EFFECTS OF SALT SUBSTITUTES ON PROTEIN STRUCTURE IN MUSCLE FOODS STUDIED BY FTIR MICROSPECTROSCOPY

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***Abstract*— Since high sodium intake has been linked to detrimental health effects, there is a necessity to decrease the content of sodium chloride (NaCl) in foods. One approach is to partially replace it by inorganic substitutes. Therefore, the effect of alternative salts to food components needs to be determined. As Fourier transform infrared (FTIR) microscopy is proven to be a powerful tool for determining the changes in protein secondary structure, it has been applied to determine the effect of salt substitutes on beef meat proteins. In the current study three different salts (NaCl, KCl and $MgSO_4$) in three different concentrations (1.5, 6 and 9 %) have been used for salting beef meat (*longissimus dorsi* muscle). Samples were taken from four different animals in order to reduce the effect of animal variation. The effect of salt combinations was also studied. By the use of FTIR microscopy it has been revealed that the influence of KCl to the protein β-structures is distinguishable from the effects of NaCl and $MgSO_4$.**

Index Terms— FTIR microscopy, protein structure, salt substitutes

I. INTRODUCTION

Excessive intake of dietary sodium has been linked to increased risk of hypertension and consequently cardiovascular disease. Since the current daily intake in developed countries is approximately threefold the recommended daily allowance (with processed meat products as a major source of sodium), public health and regulatory authorities have prompted a reduction in dietary intake of salt (NaCl) [1-3]. On the other hand, salt imparts a large number of functional properties in meat products: it increases water-holding capacity and hydration through activation of proteins, it improves the texture by increasing the binding properties of proteins, prolongs the shelf life through bacteriostatic effects, and has an essential contribution to the taste [2]. These properties, together with the fact that salt is one of the most affordable food ingredients available, constitue the most important barriers against the desired salt reduction. Even so, there are currently three different approaches in reducing salt to the optimal level in processed foods: (1) utilisation of salt substitutes, as probably the most common method, (2) addition of salty taste enhancers and (3) optimisation of physical form of salt in order to increase its bioavailability [2]. Product development and studies regarding salt substitution thus need to address all the technological, structural and sensorial effects it may have.

Since the approach of substituting a part of the NaCl content in foods with alternative salts is the most common one, there have been various studies reported, particularly regarding taste differences this may cause [4,5]. However, the effects of these salts on texture and protein structure have not been studied extensively. Vibrational spectroscopic techniques have proven to be excellent tools for studying protein secondary structure in the processing of meat muscle [6,7].

Fourier transform infrared (FTIR) spectroscopy is highly sensitive to the chemical composition and structure of molecules, and because of this feature it is widely used for the investigation of biological systems. It is particularly powerful for exploring protein folding, unfolding and misfolding [8-10]. FTIR microscopy is a technique that combines light microscopy with FTIR spectroscopy, making it possible to obtain information on minute domains commonly present in biological samples. This has been applied in meat science to study denaturation processes in myofibrillar and connective tissue proteins in beef [6] and the changes in myofibrillar protein structure of pork as affected by heating, salting and ageing [7]. These studies have revealed an increase in aggregated β-sheets and a decrease in α-helical structures upon heating, which have been observed much more pronounced for the myofibers than for the connective tissue using FTIR-imaging. Salting induced an increase in native β-sheet and a decrease in α-helical structures.

In the present study, FTIR microscopy has been applied to assess the protein structural changes in beef muscle treated with NaCl and two salt substitutes, namely KCl and $MgSO_4$, in different concentrations. KCl and $MgSO_4$ are present in some of the commercially available salt substitutes that are currently used to some extent in foods [1]. The effect of salt combinations is also studied. The aim of this study is to compare the effect of NaCl on meat protein structure to the effects of these two substitutes.

II. MATERIALS AND METHODS

Meat and sample preparation. Samples of beef muscle *longissimus dorsi* were taken from four different animals after 48 hours post rigour. Muscle blocks of approximately 4×4×1 cm in size were excised and placed in pure salt brines of NaCl, KCl and $MgSO_4$ including the 1/2-1/2 and 1/3-1/3-1/3 combinations in 1.5 %, 6 % and 9 % total salt concentration (21 different brines in total). The samples were kept in brines at 4 ºC for 48 hours with 0.05 % NaN_3 added in order to prevent any possible deterioration caused by microbial growth.

FTIR measurements. Meat samples were sectioned, frozen in liquid nitrogen and cryo-sectioned at -25 ºC with a thickness of 10 μm and finally placed on ZnSe plates for the FTIR microscopy measurements. The spectroscopic analysis was performed on Bruker Optics IRscope II IR microscope connected to an Equinox 55 FTIR spectrometer in the range of 4000-1000 cm^{-1} with a spectral resolution of 4 cm^{-1} using an MCT detector. The spectra were taken on single muscle myofibers on different sections, in total 30 spectra per one animal treated with one brine.

Data analysis. Second derivative of the spectra was taken in order to resolve the overlapping bands in the Amide I protein region (1700-1600 cm^{-1}). Afterwards, the spectra were pre-processed using extended multiplicative signal correction (EMSC), which allows the separation of physical light-scattering effects from chemical information contained in the spectra. Pre-processing and data analysis were performed using in-house developed routines written in MATLAB (version 7.10 The MathWorks, Natick, MA) and The Unscrambler (version 9.2 CAMO Process AS).

III. RESULTS AND DISCUSSION

A typical example of an FTIR spectrum collected from a single myofiber from beef muscle is shown in Figure 1a. Five of the nine amide infrared modes typically found for proteins and peptides are indicated. These bands are caused by the amide groups that are found in the protein backbone, in particular amide A and B, amide I, amide II and amide III. Amide A and B are occurring at around 3300 to 3070 cm^{-1} and are comprised mostly of N-H stretching vibrations.

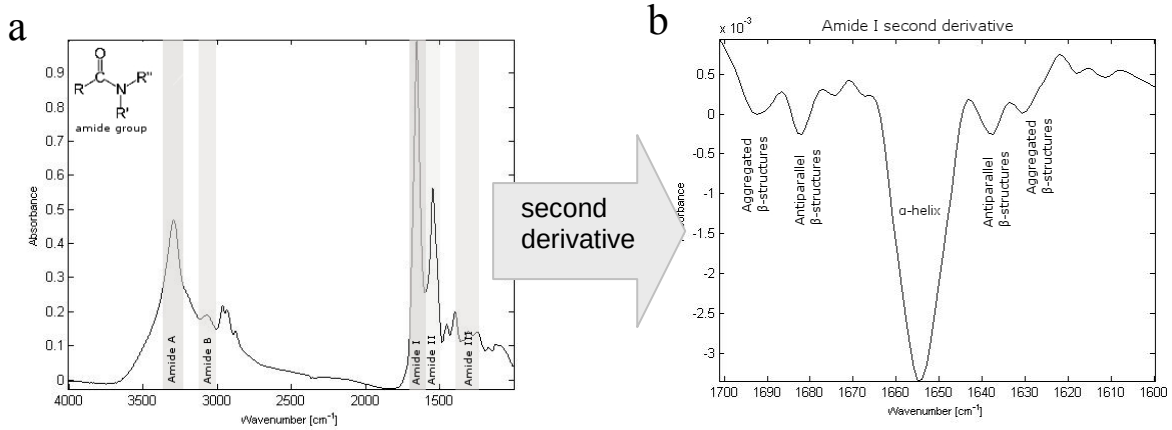

Figure 1: (a) Typical FTIR spectrum of a single muscle myofiber. Five amide modes (amide A and B, amide I, amide II and amide III) originating from the amide group are indicated. (b) The amide I band region (1700-1600 cm^{-1}) after application of the second derivative. The amide I region is commonly used for protein secondary structure investigation. The most important protein secondary structure motifs and the respective bands are indicated.

Among the amide bands, the amide I is most prominent one, occurring around 1650 cm^{-1}, comprised mainly of C=O stretching vibrations with minor contributions of the C-N out-of-plane stretching vibration, C-C-N deformation and the N-H in-plane bend. The amide II band, absorbing at around 1500 cm^{-1}, is generated from the N-H in-plane bend and C-N stretching vibration, with a small contribution of other vibrations. Finally, amide III is absorbing in the region 1400-1200 cm^{-1} and is providing information mostly about N-H and C-N stretching vibrations [9-11].

Due to the characteristics of amide I band, it is widely used for protein secondary structure analysis. This is because it is only slightly affected by the nature of side chains and is highly influenced by the configuration of secondary structure of the protein backbone [9]. By applying derivation, spectral resolution can be enhanced and underlying features in the amide I band can be resolved facilitating the identification of existing structural motifs.

In this study, FTIR microscopy was applied on the samples treated with pure salts brines (NaCl, KCl and $MgSO_4$ respectively) in all the concentrations (1.5, 6 and 9 %). The second derivatives of the spectra are presented in Figure 2. Taking into account that the minima in the second derivatives correspond to maxima in the original spectra, there are nine bands revealed in the amide I region. The peak maximum positions occur at 1693, 1682, 1665, 1660, 1654, 1638, 1630, 1618 and 1611 cm^{-1}, and spectral changes are revealed according to the tested parameters, particularly concentration of salt in brines. A summary of these bands and their tentative assignment, made in accordance to the previous work [6, 12] is summarised in Table 1.

Table 1: Band positions and approximate descriptions of vibrational modes of FTIR Amide I band (comprised of 80 % C=O stretch, 10 % C-N stretch and 10 % N-H bend vibrations) [12]

Frequency (cm^{-1})	Tentative assignment
1693	Aggregated β-sheet structures
1682	Antiparallel β-sheet structures
1667	Non-hydrogenated C=O groups
1660	Loop structures
1654	α-helical structures
1638	Antiparallel β-sheet structures
1630	Aggregated β-sheet structures
1618	Aggregated β-sheet structures
1611	n.a.

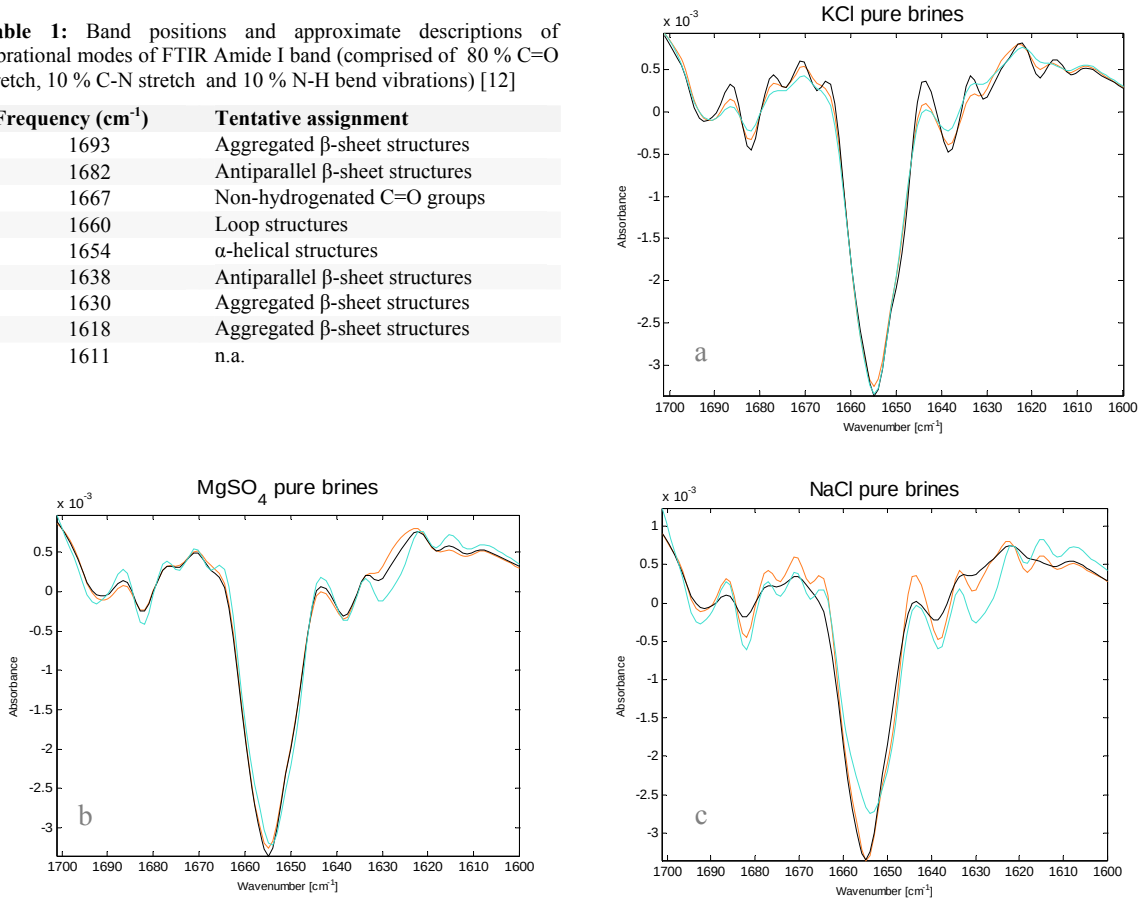

Figure 2: Second derivative of the FTIR spectra in amide I region (1700 – 1600 cm^{-1}) of the samples treated with (a) pure KCl , (b) pure $MgSO_4$ and (c) pure NaCl. The orange lines refer to spectra obtained on samples treated with concentration of 1.5 %, black to 6 % while green to samples treated with 9 % brines. The biggest difference between the samples occurs in the spectra at 1630 cm^{-1}, which can be linked to denaturation processes. Additionally, there is a difference in the intensity and position of 1654 cm^{-1} band of 9 % NaCl and $MgSO_4$ brines compared to the lower concentration ones Every line in the spectra represents an average over 120 spectra.

The most pronounced band in the spectra at 1654 cm^{-1} can be assigned to C=O stretching vibrations in α-helical structures in the myofibrillar proteins [10]. There is no significant change in the shape and the intensity of this band between the samples treated with different concentrations of KCl, while the change in intensity and position is noticeable in the spectrum of samples treated with 9 % $MgSO_4$ and even more pronounced in 9 % NaCl brine. The most significant effect of different treatments is visible in the intensity of the band occurring at 1630 cm^{-1}, which is assigned to aggregated β-structures [10]. The increase in intensity of this band corresponds to the increased amount of aggregated β-structures, as a result of a partial denaturation of native structures indicated by a decrease in intensity of the band at 1654 cm^{-1} band. In the case of $MgSO_4$ salt, the brine with the lowest concentration gives spectra with the lowest intensity of the aforementioned band at 1630 cm^{-1}. As the concentration in brine increases, the intensity simultaneously increases and with it, the amount of aggregated β-structures. This trend is not as apparent in NaCl brines where the middle range concentration (6 %) is not showing any increase in the intensity of the 1630 cm^{-1} band. The highest concentration of NaCl is causing the highest increase in intensity. This trend is completely absent in the spectra of samples treated with KCl brine, showing the lowest intensity of the mid-range concentration, followed by the highest concentration, with the brine with lowest salt concentration showing the highest intensity of the 1630 cm^{-1} band.

Considering the effect of combining two different salts on the secondary structure of the proteins, differences are again mainly apparent in the 1630 cm^{-1} band, as shown in Figure 3. By a closer inspection it is clear that the combinations of salts that contain $MgSO_4$ are giving spectra with higher intensity of the aforementioned band. The combination of $MgSO_4$ and NaCl seems to affect this band more strongly than the other salt combinations, whilst the combination of salts which doesn't contain $MgSO_4$ is affecting it the least.

Besides the intensity, positions of the bands are also affected by salt type and the applied concentration. In the case of NaCl, the bands at 1693 and 1630 cm^{-1} are changing the position moving from low salt concentration (1.5 %) to the high concentrations (6 and 9 %). The position of 1693 cm^{-1} band is moved to 1694 cm^{-1} from low to the high salt concentrations, while the 1630 cm^{-1} band is moved to 1629 cm^{-1}. These two bands originate from the same aggregated β-sheet structures.

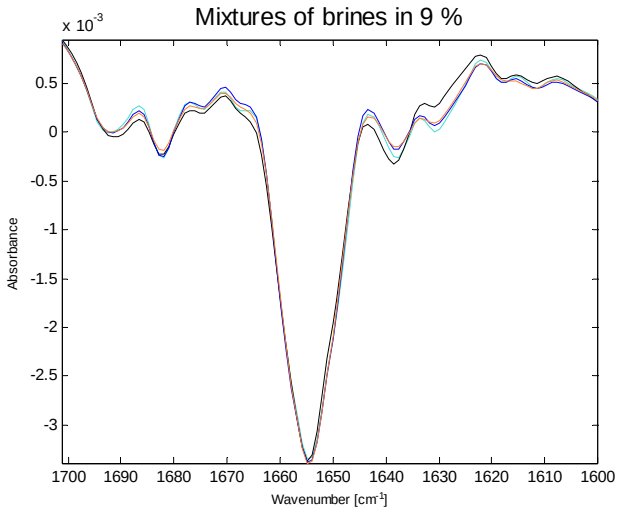

Figure 3: Second derivate FTIR spectra in Amide I protein region of samples treated with combination of salts: The green line refer to spectra obtained on samples treated combination of NaCl and $MgSO_4$, black to NaCl and KCl, blue to $MgSO_4$ and KCl, while the combination of all three salts is depicted with orange colour. It is noticeable that the combination of the salts that does not contain $MgSO_4$ (black line) is showing different features regarding the 1630 cm^{-1} band. Every line represents an average over 120 spectra.

IV. CONCLUSION

Fourier transformation infrared microscopy is an important tool for probing protein structure in partially-intact meat samples. With this investigation, it has been shown that the protein structure is affected differently by applying certain salt substitutes, particularly potassium chloride, as the first alternative to NaCl. Since the protein structure in meat is responsible for many qualities that are important for both industry and consumers, such as water holding capacity and tenderness, it is necessary to optimise the substitution with the respect to that. Because there is no panacea in terms of a single ingredient, a range of functional ingredient combinations need to be developed and/or optimised. In this study it was demonstrated that among brines containing salt combinations of NaCl, KCl and $MgSO_4$, the combinations containing $MgSO_4$ and NaCl have similar effects on protein secondary structure as brines containing pure NaCl, while brines containing KCl (pure KCl brains and in combination with other salts) are more different from brines containing pure NaCl. This is a notable result, since today, KCl is the most common inorganic salt substitute.

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