Optimisation of a simple and reliable label-free methodology for the relative quantitation of raw pork meat proteins

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mailto:**Abstract**

Recent advances in proteomics have become an indispensable tool for a fast, precise and sensitive analysis of proteins in complex biological samples at both, qualitative and quantitative level. In this study, a label-free quantitative proteomic methodology has been optimised for the relative quantitation of proteins extracted from raw pork meat. So, after the separation of proteins by one-dimensional gel electrophoresis and trypsin digestion, their identification and quantitation have been done using nanoliquid chromatography coupled to a quadrupole/time-of-flight (Q/ToF) mass spectrometer. Relative quantitation has been based on the measurement of mass spectral peak intensities, which have been described that are correlated with protein abundances. The results obtained regarding linearity, robustness, repeatability and accuracy show that this procedure could be used as a fast, simple, and reliable method to quantify changes in protein abundance in meat samples.

Keywords: Label-free, quantitation, proteins, proteomic, peak intensity, mass spectrometry, raw pork meat.

1. Introduction

Mass spectrometry has become a fundamental tool among proteomic techniques to identify and precisely quantify proteins of complex biological samples such as meat and meat products (Aebersold & Mann, 2003; Cravatt, Simon, & Yates III, 2007).

Classical methodologies using one-dimensional (1D) or two-dimensional (2D) gel electrophoresis with different detection methods such as dyes, fluorophores or radioactivity have allowed the separation and quantitation of proteins through the measurement of stained spot intensities, providing good sensibility and linearity. However, the applicability of these methods is limited to abundant and soluble proteins when the aim is to achieve high-resolution protein separation, as well as they do not reveal the identity of the underlying proteins, and neither provide accurate results on changes of protein expression levels, especially in the case of overlapping proteins (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007; Szabo, Szomor, Foeldi, & Janaky, 2012). These difficulties are overcome by modern mass-spectrometry-based quantitation techniques, which can be separated into two categories: i) the use of labelling methodologies that involve stable isotopes, and ii) the use of label-free techniques. Labelling techniques are considered to be the most accurate in quantitating protein abundances, but they present some limitations as well as require expensive isotope labels, a large amount of starting material, and an increased complexity of experimental protocols. Moreover, some of the labelling techniques cannot be used in all types of samples due to the restricted number of available labels, which is deficient for the simultaneous study of multiple samples (Aebersold & Mann, 2003; Bantscheff et al., 2007; Schulze & Usadel, 2010; Neilson et al., 2011). On the other hand, label-free methods are considered to be less accurate, but they are a simple, reliable, versatile, and cost-effective alternative to labelled quantitation. There are currently two strategies

extensively implemented as label-free approaches: 1) quantitation based on the signal intensity measurement based on precursor ion spectra; and 2) spectral counting (Zhu, Smith, & Huang, 2010; Neilson et al., 2011). Focusing quantitation on the basis of peak intensity, it has been demonstrated that ion amount and signal are linearly correlated within the dynamic range of a mass spectrometer. In fact, despite spectral counting such as Exponentially Modified Protein Abundance Index (emPAI) or Absolute Protein Expression (APEX) techniques are very useful in the estimation of the relative amounts of proteins in a single sample, MS1 quantitation results more precise and accurate when aim is to estimate changes in protein from sample to sample (Wang et al., 2003; Levin, Hradetzky, & Bahn, 2011).

Numerous recent studies describe quantitative proteomic analysis in plants (Schaff, Mbeunkui, Blackburn, Bird, & Goshe, 2008; Stevenson, Chu, Ozias-Akins, & Thelen, 2009; Mora, Bramley, & Fraser, 2013), but to the best of our knowledge, there are not many studies in meat or meat products. Thus, the purpose of the present study is the optimisation of a label-free procedure, using ion peak intensity-based comparative nLC-MS/MS, for the relative quantitation of proteins extracted from raw pork meat.

2. Materials and methods

2.1 Preparation of a mixture of protein standards for the optimisation of the methodology

The viability and practicability of the methodology were proved using a mixture of six standard proteins typically found in muscle and meat with a wide range of molecular weights, containing myoglobin (MYG, 17 kDa), tropomyosin (TPM, 33 kDa), actin (ACT, 43 kDa), troponin (TNN, 52 kDa), and alpha-actinin (ACTN, 103 kDa). Beta-lactoglobulin protein (LACB, 19 kDa) was also included in the mixture as normaliser of

data as is not naturally present in meat. All protein standards were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Working solutions of 5 nmol for each protein were prepared with 50 mM ammonium bicarbonate (ABC) at pH 8, and subsequently an in-solution digestion was carried out using trypsin enzyme (Sequencing grade modified trypsin; Promega Corp., Madison, WI, USA). Samples were reduced with dithiothreitol (DTT) and cysteins were alkylated by using iodoacetamide (IAA). Finally, the digestion was started by adding 0.125µg/µL trypsin to obtain a final enzyme:substrate ratio of 1:50 (w/w), and the sample was incubated overnight at 37 ºC. After incubation, 10% formic acid (FA; v/v) was added to stop the digestion. The digested proteins were used to prepare standard proteins mixtures at different proportions as indicate the ratios shown in Table 1. The concentration of beta-lactoglobulin was kept constant for the normalisation of quantitative data. Moreover, working solutions at concentrations of 100, 50, 20, 10, 5, 2, and 1 fmol/µL of the digested LACB were prepared to test the linearity under the experimental conditions.

2.2 Preparation of raw meat samples and extraction of proteins

Optimised methodology for protein quantitation was carried out using raw meat from 6 months old pig (Landrace x Large White) at 24 h post-mortem. Extraction of sarcoplasmic and myofibrillar proteins was done in triplicate according to Sentandreu, Fraser, Halket, Patel, and Bramley (2010), and protein concentrations were determined by using the Bradford protein assay (Bradford, 1976).

2.3 Separation of raw meat myofibrillar and sarcoplasmic proteins by 1D-SDS-PAGE

Solutions with sarcoplasmic and myofibrillar proteins were diluted at concentrations of N, N/2, and N/4 (N = 2 mg/mL) with regard to the concentration values obtained by the Bradford assay. A total of 100 µL of each dilution was mixed with 100 µL of sample

buffer (containing 0.5 M Tris-HCl pH 6.8, 10% w/v SDS, 50% v/v glycerol, 0.2 M DTT and 0.05% v/v bromophenol blue) and the homogenate was heat denatured at 95 ºC for 4 min. Then, 10 µL of each sample was loaded onto the gel, and the electrophoresis was carried out at 120 V and 50 W, using a separation gel (12% acrylamide) and a stacking gel (4% acrylamide) (Laemmli, 1970). The ProteoSilver plus silver stain kit (Sigma, St. Louis, MO, USA) was employed to develop the gel, and SDS-PAGE molecular weight standards, broad range (161-0317; Bio-Rad Laboratories, Inc., CA, USA) were used to assess them molecular weights of the proteins.

2.4 In-gel digestion of raw meat myofibrillar and sarcoplasmic proteins

After the separation by SDS-PAGE, one section from the gel of sarcoplasmic proteins and another section from the gel of myofibrillar proteins at the three concentrations assayed (N, N/2, and N/4) were selected for in-gel digestion and the posterior quantitation, as can be seen in Figure 1.

The stained bands were excised into small pieces, and then reduced and alkylated by using DTT and IAA, respectively. Gel pieces were dried three times for 10 min with 100 µL of ACN. Once the gel fragments became dry and opaque, they were placed in ice for 10 min, and 1 µL of freshly prepared LACB protein solution of 500 fmol/µL was added. The digestion was started by adding 12.5 ng/µL of trypsin enzyme dissolved in 50 mM ABC pH 8, in order to obtain an enzyme:substrate ratio of 1:50 (w/w), and maintaining the samples in ice for 30 min to allow the enzyme to come into the gel. Samples were incubated at 37 ºC overnight, and then 10% (v/v) FA was added to stop the enzyme activity. Peptides were extracted from the gel pieces after sonication for 10 min with 50 µL of 0.1% v/v TFA in ACN:H_2O (50:50, v/v), and the extract was evaporated using a vacuum concentrator. Once the samples were dried, the remaining

147 residue was reconstituted in 30 µL of loading buffer containing 0.1% v/v TFA, for
148 further MS/MS analysis.

149 **2.5 Analysis of trypsin digested samples by nLC-MS/MS**

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

154 A total of 5 µL of each sample were injected through an autosampler, and
155 preconcentrated on an Eksigent C18 trap column (3µ, 350µm x 0.5mm; Eksigent of AB
156 Sciex, CA, USA), at a flow rate of 3 µL/min for 5 min and using 0.1% v/v TFA as
157 mobile phase. Then, the trap column was automatically switched in-line onto a nano-
158 HPLC capillary column (3µm, 75µm x 12.3 cm, C18; Nikkyo Technos Co, Ltd. Japan).
159 The mobile phases were solvent A, containing 0.1% v/v FA, and solvent B, containing
160 0.1% v/v FA in 100% ACN. Chromatographic conditions were a linear gradient from
161 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at 30
162 ºC and a flow rate of 0.30 µL/min. The column outlet was directly coupled to a
163 nanoelectrospray ionisation system (nano-ESI). The Q/ToF was used in positive polarity
164 and information-dependent acquisition mode, in which a 0.25-s ToF MS scan in the
165 range from *m/z* 300 to 1250 was performed, followed by 0.05-s product ion scans from
166 *m/z* of 100 to 1500 on the 50 most intense 1 - 5 charged ions.

167 **2.6 Data analysis**

168 Automated spectral processing, peak list generation, database search, normalisation and
169 quantitative comparisons were performed using Mascot Distiller v2.4.3.3 software
170 (Matrix Science, Inc., Boston, MA, USA; hppt://www.matrixscience.com).

171 The methodology used in this study for label-free quantitation is based on replicates of
172 the relative intensities of extracted ion chromatograms (XICs) for precursors aligned
173 using mass and elution time (Silva et al., 2005; Wang, Wu, Zeng, Chou, & Shen, 2006).
174 The label-free quantitation methodology used in this study requires robust search
175 parameters because it is based on the identification at peptide level. The identification of
176 the protein origin of peptides was done using SwissProt database, and the taxonomy
177 parameter was designated as Mammalia. Moreover, the selected search parameters
178 include the use of trypsin as the enzyme, allowing up to two missed cleavage site, and
179 oxidation of methionine (M), carbamidomethyl (C), and deamidated (NQ) as variable
180 modifications.
181 Generated MS/MS spectra were searched using a significance threshold $p<0.05$, a FDR
182 of 0.5, and a tolerance on the mass measurement of 0.3 Da in MS mode and 0.3 Da for
183 MS/MS ions. Quantitation parameters were selected using the label-free option
184 provided in the Mascot search engine. Quality criteria to determine peptide ratios used
185 to quantify were established to effectively eliminate outlier points. In this sense, the
186 method of integration was optimised and a standard error of 0.2 and a correlation
187 coefficient of 0.95 with a fraction threshold value of 0.5, that is the fraction of the peak
188 area in the precursor region accounted for by the components. LACB protein was added
189 to the sample and a median of five peptides described in Figure 2, was used to
190 normalise data. Principal component analysis (PCA) and loading plot statistical analysis
191 for the control and sets of standard proteins mixture (results not shown) were performed
192 using Simca-P+ 13.0 (Umetrics AB, Sweden). Results were exported from Mascot
193 Distiller into Excel in order to perform statistical analyses of the data.
194
195 **3. Results**

3.1 Normalisation of the data

The digested protein LACB was added to the samples, and five of the peptides generated were used to normalise data (see Figure 2) allowing a more robust analysis and quantitation. A good linearity was established in a range of masses from 670 to 1250 Daltons corresponding to the five peptides selected from the trypsin digestion of beta-lactoglobulin protein. So, a plot of the LACB concentration against the mean of each peptide ratio calculated with 100 fmol/µL value as reference is shown in Figure 2. Regression coefficients obtained for all the peptides were between 0.99 and 0.97, and the limit of detection (LOD) and identification was determined as 5 fmol/µL for the five peptides used in the normalization after testing samples at lower concentrations such as 3 fmol/µL and 1 fmol/µL.

3.2 Repeatability of the procedure

The repeatability of the digestion with trypsin enzyme and analysis by using nLC-MS/MS was evaluated in triplicate for each protein mixture. So, the ratios of LACB, ACT, TPM, MYG, TNN, and ACTN, together with their standard deviations and coefficients of variation were estimated, as can be observed in Table 2. Very good repeatability was obtained with percentages of coefficients of variation smaller than 11.5% for all proteins in all samples tested. The different measurements were done by the same analyst, and the instrument worked with the same procedure and under the same experimental conditions to test the repeatability of the methodology.

3.3 Recovery of the method

The percentage of recovery was evaluated in order to compare the agreement between the values obtained by the method and the theoretical values of the protein mixtures. Table 3 shows the theoretical and calculated ratios, accuracy of the method, standard deviations and coefficients of variation obtained from the three replicates. The accuracy

values obtained comprised between 97 and 120% for all the proteins in the four samples, and the percentages of coefficients of variation were smaller than 11.5% in all instances. The mean of the median pair of 11, 9, 9, 6, 3, and 3 peptides of ACT, LACB, TPM, MYG, TNN, and ACTN proteins, respectively, have been used to calculate the ratio of the respective proteins. Same peptides were used in repeatability and recovery studies.

3.4 Identification and quantitation of meat proteins

Sarcoplasmic and myofibrillar protein extracts from raw ham meat were separated by SDS-PAGE electrophoresis at three different concentrations (N, N/2, and N/4). The selected bands (see Figure 1) were subjected to in-gel trypsin digestion, followed by nLC-MS/MS analysis in order to identify the proteins present and to assess the differences in protein concentrations.

Regarding sarcoplasmic proteins, some proteins such as beta-hemoglobin, myoglobin, and fatty acid-binding protein (accession numbers P02067, P02189, and O02772, respectively, according to Uniprot protein database) were identified from the selected bands in the SDS-PAGE gel after searching MS/MS spectra against the protein database. On the other hand, the analysis of the selected bands of myofibrillar proteins by nLC-MS/MS allowed to identify several proteins such as α-actin-1, actin cytoplasmic 1, β-actin-like protein 2, desmin, and troponin T, with Uniprot protein database accession numbers P68138, P60712, Q562R1, O62654, and P02641, respectively (see Table 4).

The recovery of protein in raw meat extracts was calculated by comparing the theoretical ratio with the calculated value for each identified protein as it is also shown in Table 4. Thus, the accuracy percentage obtained was between 99 and 123% in sarcoplasmic proteins, with coefficients of variation smaller than 10%. In myofibrillar proteins the accuracy values were between 100 and 113%, with percentages of coefficients of variation smaller than 9%.

4. Discussion

The use of the latest generation proteomic techniques for label-free quantitation like tandem mass spectrometry with electrospray ionisation (ESI), provides high resolution, mass precision, reproducibility and linearity, together with accuracy and reliability of the obtained data for complex proteomes (Wang et al., 2003; Wang et al., 2006; Zhu et al., 2010).

Traditionally, quantitation of proteins extracted from meat and meat products has been based on the measurement of electrophoretic bands density by using densitometric scanning (Giulian, Moss, & Greaser, 1983; Claeys, Uytterhaegen, Buts, & Demeyer, 1995), spectrophotometric measurements (Everitt & Maksimova, 1984), fluorescent scanning procedures (Goldberg & Fuller, 1978), or computer image analysis (Fritz, Mitchell, Marsh & Greaser, 1993; Morzel, Chambon, Hamelin, Santé-Lhoutellier, Sayd, & Monin, 2004). Quantitation using gel electrophoresis shows some limitations besides its limited dynamic range and poor specificity when extracted proteins from meat samples are analysed, showing problems with very hydrophobic proteins, those with very high or low molecular weight or proteins less abundant in unfractionated samples (Bendixen, 2005; Hollung, Veiseth, Jia, Færgestad, & Hildrum, 2007).

Recent advances in mass spectrometry have allowed protein map identification through a combination of 2D electrophoresis gel followed by peptide mass fingerprint MS (Bouley, Chambon, & Picard, 2004; Doherty et al., 2004; Bendixen, 2005), and improved quantitation of meat proteins in terms of robustness, sensitivity and dynamic range by using isotope labeling techniques and MS/MS analysis to quantify changes in protein abundance between samples (Doherty et al., 2004; Bjarnadóttir, Hollung, Høy, Bendixen, Codrea & Veiseth-Kent, 2012). However, the development of label-free

comparative proteomics would give more simplicity in sample preparation, reliability due to the high number of replicates, as well as suitability for all kinds of samples, which provides an essential value when analysing complex matrices such as meat samples.

In the present study, a label-free methodology based on the measurements of changes in chromatographic ion intensity, has been optimised for the relative quantitation of meat proteins. It is essential that sample preparation, sample injection to LC-MS/MS system, and LC separation be highly reproducible, as well as the normalisation of the data and alignment of peaks obtained of multiple LC-MS datasets to avoid possible variations between LC and MS runs (Wang et al., 2003; Zhu et al., 2010). In fact, peptide extraction procedures and trypsin digestion are critical steps in this quantitative methodology that is based on replicates, so it is important to avoid variance in efficiency that could lead to low proteomic quantitation or even make impossible the quantitation (Brownridge & Beynon, 2011; Szabo et al, 2012).

The peptide peak intensity methodology was used instead of other label-free methods like spectral counting because it is more accurate in reporting changes in protein abundance between samples and estimating ratios for proteins with large numbers of overlapping peptide ions. An uncertain linearity of response and relatively poor precision are obtained when spectral counting approach is used because the dynamic exclusion of ions usually employed for fragmentation is detrimental to obtain an accurate quantitation. Thus, the use of peak intensity measurements for the relative quantitation of large and global protein changes and the comparison between samples seems more advantageous and adequate than spectral counting methodology when complex mixtures of proteins are analysed (Old et al, 2005; Bantscheff et al., 2007; Chen, Ryu, Gharib, Goodlett, & Schnapp, 2008).

296 In this approach, four protein mixtures at different ratios were analysed by nLC-Q/ToF
297 mass spectrometry showing the linearity, repeatability and recovery of the sample
298 preparation and the nLC-MS/MS analysis. Regarding data processing, mass
299 spectrometry analysis was done by triplicate. First step was the acquisition of the data; a
300 list of survey scans for each TIC followed by groups of the MS/MS scans was created.
301 Then, peptide peaks were detected, distinguishing from neighboring peaks and
302 background noise by attempting to fit an ideal isotopic distribution to the experimental
303 data. Finally, peaks from the LC-MS runs were aligned matching the retention times
304 with the corresponding mass peaks to carry out (*i*) the identification of peptides and
305 origin proteins by using databases, and (*ii*) the quantitation of proteins by normalising
306 the mass spectral peak intensity preceding the statistical analysis. Normalisation with
307 the digested LACB protein was done to eliminate the possible variability in the
308 technical or analytical process, improving the quantitative profiling. A group of five
309 peptides was used for normalisation, which allows a more accurate matching and
310 quantitation than using only one peptide.
311 The applicability of this methodology was demonstrated using raw pork meat samples
312 for the comparative quantitation of proteins. Thus, sarcoplasmic and myofibrillar
313 proteins extracted from raw meat were separated using SDS-PAGE at three different
314 concentrations. The use of 1D gel electrophoresis, instead 2D gel, simplifies the
315 methodology for the subsequent analysis by mass spectrometry (Jafari, Primo, Smejkal,
316 Moskovets, Kuo, & Ivanov, 2012). In fact, sample preparation influence on quantitation,
317 as well as the variability introduced in the procedure due to uncontrolled changes during
318 SDS-PAGE separation or trypsin digestion were also studied by analysing the results
319 showed in Table 4.

In this study, protein identification was done matching spectra to peptides by database searching, and then protein quantitation was carried out according to the actual amount of protein instead than other less sensitive methods that are based on an estimation of the amount of protein calculated by using imaging densitometry. So, relative quantitation was done using a different number of peptides depending on the protein through the integration of their chromatographic peaks. Such procedure showed a high repeatability, linearity, and accuracy. Thus, this simple and reliable label-free quantitative methodology could be applied to study changes in protein abundance along processes such as fermentation, curing, and ripening in meat products.

5. Conclusions

A label-free methodology based on the relative intensities of extracted ion chromatograms (XICs) aligned using mass and elution time has been optimised and applied for the relative quantitation of raw pork meat proteins. After SDS-PAGE separation and in-gel digestion of proteins, nLC-MS/MS spectra were used in order to identify and quantify proteins by using ion peak intensity, as peak areas of peptides can be correlated to the concentration of the protein from which the peptide is derived. Linearity, repeatability and accuracy of the procedure have been demonstrated, and the methodology has resulted to be a simple and reliable method to quantify changes in protein abundance of meat samples. Furthermore, this procedure could be very useful in comparative proteomics in order to evaluate changes in proteins during post-mortem meat period or along the processing of meat products.

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FIGURES CAPTIONS

Figure 1. Separation of sarcoplasmic and myofibrillar proteins from raw pork meat using SDS-PAGE at three different concentrations (N, N/2, N/4). Sections indicated in rectangles contain those bands selected for the label-free quantitation of each group of proteins. Molecular weights of the standards (STD) are indicated.

Figure 2. Representation of the linearity range and regression coefficients of LACB protein (n=3), showing the five peptides obtained after trypsin digestion of this protein which were used to normalise the datasets. A digested control sample at a concentration of 100 fmol/µL was used to calculate the ratio. The limits of detection (LOD) were determined for the five peptides.

Table 1. Composition of each protein mixture containing six standard proteins.

Protein name	Control	Set 1	Set 2	Set 3
LACB	1	1	1	1
ACT	1	0.5	0.5	1.5
TPM	1	1	0.5	1
MYG	1	1.5	1	0.5
TNN	1	0.5	1.5	1.5
ACTN	1	1	1.5	0.5

LACB, beta-lactoglobulin; ACT, actin; TPM, tropomyosin; MYG, myoglobin; TNN, trop

Table 2

Table 2. Repeatibility of the method in each sample of proteins mixture (n=3).

Sample	Protein[a] name	Average[b] ratio	SD[c]	CV[d] (%)
Control	LACB	1.00	0.00	0.00
	ACT	1.06	0.02	2.27
	TPM	1.05	0.04	3.69
	MYG	1.01	0.01	1.23
	TNN	1.02	0.03	2.65
	ACTN	1.08	0.04	4.12
Set 1	LACB	1.00	0.00	0.00
	ACT	0.56	0.03	4.97
	TPM	1.06	0.04	3.49
	MYG	1.50	0.02	1.12
	TNN	0.55	0.02	3.66
	ACTN	1.05	0.03	2.88
Set 2	LACB	1.00	0.00	0.30
	ACT	0.54	0.01	1.83
	TPM	0.51	0.01	1.74
	MYG	1.01	0.02	1.81
	TNN	1.51	0.01	0.65
	ACTN	1.49	0.03	2.26
Set 3	LACB	1.00	0.00	0.00
	ACT	1.81	0.10	5.50
	TPM	1.00	0.01	0.55
	MYG	0.55	0.06	11.48
	TNN	1.46	0.10	7.09
	ACTN	0.53	0.05	9.95

[a]LACB, beta-lactoglobulin; ACT, actin; TPM, tropomyosin; MYG, myoglobin; TNN, t

[b]Calculated value obtained from the replicates (n=3).

[c]Standard Deviation.

[d]Coefficient of Variation, expressed as percentage.

Table 3. Recovery (%)of the method in each protein mixture (n=3).

Sample	Protein[a] name	Theoretical[b] value	Calculated[c] value	Accuracy[d] (%)	SD[e]	CV[f] (%)
Control	LACB	1	1.00	100.00	0.00	0.00
	ACT	1	1.06	105.54	2.40	2.27
	TPM	1	1.05	105.05	3.88	3.69
	MYG	1	1.01	101.34	1.24	1.23
	TNN	1	1.02	101.64	2.69	2.65
	ACTN	1	1.08	107.88	4.45	4.12
Set 1	LACB	1	1.00	100.00	0.00	0.00
	ACT	0.5	0.56	111.98	5.57	4.97
	TPM	1	1.06	105.99	3.70	3.49
	MYG	1.5	1.50	99.94	1.12	1.12
	TNN	0.5	0.55	110.20	4.04	3.66
	ACTN	1	1.05	105.14	3.02	2.88
Set 2	LACB	1	1.00	100.18	0.30	0.30
	ACT	0.5	0.54	108.51	1.98	1.83
	TPM	0.5	0.51	102.66	1.78	1.74
	MYG	1	1.01	100.96	1.83	1.81
	TNN	1.5	1.51	100.34	0.65	0.65
	ACTN	1.5	1.49	99.34	2.24	2.26
Set 3	LACB	1	1.00	100.00	0.00	0.00
	ACT	1.5	1.81	120.34	6.62	5.50
	TPM	1	1.00	99.92	0.55	0.55
	MYG	0.5	0.55	109.14	12.53	11.48
	TNN	1.5	1.46	97.26	6.90	7.09
	ACTN	0.5	0.53	106.66	10.62	9.95

[a]LACB, beta-lactoglobulin; ACT, actin; TPM, tropomyosin; MYG, myoglobin; TNN, troponir

[b]Theoretical ratio of each protein in each sample

[c]Average ratio obtained from the replicates (n=3).

[d]Accuracy obtained from the theoretical and calculated values, expressed as percentage.

[e]Standard Deviation.

[f]Coefficient of Variation, expressed as percentage.

Table 4

Table 4. Label-free quantitation obtained using SDS-PAGE separation at different dilutions of the raw pork meat extracts and accuracy of the method

	Protein[a] name	Molecular mass (Da)		Theoretical[b] value	Calculated[c] value	Accuracy[d] (%)	SD[e]	CV[f] (%)	No[g] peptides
Sarcoplasmic	HBB	16155	N	1	1.08	108.23	2.25	2.08	5
			N/2	0.5	0.58	115.57	8.96	7.75	8
			N/4	0.25	0.31	122.84	3.21	2.57	2
Sarcoplasmic	MYG	17074	N	1	1.04	104.01	4.61	4.44	6
			N/2	0.5	0.51	102.37	5.91	5.77	5
			N/4	0.25	0.25	101.82	2.14	2.10	4
Sarcoplasmic	FABPH	14740	N	1	1.08	107.83	2.25	2.09	4
			N/2	0.5	0.50	99.36	7.00	7.05	3
			N/4	0.25	0.26	102.34	10.02	9.79	3
Myofibrillar	ACTS	42024	N	1	1.04	104.01	4.41	4.24	36
			N/2	0.5	0.51	101.20	2.09	2.06	20
			N/4	0.25	0.25	101.53	2.10	2.07	31
Myofibrillar	ACTB	41710	N	1	1.04	104.21	4.59	4.41	21
			N/2	0.5	0.50	100.40	3.35	3.34	12
			N/4	0.25	0.26	102.20	0.68	0.67	16
Myofibrillar	ACTBL	41976	N	1	1.03	103.33	4.81	4.65	10
			N/2	0.5	0.54	107.17	6.67	6.22	6
			N/4	0.25	0.26	105.43	1.12	1.07	7
Myofibrillar	DESM	53499	N	1	1.04	103.53	5.76	5.56	5
			N/2	0.5	0.51	101.65	1.54	1.52	5
			N/4	0.25	0.26	103.34	0.91	0.88	4
Myofibrillar	TNNT3	33014	N	1	1.00	100.26	1.57	1.56	2
			N/2	0.5	0.56	112.88	1.41	1.25	2
			N/4	0.25	0.27	108.88	9.52	8.74	3
Myofibrillar	TPM2	32817	N	1	1.08	107.68	0.06	0.06	4
			N/2	0.5	0.55	109.59	3.39	3.09	8
			N/4	0.25	0.26	104.74	3.73	3.56	4

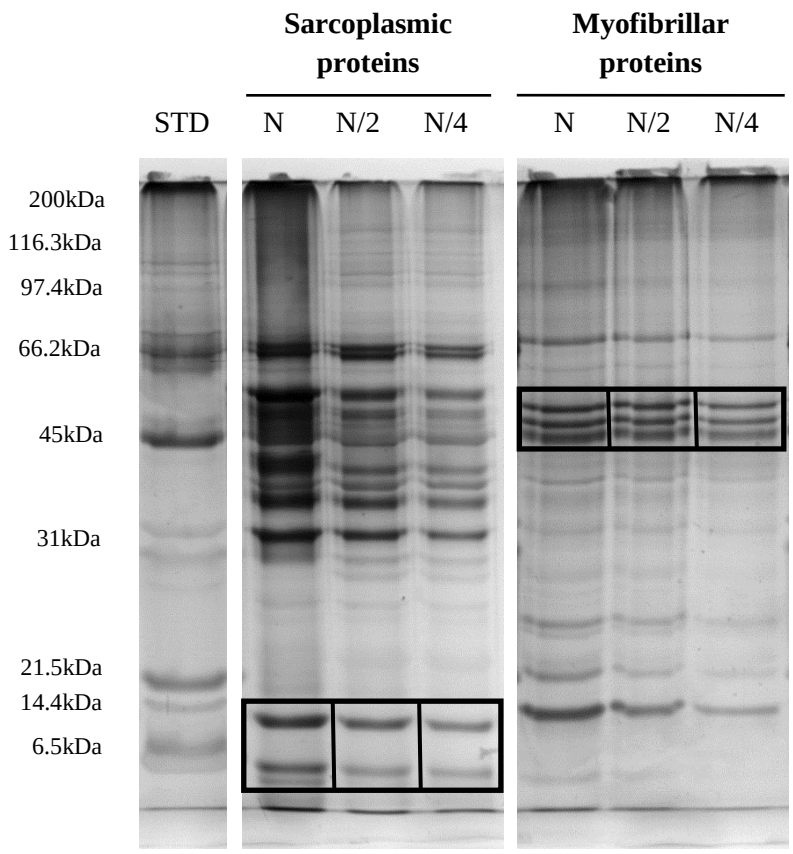
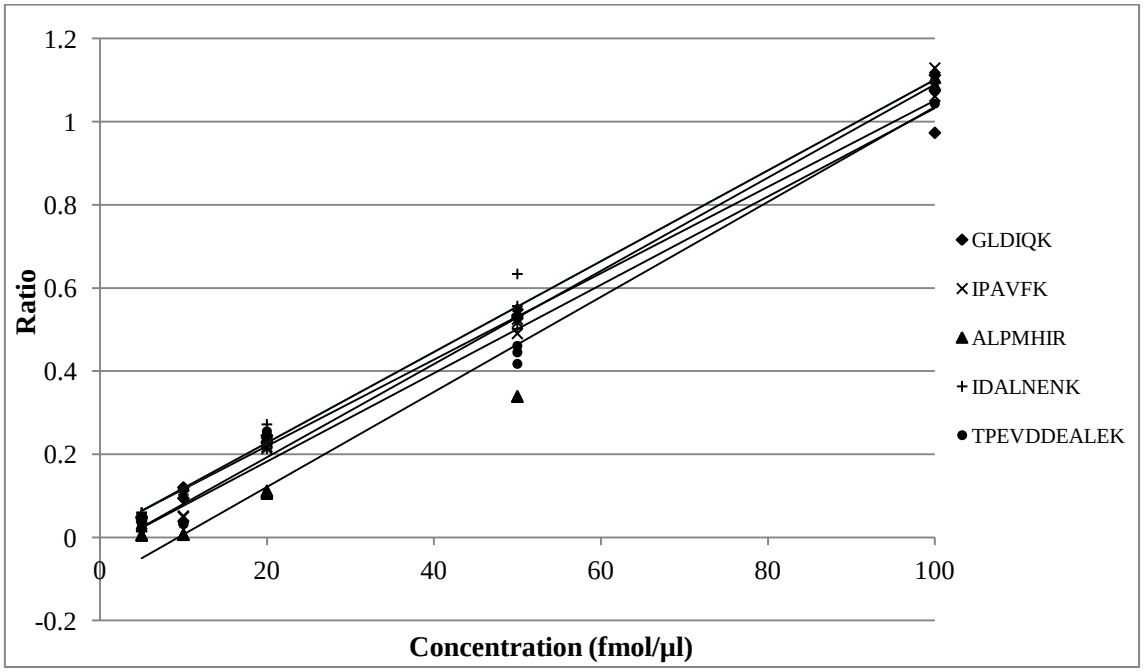

Sarcoplasmic proteins
Myofibrillar proteins
STD
N
N/2
N/4
N
N/2
N/4
200kDa
116.3kDa
97.4kDa
66.2kDa
45kDa
31kDa
21.5kDa
14.4kDa
6.5kDa

Figure 1.



Peptide number	Mass (Da)	Peptide sequence	Equation	R-squared value	LOD (fmol/µl)
1	673.388	GLDIQK	y = 0.0104x + 0.0121	R^2 = 0.99	5
2	674.423	IPAVFK	y = 0.0112x - 0.0315	R^2 = 0.99	5
3	837.476	ALPMHIR	y = 0.0114x - 0.1076	R^2 = 0.97	5
4	916.473	IDALNENK	y = 0.0109x + 0.0091	R^2 = 0.99	5
5	1245.584	TPEVDDEALEK	y = 0.0106x + 0.0304	R^2 = 0.98	5

Figure 2.