

Acrylamide Formation in Almonds (*Prunus dulcis*): Influences of Roasting Time and Temperature, Precursors, Varietal Selection, and Storage

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ABSTRACT: Acrylamide is a probable human carcinogen that is found in many roasted and baked foods. This paper describes two sensitive and reliable LC-(ESI)MS/MS methods for the analysis of (1) acrylamide and (2) common acrylamide precursors (i.e., glucose, fructose, asparagine, and glutamine) in raw and roasted almonds. These methods were used to evaluate the impact of roasting temperatures (between 129 and 182 °C) and times on acrylamide formation. Controlling the roasting temperature at or below 146 °C resulted in acrylamide levels below 200 ppb at all roasting times evaluated. Six varieties of almonds collected in various regions of California over two harvest years and roasted at 138 °C for 22 min had acrylamide levels ranging from 117 ± 5 μg/kg (Sonora) to 221 ± 95 μg/kg (Butte) with an average of 187 ± 71 μg/kg. A weak correlation between asparagine content in raw almonds and acrylamide formation was observed ($R^2 = 0.6787$). No statistical relationship was found between acrylamide formation and almond variety, orchard region, or harvest year. Stability studies on roasted almonds indicated that acrylamide levels decreased by 12.9–68.5% (average of 50.2%) after 3 days of storage at 60 °C. Short-term elevated temperature storage may be another approach for mitigating acrylamide levels in roasted almonds.

KEYWORDS: acrylamide, almond, asparagine, roasting, color, LC-MS/MS

INTRODUCTION

Acrylamide is a chemical intermediate used in the production of polyacrylamides for numerous industrial applications.^{1,2} Acrylamide has been shown to induce tumors in experimental animals, is neurotoxic, and is classified as a probable human carcinogen (group 2A) by the International Agency for Research on Cancer (IARC).^{3–6} In 2002, the Swedish National Food Administration and the University of Stockholm announced the finding of high levels (micro- to milligrams per kilogram) of acrylamide in foods cooked at elevated temperatures.^{7,8} This finding led to worldwide concern and increased research activities investigating the formation and mitigation of acrylamide in foods.^{9–14} Although the relevance to human health of dietary exposure to acrylamide is unclear, regulatory agencies such as the World Health Organization (WHO) continue to encourage food manufacturers to take measures to reduce acrylamide levels in processed foods.¹³

Acrylamide is formed through reactions between free amino acids and reactive carbonyls (e.g., reducing sugars) via the Maillard reaction typically occurring at temperatures above 120 °C.^{9–12} Stable isotope-labeling studies in potato-based foods and model systems indicate that the backbone of acrylamide arises from asparagine.¹⁰ Strong correlations between asparagine levels and acrylamide formation have been reported in almonds,^{15–17} green tea,¹⁸ and yeast-leavened wheat or rye breads.^{19,20} Alternative formation mechanisms have also been described for lipid-rich foods.^{21,22} For example, studies of California-style black ripe olives suggest that acrolein and/or acrylic acid, formed from oxidized lipids, may react with ammonia, generated through the decomposition of amino acids, to form acrylamide.²³

The amount of acrylamide in roasted almonds depends upon the content of asparagine in raw almonds, the roasting time and temperature, and water content.^{15–17} Acrylamide levels in roasted almonds range between 260 and 2000 μg/kg.^{11,15} Almonds are susceptible to acrylamide formation as they contain relatively high levels of asparagine (500–2760 mg/kg), glucose (450–35830 mg/kg), fructose (210–2580 mg/kg), and sucrose (12380–50630 mg/kg) and are a lipid-rich food.¹⁶ Almonds are typically roasted to light, medium, or dark roast color, which is measured instrumentally or visually (based on roaster operator experience). Generally, higher temperatures and longer roasting times produce increased levels of acrylamide.^{9–11,15–18} Acrylamide formation begins when the almond kernel temperature exceeds 130 °C.¹⁷ Acrylamide levels are reduced at lower temperatures (e.g., 165 °C roasting for 20–30 min), which impart the desired color but can result in unacceptable bitter off-flavors.^{9–11,17–20} Strategies for reducing the levels of acrylamide in roasted almonds include optimization of roasting conditions, identifying varieties naturally low in precursor content, identifying cultivation practices that reduce precursor content, and optimizing nut maturity.

Tandem LC-MS/MS methods have been useful in monitoring acrylamide in foods as they offer the advantage of improved selectivity in complex food matrices.^{24,25} Isolation of acrylamide

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is difficult due to its polarity and poor retention on solid-phase absorbents; improvements in sample cleanup methods are needed to minimize interference from coextractives, especially at low acrylamide levels.^{24,25} Currently, quantification of nonaromatic amino acids (e.g., asparagine and glutamine) and reducing sugars typically requires two separate chromatographic runs (one for carbohydrates and one for amino acids) and often involves derivatization to improve detection sensitivity.^{26–31} More, recently, tandem LC-MS/MS techniques employing multiple reaction monitoring (MRM) have been used to quantify or determine these compounds.^{32–34} For example, Nielsen et al.³³ developed a LC-MS/MS method to determine acrylamide, asparagine, and three sugars (glucose, fructose, and sucrose). This method required one run using positive mode ESI for acrylamide and the saccharides and one run in negative mode for the asparagine. The recoveries of the sugars varied greatly, from 173% for fructose to 120% for glucose and 80% for sucrose. Nielson and co-workers realized the potential for simultaneous monitoring of the saccharides and amino acids by switching between positive and negative voltages in one run, yet due to instrument limitations had difficulty doing this for quantification. Accordingly, we hypothesized that using MRM, optimizing transition voltages, and employing rapid switching between ESI positive and negative modes, it would be possible to develop a single method to determine both amino acids and reducing sugars and shorten the overall analysis time involved in determining acrylamide and its precursors in complex matrices.

The goals of this study were to (1) improve extraction and LC-MS/MS approaches for the analysis of acrylamide and its precursors in raw and roasted almonds; (2) investigate the influence of several time–temperature combinations used to produce light-, medium-, and dark-roast almonds on acrylamide formation; and (3) study the influence of varietal selection in terms of almond variety, harvest year, and growing region, as well as postroasting storage conditions, on precursor content and acrylamide formation.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless otherwise stated. Acrylamide (99+%), D-fructose (98.0%), D-(+)-glucose (98.0%), L-asparagine (98.0%), L-glutamine (98.0%), and formic acid (95%) were obtained from Sigma-Aldrich (St. Louis, MO). D₇-glucose (1,2,3,4,5,6,6-D₇, 98%) and D₃-labeled acrylamide (2,3,3-D₃, 98.0%) were purchased from Cambridge Isotope Laboratory (Andover, MA). HPLC-grade methanol, acetonitrile, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). All solutions prepared for LC-MS/MS analysis were passed through a 0.22 μm nylon filter before use.

Caution: Acrylamide and D₃-labeled acrylamide are potent cumulative neurotoxins in animals and humans and may be carcinogenic.³ These chemicals are hazardous and should be handled carefully. Preparation of all standards and samples should be carried out in a fume hood.

Almonds. Raw kernels of nine commercial varieties of almonds (*Prunus dulcis*) were kindly supplied by the Almond Board of California (Modesto, CA). These varieties included Carmel, Avalon, Butte, Fritz, Mission, Monterey, Nonpareil, Price, and Sonora. Eight 1 kg composite samples of each almond variety (only 6 1 kg samples of Nonpareil) were shipped to the University of California (UC), Davis, immediately after harvest. Samples of Butte, Carmel, and Nonpareil almonds were collected from the same orchards in three different locations within California (representing north, south, and central growing regions) and over two harvest years (2006 and 2007); samples of the other varieties were obtained from the central region over two harvest years. Carmel almonds were used in the initial studies of the effect of roasting time–temperature

combinations on acrylamide formation. Multiple varieties of almonds were used in the subsequent precursor content and varietal selection studies. Nonpareil almonds were used in the final acrylamide storage stability study.

Almond Roasting. Raw almond kernels were processed at UC Davis in a commercial fluidized-bed hot air roaster (Aeroglide, Cary, NC). Batches (~1 kg) of Carmel almonds were roasted at constant preset air inlet temperatures for specific times to achieve light through dark roasts: 129 °C (32–70 min), 138 °C (18–28 min), 146 °C (12–18 min), 154 °C (9–13 min), 168 °C (5–8 min), or 182 °C (3.8–5.7 min). A single roasting treatment combination (138 °C for 22 min) was used to roast almonds for studies evaluating acrylamide and precursors in the nine almond varieties and to evaluate the influences of growing region and harvest year in selected varieties. A single roasting treatment of 146 °C for 14.7 min was used to evaluate acrylamide stability in almond samples during storage. In the roaster, the nut bed thickness was maintained at ~2.5 cm. During roasting, the direction of air flow was changed three times to ensure homogeneous roasting. After the preset heating period, the almonds were cooled to <40 °C by switching the air stream to a direct inlet of room temperature air. The cooled roasted almonds were removed and held briefly at room temperature before grinding and subsequent analyses. Roasting experiments were performed in duplicate (where sample was limited) or triplicate.

For all analyses, raw and roasted almond samples were ground for ~10 s in an electric coffee grinder and manually shaken through a sieve (2 mm pore size mesh). The sieved samples were either analyzed directly or stored at –20 °C in airtight containers before analysis.

Almond Moisture and Color Measurement. The moisture content of the ground almonds was determined by drying samples (5 g) at 60 °C under vacuum until a constant weight was achieved (in ~2 days). Moisture was determined for duplicate samples, and the results were averaged. To prepare samples for color measurement, 60 g of kernels was weighed out, ground for 10 s as described previously, and mixed thoroughly. The color of the ground samples was measured with a LabScan XE spectrophotometer (HunterLab, Reston, VA). Instrument readings for color values *L** (lightness), *a** (red/green), and *b** (blue/yellow) according to the CIE *L*a*b** color scale were obtained from triplicate samples and averaged.

Preparation of Samples for Acrylamide Analysis. Sieved ground almonds (4 g) were added to a 50 mL conical tube along with 100 μL of D₃-acrylamide (10 μg/mL; internal standard) and 20 mL of ultrapure water. Each tube was capped and shaken at 3000 rpm for 30 min at room temperature (Orbit Environ-Shaker, Lab-line Instrument Inc., Melrose, IL) and subsequently centrifuged at 3000g for 15 min. Extraction resulted in the formation of an immiscible oil layer and an aqueous layer with a suspension of fine particles as well as a solid residue. An 8 mL aliquot of the aqueous layer was promptly recovered with a glass Pasteur pipet. This layer was frozen at –20 °C for at least 2 h, thawed at 4 °C overnight, and centrifuged at 3000g for 10 min to remove proteins and other contaminants. The clarified aqueous extract obtained was transferred to a clean 15 mL tube for solid phase extraction (SPE). For SPE, a Strata-X-C cartridge (3 mL, 200 mg) was preconditioned with two 1 mL aliquots of methanol, followed by two 1 mL aliquots of water at a flow rate of 2 mL/min. The clarified aqueous extract (3 mL) was then loaded onto the preconditioned cartridge at a flow rate of <0.5 mL/min. The acrylamide-containing fraction was eluted with 1 mL of mobile phase (0.1% formic acid in water/methanol, 90:10, v/v) at a flow rate of <0.5 mL/min. The eluant was filtered through a 0.22 μm membrane prior to analysis by LC-(ESI)MS/MS.

Acrylamide Analysis by LC-(ESI)MS/MS. The quantification of acrylamide was performed using reversed-phase HPLC (Shimadzu Scientific, Columbia, MD) on a 5 μm Prodigy ODS column (4.6 mm × 250 mm) with a C₁₈ guard column (Phenomenex, Torrance, CA). The mobile phase was an isocratic mixture of 0.1% formic acid in water/methanol

(90:10, v/v) at a flow rate of 0.4 mL/min. The column temperature was controlled at 25 °C. The HPLC system employed an electrospray interface (ESI) to a Z-SPRAY triple-quadrupole Micromass Quattro LC system (Beverly, MA). The LC-(ESI)MS/MS was optimized using a capillary voltage of 3.0 kV, a cone voltage of 20 V, in positive mode ESI at a source temperature of 145 °C and with a desolvation gas temperature of 400 °C at a 600 L/h flow rate. The argon collision gas pressure was adjusted to 2×10^{-3} mbar for MS/MS fragmentation. The collision energy for MRM was set at 12 eV for both acrylamide and D₃-acrylamide. Transition ions monitored for quantification were m/z 72 → 55 for acrylamide and m/z 75 → 58 for D₃-labeled acrylamide. A 50 μL aliquot of extract or standard was injected onto the LC-MS/MS, and data were acquired for 12 min.

Preparation of Samples for Precursor Analysis. Sieved ground almonds (0.5 g) were weighed out into a 50 mL centrifuge tube, and 25 mL of ultrapure water was added. Tubes were shaken for 60 min and centrifuged at 3000g for 15 min. The aqueous layer was recovered and diluted 10 times with initial mobile phase (water/acetonitrile, 15:85, v/v) containing 4 μg/mL D₇-glucose, mixed/ and left to stand for 1 h. The mixture was then filtered through a 0.22 μm membrane prior to analysis by LC-(ESI)MS/MS for acrylamide precursors free asparagine, glutamine, glucose, and fructose.

Precursor Analysis by LC-(ESI)MS/MS. LC-(ESI)MS/MS analysis of the acrylamide precursors was performed on the same instrument used for acrylamide analysis described above. Components were resolved using a Luna NH₂ column (5 μm; 2.0 × 250 mm) (Phenomenex) with the column temperature controlled at 25 °C. The binary mobile phase consisted of water (A) and acetonitrile (B). Separations were performed using a linear gradient at a flow rate of 0.5 mL/min as follows: time 0–8 min, 15% A and 85% B; time 9 min, 35% A and 65% B; time 12 min, 35% A and 65% B; time 13 min, 10% A and 90% B, which was held constant for 20 min. Two T-unions were connected in line between the column and mass spectrometer. The first was used for the post-column addition of 1% formic acid in acetonitrile at a flow rate of 0.05 mL/min using a Waters 515 binary HPLC pump. The second was for splitting the flow rate to 40% into ion source. A 20 μL aliquot of extract or standard was injected onto the LC-MS/MS, and data were acquired for 12 min. ESI was used in negative mode for the sugars from 0 to 9.5 min and in positive mode for the amino acids from 9.6 to 12 min. The capillary voltage was 3 kV. The source temperature was 145 °C. The desolvation gas flow and temperature were held at 500 L/h and 400 °C, respectively. The cone gas flow was 40 L/h. The MRM transitions monitored for quantification were m/z 179 → 89 (fructose and glucose), m/z 186 → 124 (D₇-glucose), m/z 133 → 74 (asparagine), and m/z 147 → 130 (glutamine). Glucose and fructose were quantified using isotope-labeled D₇-glucose as the internal standard. Asparagine and glutamine were quantified using external standard methods. Cone voltages and collision energies were optimized as follows: cone voltage = 20 V and collision energy = 8 eV (glucose and fructose); cone voltage = 18 V and collision energy = 14 eV (Asn and Gln).

RESULTS AND DISCUSSION

Extraction and Chromatography. The extraction methodology was improved to increase recoveries of acrylamide throughout the sample pretreatment and concomitantly reduce the coextractives in the almond samples. Ultrafiltration,¹¹ organic solvent extraction,^{35,36} Carrez reagent ($K_4[Fe(CN)_6] \cdot 3H_2O$ and $ZnSO_4 \cdot 7H_2O$),^{36–38} and freeze–thaw cycling³⁹ were evaluated for their ability to remove coextractives and fine particles in the aqueous extracts. Results indicated that freezing the sample at –20 °C for a minimum of 2 h and thawing the sample overnight at 4 °C prior to centrifugation resulted in the highest recoveries of acrylamide. Ono et al.³⁹ described a similar result with respect

to the study of acrylamide in Japanese processed foods stored at –30 °C and thawed at room temperature prior to SPE. Herein, we found that almond samples needed to be thawed at 4–8 °C to achieve clear solutions that could pass through SPE cartridges. Methanol and Carrez reagents improved SPE but decreased recoveries (20–30%), and new interferences were introduced.

Due to the polarity of acrylamide, there is no one ideal SPE adsorbent, and multiple or mixed-bed SPE matrices are often employed to improve extraction efficiencies. Even with these approaches, coextractives are a problem, and multiple SPE cartridges can result in lower absolute recoveries.⁴⁰ Bermudo et al.⁴¹ demonstrated that Strata-X-C SPE cartridges (Phenomenex application, TN-007) with a mixed-bed stationary phase composed of a strong cation exchange group on a styrene–divinylbenzene polymer performed well for the analysis of acrylamide in some foods. Using this SPE matrix, and a loading with water (maximum of 3 mL) and elution solvent of 10% methanol with 0.1% formic acid (1 mL), we minimized coextraction interferences and maximized recoveries (100%); sensitivity was increased 3–5-fold. Our modified extraction methods resulted in improved sensitivity with a limit of detection (LOD) of 2 μg/kg and a limit of quantitation (LOQ) of 6 μg/kg. Recoveries, determined for two spiking levels in raw almonds (20 and 200 μg/kg) and roasted almonds (100 and 500 μg/kg), ranged from 95.0 to 115.6% with an relative standard deviation (RSD) below 6.3%.

A fast and sensitive LC-MS/MS method, employing stable isotope dilution and MRM, was developed for acrylamide. The predominant CID fragment ion, m/z 55, corresponding to the loss of NH₃, was explored for MRM quantification.^{24,25,40} This transition was optimized at 12 eV for almond samples.

A simultaneous LC-(ESI)MS/MS method, employing both negative and positive ionization potentials, was used for quantitation of the amino acids and reducing sugars. Reducing sugars ionized best with negative potentials, and amino acids ionized best with positive potentials. Therefore, MS scans were obtained by rapid switching between negative and positive ionization modes in accordance with the eluting times of these compounds. Fructose and glucose have similar spectra with major ions at m/z 89, 119, 113, 101, 71, and 59. The most abundant fragment ion, m/z 89, was used for MRM and quantification of fructose and glucose. For the D₇-glucose internal standard, transition ions of 186 → 124 were used for quantitation. The predominant transition ions for asparagine and glutamine were m/z 133 → 74 and 147 → 130, respectively. Because the transition of m/z 179 → 89 is the same for glucose and fructose, complete separation by HPLC is necessary prior to MS detection. Formic acid enhances the ionization of sugars and especially amino acids for MS detection; however, we found that the addition of formic acid to the HPLC mobile phase resulted in coelution of glucose and fructose. Therefore, postcolumn infusion of 1% formic acid in acetonitrile was employed. This resulted in complete resolution of the two reducing sugars with high ionization efficiencies. Representative MRM chromatograms are shown in Figure 1.

A linear response was found for all precursor compounds over the range of 0.2–8 μg/mL with correlation coefficients of >0.995. The LOQs for glucose, fructose, asparagine, and glutamine were below 20 mg/kg in almond samples. Recovery was determined by spiking two levels of the precursors (400 and 2000 mg/kg) into roasted almond samples. Average recoveries ranged from 81.5 to 94.6% with a RSD below 6.7%. Repeat analyses of the

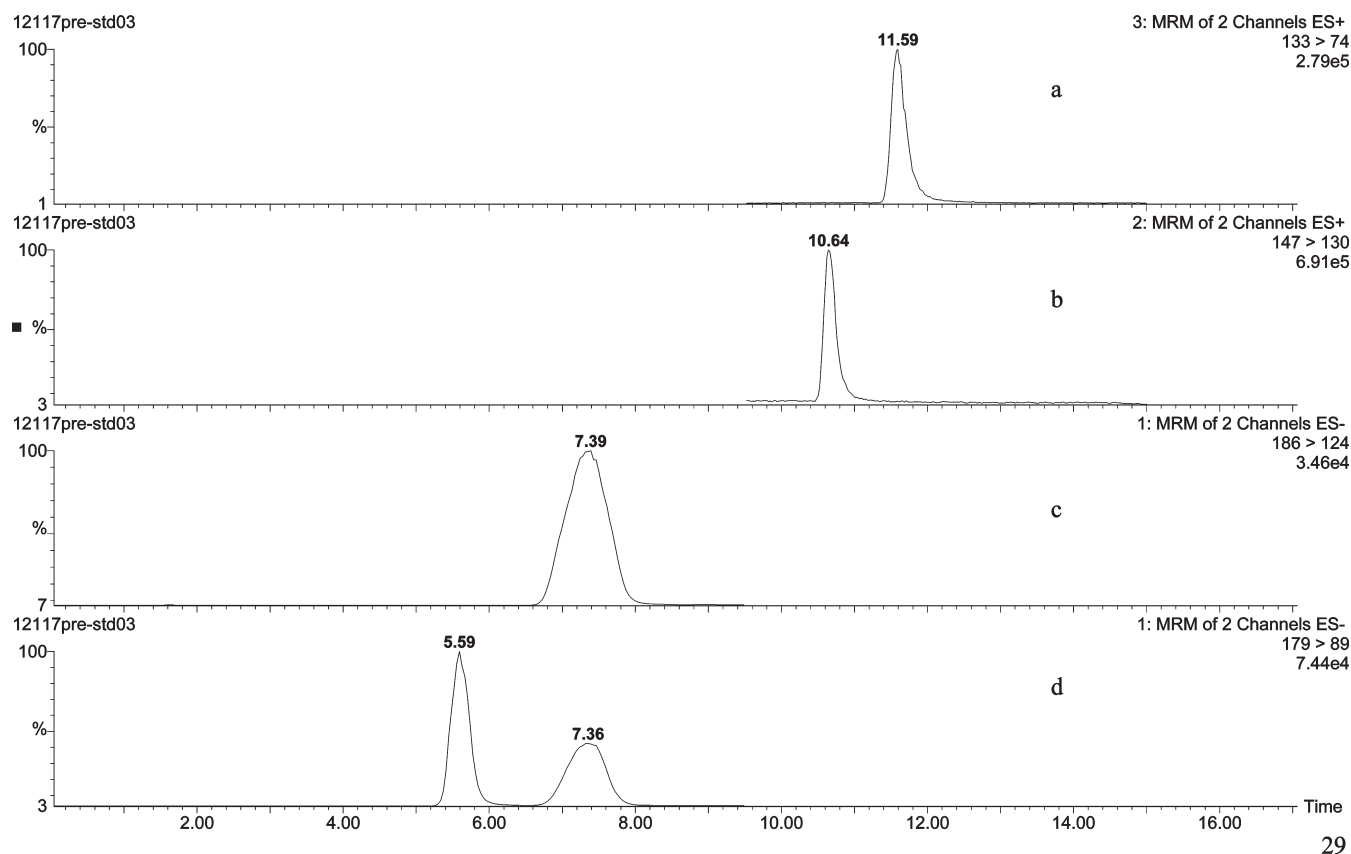


Figure 1. LC-(ESI)MS/MS MRM chromatograms of (a) 1 $\mu\text{g}/\text{mL}$ asparagine (10.64 min) monitoring the 133 \rightarrow 74 Da transition; (b) 1 $\mu\text{g}/\text{mL}$ glutamine (11.59 min) monitoring the 147 \rightarrow 130 Da transition; (c) 2 $\mu\text{g}/\text{mL}$ D₇-glucose (7.39 min) monitoring the 186 \rightarrow 124 Da transition; and (d) 1 $\mu\text{g}/\text{mL}$ fructose (5.59 min) and glucose (7.36 min) monitoring the 179 \rightarrow 89 Da transition.

Table 1. Influence of Roasting Temperature on Acrylamide Levels and Color (L^* Value) in Almonds (Carmel Variety)^a

temp ($^{\circ}\text{C}$)	acrylamide ($\mu\text{g}/\text{kg}$)			L^* value	
	min	max	av	min	max
129	146 \pm 30	343 \pm 8	235 \pm 81 ^{*b}	58.81 \pm 0.93	63.77 \pm 0.34
138	132 \pm 15	378 \pm 62	269 \pm 11 [*]	58.56 \pm 1.91	61.98 \pm 0.84
146	200 \pm 21	411 \pm 15	323 \pm 94 [*]	58.69 \pm 0.39	62.49 \pm 1.56
154	308 \pm 36	975 \pm 14	642 \pm 22	57.77 \pm 0.42	61.90 \pm 0.49
168	249 \pm 53	1469 \pm 121	778 \pm 45	54.63 \pm 0.35	62.23 \pm 0.39
182	325 \pm 28	1316 \pm 220	907 \pm 37	55.82 \pm 0.52	62.12 \pm 0.33

^a Data represent the mean \pm SD; for min and max $n = 3$; for average $n = 12$. ^b At individual roasting temperatures, average acrylamide levels marked with an asterisk (*) are significantly lower ($p < 0.01$) than the average level obtained at 182 $^{\circ}\text{C}$.

roasted samples over 5 days gave RSDs ranging between 4.6 and 8.9%.

Influence of Roasting on Acrylamide Content and Color Values. The methods described above were used to investigate the influence of a range of industrially applicable time–temperature combinations used to roast almonds. Mean levels of acrylamide in almonds (Carmel variety) roasted at these conditions ranged from 235 \pm 81 to 907 \pm 36 $\mu\text{g}/\text{kg}$ (Table 1). The most significant increase in acrylamide ($p < 0.05$) was observed

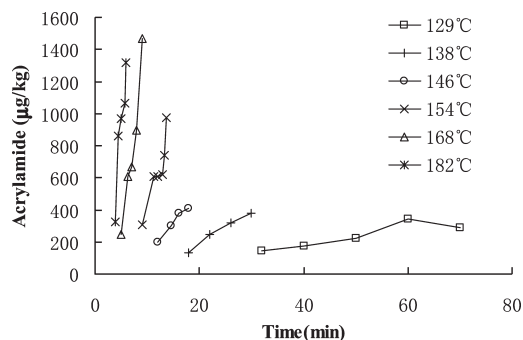


Figure 2. Influence of time at different roasting temperatures on the acrylamide content of almonds (Carmel variety).

with the temperature increase between 146 and 154 $^{\circ}\text{C}$, as the mean level of acrylamide almost doubled (from 323 to 642 $\mu\text{g}/\text{kg}$). The range of the L^* values (lightness) obtained at each roasting temperature is shown in Table 1. (In this study, L^* values of 62.41 \pm 0.70 were used to represent a typical light-roast color, whereas L^* values of 58.02 \pm 0.83 represented a typical dark-roast color.) Acrylamide levels increased in all samples as the roasting time increased at each roasting temperature (Figure 2), and this occurred within a range of L^* values between 54.63 \pm 0.35 and 63.77 \pm 0.34. It is not practical to achieve precise color development at each temperature due to the natural variation in almonds

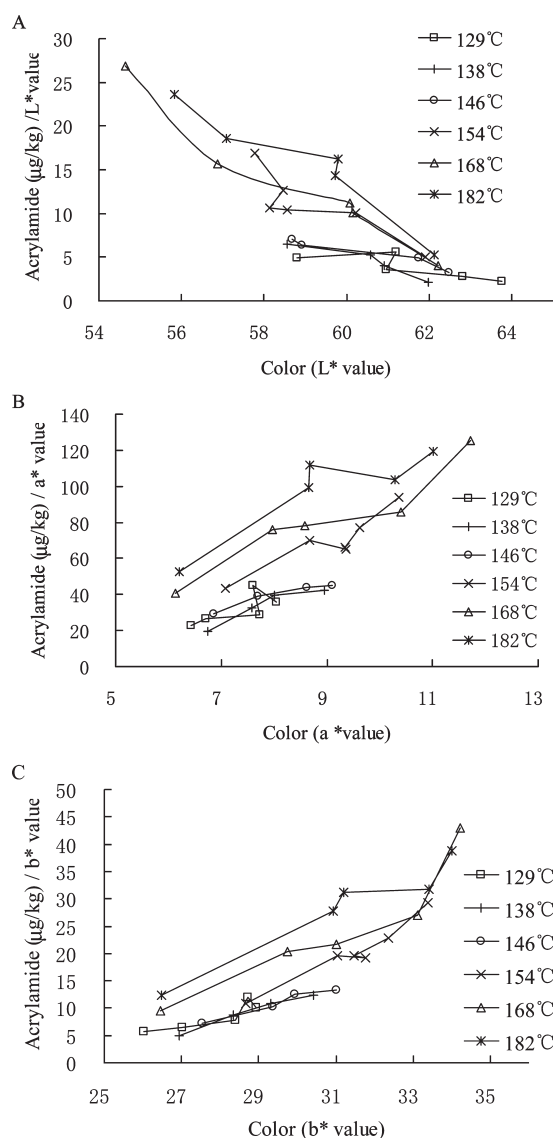


Figure 3. Relationships between the acrylamide content/color value ratios and color development for almonds (Carmel variety) at different roasting temperatures: (A) acrylamide/ L^* value; (B) acrylamide/ a^* value; (C) acrylamide/ b^* value.

and the limitations in roasting. Therefore, to minimize variation, the ratio of acrylamide content to L^* , a^* , or b^* values was used to describe the relationship between acrylamide formation and color development at different roasting temperatures.

Acrylamide levels decreased per unit of L^* value (Figure 3A) and increased in relationship to a^* or b^* values (Figure 3B,C). With respect to all color values, the plotted lines for 129, 138, and 146 °C essentially overlap and group. This finding suggested that when roasting is performed at or below 146 °C, the same roast color achieved, regardless of time, will generate similar acrylamide levels. Different correlations were observed with roasting temperatures of 154, 168, and 182 °C. For example, the acrylamide levels differed significantly at L^* values corresponding to medium or dark color ($\sim L^* < 61.5$). These results, based on almonds of the Carmel variety, indicated that controlling the roasting temperature at or below 146 °C will minimize acrylamide formation in roasted almonds. In addition, the color values

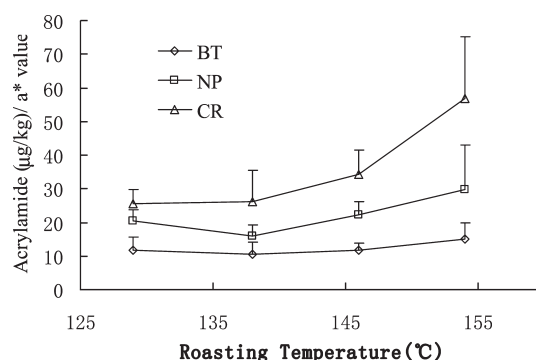


Figure 4. Comparison of acrylamide/ a^* ratios in three almond varieties roasted at different time–temperature combinations. The data represent the average of acrylamide/ a^* value ratios at each temperature. CR, Carmel; NP, Nonpareil; BT, Butte.

a^* and b^* have a stronger correlation with changes in acrylamide formation than does the color value L^* over all roasting temperatures and may be better predictors of acrylamide than the traditionally used L^* value.

Herein, almonds roasted at temperatures between 129 and 146 °C had the lowest levels of acrylamide. Roasting at 138 °C requires about half the time to achieve the same degree of color as roasting at 129 °C, yet acrylamide levels in almonds roasted at these two temperatures were not significantly different (Figure 3A). However, almond samples roasted at 121 °C to an L^* value of 63.65 required 100 min to develop the same color as those roasted at 129 °C to an L^* value of 63.77 (32 min), yet the acrylamide levels were not significantly lower ($173 \pm 6 \mu\text{g}/\text{kg}$ at 121 °C and $146 \pm 30 \mu\text{g}/\text{kg}$ at 129 °C). It appears that there is no advantage to using roasting temperatures as low as 121 or 129 °C. Use of roasting temperatures between 138 and 146 °C will minimize acrylamide formation (<200 ppb) while limiting roasting times, which has the additional advantage of energy savings. To determine if these observations would translate to other almond varieties, three varieties of almonds including Carmel, Nonpareil, and Butte were roasted at 129, 138, 146, and 154 °C and compared. For each temperature, two roasting times were used to achieve a light or medium color.

Figure 4 shows a plot of the average \pm SD of acrylamide/ a^* value ratios for each temperature for each of the almond varieties. The SD represents the variance in the almond color achieved at the individual temperatures; the greater the SD, the greater the difference between the light and medium color at that roasting temperature. As shown in Figure 4, the level of acrylamide generated per unit color value (a^*) is similar in all three varieties over the tested roasting conditions. The acrylamide/ a^* ratio increased with the roasting temperature for all three almond varieties, with an abrupt increase observed from 146 to 154 °C. Moreover, the variance (SD) between light and medium color is significantly higher at 154 °C than at the lower temperatures.

The relationship between roasting conditions and almond flavor is still not clear; however, studies indicate that the presence of acrylamide is related to the formation of nutty flavor compounds. For example, Ehling et al.⁴² used the asparagine/glucose ratio as a model to investigate the relationship between major flavor compounds (pyrazines), acrylamide, and color; the formation of pyrazines correlated strongly with acrylamide formation.

Table 2. Average Contents of Acrylamide and Precursors in Raw and Roasted Almonds^a

almond variety	in raw samples (mg/kg)					in roasted samples ^b (mg/kg)				
	acrylamide	fructose	glucose	asparagine	glutamine	acrylamide	fructose	glucose	asparagine	glutamine
Monterey	nd ^c	970 ± 78	1948 ± 178	4089 ± 287	273 ± 23	0.287 ± 0.044	107 ± 36	78 ± 21	2926 ± 374	12 ± 1
Carmel	nd	1130 ± 92	1976 ± 205	2210 ± 212	201 ± 12	0.248 ± 0.053	165 ± 27	231 ± 68	2431 ± 81	20 ± 3
Mission	nd	1646 ± 76	3154 ± 102	1656 ± 139	155 ± 15	0.229 ± 0.017	120 ± 8	96 ± 8	1638 ± 73	7 ± 1
Price	nd	1200 ± 112	2165 ± 172	2359 ± 215	320 ± 22	0.202 ± 0.026	187 ± 34	167 ± 10	2829 ± 297	26 ± 1
Fritz	nd	994 ± 65	2627 ± 312	2018 ± 96	195 ± 9	0.200 ± 0.036	142 ± 18	96 ± 25	1409 ± 22	8 ± 1
Nonpareil	nd	1409 ± 130	2141 ± 165	1208 ± 118	277 ± 12	0.159 ± 0.012	412 ± 5	161 ± 1	1227 ± 8	15 ± 1
Sonora	nd	1049 ± 115	1693 ± 131	2039 ± 106	248 ± 25	0.131 ± 0.007	189 ± 8	152 ± 7	1864 ± 162	14 ± 4
Avalon	nd	1345 ± 149	1982 ± 175	925 ± 64	209 ± 18	0.124 ± 0.01	194 ± 20	121 ± 23	1097 ± 223	18 ± 5
Butte	nd	1245 ± 97	2547 ± 96	568 ± 32	124 ± 11	0.109 ± 0.01	128 ± 7	120 ± 15	594 ± 69	6 ± 1
<i>R</i> ^{2d}		0.0399	0.0111	0.6787	0.0604					

^a Data represent the mean ± SD, *n* = 3. ^b Almonds were roasted at 138 °C for 22 min. ^c nd, not detected (<LOD, 2 μg/kg). ^d *R*² represents the correlation efficacy between acrylamide in roasted almond and individual precursor's content in raw almond.

Relationships between Precursors and Acrylamide. The levels of acrylamide, sugars, and free amino acids determined in samples of raw and roasted (138 °C for 22 min) almonds of nine varieties are shown in Table 2. (The moisture content for the nine almond varieties sampled was determined to be within a range of 3–4%.) No acrylamide was detected in the raw almond samples (LOD < 2 μg/kg). The roasted Monterey almonds contained the highest levels of acrylamide (287 ± 44 μg/kg). Roasted Butte almonds contained much lower levels (109 ± 10 μg/kg), which suggested that variety can play a role in acrylamide formation. Also, it appears that there is a correlation between acrylamide formation and asparagine content; the mean level of asparagine in raw Monterey almonds was 4089 ± 287 μg/kg as compared to 568 ± 32 μg/kg in raw Butte almonds. The loss of asparagine was limited after roasting, whereas essentially all of the reducing glucose, fructose, and free glutamine were consumed during roasting. No direct correlation was found between levels of the sugars and glutamine in raw almonds and final acrylamide levels in roasted almonds. A weak correlation was found between acrylamide and the content of free asparagine in the raw almonds (*R*² = 0.6787). This coefficient is not as high as that found in other studies for almond (*R*² = 0.843) and green tea (*R*² = 0.806).^{16,18} However, Amrein et al.¹⁶ reported that different roasting conditions can affect the correlation between asparagine content and acrylamide formation. No correlation was found when roasting was performed at or below 145 °C (14 min), whereas a correlation of *R*² = 0.843 was found for roasting at or above 165 °C (12.5 min).

Interestingly, additional experiments in the current study (data not shown) found that at roasting temperatures below 154 °C, <10% of the asparagine was consumed even in almonds roasted to a medium or dark color. At roasting temperatures of ≥ 168 °C, ~20% of the free asparagine was consumed, and at roasting temperatures of 138, 146, and 182 °C, the content of free asparagine increased slightly during the first stage of roasting. An increase in asparagine is thought to result from the loss of water and breakdown of proteins that occur during the initial stages of roasting.¹⁶

Influence of Varietal Selection on Acrylamide. The influence of variety, growing region, and harvest year on acrylamide formation in roasted almonds was investigated for six almond varieties. All samples were roasted at 138 °C for 22 min. Color was also determined and, in general, the *L*^{*} value of the roasted samples

varied from 53.44 to 65.09 (average 59.06) and the *a*^{*} and *b*^{*} values also varied significantly (data not shown). Unlike the findings with the Carmel almonds roasted at various time–temperature combinations, no significant correlation was found between any color value and acrylamide formation. These results suggested that correlation between color and acrylamide is only applicable within a sample of almonds obtained from the same raw material.

Acrylamide was not detected in any raw almond samples and varied in roasted almonds from 117 ± 5 μg/kg (Sonora) to 221 ± 95 μg/kg (Butte) on the basis of the 2-year mean for all regional samples taken over the two years (Table 3). The RSD varied significantly from 9.2 to 49% for these samples. No statistical difference in acrylamide levels was observed for almonds obtained from the different growing regions and harvest years. The overall mean acrylamide level for these samples (roasted at 138 °C for 22 min) was 187 ± 71 μg/kg. This value is significantly lower than the average acrylamide content (443 μg/kg) reported previously for 36 roasted almond samples.¹⁵

Stability of Acrylamide in Roasted Almonds. The stability of acrylamide in almonds is also of interest as studies indicate that acrylamide is stable in starchy foods such as cereal products, biscuits, and potato chips during storage but is not stable in almonds. For example, acrylamide levels in roasted almonds were shown to decrease significantly by 20–57% after 100 days of storage at room temperature in sealed containers.¹⁵ To date, little information is available on the influence of roasting temperature and the stability of acrylamide in stored almonds.

In this study, the stability of acrylamide in roasted Nonpareil almonds was investigated during storage at room temperature and 60 °C. Raw Nonpareil almonds were used as a negative control. The levels of acrylamide in the almond samples (roasted at 146 °C for 14.7 min) decreased by 0–17.7% (average decrease of 6.7%) at room temperature storage for 1 month. Acrylamide levels decreased by 12.9–68.5% (average decrease of 50.2%) upon 3 days of storage at 60 °C; by 6 days of storage the acrylamide levels were on average 55.5% lower than the initial levels. However, the acrylamide levels increased when the storage temperature was further elevated to 80 °C, which was likely due to the contribution of lipid oxidation products formed at this temperature. Short-term storage at elevated temperatures such as 60 °C may be one approach for mitigating acrylamide levels in roasted almonds. However, before this approach is adopted, the effect of short-term

Table 3. Acrylamide Levels in Roasted Almonds^a As Influenced by Almond Variety, Growing Region, and Harvest Year

almond variety	acrylamide ^b ($\mu\text{g}/\text{kg}$)								
	2006 harvest				2007 harvest				2-year mean
	south	north	central	annual mean	south	north	central	annual mean	
Butte	235 \pm 19	145 \pm 23	395 \pm 87	258 \pm 126	216 \pm 40	124 \pm 12	213 \pm 17	184 \pm 52	221 \pm 95
Carmel	213 \pm 11	178 \pm 39	204 \pm 24	198 \pm 18	179 \pm 11	312 \pm 30	152 \pm 14	214 \pm 86	206 \pm 56
Nonpareil	134 \pm 43	123 \pm 11	203 \pm 28	153 \pm 43	145 \pm 16	127 \pm 16	268 \pm 45	180 \pm 77	167 \pm 58
Fritz			99 \pm 13				194 \pm 33		147 \pm 67
Monterey			131 \pm 26				263 \pm 71		197 \pm 93
Sonora			120 \pm 34				113 \pm 17		117 \pm 5

^a Almonds were roasted at 138 °C for 22 min. ^b Data represent the mean \pm SD; for a single region, $n = 3$; for the annual mean $n = 9$; for the 2-year mean, $n = 18$.

elevated temperature storage on flavor, lipid oxidation, rancidity, and color should be investigated.

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REFERENCES

- International Programme on Chemical Safety (IPCS), Environmental Health Criteria 49, Acrylamide, 1985; <http://www.inchem.org/documents/ehc/ehc/ehc49.htm> (accessed Jan 5, 2011).
- European Commission, European Chemicals Bureau, Institute for Health and Consumer Protection, 2002. Vol 24, European Union Risk Assessment Report Acrylamide.
- International Agency for Research on Cancer (IARC). Acrylamide. In *Some Industrial Chemicals*; IARC Monographs on the Evaluation of Carcinogenic Risk to Humans 60; International Agency for Research on Cancer: Lyon, France, 1994; p 389.
- Bergmark, E.; Calleman, C. J.; He, F. S.; Costa, L. G. Determination of hemoglobin adducts in humans occupationally exposed to acrylamide. *Toxicol. Appl. Pharmacol.* **1993**, *120*, 45–54.
- Bergmark, E. Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. *Chem. Res. Toxicol.* **1997**, *10*, 78–84.
- Rice, J. M. The carcinogenicity of acrylamide. *Mutat. Res./Gen. Toxicol. Environ. Mutag.* **2005**, *580*, 3–20.
- Swedish National Food Administration (Livsmedelsverket). Acrylamide is formed during the preparation of food and occurs in many foodstuffs; press release, April 24, 2002.
- Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S.; Törnqvist, M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* **2002**, *50*, 4998–5006.
- Mottram, D. S.; Wedzicha, B. L.; Dodson, A. T. Food chemistry: acrylamide is formed in the Maillard reaction. *Nature* **2002**, *419*, 448–449.
- Stadler, R. H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, P. A.; Robert, M.-C.; Riediker, S. Acrylamide from Maillard reaction products. *Nature* **2002**, *419*, 449.
- Becalski, A.; Lau, B. P.-Y.; Lewis, D.; Seaman, S. W. Acrylamide in food: occurrences, sources, and modeling. *J. Agric. Food Chem.* **2003**, *51*, 802–808.
- Yaylayan, V. A.; Wnorowski, A.; Perez Locas, C. Why asparagine needs carbohydrates to generate acrylamide. *J. Agric. Food Chem.* **2003**, *51*, 1753–1757.
- Joint FAO/WHO Expert Committee on Food Additives (JECFA). *Evaluation of Certain Food Contaminants*; 72nd report of the JECFA meeting; Rome, Italy, Feb 16–25, 2010; JECFA/72/SC.
- Friedman, M. Chemistry, biochemistry, and safety of acrylamide. A review. *J. Agric. Food Chem.* **2003**, *51*, 4504–4526.
- Amrein, T. M.; Andres, L.; Schönbacher, B.; Conde-Petit, B.; Escher, F.; Amadò, R. Acrylamide in almond products. *Eur. Food Res. Technol.* **2005**, *221*, 14–18.
- Amrein, T. M.; Lukac, H.; Andres, L.; Perren, R.; Escher, F.; Amadò, R. Acrylamide in roasted almonds and hazelnuts. *J. Agric. Food Chem.* **2005**, *53*, 7819–7825.
- Lukac, H.; Amrein, T. M.; Perren, R.; Conde-Petit, B.; Amadò, R.; Escher, F. Influence of roasting conditions on the acrylamide content and the color of roasted almonds. *J. Food Sci.* **2007**, *72* (1), C33–C38.
- Mizukami, Y.; Kohata, K.; Yamaguchi, Y.; Hayashi, N.; Sawai, Y.; Chuda, Y.; Ona, H.; Yada, H.; Yoshida, M. Analysis of acrylamide in green tea by gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 7370–7377.
- Surdyk, N.; Rosén, J.; Andersson, R.; Åman, P. Effects of asparagine, fructose, and baking conditions on acrylamide content in yeast-leavened wheat bread. *J. Agric. Food Chem.* **2004**, *52*, 2047–2051.
- Mustafa, A.; Andersson, R.; Rosén, J.; Kamal-Eldin, A.; Åman, P. Factors influencing acrylamide content and color in rye crisp bread. *J. Agric. Food Chem.* **2005**, *53*, 5985–5989.
- Yasukura, A.; Tanaka, Y.; Hengel, M.; Shibamoto, T. Gas chromatographic investigation of acrylamide formation in browning model systems. *J. Agric. Food Chem.* **2003**, *51*, 3999–4003.
- Ehling, S.; Hengel, M.; Shibamoto, T. Formation of acrylamide from lipids. In *Chemistry and Safety of Acrylamide in Food*; Friedman, M., Mottram, D., Eds.; Springer: New York, 2005; pp 223–233.
- Casado, F. J.; Montaña, A. Influence of processing conditions on acrylamide content in black ripe olives. *J. Agric. Food Chem.* **2008**, *56*, 2021–2027.
- Wenzl, T.; De La Calle, M. B.; Anklam, E. Analytical methods for the determination of acrylamide in food products: a review. *Food Addit. Contam.* **2003**, *20*, 885–902.

(25) Zhang, Y.; Zhang, G.; Zhang, Y. Occurrence and analytical methods of acrylamide in heat-treated foods: review and recent developments. *J. Chromatogr., A* **2005**, *1075*, 1–21.

(26) Fekkes, D. State-of-the-art of high-performance liquid chromatographic analysis of amino acids in physiological samples. *J. Chromatogr., B: Biomed. Sci. Appl.* **1996**, *682*, 3–22.

(27) Romani, A.; Baldi, A.; Tattini, M.; Vincieri, F. F. Extraction, purification procedures and HPLC-RI analysis of carbohydrates in olive (*Olea europaea* L.) plants. *Chromatographia* **1994**, *39*, 35–39.

(28) Zeppa, G.; Conterno, L.; Gerbi, V. Determination of organic acids, sugars, diacetyl, and acetoin in cheese by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 2722–2726.

(29) Mawhinney, T. P.; Feather, M. S.; Barbero, G. J.; Martinez, J. R. The rapid, quantitative determination of neutral sugars (as aldonitrile acetates) and amino sugars (as *O*-methyloxime acetates) in glycoproteins by gas–liquid chromatography. *Anal. Biochem.* **1980**, *101*, 112–117.

(30) Bernal, J. L.; Del Nozal, M. J.; Toribio, L.; Del Alamo, M. HPLC analysis of carbohydrates in wines and instant coffees using anion exchange chromatography coupled to pulsed amperometric detection. *J. Agric. Food Chem.* **1996**, *44*, 507–511.

(31) Toyo'oka, T.; Kuze, A. Determination of saccharides labelled with a fluorescent reagent, DBD-ProCZ, by liquid chromatography. *Biomed. Chromatogr.* **1997**, *11*, 132–136.

(32) Piraud, M.; Vianey-Saban, C.; Petritis, K.; Elfakir, C.; Steghens, J.-P.; Morla, A.; Bouchu, D. ESI-MS/MS analysis of underivatized amino acids: a new tool for the diagnosis of inherited disorders of amino acid metabolism. Fragmentation study of 79 molecules of biological interest in positive and negative ionization mode. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1297–1311.

(33) Nielsen, N. J.; Granby, K.; Hedegaard, R. V.; Skibsted, L. H. A liquid chromatography – tandem mass spectrometry method for simultaneous analysis of acrylamide and the precursors, asparagine and reducing sugars in bread. *Anal. Chim. Acta* **2006**, *557*, 211–220.

(34) Rogatsky, E.; Tomuta, V.; Stein, D. T. LC/MS quantitative study of glucose by iodine attachment. *Anal. Chim. Acta* **2007**, *591*, 155–160.

(35) Riediker, S.; Stadler, R. H. Analysis of acrylamide in food by isotope-dilution liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *J. Chromatogr., A* **2003**, *1020*, 121–130.

(36) Hoenicke, K.; Gatermann, R.; Harder, W.; Hartig, L. Analysis of acrylamide in different foodstuffs using liquid chromatography–tandem mass spectrometry and gas chromatography–tandem mass spectrometry. *Anal. Chim. Acta* **2004**, *520*, 207–215.

(37) Delatour, T.; Périsset, A.; Goldmann, T.; Riediker, S.; Stadler, R. H. Improved sample preparation to determine acrylamide in difficult matrixes such as chocolate powder, cocoa, and coffee by liquid chromatography tandem mass spectroscopy. *J. Agric. Food Chem.* **2004**, *52*, 4625–4631.

(38) Claus, A.; Weisz, G. M.; Kammerer, D. R.; Carle, R.; Schieber, A. A method for determination of acrylamide in bakery products using ion trap LC-ESI-MS/MS. *Mol. Nutr. Food Res.* **2005**, *49*, 918–925.

(39) Ono, H.; Chuda, Y.; Ohnishi-Kameyama, M.; Yada, H.; Ishizaka, M.; Kobayashi, H.; Yoshida, M. Analysis of acrylamide by LC-MS/MS and GC-MS in processed Japanese foods. *Food Addit. Contam.* **2003**, *20*, 215–220.

(40) Andrzejewski, D.; Roach, J. A. G.; Gay, M. L.; Musser, S. M. Analysis of coffee for the presence of acrylamide by LC-MS/MS. *J. Agric. Food Chem.* **2004**, *52*, 1996–2002.

(41) Bermudo, E.; Moyano, E.; Puignou, L.; Galceran, M. T. Determination of acrylamide in foodstuffs by liquid chromatography ion-trap tandem mass-spectrometry using an improved clean-up procedure. *Anal. Chim. Acta* **2006**, *559*, 207–214.

(42) Ehling, S.; Shibamoto, T. Correlation of acrylamide generation in thermally processed model systems of asparagine and glucose with color formation, amounts of pyrazines formed, and antioxidative properties of extracts. *J. Agric. Food Chem.* **2005**, *53*, 4813–4819.