Bioactive peptides and free amino acids profiles in different types of European dry-fermented sausages

Marta Gallego, Leticia Mora, Elizabeth Escudero, and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenue Agustín Escardino 7, 46980, Paterna (Valencia), Spain

* Corresponding author: Tel: +34963900022 ext.2112; fax: +34963636301.

E-mail address: ftoldra@iata.csic.es

Abstract

A wide variety of dry-fermented sausages are produced in European countries, where are considered valued traditional products. An intense proteolysis takes place during the processing of dry-fermented sausages due to the combined action of muscle and microbial peptidases, generating large amounts of peptides and free amino acids. These compounds participate in the development of the characteristic flavour of dry-fermented products, but some peptides can also exert certain bioactivities such as antioxidant and ACE inhibitory activities. This study has evaluated the changes in peptide profile and amino acid contents of three European dry-fermented sausages produced in Spain, Italy and Belgium, proving the intense degradation of proteins, mainly myofibrillar, and the generation of high amounts of different size peptides and free amino acids. The changes observed between the profiles of European sausages could be due to differences in product formulation, processing conditions and starter cultures used, which influence the activity of enzymes, both from muscle and bacterial origin. On the other hand, the bioactivity profile of each type of dry-fermented sausage was evaluated through the measurement of the ACE inhibitory and antioxidant activities in water-soluble peptide extracts fractionated by size-exclusion chromatography. Spanish and Belgian dry-fermented sausages showed values of ACE inhibition around 85%, whereas Belgian samples presented the highest DPPH radical-scavenging activity and ferric reducing power capacity. These results evidence the potential of Spanish, Italian and Belgian dry-fermented sausages as natural sources of bioactive peptides, giving an added-value to these traditional products.

Keywords: Proteolysis, enzymes, bioactive peptides, ACE inhibition, antioxidant, dry-fermented sausages.

1. Introduction

Traditional fermented meat products have a great importance and economic impact in Europe, where they have gained a renewed popularity. A great variety of dry-fermented sausages can be found depending on raw material, formulations, processing conditions, and starter culture. The sensory quality of these products is determined by the formation of end products originated from the breakdown of proteins, lipids and carbohydrates, in which participate both endogenous muscle enzymes and microbial enzymes (Fernández et al., 2000; Ordóñez et al., 1999).

The use of starter cultures for the production of fermented foods guarantees safety and desirable technological properties. Lactic acid bacteria (LAB) and staphylococci are typical in European dry-fermented sausages and they have been described to be the most active microorganisms in the acidification and denitrification processes, lipolysis and proteolysis (Flores and Toldrá, 2011; Molly et al., 1997; Ordóñez et al., 1999). In fact, the use of well-selected strains could permit to achieve improved sensory qualities and technological advantages. For example, *S. carnosus* or *S. xylosus* strains show specific peptide uptake systems and branched-chain amino acid converting and fatty acid oxidising activities, so they could be used as functional starter cultures to obtain tastier end-products (Leroy et al., 2006). Some strains of *S. carnosus* have also been described to exert antioxidant properties that may prevent food spoilage, whereas other bacteria can generate probiotics and health-promoting molecules. On the other hand, LAB can produce a diversity of bacteriocins, which are antibacterial peptides that kill or inhibit the growth of other bacteria and foodborne pathogens for food preservation (Leroy et al., 2006; Rahman et al., 2017).

Proteolysis is one of the main mechanisms that take place during the ripening of dry-cured meats. It is generally believed that muscle peptidases, mainly cathepsins, initiate

proteolysis and play an important role in the breakdown of proteins throughout the processing, while microbial enzymes participate less intensely and mainly during the latter stages of the ripening (Berardo et al., 2017; Casaburi et al., 2008; Fadda et al., 1999). As a result, large amounts of peptides of different sizes and free amino acids are naturally generated, which are related not only to sensory characteristics of the final product but also to other properties and functionalities such as biological activities (Toldrá et al., 2017). Numerous studies have described the generation of peptides with antioxidant and antihypertensive activities in different types of European dry-cured hams (Dellafiora et al., 2015; Escudero et al., 2012; Mora et al., 2015a, 2016); however, information on bioactive peptides in European dry-fermented sausages is still limited. To our knowledge, a few studies have evaluated the influence of different processing conditions on the generation of antioxidant and ACE inhibitory peptides in different types of Spanish dry-fermented sausages (Fernández et al., 2016a,b; Mora et al., 2015b), whereas other appreciated European sausages have scarcely been studied.Considering this fact, the purpose of this study was the characterisation of the peptide and free amino acid profiles of three different types of dry-fermented sausages produced in Spain, Italy and Belgium, and the evaluation of their potential as natural sources of ACE-I inhibitory and antioxidant peptides.

2. Materials and methods

2.1 Reagents

The chemicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, ferric chloride, angiotensin-I converting enzyme (ACE-I) from rabbit lung, captopril, and amino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO,USA). o-Aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO_2)-Pro-OH)

trifluoroacetate salt was from Bachem AG. (Bubendorf, Switzerland) and butylated hydroxytoluene (BHT) was from Panreac Quimica SAU (Barcelona, Spain). Triethylamine (TEA) and phenyl isothiocianate (PITC) were obtained from Fluka (Sigma-Aldrich, Co., St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

2.2 Spanish, Italian and Belgian dry-fermented sausages

Spanish dry-fermented sausages were prepared using 75% of lean pork and 25% of pork back fat, sodium chloride (27 g/kg), dextrin (20 g/kg), glucose (7 g/kg), sodium ascorbate (0.5 g/kg), sodium nitrite (0.15 g/kg), and potassium nitrate (0.15 g/kg). Dry-fermented sausages were inoculated with a starter culture containing *Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus xylosus* and *Staphylococcus carnosus*. Traditional fermentation was carried out for 24 hours at a temperature of 3-5 ºC whereas the ripening was developed by maintaining the sausages at 10 ºC under a relative humidity (RH) of 70-85% for 60 days.

Italian dry-fermented sausages were prepared using a mixture of 74% pork meat and 26% pork fat, sodium chloride (25 g/kg), dextrose (20 g/kg), sodium ascorbate (0.5 g/kg), sodium nitrite (0.15 g/kg), potassium nitrate (0.15 g/kg), and a starter culture containing *Lactobacillus sakei* and a mixture of *Staphylococcus xylosus* strains. The sausages were fermented for 3 days at 4 °C, and then ripened in two steps: 5 days at decreasing temperatures from 25 °C to 15 °C and RH from 90% to 65%, and 15 days at 13 °C and 75-80% of RH.

Belgian dry-fermented sausages were prepared by mixing 73% lean pork meat and 27% fat with the curing agents sodium chloride (25 g/kg), dextrose (25 g/kg), sodium ascorbate (0.5 g/kg), sodium nitrite (0.15 g/kg), and a starter mixture containing *Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus*. Fermentation was performed at 24

ºC and 95% RH for 4 days, and the drying process was set at 12 ºC and 95% of RH for 15 days followed by 10 days at 80% of RH.

The study was performed from three samples of each type of European dry-fermented sausage. All analyses were done in triplicate.

2.3 Peptides extraction

The extraction of peptides was done according to the methodology described by Mora et al. (2015b). So, fifty grams of each type of dry-fermented sausage were defatted, minced and homogenised in 0.01 N HCl using a stomacher. After centrifugation (12000 *g*, 20 min, 4 ºC), samples were deproteinised by adding 3 volumes of ethanol (20 h, 4ºC) and centrifuged again. The aqueous fraction of the mixture was freeze-dried and thendissolved in 25 mL of 0.01 N HCl, filtered, and stored at -20 ºC until use.

2.4 Peptides separation by size-exclusion chromatography

Peptides from dry-fermented sausage extracts were fractionated according to their molecular masses by size-exclusion chromatography (SEC). The column was calibrated using molecular weight standards including bovine serum albumin (70 kDa), cytochrome C (13 kDa), bacitracin (1.42 kDa), carnosine (0.22 kDa) and tyrosine (0.2 kDa). Then, 5 mL of each deproteinised extract were injected on a column (2.5 x 65 cm) packed with a Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden) stationary phase. Separation was performed at a constant flow rate of 15 mL/h with 0.01 N HCl at 4 ºC. Fractions of 5 mL were collected using an automatic fraction collector, monitored at 214, 254, and 280 nm (Cari 60 UV spectrophotometer, Agilent Technologies, Palo Alto, CA) and lyophilised. Finally, samples were dissolved in 2 mL bidistilled water and stored at -20 ºC until next analysis.

155 **2.5 Mass spectrometry analysis**

156 **2.5.1 Peptide-mass mapping by MALDI-ToF MS**

157 The characterisation of the profile of peptides was performed by matrix-assisted laser
158 desorption/ionization time-of-flight mass spectrometry using a 5800 MALDI-TOF/TOF
159 (AB Sciex, MA, USA). Dry-fermented sausage extracts were diluted (1:10) in water-
160 acetonitrile (95:5) with 0.1% trifluoroacetic acid and then analysed according to the
161 procedure described by Gallego et al. (2015), with slight modifications. A total of 1 µL
162 of the peptide mixture and 0.5 µL of matrix solution (5 mg/mL of α-cyano-4-
163 hydroxycinnamic acid (HCCA); Bruker Daltonics, Germany) were spotted onto the
164 MALDI target plate and air-dried. The resulting mixture was analysed in automatic
165 positive mode in two mass ranges: 200-900 *m/z* and 900-3500 *m/z*. Spectra were obtained
166 from 3000 shots in every position with a laser intensity of 3500, and the system was
167 adjusted with voltages of 15 and 3 kV in the source and reflector detector, respectively.
168 The obtained spectra were analysed using mMass v5.5 – Open Source Mass Spectrometry
169 Tool software (Niedermeyer and Strohalm, 2012).

170 **2.5.2 Peptide identification by nLC-MS/MS**

171 The identification of the peptides was done using a nano-LC Ultra 1D Plus system
172 (Eksigent of AB Sciex, CA, USA) with a quadrupole/time-of-flight (Q/ToF) TripleTOF®
173 5600+ system (AB Sciex Instruments, MA, USA) and using a nanoelectrospray ionisation
174 (ESI) source. An aliquot of 20 µL of each peptide extract was cleaned and concentrated
175 using ZipTip C18 with standard bed format (Millipore Corporation, Bedford, MA)
176 according to manufacturer's guidelines. The analysis was done according to the procedure
177 described by Mora et al. (2016). A total of 5 µL of each sample was injected into the
178 system, where it was cleaned and concentrated using a C18 trap column (350 µm x 0.5mm,
179 3 µm) and then peptides were separated in a nano-HPLC capillary column (3µm, 75µm

180 x 12.3 cm, C18). Solvent A was 0.1% formic acid in water, and solvent B was 0.1%
181 formic acid in acetonitrile. Chromatographic conditions were a linear gradient from 5%
182 to 35% of solvent B during 120 min at a flow rate of 0.3 µL/min and running temperature
183 of 30 ºC.
184 Data was acquired using an ion spray voltage of 2.9 kV, curtain gas of 20 psi, nebulizer
185 gas of 6 psi, and an interface heater temperature of 150 °C. The Q/ToF was used in
186 positive polarity and data-dependent acquisition mode. MS1 scans were acquired from
187 *m/z* of 150 to 1250 for 250 ms and MS2 scans from *m/z* of 100 to 1500 for 50 ms on the
188 50 most intense 2-5 charged ions. Data were processed using ProteinPilot™ v5.0 software
189 (AB Sciex, MA, USA) for the identification of peptides. The Paragon algorithm of
190 ProteinPilot was used to search in Uniprot database with no enzyme specificity and
191 including all possible post-traductional modifications.

192 **2.6 Free amino acids analysis**

193 **2.6.1 Preparation of dry-fermented sausage extracts**

194 An extract from dry-fermented sausage sample of each nationality was prepared by
195 mixing 5 g of sample in 25 mL of HCl 0.01 N, homogenising in stomacher for 8 min, and
196 cooled in ice. Then, the samples were centrifuged (10.000 *g*, 20 min, 4 ºC) and the
197 supernatant filtered through glass wool prior further analysis. The amino acid standard
198 solutions were made by dissolving the amino acids tested to a final concentration of 1
199 mM each in HCl 0.1 N.

200 **2.6.2 Amino acids analysis**

201 Determination of amino acid composition in samples was done according to the Pico Tag
202 procedure (Aristoy and Toldrá, 1991). The extracts were deproteinised by adding 3
203 volumes of acetonitrile and centrifuged (10000 g, 3 min). The amino acid content of the
204 supernatant was determined by reversed phase high performance liquid chromatography

(HPLC) (Agilent 1200 series, Agilent Technologies, Palo Alto, CA), as described by Bidlingmeyer et al. (1987). Norleucine was added as internal standard, and PITC derivatives were separated using a Pico Tag C18 column (300 × 4 mm, 5 μm; Waters, Wexford, Ireland).Measurements were done in duplicate.

2.7 ACE-I inhibitory activity

The ACE-I inhibitory activity was measured in the collected SEC fractions from the different dry-fermented sausages according to the method developed by Sentandreu and Toldrá (2007). This assay is based on the ability of ACE to hydrolyse the internally quenched fluorescent substrate Abz-Gly-Phe(NO_2)-Pro. A total of 50 μL of each sample was mixed with 50 μL of ACE solution (3mU/mL in 150 mM Tris-base buffer, pH 8.3). The reaction was initiated by the addition of 200 μL of substrate (0.45 mM in 150 mM Tris-base buffer with 1.125 mM NaCl, pH 8.3), and incubated for 45 min at 37 ºC. The generation of fluorescence due to the release of *o*-aminobenzoylglycine (Abz-Gly) by the action of ACE was measured using excitation and emission wavelengths of 355 and 405 nm, respectively. Bidistilled water was used as negative control and captopril as positive control. All measurements were done in triplicate and results were expressed as ACE inhibition percentage.

2.8 Antioxidant activities

2.8.1 DPPH radical-scavenging assay

The DPPH radical-scavenging activity of the collected fractions was determined as described by Bersuder et al. (1998). Thus, 50 μL of each fraction were mixed with 250 μL of ethanol and 62.5 μL of the DPPH solution (0.02% in ethanol). The mixtures were incubated during 60 min in the dark at room temperature, and the reduction of DPPH radicals was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Bidistilled water was used as negative control and

BHT as positive control. DPPH radical-scavenging activity was calculated as: DPPH radical-scavenging activity percentage (%) = [(Control absorbance - Sample absorbance) / Control absorbance] x 100. Measurements were done in triplicate.

2.8.2 Ferric-reducing antioxidant power (reducing power)

The reducing power was determined by measuring the ability to reduce ferric iron to ferrous iron (Huang et al., 2006). Briefly, 250 µL of each fraction were mixed with 250 µL of sodium phosphate buffer (200 mM, pH 6.6) and 250 µL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50 ºC for 20 min and then, 250 µL of trichloroacetic acid (100 mg/mL) were added. After centrifugation at 200 *g* for 10 min, 500 µL of the upper layer was mixed with 500 µL of bidistilled water and 100 µL of ferric chloride (1 mg/mL). The absorbance was immediately measured at 700 nm, and higher absorbance values indicate higher reducing power. Bidistilled water was used as negative control and BHT as positive control. Measurements were done in triplicate.

2.9 Statistical analysis

Statistical analysis was performed with XLSTAT v5.01 from Excel software (Microsoft, 2011), including one-way ANOVA and Fisher's multiple range tests to analyse significant differences among mean values at $p < 0.05$.

3. Results and discussion

3.1 Characterisation of the peptide profiles

The water-soluble extracts from Spanish, Italian and Belgian dry-fermented sausages were fractionated by SEC according to the molecular mass of the peptides, measuring the absorbance of each fraction at 214, 254, and 280 nm (**Fig. 1)**. Peptide profiles at 214 nm showed three differentiated areas in the three types of European samples, corresponding to elution volumes from 100 to 175 mL, 180 to 375 mL, and 375 to 625 mL. The first

eluted peak would contain the peptide fragments with molecular weights higher than 1.5 kDa, whereas the last peak would correspond to small-size peptides (< 0.5 kDa) and free amino acids. Main differences between profiles were found in the first peak as the Spanish dry-fermented sausage presented the highest amount of large-size peptides (**Fig. 1A**), followed by the Belgian sample (**Fig. 1C**). The measurements of absorbance at 254 and 280 nm would indicate the presence of aromatic amino acids, observing small differences between samples.

The pattern of proteolysis in the different dry-fermented sausages would be determined by different variables such as product formulation, processing conditions and microbial population. It is well known that the combined action of both endogenous and microbial enzymes during sausage ripening generates high amount of peptides of different sizes (Hughes et al., 2002; Martín et al., 2007; Sun et al., 2009). In this regard, a MALDI-ToF MS analysis was performed in order to better evaluate the degree of proteolysis and determine the amount and molecular mass of the peptides generated during the dry-fermented processing of each sausage (**Fig. 1 of Supplementary material**). Two different ranges of masses (from 200 to 900 *m/z* and from 900 to 3500 *m/z*) were measured to improve the sensitivity of the analysis. Similar profiles were found in Spanish, Italian and Belgian dry-fermented sausages, showing a broad distribution of peptides in a wide range of molecular masses. In this regard, it has been reported that low molecular mass peptides (< 3 kDa) and free amino acids contribute, directly or indirectly, to the generation of flavour compounds in dry-fermented sausages (Fadda et al., 2010).

The peptide profiles were fully characterised through the identification of peptides by nLC-MS/MS analysis. More than one thousand peptides were identified in Spanish, Italian and Belgian dry-fermented sausages being originated from more than 70 different proteins. **Fig. 2** shows the percentage of peptides identified in each sample from main

proteins of origin, which were mainly myofibrillar proteins such as troponin, actin, myosin, and titin. Similar results were found in previous studies using a mixed starter culture of *Lactobacillus* and *Staphylococcus* strains for the production of dry-fermented sausages (Aro et al., 2010; Hughes et al., 2002; Mejri et al., 2017). These studies evidenced an intense degradation of myofibrillar proteins throughout the ripening due to the action of both muscle and bacterial enzymes, whereas sarcoplasmic proteins were mainly degraded by endogenous muscle peptidases during fermentation. In contrast, some studies reported higher degradation of sarcoplasmic proteins than myofibrillar proteins when only lactobacilli strains were used as starters, but also the technological conditions during sausage manufacture were determinant factors in protein degradation (Castellano et al., 2013; López et al., 2015). In the present work, the distribution of peptides (expressed as percentages) was very similar in Spanish and Italian samples, whereas 44% of the identified peptides in Belgian dry-fermented sausage came from titin protein. In this sense, some works have described the intense degradation of titin throughout the processing of dry-cured meat products, generating large amounts of peptides due to the huge size of this protein (> 3 MDa of molecular mass) (Gallego et al., 2015; Mora et al., 2015b). In addition, **Table 1 of Supplementary material** shows the sequences of the peptides derived from actin, myosin, titin and troponin proteins and the dry-fermented sausage in which they have been identified. Results evidenced the hydrolysis of peptides by the action of exopeptidases, either of muscle or microbial origin, generating shorter peptides and free amino acids as final outcome of proteolysis (Mora et al., 2015c; Toldrá et al., 1993).

The contents of free amino acids in the three types of European dry-fermented sausages were determined by HPLC. Results are shown in **Fig. 3**, obtaining total concentrations of 1854, 2235, and 2414 mg/100g dry matter, in Spanish, Italian and Belgian dry-fermented

sausages, respectively. In general, Glu, Tau, Ala, Leu and Lys were the most abundant amino acids in the three samples. When comparing between samples, the contents of Glu, Ser, Asn and Orn were significantly higher in the Belgian sausage, emphasizing the high amount of Glu amino acid (402.7 mg/100g). Italian and Belgian samples showed a higher content of amino acids Asp, His and Thr than Spanish sausage, which presented the highest amount of Gln, Arg and Tyr. On the other hand, the amino acid Arg was not found in Italian and Belgian samples probably because it is metabolised by bacteria in a higher extent than its production during the ripening (Ordóñez et al., 1999). In fact, the use of Arg through the arginine deiminase (ADI) pathway of *L. sakei* has been reported to participate in its adaptation to meat (Zagorec and Champomier-Vergès, 2017). Certain ADI activity was also observed in *S. carnosus*, whereas an alternative pathway based on arginase activity has been described for strains of *S. xylosus* (Sánchez Mainar et al., 2017). Differences between European sausages might be explained by the fact that the activity of enzymes, either of muscle and bacterial origin, is influenced by processing parameters such as the temperature, relative humidity, length of fermentation and ripening times, changes in pH, content of salt, nitrate and nitrite, and type and content of carbohydrates (Stahnke and Tjener, 2007; Toldrá, 2002). According to Díaz et al. (1997), the higher temperatures applied during the fermentation stage compared to the drying process could generate a major release of free amino acids. However, most studies evidenced an increase in the content of amino acids during ripening as the activity of microbial enzymes in the latter stage of the dry-fermented sausage processing results in a significant amino acids release (Aro et al., 2010; Hierro et al., 1999; Hughes et al., 2002). Considering the starter culture used in the dry-fermented sausages studied in this work, *L. sakei* has been described to exert high exopeptidase activity by dipeptidase, tripeptidase, aminopeptidase, x-prolyl-dipeptidylpeptidase, and arginine aminopeptidase enzymes, producing a large

330 release of free amino acids, mainly Leu and Ala (Flores and Toldrá, 2011). Coagulase
331 negative staphylococci such as *S. xylosus* and *S. carnosus* have been reported to exert, in
332 general, important proteolytic activity and conversion of amino acids in aroma
333 compounds (Mauriello et al., 2004; Sánchez Mainar et al., 2017).

334 **3.2 Bioactivity profiles of dry-fermented sausages**

335 In order to evaluate the bioactivity of the water-soluble peptide extracts from Spanish,
336 Italian and Belgian dry-fermented sausages, the ACE inhibitory and antioxidant activities
337 were measured in each fraction collected from SEC (**Fig. 4**).

338 Maximum values of ACE inhibitory activity were detected in Spanish and Belgian dry-
339 fermented sausages, both showing inhibition values around 85% in the elution volume of
340 215 mL **(Fig. 4A and 4C)**. On the other hand, Italian sample reached 53 % of activity in
341 fractions corresponding to elution volumes of 115 mL and 215 mL (**Fig. 4B)**, being the
342 sample with the lowest ACE inhibitory activity. In Belgian sample **(Fig. 4C)** is also noted
343 the ACE inhibition activity (up to 30 %) observed in the last area of the profile containing
344 low-size peptides and amino acids. In this regard, Pro, Arg, Phe, Tyr and Lys have been
345 described as common residues present in antihypertensive peptides (Mejri et al., 2017;
346 Mora et al., 2015c). A previous study carried out by Vaštag et al. (2010) evidenced an
347 increased proteolysis and ACE inhibitory activity in water-soluble extracts from Petrovac
348 sausage during ripening, reaching around 75% inhibitory activity after 90 days of
349 processing. Castellano et al. (2013) demonstrated the release of ACE inhibitory peptides
350 from porcine sarcoplasmic proteins using LAB, whereas Mora et al. (2015b) studied the
351 contribution of added casein protein to the generation of ACE inhibitory peptides in dry-
352 fermented sausages with *L. pentosus* and *S. carnosus.* On the other hand, Mejri et al.
353 (2017) revealed an increased ACE inhibitory activity during ripening in both inoculated
354 and non-inoculated dry-fermented camel sausages, being fractions with peptides below 3

355 kDa those showing the highest activity in all cases. The highest antihypertensive activity
356 in the inoculated sausages evidenced the participation of bacteria in the generation of
357 peptides showing ACE inhibition that depends on the type of culture used (Mejri et al.,
358 2017).
359 Antioxidant activity was assayed by the measurement of DPPH radical-scavenging
360 activity and reducing power, which are rapid and simple methods to measure the ability
361 of a potential antioxidant to transfer one electron for reducing an oxidant (Huang et al.,
362 2005). The peptide fractions extracted from Spanish dry-fermented sausages (**Fig. 4A**)
363 showed the highest antioxidant activity in the eluted volume between 230 mL and 265
364 mL, with maximum radical-scavenging activity ranging from 55% to 68% and values of
365 1.32 units of absorbance at 700 nm for reducing power activity. Italian dry-fermented
366 sausages (**Fig. 4B**) exerted an average DPPH activity of 55% in the fractions from 205
367 mL to 240 mL, with a maximum of 58.2% in the 205 mL fraction. The reducing power
368 assay showed values ranging from 1.12 to 1.35 units in the eluted volume from 205 to
369 270 mL. Regarding Belgian dry-fermented sausages (**Fig. 4C**), fractions corresponding
370 to the elution zone between 220 mL and 275 mL presented the highest DPPH radical-
371 scavenging activity (from 58.8% to 73.7%) and reducing power capacity, which reached
372 values up to 1.65 units of absorbance in the 245 mL and 290 mL fractions. Previous
373 studies have described the presence of antioxidants in fermented sausages, mainly at the
374 end of the ripening due to the generation of small-size peptides showing bioactivity.
375 Vaštag et al. (2010) found that the antioxidant activity in protein extracts from fermented
376 sausages was 2 or 3 fold greater in the final product than in the initial sausage mixture,
377 although the obtained values at 90 days ripening (50% DPPH radical-scavenging activity
378 and 0.97 for reducing power) were lower than those found in the present study. Sun et al.
379 (2009) reported that fractions with peptides higher than 5 kDa reached values up to 86%

of DPPH radical scavenging activity after 18 h of sausage drying, whereas the fraction with peptides lower than 5 kDa showed a gradual increase in the activity up to 92% at 72 h of drying. In this regard, Broncano et al. (2012) and Mejri et al. (2017) also reported a high capacity to scavenge DPPH radical of small peptides (< 3 kDa) from dry-fermented sausages.

Antioxidant and ACE inhibitory peptides are generally short sequences (2-20 amino acids in length), with molecular mass of approximately 400-3000 Da (Korhonen and Pihlanto, 2003). The results of the present study showed a broad distribution of ACE inhibitory and antioxidant peptides in a wide range of molecular masses, but most of them would be peptides ranging from 1500 to 500 Da (**Fig. 4**). BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) was used in order to identify possible ACE inhibitory and antioxidant peptides present in the studied dry-fermented sausages. So, several dipeptides generated from the C-terminal position of peptides have been previously identified showing ACE inhibitory bioactivity, as for example, EG (released from peptide 40), AG (peptide 97), KF (peptide 181), and EK (peptides 210 and 267), whereas IY (from peptide 39) has antioxidant activity (see **Table 1 of supplementary material**).

Despite peptides are generally more effective as antioxidants than free amino acids, they can also contribute to the bioactivity profile. Meat and fermented sausages are important sources of taurine, compound that exerts a significant antioxidant activity (Bou et al., 2017). Moreover, Glu, His, and hydrophobic amino acids such as Ala, Phe, Val, Pro, Gly, Leu and Ile, which are present in significant amounts in the assayed Spanish, Italian and Belgian dry-fermented sausages (**Fig. 3**), might be also contributing to the antioxidant activity (Sarmadi and Ismail, 2010; Sun et al., 2009). Some LAB and staphylococci can also act as antioxidant factors and contribute, together with antioxidant peptides, to

prevent the oxidation of dry-fermented sausages. Oxidative processes could reduce proteolysis and the release of amino acids during ripening, negatively affecting sensory quality of the product as they are converted into aroma compounds upon conversion by the meat microbiota (Berardo et al., 2015).

Conclusions

The pattern of proteolysis of three typical European dry-fermented sausages from Spain, Italy and Belgium has been characterised, obtaining peptide and free amino acid profiles resulting from differences in formulation, processing conditions and starter culture used in each type of sausage. Moreover, the combined action of muscle and microbial enzymes in these products would contribute to the generation of bioactive peptides showing ACE inhibitory and antioxidant activities. In this regard, the Spanish and Belgian dry-fermented sausages showed the maximum values of ACE inhibition, whereas the Belgian sample presented the highest DPPH radical-scavenging activity and reducing power capacity. These results improve the knowledge about the intense proteolysis that takes place during the processing of dry-fermented Spanish, Italian and Belgian sausages as well as evidence their potential as natural sources of bioactive peptides, giving an added value to these products scarcely reported to date. However, further studies are needed in order to identify the specific sequences responsible for the observed bioactivities.

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Figure 1. Fractionation by size-exclusion chromatography of water-soluble peptide extracts from the dry-fermented sausages. The absorbance at 214, 254, and 280 nm was measured in each collected fraction of A) Spanish sausage, B) Italian sausage, and C) Belgian sausage.

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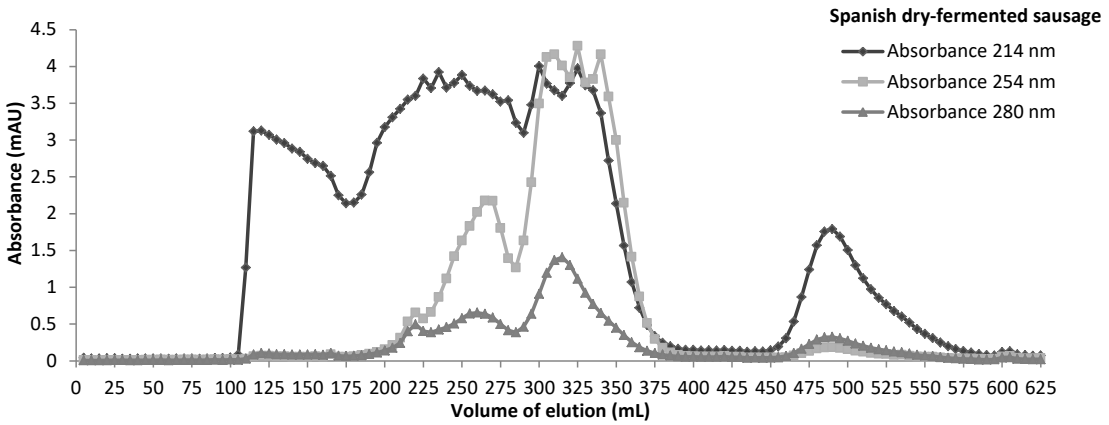

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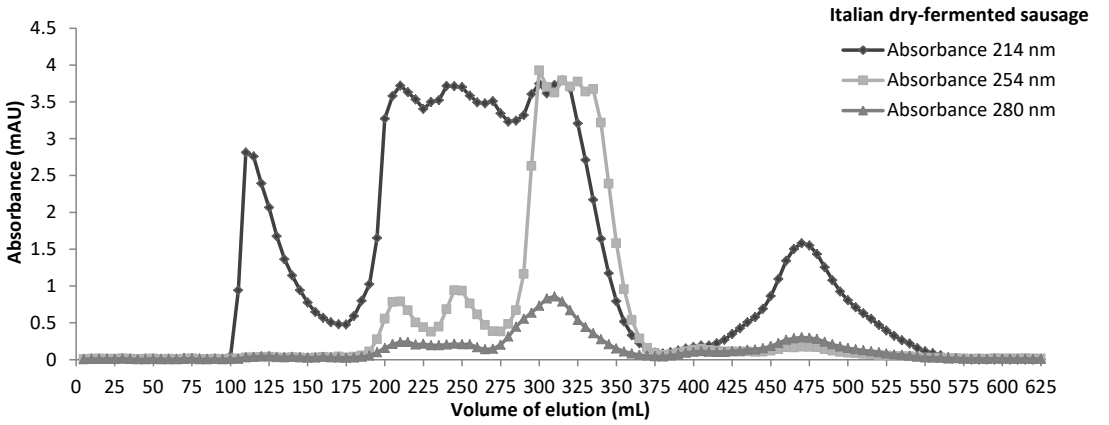

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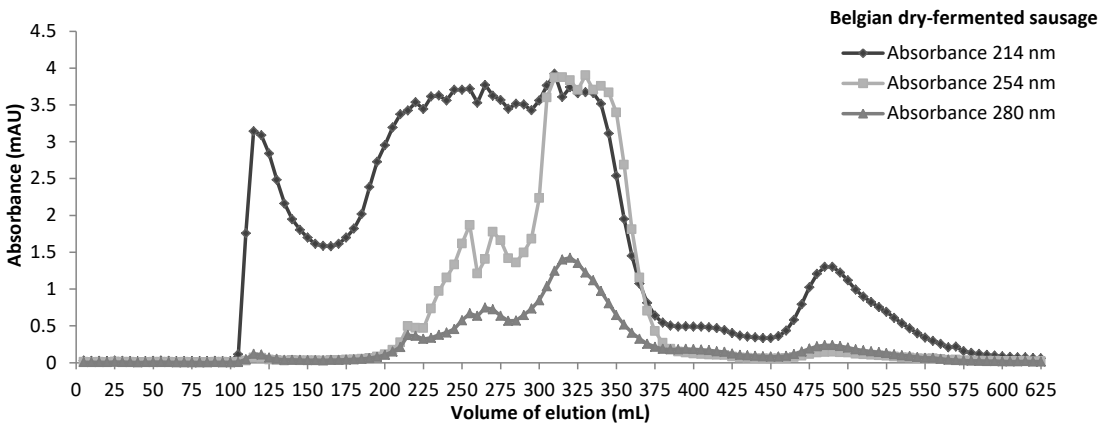

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579 **Figure 1.**

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584 **Figure 2.** Distribution of the peptides identified by nLC-MS/MS according to their
585 protein of origin in the A) Spanish dry-fermented sausage, B) Italian dry-fermented
586 sausage, and C) Belgian dry-fermented sausage. Proteins are abbreviated as ACT: actin;
587 CAH: carbonic anhydrase; CK: creatine kinase; ENO: beta-enolase; G3P:
588 glyceraldehyde-3-phosphate dehydrogenase; HB: hemoglobin; LDB3: LIM domain-
589 binding protein 3; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; MRL:
590 myosin regulatory light chain; MYG: myoglobin; MYH: myosin; MYL: myosin light
591 chain; MYOZ: myozenin; PGK: phosphoglycerate kinase; PGM: phosphoglycerate
592 mutase; SOD: superoxide dismutase; TITIN: titin; TNN: troponin; UBC: poliubiquitin;
593 others include minor proteins.
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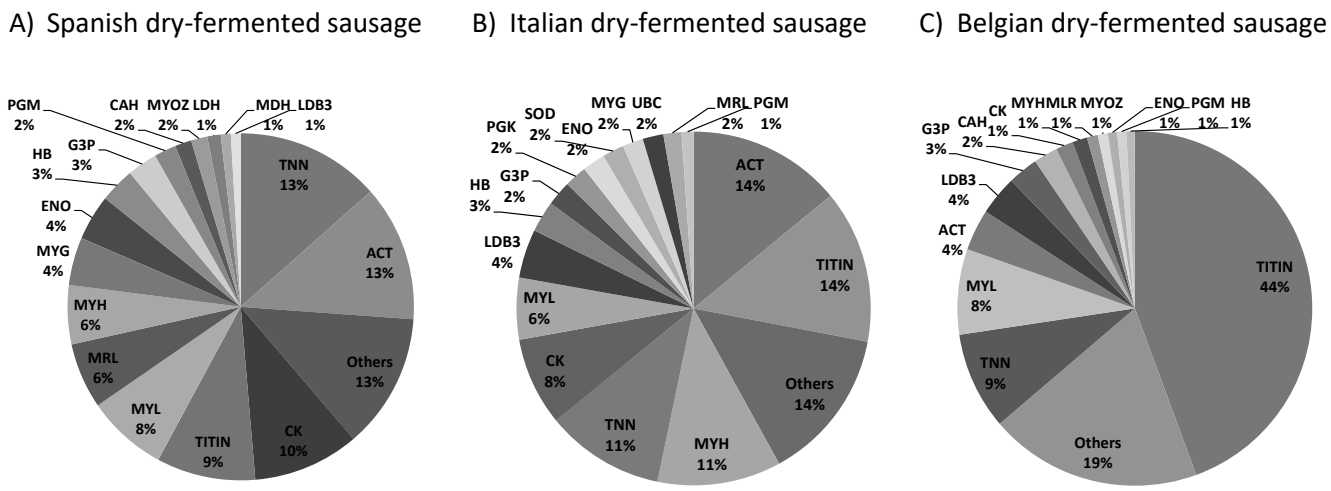

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598 **Figure 3.** Free amino acids content of Spanish, Italian and Belgian dry-fermented
599 sausages. The values represent means of three replicates ± standard deviations, expressed
600 as mg free amino acid / 100 g dry-matter sample. Bar letters indicate significant
601 differences among the values ($p < 0.05$).
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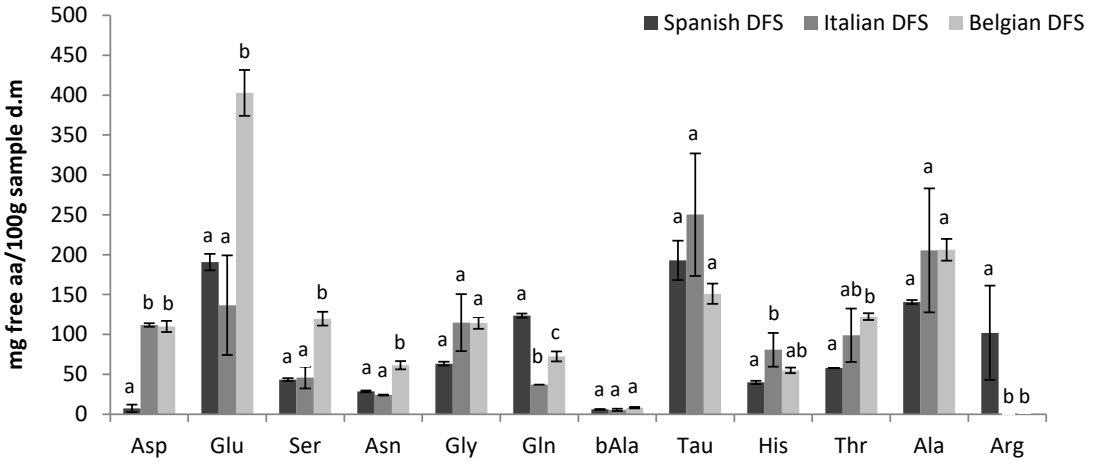

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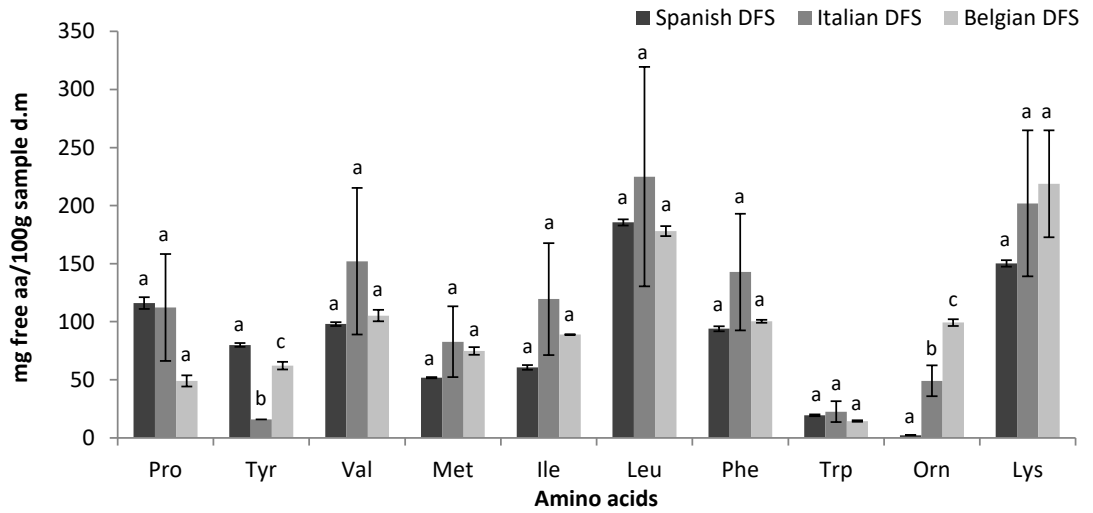

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Figure 4. ACE inhibitory activity and antioxidant activity (assayed by the measurement of DPPH radical-scavenging activity and reducing power) of the fractions collected from size-exclusion chromatography of A) Spanish dry-fermented sausage, B) Italian dry-fermented sausage, and C) Belgian dry-fermented sausage.

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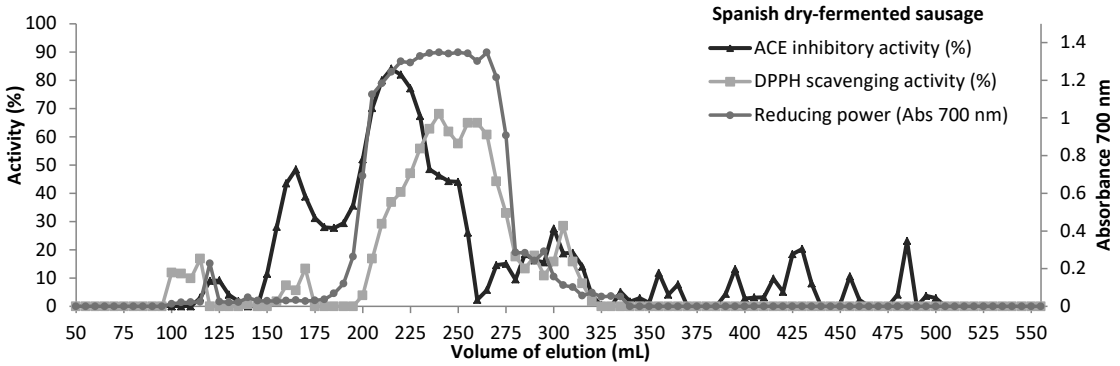

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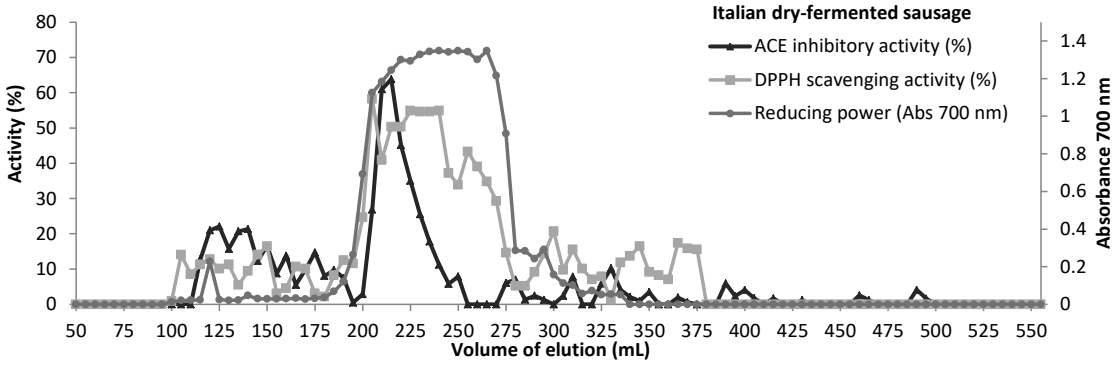
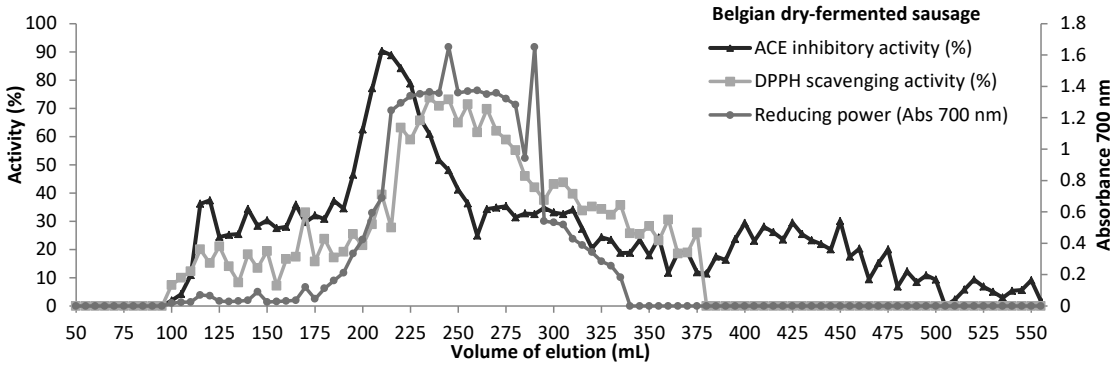


C)



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623 **Supplementary Figure 1.** MALDI-ToF spectra of Spanish, Italian and Belgian dry-
624 fermented sausages measured using two ranges: (A) from 200 to 900 *m/z* and (B) from
625 900 to 3500 *m/z*.

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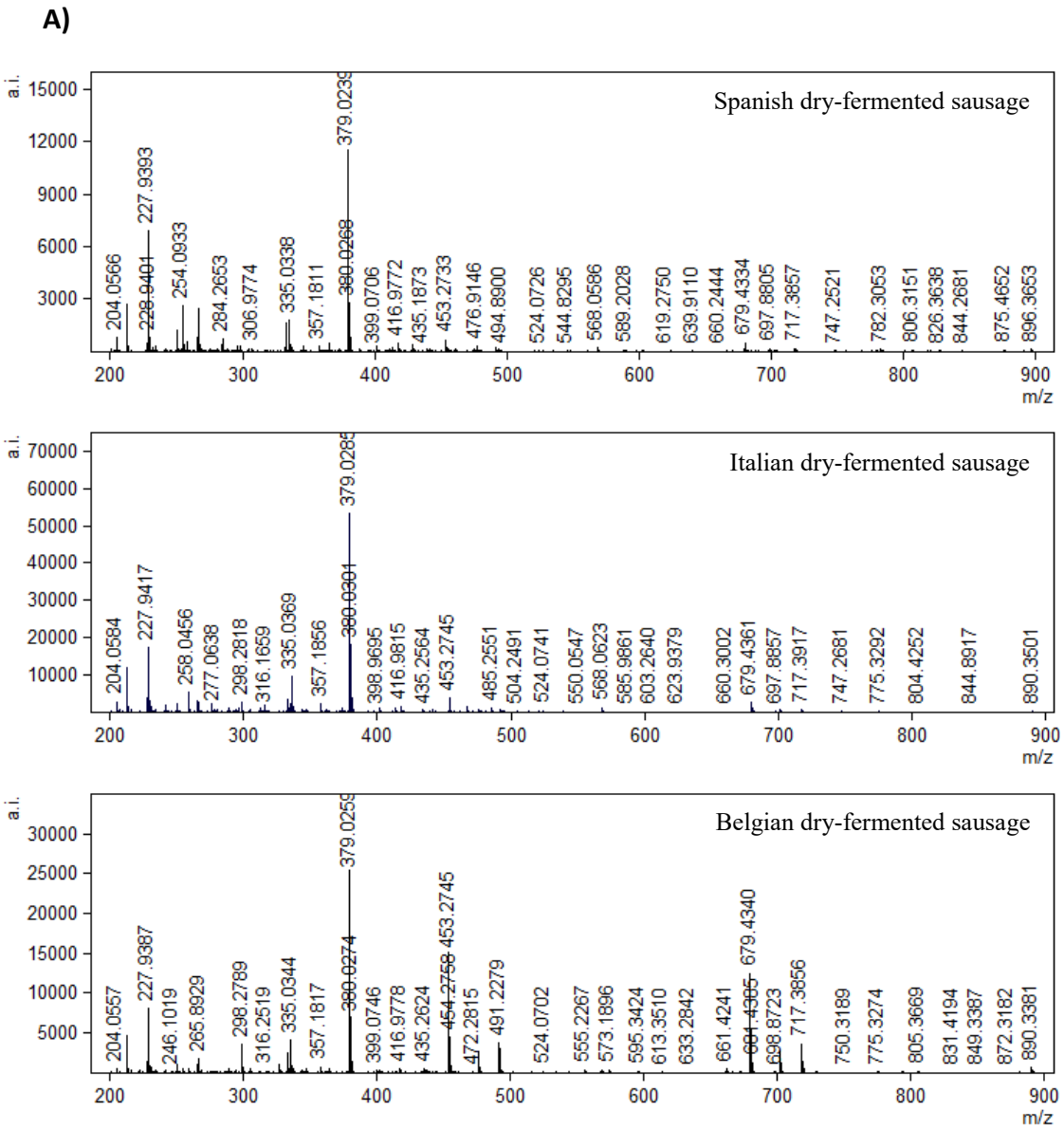

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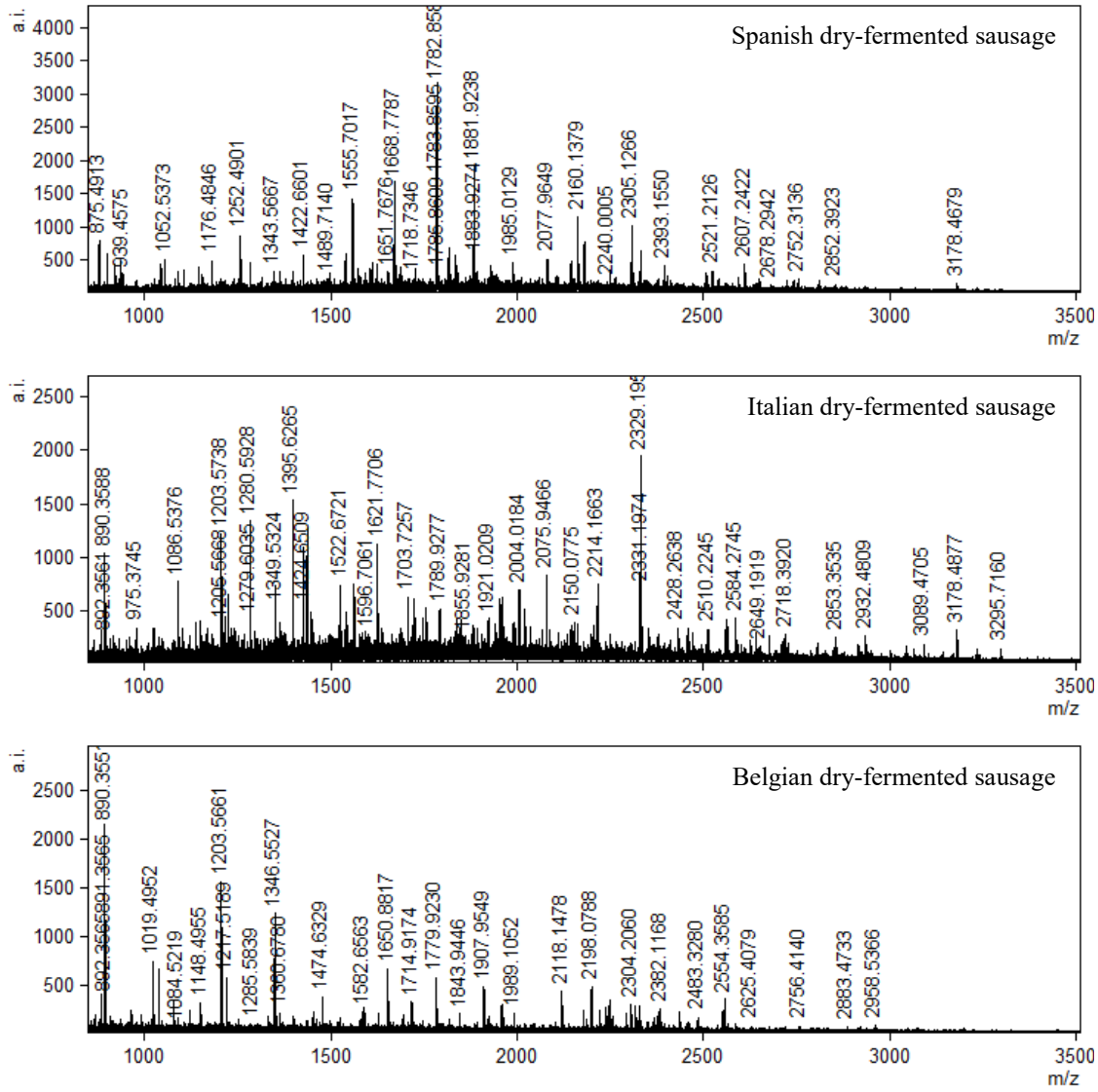
B)



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