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Antilisterial peptides from Spanish dry-cured hams: purification and identification

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ABSTRACT

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The typical Spanish dry-cured ham has a particular sensory quality that makes it a distinctive food, highly appreciated for consumers worldwide. Its particular physicochemical properties, such as high salt content and reduced water activity contribute to their shelf-stability. However, post-processing actions carried out for the commercialization of these products such as slicing may increase the risk of development of pathogenic microorganisms as *Listeria monocytogenes*. During ripening, muscle proteins are hydrolyzed by muscle peptidases releasing peptides and free amino acids. Some of these peptides have been described to exert biological activities such as antioxidant and ACE-inhibition. In this study, a peptidomic strategy using mass spectrometry techniques has been used to identify and sequence those naturally generated peptides showing antilisterial activity. One hundred and five peptides have been identified in active fractions and some synthesized and their MIC calculated. Ten peptides were able to inhibit the growth of *L. monocytogenes*, being the pentapeptide RHGYM the most effective showing a MIC value of 6.25 mM. This study proves for the first time the potential antimicrobial action against *L. monocytogenes* of certain naturally generated peptides obtained from Spanish dry-cured ham.

Keywords: *Listeria monocytogenes*, proteomics, antimicrobial peptides, mass spectrometry, dry-cured ham.

1. INTRODUCTION

44Spanish dry-cured ham is produced from white pigs crossbreeds (Losantos et al. 2000). 45Basically, the preparation method involve four main stages: conditioning of pieces, 46salting, post-salting and dry-curing. During the salting step, a microbial reservoir is 47especially created on the surface of hams produced from microorganisms present in the 48salt and environment (Cornejo et al. 1992). Some of these microorganisms together with 49endogenous enzymes contribute to the changes occurred during dry-cured processing 50such as proteolysis, lipolysis and nitrate reduction influencing the sensory quality of 51hams (Toldrá 1998; Sondergaard and Stahnke 2002).

52Dry-cured ham is a Ready-To-Eat (RTE) meat product, which is sliced and packaged 53under vacuum as an extra protection barrier prior to distribution and commercialization. 54However, these post-processing actions could favor the contamination of the product 55due to the development of pathogenic organisms such as *L. monocytogenes* 56(Chaitiemwong et al. 2014). In fact, *L. monocytogenes* is difficult to eradicate owing to 57its resistance to drying and high salt concentrations, being its survival in dry-cured 58meats a major food safety concern (Nightingale et al. 2006). Immunocompromised 59patients, pregnant women, children and elderly people are primarily affected by 60listeriosis, a rare but extremely serious zoonosis (Giovannini et al., 2007). Low 61morbidity and very high mortality rates (20%) have been related to listeriosis (EFSA 62and ECDC 2015), which makes the elimination or reduction of *L. monocytogenes* 63from food processing plants a constant challenge for RTE food producers.

64The ripening process of Spanish dry-cured ham is very long and could be up to 24 65months in drying chambers. During the processing, different chemical and biochemical 66changes occur obtaining, as a result, the characteristic color, flavor and taste of dry-67cured ham (Toldrá et al, 1992). Among all the complex biochemical reactions occurring 68during dry-cured hams processing, proteolysis is one of the most important. In this

69process, muscle proteins are hydrolyzed by muscle endogenous enzymes with the 70release of small peptides and free amino acids (Toldrá and Flores 1998; Lametsch et al. 712003). The role of sarcoplasmic and myofibrillar proteins in the generation of small 72peptides have been previously described in the final product (Sentandreu et al. 2007; 73Mora et al. 2008; 2010; 2011), and during the dry-curing process at different times 74(Gallego et al. 2014; Mora et al. 2015). On the other hand, some of the generated 75peptides have shown good antihypertensive and antioxidant activity (Escudero et al. 762012; 2013; Toldrá and Reig, 2011).

77However, to our knowledge, there are no studies describing antimicrobial peptides in 78dry-cured ham. In this sense, the identification of antilisterial activity in naturally 79generated peptides during dry-curing of Spanish ham would increase the value of this 80product by improving its safety especially when commercialized in sliced form. Thus, in 81this study, a peptidomic strategy has been used to identify and sequence those naturally 82generated peptides showing antilisterial activity derived from dry-cured ham protein 83degradation.

84 **2. MATERIALS AND METHODS**

85 **2.1. Spanish dry-cured ham material**

86Spanish dry-cured ham was produced from 6-months old pig (Landrace x Large White). 87As a pre-salting stage, hams were prepared according to traditional procedures. During 88salting, potassium nitrate was incorporated covering hams with NaCl and locating them 89in chambers at 1-3 ºC and 80-90% of relative humidity. During post-salting stage hams 90were subjected to a temperature increase (3-5ºC) and relative humidity decrease (75-9185%) for 60 days. Finally, the ripening-drying was carried out at temperatures in the 92range of 14-20ºC and lower relative humidity (up to 70%). The total processing time 93was 10 months. All analysis was done in *Biceps femoris* muscle.

94 **2.2. Bacterial strains and antimicrobial activity assay**

95 A total of eight *Listeria* strains were used as test microorganisms for the determination
96 of antilisterial activity. They include the type strain of the species *L. monocytogenes*
97 CECT 4031^{T} from the Spanish Type Culture Collection (CECT), five *L. monocytogenes*
98 food isolates (14, 49, 75, 120, 127) from the IATA (Valencia Spain) that were identified
99 in a previous study (Aznar and Elizaquível 2008), clinical isolated *L. monocytogenes*
100 FBUNT strain from Facultad de Bioquímica, Química y Farmacia, Universidad
101 Nacional de Tucumán, (Argentina) (Castellano et al. 2004) and food isolated *Listeria*
102 *innocua* 7 from the Unité de Recherches Laitieres et Genetique Appliqueé (INRA,
103 France) (Castellano and Vignolo 2006). All strains were grown overnight in tryptic soy
104 broth (TSB; BBL, Cockeysville, MD) with 0.5% added yeast extract (YE) at 30ºC.
105 *Lactobacillus curvatus* CRL705, used as a bacteriocin-producer positive control, was
106 grown in MRS at 30 °C.

107 *Listeria* cells were added at a concentration of 10^{7} CFU~~UFC~~/mL in 10 mL of TSB + YE
108 soft agar (0.7%) medium. The dry-cured ham fractions obtained from different steps of
109 chromatographic purification were added (5 µL) onto the soft agar layer inoculated with
110 different *Listeria* strains and incubated at 30°C during 24 h. The antibacterial activity
111 was measured as the diameter in mm of the clear zone of growth inhibition by
112 comparison with the bacteriocin-containing supernatant from *L. curvatus* CRL705 as
113 positive control and saline solution as negative control (Castellano and Vignolo 2006).

114 2.3. Peptides extraction from dry-cured ham

115 Fifty grams of *Biceps femoris* muscle from Spanish dry-cured ham cured for 10 months
116 were homogenized with 200 mL of 0.01 N HCl for 8 min at 4ºC. The homogenate was
117 centrifuged (12000 g during 20 min at 4°C) and filtered. The deproteinization was done
118 by adding ethanol (1:3; v:v) and keeping the mixture at 4°C overnight. Then, sample
119 was centrifuged (12000 g during 20 min at 4°C) and the supernatant lyophilized.

120 Finally, the dried extract was dissolved in 25 mL of 0.01 N HCl, and stored at -20°C
121 until use.

122 **2.4. Size-exclusion chromatography**

123 The size exclusion chromatography was done using a previously equilibrated Sephadex
124 G-25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden) with 0.01 N HCl
125 at 4 ºC and a flow rate of 15 mL/h. The collection of fractions (5 mL) was done using an
126 automatic fraction collector and their absorbance was measured at 214, 254 and 280 nm
127 (Agilent Cary 60 UV-Vis spectrophotometer, Agilent Technologies, Palo Alto, CA,
128 USA). All fractions were assayed for *L. monocytogenes* inhibitory activities. Fractions
129 showing the highest activities were put together freeze dried and dissolved in 1 mL of
130 bidistilled water. A saline solution was used as negative control.

131 **2.5. Reversed-phase high performance liquid chromatography**

132 The reversed-phase chromatographic separation was done using an Agilent 1100 HPLC
133 equipment (Agilent Tech., California, USA) with a Symmetry C18 column (4.6×250
134 mm, 5 µm) from Waters Co. (Milford, MA, USA). An aliquot of 200 µL of the most
135 active fractions (fractions 41 and 42) was injected into system. Solvent A was 0.1%
136 trifluoroacetic acid (TFA) in bidistilled water and solvent B contained acetonitrile
137 (ACN) and bidistilled water (60:40, v/v) and 0.085% of TFA. The elution of the
138 peptides was done using an isocratic gradient of 5 min with a 99% solvent A, followed
139 by a linear gradient from 0% to 45% of solvent B in 40 min at a flow rate of 1 mL/min.
140 The absorbance was measured at 214 nm and fractions of 1 mL were collected and
141 assayed for *L. monocytogenes* inhibitory activity. Those fractions showing antilisteria
142 activity were freeze dried and analysed by MS for the identification of the peptides.

143 **2.6. MALDI ToF analysis and peptide identification by LC-MS/MS**

144The most active fractions obtained after the separation using reversed-phase HPLC were
145analysed by Matrix-Assisted Laser Desorption/Ionization MS technique in order to
146obtain the *m/z* profile of the peptides in the mixture. The analysis was done in a 5800
147MALDI ToF/ToF system (ABSciex) in positive reflectron mode (3000 shots at each
148position) in a range from 200 to 3000 Da. Plate model and acquisition method were
149calibrated by a peptide mass standards calibration mixture (ABSciex) in 13 positions.
150Dried hydrolysates were dissolved in 5% ACN; 0.1% TFA, and 1 L of every sample was
151directly spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried,
1520.5 µL of matrix solution (5 mg/mL of α-Cyano-4-hydroxycinnamic acid (CHCA) in
1530.1% TFA-ACN/H_2O (7:3, v/v) was spotted.

154Those fractions obtained after RP-HPLC showing the highest values for antilisterial
155activity were analysed by nLC-MS/MS in order to identify the sequences of the peptides
156according with Gallego et al (2015). Fractions were resuspended in H_2O with 0.1% of
157trifluoroacetic acid (TFA) to obtain a final concentration of 0.2 mg/mL. Five microlitres
158of the supernatant were analysed in the LC-MS/MS system.

159The database search of peptides was done through the proteomic platform of Mascot
160Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA)
161(hppt://www.matrixscience.com). The search parameters used in the identification were:
162UniProt as protein database, with a significance threshold $p < 0.1$ and a FDR of 1.5%,
163and a tolerance of 100 ppm in MS mode and 0.3 Da in MS/MS.

164 **2.7. Peptide Synthesis**

165The most promising sequences of identified peptides were synthesized by GenScript
166Corporation (Piscataway, NJ, USA) and their purity was certified by analytical LC-MS.
167The *in vitro* inhibition of *L. monocytogenes* and minimum inhibitory concentration
168(MIC) of synthetic peptides was determined by the critical dilution assay according to

169(Vignolo et al. 1993). Serial two-fold dilutions of synthetic peptides in sterile water
170(from 100 to 0,78 mg/mL) were prepared and 5 µL volume was placed on a semi-solid
171TSB overlay inoculated with *L. monocytogenes* CECT 4031^{T} and FBUNT strains (ca.
172$10^{7}$ CFU/mL), as indicator organisms in separated experiments. The MIC value was
173determined after incubating with the diluted peptides for 24 h at 30°C. All the analysis
174were done in triplicate.

175 **2.8. Statistical analysis**

176The ANOVA procedure was used to determine significant differences in the obtained
177MIC value using the software Minitab Statistic Program, v8.21 (Minitab Inc., PA,
178USA). Each statistical analysis was done in triplicate (n=3) and the normality of the data
179was tested before applying the ANOVA procedure.

180 **3. RESULTS AND DISCUSSION**

181The application of antimicrobial natural compounds as a preservation approach has
182experienced a high interest during the last years due to the increase of consumer's
183demand for safe and fresh-tasting Ready-to-Eat products with low amounts of chemical
184preservatives. In this study, a peptidomic strategy has been used to identify and
185sequence those naturally generated peptides showing antilisterial activity derived from
186dry-cured ham protein degradation. The pathogen was chosen as the most frequently
187encountered on slices of dry-cured meat products (Vorst et al. 2006).

188 Samples of dry-cured ham were fractionated using SEC as shown in **Figure 1**. A
189total of 128 fractions were tested for antilisterial activity in Petri plates against the eight
190*Listeria* strains, including seven *L. monocytogenes*. Results showed that fractions 41
191and 42 inhibited the growth of all assayed *Listeria* strains which would indicate
192antilisterial activity; the remaining collected fractions failed to exert inhibitory effect

193against the tested strains. These results are in agreement with previously described 194antioxidant and ACE-inhibitory activity results of dry-cured ham samples after their 195fractionation using a Sephadex G-25 column in which the highest ACE inhibitory 196activity (80% of ACE inhibition) was also detected from fraction 40 (Escudero et al. 1972013). Subsequent *in vivo* studies using Spontaneously Hypertensive Rats (SHR) 198showed antihypertensive activity with a decrease of 40 mmHg in systolic blood pressure 199(Escudero et al. 2012). Regarding antioxidant activity, a similar increase in DPPH 200radical-scavenging and ferric reducing power was observed from fraction 40 in a 201Sephadex G25 in the same study (Escudero et al. 2012). This fact would be related to 202the size of sequences of the peptides contained on that elution volume as it has been 203widely described that sequences comprised between 2-20 amino acids were the most 204characteristic for bioactive peptides. However, proteolysis is very dynamic and causes 205changes in the generated peptide sequences which can be created and hydolysed 206depending on the action of a wide distribution of endogenous enzymes that are acting 207during dry-cured ham processing.

208Subsequently, these two active fractions (numbers 41 and 42) were pooled together and 209analyzed by RP-HPLC and fractions of 1 mL collected (showed in **Figure 2**). After 210measuring the antilisterial activity, those peaks eluted at 4, 5, 6, and 7 minutes showed 211inhibitory activity against *Listeria* (0.8 ±0.2 mm in diameter). The size of the peptides 212included in these RP-HPLC fractions was elucidated using MALDI-ToF mass 213spectrometry at two different levels of *m/z* (from 200 to 900 *m/z* as it is showed in 214**Figure 3** and from 900 to 3000 *m/z* in **Figure 4**) and resulted very useful in the 215characterization of the peptide profile of fractions 4, 5, 6, and 7. These results showed 216that most of the peptides contained on these fractions showed medium-low molecular 217weights, being the peptide profile showed on **Figure 4** less crowded than profiles of

218**Figure 3**. These results are in agreement with previously published information about 219the size of the most active peptides in terms of antioxidant and ACE-inhibitory 220activities, despite many of the recently described antimicrobial sequences showed a very 221wide range of chain length (MacClean et al.,2014; Trindade et al, 2015) .

222Lately, the identification of peptide sequences was done using a peptidomic approach 223based on nanoLC-MS/MS. The database search was carried out using Mascot as search 224engine and UniProt as protein database, which contains 549215 sequences and 225195767212 residues. A total of 105 peptide sequences were identified from the most 226active fractions showing molecular masses between 502 and 2065 Da and from 5 to 18 227amino acids in length (**Tables 1 to 4**). Regarding the length of bioactive peptides, 228numerous antibacterial peptides have been described as long amino acid chains, which 229can adopt an α-helical linear or circular structure organized in a β-sheet. This 230conformation is often essential considering the mechanism of action of active peptides 231against the microorganisms (Nicolas and Mor 1995). On the other hand, one group of 232antimicrobial peptides produced by lactic acid bacteria and referred as bacteriocins, 233particularly Class IIa bacteriocins, constitute a large cluster of peptides with lengths 234between 36 and 49 amino acids that have been described to be active against *Listeria* 235species (Nishie et al. 2012). Regarding this, some bacteriocins loose the activity in meat 236products due to the action of specific meat ingredients such as salt and nitrite and 237conditions such as proteolytic degradation that destabilize their biological 238activity.ccording to the previously described as optimum molecular mass and sequence 239for antilisterial inhibition, some of the identified peptides were synthesized and their 240MIC calculated against *L. monocytogenes* CECT 4031^{T} and FBUNT strains (**Table 5**). 241Among the twelve peptides synthesized, two of them failed to exert antilisterial activity. 242The remaining ten peptides were able to inhibit *L. monocytogenes* growth, being the

243pentapeptide RHGYM identified in fraction 7 of RP-HPLC (see **Table 4**) the most
244effective with a MIC value of 6.25 mM. Non-similar sequences showing antimicrobial
245activity have been found but the tripeptide RHG have been previously described as a
246potential antioxidant sequence by Saito et al (2003). Previous studies of bovine
247hemoglobin hydrolysates resulted into some peptides showing antimicrobial activity
248against *E. coli*, *Salmonella* **Enteritidis**, *L. innocua* and *Micrococcus luteus*. The peptide
249β114-145 and its peptic derivatives containing the RYH sequence exhibited the highest
250antimicrobial activity (85 µM) (Catiau et al. 2011a). In addition, (Catiau et al. 2011b)
251determined that KYR was the minimal sequence of hemoglobin alpha-chain necessary
252to exert antibacterial activity. On the other hand, the important role of Tyr (Y) in the
253interaction with membranes together with the amino acid residues Arg (R) and Lys (K),
254which are known to act as peptide anchors in membranes by interacting with negatively
255charged membrane phospholipids was reported (Lopes et al. 2005). In the present study,
256six of the synthesized and tested peptides presented tyrosine (Y) and two positive
257charged amino acids such as histidine (H) or K in their sequences, which would be
258required for antibacterial activity. Also several of the identified peptides include the
259previously described antimicrobial sequences KYR and RYH although only MDPKYR
260and HCNKKYRSEM peptides showed MIC values of 50 mM. In fact, RHGYM the
261most active peptide described in this study with a MIC value of 6.25 mM shows in its
262sequence tyrosine, arginine and histidine amino acids.

263The potential of dry-cured ham as carrier of antilisterial peptides is an added-value for
264this type of product as the joint action of these bioactive peptides could influence the
265development of possible cross-contaminations with *L. monocytogenes* during slicing
266and packaging. This would be a way to develop future natural strategies in the control of

267safer products as the use of natural antilisterial peptides as preservatives is an interesting

268alternative to chemicals compounds in the food area.

269 4. CONCLUSIONS

270In this study a highly active antilisterial peptide has been identified among the naturally

271generated peptides in Spanish dry-cured ham. A total of 105 peptide sequences were

272identified from the most active fractions against eight *Listeria* strains after RP-HPLC

273separation, showing molecular masses between 502 and 2065 Da and from 5 to 18

274amino acids in length. From them, a total of twelve peptides were chosen, synthesized

275and tested to calculate their MIC against *L. monocytogenes* CECT 4031^{T} and FBUNT

276strains. The pentapeptide RHGYM resulted to be the most effective with a MIC value of

2776.25 mM. Thus, according with the nowadays interest in food safety and food protection

278given by natural products, the results derived from this study prove the presence of

279peptides naturally generated during the processing of dry-cured ham and their

280antilisterial potential as preservative during storage and distribution of the sliced

281product.

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291CONFLICTS OF INTEREST

292Authors declare that there are no conflicts of interest

REFERENCES

Aznar R, Elizaquível E. 2008. Reliability of *Listeria monocytogenes* identification by specific PCR assessed by phenotypic and genotypic techniques. Food Anal. Methods 1:243-251.

Castellano P, Holzapfel WH, Vignolo G. 2004. The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with the bacteriocinogenic strain *Lactobacillus casei* CRL705. Food Microbiol. 21:291-298.

Castellano P, Vignolo G. 2006. Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins. Lett. Appl. Microbiol. **43:194-9.**

Catiau L, Traisnel J, Chihib N-E, Le Flem G, Blanpainb A, Melnykb O, Guillochon D, Nedjar-Arroume N. 2011. RYH: A minimal peptidic sequence obtained from beta-chain hemoglobin exhibiting an antimicrobial activity. Peptides 32:1463-1468.

Catiau L, Traisnel J, Delval-Dubois V, Chihib N-E, Guillochon D, Nedjar-Arroume N. 2011. Minimal antimicrobial peptidic sequence from hemoglobin alpha-chain: KYR. Peptides 32:633-8.

Chaitiemwong, N. Hazeleger, W.C. Beumer, R.R. Zwietering M.H. 2014.Quantification of transfer of *Listeria monocytogenes* between cooked ham and slicing machine surfaces. Food Control, 44 177–184

Cornejo I, Carrascosa AV, Marín ME, Martín PJ. 1992. Considerations about the origin of microorganisms that grow on the deep muscular tissues of dry-cured Spanish hams during processing. Fleischwirtschaft 72:1405-1407.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. EFSA J 13(1):3991.

Escudero E, Aristoy MC, Nishimura H, Arihara K, Toldrá F. 2012. Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham. Meat Sci. 91:306-311.

Escudero E, Mora L, Fraser P D, Aristoy M, Toldrá F. 2012. Identification of novel antioxidant peptides generated in Spanish dry-cured ham. Food Chem. 138:1282-1288.

332Escudero E, Mora L, Fraser PD, Aristoy M, Arihara K, Toldrá F. 2013. Purification and
333 identification of antihypertensive peptides in Spanish dry-cured ham. J.
334 Proteomics 78:499-507.

335Gallego M, Mora L, Fraser P D, Aristoy M A, Toldrá F. 2014. Degradation of LIM
336 domain-binding protein three during processing of Spanish dry-cured ham. Food
337 Chem. 149:121-128.

338Gallego, M., Mora, L., Aristoy, M.C., Toldrá, F. 2015. Titin-derived peptides as
339 processing time markers in dry-cured ham. Food Chem. 167, 326-339.

340Giovannini A, Migliorati G, Prencipe V, Calderone D, Zuccolo C, Cozzolino P. 2007.
341 Risk assessment for listeriosis in consumers of Parma and San Daniele hams.
342 Food Control 18:789-799.

343Lametsch R, Karlsson A, Rosenvold K, Andersen HJ, Roepstorff P, Bendixen E. 2003.
344 Postmortem proteome changes of porcine muscle related to tenderness. J. Agric.
345 Food Chem. 51:6992-6997.

346Lopes SC, Fedorov A, Castanho MA. 2005. Lipidic membranes are potential “catalysts”
347 in the ligand activity of multifunctional pentapeptide neokyotorphin. Chem.
348 Biochem. 6:697-702.

349Losantos A, Sanabria C, Cornejo I, Carrascosa AV. 2000. Characterization of
350 Enterobacteriaceae strains isolated from spoiled dry-cured hams. Food Microbiol.
351 17:505-512.

352McClean, S., Beggs, LB., Welch, RW. 2014. Antimicrobial activity of antihypertensive
353 food-derived peptides and selected alanine analogues, Food Chem., 146, 443-447.

354Mora L, Gallego M, Aristoy M A, Fraser P D, Toldrá F. 2015. Peptides naturally
355 generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-
356 cured ham processing time. Food Control, 48, 102-107.

357Mora L, Sentandreu MA, Fraser PD, Toldrá F, Bramley PM. 2009. Oligopeptides
358 arising from the degradation of creatine kinase in Spanish dry cured ham. J. Agric.
359 Food Chem. 57:8982-8988.

360Mora L, Sentandreu MA, Toldrá F. 2010. Identification of small troponin T peptides
361 generated in dry-cured ham. Food Chem. 123:691–697.

362Mora L, Valero ML, Sánchez del Pino MM, Sentandreu MA, Toldrá F. 2011. Small
363 peptides released from muscle glycolytic enzymes during dry-cured ham
364 processing. J. Proteomics 74:442–450.

365Nicolas P, Mor A. 1995. Peptides as weapons against microorganisms in the chemical
366 defense system of vertebrates. Annu. Rev. Microbiol. 49:277-304.

367Nightingale KK, Thippareddi H, Phebus RK, Marsden JL, Nutsch AL. 2006. Validation
368 of a traditional Italian-style salami manufacturing process for control of
369 Salmonella and *Listeria monocytogenes*. J. Food Prot. 69:794-800.

370Nishie M, Nagao J, Sonomoto K. 2012. Antibacterial peptides “bacteriocins”: an
371 overview of their diverse characteristics and applications. Biocontrol Sci. 17:1-16.

372Saito, K, Jin, DH, Ogawa, T, Muramoto, K, Hatakeyama, E, Yasuhara, T, Nokihara, K.
373 2003. Antioxidative properties of tripeptide libraries prepared by the
374 combinatorial chemistry. J Agric Food Chem. 51:3668-74.

375Sentandreu MA, Armenteros M, Calvete JJ, Ouali A, Aristoy MC, Toldrá F. 2007.
376 Proteomic identification of actin-derived oligopeptides in dry-cured ham. J. Agric.
377 Food Chem. 55:3613-3619.

378Sondergaard AK, Stahnke LH. 2002. Growth and aroma production by *Staphylococcus*
379 *xylosus*, *S. carnsous* and *S. equorum*- a comparative study in model systems. Int.
380 J. Food Microbiol. 75:99-100.

381Toldrá, F., Miralles, M-C., Flores, J. 1992. Protein extractability in dry-cured ham. Food
382 Chem., 44, 391-394.

383Toldrá F, Flores M. 1998 The role of muscle proteases and lipases in flavor development
384 during the processing of dry-cured ham. Crit. Rev. Food Sci. Nutr. 38:331-352.

385Toldrá F, Reig M. 2011. Innovations for healthier processed meats. Trends Food Sci.
386 Technol. 22:517-522.

387Toldrá F. 1998. Proteolysis and lipolysis in flavour development of dry-cured meat
388 products. Meat Sci. 49:101-110.

389Trindade, F., Amado, F., Pinto da Costa, J., Ferreira, R., Maia, C., Henriques, I., Colaço,
390 B., Vitorino, R. 2015. Salivary peptidomic as a tool to disclose new potential
391 antimicrobial peptides, Journal of Proteomics, 115, 49-57.

392Vignolo GM, Suriani F, Pesce de Ruiz Holgado A, Oliver G. 1993. Antibacterial activity
393 of Lactobacillus strains isolated from dry fermented sausages. J. Appl. Bacteriol.
394 75:344-9.

395Vorst KL, Todd ECD, Ryser ET. 2006. Transfer of *Listeria monocytogenes* during
396 mechanical slicing of turkey breast, bologna, and salami. J. Food Prot. 69:619-
397 626.

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401**FIGURE LEGENDS**

402

403FIG 1: Gel filtration chromatography of 10 months of curing dry-cured ham extract

404using a Sephadex G-25 column.

405

406FIG 2: Reversed-phase HPLC separation of fractions 41 and 42 obtained from gel

407filtration chromatography. Dotted line indicates fractions from 4 to 7 that showed

408*Listeria monocytogenes* inhibitory activity.

409

410FIG 3: MALDI-ToF spectra measured from 200 to 900 m/z [M-H+] of the most active

411fractions previously separated by RP-HPLC.

412

413FIG 4: MALDI-ToF spectra measured from 900 to 3000 m/z [M-H+] of the most active

414fractions previously separated by RP-HPLC.

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Table 1. Peptides identified in fraction 4 of RP-HPLC.

Accession Number[1]	Protein Name	Expected Mass (*m/z*)	Mass Charge[2]	Calculated Mass (Da)	Po	Identified sequence*	Pf	Modifications
TITIN_MOUSE	Titin	413.10	2	824.39	S	MDPKYR*	D	Oxidation (M)
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	309.08	2	616.27	L	DIDSAP	I	
		821.93	2	1641.76	K	DPVQEAWAEDVDLR	V	
MYOM1_BOVIN	Myomesin-1	503.06	1	502.24	A	PTTGQ	I	
		509.22	1	508.28	Q	AHPGK	Y	
		525.44	1	524.31	G	VPGRP	R	
		533.15	1	532.29	I	KAISD	E	
		542.04	1	541.30	G	VPGRN	R	
		577.10	1	576.21	K	DSNNAG	V	
		579.44	1	578.24	P	SAPMTG	Q	Oxidation (M)
		362.14	2	722.33	N	NAGVHEP	E	
		376.12	3	1125.48	P	EETGGAEITGY	Y	
DYH3_HUMAN	Dynein heavy chain 3	521.09	1	520.18	S	CNPGM	K	
		525.44	1	524.15	Y	NDSSC	C	
		526.35	1	525.25	P	NPAPQ	W	
		528.36	1	527.31	S	NGIPK	L	
		559.23	1	558.26	F	PADEK	A	
		559.24	1	558.30	L	AQDIL	S	
		755.25	1	754.38	E	TRMGYK	P	
		476.14	2	950.45	L	NMPAKEVY	G	
		336.13	3	1005.48	F	SDFSLSHTL	G	
		471.28	3	1410.66	A	LDNPYPNPAPQW	L	
		668.59	3	2002.97	I	NMLLNTGDVPNIFPADEK	A	Oxidation (M)
ENOB_PIG	Beta-enolase	613.47	1	612.30	G	SHAGNK	L	
		506.05	3	1514.87	E	KKLSVVDQEKVDK	F	
		1034.08	2	2065.93	K	NYPVVSIEDPFDQDDWK	T	
TPM4_HUMAN	Tropomyosin alpha-4 chain	622.31	2	1242.65	R	IQLVEEELDR	A	
MARH4_HUMAN	E3 ubiquitin-protein ligase	579.08	3	1733.99	L	RLCKYRDILLSEIL	M	
KCRM_BOVIN	Creatine kinase	616.30	2	1230.62	K	DLFDPIIQDR	H	
HASP_HUMAN	Serine/threonine-protein kinase	323.10	2	644.32	R	RCPGGR*	V	
RL4_PIG	60S ribosomal protein L4	514.38	1	513.33	K	KPAAK	K	
		515.37	1	514.28	K	PAAEK	K	
		580.32	1	579.35	K	KPAHK*	K	
		580.33	1	579.35	K	PAHKK	P	
		670.53	1	669.45	K	KPAVKK*	P	

1. Accession number according to UniProt protein database.
2. Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
* Sequences with an asterisc were chosen to be synthesized and tested *in vitro* .

Table 2. Peptides identified in fraction 5 of RP-HPLC.

Accession Number[1]	Protein Name	Expected Mass (m/z)	Mass Charge[2]	Calculated Mass (Da)	Po	Identified sequence*	Pf	Modifications
TITIN_MOUSE	Titin	763.63	1	762.44	Y	TKYRVP*	D	
		783.53	2	1564.86	E	IIADGLKYRIQEF*	K	
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	551.16	1	550.31	G	FLGSK	K	
		340.14	2	678.30	R	PGSGFTN	T	
		689.12	1	688.32	V	CKDPVQ	E	
MYOM1_BOVIN	Myomesin-1	515.10	1	514.24	A	GVNEP	E	
		528.47	1	527.26	T	LPPSD	Y	
		530.37	1	529.25	S	APGTGQ	I	
		533.08	1	532.27	L	PVKCS	N	
		533.16	1	532.26	V	KASNN	A	
		305.05	2	608.26	V	KMSNN	A	Oxidation (M)
		615.25	1	614.41	Y	IIITR	K	
		639.26	1	638.29	P	EIQSY	R	
		702.20	1	701.43	Y	IIITDK	Q	
DYH3_HUMAN	Dynein heavy chain 3	505.06	1	504.27	S	ARTSA	N	
		506.05	1	505.20	R	SSSEP	M	
		511.43	1	510.32	G	PPIGK	K	
		515.10	1	514.28	V	KAAEP	G	
		519.10	1	518.23	R	EADGK	K	
		521.10	1	520.21	R	EQASS	L	
		525.43	1	524.27	D	LHAAN	Q	
		538.32	1	537.29	L	PITAH	P	
		546.38	1	545.32	V	AAKEK	P	
		547.39	1	546.34	E	VKTSL	T	
		559.24	1	558.23	S	ANEEP	S	
		578.10	1	577.25	M	NPGYAG	R	
		593.13	1	592.29	R	KDMIA	P	Oxidation (M)
		617.28	1	616.35	P	IGAAASK	E	
		623.28	1	622.26	M	EMESK	E	
		339.20	2	676.22	E	NDYYC	S	
		352.27	2	702.36	L	IIPTME	T	
		404.21	2	806.35	V	NRDTNTS	I	
		737.26	2	1472.68	D	VFFRNLIMGMDD	N	Oxidation (M)
		533.16	3	1596.75	V	FVDDLNMPAKEVYG	A	
		533.26	3	1596.78	T	SPIHLAFSMMRLY	S	2 Oxidation (M)
ENOB_PIG	Beta-enolase	486.30	3	1455.81	D	LVVGLCTGQIKTGAP	C	
MUC2_HUMAN	Mucin-2	621.28	3	1860.91	L	SLAGGSELQTEGRTRYH	G	
NEK10_HUMAN	Serine/threonine-protein kinase	684.20	2	1366.61	F	TSNRYHSYPWG*	T	
CYB_ORYME	Cytochrome b	352.27	2	702.28	I	HANGASM	F	Oxidation (M)
		359.09	2	716.32	I	GGQPVEM	P	
RL4_PIG	60S ribosomal protein L9	342.97	3	1025.59	K	PAAEKKPASK	K	

1. Accession number according to UniProt protein database.
2. Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
* Sequences with an asterisc were chosen to be synthesized and tested *in vitro* .

443 **Table 3**. Peptides identified in fraction 6 of RP-HPLC.

Accession Number[1]	Protein Name	Expected Mass (m/z)	Mass Charge[2]	Calculated Mass (Da)	Po	Identified sequence*	Pf	Modifications
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	683.22	1	682.35	Q	TARQAH*	L	
		371.25	2	740.33	V	ARMNFS	H	Oxidation (M)
MYOM1_BOVIN	Myomesin-14	306.02	2	610.28	S	NNAGVH	E	
DYH3_HUMAN	Dynein heavy chain 3	503.06	1	502.28	G	LGGEK	D	
		514.15	1	513.12	N	DSSCC	A	
		301.13	2	600.27	N	CHLAAS	W	
		624.55	1	623.32	N	IIEYS	R	
		332.21	2	662.38	G	IMKQK*	K	Oxidation (M)
		339.03	2	676.34	M	VPDYAL	I	
		713.25	1	712.24	L	IMGMDD	N	2 Oxidation (M)
		329.20	3	984.55	V	ILRCLRPD	K	
		333.07	3	996.47	W	ALMIDPHGQ	A	Oxidation (M)
KCRM_BOVIN	Creatine kinase	522.10	1	521.23	K	GGNMK	E	Oxidation (M)

1. Accession number according to UniProt protein database.
2. Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.
* Sequences with an asterisc were chosen to be synthesized and tested *in vitro* .

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464 **Table 4**. Peptides identified in fraction 7 of RP-HPLC.

Accession Number[1]	Protein Name	Expected Mass (*m/z*)	Mass Charge[2]	Calculated Mass (Da)	Po	Identified sequence*	Pf	Modifications
KPYM_RABIT	Pyruvate kinase isozymes M1/M2	619.45	1	618.26	A	WAEDV	D	
		621.47	1	620.28	S	DGIMVA	R	Oxidation (M)
		666.33	1	665.29	V	ICATQM	L	
MYOM1_BOVIN	Myomesin-1	308.13	2	614.34	L	PVKASN	N	
		459.11	2	916.38	K	YGSEISDF	T	
DYH3_HUMAN	Dynein heavy chain 3	503.06	1	502.24	L	VAADQ	P	
		503.06	1	502.31	R	AVKSV	L	
		533.14	1	532.29	Q	IAKSD	S	
		326.13	2	650.38	N	LPITAH	P	
		340.12	2	678.29	V	RHGYM*	I	Oxidation (M)
		362.14	2	722.29	E	TVMENN	P	Oxidation (M)
		656.41	2	1310.59	R	HCNKKYRSEM*	E	Oxidation (M)
MARH4_HUMAN	E3 ubiquitin-protein ligase	532.24	2	1062.57	R	ELVMRVTTV	-	Oxidation (M)
HASP_HUMAN	Serine/threonine-protein kinase	394.18	3	1179.60	R	REHQEASVPK	G	
CYB_ORYME	Cytochrome b	622.39	2	1242.57	G	YVLPWGQMSF	W	Oxidation (M)

1. Accession number according to UniProt protein database.
2. Charge of the peptide according to the ionisation occured in nanoLC-MS/MS analysis.

465 * Sequences with an asterisc were chosen to be synthesized and tested *in vitro* .

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468**Table 5.** MIC values (mM) of the synthetised antibacterial peptides tested against *L.* 469*monocytogenes* CECT 4031^{T} and FBUNT strains.

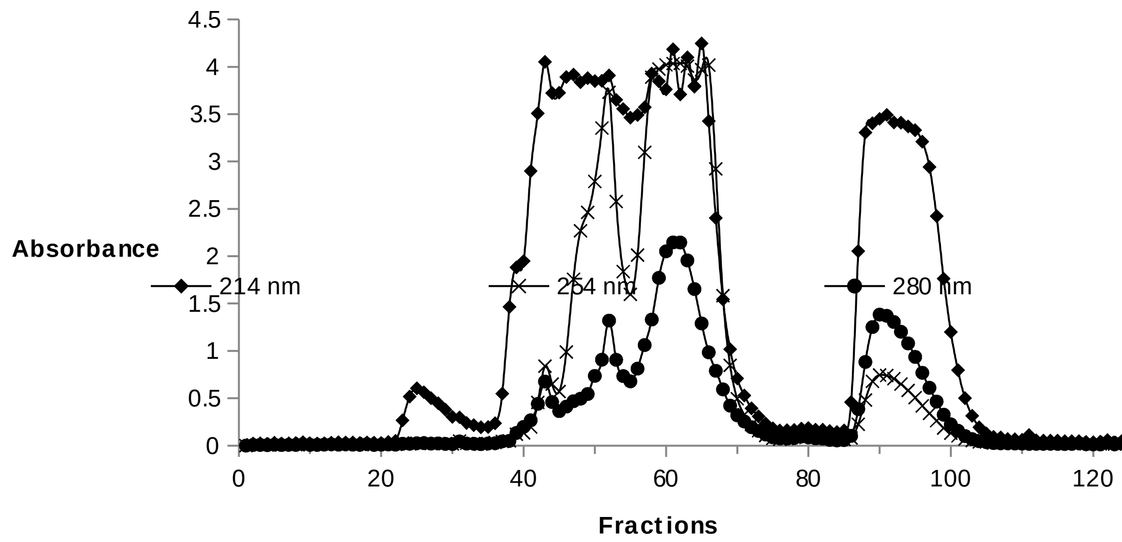
Peptide	MIC (mM)	
	FBUNT	CECT 4031^{T}
TKYRVP	100	100
TSNRYHSYPWG	100	100
IIADGLKYRIQEF	100	100
IPAVF	n.i.-*	n.i.-
MDPKYR	50	50
RCPGGR	50	50
HCNKKYRSEM	50	50
IMKQK	n.i.-	n.i.-
TARQAH	50	50
KPAHK	50	50
RHGYM	6.25	6.25
KPAVKK	50	50

470CECT, Spanish Type Culture Collection; ^{T}Type strain of the species;

471n.i. Means non–inhibitory at any of the tested concentrations.

472CEC

Figure 1. Gel filtration chromatography of 10 months of curing dry-cured ham extract using a Sephadex G-25 column.



ollection; [T]Type strain of the species; *Non inhibitory

Figure 2. Reversed-phase HPLC separation of fractions 41 and 42 obtained from gel filtration chromatography. Dotted line indicates fractions from 4 to 7 that showed *Listeria monocytogenes* inhibitory activity.

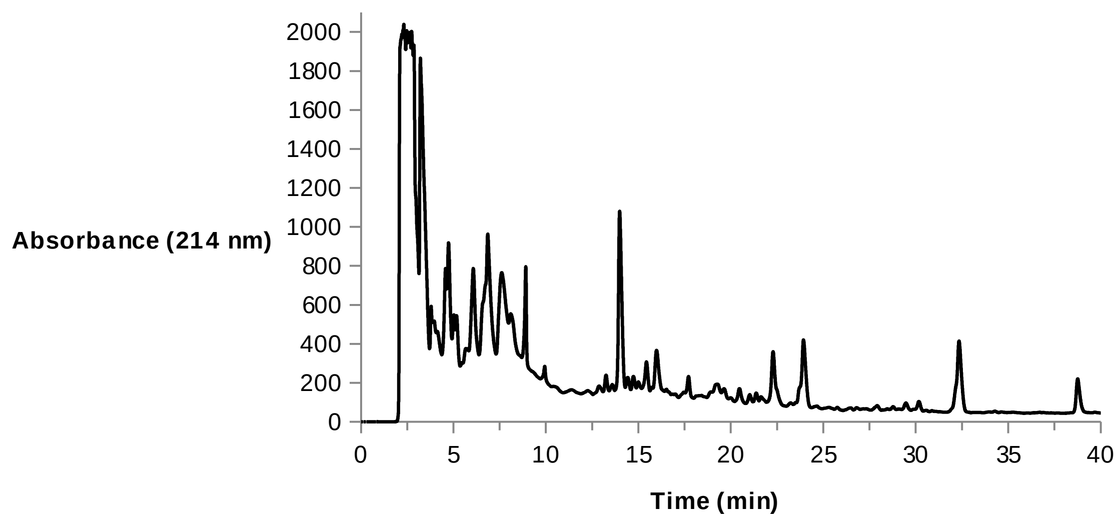


Figure 3. MALDI-ToF spectra measured from 200 to 900 *m/z* [M-H$^+$] of the most active fractions previously separated by RP-HPLC.

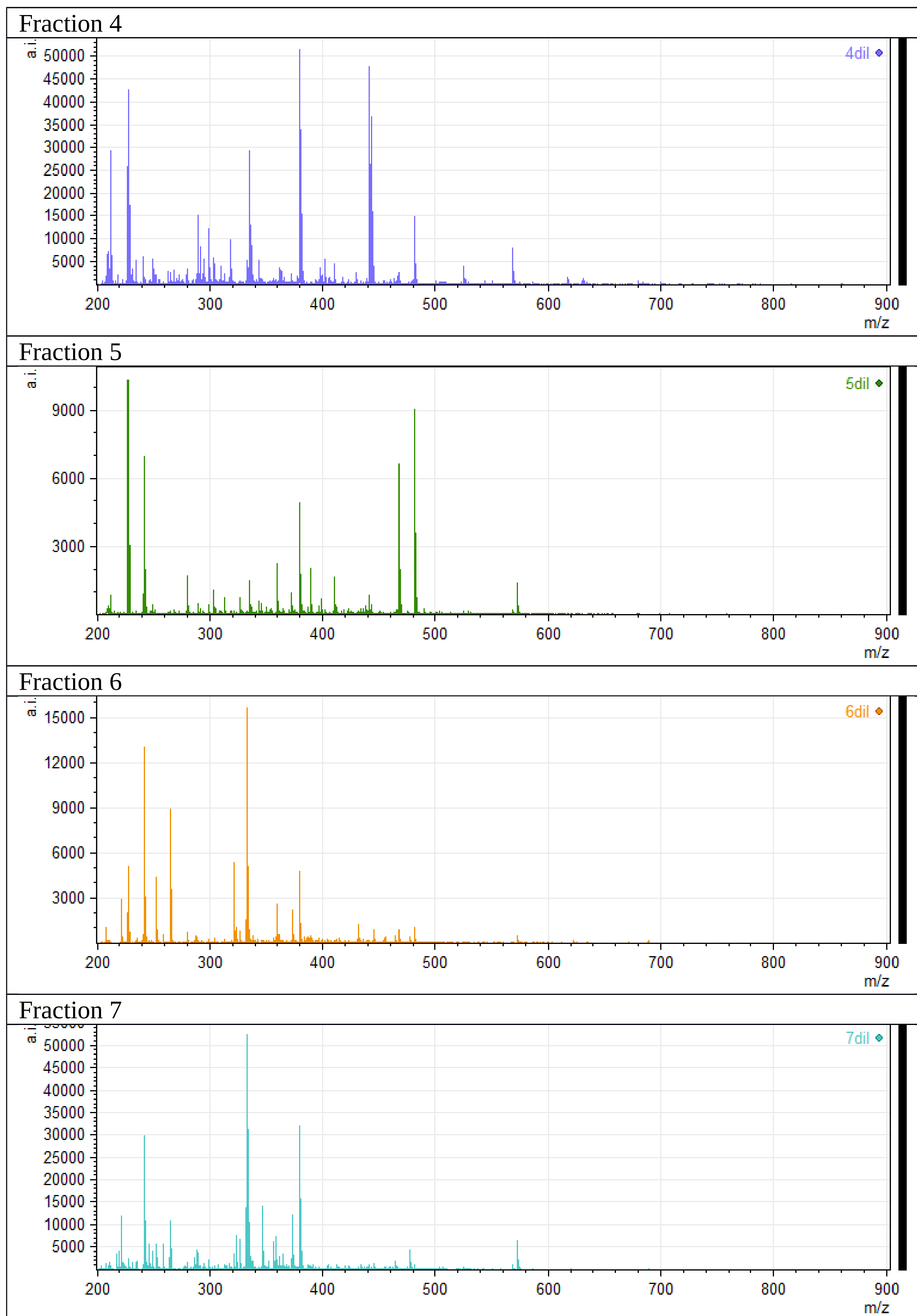


Figure 4. MALDI-ToF spectra measured from 900 to 3000 *m/z* [M-H+] of the most active fractions previously separated by RP-HPLC.