Peptidomics as a tool for quality control in dry-cured ham processing

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Abstract

Spanish dry-cured ham is a high quality product whose economic value is mainly given by its curing time. An intense proteolysis takes place throughout the dry-cured processing, which results in the generation of a high amount of peptides and free amino acids responsible for the final quality of dry-cured hams. In this work, a peptidomics approach has been used to study the evolution of peptides throughout the ham dry-curing process, identifying and quantifying the generated peptides in order to define potential quality biomarkers. For this purpose, dry-cured hams extracts at different processing times (0, 2, 3.5, 5, 6.5 and 9 months) were fractionated by size-exclusion chromatography and analysed by nanoliquid chromatography coupled to tandem mass spectrometry. Differences obtained in the relative quantification of peptides by using a label-free methodology were useful to establish differences between processing times, being peptides generated by the degradation of myosin light chain 1 protein those found as main responsible for the observed differences during the last stages of curing. In particular, APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE, exclusively identified at 9 months of curing, would be good potential markers to control the time of curing and thus the final quality of dry-cured hams.

Keywords: Peptidomics, mass spectrometry, quantification, label-free, peptides, biomarker, dry-cured ham.

1. Introduction

Peptidomics is the area of science focused on the study of composition, interactions, and properties of peptides present in biological samples. This field partially covers same research approaches and uses similar methods as proteomics, although analytic and experimental extrategies employed differ in some points [1,2]. Peptidomics follows a "top-down" approach that involves a difficult data analysis because it comprises unspecific hydrolysis resulting in peptides showing a wide range of sizes and charge states with any type of post-translational modifications. Usually its main goal is to identify as many peptides as possible, which requires the manual evaluation of the MS/MS spectra of each peptide and to verify the accuracy of the assignments. On the other hand, proteomics principally adopts a "bottom-up" approach in which proteins are enzymatically digested, mainly with trypsin enzyme, to generate peptides similar in length, charge and properties. The identification of proteins is based on automatic analysis of mass spectrometry data and database search, being not necessary the identification of all the enzymatic fragments for a conclusive identification of each protein of origin [3,4].

Peptidomics is an expanding area that was firstly applied in the medical and clinical fields for the identification of signalling molecules such as neuropeptides and hormones in biological samples [5,6] and determine peptide biomarkers of diseases as cancer [7,8], cardiovascular events [9,10] or Alzheimer's disease [11]. Later, peptidomics has also become an essential tool in food science in order to study protein digestion and identify bioactive peptides [12,13], characterise food-processing related proteolysis [14,15] or determine peptide biomarkers of food quality criteria [16,17]. In this regard, the study of peptide biomarkers may also include their relative quantification by using labelled or label-free methods [18,19]. Labelling methodologies provide the most accurate

quantitative values, but their use involves costly labelling reagents and multi-step experimental protocols. In contrast, label-free methods are a simple, versatile, reliable and cost-effective alternative for quantification, either based on the signal intensity measurements of extracted ion chromatograms or spectral counting [20-22]. In this regard, quantification on the basis of peak intensity, whereby the peak areas of peptides correlate to the concentration of the protein of origin, allows a more accurate and precise evaluation of changes in protein abundance from sample to sample in comparison with the spectral counting strategy [23,24].

Spanish dry-cured ham is a high-quality product that requires a very long ripening time, reaching in certain cases 24 months of processing or even longer, and which mainly determines its economic value. Complex and numerous biochemical reactions take place during the long dry-curing process of hams contributing to the texture, flavour and final quality of this Spanish typical product [25-27]. The control of the time of curing and thus the final quality of dry-cured hams is necessary to prevent fraudulent activities or accidental mislabelling resulting in over-valued products.

The main purpose of the present study was the relative quantification of the peptides generated during the ham dry-curing process by using a label-free methodology based on the measurement of ion peak intensities. This peptidomics approach will allow to evaluate those peptides responsible for the differences between processing times and the identification of those peptides that could be used as potential quality markers in dry-cured hams.

2. Materials and methods

2.1 Dry-cured ham processing

Spanish dry-cured hams coming from 6-months-old pigs (Landrace × Large White) were prepared according to the traditional procedure until a total processing duration of 9 months. This procedure consisted of a pre-salting stage (using a mixture of salt, nitrate and nitrite for 30 min), a salting period (the hams were buried in salt and piled up at 2-4 °C and 90-95% relative humidity for 10-12 days), a post-salting stage (at 4-5 °C and 75-85% relative humidity for 60 days), and finally a ripening-drying period (at temperatures increasing from 5 °C to 14-20 °C and relative humidity decreasing to 70%). The study was done in triplicate with samples selected at different processing times: 0 months (raw ham), 2 months (end of the post-salting stage), 3.5, 5, and 6.5 months (during the ripening-drying period), and 9 months (at the end of the dry-cured process).

2.2 Extraction of peptides

A total of 50 g sample of *Biceps femoris* muscle coming from each processing time were minced and homogeneised with 200 mL of 0.01 N HCl for 8 min in a stomacher (IUL Instrument, Barcelona, Spain). The homogenate was centrifuged at 4 °C and 12,000 *g* for 20 min and filtered through glass wool. Then, proteins were precipitated by adding 3 volumes of ethanol and maintaining the sample at 4 °C for 20 h. The resulting sample was centrifuged at 4 °C and 12,000 *g* for 10 min and the supernatant was dried in a rotatory evaporator and finally lyophilised. The dried deproteinised extract was dissolved in 25 mL of 0.01 N HCl, filtered through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA), and stored at -20 °C until use.

2.3 Size-exclusion chromatography

A 5 mL aliquot of each extract was subjected to size-exclusion chromatography in order to fractionate deproteinised ham extracts according to their molecular mass. For that, a Sephadex G25 column (2.5 x 65 cm; Amersham Biosciences, Uppsala, Sweden) was

employed, previously equilibrated with 0.01 N HCl. The separation was carried out using 0.01 N HCl as mobile phase at a flow rate of 15 mL/h in a cool room (4 °C). Fractions of 5 mL were automatically collected and monitored by ultraviolet absorption at 214 nm (Agilent 8453 UV spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). Lastly, fractions corresponding to elution volumes from 125 to 160 mL were pooled together and aliquots of 100 μL were lyophilised for the subsequent analysis.

2.4 nLC-MS/MS analysis

The identification of the peptides was done by nanoliquid chromatography-tandem mass spectrometry using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole/time-of-flight (Q-ToF) TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source (ESI).

Lyophilised dry-cured ham samples were resuspended in 100 μL of H_2O with 0.1% of trifluoroacetic acid (TFA). Then 10 μL of each sample was cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation, Bedford, MA) according to manufacturer's guidelines. Finally, 4 μL of the supernatant were injected into the nLC-MS/MS system.

Samples were preconcentrated on an Eksigent C18 trap column (3 μm, 350 μm × 0.5 mm; Eksigent of AB Sciex, CA, USA), at a flow rate of 3 μL/min for 5 min and using 0.1% v/v TFA as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3 μm, 75 μm × 12.3 cm, C18) (Nikkyo Technos Co., Ltd., Japan). The mobile phases consisted of solvent A, containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v formic acid in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.30 μL/min and running temperature of 30 °C. The column outlet was directly coupled to a

nano-electrospray ionisation system (nano-ESI). The Q/ToF was operated in positive polarity and information-dependent acquisition mode, in which a 250 ms ToF MS scan from 300 to 1250 *m/z* was performed, followed by 50 ms product ion scans from 100 to 1500 *m/z* on the 50 most intense 1 - 5 charged ions.

2.5 Data analysis

Automated spectral processing, peak list generation, database search, and relative quantification were performed using Mascot Distiller v2.5.1.0 software (Matrix Science, Inc., Boston, MA, USA; http://www.matrixscience.com).

The identification of protein of origin of peptides was done using UniProt database, the taxonomy parameter was designated as Mammalia, and oxidation of methionine (M) as variable modification and none specific enzyme were selected for the database search. Generated MS/MS spectra were searched in Mascot search engine v.2.3.0 software using a significance threshold of $p < 0.05$ and a tolerance on the mass measurement of 100 ppm in MS mode and 0.3 Da for MS/MS ions.

The relative quantification was done using the label-free methodology described by Gallego et al. [28] with slight modifications. This label-free approach is based on replicates of the relative intensities of extracted ion chromatograms (XICs) for precursors aligned using mass and elution time [23,29]. Mascot search engine assigns peptide matches to the protein of origin, requiring robust search parameters as quantification is based on the identification at peptide level. Peptides identified in dry-cured ham at 9 months of processing were used as reference in the calculation of ratios for individual peptides, which are obtained from the integration of the XICs from three replicates. The method of integration was optimised, establishing quality criteria to effectively eliminate outlier points.

Finally, statistical analysis was performed using SIMCA-P+ 13.0 (Umetrics AB, Sweden) software. In this regard, Principal Component Analysis (PCA) and loading plot analysis for dry-cured ham samples at different processing times were done.

3. Results and discussion

Control and characterisation of the quality of dry-cured hams is a difficult task since a high number of factors such as genetics, processing conditions and ripening time are involved in the process. Proteolysis is the main biochemical reaction that takes place throughout the ham dry-curing process and plays a major role in the end product quality. In this regard, the intensity of the proteolysis depends on the degree of activity exerted by endogenous muscle enzymes (endopeptidases and exopeptidases), which generate small peptides and free amino acids that contribute largely to the characteristic texture and flavour of dry-cured hams [25,30]. In the present work, a label-free methodology to relatively quantify natural peptides generated at different times during the dry-cured ham processing (0, 2, 3.5, 5, 6.5 and 9 months) have been used to establish differences between them. For this purpose, a Principal component analysis (PCA) score plot was performed to explain differences between processing times and obtain information on those peptides that mainly influence the sample similarities and differences (see Figure 1). The PCA was performed on the whole set of values (3 replicates per sample and an extra replicate for sample at 9 months as reference for quantification), showing six statistically different groups that correspond with the six processing times assayed (Figure 1A). Discriminant component 1 explains 24% of the variability in the dataset, making it possible to differentiate between the first stages of the processing (salting and post-salting stages from 0 to 3.5 months) and the later stages (from 5 to 9 months). On the other hand, Discriminant component 2 is responsible for 12.6% of the variance

within the dataset for these two discriminant components, which allows to differentiate between raw hams and hams at the beginning of the process (post-salting and first period of ripening) as well as between hams along the ripening-drying period and the final product (9 months of ripening). In addition, the loading plot (Figure 1B) revealed the proteins of origin of those peptides showing the highest influence for the description of the two discriminant components, and thus are responsible for main differences between dry-cured hams at different processing times. In this regard, the most influential peptides were identified from proteins actin (ACTS), cyclin-dependent kinase inhibitor (CDN1C), beta-enolase (ENOB), glyceraldehyde-3-phosphate dehydrogenase (G3P), LIM domain-binding protein 3 (LDB3), myosin light chain 1 (MYL1), myosin light chain 4 (MYL4), nebulin (NEBU), titin (TITIN), troponin T (TNNT1), and polyubiquitin-C (UBC).

In order to study the potential of peptides as quality markers to estimate and control the curing time of dry-cured hams, the main interest was focused on those peptides present at the end of the processing. The analysis by nLC-MS/MS revealed that the most influential and abundant peptides at 9 months of curing, located in the fourth quadrant of the PCA plot in Figure 1B, were derived from MYL1 protein. Myosin is the major myofibrillar protein in skeletal muscle, playing a key role in muscle contraction. Numerous studies have reported the intense proteolysis of myosin protein during dry-cured ham processing by using electrophoretic techniques [31,32] and MS techniques [31,33,34]. Focusing on MYL1, Mora et al. [34] identified 137 peptides resulting from this protein in dry-cured hams at 9 months of processing by using tandem mass spectrometry. In the present study, samples were analysed by nLC-MS/MS to follow the proteolysis phenomena occurring throughout the ham dry-curing process. So, Table 1 shows the identification of 211 peptides as MYL1 fragments at different processing

times (0, 2, 3.5, 5, 6.5 and 9 months). This table lists the peptide sequences, the observed and calculated masses together with the charge states, amino acids residues preceding and following each sequence, and the processing times when peptides have been identified. Among all the identified peptides, 142 of them were detected at 9 months of curing, which is similar to the previously result reported by Mora et al. [34]. However, shorter peptides than those detected in the previous work have been identified in this study, mainly resulting to be fragments of peptides earlier described. This fact could be due to variabilities in the type of dry-cured hams, genetics, and differences in the action of endopeptidases and exopeptidases during the processing [25]. Moreover, a total of 77 peptides were exclusively detected at the end of the processing, so they could be used as potential biomarkers to indicate a minimum curing time of 9 months. In fact, recent previous works were focused on the study of naturally generated peptides throughout the dry-cured ham processing from LIM domain-binding protein 3 [35], ubiquitin 60-S ribosomal protein [36] and titin [37], and the identification of those peptides useful as potential biomarkers in order to estimate and control the curing time.

Based on the results shown in Figure 1B, those peptides responsible for main differences at 9 months of curing and resulting from the degradation of MYL1 protein were selected in order to establish a new analysis. Changes in the abundance of peptides evaluated by the label-free quantitative method are mainly due to differences in the degree of action of endogenous enzymes at each processing time, which determine the amount and profile of generated peptides. Accordingly, Figure 2 shows that not all the peptides have an equal influence on the clustering of data, but peptides APAPAPAPPKEEKI, PAPAPAPAPAPAPAPPKE, APAPAPAPAPAPPKEEKID, PAPAPAPAPAPAPAPPKEEK, and VKKPAAAAAPAPAPAPAPAPAPPKE are those with higher colour density values, and thus main responsible for the observed

252 differences. In this analysis, Discriminant component 1 explains 40% of the variability
253 in the dataset, and Discriminant component 2 is responsible for 20% of the variance
254 within the dataset. These five peptides correspond, respectively, to numbers 78, 111,
255 141, 144, and 201 of Table 1, in which is indicated that peptides 78
256 (APAPAPAPPKEEKI) and 111 (PAPAPAPAPAPAPAPPKE) have been exclusively
257 identified at 9 months of processing whereas the remaining three peptides were also
258 detected at other times using nLC-MS/MS analysis. Thus, Figure 3 shows the MS/MS
259 spectra of peptides APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE. The
260 identification and relative quantification of these two peptides using the developed
261 peptidomics approach carried out in this study suggest their use as potential biomarkers
262 to control the time of curing and thus ensure the final quality of dry-cured hams.
263 Nevertheless, further analysis would be needed to confirm these peptides as biomarkers
264 of the ham dry-curing process, especially if considering that dry-cured hams with longer
265 processing times would be particularly interesting in order to avoid misleading
266 consumers and fraudulent activities regarding the quality of valuable dry-cured hams.
267
268 **4. Conclusions**
269 The use of peptidomics has resulted to be a very effective tool to study the evolution of
270 peptides during the processing of dry-cured ham as well as very useful to identify and
271 relatively quantify those peptides that could be used as potential biomarkers. This study
272 shows that differences obtained in the results of label-free relative quantification of
273 generated peptides throughout the dry-curing process are useful to establish differences
274 between processing times (0, 2, 3.5, 5, 6.5 and 9 months). Moreover, peptides derived
275 from myosin light chain 1 protein have shown to be the most influential at the end of the
276 processing, and specifically APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE,

277 exclusively identified at 9 months of curing, could be potential markers to control the
278 time of curing and thus the final quality of dry-cured hams.
279
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289 **References**
290 [1] Minkiewicz P, Dziuba J, Darewicz M, Iwaniak A, Dziuba M, Nałęcz D. Food
291 peptidomics. Food Technol Biotech 2008;46(1):1–10.
292 [2] Gašo-Sokač D, Kovač S, Josić D. Use of proteomic methodology in optimization of
293 processing and quality control of food of animal origin. Food Technol Biotech
294 2011;49(4):397-412.
295 [3] Panchaud A, Affolter M, Kussmann M. Mass spectrometry for nutritional
296 peptidomics: How to analyze food bioactives and their health effects. J Proteomics
297 2012;75(12):3546-59.
298 [4] Schrader M, Schulz-Knappe P, Fricker LD. Historical perspective of peptidomics.
299 EuPA Open Proteom 2014;3:171-82.

[5] Schulz-Knappe P, Zucht HD, Heine G, Jürgens M, Hess R, Schrader M. Peptidomics: The comprehensive analysis of peptides in complex biological mixtures. Comb Chem High Throughput Screen 2001;4(2):207-17.

[6] Svensson M, Sköld K, Svenningsson P, Andren PE. Peptidomics-based discovery of novel neuropeptides. J Proteome Res 2003;2(2):213-9.

[7] Diamandis EP. Peptidomics for cancer diagnosis: Present and future. J Proteome Res 2006;5(9):2079-82.

[8] Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB, et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. J Clin Invest 2006;116(1):271-84.

[9] Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 2006;355(25):2631-9.

[10] Jaffe AS, Babuin L, Apple FS. Biomarkers in acute cardiac disease. The present and the future. J Am Coll Cardiol 2006;48(1):1-11.

[11] Selle H, Lamerz J, Buerger K, Dessauer A, Hager K, Hampel H, et al. Identification of novel biomarker candidates by differential peptidomics analysis of cerebrospinal fluid in Alzheimer's disease. Comb Chem High Throughput Screen 2005;8(8):801-6.

[12] Bauchart C, Morzel M, Chambon C, Mirand PP, Reynès C, Buffière C, et al. Peptides reproducibly released by in vivo digestion of beef meat and trout flesh in pigs. Brit J Nutr 2007;98(6):1187-95.

[13] Dallas DC, Guerrero A, Khaldi N, Castillo PA, Martin WF, Smilowitz JT, et al. Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective antimicrobial peptides. J Proteome Res 2013;12(5):2295-304.

[14] Sforza S, Cavatorta V, Lambertini F, Galaverna G, Dossena A, Marchelli R. Cheese peptidomics: A detailed study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24 months of aging. J Dairy Sci 2012;95(7): 3514-26.

[15] Mora L, Escudero E, Aristoy MC, Toldrá F. A peptidomic approach to study the contribution of added casein proteins to the peptide profile in Spanish dry-fermented sausages. Int J Food Microbiol 2015;212:41-8.

[16] Mansor R, Mullen W, Albalat A, Zerefos P, Mischak H, Barrett DC, et al. A peptidomic approach to biomarker discovery for bovine mastitis. J Proteomics 2013;85:89-98.

[17] López CM, Bru E, Vignolo GM, Fadda SG. Identification of small peptides arising from hydrolysis of meat proteins in dry fermented sausages. Meat Sci 2015;104:20-9.

[18] Schrader M, Schulz-Knappe P. Peptidomics technologies for human body fluids. Trends Biotechnol 2001;19(10):S55-60.

[19] Fricker LD, Lim J, Pan H, Che FY. Peptidomics: Identification and quantification of endogenous peptides in neuroendocrine tissues. Mass Spectrom Rev 2006;25(2):327-44.

[20] Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: A critical review. Anal Bioanal Chem 2007;389(4):1017-31.

[21] Elliott MH, Smith DS, Parker CE, Borchers C. Current trends in quantitative proteomics. J Mass Spectrom 2009;44(12):1637-60.

[22] Neilson KA, Ali NA, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, et al. Less label, more free: Approaches in label-free quantitative mass spectrometry. Proteomics 2011;11(4):535-53.

350 [23] Wang G, Wu WW, Zeng W, Chou CL, Shen RF. Label-free protein quantification
351 using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and
352 application with complex proteomes. J Proteome Res 2006;5(5):1214-23.
353 [24] Zhu W, Smith JW, Huang CM. Mass spectrometry-based label-free quantitative
354 proteomics. J Biomed Biotechnol 2010;840518.
355 [25] Toldrá F, Flores M. The role of muscle proteases and lipases in flavour
356 development during the processing of dry-cured ham. Crc Cr Rev Food Sci
357 1998;38(4):331-52.
358 [26] Lametsch R, Karlsson A, Rosenvold K, Andersen HJ, Roepstorff P, Bendixen E.
359 Postmortem proteome changes of porcine muscle related to tenderness. J Agr Food
360 Chem 2003;51(24):6992-97.
361 [27] Sforza S, Galaverna G, Schivazappa C, Marchelli R, Dossena A, Virgili R. Effect
362 of extended aging of Parma dry-cured ham on the content of oligopeptides and free
363 amino acids. J Agr Food Chem 2006;54(25):9422-29.
364 [28] Gallego M, Mora L, Aristoy MC, Toldrá F. Optimisation of a simple and reliable
365 label-free methodology for the relative quantitation of raw pork meat proteins. Food
366 Chem 2015;182:74-80.
367 [29] Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, et al. Quantitative
368 proteomic analysis by accurate mass retention time pairs. Anal Chem 2005;77(7):2187-
369 200.
370 [30] Toldrá F. The role of muscle enzymes in dry-cured meat products with different
371 drying conditions. Trends Food Sci Tech 2006;17(4):164-8.
372 [31] Di Luccia A, Picariello G, Cacace G, Scaloni A, Faccia M, Liuzzi V, et al.
373 Proteomic analysis of water soluble and myofibrillar protein changes occurring in dry-
374 cured hams. Meat Sci 2005;69(3):479-91.

[32] Larrea V, Hernando I, Quiles A, Lluch MA, Pérez-Munuera I. Changes in proteins during Teruel dry-cured ham processing. Meat Sci 2006;74(3):586-93.

[33] Mora L, Sentandreu MA, Koistinen KM, Fraser PD, Toldrá F, Bramley PM. Naturally generated small peptides derived from myofibrillar proteins in Serrano dry-cured ham. J Agr Food Chem 2009;57(8):3228-34.

[34] Mora, L., Sentandreu, M.A., & Toldrá, F. Intense degradation of myosin light chain isoforms in Spanish dry-cured ham. J Agr Food Chem 2011;59:3884–92.

[35] Gallego M, Mora L, Fraser PD, Aristoy MC, Toldrá F. Degradation of LIM domain-binding protein three during processing of Spanish dry-cured ham. Food Chem 2014;149:121-8.

[36] Mora L, Gallego M, Aristoy MC, Fraser PD, Toldrá F. Peptides naturally generated from ubiquitin-60S ribosomal protein as potential biomarkers of dry-cured ham processing time. Food Control 2014;48:102-7.

[37] Gallego M, Mora L, Aristoy MC, Toldrá F. Titin-derived peptides as processing time markers in dry-cured ham. Food Chem 2015;167:326-39.

FIGURE CAPTIONS

Figure 1. A) Principal Component Analysis (PCA) score plot to assess the variance among the naturally generated peptides at different times of the dry-cured ham processing (0, 2, 3.5, 5, 6.5 and 9 months) in three replicates (n=3). **B)** PCA loading plot showing the proteins of origin of those peptides more responsible for main differences between processing times.

Figure 2. PCA loading plot showing peptides identified at 9 months of curing from MYL1 protein. Higher colour density values indicate those peptides more responsible for influencing the clustering of data.

Figure 3. MS/MS spectra of peptides APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE generated from MYL1 protein at 9 months of processing. The spectra are presented with their corresponding *b* and *y* ions matched by Mascot search engine.

Table

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Table 1. Peptides identified by nLC-MS/MS from myo

Peptide number	Observed (*m/z*)[a]	Charge (+)	Calculated (Da)[b]	P_o[c]
1	301.137	2	600.287	V
2	384.198	2	766.386	A
3	394.718	2	787.423	A
4	412.770	2	823.492	V
5	419.718	2	837.423	P
6	430.236	2	858.460	A
7	432.742	2	863.487	K
8	434.254	2	866.490	F
9	439.236	2	876.471	P
10	457.336	2	912.576	M
11	458.763	2	915.518	P
12	462.286	2	922.560	D
13	478.760	2	955.513	P
14	487.731	2	973.523	A
15	488.298	2	974.519	K
16	503.759	2	1005.513	P
17	507.255	2	1012.571	A
18	507.769	2	1013.525	L
19	514.283	2	1026.550	A
20	516.790	2	1031.576	K
21	522.797	2	1043.586	K
22	523.281	2	1044.560	P
23	526.264	2	1050.429	E
24	527.255	2	1052.566	A
25	528.286	2	1054.650	P
26	532.276	2	1062.619	V
27	540.276	2	1078.602	P
28	542.777	2	1083.608	P
29	546.330	2	1090.650	D
30	549.798	2	1097.587	A
31	552.287	2	1102.566	A
32	562.774	2	1123.603	P
33	378.874	3	1133.608	P
34	571.807	2	1141.613	A
35	586.846	2	1171.681	C
36	587.804	2	1173.603	P
37	588.318	2	1174.558	L
38	591.293	2	1180.660	A
39	398.881	3	1193.629	P
40	598.310	2	1194.640	A
41	598.315	2	1194.640	A
42	599.345	2	1196.724	P
43	607.326	2	1212.646	V
44	607.326	2	1212.650	P
45	611.349	2	1220.655	A
46	407.910	3	1220.596	E

Figure 1

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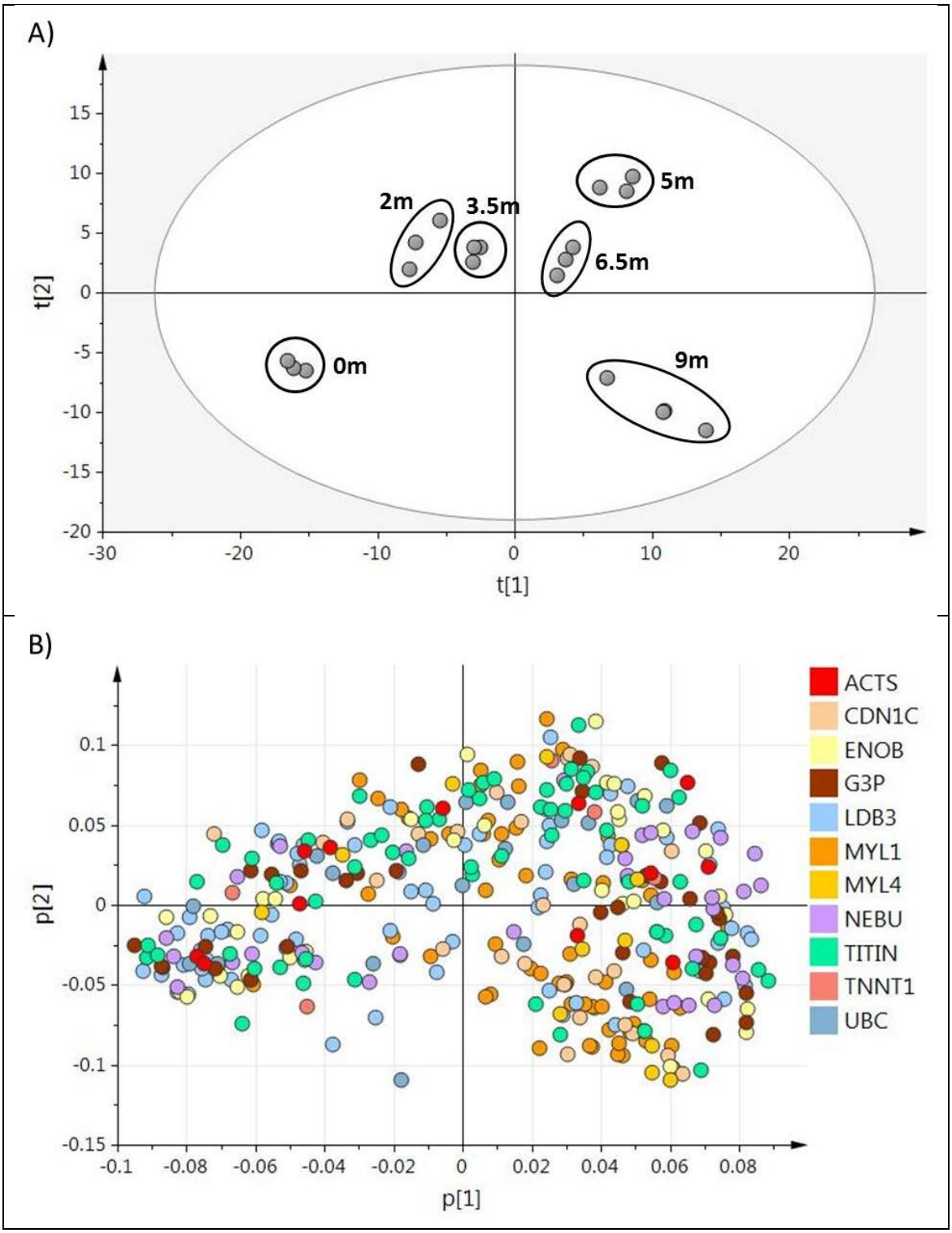

Figure 1.

Figure 2

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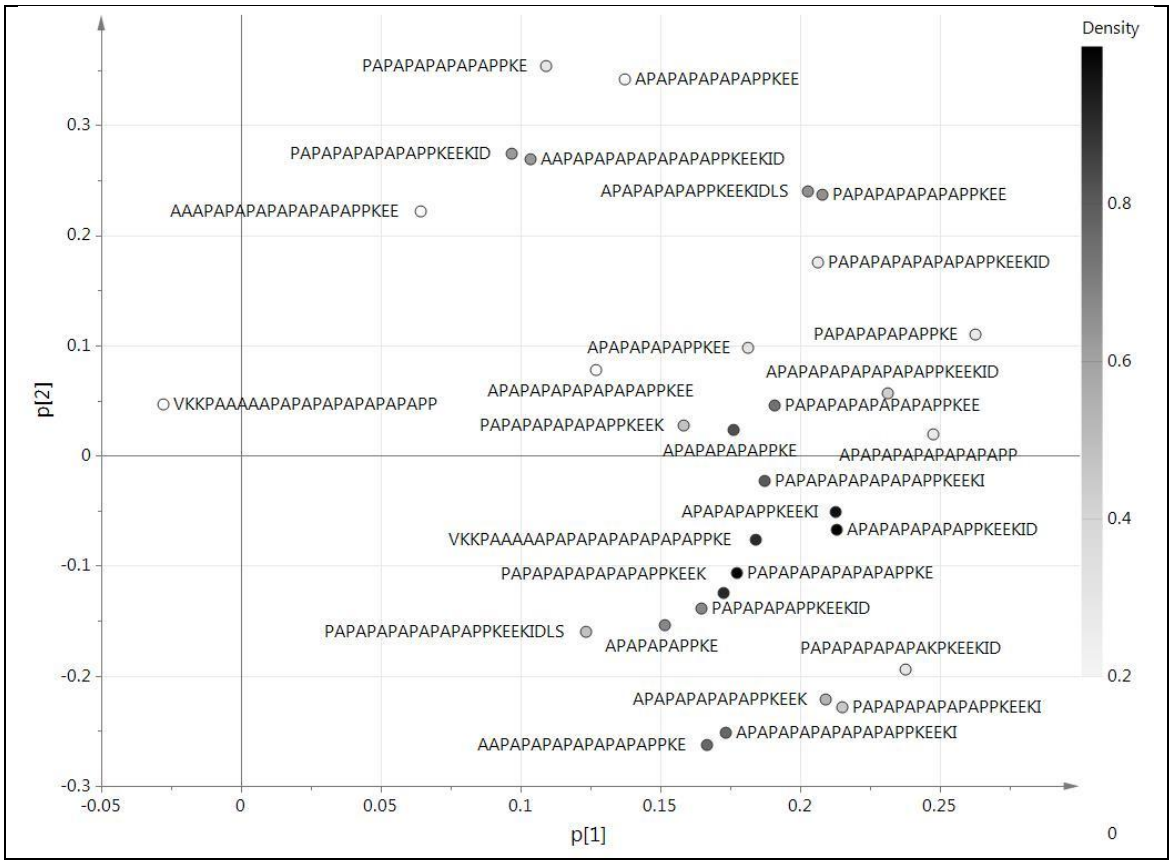

Figure 2.

Figure 3

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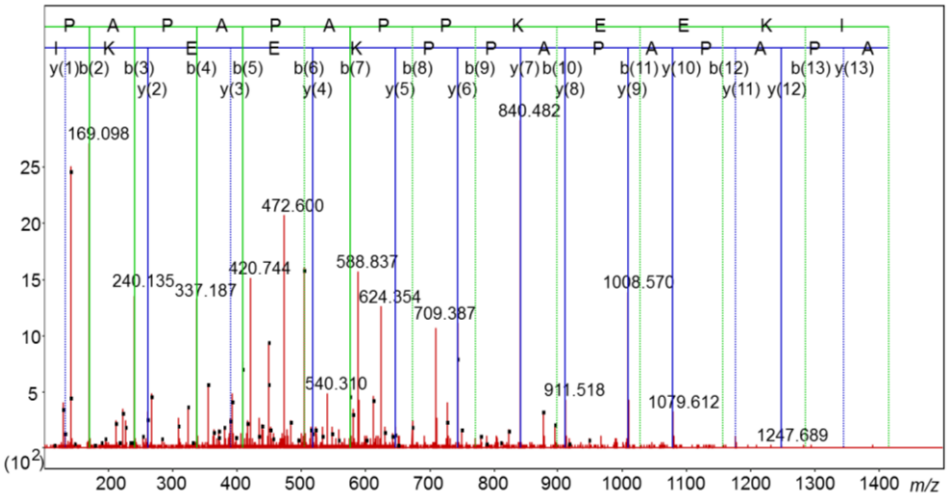
APAPAPAPPKEEKI (708.339, 2+)



PAPAPAPAPAPAPPKE (549.625, 3+)

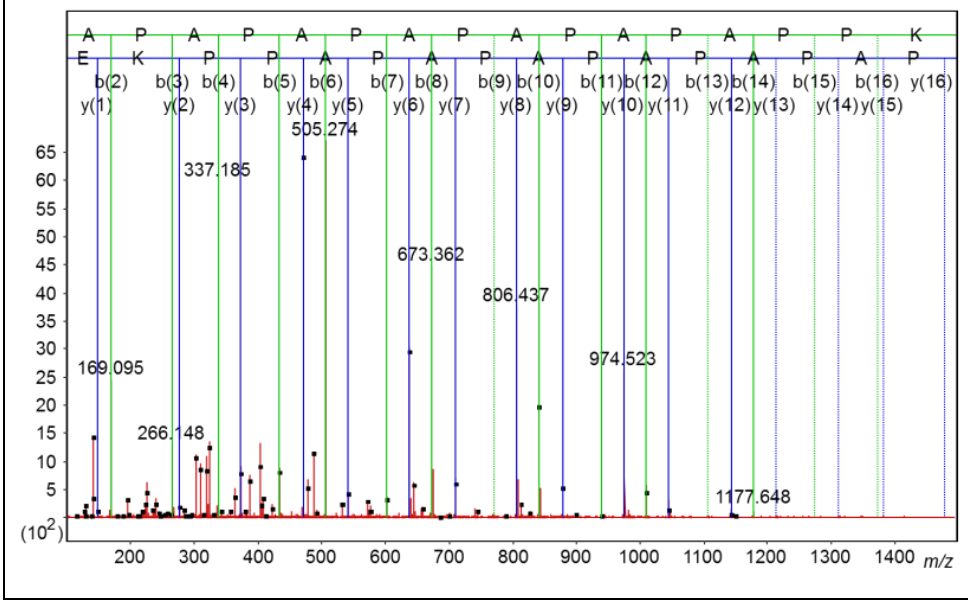


Figure 3.

***Conflict of Interest**

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