1 **Characterisation of the peptide profile of Spanish Teruel, Italian**

2 **Parma and Belgian dry-cured hams and its potential bioactivity**

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Abstract

Dry-cured hams are appreciated products in many European countries. One of the most important processes taking place during ham processing and responsible for its unique taste and flavour is the proteolysis of muscle proteins. Muscle peptidases play an important role in breaking down muscle proteins and generating small peptides and free amino acids. It is known that changes in genetics and processing conditions can result in differences in the action of endopeptidases and exopeptidases.

In this study, the peptides generated in Spanish Teruel, Italian Parma and Belgian dry-cured hams have been identified and quantified using a label-free methodology to assess main differences in proteolysis between the 3 types of hams. The identification of the peptides resulted in differential peptide sequences according to the type of ham. On the other hand, an aqueous peptide extract fractionated by size-exclusion chromatography was assayed for Angiotensin-Converting Enzyme (ACE) inhibitory and antioxidant activity. Peptide fractions of Teruel ham exhibited 93% ACE inhibition while those from Parma and Belgian hams had ACE inhibitory activity of 70% and 76%, respectively. The investigated peptide fractions exhibited similar values of DPPH scavenging activity whereas an important Fe^{2+} reducing power was also detected in the same fractions, suggesting the important presence of peptides with antioxidant activity in the three studied types of dry-cured hams.

Keywords: ACE, antioxidant activity, dry-cured ham, peptides, bioactive peptides.

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50 **1. Introduction**

51 Dry-cured hams are well-known and highly appreciated products in almost all Europe
52 and many worldwide countries. In fact, traditional dry-cured hams are considered as
53 high-quality products that can reach really expensive prices depending on the time of
54 ripening and the characteristics of the pigs of origin. Proteolysis is a very important
55 phenomenon that occurrs during dry-cured processing. The activity of endogenous
56 muscle peptidases is the responsible for the release of free amino acids and small
57 peptides (Toldrá & Flores, 1998; Virgili, Saccani, Gabba, Tanzi & Soresi, 2007), which
58 have been described to be directly related with the final quality of this product.
59 However, dry-cured ham elaboration is a traditional procedure that shows certain
60 differences in the processing parameters depending on the origin country. In fact, Teruel
61 and Parma hams have Protected Denomination of Origin (PDO), both with more than
62 12 months of processing and Belgian hams with a minimum of 9 months. On the other
63 hand, proteolysis has been described to be highly influenced by pig genetics as well as
64 parameters of the processing such as salt, temperature, humidity, and time of ripening
65 (Bermúdez et al., 2014). In fact, a recent study involving Iberian dry-cured (24 months
66 of curing) and traditional Spanish dry-cured ham (14 months) showed significant
67 differences in the generation of peptides by both genetics and time of ripening (Mora,
68 Escudero, Arihara & Toldrá, 2015a). A more in depth study considering genetics as the
69 unique differential factor between dry-cured hams confirmed the influence of genetics
70 in the generation of small peptides (Mora, Calvo, Escudero & Toldrá, 2016) which had
71 only been proved by means of enzymatic assays (Armero et al, 1999; Hernández,
72 Zomeño, Ariño, & Blasco, 2004).

73 Besides the characteristics that contribute to the unique organoleptic attributes of dry-
74 cured hams, the generated peptides during dry-curing may have special functions in

75 human body. Natural bioactive peptides are not active whereas they remain within the
76 sequence of the origin protein but show its physiological effect once released. Peptides
77 from traditional Spanish dry-cured ham have been reported to exhibit Angiotensin-I
78 Converting Enzyme (ACE) inhibitory activity *in vitro* and also a relevant
79 antihypertensive activity *in vivo* (Escudero, Mora, Fraser, Aristoy & Toldrá, 2013).
80 ACE role in blood pressure regulation is based on the conversion of angiotensin I into
81 the potent vasoconstrictor angiotensin 2 together with the degradation of the
82 vasodilative bradykininin blood vessels. In fact, peptide AAATP showed an IC_{50} value
83 of 100 μM assayed *in vitro* for ACE inhibitory activity, which also resulted in a
84 decrease of systolic blood pressure of -25.62 mmHg when tested with spontaneously
85 hypertensive rats. Also some naturally present antioxidant peptides are well-known in
86 meat although many others are produced during dry-cured processing (Sarmadi &
87 Ismail, 2010; Zhu, Zhang, Zhou, & Xu, 2016; Xing et al., 2016). Recently, the
88 antioxidant peptide SNAAC was identified in dry-cured ham showing an IC_{50} value of
89 75.2 μM and 205 μM in DPPH radical scavenging assay and ferric-reducing antioxidant
90 power analysis, respectively (Mora, Escudero, Fraser, Aristoy & Toldrá, 2014).
91 In this study, the characterisation of the peptide profile of three different types of dry-
92 cured ham using mass spectrometry techniques and a relative quantitation label-free
93 approach has been done. The aqueous extracts were also isolated and tested to evaluate
94 the ACE inhibitory and antioxidant activity of the fractions and free amino acids of the
95 extracts were analysed.
96

97 **2. Materials and methods**

98 **2.1 Reagents**

99 Trifluoracetic acid (TFA), acetic acid, ferric chloride, 1,1-diphenyl-2-picrylhydrazyl
100 (DPPH), ACE enzyme (A6778-5UN), potassium ferricyanide, and amino acid standards
101 were purchased from Sigma-Aldrich (Misuri, USA). The substrate Abz-Bly-p-nitrophe-
102 pro-OH trifluoroacetate salt was obtained from Bachen AG (Bubendorf, Switzerland).
103 Triethylamine (TEA) and phenylisothiocianate (PITC) were obtained from Fluka.
104 Formic acid (FA), water, and acetonitrile (ACN) were of mass spectrometry grade and
105 were purchased from Scharlab (Barcelona, Spain). Methanol and acetonitrile used in the
106 preparation of samples were of analytical grade and from Scharlab.

107 **2.2 Materials**

108 Dry-cured hams of Teruel PDO (Spain), Parma PDO (Italy), and Grega ham
109 (Buggenhout, East Flanders, Belgium) were used. Teruel PDO hams were from white-
110 breed pigs with a maternal line Landrace x Large White crossbreds, and a paternal line
111 purebred Duroc, and prepared for a minimum time of ripening of 14 months according
112 to the PDO regulations. Parma PDO hams were also from white-breed pigs but using
113 Italian Landrace and Italian Large White cross genotypes, and were cured a minimum
114 time of 12 months according to the Italian PDO regulation. Belgian hams were prepared
115 following the typical Grega processing: dry-salting and 9 months of ripening. All
116 sampled dry-cured hams, three from each country, had weight losses within the range
117 34-40%. Main characteristics of different dry-cured hams can be found in Petrova,
118 Aasen, Rustad, & Eikevik (2015).
119

120 **2.3 Peptide extraction**

121 Fifty grams of *Biceps femoris* muscle were excised of visible fat and connective tissues,
122 milled and mixed in 200 mL of 0.01 N HCl for 8 min. The homogenate was centrifuged
123 at 12000 g for 20 min at 4 ºC. Then, the supernatant was cleaned through glass wool

124 and the protein fraction precipitated using 3 volumes of ethanol and storing for 20 h at 4
125 ºC. The mixture was centrifuged at 12000 g for 20 min (4 ºC) and supernatant was
126 evaporated using a rotary evaporator, whereas the remaining aqueous fraction was dried
127 in a lyophiliser. The deproteinised extract was resuspended in 25 mL of 0.01 N HCl and
128 5 mL were used in the size-exclusion chromatography separation to obtain fractions
129 from the different hams to evaluate the bioactivity profile.
130

131 **2.4. Characterization of peptides profile using MALDI-ToF**

132 The characterization of the peptides profile was done using a Matrix-Assisted Laser
133 Desorption/Ionization (MALDI) system with a Time-of-Flight (ToF) detector. The
134 analysis of samples and data analysis was done according to Mora et al. (2015a) using a
135 5800 MALDI ToF/ToF instrument (ABSciex) with some minor changes. The peptides
136 profile was measured in two different ranges: from 350 to 850 Da and from 850 to 1800
137 Da in order to improve the sensitivity of the analysis. An aliquot of 20 µL of the peptide
138 extract obtained in section 2.3. was concentrated up to saturation using ZipTip C18 tips
139 according to the manufacturer. 1 µL of the eluted peptides were spotted on each position
140 of the MALDI plate together with 0.5 µL of matrix solution (5 mg/mL of α-Cyano-4-
141 hydroxycinnamic acid (CHCA) and air dried during some minutes to allow the
142 homogenous distribution of the crystals.
143

144 **2.5. Identification of peptides using LC-MS/MS**

145 The analysis of peptides was done in high resolution mode using a LC Ultra 1D Plus
146 system (Eksigent) with a quadrupole/Time-of-Flight (Q/ToF) TripleTOF® 5600+
147 detector from ABSciex Instruments (Framingham, MA, USA) to allow the

148 identification and subsequent relative quantitation of the peptides generated during the
149 processing using a label-free approach.
150 The chromatographic conditions were established according to Mora, Gallego, Aristoy,
151 Fraser, & Toldrá (2015b) using a C18 trap column (3 µ, 350 µm× 0.5 mm) to clean and
152 concentrate the sample and a nano-HPLC capillary column (3 µm, 75 µm× 12.3 cm,
153 C18) in the separation of peptides.
154 The solvent A was 0.1% formic acid (FA), and solvent B was 0.1% FA in acetonitrile.
155 Chromatographic conditions were a linear gradient from 5% to 35% of B during 120
156 min at a flow rate of 0.3 µL/min and running temperature of 30 °C.
157 Samples were ionized using an electrospray ionization source (ESI) applying 2.9 kV to
158 the spray emitter. The detector was used in positive polarity and data-dependent mode.
159 Survey MS1 scans were acquired from 150-1250 m/z for 250 ms. The quadrupole
160 resolution was set to 'UNIT' for MS2 experiments, which were acquired 100-1500 *m/z*
161 for 50 ms in 'high sentitivity' mode. The injection of the samples was done randomly to
162 avoid their clustering due to MS analysis factors. The high resolution mode of the
163 detector is necessary to register a higher number of intensity measures in the total ion
164 chromatogram which will allows to obtain a more accurate area under the curve that
165 will be used in the quantitation of the peptides. The identification of peptides was done
166 using NCBI non-redundant protein database using ProteinPilot v4.5 software from
167 ABSciex.
168

169 **2.6. Free amino acids analysis**

170 **2.6.1 Preparation of ham extracts**

171 An extract from 3 dry-cured ham samples of each nationality was prepared to analyse
172 free amino acids by mixing 5g of ham sample with HCl 0.01N in a proportion of 1:5.

173 The mixture was kept in a Stomacher at 4ºC (IUL Instrument, Barcelona, Spain) for 8
174 min, and cooled in ice. Then, the samples were centrifuged at 10000xg for 20 min in
175 cold and the supernatant was deproteinized by adding x2.5 ACN (Aristoy & Toldrá,
176 1991) and centrifuged at 10000xg for 3 min in cold. The supernatant was kept at -20ºC
177 until use. The amino acids standard solution contained 1mM of each amino acid in HCl
178 0.1N.

179 **2.6.2 Amino acids analysis by HPLC**

180 The amino acid content was determined by HPLC using an Agilent 1200 series (Palo
181 Alto, CA). Phenyl isothiocyanate derivatives were analysed using a Pico Tag C18 (3.9 x
182 300 mm) column at 52 ºC with a flow rate of 1 mL/min. Norleucine was used as internal
183 standard (IS) before derivatization. The HPLC solvents were: Solvent A, sodium acetate
184 0.07 M (pH 6.55), 10% acetic acid and 2.5% ACN, and solvent B, ACN:H_2O:MeOH
185 with a proportion of 45:40:15. The chromatographic conditions used in the separation of
186 free amino acids is described in Flores, Aristoy, Spanier & Toldrá (1997).

187

188 **2.7. Peptide separation by size-exclusion chromatography**

189 Peptides were separated depending on their molecular masses using size-exclusion
190 chromatography (SEC). 5 mL of each deproteinised extract obtained as explained in 2.3
191 from the different dry-cured hams were separated on a Sephadex column (2.5 x 65 cm).
192 The fractionation was done in isocratic gradient with 0.01 N HCl at a flow rate of 15
193 mL/h in cold. Fractions were monitored by ultraviolet (UV) absorption at 214, 254, and
194 280 nm (Cari 60 UV spectrophotometer, Agilent Technologies, Palo Alto, CA) and 5
195 mL fractions were collected using an automatic fraction collector and lyophilised. Then,
196 samples were dissolved again in 2 mL distilled water and stored at -20 ºC until use for
197 the measurement of biological activities.

198

199 **2.8. ACE inhibitory activity assay**

200 The ACE inhibitory activity was assayed in the different dry-cured hams according to
201 the method described by Sentandreu & Toldrá (2007). 50 µL of each one of the
202 obtained dry-cured ham fractions were added in a black multiplate (96 wells) and mixed
203 with 50 µL of 7.5 µg/mL of ACE in 150 mM Tris-base buffer pH 8.3. The reaction was
204 initiated by the addition of 200 µL of 0.45 mM Abz-Gly-Phe- (NO_2)-Pro in same Tris-
205 base buffer with 1.125 M NaCl. The reaction mixture was incubated during 45 min at
206 37ºC in a multiplate reader. The fluorescence was registered using excitation and
207 emission wavelengths of 355 and 405 nm, respectively. All measurements were done in
208 triplicate. Distilled water was used as negative control and captopril was used as
209 positive control.

210

211 **2.9. Antioxidant activities**

212 **2.9.1 DPPH radical-scavenging activity assay**

213 The collected fractions were assayed using the DPPH radical-scavenging method as
214 described by Bersuder, Hole and Smith (1998) with some minor changes. Thus, 250 µL
215 of each fraction were mixed with 250 µL of ethanol and 62.5 µL of 0.02% DPPH
216 prepared in ethanol and incubated for 60 min in the dark. The reduced radicals were
217 measured at 517 nm using a spectrophotometer. Calculations were as following: DPPH
218 radical-scavenging activity percentage (%) = $[(C_{abs}- S_{abs})/ C_{abs}] \times 100$, where C_{abs} is the
219 absorbance el the negative control and S_{abs} is the absorbance measured in sample.
220 Distilled water was used as control whereas the synthetic antioxidant butylated
221 hydroxytoluene (*BHT*) was used as positive control. Measurements were done in
222 triplicate.

223 **2.9.2 Ferric-reducing antioxidant power assay**

224 The measurement of antioxidant potential in samples was done through the reduction of
225 ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) according to the method described in Huang,
226 Tsai and Mau (2006). Thus, 250 µL of each fraction, 250 µL of 200 mM sodium
227 phosphate buffer at pH 6.6 and 250 µL of potassium ferricyanide (10 mg/mL) were
228 mixed and incubated at 50ºC. After 20 min of incubation, 250 µL of TCA (100 mg/mL)
229 was added and centrifuged at 200x*g* for 10 min. A total of 0.5 mL of the supernatant
230 weremixed with 0.5 mL of distilled water and 100 µL of ferric chloride (1 mg/mL). The
231 absorbance was immediately measured at 700 nm. The negative control was done using
232 distilled water and the positive control was butylated hydroxytoluene (BHT).
233 Measurements were done in triplicate.

234

235 **2.10. Statistical analysis**

236 Statistical analysis was performed with XLSTAT from Excel software (Microsoft,
237 2011). In this sense, one-way ANOVA was done to determine significant differences in
238 the content of free amino acids between types of dry-cured ham. The analysis was done
239 in triplicate and the normality of data was tested before the ANOVA procedure. Fisher´s
240 multiple range test was done to analyze significant differences among mean values at
241 $p<0.05$.

242 **2.11. Mass spectrometry relative quantitation**

243 The label-free quantitation methodology used is based on a replicate protocol that
244 consists on the measurement of relative ion intensities of extracted ion chromatograms
245 aligned using mass and elution times. Identification and quantitation was done at
246 peptide level. Mascot assigns peptide matches to protein hits and the ratios for
247 individual peptide matches were combined to determine ratios for the protein hits. The

248 label-free quantification was done through the extracted ion chromatograms of each
249 identified peptide sequence using Peak View 1.1 software from ABSciex. The quality
250 parameters of the quantitation were previously established to avoid outliners. The
251 obtained data was statistically analysed with Marker View 1.2 software from ABSciex.
252 Thus, the score plot of Principal Component Analysis, the loading plot of PCA and T-
253 test were done.

254

255 **3. Results and discussion**

256 **3.2. Proteolysis differences observed using a label-free mass spectrometry**
257 **approach**

258 Main differences in the degree of hydrolysis were analysed using a MALDI-ToF
259 approach that showed the intensities of the peptides extracted in the water soluble
260 fraction from the three types of dry-cured hams. Results are shown in **Figures 1 and 2**
261 **of Supplementary material** where important differences in the intensity of the
262 obtained spectra can be observed in both *m/z* studied ranges. The proteolysis during the
263 dry-curing period is highly influenced by genetics of raw material, time of processing,
264 and processing conditions such as the amount of salt (Mora et al., 2016). In fact, the
265 activity of the endogenous muscular enzymes that are main responsible for hydrolysis in
266 dry-cured ham products are very dependent of all the numbered factors. In order to
267 characterize main peptide differences between the studied samples, a label-free
268 quantitative mass spectrometry approach based on LC-MS/MS was used to quantify the
269 peptides generated during curing process in Spanish Teruel, Italian Parma and Belgian
270 dry-cured hams. In this respect, **Figure 1A** shows the Principal Component Analysis
271 score plot performed to assess the variance among the peptides generated at the end of
272 the curing period in the three types of European hams, showing three main clusters. The

273 loading plot in **Figure 1B** shows the sequence of the peptides and their origin proteins
274 that are causing main differences between the groups and the clustering of data. A total
275 of 9076 sequences of peptides were used in the statistical test and the observed
276 differences are mainly due to the differences in genetics and elaboration processes
277 between dry-cured hams from different countries. The knowledge of the specific peptide
278 sequences that are responsible for the differences between products could be used as an
279 authentication tool to detect possible frauds in the purchase of dry-cured hams. In fact,
280 main peptides responsible for the differences detected in Principal Component 1 (PC1)
281 were three types of myosin light chain from *Sus scrofa* (MYL1f, MYL2, and MYL6B),
282 whereas main peptides responsible for differences detected in Principal Component 2
283 (PC2) were from the proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
284 myosin heavy chain-7 (MYH7) and myosin 4 (MYO4) from *Sus scrofa*.

285 **3.1 Fractionation of peptides extract using size-exclusion chromatography and**
286 **bioactivity profile**

287 The influence of proteolytic enzymes in the generation of bioactive peptides is widely
288 known. In fact, different peptide profiles are generated when differences in the action of
289 endogenous enzymes exists, which could result in important differences in the
290 bioactivity profile of the product. For this reason, size-exclusion chromatography (SEC)
291 was used to fractionate Spanish Teruel, Italian Parma, and Belgian water-soluble
292 peptide extracts in order to characterize their bioactive profile. Similar absorbance
293 intensities were obtained in all three types of European dry-cured hams as can be
294 observed in **Figure 2**, with three main separated areas. The first peak eluting would
295 contain large-size peptides whereas the last observed peak corresponds to the shortest
296 peptide fragments and free amino acids. In fact, a previous study confirmed the
297 presence of high concentrations of free amino acids at the end of curing process in

298 traditional Italian and Spanish dry-cured hams (Virgili et al., 2007; Toldrá, Aristoy &
299 Flores, 2000).
300 In **Figure 2A** (Spanish Teruel ham), an important *in vitro* ACE inhibition was observed
301 with a maximum inhibitory activity of 93% in elution volumes of 205 to 210 mL. Other
302 fractions corresponding to an elution volume from 215 mL to 250 mL had ACE
303 inhibitory activity from 39% to 84%. On the other hand, **Figure 2B** (Italian Parma dry-
304 cured hams) resulted in a main peak of ACE inhibition from 195 mL to 205 mL with a
305 maximum inhibition from 63% to 70%. Finally, **Figure 2C** (Belgian dry-cured ham)
306 describes an ACE inhibition of almost 76% at 200 mL, being the main volume showing
307 an ACE inhibition higher than 60% from 195 mL to 220 mL.
308 According to these results, Spanish Teruel dry-cured ham exhibits higher ACE
309 inhibitory activity than Parma and Belgian hams for the same range of eluted volume.
310 This fact could be due to longer curing period of Spanish Teruel hams (14 months) in
311 comparison with Italian Parma (12 months) and Belgian hams (9 months). The
312 influence of the type of processing between dry-cured hams in Italian Parma, San
313 Daniele and Toscano dry-cured hams have also been previously studied finding
314 significant differences between their sensory characteristics (Laureati et al., 2014). Also
315 differences in raw materials affect the proteolysis and generation of free amino acids as
316 has been previously described in a study showing differences in the profiles of muscle
317 proteolytic enzymes in pigs according to their weight (Toldrá, Flores, Aristoy, Virgili &
318 Parolari, 1996).
319 Regarding the proteolysis intensity in the different types of studied dry-cured hams,
320 marked increased content of free amino acids was observed in Spanish Teruel when
321 compared to Italian Parma and Belgian hams as shown in **Figure 3**, especially in
322 aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), alanine (Ala), beta-alanine

323 (bAla), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine
324 (Phe), tryptophan (Trp), and lysine (Lys). Also the content of taurine was significantly
325 higher in Teruel ham samples. However, the only significant difference detected
326 between Parma and Belgian dry-cured ham was in aspartic acid concentration. These
327 differences in the amount of amino acids can also be observed in **Figure 2,** where the
328 third main peak (volume of elution from 425 mL to 500 mL) in Teruel dry-cured ham
329 extract showed a higher absorbance at 214 nm than Parma and Belgian dry-cured hams.
330 This fact confirms the higher amount of released free amino acids and thus, the major
331 proteolysis occurred during Teruel dry-cured ham processing, probably due to the
332 intense action of muscle exopeptidases, such as aminopeptidases and carboxypeptidases
333 (Toldrá, Cerveró & Part, 1992; Mora, Sentandreu & Toldrá, 2010) that also depends on
334 processing conditions such as temperature, relative humidity and salt content, as well as
335 the length of the ripening period. In this sense, aminopeptidase acitivity has been
336 detected in meat products even after >8 months of processing, suggesting that these
337 enzymes are involved in the last period of curing (Toldrá, Aristoy & Flores, 2000).
338 The antioxidant activity of peptides is due to their free radical scavenging and reducing
339 power capacities, among others. In this study, the antioxidant activity of fractions
340 obtained after SEC separation of the deproteinized extract of Spanish Teruel, Italian
341 Parma and Belgian dry-cured hams were evaluated by the measurement of DPPH•
342 scavenging activity and Fe^{2+} reducing power ability (**Figure 4** and **5,** respectively).
343 Spanish Teruel ham showed high DPPH scavenging activity in the elution zone
344 between 200 and 250 mL with an activity from 50% to 65% (**Figure 4A**). Fractions
345 from Italian Parma ham (**Figure 4B**) also exhibited a DPPH scavenging activity in the
346 elution area from 205 to 225 mL (antioxidant activity from 50% to 65%). On the other
347 hand, **Figure 4C** shows that Belgian dry-cured ham exerted a DPPH scavenging activity

348 from 50% to 63% but only in the elution zone from 210 to 220 mL. The values obtained
349 for these three different dry-cured hams are in agreement with those reported for
350 traditional Spanish dry-cured ham (Escudero, Aristoy, Hitoshi, Arohara, & Toldrá,
351 2012; Escudero et al., 2013), although some differences were found in the percentages
352 of DPPH scavenging activity when comparing with some Spanish dry-cured ham
353 fractions. This fact could be due to differences in the processing steps and the final
354 ripening time.
355 **Figure 5** shows the values of ferric-reducing antioxidant activity of Spanish Teruel,
356 Italian Parma and Belgian dry-cured ham fractions. Higher absorbance values indicate
357 higher reducing power potential. Spanish Teruel dry-cured ham (**Figure 5A**) had a
358 maximum of absorbance ranging from 1.21 to 1.28 units of absorbance in the elution
359 area from 200 to 260 mL. Italian Parma ham fractions (**Figure 5B**) also exhibited high
360 absorbance with values from 1.12 to 1.21 units in the eluted volume from 190 mL to
361 250 mL. Finally, Belgian dry-cured ham (**Figure 5C**) showed its maximum of
362 absorbance ranging from 1.17 to 1.24 when eluted from 190 to 260 mL. All studied
363 hams showed their maximum values of antioxidant activity in the same fractions. These
364 results are in accordance to those previously reported by Escudero et al. (2013) in
365 traditional Spanish dry-cured ham. Moreover, hydrophobic fractions extracted from
366 Jinhua ham have been described to contribute to DPPH radical scavenging activity and
367 Fe^{2+} chelating activity resulting in the identification of some natural peptides sequences
368 showing antioxidant activity (Zhu et al., 2013). More recently, some studies isolated
369 and identified different antioxidant peptides from dry-cured Xuanwei ham and Jinhua
370 ham such as the sequences DLEE, GKFNV, and LPGGGHGDL which were tested
371 using DPPH radical scavenging and hydroxyl radical scavenging assays (Zhu et al,
372 2016; Xing, et al., 2016).

373 The antioxidant activity of peptides has been described to be closely related to their
374 molecular weight, amino acid composition, sequence length and hydrophobicity
375 (Escudero et al., 2013; Rajapakse, Mendis, Jung, Je & Kim, 2005). Antioxidant peptides
376 have been widely described to consist of 2-20 amino acids with molecular weights
377 lower than 6000 Da (Sarmadi & Ismail, 2010). Apart from antioxidant peptides
378 generated during processing, dry-cured hams have been described to contain
379 antioxidants that are naturally present in meat as, for example, some free amino acids,
380 dipeptides carnosine and anserine, ubiquinone, or alpha-tocopherol among others
381 (Pearson, Wolzak & Gray, 1983). The presence of these compounds would also
382 influence the antioxidant activity of the assayed dry-cured fractions. In this study,
383 fractions eluted from 200 mL in SEC corresponded to peptides smaller than 2500 Da
384 (Mora et al., 2014). According to these results, Spanish Teruel, Italian Parma and
385 Belgian dry-cured hams might be considered a considerable source of natural
386 antioxidant peptides.

387 **4. Conclusions**

388 This study revealed for the first time the differences in the generated peptides during the
389 curing processing of Spanish Teruel, Italian Parma and Belgian dry-cured hams using a
390 relative quantitation methodology based on label-free mass spectrometry techniques.
391 The obtained clusters show significant differences between the three types of hams
392 especially due to differences in genetics and processing that differ between countries.
393 The used peptidomic approach based on mass spectrometry may result very useful in
394 the differentiation between dry-cured hams because the proteolytic profile is
395 characteristic in each type of ham as well as might be related to the final quality due to
396 the close relation between genetics and final value of this product.

397 On the other hand, the bioactive profile in ACE-inhibitory, DPPH and Fe^{2+} reducing
398 power activities provided a good evidence for the added-value of dry-cured ham as a
399 natural source of antihypertensive and antioxidant peptides. Despite the observed
400 differences in the peptides profile, ACE-inhibitory activity and antioxidant capacities of
401 the assayed fractions were very similar in the three types of hams although a significant
402 increase in ACE-inhibitory activity of Spanish Teruel dry-cured ham probably due to
403 the longer period of curing used in its processing was observed.

404

405 **Acknowledgements**

406 Authors acknowledge European Union FP7 under Grant Agreement 312090
407 (BACCHUS). This publication reflects only the author views and the Community is not
408 liable for any use made of the information contained therein. JAEDOC-CSIC
409 postdoctoral contract cofounded by European Social Fund to L.M. is also acknowledged.
410 Collaboration of Dr John van Camp (Gent, Belgium) in supplying the controlled
411 samples of Belgian hams is acknowledged.

412

413 **References**

414 Aristoy, M.C., & Toldrá, F. (1991). Deproteinization techniques for HPLC amino acid
415 analysis in fresh pork muscle and dry-cured ham. *Journal of Agricultural and Food*
416 *Chemisrty*, 39 (10), 1792-1795.

417 Armero, E., Flores, M., Toldrá, F, Barbosa, J. A., Olivet, J., Pla, M., & Baselga, M.
418 (1999). Effects of pig sire type and sex on carcass traits, meat quality and sensory
419 quality of drycured ham. *Journal of the Science of Food and Agriculture*, 79, 1147–
420 1154.

421 Bermúdez, R., Franco, D., Carballo, J., Sentandreu, M.A. and Lorenzo, J.M. (2014).
422 Influence of muscle type on the evolution of free amino acids and sarcoplasmic and
423 myofibrillar proteins through the manufacturing process of Celta dry-cured ham. *Food*
424 *Research International,* 56, 226-235.

425 Bersuder, P., Hole, M., & Smith, G. (1998). Antioxidants from heated histidine-glucose
426 model system. I. Investigation of the antioxidant role of histidine and isolation of
427 antioxidants by high performance liquid chromatography. *Journal of American Oil*
428 *Chemists' Society,* 75, 181-187.

429 Escudero, E. Aristoy, M.C., Hitoshi N., Arihara, K., & Toldrá, F. (2012).
430 Antihypertensive effect and antioxidant activity of peptide fractions extracted from
431 Spanish dry-cured ham. *Meat Science,* 306-311.
432 Escudero, E., Mora, L., Fraser, P.D., Aristoy, M.C., & Toldrá, F. (2013). Identification
433 of novel antioxidant peptides generated in Spanish dry-cured ham. *Food Chemistry,* 138,
434 1282-1288.

435 Flores, M., Aristoy,M.C., Spanier, A.M., & Toldrá, F. (1997). Non-volatile components
436 effects on quality of "Serrano" dry-cured ham as related to processing time. *Journal of*
437 *Food Science*, 62, 1235–1239

438 Hernández, P., Zomeño, L., Ariño, B., & Blasco, A. (2004). Antioxidant, lipolytic and
439 proteolytic enzyme activities in pork meat from different genotypes. *Meat Science*, 66,
440 525–529.

441 Huang, S.J., Tsai, S.Y., & Mau, J.L. (2006). Antioxidant properties of methanolic
442 extract from Agrocybe cylandrea. *LWT-Food Science and Technology,* 39, 378-386.

443 Laureati, M.; Buratti, S.; Giovanelli, G.; Corazzin, M.; Lofiego, D., & Pagliarini, E.
444 (2014). Characterization and differentiation of Italian Parma, San Daniele and Toscano
445 dry-cured hams: a multidisciplinary approach. *Meat Science,* 96, 288-294.

446 Mora, L.; Sentandreu, M.A., & Toldrá, F. (2010). Identification of small troponin
447 peptides generated in dry-cured ham. *Food Chemistry,* 123, 691-697.

448 Mora, L., Escudero, E., Fraser, P.D., Aristoy, M.C., & Toldrá, F. (2014). Proteomic
449 identification of antioxidant peptides from 400 to 2500 Da generated in Spanish dry-
450 cured ham contained in a size-exclusion chromatography fraction. *Food Research*
451 *International,* 56, 68-76.

452 Mora, L., Gallego, M., Aristoy, MC., Fraser, PD., & Toldrá, F. (2015). Peptides
453 naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of
454 dry-cured ham processing time. *Food Control*, 48, 102-107.

455 Mora, L., Escudero, E., Arihara, K., & Toldrá, F. (2015). Antihypertensive effect of
456 peptides naturally generated during Iberian dry-cured ham processing. *Food Research*
457 *International,* 78, 71–78.

458 Mora, L., Calvo, L., Escudero, E., & Toldrá, F. (2016). Differences in pig genotypes
459 influence the generation of peptides in dry-cured ham processing. *Food Research*
460 *International*, 86, 74–82.

461 Pearson, A.M., Wolzak, A.M., & Gray, J. I. (1983). Possible role of muscle protein in
462 flavor and tenderness of meat. *Journal of Food Biochemistry,* 7, 189-210.

463 Petrova, I., Aasen, I.M., Rustad, T.,& Eikevik, T.M. (2015). Manufacture of dry-cured
464 ham: a review. Part 1. Biochemical changes during the technological process. *European*
465 *Food Research and Technology,* 241 (5), 587–599.

466 Rajapakse, N., Mendis, E., Jung, W.K., Je, J.Y., & Kim, S.K. (2005). Purification of
467 radical scavenging peptide from fermented mussel sauce and its antioxidant properties.
468 *Food Res. Int.* 38, 175-182.

469 Sarmadi, B.H. & Ismail, A. (2010). Antioxidative peptides from food proteins: A
470 review. *Peptides*, 31, 1949-1956

471 Sentandreu, M. A., & Toldrá, F. (2007). Evaluation of ACE inhibitory activity of
472 dipeptides generated by the action of porcine muscle dipeptidyl peptidases. *Food*
473 *Chemistry,* 102, 511-515.

474 Toldrá, F. & Flores, M. (1998). The role of muscle proteases and lipases in flavor
475 development during the processing of dry-cured ham. *Critical Reviews in Food Science*
476 *and Nutrition*, 38 (4), 331–352.

477 Toldrá, F.; Aristoy, M-C., & Flores, M. (2000). Contribution of muscle aminopeptidases to
478 flavor development in dry-cured ham. *Food Research International,* 33, 181-185.

479 Toldrá, F., Cerveró, M-C., & Part, C. (1992). Porcine aminopeptidase activity as affected
480 by curing agents. *Journal of Food Science,* 58, 724-726.

481 Toldrá, F., Flores, M., Aristoy, M.C., Virgili, R.,& Parolari, G. (1996). Pattern of
482 muscle proteolytic and lipolytic enzymes from light and heavy pigs. *Journal of the*
483 *Science of Food and Agriculture*, 71, 124-128.

484 Virgili, R., Saccani, G., Gabba, L., Tanzi, E., & Soresi, B.C. (2007). Changes of free
485 amino acids and biogenic amines during extended aging of Italian dry-cured ham. *LWT-*
486 *Food Science and Agriculture*, 40, 871-878.

487 Xing, L-J., Hu, Y-Y., Hu, H-Y., Ge, Q-F., Zhou, G-H., & Zhang, W-G. (2016).
488 Purification and identification of antioxidative peptides from dry-cured Xuanwei ham.
489 *Food Chemistry*, 194, 951–958.

490 Zhu,C-Z., Zhang, W-G., Zhou, G-H., & Xu, X-L. (2016). Identification of antioxidant
491 peptides of Jinhua ham generated in the products and through the simulated
492 gastrointestinal digestion system. *Journal of the Science of Food and Agriculture*, 96,
493 99–108.

494 Zhu, C-Z., Zhang, W.-G., Zhou, G.-H., Xu, X.-L., Kang, Z.-L., & Yin, Y. (2013).
495 Isolation and Identification of Antioxidant Peptides from Jinhua Ham. *Journal of*
496 *Agricultural and Food Chemistry*, 61, 1265−1271

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501 **FIGURE CAPTIONS**

502 **Figure 1. A)** Principal component analysis (PCA) score plot to assess the variance
503 among the naurally generated peptides in three different types of European dry-cured
504 hams (n=10). PC1 is responsible for 26.2 % of the variability of the dataset whereas
505 PC2 explains 18 % of the variance within the dataset. **B)** Loading plot of PCA. Each
506 point corresponds to one identified peptide. MLC1f: myosin light chain 1f; MYL2:
507 myosin light chain 2; MYL6B: myosin light chain 6B; MYH7: myosin heavy chain 7;
508 MYO4: myosin 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

509 **Figure 2.** Fractionation of dry-cured ham extracts on Sephadex G-25 gel filtration
510 column. Fractions were collected and assayed for *in vitro* ACE inhibitory activity of: A)
511 Spanish Teruel ham, B) Italian Parma ham and C) Belgian ham.

Figure 3. Free amino acids content extracted from Spanish Teruel ham, Italian Parma ham and Belgian ham. Means of 3 samples (mg free amino acids/100 g dry-cured ham) ± s.d. Bars with different letters are significantly different at $P<0.05$.

Figure 4. Fractionation of dry-cured ham extracts on Sephadex G-25 gel filtration column. Fractions were collected and assayed for DPPH radical scavenging ability of: A) Spanish Teruel ham, B) Italian Parma ham and C) Belgian ham.

Figure 5. Fractionation of dry-cured ham extracts on Sephadex G-25 gel filtration column. Fractions were collected and assayed for reducing power of: A) Spanish Teruel ham, B) Italian Parma ham and C) Belgian ham.

Figure 1

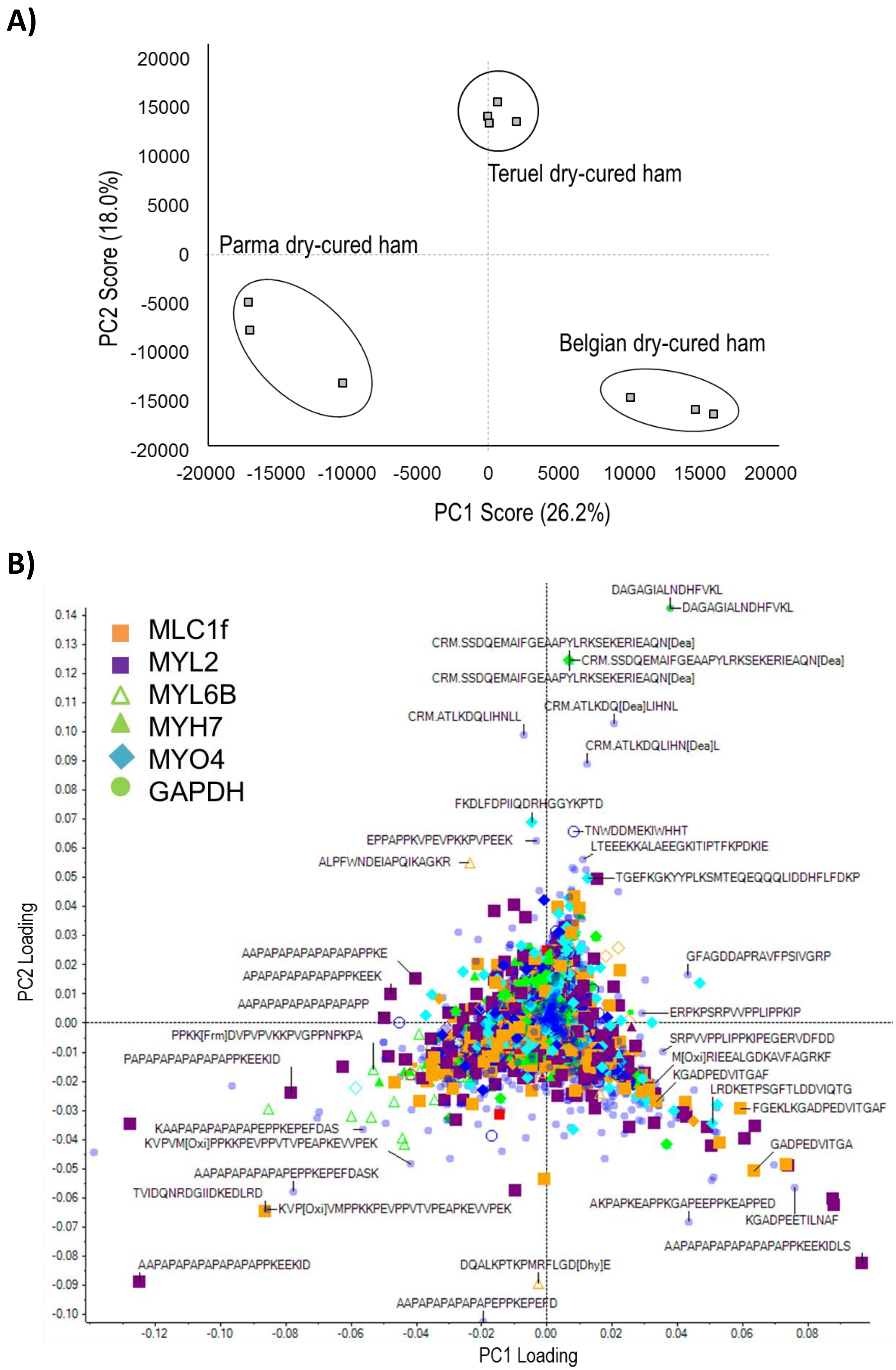


Fig. 1.

Figure 2

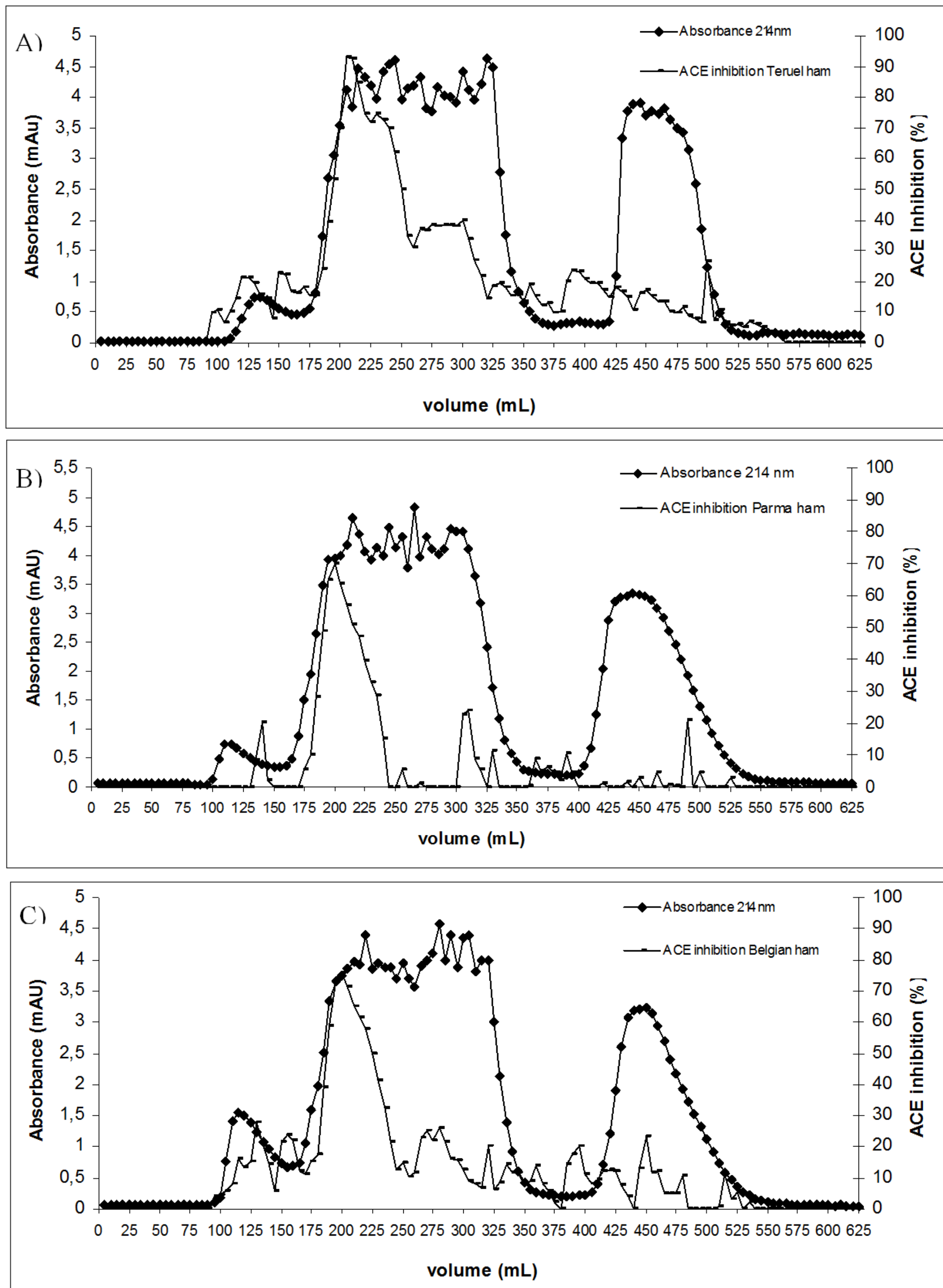


Fig.2.

Figure 3

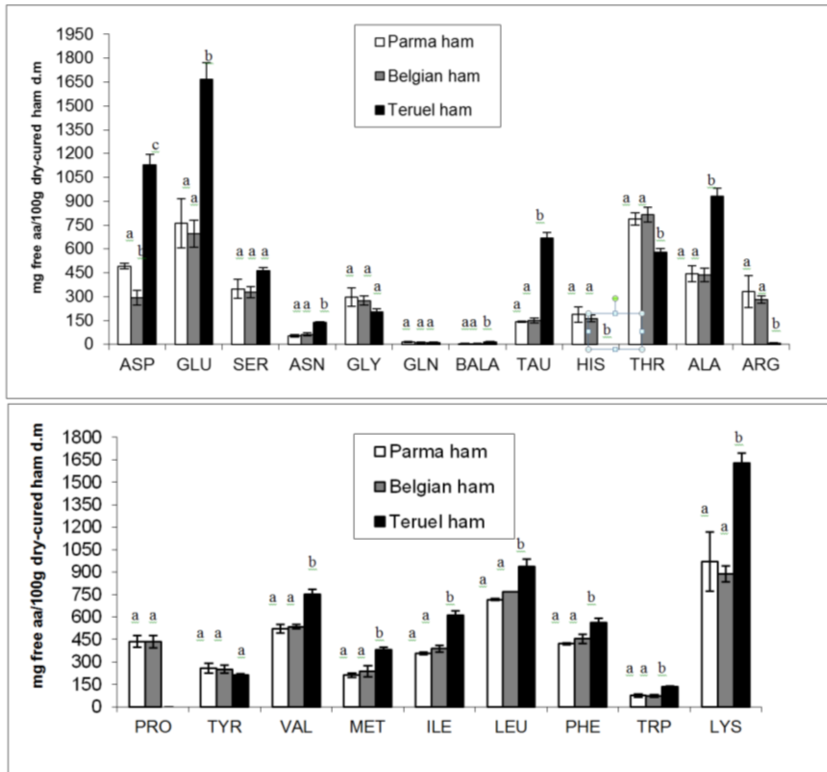
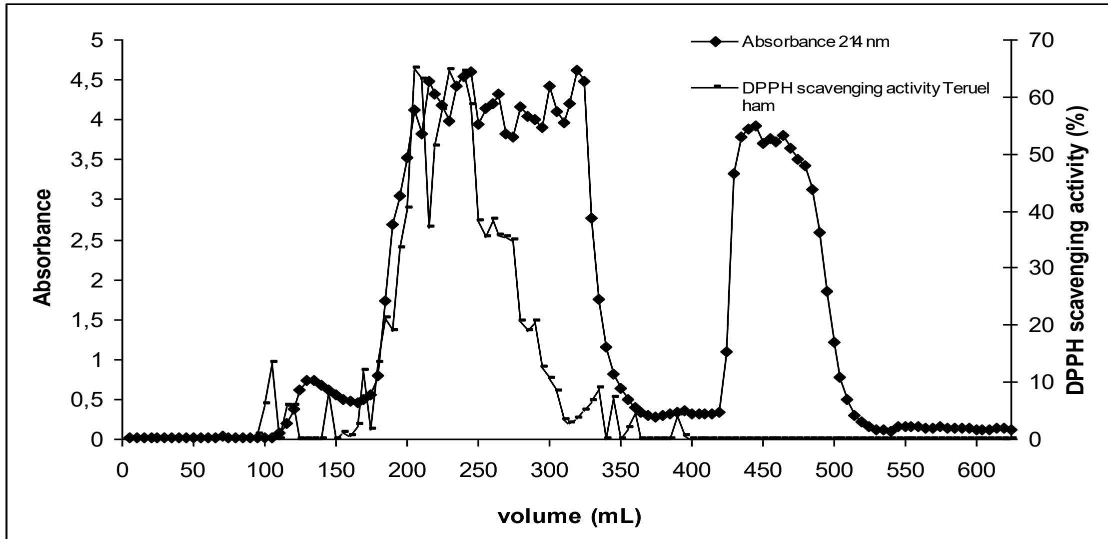


Fig.3.

Figure 4

A)



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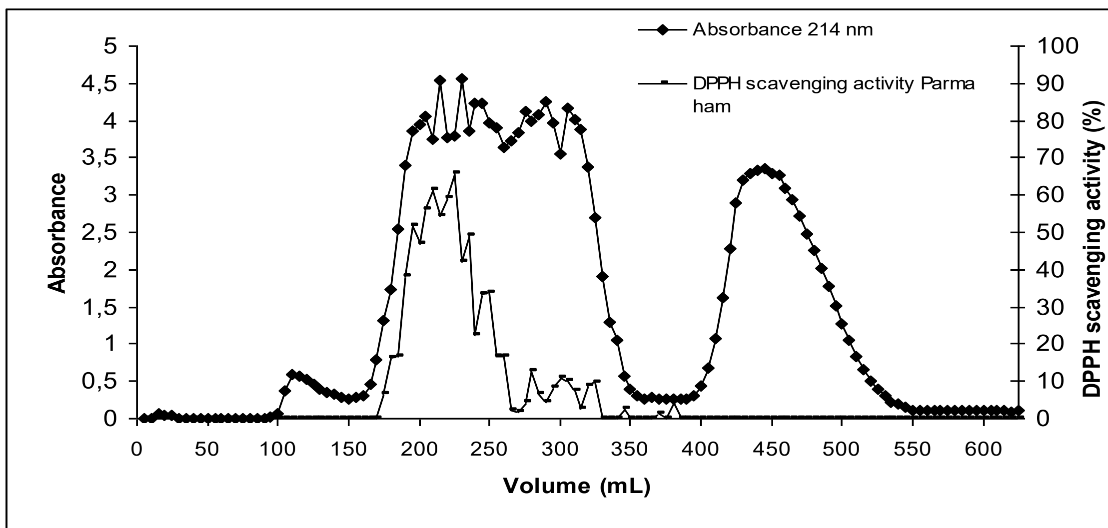

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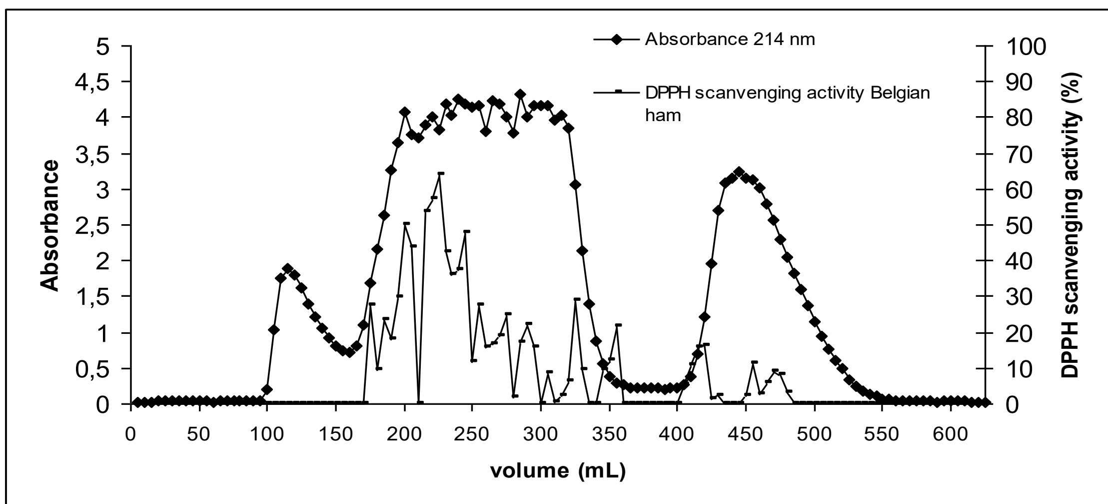
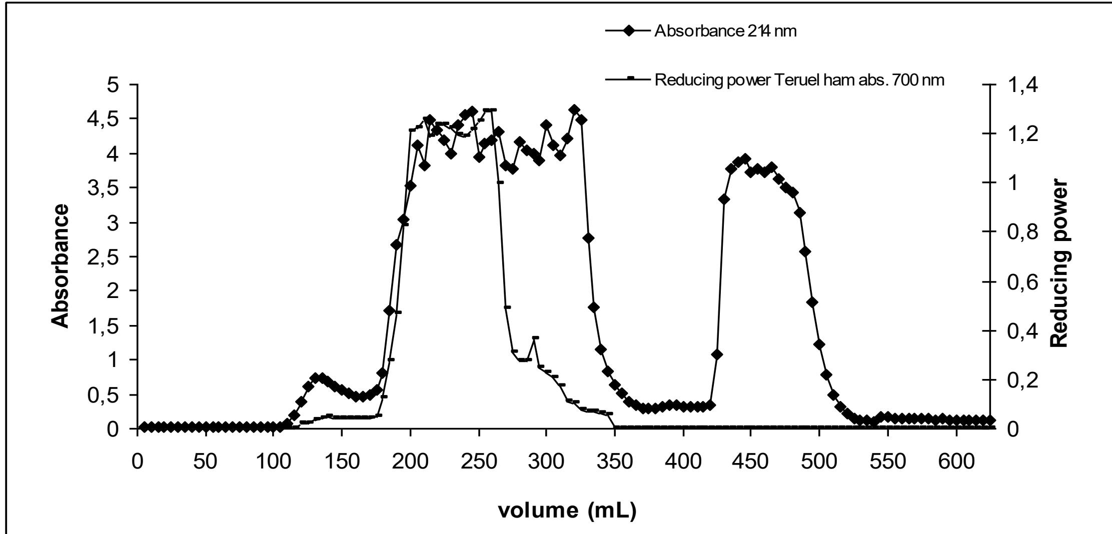


Fig.4.

Figure 5

A)



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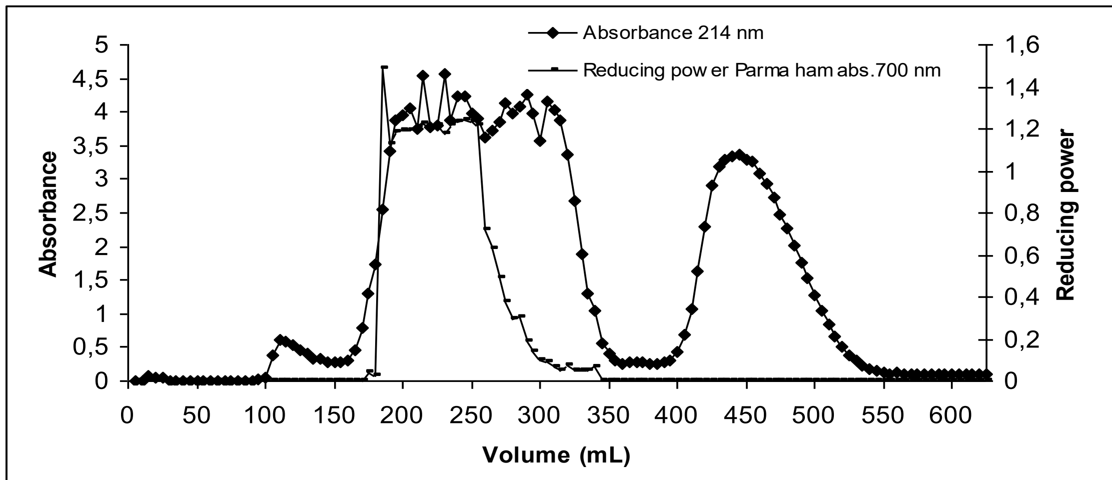

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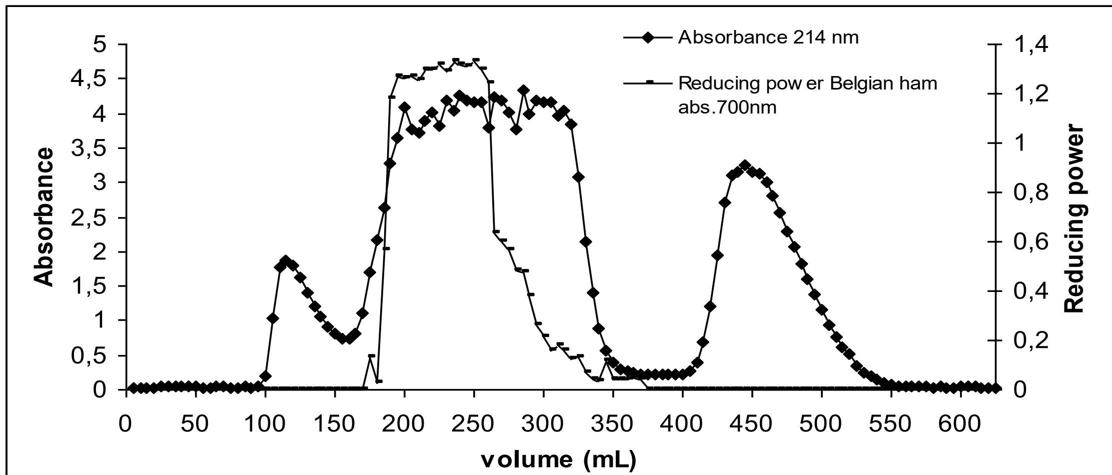

Fig.5.