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Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures

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Abstract

The influence of *postmortem* storage time and pre-slaughter conditions (transport the day before slaughter or immediately before slaughter) on proteome changes of pork meat was investigated over a 72 h ageing period. Intensities of 37 spots varied significantly ($p < 0.05$) with ageing time. Changes indicated proteolysis of troponin T, actin, α-crystallin, myokinase, creatine kinase and mitochondrial ATPase, but also of proteins constitutive of the Z-lines, namely cypher proteins and myozenin. Other modifications were the intensity increase of a full-length protein of the sarcoplasmic reticulum, which may be linked to its increased extractibility after membrane disruption, and a gradual shift in pHi towards alkaline values of some forms of myosin light chains (MLC) 2 and 3. The pre-slaughter conditions affected significantly ($p < 0.05$) 8 spots. Mitochondrial ATPase was over-expressed in the group transported immediately before slaughter, also characterised by a faster pH fall, and the shift in pHi of MLC 2 was more pronounced. The pre-slaughter conditions had no significant effect on the above proteolytic events.

Keywords: Pork; Meat ageing; Proteomics; 2D electrophoresis; Rate of pH decline

1. Introduction

Because muscle is composed almost exclusively of water and proteins, meat and seafood science cannot be dissociated from the study of structural or functional proteins, either in vivo or *postmortem*. For that purpose, the most common separating techniques are 1-D electrophoresis and chromatographic techniques. 2-D electrophoresis is gaining interest since it allows the concomitant separation of hundreds or even thousands of proteins. The number of applications related to *postmortem* study of meat and muscle foods has been somewhat limited. Thus, 2-D electrophoresis was used to characterise phenomena occurring during meat ageing and fish cold storage (Kjaesgård & Jessen, 2003; Lametsch & Bendixen, 2001; Lametsch et al., 2003; Lametsch, Roepstorff, & Bendixen, 2002; Verrez-Bagnis, Ladrat, Morzel, Noël, & Fleurence, 2001) or during processing of fish products (Martinez, Solberg, Lauritzen, & Ofstad, 1992; Morzel, Verrez-Bagnis, Arendt, & Fleurence, 2000). However, the most recent study (Lametsch et al., 2003) showed that it was possible to correlate proteome changes and tenderness of meat. In this context, proteomics appears as a promising technique to establish the link between protein changes occurring *postmortem* and sensory or technological characteristics of meat. Pre-slaughter conditions including lairage time are known to influence pork quality through a variety of incidences such as muscle temperature, glycogen depletion, rate of pH decline. (e.g., Dall Aaslyng & Barton Gade, 2000). With the objective of unveiling mechanisms by which pre-slaughter conditions influence ageing, *postmortem* proteome changes were studied in pork meat from animals slaughtered in two conditions.

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2. Materials and methods

2.1. Animals and samples

The animals used in this study were six Large White pigs, all slaughtered in the same commercial abattoir. Three pigs were slaughtered under commercial conditions (mixing of animals from different pens and transport to the abattoir 12 h before slaughter), and three were slaughtered under experimental conditions (no mixing of animals, transport immediately before slaughter). Samples were excised with a corer from the *Longissimus lumborum* (LL) at the last rib immediately after dehairing. Such samples corresponded to time 0 h. The LL was removed from the carcass at 2 h postmortem and stored at 4 °C. At 12 h *postmortem*, samples of approximately 5 g were sampled at the same level as the previously cored samples. A 4 cm LL slice was excised 24 h after slaughter, from the last rib towards the ham. The slice was vacuum-packed and stored at 4 °C. Samples of approximately 5 g were taken from that slice at 72 h *postmortem*. All samples were frozen in liquid nitrogen and stored at −80 °C until used.

2.2. pH measurement

pH was measured at 45 min *postmortem:* approximately 2 g of LL muscle, taken at the last rib, were homogenised in 18 ml of 0.005 M iodoacetate and readings were taken using a pH meter CG822 (Schott-Geräte, Hofheim, Germany). The ultimate pH was recorded on the day after slaughter: readings were taken in duplicate directly in the LL, from the 4cm slice described above, using a PH62 spear probe meter (WTW, Weilheim, Germany).

2.3. Protein extraction

The extraction buffer consisted of 7 M urea, 2 M thiourea, 1% (w/v) DTT, 2% (w/v) CHAPS, 40 mM Tris, 5 mM Pefabloc, 1 mM EDTA and 0.8% carrier ampholytes. 50 mg of muscle were added to 1 ml of extraction buffer in an Eppendorf containing a glass bead. Homogenisation was performed in Retsch MM2 agitator (Retsch, Haan, Germany) for 1 h at 4 °C. Extracts were centrifuged at 10,000*g* for 1 h at 10 °C and the supernatant was collected. The protein content was measured using a Bio-Rad RC DC protein assay kit.

2.4. 2-D electrophoresis

Immobilised pH gradient (IPG) isoelectric focusing (IEF) was carried out in a Protean IEF cell (Bio-Rad), using Bio-Rad ReadyStrip, 17 cm, pH 3–10 non-linear. 90 or 1000 μg of protein were loaded onto the strips for analytical or preparative gels, respectively. Proteins were loaded by inclusion of an adequate volume of extract in a buffer consisting of 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 5 Mm Pefabloc, 0.2% (w/v) DTT and 0.2% carrier ampholytes. Strips were rehydrated overnight. For the subsequent IEF, voltage was increased gradually to 10,000 V until a total of 60,000 Vh. Strips were immediately frozen and stored at −20 °C until further use.

Prior to SDS–PAGE, strips were equilibrated for 15 min followed by 25 min in a solution of 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 50 mM Tris, supplemented successively with 1% (w/v) DTT or 2.5% (w/v) iodoacetamide and bromophenol blue as a dye. SDS–PAGE was performed in a protean IIxi cell (Bio-Rad) on 11% polyacrylamide gels at 15 mA, until the dye track reached the end of the gels. Analytical gels were silver stained following the protocol of Yan et al. (2000). Preparative gels were stained in 0.02% colloidal Coomassie blue and destained in water. Gels were produced in triplicate and the two gels of best quality were retained for further analysis.

2.5. Image analysis

Gels images were acquired through a GS-800 densitometer and analysed using the PDQuest software (Bio-Rad). After automated detection and matching, highly saturated or ill-defined spots were manually removed and matching across gels was inspected and corrected when necessary. The statistical effects of *postmortem* storage and initial pH on spot quantities were tested for significance at the 5% level by two-way ANOVA using the GLM procedure of SAS v8.10 (SAS Institute Inc., Cary, USA).

2.6. In-gel digestion of protein spots, desalting, concentration and identification

Spots were excised from preparative gels using pipette tips. Gel pieces were placed into 96-wells plates, destained, digested and desalted using the Montage In-Gel $\text{Digest}_{96\ ZP}$ Kit (Millipore, Bedford, MA, USA) following the supplier's instructions. Resulting peptides mixtures were loaded directly onto the MALDI target. The matrix solution (5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) was added immediately and allowed to dry at room temperature. A Voyager DE-Pro model of MALDI-TOF mass spectrometer (Perseptive BioSystems, Farmingham, MA, USA) was used in positive-ion reflector mode for peptide mass fingerprinting. External calibration was performed with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from auto-digestion of porcine trypsin. Monoisotopic peptide masses were assigned and used from NCBI database

searches with the "Mascot" and "Profound" softwares (http://www.matrixscience.com and http://prowl.rockefeller.edu).

3. Results and discussion

3.1. pH values

Average $pH_{45\,min}$ values were 6.69 and 6.41 for the pigs slaughtered following commercial and experimental conditions, respectively. The mean ultimate pH was 5.50 in both groups, as typically reported for normal pork meat.

3.2. 2-D electrophoretic protein pattern

Up to 800 spots were detected per gel, which compares well with previous studies on muscle proteome (Gelfi, De Palma, Cerretelli, Begum, & Wait, 2003; Lametsch & Bendixen, 2001; Sayd, Santé-Lhoutellier, Chambon, Laville, & Monin, 2003; Yan et al., 2001). After removal of saturated or poorly reproducible zones, 252 spots were successfully matched across the whole set of images. Fig. 1 shows the image of a silver stained gel and a computer-generated "master" gel, which contains only spots retained for further analysis. Spots that will be referred to later in the article are indicated by their unique identification number, given by PDQuest.

3.3. Effect of postmortem storage

Table 1 gives relative quantities of spots evolving significantly with storage time, and their identification when possible. When there was an evident discrepancy between the theoretical MW of a protein and its MW estimated through its position in the gel, it was indicated as "fragment". The two spots 6104 and 9112, identified as troponin T, were designated as "putative fragment" since their estimated MW differed slightly from the theoretical value and they migrated slightly further in the 2nd dimension than the troponin T spot 6103. Based on a comparable observation, spot 2101 was designated as "putative fragment" possibly originating from the strand of actin isoforms indicated on Fig. 1. Concerning spot 9112, it should be noted that its observed pI (close to 10) is very different from its theoretical pI. Furthermore, based on their identical apparent MW, it is very likely that spot 9112 corresponds to the same protein as 6104 (putative troponin T fragment) that did not migrate along the 1st dimension.

Intensities of 37 spots varied significantly ($p < 0.05$) with time. 10 spots were not detected at time 0 h but appeared *postmortem*, 12 increased in intensity while 15 decreased in intensity. We propose that these changes may have three different causes, namely proteolysis, post-translational changes and release from protein structures or complexes. These three phenomena are described below.

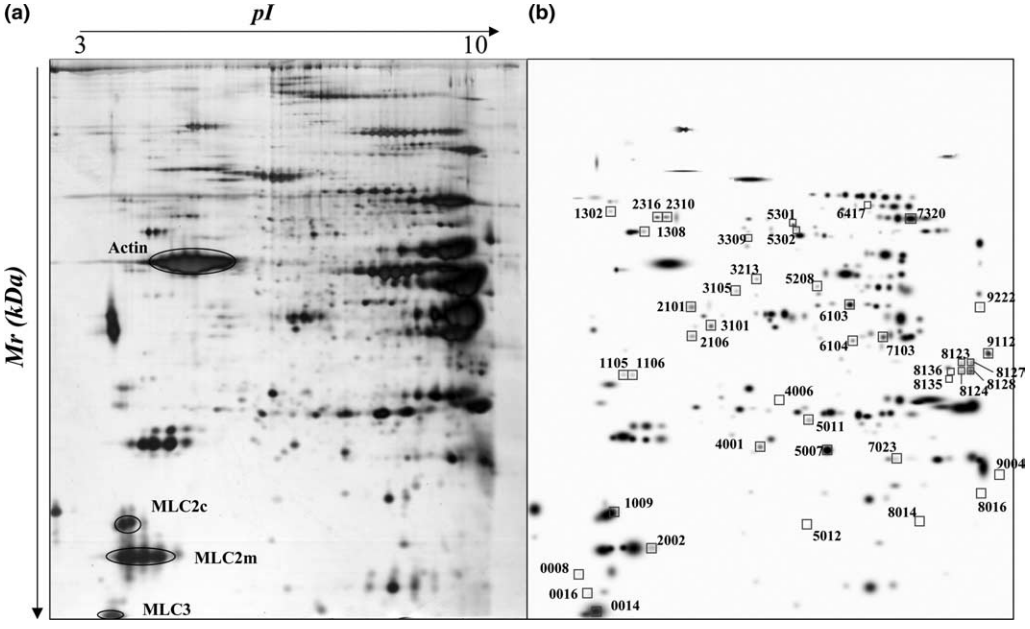

Fig. 1. Raw image of a silver-stained 2DE gel of porcine LD taken 72 h *postmortem* (a) and computer-generated master gel corresponding to all gels of samples taken at 72 h (b). Relative positions of Actin, MLC 2c (cardiac isoform), MLC 2m (skeletal muscle isoform) and MLC 3 were determined by comparison with results of Bouley, Chambon, and Picard (in press).

Table 1
Quantities (arbitrary units) of spots which levels varied significantly with time

Spot number	Identification	0 h	12 h	72 h
Increasing PM				
0008	NI	0	6.73	14.26
0014	Myosin light chain 3	0	694.26	979.78
0016	Myosin light chain	0	6.18	38.11
1009	Myosin light chain 2	122.83	402.30	451.80
1105	Actin (fragment)	13.86	32.46	29.26
1106	NI	18.05	34	28.11
2002	Myosin light chain	29.46	123.08	207.82
2101	Actin (putative fragment)	22.88	48.57	53.68
2106	Pyr. dehydrogenase (β-subunit)	11.15	19.30	26.72
3213	NI	14.38	20.75	25.68
3309	NI	9.13	11.03	23.28
4001	Myokinase (fragment)	0	27.30	128.08
5007	Myokinase (fragment)	0	175.57	625.25
5011	CK (fragment)	15.18	41.78	61.99
5012	α-Crystallin (fragment)	0	0	22.16
5208	NI	13.51	15.32	23.78
5302	SR Sarcalumenin	16.64	33.69	47.56
6104	Troponin T (putative fragment)	0	12.48	24.04
8014	CK (fragment)	0	0	12.96
9004	NI	58.24	42.66	531.28
9112	Troponin T (putative fragment)	0	181.63	384.58
9222	NI	0	3.51	9.33
Decreasing PM				
2310	ATPase F1 chain B	151.43	93.44	57.74
2316	ATPase F1 chain B	148.93	81.09	44.45
5301	T complex protein 1	101.13	76.16	47.03
6103	Troponin T	513.29	277.42	132.63
6417	NI	39.12	32.23	0
7023	NI	74.71	48.42	39.84
7103	Myozenin 1	146.74	140.28	77.37
7320	Pyruvate kinase	434.40	317.63	227.77
8016	NI	40.44	32.53	0
8123	Cypher 1, 2	106.02	35.15	30.95
8124	Cypher 2	155.34	52.99	41.34
8127	Cypher 1, 2	170.75	70.01	58.96
8128	Cypher 2	496.76	257.32	138.06
8135	Cypher 1, 2	46.21	18.53	0
8136	NI	76.44	76	0

Each value is the average of six quantities.
PM, *postmortem*; NI, non identified.

3.4. Structural proteins

As expected, several myofibrillar proteins were successfully identified among the significant spots. Thus fragments of actin and troponin T, i.e., direct results of proteolytic activity, accumulated *postmortem*. The latter result is most probably related with the decrease of spot 6103 corresponding to the full-length troponin T. These results are in accordance with previous research on proteolysis during meat ageing. For example, troponin T has been described in various species as a proteolysis substrate (Ho, Stromer, & Robson, 1996; Negishi, Yamamoto, & Kuwata, 1996; Rees, Trout, & Warner, 2003). Concerning actin, it was commonly thought until recently that it was not affected by *postmortem* proteolysis. However, using 2-D electrophoresis, Lametsch et al. (2002, 2003) showed that fragments of actin appeared during pig muscle ageing. More generally, the most frequently reported effects of proteolysis concern myofibrillar proteins situated in the I-band and that attach the Z-line either to the filaments of actin or to the sarcolemma. Thus, the degradation of the anchoring proteins titin, desmin and dystrophin has been reported on many occasions (Koohmaraie, Schollmeyer, & Dutson, 1986; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995; Uytterhaegen, Claeys, & Demeyer, 1992). Our observations bring new information related to that ultrastructural area: we observed the quantitative decrease of full-length proteins located at the Z-line, namely cypher proteins (1 and 2) and myozenin. Although it can not be formally excluded that the decrease may be due to a lesser extractability, the simplest

explanation resides in the fact that these proteins were subject to proteolysis. Cypher proteins were first described in striated muscle by Zhou, Ruis-Lozano, Martone, and Chen (1999). They are localised at the Z-line, where they maintain its structure during muscle function by binding to its major component α-actinin (Zhou et al., 1999, 2001). Similarly, myozenin can bind α-actinin in the Z-line or γ-filamin at its periphery (Takada et al., 2001). The suggested proteolysis of cypher proteins and myozenin would contribute to the weakening of the Z-line observed during meat or fish ageing (Ho et al., 1996; Papa, Alvarez, Verrez-Bagnis, Fleurence, & Benyamin, 1996), which has been correlated with the release of intact α-actinin from bovine myofibrils during in vitro incubation with proteolytic systems (Goll, Dayto, Singh, & Robson, 1991; Robert, Briand, Taylor, & Briand, 1999). It contrasts with the conclusion of Taylor et al. (1995) who suggested that weakening of the Z-line was due to proteolysis of proximate proteins in the I-band but not of those of the Z-line itself.

Interestingly, the other changes affecting myofibrillar proteins were the intensity increase of four spots corresponding to myosin light chains (MLC). With the exception of spot 0016, the others were at the alkaline extremity of a strand of MLC isoforms, indicated on Fig. 1. To give an example, spot 0014 and the concomitant spot migrated as one single spot at time 0 and gradually separated into two. In other words, the intensity increase of spots 0014, 1009 and 2002 is linked to a gradual shift in pI of MLC 3, 2c (cardiac isoform) and 2m (skeletal muscle isoform), respectively, towards more alkaline values. In our view, as an explanation for this observation, the hypothesis of dephosphorylation deserves to be considered for several reasons. Firstly, 2-D electrophoresis allowed the separation of MLCs according to their status of phosphorylation, the less acidic spots being unphosphorylated (Bozzo, Stevens, Toniolo, Mounier, & Reggiani, 2003). Furthermore, the natural course of events *postmortem*, especially the increase of sarcoplasmic Ca^{2+} and exhaustion of ATP reserves, would favour the accumulation of unphosphorylated forms. For example, Hidalgo, Craig, Ikebe, and Padrón (2001) reported that elevated free Ca^{2+} concentrations, above those required for muscle contraction, inhibited MLC phosphorylation. If free [Ca^{2+}] of approximately 10 μM are observed during muscle contraction, they can reach 200 μM in postmortem muscle (Takahashi, 1996) which would drastically inhibit MLC phosphorylation, very early *postmortem*. Finally, phosphorylation of MLC in vivo is regulated by an equilibrium between the activities of both MLC-kinase and MLC-phosphatase (Solmyo & Solmyo, 2003). Because ATP is required for kinase function, in contrast to phosphatase, it seems plausible that the balance between the two enzymes is jeopardised and favours dephosphorylation as soon as ATP reserves are consumed, i.e., when rigor mortis sets in. Further work should be performed to verify the hypothesis. As to spot 0016, it is well separated from other spots and was not detected at time 0: although its apparent MW is very close to its theoretical value, it may be a proteolytic fragment, which would be in accordance with Lametsch et al. (2003) who recently reported proteolysis of MLC.

3.5. Sarcoplasmic proteins

Several proteolytic fragments accumulated *postmortem*. Thus, the stress-related protein α-crystallin is subjected to *postmortem* proteolysis as one fragment is clearly detected, in accordance with Sayd et al. (2003). Fragments also originated from the metabolic proteins myokinase and creatine kinase, thereby confirming the degradation of these proteins involved in energy metabolism (Lametsch et al., 2002; Stoeva, Byrne, Mullen, Troy, & Voelter, 2000). Increase in intensity of a spot corresponding to pyruvate dehydrogenase (2106) was also significant. However, since the estimated apparent MW was close to the theoretical MW for that protein, it is not possible to assess whether this is a proteolytic fragment or the full-length protein that would become more extractible. As to pyruvate kinase (PK), for which one isoform (spot 7320) decreased in intensity with time, it was previously described as a substrate of proteolysis in pig muscle (Lametsch et al., 2002). However, in the latter study, the supposedly appearing fragment had a MW of 64 kDa, i.e., higher than the reported MW for the full-length protein, reported in databases. To our knowledge, the only previous report of PK proteolysis in meat related to aged Parma ham (Sforza, Boni, Ruozi, Virgili, & Marchelli, 2002), i.e., a product where extensive and prolonged proteolysis occurs. Our own results are not conclusive on the fate of PK during *postmortem* storage: although one spot of PK decreases with time, it is possible that there is a shift of this specific form towards others. The other PK spots could not be quantified successfully because of their high abundance and subsequent saturation of the spots.

Finally, it is likely that the chaperone protein T-complex protein 1 is proteolysed since its quantity decreases unambiguously without concomitant changes in neighbouring spots. Protein T-complex protein 1 has been studied in many organisms and tissues. The common function of that cytosolic chaperone protein is the folding of various newly formed or denatured proteins, essentially actin (Kubota, 2002). Tokumoto, Horiguchi, Nagahama, Ishikawa, and Tokumoto (2000) suggested that it somehow interacts with the proteasome and can be degraded by this proteolytic system. Its degradation therefore comes as no surprise during meat ageing.

Our results show that, expectedly, sarcoplasmic proteins are subjected to *postmortem* proteolysis. This does

not allow us to describe the mechanisms for meat ageing. However, in future studies correlating meat quality and proteome modifications, it would be interesting to assess whether their fragments can be used as biomarkers of meat quality.

3.6. Mitochondrial and sarcoplasmic reticulum (SR) proteins

Two F1-ATPase chain B isoforms (spots 2310 and 2316) decreased in intensity with storage time. F1-ATPase, located in the mitochondrial matrix, is a subunit of a mitochondrial enzymatic complex. Their fast reduction in intensity suggests that they are proteolysed quite rapidly, probably after depolarisation of the mitochondria membranes. In parallel, we observed an intensity increase of a spot identified as sarcalumenin (spot 5302). Sarcalumenin is a 160 kDa protein present in the lumen of the SR. It was shown that the gene encoding for sarcalumenin also encodes for a 53 kDa glycoprotein protein. Both proteins co-exist and share a common activity of Ca^{2+} sequestration within the SR (Leberer, Timms, Campbell, & MacLennan, 1990). The fact that the apparently full-length 53 kDa "sarcalumenin-like" protein increases in intensity probably means that it becomes more extractible, supposedly because of disorganisation of the membrane system of SR.

3.7. Effect of pre-slaughter conditions

Intensities of 8 spots were significantly affected by the pre-slaughter conditions, as seen in Table 2. Out of 8 spots, 7 were consistently present in higher quantities in the group of animals transported immediately before slaughter, i.e., samples with a faster pH decline. The three identified spots corresponded to F1-ATPase chain B, and to one isoform of myosin.

As previously described, F1-ATPase chain B was proteolysed in both groups. However, the full-length protein was initially over-expressed in the group of animals with a higher rate of pH drop. It remains unclear whether the difference reflects individual variations in the expression of mitochondrial ATP-ase, or is related to the difference in muscular activity engendered by variation in pre-slaughter conditions. The literature reports increased expression of F1-ATPase after chronic increased exercise (Gonzalez, Hernando, & Manso, 2000) but we did not find any report of short-term increase. In any case, this over-expression is most probably related to accelerated *postmortem* metabolism: thus, increased mitochondrial ATPase activity would contribute to enhancement of energetic pathways in order to generate protons outside of the mitochondria membranes and regenerate mitochondrial ATP levels. Furthermore, it has been shown that mitochondria, together with SR, have an important role in sequestration of excess Ca^{2+} from the sarcoplasm (Cheah & Cheah, 1978) and that the process requires ATP. F1 ATP-ase synthetizes ATP under normal conditions but reverses its action and hydrolyses ATP under specific conditions such as ischemia (Bosetti, Yu, Zucchi, Ronca-Testoni, & Solaini, 2000). *Postmortem*, higher amounts of ATPase would give more ATP hydrolysis, therefore less ATP would be available for Ca^{2+} sequestration. The consequent enhanced rise in sarcoplasm [Ca^{2+}] would also contribute to an acceleration of glycolysis and glycogenolysis.

The other effect of pre-slaughter conditions concerns spot 2002, which has been suggested to be a dephosphorylated form of MLC 2 (skeletal muscle isoform). The intensity of spot 2002 was drastically increased in samples from animals transported immediately before slaughter, reaching a 3-fold difference between the two groups at 72 h *postmortem*. If, as proposed above, dephosphorylation is a *postmortem* event related to increased sarcoplasmic [Ca^{2+}] and depletion of ATP, it is logical that dephosphorylated forms of MLC 2 are found in higher amounts in samples with accelerated *postmortem* metabolism.

In contrast, the effect of pre-slaughter conditions, and indirectly of the rate of pH decline, on proteolysis was

Table 2
Quantities (arbitrary units), at three *postmortem* sampling times, of spots whose levels varied significantly according to pre-slaughter conditions (commercial/experimental, see text)

Spot number	Identification	0 h	12 h	72 h
		Commercial/experimental	Commercial/experimental	Commercial/experimental
1302	NI	7.87/16.30	13.98/28.78	20.10/32.07
1308	ATPase F1 chain B	63.57/89.95	52.67/99.70	81.38/110.85
2002	Myosin light chain	29.10/29.82	58.10/188.07	124.53/291.10
2310	ATPase F1 chain B	120.48/182.37	51.80/137.08	47.57/67.92
3105	NI	0.18/12.00	0.02/20.67	18.55/21.43
4006	NI	16.93/7.70	13.82/9.63	18.90/7.40
6417	NI	26.78/51.45	0/64.43	0/0
9222	NI	0/0.25	0/7.00	0/18.43

Each value is the average of three quantities.
NI, non identified.

not evident from our results. In particular, we found no statistical differences in intensities of the spots identified with certainty as proteolytic fragments. Two opposite effects of accelerating pH decline have been described: it can promote earlier calcium release and activation of calpains, thereby stimulating proteolysis (Dransfield, 1994). However, when the pH decline is exceedingly fast, it favours protein denaturation including denaturation of enzymes themselves and results in proteolysis inhibition. This is for example the case in PSE pork (Boles, Parrish, Huiatt, & Robson, 1992; Hortos, Gil, & Sarraga, 1994). In the present study, the difference in rate of pH decrease was limited (≈0.3 units), which may have been insufficient to lead to different levels of proteolysis in the first 72 h.

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