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Analytical and Sensory Studies on the Release of Sodium from Wheat Bread Crumb

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Supporting Information

ABSTRACT: As a basis for sodium reduction, interactions between sodium and wheat bread ingredients and their impact on salt perception in bread crumb were examined. The theoretical sodium binding capacities of wheat proteins revealed that a maximum amount of 0.24% NaCl (based on flour) could be bound in bread crumb by ionic interactions between sodium ions and acidic amino acid side chains. However, the sodium binding capacities of wheat proteins, determined by a magnetic beads assay and a sodium-selective electrode, were only about 0.002% NaCl. They were negligible concerning the sensory perception of saltiness, as 0.075 and 0.3% NaCl were the lowest noticeable differences using bread containing 0 and 1% NaCl as a reference, respectively. Extracting bread crumb in a mastication simulator with ultrapure water, buffer solutions, and artificial and human saliva revealed that interactions between sodium and wheat bread ingredients were sufficiently weak to enable complete sodium extraction during simulated mastication.

KEYWORDS: *sodium reduction, wheat bread, salt taste, wheat proteins, sodium binding capacity, mastication simulator*

INTRODUCTION

The use of table salt (sodium chloride) in breadmaking is due to several essential sensory and technological functions. Apart from its unique salty taste, it has been reported to mask metallic or bitter off-tastes and enhance flavor.[1] Salt inhibits yeast growth and thus serves to control the fermentation rate, reduce the water activity of the baked product, thereby prolonging microbial shelf life, and lead to an improvement of bread texture by facilitating the formation of a gluten network during mixing of the dough.[2]

However, high sodium levels in the diet are a primary cause of hypertension,[3] which is a major risk factor for cardiovascular diseases that are the leading cause of death worldwide and account for 41% of mortality in Germany.[4] Furthermore, excess intake of sodium has been linked to other harmful effects on human health such as stroke, kidney stones, and bone defects.[5] Surveys indicate that the average daily salt intake of the majority of people in industrialized nations ranges from 8 to 11 g salt per day and is well in excess of 5 g salt/day, which is recommended by the World Health Organization. This is mainly attributable to the consumption of processed foods, with bread being responsible for 24% of daily sodium intake in Germany,[6] which makes it an important target for sodium reduction.

One method to reduce sodium in food is the slow and steady reduction of salt levels over a long period of time.[7] Potassium chloride is most commonly used as a salt substitute to reduce sodium levels in bread due to its similar effects on yeast activity and dough rheology. However, its metallic-bitter after-taste limits the replacement level to 20−30% of NaCl.[8,9] Several mixtures containing other inorganic salts, organic acids, amino acids, peptides, protein hydrolysates, or other salt-enhancing substances have been suggested,[2,10,11] yet the search for an adequate substitute remains a major challenge because no other known substance has the unique pure salt taste of sodium chloride.

Apart from its unique taste, sodium chloride has a strong influence on wheat gluten properties due to its ionic nature, which allows shielding of charged amino acid residues leading to reduced electrostatic repulsion.[12] This neutralization of the excess of positively charged basic amino acids at neutral and low pH in addition to an exposure of nonpolar residues in the presence of NaCl due to higher free entropy leads to increased hydrophobic protein−protein interactions and thus facilitates gluten aggregation.[13] More recently, NaCl has been found to induce gliadin aggregation during the formation of gluten and to make monomeric α/β-gliadins and γ-gliadins from gluten treated with NaCl soluble in distilled water.[14] Another effect of NaCl, in particular when added in concentrations above 1% (based on flour), is the restriction of the availability of water for the development of a gluten structure, which leads to an increased dough development time.[2] These techno-functional properties of NaCl based on ionic interactions with wheat gluten proteins have been characterized in several farinograph,[13,15,16] mixograph,[17,18] and extensigraph[13,16] studies as well as in baking tests.[19,20] However, the possible impact of NaCl−wheat protein interactions on the sensory properties of

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wheat bread has not been studied so far. Judging from the extensive interaction between NaCl and wheat proteins during dough formation, a certain amount of sodium might be irreversibly bound due to ionic interactions with negatively charged amino acid side chains. These bound sodium ions would be unavailable for salt taste perception on the tongue, because only free sodium ions in solution can elicit a salty taste. Assuming that a certain percentage of sodium is irreversibly bound, the addition of suitable additives that weaken NaCl–wheat protein interactions could reduce sodium binding while increasing the amount of free sodium available for salt perception. This could be used as a basis for new strategies to reduce the amount of NaCl in wheat bread without influencing salt taste intensity.

Therefore, the aim of this study was to analyze whether sodium binding of wheat bread crumb affects salt taste perception. Flavor profile analyses and the determination of just noticeable differences regarding salt taste perception in wheat bread crumb were used to evaluate the effect of reducing salt levels on the sensory quality of bread and to evaluate the possible impact of sodium binding. The sodium binding capacities of the different protein fractions isolated from wheat flour were measured, and the extractability of sodium from bread crumb containing various NaCl concentrations was studied to examine the interactions of sodium not only with wheat proteins but also with all other bread ingredients after baking. Additionally, the extractability of sodium from wheat bread crumb was compared to potassium and calcium to find out whether the extractability is influenced by the valence of the cations. Combining the determined sodium binding capacities of the protein fractions, the rate of sodium extractability from bread crumb, and the results of the sensory analyses allowed a judgment of the impact of sodium binding on salt taste perception in bread. This more fundamental understanding of the sensory dimension of NaCl–wheat protein interactions helped clarify if sodium binding plays a role in salt taste perception of bread crumb.

MATERIALS AND METHODS

Wheat Flour. Wheat flour (cv. Tommi) (Nordsaat, Langenstein, Germany, harvested in 2008) and commercial wheat flour (Rosenmehl Type 550) (Rosenmühle, Ergolding, Germany) were characterized as described by Selmair and Koehler.[21] Analytical characteristics of the flours were 14.2% (Tommi) and 9.7% (Rosenmehl) moisture, 0.46% (Tommi) and 0.61% (Rosenmehl) ash (dry mass), and 12.6% (Tommi) and 11.8% (Rosenmehl) protein (dry mass), respectively.

Chemicals. The quality of all solvents was pro analysi (p.a.) unless stated otherwise. α-Amylase from porcine pancreas (EC 232-565-6), bicinchoninic acid solution, Bis-Tris propane, glutaraldehyde solution (25%, w/w), potassium chloride puriss., copper(II) sulfate pentahydrate solution (4%; w/v), methanesulfonic acid, magnetic amino functionalized microparticles, and pyridine were from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (LiChrosolv), ascorbic acid, cesium chloride, calcium chloride dihydrate, dipotassium hydrogen phosphate, dithioerythritol, ethanol, glacial acetic acid, glycine, hydrochloric acid (≥37%, w/w), hydrogen peroxide (30%, w/w), magnesium chloride hexahydrate, methanol (LiChrosolv), nitric acid (65%, w/w), potassium dihydrogen phosphate, potassium hydroxide, *n*-propanol, silver nitrate, sodium chloride, sodium bicarbonate, sodium hydroxide pellets, sodium phosphate, sucrose, and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany). *n*-Pentane was from ScienTEST (Rehburg-Loccum, Germany).

Breadmaking. Breads were baked using 300 g of commercial flour. The other ingredients based on the flour weight were 1% sucrose, 7% fresh baker's yeast (Wieninger, Passau, Germany), and from 0 to 6% NaCl, 1.9% KCl, or 3.8% $CaCl_2 \cdot 2H_2O$. All ingredients were mixed with distilled water at 60 rpm and 22 °C for 8 min to an optimum consistency of 550 Brabender units (BU) in a Farinograph (Brabender, Duisburg, Germany) equipped with a 300 g Z-blade mixer, removed from the mixer, and allowed to rest for 20 min at 30 °C in a water-saturated atmosphere. The dough was molded, weighed, and placed in a loaf pan. After a second proofing time of 40 min at 30 °C in a water-saturated atmosphere, the dough was baked for 28 min at 230 °C. The breads were allowed to cool for 2 h, weighed, cut into slices of 1.5 cm thickness, and stored in airtight freezer bags at room temperature until the next day. In the following, all percentages of NaCl are given on the basis of flour weight, that is, (%) is equivalent to (g NaCl/100 g wheat flour).

Sensory Analyses. *Training of the Sensory Panel.* All sensory analyses were performed in three different sessions in a sensory panel room at 20–22 °C. The panel consisted of 15 trained (ISO 8586)[22] subjects (10 women and 5 men, aged 25–31 years) with no history of known taste or smell disorders who had given informed consent to participate in the sensory tests. At the beginning the panelists were accustomed to evaluating bread samples by learning to distinguish between two different samples of bread crumb containing 1.0 and 2.0% NaCl by means of a two-alternative forced-choice test (2-AFC test). Once all panelists had correctly identified the sample with 2.0% NaCl as tasting saltier, the difference in the salt content between the two breads was narrowed step by step from 1 to 0.67, 0.5, and 0.3%.

Procedure of the 2-AFC Tests. A covered sensory flask encrypted by a three-digit random number was filled with two 3 g pieces of bread crumb, and the panelists were instructed to rinse their mouths with Evian mineral water between tastings to eliminate interfering carry-over effects. Two sensory flasks with different bread samples were presented to each panelist in a randomized order. The panelists were asked to write down the number of the sample that tasted saltier according to the forced-choice method. The statistical evaluation of the answers of this 2-AFC test was done by reference to a table of significance for one-sided paired comparison tests in compliance with ISO 5495.[23] Depending on the number of correct answers the level of significance α was determined. All α levels above 0.05 were judged as not significant, whereas an α level of 0.05 (significant) corresponded to a statistical certainty of 95%, an α level of 0.01 (highly significant) to a statistical certainty of 99%, and an α level of 0.001 (very highly significant) to a statistical certainty of 99.9%. All 2-AFC tests were done in triplicates.

Flavor Profile Analyses. Descriptive sensory analyses were conducted by the same panel that had already participated in the 2-AFC tests. Prior to the analysis of the different flavor profiles, the 15 panelists participated in the development of a descriptive vocabulary that best described the flavor attributes associated with the breads. In three subsequent sessions, these terms were refined and reduced to the seven final descriptors sweet, salty, acidic (flavor associated with sourdough), bitter, yeasty (flavor associated with yeast fermentation), floury/watery (flavor associated with a suspension of flour in water), and musty (flavor associated with staleness). Then the panel was trained in the recognition and evaluation of these descriptors using a nonstructured 50 mm scale labeled with not perceivable (0) at one end and with strongly perceivable (5) at the other end. The samples were presented in sensory flasks encrypted with three-digit random numbers and in a randomized order. Each sample of bread crumb (containing 0, 0.7, 1.5, or 2% NaCl) was evaluated in three different sessions on consecutive days. The intensities for each of the given descriptors were recorded, and the reliability of assessors was checked by ANOVA (SigmaPlot 11.0, Systat, San Jose, CA, USA) with sample, panelist, and repetition as factors prior to averaging results for the bread samples. The statistical significance of mean panel scores from the triplicate analysis of each attribute was determined by ANOVA (Tukey test, $p < 0.05$).

Fractionation of Wheat Flour. The fractionation was performed on flour of wheat (cv. Tommi, 2008), which had been milled on a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany) to

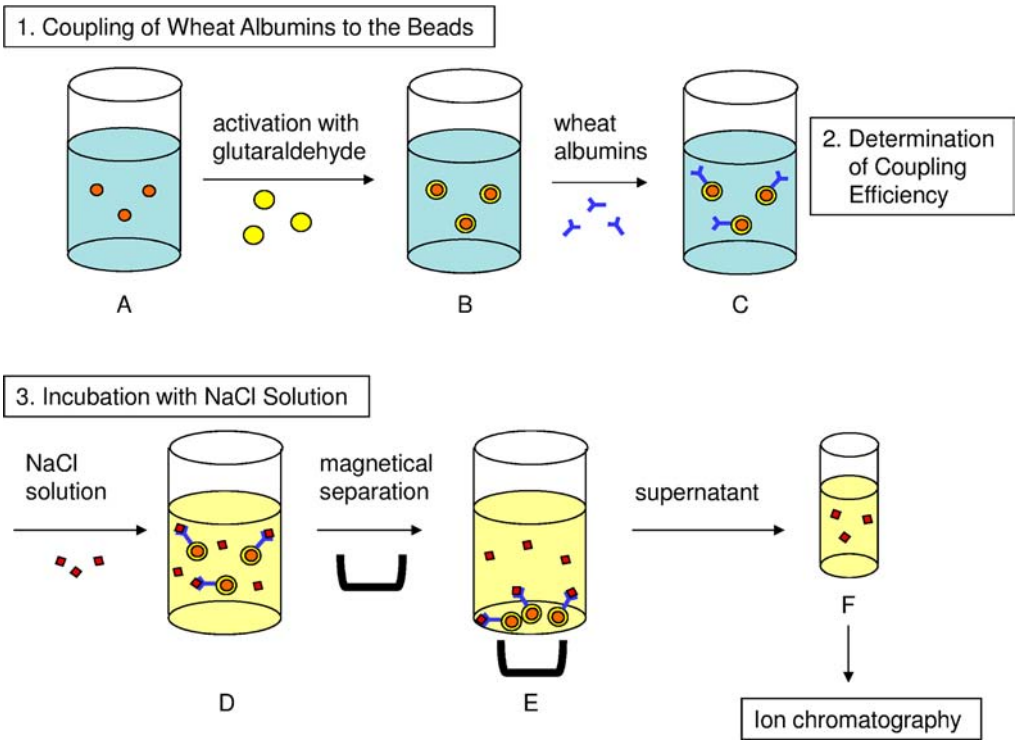

Figure 1. Schematic representation of the magnetic beads assay procedure: (A) magnetic beads suspension; (B) activated magnetic beads suspension with glutaraldehyde coupled to the free primary amino groups of the beads; (C) magnetic beads coupled with wheat albumins via glutaraldehyde as a covalent cross-link; (D) incubation of wheat albumin-laden magnetic beads with NaCl solution; (E) magnetic removal of wheat albumin-laden magnetic beads with bound sodium ions while unbound sodium ions remain in the supernatant; (F) transferral of the supernatant to a new vial and quantitation by ion chromatography.

ensure that no potentially interfering additives such as ascorbic acid were present as is the case in commercial flour.

Defatting. One hundred grams of wheat flour was stirred three times at room temperature for 30 min with 250 mL of *n*-pentane/ethanol (95:5; v/v) followed by stirring once with 250 mL of *n*-pentane. The solvent was removed by centrifugation (3750*g*, 15 min, 20 °C), and after the last extraction step, the residue was vacuum-dried overnight on a filter sheet and homogenized carefully.

Albumins. Fifty grams of defatted wheat flour was extracted three times with 200 mL of ultrapure water by homogenizing with an Ultra Turrax in a centrifuge vessel for 5 min at room temperature. The suspension was centrifuged (3750*g*, 25 min, 20 °C), and the supernatants were combined.

Albumins and Globulins. Fifty grams of defatted wheat flour was extracted three times with 200 mL of 0.067 mol/L K_2HPO_4/KH_2PO_4 containing 0.4 mol/L KCl (pH 7.6) as described above.

Gliadins. After the combined extraction of the albumins and globulins, the obtained residue was extracted three times with 200 mL of 60% (v/v) aqueous ethanol as described above.

The protein fractions extracted with salt solution (albumins/globulins) and aqueous ethanol (gliadins) were dialyzed (MW cutoff 12000−14000) against 0.01 mol/L acetic acid and in the last step against ultrapure water for 3 days. All protein fractions were freeze-dried and carefully pulverized in a mortar.

Nonreduced Glutenin Fraction. Fifty grams of defatted flour was mixed with 37.2 mL of distilled water for 12 min at 60 rpm and 22 °C to an optimum consistency of 550 BU in a Farinograph equipped with a 50 g Z-blade mixer. Gluten was washed out from this dough (16 g portions) with ultrapure water for 10 min by means of a Glutomatic system (Perten Instruments, Huddinge, Sweden), formed to small beads (diameter ≤ 4 mm), lyophilized, and ground after shock-freezing with liquid nitrogen in an ultracentrifugal mill ZM 200 (Retsch, Haan, Germany) using a 0.2 mm sieve. One and a half grams of the resulting powder was extracted three times with 60 mL of 60% (v/v) aqueous ethanol by homogenization for 2 min with a vortex mixer and magnetic stirring for 30 min at room temperature. After centrifugation (3750*g*, 20 min, 20 °C), the residue (nonreduced glutenin fraction) was again lyophilized and ground as described for gluten. The content of crude protein in each protein fraction was determined according to the Dumas method as described previously.[21] A conversion factor of 5.7 was used.

All isolated protein fractions (albumins, combined albumins and globulins, gliadins, and glutenins) were characterized by reversed-phase high-performance liquid chromatography (RP-HPLC) on C_{18} silica gel according to the procedure described by Gessendorfer et al.[24] The chromatograms (data not shown) were compared to the literature.[24,25]

Sodium Binding Capacity of Wheat Proteins. *Magnetic Beads Assay for Water-Soluble Wheat Albumins.* Amine-terminated magnetic iron oxide particles coated to provide primary amino groups (magnetic beads) allow the specific and covalent attachment of water-soluble proteins to a substrate without loss of functionality. The entire procedure is depicted in Figure 1. Bis-Tris propane/HCl was used as a buffer for all measurements because it contained only trace amounts of sodium and was thought not to compete with sodium for the protein binding sites because of its entirely different molecular structure and considerably larger size.

Coupling of Wheat Albumins to the Beads. Two and a half milliliters of magnetic beads suspension was transferred to a screw-capped glass tube, and 12.5 mL of ultrapure water was added. After vigorous shaking (vortex), the suspension was separated magnetically. The clear supernatant was aspirated and discarded. This was repeated three times with 12.5 mL of ultrapure water and then three times with 12.5 mL of 0.01 mol/L pyridine/HCl, pH 6.0 (coupling buffer). Five milliliters of glutaraldehyde solution (5%; v/v) was added, and after vortexing, the suspension was gently shaken for 3 h at room temperature. Next, the suspension was separated magnetically, the supernatant was discarded, and the beads were washed again three times with 12.5 mL of coupling buffer. Sixty milligrams of wheat albumins was dissolved in 10 mL of coupling buffer, and 2.5 mL was

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added to the beads, whereas 1 mL of the solution was set aside for the coupling efficiency determination (precoupling solution). After shaking the beads for 22 h at room temperature and magnetic separation, 200 μL of the supernatant was kept for the coupling efficiency determination (postcoupling solution), whereas the rest was discarded. Twelve and a half milliliters of 1.0 mol/L glycine/KOH, pH 8.0, was added to the beads followed by shaking for 30 min at room temperature. Then the beads were washed three times with coupling buffer, taken up in 0.1 mol/L Bis-Tris propane/HCl, pH 7.0 or 9.0, to yield a final concentration of 2×10^{-4} mmol wheat albumin/mL buffer, and stored in brown glass at 4 °C for no more than a week.

Determination of Coupling Efficiency. The amount of protein bound to the beads was determined by means of the 96-well plate BCA assay. For the external calibration 20 mg of wheat albumins was dissolved in 20 mL of coupling buffer and diluted 1:6, 1:3, 1:2, 1:1.5, and 1:1.2. The precoupling solution was diluted 1:10 and the postcoupling solution 1:2. Ten microliters of sample and calibration solutions was pipetted into the cavities of a 96-well plate, and 200 μL of copper(II) sulfate pentahydrate solution (4%, w/v)/BCA solution (0.5 + 25; v/v) was added. After an incubation time of 30 min in a water bath at 37 °C, the absorption at 562 nm was recorded with an ELISA-Reader (BMG Labtech, Ortenberg, Germany), and the amount of protein was calculated from the calibration function. The amount of bound protein was calculated from the difference between pre- and postcoupling solution. All determinations were done in duplicate.

Incubation of Albumin-Laden Beads with NaCl Solution. NaCl was dissolved in 0.1 mol/L Bis-Tris propane/HCl, pH 7.0 or 9.0, buffer to yield a concentration of 25 mg Na^+/L. Five hundred microliters of NaCl solution was pipetted into a vial, and different volumes (50, 100, 200, 300, 400, and 500 μL) of albumin-coupled beads were added and adjusted to a final volume of 1000 μL with buffer. A control was prepared with 500 μL of NaCl solution and 500 μL of buffer. All solutions were vortexed and mixed vigorously for 15 min at room temperature on a laboratory shaker. After magnetic separation of the beads, the supernatant was transferred into a new vial, and the concentration of sodium ions was quantitated by ion chromatography (IC) via external calibration. The amount of bound sodium was given by the difference between the control and the samples. All determinations were done in triplicates.

Sodium-Selective Electrode for Water-Insoluble Wheat Proteins. The combined albumin/globulin fraction, the gliadins, and the glutenins were incubated with buffered NaCl solutions (pH 7 or 9). According to their crude protein contents, 400−800 mg of each protein fraction (albumins/globulins, gliadins, glutenins) was weighed into centrifuge tubes (crude protein weight = 400 mg). After the addition of 10 mL of 3.33 mol/L NaCl/0.5 mol/L tris(hydroxymethyl)aminomethane (pH 7 or 9), the mixtures were homogenized for 30 s with a vortex mixer and stirred for 30 min at room temperature. The free sodium ions in the suspensions were quantitated by an ion-selective electrode (ISE). For the determination of control values the same procedure was carried out with 0.5 mol/L tris(hydroxymethyl)aminomethane (pH 7 or 9) without NaCl. The sodium binding capacity of each protein fraction was calculated from the difference to the initial sodium concentration. All determinations were done in triplicates.

Calculation of the Theoretical Sodium Binding Capacity of Wheat Proteins. On the basis of data on the amino acid composition of wheat proteins,[26] the theoretical sodium binding capacity of the proteins in wheat flour as a whole as well as the single protein fractions (albumins, globulins, gliadins, and glutenins) was calculated under the assumption that a maximum amount of 1 mol of sodium ions could be bound by 1 mol of acidic amino acid side chains. Moreover, the theoretical binding of chloride ions to wheat proteins was calculated assuming that a maximum amount of 1 mol of chloride ions could be bound by 1 mol of basic amino acid side chains. Considering the ionic interactions between acidic and basic amino acid side chains of wheat proteins, the theoretical sodium binding capacity of the surplus of acidic amino acids was calculated for the protein fractions albumins, globulins, gliadins, and glutenins after subtracting the percentage of basic amino acids from the percentage of acidic amino acids. To obtain the sodium binding capacities expressed in milligrams per gram protein, the amount of amino acids per gram protein was calculated using the mean molecular mass of an amino acid in wheat protein, which was obtained from the amino acid composition of wheat protein.[26]

Determination of Total Sodium in Bread Crumb. Two grams of bread crumb containing 0, 1, 2, and 4% NaCl was weighed into round-bottom flasks (250 mL). After the addition of 15−20 glass beads and 5 mL of nitric acid (65%, w/w), the flasks were heated under reflux. As soon as yellow vapors became visible, 5 mL of hydrogen peroxide (30%, w/w) was added. After 15 min of heating, another 5 mL of hydrogen peroxide was added, and the solutions were refluxed for a further 15 min. The solutions were left to cool and transferred into volumetric flasks (100 mL). After the addition of 2 mL of cesium chloride solution (50 mg cesium/mL), each flask was filled to the mark with ultrapure water. Control values were determined in the same way but without bread crumb. For the quantitation of sodium by inductively coupled plasma optical emission spectrometry (ICP-OES) and atomic absorption spectroscopy (AAS), the solutions were filtered and diluted with ultrapure water while maintaining a cesium concentration of 1 mg/mL. All determinations were done in triplicates, and statistical significances were determined by ANOVA (SigmaPlot 11.0, Systat).

Mastication Simulator. A modified Potter S Homogenizer (Braun, Melsungen AG, Germany) was used to simulate mastication. The corresponding glass cylinders were replaced by centrifugal tubes, which were placed in a water bath at 37 °C. Five milliliters of extracting agent (ultrapure water, 0.1 mol/L tris(hydroxymethyl)-aminomethane (pH 7 or 9), artificial saliva[27,28] consisting of 0.85 g/L NaCl, 0.13 g/L K_2HPO_4, 1.2 g/L KCl, 0.17 g/L $CaCl_2 \cdot 2H_2O$, and 0.107 g/L $MgCl_2 \cdot 6H_2O$; with and without α-amylase (200,000 U/L), or human saliva of two subjects) was added to 3 g of bread crumb containing 0.25, 0.5, 1, 1.5, 2, 4, and 6% NaCl. Then mastication was simulated in the centrifugal tube for 1 min by the combination of an automated rotation (150 rpm) and a manual up-and-down movement (compression/decompression rate of 1/s) of a piston (PTFE). The obtained chewing pulps were centrifuged (3750*g*, 10 min, 20 °C), and the sodium concentrations in the supernatants were quantitated by IC, AAS, and/or ISE. Control values were determined in the same way with bread crumb baked without NaCl. All determinations were done in triplicates, and statistical significances were determined by ANOVA (SigmaPlot 11.0, Systat).

Mastication in Vivo. Three grams of bread crumb was chewed in the mouth for 60 s. The resulting chewing pulps were spat out and centrifuged (3750*g*, 10 min, 20 °C). The sodium concentrations in the supernatants were quantitated by a sodium-selective electrode. Triplicate determinations were carried out by the same two subjects whose saliva had been used before in the mastication simulator, and statistical significances were determined by ANOVA (SigmaPlot 11.0, Systat).

Ion Chromatography. *Sodium.* Sodium was quantitated using a Dionex ICS-2000 apparatus with an AS autosampler, an eluent generator equipped with a RFIC EluGen cartridge EGC II MSA, a digital conductivity detector DS6, and a suppressor CSRS 300 (Dionex, Idstein, Germany). Data analysis was performed using Chromeleon software 6.80. The column used was a 250 mm × 2 mm i.d., Ion-Pac CS-18, with a 50 mm × 2 mm i.d. guard column of the same material (Dionex) at a flow rate of 0.3 mL/min and a temperature of 40 °C. The cations were eluted using 5 mmol/L methanesulfonic acid under isocratic conditions during 20 min. A cation self-regenerating suppressor operating at 5 mA was installed between the column and the conductivity detector. For quantitation, external standard calibration was done in concentrations ranging from 0.5 to 100 mg Na/L (six-point calibration).

Chloride. Chloride was quantitated with a Dionex ICS-2000 system equipped with a suppressor ARS, and the software was Chromeleon 6.50. The column used for the analyses of the bread extracts was a 250 mm × 2 mm i.d. Ion-Pac AS11-HC (Dionex) operated at 30 °C at a flow rate of 0.38 mL/min. The gradient was 1 mmol/L KOH for 8 min, increasing to 15 mmol/L KOH within 10 min, increasing to 30

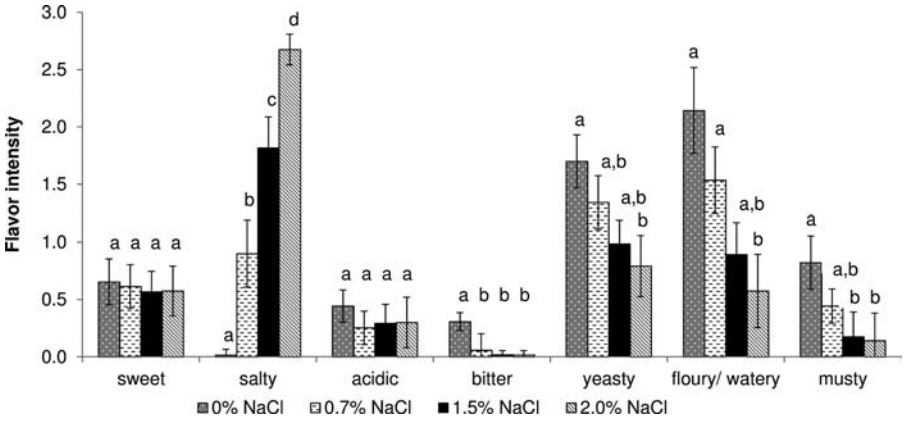

Figure 2. Flavor profiles of wheat bread as affected by the NaCl content (0, 0.7, 1.5, and 2% NaCl based on flour). Mean values associated with different letters within one flavor descriptor denote significant differences (ANOVA, Tukey test, $p < 0.05$). Error bars represent mean standard deviations of triplicate determinations by 15 panelists. The total scale ranged from 0 (not perceivable) to 5 (strongly perceivable).

mmol/L KOH within 10 min, and isocratically with 30 mmol/L KOH for another 4 min.

Ion-Selective Electrodes. Sodium, potassium, and calcium were quantitated using a Metrohm 781 pH-/ion-meter (Metrohm, Filderstadt, Germany) and specific polymer membrane-based ion-selective electrodes for sodium, potassium, or calcium in combination with a double-junction Ag/AgCl reference electrode (LL ISE Reference; reference electrolyte, 3 mol/L KCl). The standard solutions as well as the sample solutions were diluted in the ratio 1:1 with total ionic strength adjustment buffer (TISAB; for sodium, 1 mol/L tris(hydroxymethyl)aminomethane, pH 7) or ionic strength adjustment solutions (ISA; for potassium, 0.1 mol/L NaCl; for calcium, 1 mol/L KCl). For quantitation, external standard calibration was used for all cations in concentrations ranging from 5 to 5000 mg/L (eight-point calibration).

Atomic Absorption Spectroscopy. Sodium was quantitated by flame atomic absorption spectroscopy using an atomic absorption spectrometer Varian SpectrAA 100 (Agilent Technologies, Santa Clara, CA, USA). The samples were measured at a wavelength of 589.6 nm and a gap width of 1.0 nm without background correction. For quantitation, external standard calibration was done in concentrations ranging from 0.2 to 2 mg Na/L (five-point calibration).

Inductively Coupled Plasma Optical Emission Spectrometry. Sodium was quantitated using an ICP-OE spectrometer Optima 3000 (PerkinElmer Inc., Waltham, MA, USA). The plasma was created with argon. For quantitation, external standard calibration was done with a solution of 100 mg Na/L (single-point calibration).

RESULTS AND DISCUSSION

Flavor Profile Analysis of Breads with Different NaCl Levels. The impact of four different salt levels, 0, 0.7, 1.5, and 2% NaCl, on the flavor profile of bread crumb was assessed to gain a fundamental insight into the impact of NaCl reduction on the following flavor attributes: sweet, salty, acidic, bitter, yeasty, floury/watery, and musty. Breads without salt had a bland, insipid taste and were rated as predominantly yeasty, floury/watery, and musty (Figure 2). A slight, but significant, bitter note was also detected that disappeared as soon as salt was added to the recipe, which can be explained by the ability of salt to mask bitterness.[1] No significant differences were observed between the breads with regard to acidic flavor. With increasing levels from 0.7 to 1.5 and 2% NaCl, the breads were described as less and less yeasty, floury/watery, and musty, whereas the salty taste became significantly more intense with every salt level added (Figure 2). These findings correlate well with earlier results.[29] As expected, the intensity of sweetness remained unchanged, because 1% sucrose was part of the recipe for all breads. Therefore, the addition of salt had an impact on the entire flavor profile of bread crumb because it not only added saltiness itself but led to a reduced perception of unwanted flavor attributes such as musty and floury/watery.

Perception of Saltiness in Wheat Bread. *Perceptibility of Saltiness in Wheat Bread Depending on the Absolute Salt Concentration.* The perceptibility of saltiness in breads with 0−2% NaCl was assessed by means of 2-AFC tests. The absolute difference of 0.3% NaCl was the same for all pairs that were evaluated against each other, that is, 0 vs 0.3%, 0.3 vs 0.6%, 0.7 vs 1.0%, 1.0 vs 1.3%, 1.2 vs 1.5%, and 1.3 vs 1.6%. The panelists were not able to distinguish between breads containing high amounts of 1.2/1.5% NaCl ($\alpha = 0.1$) and 1.3/1.6% NaCl ($\alpha = 0.2$), respectively. However, as soon as the salt level was reduced to 1.0%, it was possible to identify the bread with 1.3% as being saltier ($\alpha = 0.01$). Once the salt level was further reduced, the difference between the two breads became even more distinct, so that the saltier bread was correctly identified in the 0/0.3% ($\alpha = 0.01$) and 0.3/0.6% ($\alpha = 0.01$) pairings, respectively. One reason, in particular for the 0/0.3% pairing, may be the characteristic bland, floury/watery, yeasty, and musty flavor of the bread without salt. Another reason is the complex relationship between chemical concentration, taste detection threshold, and suprathreshold intensity of tastants.[30] The findings agree well with Weber's law of psychophysics, which states that the amount of a physical stimulus, which is needed to increase to be just noticeably different (JND), is a constant ratio $k = \Delta I/I$ (I = intensity of the physical stimulus, i.e., salt concentration).[31−33] Therefore, the gustatory sensation may be more sensitive and the sensory resolution better at low absolute tastant concentrations so that small differences can be perceived more accurately. Consequently, as seen here, the absolute difference between two stimuli needs to be higher to be perceived as different at higher tastant concentrations.

Perceptibility of Variable Differences of Salt Compared to Bread with 1% NaCl as a Reference. Because the perceptibility of saltiness in wheat bread depended on the absolute amount of salt, this relationship was investigated in more detail by taking bread with 1% NaCl as a reference, because this seemed to be a critical concentration. The saltiness of breads with 2.0, 1.6, 1.5, 1.3, and 1.2% NaCl, respectively, was compared to this reference by means of 2-AFC tests to determine the lowest detectable difference. All breads containing 1.3% NaCl and

Table 1. Comparison of Theoretical and Experimentally Determined Sodium Binding Capacities of Wheat Protein Fractions at pH 7 and 9

	calculation				experimental determination[a]	
protein fraction	content of acidic amino acids (mol %)	theoretical sodium binding capacity by acidic−basic amino acids (mg Na/g protein)	difference acidic−basic amino acids (mol %)	theoretical sodium binding capacity by acidic-basic amino acids (mg Na/g protein)	sodium binding capacity at pH 7 (mg Na/g protein)	sodium binding capacity at pH 9 (mg Na/g protein)
albumins/ globulins	9.2	19.1	−0.8	−1.7	0.04 ± 0.00	0.09 ± 0.02
gliadins	2.3	4.6	−1.9	−3.8	0.08 ± 0.01	0.14 ± 0.02
glutenins	2.8	5.8	−3.8	−7.8	0.01 ± 0.01	0.06 ± 0.02

[a]Mean values ± standard deviations of triplicate determinations.

above could be significantly distinguished ($\alpha \leq 0.01$) from the bread with 1% NaCl. However, the difference between the bread with 1.2% NaCl and the reference with 1% NaCl was too small to be recognized ($\alpha = 0.2$), thus making 0.3% NaCl the lowest perceivable difference.

Perceptibility of Variable Differences of Salt Compared to Bread with 0% NaCl as a Reference. Similarly, the saltiness of breads with 0.7, 0.5, 0.3, 0.2, 0.1, 0.075, and 0.05% NaCl, respectively, was assessed in 2-AFC tests in comparison to bread with 0% NaCl. Here the breads containing 0.3% NaCl and more were all rated significantly ($\alpha = 0.001$) saltier. Breads containing very low amounts of only 0.2 and 0.1% salt could also be significantly distinguished ($\alpha = 0.01$) from the bread without salt. Even the slight difference of 0.075% NaCl was perceived as significantly ($\alpha = 0.05$) saltier compared to the bread containing no salt. Only the difference of 0.05% NaCl was too small to be noticed by the panel ($\alpha = 0.1$). During the sensory evaluation of these breads containing 0.1% salt and below, it became apparent that the actual definition of saltiness in bread crumb was very difficult. Although these low-salt breads could not be described as explicitly salty, there was a significant difference, which could be attributed to the entire flavor. As has been described above, bread without salt had a characteristic bland, insipid taste (Figure 2), so that already the addition of minor amounts of NaCl improved the entire flavor and allowed differentiation. Due to the design of the 2-AFC tests, which asked for saltiness as the only attribute to evaluate, differences in flavor might have been projected onto saltiness because this was the only descriptor available to the panelists. This effect, known as dumping,[34] was clearly a limitation of this part of the study. However, it is known that salt intensifies the flavor even if the level is too low to impart the salty taste itself,[1,2] which explains these findings very well. In the case that the overall salt content is to be reduced below 1% NaCl, the increased sensitivity at low tastant concentrations has to be taken into consideration, because the lowest detectable difference was only 0.075% with 0% NaCl as reference bread compared to 0.3% with 1% NaCl as reference bread.

Sodium Binding to Wheat Proteins. An important technological function of NaCl in bread is its strengthening effect on wheat gluten.[13,14] This suggests ionic interactions between charged amino acid side chains of gluten proteins and sodium or chloride ions resulting in improved aggregation, for example, due to a decrease of the net charge of gluten. Chloride ions can interact with positively charged side chains of histidine, lysine, and arginine, whereas sodium ions can interact with negatively charged side chains of aspartic and glutamic acid. To get an idea of the theoretical ion binding capacity of wheat proteins, data on the amino acid composition of wheat protein[26] were used, which also reported a content of 4.3 mol % acidic and 6.4 mol % basic amino acid side chains. On the basis of these data, a maximum amount of 0.95 mg of sodium or 2.18 mg of chloride was calculated that could be bound by 1 g of wheat flour used in this study via ionic interactions. This amount of sodium ions corresponds to a content of 0.24% NaCl in bread. Assuming complete interaction between sodium ions and negatively charged amino acid side chains in wheat proteins, a considerable portion of sodium in bread would not be available for sensory perception; that is, in bread containing 1% NaCl, only the sodium ions of 0.76% NaCl (corresponding to 76% of the added amount of sodium ions) could be perceived.

For the precise determination of sodium binding to wheat proteins, the wheat protein fractions albumins, a combined albumin/globulin fraction, gliadins, and glutenins were extracted from wheat flour cv. Tommi according to a modified Osborne fractionation.[25,35] This procedure was slightly changed to avoid sodium impurities. For this reason ultrapure water instead of desalted water was used for the extraction of the albumins and a potassium hydrogen phosphate buffer instead of a sodium-containing buffer for the extraction of the combined albumin/globulin fraction. Additionally, the albumin/globulin and gliadin fractions were extensively dialyzed against 0.01 mol/L acetic acid and in the last step against ultrapure water. Because changes in protein structures such as denaturation due to the extraction method were likely to influence sodium binding capacities, the entire procedure was carried out as gently as possible. To ascertain the integrity of the proteins, the protein patterns of the isolated albumin, albumin/globulin, gliadin, and glutenin fractions were characterized by RP-HPLC (data not shown). A comparison to the literature[24,25] revealed no significant differences, which indicated that the proteins were intact. Nevertheless, modifications of the native protein structures after isolation of the respective fractions cannot be excluded. The sodium binding capacity of the obtained protein fractions was determined by two different methods. A magnetic beads assay (MB) was used for the water-soluble wheat albumins, whereas a sodium-selective electrode (ISE) was applied for the other water-insoluble protein fractions.

Magnetic Beads Assay for Water-Soluble Wheat Albumins. The wheat albumins were capable of binding sodium ions, and the amount of bound sodium rose from 0.37 (±0.16) mg/g albumins at pH 7 to 0.55 (±0.17) mg/g albumins at pH 9. This increase, even if it is not significant, can be explained by the higher amount of deprotonated free aspartic and glutamic acid residues at pH 9, which can interact with the positively charged sodium ions. Because only water-soluble proteins can be attached to the magnetic beads, a second method was used to study sodium binding of water-insoluble wheat proteins such

Table 2. Content of Protein Fractions of Wheat Flour cv. Tommi, Sodium Binding Capacities of the Fractions, and the Corresponding NaCl Contents in Bread[a]

protein fraction	content in wheat flour (%)	Na^+ bound to fraction at pH 7 (mg/g protein)	Na^+ bound to fraction at pH 7 (mg/100 g flour)	NaCl content in bread at pH 7[b] (%)
albumins[c]	0.87 ± 0.07	0.37 ± 0.16	0.322	<0.001
albumins/globulins[d]	1.16 ± 0.02	0.04 ± 0.00	0.042	<0.001
gliadins[d]	5.73 ± 0.15	0.08 ± 0.01	0.428	0.001
glutenins[d]	3.34 ± 0.17	0.01 ± 0.01	0.016	<0.001

[a]Mean values ± standard deviations of triplicate determinations. [b]Based on flour. [c]Determined by magnetic beads assay. [d]Determined by sodium-selective electrode.

as the combined albumin/globulin fraction, the gliadins, and the glutenins.

Sodium-Selective Electrode for Water-Insoluble Wheat Proteins. The highest sodium binding capacity was measured for the gliadins, followed by the albumin/globulin fraction. No significant sodium binding was observed for the glutenins at pH 7, but at pH 9 the glutenins also bound sodium. In accordance with the results of the magnetic beads assay for the albumins, the measurements revealed a higher sodium binding capacity at pH 9 for each protein fraction as compared to pH 7 (Table 1). As the amount of dissociated acidic amino acid side chains increases with increasing pH, this dependency on pH suggests that ionic interactions with negatively charged acidic amino acid side chains are involved in the binding of sodium ions. Nevertheless, no correlation could be observed between the sodium binding capacities either with the content of acidic amino acids or with the difference of acidic and basic amino acids in the protein fractions (Table 1). This indicates that the local chemical environment of the particular amino acid residues in the complex protein fractions, the secondary and the tertiary structure, have to be taken into consideration as well. The binding of sodium ions seems to be affected by more complex mechanisms, for example, by a chelating mechanism involving carboxyl−imidazol and carboxyl−amino pairs as described for β-lactoglobulin.[36] To elucidate the exact mechanism by which sodium is bound by wheat proteins, further studies on single proteins isolated from the wheat protein fractions would be necessary.

Sodium binding to the combined albumin/globulin fraction was lower than the results for the pure wheat albumin fraction, determined by the magnetic beads assay. Considering the contents of these protein fractions in wheat flour (Table 2), this difference cannot be explained only with the assumption that no sodium could be bound by the additionally contained globulins. It seems likely that this observation is due to the two different determination methods.

Because all wheat protein fractions bound sodium, the question was to what extent this would be relevant for the perception of saltiness in bread. For this purpose, the corresponding amounts of sodium chloride in bread were calculated on the basis of the sodium binding capacities of each protein fraction at pH 7 (Table 2). According to these values, about 0.002% NaCl could be bound to wheat proteins in bread. However, as shown above, 0.3% NaCl is the lowest perceivable difference in bread crumb when bread containing 1% NaCl is used as a reference. Even when bread crumb containing no NaCl is used as a reference, a difference of 0.075% NaCl is necessary to enable sensory distinction. Therefore, it can be concluded that sodium binding by wheat proteins is negligible concerning the sensory perception of saltiness in bread crumb.

The sodium binding capacities were determined using protein fractions gained from wheat flour. Although the extraction procedure was done as gently as possible, changes in native protein structures due to the extraction from flour cannot be excluded. Because sodium binding is thought to be dependent not only on the amino acid distribution but also on the entire native protein structure, the question was if the measured sodium binding capacities of the different protein fractions correspond to the extent of sodium binding in bread crumb. Differences might occur due to modified protein structures caused by the breadmaking process or due to further interactions with other bread ingredients. Therefore, sodium binding in bread crumb was determined by extracting bread crumb in a mastication simulator.

Extractability of Sodium and Chloride from Bread Crumb during Mastication. *Extractability of Sodium Ions with Water.* Sodium ions were completely extractable in the mastication simulator from bread crumb containing 1, 2, and 4% NaCl with ultrapure water as extracting agent and after quantitation by AAS, IC, and ISE (Figure 3). The amounts of sodium measured after extraction with ultrapure water corresponded to those determined by AAS and ICP after total hydrolysis of bread crumb. No significant differences were observed between all methods used for the quantitation of sodium, with the lowest standard deviations being obtained for the measurements with ISE. In addition, the analytical results

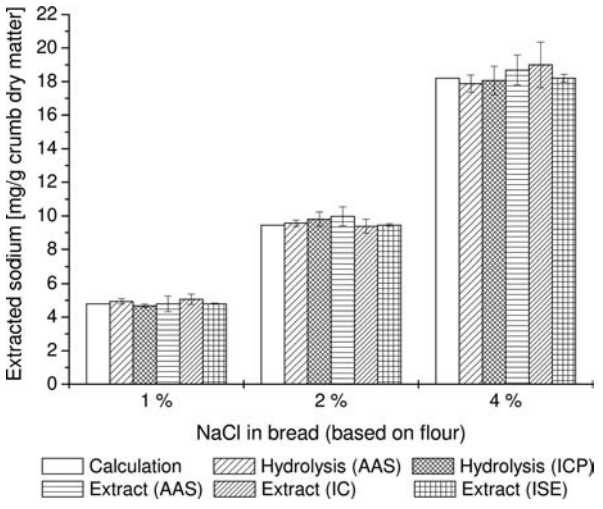

Figure 3. Sodium content of bread crumb determined by AAS and ICP after hydrolysis in comparison to the calculated sodium content and the sodium extractability in mastication experiments, determined by AAS, IC, and ISE. Error bars represent mean standard deviations of triplicate determinations.

were in agreement with the sodium contents obtained by calculation from the recipe (Figure 3). Therefore, the interactions between sodium ions and bread ingredients are weak enough to enable a complete extraction of sodium ions after 1 min of chewing in the mastication simulator. Assessing the influence of the breadmaking process on the sodium binding capacities of the wheat proteins is impossible, as they are too small to be detected with this test arrangement.

Previous studies on the recovery of sodium from wheat and rye breads after incineration and AAS determination compared to extraction with buffer solution (pH 7.8) and ISE quantitation found even higher recovery rates for the determination by ISE (about 100%) than for the determination by AAS (about 95%). As only free sodium ions are detectable by ISE, Rabe[37] concluded that only a minimal part of the sodium ions could be complexly bound in bread.

Extractability of Chloride Ions with Water. In corresponding mastication experiments bread crumb containing 1, 2, and 4% NaCl was extracted with ultrapure water. The extracted amounts of chloride were determined by IC and compared with the stoichiometric contents of sodium, assuming that the amount of chloride endogenously contained in flour is negligible. With these experiments a complete extractability of chloride ions was observed (data not shown). As saltiness is mainly evoked by sodium ions, the extractability of chloride ions was not further investigated.

Extractability of Sodium Chloride with Buffer Solutions and Artificial Saliva. To assess the influence of pH value and salivary ingredients (inorganic salts, α-amylase) on sodium extractability from bread crumb, buffer solutions and artificial saliva with and without α-amylase were used as extracting agents in addition to ultrapure water. The results depicted in Figure 4 virtually show no differences between the different

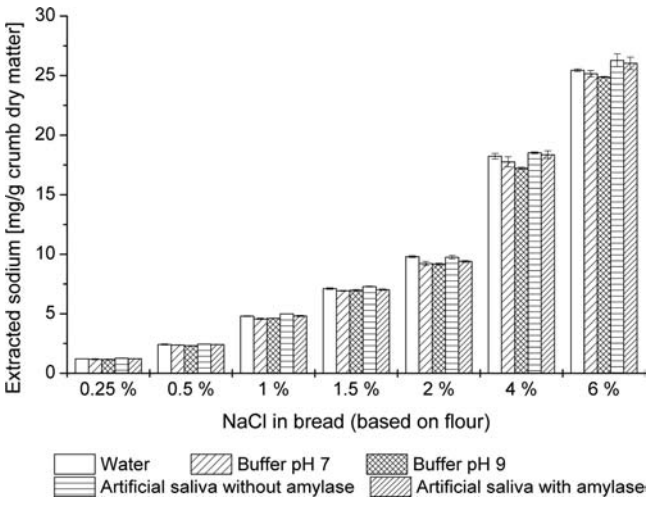

Figure 4. Sodium extractability from bread crumb in the mastication simulator with ultrapure water, buffer solutions (pH 7 or 9), and artificial saliva (without or with α-amylase). Error bars represent mean standard deviations of triplicate determinations.

extracting agents. Although the differences are partially significant by way of calculation, no consistent tendency is recognizable among the significantly different results. Even the biggest significant difference, which occurred between the sodium extraction with buffer solution (pH 9) and artificial saliva without α-amylase from bread crumb containing 6% NaCl, was <0.3%. As already discussed above, a difference of 0.3% NaCl would be necessary to detect differential saltiness of wheat bread, if bread containing 1% NaCl is used as a reference. As the perceivable difference in the content of NaCl increases with rising absolute NaCl concentration, it can be concluded that this difference between the extracted amounts of sodium from bread crumb containing 6% NaCl is not perceivable.

Extractability of Sodium Ions with Human Saliva. The extractability of sodium was additionally tested under more realistic conditions with human saliva collected from two subjects in the mastication simulator with bread crumb containing 1.5% NaCl. The results are depicted in Figure 5

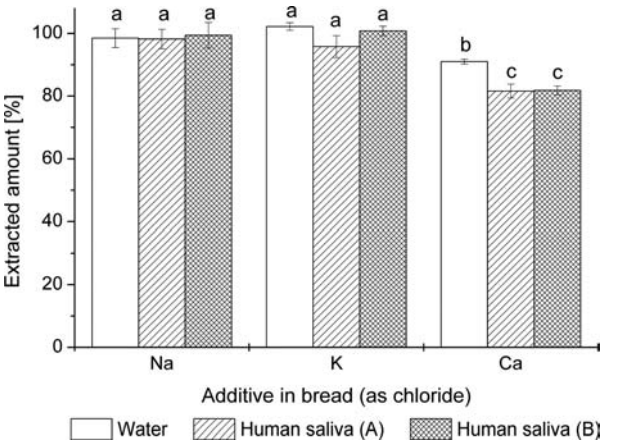

Figure 5. Extractability of sodium, potassium, and calcium from bread crumb (0.26 mol/kg flour) in the mastication simulator with ultrapure water and human saliva of two subjects (A and B). Mean values associated with different letters are significantly different (ANOVA, Tukey test, $p < 0.05$). Error bars represent mean standard deviations of triplicate determinations.

and also show complete sodium extraction. Therefore, the ingredients of human saliva appear to have no impact on the extractability of sodium ions from bread crumb during mastication.

Extractability of Sodium Ions in vivo. To compare the results of sodium extractability after simulated mastication (in vitro) to the conditions in vivo, bread crumb was chewed by the same two subjects that had already donated their saliva for the experiments with the mastication simulator. After a chewing time of 1 min, the entire amount of sodium was released in vivo and there were no significant differences between subjects A and B or between in vivo and artificial mastication. Therefore, the mastication simulator was a reliable tool to model in vivo mastication of bread crumb for the chosen time interval of 1 min.

These investigations showed that interactions between sodium ions and wheat bread ingredients were weak and did not reduce sodium extractability in the mastication simulator and in vivo. Therefore, it is not possible to enhance the saltiness of bread by increasing the overall extractability of sodium. Because mastication is a dynamic process, there is also a temporal dimension to the delivery of sodium and parameters such as food matrix and texture, and oral parameters such as chewing behavior and salivary flow rate are known to influence sodium extractability from chewing gum,[38] lipoprotein matrices,[39,40] and potato crisps.[41] Further studies on the kinetics of sodium release from bread crumb during mastication in the mouth are currently underway and will provide more fundamental information on salt perception in bread. As fast

Table 3. Recovery Rates of Calcium Dissolved in Human Saliva of Two Subjects (A and B)[a]

	subject A		subject B	
Ca^{2+} added (mg/mL saliva)	2.4	4.8	2.4	4.8
Ca^{2+} detected (mg/mL saliva)	2.17 ± 0.05	4.14 ± 0.14	2.25 ± 0.14	4.46 ± 0.10
recovery rate (%)	90 ± 2	86 ± 3	94 ± 6	93 ± 2

[a]Mean values ± standard deviations of triplicate determinations.

changes in the concentration of sodium in saliva lead to an increased salt perception,[42] the velocity of sodium release during the first seconds of mastication seems to be crucial for the perceived saltiness. Consequently, the saltiness of bread could be intensified by generating a faster release of sodium ions, which would allow a reduction of sodium in bread.

Comparison between the Extractability of Sodium, Potassium, and Calcium. As bivalent cations such as calcium ions act as cofactors in numerous enzymes, stronger interactions of proteins with bivalent cations can be expected than with monovalent ones. To evaluate the influence of ionic valence on the extractability of cations from bread crumb, breads were baked with equimolar concentrations (0.26 mol/kg flour) of NaCl (1.5%), KCl (1.9%), and $CaCl_2 \cdot 2H_2O$ (3.8%) and extracted in the mastication simulator with ultrapure water as well as with human saliva of two subjects. The extracted amounts of sodium, potassium, and calcium were determined by ISE and are depicted in Figure 5. The monovalent cations (sodium and potassium) were completely extractable with all extracting agents. In contrast, only 91% of the calcium ions were extractable with ultrapure water and about 9% of the calcium ions remained bound to bread ingredients. This lower extraction rate of calcium can be explained by stronger interactions of wheat proteins with bivalent than with monovalent cations (e.g., by complex formation). In the extracts obtained with human saliva only 82% of the calcium ions were detected. Therefore, the recovery rates of calcium in the human saliva of both subjects were determined with different calcium/saliva ratios (Table 3). The recovery rates averaged only about 91%; that is, the other 9% of calcium in the saliva extracts could not be detected with the calcium-selective electrode due to interactions of saliva ingredients either with calcium ions or with the polymer membrane of the electrode. The calculation of undetected calcium per milliliter of human saliva showed that there is no saturation of undetectable calcium, but the amount of undetectable calcium seems to correlate with the amount of added calcium. Because less calcium was detected in the saliva of subject A than in the saliva of subject B, especially at the higher calcium concentration (2.4 mg calcium added per mL saliva (p = 0.40); 4.8 mg calcium added per mL saliva (p = 0.03)), saliva ingredients seem to have an impact on the recovery rates of calcium.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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