Tracking hydrophobicity state, aggregation behaviour and structural modifications of pork proteins under the influence of assorted heat treatments

Mitra, Bhaskar.a; Rinnan, Åsmund.a; Ruiz Carrascal, Jorge.a\*;

aDepartment of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

\* Author to whom the correspondence should be sent:

Jorge Ruiz Carrascal, Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Phone: +45 23810623

* E-mail: jorgeruiz@food.ku.dk

**Abstract**

Structural modifications of pork proteins under an assortment of industrial heat treatments were studied. With raw as control, assorted heat treatments involved were 58, 80, 98 and 160 °C for 72 min, 118 °C for 8 min and 58 °C for 17 hours, resembling most common processing procedures. Protein denaturation, surface protein hydrophobicity state and protein aggregation behaviour were investigated. Modifications and molecular chemistry in protein structures were tracked by Fourier Transform Infrared Spectroscopy in order to extract relative proportions of β-sheet, α-helix and residual conformations. In comparison to uncooked samples, cooked ones showed more than two-fold increase in hydrophobicity and larger particles. Thermograms from differential scanning calorimetry showed endothermic transitions (positive enthalpy) indicating a different pattern of protein denaturation as a result of varied cooking temperatures and cooking times. Deconvolution and curve fitting procedures (R2 = 0.99) provided information on rise of the β-sheet to α-helix ratio that further confirmed aggregation with thermal rise and longer cooking time.

*Industrial Relevance*

Thermal treatments following different time-temperature combinations have been applied to meat in order to assess their protein physicochemical status. Previous reports have shown that milder treatments, like *sous vide*, are beneficial for the overall quality of the meat in terms of texture, juiciness or lower chemical modifications. However, the available reports on detecting structural modifications in meat proteins by infrared spectroscopy are quite limited. Based on our present work, a new insight has been developed by usage of Fourier Transform Infrared Spectroscopy to track protein structural alterations that occur under the influence of cooking treatments. Additionally, rise in hydrophobicity levels indicates that protein denaturation takes place on processing and these possible changes could potentially attribute to influencing functionality. Alongside, aggregation mechanisms also get triggered which might be detrimental to the overall quality of the proteins. Results from our experiment can be used to propose that *sous vide* cooking at low temperature could be an attractive and sustainable method to improve overall nutritional quality and biofunctionality thereby useful for nutrient utilization. This information could be a cutting edge concept for meat industries and cooperatives to adopt for improving their existing commercial product portfolio.

Keywords: FTIR; Structural modifications; Aggregation; Hydrophobicity; Denaturation; Meat processing.

1. **Introduction**

Meat processing has always been a topic of interest due to its potential for offering highly nutritious and palatable products for humans. This includes processes with a variety of cooking temperatures and times, from conventional cooking methods like grilling, stewing, pan-frying or roasting, to the more recent sous vide cooking at low temperature for long time (LTLT) (Boles, 2010). As a response to the different heat treatments, proteins undergo varied modifications that could alter their secondary, tertiary and quaternary structure, leading to changes in their hydrophobicity state or aggregation behaviour, which in turn has a potential effect on their nutritional and technological properties, including digestibility, water holding ability, gel formation, light scattering, solubility or extractability (Christensen, Ertbjerg, Dall, & Christensen, 2011; Lund, Heinonen, Baron, & Estévez, 2011; Traore et al., 2012). On top of the obvious impact on the quality of the products, these changes might also have a remarkable importance for those consumers in which protein intake should be optimized, as it happens to elderly, who are frequently not capable of reaching the dietary protein recommendations, and thus impaired nutritional quality of proteins could worsen this situation (Bauer et al., 2013).

There is indeed a lack of understanding about the significance of protein structure modifications in influencing protein functionality and quality and nutritional outcomes. Over recent years, vibrational spectroscopy methods like FTIR, have proved to be a rapid and non-destructive tool to track secondary structural changes and hydration affinities in meat proteins (Perisic, Afseth, Ofstad, Narum, & Kohler, 2013). This may in turn be used to identify their potential final influence on protein functionality or nutritional value. For example, a relatively high proportion of the β-sheet structure may induce a lower accessibility to the digestive enzymes, which might result in lower protein availability (Yu et al., 2005). Heating leads to an increase in the dipole coupling that indicates presence of high content of aggregated β-sheet structures (Calabrò & Magazù, 2012; Herald & Smith, 1992; Yu et al., 2005). With escalating temperatures, heat denaturation occurs with a conformational transition and geometrical alteration at around 65 °C (Zhang, Yamamoto, Ishikawa, & Carpentier, 1999). However, not much work has been carried out in the field of vibrational spectroscopy to understand how modifications in structural elements of meat could influence nutritional bioavailability or quality changes.

In this project, our objective was to elucidate the effect of different common heat processing methods on the structural modification of pork proteins. We also shed some light on the intricate chemistry behind changes in protein structures and how the folding patterns are affected by thermal processing after Fourier Self-Deconvolution (FSD) and Gaussian multicomponent peak fitting. Alongside, authors would like to put emphasis on effect of temperature-time combinations that could possibly alter the physicochemical status of pork proteins leading to either enhancement or deterioration in functionality.

1. **Materials and methods**
   1. *Chemical Reagents*

List of chemicals includes: Sodium dodecyl sulphate (SDS Ultrapure, AppliChem GmbH, Darmstadt, Germany), Bromo Phenol Blue (BPB, AppliChem GmbH, Darmstadt, Germany), Urea (Merck KGaA, Darmstadt, Germany), Cleland’s reagent (DTT, 1, 4-Dithio-DL-Threit (ol), AppliChem GmbH, Darmstadt, Germany), Trizma (Tris) Hydrochloride (Sigma Aldrich, Missouri, USA), Phosphate buffered saline (PBS, Sigma Aldrich, Missouri, USA), Potassium Chloride (KCl, Sigma Aldrich, Missouri, USA), Potassium Dihydrogen Phosphate (KH2PO4, Chemika, Girraween, New South Wales, Australia), Di-Potassium Hydrogen Phosphate (K2HPO4, Merck, Darmstadt, Germany), Magnesium Chloride (MgCl2, Sigma Aldrich, Missouri, USA) and Ethylene glycol-Bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA, Sigma Aldrich, Missouri, USA) . For dissolving chemicals, water was prepared using a Millipore-Milli-Q purification system (Milli-Q Plus Corporation, Bedford, MA). All reagents used were of analytical grade.

* 1. *Collection of meat samples*

Seven female pigs from the same vendor (Supplier No. 77752, Danish Crown) were selected with a slaughter weight of 83 – 86 kg. The animals were stunned with CO2 and dressing activities were performed within 60 min post-mortem at the abattoir (DC-Herning, Denmark). The lean percentage of the carcass varied between 59 – 63 %, with a final pH ranging from 5.5 - 5.6. Carcasses were chilled-stored at 4° C for 24 hour PM, and subsequently cut up. Loins were transported to the meat pilot plant (Danish Meat Research Institute, Taastrup, Denmark), where they were trimmed to obtain the *Longissimus dorsi* (LD) muscle. Steaks (thickness = 2 cm) were chopped from the oyster end (hip) of the right and left LD. First 10 steaks were obtained from the right loin and the next 11 steaks were from the left loin. Steaks were then coded, weighed, packed in vacuum bags (LogiCon EM-628824 - Vacuumpose 200 x 270 x 0,090 mm3, Kolding, Denmark) transported to University of Copenhagen and kept at -80 °C.

* 1. *Cooking treatments*

One hundred and forty-seven sample samples (7 Animals x 7 Treatments x 3 Steaks) were chosen for the following study. Before the samples were subjected to cooking, steaks were thawed 24 hours at 4 °C. Steaks were subjected to 7 different treatments with variation in temperature and time profiles, trying to mimic commonly used heating treatments for producing different types of meat products. The 7 treatments included: Control raw samples (RAW); *sous vide* at 58 °C for 72 min (SV5872, as used for LT-LT cooking meat recipes at caterings), *sous vide* at 80 °C for 72 min (SV8072, as used in numerous cooked meat products), braising at 98 °C for 72 min (B9872, as used for production of stews), autoclave operations at 118 °C for 8 mins holding time (AC1188, as used for canned meat products), oven roasting at 160 °C for 72 min (OV16072, mimicking grilled meat products) and *sous vide* at 58 °C for 17 hours (SV5817, LT-LT cooking, frequently used in restaurants and gastronomic playgrounds). In all the cooking treatments, probes (Testo 176 T4, 4-channel temperature data logger with TC Type T) were used to record and assess core and surface temperature. Before cooking, steaks were coded and vacuum-packed in Cryovac CN 300 bags (Sealed Air Corporation, Charlotte, North Carolina, USA) for cooking temperatures below 100 °C and in LogiCon vacuum bags (EM-62890, Kolding, Denmark) for the autoclave operations at 118 °C, while oven roasted samples were cooked unpacked. As soon as the heating processes were completed, all sample replicates were immediately submerged into ice cold water (4 °C). Pouches were then opened and samples were weighed, and subsequently repackaged and frozen at -80 °C for further analysis. Cooking loss was calculated by difference in weights before and after the treatments.

* 1. *Protein content*

# Two gram of meat sample were homogenized in 20 mL of 0.01 M phosphate saline buffer, pH 7.4, in a 50 ml centrifuge tube using an Ultra Turrax T25 equipped with a S25N-18 G dispersing element (Ikka Labortechnik, Staufen, Germany) for 30 sec with a speed of 20,500 rpm. Separate aliquots of 30 µL were taken in Eppendorf tubes, and 5 % (w/v) SDS (1148 µL) in 50 mM Tris HCl (pH 8), Urea (8 M, 20 µL) and DTT (1 M, 2 µL) were added to achieve a dilution of 40 times. Eppendorfs were then vortexed and incubated into a microplate incubator (Provocell, Model No. PV-PVC-1, Order No. BLC-1, Esco technologies Pty Ltd, Centurion, South Africa) at 80 °C for 30 min. The absorbance of this solution at 280 nm was measured (SpectraMax i3x Multi-Mode Detection Platform, Molecular Devices, Inc., Silicon Valley, USA) and used to estimate protein concentration.

* 1. *Protein Surface Hydrophobicity (H0)*

The hydrophobicity (H0) pattern of total protein pool was assessed by binding of hydrophobic chromophore bromophenol blue (BPB) as previously described by (Chelh & Gatellier, 2006) with minor modifications. Frozen meat samples (2 g) were homogenized with an Ultra Turrax T25 (30 sec x 20,500 rpm) in 20 mL of 20 mM PBS (pH 6). Total protein concentration was then adjusted to 5 mg/mL with PBS. To 1 mL of total sample suspension, 200 µL of 1 mg/mL BPB (solubilized in Millipore water) was added and vortexed. For the control, 200 µl of 1 mg/mL BPB was added to 1 mL of 20 mM PBS. Both the samples and the control were continuously agitated at room temperature in a microplate incubator and then centrifuged at 2000 g for 15 min at 4 °C. The supernatant was removed, diluted 10 times with PBS, and the absorbance at 595 nm was measured. The amount of bound BPB (hydrophobicity index) was calculated by the formula:

BPB bound (µg) = 200 µg x (OD Control – OD Sample) / OD Control

Determinations were performed in duplicate.

* 1. *Differential Scanning Calorimetry (DSC)*

Samples were thawed at 4 °C for 4 hours and minced using a coffee grinder. Meat samples (30 mg) were placed in a sample cell of a differential scanning calorimeter (DSC 1, STARe System, Mettler Toledo, Schwerzenbach, Switzerland). An empty sealed aluminium crucible (capacity 40 µL) was positioned into the reference cell. The sample was scanned from 30 °C to 90 °C with a heating rate of 2 °C /min to track major endothermic transitions that implicates protein denaturation. Three samples per treatment group were analysed.

* 1. *Particle aggregation and size distribution*

Particle aggregation and size distribution profile of total protein pool was measured by static light scattering, using a Malvern Mastersizer Micro Plus (Malvern Instruments, Worcestershire, UK) instrument, according to Liu et al. (2016) with some modifications. Meat samples (2.5 g) were solubilized in 29 mL of cold homogenization buffer, pH 7, (0.1 M KCl, 0.01 M KH2PO4, 0.01 M K2HPO4, 0.001 M EGTA and 0.001 M MgCl2) in a 50 ml centrifuge tube (Lametsch, Knudsen, Ertbjerg, Oksbjerg, & Therkildsen, 2007). Samples were homogenized using an Ultra Turrax T25 for 30 sec with a speed of 20,500 rpm. The meat homogenate was then further filtered through 400 µm nylon filter gauge in order to remove connective tissue. Pump speed, ultrasonic displacement and ultrasonic timer were monitored and evaluated in the Mastersizer. The background was measured after attaining ≥ 70% laser power. The instrument was connected to a small volume sample holder where distilled water was added for cleaning impurities, diluting sample concentration and reaching 0% beam obscuration level before sample measurements. Particle refractive index 1.414 (real part), absorption 0.001 (imaginary part) and dispersant refractive index 1.333 (water) were used respectively with the standard presentation code. Since meat homogenates are heterogeneous in nature, the instrument was operated in polydisperse mode settings. Sample replicates were vortexed and then added before the particle size measurement until obscuration reached was in the range of 15 – 20 %. From the analysed matrix, D4,3(mean diameter in volume), D3,2(mean diameter in surface “Sauter diameter”) and specific surface area (SSA, m2/g) were recorded (W. Sun et al., 2011). D v, 0.1 is the mean threshold size for which 10 % of the sample particles have a lower size. D v, 0.5 is the mean threshold size for which 50 % of the sample particles have a lower size and the rest 50 % have an upper size. D v, 0.9 is the mean threshold size for which 90 % of the sample particles have a lower size. Dispersion Index (Span) is defined by the formula:

Span = (D v, 0.9 - D v, 0.1)/ D v, 0.5.

It has to be noted that the model used for analyzing the particle size distribution is based on spherical particles and therefore the meat particles have apparent sizes. In between each measurement, the small volume sample holder was cleaned twice for eliminating any possible contamination from the prior sample analysis. Parameters were analyzed using Malvern Mastersizer software (5.12c version, Malvern Instruments Co. Ltd., Worcestershire, UK). All the analyses were performed in triplicate.

* 1. *Diamond ATR-FTIR Spectroscopy*
     1. *Sample handling*

Frozen steaks were thawed at 4 °C for 24 hours. Out of these thawed samples, small pieces (2x2x2 cm) were cut. Prior to spectroscopic measurements, samples were equilibrated at room temperature so that there was no occurrence of ice crystals which could interfere the background and real readings. Sample surfaces were wiped with paper towels and loaded onto a Durascope Dicomp Attenuated Total Reflection (ATR) accessory with a 3-bounce diamond ATR crystal (SensIR technologies). To ensure proper contact between the diamond crystal and samples, a pressure of 5 N/cm2 was applied with a pressure clamp. The radiation source used was Globar silicon carbide and was collimated to a 2.5 cm diameter beam.

* + 1. *Collecting raw spectra*

Raw spectra of the meat steaks were recorded using a Bomem MB 100 FTIR Spectrometer (ABB, Zurich, Switzerland), connected to a deuterated triglycine sulphate detector (DTGS), with a KBr beam splitter, and the analyses were performed according to (Calabrò & Magazù, 2012; Garidel & Schott, 2006; W. Sun et al., 2011; Yu, 2005) with modifications. The equipment was continuously purged with dried air to remove traces of water vapour and CO2 which could affect background reading. Spectra were obtained in the wave number range from 550 to 4000 cm˗1 with a 2 cm-1 resolution. The recorded spectrum is an average across 128 scans; multiple scans were taken in order to increase the signal to noise ratio. The preamplifier gain is kept in medium mode so that there is good signal to noise ratio without reaching the saturation limit. A smoothing operation called Norton-Beer medium function has also been applied to minimize small ripples in the spectra called apodization. All the above functions were performed using the Winbomem easy (3.04 Version) software. The scanning rate for each sample spectra was 20 scans / min and background spectrum was acquired by collecting a spectrum from the cleaned blank diamond crystal prior to sample measurement. In between the measurements, crystal was washed with distilled water, cleaned with 70 % ethanol and dried with lint free tissue paper.

* + 1. *Data Processing and Analytics*

From the raw data, a specific region of the averaged spectrum from 1600 to 1700 cm-1 (Amide I) was snipped, extracted and loaded onto the PeakFit Version 4.12 software (SPSS Inc., Chicago, IL, USA) as described in W. Sun et al. (2011) for further analysis. This particular region of the spectrum was then treated by second derivative function to inspect the position of hidden peaks. In order to retrieve more information about various bands and secondary structure of proteins, the concept of Fourier Self-Deconvolution (FSD) was applied to narrow band widths; this also helped to avoid overlapping of subcomponent bands as they broaden in the solid state. Parameters like resolution enhancement factor and full width at half-height (FWHH) were also optimized to ensure elimination of over-deconvolution within the overall band’s contour as often times noise can be amplified and misunderstood as a band as reported by Garidel and Schott (2006). In all these operations, AI Expert was used for smoothing overall spectra. Baseline correction was performed to obtain the best fit. Smoothing function like Sativsky-Golay was also used to smooth data variations and increase signal to noise ratio. Following the FSD, the multi-peak fitting procedure was performed with Gaussian area function to quantify the different peak areas within the subcomponent bands. To have the best fit, curve fitting was done by repeated adjustment of root mean square error (R2) of parameters like peak shape, offset, peak width, peak area and center (Byler & Susi, 1986). In order to reduce overfitting of the data, the above peak finding operation was only done on the average spectra for each of the seven treatments. Based on these fits, the 21 subsamples for each treatment were processed with Alternate Least Squares (ALS) constraining the peaks to Gaussian distributions. This was all done in MATLAB R2015b Software (Math Works, Inc., Natick, Massachusetts, USA).

* + 1. *Determination of subcomponent bands in the secondary structures*

Bands were assigned to the deconvoluted spectra based on adaptations from (Bouhekka & Bürgi, 2012; Jackson & Mantsch, 1995; W. Z. Sun, Zhou, Sun, & Zhao, 2013; Ngarize, Herman, Adams, & Howell, 2004; P. Wang, Bohr, Otto, Danzer, & Mizaikoff, 2015) and their respective areas were calculated to configure relative amounts of α-helixes, β-sheets and turns, non-ordered structures, and random loops. β-sheet to α-helix ratio was also computed as an approach to obtain nutritive value of proteins in its native and processed state (Calabrò & Magazù, 2012). Percentage of structural change in the secondary components was also calculated. Formulae for calculating the parameters are as follows:

Ratio of β-sheet to α-helix: Area of β-sheet / Area of α-helix

% of β-sheet: (Area of β-sheet/ Total area of spectrum) x 100

% of α-helix: (Area of α-helix / Total area of spectrum) x 100

All determinations were done in triplicates.

* 1. *Statistical Analysis*

Data were analysed using a one-way ANOVA, with cooking procedure as the independent variable, using the General Linear Model (GLM) procedure (SPSS 22.0). The significant level was set to 0.05, and if an effect was significant, the Tukey's test was used at the 5% level to make pair wise comparisons between sample means. Results are expressed as mean ± standard deviation. Principle Component Analysis (PCA) was performed using MATLAB 2015b to understand the effect of water content (1646-1650 cm-1) on the inherent secondary structures of the protein molecule that are positioned in the range of 1600 to 1700 cm-1 of the Amide I region.

1. **Results and discussion**
   1. *Cooking loss*

The effect of cooking methods on cooking loss is shown in Table 1. Sous-vide samples cooked at 58 ºC (SV5872 and SV5817) had lower cooking loss than all the other groups (*p* < 0.05), probably due to the fact that longitudinal shrinkage of myofibrils does not occur until around 60ºC, and therefore loss of water is smaller than in high temperature treatments (Sánchez del Pulgar, Gázquez, & Ruiz-Carrascal, 2012). Samples cooked between 80 °C and 118 °C (SV8072, B9872 and AC1188) showed similar cooking loss, while in oven roasted samples, cooking loss was approximately three times higher than in those cooked at 58 ºC samples and 1.5 times higher than in the other cooking groups, most likely due to both, the high evaporation of water from the surface and the high temperature causing the myofibrillar proteins to shrink rapidly. According to (Tornberg, 2005), when temperatures are in between 60 to 70 °C and higher, water holding capacity of meat reduces because connective tissue and associated fibres undergo longitudinal shrinkage; a pressure is created and water molecules are expelled out as reported by Champion, Purslow and Duance (1988). Hence the advantage of getting high juiciness in meat is likely to be derived from the temperature used rather than the cooking method.

* 1. *Analysis of protein hydrophobicity levels*

Fig.1 shows the effect of cooking methods on the hydrophobicity profile of meat proteins. Samples from the RAW group had a H0 value of 81 µg, and with any of the cooking methods the value of H0 significantly increased. Thus, for the sous vide groups (SV5872, SV8072 and SV5817), H0 values obtained were 159, 171 and 168 µg bound BPB, respectively. This is somehow in agreement with Chelh and Gatellier (2006), who reported a 2.5 fold increase in myofibrillar protein hydrophobicity between 30 and 70°C. This is most likely due to the fact that higher temperatures cause the rupture of hydrogen bonds within the protein structure, leading to protein unfolding, which in turn exposes the hydrophobic amino acids, that can bind to BPB (Chelh & Gatellier, 2006; Santé-Lhoutellier et al., 2008). Interestingly, longer cooking times at 58 ºC did not lead to any further significant increase in H0, which would reflect no further unfolding. Values for H0 for the other studied groups (B9872, AC1188, OV16072) were 173, 177 and 174 µg bound BPB respectively, and were significantly higher than those for SV5872, but not than the other two sous-vide groups. This reflects further, but limited unfolding above 58 ºC, and apparently no increase above 80 ºC, which might be possibly due to the completion of unfolding and this temperature.

A link between increased protein surface hydrophobicity and occurrence of aggregation and polymerization of meat proteins has been previously described (Filgueras et al., 2011), and in turn, protein aggregation has been shown to impair proteolytic susceptibility (Santé-Lhoutellier et al., 2008).

* 1. *Differential Scanning Calorimetry*

Major endothermic peak transitions could be detected from the DSC thermograms indicating protein denaturation patterns as illustrated in Fig. 2. Region I corresponded to myosin (53 - 58° C), Region II has been ascribed to sarcoplasmic, actomyosin complex and connective tissue (61 - 68° C) and Region III has been assigned to actin (75 - 83° C). The above mentioned denaturation regions were adapted keeping in mind that there could be variation in peak shifts and intensities due to external environmental conditions like vapour pressure and freeze-thaw process (Berhe, Engelsen, Hviid, & Lametsch, 2014; Christensen et al., 2013; Tornberg, 2005). In the RAW group, peak transitions (positive enthalpy) could be tracked for myosin (tail and head), connective tissue (collagen), water soluble sarcoplasmic proteins and actin. In samples from the SV5872 group the peak for myosin and its subunit regions is not present, but those for connective tissue, sarcoplasmic proteins and actin still remain. Nevertheless, these samples showed a decrease in collagen intensity compared to the RAW group, possibly due to contraction triggered by formation of hydrogen bonds that lose the fibrillar structure, as reported by (Tornberg, 2005). Interestingly, samples from the SV5817 treatment showed no peak for neither the myosin, nor for the collagen and sarcoplasmic proteins and only a reduced peak for actin was present. This seems to point out to a practical denaturation of most meat proteins as a result of longer cooking times, despite to the fact that the cooking temperatures did not reach the theoretical denaturation temperature. Previous research did not detected any alteration of peak III at 60 ºC after 180 min in cooked beef (Bertola, Bevilacqua, & Zaritzky, 1994). However, in agreement with our results, studying LTLT cooking of pork, Christensen, Bertram, Aaslyng, & Christensen (2011) also detected a decrease in the intensities of the 75 ºC endothermic peak after 20 h cooking of porcine meat at 53 and 59 ºC.

For the other groups (SV8072, B9872, AC1188 and OV16072), no further denaturation could be traced in DSC thermograms, possibly due to the fact that at higher cooking temperatures all the proteins have already been denatured.

* 1. *Analysis of Particle aggregation*

Values for mean surface diameter (D3,2), mean volume diameter (D4,3), mean threshold sizes (Dv,0.1, Dv,0.5, Dv,0.9) and span of total protein pool from pork cooked under different combinations of time and temperature are illustrated in Table 1. In general, results were very variable. Most of the information about this type of parameter for meat has been done on pure myofibrillar (W. Sun et al., 2011) or sarcoplasmic proteins (Li et al., 2017), where the span range is much shorter than when considering the total protein pool, as it has been done in the present study.

Overall, increased cooking temperatures led to higher values for indicators of aggregation, like D4,3 or Dv,0.9, that showed significantly lower values for raw samples than for all cooking groups, with the exception of AC1188, pointing out to a potential role of cooking time on aggregation. It seems that protein molecules might require more time span for aggregation mechanism to occur.

One of the possible explanations for heat induced protein aggregation is the compelling of thiol groups and hydrophobic residues to be exposed, resulting in the development of hydrogen bonds and increased hydrophobic interactions between diverse denatured proteins, leading to the formation of aggregates (Filgueras et al., 2011; Grossi et al., 2016). Nevertheless, many other chemical factors may play a critical role in influencing aggregation, like formation of higher amount of carbonyls with longer cooking time and higher temperature (Roldan, Antequera, Armenteros, & Ruiz, 2014), generation of disulphide bridges, oxidation, transamidation, Schiff’s bases development and production of free radicals resulting in cross linking (Santé-Lhoutellier et al., 2008; Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015). Protein aggregation is also regulated by physical parameters, like crowding effect of protein concentration, electrostatic interactions, van der Waals forces, and thermodynamic traits like reduction of activation energy, increased diffusion and enhanced frequency of molecular collisions with high momentum during thermal treatment (Wang, Nema, & Teagarden, 2010). An overall decrease in the span (width of distribution) was observed from RAW samples to the other processed groups, indicating that the uniformity of the measured distribution is lessening and has a sharper particle distribution trend towards bigger sizes depicting aggregation.

* 1. *Structural modifications tracked by FTIR spectroscopy*

Fig. 3A shows the raw spectra of the Amide I region for all the sample groups and it can be distinctly observed that there is a spectral shift that has occurred from the RAW to the groups that were heat treated. Fig. 3B presents the second derivative spectra for all sample groups, which allows the identification of various peak positions assigned to the secondary structures occurring in the protein. Garidel and Schott (2006) stated that quantitative analysis of secondary structures can be considered as a linear sum of structural elements, assuming that carbonyl molar absorption coefficient for all secondary structures are equal. Second derivative of the raw spectra were computed in order to elevate the minute differences observed in the raw readings as a functional coefficient of temperature rise.

Fig.3C elucidates an instance of deconvoluted infrared amide I region from the second derivative spectrum of a sample. FSD was used in order to configure subcomponent peak frequencies with multicomponent peak fitting by assuming that peaks would appear to be of Gaussian distribution (R2 = 0.99). The bands falling in the region between 1610 and 1620 cm-1 can be associated with formation of aggregated strands. The band between 1650 and 1660 cm-1 corresponded to α-helix structure. The band arising in the range of 1660 and 1670 cm-1 is due to 310 helix, which has slightly different internal hydrogen bonding arrangement from α-helix. The band located around 1614 cm-1 is attributed to the aromatic ring vibration of tyrosine residues and protein side chains. Band regions that fall between 1640 and 1650 cm-1 are due to random/non-ordered structures. Presence of low frequency β-sheet and high frequency β-sheet are detected in the range of 1618-1640 cm-1 and 1670-1680 cm-1 respectively. Additionally, antiparallel β-sheet and β-turns also appear between 1675 and 1695 cm-1. These information about band assignments was adapted from the scientific literature (Byler & Susi, 1986; Garidel & Schott, 2006; Jackson & Mantsch, 1995; Kong & Yu, 2007; Ngarize et al., 2004; W. Z. Sun et al., 2013)**.**

Structural modifications could be tracked between RAW and SV5872 samples via the second derivative spectrum with the relative abundance of significant structures like α-helix and β-sheet changing to a moderate extent (Table 2). It has been previously described that, even having similar protein content, the nutritional value of a protein source may vary if the ratio of α- helix to β-sheet in the secondary structures are different , due to a different susceptibility to digestion (Calabrò & Magazù, 2012). Apart from RAW and SV5872 groups, noticeable differences could be observed in the remaining groups where with increasing temperature, certain bands appeared around 1635, 1676, 1686 and 1624 cm-1, that reflects variations in the β-sheet conformations (Sun et al., 2011). At higher temperatures proteins could self-aggregate and enhanced aggregation mechanism occurs due to intermolecular β-sheet interaction. The evidence was confirmed when shoulder bands became prominent around 1625 cm-1 and 1686 cm-1 indicating antiparallel geometrical orientation. Structural modifications also occur when bands allocated in the range of 1620 to 1625 cm-1 are developed, underpinning non-linear assemblies of insoluble amyloid fibrils that are characterized by crossed β-sheet configuration, where the molecular structure is dissimilar to its native conformation (Van der Linden & Venema, 2007; Wang et al., 2015). In all groups, random/non-ordered structures were identified but such irregular and erratic orientations are inherently present in the protein systems and likely to be dependent on increasing thermal regimes. In groups like SV8072, AC1188, OV16072 and SV5817, bands around 1635 cm-1 could be found involving β turns and bends responsible for rebuilding purposes but with a high uncertainty. Interestingly, this band was not present when cooking at 88 ºC for 72 min, while it was obvious in samples cooked at the same temperature but for 17 h, pointing out to further protein structural modifications due not only to increased temperature, but also longer cooking times. Lastly, shifts in α-helix towards lowered intensity could be observed with greater degree of denaturation as temperature elevates.

On quantifying the relative proportion of secondary structures present in the protein, it could be observed that the proportion of β-sheet and turn significantly increased (*p* < 0.05) from RAW samples to all the cooking groups, except those samples cooked at 58 ºC for 72 min (SV5872). Similarly, cooking time seemed to play a role on this parameter, since β-sheet and turn proportion increased from 24.8 % in SV5872 to 48.2 % in SV5817. Moreover, AC1188 samples, despite the higher cooking temperature, showed lower proportion of β-sheet and turn than those cooked at 98 ºC or 80 ºC for 72 min, which also points out to the relevance of cooking time on the occurrence of this type of structure. Such higher proportions of β-sheet and turn due to cooking temperature and cooking time might influence protein aggregation through intermolecular interactions among β-sheet conformers. Interestingly, the proportion of this type of conformation in meat proteins decreased from RAW to SV5872 samples. Information about α-helix also provides an understanding about the effect of thermal rise: there was a reduction in the proportion from RAW samples to all the other groups.

As a consequence of the changes in the proportion of both types of structural conformations, the β/α ratio also varied (Table 2). Thus, a β/α ratio of 1.2 was obtained for RAW samples, which significantly increased to 1.8 in SV5872, 2.4 in SV5817, 2.8 in SV8072 and AC1188, 3.5 in B9872 and 4.9 in OV16072, giving a picture in which both cooking temperature and time play a role in the structural modification of meat proteins. This somehow shows that the functionality of the total protein pool might be adversely affected as a consequence of stronger cooking conditions. A higher proportion of β-sheet may partly cause a low access to gastrointestinal digestive enzymes, which may lead to lower protein nutritional value due to self-aggregation among intermolecular β-sheet conformers (Calabrò & Magazù, 2012; Yu, 2005). The detected protein structural changes are quite in agreement with those previously described about the pattern of modifications in hydrophobicity and denaturation, with clear effects of cooking time and temperature on increased levels of hydrophobicity and denaturation of different types of proteins with the assorted cooking methods.

The score values of the PCA carried out on the 2nd derivative spectra values of the range 1710-1600 cm-1 (Figure 4A), indicate a clear difference between the treatment groups. It should be noted that the values for the RAW group are not shown here, as the RAW is very different from the rest (as already noted above). OV16072 and SV5817 hold the largest variation. It is of no surprise that the OV16072 has such a large variation, as this probably is due to the less stringent procedure (open without bags). More surprisingly is the difference between the replicates of the SV5817. This difference is due to the spectra from two clusters, one containing four replicates, while the remaining 17 are close to each other. For the interpretation of the results, it is important to note that a positive loading value in the 2nd derivative pre-processed spectra, indicates low values at this wavelength in the original data. On the PC1 axis, the most different treatments were OV16072 and SV5872, the harshest and the mildest cooking procedure. This was due to an increase in the peak at 1620 cm-1 for the samples cooked in the oven compared to the SV5872, acknowledging the increase in aggregated strands. On the second axis (PC2), the two most extreme samples were B9872 and the two SV58 samples (both SV5817 and SV5872). Through inspection of the loading plot in Fig 4B, such extremity can be seen because the SV58 samples have a higher response on the 1630 and 1650 cm-1 peaks, indicating moreaffinity towards β-sheet, α-helix and microenvironment water conditions.

1. **Conclusion**

The results from this experiment indicate the effect of regular cooking time-temperature combinations on the hydrophobicity state, aggregation behaviour and secondary structural changes of meat proteins. Meat subjected to higher temperature treatments suffer more intense modification in protein structure, which in turn promote extended aggregation. The effect of cooking time is somehow in the same direction but with a lesser intensity. As a consequence, it seems that cooking procedures at lower temperatures might preserve better the functionality and nutritional value of meat proteins.

**Acknowledgements**

This project was funded by The Danish Pig Levy Fund. Mianne Tenna Darré and Hauke Valentin Hemmsen (Danish Meat Research Institute –DMRI-) support in sample preparation and collection is acknowledged. Lars Kristensen (DMRI) insightful comments are thanked. The authors would also like to thank Linda de Sparra Terkelsen, lab technician, for her assistance in the day to day activities of the laboratory.

The authors declare no conflict of interest.

**References**

Bauer, J., Biolo, G., Cederholm, T., Cesari, M., Cruz-jentoft, A. J., Mb, J. E. M., … Boirie, Y. (2013). Evidence-Based Recommendations for Optimal Dietary Protein Intake in Older People : A Position Paper From the PROT-AGE Study Group. *Journal of the American Medical Directors Association*, *14*(8), 542–559. http://doi.org/10.1016/j.jamda.2013.05.021

Berhe, D. T., Engelsen, S. B., Hviid, M. S., & Lametsch, R. (2014). Raman spectroscopic study of effect of the cooking temperature and time on meat proteins. *Food Research International*, *66*, 123–131. http://doi.org/10.1016/j.foodres.2014.09.010

Bertola, N. C., Bevilacqua, A. E., & Zaritzky, N. E. (1994). theremaof PROTEINS IN BEEF MUSCLe. *Journal of Food Processing and Preservation*, *18*(1), 31–46. http://doi.org/10.1111/j.1745-4549.1994.tb00240.x

Boles, J. A. (2010). Thermal Processing. In *Handbook of Meat Processing* (pp. 169–183). Oxford, UK: Wiley-Blackwell. http://doi.org/10.1002/9780813820897.ch8

Bouhekka, A., & Bürgi, T. (2012). In situ ATR-IR spectroscopy study of adsorbed protein: Visible light denaturation of bovine serum albumin on TiO2. *Applied Surface Science*, *261*, 369–374. http://doi.org/10.1016/j.apsusc.2012.08.017

Byler, D. M., & Susi, H. (1986). Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers*, *25*(3), 469–487. http://doi.org/10.1002/bip.360250307

Calabrò, E., & Magazù, S. (2012). Comparison Between Conventional Convective Heating and Microwave Heating: An FTIR Spectroscopy Study of the Effects of Microwave Oven Cooking of Bovine Breast Meat. *Journal of Electromagnetic Analysis and Applications*, *4*(11), 433–439. http://doi.org/10.4236/jemaa.2012.411060

Champion, A. E., Purslow, P. P., & Duance, V. C. (1988). Dimensional changes of isolated endomysia on heating. *Meat Science*, *24*(4), 261–273. http://doi.org/10.1016/0309-1740(88)90039-3

Chelh, I., & Gatellier, P. (2006). MEAT Technical note : A simplified procedure for myofibril hydrophobicity determination. *Meat Science*, *74*, 681–683. http://doi.org/10.1016/j.meatsci.2006.05.019

Christensen, L., Bertram, H. C., Aaslyng, M. D., & Christensen, M. (2011). Protein denaturation and water–protein interactions as affected by low temperature long time treatment of porcine Longissimus dorsi. *Meat Science*, *88*(4), 718–722. http://doi.org/10.1016/j.meatsci.2011.03.002

Christensen, L., Ertbjerg, P., Dall, M., & Christensen, M. (2011). Effect of prolonged heat treatment from 48 ° C to 63 ° C on toughness , cooking loss and color of pork. *MESC*, *88*(2), 280–285. http://doi.org/10.1016/j.meatsci.2010.12.035

Christensen, L., Ertbjerg, P., Løje, H., Risbo, J., van den Berg, F. W. J., & Christensen, M. (2013). Relationship between meat toughness and properties of connective tissue from cows and young bulls heat treated at low temperatures for prolonged times. *Meat Science*, *93*(4), 787–795. http://doi.org/10.1016/j.meatsci.2012.12.001

Filgueras, R. S., Gatellier, P., Ferreira, C., Zambiazi, R. C., & Santé-Lhoutellier, V. (2011). Nutritional value and digestion rate of rhea meat proteins in association with storage and cooking processes. *Meat Science*, *89*(1), 6–12. http://doi.org/10.1016/j.meatsci.2011.02.028

Garidel, P., & Schott, H. (2006). Fourier-Transform Midinfrared Spectroscopy for Analysis and Screening of Liquid Protein Formulations Part 2: Details Analysis and Applications. *BioProcess International*, *1*, 48–55.

Grossi, A., Olsen, K., Bolumar, T., Rinnan, Å., Ogendal, L. H., & Orlien, V. (2016). The effect of high pressure on the functional properties of pork myofibrillar proteins. *Food Chemistry*, *196*, 1005–1015. http://doi.org/10.1016/j.foodchem.2015.10.062

Herald, T. J., & Smith, D. M. (1992). Heat-Induced Changes in the Secondary Structure of Hen Egg S- Oval bumin. *Journal of Agricultural and Food Chemistry*, *40*, 1737–1740.

Jackson, M., & Mantsch, H. H. (1995). The use and misuse of FTIR spectroscopy in the determination of protein structure. *Critical Reviews in Biochemistry and Molecular Biology*, *30*(2), 95–120. http://doi.org/10.3109/10409239509085140

Kong, J., & Yu, S. (2007). Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures Protein FTIR Data Analysis and Band Assignment. *Acta Biochimica et Biophysica Sinica*, *39*(8), 549–559. http://doi.org/10.1186/1479-5876-10-117

Lametsch, R., Knudsen, J. C. C., Ertbjerg, P., Oksbjerg, N., & Therkildsen, M. (2007). Novel method for determination of myofibril fragmentation post-mortem. *Meat Science*, *75*(4), 719–724. http://doi.org/10.1016/j.meatsci.2006.10.002

Li, L., Liu, Y., Zou, X., He, J., Xu, X., Zhou, G., & Li, C. (2017). In vitro protein digestibility of pork products is affected by the method of processing. *Food Research International*, *92*, 88–94. http://doi.org/10.1016/j.foodres.2016.12.024

Liu, G., Jæger, T. C., Lund, M. N., Nielsen, S. B., Ray, C. A., & Ipsen, R. (2016). Effects of disulphide bonds between added whey protein aggregates and other milk components on the rheological properties of acidified milk model systems. *International Dairy Journal*, *59*(August), 1–9. http://doi.org/10.1016/j.idairyj.2016.03.002

Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition and Food Research*, *55*(1), 83–95. http://doi.org/10.1002/mnfr.201000453

Ngarize, S., Herman, H., Adams, A., & Howell, N. (2004). Comparison of changes in the secondary structure of unheated, heated, and high-pressure-treated ss-lactoglobulin and ovalbumin proteins using Fourier transform Raman spectroscopy and self-deconvolution. *Journal of Agricultural and Food Chemistry*, *52*(21), 6470–6477. http://doi.org/Doi 10.1021/Jf030649y

Perisic, N., Afseth, N. K., Ofstad, R., Narum, B., & Kohler, A. (2013). Characterizing salt substitution in beef meat processing by vibrational spectroscopy and sensory analysis. *Meat Science*, *95*(3), 576–585. http://doi.org/10.1016/j.meatsci.2013.05.043

Roldan, M., Antequera, T., Armenteros, M., & Ruiz, J. (2014). Effect of different temperature-time combinations on lipid and protein oxidation of sous-vide cooked lamb loins. *Food Chemistry*, *149*, 129–136. http://doi.org/10.1016/j.foodchem.2013.10.079

Sánchez del Pulgar, J., Gázquez, A., & Ruiz-Carrascal, J. (2012). Physico-chemical, textural and structural characteristics of sous-vide cooked pork cheeks as affected by vacuum, cooking temperature, and cooking time. *Meat Science*, *90*(3), 828–835. http://doi.org/10.1016/j.meatsci.2011.11.024

Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., Gatellier, P., Houtellier, V. S., … Atellier, P. G. (2008). Effect of Meat Cooking on Physicochemical State and in Vitro Digestibility of Myofibrillar Proteins. *Journal of Agricultural and Food Chemistry*, *56*(4), 1488–1494. http://doi.org/10.1021/jf072999g

Soladoye, O. P., Juárez, M. L., Aalhus, J. L., Shand, P., & Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, *14*(2), 106–122. http://doi.org/10.1111/1541-4337.12127

Sun, W. Z., Zhou, F. B., Sun, D. W., & Zhao, M. M. (2013). Effect of Oxidation on the Emulsifying Properties of Myofibrillar Proteins. *Food and Bioprocess Technology*, *6*(7), 1703–1712. http://doi.org/10.1007/s11947-012-0823-8

Sun, W., Zhou, F., Zhao, M., Yang, B., & Cui, C. (2011). Physicochemical changes of myofibrillar proteins during processing of Cantonese sausage in relation to their aggregation behaviour and in vitro digestibility. *Food Chemistry*, *129*(2), 472–478. http://doi.org/10.1016/j.foodchem.2011.04.101

Tornberg, E. (2005). Effects of heat on meat proteins - Implications on structure and quality of meat products. *Meat Science*, *70*(3 SPEC. ISS.), 493–508. http://doi.org/10.1016/j.meatsci.2004.11.021

Traore, S., Aubry, L., Gatellier, P., Przybylski, W., Jaworska, D., Kajak-Siemaszko, K., & Santé-Lhoutellier, V. (2012). Higher drip loss is associated with protein oxidation. *Meat Science*, *90*(4), 917–924. http://doi.org/10.1016/j.meatsci.2011.11.033

van der Linden, E., & Venema, P. (2007). Self-assembly and aggregation of proteins. *Current Opinion in Colloid and Interface Science*, *12*(4–5), 158–165. http://doi.org/10.1016/j.cocis.2007.07.010

Wang, P., Bohr, W., Otto, M., Danzer, K. M., & Mizaikoff, B. (2015). Quantifying amyloid fibrils in protein mixtures via infrared attenuated-total-reflection spectroscopy. *Analytical and Bioanalytical Chemistry*, *407*(14), 4015–4021. http://doi.org/10.1007/s00216-015-8623-4

Wang, W., Nema, S., & Teagarden, D. (2010). Protein aggregation-Pathways and influencing factors. *International Journal of Pharmaceutics*. http://doi.org/10.1016/j.ijpharm.2010.02.025

Yu, P. (2005). Protein secondary structures (alpha-helix and beta-sheet) at a cellular level and protein fractions in relation to rumen degradation behaviours of protein: a new approach. *The British Journal of Nutrition*, *94*(5), 655–665. http://doi.org/10.1079/bjn20051532

Zhang, H., Yamamoto, Y., Ishikawa, Y., & Carpentier, R. (1999). Characterization of the secondary structure and thermostability of the extrinsic 16 kilodalton protein of spinach photosystem II by Fourier transform infrared spectroscopy. *Journal of Molecular Structure*, *513*(1–3), 127–132. http://doi.org/10.1016/S0022-2860(99)00107-6