PHYSICOCHEMICAL AND HISTOLOGICAL CHANGES IN $CaCl_2$-TREATED BEEF, HORSE, RABBIT AND CHICKEN MEAT

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Summary
Meat from four animal species was treated with 150 mM $CaCl_2$ solution during 48 h. Electrophoresis was carried out in protein samples extracted from myofibrils of treated and untreated samples in order to know the molecular weight range of peptides obtained from degraded proteins. HPLC was also carried out on these samples. $CaCl_2$-treated meat, imbibed in paraffin, was examined by light-microscopy. As expected, electrophoresis showed greater range of peptides obtained from $CaCl_2$-treated meat as compared to untreated samples in the four animal species. A 30 kDa polypeptide, considered as an index of fragmentation, was found in the four species. Light microscopy showed considerable alterations in ultrastructure in beef, whereas chicken and horse meat showed only slight alteration. Rabbit meat showed no ultrastructural changes due to calcium treatment.

Objective
The aim of this work was to find out the extent of protein degradation in terms of peptide production and ultrastructural changes in meat from four different species, treated by immersion in a $CaCl_2$ solution.

Methods
Samples of *Longissimus dorsi* muscle were taken from the right side of carcasses. Half of the muscle was treated with 150 mM $CaCl_2$ during 48 h at 4°C, and the other half, stored at 4°C, was used as a control. Beef and horse meat samples were obtained from local abattoirs 18 h after slaughter. Chicken and rabbit meat samples were obtained from the animal house at the University, shortly after slaughter and evisceration. Therefore, the meat from these two species was in a pre-rigor condition. Myofibrillar protein extraction was carried out by salt precipitation using increments in the ionic strength of the extracting solution.
SDS-polyacrylamide gel electrophoresis (PAGE) was used to detect peptides from 66 to 14 kDa and 584 to 97 kDa (12% and 3.5% polyacrylamide respectively) using 3% stacking gels in a Mini Protean II system (BioRad) according to Laemmli (1970). The resulting bands were stained with 0.1% Coomassie blue. Molecular weights were also determined by gel filtration in a 15 cm protein pak 300 SW gel filtration column fitted to a Waters HPLC with a diode array detector. Five hundred µl of previously filtered (0.22 µm, Millipore) samples were injected into the column together with 10 to 300 kDa markers. Potassium phosphate buffer was used at a 0.2 ml/min constant flow. Marinated and control samples were treated with Bouin solution (75% picric acid+5% acetic acid+25% formol) during 6 h. The samples were then dehydrated with ethanol and xylol and imbibed in paraffin where they were stained with hematoxilin and eosin. Ultrastructural changes were observed using a Zeiss photomicroscope fitted with two polarizing filters.

Results and Discussion
Calpains and cathepsins B and D are the only proteases present in muscle that are known to degrade myofibrillar proteins. Cathepsins are more active in an acid environment, they undergo irreversible inactivation at pH<7.0 in the presence of Ca^{2+}. When proteins are solubilised at pH 7.0, calpains may become inactive due to the increase in pH, presence of SDS or absence of Ca^{2+} (Claeys *et al.*, 1995). After electrophoresis of low molecular weight proteins (66 to 14 kDa) more bands were observed for Ca^{+} treated samples compared to the control. The first of three main bands corresponded to 66 kDa. The second band of 29 kDa was observed mainly for horse, chicken and beef, being more intense for horse and beef. Because beef and horse meat were in a post rigor state, post-mortem degradation was evident and one of the most important changes associated with this was the presence of a 30 kDa band. This band had already been reported in post-rigor chicken, beef and pork (Chou *et al.*, 1995). It is possible that the presence of the 30 kDa polypeptide coincides with the degradation of troponin-T. This protein is considered the most sensitive subunit to proteolysis (Morimoto and Ohtsuki, 1994). Incubation of calpains with Ca^{2+} results in the degradation of troponin-T and the appearance of a 30 kDa polypeptide (Ho *et al.*, 1994). Whereas some authors (Jaarsveld *et al.*, 1997) reported that treatment of myofibrils with calpains results in troponin-T degradation and in an increase in the intensity of 27, 29, 30, 31 and 32 kDa bands, there are no reports about this on horse meat. Rabbit meat presented a 27 kDa polypeptide band in agreement with findings of Ouali and Talmant (1990) about the presence of a 30 kDa polypeptide in maturated beef. Another intense band at 23 kDa, probably corresponded to light myosin, also apparent after the $CaCl_2$. treatment. Meat tenderization mechanisms are the same for all muscles and animal species, although some qualitative differences among species have been reported (Jaarsveld *et al.*, 1997).

There is controversy about the time when post-mortem proteolysis occurs. Koohmaraie *et al* (1988) reported that tenderization starts just after slaughter while other authors report that myofibrillar proteins start to breakdown 4 to 6 h after slaughter. Electrophoresis of myofibrillar proteins in the present studies did not show any evidence of proteolysis before 12 h *post mortem*.

Treated and non-treated rabbit meat samples showed the same electrophoretic pattern. Information on proteolysis in pre-rigor meat is scarce (Wheeler and Koohmaraie, 1994). A 43 kDa band observed only in beef and rabbit meat, probably corresponds to a product of desmin degradation. It is assumed that the first protein undergoing proteolysis is desmin, a component of the intermediate filaments associated with the Z-line. In accordance with our results, Ho *et al.* (1997) reported the presence of a 43 kDa polypeptide as a desmin degradation product. This supports the view of Koohmaraie *et al.* (1984) that tenderization of post-mortem muscle is mainly due to desmin degradation.

Electrophoretic patterns for 97 to 584 kDa myofibrillar proteins showed more bands for chicken and horse meat than for the other species, whereas $CaCl_2$-treated beef had less bands than the control. Treated and untreated rabbit meat presented the same pattern. The densest bands were observed between 310 and 385 kDa for the four species, probably corresponding to degradation products from intermediate filaments such as conectin or titin as reported by Kawamura *et al.* (1995). Nebulin degradation was probably the cause of the occurrence of several bands, in agreement with the studies of Chou *et al.* (1995) and Ho *et al.* (1996). In contrast to Claeys *et al.* (1995) study, no band at 170 kDa was found. A band at 97 kDa was detected, which probably corresponds to a-actinin. The fast degradation of a-actinin can be related to the fracture of Z-lines. Gel filtration by HPLC produced few peaks, probably because of poor separation of proteins. Consequently this technique was not as efficient as SDS-PAGE for analysing the degradation of myofibrillar proteins.

Calpain activity affects ultrastructure (Uytterhaegen *et al.*, 1994). Z-lines and M-bands were detected in the inner part of the myofibrills, together with some dark zones, in proximity to the Z-lines. Myofibrillar structure was indistinct, being replaced by an amorphous band, non uniform in density and adjacent to I-band fractures. Interfibrillar gaps also increased as a result of $CaCl_2$-marination in agreement with the reports by Ho *et al.* (1996). Rabbit meat showed no ultrastructural changes.

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