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Selective extraction of napins: process optimization and impact on structural and functional properties

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21 **Abstract**

22 There is a growing worldwide demand for alternative sources of proteins for human nutrition. In this
23 context, many researches have been carried out to develop an original process for production of total
24 proteins from rapeseed meal. Interestingly, some recent works have demonstrated a possible selective
25 extraction of napins (rapeseed albumins). However, the precise impact of extraction conditions on process
26 performances has never been elucidated. The aim of this study was to establish the optimal conditions for
27 selective extraction of napins from rapeseed meal while producing a high-quality solid residue. First, the
28 effect of pH (2-4) and NaCl concentration (0-0.2 mol.L⁻¹) at 20 and 55 °C on the extraction process
29 performances was investigated by design of experiments. Second, a rational approach of multicriteria
30 optimization including genetico-evolutionary algorithm was employed. As a result, two sets of optimal
31 conditions (pH 2.0/0.11 mol.L⁻¹ of NaCl at 20 °C and pH 3.75/0.2 mol.L⁻¹ of NaCl at 55 °C) were found. They
32 allowed for > 55% of napin extraction yield, > 95% of napin proportion in the extract and < 4% of phytic acid
33 content in the solid residue. However, much greater yield of napins was noted at the higher extraction
34 temperature (55.4% vs 75.56% at 20 and 55 °C, respectively). Napins extracted under the two optimal
35 conditions and purified by ultrafiltration had a light creamy color and similar composition. Minor structural
36 modifications between the two proteins were observed, but they did not affect the functional properties
37 (foaming and emulsifying properties) that appeared to be comparable.

38

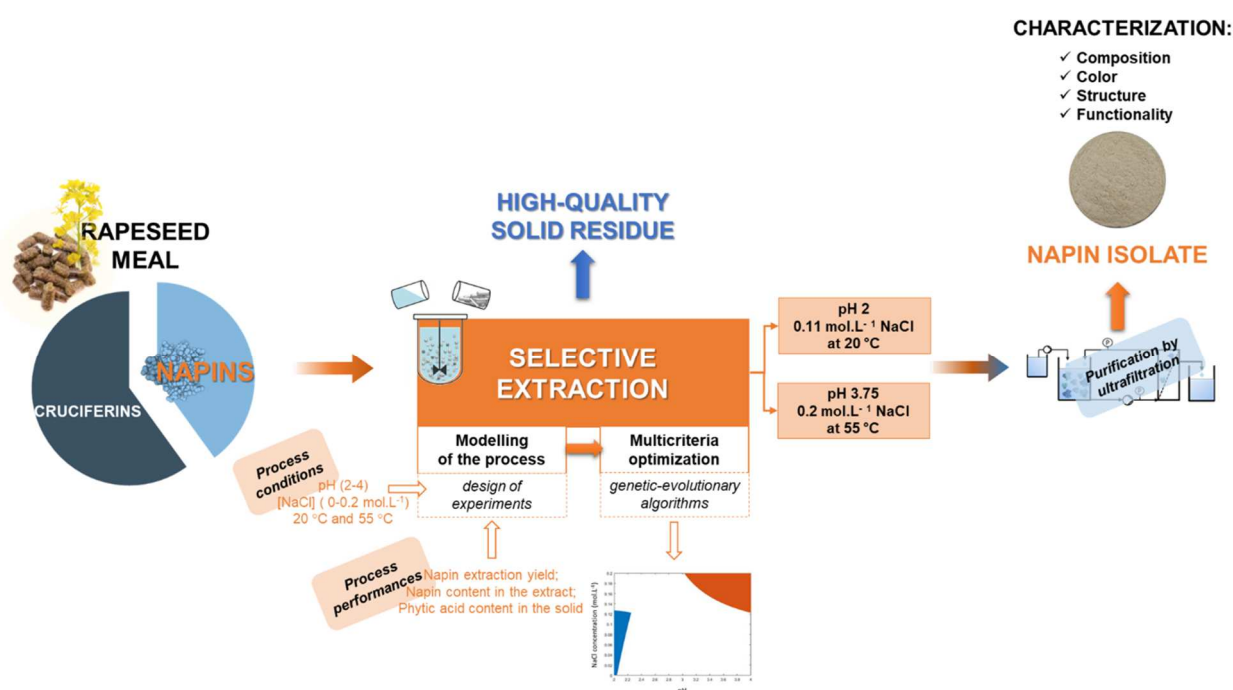
39 **Keywords:** rapeseed, napin, multicriteria optimization, extraction, structure, functionality

40

Highlights

- Selective extraction of napins while producing a high-quality solid residue
- Modeling of selective napin extraction process coupled with multicriteria optimization
- Optimal conditions were pH 2/0.11 mol.L⁻¹ NaCl/20°C and pH 3.75/0.2 mol.L⁻¹ NaCl/55°C
- Greater yield of napins observed at the higher extraction temperature (55 vs 20 °C)
- Napins extracted were light creamy, with high solubility and promising functionalities

Graphical abstract



CRediT author statement

Sara ALBE-SLABI: Roles/Writing - original draft, Formal analysis, Investigation, Data curation, Visualization ; **Claire DEFAIX:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Visualization; **Sophie BEAUBIER:** Roles/Writing - original draft, Formal analysis, Investigation, Data curation, Visualization ; **Olivier GALET:** Conceptualization, Resources, Supervision, Funding acquisition, Project administration ; **Romain KAPEL:** Roles/Writing - original draft, Writing - Review & Editing, Conceptualization, Methodology, Resources, Supervision Funding acquisition, Project administration.

61 **1. Introduction**

62 **Rapeseed including canola** is one of the largest oilseed crops worldwide with an annual production of over
63 70 million tons in 2019 ([FAOSTAT, 2019](#)). The solid residue remaining after the oil extraction process
64 (rapeseed meal) is currently used for feed due to its high protein content (30 to 50% on dry matter basis
65 ([von der Haar, Muller, Bader-Mittermaier, & Eisner, 2014](#); [Wanasundara, McIntosh, Perera, Withana-](#)
66 [Gamage, & Mitra, 2016](#); [Aider & Barbana, 2011](#); [Aluko & McIntosh, 2001](#)). However, rapeseed meal recently
67 gained a great interest as a promising alternative source of plant-based proteins that could be used as an
68 isolate in food industry.

69 Storage rapeseed proteins consist mainly of globulins and albumins. The 12S globulins, also called
70 cruciferins, are the most abundant protein fraction accounting for 50 to 70% of total rapeseed proteins.
71 They have a high molecular weight (about 300 kDa) and an isoelectric point (pI) at pH 7.2 ([Wanasundara et](#)
72 [al., 2016](#); [Hudson, 1994](#); [Cheung, Wanasundara, & Nickerson, 2015](#); [Perera, McIntosh, & Wanasundara,](#)
73 [2016](#); [Tan, Mailer, Blanchard, & Agboola, 2011](#)). The 2S albumins, also called napins, represent from 20 to
74 45% of total rapeseed proteins ([von der Haar et al., 2014](#); [Wanasundara, 2011](#)). They are low-molecular
75 weight proteins (12 to 15 kDa) composed of a small and large chain of 4.5 and 9.5 kDa, respectively ([Akbari](#)
76 [& Wu, 2015](#); [Aider, & Barbana, 2011](#); [Wanasundara et al., 2016](#)). Several isoforms of napins have recently
77 been identified ([Wanasundara et al., 2016](#)) **with an average pI around 11** ([Akbari & Wu, 2015](#)).

78 Isolates are the most refined protein products containing at least 90% of proteins on dry matter basis
79 ([Codex Alimentarius, 2019](#)). They are commonly used in food industry as nutritional, functional and
80 stabilization ingredients. In general, the production of plant protein isolate is a two-step process. The first
81 step is a solid/liquid extraction, which is based on the solubilization of proteins from vegetable solid matrix
82 in an aqueous solution. In the second step of purification, extracted proteins are separated from other
83 hydrosoluble non-protein compounds (carbohydrates, phenolic compounds, organic acids, etc.) to achieve
84 an isolate grade product.

85 A significant number of studies have proposed a production process for total protein isolate from rapeseed
86 meal. Most of them consist of alkali extraction (pH 9-12) followed by protein purification by acidic
87 precipitation (pH 2.5-5.5) (Aider, & Barbana, 2011; Tan et al., 2011; Aluko & McIntosh, 2001; Fetzer, Muller,
88 Schmid, & Eisner, 2020). However, the current technologies present several drawbacks. First is the
89 presence of non-protein compounds such as phenolic compounds, which can complex *via* covalent
90 interactions with proteins, particularly in an alkaline pH environment and contribute to unfavorable color
91 and taste (Wanasundara, 2011; Sosulski, 1979). Second is the production of protein isolates characterized
92 by limited functional and nutritional properties. Indeed, the acidic precipitation leads to an irreversible
93 denaturation of the cruciferins and an important loss of the well-soluble napins which decrease the overall
94 solubility of final protein products (Tan et al., 2011; Pedroche et al., 2004; Akbari & Wu, 2015; Chen &
95 Rohani, 1992; Blaicher, Elstner, Stein, & Mukherjee, 1983). Finally, the proposed technologies lead to the
96 by-production of a large amount of poor-quality solid residue left after protein extraction having a low
97 content of proteins (about 50% of initial content). In addition, the resulting solid residue is rich in
98 antinutritional compounds *i.e.*, phytic acid which limits the bioavailability of many essential minerals and
99 forms an insoluble phytate-protein complex (Cheryan & Rackis, 1980). Consequently, the solid residue from
100 protein processing can no longer be used for feed.

101 Interestingly, several authors analyzing SDS-PAGE gels of rapeseed extract have observed selective
102 extraction of napins at pH 2 to 4 (Nioi, Kapel, Rondags, & Marc, 2012; Perera et al., 2016; Wanasundara,
103 Abeysekara, McIntosh, & Falk, 2012; Fetzer, Herfellner, Stabler, Menner, & Eisner, 2018). The sequential
104 production of napin-rich extract from *Brassicaceae* oilseed at pH range of 2.5 to 5, followed by production
105 of cruciferin-rich extract from remaining meal residue at pH 7 to 13 was also widely described in the patent
106 of Wanasundara and McIntosh (2013). The purified napins were characterized by perfect solubility in water
107 over a wide range of pH (>90% at pH 2-10), excellent foaming properties and stable structure against
108 various pH and temperature. Napins had also a well-balanced composition in amino acids, exceptionally
109 regarding sulfur-containing amino acids (cysteine, methionine) that are limiting in other plant proteins
110 (Hudson, 1994; Young, & Pellett, 1994; Gueguen, Walrand, & Bourgeois, 2016, Wanasundara, & McIntosh,
111 2013).

112 Therefore, the selective extraction of napins from rapeseed meal would probably yield a high-quality
113 protein isolate that could be used for human nutrition. This strategy is particularly interesting since a solid
114 residue remaining after napin extraction process would be rich in cruciferins and so could still be used for
115 feed. Besides, other authors have also reported that acidic washing of rapeseed meal at pH from 3.5 to 4.0
116 prior alkali extraction can remove up to 50% of phytic acid from meal increasing its nutritional quality
117 (Akbari & Wu, 2015; Zhou, He, Yu, & Mukherjee, 1990; Serraino, & Thompson, 1984; Blaicher et al., 1983).

118 However, the acidic protein extraction from rapeseed meal is still poorly described in the literature. Indeed,
119 the precise impact of crucial protein extraction conditions such as pH, NaCl concentration and temperature,
120 and their interactions on process performances has not been addressed, so far. Furthermore, the
121 performances of the acidic protein extraction process have never been quantitatively analyzed and
122 optimized considering simultaneously the criteria of the napin extraction and the quality of the remaining
123 solid residue. Besides, the influence of selected process conditions on the composition, structure and
124 functional properties of proteins has not been described previously. This makes the rational choice of the
125 appropriate extraction conditions extremely challenging. Hence, an alternative strategy based on the
126 selective extraction of napins while producing a high-quality solid residue under acidic conditions should be
127 more explored.

128 The first step of this work was thus to investigate by design of experiments (DoE) the effect of pH (2-4),
129 NaCl concentration (0-0.2 mol.L⁻¹) at two different temperatures (20 and 55 °C) on the napin extraction
130 yield and the napin content in the liquid phase from extraction as well as the phytic acid and the protein
131 content in the solid residue. The starting material was a cold-pressed rapeseed meal which was not
132 defatted with hexane and would likely allow higher protein yield and quality. In the second step, polynomial
133 models from DoE were used in a multicriteria optimization methodology based on genetico-evolutionary
134 algorithms to establish an optimal set of pH and NaCl concentration at 20 °C and 55 °C. In the third part,
135 napins were extracted under the highlighted optimal conditions at 20 °C and 55 °C and purified by
136 ultrafiltration. The produced protein isolates were compared in terms of aggregation state, secondary and
137 tertiary structures, functional properties (solubility, foaming and emulsifying) and color.

138

139

140 2. Material and methods

141 2.1. Material

142 Cold-pressed rapeseed meal was provided by Olead (Pessac, France). Approximate composition was
143 determined as follows: 37.4% (w/w) (N x 6.25) of proteins, 6.8% of phytic acid, 3.5% (w/w) of total dietary
144 fibers, 8.7% (w/w) of carbohydrates, < 1% (w/w) of polyphenols and 15.4% (w/w) of fat on dry matter basis.
145 The particle size distribution of ground cold-pressed rapeseed meal showed the median diameter
146 D_{50} (represents 50% cumulative percentiles of the particle sizes), the surface weighted mean diameter
147 $D_{(3,2)}$, and the volume weighted mean diameter $D_{(4,3)}$ of $268.2 \pm 17.2 \mu\text{m}$, $49.97 \pm 3.21 \mu\text{m}$, and 329.2 ± 11.4
148 μm , respectively.

149 HCl was purchased from Carlo Erba (Val-de-Reuil, France). NaCl, NaOH, FeCl_3 , sulfosalicylic acid, Na_2SO_4 and
150 ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich (St. Louis, MO, USA). All reagents for
151 chromatography were of analytical grade. Acetonitrile was provided by Biosolve BV (Valkenswaard,
152 Netherlands). Trifluoroacetic acid (TFA) was from ThermoFisher (Waltham, MA, USA). Ultra-pure water (\geq
153 18.2 M Ω) was generated by a system Purelab Ultra from Elga (High Wycombe, United Kingdom).

154 2.2. Protein extraction

155 The extraction process was based on procedures used by Defaix et al. (2019). Extractions were carried out
156 in a temperature-controlled bioreactor of 200 mL at 20 or 55 °C. The mass of 15 g of rapeseed meal was
157 poured into 120 mL of NaCl solutions (0 to 0.2 mol.L⁻¹) and adjusted to pH 2.0-4.0 with 1 mol.L⁻¹ of HCl
158 solution at a 1:9 solid:liquid (w:w) ratio. After 60 min of stirring, the remaining meal was eliminated by
159 centrifugation (15 000 x *g*, 15 min, 20 °C). The liquid extract was filtered through a cellulose paper of 0.2 μm
160 pore-size membrane from Fisher Scientific (Pittsburgh, Pennsylvania, USA) and clarified by microfiltration
161 with a 0.22 μm and 50 cm² membrane (Pellicon XL, Millipore, Burlington, Massachusetts, USA) on a Cogent
162 μScale tangential filtration system (also from Millipore) at room temperature up to a volume reduction
163 factor (VRF) of 10. The clarified extract was analyzed for the total protein and napin content. The solid

164 residue left after the solid/liquid extraction was first freeze-dried and then the protein and phytic acid
165 content were characterized.

166 2.3. Design of experiments and multicriteria optimization

167 The effect of pH and NaCl on albumin extraction was studied by design of experiments (DoE) at 20 and 55
168 °C. Central composite face centered (CCF) design was chosen to study the impact of parameters (extraction
169 conditions) on performance criteria (responses). Each design matrix was composed of 11 experiments
170 including 3 repetitions of the center points (**Table A1**). Modde software (Umetrics, Umea, Sweden) was
171 used for the generation of the DoE matrix and the statistical analysis of models. The two studied
172 parameters were: (i) pH (x_1) ranking from 2.0 to 4.0 to ensure the selective extraction of napins (Nioi et al.,
173 2012) and (ii) NaCl concentration (x_2) from 0 to 0.2 mol.L⁻¹ because it was reported that in this range the
174 addition of salt had the most pronounced impact on the rapeseed protein extraction (Wanasundara et al.,
175 2012; Fetzer et al., 2018). Higher salt concentration was also avoided to limit the corrosive effect of salts on
176 industrial steel under acidic conditions and to reduce the process cost. The results were obtained from
177 design of experiments carried out at 20 °C and 55 °C. Intermediate temperatures were not included due to
178 potential microbial development, while temperatures above 55 °C could lead to protein denaturation. The
179 investigated responses were: (i) napin extraction yield, (ii) napin proportion in the extract and (iii) phytic
180 acid content in the solid residue. The model proposed for response (y) was as below (Eq. 1):

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (\text{Eq. 1})$$

181 where β_0 is the constant, β_1 and β_2 are the coefficients of linear effect, β_{11} and β_{22} are the coefficients of
182 cubic effect, β_{12} is the coefficient of interaction effect, x_1 and x_2 are the coded independents. Models were
183 statistically assessed using ANOVA test with a confidence level of 95%.

184 The multicriteria optimization of the extraction process was carried out using the predictive equations of
185 design of experiments as described previously by Muniglia et al. (2004). Genetico-evolutionary algorithms
186 were employed to identify the optimum of experimental parameters in terms of pH and NaCl concentration
187 at 20 and 55 °C. The aim of the objective function was to maximize the napin yield and the napin proportion
188 in the extract, and to minimize the phytic acid content in the solid residue. The non-dominated solutions

189 were calculated including the set of constraints: > 55% of napin yield at 20 °C and > 70% at 55 °C, > 95% of
190 napin proportion in the extract and < 4% of phytic acid on the dry matter of the solid residue left after the
191 napin extraction. The optimization runs and all data exploitation were carried out using MATLAB software
192 from MathWorks (Natick, MA, USA).

193 2.4. Napin purification

194 Napins were extracted under the optimal extraction conditions at 20 and 55 °C and purified up to the
195 isolate grade for the characterization of their structure and functionalities. The optimal conditions were
196 established at pH 2.0 and 0.11 mol.L⁻¹ of NaCl solution at 20 °C, and at pH 3.75 and 0.2 mol.L⁻¹ of NaCl at 55
197 °C. The extraction of napins was carried out as described in section 2.2. In brief, 250 g of rapeseed meal was
198 mixed with a NaCl solution and the pH was adjusted to a given value with 1 mol.L⁻¹ of HCl solution
199 respecting a solid:liquid ratio of 1:9 (w:w). The mixture was stirred during 60 min keeping the pH constant
200 (±0.05). After the extraction, the mixture was centrifuged (15000 x *g*, 30 min, 20 °C) using Lynx 6000
201 centrifuge from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The liquid phase from the
202 extraction (the extract) was filtered through a cellulose paper of 0.2 µm pore-size membrane and
203 microfiltered at a VRF of 10 using a ProScale system (Millipore, Burlington, Massachusetts, USA) coupled
204 with a 0.1 m² Pellicon XL membrane with a 0.22 µm pore size membrane (Millipore, Burlington,
205 Massachusetts, USA).

206 Napins in the clarified extracts were purified by tangential diafiltration (Äkta Flux 6, GE Healthcare, Chicago,
207 Illinois, USA) using a 3 kDa hollow fiber cartridges of 4800 cm² (GE Healthcare, Chicago, Illinois, USA). The
208 feed flow rate applied was 2 L.min⁻¹ and the transmembrane pressure was 1.5 bars (at room temperature).
209 The first step of the procedure consisted of five diavolumes (DV) with a feed solution of 0.5 mol.L⁻¹ of NaCl.
210 The pH was kept in the feed tank at value used during protein extraction with a help of 1 mol.L⁻¹ of HCl
211 solution. Then, four DV of ultrapure water were applied to remove salts. Final retentates were freeze-dried
212 (LTD type GZL-0.5, Shanghai Pharmaceutical Machinery Co., Shanghai, China).

213 2.5. Chemical analysis

214 The total protein content in the meals and the extracts were quantified according to the official AOAC
215 979.20 Kjeldahl method (1995). In calculation, a nitrogen-to-protein conversion factor of N x 6.25 was used.

216 The napin proportion in the extract in relation to the total protein content (*i.e.*, napins and cruciferins) was
217 assessed by size exclusion high performance liquid chromatography (SE-HPLC) as described by Albe-Slabi et
218 al. (2020a and 2020b). In brief, 5-10 μL of sample was injected into a Biosep-SEC-s2000 column (300 x 7.8
219 mm, 5 μm) from Phenomenex (Torrance, California, USA) connected with an HPLC system was from
220 Shimadzu (Kyoto, Japan). Samples were filtered on 0.22 μm membranes from Pall (Port Washington, NY,
221 USA) beforehand. The mobile phase was composed of 45% of acetonitrile, 54.9% of ultrapure water and
222 0.1% (v/v) of trifluoroacetic acid (TFA). Separation was achieved during 30 min at a flowrate of mobile
223 phase of 0.6 $\text{mL}\cdot\text{min}^{-1}$. The UV detection was recorded at 280 and 214 nm for proteins and 325 nm for
224 phenolic compounds. Data were collected and treated using LabSolutions software, also from Shimadzu.
225 The napin extraction yield was then calculated assuming a mean value of the initial napin content in crude
226 rapeseed meal of 35% (von der Haar et al., 2014; Wanasundara, 2011).

227 The phytic acid content in the solid residue remaining after the protein extraction was determined based on
228 the method developed by Garcia-Esteva et al. (1999) and was expressed on dry matter of the solid sample.

229 2.6. Napin characterization

230 2.6.1. Purity and solubility

231 The purity of the napins produced was determined by the Kjeldahl method and expressed on dry matter
232 basis. For measurement of the solubility, a 5 $\text{g}\cdot\text{L}^{-1}$ of napin solution was prepared and adjusted to 3.0, 5.0,
233 7.0 or 9.0 by addition of 0.1 $\text{mol}\cdot\text{L}^{-1}$ of HCl or NaOH solution. The added volumes were recorded. The
234 mixture at constant pH (± 0.05) was stirred at 300 rpm at ambient temperature during 30 min and then
235 centrifuged ($11\,000 \times g$, 15 min, 20 $^{\circ}\text{C}$). The supernatant was recovered and filtered through a 0.22 μm filter
236 and the protein content was measured by the Kjeldahl method. The solubility at a given pH was calculated
237 as the ratio of the protein mass in the supernatant on the protein mass in the initial solution.

238 2.6.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

239 The napin isolates were characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis
240 (SDS-PAGE) as described by Laemmli method (1970). The volume of 10 μ L of the protein samples reduced
241 and the molecular weight markers (ranged from 250 to 15 kDa and from 26.6 to 1.4 kDa) were deposited to 5
242 and 17% of polyacrylamide stacking and resolving SDS-PAGE gels, respectively. All reagents and
243 electrophoretic equipment were obtained from Bio-Rad (Hercules, USA).

244 2.6.3. Color analysis

245 The color of the napin isolates was determined in aqueous solution. The analysis in liquid was carried out
246 on a Lovibond PFX195 (Tintometer, Amesbury, United Kingdom). Samples were prepared as 1% (w/w)
247 solution of protein powder in 0.5 mol.L⁻¹ of phosphate buffer, pH 7.0. The system measured the color of the
248 sample considering the CIELAB uniform space scale. The term “L” related to the quantity of black/white, “a”
249 to green/red and “b” to blue/yellow. All measurements were performed in triplicate and a mean value was
250 calculated.

251 2.6.4. Amino acid composition

252 The amino acid profile was determined for the napin isolates according to ISO 13903:2005 and EU 152/2009
253 procedures. In brief, about 10 mg of sample was hydrolyzed. Depending on the amino acid analyzed, three
254 different hydrolysis methods were performed: an acidic hydrolysis for isoleucine, leucine, lysine,
255 phenylalanine, tyrosine, threonine, and valine; an alkaline hydrolysis for tryptophan; and an oxidative
256 hydrolysis for cysteine and methionine. Then, the resulting hydrolysate was injected into a C18 column. The
257 composition in these amino acids was determined by reaction with ninhydrin using photometric detection
258 at 570 nm. For the quantification of tryptophan, a HPLC system coupled with fluorescence detector
259 (excitation at 280 nm, emission at 231 nm) was used. The amino acid profile was expressed as mg of amino
260 acid per g of napins.

261 2.6.5. Circular dichroism (CD)

262 The secondary structure of napins was analyzed using far-UV between 180 and 260 nm. The napin solutions
263 at 1 g.L⁻¹ were prepared in 0.5 mol.L⁻¹ of phosphate buffer, pH 7.0. The spectral analyses were carried out at
264 20 °C using Chirascan plus system from Applied Photophysics (Leatherhead, United Kingdom). Samples

265 were prepared in cells of 0.1 mm path length. The blank signal of buffer was subtracted. All measurements
266 were performed in triplicate and a mean value was calculated. Pro-Data Chirascan software from Applied
267 Photophysics (Leatherhead, United Kingdom) was used to process the data, while CDNN software (version
268 2.1.) was used for further deconvolution of far-UV spectra and determination of α -helix, β -sheet, and
269 random coil content. The same system was used to assess tertiary structures using near-UV spectra
270 between 250 and 320 nm. A quartz cell of 10 mm path length was used.

271 2.6.6. Dynamic Light Scattering (DLS)

272 DLS was used to determine the aggregation state of the napin isolates. Protein solution at 1 g.L⁻¹ were
273 prepared in 0.5 mol.L⁻¹ of phosphate buffer, pH 7.0. Analyses were carried out with a Zetasizer Nano – S
274 from Malvern Instruments (Malvern, United Kingdom). Samples were maintained at 25 °C during
275 measurement. The number of acquisitions varied between 13 and 17 scans. Volumetric distribution of
276 particle size was determined.

277 2.6.7. Emulsifying and foaming properties

278 2.6.7.1. Emulsifying capacity and stability

279 Emulsifying properties were assessed using the method of Vioque et al. (2000) with modifications. For this
280 purpose, 5 mL of aliquot of 5% (w/v) napin solution in 0.5 mol.L⁻¹ of sodium phosphate buffer, pH 7.0 were
281 mixed with 2.5 mL of rapeseed oil at 10 000 rpm at room temperature during 30 s using an Ultra-Turrax®
282 homogenizer. After this time, 2.5 mL of oil was added and mixed again during 90 s. After this time, the
283 mixture was centrifuged at 1 100 × g during 5 min at 20 °C. The emulsified layer was recorded. The
284 percentage of the volume of the emulsified layer remaining after centrifugation in relation to the volume of
285 the mixture before centrifugation represented the emulsifying capacity. For evaluation of the emulsion
286 stability against temperature, the mixture obtained after centrifugation was placed during 15 min in a water
287 bath heated at 85 °C. After this time, the mixture was centrifuged again. The stability of the protein
288 emulsion was calculated as a ratio of the emulsified layer before and after heating. The results were
289 expressed in percentage.

290 2.6.7.2. Foaming capacity and stability

291 Foaming properties of the isolated napins were evaluated according to the method of Vioque et al. (2000)
292 with modifications. In brief, the protein powder was dissolved in 0.5 mol.L⁻¹ of sodium phosphate buffer, pH
293 7.0 at a final concentration of 1% (w/v). The volume of 20 mL of aliquot was mixed at 10 000 rpm at room
294 temperature for 5 min using an Ultra-Turrax® T25 digital homogenizer from IKA (Staufen im Breisgau,
295 Germany). The foaming capacity was calculated as a ratio between the obtained foam volume and the
296 volume of initial protein solution before mixing. The results were expressed in percentage. The foaming
297 stability was determined by measuring the percentage of the foam volume left after 5, 15, 30, 60 and 120
298 min.

299 2.7. Statistical analysis

300 All measurements were carried out at least in triplicate (n=3) and data were presented as a mean value
301 with standard deviation (\pm). Significant differences were statistically analyzed by one-way analysis of
302 variance using F-test with a confidence level of 95% (p-value < 0.05).

303

3. Results and discussion

3.1. Impact of pH and NaCl concentration on napin extraction at 20 and 55 °C

3.1.1. Statistical analysis of models

The effect of the extraction pH (2-4) and NaCl concentration (0-0.2 mol.L⁻¹) on napin extraction yield and napin proportion in the extract at 20 and 55 °C was studied by design of experiments (DoE). The influence of the extraction conditions on the phytic acid content of the post-extraction solid residue was also considered. **Table 1** displays the significant regression model coefficients from the polynomial regression at 95% of confidence level and the statistic model parameters. As a result, non-linear regression R² of all models ranged from 0.821 to 0.965, which indicated that most of the observed variations can be explained by models. The obtained models were also characterized by a reasonable reproducibility (0.723-0.982) and the difference between R² and Q² was lower than 0.3. Moreover, the ANOVA test revealed low p-values (0.001-0.004) and insignificant lacks of fit (0.173-0.793). As a conclusion, all developed models can be reliably used to interpret the obtained data.

3.1.2. Napin extraction yield

Fig. 1A and **A'** show the napin yields after solid/liquid extraction at pH ranging from 2.0 to 4.0 and NaCl concentration ranging from 0 to 0.2 mol.L⁻¹. The results were obtained from design of experiments carried out at 20 and 55 °C, respectively. As presented in the response surface in **Fig. 1A**, at pH between 2.0 and 2.5 the napin yield was high (around 55%) and rather constant whatever the NaCl concentration. Above pH 3.0 and at low NaCl concentration (<0.1 mol.L⁻¹), the increase of pH resulted in the decrease of the napin extraction yield (55 vs 42% at pH 2.0 and 4.0, respectively, without salt addition). This negative effect was overcome at a higher NaCl concentration. Indeed, at pH 4.0, the napin yield rose from 42 to 61% at 0 and 0.2 mol.L⁻¹ of NaCl, respectively. A very similar effect of the extraction conditions on the napin yield was also observed at 55 °C (**Fig. 1A'**). However, the response varied in the range from 60 to 76%. The results were thus significantly higher than those at 20 °C.

328

329 These observations are consistent with the previous results ([Wanasundara et al. 2012](#); [Wanasundara &](#)
330 [McIntosh, 2013](#); [Chen & Rohani, 1992](#)) showing the minimal solubility of total nitrogen from various
331 rapeseed species at pH 4-5. Furthermore, Wanasundara & McIntosh ([2013](#)) noted the improved
332 extractability of napins from *Brassicaceae* oilseed at pH 3 with the increase of NaCl concentration up to
333 0.13 mol.L⁻¹. The authors explained it by the disruption of the association between napins and other
334 compounds.

335 The negative effect of both pH and NaCl on the napin yield indicates that electrostatic interactions are
336 involved in the protein solubilization process. This kind of process is classically done in three steps. The first
337 one is the diffusion of the aqueous solvent in the solid meal. The second one is the protein transport from
338 the solid phase toward the imprinting aqueous phase inside the solid. The third one is a protein diffusion
339 from the impregnating liquid to the liquid bulk. The overall process may be limiting by (i) a strong affinity of
340 the proteins toward the solid phase after the liquid impregnation, (ii) a poor affinity in the liquid phase or,
341 (iii) a diffusion limitation in the liquid bulk. Napins are positively charged over the investigated pH range
342 since their isoelectric point was reported to be around 11 ([Akbari & Wu, 2015](#); [Perera et al., 2016](#)).
343 Furthermore, the solid is composed of insoluble hemicellulose polymers that contain glucuronic or
344 galacturonic acids ([Pustjens, Schols, Kabel, & Gruppen, 2013](#); [Bhattacharyya, & Rohrer, 2012](#)). The pKa of
345 the carboxylic acid function of these carbohydrates is around 3.0-3.5. Once impregnated with an aqueous
346 phase at pH higher than 3.0, these insoluble polymers expose negative charges. Hence, the limitation of the
347 napin extraction observed at pH 4.0 might be due to electrostatic interactions between the negatively
348 charged solid particles and positively charged napins. This would explain the positive effect of the NaCl at
349 high pH and the absence of effect at pH lower than 3.0, where the carboxylic functions are not or poorly
350 charged.

351 Regarding the effect of temperature, few data are currently available. Wanasundara & McIntosh ([2013](#))
352 reported the enhanced extractability of proteins from rapeseed meal at pH 4.5 with the increase of
353 temperature from 20 to 50 °C. Likewise, Radwan & Lu ([1976](#)) showed that, in general, higher temperature
354 facilitate the solubilization of the proteins from rapeseed meal and obtaining greater yields in a wide range

355 of extraction pH (1-13). This effect was attributed to the partial protein hydrolysis or the release of proteins
356 upon destabilization of the seed matrix. However, napins have a high structural stability against thermal
357 treatments (denaturation temperatures above 100 °C as reported by Nioi et al. (2012) and Perera et al.
358 (2016). As in that study, no denaturation was observed when comparing the electrophoretic profiles of
359 napins extracted at 20 and 55 °C (data not shown). Therefore, the improvement of napin extractability from
360 rapeseed meal at 55 °C is probably due to the reduction of the electrostatic interactions between proteins
361 and carbohydrates upon higher extraction temperatures.

362 3.1.3. Napin proportion in the extract

363 **Fig. 1B** shows the response surface for the proportion of napins, related to the total rapeseed proteins (*i.e.*,
364 cruciferins and napins), in the extracts at 20 °C. As it can be seen, the napin proportion in the extract
365 depended primarily on the extraction pH. This effect was negative. Indeed, the maximum proportion of
366 napins were found around pH 2.0 without salt addition (97.9%), whereas lower values were observed at
367 higher pH (89.0% at pH 4.0). The NaCl concentration had no significant impact on the studied response, as
368 also shown in **Table 1**. Regarding the response surface of the proportion of napins extracted at 55 °C (**Fig.**
369 **1B'**), the values ranged from 93.0 to 96.5%. Similar to the extraction at 20 °C, this response was mainly pH-
370 dependent. No effect of the addition of NaCl was noticed. However, in opposite to the results at 20 °C, the
371 pH had a slight positive impact on the napin proportion. The lowest value (93.0%) was found at pH 2.0,
372 whereas the highest value (96.5%) was between pH 3.0 and 3.7.

373 Interestingly, the pronounced bands of napins on SDS-PAGE gels have also been previously noted for the
374 rapeseed extract at pH 2-4 (Nioi et al., 2012; Akbari & Wu, 2015; Perera et al., 2016). The selective
375 extraction of napins under acidic conditions was probably related to the difference in structural stability
376 between napins and cruciferins. Those last are known to precipitate irreversibly at strong acidic medium
377 (pH 2-3) due to the destruction of their native structure. In contrast, napins exhibit an exceptionally stable
378 structure in a large range of pH (Perera et al., 2016). Thus, contrarily to cruciferins, they remain soluble in
379 the liquid phase from acidic extraction. A slight reduction in the proportion of napins in the extract at higher
380 pH (98% vs 89 % at pH 2.0 and 4.0, respectively) may indicate a gentle increase of the cruciferins

extractability from the meal. Another reason could be also attributed to the distinct extraction behaviors of various napin isomers. Indeed, as it was mentioned in the work of Perera et al. (2016) lower pH values would favor the extraction of most napin isoforms from *Brassicaceae* seed meals. The slight discrepancies, comparing the extraction at 20 and 55 °C, could be explained by a slightly better extractability of cruciferins at elevated temperatures. These results therefore support the hypothesis of Wanasundara & McIntosh (2013) who highlighted that higher extraction temperatures may resulted in the increase of globulin recovery from the rapeseed meal. Also, Radwan & Lu (1976) have reported that the total proteins from rapeseed meal were more soluble at 55 °C than at 25 °C. The same study showed a shift of the minimum of solubility from pH 4-5 at 25 °C (which corresponds to the pI of cruciferins) to pH 7 at 55 °C.

3.1.4. Phytic acid content in the solid residue

The objective of the extraction step was to selectively extract the napins at the highest yield while keeping a high cruciferins level in the solid residue generated after the protein extraction. The overall protein content in the residual solid under the studied extraction conditions ranged from 28 to 34% of the dry matter content (data not shown). This corresponded to the usual range of protein content in meal. Therefore, this criterion was considered very satisfying whatever the extracting conditions.

Apart from proteins, the phytic acid content is another important indicator that defines the quality of the residual meal. This molecule has been recognized over the years as an antinutritional compound that negatively affects the meal nutritional property (Cheryan & Rackis, 1980) Therefore, the impact of the extraction conditions on the phytic acid content was also studied.

As presented in **Fig. 1C**, the phytic acid content increased gradually from pH 2.0 to 2.4 and reach a maximum between pH 2.4 and 3.3 (> 4.6% on dry matter basis). Then, it decreased toward higher pH values. NaCl concentration had a weak negative impact on the phytic acid content in the solid, especially in the pH area around pH 3. The lowest value was found at pH 4 and 0.1 mol.L⁻¹ of NaCl (2.8% on dry matter basis). At 55 °C (**Fig. 1C'**), the phytic acid content in the residual meal also depended on both pH and NaCl concentration. In general, these effects were similar. From pH 2.5 and 0 mol.L⁻¹ of NaCl to 4.0 and 0.2 mol.L⁻¹ of NaCl, the content of phytic acid decreased from 4.5 to 2.8% on dry matter basis. Nevertheless, different

407 extraction behaviors were noted at low pH value. Indeed, the highest level of phytate in solid was found at
408 pH 2.0 and 0.2 mol.L⁻¹ of NaCl. These results were hardly dependent on pH neither NaCl addition. The
409 current observations coincide well with the findings of Zhou et al. (1990), who highlighted a significant
410 correlation between the pH and the concentration of phytic acid in napin-containing extract. According to
411 them, the lower solubility of phytic acid from rapeseed meal was also around pH 3 and increased at pH 4.
412 Similar conclusion has been drawn by Akbari & Wu (2015) and Blaicher et al. (1983), showing that the acidic
413 washing at pH 4-4.5 at room temperature can remove from 47 to 70% of phytic acid from the meal. The
414 first pKa of phytic acid was found at pH 1.5-2 (Cheryan & Rackis, 1980). Therefore, from pH 3.5-4 the six
415 phosphoric groups of phytic acid start to be negatively charged which facilitates their solubilization in the
416 aqueous phase. This probably also explains a slight increase in the solubility of phytic acid from the meal
417 upon salt addition and consequently in the observed reduction of phytic acid content in the solid residue
418 remaining after solid/liquid extraction.

419 3.2. Multicriteria optimization of extraction process at 20 and 55 °C

420 The objective of this part was to find the optimal set of extraction conditions, in terms of pH and NaCl
421 concentration at 20 and 55 °C, to maximize on the one hand the napin yield and selectivity, and on the
422 other hand, to maximize the removal of phytic acid from the residual solid.

423 To do so, the previously developed methodology of multicriteria optimization based on genetical-
424 evolutionary algorithms was used (Muniglia, Kiss, Fonteix, & Marc, 2004). To narrow the selection of the
425 solutions, some constraints were included into the algorithm. For this purpose, a napin proportion in the
426 extract of at least 95% was searched whatever the temperature applied. Because extraction yields were
427 very different for the two temperatures, the limits of the napin yield were set at, at least, 55% and 70% at
428 20 °C and 55 °C, respectively. Finally, the phytic acid content in the solid residue was chosen lower than 4%
429 on the dry matter content, which corresponds to the elimination of almost 50% of the initial value in the
430 rapeseed meal.

431 **Fig. 2** depicts the extraction conditions areas fitting these constraints (blue at 20 °C and orange at 55 °C).
432 Surprisingly, these results highlighted two distinct zones of optimal conditions corresponding to pH values

433 and NaCl concentrations totally different as a function of the extraction temperature. The first zone for
434 extraction at 20 °C was located below pH 2.3 with up to 0.13 mol.L⁻¹ of NaCl. On the contrary, the optimal
435 extraction conditions for the napin extraction at 55 °C were found when pH values were above 3.0 with
436 0.13 to 0.2 mol.L⁻¹ of NaCl. Consequently, two sets of extraction conditions were chosen inside these zones:
437 pH 2.0 with 0.11 mol.L⁻¹ of NaCl for the napin extraction at 20 °C and pH 3.75 with 0.2 mol.L⁻¹ of NaCl at 55
438 °C. The first optimal set yielded a napin extraction yield of 55.4%, a napin proportion in the extract of
439 97.9%, and a phytic acid content in the residual meal of 3.59%. The second one allowed to achieve 75.6% of
440 napin extraction yield, 96.6% of napin proportion in the extract and 2.99% of phytic acid content in the
441 residual meal.

442 Regarding previous works, the systematic approach for the optimization of the rapeseed protein extraction
443 involved holding the extraction conditions constant (pH, salt concentration, temperature, etc.) and varying
444 the levels of another one (Fetzer et al., 2018; Nioi et al., 2012). However, this “one-by-one” strategy is often
445 ineffective because it presents a high risk of missing significant interactions between the different
446 extraction conditions. In the present study, we modelled the effect of independent extraction conditions
447 (pH and NaCl concentration) by modifying their levels simultaneously for two different temperatures. This
448 strategy coupled with the multicriteria optimization allowed for the achievement of fully satisfactory results
449 given the high complexity of the process, due to all the possible interactions of the individual extraction
450 conditions and between them.

451 3.3. Characterization of napin isolates

452 3.3.1. Purity and color

453 The napin extractions under both highlighted sets of optimal conditions (pH 2.0/0.11 mol.L⁻¹ of NaCl at
454 20 °C and pH 3.75/0.2 mol.L⁻¹ of NaCl at 55 °C) were performed and purification was implemented by
455 ultrafiltration process. It yielded powders with an isolate grade (91.6 and 92.9% of purity on dry matter
456 basis for 20 and 55 °C, respectively). In terms of protein composition, both isolates were rich in napins (>
457 96.5% of total proteins). The high proportion of napins in the isolates was also confirmed by SDS-PAGE gel
458 (Fig. 3A) showing three low-molecular bands between 5 and 15 kDa. Two major bands of ~5 and 10 kDa

459 were assigned to a small and large polypeptide of napin, respectively. The band of ~15 kDa corresponded to
460 the residue of napin not completely dissociated in the presence of β -mercaptoethanol. The obtained
461 electrophoretic profile was similar to the previously described pattern of napins (Akbari & Wu, 2015; Perera
462 et al., 2016; Cheung et al., 2015).

463 Concerning the color of the isolates obtained, both napin products were light creamy powders (Fig. A1A
464 and A'). In solution, the resulting index of lightness (L^*) and the two coordinates of color (a^* and b^*) were
465 92, -4.2 and 24.3, and 96.3, -3.3 and 23.1, respectively for napins extracted at 20 and 55 °C. Most
466 concentrates of total rapeseed proteins have been characterized by much darker color (L^* around 50), as
467 described by Fetzer et al. (2019). The dark colors previously observed of rapeseed protein isolates are
468 thought to be due to the presence of phenolic compounds, known to interact covalently with proteins
469 forming a colored complex even at very low concentrations (Sosulski, 1979). Among both proteins, stronger
470 phenolic complexations with napins than cruciferins has previously been noted (Albe-Slabi et al., 2020a). To
471 verify this with the isolates obtained, the phenolic complexation was investigated by the SE-HPLC method
472 at 280 and 325 nm. As it is shown on the chromatograms (Fig. 3B and B'), the signal of polyphenols at 325
473 nm represented about 13% of the signal at 280 nm. This ratio was thus low and similar for both products. It
474 suggested that the selected sets of conditions, in particular the extraction temperature, did not influence
475 the covalent interaction between napins and phenolic compounds during the protein extraction. Hence, the
476 phenolic complexation occurs probably much earlier, in the stage of seed formation and/or the processing
477 of the meals (grinding, pressing, etc.).

478 Altogether, the color is essential to define the field of applications of proteins in food industry. The results
479 obtained were thus very encouraging toward a possible use of napin isolates in human nutrition.

480 3.3.2. Amino acid composition

481 The aminograms of the napin isolates produced are displayed in Table 2. In general, the amino acid
482 compositions of both napin isolates (extracted at 20 and 55 °C) were almost identical. It suggests that the
483 temperature of protein extraction had no significant impact on the overall amino acid profile. Considering
484 the reference protein pattern, established by FAO/WHO/UNU (2007), both napin isolates met the essential

485 amino acid nutritional requirements for adults, expect for isoleucine (about 28 mg.g⁻¹ of protein). However,
486 a particularly high content of the sulfur amino acid *i.e.*, cysteine (580-650% of the FAO/WHO/UNU
487 requirements) as well as histidine, lysine, and tryptophan (about 200% of the reference) was observed. This
488 was in line with previous results (Wanasundara et al., 2016; Wanasundara & McIntosh, 2013).

489 These authors have also pointed out on the high level of cysteine in napin-rich isolates. Baszczynski & Fallis
490 (1990) and Monsalve et al. (1991) have previously drawn similar conclusions. The high content of sulfur-
491 containing amino acids is particularly interesting because they are considered to be limiting for many other
492 plant proteins (Hudson, 1994; Young, & Pellett, 1994; Gueguen et al., 2016). Noteworthy napins contain
493 also, unlike cruciferins, the adequate amount of lysine. The presented results confirmed thus that napins
494 are a well-balanced source of amino acids that can be used in human nutrition. In addition, similar
495 aminograms also suggested that both isolates were composed of the same napin isoforms, regardless of the
496 applied isolation procedures.

497 3.3.3. Structural properties

498 The secondary and tertiary structures of napins extracted at 20 and 55 °C were studied at pH 7 by circular
499 dichroism. In general, the far-UV spectra of both isolates (**Fig. 3C**) had a typical shape of an α -helical protein
500 with negative bands around 220 and 210 nm and a positive band around 190 nm. However, the far-UV
501 spectra of napins extracted at 55 °C was altered showing a significant lower ellipticity between 190 and 200
502 nm corresponded to α -helix structure and slightly greater signal between 210 and 230 nm that belongs to
503 β -sheets. As majority of authors (Nioi et al., 2012; Hudson, 1994; Schwenke, 1990; Schmidt et al., 2004)
504 previously indicated, the native structure of napins is characterized by the predominant content of α -helix
505 (37-59%) with a significant lower amount of β -sheet conformation (7-20%). In this work, the conversion of
506 signal into a proportion of each type of secondary structures revealed that the napins extracted at 20 °C
507 were composed of 49.7% of α -helix, 7.8% of β -sheets, 14.3% of β -turn and 21.3% of random coil. The napins
508 extracted at 55 °C consisted of 44.2% of α -helix, 9.9% of β -sheets, 15.3% of β -turn and 22.9% of random
509 coil. These results demonstrated that the production process at 20 °C applied yielded a well-structured
510 napin isolate, whereas the extraction at 55 °C caused a partial denaturation of the napin native structure.

Concerning the near-UV spectrum (**Fig. 3C'**), pronounced signals of tryptophan at 290 nm, tyrosine from 275 and 282 nm and phenylalanine from 255 to 270 nm were observed for both isolates. The spectrum was almost identical to this previously presented for napins at pH 7 by Perera et al. (2016). However, as it was noted before, slight differences were observed for the two napin isolates. Indeed, the near-UV spectrum of napins extracted at 55 °C was characterized by more pronounced signal, in particular in the region of phenylalanine and tyrosine. This confirmed the above hypothesis about possible modifications in conformation of napin structure caused by the conditions of the production process. Evidently, in higher extraction temperature, the napins have probably partially unfolded exhibiting aromatic amino acid residues.

Dynamic light scattering (DLS) was used to measure the size distribution of the isolates produced. As displayed at **Fig. 3D** and **D'**, the size distribution according to the volume for both napin isolates showed one single peak. The average hydrodynamic diameter of the detected particles was 5.60 ± 0.20 d.nm and 6.34 ± 0.39 d.nm for napins produced at 20 and 55 °C, respectively. Comparable results were previously reported by Barciszewski et al. (2000) determining the hydrodynamic diameter of rapeseed albumins at 5.12 nm. Thus, the napins produced in this study were apparently in the form of monomers. However, the larger diameter of the napins extracted at 55 °C comparing to those at 20 °C suggested the partial unfolding of the native structure upon thermal treatment. This is in line with the previous observations of structural modifications studied by circular dichroism.

3.3.4. Functional properties

3.3.4.1. Solubility

Fig. 4A presents the solubility of both napin isolates as a function of the pH value (3-9). As a result, both napin isolates were largely soluble in the whole pH range. Their solubility at pH 3, 7 and 9 was high (89-99%) and did not significantly differ between isolates (p-value > 0.05). At pH 5, the solubility of the napins extracted at 20 °C decreased slightly ($82.87 \pm 2.66\%$). Similar observation was made in the case of the napin isolate extracted at 55 °C for which the minimum solubility was also noted at pH 5 ($62.98 \pm 2.78\%$). Cheung

et al. (2015) have previously reported that napins have generally an elevated solubility in water (> 90%) from pH 2 to 10.

Therefore, a possible explanation of the lower solubility at pH 5 might be attributed to the complexation between napins and phytic acid. Indeed, phytic acid exhibits a strong ability to interact with proteins reducing their solubility in water (Cheryan & Rackis, 1980; Bye, Cowieson, Cowieson, Selle, & Falconer, 2013). In the detailed study of Kroll (1991), rapeseed proteins have also demonstrated a minimum solubility at pH 5 when mixed with 5% of phytic acid (w/w). At this pH, the opposite charge of napins (positive) and phytic acid (negative) facilitates the interaction between both molecules and the formation of insoluble complex. Moreover, the protein/phytic acid interaction is promoted when the protein structure is unfolded, such as in the case of napins extracted at 55 °C. This is due to the exposure of new binding sites that are normally buried with hydrophobic residues inside the core of the protein (Wang & Guo, 2021). Besides, during denaturation the protein charge becomes more positive increasing the attractions of negatively charged phytic acid. This explains the significant difference in the solubility of both proteins in water.

Nevertheless, such solubility of protein isolate remains still exceptionally high comparing to other plant proteins currently used in food industry. Indeed, most proteins have an isoelectric point between pH 5 and 7 and a very low solubility in this area (Gonzalez-Perez, S., & Vereijken, 2007; Tang et al., 2006). This considerably reduces the field of applications of such isolates and confirms the great interest of the napins obtained in this study for food applications.

3.3.4.2. Emulsifying and foaming

The emulsifying properties of the napin isolates extracted at 20 and 55 °C are presented in **Fig. 4B** and **B'**. As shown in **Fig. 4B**, the napin isolates were characterized by similar emulsifying capacity, whatever the extraction conditions (32.7±2.5 and 30.1±1.3% for 20 and 50 °C, respectively). These values were not statistically different (p-value > 0.05). The emulsifying stability against heating (**Fig. 4B'**) was also high and comparable between isolates (100%). Similarly, Chabanon et al. (2007) have reported about 35% of emulsifying activity for a napin isolate. However, the stability of the emulsion formed by these published

561 proteins was significantly lower (48%) than in our study. In comparison with the reference proteins, the
562 emulsifying properties obtained were close to those of commercial soy proteins (about 40% of capacity and
563 100% of stability) measured by the same procedures (Albe-Slabi et al., 2020a).

564 Regarding the foaming capacity (**Fig. 4C**), the results shown that both napin isolates produced an almost
565 identical foam volume (263.7 ± 18.5 and $320.0 \pm 32.1\%$ of foaming capacity for napins extracted at 20 and 50
566 °C, respectively). Similar to the emulsifying properties, these values did not statistically differ (p-value >
567 0.05). The stability of the foam produced with both napin isolates was good (**Fig. 4C'**), with about 70% of
568 the initial foam volume remaining after 120 min. The present results were comparable to the observations
569 of other authors. Indeed, Nitecka et al. (1986) studying functional properties of napins at pH 7, have found
570 above 400% of foaming capacity and 90% of foam stability after 10 min from whipping. Akbari et al. (2015)
571 have also showed a good foaming capacity (134%) and foam stability of a napin isolate after 120 min (79%).
572 A slight lower foaming stability of albumin-based isolate (41% after 120 min) was presented by Chabanon et
573 al. (2007). Interestingly, according to the previous measurements of Albe-Slabi et al. (2020b), napins had a
574 similar foaming capacity and a greater emulsion stability compared to soy proteins used as a commercial
575 reference.

576 Overall, napin is a high helical protein with globular and rigid structure. However, as presented in the
577 previous part of the study, heating during extraction resulted in partial unfolding of napins associated with
578 a decrease of alfa-helix content and exposure of hydrophobic amino acids on the external surface. In
579 general, such changes in protein structure make it more flexible, which can facilitate the adsorption of
580 protein at the interface and thus improve its emulsifying and foaming properties. Apparently, the structural
581 modifications of napins observed in the present study were not strong enough to considerably affect their
582 interfacial behavior. Besides, the functional properties were measured at pH 7, where the solubility was
583 high and almost identical for both proteins. This may explain the lack of significant differences in the
584 emulsifying and foaming properties between them.

585

Conclusion

This study established optimum conditions for selective extraction of napin from cold-pressed rapeseed meal by optimizing multiple factors. Our results showed that the pH value, the NaCl concentration and the temperature considerably influenced the extraction yields of napins, their proportions in extract and the phytic acid contents in the solid residue generated after the extraction process. Two optimal extraction conditions were proposed: pH 2.0/0.11 mol.L⁻¹ of NaCl at 20 °C and pH 3.75/0.20 mol.L⁻¹ of NaCl at 55 °C allowing for a selective extraction of napins (> 97% in relation to total proteins), a satisfactory extraction yield (>55% at 20 °C and >75% at 55 °C) and a significant reduction of phytate from the solid residue (50-45% of the initial value). Furthermore, both napin isolates obtained under the optimal conditions and purified by ultrafiltration showed a similar amino acid composition, color and solubility (except of pH 5). Although some structural variations were observed in the two napin isolates, no significant impact on the functional properties was evident from their comparable for emulsifying and foaming properties. Extraction process at 55 °C allowed significantly higher yield of napin (1.4 fold), maintaining selectivity for napin while preserving the native functional properties of the protein.

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740 **FIGURES**

741 **Table 1.** Uncoded regression coefficients of significant predicted polynomial models (process conditions: pH
742 (x_1) and NaCl concentration (x_2)) and statistical analysis results of the models for the prediction of napin
743 extraction yield (%), napin proportion in the extract (%), and phytic acid content in the solid residue (%) at
744 20 and 55 °C.

	Response (y)					
	Napin extraction yield (%) [*]		Napin proportion (%)		Phytic acid content (%)	
	20 °C**	55 °C	20 °C**	55 °C	20 °C	55 °C
Uncoded regression coefficients						
β_0	-1.48	72.34	-0.48	73.14	-5.04	11.43
β_1	-0.07	-3.53	0.30	14.02	6.87	-4.36
β_2	-1.32	-127.4	-	-	-2.99	13.11
β_{11}	-	-	-0.11	-2.08	-1.19	0.67
β_{22}	-	-	-	-	-	-
β_{12}	0.54	55.90	-	-	-	-5.58
Statistic parameters						
R^2	0.847	0.840	0.940	0.965	0.836	0.827
Q2	0.563	0.566	0.896	0.944	0.689	0.612
Reproducibility	0.852	0.723	0.982	0.929	0.776	0.805
p-value of F-test	0.003	0.004	<0.001	<0.001	0.001	<0.001
Lack of fit	0.418	0.654	0.173	0.793	0.531	0.442

754 *assuming a mean value of total napin content in crude reapseed meal of 35% as decribed by von der Haar et al.
755 (2014) and Wanasundara (2011)

756 **negative log transformed response: $-10\log(100-y)$

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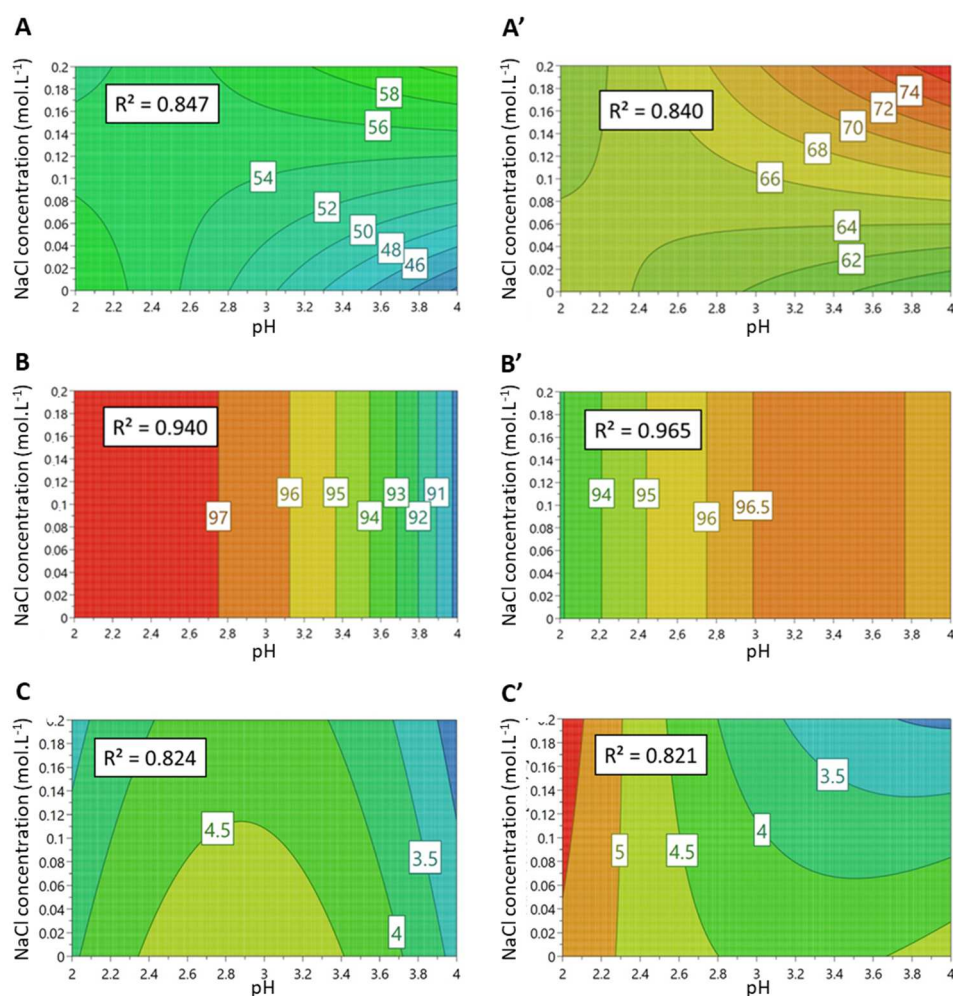


Fig. 1. Response surfaces of the napin extraction yield at 20 °C (A) and 55 °C (A'); the proportion of napins in relation to the total proteins in extract at 20 °C (B) and 55 °C (B') and the phytic acid content in the solid residue after napin extraction at 20 °C (C) and 55 °C (C').

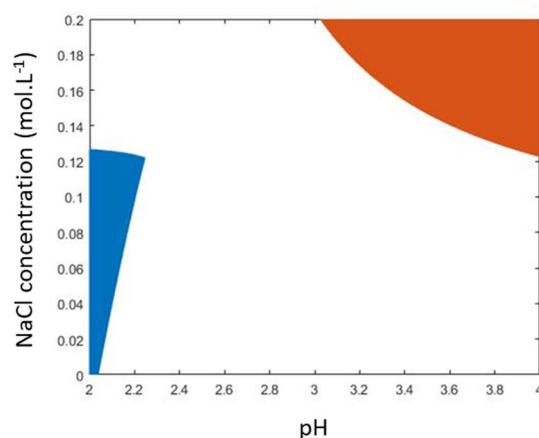


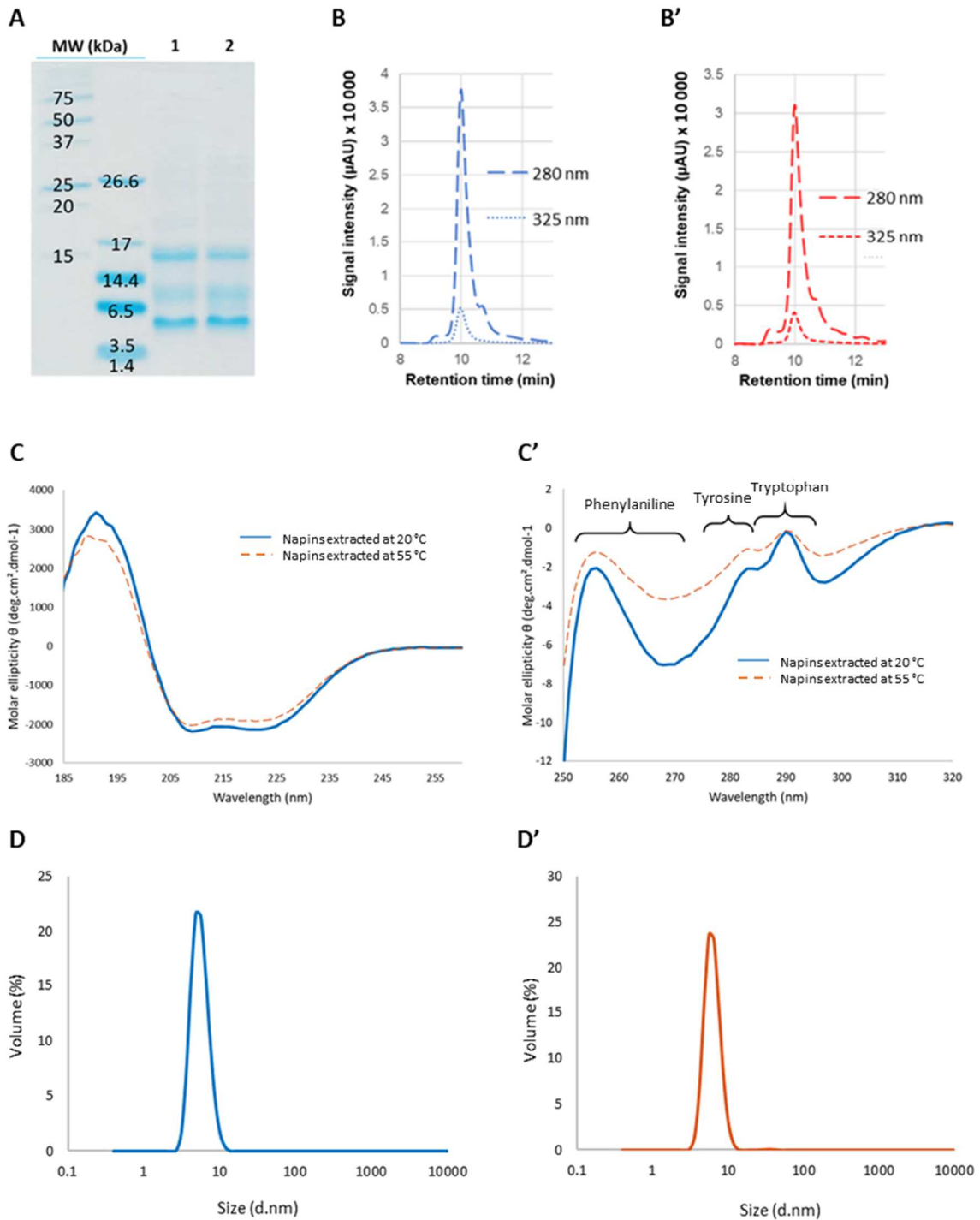
Fig. 2. Sets of the optimal conditions (pH/NaCl concentration) for selective napin extraction. The blue zone corresponds to extraction at 20 °C (constraints: napin extraction yield > 55%, napin proportion in extract > 95% and phytic acid content in the solid residue < 4% on dry matter basis). The orange zone corresponds to extraction at 55 °C (constraints: napin extraction yield > 70%, napin proportion in extract > 95% and phytic acid content in the solid residue < 4% on dry matter basis).

770 **Table 2.** Aminograms of both napin isolates produced in the optimal conditions at 20 and 55 °C compared
 771 to the recommendations of World Health Organization.

Amino acid	Content (mg.g ⁻¹ of protein)		
	Napin isolate extracted at 20 °C	Napin isolate extracted at 55 °C	FAO/WHO/UNU recommendation
	Essential amino acids		
Cysteine	39.0	35.0	6
Histidine	33.9	32.9	15
Isoleucine	28.1	27.7	30
Leucine	58.2	55.6	59
Lysine	74.8	73.6	45
Methionine	19.2	18.3	16
Phenylalanine + Tyrosine	41.8	41.7	38
Threonine	31.3	34.2	23
Tryptophan	10.7	10.4	6
Valine	40.3	39.9	39
	Nonessential amino acids		
Alanine	38.0	38.4	-
Arginine	55.5	56.2	-
Aspartic acid	27.8	35.0	-
Glutamic acid	242.0	230.0	-
Glycine	44.1	45.6	-
Hydroxyproline	1.18	1.98	-
Proline	79.8	74.4	-
Serine	35.3	36.3	-

772

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774

775 **Fig. 3.** SDS-PAGE gel under reducing conditions of the molecular weight markers (MW), the napin isolate
 776 extracted at 20 °C (lane 1) and the one at 55 °C (lane 2) (**A**).SE-HPLC chromatograms of the napin isolates
 777 extracted at 20 °C (**B**) and 55 °C (**B'**) recorded at 280 and 325 nm. Far-UV (**C**) and near-UV CD (**C'**) spectra of
 778 both napin isolates at pH 7. The volume size distribution of napin isolates extracted at 20 °C (**D**) and 55 °C
 779 (**D'**) analyzed by DLS at pH 7.

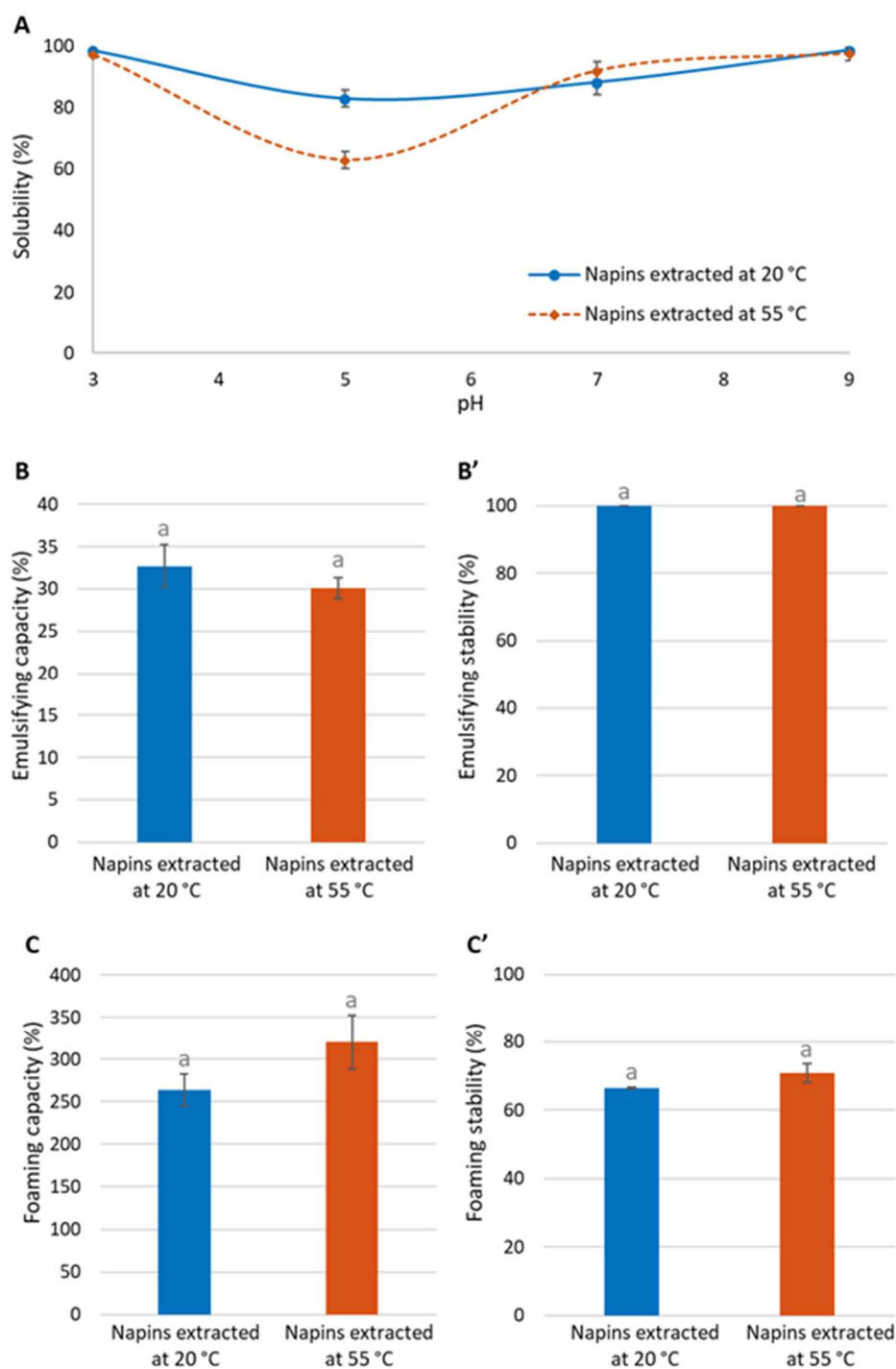


Fig. 4. Functional properties of the napins extracted in the optimal conditions at 20 °C (blue) and 55 °C (orange): solubility as a function of pH 3-9 (A), emulsifying capacity (B) and stability (B'), foaming capacity (C) and stability after 120 min (C'). Within each set of measurements, means with different superscripts are significantly different (p -value ≤ 0.05).