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1 **Optimization of large-scale purification of omega gliadins and other wheat gliadins**

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17 **Abstract**

18 Among wheat storage proteins, omega-gliadins display a singular amino acid sequence only
19 composed by repetitive sequences. They have been described as a major wheat allergen and
20 also contain T-cell epitopes implicated in coeliac disease. To study the structural and
21 biophysical properties of omega-gliadins and other gliadins (e.g., gluten network formation,
22 allergic response), highly purified and concentrated fractions are needed. In the present work,
23 we used chromatography media screening to improve their fractionation. A S Ceramic Hyper
24 D (Pall) resin was selected for its ability to concentrate omega-gliadins in the first elution peaks.
25 Gamma- and beta-gliadins were then separated by hydrophobic interaction chromatography and
26 eluted with an ethanol gradient. Alpha-gliadins were purified by gel filtration. Each fraction
27 was characterized by electrophoresis and reverse phase HPLC. The developed preparative
28 protocol enabled the purification of several grams of highly purified gliadins to the detriment
29 of the yield.

30

31 **Keywords**

32 Wheat protein fractionation, gluten proteins, omega-gliadins, cation exchange chromatography

33

34 1. INTRODUCTION

35 Wheat is overwhelmingly present in the diets of Western civilizations. Unfortunately, wheat-
36 derived products are also responsible for adverse reactions such as coeliac disease and food
37 allergies. The largest number of allergenic plant proteins is found among the prolamin super-
38 family, which notably contains cereal storage proteins such as gliadins (Tatham and Shewry,
39 2008). Gliadins are monomeric proteins characterized by their solubility in alcohol-water
40 media, with molecular weights (MW) ranging from 30 to 45 kDa (major fraction) and up to 75
41 kDa (minor fraction) and isoelectric points (pI) from 5 to 8 (Shewry, 2019). Gliadins consist
42 of highly polymorphic polypeptides divided into four groups: alpha, beta, gamma, and omega,
43 according to their decreasing electrophoretic mobility at low pH. Because of their molecular
44 weight, their low sulfur content and their particular amino acid sequence based on only
45 repetitive motives, omega-gliadin differ from other gliadins (Shewry, 2019). According to their
46 N-terminal and repetitive sequences, to their elution order in reverse phase high performance
47 liquid chromatography (RP-HPLC) and to their electrophoretic mobility, they are separated into
48 2 subgroups: omega 5-gliadin, also called fast omega-gliadins (eluted first in RP-HPLC), and
49 omega 1,2-gliadins, slow omega-gliadins (eluted second in RP-HPLC) (Schalk et al., 2017; van
50 Eckert et al., 2006). Omega 5 gliadins have been reported as a major allergen in Wheat-
51 Dependent Exercise-Induced Anaphylaxis (WDEIA) (Morita et al., 2003), while omega 1,2
52 gliadins have been identified as one of the dominant allergens in allergic reactions to
53 deamidated gluten (Denery-Papini et al., 2012). Gliadins are the main triggers of coeliac
54 disease. In particular, T-cells from coeliac patients recognize epitopes containing the repetitive
55 sequences present in omega-gliadins (Sollid et al., 2012). Yet, omega-gliadins are only a minor
56 group among prolamins, amount-wise, and have been until recently less well characterized than
57 other prolamin classes for their polymorphism and contribution to gluten properties (Altenbach
58 et al., 2018).

59 The storage proteins of wheat grains have the unique property of forming a viscoelastic network
60 called gluten, which is essential for the preparation of a wide diversity of wheat-based foods.
61 Gliadins are essential in the formation of the gluten network in combination with glutenins: in
62 dough, hydrated gliadins are commonly thought to contribute to the viscosity and extensibility
63 of the network (Cornec et al., 1994; Shewry, 2019). Baking quality has been correlated
64 positively with the contents in glutenin polymeric proteins and omega-gliadin (Malalgoda et
65 al., 2018). The repeated sequences present in omega-gliadins and glutenins are believed to form
66 inter-protein hydrogen bonds resulting in the particular viscoelastic properties of the gluten
67 network (Belton, 1999). Besides, omega-gliadins were recently found to form supramolecular
68 assemblies with glutenin through non-covalent interactions (Morel et al., 2020).

69 To study the structural, biophysical or immunochemical properties of omega-gliadins, highly
70 purified and concentrated fractions are needed. Gliadins comprise several groups of proteins
71 encoded by multigene families responsible for heterogeneity within each group. This
72 complexity and the variable amount of each gliadin within wheat varieties makes their
73 fractionation and study difficult. For example, the proportion of omega-gliadin may vary from
74 10 to 20 % of the total gliadins as recently reviewed by Shewry (Shewry, 2019). Their
75 proportion is increased under increased nitrogen availability or sulfur deficiency in the
76 cultivation conditions (Wan et al., 2014). Several methods have been described to obtain more
77 or less purified gliadins but, generally, they yielded fractions comprising several components
78 or only small quantities of individual proteins.

79 Recombinant proteins or peptides have been produced to study the role of the immune-
80 dominant part of gliadins (Denery-Papini et al., 2000; Mameri et al., 2015; Matsuo et al., 2005;
81 Tamás and Shewry, 2006). Such a strategy ensures the authenticity of the expressed proteins
82 but masks the heterogeneity of the group and yields only low amounts of proteins. Moreover,

83 differences may be observed between purified and recombinant gliadin fractions in their
84 secondary structure contents and antigenic activities (Mameri et al., 2015).

85 Classically, purification of gliadins starts with obtaining gluten by extensive washing of
86 hydrated flour with water followed by successive extraction according to Osborne fractionation
87 (Marsh et al., 2003; Schalk et al., 2017). These conditions of extraction separate gliadins from
88 glutenins, even though gliadin fractions may still contain traces of glutenins (Shewry, 2019).

89 Usually, omega-gliadins were fractionated with a combination of cation exchange
90 chromatography at acidic pH (Charbonnier, 1974). All cation exchange chromatography resins
91 are effective in separating omega-gliadins from other groups of gliadins (Charbonnier and
92 Mossé, 1980; Larre et al., 1991; Patey and Evans, 1973; Popineau et al., 1986). A low salt
93 concentration makes it possible to selectively elute omega-gliadins. They are thus obtained in
94 the first eluted fractions, but many authors noted that some of these omega-gliadins were eluted
95 later, thus scattering the omega fraction and contaminating the other fractions (Charbonnier and
96 Mossé, 1980; Larré et al., 1991). The yield is often not specified in literature but is generally
97 around 60 % for all gliadins groups corresponding to a few hundreds mg for omega-gliadins
98 (Patey and Evans, 1973; Schalk et al., 2017). To produce more than one gram of pure omega-
99 gliadins, the fractionation should run over several hours up to several days when large gliadin
100 amounts are treated (Patey and Evans, 1973). A regeneration step in harsh conditions is
101 necessary to eliminate proteins clogged onto the column to keep a good yield and
102 reproducibility (Larre et al., 1991; Popineau et al., 1986).

103 This work aims to identify a cation exchange chromatography medium able to separate omega-
104 gliadins from other gliadins, with a high yield. Different chromatography media were
105 compared: strong (sulfoethyl, S and sulfopropyl, SP) and weak cation (carboxymethyl, CM)
106 exchanger resins and the following parameters were explored: resolution, yield and duration.
107 Several chemical features for the support matrix were tested to evaluate possible interactions

108 with the resin. A resin was selected and applied at a preparative scale to obtain highly purified
109 fractions. This procedure helped us to purify omega-gliadins and all other gliadin groups for
110 future physico-chemical investigations.

111

112 **2. MATERIALS AND METHODS**

113 **2.1. Materials**

114 Bread wheat grains (7 kg) were obtained from INRAE, Le Rheu, France (cv. Récital harvested
115 in 2018) and were grounded into flour (5 kg) by Livrac (SA) (France). Protein content of wheat
116 flour were 13.6 % on a dry basis.

117

118 **2.2. Gliadins extraction**

119 The flour was hydrated, kneaded into a dough and washed with water using a Martin-type
120 process on a preparative scale (Popineau and Pineau, 1985). The recovered gluten was freeze-
121 dried, ground with a Waring blender and defatted with dichloromethane during 2 hours at room
122 temperature (gluten/solvent ratio (m/v) : 1/4). After filtration (Whatman filter paper n°3), two
123 rinsing steps with the same gluten/solvent ratio were performed. Then, the solvent was
124 evaporated under vacuum and defatted gluten was freeze-dried.

125 Total gliadins were extracted from dried gluten powder (514 g) with ethanol 70% (v/v)
126 (gluten/solvent ratio (m/v): 1/6) during 3 hours with a propeller mixer at 850 rpm. The
127 suspension was centrifuged at 10 000 g during for 30 min (Avanti J-26 XP, Beckman Coulter)
128 to pellet glutenins not soluble in alcohol. The pellet was discarded and the supernatant was
129 extensively dialyzed against water and then acetic acid 0.01 M and freeze-dried. It contained
130 total gliadins and glutenins in a small amount.

131

132 **2.3. Chromatography media screening**

133 Different ion exchange chromatography resins were tested under fixed conditions: Toyopearl
134 CM 650 M (Tosoh), Cellufine CM C500 (Amicon), CM Ceramic hyper D, S Ceramic Hyper D
135 and SP Trisacryl M (Pall). Each resin (30 mL) was packed in XK 16/15 support following the
136 manufacturer's instructions. Gliadins (1 g) were dispersed in 50 mL buffer (Na lactate 0.01 M
137 pH 3.6, urea 2 M) overnight on a rotary shaker. The extract was centrifuged 30 min at 20 000

138 g (SR 12.22, Jouan Centrifuge, France) and 5 mL supernatant were injected onto the column at
139 1 mL/min. First, the column was equilibrated with the extraction buffer and washed with 60
140 mL (corresponding to twice the column volume). Proteins were eluted in a 420ml gradient from
141 0 to 30% of the same buffer pH 3.6 with NaCl 0.5 M using a total volume corresponding to 14
142 times the column volume. Protein absorbance was recorded at 280 nm. Fractions of 5 mL were
143 collected and analyzed with polyacrylamide gel electrophoresis.

144

145 **2.4. Preparative fractionation**

146 Total gliadins (10 g) per run were dissolved in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M) and
147 stirred for 2 hours at room temperature on a rotary shaker. They were then centrifuged for 30
148 min at 17 000 g (Beckam, Avanti JSP26, rotor JSP F500). The supernatant was injected on an
149 S Ceramic Hyper D column (5 x 15 cm) using a preparative purification system (Pharmacia
150 biotech, Sweden). The column was first equilibrated with buffer (Na lactate 0.01 M, pH 3.6,
151 urea 2 M) and gliadins were eluted with a step gradient of NaCl 0.5 M. The absorbance of
152 proteins was recorded at 280 nm. This chromatography step allowed for separating the different
153 groups of gliadins. Omega-gliadins were eluted in the first peaks. They were dialyzed first in
154 water, then in acetic acid 0.01 M and freeze-dried.

155 Eluted fractions containing alpha gliadins (2 g) were purified on a Sephacryl S100 HR column
156 (5 x 100 cm) equilibrated in buffer (Na lactate 0.01 M, pH 3.6, urea 0.5 M). Different fractions
157 were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

158 The other eluted fractions containing beta and gamma gliadins (10 g) were loaded on SP
159 Sepharose FF (5 x 15cm) in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M). Proteins were eluted
160 using a gradient from 0 to 25 % of the same buffer with NaCl 0.5 M. Different peaks were
161 pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried. This

162 chromatography step separated gamma- 44 and 46 gliadins forms, and removed traces
163 of alpha/beta gliadins.

164 Gamma gliadins were last purified on a phenyl sepharose FF column (5 x 15cm). The column
165 was equilibrated in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M), then the sample (1.5 g)
166 dissolved in the same buffer was injected. The column was washed with buffer (ammonia 0.02
167 M pH 11), and proteins were eluted in a gradient from 0 to 100 % of the same buffer with
168 ethanol 70 % (v/v). Different fractions were pooled, dialyzed first in water, then in acetic acid
169 0.01 M and freeze-dried.

170 Beta gliadins were purified from the flowthrough peak of phenyl sepharose step. Different
171 fractions were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

172

173 **2.5. Analytical characterization**

174 **2.5.1. Determination of water, ash, and protein contents**

175 Prior to analysis, protein powders were stored at 20 °C in a desiccator containing K₂CO₃
176 saturated salt to ensure constant moisture content. The water content was measured on
177 equilibrated powders using thermogravimetric analysis under nitrogen atmosphere (TGA 2050,
178 TA-Instrument). About 10 mg of powder were heated from 20 to 105 °C at 3 K/min and at 105
179 °C for at least 80 min until constant weight was achieved.

180 Protein content was determined according to the Dumas method with an elemental analyzer for
181 nitrogen (Vario Micro Cube, Elementar, Frankfurt, Germany) with nitrogen-to-protein
182 conversion factor of 5.4 as recommended for wheat proteins (Mariotti et al., 2008).

183

184 **2.5.2. Polyacrylamide gel electrophoresis at acid pH (A-PAGE)**

185 Gliadins were separated onto acid polyacrylamide gel according to the method described by
186 Morel (1994). Briefly protein solutions were loaded onto a 12 % polyacrylamide gel composed

187 of urea 2 M, ascorbic acid 0.1 %, ferrous sulfate 7 H₂O 0.0014% (w/v) and acetic acid 1 N 13
188 %, pH 3.1 polymerized by 60 µL H₂O₂ 0.6 % (v/v). Migration in acetic acid buffer pH 3.1 was
189 run during 1 h 40 under 250 V. Then gels were fixed in trichloroacetic acid 10 % and stained
190 with Coomassie Brilliant blue R250 0.4 % (w/v).

191

192 **2.5.3. Polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS-PAGE)**

193 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under
194 reducing conditions. Proteins were dissolved at 1 mg/mL in a pH 6.8-buffer constituted of Tris
195 0.5 M, SDS 10%, glycerol 30%, β-mercaptoethanol 8% and bromophenol blue buffer. Samples
196 were incubated at room temperature overnight and heated at 95 °C for 5 min before being loaded
197 onto 4–12% Bis–Tris precast gels Novex Nupage™ (Invitrogen, Thermo Fisher Scientific)
198 with MES SDS running buffer (Invitrogen, Thermo Fisher Scientific) and Recombinant
199 Precision Plus unstained protein standards (Biorad). Electrophoresis was performed at constant
200 voltage (150 V) and the gels were stained with a colloidal Coomassie blue solution. Gels were
201 washed with water and scanned.

202

203 **2.5.4. RP-HPLC**

204 Purity of gliadin fractions was probed by RP-HPLC (Bietz and Simpson, 1992). Briefly, 120
205 µg of proteins were dissolved in acetonitrile 25 % TFA 0.1 % and injected onto a C18 analytical
206 column (Nucleosil C18, 4 x 250 mm, 5µm, 300 Å). They were separated at 50 °C using a
207 gradient running from acetonitrile 25 % TFA 0.1 % to acetonitrile 55 % TFA 0.1 % during 70
208 min at 0.8 mL/min. Monitoring the protein was carried out using the absorbance at 214 nm.

209

210 **3. RESULTS AND DISCUSSION**

211 **3.1. Total gliadins extraction**

212 First, 514 g of gluten were obtained from the wheat dough prepared from 5 kg of flour
213 extensively washed with water to remove starch and water-soluble constituents. This process
214 concentrated proteins: the resulting dry gluten contained 73 % proteins of the flour and
215 corresponds to 10.2 % of the flour and 79% of the flour proteins. This quantitative value was
216 in good agreement with the isolation protocol from Popineau et al. (1985) which yielded
217 routinely 10.8 % of gluten from flour. Then, gliadins were separated from glutenins following
218 Osborne fractionation by solubilisation in 70% v/v ethanol. 84 g of total gliadins from 5 kg of
219 flour were produced with 86 % protein content. The extracted gliadins represent 1.7 % of the
220 initial flour. In comparison with the yield of 2.8% for the preparation of Prolamin Working
221 Group (PWG) gliadin reference (van Eckert et al., 2006), a large amount of gliadins may be
222 lost in the pellet during alcohol extraction or the initial gliadin content of our flour was lower.
223 The total gliadins (TG) extract was composed by the four types of gliadins : alpha-, beta-,
224 gamma-, omega-gliadins and small amount of glutenins as revealed by electrophoresis at low
225 pH (Figure 1 A, B, C, D bottom, lines TG).

226

227 **3.2. Chromatography media screening**

228 The different groups of gliadins were fractionated by cation exchange chromatography
229 according to their charge at acidic pH (pH 3.6) under denaturing conditions (urea 2 M). Five
230 different cation exchanger chromatography resins varying in their electrostatic and chemical
231 characteristics were tested under the same conditions to separate the different groups of wheat
232 gliadins. The fractionation of omega-gliadin and the loading capacity were compared for each
233 resin.

234 Peak area is used to evaluate the protein amount bound to the resin. The largest area under the
235 peaks eluted between between 0 and 600 min was obtained with with Toyopearl CM 650 M, S
236 Ceramic Hyper D and Cellufine C-500 resin, respectively 36, 34 and 34 AU*min. CM Ceramic

237 Hyper D and SP Trisacryl M had the smallest peak area (respectively 27 and 30 AU*min),
238 indicating that they bind fewer proteins than the other resins. Moreover, CM Ceramic Hyper D
239 did not lead to any gliadin separation (Figure 1A). All gliadins were eluted in the first unbound
240 peak during the initial washing step and few proteins were eluted by the NaCl gradient. This
241 resin was therefore eliminated. SP Trisacryl M (Figure S1) having the smallest peaks area under
242 curve was not retained.

243 Omega-gliadins were eluted in the first peaks of the chromatograms. They were either unbound
244 or eluted at the very beginning of the gradient, which is in line with other studies (Patey and
245 Evans, 1973; Popineau et al., 1986). Then, the salt gradient induced the joint elution of gamma
246 and beta-gliadins, followed by alpha-gliadins (Figure 1B, C, D). Only the resin S Ceramic
247 Hyper D (Figure 1B, fractions 2 - 40) allowed for a clear split of the omega gliadins from other
248 gliadins, all of them were eluted before 7 mS/cm conductivity in three separated peaks. The
249 well-resolved peaks containing omega-gliadins for Cellufine C500 (Figure 1C fractions 4 - 6)
250 and for Toyopearl CM 650 M (Figure 1D fractions 4 - 6) were smaller than for S Ceramic Hyper
251 D. Furthermore, it can be noticed that omega-gliadins were not separated (Figure 1C, fractions
252 25 - 29) and partly co-eluted with gamma gliadins with Cellufine C500 (Figure 1C, fractions
253 33 - 35). The same observation applies to Toyopearl CM 650 M (Figure 1D, fractions 29 - 35).
254 Since S Ceramic Hyper D (Figure 1 B) had a good loading capacity (34 AU*min) and
255 concentrated omega-gliadins in the first peaks of elution, this resin was selected.

256 Omega-gliadins are mainly composed of glutamine (40 - 50 % mol) and proline (20 - 30% mol)
257 (Shewry, 2019). Therefore, they have few charges on their surface, and their binding capacity
258 to cation exchanger resin is expected to be low. S Ceramic Hyper D has particular properties:
259 it is a hybrid resin called “gel-in-a-shell”. The functionalized hydrogel is entrapped in a rigid
260 ceramic bead (Boschetti, 1994). Large pores at the surface of the beads allow proteins to diffuse
261 rapidly and bind to the hydrogel throughout its volume, not just on the surface (Rendueles De

262 La Vega et al., 1998). The pore size of S Ceramic Hyper D is the largest among the tested resins
263 (Table 1). Its binding sites would be more accessible than in the other resins, thus improving
264 the sorption capacity. Moreover, omega-gliadins could have a compact structure even in 8 M
265 urea while the other gliadins are unfolded in 2 M urea (Paananen et al., 2006), it could be
266 speculated that omega-gliadins could diffuse faster into the ceramic bead. Altogether, this could
267 explain the better performance of S Ceramic Hyper D resin.

268

269 **3.3. Preparative fractionation**

270 Following the analytical screening, the S Ceramic Hyper D column was retained for the first
271 step of gliadin fractionation to reach a good separation of omega-gliadins. The process scaled-
272 up involved the same extraction and purification buffers as at analytical scale. The general
273 purification process is presented in Figure 2 A. S Ceramic Hyper D could fractionate roughly
274 the four gliadins groups, and concentrate omega-gliadins in the first peaks of elution with the
275 same efficiency as in the optimization step. At preparative scale, a stepwise elution of increasing
276 NaCl concentration was preferred to speed up the separation time and reduce buffer
277 consumption while retaining the required purity level. An extension of the washing step at 5%
278 of elution buffer corresponding to 25 mM NaCl was necessary to avoid contamination of gliadin
279 peaks by omega-gliadins. Two fractions of omega-gliadins were produced according to the salt
280 elution gradient. They contained both a mixture of omega 5- and omega 1,2-gliadins. The
281 fraction of omega-gliadins eluted with 25 mM NaCl was further called “Omega NaCl25” and
282 the fraction eluted with 50 mM NaCl “Omega NaCl50”. Then gamma and beta gliadins were
283 eluted with 100 mM NaCl and finally alpha-gliadins and traces of glutenins with 500 mM NaCl
284 (Figure 2 B). The preparative fractionation allowed to produce very concentrated protein
285 fractions with a protein content greater than 92% for each gliadin fraction (Table 2).

286 Eighty four grams of total gliadins were applied to the column in nine runs. A wash with NaOH
287 1 M was necessary after five runs to avoid loss of capacity. Omega-gliadin recovery including
288 chromatography, dialysis and freeze-drying was 1.5 g of Omega NaCl25 and 3 g of Omega
289 NaCl50 (Table 2). This recovery was in accordance with large scale fractionation of gliadins
290 which produced from 4 g to 6 g of omega-gliadins from a greater amount of total gliadins (Patey
291 and Evans, 1973; Popineau et al., 1986). It was noticed that omega gliadin powders were highly
292 sensitive to water content. A change in relative humidity induced a solid-to-liquid transition at
293 ambient temperature in a few hours after removal from the freeze dryer. To avoid such a
294 transition, powders were stored in a desiccator containing K_2CO_3 saturated salt to avoid an
295 increase of moisture content during storage. Such a transition was not observed with other
296 gliadin powders.

297 The next separation step was another cation exchanger chromatography (Figure 2B). A SP
298 sepharose FF resin had previously been shown to fractionate different isoforms of gamma
299 gliadins according to charge (Popineau and Pineau, 1985). The fraction obtained from the S
300 Ceramic Hyper D column, containing gamma and beta gliadins, was injected onto SP FF with
301 the same buffers as the first step in four runs. A NaCl gradient from 25 mM to 125 mM was
302 used to separate gamma 46 and gamma 44 (Figure 2B). These isoforms could be detected in A-
303 PAGE and correspond to the two major bands at the level of gamma-gliadin migration in the
304 total gliadin lane (Figure 1B). They were not well separated with S Ceramic Hyper D column
305 because of step elution. Gamma and beta gliadins were separated by hydrophobic interaction
306 chromatography (HIC) with a Phenyl Sepharose FF column and eluted with an ethanol gradient
307 (Popineau and Pineau, 1985). Briefly, the whole gamma fraction solubilized in urea lactate
308 buffer was injected on the Phenyl sepharose FF column, then was eluted by a linear gradient of
309 ethanol in ammonia buffer pH 11. Beta-gliadins were eluted by 10 % (v/v) ethanol and gamma
310 46 and 44 were eluted respectively with 39% and 46% (v/v) ethanol (Figure 2B). They were

311 separated according to their surface hydrophobicity. A similar elution pattern was observed in
312 literature, confirming that separation by HIC is highly reproducible (Popineau and Pineau,
313 1985). The gamma gliadin recovery was 1.6 g of gamma 46, 0.6 g of gamma 44 and 0.2 g of
314 gamma 44 and gamma 46 mixture (Table 2). Three grams of beta-gliadin were purified (Table
315 2). The overall recovery for gamma and beta was 3 and 4 % respectively. Such very low
316 recovery, which are encountered when targeting fractions with high purity (Figure 3 - 4) were
317 due to the numerous steps and a technical problem with one batch at the freeze-drying step
318 which led to losses. From batch to batch, the mean recovery for HIC was around 65% which
319 gave a final yield of 7 %.

320 Alpha-gliadins were last purified by gel filtration. They represent the major protein in total
321 gliadins. Whole alpha gliadins were collected in the first chromatographic step and
322 corresponded to the last peak eluted with 500 mM NaCl. This fraction contained traces of
323 glutenins eliminated by size exclusion chromatography (Charbonier and Mossé, 1980; Patey
324 and Evans, 1973). Glutenins are composed of sub-units stabilized by disulphide bonds. Their
325 apparent molecular weight is much larger than that of gliadins in non-reducing conditions
326 (Shewry, 2019). The sephacryl S100-HR column had a separation range adapted to the size of
327 the targeted proteins, and was equilibrated with the same buffers as in the first step. Glutenins
328 were excluded from the gel (27 % peak area at 280 nm) and alpha-gliadins were eluted at elution
329 volume of 1000 mL in the main peak (62% peak area at 280 nm) (Figure 2B). More than 4 g of
330 alpha gliadin were purified (Table 2) with a final recovery of 11 %. Only 11.8 g over 22 g of
331 fraction was purified by Sephacryl S100-HR. This purification step had a 40 % yield. If the
332 entire fraction containing alpha-gliadins had been processed, 9 g of alpha could have been
333 purified in total.

334 The overall procedure was rather long but highly purified fractions (over 85% for each gliadin)
335 were recovered in the gram-range. Due to the purification step series, the final recovery was

336 relatively low (< 25%). Protein losses may also be due to protein bonding onto the columns.
337 Indeed, regular cleaning of the columns with sodium hydroxide was necessary to keep
338 reproducible results.

339

340 **3.4. Analysis of the purified fractions**

341 The purity of each gliadin fraction was evaluated by electrophoresis and analytical reversed
342 phase HPLC. In SDS-PAGE, proteins are only separated according to their size, as negatively
343 charged SDS masks the protein charges. The characteristic bands for each gliadin are expected
344 at the corresponding MW ranges of 60 000-80 000 Da for omega-gliadins, and 32 000 to 45
345 000 Da for alpha, beta and gamma-gliadins (Shewry, 2019). In the present work, purified
346 gliadins showed a typical electrophoretic profile with only one band for highly pure proteins.
347 Omega-gliadins showed one band at 70 kDa in SDS-PAGE (Figure 3A). Omega-gliadins
348 showed a weak Coomassie staining, probably due to the repetitive pattern of their sequence and
349 lack of basic amino acids as reviewed by Shewry (Shewry, 2019). Beta-gliadins (Figure 3A)
350 and Gamma (Figure 3A) migrated around 40 kDa. Alpha-gliadins (Figure 3A) had an intense
351 band at 37 kDa with minor bands at lower MW. These bands, around 10 – 15 kDa, could
352 correspond to alpha-amylase/trypsin inhibitors (ATIs) (Shewry, 2019).

353 Gliadins could be subclassified as omega, gamma, beta and alpha based on increasing mobility
354 in electrophoresis at acidic pH (Shewry, 2019). In A-PAGE, proteins are separated according
355 to their molecular weight and their apparent charge at pH below 3 (Bietz and Simpson, 1992).
356 Migration profiles showed characteristic bands for each gliadin according to the expected
357 profiles, with only traces of contaminants (Figure 3B) (Bietz and Simpson, 1992). Omega
358 NaCl25 fraction showed two bands with low electrophoretic mobility in A-PAGE
359 corresponding to omega1,2-gliadins, whereas Omega NaCl50 fraction presented three bands
360 corresponding to a mixture of omega1,2 and omega5-gliadins (Figure 3B). Gamma-gliadins

361 (Figure 3B) had a similar profile. They migrated to the middle of the gel with an intense band
362 and another weak band just above, meaning that these fractions had homogeneous charges. The
363 beta-gliadin fraction (Figure 3B) migrated between gamma- and alpha-gliadins. Alpha-gliadins
364 (Figure 3B) migrated to the migration front with several bands. It could be different isoforms
365 of proteins (Mameri et al., 2015).

366 Analytical C18 columns have also been widely used to characterize gluten proteins since 1980's
367 (Bietz and Simpson, 1992). Proteins are separated by surface hydrophobicity and are eluted
368 with an acetonitrile gradient. Figure 4 presents the elution pattern of purified gliadins on C18
369 column. Each fraction was eluted in a single major peak which highlights a high protein purity
370 rarely obtained because of gliadin polymorphism. Omega5-gliadins were eluted between 30
371 min and 35 min, omega1,2-gliadins around 38 min (Figure 4). Both omega-gliadin fractions are
372 composed of a mixture of omega5 and omega1,2. Based on the respective peak areas, Omega
373 NaCl25 fraction contained 71% of omega1,2 and 29% of omega5-gliadin, while Omega NaCl50
374 fraction contained 69% of omega1,2 and 31% of omega5-gliadin . When both types of omega
375 1,2 and omega 5 need to be separated from the omega-gliadin fraction, preparative RP-HPLC
376 is the most appropriate technique. Beta gliadins were eluted around 42 min (Figure 4) in a major
377 peak, which confirms the purity (88 % of total peak area). Only a slight peak was visible at 45
378 min, which corresponds to gamma gliadins and represents 7 % of the fraction. Alpha-gliadins
379 were eluted between 44 min and 49 min (Figure 4). Two main peaks were eluted at 46 min and
380 47.5 min (76% peak area) meaning that alpha-gliadins do not have a homogeneous composition
381 in terms of polarity. Gamma44-gliadins (Figure 4) were eluted at 50 min (93 % peak area).
382 Gamma46-gliadins (Figure 4) were eluted at 51 min (92 % peak area) and a more polar fraction
383 is eluted at 59 min (8 % peak area). These gamma-gliadin fractions are highly pure.

384

385 4. CONCLUSION

386 The developed preparative protocol enabled the purification of several grams of highly purified
387 gliadins. Analytical techniques confirmed that each gliadin fraction was purified near
388 electrophoretic and HPLC homogeneity. Gliadins with high purity at the level of grams are
389 rarely obtained because of the considerable polymorphism of gliadins. We choose a S Ceramic
390 Hyper D (Pall) column for its ability to concentrate omega-gliadins in the first elution peaks
391 and to separate different groups of gliadins. Its specific chemistry “gel in a-shell” could explain
392 the improved resolution of this resin compared to the other tested. The mass transfer mechanism
393 in Ceramic hyper D resin is described as intraparticle convection rather than diffusion which
394 allows larger proteins as omega gliadins to enter the pores of the gel.

395

396 **ABBREVIATIONS**

397 A-PAGE, acidic polyacrylamide gel electrophoresis

398 CM, carboxy methyl

399 CV coefficient variation

400 FF fast flow

401 HIC hydrophobic interaction chromatography

402 HMW high molecular weight

403 HPLC, high performance liquid chromatography,

404 RP, reversed phase,

405 RP-HPLC reversed phase high performance liquid chromatography

406 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

407 SP sulfo propyl

408 TFA, trifluoroacetic acid,

409 TG total gliadin

410 TGA thermogravimetric analysis

411 UV ultraviolet

412

413 **DECLARATION OF COMPETING INTEREST**

414 The authors declare to have no conflicts of interest.

415

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421

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425

426 **AUTHORS CONTRIBUTIONS**

427 **Véronique Solé-Jamault:** conceptualization, formal analysis, original draft, review &
428 editing; **Joëlle Davy:** investigation; **Rémy Cochereau:** investigation review & editing;
429 **Adeline Boire:** supervision, funding acquisition review & editing; **Colette Larré:**
430 supervision, review & editing; **Sandra Denery-Papini:** supervision, review & editing

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432 **REFERENCES**

- 433 Altenbach, S.B., Chang, H.C., Simon-Buss, A., Jang, Y.R., Denery-Papini, S., Pineau, F., Gu,
434 Y.Q., Huo, N., Lim, S.H., Kang, C.S., Lee, J.Y., 2018. Towards reducing the immunogenic
435 potential of wheat flour: Omega gliadins encoded by the D genome of hexaploid wheat may
436 also harbor epitopes for the serious food allergy WDEIA. *BMC Plant Biol.* 18, 291.
437 <https://doi.org/10.1186/s12870-018-1506-z>
- 438 Belton, P.S., 1999. Mini Review: On the Elasticity of Wheat Gluten. *J. Cereal Sci.* 29, 103–
439 107. <https://doi.org/10.1006/jcrs.1998.0227>
- 440 Bietz, J.A., Simpson, D.G., 1992. Electrophoresis and chromatography of wheat proteins:
441 available methods, and procedures for statistical evaluation of the data. *J. Chromatogr. A* 624,
442 53–80. [https://doi.org/10.1016/0021-9673\(92\)85674-I](https://doi.org/10.1016/0021-9673(92)85674-I)
- 443 Boschetti, E., 1994. Advanced sorbents for preparative protein separation purposes. *J.*
444 *Chromatogr. A*. [https://doi.org/10.1016/0021-9673\(94\)80017-0](https://doi.org/10.1016/0021-9673(94)80017-0)
- 445 Charbonier, L., Mossé, J., 1980. Large Scale Isolation of Gliadin Fractions. *J. Sci. Food Agric*
446 31, 54–61.
- 447 Charbonnier, L., 1974. Isolation and characterization of ω -gliadin fractions. *BBA - Protein*
448 *Struct.* 359, 142–151. [https://doi.org/10.1016/0005-2795\(74\)90139-1](https://doi.org/10.1016/0005-2795(74)90139-1)
- 449 Cornec, M., Popineau, Y., Lefebvre, J., 1994. Characterisation of Gluten Subfractions by SE-
450 HPLC and Dynamic Rheological Analysis in Shear. *J. Cereal Sci.* 19, 131–139.
451 <https://doi.org/10.1006/JCRS.1994.1018>
- 452 Denery-Papini, S., Bodinier, M., Larré, C., Brossard, C., Pineau, F., Triballeau, S., Pietri, M.,
453 Battais, F., Mothes, T., Paty, E., Moneret-Vautrin, D.-A., 2012. Allergy to deamidated gluten
454 in patients tolerant to wheat: specific epitopes linked to deamidation. *Allergy* 67.
455 <https://doi.org/10.1111/j.1398-9995.2012.02860.x>
- 456 Denery-Papini, S., Samson, M.F., Autran, J.C., 2000. Anti-Peptide Antibodies Directed

457 Against Omega-Gliadins for the Detection of Sequences from Bread and Durum Wheats.
458 Food Agric. Immunol. 12, 67–75. <https://doi.org/10.1080/09540100099634>

459 Larre, C., Popineau, Y., Loisel, W., 1991. Fractionation of gliadins from common wheat by
460 cation exchange FPLC. J. Cereal Sci. 14, 231–241. <https://doi.org/10.1016/S0733->
461 5210(09)80042-8

462 Malalgoda, M., Ohm, J.-B., Meinhardt, S., Simsek, S., 2018. Association between gluten
463 protein composition and breadmaking quality characteristics in historical and modern spring
464 wheat. Cereal Chem. 95, 226–238. <https://doi.org/10.1002/cche.10014>

465 Mameri, H., Brossard, C., Gaudin, J.-C., Gohon, Y., Paty, E., Beaudouin, E., Moneret-
466 Vautrin, D.-A., Drouet, M., Solé, V., Wien, F., Lupi, R., Larré, C., Snégaroff, J., Denery-
467 Papini, S., 2015. Structural Basis of IgE Binding to α - and γ -Gliadins: Contribution of
468 Disulfide Bonds and Repetitive and Nonrepetitive Domains. J. Agric. Food Chem. 63.
469 <https://doi.org/10.1021/acs.jafc.5b01922>

470 Mariotti, F., Tomé, D., Mirand, P.P., 2008. Converting nitrogen into protein - Beyond 6.25
471 and Jones' factors. Crit. Rev. Food Sci. Nutr. 48, 177–184.
472 <https://doi.org/10.1080/10408390701279749>

473 Marsh, M.N., Tatham, A.S., Gilbert, S.M., Fido, R.J., Shewry, P.R., 2003. Extraction,
474 Separation, and Purification of Wheat Gluten Proteins and Related Proteins of Barley, Rye,
475 and Oats, in: Celiac Disease. Humana Press, pp. 055–073. <https://doi.org/10.1385/1-59259->
476 082-9:055

477 Matsuo, H., Kohno, K., Morita, E., 2005. Molecular cloning, recombinant expression and
478 IgE-binding epitope of omega-5 gliadin, a major allergen in wheat-dependent exercise-
479 induced anaphylaxis. FEBS J. 272, 4431–4438. <https://doi.org/10.1111/j.1742->
480 4658.2005.04858.x

481 Morel, M., 1994. Acid-Polyacrylamide gel-electrophoresis of wheat glutenins – a new tool for

482 the separation of high and low-molecular-weight subunits. *Cereal Chem.* 71, 238–242.

483 Morel, M.H., Pincemaille, J., Chauveau, E., Louhichi, A., Violleau, F., Menut, P., Ramos, L.,
484 Banc, A., 2020. Insight into gluten structure in a mild chaotropic solvent by asymmetrical
485 flow field-flow fractionation (AsFIFFF) and evidence of non-covalent assemblies between
486 glutenin and ω -gliadin. *Food Hydrocoll.* 103. <https://doi.org/10.1016/j.foodhyd.2020.105676>

487 Morita, E., Matsuo, H., Mihara, S., Morimoto, K., Savage, A.W.J., Tatham, A.S., 2003. Fast
488 ω -gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis. *J. Dermatol.*
489 *Sci.* 33, 99–104. [https://doi.org/10.1016/S0923-1811\(03\)00156-7](https://doi.org/10.1016/S0923-1811(03)00156-7)

490 Paananen, A., Tappura, K., Tatham, A.S., Fido, R., Shewry, P.R., Miles, M., McMaster, T.J.,
491 2006. Nanomechanical force measurements of gliadin protein interactions. *Biopolymers* 83,
492 658–667. <https://doi.org/10.1002/bip.20603>

493 Patey, A.L., Evans, D.J., 1973. Large-Scale preparation of gliadin proteins. *J. Sci. Food*
494 *Agric.* 24, 1229–1233. <https://doi.org/10.1002/jsfa.2740241011>

495 Popineau, Y., Guerroué, J.L. 1., Pineau, F., 1986. Purification and characterisation of ω -
496 gliadin components from common wheat. *LWT - Food Sci. Technol.* 19, 266–271.

497 Popineau, Y., Pineau, F., 1985. Fractionation and characterisation of γ -Gliadins from bread
498 wheat. *J. Cereal Sci.* 3, 363–378. [https://doi.org/10.1016/S0733-5210\(85\)80009-6](https://doi.org/10.1016/S0733-5210(85)80009-6)

499 Rendueles De La Vega, M., Chenou, C., Loureiro, J.M., Rodrigues, A.E., 1998. Mass transfer
500 mechanisms in Hyper D media for chromatographic protein separation. *Biochem. Eng. J.* 1,
501 11–23. [https://doi.org/10.1016/S1369-703X\(97\)00003-X](https://doi.org/10.1016/S1369-703X(97)00003-X)

502 Schalk, K., Lexhaller, B., Koehler, P., Scherf, K.A., 2017. Isolation and characterization of
503 gluten protein types from wheat, rye, barley and oats for use as reference materials. *PLoS One*
504 12. <https://doi.org/10.1371/journal.pone.0172819>

505 Shewry, P., 2019. What is gluten—Why is it special? *Front. Nutr.*
506 <https://doi.org/10.3389/fnut.2019.00101>

507 Sollid, L.M., Qiao, S.W., Anderson, R.P., Gianfrani, C., Koning, F., 2012. Nomenclature and
508 listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules.
509 Immunogenetics 64, 455–460. <https://doi.org/10.1007/s00251-012-0599-z>

510 Tamás, L., Shewry, P.R., 2006. Heterologous expression and protein engineering of wheat
511 gluten proteins. J. Cereal Sci. <https://doi.org/10.1016/j.jcs.2006.02.001>

512 Tatham, A.S., Shewry, P.R., 2008. Allergens to wheat and related cereals. Clin. Exp. Allergy
513 38, 1712–1726. <https://doi.org/10.1111/j.1365-2222.2008.03101.x>

514 van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirido, F., Denery-Papini, S., Ellis, H.J.,
515 Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Méndez, E., Mothes, T., Novalin, S.,
516 Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., Wieser, H., 2006. Towards a new
517 gliadin reference material-isolation and characterisation. J. Cereal Sci. 43, 331–341.
518 <https://doi.org/10.1016/j.jcs.2005.12.009>

519 Wan, Y., Gritsch, C.S., Hawkesford, M.J., Shewry, P.R., 2014. Effects of nitrogen nutrition
520 on the synthesis and deposition of the ω -gliadins of wheat. Ann. Bot. 113, 607–615.
521 <https://doi.org/10.1093/aob/mct291>

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524 **Table 1 List of tested cation exchanger chromatography resins**

Functional group	Commercial name and manufacturer	Particle size μm	Pore size \AA	Resin chemistry
Carboxy methyl (CM)	Toyopearl CM 650 M, Tosoh	65	1000	hydroxylated methacrylic polymer
	Cellufine C-500, Chisso	53-125	/	cross-linked cellulose
	CM Ceramic Hyper D, Pall	50	2000	ceramic, gel-in-a-shell
	S Ceramic Hyper D, Pall	50	2000	ceramic, gel-in-a-shell
Sulfo ethyl (S)	S Ceramic Hyper D, Pall	50	2000	ceramic, gel-in-a-shell
SulfoPropyl (SP)	SP Trisacryl M, Pall	40-80	/	acrylic copolymer

525

526 **Table 2 Recoveries of purified gliadins at the end of the optimized process.**

Gliadins fraction	Protein recovered weight (g)	Protein content % (dry mass)	Chromatography steps number	Final yield (%) From 84 g of total gliadins treated
Omega NaCl25	1.5	92.3 \pm 1.3	1	2
Omega NaCl50	3.0	94.1 \pm 1.8	1	4
Gamma	2.4	93.4 \pm 1.3	3	3
Beta	3.0	96.6 \pm 1.0	3	4
Alpha	4.7 *	96.7 \pm 1.1	2	11

527 *. If the entire fraction containing alpha-gliadins had been processed, 9 g of alpha could have
 528 been purified in total

529 **LIST OF FIGURE CAPTIONS**

530

531 **Figure 1 Gliadins fractionation on (A) CM Ceramic Hyper D, (B) S Ceramic Hyper D (C)**
532 **Cellufine C 500 and (D) Toyopearl CM 650 M with associated A-PAGE analysis (bottom).**

533 Numbers above the peaks correspond to numbered lanes in A-PAGE. TG stands for total
534 gliadins. Fractions signalized by an arrow on the chromatogram ($\approx 10 \mu\text{g}$) were analyzed with
535 A-PAGE stained by Coomassie Brilliant blue. Chromatogram of SP Trisacryl M is presented
536 in supplementary S1.

537

538 **Figure 2 : Purification process and associated representative chromatograms**

539 A. Chromatography series are represented with operating conditions and the subsequent
540 purified fractions numbered in the elution order (SCHD for S Ceramic HyperD fractions, SP
541 for Sepharose FF fractions, Phen for Phenyl Sepharode FF fractions and S100 for Sephacryl
542 S100 fractions). B. Representative chromatograms are presented. The absorbance of proteins is
543 recorded at 280 nm (black line). Proteins are eluted in a elution of buffer B (concentration B %,
544 grey line) and real gradient is measured by conductivity (grey dashed line). Please note the
545 differences in the range of the UV axes between chromatograms. The peaks of interest are
546 colored in blue for omega-gliadin, green for gamma-gliadin, yellow for alpha-gliadin and red
547 for beta gliadin. For interpretation of the references to color in this figure legend, the reader is
548 referred to the web version of this article.

549

550 **Figure 3 SDS-PAGE and A-PAGE of purified gliadins fractions.**

551 Each fraction ($10 \mu\text{g}$) is analyzed by electrophoresis and purity is check by Coomassie Brilliant
552 blue staining: (M) indicates molecular weight marker, (TG) total gliadins, (1) omega NaCl25
553 fraction, (2) omega NaCl50 fraction, (3) gamma46-gliadins, (4) gamma44-gliadins, (5) beta-
554 gliadins, (6) alpha-gliadins. Two images of gels are combined to present all of the fractions in
555 a single figure.

556

557 **Figure 4 RP-HPLC chromatograms of purified gliadins fractions.**

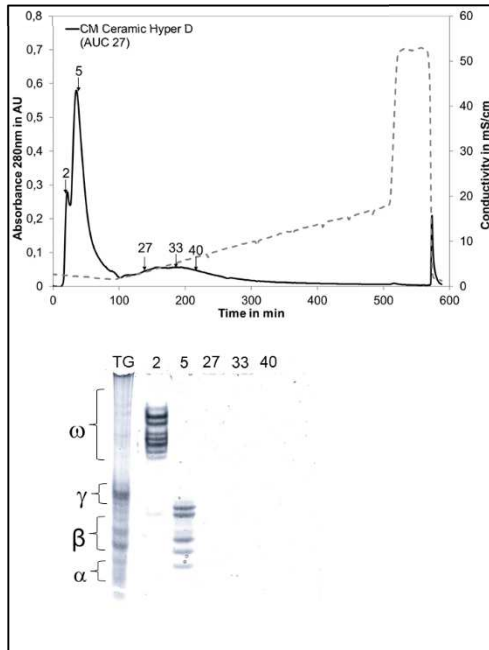
558 Each fraction (120 µg) is injected on analytical C18 column. Proteins are eluted in a gradient
559 from 25 to 55% acetonitrile-TFA 0.1% in 58 min at 0.8 mL/min. The absorbance of proteins
560 recorded at 214 nm is represented in light blue for omega NaCl25, dark blue for omega NaCl50,
561 red for beta-gliadins, yellow for alpha-gliadins, light green for gamma44-gliadins and dark
562 green for gamma46-gliadins. For interpretation of the references to color in this figure legend,
563 the reader is referred to the web version of this article.

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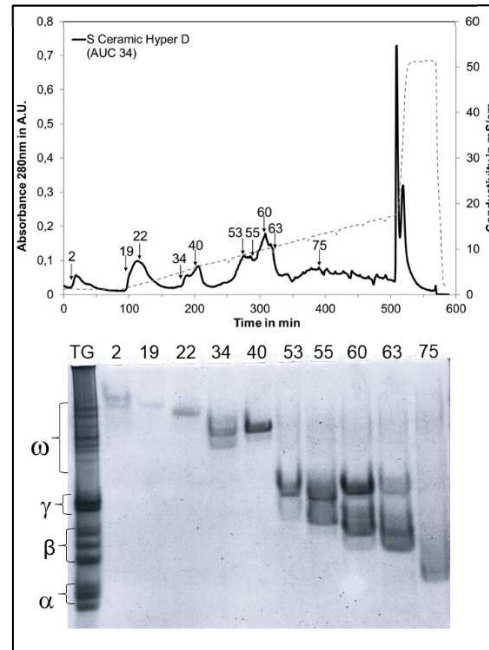
565 **FIGURE 1**

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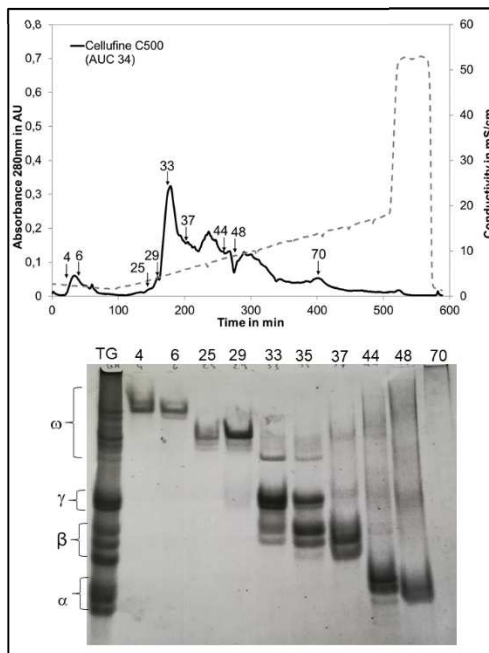
A. CM Ceramic Hyper D



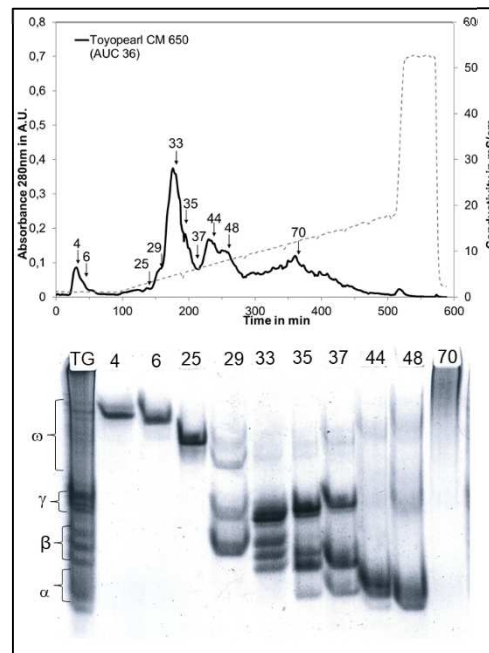
B. S Ceramic Hyper D



C. Cellufine C 500



D. Toyopearl CM 650



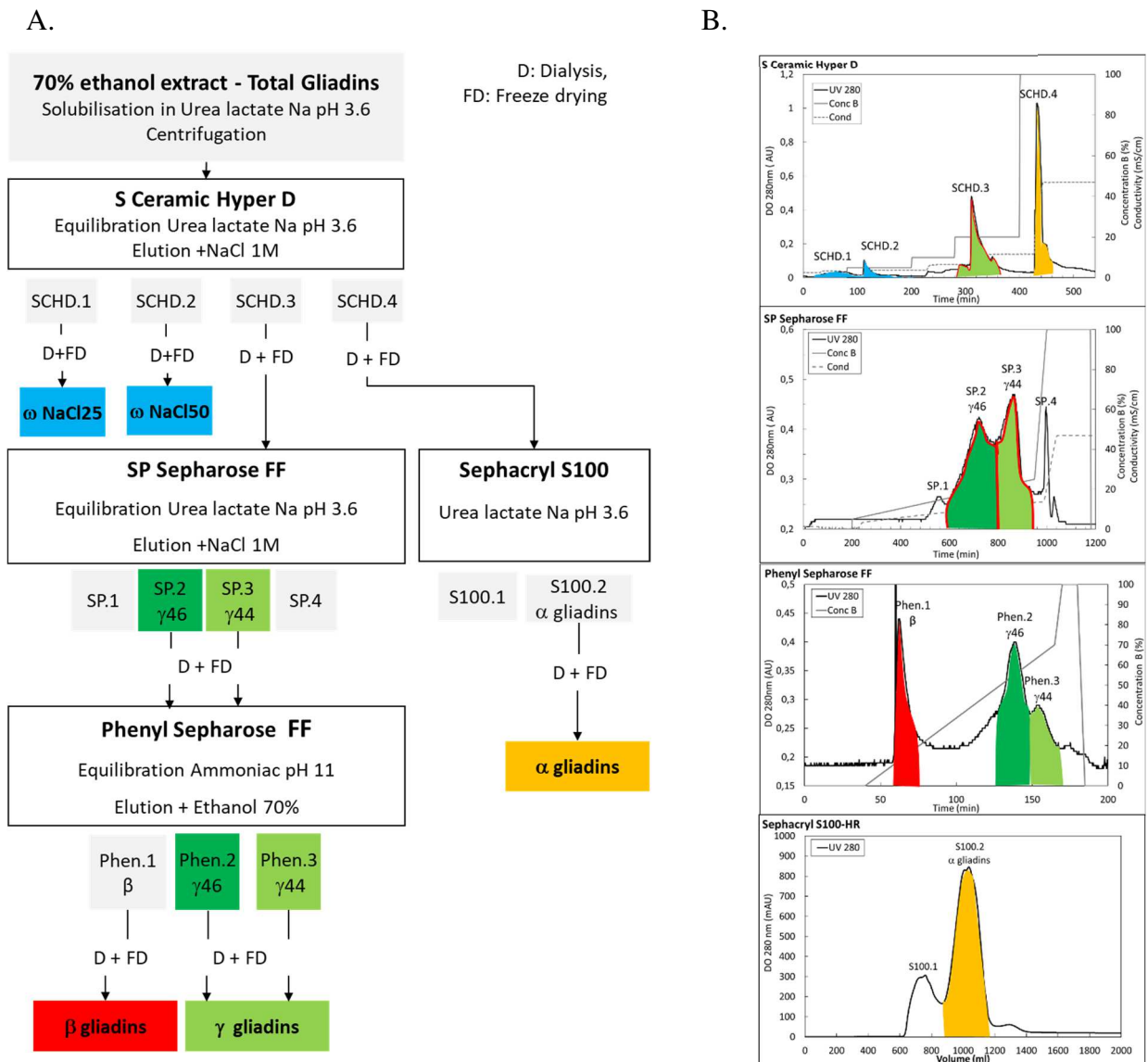
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Figure 1 Gliadins fractionation on (A) CM Ceramic Hyper D, (B) S Ceramic Hyper D (C) Cellufine C 500 and (D) Toyopearl CM 650 M with associated A-PAGE analysis (bottom). Numbers above the peaks correspond to numbered lanes in A-PAGE. TG stands for total gliadins. Fractions signaled by an arrow on the chromatogram ($\approx 10 \mu\text{g}$) were analyzed with A-PAGE stained by Coomassie Brilliant blue. Chromatogram of SP Trisacryl M is presented in supplementary S1.

591 **FIGURE 2**

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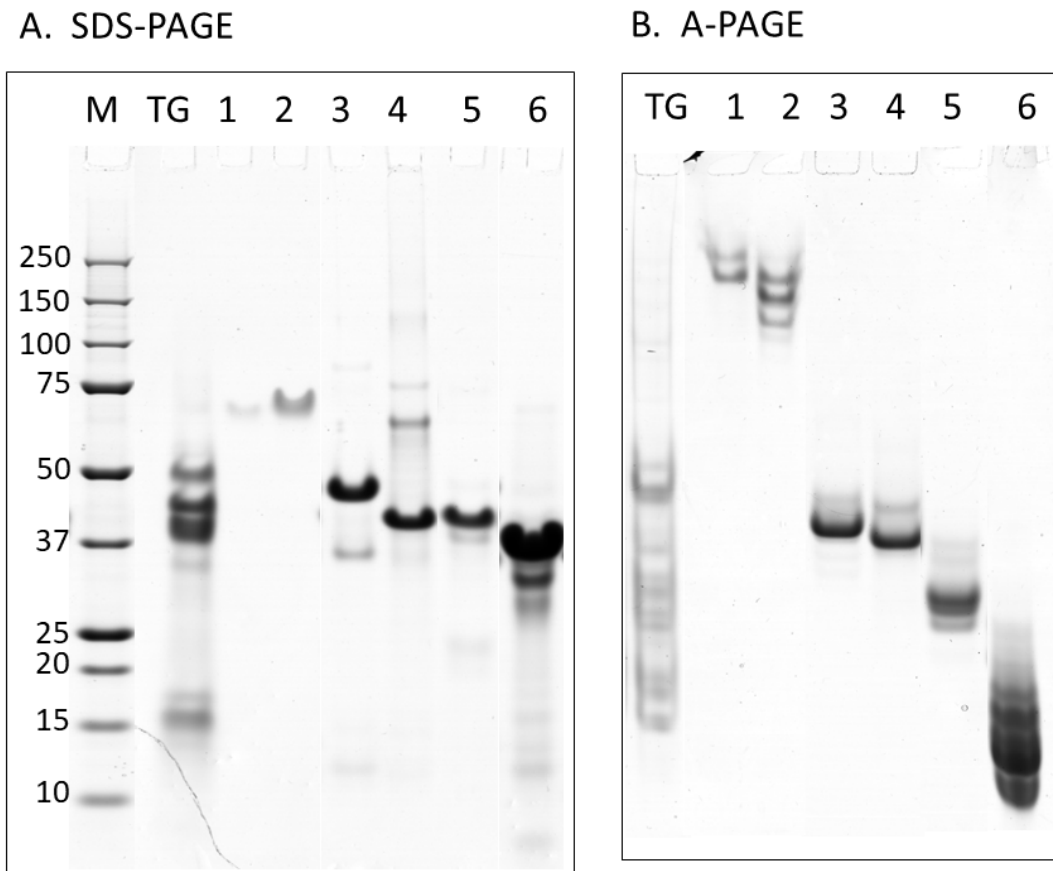
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596 purified fractions numbered in the elution order (SCHED for S Ceramic HyperD fractions, SP
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598 S100 fractions). B. Representative chromatograms are presented. The absorbance of proteins is
599 recorded at 280 nm (black line). Proteins are eluted in a elution of buffer B (concentration B %,
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601 differences in the range of the UV axes between chromatograms. The peaks of interest are
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605 **FIGURE 3**

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607

608 **Figure 3 SDS-PAGE and A-PAGE of purified gliadins fractions.**

609 Each fraction (10 μ g) is analyzed by electrophoresis and purity is check by Coomassie Brilliant
610 blue staining. (M) indicates molecular weight marker, (TG) total gliadins, (1) omega NaCl25
611 fraction, (2) omega NaCl50 fraction, (3) gamma46-gliadins, (4) gamma44-gliadins, (5) beta-
612 gliadins, (6) alpha-gliadins. Two images of gels are combined to present all the fractions in a
613 single figure.

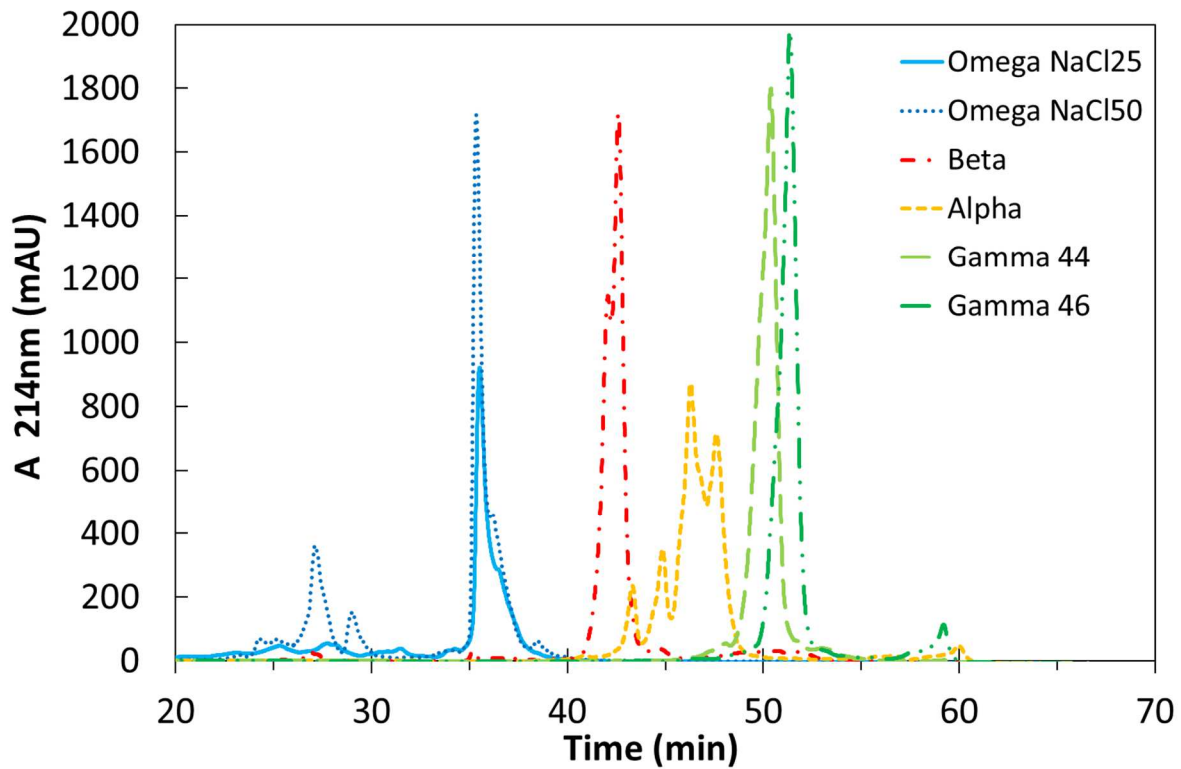
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618 **FIGURE 4**



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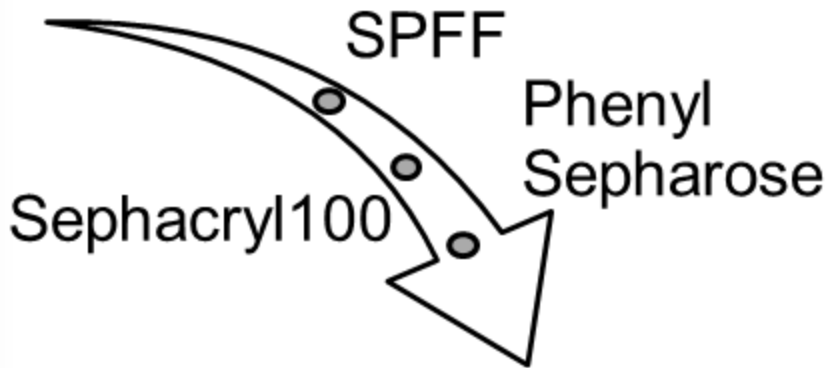
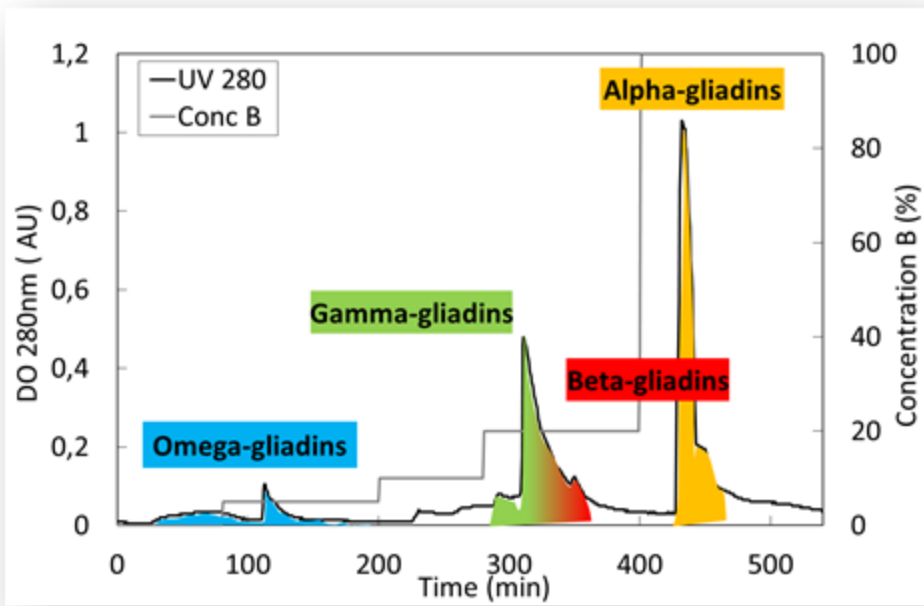
620 **Figure 4 RP-HPLC chromatograms of purified gliadins fractions.**

621 Each fraction (120 μ g) is injected on analytical C18 column. Proteins are eluted in a gradient
622 from 25 to 55% acetonitrile-TFA 0.1% in 58 min at 0.8 mL/min. The absorbance of proteins
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624 red for beta-gliadins, yellow for alpha-gliadins, light green for gamma44-gliadins and dark
625 green for gamma46-gliadins. For interpretation of the references to color in this figure legend,
626 the reader is referred to the web version of this article.

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Optimized separation of gliadins on S Ceramic Hyper D - Pall



Final Purification

Gliadins fraction	Chromatography steps number	Final recovery g
Omega	1	4.5
Gamma	3	2.4
Beta	3	3
Alpha	2	9*