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Determination of Advanced Glycation Endproducts by LC-MS/MS in Raw and Roasted Almonds (*Prunus dulcis*)

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ABSTRACT: A sensitive and reliable LC-(ESI)MS/MS method was developed and validated for the simultaneous analysis of five common advanced glycation endproducts (AGEs) after enzymatic digestion in raw and roasted almonds. AGEs included carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), pyralline (Pyr), argpyrimidine (Arg-p), and pentosidine (Pento-s). This method allows accurate quantitation of free and AGE−protein adducts of target AGEs. Results indicate that CML and CEL are found in both raw and roasted almonds. Pyr was identified for the first time in roasted almonds and accounted for 64.4% of free plus bound measured AGEs. Arg-p and Pento-s were below the limit of detection in all almond samples tested. Free AGEs accounted for 1.3−26.8% of free plus bound measured AGEs, indicating that protein-bound forms predominate. The roasting process significantly increased CML, CEL, and Pyr formation, but no significant correlation was observed between these AGEs and roasting temperature.

KEYWORDS: advanced glycation endproducts, AGEs, enzymatic hydrolysis, LC-MS/MS, almond, roasting

INTRODUCTION

Advanced glycation endproducts (AGEs) are generated in some foods and biological systems as advanced reaction products derived from the Maillard reaction.[1] Absorption of dietary (or food-derived) AGEs may accelerate oxidative stress and inflammation and alter glucose regulation, especially in diabetics and renal failure patients.[2−5] Dietary AGEs may be directly absorbed into the circulation and contribute significantly to the body's AGE pool, and restriction of dietary AGE intake is associated with an average decrease of serum AGE levels by 30−40%.[6] Measures of both serum AGEs and oxidative stress in vivo are directly influenced by the intake of dietary AGEs, independent of age or energy intake.[7] In addition, serum AGEs in maternal blood and food-derived AGEs can prematurely raise AGEs in children to norms, preconditioning them to abnormally high oxidant stress and inflammation and thus possibly to early onset of disease, such as diabetes.[8]

The Maillard reaction is important for the color and flavor formation in many heat-treated foods, resulting in improved quality attributes.[9−11] However, the formation of AGEs increases significantly during thermal processes such as pasteurization, sterilization, frying, broiling, roasting, and boiling.[12−16] Dry heat has been shown to promote AGE formation by 10−100-fold above the uncooked state across food categories.[16] Also, foods high in fat and protein are generally AGE-rich and prone to new AGE formation during cooking, whereas carbohydrate-rich foods, such as vegetables, fruits, whole grains, and milk, contain relatively few AGEs, even after cooking.[16] Storage of foods at elevated temperatures may also result in increased AGE levels.[13]

Despite the great variety of AGEs that could be formed during heating processes, only a few have been identified unequivocally and quantified in foods. Well-characterized AGEs in foods include *N*-ε-(carboxylmethyl)lysine (CML), *N*-ε-(carboxylethyl)lysine (CEL), and pyrraline (Pyr), which all arise from lysine derivatization. Information about arginine derivatization and cross-linked AGEs in foods is very limited.[1,17,18]

Methods for AGE quantification in foods or biological samples can be categorized into two groups: (1) enzyme-linked immunosorbent assay (ELISA) methods based on antibodies for CML or methylglyoxal derivatives[14,17] and (2) instrumental analysis after protein digestion, including HPLC-DAD,[13] HPLC-FLD,[20−22] LC-MS/MS,[12,15,23−27] and GC-MS[19,28,29] methods. Compared with instrumental analysis, ELISA methods are simple and rapid, but results are expressed in arbitrary units (e.g., kilounits (kU) per gram of food) rather than actual concentrations. Moreover, specific antibodies are required for ELISA measurement of each compound, and the specificity of the assay is affected significantly by the sample matrix, which can lead to incorrect estimation of AGE levels.[28] Instrument methods can provide accurate results; however, these require either the acid or enzymatic hydrolysis of AGEs bound to proteins or peptides prior to analysis. Acid hydrolysis is generally applied for the analysis of CML in food samples because it is a simple, inexpensive, and reliable procedure.[17] However, this treatment is not suitable for acid-labile AGEs (e.g., Pyr) that need to be reduced prior to digestion and can lead to an overestimation of AGE concentration in samples. The use of a cocktail of proteolytic enzymes for hydrolysis of AGEs has been successfully applied in biological and some food samples.[12,27] This approach can overcome the disadvantages associated with acid hydrolysis and possibly allow for the determination of a more extensive range of AGEs in foods.

Almonds are rich in lipids, protein, and carbohydrates, making them susceptible to the formation of various AGEs during thermal

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treatment or even in storage. However, only a few studies have examined AGEs in almonds, and sample analysis has been limited. Studies employing ELISA methods indicate that blanched and roasted almonds contained relatively high levels of CML (5473 and 6650 kU/100 g, respectively).[14,16] In other studies in which acid hydrolysis was coupled with GC-MS/MS analysis, no CML was detected, whereas 1.2 mg/100 g was detected in almonds roasted at 180 °C (8 min).[19]

In the present study, five representative AGEs were evaluated in raw and roasted almonds. These include CML, CEL, Pyr, Arg-p, and pentosidine (Pento-s). CML and CEL are formed by a reaction between lysine and glyoxal and methylglyoxal, respectively. Both can be derived from protein glycoxidation and protein lipoxidation, and both are considered to be AGEs and advanced lipoxidation endproducts (ALEs).[30,31] Pyr, an acid-labile pyrrole derivative of lysine, is formed from the reaction of the ε-amino group of lysine and 3-deoxyglucosone and has been quantified in milk, bakery products, and pasta.[1,18] Argpyrimidine (Arg-p) is an AGE derived from the reaction of methylglyoxal with arginine residues, and Pento-s is a cross-linker formed by the reaction of pentose with lysine and arginine residues of proteins.[1,18] It is important to distinguish between free and bound AGEs as free AGEs are more bioavailable than protein-bound AGEs.

The objectives of this study were to (1) develop an LC-MS/MS method to simultaneously analyze five representative AGE compounds (CML, CEL, Pyr, Arg-p, and Pento-s) and quantify the free and free plus bound measured AGEs in raw and roasted almonds; (2) apply the developed method to evaluate the influence of roasting temperature and time on the formation of these AGEs in almonds; and (3) evaluate the levels of these AGEs in raw and roasted samples of nine varieties of almonds.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless otherwise stated. CML, CEL, Pyr, Arg-p, Pento-s, and *N*-ε-(carboxy[$^2H_2$]methyl)-lysine (CML-d_2) were purchased from NeoMPS (San Diego, CA). The purity of the standards is as follows: CML, >99.0%; CEL, >99.0%; pyrraline, 99.0%; pentosidine, 98.8%; argpyrimidine, 99.3%; CML-d_2, 95.7%. Pepsin, Pronase E (protease, type XIV), aminopeptidase (type VI-S), prolidase, and thymol were purchased from Sigma-Aldrich (St. Louis, MO). Nonafluoropentanoic acid (NFPA, 95+%) was obtained from Fluka (Sigma-Aldrich). HPLC grade methanol, acetonitrile, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ).

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 University of California, Davis (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 northern, southern, and central growing regions) and over two harvest years (2006 and 2007); samples of the other almond varieties were collected from the central region over two harvest years. Carmel almonds were used in developing the AGE analysis method and in assessing the impact of various roasting treatments (described below) on AGE formation. All almond varieties were used in the initial protein extraction efficiency experiments and in evaluating the effect of variety on AGE contents in raw and roasted samples.

Almond Roasting. Raw almond kernels were processed at UC Davis in a commercial fluidized-bed hot air roaster (Aeroglide, Cary, NC).

Table 1. Roasting Time at Different Temperatures Used To Achieve Light- and Dark-Roast Almonds

	roasting temperature					
	129 °C	138 °C	146 °C	154 °C	168 °C	182 °C
light roast[a] (min)	32	18	12	9	5	3.8
dark roast[b] (min)	70	28	18	13	8	5.7

[a] L^* = 62.41 ± 0.70 for light roast color. [b] L^* = 58.02 ± 0.83 for dark roast color.

Batches (~1 kg) of Carmel almonds were roasted at constant preset air inlet temperatures of 129, 138, 146, 154, 168, or 182 °C for specific times (ranging from 3.8 to 70.0 min) to achieve light through dark roasts (Table 1). A single roasting treatment combination (138 °C for 22 min) was used to roast almonds for the study evaluating AGEs in the nine almond varieties. The nut bed thickness was maintained at ~2.5 cm in the roaster. During roasting, the direction of airflow 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 (when 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). Sieved ground 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 L^* (lightness) according to the CIE $L^*a^*b^*$ color scale were obtained from triplicate samples and averaged.

Preparation of Samples for Free AGEs. Ground almonds (2 g) were added to a 50 mL conical tube along with 50 μL of CML-d_2 (10 μg/mL, internal standard) and 20 mL of ultrapure water. The mixture was homogenized for 1 min (model M133/1281-O, Biospec Products, Switzerland) and then centrifuged at 4000 rpm for 20 min, resulting in the formation of an immiscible oil layer and an aqueous layer with a suspension of fine particles as well as a solid residue. A 12 mL aliquot of the aqueous layer was promptly recovered with a glass Pasteur pipet into a 15 mL conical tube. NFPA (95%+) was then added at levels (120 μL) to give a final concentration of 1% NFPA. After the addition of NFPA, the tube was shaken by hand for 10 s, allowed to stand for 20 min, and then centrifuged at 4000 rpm for 10 min. The clarified aqueous extract obtained was transferred to another clean 15 mL tube for solid phase extraction (SPE). For SPE, a Strata-X-C cartridge (3 mL, 200 mg; Phenomenex, Torrance, CA) was preconditioned with two 1 mL aliquots of acetonitrile, followed by two 1 mL aliquots of 5 mM NFPA at a flow rate of 2 mL/min. The clarified aqueous extract (10 mL) was loaded onto the preconditioned cartridge at a flow rate of <0.5 mL/min. The cartridge was washed, in sequence, with 2 mL of 5 mM NFPA, 2 mL of water, and 2 mL of 50% acetonitrile/water (v/v). Finally, the AGE-containing fraction was eluted with 2 mL of 50% ammonia/acetonitrile (v/v) at a flow rate of <0.5 mL/min. The eluant was concentrated gently with a stream of nitrogen gas to dryness and to remove volatile ammonia, resolved in 1 mL of the initial mobile phase (5 mM NFPA in 15:85 water/acetonitrile, v/v), vortexed for 10 s, and then filtered through a 0.22 μm membrane prior to analysis by LC-MS/MS.

AGEs were quantified using standard addition methods employing CML-d_2. Raw and roasted almond samples were spiked with different levels of the five AGE standards, and sample preparation was carried out as described above. The peak area ratio of each standard was compared to the CML-d_2 internal standard and used to establish the standard curve. CML-d_2 was spiked into all samples to account for matrix effects. Recovery experiments were performed with five to seven replicate samples.

Preparation of Samples for Free Plus Bound Measured AGEs. Ground almonds (2 g) were added to a 50 mL conical tube and extracted three times with hexane to remove the almond oil. For each extraction, 20 mL of hexane was added to the tube, the mixture was homogenized for 1 min and centrifuged at 4000 rpm for 10 min, and the hexane extract was removed with a Pasteur pipet. After the three extractions, the residue was dried completely with a stream of nitrogen gas. Ultrapure water (20 mL) was added to the residue, and the mixture was homogenized for 1 min as described above, followed by filtering under vacuum in a Buchner funnel with Whatman no. 1 qualitative filter paper (70 mm). The effect of filtration on AGEs was determined in a subsample of three almond samples (one raw and two roasted, light and dark) treated with and without filtration. There were no statistically significant differences between the AGE levels and protein content for the two methods (e.g., CML in raw almonds before filtration (1.52 ± 0.06 mg/kg) and after filtration (1.49 ± 0.12 mg/kg)).

To evaluate the effect of centrifugation on the protein content and AGE levels, a portion of the filtered extract was removed and centrifuged at 4000 rpm for 20 min. Aliquots (1 mL) of the centrifuged portion and the original filtered extract (noncentrifuged portion) were subjected to analysis: (1) for protein content on the basis of the Bradford method (Bio-Rad, Hercules, CA) and (2) for free plus bound measured AGEs by hydrolysis with an enzyme cocktail, as described below. These analyses were carried out in duplicate.

A 20 μL aliquot of the filtered extract was hydrolyzed using a cocktail of proteolytic enzymes as described by Ahmed et al.[12] Briefly, aliquots of 40 mM HCl (25 μL), pepsin solution (900 U/mL in 20 mM HCl; 5 μL), and thymol solution (2 mg/mL in 20 mM HCl; 5 μL) were added to the filtered extract, and the sample was incubated at 37 °C for 24 h. The sample was then neutralized and buffered at pH 7.4 by the addition of 25 μL of 0.5 M potassium phosphate buffer (pH 7.4) and 5 μL of 260 mM KOH. Subsequently, a number of steps were performed under nitrogen to inhibit the degradation of fructosyl-lysine and prevent overestimation of CML residues. First, 5 μL of Pronase E solution (10 U/mL in 10 mM potassium phosphate buffer (pH 7.4)) was added, and the sample was incubated at 37 °C for 24 h. Then 5 μL each of aminopeptidase and prolidase solutions (20 and 200 U/mL, respectively, in 10 mM potassium phosphate buffer (pH 7.4)) was added, and the sample was incubated at 37 °C for 24 h. Finally, the hydrolyzed sample was spiked with 10 μL of CML-d_2 (1 μg/mL, prepared with 30% NFPA water) and then filtered through a 0.22 μm membrane prior to analysis by LC-MS/MS.

A calibration curve was also prepared for determining free plus bound measured AGEs. At the end of the digestion step, different levels of the AGE standards and 50 μL of the internal standard CML-d_2 (10 μg/mL) were added to blank samples, which included all of the buffers and enzymes, but the almond sample was replaced with 20 μL of water.

Comparison between Acid and Enzymatic Hydroylsis. Ground almonds (2 g) were extracted with 20 mL of hexane three times, and the residue was dried under a stream of nitrogen gas. Ultrapure water (20 mL) was added to the residue, and the mixture was homogenized for 1 min, followed by filtering under vacuum in a Buchner funnel with Whatman no. 1 qualitative filter paper (70 mm). Acid hydrolysis was achieved by the addition of 500 μL of concentrated hydrochloric acid (12.1 M) to an aliquot (500 μL) of the mixture. This was hydrolyzed at 110 °C for 20 h. Enzymatic hydrolysis was achieved using methods described above.

A 50 μL aliquot of internal standard (L-norleucine, 10 mg/mL) was added to the acid hydrolysates, mixed, and filtered using a 0.22 μm syringe filter. A 50 μL aliquot was then transferred to a glass vial and dried at 40 °C under a stream of nitrogen gas. The residue was redissolved in 100 μL of ultrapure water. A 20 μL aliquot of internal standard (L-norleucine, 1 mg/mL) was added to the enzymatic hydrolysates, mixed, and filtered using a 0.22 μm filter. An aliquot of 50 μL was transferred to a glass vial, and 5 μL of 260 mM KOH and 45 μL of ultrapure water were added to achieve a final volume of 100 μL (pH 9.5). Both digested hydrolysates were then derivatized using dansyl chloride as follows. An aliquot of 100 μL of 0.5 M $NaHCO_3$ buffer (pH 9.5) and 100 μL of dansyl chloride (20 mg/mL in acetonitrile) was added. The mixture was kept at 80 °C in the dark for 30 min, and the reaction was stopped by adding 40 μL of acetic acid. Sample solutions (5 μL) were analyzed at ambient temperature using reversed-phase HPLC (Shimadzu Scientific, Columbia, MD) on a Zorbax Eclipse Plus C_{18} column (2.1 × 100 mm, 1.8 μm, Agilent, USA). The binary mobile phase consisted of solvent A, composed of 10 mM ammonium formate (pH 3.4) in water, and solvent B, which was 100% acetonitrile. Separations were performed using a linear gradient of B into A at a flow rate of 0.25 mL/min as follows: 0−10 min, 40% B; 10−15 min, 40−85% B; 15−18 min, 85% B; 18−19 min, 85−40% B; and 19−23 min, 40% B. The UV detector was set to monitor 254 nm.

LC-MS/MS Analysis. Sample solutions were analyzed at ambient temperature using reversed-phase HPLC (Shimadzu Scientific) on a 5 μm Prodigy ODS column (2.0 × 150 mm) with a C_{18} guard column (Phenomenex). The binary mobile phase consisted of solvent A, composed of 5 mM NFPA in water, and solvent B, composed of 5 mM NFPA in acetonitrile. Separations were performed using a linear gradient of B into A at a flow rate of 0.2 mL/min as follows: 0−15 min, 15−45% B; 15−20 min, 45% B; 20−22 min, 45−15% B; and 22−30 min, 15% B. To maintain the sensitivity within the mass spectrometer, only HPLC eluents between 4.5 and 12 min were introduced into the mass spectrometer. All other column eluents were diverted to waste via a control diverter valve. 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, in positive mode ESI at a source temperature of 120 °C, and with a desolvation gas temperature of 380 °C at a 500 L/h flow rate. The argon collision gas pressure was adjusted to 3×10^{-3} mbar for MS/MS fragmentation. Data acquisition was performed in multiple-reaction monitoring (MRM) mode with a dwell time of 100 ms for each transition. Transition ions monitored for quantification, optimized collision energy, and cone voltages are summarized in Table 2. For each compound, two characteristic ions were chosen to improve the selectivity, with one ion labeled for quantification and the other for confirmation. Calibration curves, obtained by linear regression of a plot of the analyte/internal standard peak-area ratio against analyte concentration, were used to calculate compound concentrations in the samples. Quality control samples and calibration curves were included in each analysis run.

RESULTS AND DISCUSSION

Mass Spectra and Chromatography. The ESI positive mode was used in LC-MS/MS analysis of AGEs because of the strong response induced by amine groups ($-NH_2$, $-NH-$, $-N=$) in the acidified mobile phase. The collision-induced decomposition (CID) of CML, CEL, and Pyr was described in previous studies.[23−26] Findings from our study were consistent with reported fragmentation patterns. In general, CID of CML, CEL, and Pyr resulted in only two to four major product ions, and the two most prominent major ions accounted for >80% of ion intensities of the total product ions. For each compound,

Table 2. Mass Spectrometric Settings for Multiple-Reaction Monitoring of AGEs

AGE compd	cone voltage (V)	precursor ion (m/z)	product ion[a]/collision energy ((m/z)/eV)	neutral fragment loss
CML	20	205	130/12	$NH_2CH_2CO_2H$
			+ 84/18	$NH_2CH_2CO_2H$, H_2CO_2
CML-d_2	20	207	130/12	$NH_2CD_2CO_2H$
			+ 84/18	$NH_2CD_2CO_2H$, H_2CO_2
CEL	25	219	130/12	$NH_2CH(CH_3)CO_2H$
			+ 84/18	NH_2 $CH(CH_3)CO_2H$, H_2CO_2
Pyr	15	255	237/8	H_2O
			+ 175/12	H_2O, H_2O, CO_2
Arg-p	25	255	192/15	H_2CO_2, NH_3
			+ 140/15	$NH_2CH(CO_2)CH_2CH{=}CH_2$
Pento-s	45	379	250/22	$NH_2CH(CO_2)CH_2$ $CH_2CH{=}CH_2$
			+ 187/30	$NH_2CH(CO_2)CH_2$ $CH_2CH{=}CH_2$, H_2CO_2, NH_3

[a] Product ion labeled with + is for quantification; the other ion is for confirmation.

Table 3. Calibration, Sensitivity, and Recovery Data for LC-MS/MS Analysis Method for Free AGEs

	calibration		sensitivity[a]		recovery[b] (%)		
AGE compd	range (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)	200 μg/kg	500 μg/kg	1000 μg/kg
CML	500−2000	0.998	*3	*9		92.3−106.2	95.4−104.3
CEL	500−2000	0.992	*4	*12		86.7−110.3	88.2−121.1
Pyr	100−2000	0.999	6	16	86.7−110.2	92.4−105.7	91.6−108.2
Arg-p	100−2000	0.996	9	28	84.2−121.2	88.3−110.2	90.4−107.8
Pento-s	100−2000	0.982	12	38	92.6−108.5	94.5−102.4	90.6−102.3

[a] * indicates limit of detection (LOD) and limit of quantitation (LOQ) for CML and CEL dissolved in ultrapure water. Values were determined in ultrapure water as the level of these two compounds in all almond samples exceeded the LOD. [b] Recovery studies were determined seven times for spiking level of 200 μg/kg and five times for spiking levels of 500 and 1000 μg/kg.

the two most prominent product ions were chosen as MRM transition ions for quantification and confirmation. However, CID of Arg-p resulted in more than seven product ions, and even more complicated fragmentation was observed for CID of Pento-s. All of the settings for MRM are summarized in Table 2. In comparison with the other three AGE compounds, the transition ions used for quantitation for Arg-p and Pento-s showed relatively low response ion intensity, which consequently led to a higher limit of detection (LOD) or limit of quantitation (LOQ) (Table 3). The typical LC-MS/MS chromatographs of the five AGE compounds and the internal standard (CML-d_2) (in spiked ground almond samples) are shown in Figure 1.

Due to the high polarity of CML and CEL, both are usually not retained on common reversed-phase columns and the ion suppression effects from coelution of polar matrix components may significantly affect the analysis sensitivity. To avoid such interference, hydrophilic ion liquid chromatography (HILIC)[25] or NFPA as an ion-paring reagent has been used.[15,24,26] Although NFPA is not an ideal ion-pairing reagent due to the strong acidity it produces in the mobile phase (pH ~2), it greatly improves resolution and sensitivity. In general, we found that flushing the column with 100% solvent at the end of each day of use resulted in little, if any, stationary phase deterioration due to the acid nature of NFPA.

Quantification of unknown samples was achieved using an internal standard and by comparing the sample with standards in terms of relative retention time and relative abundance of the two selected product ions.

Sample Preparation for AGE Analysis. *Free AGEs.* To evaluate sample preparation for free AGE analysis, various concentrations of NFPA were added to aqueous extracts of spiked almond samples to facilitate AGE extraction and to precipitate proteins. The peak area for the added CML-d_2 standard in the aqueous extract was used as an indicator of extraction percent. NFPA concentrations at 0, 0.1, and 0.2% resulted in minimal extraction of AGEs, 0.5% NFPA resulted in 50−70% extraction, and 1 and 2% NFPA yielded >95% extraction efficiency. Similar results were obtained for aqueous extracts spiked with all five AGE standards (data not shown). These results indicate that acidified treatment by 1% NFPA is essential for free AGE extraction from almond samples. It is possible that AGEs are bound to protein reversibly and are released by NFPA acidification.

SPE cartridges (Strata-X-C) were effective in concentrating the free AGEs in the NFPA extracts. The cartridges could

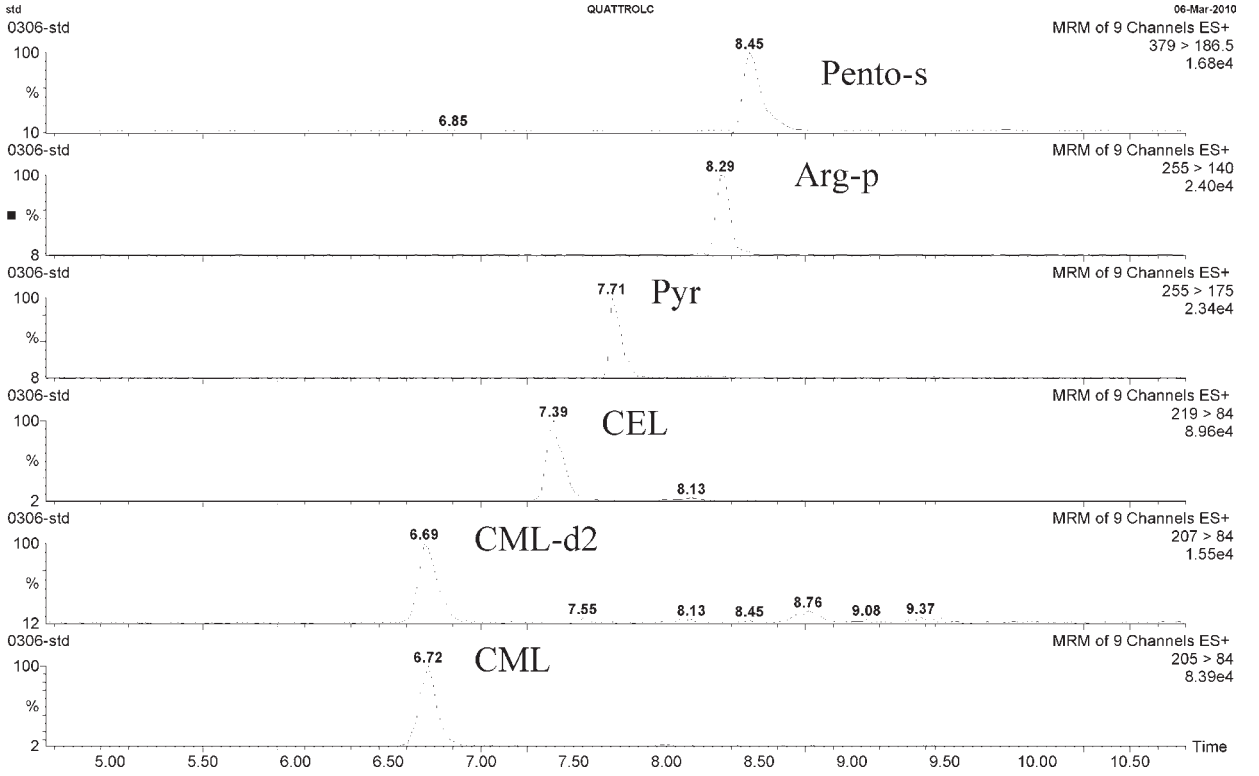

Figure 1. LC-MS/MS chromatograms obtained for simultaneous analysis of five free AGE standards in ground raw almond sample spiked with standards prior to extraction. MRM transitions and retention time (RT) are as follows: CML, m/z 205 → 84 at 6.72 min; CML-d_2, m/z 207 → 84 at 6.69 min; CEL, m/z 219 → 84 at 7.39 min; Pyr, m/z 255 → 175 at 7.71 min; Arg-p, m/z 255 → 140 at 8.29 min; Pento-s, m/z 379 → 187 at 8.45 min.

accommodate up to 10 mL of NFPA extract with complete retention of all AGE compounds. Sequential washing of the cartridge removed most interfering compounds and had no effect on AGE retention. The ability of an ammonia/acetonitrile solution (>50% ammonia) to completely elute the AGEs was confirmed. Final reconstitution of the sample in the initial mobile phase (5 mM NFPA in 15: 85 water/acetonitrile, v/v) gave clean LC-MS/MS chromatograms and satisfactory sensitivity (Table 3).

For sample preparation for free plus bound AGE extraction, defatted almond samples were homogenized with water to extract protein prior to hydrolysis with the enzyme cocktail. Preliminary experiments on protein extraction efficiency were carried out to compare pure water extraction and protein extraction, on the basis of the Bradford method, with Tris/glycine/SDS buffer (Bio-Rad), as described earlier. However, this buffer did not result in greater protein extraction from almond samples as compared with pure water (data not shown). Therefore, pure water was used for protein extraction to avoid possible interference from buffer during subsequent enzymatic hydrolysis and analysis by LC-MS/MS.

Instead of purifying the extracted protein by centrifugal ultrafiltration or other treatments, we simply filtered the extract with qualitative filter paper to remove the solid portion prior to enzymatic hydrolysis. Preliminary experiments showed that even low-speed centrifugation (4000 rpm for 20 min) could significantly affect protein content and AGE levels. For the 10 samples of raw and roasted Carmel almond samples tested, AGEs and protein content were found to be significantly higher in the noncentrifuged (NC) portion than in the centrifuged (C) portion. In the NC portions, CML levels in almonds ranged from

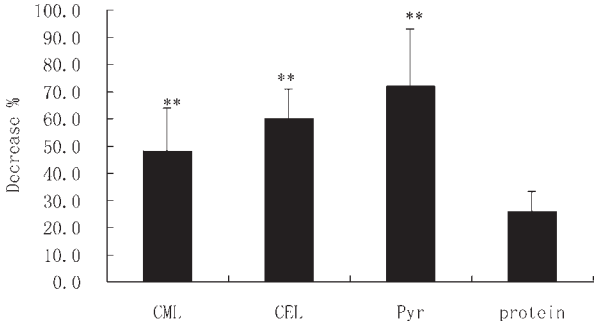

Figure 2. Decreased percentage (D%) of AGEs and protein content in roasted almond sample extracts prepared with centrifugation compared to without centrifugation (** indicates significant differences at $p < 0.01$ with respect to mean values for each AGE.)

2.87 to 4.51 mg/kg (average, 3.73 ± 0.50 mg/kg), CEL from 4.56 to 8.82 mg/kg (average, 6.28 ± 1.63 mg/kg), Pyr from 4.34 to 48.72 mg/kg (average, 16.84 ± 13.41 mg/kg), and protein content from 67 to 124 mg/g (average, 105 ± 15 mg/g).

The following equation was used to calculate the decreased percentage (D%) of AGEs and protein after centrifugation:

$$D\% = (C_{NC} - C_C)/C_{NC} \times 100\%$$

C_{NC} is the concentration in the noncentrifuged portion, and C_C is the concentration in the centrifuged portion. Results for percent decrease (D%; calculated from mean values ± SD) are illustrated in Figure 2. On average, the protein content decreased by 25.8% (15.1−35.9%) after centrifugation. The content of CML, CEL, and Pyr decreased significantly ($p < 0.01$, t test) after

centrifugation, with average D% values of 48.2% (18.4−70.4%), 59.9% (32.3−72.3%), and 72.0% (30.2−96.0%), respectively. It is clear that the decrease in protein content correlates with significant decreases in AGE levels, especially Pyr, which could lead to marked underestimation of total AGE levels. Even when the concentration of each AGE was calculated in terms of protein content, the AGE levels in the noncentrifuged portion were still significantly higher than those in the centrifuged portion (data not shown).

The isolation of protein is usually required prior to hydrolysis in foods for AGE analysis and can influence levels of reported AGEs.[17] Protein precipitation, ultrafiltration, and washing procedures can lead to the underestimation of protein due to the loss of small peptides and proteins. Sheoran et al.[32] demonstrated this with tomato pollen protein extracted using four different extraction media: trichloroacetic acid (TCA)/acetone, phenol, direct isoelectric focusing (IEF) buffer, and Tris-HCl buffer. This group found that the direct IEF method extracted the greatest amount of protein, whereas the other methods resulted in significantly lower protein extraction with values ranging from 57.8 to 76.7% as compared with direct IEF.[32] Interestingly, Assar et al.[15] found that protein isolation methods influence CML analysis, such that the CML level in skimmed milk increased consistently in the following order: ultrafiltration (0.04 ± 0.01 mmol/mol Lys) < TCA precipitation (0.06 ± 0.02 mol/mol Lys) < Folch extraction (0.08 ± 0.02 mmol/mol Lys). These researchers suggested that the presence of trace impurities (e.g., heavy metals) promotes the oxidation of lipid, sugar, or Maillard reaction intermediates to form CML during sample workup. However, this cannot entirely explain the significantly lower level of CML extracted using ultrafiltration as compared with the other methods. A more reasonable explanation might be that the composition of isolated total protein varied between methods, especially in terms of the composition of small protein or peptides. In the current study, to avoid loss of protein, the defatted aqueous extract solutions were subjected directly to enzymatic hydrolysis after filtration without any further purification treatments.

Enzymatic hydrolysis was carried out using a cocktail of enzymes as reported by Ahmed et al.[12] with one modification: the last incubation step (with aminopeptidase and prolidase) was changed from 48 to 24 h after our trials showed that this change had no effect on AGE analysis in almond samples (data not shown). Initially, each defatted roasted almond sample was extracted 5:1 with water to give extracts containing 24 mg of protein/mL. Extract solutions were then sequentially diluted with pure water to obtain 12 and 6 mg/mL protein, respectively. Because 20 μL of each solution was subjected to enzymatic digestion, the protein amounts were 480, 240, and 120 μg, respectively. A linear correlation ($R^2 > 0.999$) was found between AGE level and the protein amount in the sample extracts (Figure 3). CML, CEL, and Pyr levels increased proportionally with protein, indicating that the enzymatic digestion conditions were sufficient for samples containing up to 480 μg of almond protein. In the current study, the protein amount in the 20 μL extracts ranged from 90 to 360 μg, which was well below 480 μg.

Enzymatic digestion is never complete, and roasting temperatures could denature and/or degrade proteins and influence enzymatic hydrolysis. For example, Hegele et al. evaluated the enzyme cocktail used herein for protein cleavage and compared results with those obtained via acid hydrolysis in dairy products.[23] The two proteolysis methods yielded comparable quantitative results (slope = 0.97). Another study comparing

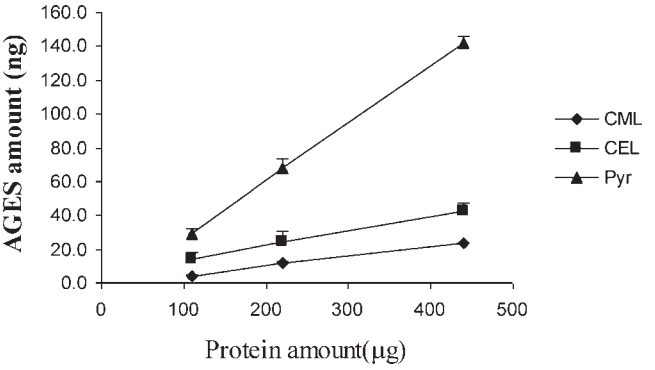

Figure 3. Correlation between level of AGE and protein content in roasted almond sample extracts obtained after enzymatic hydrolysis and diluted to different protein concentrations.

Table 4. Comparison of Acid Hydrolysis (A) with Enzymatic Hydrolysis (E) in Raw and Light- and Dark-Roasted Carmel Almonds[a]

	enzyme/Lys (μg/mg protein)	acid/Lys (μg/mg protein)	E/A%
raw	34.5 ± 5.5	49.7 ± 0.4	69.5
light roast	38.5 ± 2.0	53.9 ± 1.3	71.4
dark roast	34.8 ± 1.8	50.4 ± 2.7	68.9

[a] Almonds were roasted to light color at 154 °C for 12 min and to dark color at 154 °C for 18 min.

enzymatic hydrolysis with acid hydrolysis for 3-nitrotyrosine (NY) in BSA[34] showed that acid hydrolysis gave 95% digestion and the cocktail method gave 75%. Therefore, an acid digestion was compared to an enzymatic digestion protein digestion in raw and light- and dark-roasted almonds (Table 4). The results indicate that the enzymatic hydrolysis was 69.5% efficient in raw almonds (lys μg/mg protein), 71.4% in light-roasted almond and 68.9% in dark-roasted almond.

Calibration, Sensitivity, and Recovery. *Free AGEs.* The unspiked raw almond samples tested contained some CML and CEL, so these background values were subtracted from spiked samples. The method validation results and recoveries of AGE standards are summarized in Table 3. The correlation coefficients (R^2) were >0.99 (except Pento-s, R^2 = 0.982). The linear range was large enough to accommodate free AGEs in real almond samples, and recoveries at all three spiking levels (200, 500, and 1000 μg/kg) fell in the range of 84−110%. Recoveries >120% were observed for CEL and Arg-p at spiking levels of 1000 and 200 μg/kg, respectively. Validation of method sensitivity indicated that the AGE compounds could be detected in samples at levels as low as 3−12 μg/kg (LOD) and could be quantified at levels of 9−38 μg/kg (LOQ).

Free plus Bound Measured AGEs. Arg-p and Pento-s were not detected in almond samples in free or adduct form; therefore, the calibration curve for total AGE determination was established for only CML, CEL, and Pyr. The linear range determined for these AGE compounds was from 0.02 to 0.4 μg/mL, and R^2 values were >0.99 (data not shown). Correspondingly, as calculated by sample weight, this method could be used to quantify these AGEs over a wide range, from 1 to 20 mg/kg in almond samples. Validation of method sensitivity (using the same calibration ranges as for free AGEs) showed that, in the final

enzyme-hydrolyzed solutions, this method was able to detect levels as low as 8.4 μg/mL CML, 9.4 μg/mL CEL, and 15.6 μg/mL Pyr; at these concentrations the S/N was ≥3. Calculated in terms of sample weight, the LOD of the method corresponded to 0.42 mg/kg for CML, 0.47 mg/kg for CEL, and 0.78 mg/kg for Pyr. Thus, the sensitivity of the method for free plus bound measured AGEs was not as great as for free AGEs, which was likely due to the multiple dilution steps introduced by the extraction and enzymatic hydrolysis treatment. Also, prior to analysis by LC-MS/MS, no further concentration treatment was applied due to the very small final volume of 100 μL.

Recovery was tested by spiking the hydrolyzed sample solutions with three levels of AGE standards (5, 10, and 20 mg/kg; concentration calculated by sample weight), with five to seven replicates for each level. The recovery varied for the individual AGE compounds at different spiking levels as follows: CML recovery ranged from 90.3 to 103.9% (RSD < 9.9%); CEL ranged from 82.8 to 96.7% (RSD < 9.3%); and Pyr ranged from 83.3 to 112.3% (RSD < 15.9%).

Matrix Interference Effect. A matrix interference effect from the almond sample was observed in this study for recovery tests of free plus bound measured AGEs and some free AGEs. For CML and CEL, there was no difference in recovery whether the

Table 5. Influence of Roasting Treatment on Free and Total AGE Levels in Almonds (Carmel)

roasting treatment			free AGEs (mg/kg)			free + bound AGEs (mg/kg)		
temp (°C)	time (min)	color L^*	CML	CEL	Pyr	CML	CEL	Pyr
raw	0	67.34 ± 0.38[a]	0.37 ± 0.018	0.416 ± 0.022	nd[b]	1.4 9 ± 0.12	1.30 ± 0.13	nd
129	32	63.77 ± 0.34	0.655 ± 0.044	1.065 ± 0.043	0.492 ± 0.040	4.1 ± 0.22	4.2 ± 0.32	25.7 ± 2.3
	40	62.82 ± 0.40	0.582 ± 0.035	1.156 ± 0.079	0.703 ± 0.041	4.3 ± 0.24	4.6 ± 0.74	29.5 ± 2.8
	50	60.95 ± 1.54	0.601 ± 0.054	0.854 ± 0.091	0.937 ± 0.056	5.0 ± 0.48	5.5 ± 0.86	39.9 ± 3.1
	60	61.20 ± 0.74	0.576 ± 0.049	1.077 ± 0.080	0.807 ± 0.086	6.5 ± 0.77	6.3 ± 0.65	48.6 ± 3.0
	70	58.81 ± 0.93	0.589 ± 0.038	0.930 ± 0.085	0.796 ± 0.103	4.8 ± 0.56	5.1 ± 0.37	42.3 ± 1.9
mean ± SD[c]			0.601 ± 0.032	1.0 ± 0.12	0.747 ± 0.165	4.93 ± 0.95	5.13 ± 0.81*[d]	37.2 ± 9.4
138	18	61.98 ± 0.84	0.584 ± 0.059	0.912 ± 0.076	0.509 ± 0.063	2.9 ± 0.19	4.9 ± 0.37	4.3 ± 0.2
	22	60.92 ± 2.27	0.555 ± 0.039	0.771 ± 0.057	0.544 ± 0.045	3.9 ± 0.23	5.9 ± 0.79	4.8 ± 0.4
	26	60.57 ± 2.02	0.579 ± 0.050	0.908 ± 0.048	0.547 ± 0.032	3.9 ± 0.35	5.9 ± 0.96	11.1 ± 1.1
	30	58.56 ± 1.91	0.546 ± 0.043	0.893 ± 0.048	0.568 ± 0.034	4.1 ± 0.35	5.3 ± 0.86	12.4 ± 1.2
mean ± SD			0.566 ± 0.018	0.87 ± 0.07	0.542 ± 0.024**	3.66 ± 0.54	5.51 ± 0.51*	8.2 ± 4.2**
146	12	62.49 ± 1.56	0.601 ± 0.042	1.004 ± 0.097	0.589 ± 0.068	3.7 ± 0.24	6.7 ± 0.62	6.3 ± 0.4
	14.7	61.76 ± 0.30	0.625 ± 0.031	1.049 ± 0.122	0.668 ± 0.036	3.8 ± 0.38	6.3 ± 0.52	47.9 ± 2.4
	16	58.92 ± 2.02	0.528 ± 0.027	0.864 ± 0.101	1.054 ± 0.127	4.2 ± 0.30	6.4 ± 0.80	50.3 ± 3.8
	18	58.69 ± 0.39	0.594 ± 0.054	1.074 ± 0.072	1.132 ± 0.085	4.7 ± 0.41	8.3 ± 0.98	66.8 ± 4.7
mean ± SD			0.587 ± 0.041	1.00 ± 0.09	0.861 ± 0.272	4.09 ± 0.47	6.91 ± 0.94	42.8 ± 25.8
154	9	61.90 ± 0.49	0.573 ± 0.063	0.993 ± 0.060	0.576 ± 0.038	3.2 ± 0.26	5.3 ± 0.63	5.4 ± 0.4
	11.3	60.23 ± 0.58	0.577 ± 0.061	1.028 ± 0.092	1.155 ± 0.115	4.2 ± 0.30	5.7 ± 0.39	19.4 ± 1.5
	12	58.56 ± 0.71	0.573 ± 0.049	0.939 ± 0.080	1.231 ± 0.116	4.5 ± 0.23	5.4 ± 0.33	48.7 ± 2.9
	13.3	58.47 ± 0.47	0.561 ± 0.070	0.969 ± 0.063	1.269 ± 0.091	4.4 ± 0.23	7.2 ± 0.65	67.6 ± 6.3
mean ± SD			0.571 ± 0.007	0.98 ± 0.04	1.058 ± 0.325	4.08 ± 0.60	5.90 ± 0.89	35.3 ± 28.1
168	6.3	60.15 ± 1.13	0.532 ± 0.018	0.849 ± 0.086	0.821 ± 0.092	4.5 ± 0.41	6.3 ± 0.54	16.1 ± 1.0
	7	60.09 ± 0.17	0.485 ± 0.044	0.853 ± 0.060	0.882 ± 0.069	4.9 ± 0.54	7.1 ± 0.46	19.7 ± 1.6
	8	56.89 ± 1.18	0.492 ± 0.053	0.844 ± 0.073	1.225 ± 0.117	4.7 ± 0.50	7.2 ± 0.73	46.8 ± 3.4
	9	54.63 ± 0.79	0.466 ± 0.064	0.746 ± 0.059	1.291 ± 0.113	5.7 ± 0.46	19.8 ± 1.41	56.6 ± 3.6
mean ± SD			0.494 ± 0.028	0.82 ± 0.05	1.055 ± 0.238	4.94 ± 0.54	10.09 ± 6.50	34.8 ± 20.0
182	3.8	62.12 ± 0.52	0.630 ± 0.034	0.735 ± 0.051	0.290 ± 0.022	3.8 ± 0.48	4.6 ± 0.40	8.6 ± 0.4
	4.5	59.73 ± 1.61	0.615 ± 0.063	1.010 ± 0.050	0.548 ± 0.030	3.3 ± 0.11	8.5 ± 0.68	23.4 ± 1.1
	5.7	57.09 ± 1.21	0.472 ± 0.033	0.634 ± 0.032	0.460 ± 0.026	4.0 ± 0.37	8.4 ± 0.59	22.0 ± 1.8
	6	55.82 ± 1.32	0.473 ± 0.014	0.573 ± 0.052	0.752 ± 0.076	3.5 ± 0.38	8.8 ± 0.44	19.5 ± 2.0
mean ± SD			0.548 ± 0.087	0.74 ± 0.19	0.512 ± 0.19*	3.65 ± 0.30**	7.59 ± 2.02	18.4 ± 6.7*

[a] Represents the individual data obtained in triplicate roasting experiments. [b] Mean ± SD represents the average level obtained from different roasting times at individual roasting time. [c] nd, not detected. [d] * and ** indicate significant differences with respect to mean values within a column at $p < 0.05$ and $p < 0.01$, respectively, as determined by one-way ANOVA analysis ($n = 5$ for temperature 129 °C, $n = 4$ for other roasting temperature).

standards were spiked in the aqueous solutions or almond extracts. The application of isotope-labeled CML-d_2 as the internal standard was effective in correcting any matrix interference for CML determination, and CEL is similar to CML in terms of structure and other chemistry characteristics. However, for Pyr, Arg-p, and Pento-s there was significant matrix interference when recovery was calibrated by standard curves established by spiking standards in aqueous solutions; the recovery of these compounds could vary from 50 to 200%. As shown by the recovery data in Table 3, matrix interference was overcome when the calibration curve was made by spiking standards in almond samples and following all of the extraction treatments as for unknown samples.

Impact of Roasting Treatment on AGE Formation. As shown in Table 5 for Carmel almonds, only CML and CEL were detected in both raw and roasted almond samples, and Pyr was absent in raw almonds but identified in all roasted samples. CML and CEL are formed from lysine modified by glyoxal and methylglyoxal. These 1,2-dicarbonyls are formed by the Maillard reaction but may also result from lipid oxidation.[30,31] During a typical harvest in California, almonds are stored in the field with temperatures frequently >35 °C (daytime), until a kernel moisture of 5−8% is reached. Almonds are rich in lipids; therefore, it is plausible that the initial CML and CEL may have come from lipid oxidation generated during in-field drying. Pyr is formed from lysine and 3-deoxyglucosulose, which is a major product of the Maillard reaction. Maillard chemistry normally requires high temperatures and low moisture, which might explain the presence of Pyr in only the roasted almond samples. Arg-p and Pento-s were not detected in either raw or roasted almonds and so are not included in the table. For each AGE shown, the average levels of free and free plus bound measured AGEs were higher in all roasted samples compared with the raw samples. After roasting, free CML levels increased by about 50% (23.3−73.2%) and free CEL levels increased by about 120% (37.7−177.9%); total CML and total CEL levels increased by 186% (91.1−333.6%) and about 413% (187.5−1425%), respectively. Pyr was the major AGE in roasted almonds, accounting for about 64% (30.7−84.4%) of the three compounds; CML and CEL accounted for only about 13 and 20%, respectively.

Average free CML or CEL levels in almonds from the various roasting temperature treatments were not significantly different. By contrast, average total CML levels were significantly lower ($p < 0.01$) in almonds roasted at 182 °C, and average total CEL levels were significantly lower ($p < 0.05$) in almonds roasted at 129 and 138 °C. Both free and total Pyr levels were significantly lower in almonds roasted at 138 and 182 °C for specific times compared with the other roasting treatments.

At the individual roasting temperatures, no correlation was found between CML or CEL levels in almonds and roast color (light and dark). The maximal level of CML and CEL formation was attained even at the roast condition that resulted in a lighter color (higher L^* value) at the individual roasting temperatures (not shown). Total Pyr levels increased in samples roasted to a dark roast color (L^* values of 58.02 ± 0.83) at roasting temperatures of 154 and 168 °C, but not at 182 °C, as illustrated in Figure 4. Total Pyr levels were relatively low in all samples roasted at 138 °C, regardless of the roast color or roasting time. Roast color is highly correlated to the formation of flavor compounds during roasting, and shorter roasting times may affect product quality in terms of preferable flavor and color.[33]

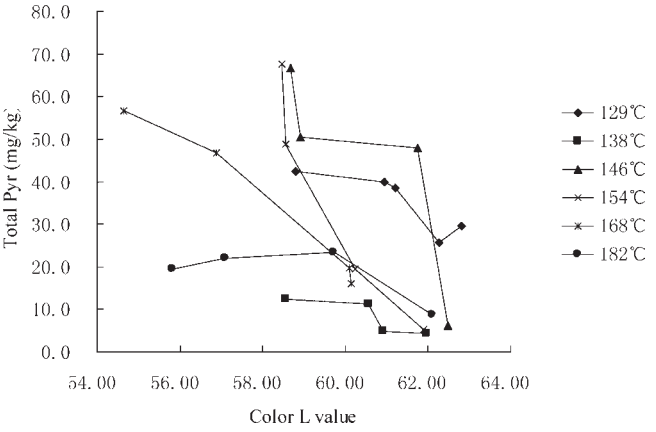

Figure 4. Relationship between total Pyr levels and color (L^* value) of almonds roasted at various temperatures to achieve light and dark roasts. (Roasting times used to achieve a light or dark roast at each treatment temperature are provided in Table 1.)

Correlation between Free AGEs and Free plus Bound Measured AGEs. In all almond samples, the total AGE level consisted mainly of the protein adducts of each AGE (Table 5). In raw almonds, free CML and CEL made up 25.4 and 31.9% of total CML and CEL, respectively. Roasted almonds contained a lower proportion of free AGEs to free plus bound measured AGEs: free/total percentages for CML, CEL, and Pyr averaged 13.6, 15.4, and4.1%, respectively. Similar reductions in the proportion of free to free plus bound measured AGEs after heat processing were reported by Ahmed et al.,[12] who found that free CML made up 30% of total CML adducts in raw milk and ∼12 or 24% of total CML in sterilized or pasteurized milk and that free CEL made up ∼21% of total CEL adducts in raw milk and ∼10% of total CEL in sterilized or pasteurized milk. Herein a plot of the percentage values for free to total AGE against the total AGE concentration (mg/kg) demonstrates that the percentage of each free AGE decreased with the increase in total AGE concentration (CML (R^2 = 0.8555), CEL (R^2 = 0.8935), and Pyr (R^2 = 0.7647)).

Impact of Almond Variety on AGE Formation in Roasted Almonds. The moisture content for the nine almond varieties sampled was determined to be within a range of 3−4%. A single roasting treatment was used in evaluating AGE formation in the nine varieties of almonds, and this was compared to Carmel almonds. Hunter L^* values (lightness) of the roasted ground samples varied from 57.26 to 64.44, with an average L^* value of 61.40 ± 2.20 for the nine varieties. Table 6 summarizes the average free and total AGE levels found in these raw and roasted samples. CML and CEL were detected in both raw and roasted almonds of all tested varieties, and Pyr was detected only in roasted almonds. After roasting, the free CML increased by about 81% and CEL by 73%. Total average AGE levels increased by about 149% for CML and by about 356% for CEL. The total Pyr level ranged over the nine varieties of roasted almonds from 4.8 mg/kg in Carmel samples to 21.6 mg/kg in Avalon samples. Thus, almond variety might play an important role in Pyr formation introduced by thermal processing. Because Pyr was the predominant AGE among the three detected AGEs (50.1 ± 10.0% in roasted almonds, Table 6) and not detected in raw almonds, it may be a better marker of thermal processing-induced damage to proteins than CML/CEL in almonds.

Table 6. Average Variability in Free and Total AGE Levels in Raw and Roasted Almonds (Nine Varieties)

		free AGE (mg/kg)			total AGE[b] (mg/kg)			free/total (%)		
almond treatment[a]		CML	CEL	Pyr	CML	CEL	Pyr	CML	CEL	Pyr
raw	av	0.298	0.479	nd[c]	1.77	1.92	nd	17.4	28.1	
	SD	0.059	0.105		0.28	0.65		5.3	12.7	
roasted	av	0.520	0.785	1.100	4.26	7.70	12.6	13.4	11.4	10.0
	SD	0.082	0.133	0.512	1.38	2.56	4.9	4.1	4.2	6.4
av increase (%)		80.6	72.7		149.2	355.7				
SD		21.3	23.6		116.4	284.1				

[a] Almonds were roasted at 132 °C for 22 min; duplicate samples of nine varieties were analyzed to obtain average values for AGEs. [b] Total AGE = free AGE + protein-bound AGE. [c] nd, not detected.

Levels of CML reported herein (4.26 ± 1.38 mg/kg, Table 6) are lower than levels reported previously (12 mg/kg CML) in almonds roasted at 180 °C for 8 min.[19] However, this group used acid hydrolysis without previous reduction, and levels of CML have been shown to increase 2−10-fold by the conversion of FL (N^{ϵ}-fructosyl-lysine) to CML during acid hydrolysis.[23]

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