer's suggestion. Lines 130-132.

## Materials and methods

1. Revise English language very carefully in the whole section.

The whole section was revised according to the reviewer's suggestion.

2. Revis all methods for making them clear in a technical scientific way.

The methods were revised according to the reviewer's suggestion.

3. Lines 120-121. ... USA) buffer solution? Authors should revise all paragraphs in the whole manuscript to make them clearer and complete.

The text was modified according to the reviewer's suggestion. Line 141.

4. Revis that all paragraphs in the Materials and methods section be clear.

The whole section was revised according to the reviewer's suggestion.

5. Lines 119-124. Revise the entire paragraph. In its present form is not clear.

The entire paragraph was revised according to the reviewer's suggestion. Lines 140-147.

6. Line 123. Therefore, how many combinations were prepared?

Regarding protein content and calcium/protein ratio, 9 combinations were prepared. After HHPT, the number of combinations was 18.

7. Line 126. How many milliliters were packaged to be pressurized?

The text was modified according to the reviewer's suggestion. Line 149.

8. Line 127. Did authors use only one HHP for pressurizing the dispersions? Why?

Taking into account the data from previous works, treatment with 600 MPa produces a significant increase in the solubility in samples added with calcium and a high degree of protein denaturation. The text was modified according to the reviewer's suggestion. Lines 152-154.

9. Lines 130-131. Unclear.

The sentence refers to the compression heating that occurs during the pressure rise stage of HHPT. All compressible substances change temperature during physical compression. This phenomenon is induced by compression work against intermolecular forces. The text was modified according to the reviewer's suggestion. Lines 156-158.

10. Line 142 ... UK) equipment.? Authors should revise all paragraphs in the whole manuscript to make them clearer and complete.

The text was modified according to the reviewer's suggestion. Line 176.

11. Again, authors should revise all methods, they should be reported in a scientific way.

The whole section was revised according to the reviewer's suggestion.

12. ...

13. Authors should continue revising according to the above suggestions.

The whole manuscript was revised according to the reviewer's suggestion.

#### Results and discussion

1. Revise English language very carefully in the whole section.

The whole section was revised according to the reviewer's suggestion.

2. Line 178. Why authors used a statistical decision point to 0.0001; it is very strict. Why authors did not try to use a decision p value of 0.05 which is not so strict?

To set the tests, a value of 0.05 was chosen, but then the software reports (for each comparison or effect) the value of the parameter p that corresponds to each effect, which in some cases is less than 0.05, indicating greater significance.

3. Revis each paragraph in the whole section for making them clearer.

The whole section was revised according to the reviewer's suggestion.

4. Authors should continue revising according to the above suggestions.

The whole section was revised according to the reviewer's suggestion.

#### Conclusions

1. Revise English language very carefully in the whole section.

The whole section was revised according to the reviewer's suggestion.

2. Revise conclusions for making more straightforward form relevant results, non-theoretical statements.

The text was modified according to the reviewer's suggestion.

3. Authors should continue revising according to the above suggestions.



## References

1. Revise to be in accordance with the journal guidelines.

## Tables

1. Revise to be in accordance with the journal guidelines.

## Figures

1. Revise to be in accordance with the journal guidelines.

The references, tables and figures were checked according to the reviewer's suggestion.

### **Reviewer #2:** Comments to the manuscript IFSET-D-24-01047

"Improving foam stability of soybean protein isolates by combining high hydrostatic pressure with calcium addition"

by Speroni F., De Lamballerie M., & Anton M.

This article is a study dealing with the influence of different parameters (HPPT /Ca/[Prot]) on the foaming properties of soybean protein isolates. The study is consistent with the fields of this journal. It provides interesting data on how improving the functional properties of soybean proteins. PCA analysis presented in the study is particularly relevant to better understand the influence of each parameters (alone or in combination). However, revision is needed since some methodological aspects should be clarified

### Highlights

The first highlight should be change as it is too general and rather repetitive of the others. The highlights need to be more relevant.

The highlights were modified according to the reviewer's suggestion

### Industrial relevance

Line 31, why authors mentioned that the findings of this work would serve as input for microbiologically save foods? Please clarify the link between the present work and microbiological effect since HHPT was applied before foaming and not on the final product.

The authors agree with the Reviewer that the foam will be made after HHPT, so in most cases, the treatment serves to modify the protein structure but not to generate a microbiologically safe foam. Therefore, in most cases the interest is in the changes in the protein structure that could allow the development of innovative products. However,

under certain conditions, such as those of aerosol whipped creams, high hydrostatic pressure would simultaneously improve techno-functional properties of proteins and pasteurize the dispersions.

The text was modified according to the reviewer's suggestion. Lines 34-37.

#### Introduction

- Line 50, plant proteins present a large range of sizes and structures, please precise which plant proteins Zhan et al. 2022 are talking about, and their main characteristics (size, solubility, structure ...globular, random coil, ...);

Zhan et al. (2022) refer to the 7S and 11S globulins (storage proteins), the text was modified according to the reviewer's suggestion. Lines 53-56.

- Line 48-49, 57, 65 authors cited "Pickering mechanisms" and "plateau borders system" to describe foam stabilization by proteins. Pickering phenomenon usually refers to solid particles, so can protein aggregates be considered as solid particles? in order to improve understanding of this section, the authors need to clarify this point and to add more information/definition/description of these two mechanisms (pickering & plateau borders) correlated to foam stability.

Sarkar and Dickinson (2020, <https://doi.org/10.1016/j.cocis.2020.04.004>) stated that originally, the research on Pickering mechanism was dominated by studies of inorganic particles, but now a growth of research output numbers in the area of food-grade Pickering emulsion has occurred. Regarding plant-based particles, the low solubility of some plant proteins in both aqueous media and edible oils is now likely to be considered as a valued functional attribute by researchers working on Pickering system.

Colloidal particles can act as Pickering stabilizers (Dickinson, 2010, <https://doi.org/10.1016/j.cocis.2009.11.001>; Lazidis et al., 2017; <http://dx.doi.org/10.1016/j.foodhyd.2017.07.030>). In our case, insoluble aggregates (SPI added with calcium without HHPT) seem to be candidates to be considered as particles that exert the Pickering mechanism, their high size favors the aggregates remain adsorbed, when compared with smaller proteins (Lazidis et al., 2017). Particles can also contribute to foam stabilization by accumulating in Plateau borders, these are particles that do not necessarily adsorb, but are present in the Plateau borders because of a percolation process, and create a gel-like network or act as corks reducing the drainage of the continuous phase due to gravity (Lazidis et al., 2017).

The text was modified according to the reviewer's suggestion. Lines 58-67.

- Lines 67-70 should be placed line 59 to introduce soybean proteins before description of Murray (2020) study.

The text was modified according to the reviewer's suggestion. Lines 72-75.

- Line 82, please precise "pasteurization level not sterilization".

The text was modified according to the reviewer's suggestion. Lines 100-101.

- Line 87, please precise the level of pressure applied and the time of treatment.

The text was modified according to the reviewer's suggestion. Lines 105-106.

- Authors must add, in this section, supplementary data on the foaming properties of SPI (i.e. Li et al (2022), Soy protein isolate: an overview on foaming properties and air-liquid interface, IJFST). This would enable the reader to situate this work in relation to the studies already published on this subject and would also help to highlight the scientific originality of the present study.

The text was modified according to the reviewer's suggestion. Lines 83-88.

#### Materials and method

- Line 116-118, has the sample been centrifuged to remove any dust or no soluble particles? Please specify

Samples were not centrifuged before HHPT or before making the foams. The text was modified according to the reviewer's suggestion. Lines 146-147.

- Line 126, please specify the volume of the high-pressure vessel and the volume of processed samples (volume of vacuum-packed dispersion).

The text was modified according to the reviewer's suggestion. Lines 149 and 151.

- Line 127, please justify the HHPT conditions (600 MPa, 5 min) used in the study.

Based on previous works, with a treatment at 600 MPa for 5 min, a high degree of denaturation can be reached, and the solubilizing effect occurred. The text was modified according to the reviewer's suggestion. Lines 152-154.

- Line 131, HHPT can induce buffer dissociation and a modification of the pH under and/or after HP processing. Authors must specify if the pH of each samples was measured/ checked after treatment.

This is a very interesting topic, in fact during high pressure treatment, an effect called electrostriction occurs by which some electrostatic bonds are dissociated (Boonyaratanakornkit et al., 2002, 10.1016/s0167-4838(01)00347-8). While this is reversible when the pressure decreases, exchanges can occur leading to the final effect. This could occur in buffer systems and transient pH changes may occur during treatment. At the exit of the HHPT device, the pH was checked and it had not changed. The text was modified according to the reviewer's suggestion. Lines 158-159.

- Line 133, to determine protein solubility samples were centrifuged at 3000 g, that seems quite low to separate insoluble particles. Stronger centrifugation conditions were usually used (up to 10 000 or 15 000 g). Authors should clarify this point.

The authors agree that in general, higher accelerations are used to determine protein solubility, even in other studies of our group, we have used 10.000xg (Añón et al., 2012, Manassero et al., 2018) and the results were similar to those of the present study. In any case, as discussed below, "solubility" is a value that depends on the centrifugation conditions, since the particles are too large to be really soluble.

Other authors that studied plant protein solubility also centrifuged in the range 700-5000xg: Bogahawaththa et al., 2019, 10.1016/j.lwt.2018.12.034 (Pea), Malhotra et al., 2004, 10.1016/S0268-005X(03)00047-X (Soy), Avanza et al., 2012, 10.1016/j.lwt.2012.04.015 (Cowpea), Tavano et al., 2008, 10.1016/j.lwt.2007.08.003 (Chickpea), Jiang et al., 2010, 100.1021/jf101045b (Soy).

The most important thing is that all samples are compared strictly in the same conditions and, given the sizes of the aggregates, 3000xg was an appropriate level to properly discriminate our samples.

- Line 142-143, please specify if the samples were diluted before size measurement and/or filtered (dilution factor, buffer ...). Size distributions from (no dynamic) light scattering such as Zetasizer are usually determined in % of intensity, why authors chose to express in % of volume? In addition, it would have been relevant to represent the size distributions in numbers. This would have provided factual arguments for the discussion of the foam stability results.

Samples were evaluated without dilution or filtration. The text was modified according to the reviewer's suggestion. Lines 177-178.

Each type of distribution (intensity, number, surface, volume) emphasizes some aspect. The authors chose the volume distribution because it better represents the mass of proteins that are in each size to participate in the foam. The intensity distribution overestimates the abundance of the largest particles. For this work, we think that with the volume distribution, the Z-average and the polydispersity index, a good characterization of the dispersions is achieved.

#### Results and discussion

- Line 178 please introduce first the Figure 1 before describing the results.

The text was modified according to the reviewer's suggestion. Line 213.

- Line 185-186, authors should clarify the term "operational" related to solubility. As already mentioned the centrifugation speed (3000 g) used in the present study to determine protein solubility is low, authors should nuance their results by using

"apparent solubility" instead of "solubility" in the whole text. The term "Pseudo- soluble" aggregates was used line 306 but should be used earlier in the text, especially in the "solubility section"

We used the term "operational solubility" because these proteins are colloidal particles in dispersion. Thus, the amount of protein that remains in the supernatants depends on the conditions during centrifugation (speed, time, temperature, and also pH, ionic strength, cosolvents, etc.).

The text was modified according to the reviewer's suggestion. Lines 160-169, 239, 249.

- Figure 1, 5, 6, 7: I don't think the 3D graphic type chosen is relevant (some bars are hidden by others...), a classical 2D bar charts (with the 3 concentrations and 3 histograms per [Prot] (representing Ca/Prot ratio)) would be easier to read /analyze and will allow to add standard deviation bars.

Figures were changed according to the reviewer's suggestion.

- Figure 2 / Figure 1: the solubility of unpressurized sample without calcium addition (control) was about 53% but the size distribution of the dispersion (non centrifugated) was quite monomodal (around 40 nm). Were the untreated SPI dispersions turbid or clear ? Large particles are also expected in the nontreated dispersions (since they are not centrifuged before size measurements). To avoid any confusion between size measurement and solubility results authors should mentioned for each conditions if the SPI dispersions were turbid or clear and if there was decantation. Authors clarify/specify this point.

The untreated non-centrifuged SPI dispersions without calcium addition were barely turbid (absorbance at 630 nm was in the range 0.043-0.075, depending on protein content). Non-centrifuged samples with calcium addition had absorbance values between 0.232 (1g/L) and 1.798 (10 g/L).

The turbidity of the samples was evaluated as absorbance at 630 nm. The data had not been shown in the original version because they behaved similarly to the results of a previous work (Manassero et al., 2018a). The text was modified according to the reviewer's suggestion and the absorbance data were included in the supplementary material (ST1).

- Figure 2 : Size distributions of SPI dispersions with Ca (no pressurization) showed peaks around 5000  $\mu\text{m}$ , that is close to maximum limit of the Zetasizer. Did authors carry out measurements with a dynamic light scattering device (as Mastersizer) that is more suitable for micrometers particles ?

Unfortunately, we have no used other devices to complete these results because we wanted to have access to the molecular size and to the first state of aggregation of the samples which the mastersizer does not allow (only focused on the bigger aggregates).

- Line 223, please change "in accord with" by "in accordance with"

The text was modified according to the reviewer's suggestion. Line 265.

- To improve the clarity of the results, data from Figure 3 and 4 should be pooled together in a Table with statistical analysis and not on 3D graphs.

Data from Figures 3 and 4 were presented according to the reviewer's suggestion (Table 1).

- Lines 241-246, the section on foam formation (MFLI) appeared quite short. Authors should develop the analysis of the results especially by discussing the effect of HHPT (as compared to non-pressurized samples) or they should merge this section with the next one on foam stability.

The sections were merged according to the reviewer's suggestion. Line 382.

- Foam stability section: did authors measure the viscosity of the different dispersions (pressurized or not...) before foam production? Viscosity is also an important parameter for foam stability but also for colloidal stability of turbid dispersions. Authors should clarify this point.

Unfortunately, we have not measured the viscosity of dispersions.

- Line 307, please add a reference concerning unfolding and charge modification of SPI by HHPT.

The text was modified according to the reviewer's suggestion. Lines 347-348.

## Highlights

Large and non-denatured Ca-induced soy protein aggregates stabilized foams

Insoluble soy protein stabilized foam by Pickering and/or blocking Plateau borders

At low protein content, denatured soy protein stabilized foam without Ca addition

Combination of Ca and high pressure improved foam stability

1 **Improving foam stability: calcium addition, high hydrostatic pressure, and their**  
2 **combinations on soybean protein isolates**

3  
4 Francisco Speroni<sup>a</sup>, Marie de Lamballerie<sup>b</sup>, Marc Anton<sup>c</sup>

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9  
10  
11 **Abstract**

12 **Various** combinations of high hydrostatic pressure treatment (HHPT, **0.1 or 600 MPa**), calcium  
13 addition (**0, 0.50, or 0.75 mmol CaCl<sub>2</sub>/g protein**), and protein content ([Prot.], **1, 5, or 10 g/L**)  
14 were applied to soybean protein isolate (SPI) dispersions to analyze their effects on the  
15 formation and stability of foams. The significant interactions between these factors led to  
16 several combinations that improved foam stability **via** different mechanisms that involved  
17 different solubilities, aggregate sizes, and molecular structures **of proteins**. **Thirty 30 min after**  
18 **foam formation**, calcium combined with HHPT improved volume of foam (VF<sub>30</sub>) and volume of  
19 liquid retained (VLR<sub>30</sub>), by **the** formation of strong interfacial films **owing** to the increased cross-  
20 linking **ability** of soluble and intermediate-sized aggregates. Calcium without HHPT had a greater  
21 effect on VLR<sub>30</sub> than on VF<sub>30</sub>, which could be **owing to the** insoluble and large aggregates that  
22 blocked Plateau borders. HHPT without calcium led to small denatured aggregates that were  
23 soluble and improved **the** VLR<sub>30</sub> when [Prot.] was lower.

24  
25 **Keywords**

26 Soy protein; calcium addition; **foam formation**; foam stability; high hydrostatic pressure



27

## 28 **Industrial relevance**

29 **Calcium enriched** plant proteins with good techno-functional properties is a prevailing need,  
30 **considering the current** cultural, economic and environmental changes that occur. The findings  
31 of this **study could** serve as **an** input for the incorporation of high biological value proteins and  
32 calcium **into** aerated foods, **and would contribute to develop innovative products**. Treatment  
33 with high hydrostatic pressure **could** be advantageous **on account of** modifying the protein  
34 structure and **thereby** improving the stability of the foams. **Under certain conditions, such as**  
35 **those of aerosol whipping creams, high hydrostatic pressure would simultaneously improve**  
36 **techno-functional properties of proteins and pasteurize** the dispersions, **which would then have**  
37 **to be safely canned**.

38

## 39 **1. Introduction**

40 **Owing to** environmental, economic, health-related, and cultural factors **the use of plant proteins**  
41 **for human consumption is increasing**. In **this** context, **soybeans are an inexpensive** source of  
42 proteins with high biological value and health-improving compounds; their techno-functional  
43 properties have been widely studied and they are used in different food applications such as  
44 emulsions, foams, **and gels** (Speroni et al., 2007; Nishinari et al., 2014).

45 Edible foams are unstable heterogeneous systems that provide specific organoleptic  
46 characteristics to **foods** such as ice creams **and** whipped creams. Different chemical species,  
47 from relatively small molecules to solid particles, are used to stabilize them. In the case of  
48 proteins, the **size of these** species vary from tens (for monomeric globular proteins) to thousands  
49 of nanometers (for macroaggregates). **Importantly**, monomeric globular proteins can decrease  
50 interfacial tension between air and water and **thereby** facilitate the formation of foams;  
51 **however**, particles (often aggregated proteins) can stabilize them either by direct adsorption to  
52 the interface (Pickering mechanism, Yan et al., 2020), or by blocking the system in **the** Plateau

53 borders (Bolontrade et al., 2016; Lazidis et al., 2017). Regarding molecular state, Zhan et al.  
54 (2022) reported that the storage proteins of seeds, such as the globulins 7S and 11S  
55 (approximately 150 and 380 kDa, respectively) that are present in various species and  
56 consequently in plant proteins isolates, exhibited lower foamability and foam stability than  
57 animal proteins due to their higher molecular weight (although there are high molecular weight  
58 animal proteins, such as myosin), and their more rigid structure. Regarding the aggregated state,  
59 they proposed that proteins play a central role in the Pickering stabilization mechanisms  
60 because their shape, size, charge, and surface properties can easily be modified. The low  
61 solubility and rigid structure of some plant proteins are now likely to be considered as valued  
62 functional attributes by researchers working on Pickering system (Sarkar and Dickinson, 2020).  
63 Moreover, the higher the size of particles, the higher the detachment energy required for their  
64 dislodging (Lazidis et al., 2017). Thus, insoluble macroaggregates are candidates to exert  
65 Pickering mechanism. Besides, stabilization can be exerted by particles that do not necessarily  
66 adsorb, but are present in the Plateau borders because of a percolation process, and create a  
67 gel-like network or act as corks reducing the drainage of the continuous phase due to gravity  
68 (Lazidis et al., 2017). Consequently, a balance is sought between the proportions and functions  
69 of soluble (molecular state) and aggregated (particulate state) proteins in a dispersion to, at the  
70 same time being surface active and adsorb at interface, and coating the interface via Pickering  
71 stabilization and/or blocking the Plateau borders (Lazidis et al., 2017).

72 Soybean protein isolate (SPI) is a blend of proteins, mainly composed of glycinin and  $\beta$ -  
73 conglycinin (globulins of approximately 360 and 180 kDa, respectively), which behave differently  
74 at the air-water interface (Rodríguez Patino et al., 2004) and form a variety of aggregates  
75 depending on the media and processing conditions (Nishinari et al., 2014). Concerning the  
76 foaming properties of soybean proteins, Murray (2020) demonstrated that they may be  
77 improved by heat-induced denaturation as this process leads to a more flexible structure and a  
78 higher hydrophobicity. Moreover, aggregation of the globular proteins modifies the bulk

79 rheology, and plays a role at the interface. For improving the stability of emulsions via Pickering  
80 mechanism, calcium-induced aggregation of soybean proteins followed by crosslinking with  
81 glutaraldehyde and the hydrophobic modification of insoluble soybean peptide aggregates were  
82 used to prepare particles (Liu et al., 2017; Jing et al., 2022). However, there are very few studies  
83 on foam stabilization via the Pickering mechanism using soybean proteins (Han et al., 2023). Li  
84 et al. (2022) analyzed the factors that determine the foamability and foam stability of soybean  
85 proteins and reported that heat induced denaturation (by increasing hydrophobicity), ionic  
86 strength (by weakening electrostatic repulsion and consequently accelerating the rate of  
87 absorption at the interface) can improve those parameters; they also found that foam  
88 characteristics and protein solubility correlate positively.

89 Replacing animal-based products is a challenge because of their desired techno-functional and  
90 nutritional properties. For example, calcium, primarily provided by animal sources, such as dairy  
91 foods, must be supplemented in plant-based products if they are to function as alternatives or  
92 substitutes. However, the incorporation of salts exerts specific and non-specific effects on food  
93 components that affect their functionality (Kharlamova et al., 2018). In particular, calcium  
94 modifies the techno-functional properties of soybean proteins; the most noticeable effect is the  
95 decrease in solubility, mediated via calcium-induced aggregation (Scilingo & Añón, 1996; Peng  
96 et al., 2020); besides, under certain conditions, calcium promotes polypeptide associations  
97 between soybean proteins that can be used to form gels (Maltais et al., 2005; Marinacci et al.,  
98 2024). Complexity of the formulation makes it challenging to achieve dispersed food products  
99 that are soybean-stabilized and equilibrated in calcium.

100 High hydrostatic pressure treatment (HHPT) aids in obtaining microbiologically safe food (by  
101 achieving pasteurization, but not sterilization) without the use of heat, although it might affect  
102 the structure of macromolecules, such as proteins (Balny & Masson, 1993). The application of  
103 appropriate chemical conditions of the medium, pressure level, duration of treatment, and  
104 other variables, these changes can be used to optimize the techno-functionality of proteins,

105 particularly soybean ones (Manassero et al., 2019; Bernasconi et al., 2020). HHPT (400 – 600  
106 MPa, 5 – 10 min) increases protein solubility in SPI dispersions with the addition of CaCl<sub>2</sub> (Añón  
107 et al., 2012; Manassero et al., 2018a). Manassero et al. (2018a) noted that HHPT induced an  
108 increase in solubility, which was strongly related to a decrease in aggregate size.

109 The effects of salts (MgCl<sub>2</sub>, CaCl<sub>2</sub>, and NaCl) on whey protein-stabilized foams were studied by  
110 Zhu and Damodaran (1994), who concluded that the enhancement of foam properties of MgCl<sub>2</sub>  
111 and CaCl<sub>2</sub> was not due to their non-specific ionic strength effect, but rather a result of the  
112 binding-induced structural changes and polymerization via ionic bridges. They suggested that  
113 interactions of divalent ions and proteins at the interface led to the formation of a viscoelastic  
114 film that provided foam stability. Henao Ossa et al. (2020) studied tofu whey concentrate and  
115 found that samples with high calcium content formed foams with increased stability. Large  
116 aggregates promote foam stability by blocking the Plateau channels for drainage and/or  
117 preventing coalescence owing to the formation of an interlamellar structure (Völp et al., 2021).

118 Additionally, Dickinson (2010) stated that the relationship between protein molecular structure  
119 and foaming behavior is complex, and that the conditions (and consequently the interactions)  
120 that favor aggregation, favor the formation of stable foams as well.

121 The combination of HHPT and calcium addition to SPI on gelation, emulsification, and colloidal  
122 stabilization of plant-based beverages was studied (Speroni & Añón, 2013; Manassero et al.,  
123 2018b; Manassero et al., 2019). It was found that the modified proteins form mixtures of soluble  
124 and insoluble proteins (in which a notable fraction of insoluble protein is colloidally stable), that  
125 under certain conditions exhibit an increased ability to associate with each other (Speroni et al.,  
126 2010; Manassero et al., 2018b). Under different conditions, such as calcium concentration,  
127 protein content, and HHPT-induced denaturation, different SPI dispersions could be obtained  
128 (with protein species in varied sizes, hydrophobicities, ζ-potentials, and other physicochemical  
129 characteristics), which consequently could positively affect the formation and stabilization of  
130 foams. Consequently, the aim of this study was to evaluate the behavior of these dispersions

131 and the protein fractions that constitute them, under the combined effect of calcium addition  
132 and high hydrostatic pressure, in the formation and stabilization of foams.

133

## 134 **2. Materials and methods**

### 135 **2.1. Preparation of soybean protein isolate (SPI)**

136 SPI was prepared from defatted soybean flour obtained from The Solae Company (Brazil).

137 Alkaline extraction (pH 8.0) was followed by isoelectric precipitation (pH 4.5) and finally  
138 neutralization (pH 7.0) as described by Speroni et al. (2007).

### 139 **2.2. Preparation of protein dispersions**

140 SPI dispersions were prepared at three protein contents: 1, 5, or 10 g protein/L in 50 mmol/L

141 TRIS-HCl pH 7.0 (Sigma, St Louis, USA) buffer solution. CaCl<sub>2</sub> was added to protein dispersions

142 from a stock solution at 1 mol/L prepared from CaCl<sub>2</sub>·2H<sub>2</sub>O (Sigma, St Louis, USA). Two

143 calcium/protein ratios (Ca/Prot.) (0.50 or 0.75 mmol CaCl<sub>2</sub>/g protein) were applied for each

144 protein content ([Prot.]). Samples without added calcium were also prepared at each [Prot.].

145 The dispersions were magnetically stirred for 60 min, the pH was checked and adjusted with

146 NaOH when appropriate (i.e.g. in the samples with the highest Ca/Prot. ratio). Samples were not

147 centrifuged before the HHPT or to make the foams.

### 148 **2.3. High hydrostatic pressure treatment**

149 HHPT was applied to vacuum-packed dispersions (samples of 50 mL, polyamide/polyethylene

150 bags obtained from La Bovida, France) at 600 ± 5 MPa for 5 min in a high-pressure system (Nova

151 Swiss, Cesson, France), in a 6.4 L chamber and equipped with a temperature sensor (Julabo,

152 Seelbach, Germany). HHPT was chosen at a level of 600 MPa because it produces a significant

153 increase in protein solubility in calcium-added samples and a high degree of protein

154 denaturation (Speroni et al., 2010; Añón et al., 2012). The compression fluid was water. The

155 pressure was reached at 3.4 MPa/s and released almost instantaneously. The initial temperature

156 of the compression fluid and samples was 20 °C. The compression heating (induced by

157 compression work against intermolecular forces) provoked a reversible increase in sample  
158 temperature to 25 °C. The pH (7.0) of samples was checked after HHPT and no change was  
159 detected.

#### 160 **2.4. Apparent protein solubility**

161 Protein dispersions were centrifuged at 3,000 xg for 20 min at 4 °C in an air-cooled centrifuge  
162 Universal 320R (Hettich, Germany). The [Prot.] in the supernatant was determined by the  
163 Bicinchoninic Acid Protein Assay (Sigma, USA), using bovine serum albumin as a standard (Sigma,  
164 USA). Absorbance was measured at 562 nm in an Epoch™ Multi-mode Microplate Reader  
165 (BIOTEK Instruments Winooski, USA). The [Prot.] in supernatant was determined in duplicate for  
166 each sample. Since the [Prot.] in the supernatant depends on operational conditions (angular  
167 velocity, temperature, time) and supernatants were turbid in some cases, the parameter was  
168 called "apparent protein solubility" (APS).

169  $APS (\%) = \text{protein content in the supernatant} / \text{initial protein content} \times 100$

#### 170 **2.5. Turbidity**

171 The absorbance at 630 nm was used as an indicator of turbidity and it was measured in whole  
172 samples and in the supernatants obtained to determine APS. The absorbance was measured in  
173 an Epoch™ Multi-mode Microplate Reader.

#### 174 **2.6. Particle size distribution**

175 Particle size distributions were determined by dynamic light scattering experiments using a  
176 Zetasizer Nano Zs equipment (Malvern Instruments Ltd., UK). The scans were performed in  
177 triplicate for each sample. The samples were not filtered or diluted to carry out the  
178 determinations. The particle size distributions of the samples were based on volume  
179 measurements. Data analysis was performed by the Zetasizer Nano software employing the  
180 Stokes-Einstein equation. Z-average was the intensity-weighted mean hydrodynamic size of the  
181 ensemble collection of particles. The polydispersity index (Pdl) ranged between 0  
182 (monodisperse) and 1.0 (100 % polydisperse).

183 **2.7. Preparation and characterization of foams**

184 The foams were prepared and characterized using FoamScan (Longessaigne, France). The foam  
185 formation and stability were followed by conductivity and optical measurements. Foams were  
186 produced by injection of gaseous N<sub>2</sub> through a porous glass, using 12 mL of protein dispersion.  
187 The N<sub>2</sub> flow rate was 35 mL/min. All the foams were allowed to reach a final volume of 37 mL,  
188 after which the N<sub>2</sub> flow was stopped. The foam volume was determined using a CCD camera  
189 (Sony Hexwave HAD). The liquid volume in the foam was assessed using the mean of  
190 conductivity measurements along the foam column.

191 -Foamability was analyzed in terms of the time required to reach the final volume of foam and  
192 of the maximum fraction of liquid incorporated (MFLI).

193 MFLI = liquid volume in foam at the time of maximum foam volume/maximum foam volume

194 -Foam stability was analyzed in terms of the volume of foam remaining after 30 min (VF<sub>30</sub>) and  
195 the relative volume of liquid retained in the foam after 30 min (VLR<sub>30</sub>).

196  $VLR_{30} = VLi_{30min} / VLi_{max} \times 100$

197 Where VLi<sub>30min</sub> is the volume of liquid in the foam after 30 min and VLi<sub>max</sub> is the volume of  
198 liquid in the foam when the volume of foam was maximum.

199 **2.8. Denomination of samples**

200 Each sample condition is specified using the [Prot.], the Ca/Prot. ratio, and the pressure level, to  
201 simplify reading and systematize the nomenclature of the samples. For example, 1g/L-0Ca/Prot.-  
202 0.1MPa and 10g/L-0.75Ca/Prot.-600MPa were the extreme points.

203 **2.9. Statistical analysis**

204 Data were analyzed using a factorial design with [Prot.] (1, 5, or 10 g/L), Ca/Prot. ratio (0, 0.50,  
205 or 0.75 mmolCa/g protein), and HHPT (0.1 or 600 MPa) as the main factors. Analysis of variance  
206 was used to evaluate the effects of the main factors. Duncan's test was used to determine  
207 differences among means (p = 0.05). The Infostat software was used (Di Rienzo et al., 2011).

208 **Additionally**, the data were analyzed by principal component analysis (PCA) using Minitab 19.1  
209 software (Minitab LLC, USA).

210

### 211 **3. Results and discussion**

#### 212 **3.1. Apparent protein solubility**

213 **Figure 1 shows the values of APS, which** is significantly affected by [Prot.] ( $p < 0.0001$ ), Ca/Prot.  
214 ( $p < 0.0001$ ), and HHPT ( $p < 0.0001$ ), and by the interaction between Ca/Prot. and HHPT ( $p <$   
215  $0.0001$ ). Clearly, **addition of calcium in** non-pressurized samples drastically **decreases the APS,**  
216 **regardless of** the [Prot.]. However, the application of HHPT increases **APS regardless of** the  
217 sample, **particularly** inhibiting the negative impact of calcium. The range of **APS** is between 11 %  
218 and 53 % for samples **that are** not subjected to HHPT, whereas **the range** is very narrow (53 % -  
219 67 %) for **the** pressurized **samples.**

220 The HHPT-induced increase in **APS of the present study is** in accordance with the results of Añón  
221 et al. (2012) in which, as in the present work, the definition of solubility was operational. A  
222 previous **study** (Manassero et al., 2018a) **reported** that large amounts of **proteins** remained in  
223 turbid dispersions after centrifugation, **and therefore**, pressurized and calcium-added SPI  
224 dispersions **have** a fraction of **the** insoluble **proteins** in a stable colloidal state. The same behavior  
225 was observed in **this study** (turbidity data **are** shown in **ST1**), which indicates that the samples  
226 are a mixture of soluble and insoluble protein species, **that** exhibit increased colloidal stability  
227 **after HHPT.** Regarding [Prot.], **the** unpressurized samples at 1 g/L are less sensitive to **the**  
228 **addition of** calcium than samples at 5 and 10 g/L. **Moreover, the** pressurized samples **at 1 g/L**  
229 without calcium addition have higher protein **APS** than **that of the** samples at 10 g/L. Thus, in  
230 this range of values, [Prot.] **is** an important factor **in** protein-calcium **interactions** and **APS.**

#### 231 **3.2. Protein aggregation**

232 **Figure 2 presents** the particle size distribution of dispersions as measured by dynamic light  
233 scattering. **Unpressurized** samples without **the addition of** calcium **exhibit** a main peak at 43 nm,



234 which indicates that SPI proteins are in the form of aggregates, as the molecular size for of  $\beta$ -  
235 conglycinin and glycinin is approximately 11 and 15 nm, respectively (Pizones Ruíz-Henestrosa  
236 et al., 2012; Liu et al., 2017). The particle size distribution confirms the results obtained on APS,  
237 as we can see that, without HHPT (black lines), the addition of calcium shifts the characteristics  
238 of the dispersion from a single-mode population centered at 43 nm (pseudosoluble aggregates)  
239 toward appearance of two peaks centered approximately at 1 and 5  $\mu$ m. This phenomenon is  
240 observed regardless of the [Prot.], amplifying with the increase thereof. This change in particle  
241 size distribution was accompanied by an increase in turbidity (Table S1). With a Ca/Prot. of 0.50,  
242 the aggregates were distributed in two populations with similar abundance, with peaks at 740  
243 and 4359 nm. Meanwhile for the highest Ca/Prot. ratio (0.75), unpressurized samples exhibited  
244 a major peak at 5002 nm (which was the only peak for the samples with [Prot.] at 10 g/L), and a  
245 small peak at 999 nm (which appeared in the samples at 1 and 5 g/L).

246 When HHPT is applied (red lines), the largest populations shifted to the smallest (approximately  
247 50 and 34 nm for samples with and without calcium addition, respectively, which also  
248 correspond to pseudosoluble aggregates). This treatment permitted to disrupt the aggregating  
249 effect of calcium with only the persistency of a shoulder around 350 nm whose population is  
250 proportional to the [Prot.]. The turbidity of the non-centrifuged calcium-added samples  
251 decreases drastically with the HHPT, which is related to the breakdown of the largest aggregates.  
252 On the other hand, the turbidity of the supernatants of samples added with calcium after HHPT  
253 is relatively high (compared with the samples without calcium addition) and increases with the  
254 [Prot.], as does the population of aggregates of approximately 350 nm (Table S1).

255 When the Z-average is analyzed (Table 1), it is observed that this parameter is significantly  
256 affected by [Prot.] ( $p < 0.0001$ ), Ca/Prot. ( $p < 0.0001$ ), and HHPT ( $p < 0.0001$ ), and by the  
257 interactions between these factors ( $p < 0.0001$ ).

258 In samples without HHPT, calcium leads to an increase in Z-average, from values approximately  
259 200 nm (without calcium addition) to approximately 1400 and 4700 nm for Ca/Prot. ratio of 0.50

260 and 0.75, respectively. Nevertheless, in samples with HHPT, calcium achieves no significant  
261 effect, but reveals a trend toward higher Z-average values for [Prot.] of 5 and 10 g/L. The Z-  
262 average values of the pressurized samples were within a small range (84 - 242 nm), which in turn  
263 partially explains the APS values of these samples that, regardless of Ca/Prot. and [Prot.], were  
264 observed in a narrow range (53-67%), This result is in accordance with the findings of Manassero  
265 et al. (2018a) who noted that the colloidal stability of pressurized SPI is governed mainly by the  
266 size of the aggregates.

267 Polydispersity index (Table 1) was significantly affected by Ca/Prot. ( $p < 0.0001$ ), by the  
268 interactions between Ca/Prot. and HHP ( $p < 0.0001$ ), and between Ca/Prot. and [Prot.] ( $p < 0.05$ ).

269 All samples were polydisperse, given that SPI is a mixture of globulins, which in turn are involved  
270 in the formation of aggregates. Among the unpressurized samples, those with the highest  
271 Ca/Prot. ratio (0.75) have the lowest Pdl values (and the highest Z-averages, Table 1). However,  
272 some pressurized samples (all three containing Ca/Prot. ratio of 0.75 and 10g/L-0.50Ca/Prot.-  
273 600MPa) exhibited higher Pdl than the corresponding to their unpressurized samples. This is a  
274 consequence of the HHPT-induced split of aggregates (Figure 2; Manassero et al., 2018a) that  
275 led to distributions with more heterogeneous sizes.

276 Our results indicate that in unpressurized samples, the Ca/prot. ratio governs the size  
277 distributions and as this ratio increases, it leads to dispersions with higher proportions of  
278 insoluble macroaggregates. In the pressurized samples, the size distributions are less dependent  
279 on the level of Ca/Prot. and the aggregates are smaller and more soluble than those in the  
280 unpressurized samples.

### 281 3.3. Foam formation and stability

282 Notably, no differences were observed in the time required to reach the maximum volume of  
283 foam, which was  $64.0 \pm 0.5$  s.

284 Regarding MFLI (Figure 3), the only significant factor is [Prot.] ( $p < 0.0001$ ). The sample with the  
285 highest MFLI, which occurred at the end of foam formation, was 10g/L-0.75Ca/Prot.-0.1MPa. A

286 high MFLI would lead to the availability of a large number of molecules to reach the air-liquid  
287 interface, especially in our case, where samples with high MFLI were those with high [Prot.] as  
288 well.

289 For  $VF_{30}$  (Figure 4), the [Prot.] ( $p < 0.001$ ), and Ca/Prot. ( $p < 0.001$ ) are considered significant  
290 factors; the interactions Ca/Prot. x HHPT, and Ca/Prot. x HHPT x [Prot.] are also significant ( $p <$   
291  $0.001$ ). In the unpressurized samples,  $VF_{30}$  increases with the increase in [Prot.] and the increase  
292 in Ca/Prot. (0 vs. 0.75). When HHPT is applied, the  $VF_{30}$  increases in the samples without calcium  
293 at 1 and 5 g/L (compared with the unpressurized samples), and this parameter achieves its  
294 highest value when pressurization is combined with the highest Ca/Prot. and [Prot.] (10g/L-  
295 0.75Ca/Prot.-600MPa).

296 In  $V_{Lr_{30}}$  (Figure 5), Ca/Prot. ( $p < 0.001$ ), HHPT ( $p < 0.001$ ), and [Prot.] ( $p < 0.05$ ) are significant  
297 factors; the interactions between these factors are also significant ( $p < 0.005$ ).  $V_{Lr_{30}}$  is increased  
298 by the Ca/Prot. (ratios of 0.50 and 0.75) in the unpressurized samples, with higher increases for  
299 0.75 than for 0.50 mmolCa/g protein. HHPT induces an increase in  $V_{Lr_{30}}$  expression in samples  
300 without calcium. Moreover, the combination of calcium addition and HHPT induces an increase  
301 in  $V_{Lr_{30}}$  at 5 and 10 g/L.

302 Without HHPT, the addition of calcium at a ratio of 0.75 promotes a similar effect (increases at  
303 each [Prot.]) on  $VF_{30}$  and  $V_{Lr_{30}}$ . Since both parameters increase simultaneously, this fact suggests  
304 that, in unpressurized samples, a high Ca/Prot. stabilizes the foam owing to the effects of the  
305 abundance of macroaggregates (Figure 2), such as retarding drainage (blocking Plateau borders),  
306 possibly by preventing coalescence (by formation of interlamellar structure), as proposed by  
307 Völöp et al. (2021) and/or the Pickering mechanism (Yan et al., 2020). Regarding the effect of  
308 Ca/Prot ratio, the increase from 0.50 to 0.75 exerts a greater increase on  $V_{Lr_{30}}$  than on  $VF_{30}$ .  
309 Therefore, considering the increase in the abundance of macroaggregates (around 5  $\mu\text{m}$ ) that  
310 was observed from ratio 0.50 to 0.75, these data suggest that, without HHPT, the increase in  
311  $V_{Lr_{30}}$  heavily depends on the size of aggregates.

312 Without calcium addition, the effect of HHPT consisted of an increase in both  $V_{Lr30}$  and  $V_{F30}$ ; this  
313 effect depends on [Prot.], and it had a greater magnitude at the lowest [Prot.], and even no  
314 increase in  $V_{F30}$  was observed at the highest [Prot.].

315 The combination of HHPT and calcium increases  $V_{Lr30}$  and  $V_{F30}$  when compared with  
316 unpressurized and non-calcium-added samples at each [Prot.]. Moreover, when  $V_{F30}$  values are  
317 compared with those of pressurized samples without calcium, the combination of HHPT and  
318 calcium addition induces no changes. For  $V_{Lr30}$ , the combination of HHPT and calcium addition  
319 leads to higher values than those corresponding to HHPT alone at 5 and 10 g/L, but not at the  
320 lowest [Prot.] (1g/L-0Ca/Prot.-600MPa has an outstandingly high value of  $V_{Lr30}$ ).

### 321 3.5. PCA and discussion

322 The projection graph for the first two principal components, which explains 74.1% of cumulative  
323 variation, is presented in Figure 6. First, it distinguishes the unpressurized samples from those  
324 subjected to HHPT. The unpressurized samples are located in the regions of low  $V_{Lr30}$  and  $V_{F30}$   
325 values, with the exception of 10g/L-0.75Ca/Prot.-0.1MPa (which appeared in the region of high  
326  $V_{Lr30}$ ,  $V_{F30}$ , Z-average, and MFLI, and of low APS). Moreover, the unpressurized samples exhibit  
327 an ordering in which the higher the [Prot.], the greater the proximity to the regions with high  
328  $V_{Lr30}$  and  $V_{F30}$  values, indicating that in the non-denatured samples, higher [Prot.] favors foam  
329 stability (red, black, and green ovals). Additionally, in the unpressurized samples, as the calcium  
330 content increases, the samples move to the regions of low APS and high Z-average, in  
331 accordance with the data from Figure 1 and Table 1.

332 The pressurized samples are found in regions with high solubility and low Z-average. The  
333 closeness between protein solubility and PdI (and the opposition of these parameters to the Z-  
334 average) can be explained as the effect of the HHPT, which breaks a fraction of the protein  
335 aggregates, generating species of smaller and varied sizes, with greater colloidal stability  
336 (Manassero et al., 2018a). Samples 1g/L-0.75Ca/Prot.-600MPa, 5g/L-0.75Ca/Prot.-600MPa, and  
337 10g/L-0.75Ca/Prot.-600MPa are located in regions of high foam stability, which corresponded

338 to the observations from Figures 4 and 5. These samples share a high Ca/Prot. ratio, underwent  
339 HHPT, and behave similarly in terms of  $V_{Lr_{30}}$  (values between 16.6 and 18.9 %). Their  $V_{F_{30}}$  values  
340 were high (between 27.3 and 35.3 mL), and were directly proportional to [Prot.]. The HHPT  
341 induces the breaking of large aggregates (especially with the increase in Ca/Prot.) to obtain a  
342 large population with a mode at 50 nm and a shoulder with a mode at 350 nm; the latter was  
343 more populated as the [Prot.] increased. This could explain the better stability of foams  
344 constructed with these samples due to the high functionality of pseudosoluble aggregates (50  
345 nm) that can be adsorbed and spread at the air-water interface. Furthermore, these proteins  
346 are partially unfolded (owing to HHPT, Speroni et al., 2010), and have a decreased surface  
347 electric charge (owing to the calcium addition, Manassero et al., 2018a), which favors protein  
348 absorption at the interface and formation of entangled interfacial films. Manassero et al.  
349 (2018b) concluded, with similar dispersions in oil/water emulsions, that the combination of  
350 calcium (Ca/Prot. ratio of 0.5 mmol Ca/g protein) and HHPT (600 MPa) improved the ability of  
351 SPI to associate and form denser and thicker interfacial films. Thus, the higher [Prot.] would also  
352 favor the stabilization of the interfacial film, which explains the increase in  $V_{F_{30}}$  as the [Prot.]  
353 increased. Desfougères et al. (2008) noted that the presence of bulk aggregates in solution was  
354 not essential for the improvement of the foaming properties; although, the conditions that favor  
355 aggregation also favor the formation of stable foams. Finally, the presence of intermediate sized  
356 aggregates (shoulder at 350 nm) could also increase foam stability as reported by Lazidis et al.  
357 (2017), who proposed that protein particles can stabilize foams by accumulating in the junction  
358 zones of the bubbles either due to the affinity of these particles for the interface itself or for  
359 what is on the interface already.

360 The samples 10g/L-0.75Ca/Prot.-0.1MPa and 1g/L-0Ca/Prot.-600MPa (Figure 6, dark green)  
361 were located almost diametrically opposite on the “protein solubility and Z-average axis” and  
362 exhibits high  $V_{Lr_{30}}$  and  $V_{F_{30}}$  values. These samples correspond to the opposite extremes of the  
363 levels of the three factors assayed, which leads to important differences in dispersion

364 characteristics (protein solubility, aggregate size,  $\zeta$ -potential, and degree of denaturation). This  
365 phenomenon suggests that stabilization of foams by proteins is a complex phenomenon and  
366 that, depending on the protein aggregation, it can be performed globally either by non- or poorly  
367 aggregated proteins at the interface (1g/L-0Ca/Prot.-600MPa case) or by protein aggregates  
368 blocking Plateau borders and/or through Pickering mechanism (10g/L-0.75Ca/Prot.-0.1MPa  
369 case). The sample 1g/L-0Ca/Prot.-600MPa has the highest protein solubility detected in this  
370 study (67%); the protein species constituted a monomodal population of small particles (mode  
371 = 34 nm, Z-average = 91 nm). Following HHPT, denatured soybean proteins exhibit enhanced  
372 hydrophobicity (Puppo et al., 2004). Possibly, the high protein solubility and low molecular  
373 weight of aggregates allowed a quick absorption at the interface, once at the interface, the high  
374 hydrophobicity could favor protein-protein associations that led to stable interfacial films.  
375 Manassero et al. (2018a) reported that pressurized SPI (with or without calcium addition) at pH  
376 7.0 exhibited improved interfacial activity. Remarkably, in this study the fraction of liquid  
377 incorporated during foam formation was low (Figure 3), but remained stable (Figure 5) in foams  
378 made from samples similar to those reported by Manassero et al., suggesting that the lamellae  
379 are thin but well structured. In the other extreme, the sample 10g/L-0.75Ca/Prot.-0.1MPa  
380 exhibited the lowest protein solubility detected in this work (11%) and the dispersion is mainly  
381 composed of large aggregates (mode = 5002 nm, Z-average = 5143 nm). Regarding aggregate  
382 structure, Scilingo and Añón (1996) reported that SPI aggregates had a compact structure when  
383 calcium was present. Additionally, Peng et al. (2022) proposed that the calcium-induced  
384 associations were responsible for the dense and compact structure of soybean aggregates,  
385 which in turn decreased their interaction with water. Insoluble and inflexible aggregates exhibit  
386 improved wettability (i.e., an improved ratio between hydrophobicity and hydrophilicity), which  
387 enhances the foam stabilization. Henao Ossa et al. (2020) reported that compact aggregates  
388 with low net charges had an increased ability to adsorb at the air-water interface and form an  
389 interfacial film with high viscoelasticity. An advantage of large particles or compact aggregates

390 is that their adsorption is practically irreversible, and once adsorbed, they remain at the  
391 interface (Dickinson, 2010). Wierenga et al. (2005) reported that for ovalbumin, the rate of  
392 adsorption decreased with increasing net charge, and noted that the surface layer was less  
393 densely packed when the proteins had a higher charge. Davis et al. (2004) also reported that the  
394 addition of calcium to whey proteins at pH 7.0 increased the rate of drop in surface tension due  
395 to the screening of the protein charges, which allowed protein concentration at the interface.  
396 In our samples, calcium decreases the negative charge of soybean proteins (Manassero et al.,  
397 2018a), which in turn would facilitate their adsorption. Besides the effects that calcium may  
398 exert on the aggregates at the interface, these compact aggregates can stabilize the foam by  
399 accumulating in the Plateau borders. The high [Prot.] and high MFLI (Figure 3) also contributed  
400 to foam stabilization by increasing the amount of protein available to adsorb at the interface  
401 and/or accumulate in Plateau borders.

#### 402 **4. Conclusions**

403 The combination of [Prot.], calcium, and HHPT generated soybean protein dispersions with  
404 different physicochemical characteristics such as protein solubility and particle size distribution.  
405 These dispersions were composed of mixtures of small, intermediate, or large aggregates. Each  
406 fraction (molecular, pseudosoluble, and insoluble aggregates) played different roles in foam  
407 formation and stabilization. Since the interactions between these factors were significant, the  
408 effect of each depended on the level of the others and the effects were not simply additive.

409 The combination of calcium and HHPT improved  $V_{F_{30}}$  and  $V_{Lr_{30}}$  values, which can be explained  
410 by the formation of strong interfacial films due to the increased ability of these modified  
411 proteins (most aggregate were approximately 50 nm, with a small population approximately 350  
412 nm) to cross-linking. Calcium without HHPT had a greater effect on  $V_{Lr_{30}}$  than on  $V_{F_{30}}$ , which  
413 could be attributed to the formation of compact and large aggregates that would block Plateau  
414 borders and therefore retard the drainage; large aggregates could also contribute to the  
415 formation of a structured interfacial film that protected towards disproportionation and

416 coalescence. Remarkably, HHPT without calcium further improved stability at lower [Prot.]. **This**  
417 **may be** related to (1) molecular characteristics of HHPT-denatured proteins such as flexibility  
418 and hydrophobicity that **improved** surface activity and (2) the formation of pseudosoluble  
419 aggregates (**approximately 34 nm**), **which can** adsorb and spread at the air-water interface.  
420 **Further studies** are needed to elucidate the mechanisms by which stability was increased in each  
421 condition, especially **when analyzing** the behavior of proteins at the interface.

422

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426

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548

549

**Table 1**

550

Z-average and polydispersity index of unpressurized and pressurized dispersions with different

551

protein contents and Ca/protein ratios.

Ca/Prot. (mmolCa/gProt.)	HHPT (MPa)	[Prot.] (gProt./L)	Z-average (nm)	Polydispersity index
0	0.1	1	169.3 ± 16.9 <sup>f</sup>	0.47 ± 0.03 <sup>def</sup>
0	0.1	5	268.2 ± 29.6 <sup>f</sup>	0.47 ± 0.03 <sup>cdef</sup>
0	0.1	10	158.2 ± 8.6 <sup>f</sup>	0.64 ± 0.06 <sup>a</sup>
0	600	1	91.3 ± 6.8 <sup>f</sup>	0.53 ± 0.02 <sup>bcd</sup>
0	600	5	83.5 ± 2.9 <sup>f</sup>	0.46 ± 0.01 <sup>def</sup>
0	600	10	89.9 ± 2.8 <sup>f</sup>	0.48 ± 0.01 <sup>cdef</sup>
0.50	0.1	1	1134.2 ± 80.5 <sup>e</sup>	0.58 ± 0.06 <sup>abc</sup>
0.50	0.1	5	1272.9 ± 110.9 <sup>e</sup>	0.59 ± 0.03 <sup>ab</sup>
0.50	0.1	10	1741.5 ± 181.9 <sup>d</sup>	0.42 ± 0.03 <sup>def</sup>
0.50	600	1	97.8 ± 4.5 <sup>f</sup>	0.44 ± 0.01 <sup>def</sup>
0.50	600	5	146.6 ± 2.5 <sup>f</sup>	0.41 ± 0.01 <sup>ef</sup>
0.50	600	10	209.3 ± 7.6 <sup>f</sup>	0.45 ± 0.06 <sup>def</sup>
0.75	0.1	1	4817.7 ± 552.8 <sup>b</sup>	0.31 ± 0.02 <sup>g</sup>
0.75	0.1	5	4169.2 ± 475.3 <sup>c</sup>	0.30 ± 0.03 <sup>g</sup>
0.75	0.1	10	5143.4 ± 264.0 <sup>a</sup>	0.26 ± 0.01 <sup>g</sup>
0.75	600	1	111.4 ± 6.4 <sup>f</sup>	0.43 ± 0.01 <sup>def</sup>
0.75	600	5	209.3 ± 4.7 <sup>f</sup>	0.51 ± 0.02 <sup>bcde</sup>
0.75	600	10	244.2 ± 5.2 <sup>f</sup>	0.37 ± 0.02 <sup>fg</sup>

552

553

[Prot.], protein content; HHPT, high hydrostatic pressure treatment. Results are expressed as

554

mean values ± standard deviation. Values in the same column with different superscripts

555

indicate significant differences (p &lt; 0.05).

556

557

558 **Figure captions**

559 **Figure 1.** Protein solubility of unpressurized and pressurized samples **with** different protein  
560 **contents** and Ca/protein **ratios**.

561 **Figure 2.** Particle size distributions of unpressurized (black lines) and pressurized (red lines)  
562 samples **with** different protein **contents** and Ca/protein **ratios**.

563 **Figure 3.** Maximum fraction of liquid incorporated (MFLI) to foams made from unpressurized  
564 and pressurized samples **with** different protein **contents** and Ca/protein **ratios**.

565 **Figure 4.** Volume remaining after 30 min of foams made from unpressurized and pressurized  
566 samples **with** different protein **contents** and Ca/protein **ratios**.

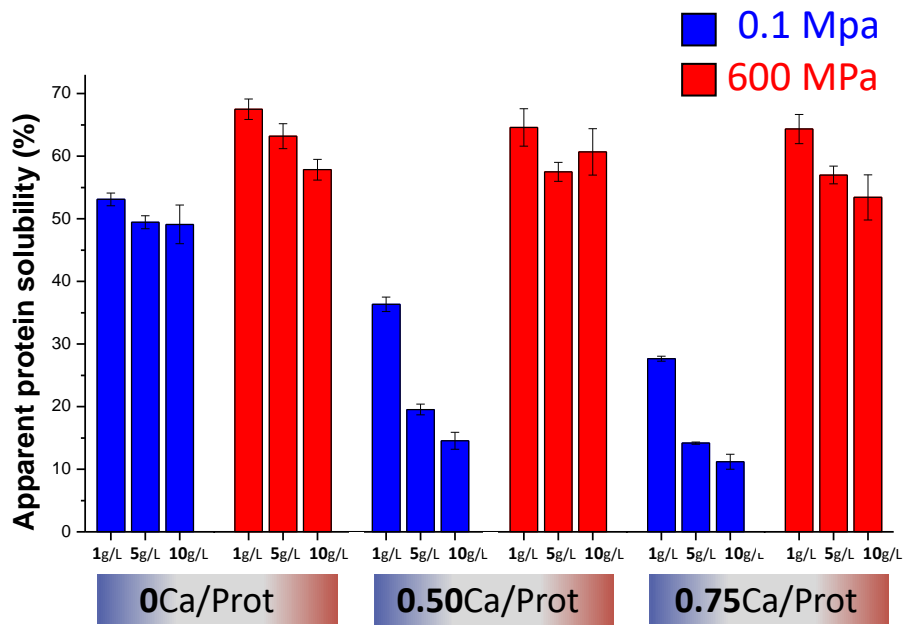
567 **Figure 5.** Relative volume of liquid retained after 30 min in foams made from unpressurized and  
568 pressurized samples **with** different protein **contents** and Ca/protein **ratios**.

569 **Figure 6.** Principal Component Analysis projection for the first two principal components. **APS**,  
570 **apparent protein solubility**; PDI, polydispersity index; MFLI, maximum fraction of liquid  
571 incorporated;  $VF_{30}$ , volume of foam after 30 min;  $VLr_{30}$ , relative volume of liquid retained in foam  
572 after 30 min.

573 Figure 1

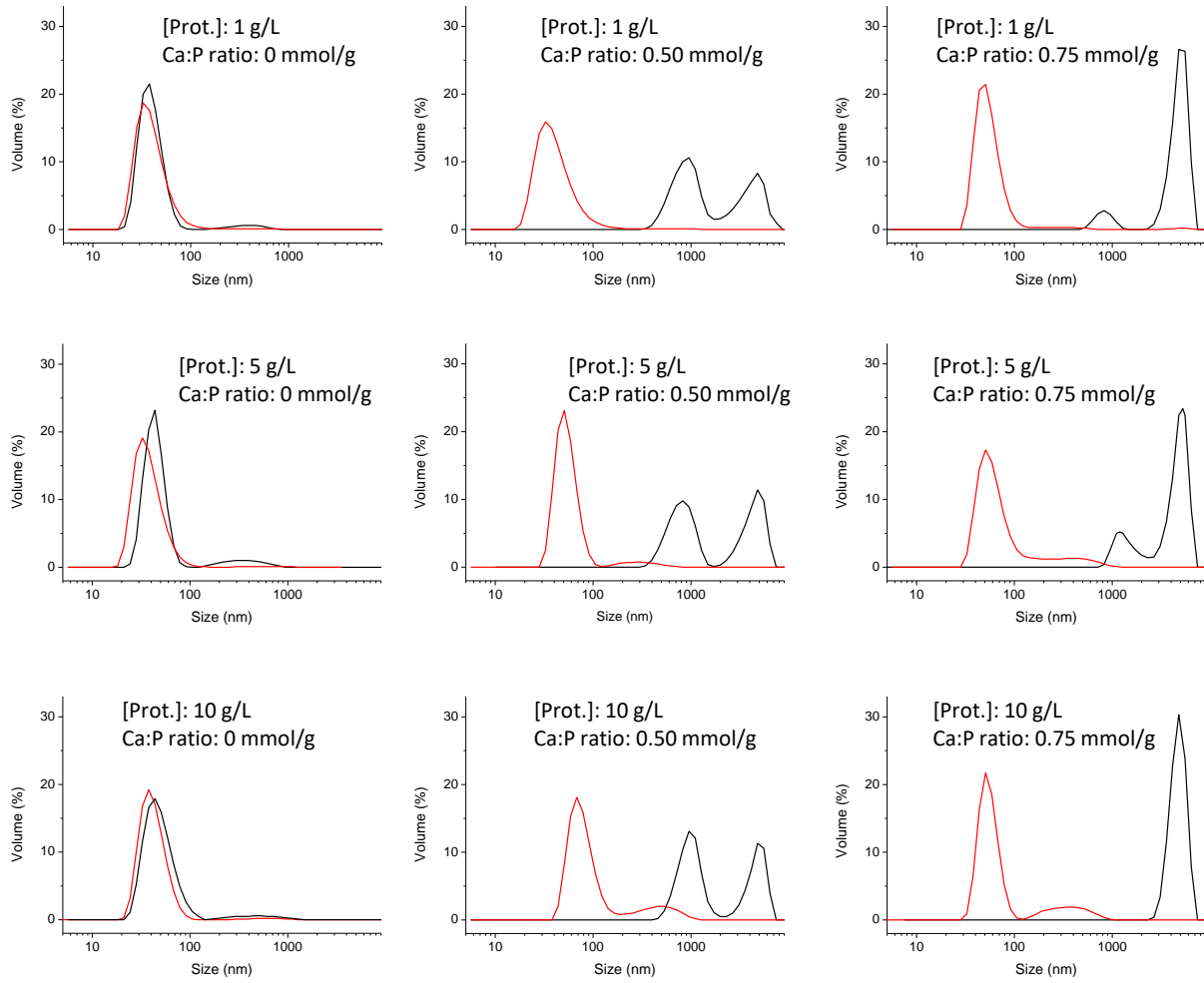
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576 Figure 2



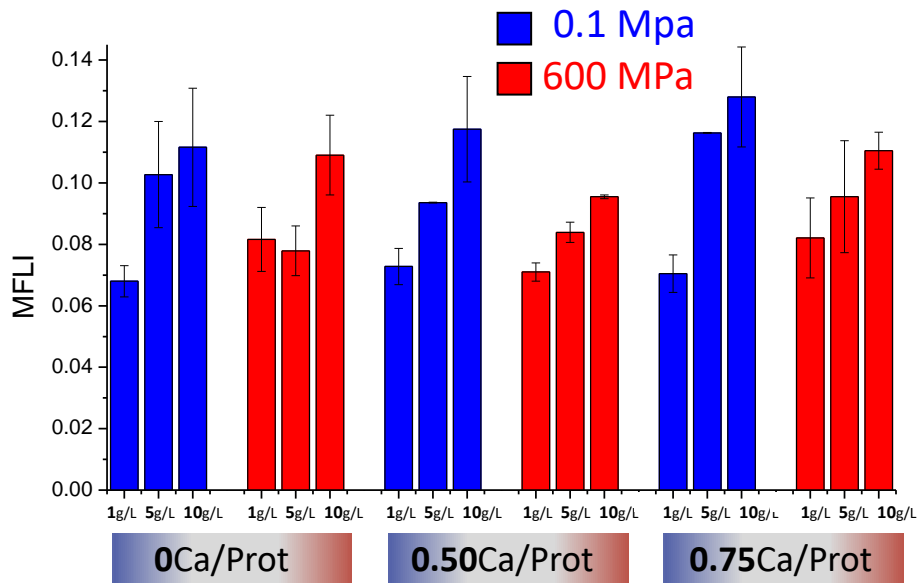
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579 Figure 3

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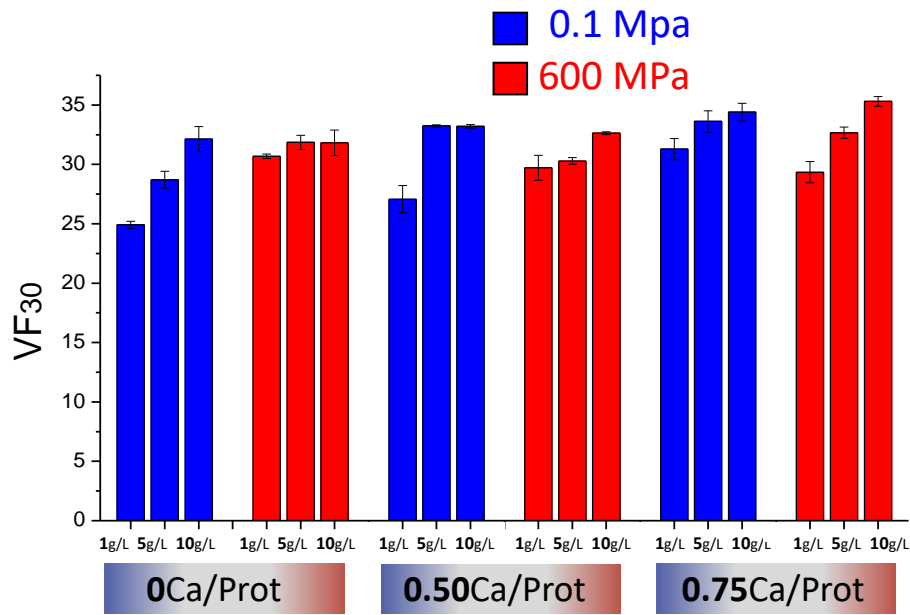
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582 Figure 4

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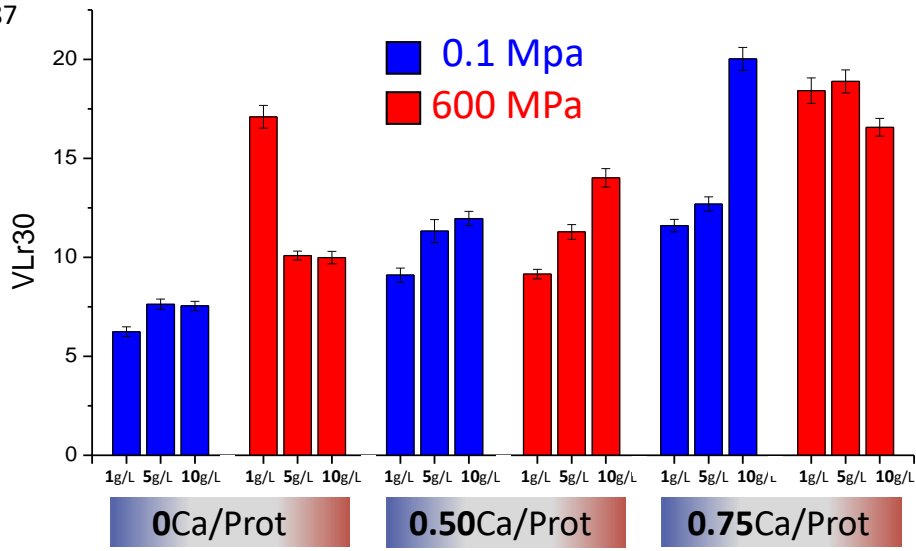
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585 Figure 5

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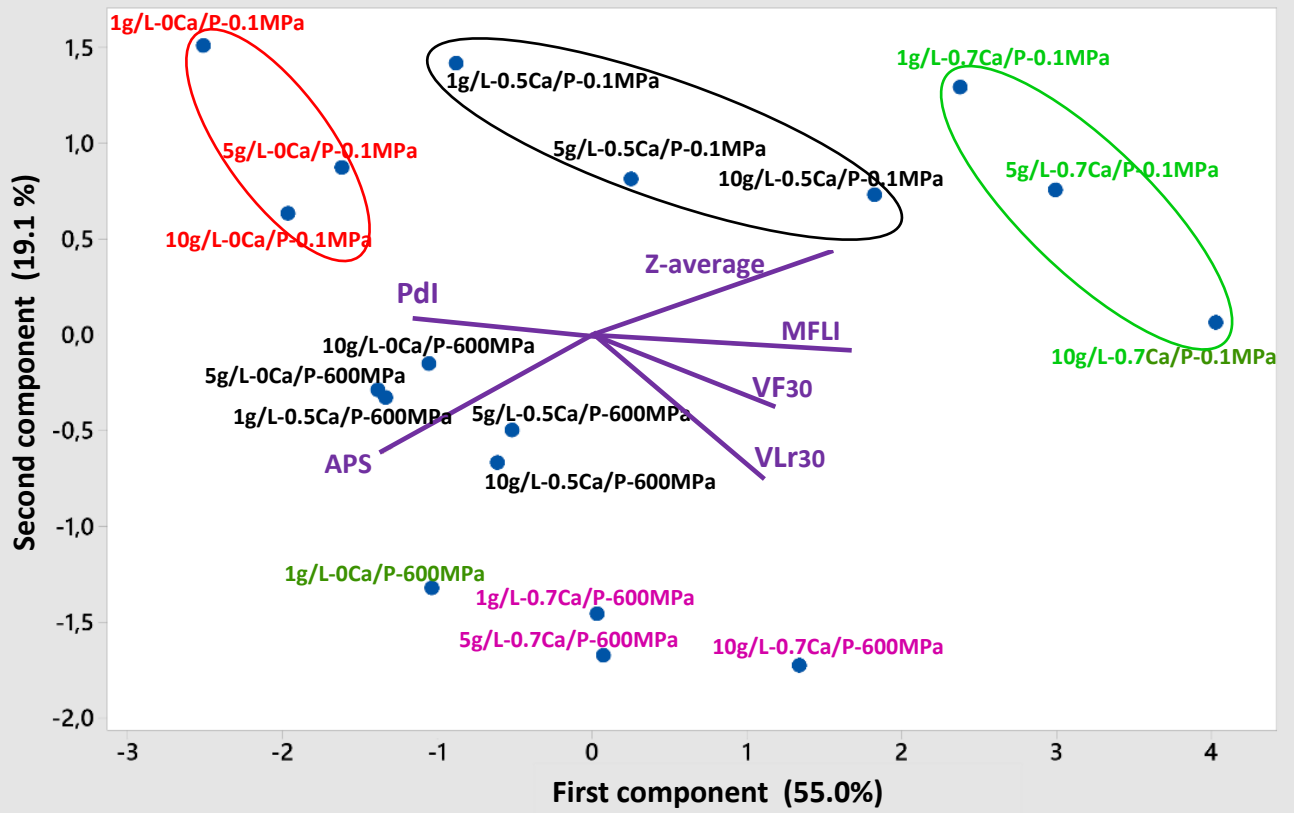
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588 Figure 6

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590



591 **Declaration of interest**

592 There is no financial or personal interest that could affect the objectivity of this **study**.

593

## Improving foam stability: calcium addition, high hydrostatic pressure, and their combinations on soybean protein isolates

Speroni, de Lamballerie, Anton

### Supplementary material

#### 1) Table ST1 Turbidity (absorbance at 630 nm)

#### 2) Analysis of variance and means comparisons

**Table ST1**

Absorbance at 630 nm of unpressurized and pressurized dispersions with different protein contents and Ca/protein ratios.

Ca/Prot. (mmolCa/gProt.)	HHPT (MPa)	[Prot.] (gProt./L)	Before centrifugation	After centrifugation
0	0.1	1	0.043 ± 0.001	0.038 ± 0.000
0	0.1	5	0.075 ± 0.002	0.054 ± 0.010
0	0.1	10	0.065 ± 0.001	0.049 ± 0.002
0	600	1	0.044 ± 0.002	0.042 ± 0.001
0	600	5	0.057 ± 0.003	0.047 ± 0.002
0	600	10	0.075 ± 0.003	0.053 ± 0.001
0.50	0.1	1	0.133 ± 0.005	0.038 ± 0.001
0.50	0.1	5	1.060 ± 0.019	0.054 ± 0.001
0.50	0.1	10	1.681 ± 0.105	0.070 ± 0.002
0.50	600	1	0.044 ± 0.003	0.036 ± 0.001
0.50	600	5	0.082 ± 0.003	0.066 ± 0.000
0.50	600	10	0.159 ± 0.003	0.112 ± 0.001
0.75	0.1	1	0.232 ± 0.021	0.038 ± 0.001
0.75	0.1	5	1.314 ± 0.043	0.044 ± 0.001
0.75	0.1	10	1.798 ± 0.004	0.044 ± 0.002
0.75	600	1	0.045 ± 0.003	0.041 ± 0.001
0.75	600	5	0.120 ± 0.001	0.087 ± 0.001
0.75	600	10	0.317 ± 0.035	0.175 ± 0.004

[Prot.], protein content; HHPT, high hydrostatic pressure treatment.

Centrifugation was carried out at 3000 g, for 20 min, and at 4 °C.

### Analysis of variance of Protein Solubility

Source	SS	CM	Degree of freedom	F	p-value
Model	13123.01	771.94	17	116.02	<0.0001
[Prot.]	814.71	407.35	2	61.22	<0.0001
Ca/Prot.	2369.97	1184.99	2	178.09	<0.0001
HHPT	8229.82	8229.82	1	1236.88	<0.0001
[Prot.] x Ca/Prot.	98.37	24.59	4	3.70	0.0229
[Prot.] x HHPT	65.81	32.90	2	4.95	0.0194
Ca/Prot. x HHPT	1402.03	701.01	2	105.36	<0.0001
[Prot.] x Ca/Prot. x HHPT	142.31	35.58	4	5.35	0.0051
Error	119.77	6.65	18		
Total	13242.78		35		

### Means comparison

Test: Duncan  $\alpha = 0.05$

Error: 6.6537 DF: 18

[Prot.]	Ca/Prot.	HHPT	Mean	SE						
1	0	600	67.49	0.86	A					
1	0.50	600	64.59	2.99	A	B				
5	0	600	64.40	1.00	A	B				
1	0.75	600	64.34	2.34	A	B				
10	0.50	600	60.73	3.77		B	C			
10	0	600	57.84	0.66			C	D		
5	0.50	600	57.50	1.50			C	D		
5	0.75	600	57.00	1.40			C	D		
10	0.75	600	53.42	3.60				D	E	
1	0	0.1	53.13	1.03				D	E	
5	0	0.1	49.44	1.04					E	
10	0	0.1	49.12	2.10					E	
1	0.50	0.1	36.34	1.14						F
1	0.75	0.1	27.67	0.13						G
5	0.50	0.1	19.55	0.85						H
10	0.50	0.1	14.54	1.36						H I
5	0.75	0.1	14.18	0.18						H I
10	0.75	0.1	11.19	1.22						I



**Analysis of variance of Polydispersity Index**

Source	SS	CM	Degree of freedom	F	p-value
Model	0.96	0.06	17	7.77	<0.0001
[Prot.]	0.01	0.01	2	0.86	0.4250
Ca/Prot.	0.37	0.18	2	25.55	<0.0001
HHPT	4.9E-4	4.9E-4	1	0.07	0.7950
[Prot.] x Ca/Prot.	0.14	0.04	4	4.92	0.0010
[Prot.] x HHPT	3.0E-3	1.5E-3	2	0.21	0.8119
Ca/Prot. X HHPT	0.28	0.14	2	19.22	<0.0001
[Prot.] x Ca/Prot. X HHPT	0.22	0.06	4	7.72	<0.0001
Error	0.87	0.01	120		
Total	1.82		137		

**Means comparison**

Test: Duncan  $\alpha = 0.05$

Error: 0.0072 DF: 120

[Prot.]	Ca/Prot.	HHPT	mean	SE							
10	0	0.1	0.64	0.04	A						
5	0.50	0.1	0.59	0.03	A	B					
1	0.50	0.1	0.58	0.04	A	B	C				
1	0	600	0.53	0.03		B	C	D			
5	0.75	600	0.51	0.03		B	C	D	E		
10	0	600	0.48	0.02			C	D	E	F	
5	0	0.1	0.47	0.04			C	D	E	F	
1	0	0.1	0.47	0.04				D	E	F	
5	0	600	0.46	0.03				D	E	F	
10	0.50	600	0.45	0.03				D	E	F	
1	0.50	600	0.44	0.03				D	E	F	
1	0.75	600	0.43	0.04				D	E	F	
10	0.50	0.1	0.42	0.02				D	E	F	
5	0.50	600	0.41	0.03					E	F	
10	0.75	600	0.37	0.03						F	G
1	0.75	0.1	0.31	0.05							G
5	0.75	0.1	0.30	0.03							G
10	0.75	0.1	0.26	0.05							G

### Analysis of variance of Z-average

Source	SS	CM	Degree of freedom	F	p-value
Model	312814442.04	18400849.53	17	690.53	<0.0001
[Prot.]	1312539.75	656269.88	2	24.63	<0.0001
Ca/Prot.	121467344.05	60733672.02	2	2279.16	<0.0001
HHPT	126473321.13	126473321.13	1	4746.18	<0.0001
[Prot.] x Ca/Prot.	1598358.35	399589.59	4	15.00	<0.0001
[Prot.] x HHPT	869225.40	434612.70	2	16.31	<0.0001
Ca/Prot. X HHPT	112202466.55	56101233.27	2	2105.32	<0.0001
[Prot.] x Ca/Prot. X HHPT	1479045.65	369761.41	4	13.88	<0.0001
Error	3384221.09	26647.41	127		
Total	316198663.13		144		

### Means comparison

Test: Duncan  $\alpha = 0.05$

Error: 26647.4102 DF: 127

[Prot.]	Ca/Prot.	HHPT	mean	SE	
10	0.75	0.1	5143.40	73.00	A
1	0.75	0.1	4817.75	81.62	B
5	0.75	0.1	4169.22	54.41	C
10	0.50	0.1	1741.50	57.71	D
5	0.50	0.1	1272.88	57.71	E
1	0.50	0.1	1134.22	54.41	E
5	0	0.1	268.23	54.41	F
10	0.75	600	244.20	66.64	F
10	0.50	600	209.33	81.62	F
5	0.75	600	200.63	57.71	F
1	0	0.1	169.31	51.62	F
10	0	0.1	158.20	57.71	F
5	0.50	600	146.59	57.71	F
1	0.75	600	111.43	47.12	F
1	0.50	600	97.77	49.22	F
1	0	600	91.27	51.62	F
10	0	600	89.87	57.71	F
5	0	600	83.52	57.71	F

**Analysis of variance of Maximum Fraction of Liquid Incorporated**

Source	SS	CM	Degree of freedom	F	p-value
Model	0.02	1.1E-3	17	5.27	<0.0001
[Prot.]	0.01	0.01	2	30.37	<0.0001
Ca/Prot.	7.6E-4	3.8E-4	2	1.91	0.1647
HHPT	3.6E-4	3.6E-4	1	1.80	0.1893
[Prot.] x Ca/Prot.	3.6E-4	8.9E-5	4	0.45	0.7727
[Prot.] x HHPT	1.1E-3	5.3E-4	2	2.65	0.0859
Ca/Prot. X HHPT	2.4E-5	1.2E-5	2	0.06	0.9413
[Prot.] x Ca/Prot. X HHPT	7.9E-4	2.0E-4	4	0.99	0.4265
Error	0.01	2.0E-4	32		
Total	0.02		49		

**Means comparison**

Test: Duncan  $\alpha = 0.05$

Error: 0.0002 DF: 32

[Prot.]	Ca/Prot.	HHPT	mean	SE							
10	0.75	0.1	0.13	0.01	A						
10	0	0.1	0.11	0.01	A	B					
10	0.75	600	0.11	0.01	A	B	C				
10	0	600	0.11	0.01	A	B	C				
10	0.50	0.1	0.11	0.01	A	B	C				
5	0	0.1	0.10	0.01		B	C	D			
10	0.50	600	0.10	0.01		B	C	D	E		
5	0	0.1	0.10	0.01		B	C	D	E	F	
5	0	0.1	0.10	0.01		B	C	D	E	F	G
5	0.50	600	0.08	0.01		B	C	D	E	F	G
5	0.50	600	0.08	0.01		B	C	D	E	F	G
1	0.75	600	0.08	0.01		B	C	D	E	F	G
1	0.50	600	0.08	0.01			C	D	E	F	G
5	0.50	600	0.41	0.01				D	E	F	G
1	0.75	0.1	0.07	0.01					E	F	G
1	0.75	600	0.07	0.01					E	F	G
1	0.75	0.1	0.07	0.01						F	G
1	0	0.1	0.07	0.01							G



### Analysis of variance of VLr30

Source	SS	CM	degree of freedom	F	p-value
model	968.22	56.95	17	18.57	<0.0001
[Prot.]	23.79	11.89	2	3.88	0.0317
Ca/Prot.	468.61	234.30	2	76.41	<0.0001
HHPT	85.85	85.85	1	28.00	<0.0001
[Prot.] x Ca/Prot.	88.71	22.18	4	7.23	0.0003
[Prot.] x HHPT	35.84	17.92	2	5.84	0.0072
Ca/Prot. X HHPT	80.56	40.28	2	13.14	0.0001
[Prot.] x Ca/Prot. X HHPT	76.68	19.17	4	6.25	0.0009
Error	91.99	3.07	30		
Total	1060.22		47		

### Means comparison

Test: Duncan  $\alpha = 0.05$

Error: 2.6669 DF: 30

[Prot.]	Ca/Prot.	HHPT	mean	SE									
10	0.75	0.10	20.02	0.82	A								
5	0.75	600	18.89	1.15	A	B							
1	0.75	600	18.04	0.94	A	B							
1	0	600	17.10	1.15	A	B	C						
10	0.75	600	16.57	0.82		B	C						
10	0.50	600	14.01	1.15			C	D					
1	0.75	0.10	12.71	0.94				D	E				
5	0	600	12.30	1.15				D	E	F			
5	0.75	0.10	11.99	0.94				D	E	F			
10	0.50	0.10	11.96	1.15				D	E	F			
5	0.50	0.10	11.34	1.15				D	E	F			
5	0.50	600	11.28	1.15				D	E	F			
10	0	600	10.11	0.94					E	F	G		
1	0.50	0.10	9.10	1.15						F	G	H	
10	0	0.10	7.30	0.82							G	H	
5	0	0.10	6.98	0.94							G	H	
1	0.50	600	6.60	1.15								H	
1	0	0.10	5.99	0.94								H	

**Declaration of interest**

There is no financial or personal interest that could affect the objectivity of this work.

On behalf of the authors,

Francisco Speroni

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