Determining the Effect of Calcium Cations on Acrylamide Formation in Cooked Wheat Products Using a Model System

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A model system was used to cook wheat flour and water dough pieces in sealed pressure tubes under controlled pH conditions and with various additives in the recipe water to determine acrylamide (AA) formation and elimination. The potential effectiveness of calcium as $CaCl_2$ or $CaCO_3$ salts to reduce the formation of AA in wheat based food products was assessed. Since the divalent Ca^{2+} was capable of inducing significant pH reduction in the dough, and pH lowering is known to reduce AA formation, it was necessary in some cases to adjust the pH before cooking or use a pH matched control. For comparison, the effect of NaCl on AA formation was also determined. It was found that AA reduction up to 36% was obtained by adding $CaCl_2$ to the recipe water at a 0.04 M concentration, compared to 23% for 0.04 M NaCl, and there was no reduction when $CaCO_3$ was added to simulate a calcium enriched flour.

KEYWORDS: Acrylamide; cereal; wheat; flour; baking; calcium chloride; divalent cations; GC-MS

INTRODUCTION

Acrylamide (AA), a suspected human carcinogen, has been detected in cooked foods containing asparagine and sugar, including potatoes and wheat, and mitigation strategies have been reviewed (*1−3*). Some reviews have focused specifically on the formation and methods of reduction of AA in wheat based foods (*4−8*). Recently, there has been interest in studying the use of mono-, di-, and trivalent metal cations, and in particular Na^+ and Ca^{2+}, to prevent formation of AA in model systems with asparagine and sugar (*9−11*), potatoes (*12−15*), and wheat (*6, 7*).

AA formation and elimination are known to be pH sensitive with a maximum net formation around pH 7 to 9 (*7, 16−18*), and lowering pH has been suggested as a way to reduce AA in foods (*19*). Therefore, the study of potential mitigating factors, such as Ca^{2+} and other cations, should properly control for pH if comparable data is desired. Recent studies of the effect of $CaCl_2$ on AA formation in a potato model system included data on the pH of the model (*13, 15*).

Metal cations have long been known to cause pH reduction when added to certain foods or food components. A 1927 report noted that when several plant tissues were placed in contact with various mono- or divalent cationic salts of varying molarities, the solutions became more acidic to different degrees that depended on salt concentration and the nature of the cation, and an emphasis was placed on the study of $CaCl_2$ (*20*). The mechanism for metal ion induced pH lowering has been generally attributed to the competitive displacement of protons from ionizable functional groups, containing oxygen, nitrogen, or sulfur atoms that share electrons with hydrogen atoms, when the pH is high enough

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to permit mass action competition between protons and the metal cations (*21*), but the pH must not be so high that most protons have been removed prior to adding the metal ions. Lowering of pH in spinach chloroplast suspension was found to be related to cation valence, the order was tri- > di- > monovalent based on the magnitude of pH change, and the effect was attributed to protonated carboxyl groups in proteins and lipids (*22*). When diced tomatoes were dipped in a $CaCl_2$ solution, the requirement for a separate acidification step was eliminated (*23*). It was shown that cooking potato disks in solutions of varying concentrations of $CaCl_2$ resulted in increased compressive strength at high concentrations, principally due to pH lowering induced by calcium binding to potato tissue rather than a direct effect of the Ca^{2+} cations, and the pH after cooking was the key parameter needed to reach that conclusion (*24*). Vadlamani and Seib reported that zinc chloride and aluminum sulfate salts at 50 to 500 ppm (flour basis) reduced the initial pH of dough used for breadmaking from 5.9 to as low as 4.5 (*25*).

The pH of cooked wheat is known to decrease significantly during cooking, and the magnitude of the decrease can increase with either the initial pH or cooking temperature (*26, 27*). This has been attributed to the Maillard reaction, which shows similar pH change properties (*17, 28*). Therefore, studies of wheat based models need to consider pH before and also after cooking in order to obtain comparable data.

The objective of this study was to determine AA formation and elimination of $^{13}C_3$-enriched AA (^{13}C-AA) in a model system based on flour and water dough to determine the effects of $CaCl_2$ and NaCl under comparable pH conditions measured before and after cooking. A sealed reactor minimized the loss of moisture which would otherwise have made pH control and measurement difficult (*29*).

MATERIALS AND METHODS

The 1,2,3-^{13}C$_3$-acrylamide (^{13}C-AA, 99%) was from Cambridge Isotope Laboratories (Andover, MD). A stock solution was prepared by pipetting exactly 1 mL of the 1 mg/mL methanol solution into a 100 mL volumetric flask, the methanol was carefully evaporated to near dryness without heat under nitrogen flow, and then the labeled AA was diluted with water for the stock and working solutions. Pastry flour (100% soft wheat, *Triticum aestivum* L., same brand and lot used throughout) was obtained from the manufacturer. Solvents were of pesticide residue grade: ethyl acetate from EMD Chemicals (Gibbstown, NJ), deionized water prepared in house, and other solvents from Burdick and Jackson (Muskegon, MI). Chemicals were from Sigma Aldrich (St. Louis, MO) and were greater than 98% purity. The 10 mL unbuffered Chem Elut Hydromatrix (diatomaceous earth) columns were from Varian (Lake Forest, CA).

The concentrated nitric acid to digest samples for elemental analysis was of GFS Chemicals (Columbus, OH) redistilled trace metal grade, and the 30% hydrogen peroxide, 99.999% purity, was from Sigma Aldrich. For ICP-OES, stock solutions of yttrium for internal standard and other elements for standard curves were from CPI International (Santa Rosa, CA) and diluted according to appropriate schemes to match the sample matrix.

Measurement of pH. The pH values of solutions and doughs were measured with an IQ150 hand-held meter and an ISFET stainless steel probe from IQ Scientific Instruments (Carlsbad, CA). A 2-point calibration with the appropriate bracketing buffers was performed before every analysis. For dough pH measurements, a method similar to that of Miller et al. (*30*) was followed. About 10 g of dough was placed in a 20 mL vial, and the probe was pressed firmly against the dough to maintain good contact with the solid state sensor. Repeated measurements were made until a stable reading was obtained. In between dough pH measurements, the probe calibration was checked, and the sensor was cleaned and recalibrated as necessary.

Preparation of Dough Model. Two milliliters of 5 μg/mL ^{13}C-AA and appropriate volumes of 2 M CaCl$_2$, 1 M NaCl, 1 N HCl, or 1 N NaOH were added to water, making a final volume of 25 mL. In some cases where the final CaCl$_2$ concentration was 0.02 M or higher, an amount of NaOH solution was also added to give a final normality in 25 mL that was half or one-quarter the CaCl$_2$ molarity, as required, to adjust the dough pH near 6. For controls, 25 mL of water only was used. In one experiment, CaCO$_3$ dry powder was mixed with the flour. Then 40 g of flour was added to the water and mixed using a hand-held mixer with dough hooks at low speed for 2 min and then rested at room temperature for 30 min. The dough was kneaded by hand for 2 min, and pieces of 4.25 g were weighed, each rolled into a cylindrical shape about 5 cm long.

Cooking the Dough Model. The 15 mL pressure tube equipment was from Ace Glass (Vineland, NJ). The heavy wall, threaded glass tubes were sealed with solid PTFE plugs and Viton O-rings. For temperature measurements, the solid plug was replaced with a 1/8 in. NPT adaptor (Ace Glass), a 12 in. × 1/16 in. type-T thermocouple was inserted into the sample center through an NPT to 1/16 in. compression fitting, both from Omega Engineering (Stamford, CT), and the measurements were stored on a Digi-Sense DualLogR thermocouple thermometer from Cole Parmer (Vernon Hills, IL) at 30 s intervals.

Each cylindrical dough piece was inserted into a 15 mL pressure tube. The dough was gently pressed against the side of the glass tube with a spatula where the slight dough stickiness held it in place; this increased the dough surface area in contact with the glass and also prevented the dough from falling to the bottom of the tube when it was held vertically. Then 150 μL of water was added to the bottom of the tube, to saturate the air inside with water vapor during cooking and minimize drying of the exposed dough surface, and then the tube was sealed with a plug. The pressure tubes were heated in a surplus GC oven. In every case, the oven was preheated to a temperature 40 °C above the desired cooking temperature and held at that temperature during the first 2 min of sample heating, then the temperature was lowered to the cooking temperature, which is the value reported. After completion of the heating period, the glass tubes were air cooled by a fan at room temperature. Dough samples were cooked at 140 °C for 40 min or other conditions as stated.

Extraction, Cleanup, and GC-MS Analysis of AA from Dough Model. Each dough piece was removed from the pressure tube after cooling to room temperature, cut into smaller pieces with a spatula, and then transferred to a nominally 50 mL Teflon 28.5 × 107 mm centrifuge tube from Nalgene Labware (Rochester, NY), and the dough pH was measured. The pressure tube was rinsed with 5 mL of water that was then transferred to the centrifuge tube. A thick glass rod was used to mix the cut dough pieces and water until a lumpy paste formed, and an additional 10 mL of water was then added and mixed to form a slurry. The tube was centrifuged in an IEC model HT with 856 rotor at 17,000g for 60 min. The supernatant was decanted to fill a 10 mL graduated cylinder to the mark and poured onto a 10 mL Hydromatrix column, and after 15 min, the column was eluted with 125 mL of ethyl acetate. The ethyl acetate was heated in a 60 °C water bath using appropriate glassware and evaporated under a stream of nitrogen until the volume was about 15 mL; the evaporation was continued in a 15 mL graduated conical tube to final 1 mL volume, being careful not to let the sample go dry. The GC-MS method was identical to that reported earlier (*31*) except for minor changes in the column temperature program. In short, 1 μL of ethyl acetate was injected into a Varian GC with a dbWAXETR column (15 m × 0.25 μm × 0.25 mm) from Agilent/J&W (Wilmington, DE), and the temperature program after holding at 60 °C for 1 min was 60 to 170 °C at 12 °C/min, followed by 170 to 200 °C at 50 °C/min and held 200 °C for 5 min. A Saturn 4D ion trap mass spectrometer operating in the chemical ionization mode and a mass range of 60−80 was used, and the peak areas of the [M + 1]$^+$ ions, m/z 72 and 75, were integrated to determine AA and ^{13}C-AA, respectively. In one experiment to determine the extraction efficiency of ^{13}C-AA from cooked dough, the ^{13}C-AA was omitted from the recipe, and after cooking the dough, a syringe was used to make multiple injections of 5 to 10 μL each of the 5 μg/mL aqueous ^{13}C-AA solution into the chopped pieces of cooked dough until a total of 131 μL was injected, and then the extraction and cleanup proceeded as described above.

Digestion and ICP-OES Analysis of Wheat. Digestion of wheat flour was performed with a CEM Corporation (Matthews, NC) MARS-5 microwave digester equipped with an HP-500 rotor. About 2 g of flour was accurately weighed and placed in a Teflon-lined vessel, 7 mL concentrated nitric acid and 2 mL 30% hydrogen peroxide were added, and the vessels were capped and torqued with the venting nut loosened and allowed to sit overnight in a fume hood. The next day, the venting nuts were tightened and vessels placed on the turntable, and the temperature and pressure probes were attached. After the digestion program was carried out and the vessels cooled to 50 °C, they were vented in a fume hood, and the contents were poured into an appropriate volumetric flask and diluted to volume with deionized water.

A Varian (Walnut Creek, CA) Vista-Pro ICP-OAS (axial view) instrument with CCD detector was used to determine elemental composition. The elements calcium, iron, potassium, magnesium, manganese, and sodium were determined at the appropriates wavelengths with background correction on both sides of the peaks. Power was 1.25 kW. Plasma flow rate was 15.0 mL/min argon with a nebulizer flow of 1.0 L/min argon and using an auxiliary flow of 1.5 L/min argon. The integration time was 3.0 s, and 3 replicates were performed. A Sea-Spray nebulizer was used with a Twister style spray chamber.

The elemental determinations of the wheat flour, in mg/kg (14% moisture basis) were K, 3050 ± 47; Mg, 1040 ± 33; Ca, 405 ± 3.7; Na, 36.0 ± 0.81; Mn, 27.9 ± 0.27; and Fe, 19.6 ± 0.57; with standard deviations based on $n = 2$.

RESULTS AND DISCUSSION

ICP-OAS Analysis of Wheat. Results of elemental analysis are in general agreement with wheat composition data reported in the literature (*32−34*).

For the dough model system, determination of the actual concentrations of free ionized cations in the aqueous phase of dough during cooking at the temperature where AA can form from water-soluble precursors would be difficult and was not attempted. In this article, only the elemental composition of flour and the exact initial recipe conditions are stated, and they do not directly determine the true concentrations of metal ions in the

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dough when volume of free water, cation binding, and other important factors are taken into account.

Some general observations can be made about the amounts and relative solubilities of the elements determined. Potassium is the cationic element present in greatest amount and also has a relatively high water solubility. Potassium in oat bran was found to be 80% soluble when starches and proteins were digested (*35*). The calcium and magnesium in wheat flour were found to have low water solubilities, about 5 and 15% soluble, respectively (*36*), presumably due to their high binding levels and precipitation as insoluble phytate or other salts. But note that although water solubilities have been studied, the actual concentrations of calcium and magnesium cations in solution as free ions in the aqueous dough phase, as opposed to water-soluble complexes of cations that are not able to affect the AA formation mechanisms, have not been determined. Another complication was that magnesium concentrations were not likely to remain constant when varying amounts of $CaCl_2$ were added to the recipes because of the interactions between the minerals (*21*).

Dough Model. The use of sealed, heated reactor tubes has been reported in models based on potato (*13*) and dough pieces (*27*), and a modified method was followed. Since the mass of the thick walled glass pressure tube used here made it somewhat difficult to heat quickly, especially when run in sets of 8 at a time, the oven was heated 40 °C above the desired cooking temperature for the first 2 min to speed the temperature rise inside the dough pieces without any danger of overshooting the cooking temperature, which resulted in temperatures measured at the center of a typical sample during cooking of 121, 136, and 140 °C at times of 5, 10, and 15 min, respectively, a curve shape similar to that reported for cooked samples in a stainless steel reactor using an oil bath at constant temperature (*17*).

Previous research on model systems was consulted before selecting time and temperature conditions for the dough model. It has been shown that optimum pH for AA formation is in the range of 7 to 9 (*7, 16−18*). But maintaining a relatively constant pH in a sealed system during the cooking process tends to become more difficult as the initial pH increases or the temperature increases. For example, when ground wheat samples, after pH adjustment with benzoic acid or NaOH, were cooked in sealed glass ampules for 70 min at 121 °C, the pH decreased by 0.24 and 1.49 units from initial pH values of 6.12 and 7.81, respectively (*26*). Using a basic flour, water and salt dough, the pH dropped from 5.91 to 4.73 in a pressure-cooking apparatus at 180 °C for 20 min (*27*). Reductions in pH are often attributed to the Maillard reaction. When initial pH values and cooking temperatures for pH buffered asparagine−glucose closed reactor systems were varied independently to model AA formation, decreases in pH measured after heating were increased significantly in magnitude either by increasing initial pH from 6 to 8 at constant temperature or by increasing temperature from 120 to 200 °C at constant pH (*17*).

Preliminary tests for this project (data not shown) indicated similar results for the sealed tube dough model system; for example, although it was easier to form AA at pH above 7, the control of pH was problematic even with buffer added to the dough and cooking temperature reduced to 120 °C. Major objectives of this study were to minimize pH changes during cooking, add minimal amounts of salts to control pH, and not eliminate entirely the ^{13}C-AA spike. Compromise conditions were selected by using the considerable intrinsic buffer capacity of dough near pH 6 (*37*) and limiting the cooking parameters to 140 °C at 40 min, which permitted a significant net amount of AA to be formed while keeping the pH changes to about 0.5 to 0.7 units for controls and the lower concentrations of $CaCl_2$. **Table 1**

Table 1. Acrylamide Formation and Elimination in Dough Model Control Affected by Cooking Time at 140 °C

cooking time (min)	pH[a] before	pH after	AA		^{13}C-AA		
			μg/kg	SD[b]	% rec.[c]	SD	n[d]
0	6.03		nd[e]		96.8	5.8	8
10	6.00	5.75	8.1	1.4	73.6	3.3	4
20	6.02	5.63	46.1	6.4	61.4	3.3	4
30	6.03	5.55	65.1	6.8	35.3	1.7	4
40	6.03	5.53	110.5	10.5	29.0	3.3	22

[a] Estimated uncertainty of pH measurements: pH before ± 0.02; pH after ± 0.05. [b] Standard deviation. [c] AA elimination during heating expressed as percent recovery of ^{13}C-AA added to recipe water; for comparison, the recovery from dough spiked after heating was 85.1 ± 1.7 with n = 4, and note the recovery from dough at 0 min cooking. [d] Number experiments. [e] Not detected.

shows that a minimal change of about 0.1 pH unit in controls occurred between 20 and 40 min and there was a significant amount of AA formed in that time period. Therefore, since it was not possible to measure the actual pH of the sample inside the pressure tube at the 140 °C cooking temperature, the pH of the room temperature sample after cooling was considered to be the closest available indication of relative pH conditions that existed during cooking while the AA was forming and being eliminated at the highest rates, and net formation was also increasing. A similar conclusion that final pH is significant in a pressure-cooked dough model system was reached from a study of formation of mono-chloropropanediols (*38*) because final pH coincided with maximum temperature and formation rate.

The method recovery was determined by using a syringe to spike samples with ^{13}C-AA solution after cooking and was found to be 85.1% ± 1.7 (*n* = 4), while the recovery from uncooked dough where the spike was included in the recipe water was 96.8% ± 5.8 (*n* = 8). The somewhat reduced recovery from cooked dough may have been caused by the rubbery nature that made it more difficult to get a complete penetration by water into the entire sample during extraction. The amount of AA impurity in the ^{13}C-AA spike was below the detection limit of 2 μg/kg after dilution in the dough.

Experimental data is presented in **Table 2**. The data for the control was based on 6 batches of dough prepared throughout the time period of the project for a total of 22 samples in order to include batch-to-batch as well as sample-to-sample variation in the statistics. The mean AA formation in 4.25 g of flour and water dough pieces cooked in sealed tubes at 140 °C for 40 min was 110.5 μg/kg ± 10.5, and the mean ^{13}C-AA recovery was 29.0% ± 3.3. Since the method recovery was good, it was clear that the lower recoveries when the dough was spiked prior to cooking were caused by elimination of ^{13}C-AA during the heating process rather than by a loss during the extraction and cleanup. This was further confirmed by the recovery versus cooking time data in **Table 1** showing that elimination increased with time. Although there was a loss of ^{13}C-AA in control dough during the last 20 min of cooking, the final recovery near 30% was high enough to test for possible effects of salts and pH on elimination.

It appeared that pH after heating was the only measured factor that had a slight correlation with ^{13}C-AA elimination (data from **Table 2** plotted in **Figure 1**) with a linear regression coefficient R^2 of 0.82; the highest concentration of NaCl was not included because the apparent pH reduction at high ionic strength compared to that of the control was likely the result of lowered activity coefficients. Although not conclusive, the plot suggests that the reduced elimination at higher calcium concentrations was due primarily to the decrease in final pH that accompanied calcium increase rather than any other effect of the calcium because the

samples where pH values were changed by adding HCl or NaOH showed a similar trend.

Effect of pH. The effect of pH on AA formation in the dough model system, for each sample where pH was adjusted with HCl or NaOH, plus the control, is shown in **Figure 2**. Error bars on the control and the dotted lines represent expanded uncertainty with a coverage factor of 2 and a level of confidence of approximately 95%. None of the individual pH adjusted samples differed from the control in AA formation by more than experimental uncertainty, but the total set of data showed a correlation with final pH values in the range from 5.32 to 5.71 with a linear regression

Table 2. Acrylamide Formation and Elimination in Dough Model Affected by Calcium, Sodium, and pH, Heated at 140 °C for 40 min

added to recipe water	pH[a] before	pH after	AA			^{13}C-AA		
			μg/kg	SD[b]	% diff.[c]	% rec.[d]	SD	n[e]
control	6.03	5.53	110.5	10.5		29.0	3.3	22
CaCl$_2$ 0.005 M	6.00	5.52	98.3	11.4	−11.0	28.3	4.7	8
CaCl$_2$ 0.01 M	5.97	5.52	93.6	4.9	−15.3	29.2	1.5	7
CaCl$_2$ 0.02 M + NaOH 0.01 N	6.03	5.56	81.1	6.4	−26.6	29.8	2.5	4
CaCl$_2$ 0.02 M	5.88	5.41	83.1	6.5	−24.8	33.1	2.0	4
CaCl$_2$ 0.04 M + NaOH 0.02 N	6.09	5.37	71.3	6.3	−35.5	31.0	3.3	6
CaCl$_2$ 0.04 M	5.70	5.05	65.9	11.5	−40.4	39.4	5.0	3
CaCl$_2$ 0.08 M + NaOH 0.02 N	6.00	5.05	58.8	4.5	−47.1	45.5	2.7	8
NaCl 0.02 M	6.03	5.52	113.9	11.0	3.1	25.6	1.7	8
NaCl 0.04 M	6.08	5.51	85.5	7.4	−22.6	23.5	2.2	8
NaCl 0.08 M	6.09	5.65	85.6	7.9	−22.5	26.3	1.5	4
NaCl 0.44 M	5.85	5.33	80.1	2.4	−27.5	32.1	1.5	3
HCl 0.005 N	5.93	5.51	109.9	10.1	−0.5	30.5	2.8	4
HCl 0.01 N	5.87	5.39	101.1	7.8	−8.5	29.2	2.9	4
HCl 0.02 N	5.68	5.32	93.9	7.0	−15.0	36.0	3.4	4
NaOH 0.005 N	6.06	5.57	114.2	13.3	3.4	25.0	1.8	8
NaOH 0.01 N	6.19	5.60	112.9	13.7	2.2	22.2	1.8	4
NaOH 0.02 N	6.35	5.71	120.5	8.9	9.1	22.2	1.1	4
CaCO$_3$ 0.21 g[f]	5.99	5.52	105.7	5.3	−4.3	31.3	1.9	8

[a] Estimated uncertainty of pH measurements: pH before ± 0.02; pH after ± 0.05. [b] Standard deviation. [c] Difference in AA formation compared to control in percent. [d] AA elimination during heating expressed as percent recovery of ^{13}C-AA added to recipe water; for comparison, the recovery from dough spiked after heating was 85.1 ± 1.7 with n = 4. [e] Number of experiments. [f] Solid CaCO$_3$ mixed with the flour component of the recipe.

R^2 of 0.97 (straight line not plotted; regression parameters calculated from data in **Table 2**). Using the linear regression equation (AA = 66.7 × pH − 259), we determined that the AA formed at the lowest pH was reduced 21.4% compared to the highest pH over a range covering 0.39 pH units. The correlation was consistent with literature reports demonstrating that lowering pH caused reduction in AA formation, although the results differed quantitatively due to recipes and cooking conditions being changed; for example, it was found that pH in dough before cooking had a linear correlation with the net AA formed in bread over a range from pH 3 to 8 and that there was a 12% decrease in AA for each drop of 1 pH unit (7).

Selection of Comparable Data. Figure 3 shows the effects of recipe CaCl$_2$ and NaCl concentrations on AA formation. The figure includes a subset of data from **Table 2** where samples and the control had comparable pH values, each with initial and final pH within ranges of 0.06 and 0.16 pH units, respectively. The 0.08 M CaCl$_2$ and 0.44 M NaCl pH values fell outside those ranges. Again, error bars on the control and the dotted lines represent 95% confidence limits, which makes it possible to ascertain which samples were significantly different from the control.

Effect of NaCl. The NaCl samples were evaluated because some of the CaCl$_2$ samples also had NaOH added as an adjustment to keep the initial pH near 6 to compensate for the changes in pH caused by Ca^{2+} ions, and it was known that added Na$^+$ cations affected AA formation in other model systems (6, 10, 12, 15). We also desired the evaluation of NaCl because it is a commonly used ingredient in bread and other wheat based products in relatively high amounts. The dough model with NaCl concentration of 0.02 M in the recipe water did not differ from the control, while model samples prepared from concentrations of 0.04 and 0.08 M showed a marginally significant reduction in AA formation of about 23% (**Figure 3**). The 0.44 M recipe concentration (**Table 2**) corresponded to about 1% NaCl based on the dough weight, which is a typical value used in bread baking. Although the high ionic strength of that sample complicated interpretation, it was noted that the increase in NaCl from 0.08 to 0.44 M made only a small difference in AA formation. In comparison with the literature, it was found that optimum concentrations of 1 to 2% NaCl minimized AA in bread and roll production (6) and a flour and water dough with 1.3% NaCl
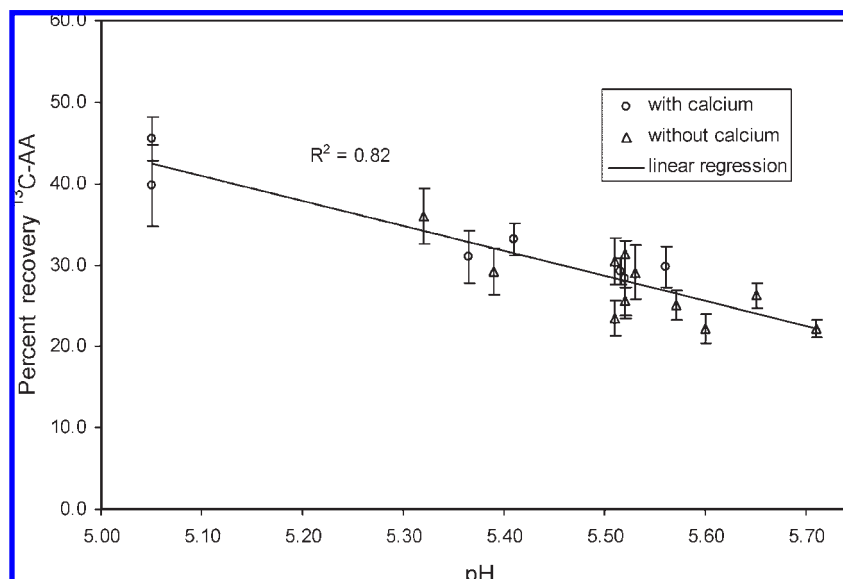

Figure 1. Correlation between percent recovery of ^{13}C-AA and the pH after cooking for dough pieces with various amounts of calcium or sodium chloride, or acid or base that had been added to the recipe. See **Table 2** for data. Error bars are standard deviations. The 0.44 M NaCl sample is not included.
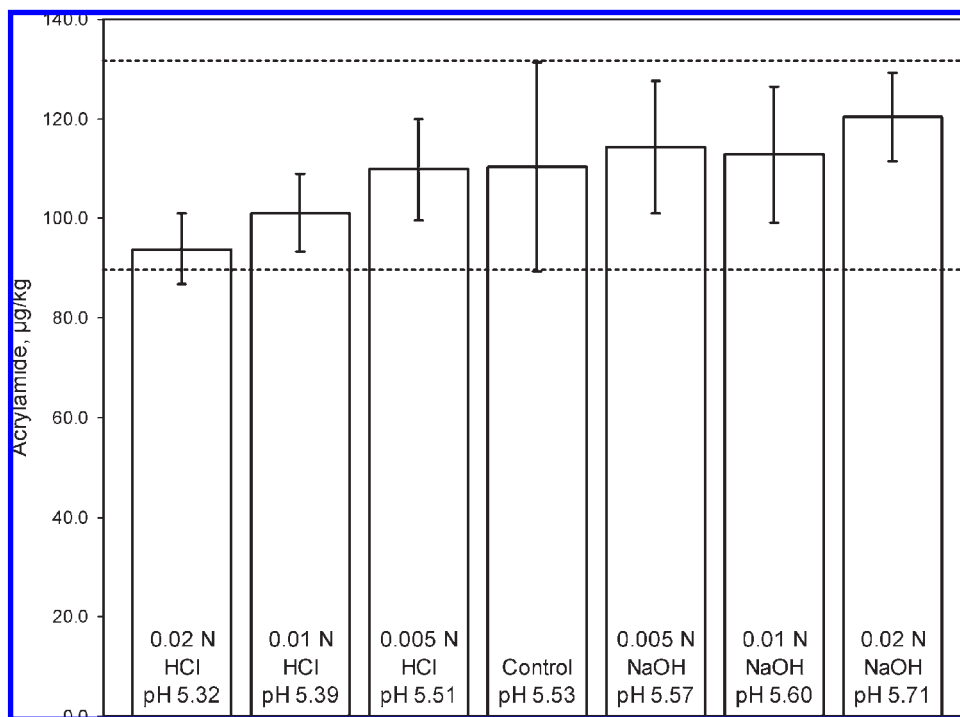
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Figure 2. Graph represents the effect of pH on acrylamide formation in the dough model system. The sample pH was adjusted with varying HCl or NaOH normality in the recipe as shown, plus the unadjusted control. Final dough pH values after heating are shown. Control error bars and the dotted lines represent expanded uncertainty with a coverage factor of 2 and a level of confidence of approximately 95%. Error bars on the other samples represent standard deviations.
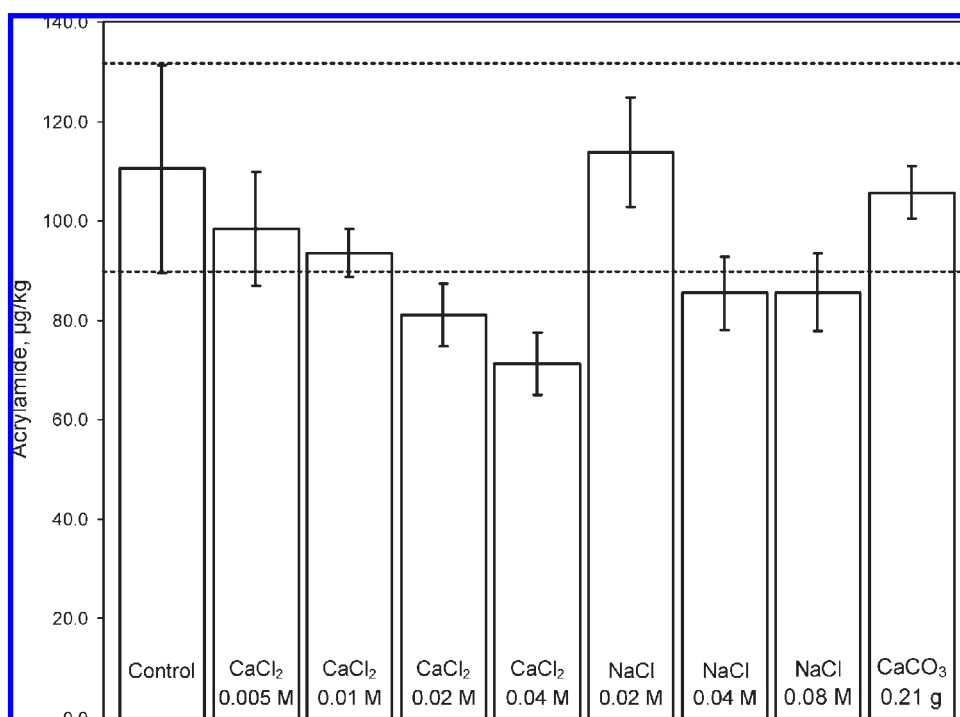

Figure 3. Graph represents the effect of salt concentration on acrylamide formation in the dough model system, subset of samples with comparable initial and final pH. Concentrations of added salts are expressed as molarity of the water component of the recipe, except for insoluble $CaCO_3$ which was mixed as a solid with the wheat flour. Dough samples with 0.02 and 0.04 M $CaCl_2$ also had 0.01 and 0.02 N NaOH, respectively. Control error bars and the dotted lines represent expanded uncertainty with a coverage factor of 2 and a level of confidence of approximately 95%. Error bars on the other samples represent standard deviations.

decreased AA formation by about 40% in a cracker model baked at 180 °C for 15 min (*31*), but salt levels below 1% were not considered in either study.

Effect of $CaCl_2$. As the $CaCl_2$ in the dough recipe water increased from 0.005 to 0.04 M, there was a trend of decreasing AA formation during cooking that reached statistical significance

compared to the control for the 0.02 and 0.04 M samples with AA reduced by 26.6 and 35.5%, respectively (**Table 2** and **Figure 3**). At recipe concentrations up to 0.01 M $CaCl_2$, no pH adjustments were made to match the control since the initial dough pH values stayed within 0.06 units. In order to keep the other $CaCl_2$ samples within 0.06 units initial pH compared to that of the control, NaOH normalities equal to half the $CaCl_2$ concentrations were used for doughs where the recipe water had 0.02 and 0.04 M $CaCl_2$, while only 0.02 N NaOH was required to adjust the 0.08 M $CaCl_2$ dough pH; the need for relatively less NaOH to adjust pH at the higher Ca^{2+} concentration was presumed to be due to fewer protons in the food matrix being available for displacement by cations when binding sites become increasingly saturated with cations. None of these recipes had a Na^+ concentration above the 0.02 M NaCl level that was shown to have no significant effect compared to the control. For additional confirmation, samples with 0.02 and 0.04 M $CaCl_2$ in the recipe water were also prepared without NaOH pH adjustment. The 0.01 N HCl sample was observed to be a matched control for the 0.02 M $CaCl_2$ sample because pH values before and after cooking were nearly equal, and the sample containing calcium lowered AA by a significant amount (24.8%), while the HCl control did not (8.5%).

At the 0.04 M $CaCl_2$ level, the buffer capacity of the dough and the NaOH added to adjust the initial pH were just marginally adequate to keep the pH after cooking within 0.16 units of the control, while at 0.08 M, the pH dropped during cooking by almost a full pH unit or 0.48 units below that of the control. At 0.08 M, therefore, the level of AA reduction was no longer attributed mostly to direct effects of Ca^{2+} on the reaction mechanisms since the larger pH drop likely became a more important factor as an indirect effect. It also showed the importance of measuring pH after cooking since, based only on the initial pH values being equal, the decrease in AA in the 0.08 M $CaCl_2$ dough compared to 0.04 M would have been attributed solely to calcium ions. The pH drop during cooking was found to be a function of the $CaCl_2$ concentration even when the calcium induced pH drop before cooking was compensated with NaOH.

In a comparison with the literature for wheat based models, when 1% $CaCl_2$ was added to flour, salt, and water bread dough or cracker dough, the AA formation during cooking was reduced about 35% or 60%, respectively; when added at the 2% level in sweet and savory biscuits, the reduction was about 60%, and it was suggested that $CaCl_2$ can be applied more efficiently to baked products by adding it to a commercial tin-release agent (7).

Dough prepared with 0.02 M $CaCl_2$ in the water component of the recipe corresponded to about 300 ppm calcium in the dough on a weight basis, which is a very high level of soluble calcium salt that is not likely to be used in a wheat based food product. However, the 0.04 M recipe concentration of NaCl, considerably less than the amount of salt normally used in baking, suppressed AA formation by 22.6%, which was nearly as much as the 26.6% reduction with 0.02 M $CaCl_2$, and demonstrated in this model system that calcium provided little additional AA suppression over sodium when the calcium salt was limited to levels that permitted pH control. Although these effective concentrations of the sodium and calcium salts with similar AA reduction have molarities that differed by only a factor of 2, the actual concentrations of the respective free cations undoubtedly differed by a larger amount because binding of divalent calcium cations in a food matrix is greater than binding of monovalent sodium cations. Additional studies of the dough aqueous phase, either with ion selective electrodes or with other means, would be required to estimate the actual concentrations of free sodium and calcium ions that are assumed to be the species that suppress

AA formation. Even without knowing the exact free calcium concentration, it was clear that on a molar basis the amount of added free calcium must have been very high compared to the AA reduction. In comparison, the data from samples where pH was lowered by HCl suggested that a similar reduction was attained by adding relatively fewer H^+ ions. It is therefore difficult to speculate about possible mechanisms for such a weak calcium effect.

Effect of $CaCO_3$. $CaCO_3$ is among the preferred sources of calcium for the fortification of cereal-based foods since, being water insoluble, it minimally affects product quality. On the basis of the solubility product of $CaCO_3$ at room temperature in pure water, the concentration of Ca^{2+} ions in the dough model was expected to be too low to affect AA formation. However, there are several possible factors in a cooked food model that might shift the equilibrium and change solubility, such as heat, acidity, partial pressure of CO_2 (39), and binding of free Ca^{2+} ions with other food components. Therefore, an experiment was performed with 0.21 g of $CaCO_3$ mixed with the 40 g of unenriched flour in the dough recipe (0.21% calcium in flour by weight), which corresponded to the level of calcium in enriched flour as specified in the U.S. Code (21 CFR 137.165), which is stated as 960 mg calcium per pound. It was found (**Figure 3**) that that amount did not significantly affect AA formation in the dough model. The negative result may have been partly due to the dough being cooked in a sealed tube that maintained a higher partial pressure of CO_2 with corresponding reduced solubility of $CaCO_3$ compared to an open system, although partial pressure of CO_2 was found to have a relatively small effect on solubility at pH 6 in simulated gastrointestinal environment at room temperature with a greater effect only at pH 7.5 (39). In an earlier report of a bread system, dough with pure base flour had 30% more AA formation than dough with 0.3% $CaCO_3$ added when they were both cooked, and there was about a 10% reduction in sweet and savory biscuits prepared with 1% $CaCO_3$ in the recipe (7). The relatively small differences compared with this report may be due to the bread and biscuit doughs being cooked in open systems and the different recipes.

In conclusion, for this dough model system, in some cases where samples with added $CaCl_2$ in the recipe had comparable pH with that of the control both before and after cooking, the effect was to reduce AA formation by an experimentally significant amount, up to 36% for 0.04 M $CaCl_2$. But that concentration was relatively high for wheat products that do not generally include soluble calcium salts in the recipes. The AA reduction was not much greater than that in samples with 0.04 M NaCl, a salt concentration less than one-tenth that used in a typical bread recipe. Adding $CaCO_3$ to simulate enriched flour was found to have no effect on AA under the conditions of this model. The advantages of the model included simplicity, resemblance to an actual food, constant and known temperature throughout the sample, constant water content, comparable pH levels before and after cooking, and the fact that a single recipe variable was changed at a time to determine the effect. Disadvantages included unknown concentrations of soluble, uncomplexed cations in the aqueous phase of the dough and the unknown pH value at the temperature where AA formation and elimination were taking place.

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