DOI: 10.1016/j.jcs.2015.07.004

1

Quinoa (*Chenopodium quinoa* Willd.) protein hydrolysates with *in vitro* dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties

2

3

4 Alice B. Nongonierma[1,2], Solène Le Maux[1,2], Cecile Dubrulle[1], Chloé Barre[1] & Richard J.
5 FitzGerald[1,2]*

6

7 *[1]Department of Life Sciences and [2]Food for Health Ireland (FHI), University of Limerick,*
8 *Limerick, Ireland.*

9

10

Please cite as:

A.B. Nongonierma, S. Le Maux, C. Dubrulle, C. Barre & R.J. FitzGerald (2015). Quinoa (*Chenopodium quinoa* Willd.) protein hydrolysates with *in vitro* dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties. Journal of Cereal Science. 65,112-118.

11

12

13

14

15

16

17

18 *Corresponding author: dick.fitzgerald@ul.ie
19 Tel: +353 (0) 61 202598
20 Fax: + 353 (0) 61 331490

21

22

23 ## Abstract

24 The potential of quinoa to act as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory and
25 antioxidant peptides was studied. A quinoa protein isolate (QPI) with a purity of 40.73 ± 0.90%
26 was prepared. The QPI was hydrolysed at 50°C for 3 h with two enzyme preparations: papain (P)
27 and a microbial papain-like enzyme (PL) to yield quinoa protein hydrolysates (QPHs). The
28 hydrolysates were evaluated for their DPP-IV inhibitory and oxygen radical absorbance capacity
29 (ORAC) activity. Protein hydrolysis was observed in the QPI control, possibly due to the activity
30 of quinoa endogenous proteinases. The QPI control had significantly higher DPP-IV half
31 maximal inhibitory concentrations (IC_{50}) and lower ORAC values than QPH-P and QPH-PL ($P <$
32 0.05). Both QPH-P and QPH-PL had similar DPP-IV IC_{50} and ORAC values. QPH-P had a DPP-
33 IV IC_{50} value of 0.88 ± 0.05 mg mL^{-1} and an ORAC activity of 501.60 ± 77.34 µmol Trolox
34 equivalent (T.E.) g^{-1}. To our understanding, this is the first study demonstrating the *in vitro* DPP-
35 IV inhibitory properties of quinoa protein hydrolysates. QPHs may have potential as functional
36 ingredients with serum glucose lowering properties.

37

38 **Key words:** *dipeptidyl peptidase IV inhibition, antioxidant, bioactive peptides, quinoa.*

39

40 **Abbreviations:** AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; ACE, angiotensin
41 converting enzyme; ACN, acetonitrile; AN, free amino group content; ANOVA, analysis of
42 variance; BCA, bicinchoninic acid ; BSA, bovine serum albumin; DPPH, 2,2-diphenyl-1-
43 picrylhydrazyl; DPP-IV, dipeptidyl peptidase IV; DPP-IV PI, DPP-IV inhibitory potency index;
44 E:S, enzyme to substrate ratio; GIP, glucose dependent insulinotropic polypeptide; GLP-1,
45 glucagon-like peptide-1; GP-HPLC, gel permeation high-performance liquid chromatography;
46 HCl, hydrochloric acid; HPLC, high performance liquid chromatography; IC_{50}, half maximal
47 inhibitory concentration; NaOH, sodium hydroxide; ORAC, oxygen radical absorbance capacity;

48 P, papain; PL, papain-like enzyme; QPH, quinoa protein hydrolysate; QPH-P, QPH obtained with
49 P; QPH-PL, QPH obtained with the PL; QPI, quinoa protein isolates; RP-HPLC, reverse-phase
50 high-performance liquid chromatography; RuBisCo, ribulose-1,5-bisphosphate
51 carboxylase/oxygenase; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate
52 polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; T.E.,
53 Trolox equivalent; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TRIS,
54 tris(hydroxymethyl)aminomethane; T2D, type 2 diabetes.
55

1. Introduction

Due to the increasing prevalence of diabetes worldwide, the investigation of natural strategies to slow down the progress of this disease is a subject of interest to the scientific community. It has been suggested that natural components originating from foods can affect different biomarkers of type 2 diabetes (T2D). Among these, amino acids, peptides and food-derived proteins have been shown to affect serum glucose levels in normoglycaemic and T2D subjects (Manders et al., 2014). Although milk proteins appear to be one of the most studied substrates for the generation of insulinotropic components, selected studies have also demonstrated the benefit of ingesting plant proteins or plant protein hydrolysates in the regulation of serum glucose in humans (Méric et al., 2014). The antidiabetic properties of dietary proteins and peptides have been attributed to their direct insulinotropic properties or to the inhibition of metabolic enzymes such as dipeptidyl peptidase IV (DPP-IV) or α-glucosidase (Lacroix and Li-Chan, 2013; Mojica et al., 2015).

DPP-IV is responsible for the degradation of the incretin hormones such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Cleavage of the incretins by DPP-IV leads to a diminution of insulin secretion in pancreatic beta cells, in the post prandial phase (Juillerat-Jeanneret, 2014). DPP-IV inhibitory drugs, or gliptins, are currently being used for the treatment of T2D. DPP-IV inhibitors are also naturally found within a wide range of dietary proteins in the format of peptide fragments as demonstrated *in silico* (Lacroix and Li-Chan, 2012; Nongonierma and FitzGerald, 2014). These DPP-IV inhibitory peptides may be released during the enzymatic digestion of food proteins.

A relatively high oxidative status is generally found in individuals suffering from T2D as a consequence of the onset of secondary diseases including cardiovascular and renal complications (Hayden and Tyagi, 2001). Several studies have demonstrated that specific peptides from foods display an antioxidant activity *in vitro*. This can be seen through the scavenging of free radicals (Di Pierro et al., 2014; Nongonierma and FitzGerald, 2013) or through the inhibition/activation of

certain pro- or anti-oxidative metabolic enzymes (Nongonierma and FitzGerald, 2012; O'Keeffe and FitzGerald, 2014). However, to date, a clear relationship between the consumption of dietary antioxidants and a reduction of *in vivo* oxidative status has not been established (Lacroix and Li-Chan, 2014).

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal originating from South America which has gained increasing interest in other regions of the world over the past number years. This is linked with its high protein content and a balanced amino acid profile. It has been reported that quinoa contains higher content of proteins than other dietary grains such as wheat, rice, maize, oat and barley (González Martín et al., 2014). It is also becoming popular as a gluten-free grain. Only a restricted number of studies have demonstrated that quinoa potentially contains bioactive peptides. To date, it appears that quinoa peptides have mainly been studied for their *in vitro* angiotensin converting enzyme (ACE) inhibitory and antioxidant properties (Aluko and Monu, 2003). Recently, an *in silico* study has shown that quinoa proteins contain previously identified DPP-IV inhibitory peptides. A model was used to rank dietary proteins in terms of their DPP-IV inhibitory potency index (DPP-IV PI). It was shown that the large ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) chain from quinoa had a higher DPP-IV PI (2.44 10^{-6} μM^{-1} g^{-1}) than selected milk proteins such as bovine serum albumin (BSA) and α_{s2}-casein (0.93 and 1.93 10^{-6} μM^{-1} g^{-1}, respectively) (Nongonierma and FitzGerald, 2014). This suggests that quinoa protein hydrolysates may have potential as a source of DPP-IV inhibitory peptides.

To our knowledge, no studies to date have shown that quinoa protein hydrolysates contain DPP-IV inhibitory properties. Therefore, the aim of this study was to generate quinoa protein hydrolysates which could inhibit DPP-IV. This was achieved by preparing a quinoa protein isolate (QPI). The QPI was hydrolysed with two food-grade enzymatic preparations. The peptide profiles of the resulting hydrolysates were then analysed. Finally, the samples were tested *in vitro* for their DPP-IV inhibitory and also for their antioxidant properties.

106 ## 2. Materials and methods

107 ### 2.1. Reagents

108 Organic Real quinoa seeds from Priméal (Paugres, France) containing 12.8% (*w/w*) protein were
109 purchased in a local store (Limerick, Ireland). Trifluoroacetic acid (TFA), trichloroacetic acid
110 (TCA), tris(hydroxymethyl)aminomethane (TRIS), azocasein, sodium phosphate monobasic,
111 sodium phosphate dibasic, Gly-Pro-pNA, Leu, diprotin A (Ile-Pro-Ile), Trolox, 2,2'-azobis (2-
112 amidinopropane) dihydrochloride (AAPH) radical, porcine DPP-IV ($\geq$ 10 U mg^{-1} protein) were
113 obtained from Sigma Aldrich (Dublin, Ireland). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was
114 from Pierce Biotechnology (Medical Supply, Dublin, Ireland). Asp-Glu and Leu-Trp-Met-Arg
115 were from Bachem (Bubendorf, Switzerland). Hydrochloric acid (HCl), sodium hydroxide
116 (NaOH), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN)
117 were from VWR (Dublin, Ireland). All other chemicals were of analytical grade and obtained
118 from Sigma Aldrich.

119 ### 2.2. Quinoa protein isolates (QPI)

120 QPI was prepared according to the method described by Aluko and Monu (2003) with
121 modifications. Briefly, the quinoa seeds (300 g) were soaked for 60 min in 900 mL of distilled
122 water. The quinoa seeds were then rinsed three times with the same volume (900 mL) of distilled
123 water to remove saponins. The grains were reduced to a puree with an Ultraturrax homogeniser
124 (IKA, Staufen, Germany) set at 6,500 rpm for 20 min at room temperature (25°C). The mixture
125 was further diluted in distilled water at a 1:1 (*w/w*) ratio. The pH was adjusted to 9.0 using 0.5 M
126 NaOH to solubilise the proteins under continuous agitation for 60 min at room temperature. The
127 sample was then centrifuged (10,000 g, 30 min, 4°C, Sorvall RC-5, Fisher Scientific, Dublin,
128 Ireland). The supernatant was retained and subsequently adjusted to pH 4.6 with 0.1 N HCl and
129 then centrifuged (10,000 g, 30 min, 4°C). The proteins collected in the pellet were resuspended in
130 distilled water (1:1 (*w/w*)) and adjusted to pH 7.0 with 0.5 M NaOH. The QPI sample was freeze-

131 dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C until utilisation.

132 The protein content of the QPI was determined with the bicinchoninic acid (BCA) method using
133 a micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Absorbance at 562 nm
134 was determined using a plate reader (Biotek Synergy HT, Winoosky, VT, USA) controlled by
135 Gen 5 software (Biotek) and protein content was estimated by reference to a standard curve with
136 bovine serum albumin (BSA) in the range of 25-2,000 µg mL^{-1}. All samples were analysed in
137 triplicate. The extraction yield (equation 1) and purity (equation 2) of the QPI were calculated as
138 follows:

139 $$Yield = \frac{mass \quad of \quad protein \quad in \quad the\, QPI}{initial \quad mass \quad of \quad protein} \times 100 \qquad \text{(Equation 1)}$$

140 $$Purity = \frac{mass \quad of \quad protein \quad in \quad the\, QPI}{mass \quad of \quad QPI} \times 100 \qquad \text{(Equation 2)}$$

141 **2.3. Determination of the general proteinase activity of the enzyme preparations using the**
142 **azocasein assay**

143 The azocasein assay was used to determine the general proteolytic activity as described by
144 Kilcawley et al. (2002). Briefly, the enzyme preparations were diluted at 1 g L^{-1} in 50 mM
145 phosphate buffer, pH 7.0. A volume of 100 µL of the enzyme solution was incubated at 37°C for
146 30 min with 1 mL of a 0.5% (*w/v*) azocasein solution in the phosphate buffer. The reaction was
147 terminated by the addition of 100 µL of 2 M TCA. The samples were then centrifuged at 21,255
148 g for 5 min (Hettich Universal 320R, Hettich, Tuttlingen, Germany). The supernatant (750 µL)
149 was mixed with 250 µL of 0.5 M NaOH and the absorbance was determined at 440 nm (UV mini
150 1240 spectrophotometer, Shimadzu, Kyoto, Japan).

151 **2.4. Enzymatic hydrolysis of the QPI**

152 Hydrolysis was carried out essentially as described by Nongonierma and FitzGerald (2015), with
153 modifications. The QPI was resuspended in distilled water at 25 g L^{-1} on a protein basis, adjusted
154 to pH 7.0 with 0.5 M NaOH and allowed to hydrate for 30 min at 50°C. Two different enzyme

155 preparations were used to hydrolyse the QPI, they consisted of a food-grade proteolytic
156 preparation from *Carica papaya* latex (papain, P) and a microbial-derived alternative to papain
157 (papain-like, PL) both preparations obtained from Biocatalysts (Cefn Coed, Wales, UK). The
158 enzyme was added at 2% (*v/w*) enzyme:substrate (E:S) ratio and hydrolysis was performed at
159 50°C for 180 min. A control sample (QPI control), without enzyme, was maintained in the same
160 conditions as the reaction sample. The enzymes were heat inactivated in a water bath at 90°C for
161 20 min or 100°C for 40 min for PL and P, respectively. The hydrolysates generated with the
162 papain (QPH-P) and with the papain-like enzyme (QPH-PL) were freeze-dried and stored at -
163 20°C prior to further analysis. Each hydrolysis reaction was carried out in triplicate (n = 3).

164 **2.5. Dipeptidyl peptidase IV (DPP-IV) inhibition assay**

165 The protein hydrolysates were dispersed in HPLC water at concentrations ranging from 25.5 ×
166 10^{-3} to 2.0 mg mL^{-1} (final concentration in protein equivalents). The DPP-IV inhibition assay was
167 carried out as described by Nongonierma and FitzGerald (2013). Briefly, the test samples (25 µL)
168 were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA
169 (final concentration 0.200 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0
170 (25 µL) and Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final
171 concentration 0.0025 U mL^{-1}). All the reagents were diluted in 100 mM Tris-HCl buffer pH 8.0.
172 Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate
173 was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT) and absorbance of
174 the released pNA was monitored at 405 nm. The DPP-IV half maximal inhibitory concentration
175 (IC_{50}) values were determined by plotting the percentage inhibition as a function of the test
176 compound concentration expressed in mg protein equivalents mL^{-1}.

177 **2.6. Determination of the antioxidant capacity with the oxygen radical absorbance capacity**
178 **(ORAC) assay**

179 The samples' antioxidant capacity was determined using the ORAC assay as per Zulueta et al.

180 (2009), with some modifications. Briefly, the samples were dissolved at 0.02 and 0.03 mg protein
181 equivalents mL^{-1} (final concentration) in 75 mM phosphate buffer pH 7.0. Trolox standard was
182 prepared as reference at concentrations ranging from 0 to 8 μM (final concentration). Samples or
183 Trolox (50 μL) were added to 50 μL of fluorescein (final concentration 0.1 μM) in a black 96
184 well microplate. The plate was incubated for 15 min at 37°C and the reaction was initiated with
185 the addition of 25 μL of AAPH radical (final concentration 14.63 mM). The fluorescence was
186 recorded every min for 60 min at excitation and emission wavelengths of 485 and 520 nm,
187 respectively (Biotek Synergy HT). ORAC activity was expressed as μmol of Trolox equivalents
188 (T.E.) per g of protein equivalents. Each sample was analysed in triplicate.

189 **2.7. Determination of the free amino group content of the hydrolysates**

190 The free amino group content of the hydrolysates was determined with the method of Adler-
191 Nissen (1986) using TNBS. Absorbance values were measured at 340 nm (UV mini 1240
192 spectrophotometer) which allowed determination of the free amino group content (AN) using the
193 following formula:

$$AN = AN_2 - AN_1$$

194 With AN_1, the amino group content of the unhydrolysed protein isolate (mg N g^{-1} protein) and
195 AN_2, the amino group content of hydrolysed proteins (mg N g^{-1} protein equivalents).

196 **2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

197 The QPI and QPH samples were analysed using SDS-PAGE. Mini-PROTEAN TGX precast gels
198 (4-20% resolving gel, Bio-Rad Laboratories, Hercules, CA, USA) were used on a Mini-PROTAN
199 Tetra Cell system (Bio-Rad Laboratories) according to the manufacturer's instructions. Samples
200 were resuspended in distilled water at a concentration of 8.1 g protein equivalents L^{-1}, then mixed
201 1:1 (*v/v*) with loading buffer (protein loading buffer blue 2X - National Diagnostics, Atlanta, GA,
202 USA) under reducing conditions (with β-mercaptoethanol). Proteins were visualized by staining
203 with Coomassie brilliant blue (0.025% (*w/v*) in 10% acetic acid) and destained in 40% methanol

204 and 10% acetic acid. A wide range molecular weight calibration kit (6,500 to 200,000 Da, Sigma-
205 Aldrich) was used as molecular weight standards.

206 **2.9. Gel permeation (GP-) and reverse-phase high-performance liquid chromatography**
207 **(RP-HPLC)**

208 GP-HPLC was used to determine the molecular mass distribution of the peptides within the
209 hydrolysates as described by Nongonierma and FitzGerald (2012). Samples were resuspended at
210 0.10% (*w* protein equivalents/*v*) in 0.1% TFA and 30% HPLC grade ACN in HPLC grade water.
211 A 600 × 7.5 mm I.D. TSK G2000 SW column mounted with a 75 × 7.5 mm I.D. TSKGEL SW
212 guard column (Tosoh Bioscience, Stuttgart, Germany) was used for separation on a HPLC
213 (Model 1525 binary pump, Model 717 Plus autosampler and a Model 2487 dual λ absorbance
214 detector interfaced with a Breeze™ data-handling package, Waters, Dublin, Ireland). A 20 µL
215 sample volume was injected onto the column. The absorbance was monitored at 214 nm. The
216 standards used to calibrate the GPC consisted of BSA, β-lactoglobulin, α-lactalbumin, aprotinin,
217 bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr.
218 The peptide profiles of different samples were determined as per Nongonierma and FitzGerald
219 (2012) by RP-HPLC using a 250 × 4.6 mm I.D., 5.0 µm Jupiter C18 column coupled to a C18
220 guard column (4 × 3 mm I.D., Phenomenex, Cheshire, UK). The absorbance was monitored at
221 280 and 214 nm. Samples were resuspended at 0.3 % (*w* protein equivalents/*v*) in 0.1% TFA in
222 HPLC water. A 70 µL sample volume was injected onto the column.

223 **2.10. Statistical analysis**

224 Results are presented as the mean of triplicate (n = 3) determinations ± SD. They were compared
225 using R®software 3.1.0 package (R Foundation for Statistical Computing, Vienna, Austria) and
226 significant differences were verified by ANOVA for means comparison, followed by a post-hoc
227 Tukey's test at a significance level $P < 0.05$.

228 ## 3. Results and Discussion

229 ### 3.1. Characteristics of the QPI

230 The yield of the QPI was of 9.24 ± 0.16% and the protein purity was of 40.73 ± 0.90%. The
231 protein content reported in the QPI was lower than previous values reported in the literature, i.e.,
232 65.5 (Aluko and Monu, 2003) and 72.2-83.5% (Abugoch et al., 2008). This may be attributed to
233 the fact that the extraction was carried out directly on whole quinoa grains herein and not on a
234 milled quinoa flour, resulting in larger particle size and lower volume:surface ratio, which is
235 generally associated with a lower mass transfer of solutes during extraction procedures. In
236 addition, at higher extraction pH (11.0), a higher protein purity was achieved (Abugoch et al.,
237 2008). However, high extraction pHs > 10.0 may be detrimental to the integrity of the quinoa
238 proteins, causing for instance denaturation, aggregation and dissociation of the native proteins
239 (Valenzuela et al., 2013). For this reason, a less denaturing pH value of 9.0 was used to obtain the
240 QPI herein, even though the purity and yield were relatively low. Compositional analysis of
241 quinoa seed flour has shown that it contains relatively large amounts of carbohydrates, i.e., up to
242 74% (*w*/*w*) (Chauhan et al., 1992). It is likely that besides the proteins, the other components
243 within the QPI obtained herein are mostly composed of carbohydrates.

244 The protein profile of the QPI was determined by SDS-PAGE in reducing conditions (Fig. 1A).
245 Different protein bands were found ranging from < 6.5 to ~ 100 kDa. This is in agreement with
246 previous studies which detected bands ranging from between 8.8 to 72.0 kDa (Valenzuela et al.,
247 2013) and between 8 to 92 kDa (Brinegar and Goundan, 1993) using non-reducing SDS-PAGE
248 for quinoa protein extracts. Brinegar and Goundan (1993) have shown that the total extractable
249 proteins from quinoa at pH 8.0 ranged from 8-100 kDa, which suggested that the QPI herein
250 contained all the major quinoa proteins. It has been reported that polypeptides between 8-9 kDa
251 correspond to 2S-type proteins commonly found in a wide range of seeds. Protein bands eluting
252 between 22-23 and 32-39 kDa have previously been reported as Chenopodin subunit A and B,

253 respectively (Brinegar and Goundan, 1993). Chenopodins, which represent the major quinoa
254 proteins, are storage proteins belonging to the globulin family. Protein bands eluting at 55 kDa
255 have previously also been identified as globulins (Valenzuela et al., 2013).

256 **3.2. Physicochemical characteristics of the QPI and QPHs**

257 The concentration of free amino groups in the QPI control was higher ($P < 0.05$) than that of the
258 non-heated QPI sample (Table 1). Similar SDS-PAGE profiles were observed for the QPI and
259 QPI control (Fig. 1A), although the QPI control profile displayed bands of lower intensity. The
260 QPI control also had different characteristics compared to the QPI, notably in terms of peptide
261 profile (Fig. 2A) and molecular mass distribution (Fig. 2B). Within the first 30 min of the ACN
262 gradient, more intense peptides peaks were seen in the QPI control as compared to the QPI (Fig.
263 2A). Peaks seen in the QPI control profile eluted at similar retention times as those found within
264 the QPHs. The proportion of material > 10 kDa decreased from 61 to 34% in the QPI vs. QPI
265 control. This resulted in an increase in components < 1 kDa from 20 to 38% in the QPI vs. QPI
266 control (Fig. 2B). These results showed that a significant level of protein hydrolysis occurred
267 when the QPI was incubated at 50°C for 180 min (QPI control). It has previously been reported
268 that quinoa grains contain various proteinases (cysteine, aspartic, serine and metallo-proteases),
269 which are active between pH 3.0-6.5. It was shown that these proteinases were responsible for
270 quinoa protein breakdown after 24 h incubation (Mäkinen et al., 2014). These results are
271 consistent with the degradation of quinoa proteins seen in the QPI control (Table 1, Fig. 1 and
272 Fig. 2). The azocasein assay was used to measure the endogenous proteolytic activity of the QPI
273 after the 180 min incubation at 50°C, however, no activity was found (data not shown). This may
274 be related to the lack of sensitivity of the azocasein test.
275 The hydrolysates had a significantly higher concentration of free amino groups than that of the
276 QPI and the QPI control ($P < 0.05$, Table 1). This indicated that quinoa proteins were further
277 hydrolysed as a consequence of the hydrolytic activity of the enzyme preparations. There was no
278 significant difference ($P > 0.05$) in the free amino group concentration for the QPH-P and QPH-

279 PL (7.95 ± 0.70 and 8.55 ± 0.88 mg N g^{-1}, respectively). Protein hydrolysis in QPH-P and QPH-
280 PL was also seen on the SDS-PAGE profiles (Fig. 1B). The bands corresponding to the intact
281 proteins were fainter for the hydrolysate profiles as compared to that of the QPI. In addition,
282 QPH-P presented fainter bands between 20-24 kDa and a longer smear in the low molecular mass
283 range than QPH-PL (Fig. 1B). Protein breakdown in the QPHs was further confirmed by the
284 molecular mass distribution profile of these samples, showing a reduction in the higher molecular
285 mass (> 10 kDa) components in both QPH-P and QPH-PL (Fig. 2B) as compared to QPI. The
286 peptide profiles for both hydrolysates were very similar even though they were generated with
287 two different enzyme preparations. Interestingly, both preparations differed in their proteolytic
288 activity, which was reflected by significant differences ($P < 0.05$) in the azocasein activity of
289 0.076 ± 0.004 and 0.403 ± 0.005 Abs mg^{-1} protein min^{-1} for P and PL, respectively. However, the
290 RP-HPLC of the hydrolysates indicated that both enzyme preparations yielded hydrolysates
291 which had a similar peptide profile (Fig. 2A).

292 **3.3. DPP-IV inhibitory and ORAC activity of the QPI and QPHs**

293 The IC_{50} values for QPH-P and QPH-PL were significantly lower ($P < 0.05$) than that of the QPI
294 control. In addition, no significant difference was seen between the two hydrolysates. The QPH-P
295 had an IC_{50} value of 0.88 ± 0.05 mg mL^{-1}. Many studies have reported the *in vitro* DPP-IV
296 inhibitory activity of food protein hydrolysates, especially milk protein hydrolysates (Lacroix and
297 Li-Chan, 2013; Nongonierma and FitzGerald, 2013). However, to date, a limited number of
298 studies have shown that selected plant proteins hydrolysates display DPP-IV inhibitory properties
299 *in vitro* (Hatanaka et al., 2012; Mojica et al., 2015; Nongonierma and FitzGerald, 2015). To our
300 knowledge, this is the first time that quinoa hydrolysates are reported for their DPP-IV inhibitory
301 properties. The QPI and QPHs herein had IC_{50} values of the same order of those previously
302 reported for grain protein hydrolysates. The DPP-IV IC_{50} values for other plant protein
303 hydrolysates described in previous studies ranged from 0.09 to 26.4 ± 2.3 mg mL^{-1}, for example,
304 for a simulated gastrointestinal digest of navy beans and a rice protein hydrolysate generated with

305 Bioprase SP, respectively (Hatanaka et al., 2012; Mojica et al., 2015).

306 The antioxidant capacity of the samples obtained using the ORAC assay is summarised in Table
307 1. The antioxidant capacity of the QPHs was significantly higher ($P < 0.05$) than that of the QPI
308 control. The ORAC values for QPH-P and QPH-PL (501.60 ± 77.34 and 514.36 ± 77.34 µmol
309 T.E. g^{-1}, respectively) were not significantly different ($P > 0.05$). The antioxidant capacity
310 reported herein was of the same order as in previous studies with other food protein hydrolysates.
311 ORAC values of 180 and 468 ± 25 µmol T.E. g^{-1} for a sodium caseinate and a β-lactoglobulin
312 hydrolysate, respectively, have been reported (Di Pierro et al., 2014; Power et al., 2014). To our
313 knowledge, only one other study has shown that quinoa protein hydrolysates contain antioxidant
314 peptides which are able to scavenge radical species (Aluko and Monu, 2003). In contrast with the
315 study from Aluko and Monu (2003), where the radical scavenging was seen only after
316 fractionation by ultrafiltration, the unfractionated QPH's herein had antioxidant activity.
317 However, different antioxidant assays (2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging vs.
318 ORAC activity) and enzyme preparations (Alcalase vs. P and PL) have been employed in both
319 studies and therefore the results cannot be directly compared. It has been shown that quinoa
320 peptides with a lower molecular mass had a higher DPPH radical scavenging activity than those
321 with a higher molecular mass (Aluko and Monu, 2003). This result is in agreement with our study
322 showing that the QPHs which had a higher content of lower molecular mass components than the
323 QPI control also had a higher ORAC capacity.

324 The identity of the peptides within the QPHs which are responsible for both the DPP-IV
325 inhibitory and the ORAC activity were not investigated herein. Different peptide sequences have
326 been identified to date within food protein hydrolysates having DPP-IV inhibitory (Hatanaka et
327 al., 2012) and antioxidant activity (Di Pierro et al., 2014; Power et al., 2014). For DPP-IV
328 inhibition, a peptide alignment strategy has recently shown that a Trp at the N-terminus and/or a
329 Pro at position 2 of the peptide were generally found within the sequence of relatively potent
330 DPP-IV inhibitory peptides with an IC_{50} value < 200 µM (Nongonierma and FitzGerald, 2014).

331 This was further supported by a positive correlation between the presence of Trp-containing
332 peptides within plant (hemp, pea, rice and soy) protein hydrolysates and their DPP-IV inhibitory
333 properties (Nongonierma and FitzGerald, 2015). Antioxidant activity has been described with
334 short peptides (< 11 amino acids) containing hydrophobic residues such as Pro, His, Tyr and Trp
335 or sulphur (Cys and Met) residues (Pihlanto, 2006). Papain is a serine proteinase displaying a
336 relatively broad substrate specificity, which has notably been reported to hydrolyse at the C
337 terminal side of Lys, Arg and Phe residues (Nongonierma and FitzGerald, 2011). The enzyme
338 specificity of PL is not known. It is therefore difficult to predict the type of peptides which are
339 likely to be released by this enzyme preparation. The amino acid percentage of Pro (0.84-2.74%
340 (*w/w*)), Trp (0.69-1.71% (*w/w*)), His (0.63-3.08% (*w/w*)), Tyr (0.53-1.87% (*w/w*)), Cys (0.05-
341 0.82% (*w/w*)) and Met (0.05-4.48% (*w/w*)) have been determined in quinoa seeds of different
342 genotypes (Escuredo et al., 2014). Based on the amino acid composition of quinoa, it can be
343 suggested that Pro-, Trp-, His-, Tyr- and sulfur-containing peptides are likely to be released
344 following enzymatic hydrolysis of the QPI. These peptides may have contributed to the overall
345 DPP-IV inhibition and antioxidant activity of the hydrolysates herein.
346 Although several studies have hypothesized the positive role of dietary antioxidant peptides in
347 improving biomarkers linked with enhanced sports performance (Lollo et al., 2014), the *in vivo*
348 antioxidant mechanisms have not been fully elucidated (Lacroix and Li-Chan, 2014). Only two
349 studies to date have demonstrated that porcine and Atlantic salmon skin gelatin hydrolysates were
350 able to inhibit plasma DPP-IV in rats and therefore lower serum glucose in the post-prandial
351 phase (Hsieh et al., 2015; Huang et al., 2014).

352 Conclusion

353 The antioxidant capacity and DPP-IV inhibitory properties of QPHs were demonstrated herein.
354 Although the QPI control was degraded to lower molecular mass peptides, possibly by
355 endogenous enzymes (Mäkinen et al., 2014), this resulted in less potent antioxidant and DPP-IV

356 inhibitory peptides than those found within the QPHs. The ORAC activity of the QPHs was
357 approximately twice as high as that of QPI control. This demonstrated the benefits of utilizing
358 exogenous enzyme preparations to release bioactive peptides from quinoa proteins. Despite
359 physicochemical differences, the bioactivity of the QPHs generated with papain and microbial-
360 derived papain-like enzyme preparations was similar. Further characterization of the peptide
361 composition of the QPHs could help to better understand which peptide sequences within both
362 hydrolysates are responsible for the DPP-IV inhibition and ORAC activity seen therein. In
363 addition, assessment of these hydrolysates in humans is needed to verify that these bioactive
364 properties also translate *in vivo*.
365

366 ## Acknowledgements

367 The work described herein was partially supported by Enterprise Ireland under Grant Number
368 TC2013-0001. Cecile Dubrulle and Chloé Barre were funded by the ERASMUS Program.

369

370 ## Conflicts of interests

371 The authors declare that they have no conflict of interest.

372

373 ## References

374 Abugoch, L.E., Romero, N., Tapia, C.A., Silva, J., Rivera, M., 2008. Study of some
375 physicochemical and functional properties of quinoa (*Chenopodium Quinoa* Willd) protein
376 isolates. Journal of Agricultural and Food Chemistry 56, 4745-4750.

377 Adler-Nissen, J., 1986. Enzymic hydrolysis of food proteins Elsevier Applied Science Publishers,
378 London.

379 Aluko, R.E., Monu, E., 2003. Functional and bioactive properties of quinoa seed protein
380 hydrolysates. Journal of Food Science 68, 1254-1258.

381 Brinegar, C., Goundan, S., 1993. Isolation and characterization of chenopodin, the 11S seed
382 storage protein of quinoa (*Chenopodium quinoa*). Journal of Agricultural and Food Chemistry 41,
383 182-185.

384 Chauhan, G., Eskin, N., Tkachuk, R., 1992. Nutrients and antinutrients in quinoa seed. Cereal
385 Chemistry 69, 85-88.

386 Di Pierro, G.D., O'Keeffe, M.B., Poyarkov, A., Lomolino, G., FitzGerald, R.J., 2014.
387 Antioxidant activity of bovine casein hydrolysates produced by *Ficus carica* L-derived
388 proteinase. Food Chemistry 156, 305–311.

389 Escuredo, O., González Martín, M.I., Wells Moncada, G., Fischer, S., Hernández Hierro, J.M.,
390 2014. Amino acid profile of the quinoa (*Chenopodium quinoa* Willd.) using near infrared
391 spectroscopy and chemometric techniques. Journal of Cereal Science 60, 67–74.

392 González Martín, M.I., Wells Moncada, G., Fischer, S., Escuredo, O., 2014. Chemical
393 characteristics and mineral composition of quinoa by near-infrared spectroscopy. Journal of the
394 Science of Food and Agriculture 94, 876-881.

395 Hatanaka, T., Inoue, Y., Arima, J., Kumagai, Y., Usuki, H., Kawakami, K., Kimura, M.,
396 Mukaihara, T., 2012. Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice
397 bran. Food Chemistry 134, 797-802.

398 Hayden, M.R., Tyagi, S.C., 2001. Uric acid: a new look at an old risk marker for cardiovascular
399 disease, metabolic syndrome, and type 2 diabetes mellitus: the urate redox shuttle. Nutrition and
400 Metabolism 1, 1-10.

401 Hsieh, C., Wang, T., Hung, C., Chen, M., Hsu, K., 2015. Improvement of glycemic control in
402 streptozotocin-induced diabetic rats by Atlantic salmon skin gelatin hydrolysate as the dipeptidyl-
403 peptidase IV inhibitor. Food & Function 6, 1887-1892.

404 Huang, S.-L., Hung, C.-C., Jao, C.-L., Tung, Y.-S., Hsu, K.-C., 2014. Porcine skin gelatin
405 hydrolysate as a dipeptidyl peptidase IV inhibitor improves glycemic control in streptozotocin-
406 induced diabetic rats. Journal of Functional Foods 11, 235-242.

407 Juillerat-Jeanneret, L., 2014. Dipeptidyl peptidase IV and its inhibitors: Therapeutics for type 2
408 diabetes and what else? Journal of Medicinal Chemistry 57, 2197–2212.

409 Kilcawley, K., Wilkinson, M., Fox, P., 2002. Determination of key enzyme activities in
410 commercial peptidase and lipase preparations from microbial or animal sources. Enzyme and
411 Microbial Technology 31, 310-320.

412 Lacroix, I.M., Li-Chan, E.C.Y., 2013. Inhibition of dipeptidyl peptidase (DPP)-IV and α-
413 glucosidase activities by pepsin-treated whey proteins. Journal of Agricultural and Food
414 Chemistry 61, 7500–7506.

415 Lacroix, I.M.E., Li-Chan, E.C.Y., 2012. Evaluation of the potential of dietary proteins as
416 precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. Journal of

417 Functional Foods 4, 403-422.

418 Lacroix, I.M.E., Li-Chan, E.C.Y., 2014. Overview of food products and dietary constituents with
419 antidiabetic properties and their putative mechanisms of action: a natural approach to
420 complement pharmacotherapy in the management of diabetes. Molecular Nutrition & Food
421 Research 58, 61-78.

422 Lollo, P., Amaya-Farfan, J., Faria, I., Salgado, J., Chacon-Mikahil, M., Cruz, A., Oliveira, C.,
423 Montagner, P., Arruda, M., 2014. Hydrolysed whey protein reduces muscle damage markers in
424 Brazilian elite soccer players compared with whey protein and maltodextrin. A twelve-week in-
425 championship intervention. International Dairy Journal 34, 19-24.

426 Mäkinen, O.E., Hager, A.-S., Arendt, E.K., 2014. Localisation and development of proteolytic
427 activities in quinoa (*Chenopodium quinoa*) seeds during germination and early seedling growth.
428 Journal of Cereal Science 60, 484-489.

429 Manders, R.J., Hansen, D., Zorenc, A.H., Dendale, P., Kloek, J., Saris, W.H., van Loon, L.J.,
430 2014. Protein co-ingestion strongly increases postprandial insulin secretion in type 2 diabetes
431 patients. Journal of Medicinal Food 17, 758-763.

432 Méric, E., Lemieux, S., Turgeon, S.L., Bazinet, L., 2014. Insulin and glucose responses after
433 ingestion of different loads and forms of vegetable or animal proteins in protein enriched fruit
434 beverages. Journal of Functional Foods 10, 95-103.

435 Mojica, L., Chen, K., de Mejía, E.G., 2015. Impact of commercial precooking of common bean
436 (*Phaseolus vulgaris*) on the generation of peptides, after pepsin–pancreatin hydrolysis, capable to
437 inhibit dipeptidyl peptidase-IV. Journal of Food Science 80, H188-H198.

438 Nongonierma, A.B., FitzGerald, R.J., 2011. Enzymes exogenous to milk in dairy technology |
439 Proteinases. In: Fuquay, J.W., Fox, P.F., McSweeney, P.L.H. (Eds.), Encyclopedia of Dairy

440 Sciences (Second Edition). Academic Press, San Diego, pp. 289-296.

441 Nongonierma, A.B., FitzGerald, R.J., 2012. Tryptophan-containing milk protein-derived
442 dipeptides inhibit xanthine oxidase. Peptides 37, 263-272.

443 Nongonierma, A.B., FitzGerald, R.J., 2013. Dipeptidyl peptidase IV inhibitory and antioxidative
444 properties of milk-derived dipeptides and hydrolysates. Peptides 39, 157-163.

445 Nongonierma, A.B., FitzGerald, R.J., 2014. An *in silico* model to predict the potential of dietary
446 proteins as sources of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. Food Chemistry 165,
447 489-498.

448 Nongonierma, A.B., FitzGerald, R.J., 2015. Investigation of the potential of hemp, pea, rice and
449 soy protein hydrolysates as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides.
450 Food Digestion: Research and Current Opinion 6, 19-29.

451 O'Keeffe, M.B., FitzGerald, R.J., 2014. Antioxidant effects of enzymatic hydrolysates of whey
452 protein concentrate on cultured human endothelial cell. International Dairy Journal 36, 128-135.

453 Pihlanto, A., 2006. Antioxidative peptides derived from milk proteins. International Dairy Journal
454 16, 1306-1314.

455 Power, O., Fernández, A., Norris, R., Riera, F.A., FitzGerald, R.J., 2014. Selective enrichment of
456 bioactive properties during ultrafiltration of a tryptic digest of β-lactoglobulin. Journal of
457 Functional Foods 9, 38-47.

458 Valenzuela, C., Abugoch, L., Tapia, C., Gamboa, A., 2013. Effect of alkaline extraction on the
459 structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence on film
460 formation. International Journal of Food Science & Technology 48, 843-849.

461 Zulueta, A., Esteve, M.J., Frígola, A., 2009. ORAC and TEAC assays comparison to measure the

462 antioxidant capacity of food products. Food Chemistry 114, 310-316.

463

464

Table captions

Table 1. Concentration of free amino groups, half maximal inhibitory concentration (IC_{50}) for dipeptidyl peptidase IV (DPP-IV) and oxygen radical absorbance capacity (ORAC) of the quinoa protein isolates after 180 min incubation at 50°C and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL). All values are expressed in protein equivalents. Values represent the mean ± SD of three replicates (n = 3). For each assay, values with different superscript letters are significantly different ($P < 0.05$).

Table 1

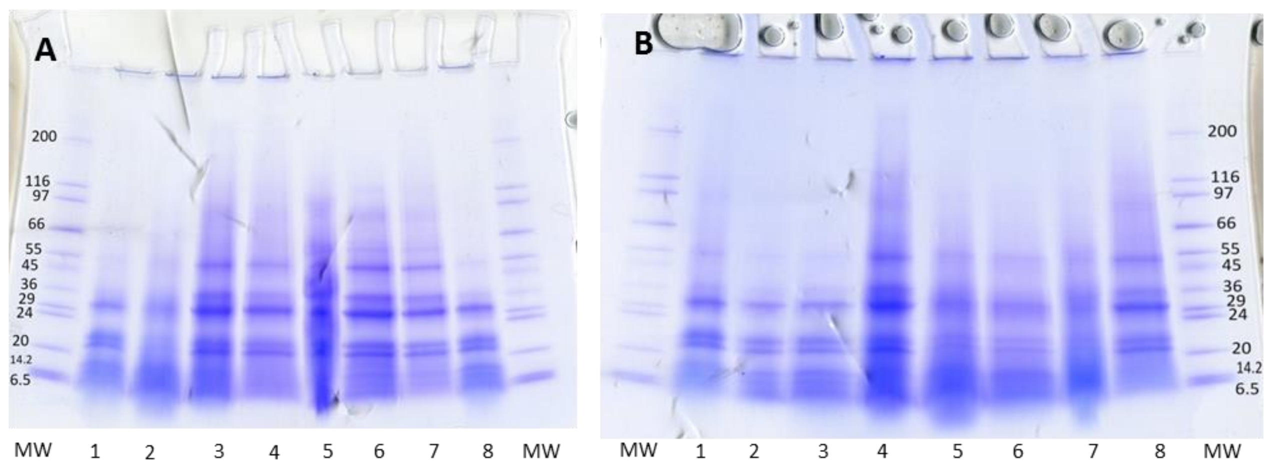
Sample	Concentration of free amino groups (mg N g^{-1})	DPP-IV IC_{50} (mg mL^{-1})	ORAC activity (µmol T.E. g^{-1})*
QPI control	5.98 ± 0.83[a]	> 2.00	264.42 ± 65.31[a]
QPH-P	7.95 ± 0.70[b]	0.88± 0.05[a]	501.60 ± 77.34[b]
QPH-PL	8.55 ± 0.88[b]	0.98 ± 0.04[a]	514.36 ± 77.34[b]

*T.E.: Trolox equivalent; Samples were tested at 0.030 mg protein equivalents mL^{-1}.

Figure captions

Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the quinoa protein isolates (QPI) before and after 180 min incubation at 50°C and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL).

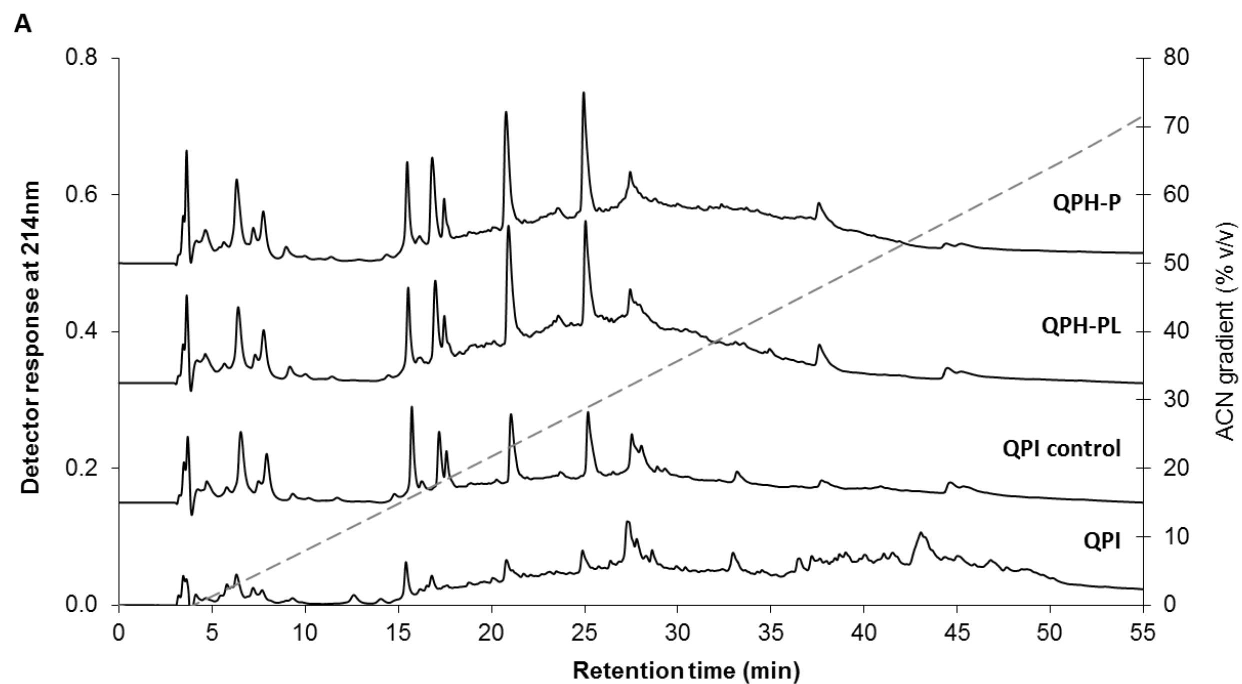
Figure 2. (A) Reverse-phase high performance liquid chromatographic (RP-HPLC) profile of the quinoa protein isolates (QPI) before and after 180 min at 50°C incubation and heat treatment (QPI control) and of the QPI hydrolysed with papain (QPH-P) and the papain-like enzyme (QPH-PL) and (B) Molecular mass distribution determined by gel permeation high-performance liquid chromatography (GP-HPLC) of QPI, QPI control, QPH-P and QPH-PL. Values represent the mean ± SD of three replicates (n = 3). Bovine serum albumin (BSA), β-lactoglobulin, α-lactalbumin, aprotinin, bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr were used as standards for the GP-HPLC.



Lane	**Sample**
MW	Molecular weight marker
1	QPH-PL
2	QPH-P
3	QPI control
4	QPI control
5	QPI
6	QPI
7	QPI
8	QPH-PL

Lane	**Sample**
MW	Molecular weight marker
1	QPH-PL
2	QPH-PL
3	QPH-PL
4	QPI control
5	QPH-P
6	QPH-P
7	QPH-P
8	QPI control

Fig. 1



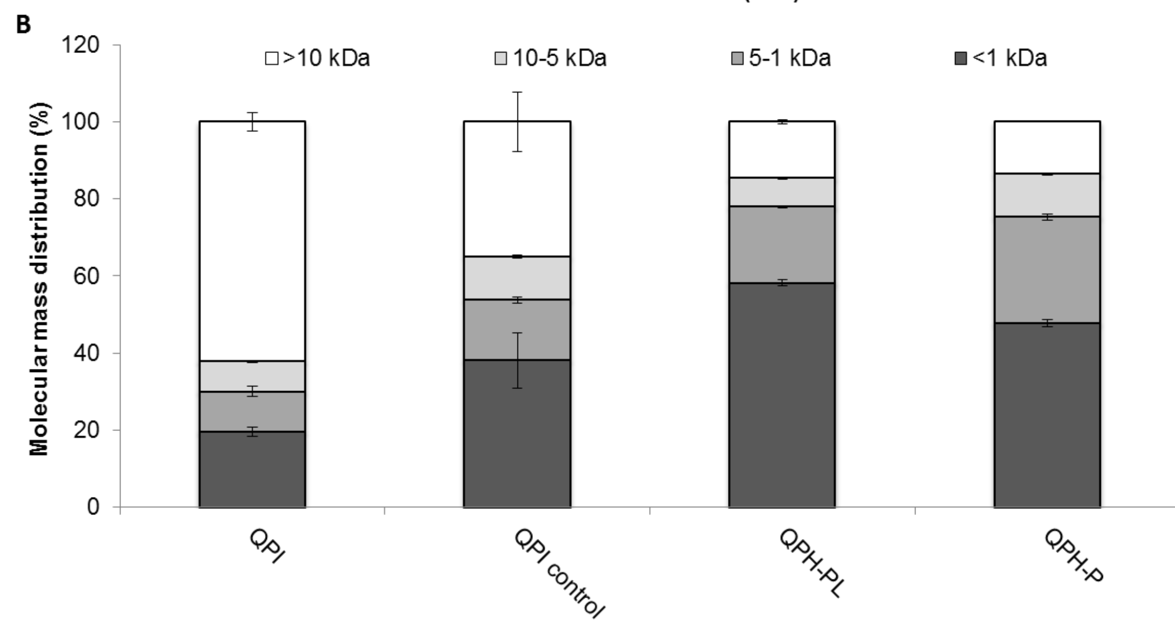


Fig. 2