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Nitrogen compounds as potential biochemical markers of pork meat quality

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Abstract

Muscle proteins, peptides and free amino acids were analysed at 2 h post-mortem as potential markers of different pork meat qualities. The meat quality classification was based on the measurements: pH_{2h}, pH_{24h}, the colour parameter L, and drip loss. The measurement of proteolytic fragments by SDS-PAGE revealed that the nebulin band was different among classes being more defined in the dark, firm and dry meat class. The detection and characterisation of small peptide fragments resulting from protein breakdown was done by isolation through cationic and reverse-phase chromatography. The analysis of peptide fractions isolated by reverse-phase chromatography revealed that could be used as potential markers of meat quality. So, peptide fraction 1 could be used to distinguish exudative from non-exudative meats and peptide fraction 4 distinguished dark, firm and dry meat from the rest. The concentration of free amino acids present in muscle was not different among qualities, probably due to the early post-mortem time (2 h) used in the study.

Keywords: Proteins; Peptides; Aminoacids; Meat quality; Pork; Markers

1. Introduction

One of the most important quality defects of pork is the pale, soft and exudative (PSE) meat. This PSE meat affects the quality perceived by consumers and also to meat processors because of high losses during processing. Also the high incidence of RSE (reddish-pink, soft and exudative) meat has an economic impact in the pig industry (Cheah, Cheah & Just, 1998). This RSE meat is characterised by an exceptionally high drip loss with normal red colour (Kauffman, Cassens, Scherer & Meeker, 1992; Laack, Kauffman, Sybesma, Smulders, Eikelenboom & Pinheiro, 1994, Warner, Kauffman & Russell, 1997). Another quality that affects consumer and processors although in low proportion, is the dark, firm and dry pork meat (DFD), due to its high susceptibility to contamination. On the other hand, the reddish pink, firm and non-exudative (RFN) meat is considered the ideal meat quality class (Kauffman et al., 1992). The prediction of quality early post-mortem is a difficult process because some of the biochemical quality properties have not yet been fully expressed.

The eating quality of meat has been mainly focused on tenderness, but two factors control it; the degree of proteolytic breakdown of myofibrillar proteins and the concentration of intramuscular lipids (Wood, Brown, Nute, Whittington, Perry, Johnson & Enser, 1996). The rate and extent of this tenderisation process is influenced by the post-mortem pH, because the two systems involved in the process, calpain and lysosomal cathepsins systems, depend on the pH. This is the case of high pH meat that has been associated with an increase tenderness or higher tenderisation rate through ageing (Watanabe, Daly & Devine, 1996) probably due to the high pH that enhances the activity of calpains (Beltrán, Jaime, Santolaria, Sañudo, Alberti & Roncales, 1997). On the other hand, Koohmaraie (1996) reported that tenderness is very highly dependent on shortening in slow-glycolysing muscles, but it is completely independent in muscles of more rapid pH decline where tenderness will be due to more rapid ageing. The study of the

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small products resulting from the action of these proteolytic systems could be useful to obtain antibodies directed to specific fragments that could be predictors of tenderness.

The objective of this study is to determine how the post-mortem pork quality affects the proteolytic breakdown process through the study of nitrogen compounds such as proteins, peptides and free amino acids in meat, in order to study differences among pork qualities at early post-mortem.

2. Materials and methods

2.1. Animals

Fifty-nine pork carcasses (mother: Large White × Landrace, father: Large White) from 6 month old pigs representing a broad array of pork quality were selected and processed in two commercial slaughterhouses. At 2 h post-mortem the muscle *Longissimus dorsi* was removed, vacuum packaged and stored at −80°C until further analysis.

2.2. Meat quality measurements

The measurements were made on the right hand side of the carcass. The pH was measured at 2 and 24 h in the muscle *Longissimus dorsi* at the fifth rib level and with a portable pH-meter Hanna HI 8424 (Hanna Instruments, Portugal). The color, L^*, a^*, b^* coordinates, were measured at 24 h post-mortem with a Hunter chromameter model labscan (Hunter, VA, USA). The drip loss (DL) was measured by the method of Warris (1982). The intramuscular fat content (IMF) was analysed by the method of Folch, Lees and Stanley (1957). The moisture content was determined after dehydration at 100°C to a constant weight (ISO, 1973) and protein content was analysed using the Kjeldahl method (Association of Official Analytical Chemists [AOAC], 1990).

2.3. Protein analysis. SDS-PAGE electrophoresis

The procedure was based on the method described by Toldrá, Miralles and Flores (1992). One gram of minced muscle *Longissimus dorsi* with no visible fat or connective tissue, was homogenised during 4 min in 10 ml of 50 mM Tris buffer, pH 6.8, containing 8 M urea, 2 M thiourea, 75 mM dithiothreitol, 3% (w/v) SDS and 0.05% bromophenol blue by using a mini-polytron PT 1200 (Kinematica, Bubendorf, Switzerland) homogenizer. The extract was centrifuged at 10,000 *g* for 20 min at 4°C and the supernatant, filtered through glass wool (soluble fraction) and was used for electrophoresis. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. The amount of protein injected in the electrophoresis gels was 12 μg in each lane. The molecular mass of the extracted proteins and polypeptides was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels. The elution buffer was 50 mM Tris (Trizma base), pH 8.8, 0.384 glycine and 0.1% SDS. Migration rates and patterns of titin and nebulin were improved by inclusion of 5 mM β-mercaptoethanol in the upper buffer according to Fritz, Swartz and Greaser (1989). Gels were stained with coomassie brilliant blue R-250 (Laemmli, 1970) and unstained overnight. Standard proteins from BioRad were simultaneously run for molecular weight identification.

2.4. Peptide analysis

The extraction and fractionation of meat peptides were done by homogenising the muscle *Longissimus dorsi* with 0.01 N HCl (dilution 1:5) in a Stomacher during 4 min at 4°C and then centrifuged in the cold at 10,000 *g* for 20 min. The supernatant was filtered through glass wool and 20 ml of this extract was loaded in an activated Bond Elut Jr C-18 cartridge (Varian, CA, USA). The cartridge was washed with 20 ml of 0.1% TFA in water. Then, 10 ml of 0.1% TFA in 15% ACN was passed through, eluting a fraction containing hydrophobic peptides. This fraction was evaporated under vacuum (35°C) till dryness and diluted with 300 μl of 0.1% TFA in water.

An internal standard (cytidine 0.012 mg) was added to the fraction, which was proved by SDS-PAGE to contain peptides of molecular weight lower than 10 KDa. This fraction was injected in a 1050 Hewlett–Packard HPLC system. The separation was done in a Spherisorb S10SCX (10×250 mm) column from Waters (Water Corporation, MA, USA) at 40°C using a sodium chloride gradient between two solvents: (A) 20% ACN in 6 mM HCl, (B) 20% ACN in 6 mM HCL cointaining 1 M NaCl. The flow rate was 3.6 ml/min and the gradient was initial 0% B and 20 min linear change to 50% B. The detection was at 214 nm using a variable wavelength uv detector incorporated in the 1050 Hewlett–Packard HPLC system. Peaks eluted in the range 0.28–0.35 M NaCl (including the internal standard) were collected, concentrated and injected in a reverse phase column Symmetry C-18 (4.6×250 mm) from Waters (Water Corporation, MA, USA). The separation was achieved in 30 min at 40°C, using a gradient between two solvents: 0.1% TFA in water (solvent A) and 0.085% TFA in water: ACN, 40 : 60 v/v (solvent B). The flow rate was 0.9 ml/min and the gradient was initial 0% B and maintained 4.0 min at 0% B, then 26 min linear change to 60% B. The detection was monitored at 214 nm using a variable wavelength uv detector.

2.5. Acid hydrolysis of chromatographic peaks

The chromatographic peaks separated by reverse phase HPLC were collected and acid-hydrolysed using 6 N HCl containing 1% phenol at 110°C for 22 h in vacuum.

2.6. Free amino acids analysis

Samples for the amino acid analysis were extracted and deproteinised following the method described by Aristoy and Toldrá, (1991). Thus, samples were homogenised with 0.01 N HCl (dilution 1:5) in a Stomacher during 4 min at 4°C and centrifuged in the cold at 10,000 *g* for 20 min. The supernatant was filtered through glass wool and stored at −80°C until use. Two hundred and fifty microlitres of thawed samples plus 50 μl of an internal standard solution (hydroxyproline, 1.3 mg/ml), were deproteinised with 750 μl of acetonitrile. Two hundred microlitres of the supernatant were derivatised to their phenylthiocarbamyl derivatives according to the method of Bidlingmeyer, Cohen, Tarvin and Frost (1987). The derivatised aminoacids were analysed by reverse-phase HPLC in a Nova Pak C18 column (3.9×300 mm) (Waters Corporation, MA, USA). The separation was achieved in 65 min at 52°C, using a gradient between two solvents: 70 mM sodium acetate at pH 6.55 with 10% acetic acid, containing 2.5% of acetonitrile (solvent A) and water-acetonitrile-methanol, 40:45:15 v/v (solvent B) (Flores, Aristoy, Spanier & Toldrá, 1997). The detection was monitored at 254 nm.

2.7. Statistical methods

The Fisher's least significant difference (LSD) procedure was used to discriminate among the means of technological parameters, composition, peptide and amino acids concentrations in the different post-mortem meat quality groups using the software Statgraphics plus (v 2.0) (Steel & Torrie, 1980).

3. Results and discussion

The carcasses were classified based upon pH, *L*, and drip loss in four different classes:

- PSE: pH_{2h} lower than 5.8, *L* higher than 50 and drip loss higher than 6%;
- RSE: pH_{2h} lower than 5.8, *L* between 44 and 50 and drip loss higher than 6%;
- RFN: pH_{2h} higher than 5.8, *L* between 44 and 50 and drip loss lower than 6%;
- DFD: pH_{24h} higher than 6.0, *L* lower than 44 and drip loss lower than 3%.

These quality classes have been previously reported by other authors (Flores, Armero, Aristoy & Toldrá, 1999; Garrido & Honikel, 1996; Warner, Kauffman & Russell, 1993, 1997). Table 1 shows the means of the 59 carcasses for each quality category. The pH_{2h} was enough to differentiate between exudative (PSE and RSE) and non-exudative (RFN and DFD), although it was necessary to use the pH_{24h} to distinguish between RFN and DFD. On the other hand, the *L* was useful to establish a difference between PSE and RSE. Also, the drip loss can be used to distinguish the classes but it takes as long as 4 days to have the results. Therefore, the classification was performed based on pH_{2h}, pH_{24h}, *L*, and drip loss (DL) as the chemical parameters for meat quality classification (Table 1).

The composition of meat is very important for meat quality (Wood et al., 1996). No significant differences ($P < 0.05$) were detected in composition among classes (Table 2). The SDS-PAGE profile of the different pork qualities at 2 h postmortem is shown in Fig. 1. The major significant changes in protein profile have been found for a nebulin band (N in Fig. 1). The DFD quality class showed a low protein breakdown with a clear nebulin band. The other classes, RFN, RSE and PSE showed a complete nebulin breakdown, although in some cases the RFN class showed a nebulin band

Table 1
Classification of pork carcasses according to the four defined quality classes[a]

	PSE ($n=22$)[b]		RSE ($n=7$)		RFN ($n=20$)		DFD ($n=10$)	
	M[c]	SE	M	SE	M	SE	M	SE
pH_{2h}	5.76b	0.04	5.87b	0.07	6.12a	0.04	6.11a	0.06
pH_{24h}	5.51b	0.03	5.30c	0.05	5.50b	0.03	5.76a	0.04
L	52.1a	0.8	46.3b	1.3	47.2b	0.9	44.9c	1.6
DL (%)	7.3b	0.2	8.5a	0.3	4.9c	0.2	2.7d	0.3

[a] Results are expressed as means (M) and standard error (S.E.).
[b] n = Number of samples.
[c] Means in a row with different letters are significantly different ($P < 0.05$).

Table 2
Means of muscle composition (expressed as a %) of the four pork quality classes[a]

	PSE ($n=22$)		RSE ($n=7$)		RFN ($n=20$)		DFD ($n=10$)	
	M	SE	M	SE	M	SE	M	SE
IMF	3.2	0.2	2.7	0.4	3.2	0.2	3.2	0.3
Moisture	74.5	0.2	74.9	0.4	74.7	0.2	74.4	0.3
Protein	21.8	0.2	21.9	0.4	21.7	0.2	22.1	0.3

[a] Results are expressed as means (M) and standard error (S.E.).

slightly marked. In the case of titin (T in Fig. 1), another major myofibrilar protein, no significant differences could be established among quality classes at 2 h post-mortem. In addition, no changes were detected among qualities classes at molecular weights below 200 KDa.

Titin and nebulin are constituents of the N2 line being this line one of the first structures degraded by calpain (Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995; Huff-Lonergan, Mitsuhshi, Beekman, Parrish, Olson & Robson, 1996). A rapid pH fall in PSE muscle results in denaturation/precipitation and adherence of part of the sarcoplasmic protein fraction to the surface of myofibrils and, reduced solubility of myofibrilar proteins (Boles, Parriah, Huiatt & Robson, 1992). They also detected that titin was degraded more slowly in muscle of PSE meat than in normal meat. The reduced solubility of myofibrilar proteins at low pH could be the reason for the lower quantity of nebulin extracted for RSE and PSE qualities (see Fig. 1). The appreciable band of nebulin in the DFD samples could be due to the high pH of this pork meat. In the RFN class the nebulin shows an intermediate behaviour because of its intermediate pH values. On the other hand, titin is very sensitive to degradation during purification (Greaser & Frizt, 1995) and, therefore, the use of this protein as predictor of meat tenderness is very difficult but they can be useful as indicator of meat quality. More work needs to be done to establish clearer differences among qualities.

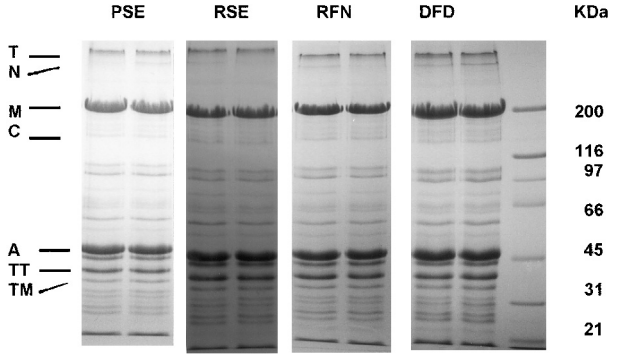

Fig. 1. SDS-polyacrylamide gel electrophoretic profile of the whole muscle proteins from the different quality classes at 2 h post-mortem. T: Titin, N: Nebulin, M: Myosin, C: Protein C, A: Actin, TT: Tropomyosin, and TM: Tropomyosin.

The detection and characterisation of small fragments resulting from protein breakdown could be very useful to obtain antibodies to predict quality. Therefore, the peptides obtained from the C-18 cartridge were separated in a cation-exchange column (Fig. 2A). The chromatogram obtained was too complex to be analysed, so it was necessary to continue the isolation of peptides. To this aim, the fraction eluted in the range 0.28–0.35 M NaCl including the internal standard (cytidine) was collected, concentrated and re-chromatographied in a reverse-phase column obtaining a peptide chromatogram with a good peak separation (Fig. 2B).

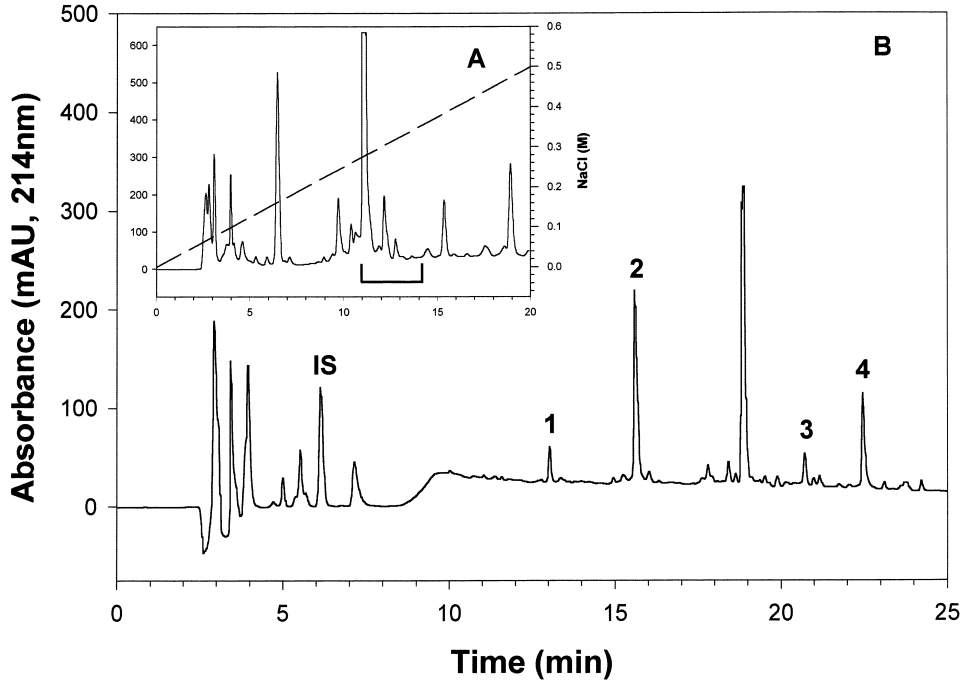

Fig. 2. (A) Cation exchange chromatogram of the C-18 cartridge fraction containing the hydrophobic peptides. (B) Reverse-phase chromatogram of the fraction eluted in the range 0.28–0.35 M NaCl (indicated with a bracket) from the cation exchange column. Chromatographic conditions were described in Materials and Methods. The numbering corresponds to that used in Tables 3 and 4. IS: internal standard (cytidine).

Four chromatographic peaks (Fig. 2B) were quantified and collected. After acid hydrolysis of these peaks their amino acid composition was analysed and appears in Table 3. Fraction 4 contained all the amino acids except His and Lys and is the only fraction containing Asp and Met. In the case of fraction 2, this contained all the amino acids except Asp, Met and Orn. However, the amino acid Phe was found in high concentration before the hydrolysis of this fraction interfering in the detection at 214 nm. On the other hand, fraction 1 contained all the amino acids except Asp, His, Ala, Met and Phe. Finally, fraction 3 lacks in Asp, His, Ala, Pro, Tyr and Met.

The possible relationship between the four chromatographic peaks and meat quality (Fig. 2B) was quantified in the different classes previously described and shown in Table 4. Fraction 1 could be used to distinguish between exudative (RSE and PSE) and normal (RFN) samples, but with a low significance level ($P < 0.15$). Fraction 2 was significantly ($P < 0.10$) lower in DFD class than in PSE class. Fraction 3 was also significantly ($P < 0.05$) lower in DFD than in RFN and PSE classes. Finally, fraction 4 was significantly ($P < 0.05$) lower in DFD class than in RFN, RSE and PSE classes. There are several studies about peptides in meat, most of them trying to establish the relationship among the specific peptides and sensory attributes due to their possible contribution to meat flavour (Aristoy & Toldrá, 1995; Spanier & Edwards, 1987). However, there are few reports about the relationship between peptides and meat quality parameters. We ascertained that fraction 1 was significantly correlated with Drip Loss ($r = 0.455$, $P < 0.05$), pH_{2h} ($r = -0.497$, $P < 0.05$) and pH_{24h} ($r = -0.532$, $p < 0.05$). Fraction 1 could constitute a possible marker of exudative classes, but due to the low significance level obtained, it is necessary to increase the number of samples. The low pH of the exudative quality groups favours the generation of peptides probably due to the cathepsin system activated at low pH (Toldrá & Etherington, 1988; Toldrá, Rico & Flores, 1993). From our results, the content of the peptidic fractions seems to be lower in the DFD class than in the other classes. The fraction 4 showed also a negative correlation with pH_{24h} ($r = -0.546$, $P < 0.05$) as expected by the low concentration of this fraction in the DFD class. Furthermore, Feidt, Brun-Bellut and Dransfield (1998) observed a slower increase in peptides at higher pH during meat storage that would produce more tender meat than at normal ultimate pH. In this sense, the fraction 4 would distinguish the most possible tender class (DFD).

The free amino acid concentrations in the different quality classes were also determined (Table 5). Only Asp, Glu, Ser, Gly and Pro were significantly different ($P < 0.05$) in the pork quality classes. Asp was significantly ($P < 0.05$) lower in PSE than in DFD pork meat. Glu was also significantly lower ($P < 0.05$) in RFN than in PSE classes. Ser was significantly lower ($P < 0.05$) in DFD than in RSE and PSE classes. Gly was significantly ($P < 0.05$) lower in RFN and DFD than in RSE class and Pro was also lower in DFD than in RSE class. Finally, the concentrations of natural

Table 3
Net peptidic amino acid content (μmol) for each fraction collected from a reverse-phase column resulting from initial 100 g of raw pork meat

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Asp	–	–	–	1.19
Glu	1.63	3.05	1.70	4.01
Ser	4.40	2.29	1.6	3.23
Gly	2.18	5.1	2.06	3.84
His	–	0.35	–	–
Thr	1.55	7.30	3.41	3.39
Ala	–	2.01	–	1.08
Arg	1.33	1.35	1.43	1.24
Pro	2.67	0.71	–	0.62
Tyr	0.70	1.78	–	1.08
Val	0.96	2.44	2.21	1.55
Met	–	–	–	4.40
Ile	1.73	1.28	5.78	1.04
Leu	1.49	1.36	5.64	1.03
Phe	–	2.83	2.31	6.29
Orn	0.65	–	0.87	1.38
Lys	0.82	1.35	0.86	–

Table 4
Comparison of the four quality classes based on peptidic fraction areas[a,b]

Frac	PSE ($n = 14$)		RSE ($n = 7$)		RFN ($n = 10$)		DFD ($n = 6$)		P
	M[c]	SE	M	SE	M	SE	M	SE	
1	0.212ab	0.025	0.223a	0.035	0.156c	0.029	0.147bc	0.038	$P < 0.15$
2	2.294a	0.116	2.034ab	0.164	2.148ab	0.137	1.885b	0.177	$P < 0.10$
3	0.249a	0.026	0.223ab	0.037	0.259a	0.031	0.129b	0.040	$P < 0.05$
4	0.423a	0.073	0.609a	0.103	0.590a	0.086	0.143b	0.112	$P < 0.05$

[a] Results are expressed as means (M) in peptidic fraction areas normalized by the internal standard and standard error (S.E.).
[b] Peptidic fractions (expressed as normalised areas) are shown in Fig. 2B.
[c] Peak areas in a row with different letters are different at level of significance (P) expressed in the column.

Table 5
Concentration of free amino acids and dipeptides by the four quality groups[a]

	PSE ($n=12$)[b]		RSE ($n=7$)		RFN ($n=15$)		DFD ($n=5$)	
	M[c]	SE	M	SE	M	SE	M	SE
Amino acids								
Asp	0.18b	0.05	0.19ab	0.06	0.30a	0.19	0.29ab	0.21
Glu	3.05a	1.11	2.93ab	1.01	2.09b	0.63	2.51ab	0.70
Ser	2.05a	0.39	2.17a	0.46	1.79ab	0.32	1.66b	0.42
Asn	1.07	0.24	1.04	0.11	1.04	0.25	1.21	0.19
Gly	7.51ab	2.36	9.03a	3.98	6.33b	1.33	5.15b	0.84
Gln	29.31	7.13	32.49	8.89	28.19	6.58	28.96	13.37
β-Ala	2.91	1.91	2.97	1.69	2.56	1.34	2.36	1.12
Tau	25.20	7.60	24.80	4.92	24.25	7.73	22.64	9.06
His	1.71	0.43	1.77	0.51	1.66	0.44	1.79	0.12
Thr	1.72	0.48	1.84	0.26	1.75	0.44	1.56	0.33
Ala	11.83	2.88	11.43	2.27	11.36	3.24	11.86	2.18
Arg	3.04	1.68	4.96	1.00	4.85	1.43	5.06	0.58
Pro	2.78ab	1.14	3.06a	1.01	2.35ab	0.55	1.94b	0.44
Tyr	1.57	0.39	1.52	0.19	1.60	0.44	1.44	0.38
Val	2.64	0.64	2.62	0.41	2.63	0.62	2.53	0.57
Met	0.82	0.20	0.77	0.14	0.76	0.24	0.78	0.25
Ile	1.49	0.38	1.39	0.22	1.57	0.37	1.50	0.32
Leu	2.87	0.68	2.93	0.56	2.78	0.73	2.31	0.39
Phe	1.88	0.37	1.81	0.27	1.74	0.47	1.71	0.47
Trp	0.98	0.31	1.09	0.11	0.90	0.31	0.77	0.26
Orn	0.41	0.17	0.42	0.01	0.53	0.39	0.35	0.14
Lys	2.59	0.70	2.33	0.47	2.98	0.85	2.58	0.74
Taac	107.59	31.23	113.57	27.56	104.03	28.90	100.95	33.08
Dipeptides								
Car	492.02	88.76	540.91	94.37	479.75	71.65	511.18	77.41
Ans	20.77	3.18	21.38	5.36	20.83	2.99	21.17	3.06

[a] Results are expressed as means (M) in mg/100 g of meat and standard error (S.E.).
[b] n, Number of pork loins.
[c] Means in a row with different letters are significantly different ($P<0.05$).

dipeptides, carnosine and anserine, did not show significant differences ($P<0.05$) among quality classes. Probably, the free amino acid concentration is not showing any pattern among quality classes due to the early post-mortem time of sampling (2 h post-mortem). At this time, it is too early to obtain differences in the latest products of the proteolytic chain. In fact, free amino acid content in the different classes was similar to the content reported for fresh porcine muscle (Aristoy & Toldrá, 1998).

In summary, the use of the nebulin band as a predictor of meat tenderness could be useful. The detection and characterisation of small fragments from proteins will be more adequate to obtain antibodies as markers of meat quality. So, fraction 1 could be used to distinguish exudative from non-exudative meats and fraction 4 to distinguish DFD samples from the rest. The sequence of these peptidic fractions is necessary in order to obtain antibodies. Further studies in this area are currently being done in our laboratory. On the other hand, the free amino acid concentration present in muscle at 2 h post-mortem was not different among qualities and can not be used as markers, probably due to the early post-mortem time used in the study.

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