Analysis of Protein Structures and Interactions in Complex Food by Near-Infrared Spectroscopy. 2. Hydrated Gluten

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Hydrated gluten, treated with various salts, was analyzed by near-infrared (NIR) spectroscopy to assess the ability of this method to reveal protein structure and interaction changes in perturbed food systems. The spectra were pretreated with second-derivative transformation and extended multiplicative signal correction for improving the band resolution and removing physical and quantitative spectral variations. Principal component analysis of the preprocessed spectra showed spectral effects that depended on salt type and concentration. Although both gluten texture and the NIR spectra were little influenced by treatment with salt solutions of low concentrations (0.1−0.2 M), they were significantly and diversely affected by treatment with 1.0 M salt solutions. Compared to hydration in water, hydration in 1.0 M sulfate salts caused spectral effects similar to a drying-out effect, which could be explained by salting-out.

INTRODUCTION

In the food industry, assessment of food quality has become a highly important issue and requires monitoring throughout the production line. Often, the food quality parameters are determined by macromolecular structures and interactions (*1*), demanding methods that can quickly, noninvasively, and nondestructively give information on this topic. In this respect, spectroscopic methods may be valuable, as they are useful for on-line monitoring. Protein secondary structures are, for example, provided by Fourier transform infrared (FTIR) spectroscopy (*2*), which allows measurement of often opaque and water-rich food samples by use of attenuated total reflectance (ATR). For example, ATR-FTIR spectroscopy has been used for the analysis of hydrated gluten (*3*, *4*) and given basis for a molecular explanation of the viscoelastic properties of the gluten network, which are essential to the breadmaking potential of dough (*5*). ATR-FTIR has also given information on molecular changes during dough mixing and mechanical work input (*6*, *7*).

Although the related method near-infrared (NIR) spectroscopy has been used to little extent for protein structure analysis, we have previously (*8*) investigated the performance of this method for monitoring protein changes during moistening of dry gluten, which is the first step in dough development. We found that the complex NIR spectrum (with overlapping overtone and combination bands) from proteins is sensitive to water-binding

and probably to the structures and intermolecular interactions in gluten. A relationship between protein secondary structure and the NIR absorption bands has previously been established for proteins in the freeze-dried state (*9*, *10*), but NIR spectroscopy has also been applied for analyzing proteins in solution, for example, for studying structural changes in ovalbumin and human serum albumin induced by heating (*11*, *12*). In the present work, we evaluate NIR spectroscopy for structure and interaction analysis in the water-rich hydrated gluten system, containing a low amount of protein compared to the gluten powder system (*8*).

The developed gluten complex is rather insoluble in water due to large molecular sizes and extensive intermolecular interactions. The gluten proteins, gliadins and glutenins, have distinct functionalities, and only glutenins can form intermolecular S−S bridges and provide an entangled polymer network (*13*). However, both proteins participate in non-covalent intermolecular interactions, which are critical to the properties of the network (*13*). ATR-FTIR studies have shown intermolecular β-sheets in hydrated gluten (*3*, *7*), and the presence of this structure in "train" regions has been proposed in the "loop and train" model (*5*). According to this model, the long glutenin polymers establish intermolecular linkages in the "train" regions by hydrophobic and intermolecular β-sheet interactions, whereas other more mobile "loop" regions (containing β-turns) interact with the solvent. Glutenin and gliadin both contain a low amount of basic amino acids with a positive charge, and besides hydrophobic and hydrogen bonding interactions, salt bridges

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and electrostatic interactions are important to the network properties as well (*14*).

The non-covalent interactions are modified by the addition of salts, and NaCl is often added to the dough to increase the dough strength and extensibility and improve loaf volume. However, the salt effects depend much on both salt type and concentration, and salts may as well disrupt the gluten functionality and deteriorate the bread quality (*15*–*17*). In this work, the gluten proteins are perturbed by different salts at 0.1–1.0 M to give diverse effects, and the resulting spectral changes in NIR are investigated with the attempt to assign them to changes in the gluten proteins.

Because NIR spectroscopy can measure deeper into the sample and has better potential for on-line monitoring compared to ATR-FTIR, the success of NIR spectroscopy in the present application could lead to many more applications in food quality assessment, as protein networks constitute the basis of many foods such as meat, egg, and milk products.

MATERIALS AND METHODS

Gluten powder was obtained from Sigma-Aldrich (catalog no. G5004).

Salt Treatments. Solutions of Na_2SO_4, $MgSO_4$, $MgCl_2$, NaCl, KBr, and $MgBr_2$ were prepared in Milli-Q water at concentrations of 0.1, 0.2, 0.5, and 1.0 M. For preparation of each gluten sample, 25 mL of Milli-Q water or salt solution was added to an aliquot of 10 mg of gluten powder, and the sample was mixed briefly until homogeneity (<20 s). After resting for ~4 h at room temperature, the sample was centrifuged for 15 min at ~340g, and excess solution was discarded. Immediately thereafter, the sample was weighed, to obtain the water uptake, and stored at 4 °C in a sealed container until further use. The treatments of gluten in water and in 1.0 M salt solutions were carried out in two to four replicates (prepared and measured on different days).

NIR Analysis. The NIR measurements were for practical reasons performed 1 or 2 days after sample preparation. During the time before measurements, there was a small loss of water from the samples, but the overall differences in appearance and texture between samples did not change in this time. The samples were equilibrated to room temperature, and slices of ~1 cm thicknesses were cut from each lump of gluten with a razor blade (the slices were kept in sealed containers until measurement). NIR spectra of the optically thick gluten slices were obtained in reflectance mode from 790 to 2500 nm on a Perkin-Elmer, Spectrum One, FT-NIR instrument, equipped with a reflectance assessory and an InGaAs detector. The nominal resolution was 16 cm^{-1}, and 100 scans were co-added. The data interval was 1.67 nm. Each slice was measured several times by placing it directly on the NIR reflectance assessory in different positions, and at least five measurement replicates were obtained for each gluten treatment. Salt solution spectra were obtained in transflectance mode: A drop of solution was added on the reflectance assessory, and a reflector plate was placed on the top. Two measurement replicates were obtained for each salt solution.

Spectral Pretreatment. Second-derivative transformation (Savitzky–Golay derivation, with 13 data point smoothing) and extended multiplicative signal correction (EMSC) (*18*) were performed in Unscrambler version 9.2 (Camo, Oslo, Norway). The two methods were used in combination, as described under Results and Discussion, for band-narrowing and for removal of the light scattering effects as well as the quantitative variations (different gluten/water ratios). EMSC is based on an approximation of the absorbance spectrum z_i as "physical" modifications of the chemically based spectrum $z_{chem,i}$. These modifications are represented by both an additive polynomial baseline effect and a multiplicative scaling effect, as shown in eq 1.

$$z_i \approx b_i \cdot z_{chem,i} + a_i \cdot 1 + d_i \cdot \tilde{\nu} + e_i \cdot \tilde{\nu}^2 \qquad (1)$$

Here, the coefficient b_i represents the multiplicative scaling effect, the coefficients a_i, d_i, and e_i represent the additive baseline deviations, and
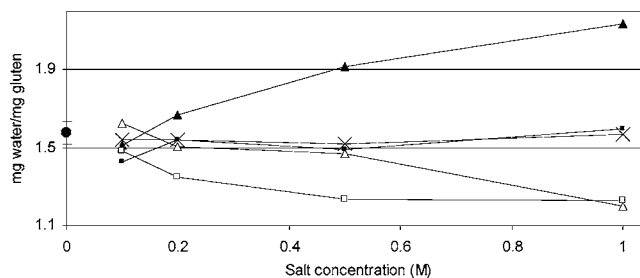

Figure 1. Water uptake in freeze-dried gluten powder hydrated in water (●) or salt solutions of $MgCl_2$ (▲), KBr (■), NaCl (×), $MgSO_4$ (△), or Na_2SO_4 (□).

$\tilde{\nu}$ is the linear wavelength range ν between −1 and +1. In the simplified physical version of EMSC used here, the chemical spectrum is taken as the average spectrum, and after estimation of the coefficients by least-squares regression for each spectrum, the corrections are done according to eq 2.

$$z_{i,corrected} = (z_i - \hat{a}_i 1 - \hat{d}_i \tilde{\nu} - \hat{e}_i \tilde{\nu})/\hat{b}_i \qquad (2)$$

Data Analysis. Principal component analysis (PCA) was carried out in Unscrambler version 9.2. The resulting score and loading vectors were used for approximation of the spectrum for sample i (x_i), according to eq 3.

$$x_i = m + t_{1,i}p_1 + t_{2,i}p_2 + \dots t_{a,i}p_a \dots + t_{A,i}p_A \qquad (3)$$

where m is the mean spectrum, p_a is the loading vector for component a, $t_{a,i}$ is sample i's score value for component a, and A is the selected number of principal components (PCs).

RESULTS AND DISCUSSION

Appearance and Water Uptake. The gluten consistency was affected in different ways by the various salts, but the salt effects were most noticeable at high salt concentrations (0.5–1.0 M). Treating gluten with 1.0 M $MgCl_2$ caused a jelly-like appearance, whereas treatments with 1.0 M sulfate salts (Na_2SO_4/$MgSO_4$) caused a firm and elastic appearance. $MgBr_2$ (1.0 M) had effects similar to those of $MgCl_2$, although it affected gluten much more severely and even caused the sample to be too sticky and too difficult to handle for further measurements. Samples treated with $MgBr_2$ will therefore not be dealt with in the following NIR analysis. The other salts (NaCl, KBr) had only small visible effects.

The water uptake depended on the salt concentrations and salt types as shown in **Figure 1**. ($MgBr_2$ had an extremely high water uptake of ~4 mg of water/mg of gluten.) Already at 0.2 M, the water uptakes reflect the above finding of opposite effects of $MgCl_2$ and the sulfate salts and virtually no effects of NaCl and KBr compared to water. From these results, it is obvious that both anions and cations affect the gluten proteins and that they act together to give a mutual effect.

The water uptakes can be related to the different abilities of the salts to increase/decrease water–protein interactions and thus decrease/increase protein–protein interactions. An observed increase of farinograph water absorption after the addition of some salts at high concentrations (>0.5 M) has been associated with a destabilization of the dough, for example, seen as decreased dough extensibility and decreased tolerance to overmixing (*16*). Thus, increased water uptake is here taken as indication of a destabilizing effect (decreased protein–protein interaction) of the salt, whereas decreased water uptake is thought to reflect a stabilizing effect (increased protein–protein interaction).

The diverse salt effects result from the different abilities of the ions to interact with water and with the folded/unfolded states of the proteins. For anions, the degree of ion−water interaction is found to be central to the salt effect at high concentrations (*19*, *20*), and they can be divided into kosmotropes and chaotropes, showing higher and lower interaction with water than water with itself, respectively (*20*). The kosmotropic anions exclude hydrophobic solutes from their hydration shells, promote increased hydrophobic interaction in proteins, and lead to salting-out, as they stabilize the native conformation and the oligomeric state (*19*, *20*). On the contrary, the chaotropic anions are able to associate with the protein surfaces (in the denatured state) and cause salting-in, as they promote unfolding, hydration, and solubilization (*19*, *20*). The Hofmeister series arranges ions according to these effects, which are observed only at high salt concentrations (>0.3 M). The stabilizing effect of the involved anions decreases in the order $SO_4^{2-} > Cl^- > Br^-$, with SO_4^{2-} being regarded as kosmotropic, Cl^- as neutral, and Br^- as chaotropic.

Thus, the decreased water uptake seen for both sulfate salts at 1.0 M (**Figure 1**) agrees with the stabilizing effect of sulfate and with observations by He et al. (*17*) of improved dough extensibility and elasticity by the addition of sulfate to weak doughs. However, the breadmaking properties were adversely affected already at 0.1 M, because the elasticity was increased too much. The neutral NaCl, although promoting structure changes (*21*) and aggregation and improving dough strength (*22*), has been found to affect the water absorption only little (*16*), as is also seen in **Figure 1**.

The increased water uptakes seen at high concentrations of $MgCl_2$ are in agreement with this salt being known as a salting-in salt (*23*). The large effect of $MgCl_2$ compared to NaCl must be ascribed to the cation effect (although the destabilizing effect of Mg^{2+} apparently is suppressed when sulfate is the counterion). As for anions, the effect of cations at high concentrations can be partly related to their ability to bind water. However, opposite to the anions, divalent cations with strong water-binding ability may also interact strongly with peptide groups and cause protein destabilization (*23*). Thus, the stabilizing effect of the involved cations decreases in the order $K^+ > Na^+ > Mg^{2+}$, with K^+ being regarded as weakly chaotropic, Na^+ as neutral, and Mg^{2+} as kosmotropic. In agreement with the Hofmeister series, Butow et al. (*22*) found the most dough-strengthening effect of K^+ compared to Na^+ and the weakly kosmotropic Li^+. However, they explained this from the low hydration of K^+, allowing it to penetrate into the gluten matrix and coordinate to functional groups on the gluten proteins and thereby cause stabilization of the large glutenin polymers. Also, He et al. (*17*) have shown a positive effect of KCl on loaf volume at an optimum salt concentration, whereas only negative effects of the kosmotropic $CaCl_2$ and LiCl were seen at all concentrations. However, the cation effects seem to depend on the protein content, as Butow et al. (*22*) observed increased breakdown of the large glutenin polymers upon addition of K^+ to wheat flours of high protein content. Thus, the cation effects are complex and unclear, but the effects of Mg^{2+}, when $MgCl_2$ are added, are investigated further in the following NIR analysis.

As seen from **Figure 1**, the salts have little effect on the water uptakes at low salt concentrations (<0.2 M). At these low concentrations, most salts induce aggregation of the gluten proteins by shielding of positive charges and thereby suppression of the electrostatic repulsions (*16*). The ion type has little importance to this effect, which is also exerted by chaotropic anions (*16*, *17*). Due to the relatively high uncertainty in the
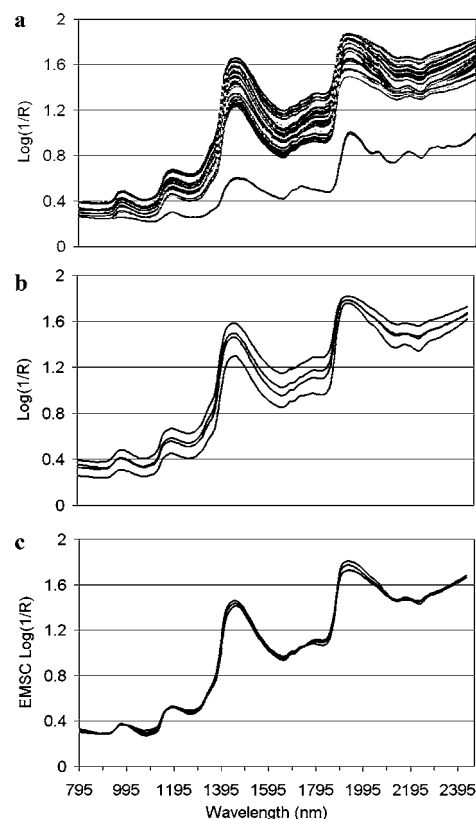

Figure 2. NIR reflectance spectra: (**a**) raw spectra of gluten fully hydrated in water or in different salt solutions (highest) and of moist gluten powder (lowest); (**b**) raw spectra of hydrated gluten at water contents of ∼1.2, 1.6, 1.9, and 2.2 mg/mg gluten (lowest to highest), resulting from hydration in water, or different $MgSO_4$ or $MgCl_2$ solutions; (**c**) spectra in (**b**) corrected by EMSC.

water uptake data compared to the small effects of the low salt concentrations, these data will not be interpreted further.

Spectral Pretreatments. The NIR reflectance spectra of fully hydrated gluten are highly dominated by the water signal (**Figure 2a**), and compared to gluten at low moisture contents, fully hydrated gluten causes much higher baselines. This is likely the result of decreased scattering properties of the fully hydrated samples due to the replacement of air in the voids for water, whereby refractive index differences are decreased. Also, baseline variations between the fully hydrated samples are noted: There is a tendency that higher water content causes a higher baseline offset, whereas it affects the baseline slope less. The water/gluten ratio is also reflected in the spectra, such that protein peaks are more pronounced, the lower the water content (as expected).

Because this study focuses on the qualitative spectral variations that have a chemical basis, it could be advantageous to remove the physical and quantitative variations before further analysis to ease the interpretation. To get rid of the physical effects, EMSC is often useful (*18*). However, in the present case, this preprocessing method could not be applied for correction of the raw data, because the spectral shape seemed to be affected in a peculiar way by an additional nonchemical factor: There is an increase in the A_{1450nm}/A_{1930nm} ratio as well as a flattening of the large water band at 1930 nm at increasing water content (**Figure 2b**). This phenomenon may be caused by nonlinearities due to a high absorbance (A_{1930nm} is above 1.6 AU), and it produces curvatures when two spectra of very different water content are plotted against each other. The effect cannot be removed by EMSC (**Figure 2c**), which can remove only the
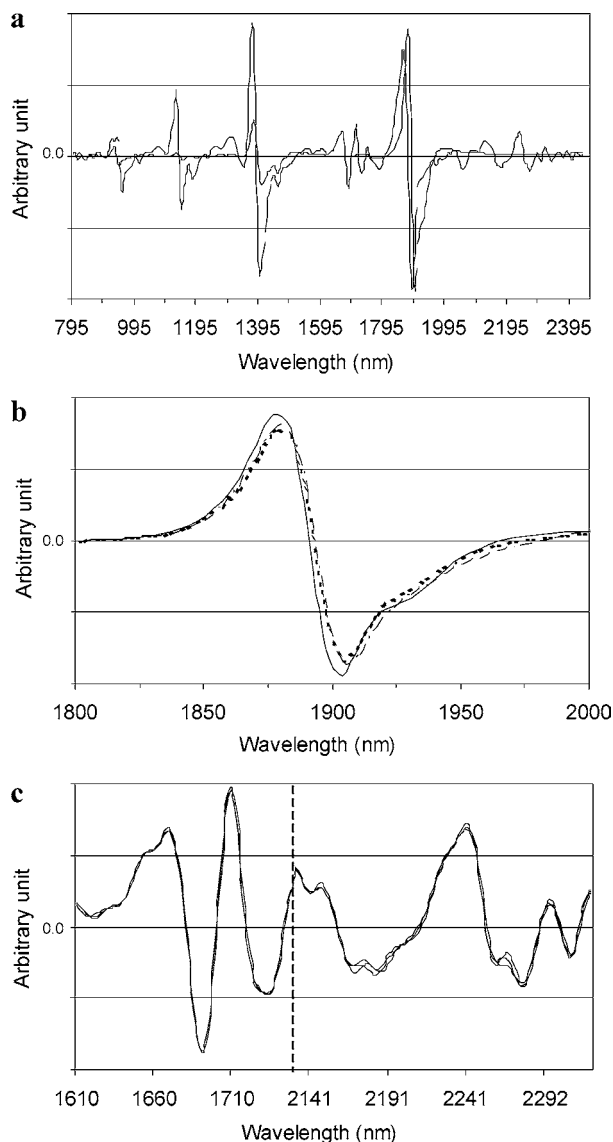
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Figure 3. Second-derivative NIR spectra obtained in reflectance mode (gluten samples) or transflectance mode (water and salt solutions): (**a**) full spectrum of pure water (thin line) and of gluten hydrated in water (normal line); (**b**) 1930 nm water band of pure water (thin line) and of salt solutions: 1.0 M $MgSO_4$ (dotted line) and 1.0 M $MgCl_2$ (dashed line); (**c**) second-derivative spectra additionally corrected by EMSC, performed separately in two regions, 1610–1748 and 2130–2320 nm (the two regions are divided by the dotted line). Samples include gluten hydrated in water and in 1.0 M salt solutions.

Table 1. NIR Combination Bands and Some Proposed Assignments[a]

wavelength (nm)	assignment
2100–2120	amide B/I
2167	amide B/II
2184	glutamine, asparagine amide A/III
2209	amide A/III
2259	OH str/HOH def
2278	amide III/CH_2 str
2308	CH str/CH def

[a] References are shown in Bruun et al. (*8*).

Even though a PCA on the raw, EMSC, or second-derivative spectra leads to grouping of the samples in the score plot according to the salt types (not shown), it is obvious that the groupings are not based solely on chemical changes in gluten. Instead, the PCA parameters contain intermingled information on the physical effects, the water spectrum variations, the water/gluten ratio, and the qualitative gluten spectrum variations, of which only the latter is of interest.

A method found to be useful in a former NIR study for removing the physical effects as well as the effect of different gluten/water ratios was termed "physical−chemical" EMSC and was applied after second-derivative transformation (*8*). In this EMSC procedure, a water spectrum was included in the representation of the chemically based spectrum $z_{i,chem}$, and its concentration in each spectrum was estimated, whereafter the water spectrum contributions were subtracted from all spectra. However, this EMSC method could not remove the gluten/water ratio or the salt-induced water spectrum variations in the present second-derivative data. This relates to the fact that the water spectrum appears to be very different in the second-derivative gluten spectrum and in the corresponding second-derivative solution spectrum (**Figure 3a**), wherefore the latter cannot serve as model spectra in EMSC. The most pronounced difference between the water absorptions seen in the second-derivative spectra in **Figure 3a** is the wavelength shift of the 1910 nm band. This shift is related to different shapes of the 1930 nm band in the two nontransformed spectra. Gluten−water interactions are thought to be of little importance to this difference, and it is more likely caused by nonlinearities in the gluten spectra due to their high absorbance level and the light scattering. These effects cause the peak maximum to shift.

Due to the above difficulties, another approach was chosen: Regions well outside the water bands were selected and subjected to further pretreatments after second-derivative transformation. Thus, the protein band regions at 1610−1748 and 2130−2320 nm were corrected by EMSC, performed separately in each of the two regions. Replicate spectra were averaged, and some resulting spectra are shown in **Figure 3c**. The selected regions contain the first overtone of CH_x stretching vibrations from amino acid side chains (1610−1748 nm) as well as the combinations of amide and side-chain vibrations (2130−2320 nm) (**Table 1**). The amide vibrations are specific for proteins and polyamides, and the five fundamental amide bands that are of importance in FTIR spectroscopy (amide I−III, amide A, and amide B) all depend on the protein secondary structure, because their vibrational frequencies are determined by dipolar couplings and hydrogen-bonding strength of the involved groups (*2*). Combinations of amide modes are present in the NIR combination band region, as outlined in **Table 1**, showing the possible assignments of the observed gluten peaks. The amide I band (mostly C=O stretching), which shows the clearest relationship between band shape and protein secondary structure in MIR, results in very weak bands in NIR. However, also the
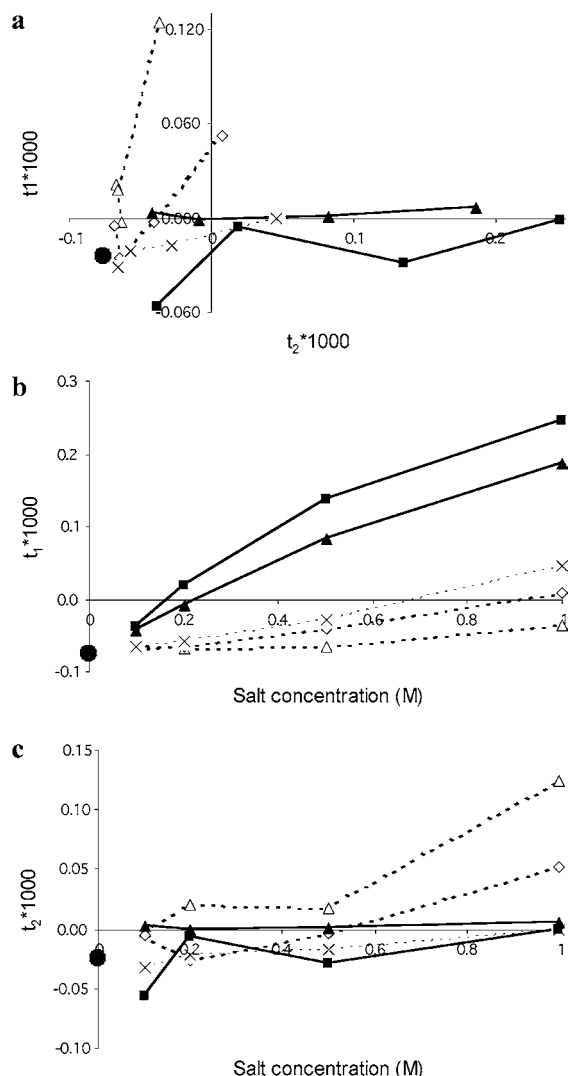
additive baseline variations and the multiplicative scaling effects as described under Materials and Methods.

Second-derivative transformation was performed on the raw gluten spectra to narrow the bandwidths and also remove some of the baseline variations. This transformation made the absorption bands much more evident (**Figure 3a**) and revealed a number of gluten bands, which are the result of different vibrations in either the protein backbone or the amino acid side chains. Still, physical and quantitative variations are left in the second-derivative spectra as this method cannot remove multiplicative effects. It is also noted that the salt types and concentrations give rise to spectral effects (**Figure 3b**) that originate from the different degrees of ion−water interactions for the various salts, affecting the positions and intensities of the water bands (*24*).

a

b

c

Figure 4. PCA score plots resulting from analysis of pretreated NIR spectra (1610–1748, 2130–2320 nm) of gluten fully hydrated in water (●) or in different salt solutions: (0.1–1.0 M) MgCl$_2$ (△), KBr (◇), NaCl (×), MgSO$_4$ (▲), Na$_2$SO$_4$ (■). The spectra were second-derivative transformed, and thereafter physical EMSC in each of the selected regions was performed: (**a**) PC1 vs PC2 scores [for each salt type, a line connects the samples in the order 0.1, 0.2, 0.5, and 1.0 M salt concentration (left to right)]; (**b**) salt concentration vs PC1 scores; (**c**) salt concentration vs PC2 scores.

amide II (N−H deformation coupled with C−N stretching) and amide III bands (N−H deformation coupled with CH$_2$ deformation) have provided valuable information on the secondary structures in MIR studies (*25, 26*). In addition, amides A and B, reflecting NH stretching and NH deformation in the peptide groups, are sensitive to the hydrogen-bonding strengths.

PCA. The two pretreated spectral regions were submitted to a PCA. The resulting score plots are shown in **Figure 4**. PC1 and PC2 explain ∼61 and ∼3%, respectively, of the spectral variance. There seems to be an almost linear relationship between the salt concentration and the PC1 score, although with different slopes for each salt type (**Figure 4b**). On the contrary, the course of PC2 scores indicates somewhat different modes of action of low and high salt concentration (**Figure 4c**). However, some noise may also influence this PC. PC3 explained 1.5% of the spectral variance and showed mostly an effect of 0.2 M Na$_2$SO$_4$ compared to the other salts, and although this
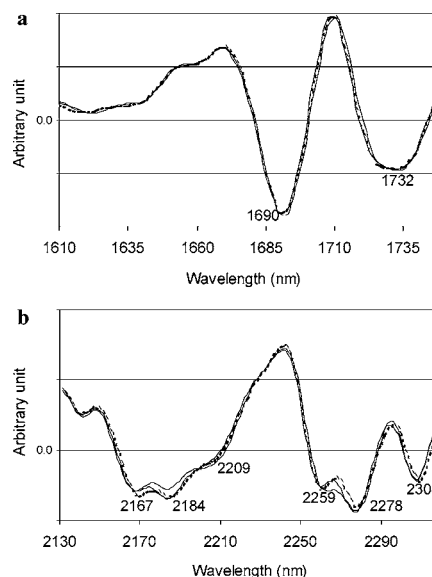

Figure 5. Calculated second-derivative NIR spectra, representing gluten fully hydrated in water (thin line), in 1.0 M Na$_2$SO$_4$ (thick line), and in 1.0 M MgCl$_2$ (dotted line): (**a**) first CH– str overtone region, 1610–1748 nm; (**b**) combination band region, 2130–2320 nm. The spectra were constructed from PC1 and PC2 loadings and scores (shown in **Figure 4**), which were obtained in a PCA on the pretreated NIR spectra of gluten in the different salt solutions.

PC may reflect some salt-specific variations, it is considered too small and noisy to be interpreted further.

Figure 4b shows that the PC1 scores increase for the sulfate salts, whereas the chloride and bromide salts produce only small increases in this PC as compared to the water sample. On the contrary, the PC2 scores increase for increasing concentrations of MgCl$_2$, whereas this PC is only little affected by the sulfate salts. The salts with less striking effects on gluten, KBr and NaCl, result in only small increases in both PC1 and PC2 scores (**Figure 4a−c**). However, the course of scores for NaCl samples is most similar to that of the sulfate-treated samples. The score patterns highly indicate that the spectral variations reflect complex changes in the system and are not simply related to the water content.

To verify that the two PCs reflect some qualitative differences in the gluten proteins and not just the different gluten/water ratios, the loading vectors were inspected for similarities with the mean spectrum and with the dry gluten spectrum (both second-derivative transformed). The highest correlation was found between p_1 and a dry gluten spectrum, but the correlation was only 0.29, and we therefore consider that the PCs may be interpreted with respect to protein structure and hydration changes.

Spectral Interpretations. From the scores and loadings of the first two PCs, the constructed spectra of gluten hydrated in 1.0 M Na$_2$SO$_4$, 1.0 M MgCl$_2$, or water were calculated according to eq 3 as $x_i = m + t_{1,i}p_1 + t_{2,i}p_2$ for sample i, where the score values ($t_{1,i}$, $t_{2,i}$) are indicated in **Figure 4a**. These simplified spectra, shown in **Figure 5**, contain less noise and less irrelevant variation than the measured spectra, and their interpretation is therefore easier. The simplification is especially important due to a rather high noise level in the original spectra compared to the minor salt effects. This is demonstrated by the replicate variations in **Figure 6**.

In the CH str overtone region (**Figure 5a**), the effect of the kosmotropic sulfate salts seems to be minor high-wavelength shifts of the 1690 and 1732 nm peaks. These shifts are in the
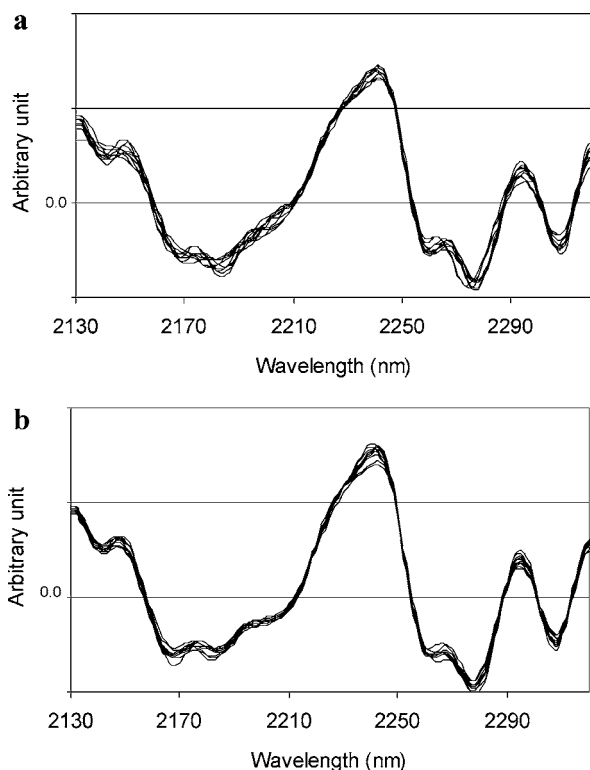
Figure 6. Second-derivative NIR spectra (2130–2320 nm) of hydrated gluten: (**a**) gluten hydrated in water; (**b**) gluten hydrated in 1.0 M Na_2SO_4. Several replicate spectra are shown after pretreatment as in **Figure 3c**.

Figure 7. Calculated second-derivative NIR spectra, representing a freeze-dried (—), a 12% mc (- - -), and a 33% mc (——) gluten sample: (**a**) first overtones of CH_x str, 1610–1760 nm; (**b**) amide and side-chain combination bands, 2130–2320 nm. The spectra were constructed from PC1 and PC2 loading weights and scores, obtained in a PLS regression for prediction of the moisture contents (**y**) from the preprocessed NIR spectra (**X**). The figure is reproduced from the companion paper (*8*).

opposite direction of those seen in a former study (*8*), caused by moistening of dry gluten (**Figure 7**), and are suggested to reflect changing microenvironments and interactions of the amino acid residues upon water binding. Thus, the present study indicates that sulfate salts cause low water binding of gluten, in concordance with the decreased water uptakes. Decreased gluten−water interaction was also suggested by Kinsella et al. (*15*) to explain the harmful effects of a kosmotropic anion (F⁻) on the dough consistency. They proposed that the anion promoted strong hydrophobic and hydrogen bonding interactions in the dry gluten proteins and caused impeded unraveling and hydration of the aggregated gluten polymers, thereby preventing proper network development (e.g., loop formation). The reinforcement of the extensive interactions in the dry gluten proteins at the expense of gluten−water interactions could likely explain the particulate nature of the sulfate-treated samples observed in the present study. The molecular arrangement of glutenin subunits in sulfate-treated gluten may be represented by the upper outline in **Figure 6**, showing the effect of hydration according to the loop and train model (*28*).

In the combination band region (**Figure 5b**), a more remarkable effect of the sulfate salts is seen: a decrease in the 2184 nm peak (glutamine, asparagine, or amide A/III) and an increase in the shoulder at ∼2209 nm (amide A/III). Again, on the basis of previous observations from a gluten moistening study (**Figure 7b**), the observed changes may be interpreted as an opposite hydration effect, that is, a drying-out effect. For example, the decreased intensity at 2184 nm could mean decreased interaction of the glutamine and asparagine side chains with water (*8*), and the increased intensity at 2209 nm could, in view of the assignments by Robert et al. (*9*) (**Table 2**), stem from an increased amount of β-sheet, explained from the impeded protein unfolding. Because NaCl is found to give a spectral effect similar to that of the sulfate salts (as indicated in the score plot, **Figure**
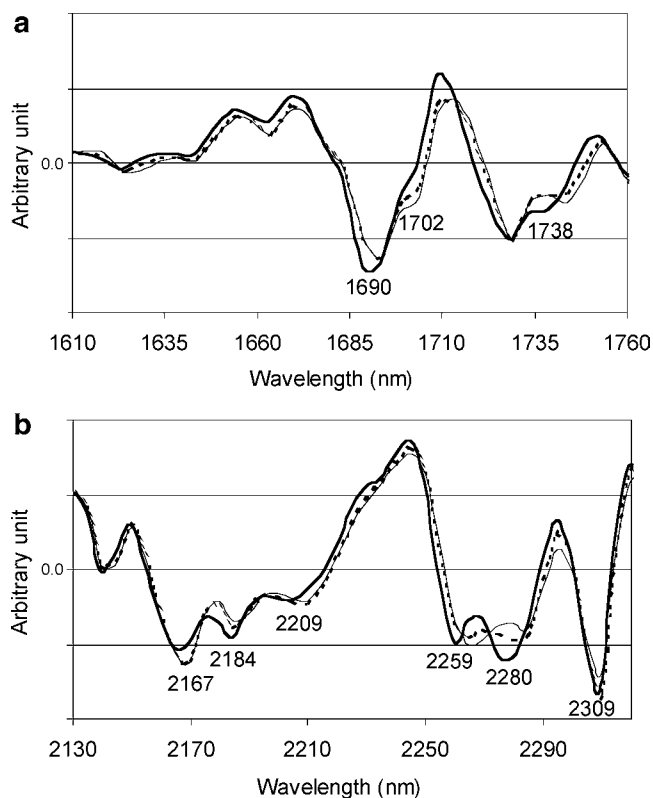
Table 2. Assignments of NIR Regions to Secondary Structures[a]

structure	characteristic wavelengths (nm)
α-helix	2056, 2172,[b] 2239, 2289,[b] 2343
β-sheet (inter- and intra-)	2205, 2264, 2313
random	2265

[a] Taken from Robert et al. (*9*). [b] Most important wavelengths for α-helix.

4a), the increase at 2209 nm could also reflect an increase of intermolecular β-sheet, which is promoted by this dough-strengthening salt (*27*). A more elaborate discussion on protein band changes in gluten and their relationship to secondary structures is given in ref *8*.

From comparisons **Figures 5b** and **7b**, the sulfate effect at higher wavelengths is again found to be similar to a drying-out effect: There is a high-wavelength shift and broadening of the 2259 nm peak, which is assigned to OH str/HOH def from amino acid side chains (e.g., tyrosine). The increase at 2264 nm may be ascribed to an increased amount of random structure or β-sheet, according to the assignments in **Table 2** and in agreement with the previous findings. However, the altered shape of this peak does not necessarily result from secondary structure changes, as other phenomena, affecting interactions and microenvironments of amino acid side chains (e.g., from tyrosine), may be reflected in this peak.

The sulfate effect is not exactly similar to a drying-out effect, as there is no large intensity change in the 2278 nm peak as was seen upon moistening (**Figure 7b**). The above indications

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of a high content of both random structure and β-sheet and a low content of hydrated structure in the sulfate-treated gluten samples was supported by our ATR-FTIR amide I studies (not presented).

On the basis of the increased water uptake for $MgCl_2$-treated samples, the expected effects of this salt would be an increase at 2184 nm, reflecting increased interaction with water (**Figure 7b**). However, this is not seen in the present study (**Figure 5b**). Instead, $MgCl_2$ increases the 2167 nm peak (α-helix) and causes it to shift slightly to 2169 nm. A decrease at 2264 nm is similar to the hydration effect and suggests a decreased amount of random structure, but otherwise the minor $MgCl_2$ effects in this region are different from the hydration effects. Thus, the NIR spectra suggest that the gluten proteins are not extensively denatured by $MgCl_2$ and also that the normal protein−water interactions are not increased, although the glutenin chains are allowed to fully unfold as in water.

The effect of $MgCl_2$ seems like a salting-in effect exerted by Mg^{2+} in combination with a chaotropic anion (Cl^- is a very weak chaotrope), because an enhanced effect was observed by pairing Mg^{2+} with the chaotropic Br^-. Chaotropic anions have been shown to cause increased water absorption and decreased dough stability (*15*, *16*), and their effect has been associated with an increase of the β-turns in the hydrated loop regions and a decrease of the β-sheet in the train regions, based on an ATR-FTIR study (*27*). This situation would be represented by the loop and train model (*28*). Because these anion effects can hardly explain the observed spectral changes exerted by $MgCl_2$, we propose instead that the Mg^{2+} binding to gluten causes formation of an unusual protein network, in which Mg^{2+} links the proteins and in which the excess water is bound to Mg^{2+}. This network formation could be hindered if unfolding of the glutenin chains is impeded, for example, by sulfate.

Final Remarks. Due to the weaker protein signals and the relatively high noise (due to the high absorbance), the spectra of hydrated gluten are expected to contain much less qualitative information on protein structures than the spectra of dry and moist gluten. However, we have shown that the NIR spectra can give information on protein−water interactions in fully hydrated gluten, although a full interpretation of the spectral changes remains.

Because the gluten system is otherwise hard to characterize at a molecular level, the possibility to quickly and easily obtain a detailed molecular picture of the gluten system by NIR would be highly valuable in the investigation of gluten functionality, which is of interest to several applications of gluten (e.g., baking and bioplastic formation). In contrast to chemical measurements, the spectroscopic measurements are nonperturbing and therefore less prone to mistaken interpretation (*4*).

Even though the absorptions in the NIR spectra are overtones and combinations of the fundamentals seen in FTIR spectra, somewhat distinct information is contained in the two infrared regions. Whereas FTIR spectra show the most intense absorptions from groups of high polarity (e.g., C=O), the NIR spectra are dominated by absorptions from groups of high anharmonicity (e.g., NH, CH, and OH) (*29*). For example, the amide II mode causes much stronger absorptions than the amide I mode in NIR spectra, whereas the opposite applies to FTIR spectra. As another distinction, the overtone and combination bands respond to hydrogen bonding in a different way from the fundamental absorptions. In NIR, the monomers, which are of higher anharmonicity than the polymeric counterpart, produce the most intense bands, whereas in MIR, these free groups cause the weakest absorptions (*30*). Therefore, NIR could be useful as a complementary method to FTIR.

Several authors (*31*, *32*) have used the NIR water band regions for monitoring dough development, as the water bands reflect the immobilization of water, but these types of studies have not provided increased information about the molecular network in gluten, as was the goal of our NIR application. However, the low protein concentration in dough will be a hindrance for protein studies in dough by NIR, and from the present gluten analysis, we cannot draw any conclusions upon the performance of NIR in a comparable dough analysis.

ABBREVIATIONS USED

ATR, attenuated total reflectance; EMSC, extended multiplicative scatter correction; FTIR, Fourier transform infrared; NIR, near-infrared; PCA, principal component analysis; PC, principal component.

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