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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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# Innovative Food Science and Emerging Technologies

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

--Manuscript Draft--

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<b>Abstract:</b>	<p>Fava bean ( <i>Vicia faba</i> 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.</p>

**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 ( $\geq 75$  °C) of fava bean concentrate at  $\text{pH} \leq 4$  and  $\text{pH} \geq 6.4$  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



1 *Research Article*

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

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11 **Abstract**

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

## 24 *Industrial Relevance*

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

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

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

## 42 **1 Introduction**

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

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

62 Protein modifications by physical, chemical and biological process techniques can facilitate foams  
63 and emulsions by influencing a balance between protein solubility, charge distribution and  
64 protein folding (Damodaran, 2006; Akharume, Aluko, & Adedeji, 2021). During ingredient  
65 processing, fava proteins have been modified by temperature and pH (Arntfield & Murray, 1981;  
66 Arntfield, Murray, & Ismond, 1985), mechanolysis (Husband, Wilde, Clark, Rawel, & Muschiolik,  
67 1994), high-intensity ultrasound treatment (Martínez-Velasco et al., 2018), succinylation

68 (Schwenke, Rauschal, & Robowsky, 1983), acetylation (Krause & Buchheim, 1994), and enzymatic  
69 treatment (Eckert et al., 2019). The effect of any treatments on protein structure and the related  
70 effect on functionalities at application conditions is not well understood (Sharan, Zotzel, et al.,  
71 2021). Amongst different protein modifications, protein-protein aggregation and hydrolysis have  
72 shown to improve functionalities (Krause & Buchheim, 1994; Cepeda, Villarán, & Aranguiz, 1998;  
73 Martínez-Velasco et al., 2018; Eckert et al., 2019). Protein aggregation and hydrolysis can be of  
74 different types and extent that result in a variety of effects on functional properties (Martínez-  
75 Velasco et al., 2018; Yang, Liu, Zeng, & Chen, 2018; Eckert et al., 2019). In addition, fava bean  
76 contain not only proteins but also various non-protein constituents, including starch, dietary  
77 fibers and fats, along with certain anti-nutritional factors (Multari et al., 2015; Sharan, Zanghelini,  
78 et al., 2021). Hence the reactions occurring during ingredient processing may be a result of  
79 proteins and/ or non-protein constituents (Kosińska, Karamać, Penkacik, Urbalewicz, &  
80 Amarowicz, 2011; Zha, Rao, & Chen, 2021). For now, there is no clear overview of all the possible  
81 reactions occurring during processing of fava ingredients that can evidently explain the changes  
82 in functional and physico-chemical properties. This investigation is a continuation of a previous  
83 paper (Sharan, Zotzel, et al., 2021) and further attempts to clarify the interplay between fava  
84 protein-associated reactions, protein properties and functional properties and brings forth the  
85 ambiguities in the relationship between them. The impact of industrially relevant process  
86 conditions such as pH, temperature and treatment duration on fava bean concentrate was  
87 evaluated in regards to: (1) fava protein aggregation and hydrolysis during ingredient  
88 modification, (2) physico-chemical properties of fava proteins at utilization conditions (charge,

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

## 91 **2 Materials & Methods**

### 92 *2.1 Sample preparation*

#### 93 *2.1.1 Starting material*

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

#### 97 *2.1.2 Modified-suspensions*

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

### 108 2.1.3 *Modified ingredients*

109 The different *modified-suspensions* were frozen at  $-20\text{ }^{\circ}\text{C}$ , followed by freeze-drying and milling  
110 to 0.08 mm mesh size by an ultra-centrifugal mill ZM 200 (Retsch GmbH, Germany). This resulted  
111 in different modified ingredient powders, which are named as  $\text{pH}_{\text{process}}\text{-T}_{\text{process}}\text{-t}_{\text{process}}$  (e.g.  
112  $\text{pH2}_{55\text{ }^{\circ}\text{C}}\text{-Low}$ ) based on the conditions used to modify them.

### 113 2.1.4 *Ingredient-aqueous-suspensions*

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

### 119 2.1.5 *Ingredient-buffered-suspensions*

120 1% (w/w) protein suspension of all ingredients (FBIC + modified ingredients) were prepared in  
121 triplicates in citrate phosphate buffers (prepared from 0.1 M citric acid and 0.2 M dibasic sodium  
122 phosphate) at two  $\text{pH}_{\text{utilization}}$  (4 and 7) and stirred for 30 min at ambient temperature to produce  
123 *ingredient-buffered-suspensions*. Ionic strength of the buffer solutions used was calculated by the  
124 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  
125 of that ion (H.-M. Eun, 1996).

## 126 2.2 *Protein-associated reactions*

### 127 2.2.1 *Protein aggregation*

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

130 deionized water used as the dispersant. The particle size distribution (PSD) from 0.005 to 5000  
131  $\mu\text{m}$  as a function of volume was recorded and the volumetric mean particle diameter,  $D[4;3]$ , was  
132 used to compared the level of particle aggregation after the different ingredient modification  
133 treatments.

### 134 2.2.2 Protein acid-hydrolysis (SDS-PAGE)

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

## 148 2.3 Physico-chemical properties

### 149 2.3.1 Nitrogen solubility

150 The soluble fractions of the *ingredient-buffered-suspensions* (section 1.1.5) were separated at  
151 8,000 g for 20 min and its total nitrogen content was determined by the Dumas method using

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

### 155 2.3.2 *Surface charge*

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

### 160 2.3.3 *Intrinsic protein fluorescence*

161 Protein folding nature of the *ingredient-buffered-suspensions* was analyzed by fluorescence using  
162 a FS 920 fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, United Kingdom).  
163 Additional experiments with 0.1% (w/w) protein concentration were conducted to observe any  
164 changes in fluorescence signals due to the dilution. The excitation-emission map of the protein  
165 region was developed by varying excitation wavelengths from 250 to 340 nm at 5 nm increments  
166 and by varying emission wavelengths from 300 to 360 nm at 2 nm increments for a dwell time of  
167 0.05 s, using excitation and emission slits of 5 nm.

### 168 2.3.4 *Protein thermal integrity (DSC)*

169 FBIC and the modified ingredients treated either very gently (pHX\_55 °C\_Low) or vigorously  
170 (pHX\_95 °C\_High) at different pH (noted pHX) were taken to assess their protein integrity due to  
171 process conditions. *Ingredient-aqueous-suspensions* of 10% (w/w) were prepared by stirring  
172 overnight at 4 °C, followed by adjustment to pH 4 and 7 and overnight stirring at 4 °C. The  
173 concentration was brought to 6% (w/w) with Milli-Q water (Millipore, France) and approximately



174 60 mg was transferred to a 120 µl medium pressure crucible and run in a DSC (Mettler Toledo,  
175 Ohio, United States). The crucible was heated from 50 to 120 °C at 5 °C/min, with an empty  
176 reference crucible. The denaturation temperature and enthalpy were determined using the DSC  
177 software package (STARe SW 16.00).

## 178 2.4 Functional Properties

### 179 2.4.1 Foaming

180 150 ml of the *ingredient-aqueous-suspension* was whipped mechanically at room temperature  
181 using a WMF Mechanical Frother (Württembergische Metallwarenfabrik GmbH, Geislingen,  
182 Germany) for 2.5 min and the foam was transferred to a graduated cylinder (inner diameter =  
183 48.9 mm and height = 400 mm measured using a digital caliper). Foam height and liquid height  
184 were recorded manually to calculate the foam and liquid volume, respectively. Foaming capacity  
185 (FC, %) was calculated as the ratio of volume of foam generated after whipping and liquid volume.  
186 Foam stability (FS, %) was foam capacity measured after 30 min (Muschiolik et al., 1986). Foam  
187 was categorized unstable when FS was below 50%.

$$188 \quad FC (\%) = \frac{\text{Foam Volume } 0_{min}}{\text{Liquid Volume}} \times 100 ; FS (\%) = \frac{\text{Foam Volume } 30_{min}}{\text{Liquid Volume}} \times 100$$

### 189 2.4.2 Emulsification

190 The *ingredient-aqueous-suspensions* were added with palm oil medium chain triglycerides (90:10  
191 w/w) and homogenized for 1 min at 8000 rpm using T-10 Basic ULTRA-TURRAX homogenizer (IKA  
192 Works, Germany) fitted with an S-10N-10G dispersing element. The coarse emulsions formed  
193 were passed twice through a Niro-Soavi NS 1001L Panda homogenizer (Gea Group, Germany) at  
194 200 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  $\mu\text{m}$  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 ( $\text{pH}_{\text{process}}$ ,  $T_{\text{process}}$ ,  $t_{\text{process}}$  and  $\text{pH}_{\text{utilization}}$ ) 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  $\alpha = 0.05$ .

## 216 3 Results & Discussion

### 217 3.1 Effect of processing (modification conditions) on fava proteins

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

233 The non-reduced SDS-PAGE analysis revealed changes in type and molecular weight distribution  
234 of the soluble proteins extracted from the *modified-suspensions*. The typical protein profile of  
235 fava bean (in FBIC) is seen in lane T0 (Figure 2) representing the globulins consisting of legumin  
236 minor subunit (80 kDa), convicilin subunit (70 kDa), legumin major subunit (60 kDa), vicilin  
237 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 ( $\leq$  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  $pH_{process}$  ( $\leq$  4), at higher  $T_{process}$  ( $\geq$  75 °C) and at 'High'  $t_{process}$  (360 min) during  
250 modification. Modification at higher  $pH_{process}$  ( $\geq$  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 ( $\mu =$   
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  $\mu \leq 0.4$  ionic strength. Thus, the change in ionic

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

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

266 Considering all the ingredients, the zeta potential varied significantly only as a function of  
267  $\text{pH}_{\text{utilization}}$  ( $p = 0.0001$ ) and not by  $\text{pH}_{\text{process}}$  ( $p = 0.532$ ),  $T_{\text{process}}$  ( $p = 0.438$ ), nor  $t_{\text{process}}$  ( $p = 0.075$ ).

268 There was an overall shift of charge at  $\text{pH}_{\text{utilization}}$  4 towards a more negative charge in the  
269 modified ingredients compared to FBIC, with an exception of the ingredients  $\text{pH}2_{55} \text{ } ^\circ\text{C}_{\text{High}}$ ,  
270  $\text{pH}6.4_{55} \text{ } ^\circ\text{C}_{\text{Low}}$  and  $\text{pH}6.4_{55} \text{ } ^\circ\text{C}_{\text{High}}$ . Comparing surface charges with the ingredients  
271 containing aggregated or hydrolyzed fava proteins, no specific trend was seen (Figure 1, 2 and 3).

272 It would have been expected that the severe process conditions might have further unfolded the  
273 globular proteins, exposing previously hidden polar groups that lead to a changed surface charge.

274 But interestingly, the charge of fava proteins seemed robust during ingredient modification, but  
275 significantly changed only during ingredient utilization.

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

281 solubility. The solubility of the modified ingredients varied significantly due to  $\text{pH}_{\text{process}}$  ( $p = 0.035$ )  
282 and  $\text{pH}_{\text{utilization}}$  ( $p = 0.0001$ ) but not by  $T_{\text{process}}$  ( $p = 0.070$ ) or  $t_{\text{process}}$  ( $p = 0.195$ ). However, this overall  
283 insignificant effect of  $T_{\text{process}}$  on protein solubility seemed to heavily weighted by the result from  
284 the different  $\text{pH}_{\text{process}}$  and  $\text{pH}_{\text{utilization}}$ , and hence should not be dismissed as a factor. On the  
285 ingredients modified at  $\text{pH}_{\text{process}}$  6.4, a notable decrease in solubility at increasing  $T_{\text{process}}$  can be  
286 seen (Figure 4). For instance, at  $\text{pH}_{\text{utilization}}$  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  $\text{pH}_{\text{utilization}}$  7 to a greater degree (but not at  $\text{pH}_{\text{utilization}}$  4).

291 Solubility of fava proteins varied more compared to the surface charge property at the two  
292 different  $\text{pH}_{\text{utilization}}$ , 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  $\text{pH}_{\text{utilization}}$ . 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  $\text{pH}_{\text{utilization}} 4$  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

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

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

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



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

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

369 Calorimetric analysis of nine specific ingredients supported the results of protein charge,  
370 solubility and protein folding; a predominance of pH<sub>utilization</sub> was observed (Table 2). As seen, FBIC  
371 had lower T<sub>p</sub> at pH<sub>utilization</sub> 4 than at pH<sub>utilization</sub> 7, indicating a relatively higher heat stability of fava  
372 proteins at pH<sub>utilization</sub> 7. This lower denaturation temperature at isoelectric pH is due to lower  
373 structural integrity corresponding to the difference in protein folding complexity, the change in  
374 surface charge resulting in precipitation in effect decreased solubility. On the contrary, the  
375 proteins in FBIC at neutral pH had a net negative surface charge (and perhaps buried hydrophobic  
376 areas), which are typical for native folded proteins, thereby exhibiting higher thermal stability.  
377 The enthalpy of FBIC at the isoelectric pH was (numerical) lower than at neutral pH, reflecting  
378 greater structural integrity at pH<sub>utilization</sub> 7. Fava proteins from ingredients that were vigorously  
379 modified at high T<sub>process</sub> and t<sub>process</sub>, i.e. pHX\_95 °C\_High, were all completely denatured ( $\Delta H = 0$ ),  
380 in accordance with results that these ingredients contained either hydrolyzed proteins and  
381 intensively aggregated proteins (Figures 1 and 2; Table 2). Ingredients modified rather gently at  
382 low T<sub>process</sub> and t<sub>process</sub>, i.e. pHX\_55 °C\_Low, had very different fractions of denaturation between  
383 27 and 100% at both pH<sub>utilization</sub>, showing that under these conditions, the fava protein structures  
384 were affected differently. The ingredient pH2\_55 °C\_Low contained extremely modified proteins  
385 through an undetermined reaction. The proteins in the ingredient pH6.4\_55 °C\_Low were least  
386 affected, but with increasing  $\Delta H$  with pH<sub>utilization</sub>, i.e. from isoelectric to neutral pH similar to FBIC.  
387 For the other gently modified ingredients, pH4\_55 °C\_Low and pH11\_55 °C\_Low, the  $\Delta H$  did not  
388 change with pH<sub>utilization</sub> (Table 2). Typically, native proteins unfold and refold with the changes in  
389 the medium due to conformational flexibility. The rigidity in  $\Delta H$  indicated that the proteins in  
390 some of these modified ingredients have lost their potential to refold between the two pH<sub>utilization</sub>

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

### 398 *3.3 Effect of modification & utilization conditions on fava protein functionality*

399 Foaming parameters, i.e. foam capacity (FC) and foam stability (FS), were high for FBIC (> 100%)  
400 at both  $pH_{utilization}$  4 and 7 (Figure 6), though both FC and FS were higher at  $pH_{utilization}$  4 compared  
401 to  $pH_{utilization}$  7 (by 15%). Also, as seen in Figures 6A and 6B, the FC of all modified ingredients at  
402 both  $pH_{utilization}$  4 and 7 was very high (> 100%). Despite the high FC values, there was still a  
403 significant impact on FC by  $pH_{process}$  ( $p = 0.006$ ),  $t_{process}$  ( $p = 0.0001$ ),  $pH_{utilization}$  ( $p = 0.010$ ), but not  
404 by  $T_{process}$  ( $p = 0.901$ ). The FS, however, was affected heavily by conditions of ingredient  
405 modification and utilization. The FS was significantly impacted by  $pH_{process}$  ( $p = 0.012$ ) and  
406  $pH_{utilization}$  ( $p = 0.0001$ ) but not by  $t_{process}$  ( $p = 0.200$ ) or  $T_{process}$  ( $p = 0.165$ ). All modified ingredients  
407 maintained high FS (> 100%) at  $pH_{utilization}$  7, but at  $pH_{utilization}$  4, FS changed severely as a function  
408 of  $pH_{process}$  (Figure 6). In fact, twelve modified ingredients gave unstable foams (< 50%) which  
409 were labelled *foam-breakers* (Figure 6A). Apparently, these *foam-breakers* were produced at  
410 treatment  $pH_{process}$  2 ( $pH2_{55} \text{ } ^\circ\text{C}_{Low}$ ,  $pH2_{55} \text{ } ^\circ\text{C}_{High}$ ,  $pH2_{75} \text{ } ^\circ\text{C}_{Low}$  and  $pH2_{95} \text{ } ^\circ\text{C}_{Low}$ ),  
411  $pH_{process}$  4 (all ingredients), and  $pH_{process}$  11 ( $pH11_{55} \text{ } ^\circ\text{C}_{Low}$  and  $pH11_{55} \text{ } ^\circ\text{C}_{High}$ ). The  
412 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<sub>utilization</sub> 4, whereas all ingredients retained their  
415 high FS (> 100%) at pH<sub>utilization</sub> 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<sub>utilization</sub> (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<sub>utilization</sub> 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<sub>utilization</sub>  
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<sub>utilization</sub> 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<sub>utilization</sub>, with slightly lower FC (7-17% decrease) and FS (4-34%

435 decrease, 30 min) at  $\text{pH}_{\text{utilization}}$  4 (Figure 7A) compared to FC (0-7% decrease) and FS (<6%  
436 decrease, 30 min) at  $\text{pH}_{\text{utilization}}$  7 (Figure 7B). Two states of non-thermally aggregated fava  
437 proteins, large ( $>1\mu\text{m}$ ), insoluble and supra-molecular ( $<1\mu\text{m}$ ), 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  $> 1\mu\text{m}$   
440 (Figure 1).

441 Emulsion capacity and stability, denoted by  $D[4;3]_{\text{Day0}}$  and  $D[4;3]_{\text{Day7}}$ , were impacted significantly  
442 by  $\text{pH}_{\text{process}}$  ( $p = 0.000$  and  $0.000$  respectively) and  $\text{pH}_{\text{utilization}}$  ( $p = 0.000$  and  $0.000$  respectively).  
443 But these were governed majorly by the  $\text{pH}_{\text{utilization}}$  and less by the  $\text{pH}_{\text{process}}$  (Figure 8). At  $\text{pH}_{\text{utilization}}$   
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  $\mu\text{m}$  (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  $T_{\text{process}}$  dependent aggregation  
450 reactions occurring during modification (Figure 1). At  $\text{pH}_{\text{utilization}}$  7 (Figure 8B), a considerable  
451 difference was seen in emulsion capacity and stability. Unlike  $\text{pH}_{\text{utilization}}$  4, all emulsions produced  
452 at  $\text{pH}_{\text{utilization}}$  7 were homogeneous immediately after production (from blue to yellow). The  
453  $D[4;3]_{\text{Day0}}$  ranged between 4-81  $\mu\text{m}$ , with  $\text{pH}_{\text{process}}$  11 modified ingredients producing emulsions  
454 with the lowest  $D[4;3]_{\text{Day0}}$ . Despite some changes in  $D[4;3]$  of certain ingredient emulsions with  
455 time (Figure 8B), the values restored back to initial at Day7.  $\text{pH}_{\text{process}}$  had significant effect on the  
456  $D[4;3]_{\text{Day0}}$  and  $D[4;3]_{\text{Day7}}$  values and this was clear as although most of the emulsions were stable

457 during storage, all the emulsions from  $\text{pH}_{\text{process}}$  2 modified ingredients creamed and clarified at  
458 Day1. Therefore at  $\text{pH}_{\text{utilization}}$  7, emulsion capacities were equivalent to each other, with  
459 differences in  $D[4;3]$  values, but during storage, the stability was affected by  $\text{pH}_{\text{process}}$ .

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  $\text{pH}_{\text{utilization}}$  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  $> 200 \mu\text{m}$  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<sub>utilization</sub> 4 were expected, but even at pH<sub>utilization</sub> 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<sub>process</sub> 2 all gave  
482 unstable emulsions, including those having hydrolyzed proteins. Detrimental effects of pH<sub>process</sub>  
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<sub>utilization</sub> 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<sub>utilization</sub> (Table 3). However, within each pH<sub>utilization</sub>,  
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-



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

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## 533 **Author Contributions**

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

## 539 **Conflicts of Interest**

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

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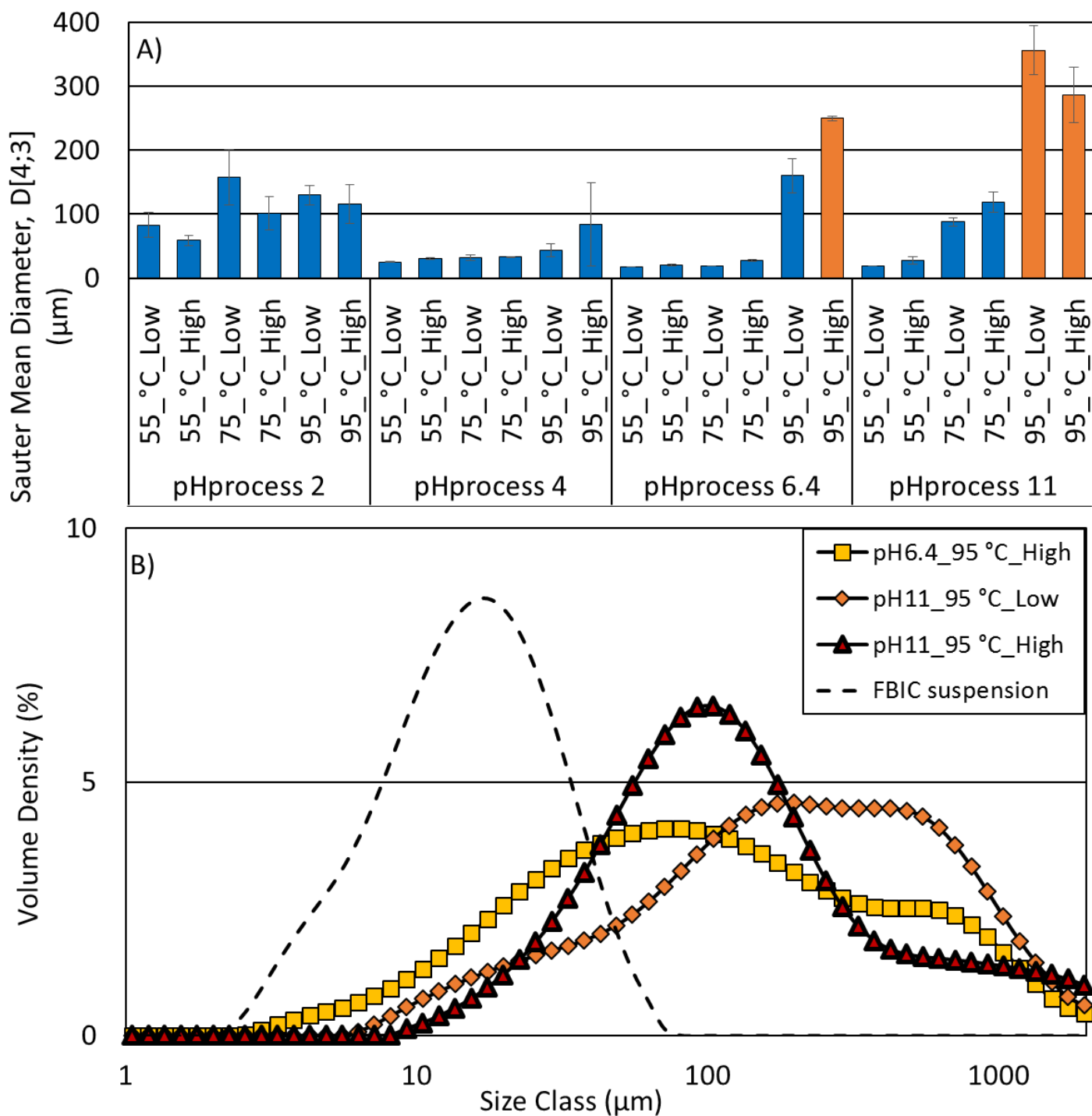
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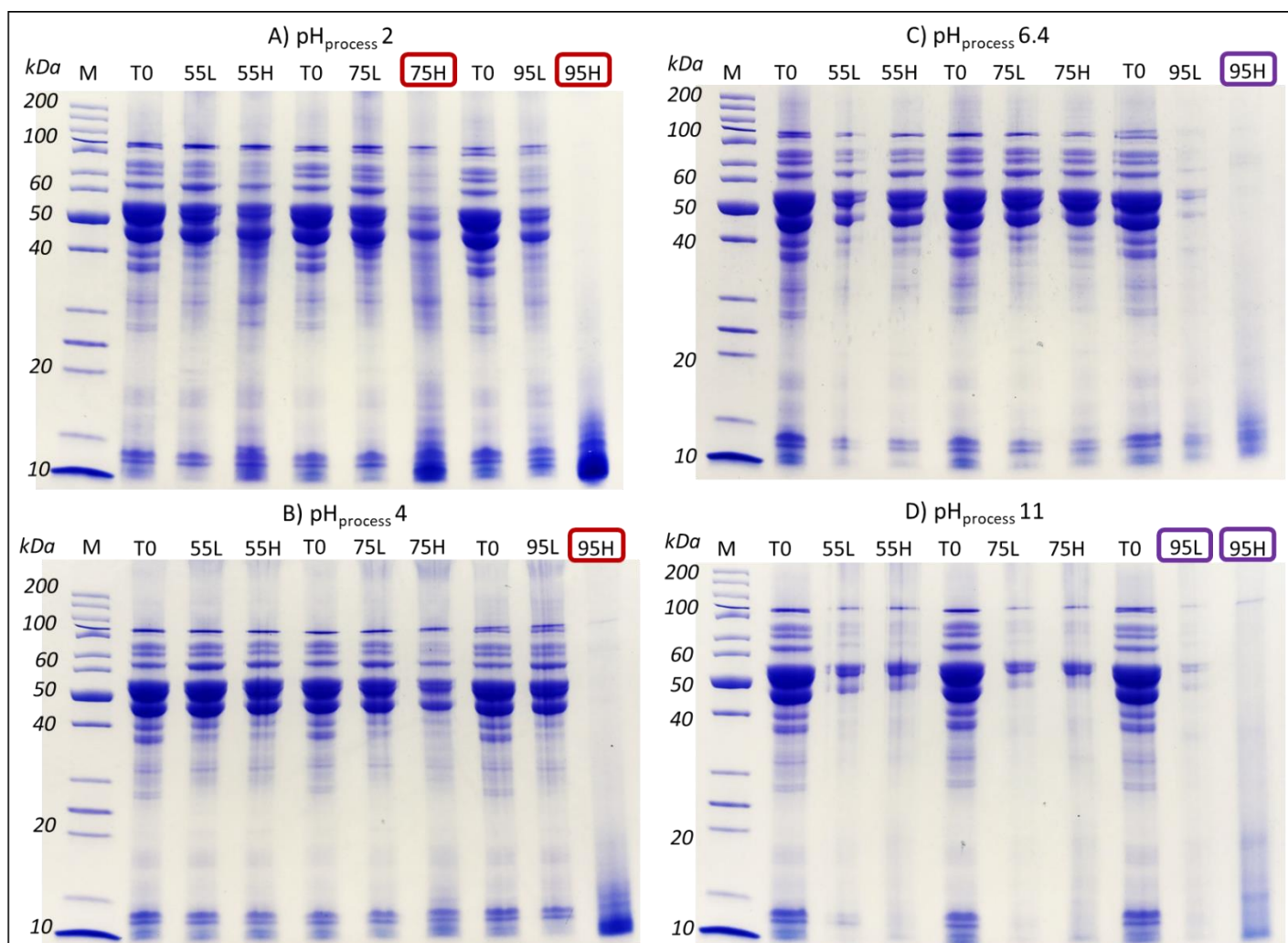
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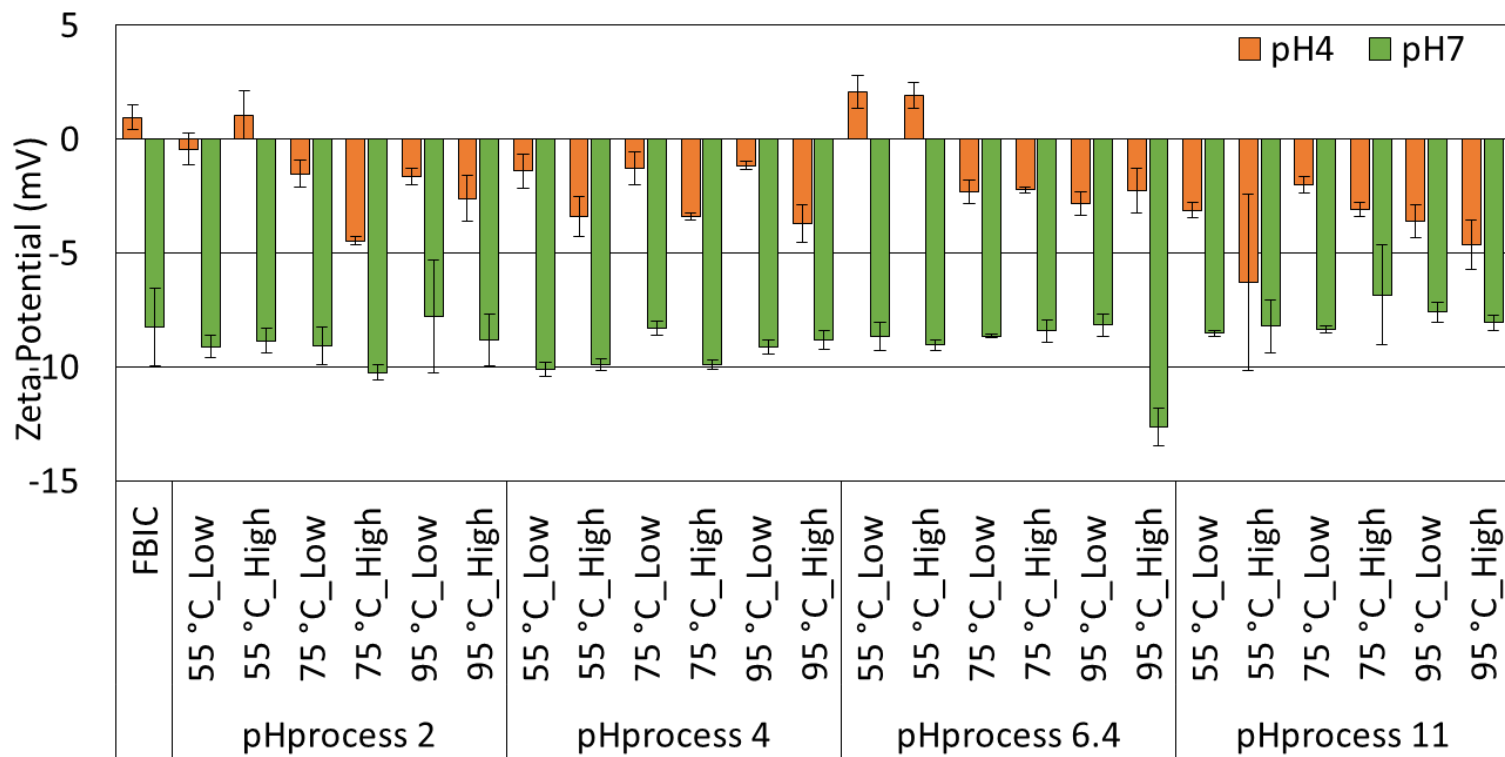
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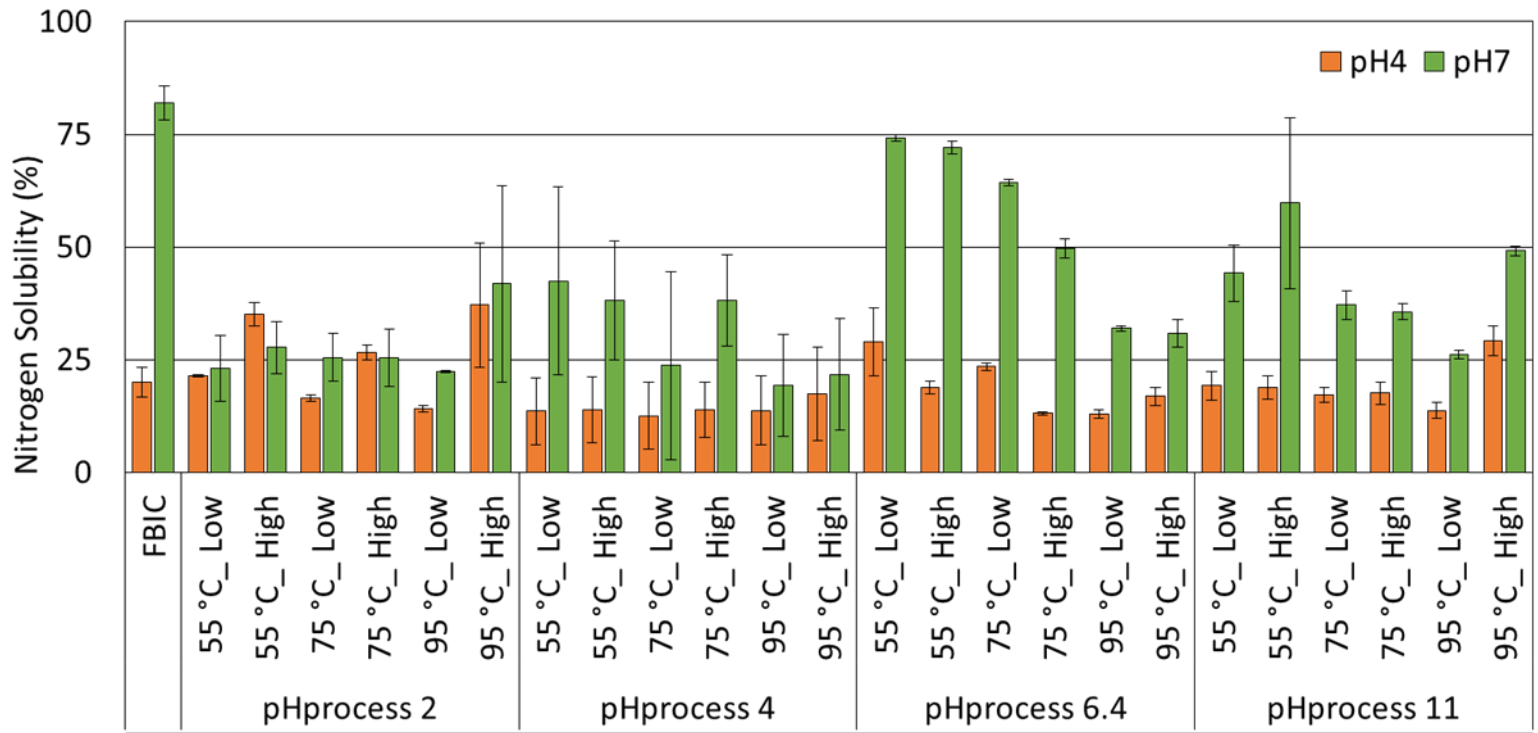
**Figure 1.** A) The volumetric mean particle diameter,  $D[4;3]$  of particles in *modified-suspensions*, B) PSD of the three *modified-suspensions* with aggregation reactions. These are compared to the fava bean initial concentrate (FBIC) suspension at the same concentration.



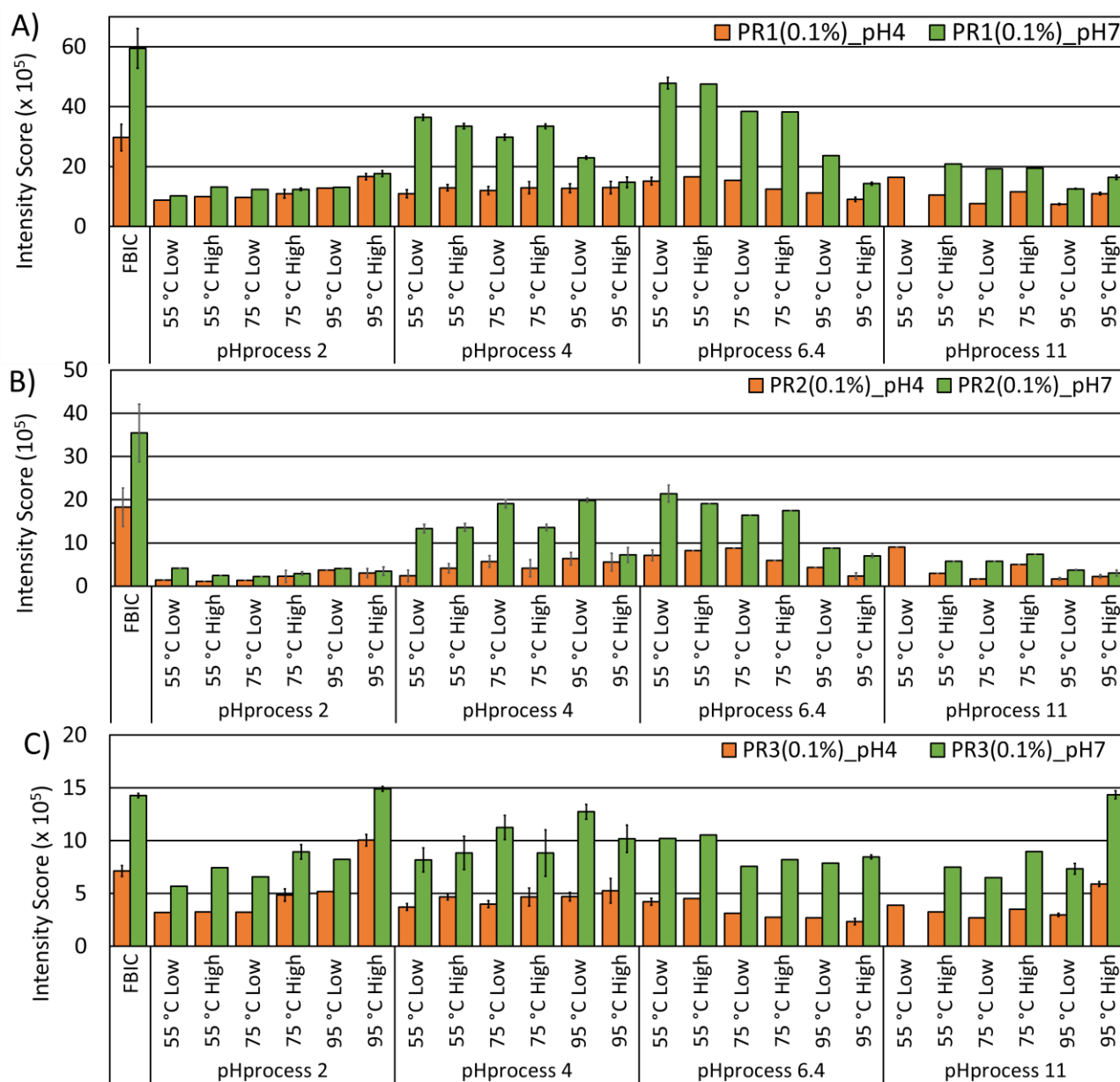
**Figure 2.** Non-Reduced SDS-PAGE of *modified-suspensions* at different  $\text{pH}_{\text{process}}$ : (A) 2, (B) 4, (C) 6.4, and (D) 11. Each gel column represents samples produced at different  $T_{\text{process}}$  (55, 75 and 95 °C) and at different  $t_{\text{process}}$ , i.e. Low = 30 min (L)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



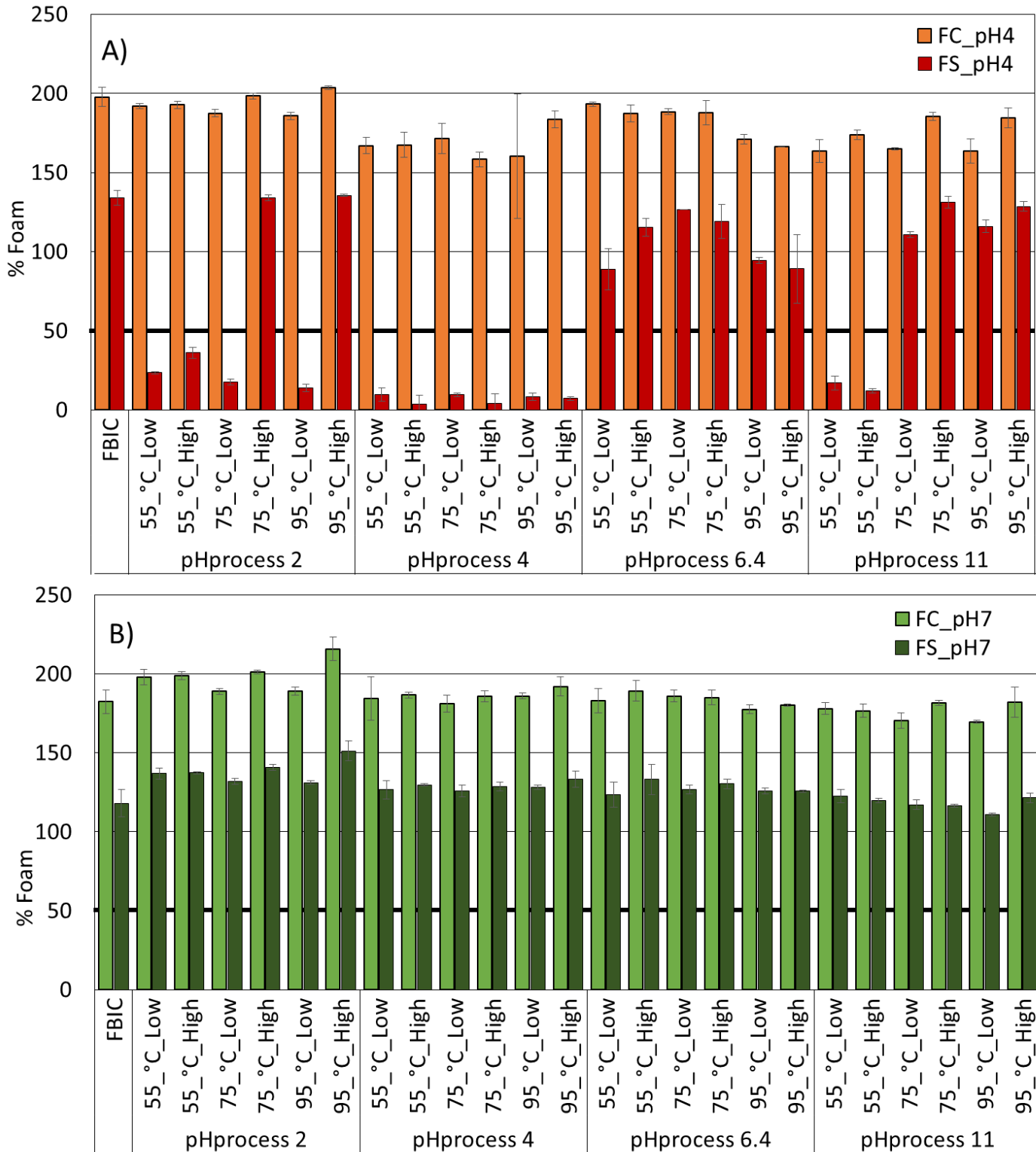
**Figure 3.** Zeta potential of FBIC and all *ingredient-buffered-suspensions* at pH<sub>utilization</sub> 4 and 7.



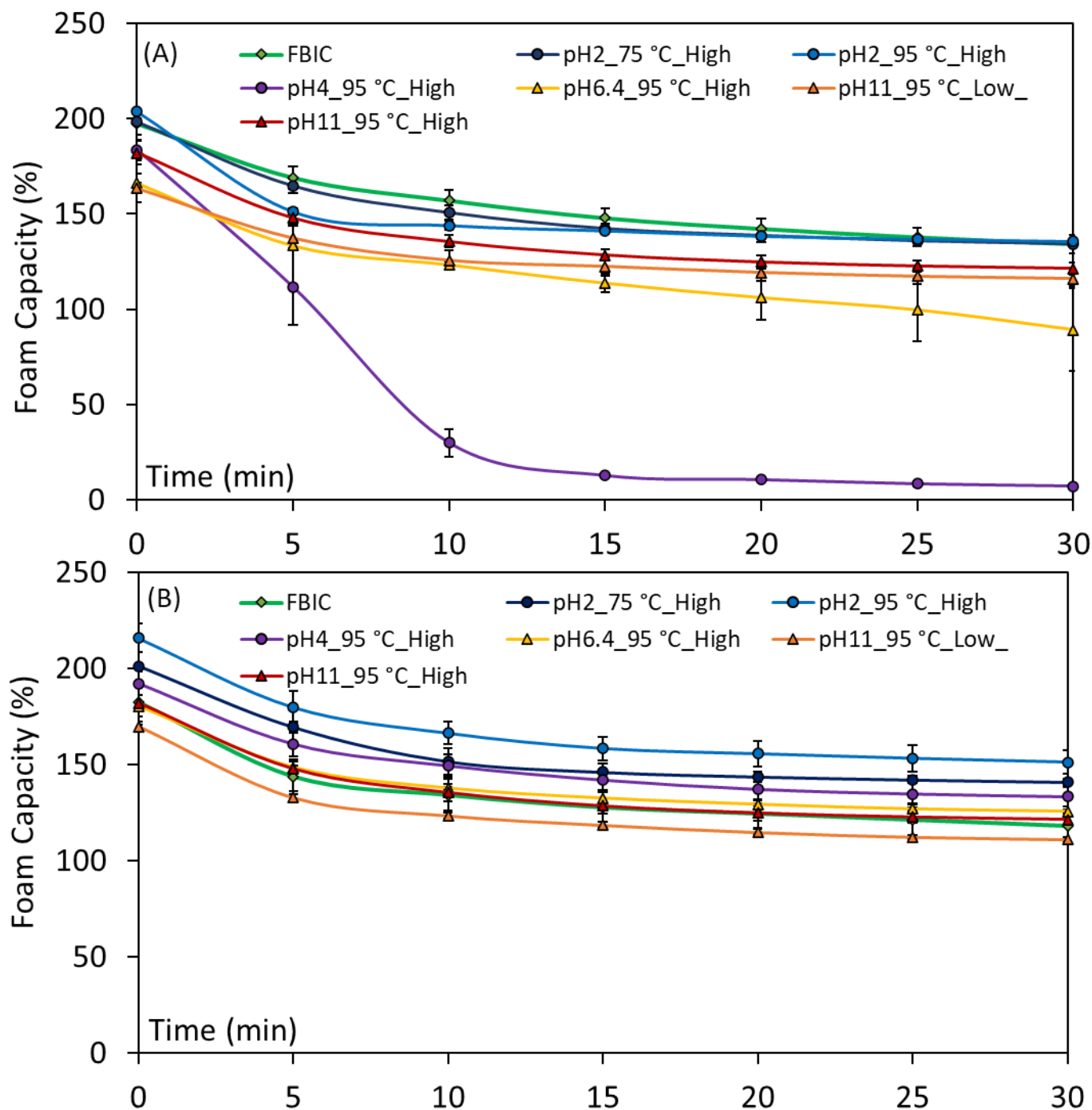
**Figure 4.** Nitrogen solubility of FBIC and all *ingredient-buffered-suspensions* at  $pH_{utilization}$  4 and



**Figure 5.** Intrinsic Protein Fluorescence by PARAFAC shown as score intensities of all *ingredient-buffered-suspensions* measured at  $\text{pH}_{\text{utilization}}$  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.

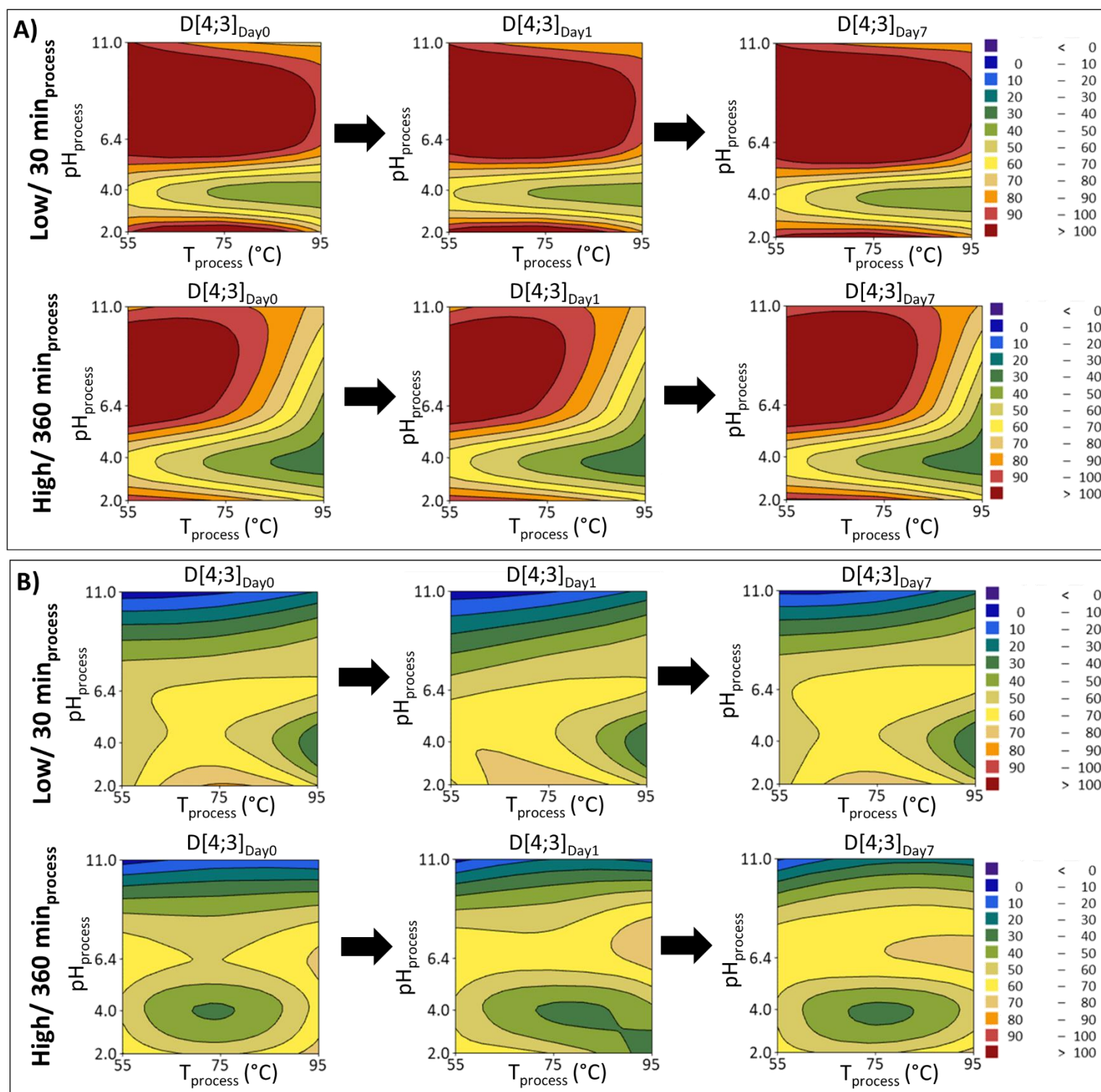


**Figure 6.** Foam capacity (FC) and foam stability (FS) of FBIC and the *ingredient-buffered-suspensions* at A)  $pH_{utilization}$  4 and B)  $pH_{utilization}$  7. FC and FS  $\geq 50\%$  were considered as 'stable', whereas FS  $< 50\%$  represent *foam-breakers*.



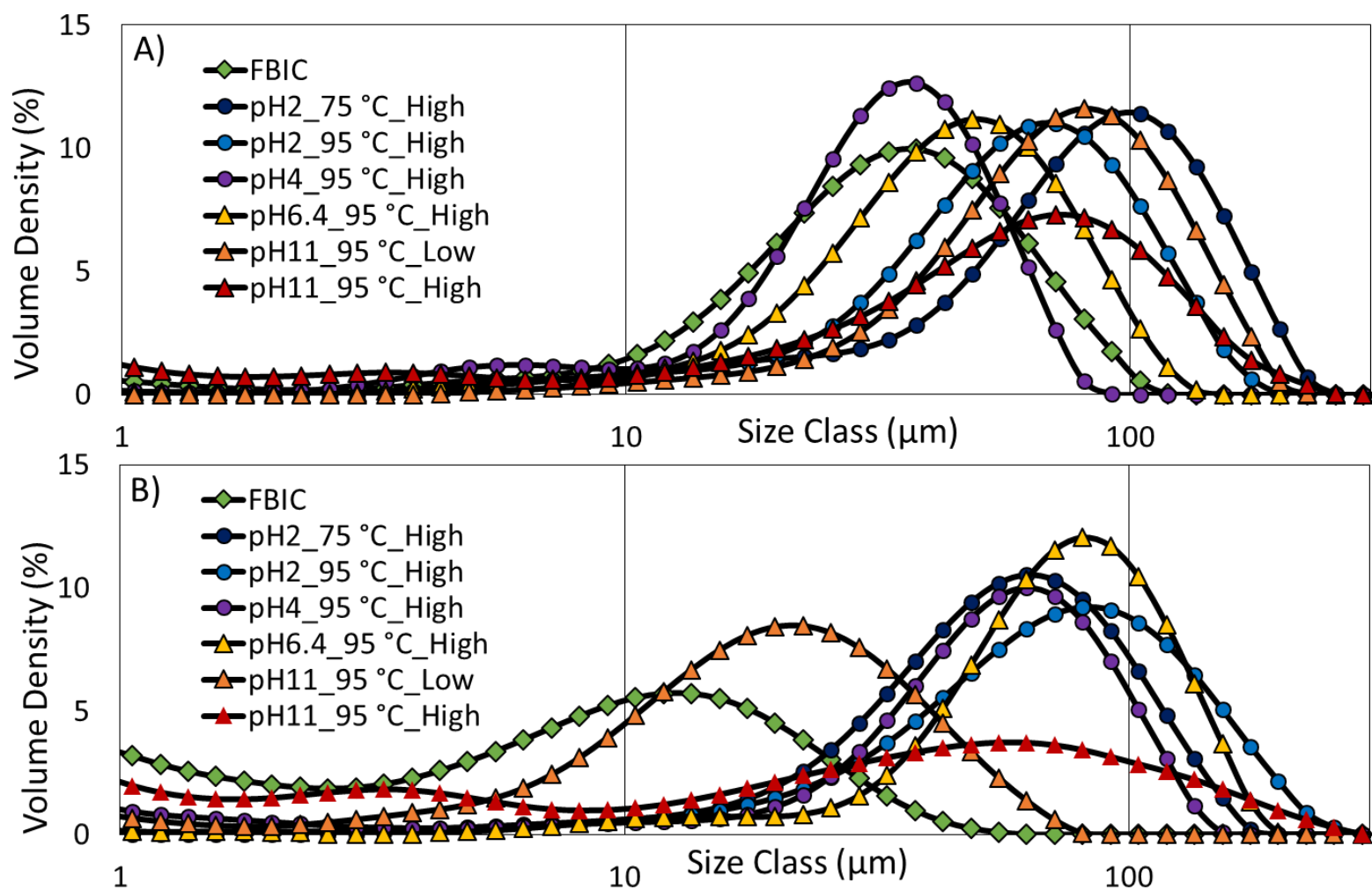
**Figure 7.** Foam capacity development during 30 min of the *ingredient-aqueous-suspensions* containing hydrolyzed proteins (pH2\_75 °C\_High, pH2\_95 °C\_High and pH4\_95 °C\_High) and those containing intensively aggregated proteins (pH6.4\_95 °C\_High, pH11\_95 °C\_Low and pH11\_95 °C\_High), compared to FBIC at A) pH<sub>utilization</sub> 4 and B) pH<sub>utilization</sub> 7.





**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  $\mu m$ .



**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<sub>utilization</sub> 4 and B) pH<sub>utilization</sub> 7.

**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
<i>0.1%(w/w) Protein Suspension</i>				
PR1	285	322	I	Tyrosine
PR2	295	336	II	Tryptophan, buried
PR3	290	366	III	Tryptophan, exposed
<i>1%(w/w) Protein Suspension</i>				
PR1	285	330	I	Tryptophan, buried <sup>β</sup>
PR2	295	354	II	Tryptophan, exposed <sup>β</sup>

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

<sup>β</sup>: 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)

**Table 2.** Thermal properties (DSC) of FBIC and less or extremely modified ingredients<sup>§</sup> at the two pH<sub>utilization</sub> (4 and 7).

Ingredient	Fava Protein modification	Enthalpy of Denaturation, $\Delta H$ (J/ g protein)		Denaturation Peak, T <sub>p</sub> (°C)	
		pH <sub>utilization</sub> 4	pH <sub>utilization</sub> 7	pH <sub>utilization</sub> 4	pH <sub>utilization</sub> 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<sub>utilization</sub>*

*§ = 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).*

**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
<b>pH<sub>utilization 4</sub></b>	Absolute Zeta Potential	+++	+++++	+++++	+++++	++++	+++++
	Nitrogen Solubility	-	-	+	+	++	-
	Protein Folding I <sup>α</sup>	--	--	--	-	--	--
	Protein Folding II <sup>α</sup>	---	---	---	--	---	---
	Protein Folding III <sup>α</sup>	-	--	--	-	--	-
	Foaming capacity (FC)	-	-	-	=	+	-
	Foaming stability (FS)	-	-	-	=	+	--
	D[4;3] <sub>Day0</sub> <sup>β</sup>	--	-	-	-	-	--
	D[4;3] <sub>Day7</sub> <sup>β</sup>	--	-	-	-	-	--
<b>pH<sub>utilization 7</sub></b>	Absolute Zeta Potential	++	-	-	+	+	+
	Nitrogen Solubility	--	---	-	--	-	--
	Protein Folding I <sup>α</sup>	--	--	--	+	--	--
	Protein Folding II <sup>α</sup>	---	---	---	+	---	---
	Protein Folding III <sup>α</sup>	--	-	--	-	--	-
	Foaming capacity (FC)	-	-	=	+	+	+
	Foaming stability (FS)	+	-	+	+	+	+
	D[4;3] <sub>Day0</sub>	++	+	+	++	++	+
	D[4;3] <sub>Day7</sub>	++	+	+	++	++	+

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

<sup>β</sup> Failure of emulsion formation at pH<sub>utilization4</sub>

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.