



# The effect of adding eggshell membrane to emulsified meat models in terms of structure, water-holding, and texture

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## ABSTRACT

This study was conducted to evaluate the effect of adding eggshell membrane (ESM), a by-product of the chicken egg processing industry, to emulsified meat models with different NaCl concentrations. The aim was to examine if ESM could facilitate a reduction in the amount of NaCl added to this type of product. The effects of ESM addition were investigated in terms of cooking loss, water distribution, color, and texture properties using a simplified meat emulsion model with no other additives than ESM. Emulsified meat samples were made with three different NaCl concentrations (0.5, 0.1, 1.5% w/w), without and with three levels of ESM (0.5, 1.0, 1.5% w/w). Addition of ESM reduced cooking loss (CL), improved texture, and increased redness. Effects of ESM were explained by structural changes in the protein matrix, as shown by two different histological methods. The addition of ESM explained 86% of the variation in low-field Nuclear Magnetic Resonance (LF-NMR)  $T_2$  relaxation times in uncooked samples, while 97% of the variation in cooked samples was explained by the NaCl concentration. Fourier transform Infrared (FTIR) micro-spectroscopic measurements revealed that samples supplemented with ESM had a higher proportion of  $\alpha$ -helical structures and reduced amount of protein  $\beta$ -sheet aggregation in samples with 1.0 and 1.5% NaCl. It was shown that ESM increased the pH of the emulsified meat, and it was, therefore suggested that increased negative repulsion effects had a positive heat stabilizing effect on the protein network. The fact that the cooked samples were redder was probably related to the antioxidant effect of ESM which was measured as MDA (malondialdehyde) equivalents after in vitro digestion of the samples. ESM can thus help reduce the salt content in sausages while ESM also has a positive antioxidant effect improving color.

## 1. Introduction

Emulsified meat products, such as sausages, play a significant role in modern food consumption (Santhi, Kalaikannan & Sureshkumar, 2017). Meat products have been related to non-communicable diseases associated with high sodium chloride content (Cappuccio, Capewell, Lincoln & McPherson, 2011; Tuomilehto et al., 2001). Development of healthier meat products is needed to meet consumers' requests. To succeed, there is a need to identify new ingredients that can replace salt (Deng, Shao, Sun, Liu & Song, 2015; Grossi, Soltoft-Jensen, Knudsen, Christensen & Orlien, 2012). Eggshell membrane (ESM) is a protein-rich membrane between the eggshell and egg white, which is available as a by-product of the chicken egg processing industry. Fermented ESM was found to have functional, antioxidant and antihypertensive activities as well as antibacterial activity (Jain & Anal, 2017). Due to its physical, chemical, and thermal properties, ESM could have potential as a functional ingredient (Park et al., 2016; Wangrunroj, Soontorntepwarakul, Samattai & Tangboriboon, 2018). ESM consists of a network of collagen fibers,

and glycosaminoglycans such as dermatan sulfate, chondroitin sulfate, sulfated glycoproteins, and hyaluronic acids (Park et al., 2016). Addition of collagen in finely comminuted meat emulsion-type products improved water-holding and reduced cooking loss (Cheng & Sun, 2008; Lee & Chin, 2019). On the other hand, addition of dermatan sulfate and glucosamine showed some detrimental effect on ham texture and cooking loss when incorporated in restructured ham via the brine (Day, Seymour, Pitts, Konczak & Lundin, 2009). To the authors' knowledge, there are no previous studies that have examined the potential functionalities ESM can provide to emulsified meat products and whether it may help reduce salt in these products.

Emulsified meat products are composed of protein-coated fat globules (oil droplets) dispersed in a myofibrillar protein gel matrix (Dickinson, 2012). Water holding is an important quality trait for emulsified meat products influencing yield, textural properties, and juiciness (Grossi et al., 2012). Functional properties such as water holding capacity (WHC), texture and emulsifying ability of processed meat are mainly due to myofibrillar proteins depolymerized with the aid of salt

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and converted to a gel through heat setting (Asghar, Samejima & Yasui, 1985). When NaCl is added to minced meat, the myofibrillar proteins are unfolded, which results in the establishment of a dense protein network upon heating. Ionic strength, pH, and heat-treatment regime affect the heat-induced aggregate of the proteins and thus the microstructure of the product (Hermansson, 1986; Liu, Zhao, Xiong, Die & Qin, 2008; Ofstad, Kidman, Myklebust, Olsen & Hermansson, 1995; Sun & Holly, 2011). Both texture and water holding properties depend on the microstructure and degree of protein aggregation (Hermansson, 1986; Liu et al., 2008). Gels with coarse, aggregated network structures and big pores held the water less firmly than gels with fine stranded and continuous matrices (Hermansson, 1994; Herrero, Carmona, Garcia, Solas & Careche, 2005).

Nuclear magnetic resonance (NMR) relaxation provides unique qualitative and quantitative information about the physical state of water (Bertram, 2016, 2004). A good correlation was found between WHC, NMR  $T_2$  relaxometry and the microstructural characteristics of heat-induced myofibrillar gels (Han, Wang, Xu & Zhou, 2014). FTIR microspectroscopy has been extensively used to study protein denaturation in meat and fish muscle tissues (Bertram, Kohler, Bocker, Ofstad & Andersen, 2006; Böcker et al., 2007; Böcker, Kohler, Aursand & Ofstad, 2008, 2006; Wu, Bertram, Böcker, Ofstad, & Kohler, 2007). These studies showed that salting in combination with heat treatment, induced changes in the protein secondary structure, characterized by a transformation of  $\alpha$ -helical structures to aggregated  $\beta$ -sheets and that this transformation was correlated to water distribution (Bertram et al., 2006; Böcker et al., 2007). Increased pH is related to increased water binding in meat and fish products (Bertram et al., 2004; Ofstad et al., 1995). ESM has a high pH (Wangrungrroj et al., 2018). It is therefore of interest to test whether ESM affects a meat emulsion in terms of pH and functional properties and whether addition of ESM can reduce sodium chloride in emulsion-based meat products. This, in combination with a potential antioxidant effect of ESM (Jain & Anal, 2017) can help reduce the negative health effects of such meat products.

The main objective of this study was to investigate the effect of adding ESM to emulsified meat products with various levels of NaCl in terms of techno-functional properties. This is to reveal whether ESM has potential to reduce NaCl level required in meat emulsion products. To elucidate the effect of ESM on the protein matrix structure, emulsified meat without additives other than NaCl and ESM was used as a model system. Cooking loss, texture, and color were measured on cooked samples, while the water distribution was analyzed by NMR  $T_2$  relaxometry on both uncooked and cooked emulsified meat samples. To examine the effect of ESM on the microstructure, both uncooked and cooked samples were examined by light microscopy, while changes in the secondary structure of the proteins were determined using FTIR micro-spectroscopy. In addition, an initial study of a possible antioxidant effect of ESM was performed using an *in vitro* digestive model.

## 2. Materials and methods

### 2.1. Raw material

Fresh ground bovine meat (14% fat) and pork back fat (70% fat, 23% water, 6.5% protein) were purchased from a commercial slaughterhouse (Furuseth, Norway). Powdered eggshell membrane (ESM) was manufactured using a patented process (Biovotec, international application: WO 2015/058,790) and supplied by Nortura, Norway. The ESM composition is typically 90% protein, of which 10% is collagen and 30% is cysteine-rich eggshell membrane proteins (CREMPs), and it contains approximately 0.05% glycosaminoglycans (GAGs) (Ahmed, Suso, Maqbool & Hincke, 2019; Liu et al., 2014). Harvested ESM was further washed by distilled water, freeze-dried, and milled to small particles under 250  $\mu$ m (Helium-Neon Laser Optical System, Sympatic Inc., Clausthal-Zellerfeld,

Germany) using a Retsch Mill (ZM100, Retsch, Haan, Germany). NaCl (Alimenta, Oslo, Norway) was used as salt.

### 2.2. Preparation of emulsified meat models

Minced meat was mixed with NaCl in a small-sized Stephan mixer (UMC 5, Stephan Food Service Equipment GmbH, Germany) at 4 °C. ESM powder, which was soaked in distilled water at room temperature overnight, was mixed into the batter. The initial pH of ESM was 9.93 when 0.5 g ESM was soaked in 25 mL distilled water. Then fat was mixed into the minced meat and the temperature was increased to 14–16 °C. In the final mixture, there was 16% fat and 12% protein. 12 different recipes were made with 0.5%, 1.0% and 1.5% NaCl (w/w), respectively. These are lower salt concentrations than those commonly used commercially. For each salt level 0.5%, 1.0%, or 1.5% ESM (w/w) were added, and control samples were made without ESM. To better elucidate the effect of ESM no other additives like starch, spices, or nitrite were used. All recipes were made in duplicate. A total of 24 formulations were made in randomized order. Approximately 40 g (exact sample weights were recorded) of the final mixtures were filled in 50 mL Falcon plastic tubes using individual plastic bags per batch for filling; 9 tubes were made per batch, centrifuged at 2100 rpm for 10 min at 10 °C to remove air bubbles and stored at 4 °C overnight until further analyses. The next day, 8 tubes were cooked in a water bath until a core temperature of 70 °C, cooled in ice water for 30 min, and consecutively stored at 4 °C until further analysis. Proximate composition and *in vitro* analysis were measured on a mixture of the cooked samples (tubes 1–6) made with the same recipe; these samples were stored at –20 °C before the analyses were performed. Cooking loss, pH, texture profile analysis and color measurements were performed on cooked samples (tubes 1–6) in duplicate, whereas histology, LF-NMR  $T_2$  relaxometry and FTIR microspectroscopy were performed on both uncooked (tube 9) and cooked (tube 7) samples.

### 2.3. Proximate analyses and pH

Gross chemical components were analyzed by a commercial laboratory (Eurofins Food & Feed Testing, Lidköping, Sweden). Protein contents were estimated as 6.25 times the nitrogen content determined by Kjeldahl's method (NMKL 6). Moisture content was determined by drying (NMKL 23) and fat content by extraction (NMKL 160 mod). Proximate analyses were performed in duplicate. The results are presented in Supplementary Table S1. pH measurements were performed in triplicate with a glass-stick probe (InLab Solids, Mettler Toledo Intl. Inc., Greifensee, Switzerland) connected to a Beckman pH meter (model PHI 31, Beckman Instruments Inc., Irvine, CA, USA). pH (Table 2) is given as the average of three replicated measurements per formulation.

### 2.4. *In vitro* digestion

*In vitro* digestion was performed according to the INFOGEST model (Brodkorb et al., 2019) with minor adjustments. One gram of sample was blended with 1 mL of simulated saliva fluid (SSF) simulating the oral phase. Amylase was not added because there were no carbohydrates in the samples. Then 2 mL of simulated gastric fluid (SGF) was added resulting in a final pepsin concentration of 2000 U/mL. The pH was adjusted to 3.0 with HCl before incubation in a shaking incubator (37 °C, 250 rpm) for 120 min. Pepsin was inactivated after the gastric phase by raising the pH to 7 using 4 mL of simulated intestinal fluid (SIF) and NaOH. The samples were incubated for another 80 min before the enzymatic digestion was terminated by boiling the tubes in water for 5 min. After inactivation, samples were centrifuged at 4000 rpm for 10 min. The supernatant was used directly for TBARS measurement.

## 2.5. Determination of malondialdehyde (MDA) equivalents

The analysis of TBARS (thiobarbituric acid reactive substances) was performed as described by Steppeler, Haugen, Rødbotten and Kirkhus (2016). The assay is based on the formation of a stable chromophore through the binding of aldehydes like malondialdehyde (MDA) to thiobarbituric acid (TBA) under acidic conditions and heat. To precipitate the proteins and stop the enzymatic activity, trichloroacetic acid (TCA), propyl gallate, and EDTA were added to the samples. MDA equivalents ( $\mu\text{mol}/\text{kg}$  sample) were quantified as the difference in absorbance between 532 and 560 nm (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). TBARS analyses were performed in triplicate.

## 2.6. Cooking loss

After being cooled in ice water for 30 min, the samples were removed from the plastic tubes and liquid release on the surface of the cooked sausage was wiped off using tissue paper before weighing. Sample weights were recorded using the same scale as when sampling the uncooked meat models. Cooking loss (CL) was measured by subtracting post-cooked weight (W1) from the pre-cooked weight of the samples (W2), expressed as a percentage (w/w) of the liquid released per gram of uncooked samples (Han & Bertram, 2017). CL (Table 2) is given as the average of six replicated measurements per formulation (tubes 1–6).

## 2.7. Texture profile analysis

The texture of the samples was determined 2 days after production on cooked samples, using a TA.XTplus (Stable Micro Systems, Godalming, UK) as described by Herrero et al. (2008). Six cores (diameter = 2 cm; height = 1.5 cm) were cut from each sample and were axially compressed by a two-cycle compression test to 60% of their original height using an aluminum 50 mm cylindrical probe. The measurements were performed at room temperature. Force–time deformation curves were recorded with a 25 kg load cell at a crosshead speed of  $1 \text{ mm s}^{-1}$ . Attributes were calculated as follows: hardness (N), peak force required for the first compression; adhesiveness (N) is the negative force area after the first compression; springiness (dimensionless), considered as the distance the sample recovers after the first compression is related to the original height; cohesiveness (dimensionless), as the ratio of positive force peak area of the second and the first compression; and chewiness (N), the product of hardness by cohesiveness and springiness. TPA parameters (Table 2) are given as the average of six replicated measurements per formulation (tubes 1–6).

## 2.8. Color

The surface color was measured using a digital color imaging system (DigiEye, VeriVide., Leicester, UK) equipped with a digital camera (Nikon D7000, 35 mm lens, Nikon, Japan) and a lightbox illuminated by standardized daylight (CIE D65) as described by Aaby, Martinsen, Borge and Roen (2020). For each recipe slices from 6 individual samples were recorded. Color measurements of the pictures were made in the CIE color ( $L^*a^*b^*$ ). Color (Table 2) is given as the average of six replicated measurements per formulation (tubes 1–6).

## 2.9. Histology

Histological analyses were performed by cryo-sectioning according to the method described by Ofstad, Kidman, Myklebust and Hermanson (1993). Samples less than  $0.5 \text{ cm}^3$  were frozen in liquid nitrogen, sectioned in  $5 \mu\text{m}$  slides in a Leica cryostat (CM 3050S, Leica Biosystems, IL, USA), fixed in Bouin's fixative (Polysciences, Warrington, PA, USA) stained with Aniline Blue-Orange G Solution (26,450–04, Electron Microscopy Sciences, Hatfield, PA, USA) in the ratio 1:10 for 4 min and mounted in glycerol. The slides were scanned with Aperio CS 2 slide

scanner (Leica Biosystems, Buffalo Grove, IL, USA) and digital images were taken with ImageScope.64 (Leica Biosystems, Buffalo Grove, IL, USA). For assessment of the fat distribution, sections from each sausage formulation were stained by Nile Red (Merck, Darmstadt, Germany) according to the method described by Ofstad et al. (Ofstad et al., 1995). Nile Red is highly soluble in oils and fluoresces when excited in the range 450–500 nm. The sections were stained at  $4^\circ\text{C}$  overnight, mounted using a pre-cooled glycerol solution, and the slides were kept cold until examination. The slides were examined by using a light microscope (DM6000 B) and the Leica Application Suite X and DMC 4500 (Leica Microsystems, Wetzlar, Germany) were used to snap images. The images were visually inspected.

## 2.10. Low-field NMR

For the proton NMR measurements, 3–3.5 g of uncooked or cooked emulsified meat model was placed in a custom-made Teflon container (16 mm in diameter) with a Teflon screw top and a rubber seal. The samples were equilibrated to  $25^\circ\text{C}$  for 30 min. using a heat block (Dri-block heater DB-3D, Techne, Staffordshire, UK). The transverse relaxation measurements were performed using a 20 MHz benchtop R4-spectrometer supplied by Advanced Magnetic Resonance (AMR, Abingdon, UK). The Carr-Purcell-Meiboom-Gill pulse sequence (CPMG) (Carr & Purcell, 1954; Meiboom & Gill, 1958) was run using an interecho spacing ( $2^*\tau$ ) of  $400 \mu\text{s}$ , 6000 echoes and 4 scans. The relaxation curves were processed fitting a T2 relaxation time distribution with 200 logarithmically distributed axis points from 1 to 10 000 ms (Provencher, 1982). The resulting  $T_2$  relaxation distributions were imported into MATLAB R2020b (MathWorks, Natick, MA, USA) cropped to only include the 8–3000 ms region for the uncooked samples and 1–3000 ms region for the cooked samples and normalized to sum before the multivariate analyses. The uncooked and the cooked emulsified meat models were treated as two separate data sets for the multivariate analyses. Spectra in Fig. 3 are the average of the measurements in duplicated formulations.

## 2.11. FTIR micro-spectroscopy

For FTIR spectroscopic analyses, cryo-sections,  $10 \mu\text{m}$  in thickness, parallel to those used for histological examination were sectioned and mounted on BaF<sub>2</sub> slides. FTIR spectra were acquired with a PerkinElmer Spectrum Spotlight 400 FTIR system (PerkinElmer, Buckinghamshire, UK) using the point function. The size of each point was  $13 \times 41 \mu\text{m}$ . All spectra were collected in transmission mode in the mid-infrared region between  $4000$  and  $750 \text{ cm}^{-1}$  with 32 scans per pixel and a spectral resolution of  $4 \text{ cm}^{-1}$ . Before each spectrum was recorded, a background spectrum of the BaF<sub>2</sub> was obtained. A minimum of ten spectra were obtained in the continuous myofibrillar matrix and six for the muscle fibers. Large variations between the spectra in the protein matrix meant that more spectra were recorded for the matrix than for the muscle fibers. Areas rich in collagen and fat were avoided. Based on visual inspection spectra dominated by spectral signatures related to fat were removed before data analysis. To increase the resolution of the peaks, the second derivative was calculated by using the Savitzky-Golay algorithm with a window size of eleven smoothing points, and thereafter for normalization, extended multiplicative signal correction (EMSC) was applied (Afseth & Kohler, 2012). Spectra for both the myofibrillar matrix and for the muscle fibers were averaged in the range  $1700$ – $1500 \text{ cm}^{-1}$  (amide I / II) and used for presentation, while the total number of 192 spectra, was utilized for the Principal Component Analysis (PCA). The ratio between  $\alpha$ -helical structures and aggregated  $\beta$ -sheets was calculated as the ratio between the area in the range  $1658$ – $1652 \text{ cm}^{-1}$  and the area in the range  $1628$ – $1622 \text{ cm}^{-1}$  according to the method described by Perisic, Afseth, Ofstad, Scheel and Kohler (2013). However, a more restricted range of wavenumbers was used; bands that occur in the area between  $1652$  and  $1658 \text{ cm}^{-1}$  are assigned to  $\alpha$ -helical structures and



non-hydrogenated C = O groups while bands between 1622 and 1628  $\text{cm}^{-1}$  are assigned to C = O groups involved in aggregated  $\beta$ -sheets. In the second derivative spectra, peaks appear negative; to get positive values, the spectra were multiplied by (-1). Since intensity values of some of the bands in the second derivative spectra were positive and thus became negative, 0.1 was added to all the 192 spectra. In this way, all the spectra were converted to positive values to obtain appropriate values of area ratios.

### 2.12. Data analysis

Analysis of variance was performed using GLM models followed by Tukey's pairwise comparison test with either 95% or 99% confidence intervals to calculate differences in mean values of MDA/TBARS, CL, TPA parameters and FTIR micro-spectroscopy peak heights (1658–1652/1628–1622) with NaCl and ESM levels as factors using Minitab software (version 19, Minitab Ltd., Coventry, UK).

Principal component analyzes (PCA) were used to find the underlying causes of the distinction between sample formulations and measured variables. PCA of NMR relaxation times was performed using PLS Toolbox v. 8.9.1 (Eigenvector Research, Manson, WA, USA). PCA of FTIR spectra was analyzed using Unscrambler (version 11.0, CAMO Analytics, Norway) and the in-house routines written in MATLAB (version 6.5, The MathWorks, Natick, MA).

## 3. Results and discussion

### 3.1. In vitro anti-oxidative effect of ESM

To the authors' knowledge, there are no previous studies that have examined the antioxidant effect of ESM in meat mixtures. Although the main purpose of this study was to examine the effect of ESM concerning the techno-functional properties of a meat emulsion, in vitro studies were performed on some selected samples of undigested and digested cooked emulsified meat. Antioxidative effect measured as TBARS values, expressed as MDA equivalents, for emulsified meat models with 0.5 and 1.5% NaCl without and with 0.5 and 1.5% ESM are shown in Table 1. Higher MDA values ( $p < 0.001$ ) were found for the digested samples compared with control samples, without digestion. Addition of ESM reduced the formation of oxidative products measured as MDA equivalents both in undigested and digested emulsified meat models. The effect of ESM was most evident in undigested samples, and the effect increased ( $p < 0.01$ ) with the increased addition of ESM. In the digested samples, ESM had a significant ( $p < 0.05$ ) antioxidant effect in samples with 0.5% NaCl added 1.5% ESM and in samples, with 1.5% NaCl added both 0.5 and 1.5% ESM compared to those without ESM.

### 3.2. pH, cooking loss, texture, and color

To avoid other ingredients affecting pH, cooking loss, texture and color measurements, the model products were made only with the addition of NaCl and ESM. Data for pH, CL, hardness, springiness, redness, and lightness are given in Table 2, and data for all the measured

**Table 1**

MDA equivalents ( $\mu\text{mol}/\text{kg}$  sample) measured in undigested and digested cooked samples of emulsified meat models with 0.5% and 1.5% NaCl without and with 0.5% and 1.5% ESM. Mean values ( $n = 3$ ) and (standard deviation). Different letters indicate significant differences ( $p < 0.05$ ).

NaCl (%)	ESM (%)	Undigested MDA equivalents	Digested MDA equivalents
0.5	0.0	115.6 (7.8) <sup>a</sup>	188.5 (28.5) <sup>b</sup>
0.5	0.5	65.2 (0.1) <sup>b</sup>	186.0 (0.95) <sup>b</sup>
0.5	1.5	25.1 (5.7) <sup>c</sup>	144.5 (9.3) <sup>c</sup>
1.5	0.0	101.6 (9.3) <sup>a</sup>	232.0 (16.3) <sup>a</sup>
1.5	0.5	55.9 (2.1) <sup>b</sup>	181.1 (10.1) <sup>bc</sup>
1.5	1.5	25.3 (1.0) <sup>c</sup>	78.7 (7.2) <sup>d</sup>

TPA and color parameters are in Supplementary materials, Tables S2–S3. The cooked emulsified meat models made with ESM had higher pH ( $p < 0.001$ ) than those prepared without ESM (Table 2). By adding ESM, pH increased from approximately 6.1 to 6.4 for all three NaCl levels. This is probably due to the high pH (9.93) of ESM soaked in distilled water. It is well known that the functional properties of muscle proteins depend both on ionic strength and pH (Sun & Holley, 2011). In meat systems, high pH favors water binding ability and emulsion stability (Chan, Omana & Betti, 2011; Liu et al., 2008b; Young, Zhang, Farouk & Podmore, 2005). By adjusting pH to 6.36 by adding alkaline additives in finely minced meat, both cooking yield and emulsion stability were improved regardless of the initial pH of the meat (Young et al., 2005). Accordingly, in the GLM model for cooking loss both NaCl and ESM and their interaction, were highly significant ( $p < 0.001$ ) factors reducing CL. Emulsified meat models with 0.5% NaCl added 1.0% or 1.5% ESM had the same CL as samples with 1.0% NaCl added 0.5% ESM. The effect of ESM was greatest at 1.5% NaCl, and CL was reduced by 50% when 0.5% ESM was added and further reduced by about 20% when 1.0% or 1.5% ESM was added. Addition of ESM increased pH, which improved some technological properties, but elevated pH may also facilitate growth of undesired bacteria particularly if salt content is decreased. However, ESM has anti-microbiological effects which may suppress bacterial growth (Yoo et al., 2014). Since microbiological recording was not included in the present work no conclusions regarding food safety can be made here.

TPA analysis of hardness and springiness, important quality parameters for emulsified meat products are shown in Table 2. The GLM model showed that both ESM and NaCl and the interaction between these factors increased springiness ( $p < 0.001$ ). Samples with 1.5% NaCl and ESM had the highest springiness. For hardness, there was some effect of NaCl, and the samples with 1.5% NaCl and ESM had lower hardness than those without ESM. These samples also had less CL and thus a higher water content than the other samples (Table 2). Liu et al. (Liu et al., 2008) reported that the gel strength of myosin from pork was reduced as water holding capacity (WHC) increased with increasing pH away from pH (5.5). In another study, it was shown that Turkey meat gels with high pH ( $> 6.1$ ) and the same moisture content had both improved deformation ability and gel springiness compared to those with lower pH (Chan et al., 2011). That the emulsified meat models with 1.5% NaCl and ESM in our study had higher springiness and lower hardness than the other samples, can therefore be explained by the fact that these samples had less CL and thus higher water content. The results as shown in Table 2 thus indicate that the differences in CL and texture properties are mainly due to differences in salt concentration, but also that ESM had some effect probably due to higher pH in samples with ESM.

It has been shown that addition of 0.5–3% collagen in sausages with less salt or fat had significant effects on water holding capacity and textural attributes (Hjelm, Mielby, Gregersen, Eggers & Bertram, 2019; Schmidt et al., 2017). The higher addition of collagen, the greater water holding capacity (WHC), product stability after cooking and therefore texture properties such as hardness and chewiness (Sousa et al., 2017). Addition of 1% pork skin gelatin into myofibrillar gels increased the cooking yield even at low salt concentrations, whereas gel strength was not affected by adding gelatin Lee and Chin, (2019). On the other hand, addition of 0.2% dermatan sulfate had some negative effect on the water-holding capacity of meat products (Day et al., 2009). ESM contains about 9% collagen and about 0.05% glycosaminoglycans (Ahmed et al., 2019; Liu et al., 2014). Since ESM makes up only a small proportion of the total protein in the sausages, it is therefore unlikely that these components have affected the observed functional properties.

The GLM-analysis revealed that both NaCl and ESM affected color. As shown in Table 2, increased NaCl levels decreased ( $p < 0.001$ ) brightness ( $L^*$ ) and increased ( $p < 0.001$ ) redness ( $a^*$ ). Decreased brightness is consistent with previous findings that high water content leads to lower  $L^*$  in emulsified meat products (Gimeno, Ansorena, Astiasaran & Bello, 2000). This is probably due to light scattering effects due to the higher

**Table 2**

pH, Cooking loss, TPA parameters (hardness and springiness), and color (brightness (L<sup>\*</sup>) and redness (a<sup>\*</sup>) measured on cooked emulsified meat models. Mean values (n = 2) and (standard deviation). Different letters indicate significant differences (p < 0.05).

NaCl (%)	ESM (%)	pH	CL (%)	Hardness (N)	Springiness (N)	L <sup>*</sup>	a <sup>*</sup>
0.5	0.0	6.07 (0.01) <sup>f</sup>	17.3 (0.4) <sup>a</sup>	5611 (640) <sup>abc</sup>	52.8 (2.3) <sup>b</sup>	67.4 (0.8) <sup>abc</sup>	7.4 (0.4) <sup>d</sup>
0.5	0.5	6.31 (0.02) <sup>cde</sup>	15.6 (0.2) <sup>ab</sup>	6528 (645) <sup>a</sup>	53.6 (2.0) <sup>b</sup>	68.6 (0.7) <sup>a</sup>	8.8 (0.7) <sup>bcd</sup>
0.5	1.0	6.36 (0.01) <sup>abc</sup>	14.6 (0.4) <sup>bc</sup>	5689 (800) <sup>abc</sup>	52.7 (2.0) <sup>b</sup>	67.2 (0.5) <sup>abc</sup>	8.6 (0.2) <sup>bcd</sup>
0.5	1.5	6.40 (0.02) <sup>a</sup>	14.1 (0.3) <sup>bcd</sup>	6136 (863) <sup>ab</sup>	53.9 (2.0) <sup>b</sup>	68.2 (0.9) <sup>ab</sup>	9.4 (0.4) <sup>abc</sup>
1.0	0.0	6.06 (0.02) <sup>f</sup>	15.2 (0.6) <sup>b</sup>	6078 (401) <sup>ab</sup>	51.3 (2.8) <sup>b</sup>	63.6 (0.6) <sup>d</sup>	8.0 (0.3) <sup>cd</sup>
1.0	0.5	6.28 (0.02) <sup>de</sup>	14.3 (0.5) <sup>bcd</sup>	6286 (587) <sup>ab</sup>	53.4 (2.2) <sup>b</sup>	65.3 (1.4) <sup>bcd</sup>	8.8 (0.4) <sup>bcd</sup>
1.0	1.0	6.35 (0.02) <sup>abcd</sup>	12.4 (0.4) <sup>de</sup>	6122 (1008) <sup>ab</sup>	53.9 (2.2) <sup>b</sup>	64.9 (0.9) <sup>cd</sup>	9.5 (0.3) <sup>abc</sup>
1.0	1.5	6.38 (0.01) <sup>ab</sup>	11.4 (0.3) <sup>e</sup>	5846 (563) <sup>ab</sup>	53.5 (1.9) <sup>b</sup>	64.6 (1.0) <sup>cd</sup>	9.8 (0.5) <sup>ab</sup>
1.5	0.0	6.03 (0.03) <sup>f</sup>	13.0 (0.5) <sup>cde</sup>	6725 (544) <sup>a</sup>	52.9 (2.4) <sup>b</sup>	62.3 (0.4) <sup>de</sup>	8.3 (0.2) <sup>bcd</sup>
1.5	0.5	6.27 (0.01) <sup>e</sup>	7.0 (0.6) <sup>f</sup>	4469 (539) <sup>c</sup>	61.1 (2.1) <sup>a</sup>	60.3 (1.0) <sup>e</sup>	9.8 (0.4) <sup>ab</sup>
1.5	1.0	6.29 (0.01) <sup>de</sup>	5.4 (0.2) <sup>f</sup>	4980 (422) <sup>bc</sup>	61.2 (1.8) <sup>a</sup>	62.3 (0.6) <sup>de</sup>	10.4 (0.4) <sup>a</sup>
1.5	1.5	6.33 (0.01) <sup>bcd</sup>	5.4 (0.6) <sup>f</sup>	5138 (400) <sup>bc</sup>	61.2 (1.2) <sup>a</sup>	62.4 (0.6) <sup>de</sup>	10.8 (0.7) <sup>a</sup>

water content and pH-induced structural differences (Hughes, Oiseth, Purslow & Warner, 2014). Young et al. (2005) reported only a subtle effect on the red color of pH-adjusted minced meat. Whereas addition of 1.5% ESM increased redness (a<sup>\*</sup>) by approximately 25% in cooked samples compared to those without ESM (Table 2). As shown in Table 1, ESM has an antioxidant effect which may be related to increased redness in the cooked samples. Sebranek, Sewalt, Robbins and Houser (2005) reported that the addition of natural antioxidants from rosemary led to increased redness in sausages by preventing oxidation of myoglobin.

The results shown in Table 2 indicate that the differences in CL, texture properties and color are mainly due to differences in salt concentration, but also that ESM had an effect probably due to higher pH in the emulsified meat models with ESM. The effect of ESM was greatest in samples with 1.5% NaCl and most pronounced on the CL.

### 3.3. Histology

Both texture and water holding properties depend on the microstructure and degree of protein aggregation. To examine the effect of ESM on the protein matrix, microstructural changes were monitored by light microscopy on those samples that had the largest differences in CL and springiness (Table 2). Examples of micrographs of uncooked and cooked samples with 0.5, 1.0 and 1.5% NaCl without and with 1.5% ESM are shown in Figs. 1 and 2. The microstructure of the emulsified meat models is like that previously described for emulsified and/or finely ground meat and fish products (Hermansson, 1987; Ofstad et al., 1995; Ofstad, Kidman, Myklebust, Olsen & Hermansson, 1996). Emulsified meat consists of a continuous matrix that encloses intact and partially dissolved muscle fibers, pieces of connective tissue in uncooked samples (Fig. 1A), and in cooked samples gelatin dissolved in the water phase (Fig. 1B). In samples prepared with 1.5% ESM, fragments of ESM, seen as dark-blue spots, are scattered throughout the yellow protein matrix. Parallel sections to Fig. 1A stained with Nile red (Fig. 2) disclose the high density of fat globules in the protein matrix. Water holding properties of minced products depends on their microstructure; gels with coarse, aggregated network structures and big pores hold the water less firmly than gels with fine and continuous matrices (Hermansson, 1986, 1994; Herrero et al., 2005; Ofstad et al., 1995). Accordingly, the most striking differences between the micrographs in Figs. 1 and 2 are the shape, size, and distribution of the liquid- or fat-filled pores in the protein matrix.

Compared to the other samples, samples made with 1.5% NaCl and 1.5% ESM with the least CL and most springiness, have smaller and more spherical pores that are more evenly distributed in the protein matrix than in the other samples (Figs. 1 and 2). The pores consisting of fat or liquid become increasingly larger and more angular with lower NaCl concentrations, these following previous findings for emulsified fish muscle (Ofstad et al., 1995). The larger pores with irregular shape

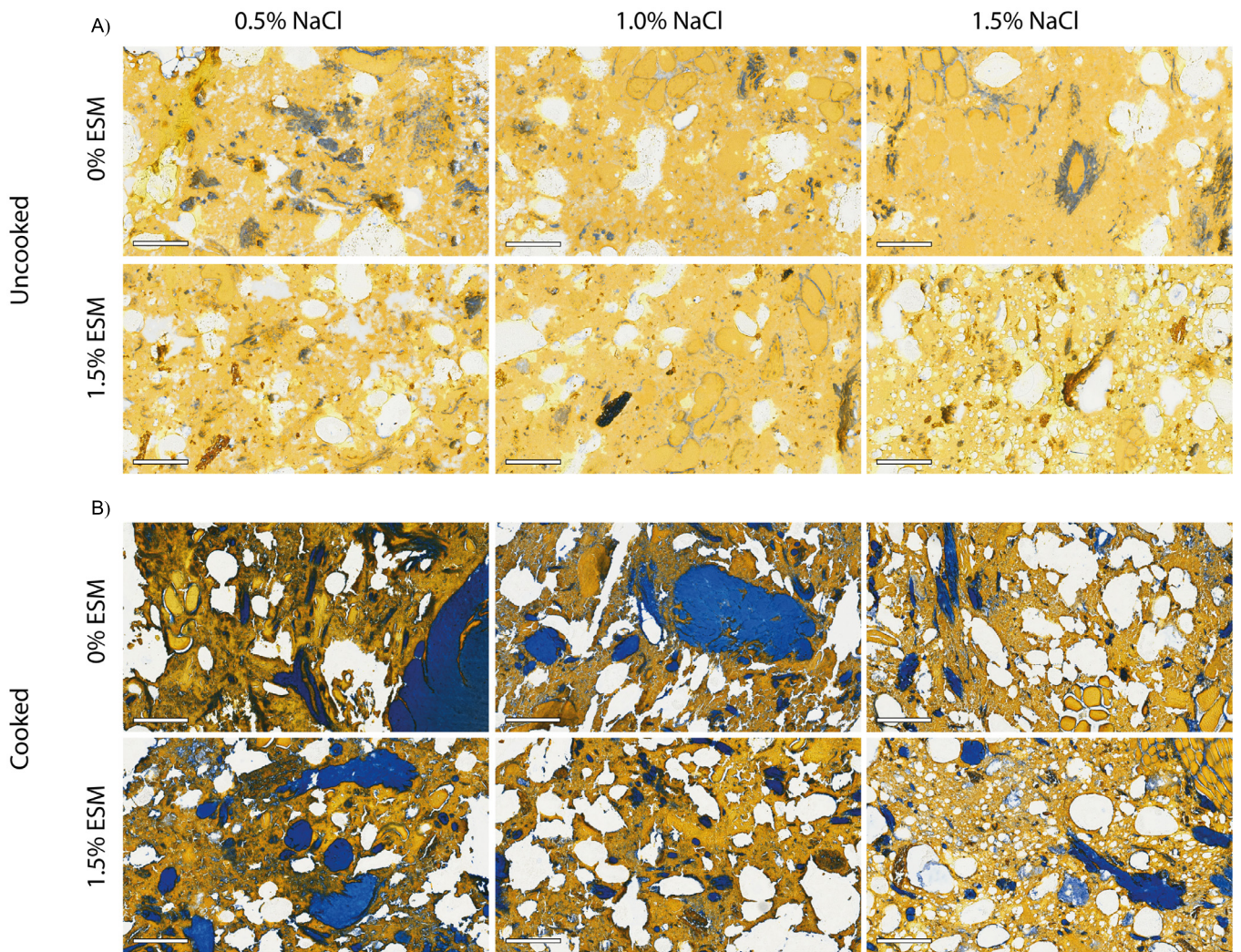
may be due to the myofibrillar protein being insufficiently solubilized, the fat becoming less emulsified, and/or the protein matrix becoming more aggregated during heating. This may indicate that ESM also affects the microstructure formed by the myofibrillar proteins solubilized with the aid of salt.

### 3.4. Low-field NMR

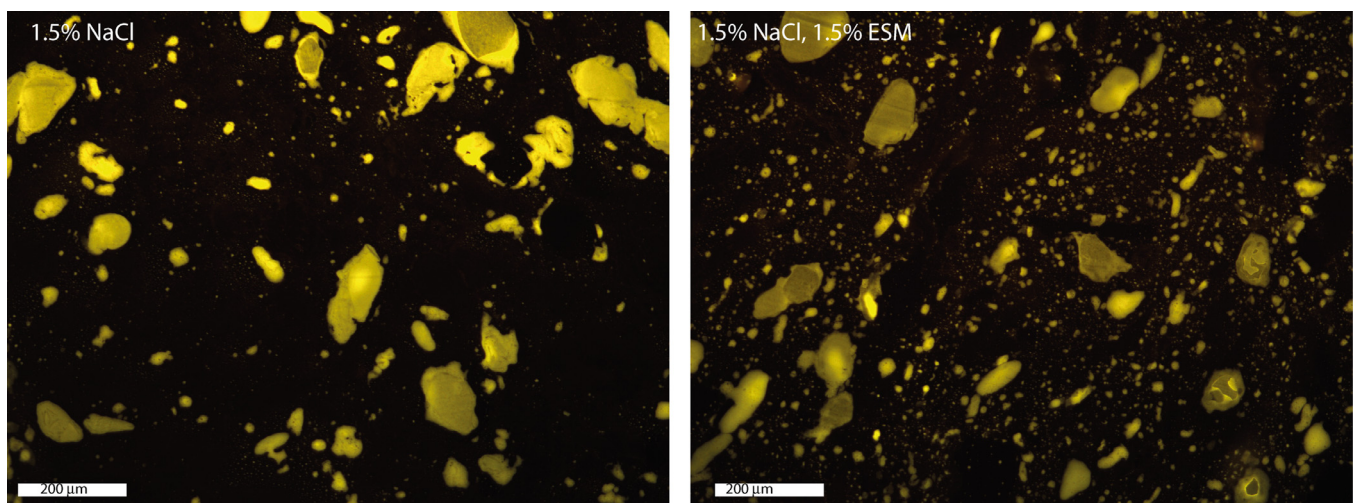
To elucidate the effect of ESM addition to a meat emulsion model intrinsic water distribution and water-ingredient interactions were studied by low-field NMR relaxometry. Low-field NMR T<sub>2</sub> relaxation times were measured on uncooked (Fig. 3, left) and cooked (Fig. 3, right) emulsified meat models. The NMR signal decay could be fitted into two or three separate peaks. In uncooked samples, the two water populations were centered around 1–10 ms (T<sub>2B</sub>) and around 30–200 ms (T<sub>21</sub>), and in cooked samples, the three water populations were centered around 1–8 ms (T<sub>2B</sub>), 10–300 ms (T<sub>21</sub>) and 250–1300 ms (T<sub>22</sub>). For gel systems these T<sub>2</sub> populations have been assigned to bound water (T<sub>2B</sub>) associated with macromolecules, immobilized water (T<sub>21</sub>) located within highly organized protein structures and unbound or free water (T<sub>22</sub>), respectively (Bertram et al., 2001; Han et al., 2014; Ruan et al., 1999). That the T<sub>22</sub> peak is quite small is probably because free water was centrifuged off before the T<sub>2</sub> relaxation times were measured. When heated to 70 °C, the T<sub>21</sub> relaxation time shifted to lower values and the distribution became broader (Fig. 3, right). The shorter T<sub>21</sub> relaxation time reflects a smaller size of the internal structures due to denaturation and aggregation of myofibrillar proteins entrapping water, in addition to limited water mobility in the gel system formed during heating (Bertram et al., 2006). A broader relaxation T<sub>21</sub> top reflects inhomogeneity in the gel microstructure and/or different physical environments causing a greater variation in the environment of the water protons (Salomonsen, Sejersen, Viereck, Ipsen & Engelsen, 2007). Broadening of the T<sub>21</sub> top (Fig. 3, right) is thus in accordance with the difference in microstructure among the sausage samples shown in Fig. 1. In heated Frankfurter type sausages added collagen an additional T<sub>2</sub> population characterized by a relaxation time in the region between 150 and 400 ms appeared as a shoulder on the myofibrillar water population, T<sub>21</sub> (Hjelm et al., 2019). A similar shoulder could not be seen for any of the samples in Fig 3.

Due to the small differences in relaxation decay between the samples, PCA was used to reveal if ESM affected the relaxation decay. PCA of the distributed T<sub>2</sub> relaxation data are shown in Fig. 4 and 5. Due to significant differences in the T<sub>2</sub> relaxation time distribution between uncooked and cooked samples, PCA was performed on uncooked and cooked samples separately. The score plot in Fig. 4 (left) of the uncooked samples revealed that the first PC, explaining 86.2% of the variations, is mainly related to the effect of ESM. The corresponding loading plot of PC1 (Fig. 4, right) shows that increased ESM addition reduces the T<sub>2</sub> relaxation time indicating that the water is increasingly immo-





**Fig. 1.** Cryo-sections of uncooked (A) and cooked emulsified meat models (B) comminuted with 0.5% NaCl, 1.0% NaCl, without or with 1.5% ESM. The muscle proteins are yellow, and collagen is blue. The scale bar represents 200  $\mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Cryo-sections of uncooked emulsified meat models comminuted with 1.5% NaCl without or with 1.5% ESM. Fat is stained yellow, and protein is unstained. The scale bar represents 200  $\mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



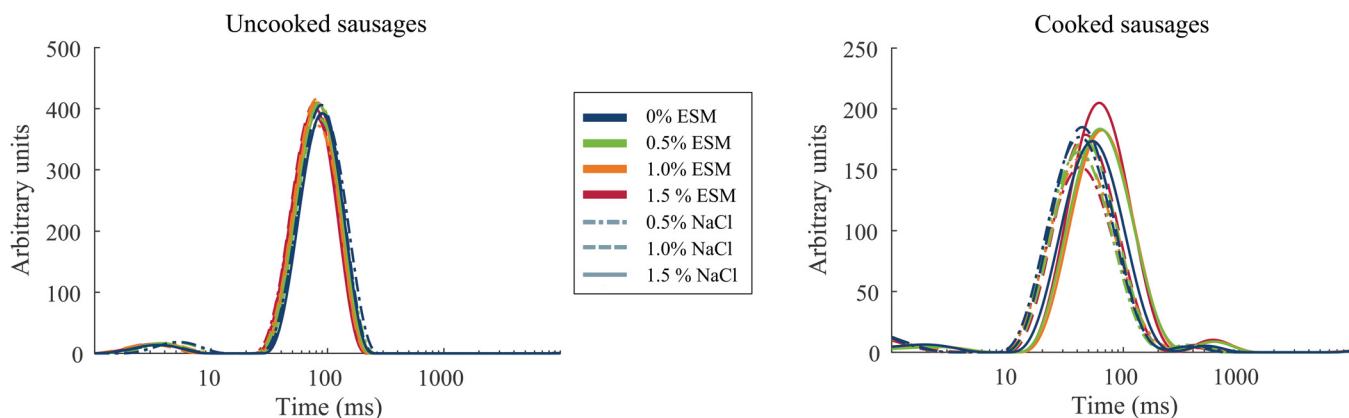


Fig. 3. Distribution of  $T_2$  relaxation times of uncooked (left) and cooked emulsified meat models (right) with three levels of salt (0.5, 1.0, 1.5%) without ESM and with three levels of ESM (0.5, 1.0, 1.5%) added. Each curve represents an average of the duplicated batches ( $n = 2$ ).

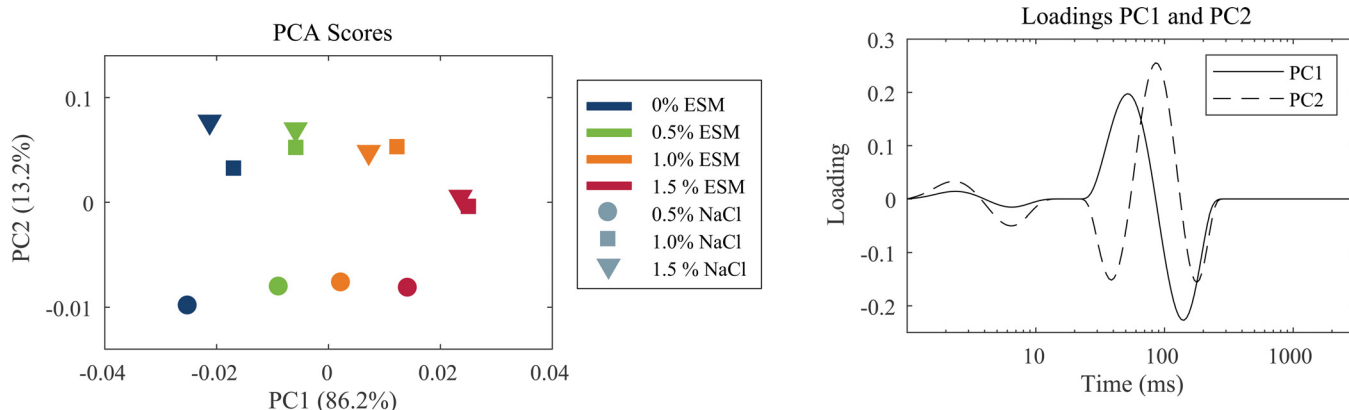


Fig. 4. Principal component analysis (PCA) of the low-field NMR  $T_2$  relaxation decay data of uncooked emulsified meat models. Score plot for PC1 vs. PC2 (left) and corresponding loading plots (right). The explained variance by PC1 and PC2 is 86.2% and 13.2%, respectively.

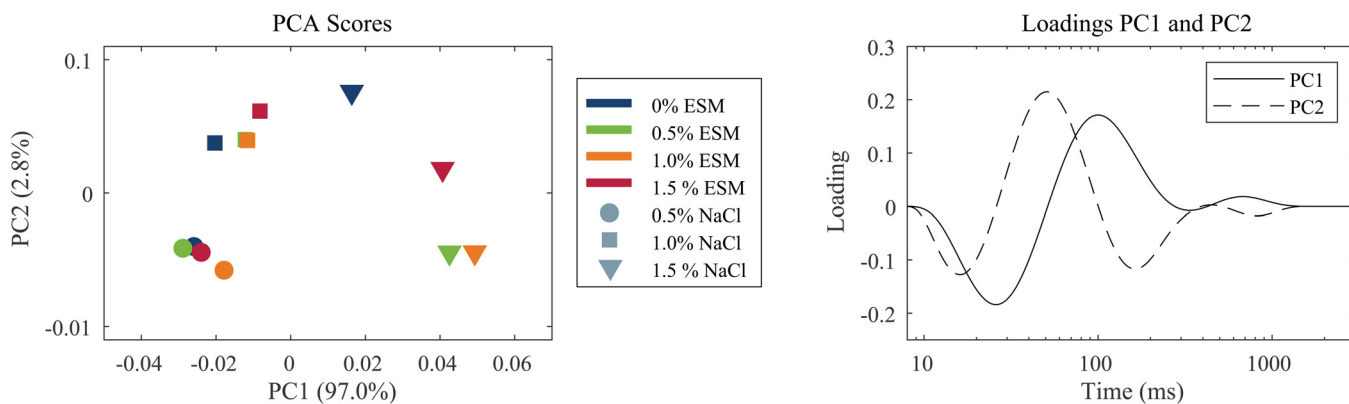
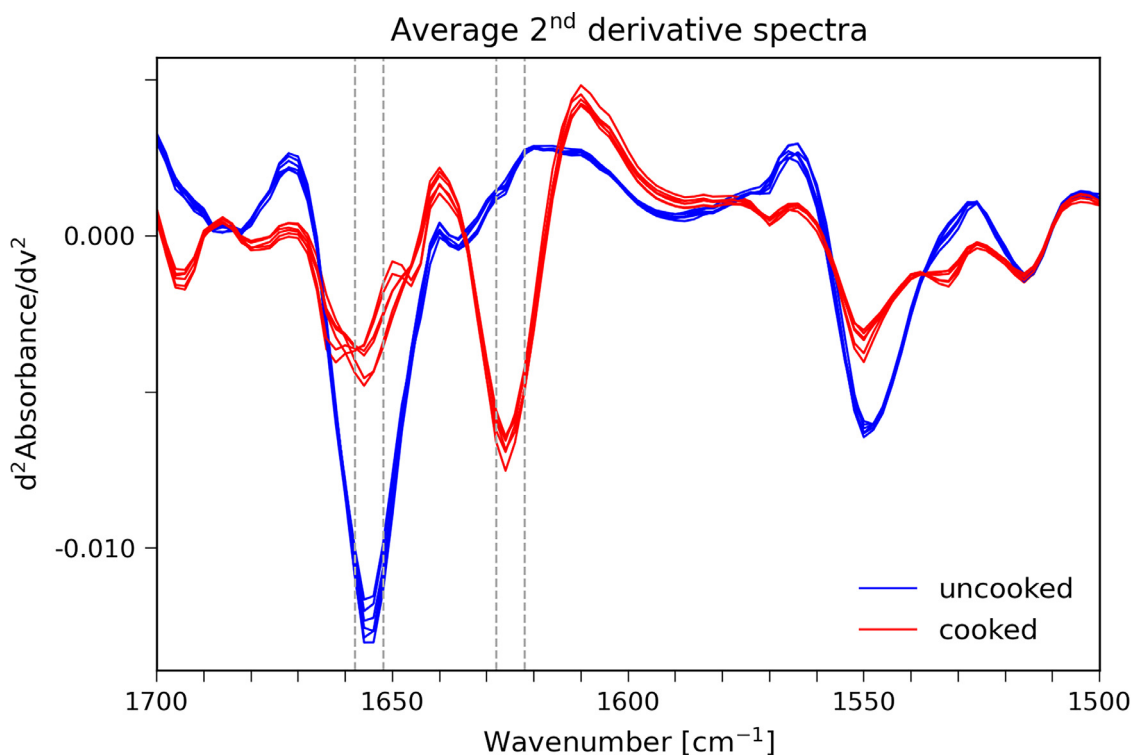


Fig. 5. Principal component analysis (PCA) of the low-field NMR  $T_2$  relaxation decay data of cooked emulsified meat models. Score plot for PC1 vs. PC2 (left) and corresponding loading plots (right). The explained variance by PC1 and PC2 is 97% and 2.8%, respectively.

bilized. Since  $T_{21}$  is the largest peak most of the variation is due to changes in this peak. A shorter  $T_{21}$  relaxation time reflects a smaller size of the intrinsic structures in which both myofibrillar and added water are entrapped (Bertram, Purslow & Andersen, 2002). The influence of NaCl was reflected in the second PC, explaining 13.2% of the variation in the  $T_2$  relaxation times (Fig. 4, left). The variation is mainly due the differences between samples with 0.5% NaCl and those with 1.0% and 1.5% NaCl. The loading plot of PC2 (Fig. 4, right) shows that the higher salt concentrations result in narrower water peaks, indicating that the water was more homogeneously distributed in the protein structure (Salomonsen et al., 2007). The lower  $T_{21}$  relaxation time and the

narrower peak in the emulsified meat models with 1.5% ESM and 1.5% NaCl agreed with the finer and more homogeneous microstructure in these samples (Fig. 1). Smaller pores and a more homogeneous myofibrillar network structure inhibit the movement of water molecules which thus exchange the spin energy with surrounding water molecules faster (Han et al., 2014; Salomonsen et al., 2007). Since no water population related to collagen was visible in Fig 3, the effect of ESM on the  $T_2$  relaxation decay is most likely related to the pH effect of ESM causing a homogeneous microstructure.

The score plot of the cooked samples is shown in Fig. 5 (left). PC1 explains 97.0% of the variation which is mainly affected by the NaCl



**Fig. 6.** Averaged second derivative EMSC-corrected FTIR-spectra ( $n = 192$ ) obtained from both single muscle fibers ( $n = 6$ ) and myofibrillar protein matrix ( $n = 10$ ) of uncooked and cooked emulsified meat models prepared with 0.5, 1.0 and 1.5% NaCl without and with 1.5% ESM added. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration. The loading plot of PC1 (Fig. 5, right) revealed that the relaxation time increased with higher NaCl concentrations. PC2, explaining 2.7% of the variations is related to the narrowing of the  $T_{21}$  peak. Relaxation times measured at the peak of the  $T_{21}$  water population ( $T_{21max}$ ) were 42 ms, 42 ms and 54 ms for 0.5%, 1.0% and 1.5% NaCl, respectively. Samples with 1.5% ESM had slightly higher  $T_{21max}$  than samples with the same salt level without ESM, 44 ms, 47 ms and 62 ms at 0.5%, 1.0% and 1.5% NaCl, respectively. The effect of ESM was most pronounced in samples with 1.5% NaCl and 1.5% ESM. These findings are in accordance with the more homogeneous and less aggregated microstructure of cooked emulsified meat models with increased salt content and ESM as shown in Fig. 1. The  $T_{21}$  relaxation time reflects to which extent water molecules interact with the highly organized protein structures in the myofibrillar matrix. The increased  $T_{21}$  relaxation time in the cooked samples with ESM may indicate an effect of ESM also on the heat-induced gel-formation of the salt solubilized myofibrillar proteins. Previously, it has been shown that both the addition of 1% gelatin and 0.2% dermatan sulfate had some effect on the water-holding capacity of meat products (Day et al., 2009; Lee & Chin, 2019). ESM contains about 9% collagen and about 0.05% glycosaminoglycans (Ahmed et al., 2019; Liu et al., 2014). Since ESM makes up only a small proportion (<10%) of the total protein in the emulsified meat models, it is unlikely that these components have affected the observed  $T_2$  relaxation times.

### 3.5. FTIR micro-spectroscopy

In purified pork myosin solutions unfolding of  $\alpha$ -helices and the formation of  $\beta$ -sheets played a significant role in gel formation (Liu et al., 2008). FTIR analyses were thus performed on the same six emulsified meat samples as used for histological examination, formulations without and with 1.5% ESM for all three levels of NaCl and of both uncooked and cooked emulsified meat models, in total 12 samples. Due to their high sensitivity to protein secondary structure, the amide I and II bands (1700–1500  $\text{cm}^{-1}$ ) are often used to study protein folding, un-

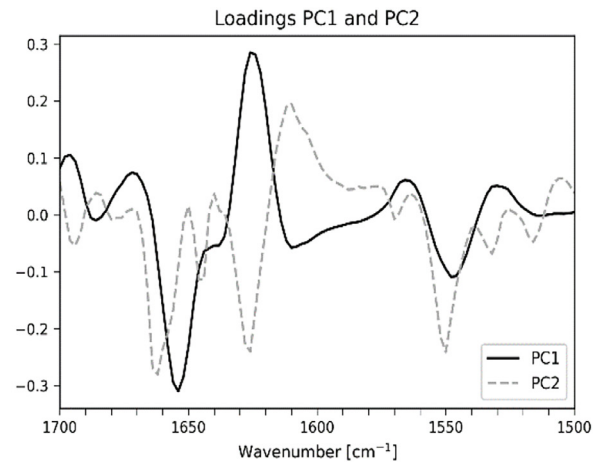
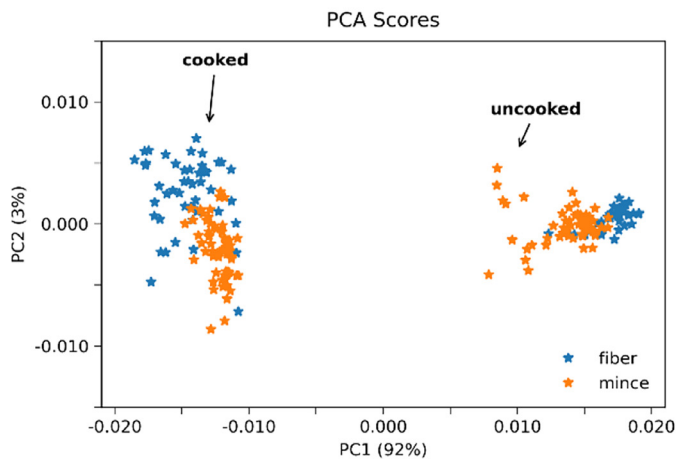
folding, and aggregation. Fig. 6 shows the average of the 2. derivative FTIR spectra in this region obtained from both the muscle fibers and the myofibrillar protein matrix of uncooked and cooked samples. The band between 1658  $\text{cm}^{-1}$  and 1652  $\text{cm}^{-1}$  decreased in magnitude upon heating for all samples, implying a loss of the native  $\alpha$ -helical structure of the myofibrillar proteins. This band has previously been assigned to C = O stretching vibrations of native  $\alpha$ -helical structure in pork muscle myofibrillar proteins (Bertram et al., 2006; Böcker et al., 2006). The bands at 1628 and 1622  $\text{cm}^{-1}$  are most pronounced in the cooked samples and are related to denatured and aggregated myofibrillar proteins having a larger amount of aggregated  $\beta$ -sheet structures (Bertram et al., 2006; Böcker et al., 2006).

To reveal structural differences among the different emulsified meat formulations, all the spectra were compared using PCA. The score plot (left) and the loading plot (right) of the first and second principal components of the PCA of the amide I and II region from 1700 to 1500  $\text{cm}^{-1}$  are shown in Fig. 7. The score plot proved that the samples were clustered according to process (uncooked vs cooked); PC1 explained 92% of the variation.

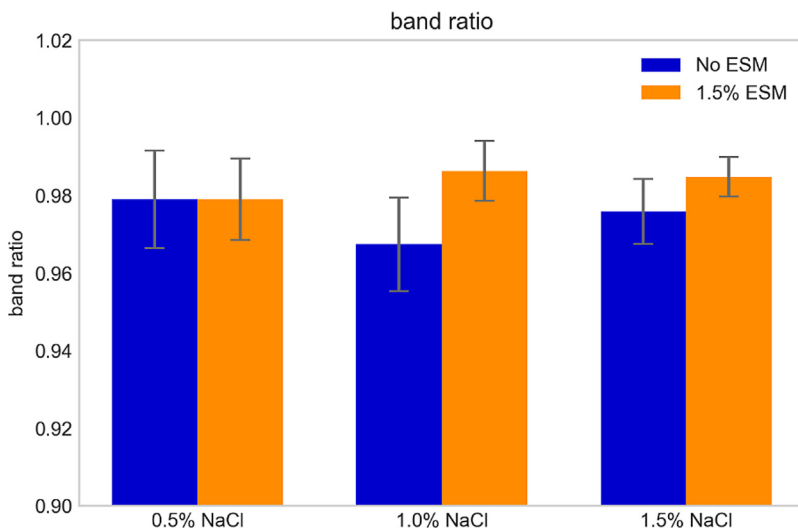
The corresponding loading plot confirmed that the difference between uncooked and cooked samples was mainly related to the bands at about 1655  $\text{cm}^{-1}$  and about 1624  $\text{cm}^{-1}$ . PC2, which explained 3% of the variation, was mainly affected by the structural differences between the myofibrillar proteins that make up the muscle fibers and those that are solubilized by NaCl and make up the myofibrillar matrix. The difference was most pronounced in the cooked samples (Fig. 7, left). The loading plot for PC2 indicates that the cooked fibers are positively correlated to the bands at about 1624  $\text{cm}^{-1}$  representing aggregated  $\beta$ -sheet structures. This can mean that the myofibrillar proteins of the fibers are more aggregated than the salt-solubilized proteins.

In a previous study of emulsified meat, prepared without starch and spices, the ratio between aggregated  $\beta$ -sheet structures and  $\alpha$ -helical structure varied with the type and amount of salt used in the recipe (Perisic et al., 2013). To find possible structural differences caused by





**Fig. 7.** PCA score and loading plot of second derivative EMSC-corrected FTIR-spectra ( $n = 192$ ) of uncooked and cooked emulsified meat models prepared with 0.5, 1.0 and 1.5% NaCl without and with 1.5% ESM added, using the IR spectra in the region of  $1700 - 1500 \text{ cm}^{-1}$  as variables. The explained variance by PC1 and PC2 is 92% and 3%, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 8.** Average band ratios ( $1658-1652/1628-1622$ ) of the second derivative EMSC-corrected FTIR-spectra ( $n = 16$ ) obtained from both single muscle fibers ( $n = 6$ ) and myofibrillar protein matrix ( $n = 10$ ) of cooked emulsified meat models prepared with 0.5, 1.0 and 1.5% NaCl without and with 1.5% ESM added. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different NaCl and ESM additions, the ratio of the bands in the range  $1658-1652 \text{ cm}^{-1}$  and  $1628-1622 \text{ cm}^{-1}$ , representing  $\alpha$ -helical and aggregated  $\beta$ -sheet structures, respectively, were calculated from FTIR spectra as described above. In this way, it was possible to determine whether there were differences between the samples concerning the proportion of helical structures versus aggregated protein structures. The average band ratios ( $1658-1652/1628-1622$ ) of the cooked emulsified meat models are shown in Fig. 8. Pairwise comparison of the band ratios showed that cooked samples added 1.5% ESM had a significant ( $p < 0.05$ ) larger mean ratio ( $0.98 \pm 0.009$ ) than those without ESM ( $0.97 \pm 0.01$ ). The effect of ESM was merely related to a reduced amount of protein aggregation in samples with 1.0 and 1.5% NaCl (Fig. 8). The reduced CL of samples added ESM having a higher portion of  $\alpha$ -helical structures agrees with Choi and Ma (2007) who suggested that hydration strength of  $\beta$ -sheets is weaker than that of  $\alpha$ -helices. In the uncooked samples, there were no differences between samples without and with 1.5% ESM added. These results indicate that ESM influenced the secondary structure of the salt solubilized myofibrillar proteins forming the matrix structure of the emulsified meat models and that these proteins were less aggregated during heating. This may be due to a higher pH in emulsified meat models with added ESM, increasing negative repulsion forces between the negatively charged myofibrillar proteins and thus promoting a heat-stabilizing effect on the protein network (Liu et al., 2008). This finding is contrasting to a previous study (Bertram et al., 2006). In that

study, there was no significant effect on FTIR spectra in the region  $1700-1600 \text{ cm}^{-1}$  measured on heated pork meat with pH between 5.4 and 6.5. This discrepancy may be related to differences between the heat-induced denaturation effect on proteins in intact myofibers versus salt-solubilized matrix proteins. PCA (Fig. 7) revealed that there were some differences between FTIR spectra obtained in muscle fibers and matrix. The mean value of the ratio ( $1658-1652/1628-1622$ ) was  $0.97 \pm 0.01$  and  $0.985 \pm 0.005$  for fibers and matrix, respectively, indicating that the fibers became more aggregated by heat denaturation than the matrix.

#### 4. Conclusion

The present study shows that emulsified meat models with ESM had less CL and a more elastic texture than those without ESM. The effect of 1.5% ESM on water binding, texture, and color was most evident in emulsified meat models added 1.5% NaCl, but there was also a noticeable effect in samples with 1.0% NaCl. In agreement with this, visual inspection of histological images of cryo-sectioned sausages revealed that the structure of the samples with 1.5% NaCl and 1.5% ESM had a fine and continuous matrix with small and spherical fat or liquid-filled pores which was evenly distributed. Moreover, ESM reduced the  $T_2$  relaxation time in uncooked samples, indicating that the water was increasingly immobilized. The reduced  $T_{21}$  relaxation time is consistent with the more homogeneous organized microstructure of the ESM samples and

thus the reduction in CL shown by adding ESM. The higher pH of these samples may aid the NaCl-induced solubilization of the myofibrillar proteins during grounding as well as stabilizing the network structure due to increased negative repulsion forces between the myofibrillar proteins, increasing the surface available for hydration. FTIR spectra, measuring changes in the protein secondary structures, revealed that the cooked samples with ESM had a higher portion of  $\alpha$ -helical versus aggregated  $\beta$ -sheet structures than those added 1.0 and 1.5% NaCl without ESM. It was suggested that higher pH and increased negative repulsion effects had a positive heat stabilizing effect preventing aggregation of the protein network. The effect of ESM on water binding and texture was related to increased pH, while increased redness was related to the fact that ESM also had an antioxidant effect. Addition of ESM increases pH in the emulsion, reduces cooking loss and thus improves texture properties such as hardness and springiness in emulsified meat products with reduced salt.

### Supplementary Materials

The following are available online, Table S1: Proximate analyses Table S2: TPA values; two replicates were made on cooked sausages per recipe. Table S2. Color measured with DigiEye; two replicates were made on cooked sausages per recipe.

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### Ethical statement

This article does not require IRB/IACUC approval because there are no human and animal participants.

### Data availability statement

Available on request due to restrictions.

### Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

### Author contributions

Conceptualization: R.R., M.E.P. and R.O., methodology: R.R.; U.B., R.O., V.H.; K.W.S. software, R.R.; U.B., K.D., and R.O., validation, U.B.; M.E.P. and R.R., formal analysis, R.R., V.H., K.W.S., K.D., and R.O., investigation, R.R., U.B. and R.O., resources, M.E.P., data curation, R.R. and R.O. writing original draft preparation, R.R., writing, review and editing, R.R.; U.B., M.E.P. and R.O., visualization, U.B., V.H., and K.D. supervision, U.B., M.E.P.; R.O., project administration, M.E.P., Funding acquisition, M.E.P. All authors have read and agreed to the published version of the manuscript.

### Conflicts of interest

The authors declare no conflict of interest with respect to this manuscript. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.afres.2022.100151](https://doi.org/10.1016/j.afres.2022.100151).

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