

Effect of industrial process conditions of fava bean (Vicia faba L.) concentrates on physico-chemical and functional properties

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Effect of industrial process conditions of fava bean (Vicia faba L.) concentrates on physico-chemical and functional properties

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Abstract:	Fava bean (Vicia faba L.) is a promising source of proteins owing to its benefits on health and environmental sustainability. Thus, fava protein-rich ingredients have a great potential in industrial food applications since processing of such ingredients can modify proteins and their functional properties. This study shows that there is no straightforward relationship between fava protein-associated reactions (hydrolysis and aggregation), protein properties and functional properties. For this study, an air-classified fava protein concentrate was processed at different combinations of pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and duration of treatment (30 and 360 min) to produce several modified fava concentrates. It was found that during ingredient modification: (1) protein hydrolysis was favored by low pH and high temperature, while (2) protein aggregation occurred at high pH and temperature. These reactions influenced foam and emulsion properties differently, emphasizing the differences in their individual stabilizing mechanisms. Despite the modifications in fava proteins, their physico-chemical and functional properties in the processed ingredients were nevertheless primarily governed by the pH of beverage application. The surprising interplay shown between properties encourages the need to dive further into the different protein-associated interactions that can occur in fava concentrate.

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To the Editorial Board,

Subject: Submission of a manuscript titled: *Effect of industrial process conditions of fava bean (Vicia faba L.) concentrates on physicochemical and functional properties* in the journal *Innovative Food Science & Emerging Technologies*.

Dear Prof. Dr. Dietrich Knorr,

We request you to consider our manuscript for publication. We believe this manuscript is very relevant to researchers working with i) plant-based ingredients and those particularly from fava bean (*Vicia faba*); ii) processing of protein-rich pulse ingredients for industrial food applications; iii) ingredient processing which can drive changes in protein-associated physicochemical and functional properties.

The manuscript attempts to understand mechanisms that explain fava bean functional (foam and emulsions in particular) modifications through ingredient processing, followed by an establishment of relationship between them and protein properties. The manuscript highlights the importance of (i) process conditions, especially pH during ingredient processing and application; (ii) the level of processing, which eventually drives structural changes in proteins, e.g. protein aggregation and/ or protein hydrolysis; and (iii) the interplay between protein and functional properties, but also the possible impact of the multicomponent character of an ingredient that can create ambiguities in the relationships between them.

The manuscript explains that by tailoring product application conditions, protein and functional properties can be favored, despite the processing levels. This paper can bring a high impact in the field of food science, food chemistry and sensory science of plant-based foods, and thus the authors believe this journal to be a suitable platform for this manuscript.

We propose, based on their expertise, following possible reviewers:

i. Peter Wierenga, Assistant Professor, Wageningen University, NL (Peter.Wierenga@wur.nl)
ii. Carmen Moraru, Professor, Cornell University, USA (cim24@cornell.edu)
iii. Simon M. Loveday, Senior Scientist, AgResearch Ltd, NZ (simon.loveday@agresearch.co.nz)
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We thank you in advance for having considered this manuscript for publication.

Best Regards,

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Highlights:

- High temperature processing (≥ 75 °C) of fava bean concentrate at pH ≤ 4 and pH ≥ 6.4 results in two different protein modifications
- Fava protein hydrolysis and aggregation induce relevant and impactful functional modifications
- Physico-chemical properties of fava proteins is greatly influenced by final application pH
- Foaming and Emulsification are highly influenced by process conditions

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Research Article

Effect of industrial process conditions of fava bean (*Vicia faba* L.) concentrates on physico-chemical and functional properties

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Abstract

Fava bean (*Vicia faba* L.) is a promising source of proteins and has a potential in industrial food applications. Processing of fava ingredients modifies proteins and their functional properties. This study shows that there is no straightforward relationship between fava protein-associated reactions, physico-chemical properties and functional properties. For this study, an air-classified fava protein concentrate was processed at different combinations of pH (2, 4, 6.4 and 11), temperature (55, 75 and 95 °C) and duration of treatment (30 and 360 min) to produce several modified fava concentrates. Ingredient processing led to protein hydrolysis and protein aggregation, which influenced foaming and emulsification differently due to the differences in their stabilizing mechanisms. Despite the protein modifications, their physico-chemical and functional properties were primarily governed by the beverage application pH. The surprising interplay shown between properties encourages the need to dive further into the different protein-associated interactions that can occur in fava concentrate.

Industrial Relevance

The focus of this work was to understand the impact of processing conditions on the functionality of fava bean protein-rich ingredients – in particular in systems close to industrial beverage applications. Ingredient processing, especially ingredient modification is meant to render ingredients more suitable for food applications – by improving their functional, organoleptic, safety properties. The process conditions (pH, temperature, treatment duration) were thus chosen for their industrial simplicity and accessibility. Despite some evidences on the effects of these conditions on fava proteins and associated functionalities, is it necessary to further understand the mechanisms at the origin of functional properties. In this manner, the food industry will be able to optimize the appropriate process conditions and their levels with the help of suitable assessment methods.

Keywords: Protein functionality**,** foaming, emulsification**,** modification, hydrolysis, aggregation

Abbreviations: A/W, air-in-water; ANOVA, analysis of variance; DSC, differential scanning calorimetry; EC, emulsion capacity; ES, emulsion stability; FBIC, fava bean initial concentrate; FC, foaming capacity; FS, foam stability; HIUS, high intensity ultrasound; MW, molecular weight; O/W, oil-in-water; PARAFAC, parallel factor analysis; PSD, particle size distribution; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulphate; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

1 Introduction

Fava bean (*Vicia faba* L.) has a great potential for human consumption due to its nutritional, functional and agronomic aspects (Multari, Stewart, & Russell, 2015). Fava bean can be processed to form ingredients (ingredient fabrication) and these ingredients can further be modified using

process conditions (ingredient modification) and eventually be utilized in food applications (ingredient utilization) (Sharan, Zanghelini, et al., 2021). In fava bean, various protein types, majorly globulins (legumin, vicilin, convicilin) exist in different conformations. Any changes in these conformations during ingredient fabrication, modification and utilization affects the functional property of the ingredient (Arntfield & Murray, 1981; Muschiolik, Hörske, Schneider, M., & Schmandke, 1986; Sharan, Zanghelini, et al., 2021). Functionalities such as foaming and emulsification, play a key role in beverage applications such as ice-cream, pudding, mousse, etc. (Damodaran, 2006; Alu'datt et al., 2017; Jarpa-Parra, 2018; Mustafa, He, Shim, & Reaney, 2018). While foams are formed from adsorbed air-in-water (A/W) interfaces, most food emulsions are produced from that of oil-in-water (O/W). Generally, proteins are effective surfactants, and thus play an essential role in the foaming and emulsification properties of plant-based ingredients. Though foams and emulsions are based on the same structure-function relationship of proteins, differences may occur because of changes in the ingredient's effectiveness or functionality due to variances in the dispersed phase, its interactions with proteins, and/or modifications in the proteins themselves (Damodaran, 2006; Mirmoghtadaie, Shojaee Aliabadi, & Hosseini, 2016; Wang et al., 2019).

Protein modifications by physical, chemical and biological process techniques can facilitate foams and emulsions by influencing a balance between protein solubility, charge distribution and protein folding (Damodaran, 2006; Akharume, Aluko, & Adedeji, 2021). During ingredient processing, fava proteins have been modified by temperature and pH (Arntfield & Murray, 1981; Arntfield, Murray, & Ismond, 1985), mechanolysis (Husband, Wilde, Clark, Rawel, & Muschiolik, 1994), high-intensity ultrasound treatment (Martínez-Velasco et al., 2018), succinylation

(Schwenke, Rauschal, & Robowsky, 1983), acetylation (Krause & Buchheim, 1994), and enzymatic treatment (Eckert et al., 2019). The effect of any treatments on protein structure and the related effect on functionalities at application conditions is not well understood (Sharan, Zotzel, et al., 2021). Amongst different protein modifications, protein-protein aggregation and hydrolysis have shown to improve functionalities (Krause & Buchheim, 1994; Cepeda, Villarán, & Aranguiz, 1998; Martínez-Velasco et al., 2018; Eckert et al., 2019). Protein aggregation and hydrolysis can be of different types and extent that result in a variety of effects on functional properties (Martínez-Velasco et al., 2018; Yang, Liu, Zeng, & Chen, 2018; Eckert et al., 2019). In addition, fava bean contain not only proteins but also various non-protein constituents, including starch, dietary fibers and fats, along with certain anti-nutritional factors (Multari et al., 2015; Sharan, Zanghelini, et al., 2021). Hence the reactions occurring during ingredient processing may be a result of proteins and/ or non-protein constituents (Kosińska, Karamać, Penkacik, Urbalewicz, & Amarowicz, 2011; Zha, Rao, & Chen, 2021). For now, there is no clear overview of all the possible reactions occurring during processing of fava ingredients that can evidently explain the changes in functional and physico-chemical properties. This investigation is a continuation of a previous paper (Sharan, Zotzel, et al., 2021) and further attempts to clarify the interplay between fava protein-associated reactions, protein properties and functional properties and brings forth the ambiguities in the relationship between them. The impact of industrially relevant process conditions such as pH, temperature and treatment duration on fava bean concentrate was evaluated in regards to: (1) fava protein aggregation and hydrolysis during ingredient modification, (2) physico-chemical properties of fava proteins at utilization conditions (charge,

solubility, intrinsic fluorescence and thermal integrity), and (3) functional properties (foam and emulsion capacity and stability) at conditions simulating beverage applications.

2 Materials & Methods

2.1 Sample preparation

2.1.1 Starting material

Fava bean initial concentrate (FBIC) containing 65% (w/w d.b.) proteins was procured by Döhler GmbH (Darmstadt, Germany). The concentrate was produced by milling of dried and dehulled beans followed by air classification (Felix, Lopez-Osorio, Romero, & Guerrero, 2018).

2.1.2 Modified-suspensions

The FBIC was modified as follows: 20% (w/w) suspensions were prepared with deionized water and agitated for 30 min at 500 rpm (~30 g) using an overhead dissolver stirrer (IKA Works, Inc., Staufen, Germany), followed by pH adjustment ($pH_{process}$) to 2, 4 or 11 using 6 M hydrochloric acid or 3 M sodium hydroxide (Sigma Aldrich, Missouri, United States) and further stirred for 30 min at 500 rpm. Additionally, a series with the natural suspension pH was prepared ($pH_{process}$ 6.4) by stirring for 30 min at 500 rpm. The suspensions were heated ($T_{process}$) in a temperature-controlled bath (Lochner Labor+Technik GmBH, Germany) at 55, 75 or 95 °C and agitated at 700 rpm for a duration ($t_{process}$) of either 30 (Low) or 360 (High) min. The suspensions produced after these treatments are denoted as *modified suspensions*. All the treatments at $pH_{process}$ 4 were performed in triplicates in order to assess reproducibility.

2.1.3 Modified ingredients

The different *modified-suspensions* were frozen at −20 °C, followed by freeze-drying and milling to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). This resulted in different modified ingredient powders, which are named as $pH_{process}_T_{process}_t_{process}$ (e.g. pH2_55 °C_Low) based on the conditions used to modify them.

2.1.4 Ingredient-aqueous-suspensions

All ingredients were suspended in deionized water in triplicates to 1% (w/w) protein concentration and stirred for 30 min at ambient temperature at the two $pH_{utilization}$ (4 and 7) to prepare *ingredient-aqueous-suspensions.* The pH was adjusted either using 6 M hydrochloric acid or 6 M sodium hydroxide. These systems were chosen as mimicking realistic beverage applications.

2.1.5 Ingredient-buffered-suspensions

1% (w/w) protein suspension of all ingredients (FBIC + modified ingredient*s*) were prepared in triplicates in citrate phosphate buffers (prepared from 0.1 M citric acid and 0.2 M dibasic sodium phosphate) at two $pH_{utilization}$ (4 and 7) and stirred for 30 min at ambient temperature to produce *ingredient-buffered-suspensions*. Ionic strength of the buffer solutions used was calculated by the formula $\sum C_i z_i^2/2$, where C_i is the molar concentration of the ion species 'i' and z_i is the net charge of that ion (H.-M. Eun, 1996).

2.2 Protein-associated reactions

2.2.1 Protein aggregation

Particle aggregation in the *modified-suspensions* (section 1.1.2) was measured using laser light scattering by Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, U.K.) with degassed,

deionized water used as the dispersant. The particle size distribution (PSD) from 0.005 to 5000 µm as a function of volume was recorded and the volumetric mean particle diameter, D[4;3], was used to compared the level of particle aggregation after the different ingredient modification treatments.

2.2.2 Protein acid-hydrolysis (SDS-PAGE)

The *modified-suspensions* were diluted to 2.25 mg protein/ml with Milli-Q water (Millipore, France) with a mixture containing 1% (w/v) SDS and 1.4% (w/v) glycine, then submitted to sonication for 30 min and centrifugation at 10,000 g for 2 min to obtain a supernatant of dissolved polypeptides. Protein concentration of the supernatants were determined at this stage by Dumas method using Rapid MAX N Exceed (Elementar, Langenselbold, Germany). Aliquots of 22.5 µg of proteins were loaded along with peqGOLD protein marker II (VWR International, Pennsylvania, United States) into 12% (w/v) Bio-Rad Mini-PROTEAN®TGX™ gel (Bio-Rad Laboratories, California, United States) and run at 200 V for 45 min. The polypeptide bands were stained by 0.25% (w/v) coomassie brilliant blue dye. Electrophoresis was performed under non-reducing conditions. The resultant gel band-size intensities of larger (40–100 kDa) and smaller (< 40 kDa) subunit groups were analyzed by semi-quantitative comparison of their pixel intensities in the gel using GelAnalyzer (Lazar & Lazar, 2010). The change in band-size intensity (%) was calculated in relation to the subunit groups found in FBIC.

2.3 Physico-chemical properties

2.3.1 Nitrogen solubility

The soluble fractions of the *ingredient-buffered-suspensions* (section 1.1.5) were separated at 8,000 g for 20 min and its total nitrogen content was determined by the Dumas method using

Rapid MAX N Exceed (Elementar, Langenselbold, Germany). The solubility (%) of proteins at each pH was presented as the ratio between the total nitrogen content of the supernatant and the total nitrogen content of the initial suspension.

2.3.2 Surface charge

Surface charge represented by the zeta potential of the undiluted soluble fractions of the *ingredient-buffered-suspensions* was determined by dynamic light scattering in DTS1070 folded capillary cells equilibrated for 120 s at 25 °C using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, U.K.).

2.3.3 Intrinsic protein fluorescence

Protein folding nature of the *ingredient-buffered-suspensions* was analyzed by fluorescence using a FS 920 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, United Kingdom). Additional experiments with 0.1% (w/w) protein concentration were conducted to observe any changes in fluorescence signals due to the dilution. The excitation-emission map of the protein region was developed by varying excitation wavelengths from 250 to 340 nm at 5 nm increments and by varying emission wavelengths from 300 to 360 nm at 2 nm increments for a dwell time of 0.05 s, using excitation and emission slits of 5 nm.

2.3.4 Protein thermal integrity (DSC)

FBIC and the modified ingredients treated either very gently (pHX_55 °C_Low) or vigorously (pHX_95 °C_High) at different pH (noted pHX) were taken to assess their protein integrity due to process conditions. *Ingredient-aqueous-suspensions* of 10% (w/w) were prepared by stirring overnight at 4 °C, followed by adjustment to pH 4 and 7 and overnight stirring at 4 °C. The concentration was brought to 6% (w/w) with Milli-Q water (Millipore, France) and approximately

60 mg was transferred to a 120 µl medium pressure crucible and run in a DSC (Mettler Toledo, Ohio, United States). The crucible was heated from 50 to 120 °C at 5 °C/min, with an empty reference crucible. The denaturation temperature and enthalpy were determined using the DSC software package (STARe SW 16.00).

2.4 Functional Properties

2.4.1 Foaming

150 ml of the *ingredient-aqueous-suspension* was whipped mechanically at room temperature using a WMF Mechanical Frother (Württembergische Metallwarenfabrik GmbH, Geislingen, Germany) for 2.5 min and the foam was transferred to a graduated cylinder (inner diameter = 48.9 mm and height = 400 mm measured using a digital caliper). Foam height and liquid height were recorded manually to calculate the foam and liquid volume, respectively. Foaming capacity (FC, %) was calculated as the ratio of volume of foam generated after whipping and liquid volume. Foam stability (FS, %) was foam capacity measured after 30 min (Muschiolik et al., 1986). Foam was categorized unstable when FS was below 50%.

$$\textbf{\textit{FC (\%)}} = \frac{\textit{Foam Volume } 0_{min}}{\textit{Liquid Volume}} \text{X } 100 \textit{ ; } \textbf{\textit{FS (\%)}} = \frac{\textit{Foam Volume } 30_{min}}{\textit{Liquid Volume}} \text{X } 100$$

2.4.2 Emulsification

The *ingredient-aqueous-suspensions* were added with palm oil medium chain triglycerides (90:10 w/w) and homogenized for 1 min at 8000 rpm using T-10 Basic ULTRA-TURRAX homogenizer (IKA Works, Germany) fitted with an S-10N-10G dispersing element. The coarse emulsions formed were passed twice through a Niro-Soavi NS 1001L Panda homogenizer (Gea Group, Germany) at 200 bars. The emulsions were pasteurized at 80 °C for 10 min just after the emulsion preparation

195 to prevent microbial growth during storage. The pasteurized emulsions were stored at 4 °C for
196 seven days to evaluate emulsion stability (Karaca, Low, & Nickerson, 2011). The emulsion oil
197 droplet size at day 0, 1 and 7 was measured using laser light scattering (Mastersizer 3000,
198 Malvern Instruments Ltd., U.K.) with degassed, deionized water used as the dispersant. The
199 particle size distribution from 0.005 to 5000 µm as a function of volume was recorded followed
200 by the estimation of the volumetric mean diameter (D[4;3]), which was used to assess the
201 emulsion capacity and stability (Makri, Papalamprou, & Doxastakis, 2005; Felix et al., 2018).
202 Contour plots of the D[4;3] values were generated by Minitab (Minitab Inc., Pennsylvania, United
203 States) using distance method of interpolation.

204 ## *2.5 Statistical Analyses*

205 Fluorescence data was processed by parallel factor analysis (PARAFAC) (Bro, 1997). The
206 fluorescence landscapes were first pre-processed by removing the Rayleigh scatter according the
207 procedure suggested by Thygesen, Rinnan, Barsberg, & Møller, 2004. This was then analyzed by
208 PARAFAC into three matrices: score matrix, an excitation loading matrix and an emission loading
209 matrix. The two suspensions at 0.1 and 1% (w/w) were analyzed separately, and the data were
210 decomposed with three and two factors, respectively. The fluorescence landscapes were
211 processed and analyzed in MATLAB (Mathworks, Massachusetts, United States).
212 Four-way ANOVA followed by Bonferroni's multiple comparison analysis to evaluate the effect of
213 factors ($pH_{process}$, $T_{process}$, $t_{process}$ and $pH_{utilization}$) on ingredient properties (protein charge, protein
214 solubility, protein fluorescence intensity scores and functional parameters) was conducted using
215 XLSTAT 2021.1. (Addinsoft, France). The threshold for statistical significance was $\alpha = 0.05$.

3 Results & Discussion

3.1 Effect of processing (modification conditions) on fava proteins

Protein associated reactions like protein aggregation and protein hydrolysis occurred during ingredient modification. The volumetric mean diameter extracted from the particle size distribution (PSD) of all the *modified-suspensions* showed different degrees of aggregation reactions as a function of the process conditions (Figure 1A). In general, a gradual increase in the aggregate size as a function of temperature ($T_{process}$) and time ($t_{process}$) was observed for the modification at $pH_{process}$ 4, 6.4 and 11. As seen, intensive aggregation (> 200 nm) took place as a result of especially three ingredient modifications (orange bars) at 'High' $T_{process}$ (Figure 1A). The PSD of these special *modified-suspensions* (Figure 1B) confirmed that these contained large aggregates of different sizes (up to 1000 µm) indicating protein associated reactions. This was compared to the monomodal distribution of FBIC suspension, which was unmodified by the process conditions. This inference corresponded well with an earlier report on fava protein aggregation that yielded similar polymodal distribution of aggregates reaching sizes of 1000 µm (Yang et al., 2018; Vogelsang-o'Dwyer et al., 2020). Interestingly, there was an indistinct trend of aggregation observed at $pH_{process}$ 2 showing some extent of aggregation for all $T_{process}$ (Figure 1A). At $pH_{process}$ 4 and 6.4, the lower $T_{process}$ had only minor impact on protein aggregation.

The non-reduced SDS–PAGE analysis revealed changes in type and molecular weight distribution of the soluble proteins extracted from the *modified-suspensions*. The typical protein profile of fava bean (in FBIC) is seen in lane T0 (Figure 2) representing the globulins consisting of legumin minor subunit (80 kDa), convicilin subunit (70 kDa), legumin major subunit (60 kDa), vicilin subunit (50 kDa), and albumin (10-20 kDa) (Bailey & Boulter, 1970; Bassuner, Hail, Jung, Saalbach,

238 & Muntz, 1987; Sáenz de Miera, Ramos, & Pérez de la Vega, 2008). It is emphasized that in the
239 SDS–PAGE analysis, the same total protein concentration is loaded in each lane, hence the
240 electrophoretic result shows the relative distribution of the individual solubilized proteins in a
241 comparable mode. Figure 2 shows severe changes in the extracted protein fractions from the
242 *modified-suspensions* owing to the differences in the band intensities obtained. Band-size of
243 larger subunits (40–100 kDa) decreased by around 37% during modification at pH2_75 °C_High
244 (marked in red in figure 2A). A total band disappearance (> 96% decrease) of the large subunits
245 occurred at acidic conditions (≤ pH 4), treated at 95 °C for 360 min (i.e. pH2_95 °C_High and
246 pH4_95 °C_High, marked in red in figures 2A and 2B). A simultaneous band-size increase (> 31%
247 increase) in smaller peptides (< 40 kDa) indicated occurrence of protein hydrolysis to a certain
248 extent (pH2_75 °C_High, marked in red in figure 2A). Thus, acid-hydrolysis of fava proteins
249 occurred at lower $pH_{process}$ (≤ 4), at higher $T_{process}$ (≥ 75 °C) and at 'High' $t_{process}$ (360 min) during
250 modification. Modification at higher $pH_{process}$ (≥ 6.4) and at higher $T_{process}$ (95 °C) resulted in no
251 visual band change of either larger subunits (> 82% decrease) or smaller subunits (> 8% decrease)
252 due to protein aggregation (purple, Figures 2C and 2D) in agreement with results from PSD
253 (Figures 1A and 1B).

254 *3.2 Effect of utilization conditions on fava proteins*

255 The fava proteins after being modified by the process conditions showed further distinction in
256 properties when suspended at two $pH_{utilization}$ (4 and 7). The highest ionic strength change (μ =
257 0.07) was at protein concentration 1% (w/w) due to changes in the $pH_{utilization}$. Functional
258 properties of fava proteins are often favored at μ ≤ 0.4 ionic strength. Thus, the change in ionic

strength was concluded not to affect the functional properties (Arogundade, Tshay, Shumey, & Manazie, 2006).

The zeta potential, representing protein surface charge of fava bean proteins, was close to 0 (0.96 ± 0.53 mV) for FBIC at $pH_{utilization}$ 4, indicating that the overall isoelectric pH of fava proteins (predominantly legumin and vicilin) was close to pH 4 (Figure 3). However, at $pH_{utilization}$ 7, the surface charge was highly negative (-8.24 ± 1.70 mV) due to effects of proteolytic active side residues.

Considering all the ingredients, the zeta potential varied significantly only as a function of $pH_{utilization}$ ($p = 0.0001$) and not by $pH_{process}$ ($p = 0.532$), $T_{process}$ ($p = 0.438$), nor $t_{process}$ ($p = 0.075$). There was an overall shift of charge at $pH_{utilization}$ 4 towards a more negative charge in the modified ingredients compared to FBIC, with an exception of the ingredients pH2_55 °C_High, pH6.4_55 °C_Low and pH6.4_55 °C_High. Comparing surface charges with the ingredients containing aggregated or hydrolyzed fava proteins, no specific trend was seen (Figure 1, 2 and 3). It would have been expected that the severe process conditions might have further unfolded the globular proteins, exposing previously hidden polar groups that lead to a changed surface charge. But interestingly, the charge of fava proteins seemed robust during ingredient modification, but significantly changed only during ingredient utilization.

The nitrogen solubility representing the solubility of fava proteins at $pH_{utilization}$ 4 and 7 (Figure 4) showed that FBIC had a very low protein solubility at $pH_{utilization}$ 4 as expected due to an overall neutral charge (Figure 3), thus, disfavoring repulsion between residues. On the contrary, FBIC proteins were highly soluble (82 ± 4%) at neutral pH, which could be attributed to the higher overall negative charge and thus enhanced repulsion, hindering the precipitation and favoring

281 solubility. The solubility of the modified ingredients varied significantly due to $pH_{process}$ ($p = 0.035$)
282 and $pH_{utilization}$ ($p = 0.0001$) but not by $T_{process}$ ($p = 0.070$) or $t_{process}$ ($p = 0.195$). However, this overall
283 insignificant effect of $T_{process}$ on protein solubility seemed to heavily weighted by the result from
284 the different $pH_{process}$ and $pH_{utilization}$, and hence should not be dismissed as a factor. On the
285 ingredients modified at $pH_{process}$ 6.4, a notable decrease in solubility at increasing $T_{process}$ can be
286 seen (Figure 4). For instance, at $pH_{utilization}$ 7, the ingredient pH 6.4_55 °C_Low with 74.3%
287 solubility was comparable to FBIC with 81.9% solubility, while the solubility decreased to 30.9%
288 for pH 6.4_95 °C_High. Thus, the native proteins were affected by the processing temperature
289 differently than at other modification temperatures, which in turn affect the solubility at
290 $pH_{utilization}$ 7 to a greater degree (but not at $pH_{utilization}$ 4).
291 Solubility of fava proteins varied more compared to the surface charge property at the two
292 different $pH_{utilization}$, indicating that the ingredient modification process has more impact on this
293 property. It is well known that the magnitude of the solubility is determined mostly by two
294 opposing contributions: 1) structural changes exposing previously hidden polar groups in effect
295 increasing protein/solvent interactions and facilitating solubility and/or 2) structural changes
296 exposing reactive side chains, in effect increasing protein/protein association resulting in
297 aggregation and reduced solubility (Sathe, Zaffran, Gupta, & Li, 2018). Solubility and surface
298 charge of proteins are often related (Kramer, Shende, Motl, Pace, & Scholtz, 2012), but as seen
299 comparing results in Figures 3 and 4, the two properties were affected differently by the process
300 conditions at the two $pH_{utilization}$. The surface charge and, thereby, any effect on ionizable side
301 groups in the proteins due to different molecular microenvironments (e.g. denaturation), did not
302 completely explain the change in solubility.

303 Digging further into the observed solubility, fava proteins from the modification and utilization
304 conditions were characterized by their intrinsic fluorescence. This was first done at 1% (w/w)
305 protein concentration, but possible inner filter effects were expected due to the physical nature
306 of the suspensions. To give an example, all the 1% (w/w) protein suspensions were visually cloudy
307 at $pH_{utilization}$ 4 due to the formation of protein precipitates. In addition, presence of quenchers at
308 this concentration could also lead to attenuation of the fluorescence signals (Bevilacqua, Rinnan,
309 & Lund, 2020). Thus, fluorescence at a dilution of 0.1% (w/w) protein suspensions was also
310 considered to avoid obscurity in comprehending the results. Eventually, the PARAFAC model
311 constructed from the fluorescence data yielded three components for the 0.1% (w/w) samples,
312 while only two for the higher, 1% (w/w) *ingredient-buffered-suspensions*.
313 PARAFAC is a rapid and efficient tool that decomposes the fluorescence signals into its individual
314 contributions. PARAFAC models conform to the Beer's Law and has been well established for
315 organic chemicals (Murphy, Stedmon, Graeber, & Bro, 2013). Models explaining intrinsic
316 fluorescence of protein and protein interactions are gaining popularity (Bruun, Holm, Hansen,
317 Andersen, & Nørgaard, 2009; Simpson, Burke, & Jiji, 2011; Steiner-Browne, Elcoroaristizabal, &
318 Ryder, 2019). The constructed PARAFAC model in this investigation consisted of a score matrix
319 and two loadings matrices. The loadings contained suggested information on protein chemistry
320 (Table 1), whereas the scores indicated the relative contribution of each of the extracted
321 fluorescence signals (given by a loading pair; excitation and emission loading) (Figure 5). The
322 PARAFAC loadings representing excitation and emission wavelengths were comparable between
323 the two concentrations. It was clear that PR1 of 0.1% (w/w) protein suspension is similar to PR1
324 of 1% (w/w) protein suspension, however, the latter is shifted slightly to higher wavelengths in

the emission mode, suggesting that the 1% solution suffers from some inner-filter effects. This is further corroborated by comparing the two factors from the 1% model, with that of the three factor 0.1% model. In the former, the factors could indicate buried and exposed tryptophan (Lakowicz, 2006; Royer, 2006), while for the three factor model, in addition to the two signals from tryptophan, it seems that tyrosine now also gives a strong, separate signal (λ_{ex} = 285 nm, λ_{em} = 322 nm). By inspection of Table 1, it becomes apparent that the two factors in the 1% model are in between the three factors from the 0.1% model, again strongly indicating that the 1% suffers from inner-filter effects.

While the PARAFAC loadings represented complexities in protein polypeptide folding, the PARAFAC scores represented effects of modification and utilization conditions on the polypeptide folding. However, upon comparing scores from 1% and 0.1% protein concentrations, it becomes evident that the scores, on average, is only ~6 times higher at 1% while it should be around 10, if the system follows the Lambert-Beer's law. Furthermore, at lower dilutions, presence of quenchers and their concentrations may add to complexity and the interpretation of the results (Bevilacqua et al., 2020). We will therefore focus our discussion on the fluorescence based on the 0.1% protein concentration samples.

For the modified ingredients at 0.1% (w/w) protein suspensions, the protein folding complexity was impacted significantly by different process conditions. For instance, $pH_{utilization}$ significantly impacted all three PR1 (p = 0.001), PR2 (p = 0.001) and PR3 (p = 0.001) scores. The $pH_{process}$ significantly impacted PR1 (p = 0.001) and PR2 (p = 0.001) scores, which possibly represented tyrosine and buried tryptophan residues respectively. Significant effects of $T_{process}$ and $t_{process}$ were only found in PR3 (p = 0.010) scores which were possibly linked to exposed tryptophan residues.

For FBIC (0.1%), all the scores are about twice as high at $pH_{utilization}$ 7 compared to $pH_{utilization}$ 4 (Figure 5A, B, C). This is in agreement with the N-solubility measurements, indicating that there are more proteins in soluble fraction at $pH_{utilization}$ 7 compared to $pH_{utilization}$ 4. This same effect can be seen for PR1 and PR2 at native pH ($pH_{process}$ 6.4), possibly indicating that the intensity of PR1 and PR2 are linked to the nitrogen solubility in the system. In general, the largest variability in the fluorescence data is caused by the difference in $pH_{utilization}$. As seen, the effect of $pH_{process}$ on the resultant PR1 and PR2 intensities gives an overall U-shaped behavior with the extreme conditions at $pH_{process}$ 2 and 11 causing severe fava protein modifications. However, it is not likely that this means that the protein is behaving in the same way at these two pH values – as seen from the protein hydrolysis and aggregation evidences (Figure 1 and 2). Additionally, effects of $T_{process}$ seem to be notable for ingredients modified at $pH_{process}$ 6.4 once again, just as in the case of solubility data, indicating that the effect of $T_{process}$ may be overshadowed by the variability in the data due to $pH_{process}$ in the ANOVA. Thus, all these observations suggest that the $pH_{process}$ could drive how the protein properties are impacted by temperature and time during ingredient processing, and additionally the impact on properties may be further exposed as a function of $pH_{utilization}$.

Overall, it was clear that the all process conditions during ingredient modification affected protein folding complexity with a large dependency of the $pH_{utilization}$. It is stressed, though, that caution regarding essential conclusions must be taken, since chemical and physical changes with non-protein components and potential quenchers may affect fluorescent data of protein concentrates. As is shown here, diluting the sample will reduce the impact of inner-filter effects and quenching, and improves the interpretability of the data.

Calorimetric analysis of nine specific ingredients supported the results of protein charge, solubility and protein folding; a predominance of $pH_{utilization}$ was observed (Table 2). As seen, FBIC had lower T_p at $pH_{utilization}$ 4 than at $pH_{utilization}$ 7, indicating a relatively higher heat stability of fava proteins at $pH_{utilization}$ 7. This lower denaturation temperature at isoelectric pH is due to lower structural integrity corresponding to the difference in protein folding complexity, the change in surface charge resulting in precipitation in effect decreased solubility. On the contrary, the proteins in FBIC at neutral pH had a net negative surface charge (and perhaps buried hydrophobic areas), which are typical for native folded proteins, thereby exhibiting higher thermal stability.

The enthalpy of FBIC at the isoelectric pH was (numerical) lower than at neutral pH, reflecting greater structural integrity at $pH_{utilization}$ 7. Fava proteins from ingredients that were vigorously modified at high $T_{process}$ and $t_{process}$, i.e. pHX_95 °C_High, were all completely denatured ($\Delta H = 0$), in accordance with results that these ingredients contained either hydrolyzed proteins and intensively aggregated proteins (Figures 1 and 2; Table 2). Ingredients modified rather gently at low $T_{process}$ and $t_{process}$, i.e. pHX_55 °C_Low, had very different fractions of denaturation between 27 and 100% at both $pH_{utilization}$, showing that under these conditions, the fava protein structures were affected differently. The ingredient pH2_55 °C_Low contained extremely modified proteins through an undetermined reaction. The proteins in the ingredient pH6.4_55 °C_Low were least affected, but with increasing ΔH with $pH_{utilization}$, i.e. from isoelectric to neutral pH similar to FBIC. For the other gently modified ingredients, pH4_55 °C_Low and pH11_55 °C_Low, the ΔH did not change with $pH_{utilization}$ (Table 2). Typically, native proteins unfold and refold with the changes in the medium due to conformational flexibility. The rigidness in ΔH indicated that the proteins in some of these modified ingredients have lost their potential to refold between the two $pH_{utilization}$

as a consequence of the modification conditions. Different extent of protein denaturation (complete and partial) due to the modification conditions were identified, while protein renaturation ($\Delta H_{pH7} - \Delta H_{pH4} > 0$ J/g) or structural rigidity ($\Delta H_{pH7} - \Delta H_{pH4} \sim 0$ J/g) between utilization conditions were observed for the selected ingredients. The results indicate the possibility of other protein-associated modifications of various degrees aside from acid-hydrolysis and intensive aggregation leading to different states of partially or completely denatured proteins, that could impact protein and functional properties.

3.3 Effect of modification & utilization conditions on fava protein functionality

Foaming parameters, i.e. foam capacity (FC) and foam stability (FS), were high for FBIC (> 100%) at both $pH_{utilization}$ 4 and 7 (Figure 6), though both FC and FS were higher at $pH_{utilization}$ 4 compared to $pH_{utilization}$ 7 (by 15%). Also, as seen in Figures 6A and 6B, the FC of all modified ingredients at both $pH_{utilization}$ 4 and 7 was very high (> 100%). Despite the high FC values, there was still a significant impact on FC by $pH_{process}$ ($p = 0.006$), $t_{process}$ ($p = 0.0001$), $pH_{utilization}$ ($p = 0.010$), but not by $T_{process}$ ($p = 0.901$). The FS, however, was affected heavily by conditions of ingredient modification and utilization. The FS was significantly impacted by $pH_{process}$ ($p = 0.012$) and $pH_{utilization}$ ($p = 0.0001$) but not by $t_{process}$ ($p = 0.200$) or $t_{process}$ ($p = 0.165$). All modified ingredients maintained high FS (> 100%) at $pH_{utilization}$ 7, but at $pH_{utilization}$ 4, FS changed severely as a function of $pH_{process}$ (Figure 6). In fact, twelve modified ingredients gave unstable foams (< 50%) which were labelled *foam-breakers* (Figure 6A). Apparently, these *foam-breakers* were produced at treatment $pH_{process}$ 2 (pH2_55 °C_Low, pH2_55 °C_High, pH2_75 °C_Low and pH2_95 °C_Low), $pH_{process}$ 4 (all ingredients), and $pH_{process}$ 11 (pH11_55 °C_Low and pH11_55 °C_High). The modification conditions associated with the *foam-breakers* seemed inconsistent (Figure 6),

413 suggesting that there might be more than one single phenomenon causing foam instability at pH
414 4. Overall, *foam-breakers* were only formed at $pH_{utilization}$ 4, whereas all ingredients retained their
415 high FS (> 100%) at $pH_{utilization}$ 7.
416 The time dependency of FC was monitored for ingredients containing acid-hydrolyzed and
417 aggregated fava proteins (Figure 7). The ingredients pH2_75 °C_High and pH2_95 °C_High
418 containing acid-hydrolyzed proteins (lines with circled markers in Figure 2), did not show a
419 remarkable difference in the foaming property compared to FBIC at the two $pH_{utilization}$ (Figure 6
420 and 7A). However, one of these ingredients was a *foam-breaker* (pH4_95 °C_High) due to FS <<
421 50% (Figure 6A), and as seen the foam destabilization occurred fast within the first 10 min at
422 $pH_{utilization}$ 4 (Figure 7A). This ingredient contained hydrolyzed proteins and was expected to be a
423 foam stabilizer, hence, the reason for this surprising foam breakage is still unclear. At $pH_{utilization}$
424 7, the ingredients with acid-hydrolyzed proteins showed a slight increase in FC (5-18%) and FS
425 (13-28%, 30 min) compared to FBIC (Figure 7B). It seems that acid-mediated protein hydrolysis
426 had an improving role in FC and FS but only at $pH_{utilization}$ 7. Previous reports on fava protein
427 hydrolysis (however enzymatic), releasing buried amino acid residues led to a decrease in surface
428 tension and thus, resultant improvement in foam stability at isoelectric and neutral pH (Dudek,
429 Horstmann, & Schwenke, 1996; Krause & Schwenke, 1996; Eckert et al., 2019). Owing to the
430 differences between acid and enzymatic hydrolyses of proteins, it is not surprising why a
431 difference in the results were noticed here (Silvestre, 1997; Hou, Wu, Dai, Wang, & Wu, 2017).
432 Effects of acid-hydrolysis needs to be studied in greater detail for fava proteins.
433 The time dependency of the ingredients containing intensively aggregated proteins was
434 comparable to FBIC at both $pH_{utilization}$, with slightly lower FC (7-17% decrease) and FS (4-34%

435 decrease, 30 min) at $pH_{utilization}$ 4 (Figure 7A) compared to FC (0-7% decrease) and FS (<6%
436 decrease, 30 min) at $pH_{utilization}$ 7 (Figure 7B). Two states of non-thermally aggregated fava
437 proteins, large (>1µm), insoluble and supra-molecular (<1µm), soluble aggregates, have been
438 identified, where the latter was found to have superior foam properties (Yang et al., 2018).
439 Interestingly, the sizes of all the aggregates, before or after modification were always > 1µm
440 (Figure 1).
441 Emulsion capacity and stability, denoted by $D[4;3]_{Day0}$ and $D[4;3]_{Day7,}$ were impacted significantly
442 by $pH_{process}$ (p = 0.000 and 0.000 respectively) and $pH_{utilization}$ (p = 0.000 and 0.000 respectively).
443 But these were governed majorly by the $pH_{utilization}$ and less by the $pH_{process}$ (Figure 8). At $pH_{utilization}$
444 4, emulsions of all the ingredients at Day0 immediately creamed after production, indicating the
445 detrimental impact of the isoelectric pH on protein-stabilized emulsions. Obviously, emulsions
446 were still creamed throughout the storage period, and emulsion instability was as expected. The
447 range of particle sizes was between 35 - 130 µm (from green to red) for the emulsions, which
448 remained rather constant for every emulsion throughout Day1 and Day7 (Figure 8A).
449 The differences in the values could have been a function of $T_{process}$ dependent aggregation
450 reactions occurring during modification (Figure 1). At $pH_{utilization}$ 7 (Figure 8B), a considerable
451 difference was seen in emulsion capacity and stability. Unlike $pH_{utilization}$ 4, all emulsions produced
452 at $pH_{utilization}$ 7 were homogeneous immediately after production (from blue to yellow). The
453 $D[4;3]_{Day0}$ ranged between 4-81 µm, with $pH_{process}$ 11 modified ingredients producing emulsions
454 with the lowest $D[4;3]_{Day0}$. Despite some changes in D[4;3] of certain ingredient emulsions with
455 time (Figure 8B), the values restored back to initial at Day7. $pH_{process}$ had significant effect on the
456 $D[4;3]_{Day0}$ and $D[4;3]_{Day7}$ values and this was clear as although most of the emulsions were stable

457 during storage, all the emulsions from $pH_{process}$ 2 modified ingredients creamed and clarified at
458 Day1. Therefore at $pH_{utilization}$ 7, emulsion capacities were equivalent to each other, with
459 differences in D[4;3] values, but during storage, the stability was affected by $pH_{process}$.
460 Creaming of emulsions did not correspond to their D[4;3] values. Thus, no specific relationship
461 between emulsion stability and D[4;3] was noticed in these experiments as expected from Stokes
462 law (Mileva & Radoev, 2004; Tadros, 2013). As Stokes relationship plays well for oil droplet
463 diameter, the distortion observed could be due to the presence of precipitates formed either due
464 to protein precipitation at $pH_{utilization}$ 4, but also due to the aggregation reactions during
465 ingredient modification (as in Figure 1). The effect of isoelectric point, as seen as an effect on
466 protein charge, solubility, fluorescence and thermal integrity, can be well related to the
467 formation of protein precipitates, and thus preventing the proteins to form a stable O/W
468 interface to create an emulsion. During ingredient modification, intensive aggregation reaction
469 leads to formation of particles of size > 200 µm as seen in Figure 1. These aggregates were
470 produced in the *modified-suspensions*, which were then freeze-dried and subsequently milled.
471 Nevertheless, the aggregates can be still seen in the emulsions by virtue of their high D[4;3]
472 detected, along with the presence of smaller oil droplet sizes in their bimodal PSD (Figure 9).
473 Unlike the case of isoelectric precipitation, the emulsions formed from ingredients containing
474 protein aggregates were not destabilized. Rather, these emulsions existed as a stable system of
475 both the oil droplets as well as the protein aggregates together. Importance of retention of
476 emulsion stability by protein aggregates also have been previously reported (Wang et al., 2019;
477 Sharan, Zanghelini, et al., 2021). Acid-mediated protein hydrolysis did not show any notable
478 differences in the emulsion properties (Figure 8). No differences in the failed emulsions at

479 $pH_{utilization}$ 4 were expected, but even at $pH_{utilization}$ 7, the size distribution of the emulsions from
480 ingredients containing hydrolyzed proteins showed less of a bimodal distribution with higher
481 presence of larger aggregates (Figure 9). Also, the ingredients modified at $pH_{process}$ 2 all gave
482 unstable emulsions, including those having hydrolyzed proteins. Detrimental effects of $pH_{process}$
483 2 in ingredient emulsion stability needs to be investigated. Limited hydrolysis of fava proteins has
484 been favorable, but complete hydrolysis has been detrimental to emulsion properties (Dudek et
485 al., 1996; Eckert et al., 2019). Stability of emulsions from pH4_95 °C_High and instability of
486 emulsions from pH2_75 °C_High and pH2_95 °C_High, despite all containing hydrolyzed proteins,
487 calls for an interest to look deeper into the degree of hydrolysis and effects on structural changes
488 and functionalities of fava proteins.
489 To sum up, modified fava proteins' physico-chemical and functional properties were influenced
490 by the $pH_{utilization}$ to a great extent. Identified specific protein modifications, aggregation and
491 hydrolysis, had different relationships to the functionalities, foaming and emulsification and, also,
492 with very different dependency on the $pH_{utilization}$ (Table 3). However, within each $pH_{utilization}$,
493 associations between charge and solubility were not clear. Interpretation and use of the protein
494 fluorescence were greatly dependent on a subsequent dilution step posterior to the utilization.
495 Foam capacity and stability measurements were well associated with each other, where the
496 hydrolysis of fava proteins positively influenced the foaming properties. Emulsion oil droplet
497 diameter (D[4;3]) measurements did not correspond well to their visual inspection, i.e. an
498 increase or decrease in the D[4;3] did not correspond necessarily to higher emulsion capacity nor
499 stability. For instance, fava protein aggregation forming larger particles increased the D[4;3], but
500 this did not disturb their emulsifying ability at favorable pH. Additionally, higher foaming property

501 did not correspond to higher emulsification. These aggregated proteins that stabilized emulsions,
502 did not necessarily improve foamability in all ingredients. Therefore, it is difficult to predict
503 functionality from another, and also just by measuring the protein properties. Protein
504 modifications thus need to be monitored during ingredient processing to predict changes in
505 functionalities to a certain degree.
506 Lastly, the effects of protein modifications were not reflected on the protein properties
507 measured. Thermal stability evaluation by DSC suggested possibility of other reactions occurring
508 at different conditions. Since fava concentrate is a complex matrix of macro- and micro-
509 constituents, other non-protein associated reactions could influence the inter-dependence
510 between the properties. Therefore, it might be essential to monitor protein as well as non-
511 protein interactions and reactions during modification and utilization of the ingredients
512 (Carbonaro, Virgili, & Carnovale, 1996; Singhal, Karaca, Tyler, & Nickerson, 2016; Zha et al., 2021).

513 ## 4 Conclusion

514 Processing of fava bean concentrate at industry simulated conditions resulted in two opposite
515 protein modifications: acid mediated hydrolysis and protein aggregation. Their effects were not
516 mirrored in the physico-chemical properties. Though certain trends were observed in foam and
517 emulsion properties, their effects were to a large extent governed by pH during ingredient
518 utilization. Protein acid-hydrolysis improved foaming only at neutral pH, but had an unclear trend
519 regarding emulsification. Aggregation did not improve foaming, but retained emulsion stability
520 at neutral pH. In general, isoelectric pH during application was not suitable for foam stability,
521 emulsion capacity nor emulsion stability. There may be other unexplored reactions leading to
522 protein modifications, and causing differences in their thermal integrity. Considering physico-

chemical and functional properties, their relationship is also mostly dependent on the application pH. The current investigation shows this inter-dependence, but encourages the need to dive further into the different protein-associated interactions that can occur in fava concentrate. Fava bean concentrate exhibits a multi-component character and thus can be of functional value for the food industry.

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Author Contributions

Conceptualization, S.S., M-N.M., A.S-E., J.Z.; methodology, validation, formal analysis, investigation and data curation, S.S., Å.R., J.S.; resources, J.Z., D.B., J.A.; writing—original draft preparation, S.S.; writing—review and editing, All.; visualization, S.S.; supervision, Å.R., V.O., K.O., J.Z., A.S-E. and M-N.M.; project administration, M-N.M., A.S-E., J.Z.; funding acquisition, M-N.M., D.B., J.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare potential conflict of interest. Jens Zotzel, Julian Aschoff and Daniel Bonerz work at Döhler GmbH. Döhler GmbH is a global producer of natural ingredients, ingredient systems and integrated solutions, including plant-based products containing fava bean. Döhler

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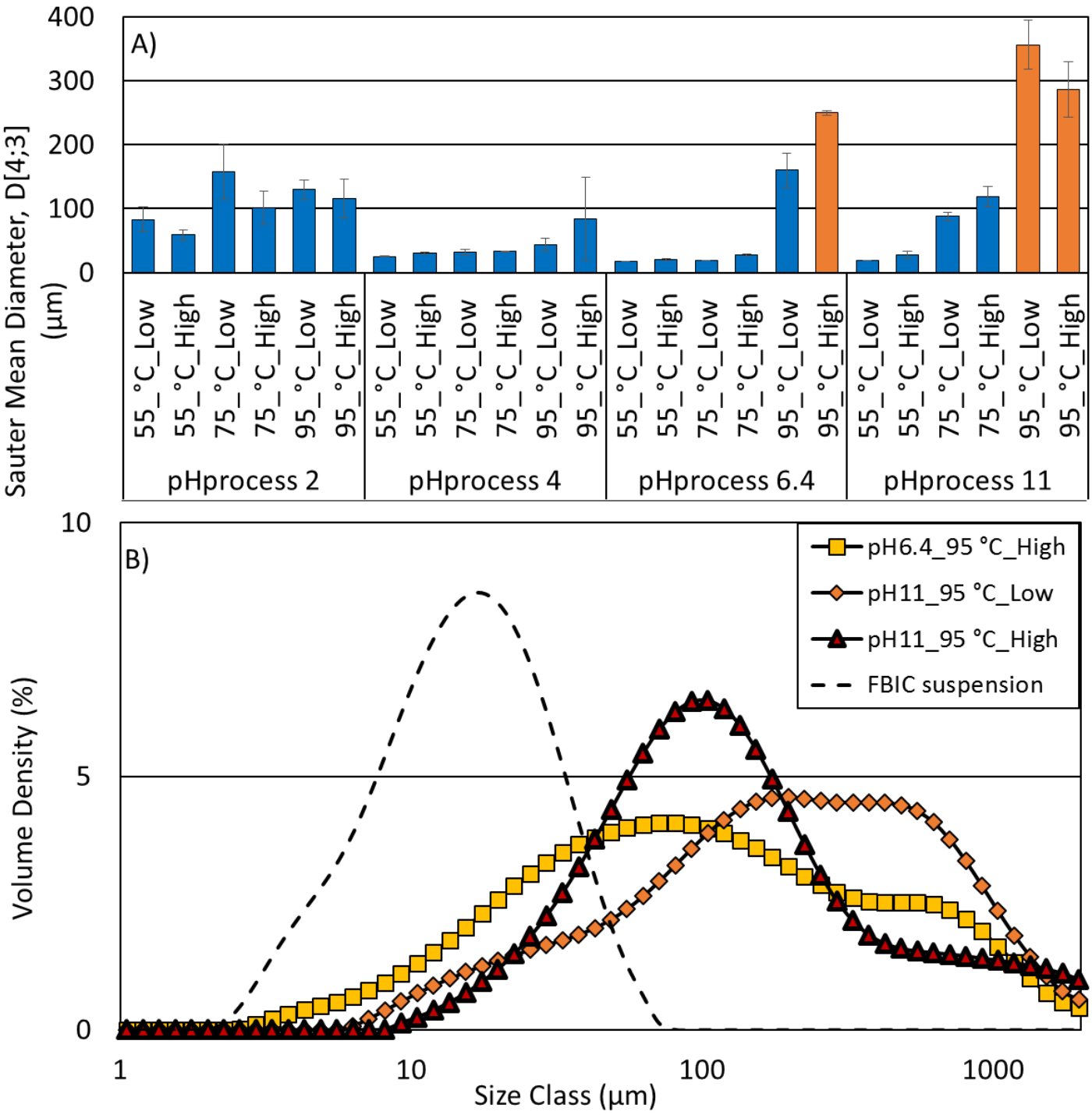


Figure 1. A) The volumetric mean particle diameter, D[4;3] of particles in *modified-suspensions*, B) PSD of the three *modified-suspensions* with aggregation reactions. These are compared to the fava bean initial concentrate (FBIC) suspension at the same concentration.

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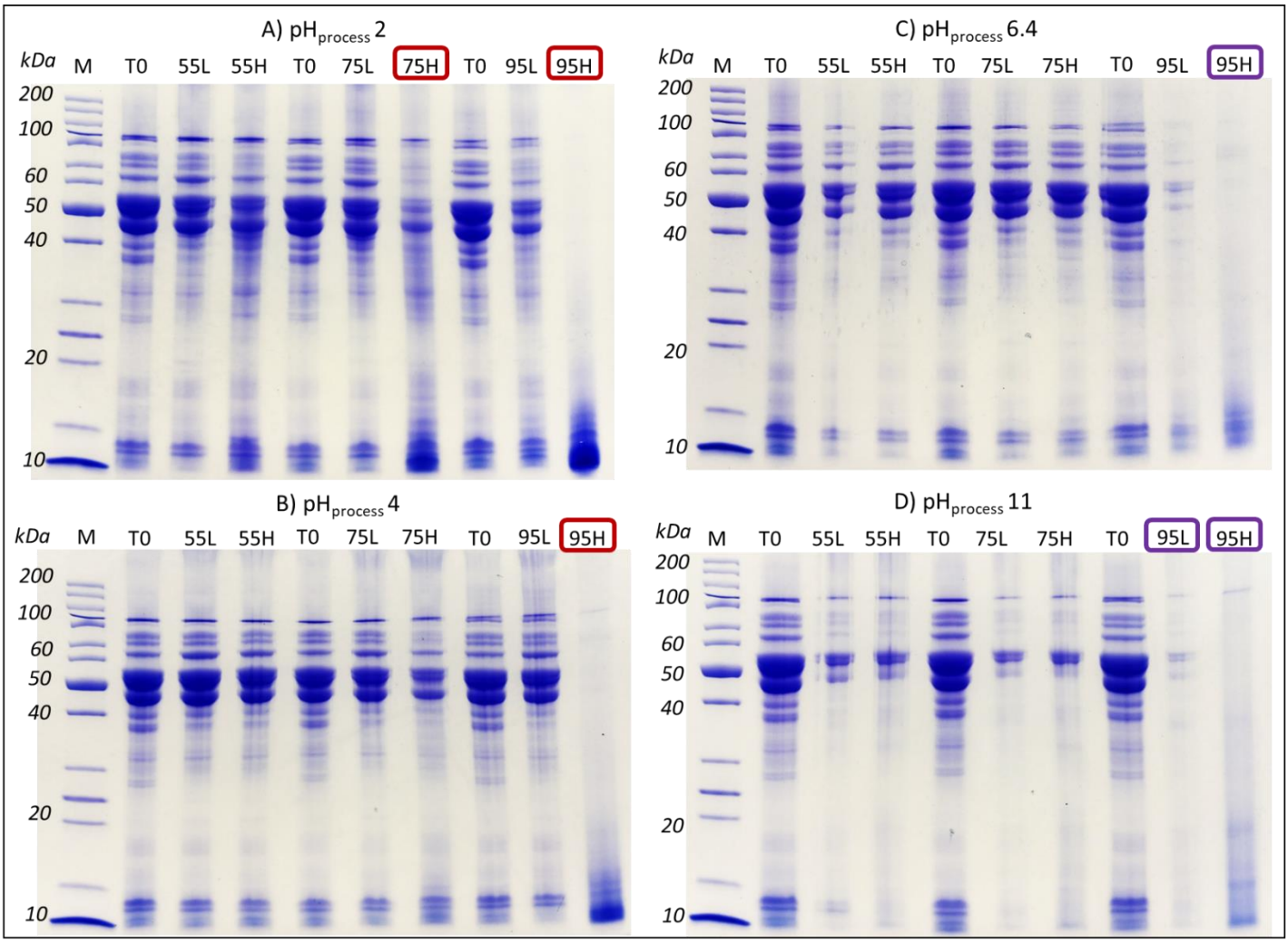


Figure 2. Non-Reduced SDS-PAGE of *modified-suspensions* at different $pH_{process}$: (A) 2, (B) 4, (C) 6.4, and (D) 11. Each gel column represents samples produced at different $T_{process}$ (55, 75 and 95 °C) and at different $t_{process}$, i.e. Low = 30 min (L) or High = 360 min (H) at a particular $pH_{process}$. Included are FBIC suspension (T0) as reference and protein marker (M).

Figure 3

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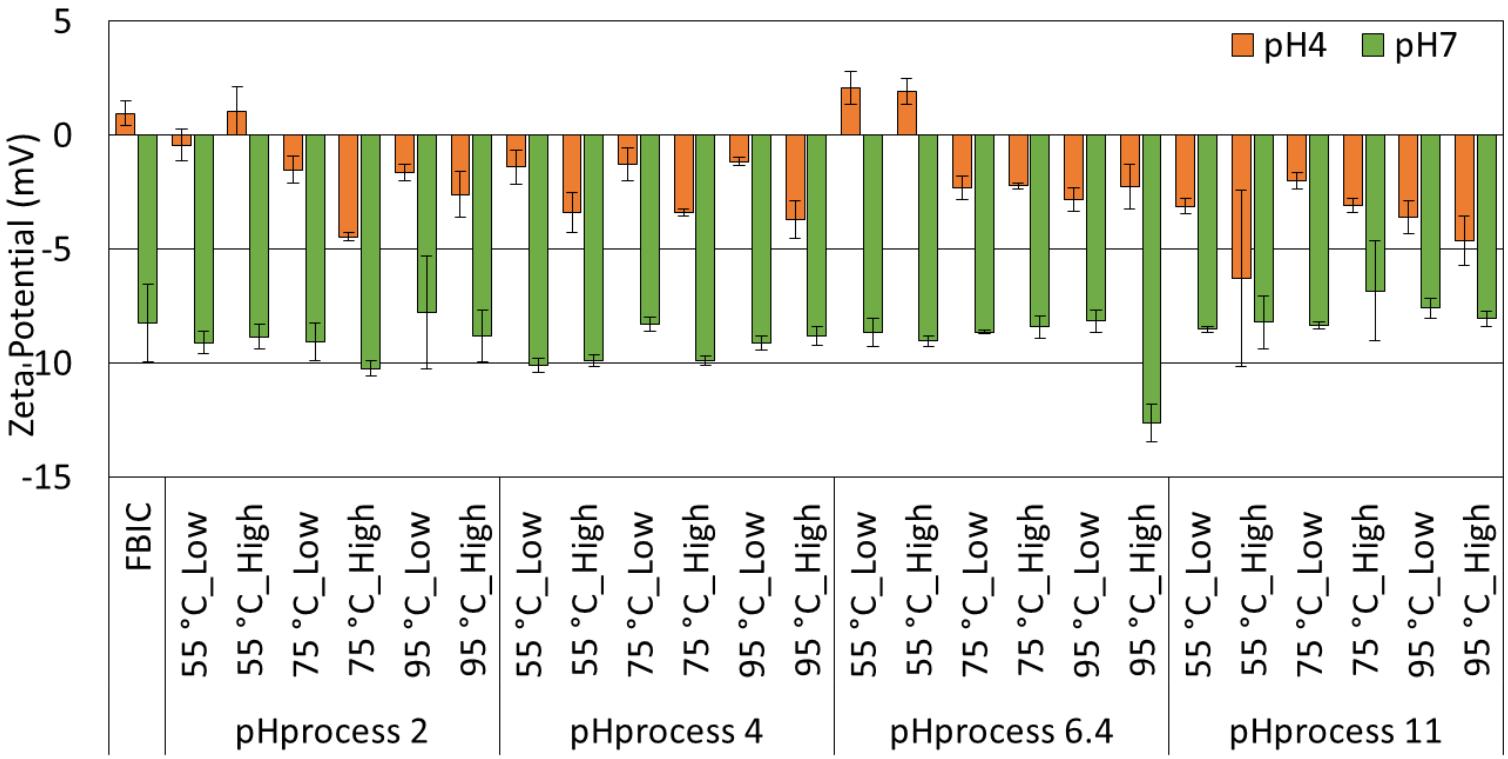


Figure 3. Zeta potential of FBIC and all *ingredient-buffered-suspensions* at $pH_{utilization}$ 4 and 7.

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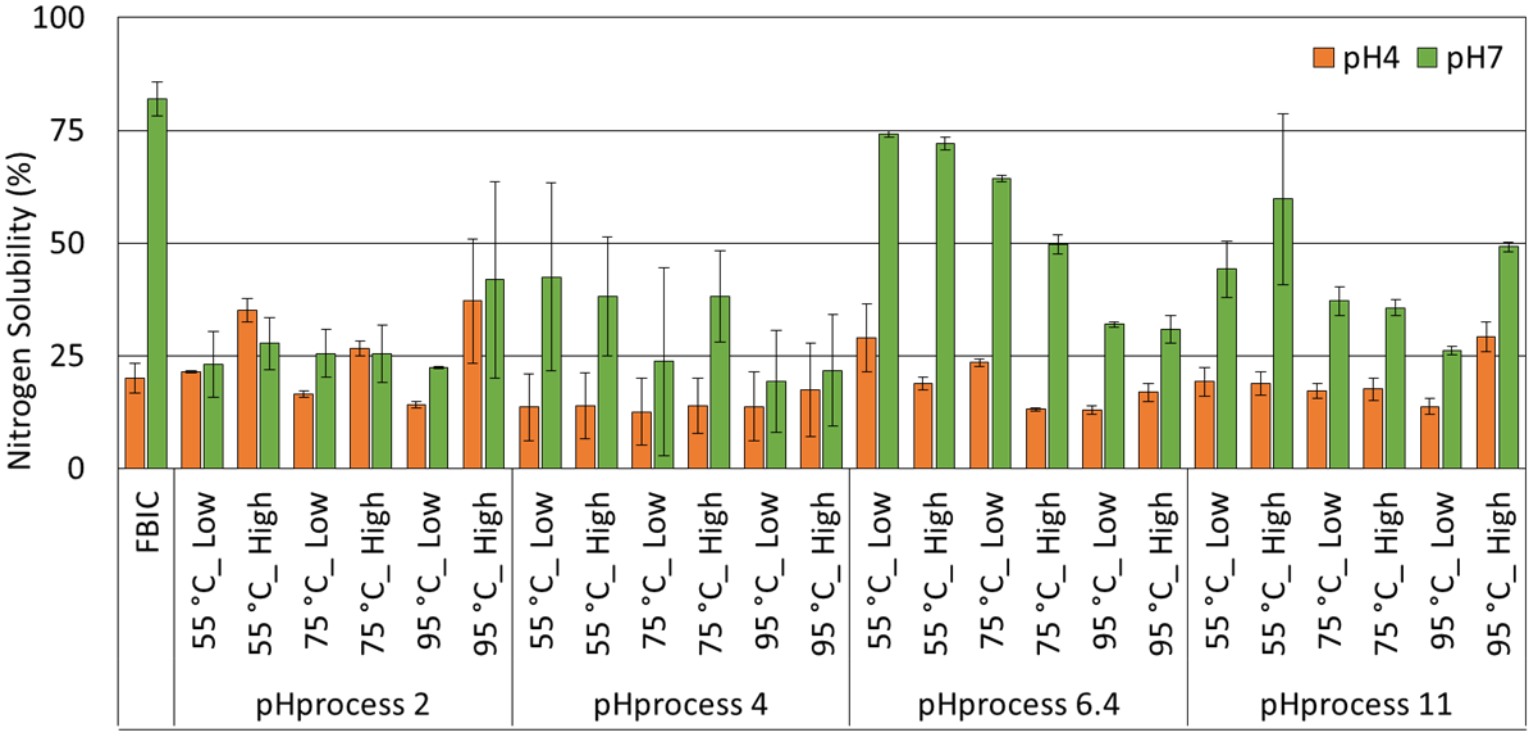


Figure 4. Nitrogen solubility of FBIC and all *ingredient-buffered-suspensions* at $pH_{utilization}$ 4 and 7.

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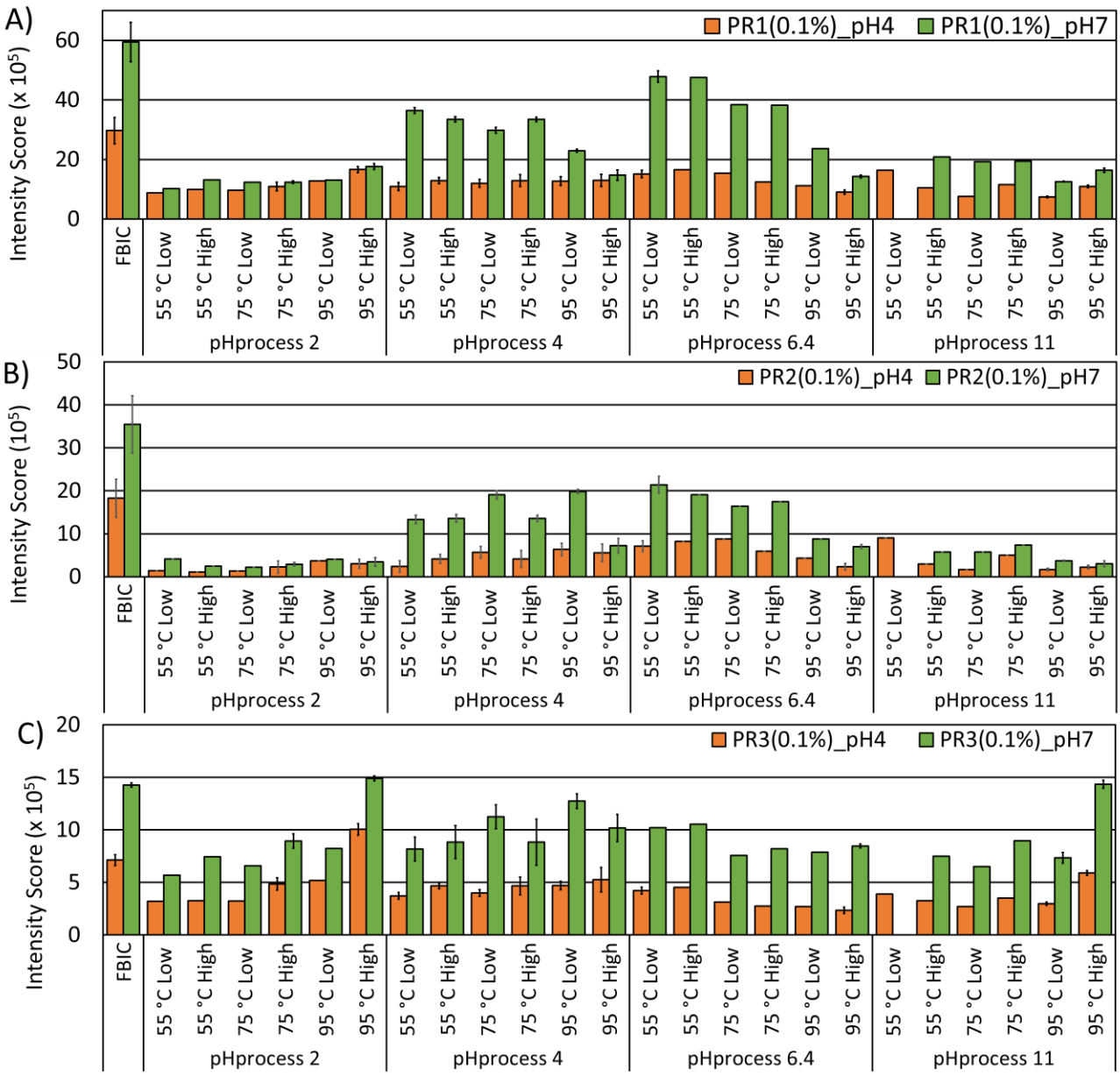


Figure 5. Intrinsic Protein Fluorescence by PARAFAC shown as score intensities of all *ingredient-buffered-suspensions* measured at $pH_{utilization}$ 4 and 7. A (PR1), B (PR2) and C (PR3) show scores of protein-associated components detected by the PARAFAC loadings at 0.1% (w/w) protein concentration.

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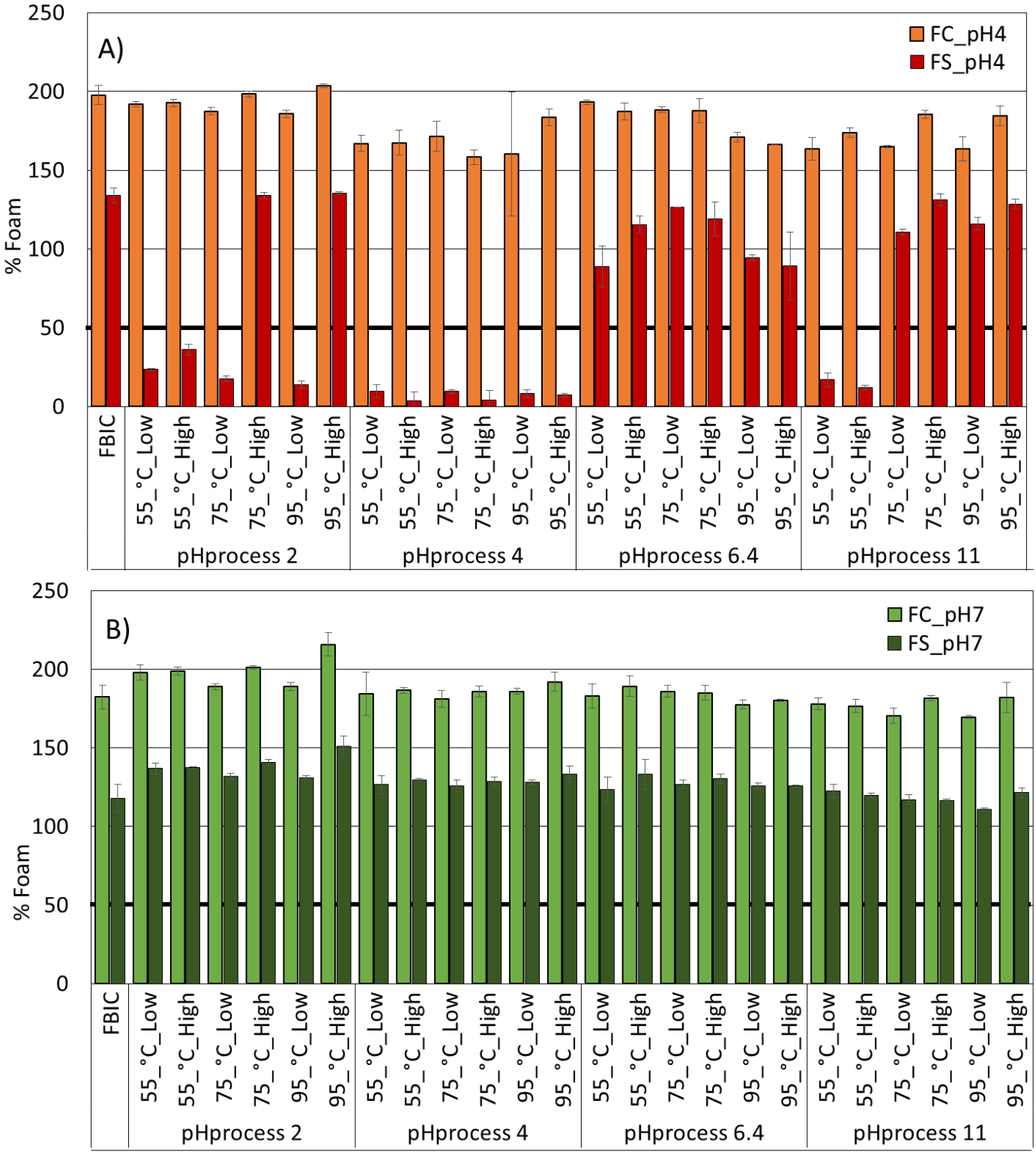


Figure 6. Foam capacity (FC) and foam stability (FS) of FBIC and the *ingredient-buffered-suspensions* at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7. FC and FS ≥ 50% were considered as 'stable', whereas FS < 50% represent *foam-breakers*.

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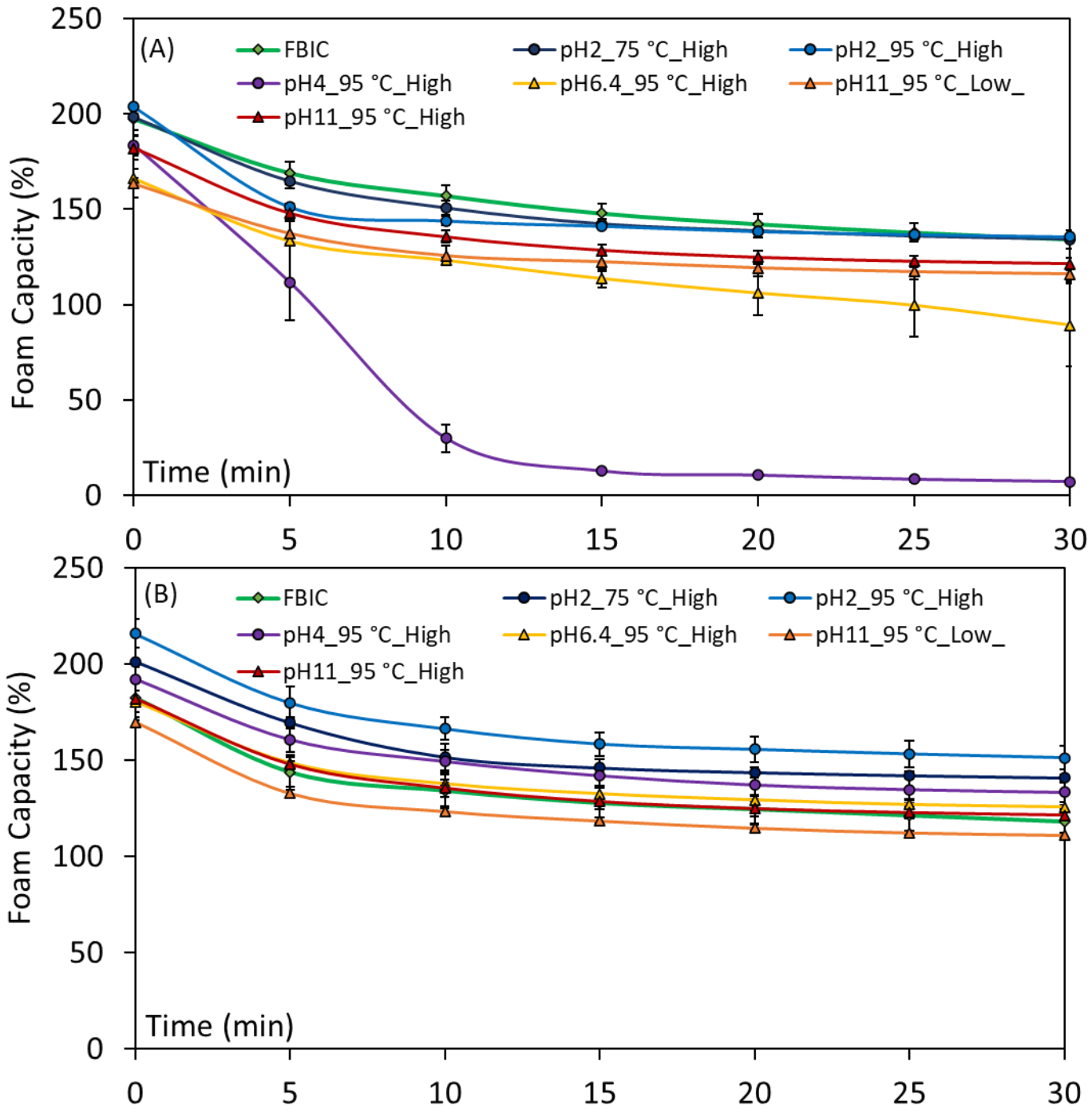


Figure 7. Foam capacity development during 30 min of the *ingredient-aqueous-suspensions* containing hydrolyzed proteins (pH2_75 °C_High, pH2_95 °C_High and pH4_95 °C_High) and those containing intensively aggregated proteins (pH6.4_95 °C_High, pH11_95 °C_Low and pH11_95 °C_High), compared to FBIC at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7.

Figure 8

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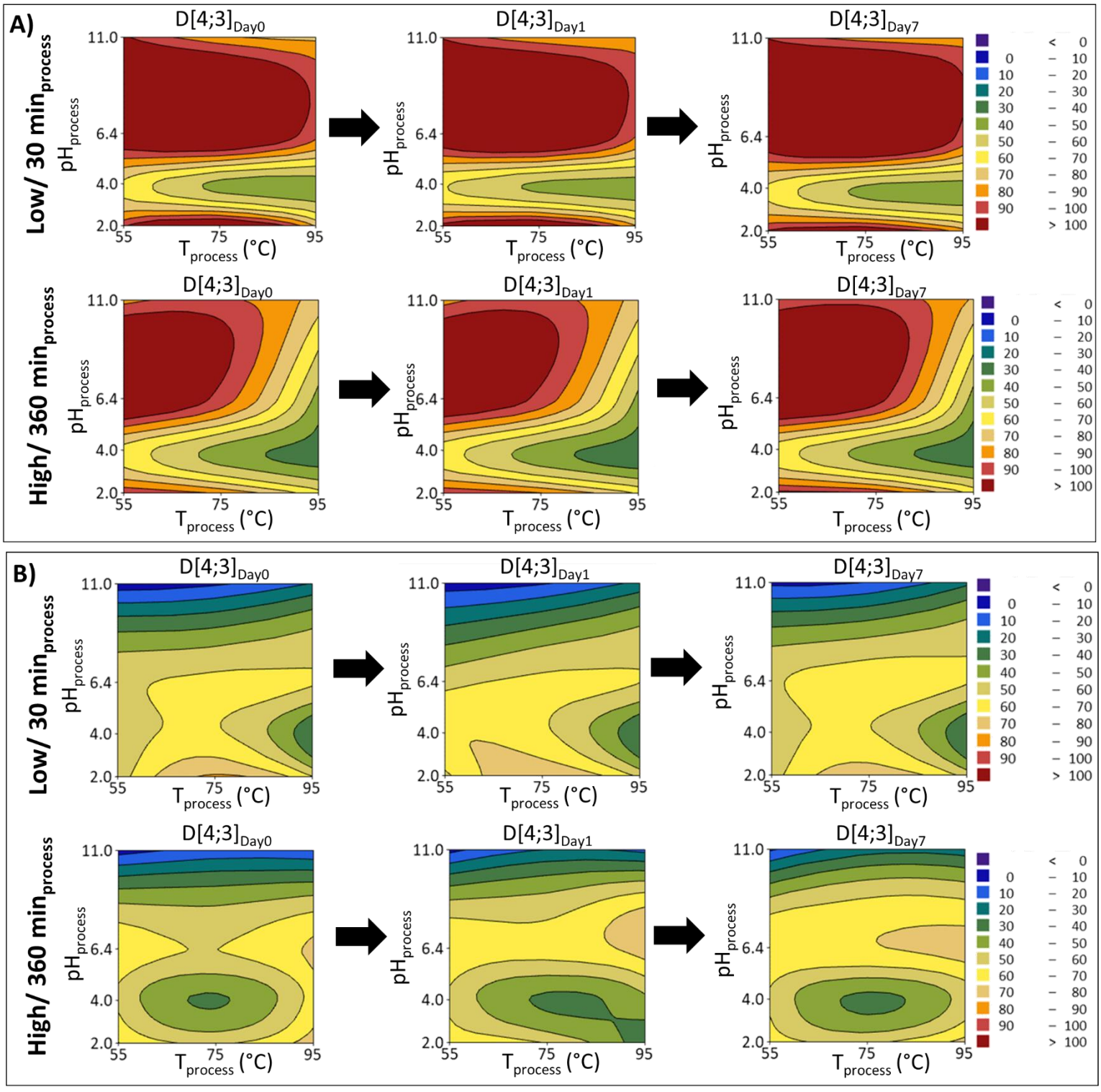


Figure 8. Contour Plot (Interpolation Method) of oil droplet Sauter mean diameter D[4;3] of the emulsions formed from *ingredient-aqueous-suspensions* of different modified ingredients, separated by $t_{process}$ i.e. Low/ 30 min and High/ 360 min, at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7. Color scale represents particle size in µm.

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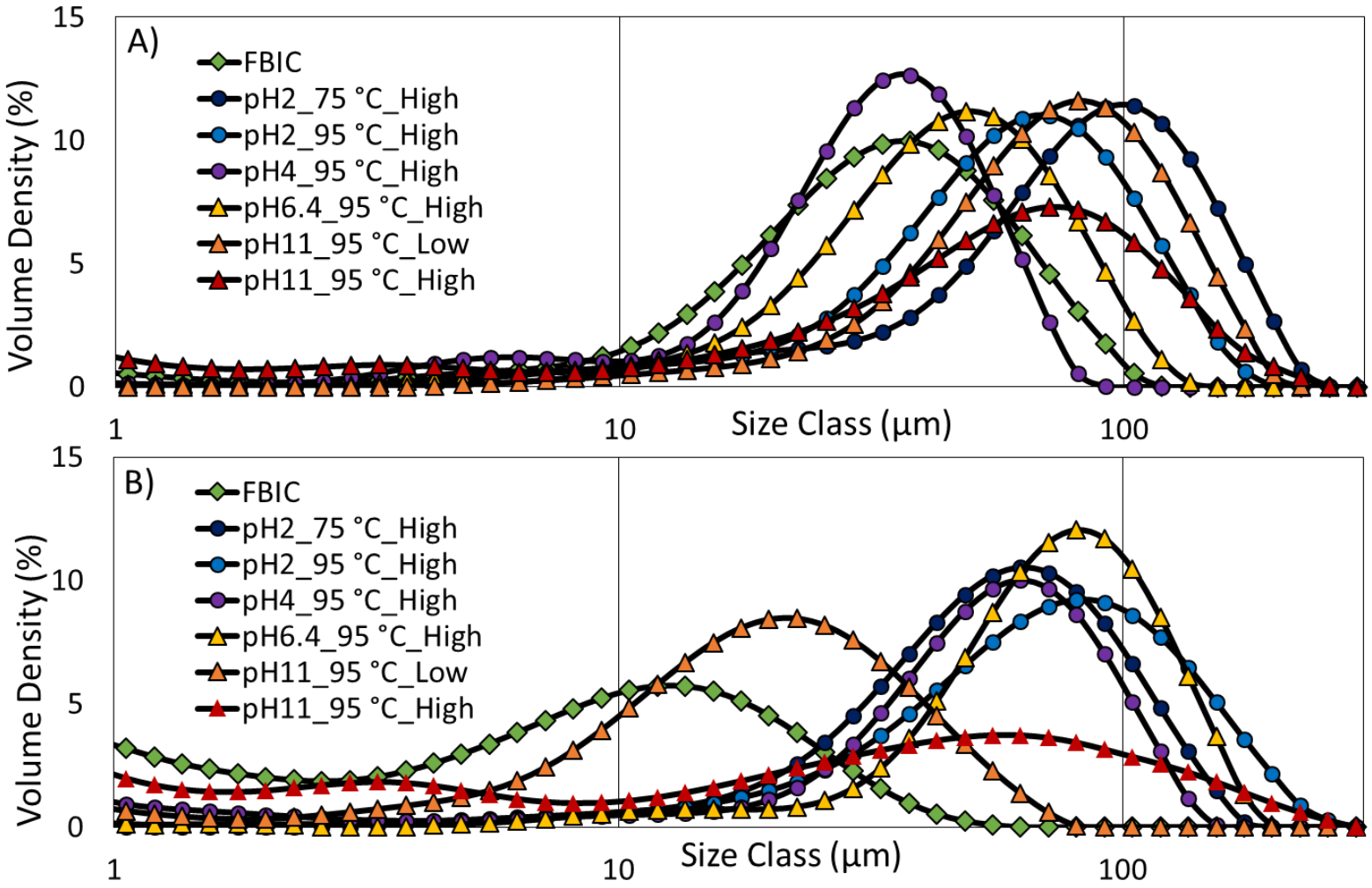


Figure 9. Comparison of particle size distribution of the emulsions produced from *ingredient-aqueous-suspensions* (Day 0) containing hydrolyzed proteins (pH2_75 °C_High, pH2_95 °C_High and pH4_95 °C_High) and those containing intensively aggregated proteins (pH6.4_95 °C_High, pH11_95 °C_Low and pH11_95 °C_High), compared to that of the fava bean initial concentrate (FBIC) at A) $pH_{utilization}$ 4 and B) $pH_{utilization}$ 7.

Click here to access/download;Table;Table 1.docx

Table 1. Intrinsic fluorescence of fava proteins with excitation and emission Loadings of *ingredient-buffered-suspensions* (0.1% and 1% (w/w) protein)

PARAFAC Component	Excitation Loading Peak (nm)	Emission Loading Peak (nm)	Protein Folded Complexity	Suggested Chemistry
0.1%(w/w) Protein Suspension				
PR1	285	322	I	Tyrosine
PR2	295	336	II	Tryptophan, buried
PR3	290	366	III	Tryptophan, exposed
1%(w/w) Protein Suspension				
PR1	285	330	I	Tryptophan, buried$^{\beta}$
PR2	295	354	II	Tryptophan, exposed$^{\beta}$

PR1, 2 and 3 represent 1st, 2nd and 3rd protein-associated components detected by the PARAFAC loadings.

$^{\beta}$*: Tryptophan excitation and emission peak with possible interference of quenching by other components*

Note: *Chemical hypothesis based on previous literature on tryptophan intrinsic fluorescence* (Lakowicz, 2006; Royer, 2006)

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Table 2. Thermal properties (DSC) of FBIC and less or extremely modified ingredients[§] at the two $pH_{utilization}$ (4 and 7).

Ingredient	Fava Protein modification	Enthalpy of Denaturation, ΔH (J/ g protein)		Denaturation Peak, T_p (°C)	
		$pH_{utilization}$ 4	$pH_{utilization}$ 7	$pH_{utilization}$ 4	$pH_{utilization}$ 7
FBIC	-	−4.54 ± 0.39 (0%)	−6.15 ± 0.65 (0%)	83.82 ± 0.09	91.07 ± 0.17
pH 2_55 °C_Low	Undetermined	0 (100%)	0 (100%)	-	-
pH 2_95 °C_High	Hydrolysis	0 (100%)	0 (100%)	-	-
pH 4_55 °C_Low	Undetermined	−3.32 ± 0.39 (~27%)	−3.32 ± 0.93 (~46%)	83.63 ± 1.93	94.17 ± 0.22
pH 4_95 °C_High	Hydrolysis	0 (100%)	0 (100%)	-	-
pH 6.4_55 °C_Low	Undetermined	−3.30 ± 0.39 (~27%)	−6.44 ± 1.13 (~0%)	83.37 ± 0.14	91.57 ± 0.20
pH 6.4_95 °C_High	Intense Aggregation	0 (100%)	0 (100%)	-	-
pH 11_55 °C_Low	Undetermined	−1.23 ± 0.04 (~73%)	−1.87 ± 0.13 (~70%)	87.19 ± 0.22	95.30 ± 0.20
pH 11_95 °C_High	Intense Aggregation	0 (100%)	0 (100%)	-	-

Note: % values show the extent of fava protein denaturation i.e., the enthalpy difference between the specific ingredient and fava bean initial concentrate (FBIC) at the respective particular $pH_{utilization}$

[§] *= ingredients corresponding to the lowest i.e., pH2, 4, 6.4 or 11 at 55 °C for 30 min (pHX_55 °C_Low) and highest level of modification i.e. pH2, 4, 6.4 or 11 at 95 °C for 360 min (pHX_95 °C_High).*

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Table 3. Interplay between fava protein modifications, properties and functionality

		Intensive Aggregation			Acid-Hydrolysis		
		pH6.4_95 °C_High	pH11_95 °C_Low	pH11_95 °C_High	pH2_75 °C_High	pH2_95 °C_High	pH4_95 °C_High
pH$_{utilization}$ 4	Absolute Zeta Potential	+ + +	+ + + + + +	+ + + + + + + +	+ + + + + + + +	+ + + +	+ + + + + +
	Nitrogen Solubility	-	-	+	+	+ +	-
	Protein Folding I [α]	- -	- -	- -	-	- -	- -
	Protein Folding II [α]	- - -	- - -	- - -	- -	- - -	- - -
	Protein Folding III [α]	-	- -	- -	-	- -	-
	Foaming capacity (FC)	-	-	-	=	+	-
	Foaming stability (FS)	-	-	-	=	+	- -
	D[4;3]$_{Day0}$ [β]	- -	-	-	-	-	- -
	D[4;3]$_{Day7}$ [β]	- -	-	-	-	-	- -
pH$_{utilization}$ 7	Absolute Zeta Potential	+ +	-	-	+	+	+
	Nitrogen Solubility	- -	- - -	-	- -	-	- -
	Protein Folding I [α]	- -	- -	- -	+	- -	- -
	Protein Folding II [α]	- - -	- - -	- - -	+	- - -	- - -
	Protein Folding III [α]	- -	-	- -	-	- -	-
	Foaming capacity (FC)	-	-	=	+	+	+
	Foaming stability (FS)	+	-	+	+	+	+
	D[4;3]$_{Day0}$	+ +	+	+	+ +	+ +	+
	D[4;3]$_{Day7}$	+ +	+	+	+ +	+ +	+

[α] Protein folding I-III represents fluorescence PARAFAC components 1-3 determined for 0.1% (w/w) protein suspensions

[β] Failure of emulsion formation at pH$_{utilization}$4

0% Change in Property: ''='';

0-50% Increase / Decrease in Property: ''+/ -'';

50-100% Increase / Decrease in Property: ''++/ - -'';

100-150% Increase / Decrease in Property: ''+++/ - - -'';

150-200% Increase / Decrease in Property: ''++++/ - - - -'';

200-250% Increase / Decrease in Property: ''+++++/ - - - - -'';

250-300% Increase / Decrease in Property: ''++++++/ - - - - - -'';

> 300% Increase / Decrease in Property: "+++++++/ - - - - - - -";

All % changes are calculated with respect to the fava bean initial concentrate (FBIC)

Author Statement

Conceptualization, S.S., M-N.M., A.S-E., J.Z., V.O.; methodology, validation, formal analysis, investigation and data curation, S.S., Å.R., J.S.; resources, J.Z., D.B., J.A.; writing—original draft preparation, S.S.; writing—review and editing, All.; visualization, S.S.; supervision, Å.R., V.O., K.O., J.Z., A.S-E. and M-N.M.; project administration, M-N.M., A.S-E., J.Z.; funding acquisition, M-N.M., D.B., J.A. All authors have read and agreed to the published version of the manuscript.

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors declare potential conflict of interest. Jens Zotzel, Julian Aschoff and Daniel Bonerz work at Döhler GmbH. Döhler GmbH is a global producer of natural ingredients, ingredient systems and integrated solutions, including plant-based products containing fava bean. Döhler GmbH is a member of the European Union's Horizon 2020 research and innovation program, grant agreement no. 765415 (acronym FOODENGINE) and hosted two PhD fellows, including Siddharth Sharan.