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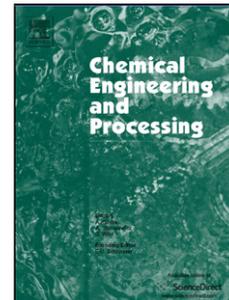
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INTENSIFIED SOY PROTEIN EXTRACTION BY ULTRASOUND

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Graphical abstract

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Highlights

- Ultrasound improves protein extraction yield during soymilk production.
- Ultrasound did not cause cell disruption.
- Solubility and separation efficiency are both accountable for improved yields.
- Particle size regime of 2–35 μm experienced greatest impact of ultrasound.
- Phytic acid stores were localised in protein bodies of dry soybeans using SEM.

22 **Abstract**

23 During soymilk production, aqueous extraction conditions are utilised resulting in suboptimal protein
 24 extraction yields. This research focuses on the intensification of extraction yields from soybeans using
 25 ultrasound and understanding the reasoning behind the results. Milled soybean slurry and okara samples
 26 were treated with ultrasound using a lab-scale probe system (20 kHz, 400 watts) for 0, 0.5, 1, 5 and 15
 27 min. Ultrasound increased the protein, oil and solids extraction yield from soy slurry by ca. 10% after
 28 1 min treatment, especially due to improved solubility and in a less extent to enhanced separation
 29 efficiency. Particles in the size range of 2-35 μm , corresponding to insoluble protein bodies in the
 30 continuous phase, were reduced in frequency but surprisingly not a stepwise decline in size upon
 31 ultrasound treatment, as shown by both laser diffraction and confocal laser scanning microscopy. No
 32 effects of ultrasound were observed on intact cells present in okara solution and soy slurries. Scanning
 33 electron microscopy could not reveal a hypothesised internal organisation of protein bodies within cells,
 34 although phytic acid stores were localised which have not been reported before. In conclusion,
 35 ultrasound has been identified as a technology with promise in soybean extraction systems where
 36 solubility requires improvement.

37 **Keywords**

- 38 • Soybeans
- 39 • Ultrasound-assisted extraction
- 40 • Acoustic cavitation
- 41 • Process intensification
- 42 • Confocal laser scanning microscopy

43 **Nomenclature**

44	S	Soybase mass
45	O	Okara mass
46	$Y I$	Primary extraction yield
47	$Y II$	Secondary extraction yield, resulting from okara treatment
48	x_i	Mass fraction of component i
49	$x_{i,j}$	Mass fraction of component i in stream j
50	i	p Protein

51	o	Oil
52	w	Moisture
53	s	Solids
54	j	S Soybase
55	O	Okara

56 **Abbreviations**

57 CLSM – confocal laser scanning microscopy

58 EDX – energy dispersive X-ray spectroscopy

59 SEM – scanning electron microscopy

60 **1. Introduction**

61 Plant-based protein products are currently gaining much interest as a more sustainable alternative to
62 animal-based protein products. One such product gaining popularity across the world is soymilk, due
63 to its complete set of essential amino acids, cholesterol-lowering attributes and lactose free nature [1].

64 Soymilk production consists of aqueous extraction from soybeans using alkaline conditions at elevated
65 temperatures, followed by removal of insoluble material to produce the resulting soybase. This soybase
66 is then used as a precursor to produce soymilk by adding other ingredients such as sugar, gums, flavours,
67 minerals and vitamins. The extraction of various components from soybeans is suboptimal; after
68 extraction, a significant amount of protein resides in the insoluble fraction, termed okara. Thermal
69 treatment during processing is often employed to reduce the activity of lipoxygenase; which, if left in
70 its native state, results in off-flavour production [2]. Vishwanathan et al. [3] show that alkaline
71 conditions (optimal pH 8+) gave enhanced protein solubility when compared to acidic conditions due
72 to the proteins isoelectric points. Assistance during protein extraction from soybeans is supported by
73 industry for reasons including: less expenditure on raw materials, less waste and lower costs associated
74 with its subsequent treatment.

75 An alternative energy source that has been commonly studied for laboratory scale extraction assistance
76 in the food industry is ultrasound [4-12]. The mechanism involved in enhancing extraction yields is
77 attributed to the cavitation phenomenon. Upon the application of ultrasound, alternating mechanical

78 waves cause microbubbles located in the liquid medium to form and grow up to a sufficiently negative
79 threshold pressure, where bubble collapse occurs [5]. As a consequence of bubble implosion, local
80 physical effects may result in very high temperatures (5000 K) and pressures (2000 atm) [6]. Local
81 regions of turbulence occur as a result of cavitation aiding mass transfer in solid-liquid extraction [7].
82 Many lab-scale studies claim that ultrasound can also enhance extraction yields of intracellular materials
83 from vegetal tissue due to cell disruption [5;6;10;13-15]. This intensification of extraction yield caused
84 by cell disruption is attributed to liquid jets of solvent resulting from asymmetric microbubble collapse
85 [16].

86 More recently, this technology has begun to show promise for implementation at industrial scale [17;18].
87 Pilot scale studies have shown the positive effects of ultrasound on a number of food extraction systems.
88 One such study by Pingret et al. [19] show the comparable results for ultrasound-assisted aqueous
89 extraction of polyphenols from apple pomace at pilot-scale to those improvements observed at lab-scale.
90 Boonkird et al. [20] showed the positive effects of ultrasound treatment on the extraction of
91 capsaicinoids from chilli peppers at pilot scale. Within the food industry, there has been implementation
92 of ultrasonic processing on an industrial scale for assistance during extraction from vegetal materials
93 [10]. Ultrasound-assisted extraction has been regarded as a green extraction process, for reasons
94 including reductions in processing times, energy consumption and enhanced rates of extraction [21;22].
95 These factors are of interest when considering protein extraction during soymilk production: protein that
96 is currently used for low quality functions, such as animal feed, is made available for human
97 consumption.

98 A key factor to be explored when considering the effects of ultrasound is the microstructure of the
99 processing materials. It is important to understand the matrix from which the extraction occurs and the
100 diffusion pathway by which protein can escape the solid, but so far little information is available in the
101 literature about processed soybean microstructures. The soybean is composed of approximately 90%
102 cotyledon cells, with the length range of 70-80 μm and a diameter of 15-20 μm once hydrated [23;24].
103 These cells contain protein bodies (5-20 μm) and a cytoplasmic network containing oil bodies (0.2-0.5
104 μm) stabilised by proteins termed oleosins [24]. The physical restraints for non-optimal protein

105 extraction yields upon preparation of soybase in an aqueous environment have been studied in previous
106 work [25]. Barriers for extraction included intact cotyledon cells, aggregated protein bodies within the
107 extraction medium caused by thermal treatment and a considerable amount of okara containing 80%
108 moisture in which soluble proteins reside [25].

109 Earlier studies of soy-based systems investigating ultrasonically-assisted extraction have been shown to
110 improve extraction yields or to enhance the functionality of components [26-32]. Fukase et al. [24]
111 investigated the effects of ultrasound on soybeans that underwent defatting using ether prior to protein
112 extraction. The ultrasound-assisted extraction from defatted soybean flakes yielded 50% more protein
113 versus the control sample (no US) after 10 min treatment at ultrasonic pressure of 106 kPa in an aqueous
114 system [24]. Another system showed the extraction of oil from soybeans using hexane was enhanced
115 by 20% with the application of ultrasound for 30 min (20 kHz) compared to a control sample [31].
116 However, there are very limited studies investigating the effects of ultrasound on aqueous extraction
117 from soybean as the starting material, without pre-treatment. One study by Fahmi et al. [33] investigated
118 the effects of ultrasound treatment (35 kHz, up to 60 min) on soy slurry protein extraction from pre-
119 soaked soybeans. The protein extraction was intensified: the protein content of soymilk increased by
120 6.3% [33].

121 This study investigates the effects of ultrasound-assisted extraction, after initial grinding of soybeans at
122 elevated temperatures. We hypothesise that ultrasound assistance will improve the extraction yields of
123 protein, oil and solids due to increased cell disruption, as discussed above. Ultrasonic treatment of both
124 the soy slurry and okara solution is investigated and extraction yields, solubilisation and separation
125 efficiencies will be discussed. In addition, confocal laser scanning microscopy (CLSM) and scanning
126 electron microscopy (SEM) has been used to study the microstructure of the soybeans, to understand
127 the target of ultrasound in our soybean extraction system.

128 **2. Experimental**

129 **2.1 Slurry preparation**

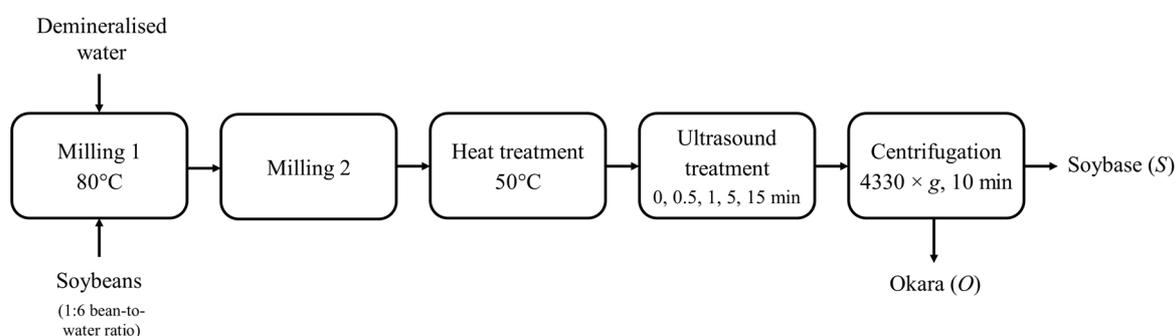
130 Preece et al. [25] describe a method for aqueous extraction to produce soy slurry and okara for
 131 subsequent treatments. Figures 1 (A) and (B) show the process schematically. Firstly, ('Milling 1' in
 132 Figure 1(A) and (B)) commercially available soybeans were ground in demineralised water at a ratio of
 133 1:6 (w/w) and at 80°C using a commercial blender (Varoma Thermomix, Vorwerk, Germany) for 10
 134 min (stepwise levels 2-8). Then the ground soybeans were treated ('Milling 2') with a high shear mixer
 135 (Silverson L4RT, Silverson Machines International, UK) for 20 min (stepwise 3000-6500 rpm) to
 136 produce a slurry with a volume-weighted mean diameter ($D_{4,3}$) of less than 300 μm .

137 **2.2 Okara solution preparation**

138 The slurry was centrifuged ($4330 \times g$, 10 min) to produce soybase *S* (supernatant) and okara *O* (pellet).
 139 The solid content of okara was measured using methods described in Section 2.4. A solution of 2.85%
 140 solids was then made by diluting the okara with demineralised water.

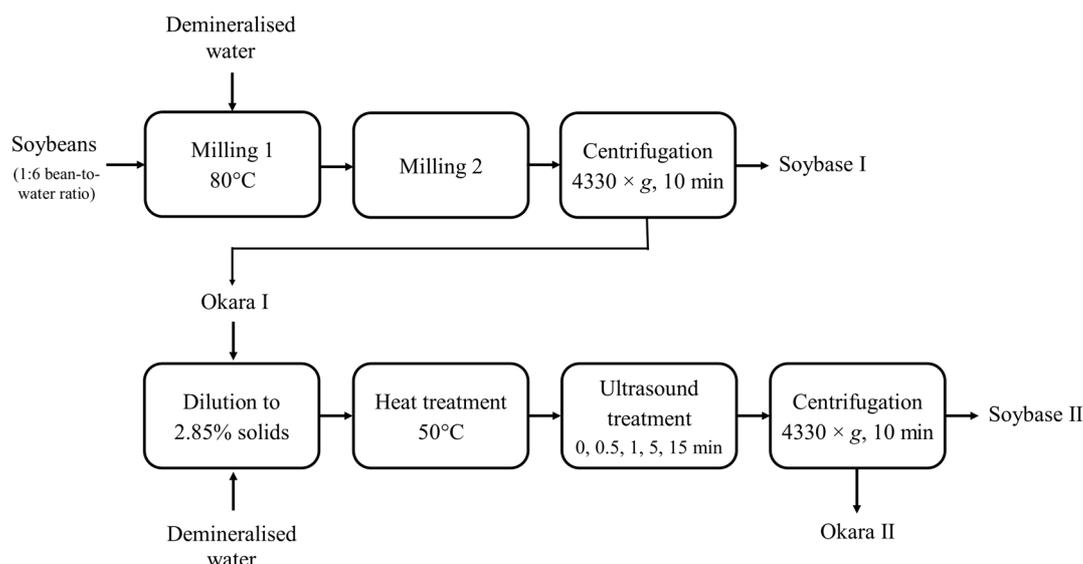
141 **2.3 Ultrasonic treatment**

142 After sample preparation, 100 g of sample (slurry or okara solution) was weighed and added into in a
 143 water bath at $50 \pm 1^\circ\text{C}$ whilst stirring at 200 rpm using a magnetic stirrer bar (25 mm in length, 10 mm
 144 in diameter cylindrical bar with central ring). Once the sample achieved the desired temperature, the
 145 sample was either subjected to sonication or held at 50°C in the water bath (control). Various times of
 146 exposure to ultrasound were investigated to understand the effects of sonication on extraction.



147

148 **Figure 1A. Schematic diagram of preparation and treatments applied to slurry during processing.**



149

150 **Figure 1B. Schematic diagram of okara preparation and subsequent treatments.**

151

152 Sonicated samples were treated with an ultrasonic probe (Branson Sonifier 450, Branson Ultrasonics
 153 Corporation, Danbury, CT), (400 watts, 20 kHz, output level 7 which translates to a power output of 65
 154 W, 13 mm probe tip) for various time periods; 0.5 min, 1 min, 5 min & 15 min. In the data presented,
 155 no temperature control was employed during ultrasonic treatment, however the temperature was
 156 recorded prior and post treatment (Table 1). From the recorded temperature increase, it was possible
 157 to calculate the actual ultrasonic energies and power inputs introduced using calorimetry: $Q =$
 158 $mC_p\Delta T$, where Q is the energy input as heat (J), m is the mass of sample (kg), CP is the specific heat
 159 capacity (assumed to be that of water, (4181 J kg⁻¹ K⁻¹) [22]) and ΔT is the temperature change (Table
 160 1). The power input was calculated by dividing the energy input by the treatment time (in s), assuming
 161 that all energy was transferred to heat energy in the system. Those powers quoted are less than those
 162 detailed by the probe manufacturer as 65 W, which might indicate that especially at high temperatures
 163 some of the heat energy was transferred from the system into the environment.

164

165 **Table 1. Temperature increase reported during ultrasound (US) treatment of soy slurry and corresponding energy**
 166 **and power input calculated using calorimetry.**

US treatment time (min)	Start T (°C)	End T (°C)	ΔT (°C)	Energy input (J)	Power input (W)
0	49	-	-	-	-
0.5	49.7	49.9	0.2	84	3
1	50.2	54.8	4.6	1923	32
5	50	80.5	30.5	12752	43
15	50.2	90.4	40.2	16808	19
15 (No US)	49.5	49.8	0.3	-	-

167

168 After reaching the desired process time, the samples were immediately centrifuged ($4330 \times g$, 10 min)
 169 to prevent further extraction occurring. Pellets and supernatants were weighed and analysed to
 170 determine extraction yields.

171 **2.4 Protein & solids content determination**

172 To determine protein extraction yields, the protein content on a wet basis (w.b.) was defined in the
 173 pellets and supernatants using the Dumas method (Vario MAX CNS, Elementar Analysensysteme
 174 GmbH, Germany). L(+)-glutamic acid (VWR International BVBA, Belgium) was used as a standard
 175 sample and UHT milk (3.5% fat) (muva kempten, Germany) as a reference material. For soy samples,
 176 a protein conversion factor of $6.25 \times N$ was utilised to determine protein content from the measured
 177 nitrogen content. From the protein concentrations and masses of streams, the protein extraction yield
 178 into the soybase could be calculated using equation 1.

$$179 \text{ Protein extraction yield} = Y (\%) = \left[\frac{S \cdot x_{p,s}}{(S \cdot x_{p,s} + O \cdot x_{p,o})} \right] \times 100 \quad (1)$$

180 Here S (soybase) and O (okara) represent the total weight of samples and x_p is the mass fraction of
 181 protein. To analyse the effects of ultrasound on okara solution, it was necessary to consider the total
 182 protein extraction yield calculated using equation 2. In this equation the nomenclature is that shown in
 183 Figure 1(B); yield I refers to the primary extraction and centrifugation for the production of soybase and
 184 okara; yield II corresponds to the okara solution treatment described.

185 Total protein extraction yield (%) = $Y I + (100\% - Y I) \times Y II$ (2)

186 In addition to the extraction yields, the separation efficiency (equation 3) was derived to show the
 187 efficiency of deliquoring of okara during centrifugation. The solubility of protein was also calculated
 188 using equation 4. In these calculations, it was assumed that the moisture content found in okara retained
 189 the same protein concentration ($x_{p,s}$) as the soybase, so that ($O \cdot x_{w,o} \cdot x_{p,s}$) is the amount of protein in the
 190 water fraction of the okara.

191 Separation efficiency (%) = $\left[\frac{S \cdot x_{p,s}}{(S \cdot x_{p,s} + (O \cdot x_{w,o} \cdot x_{p,s}))} \right] \times 100$ (3)

192 Solubility of protein (%) = $\left[\frac{S \cdot x_{p,s} + (O \cdot x_{w,o} \cdot x_{p,s})}{S \cdot x_{p,s} + O \cdot x_{p,o}} \right] \times 100$ (4)

193 Note that the extraction yield (equation 1) is equal to separation efficiency multiplied by the solubility
 194 of protein.

195 Fat and solid contents were measured using a microwave moisture analysis system equipped with NMR
 196 for direct detection of fat content (SMART System5, CEM GmbH, Germany). Oil and solid extraction
 197 yields were also determined using equation 1, replacing the masses of protein, with the respective
 198 masses.

199 **2.5 Particle size analysis**

200 The particle sizes of soy slurries after extraction were determined using laser diffraction (Mastersizer
 201 2000 Hydro S, Malvern Instruments Ltd, UK). To determine particle size distributions, refractive
 202 indices of 1.33 and 1.45 were used for the water and the particles, respectively [19]. Protein, moisture
 203 and particle sizes were measured in triplicate for each sample.

204 **2.6 Confocal laser scanning microscopy (CLSM)**

205 A Leica TCS-SP5 microscope in conjunction with DMI6000 inverted microscope (Leica Microsystems
 206 Inc., Germany) was used with the dye Nile blue A (Janssen Chimica, Belgium) to visualise the effects
 207 of ultrasound treatment on soy slurries. One drop of dye stock solution (1% w/v Nile blue) was added
 208 to 1-1.5 mL of sample and mixed well before adding the sample to the slide. For visualisation using

209 Nile blue, sequential scanning was employed to prevent the excitation laser occurring in the emission
 210 signals. Table 2 shows the scans utilised and the corresponding colours assigned to the emission
 211 channels.

212 **Table 2. Excitation and emission conditions when acquiring CLSM images**

Sequential scan	Excitation wavelength (nm)	Emission wavelengths (nm)	Illustrated colour in micrograph
1	488	520-626	Green
2	633	662-749	Red

213

214 **2.7 Cryo-scanning electron microscopy (cryo-SEM)**

215 A soy bean was cut into 2 pieces using a razorblade. One piece was placed in an aluminium sample cup
 216 and plunged into liquid nitrogen. The sample was then cryo-planed using a cryo-ultramicrotome
 217 (Ultracut UCT EM FCS, Leica Microsystems Inc., Germany), to obtain a freshly prepared cross-section.
 218 The sample was freeze-etched for 2 min at -90°C to reveal the microstructure and then sputter coated
 219 with platinum (120 s) in order to obtain a better image contrast. Samples were imaged using a Zeiss
 220 Auriga field emission SEM (Carl Zeiss Microscopy GmbH, Germany) at -125°C and an accelerating
 221 voltage of 3 kV. The microscope was equipped with an energy dispersive X-ray spectroscopy (EDX)
 222 unit; therefore, it was possible to chemically characterise regions visualised using the microscope.

223

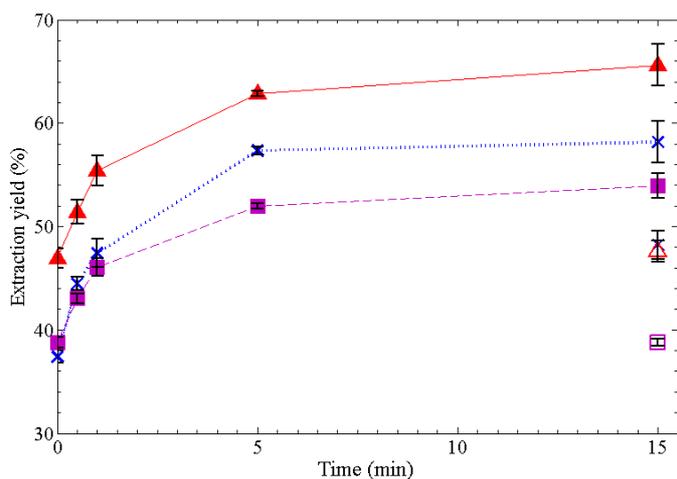
224 3. Results & Discussion

225 3.1 Extraction yields

226 3.1.1 Soy slurry treatment

227 To understand the mechanisms of ultrasound on the soy slurry matrix, it was necessary to determine the
228 extraction yields for the components of interest. Extraction yields were calculated from measurements
229 of oil, protein and solid contents of samples after treatment. Figure 2 shows the effect of ultrasound
230 treatment time versus extraction yield for the treatment of soy slurry. Ultrasound was shown to improve
231 the extraction of oil, proteins and solids vs. the control sample. After 1 min treatment time, protein and
232 oil extraction yields had improved by approximately 10% versus the 0 time point. It was shown that
233 there was no benefits to perform ultrasound-assisted extraction for more than 5 min as the maximum
234 yields had been achieved. A control sample was also analysed at 15 min to show the thermal treatment
235 with stirring was not responsible for the increases in extraction yields observed. An improvement in
236 extraction yields was also observed for control samples; however, not as much as those observed for
237 respective ultrasound treatments.

238 Temperature control was not employed for the data shown within this study. Without temperature
239 control, 15 min ultrasonic treatment caused the temperature of the solution to increase by $40.2 \pm 0.8^\circ\text{C}$.
240 In a separate study, the effect of temperature was determined by controlling the temperature of the
241 sample using a jacketed vessel cooled using counter-current flow of water at $20 \pm 1^\circ\text{C}$. The protein
242 extraction yields for US-treated samples (0.5-5 min, without temperature control) yielded insignificant
243 differences when compared to US-treated samples with temperature control (data not shown).
244 Considering the effects of ultrasound with temperature control for the 15 min US-treated sample, the
245 protein extraction yield was approximately 5% lower (absolute value) when the temperature of the
246 sample was held at 50°C .

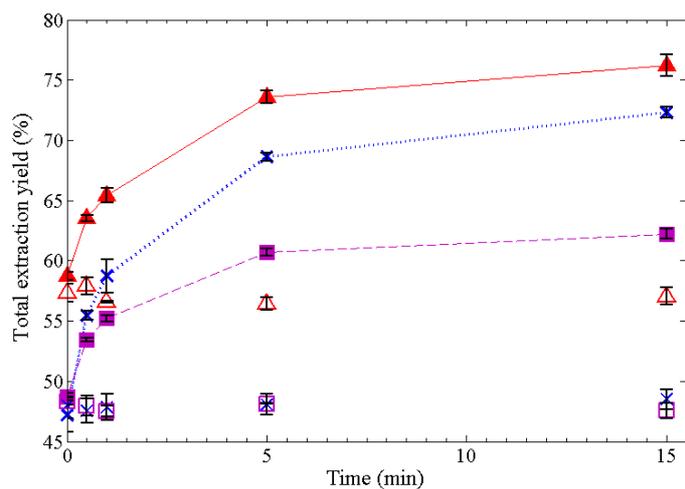


247
 248 **Figure 2. Improvement of extraction yields of slurry, oil (▲), protein (×) and solids (■) at various sonication times.**
 249 **Non-filled shapes correspond to control samples with corresponding component labels. Each data point is an average**
 250 **of three separate experiments and the error bar represents its standard error.**

251

252 **3.1.2 Okara solution treatment**

253 It has been previously reported that ultrasound has a significant effect on the extraction yield of protein
 254 from okara during soymilk production [34]. Total extraction yield refers to the addition of initial
 255 extraction yield during soybase production (soybase I and okara I production in Figure 1B) plus the
 256 extra materials which solubilised after subsequent okara treatments. Figure 3 shows that an increase in
 257 protein extraction yield upon ultrasound treatment was indeed achievable in comparison to the control
 258 samples, recorded for each time point in this instance. Total oil and solid extraction yields were also
 259 intensified during ultrasound treatment. In contrast, the total extraction yields from the control samples
 260 (no ultrasound) remained unchanged during all time periods. In this study, no temperature control was
 261 employed during the ultrasonic treatment of okara solution. It was previously shown that the 80°C
 262 thermal treatment included with the milling of the soybeans in water (detailed in section 2.1) for okara
 263 preparation affected the protein extraction yield [25]. Based on the limited effect of temperature control
 264 shown for the slurry data, the effect of subsequent thermal treatments after sample preparation was
 265 considered to be negligible.



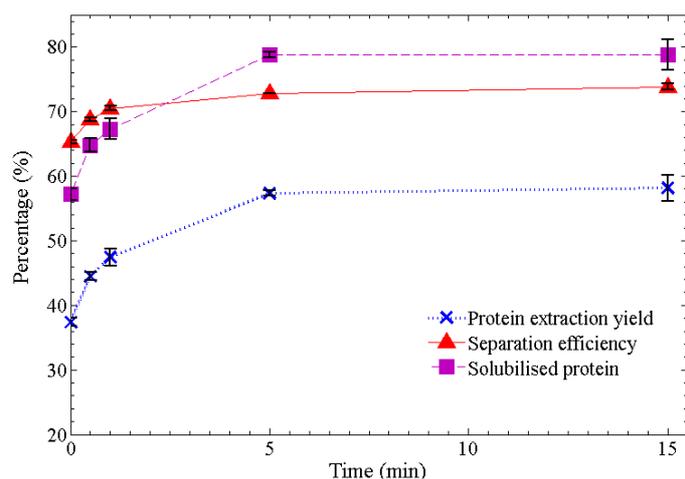
266

267 **Figure 3. Effect of ultrasound treatment when applied to okara solution. Oil (▲), protein (X) and solids (■) extraction**
 268 **yields are presented. Non-filled shapes are corresponding control samples. Data points are averages of three separate**
 269 **experiments. Error bars represent standard error of the mean from 3 separate experiments.**

270

271 3.2 Protein solubility and separation efficiencies

272 During treatments of soy slurry using ultrasound, it was possible to identify whether the solubility of
 273 protein was attributed to the increase in protein extraction yield and/or in the separation efficiency
 274 (deliquoring of okara). For the control sample, it was observed that the solubility and separation
 275 efficiency of protein were approximately 60% and 65%, respectively. As can be seen from Figure 4,
 276 the solubility of protein had the greatest impact on the protein extraction yield during ultrasound
 277 treatment. Separation efficiency was also positively influenced (to a lesser extent) by increasing
 278 ultrasonic treatment time; less water was present within the waste stream (okara), reducing the amount
 279 of soluble proteins entrapped within the matrix.



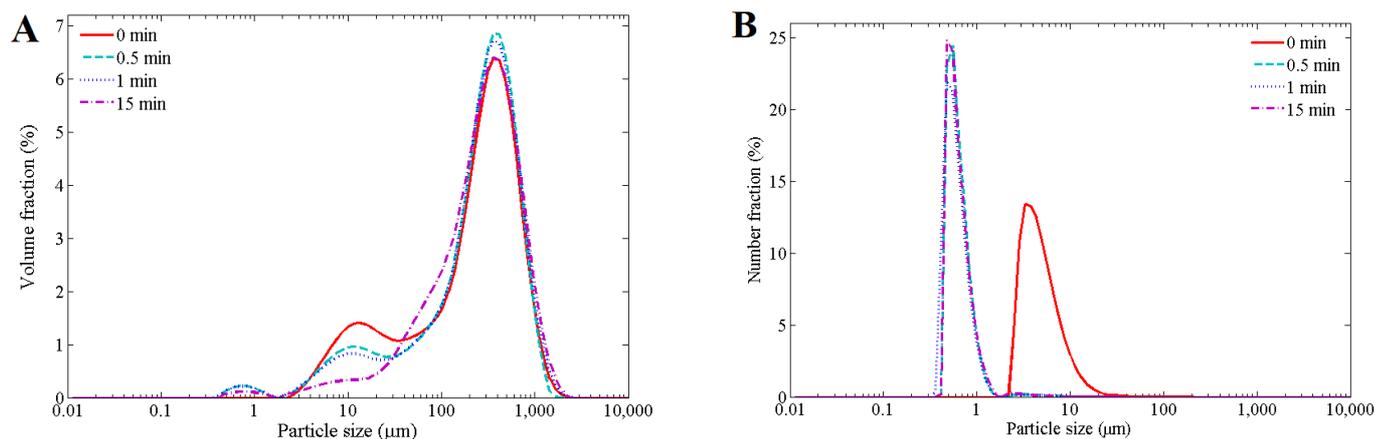
280

281 **Figure 4. Protein extraction yield, solubility of protein and separation efficiency as a function of ultrasonic treatment**
 282 **time on soy slurry. Error bars represent standard error from 3 separate experiments.**

283

284 3.3 Particle size distribution

285 During ultrasound treatment, it has been well-documented that a reduction in particle size is observed
 286 for many systems [5;11;14;35]. During the present study, particle size distributions of the treated
 287 samples were recorded and are shown in Figure 5A. The control slurry sample (0 min) showed a bimodal
 288 distribution of particles in the size range 2.5-2000 μm . The peak in the range of 2.5-35 μm is caused by
 289 the insoluble protein bodies located in the continuous phase of the sample, which have been visualised
 290 previously under the same processing conditions [25]. The larger size particles include fibres, intact
 291 cells and seed coat materials. Upon treatment with ultrasound, the peak in the 2.5-35 μm range (Figure
 292 5A) containing insoluble protein bodies was visibly reduced after 0.5 min and a stepwise reduction was
 293 observed with increasing treatment time. Interestingly, no stepwise peak shift to smaller sizes was
 294 observed. Using the Malvern Mastersizer software, it was possible to perform a number transformation
 295 on the particle size data, resulting in a plot of number fraction versus particle size (Figure 5B). The
 296 greatest number of particles in the control sample (0 min) were within the size range of 2.5-30 μm . The
 297 number-based particle size distribution confirms that ultrasound caused the particles to disintegrate;
 298 even after 0.5 min ultrasound treatment, the number of particles shifted to a smaller particle size (in the
 299 range 0.3-1.1 μm). Particles within this size range can be found within the soybase after centrifugation.



3

301 **Figure 5. Particle size distributions of soy slurry after ultrasound treatment for 0 min (control), 0.5 min, 1 and 15 min**
 302 **based on volume fraction (A) and number fraction (B).**

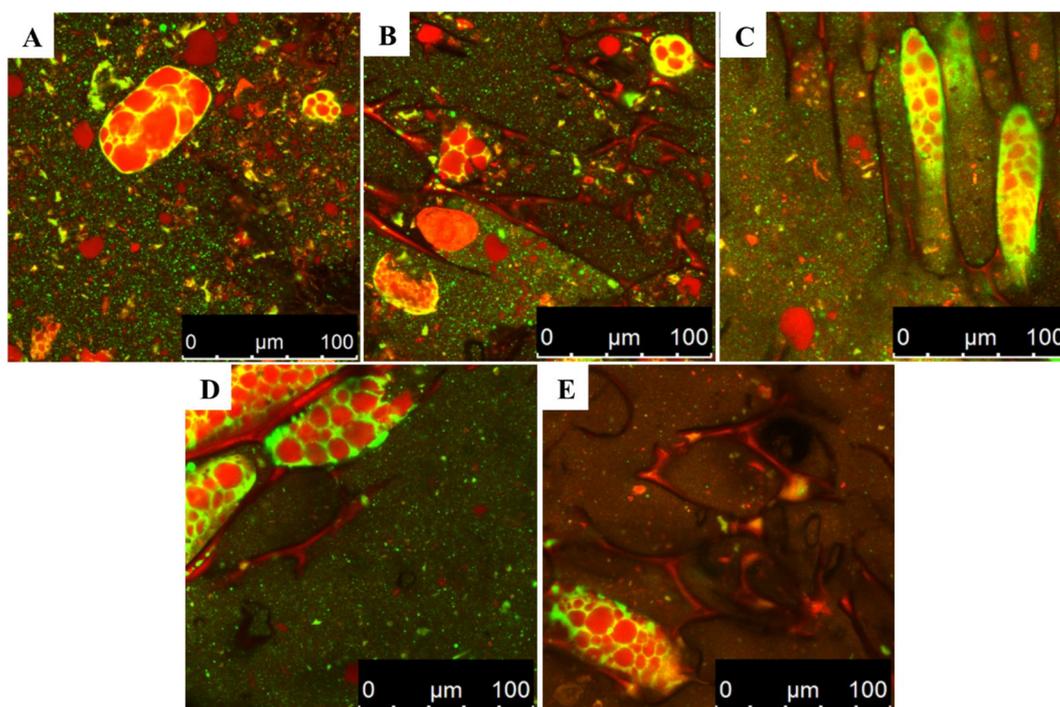
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304 The instrumentation used to determine the particle size was based on laser diffraction technology.
 305 During the determination of the size, the particles are estimated to be spherical, which was not the case
 306 for this system, as confirmed by laser scanning confocal microscopy (detailed in section 3.4). Particle
 307 size measurements are thus to be used for comparisons to one another, and not as absolute values.

308 3.4 CLSM

309 Confocal laser scanning microscopy was employed to observe the visual effects of the ultrasound
 310 treatment. To highlight the apolar features in the soy samples of interest, Nile blue was employed. Nile
 311 blue can be excited by two excitation wavelengths; emission at shorter wavelengths (520-626 nm) was
 312 highlighted green in this study, visualising oil. Longer wavelengths (662-749 nm) of emission are
 313 depicted in red and correspond to protein and fibrous materials. Figure 6 shows typical images that were
 314 observed by CLSM. Within the control micrograph (i.e. the unprocessed material) intact cells were
 315 seen, each containing complete protein bodies, shown as red within the cells (Figure 6A). Protein was
 316 also present in the continuous phase of the sample with the same size range observed within the intact
 317 cells (again coloured red in this instance). Submicron oil droplets were also observed in the continuous
 318 phase (highlighted in green). With increasing ultrasonic treatment time, the presence of the protein
 319 bodies free in solution reduced in concentration. Interestingly, no reduction in particle size of the protein
 320 bodies was observed, which correlates with the particle size distribution data (Figure 5). Intact cells
 321 were observed throughout all samples, even after 15 min of treatment (Figure 6E). If the cells are intact,

322 the materials present within are unavailable for extraction. Protein bodies within the intact cells were
 323 not affected by the ultrasound treatment; CLSM visualises intact cells containing protein bodies
 324 including after 15 min US treatment (Figure 6E). Upon the application of ultrasound at a frequency of
 325 20 kHz, it is proposed that transient cavitation will be the main cause of effects in a liquid system [4].
 326 Liquid jet formation occurs as a result of asymmetric bubble collapse during transient cavitation and
 327 this phenomena is independent of the frequency of applied ultrasound [5]. In the soybean slurry system,
 328 the cell wall disruption force was apparently much higher than that supplied with liquid jet impingement
 329 on the cell wall surface, as no change in the number of intact cells was observed via CLSM. The force
 330 required to overcome the energy holding together the insoluble protein must be lower than supplied with
 331 liquid jet impingement.



332
 333 **Figure 6.** CLSM images of soy slurry after various ultrasound treatments visualised with Nile blue A: (A) Control (0
 334 min); (B) 0.5 min; (C) 1 min; (D) 5 min; (E) 15 min. Oil is presented in the green channel and other apolar material
 335 such as agglomerated protein, protein entrapped within intact cells and fibres are highlighted in red.

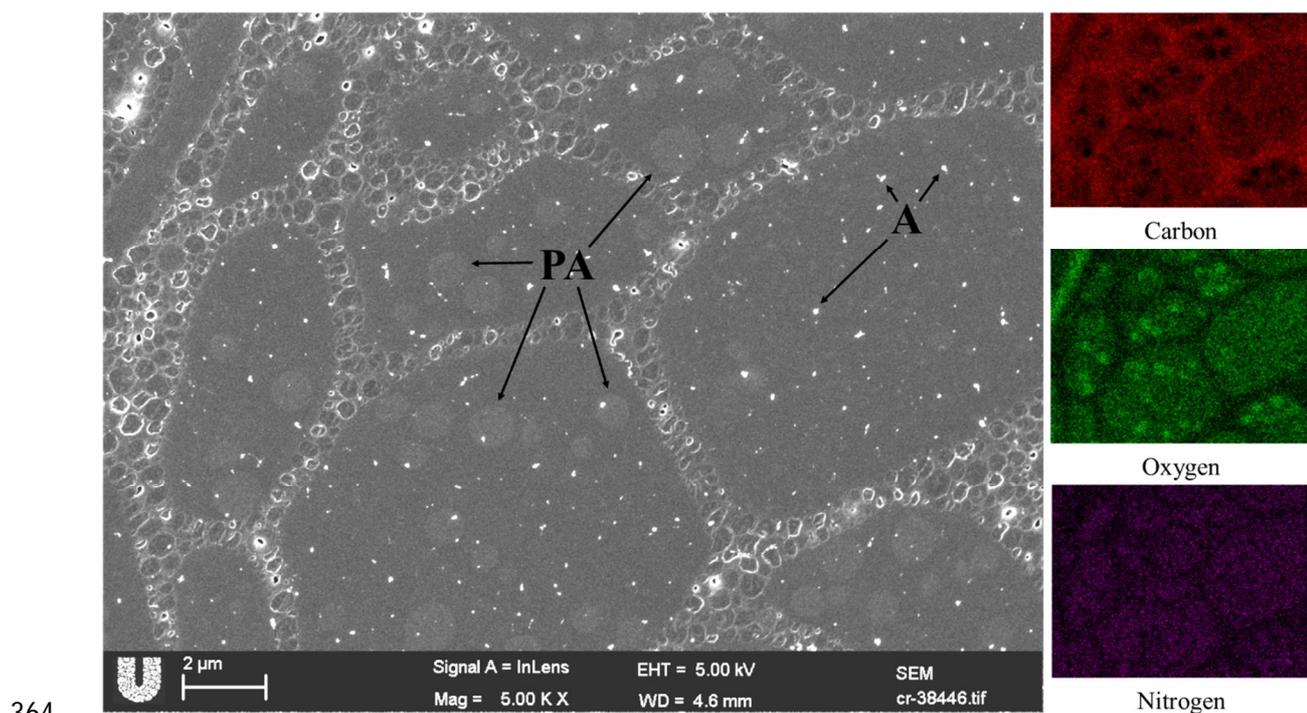
336

337 3.5 Cryo SEM-EDX

338 Surprisingly, no stepwise reduction in particle size of the protein bodies was found upon ultrasound
 339 treatment during soybase production (see Figures 5 and 6). We hypothesise that internal compartments
 340 within the protein bodies of the soybean are responsible for this ‘all or nothing’ effect, and that

341 ultrasound is either able to disrupt this internal organisation holding these internal compartments
342 completely, or not at all. In a study by Krishnan et al. [36] compartmentalisation of protein bodies
343 within rice seeds (*Oryza sativa* L.) was indeed shown. Storage proteins made in the endoplasmic
344 reticulum within plant cells may accumulate in the form of smaller protein bodies primarily into so-
345 called protein storage vacuoles by autophagy [37]. The limiting membrane of the sequestered protein
346 bodies is then digested by vacuolar enzymes, resulting in aggregated, larger protein bodies (those visible
347 in Figure 6).

348 SEM-EDX was utilised to investigate the structure and composition of the protein bodies within dry
349 soybeans (Figure 7). The Figure shows protein bodies surrounded by oil bodies, which were lighter in
350 appearance. The protein bodies ranged in size (2.4-13.5 μm), which fall in the lower part of the size
351 range quoted in the literature of 2-20 μm , derived from imaging hydrated samples by transmission
352 electron microscopy [24]. Bright white spots are artefacts arising from cryo-planing during sample
353 preparation and sample transfer (labelled on Figure 7). It was possible to visualise spherical features
354 within the protein bodies, these show as a lighter grey signal and annotated in Figure 7. EDX analysis
355 (insets to Figure 7) showed these were carbon-deficient, oxygen-rich spherical structures within the
356 protein bodies; EDX also clearly shows the difference in oxygen and carbon composition between the
357 protein and oil bodies. Nitrogen was difficult to observe using EDX analysis due to its low abundance
358 throughout the soybean; therefore, little difference in spatial arrangement was not apparent in the signal
359 (Figure 7). These spherical structures (annotated on Figure 7 as PA) are most likely phytic acid, which
360 act as a store of phosphorus and other cations during germination of the soybean [38;39]. Magnesium
361 was also present within these external structures (data not shown), which is explained by the chelating
362 ability of the phytic acid. The hypothesised compartmentalisation of soybean protein bodies was not
363 observed in Figure 7.



365 **Figure 7.** SEM image of dry soybean with examples of phytic acid (PA) and artefacts (A) annotated. Scale bar represents 2
 366 μm . Red, green and purple colour channels correspond to carbon, oxygen and nitrogen signals, respectively, during EDX
 367 analysis.

368 4. Conclusions

369 Soymilk production consists of aqueous extraction from soybeans, followed by removal of insoluble
 370 materials. The conventional extraction of various components from soybeans is suboptimal. The effect
 371 of ultrasound on separation and extraction has been studied. Ultrasound intensifies the extraction of
 372 valuable components from soybeans, leading to improved yields of protein, oil and solids of ca. 10%
 373 after 1 min treatment. It is important to understand the effects of ultrasound on the aqueous extraction
 374 system for its industrial application during soymilk production, which has not been extensively covered
 375 within recent literature. The microstructural analysis undertaken in this study indicates improved
 376 solubility as the main cause of the improved yields upon ultrasound treatment, and not cell disruption
 377 as is frequently stated in the literature. The amounts of particles in the size range of 2.5-35 μm , most
 378 likely protein bodies, were found to reduce for all ultrasound treatments investigated in an ‘all or
 379 nothing’ effect as no intermediately sized features were observed.

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387

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