JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

N-Acetylglutamate and *N*-Acetylaspartate in Soybeans (*Glycine max* L.), Maize (*Zea maize* L.), and Other Foodstuffs

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N-Acetylglutamate (NAG) and *N*-acetylaspartate (NAA) are amino acid derivatives with reported activities in a number of biological processes. However, there is no published information on the presence of either substance in foodstuffs. We developed a method for extracting and quantifying NAG and NAA from soybean seeds and maize grain using ultra performance liquid chromatography−electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). The lower limit of quantification for both NAG and NAA was 1 ng/mL. The method was then utilized to quantify NAG and NAA in other foodstuffs (fruits, vegetables, meats, grains, milk, coffee, tea, cocoa, and others). Both NAG and NAA were present in all of the materials analyzed. The highest concentration of NAG was found in cocoa powder. The highest concentration of NAA was found in roasted coffee beans. Both NAG and NAA were found at quantifiable concentrations in all foods tested indicating that these two acetylated amino acids are common components of the human diet.

KEYWORDS: *N*-Acetyl-L-glutamate; *N*-acetylglutamate; *N*-acetyl-L-aspartate; *N*-acetylaspartate; soybean (*Glycine max* L.); maize (*Zea maize* L.); UPLC-ESI-MS/MS

INTRODUCTION

N-Acetylglutamate (NAG) has several known roles in biological processes across phyla. It is an intermediate in arginine biosynthesis in plants, lower eukaryotes, and prokaryotes (*1, 2*). During the formation of a nitrogen-fixing association between white clover and *Rhizobium trifolii*, NAG can function as an extracellular signal that is released from the bacteria, which then affects plant root hair and subsequent nodule development (*3*). In addition, NAG serves as an essential allosteric cofactor for carbamoyl phosphate synthetase I (EC 6.3.4.16), the first enzyme of the urea cycle, in ureotelic animals (*1*). NAG is present in the brain of both ureotelic mammalian and nonureotelic bird species (*4*). NAG in the brain has been proposed to be an intermediate in the turnover of the neuropeptide N-acetylaspartylglutamate (NAAG; *4*).

Another acetylated amino acid, *N*-acetylaspartate (NAA), is present in the mammalian central nervous system (CNS; *5*, *6*). NAA is believed to be among the most abundant free amino acids in the CNS, second in concentration only to that of free glutamate (*7*). NAA is the primary source of acetate for the biosynthesis of myelin lipid fatty acids (*8, 9*). It has also been proposed to play a bioenergetic role in neuronal mitochondria, be a reservoir for the storage of aspartate, be involved in brain nitrogen balance in general, be implicated in neuronal osmoregulation and axon-glial signaling, and serve as a precursor in the biosynthesis of NAAG (*6, 7*).

Both NAA and NAG have been quantified utilizing a number of analytical procedures. Within the mammalian CNS, NAA is restricted to the neurons and can be measured clinically as an indicator of neuron integrity using noninvasive proton NMR because of the presence of a strongly resonating proton (*6, 10*). Quantitative NAA and NAG methods included capillary electrophoresis (*11*), ion exchange chromatography followed by deacetylation, derivatization and separation of the deacetylated amino acid on reverse phase HPLC (*12*), ion-pairing HPLC (*13, 14*), reversed phase-HPLC with UV detection (*15*), GC-MS (*4, 16*−*19*), HPLC-APCI-MS (*20*), and HPLC-ESI-MS/MS (*21*). NAG was measured semiquantitatively in plants as part of metabolomics studies using GC-MS (*18, 19*).

The concentrations of NAA and NAG have not been reported for any foodstuffs. These substances, as is the case with many other minor dietary constituents, are not typically included in the compositional analyses conducted on whole grains and other foods. In the current article, we describe a method for extracting and quantifying NAG and NAA in soybeans and maize using ultra performance liquid chromatography−electrospray ionization tandem quadrupole mass spectrometry (UPLC-ESI-MS/MS). We also report the concentrations of NAA and NAG from an application of this method to quantify these compounds in other foodstuffs.

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10.1021/jf801523c CCC: $40.75
Published on Web 09/10/2008

MATERIALS AND METHODS

Reagents. *N*-Acetyl-L-aspartic acid (>99%) and *N*-acetyl-L-glutamic acid (>99%) were purchased from Fluka (Saint Louis, MO). *N*-Aspartic-2,3,3-d_3 acid, acetyl derivative (>98%), was purchased from Isotec (Miamisburg, OH). *N*-Acetyl-L-glutamic-2,3,3,4,4-d_5 acid (>99%) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Omnisolv LC-MS grade water, Omnisolv LC-MS grade methanol, Omnisolv chloroform, and Suprapur formic acid (98−100%) were purchased from EMD Chemicals Inc. (Gibbstown, NJ).

Foodstuff Materials. Soybean seed (*Glycine max* L., Pioneer varieties 93B87, 93B86, 93B15, and 93M40) and maize grain (*Zea mays* L., Pioneer hybrids 33J56, 33P66, and 33R77) were obtained from Pioneer Hi-Bred International (Johnston, IA). Other foodstuffs were purchased from local markets, except for coffee beans from Coffee Bean Direct (Titusville, NJ) which were mail ordered.

Green (unroasted) Arabica coffee beans including Sumatra Mandheling, French/Italian Blend, House Blend, Costa Rican Tarrazu, Columbian Supremo, and Six Bean Espresso were purchased from Coffee Bean Direct. Additional coffees were Columbian Rich Premium Roast Arabica coffee beans (Shop Rite Supermarkets, Waken Food Corporation, Elizabeth, NJ), Arabica espresso (Pilon, Rowland Coffee Roasters, Inc., Miami, FL), and a ground coffee blend of Robusta and Arabica (Maxwell House, Kraft Foods, Inc., Northfield, IL). Organic China black tea leaves and organic green tea leaves were from Two Hills Tea Company (Nelson, BC, Canada).

Medium brown rice, pearled barley, hulled barley, buckwheat groats, roasted buckwheat, soft spring wheat berries, and Adzuki beans were purchased from Newark Natural Foods (Newark, DE).

Fresh fruits and fresh vegetables included bananas (Del Monte Fresh Produce S.A., San José, Costa Rica), spinach (Albertson's, Inc., Boise, ID), Romaine lettuce hearts, (Acme Markets, Eden Prairie, MN), green beans (Procacci Brothers Sales Corp, Philadelphia, PA), Roma tomatoes (Euro Fresh Farms, Wilcox, AZ), Royal Gala apples (Frusan, Chile), carrots (Albertson's, Inc.), Valencia oranges (Sunkist Growers, Inc., Sherman Oaks, CA), broccoli crowns (Acme Markets), sweet potato (Blue Denim Sweet Bakables, Sherman Masser, Inc., Sacramento, CA), and baking potato (Dole Fresh Vegetables, Inc., Salinas, CA). Shelled walnuts were from Diamond of California (Stockton, CA).

Meats consisted of ground beef (80% lean) (Albertson's, Inc.), ground pork (Smithfield, Smithfield, VA), ground turkey (Shadybrook Farms, Wichita, KS), ground chicken (Perdue, Salisbury, MD), and canned sardines with bone (King Oscar Brisling, Bumblebee Seafoods, San Diego, CA). Additional foodstuffs were whole milk with vitamin D (Acme Markets), eggs (Acme Markets), milk-based infant formula powder (Enfamil Lipil, Mead Johnson & Co., Evansville, IN), soy-based infant formula powder (Nestle Good Start, Vevey, Switzerland), cocoa (Nestle Toll House), dark chocolate (Dove, Mars Incorporated, McLean, VA), and stout beer (Guinness, Dublin, Ireland).

Soybean and Maize Sample Preparation. Soybean seed and maize grain were pulverized into a fine powder using a 2000 Geno/Grinder (Spex CertiPrep, Inc., NJ). Approximately 100 mg of powdered sample was accurately weighed into a micro centrifuge tube (2 mL, screw cap with O-ring, Bio Plas, Inc., San Rafael, CA) and in some cases fortified with NAA and NAG at a final concentration of 0.3 μg/g dry weight. A 5/32 inch diameter stainless steel ball (McMaster-Carr, Elmhurst, IL) and 1.0 mL of extraction solvent (1:2 v/v methanol/chloroform) were added to the tube, which was then placed in a Geno/Grinder for 1 min at 1500 strokes/min. Sample tubes were subsequently placed on an end-over-end mixer (Glas-Col, Terre Haute, IN) for 15 min. Water (0.3 mL) was added to the extract, which was again placed in the GenoGrinder for 1 min and mixed for an additional 15 min. Samples were centrifuged at 14,000 rpm to facilitate phase separation. The aqueous layer was collected, the organic phase discarded, and the material originally at the interphase and tube bottom was extracted again with 1 mL of extraction solvent and 0.3 mL of water. The aqueous fractions were combined, dried (about 2 h) with medium heat (approximately 43 °C) in a SpeedVac (Thermo Fisher Scientific, Inc., Waltham, MA), and resuspended in 0.2% (v/v) aqueous formic acid with 5 min in a bath sonicator (VWR International, West Chester, PA) at the highest power setting. Internal standards (deuterated NAA (d_3-NAA) and deuterated NAG (d_5-NAG)) were added to a final concentration of 50 ng/mL to allow for correction of any ion suppression, or enhancement, during mass spectrometry. Samples were filtered through a Costar Spin-X 0.22 μm nylon filter (Corning, Inc., Corning, NY) and then analyzed by UPLC-ESI-MS/MS.

Foodstuff Sample Preparation. Foodstuffs with high water content (fresh fruits, fresh vegetables, meats, eggs, and milk) were dried in a freeze-dryer (Freeze Dry Company, Inc., Nisswa, MN) prior to extraction. Fresh fruits and fresh vegetables were washed with water and then blotted dry. Bananas and oranges were peeled, with peels discarded prior to drying, and apples were cored but not peeled. Carrots were purchased already peeled. Fruits and vegetables were sliced and then immediately frozen on dry ice. A portion of each frozen fruit and vegetable sample was stored at approximately −20 °C for later extraction (below). The rest of each frozen fruit and vegetable sample was loaded into the freeze-dryer and remained frozen until dry. Meat, egg, and milk samples were frozen on dry ice prior to loading into the freeze-dryer and remained frozen until dry. Freeze-dried materials were stored at approximately −20 °C. Sample weights were measured before and after freeze-drying to determine moisture content. Dry materials, both freeze-dried and as purchased, were extracted as described for soybean and maize.

Fresh fruits and fresh vegetables with high water content were also extracted directly without freeze-drying. Frozen slices (from above) of fruits and vegetables were chopped in a food processor (Super Chopper, Black & Decker Corporation, Towson, MD) and then approximately 1 g of fresh produce was mixed for 1 h with 3.0 mL of extraction solvent (1:2 v/v methanol/chloroform) in glass tubes with PTFE-lined caps (13 × 100 mm, Kimble/Kontes, Vineland, NJ). Samples were centrifuged to facilitate phase separation. The aqueous layer was collected, and the organic phase was discarded. The samples were extracted again with 1 mL of extraction solvent and 0.3 mL of water. The aqueous fractions were combined, dried and resuspended in 1 mL of 0.2% formic acid containing internal standard (50 ng/mL d_3-NAA and d_5-NAG).

Brewed liquid coffee and espresso were also prepared and analyzed. Roasted Arabica coffee beans were ground and brewed (57 g in 1244 mL of water) using a drip coffee maker (Mr. Coffee, Sunbeam Products, Boca Raton, FL). Arabica espresso beans were purchased already ground and were brewed (28 g in 178 mL of water) in an espresso maker (Crusinallo, Italy). An aliquot of the brewed coffee or espresso (0.9 mL) was mixed for 1 h with 3.0 mL of extraction solvent in glass tubes with PTFE-lined caps. Samples were centrifuged to facilitate phase separation. The aqueous layer was collected and the organic phase discarded, and material originally at the interphase and tube bottom was extracted again with 1 mL of extraction solvent and 0.3 mL of water. The aqueous fractions were combined, dried, and resuspended as described above. Aliquots of fresh milk and stout beer were extracted as described for aliquots of liquid coffee and liquid espresso.

UPLC-ESI-MS/MS Analysis. Separations were performed on an ACQUITY UPLC equipped with a thermostatted autosampler and column compartment (Waters Corporation, Milford, MA) and fitted with an HSS T3, 1.8 μm 2.1 × 100 mm column, and a column in-line frit filter, 0.2 μm, 2.1 mm (Waters Corporation). The chromatographic conditions were as follows: sample temperature, 15 °C; injection volume, 5 μL; column temperature, 40 °C; flow rate, 0.4 mL/min; mobile phase A, 0.2% aqueous formic acid; mobile phase B, methanol. Separations were performed by holding initial conditions of 100% mobile phase A for 0.75 min and then increasing linearly to 3% mobile phase B in 2.25 min. The gradient was then increased linearly to 90% mobile phase B in 1 min, held for 1 min, and then returned to initial conditions for 3 min. Eluant from the column flowed to a divert valve and was directed to waste for the first min then directed into an ESI source on a Micromass Quattro Premier XE (Waters Corporation) tandem quadrupole mass spectrometer for 1.6 min. The source conditions were as follows: positive polarity; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow, 192 L/h; desolvation gas flow 806 L/h. From 1.10 to 1.75 min the mass spectrometer was set to perform multiple reaction monitoring of m/z 175.70 → 134.03 and 179.00 → 137.90 with dwell times of 0.300 s, collision energies of 9.00 eV, and cone voltages of 15 and 11 V, respectively. From 1.80 to 2.70 min, the mass spectrometer was set to perform multiple reaction

monitoring of *m/z* 189.90 → 129.90 and 195.07 → 135.34 with dwell times of 0.300 s, collision energies of 13.00 and 14.00 eV, and cone voltages of 13 and 14 V, respectively. Authentic standards of NAA and NAG were prepared at concentrations ranging from 1 ng/mL to 10 μg/mL in 0.2% aqueous formic acid containing 50 ng/mL d_3-NAA and 50 ng/mL d_5-NAG as internal standards. Each calibration curve contained at least five concentration levels in duplicate. MassLynx V4.1, QuanLynx V4.1 (Waters Corporation) software was used for the analyses. The ratio of peak area of analyte to area of the corresponding internal standard was calculated, and using linear regression with 1/*x* weighting, the slope, *y*-intercept, and correlation coefficient (r^2) were determined. A calibration curve was acceptable if $r^2 \geq 0.999$, and the standard value calculated from its standard curve was within 65.0 to 135% of the actual value. The limit of detection and lower limit of quantification were calculated by using the signal (analyte response) to noise (blank response) ratio of 3 and ≥10, respectively. For method accuracy and precision, percent relative standard deviation (RSD) was calculated. Mean values ± standard error of two determinations were reported for samples. Statistical analysis was performed using Microsoft Office Excel 2003 descriptive statistics tools.

RESULTS

Positive ESI (electrospray ionization) was more effective than negative ESI for quantifying NAA and NAG with the Micromass Quattro Premier XE tandem quadrupole mass spectrometer. This is in contrast with what was observed with a different make of mass spectrometer where negative ESI gave best results for NAA (*21*). A series of MS/MS product ion scans using positive ESI identified base peak ions of 134 *m/z* and 130 *m/z* from the $[M + H]^+$ precursor ions of NAA (176 *m/z*) and NAG (190 *m/z*), respectively. The loss of 42 *m/z* from the 176 *m/z* precursor ion of NAA to form the 134 *m/z* base peak is comparable to a loss of 42 *m/z* from the 179 *m/z* $[M + H]^+$ precursor ion of d_3-NAA, resulting in the formation of a base peak of 137 *m/z*, and is consistent with the loss of the acetyl group from the nitrogen. A 148 *m/z* product ion is formed with a similar loss of 42 *m/z* from the 190 *m/z* precursor ion of NAG, but it is only about a fourth of the 130 *m/z* base peak abundance. A 135 *m/z* base peak is derived from the 195 *m/z* $[M + H]^+$ ion of d_5-NAG indicating that all of the deuterium stays with the base peak. These results are consistent with the NAG base peak ion being formed by a loss of 60 *m/z* and rearrangement, probably to a six-membered heterocylic ring. In-source fragmentation of NAG on a time-of-flight mass spectrometer (Waters Corporation) generated the 130 *m/z* product ion with a suggested empirical formula of C6H11NO2 (data not shown).

Using the 134 *m/z* base peak ion derived from the NAA $[M + H]^+$ ion and the 130 *m/z* base peak ion derived from the NAG $[M + H]^+$ ion, standard curves for both NAA and NAG were linear over 4 orders of magnitude from the lower limit of quantification ($S/N \geq 10$) of 1 ng/mL up to 10 μg/mL. The limit of detection ($S/N = 3$) was 0.3 ng/mL. Both compounds could be clearly observed in soybean seed extracts using UPLC-ESI-MS/MS (**Figure 1**). The soybean NAA had the same retention time on UPLC as authentic standards of NAA and d_3-NAA, while soybean NAG had the same retention times as authentic standards of NAG and d_5-NAG. Fortification experiments using 0.3 μg NAA/g soybean and 0.3 μg NAG/g soybean resulted in recoveries close to 100% in three separate determinations with an average of 106 and 96.2% for NAA and NAG, respectively. Similar results were obtained using the same level of fortification in maize with average recoveries of 86.5 and 100% for NAA and NAG, respectively. The intra-assay variability of 20 injections of fortified soybean extract was 1.2 and 1.3% RSD for NAA and NAG, respectively. The interassay variability was 6.5 and 5.5% RSD for NAA and NAG, respectively, for 19 different determinations over a 3 month period.

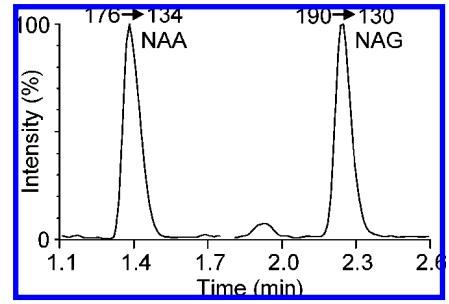

Figure 1. Detection of NAA and NAG in a soybean extract using UPLC-ESI-MS/MS. The 176 *m/z* and 190 *m/z* $[M + H]^+$ precursor ions were fragmented to produce the 134 *m/z* and 130 *m/z* base peaks for NAA and NAG, respectively.

Both soybean and maize had quantifiable concentrations of NAA and NAG (**Table 1**). Using the extraction procedures developed for soybean and maize on other foodstuffs, the highest quantities of NAA were found in roasted coffee beans (**Table 1**). Concentrations of NAA in brewed coffee and espresso were consistent with those found in the roasted coffee when taking into account added moisture. In contrast, different varieties of green (nonroasted) coffee beans contained much lower concentrations of NAA. Cocoa had the highest concentrations of NAG, and this was consistent with concentrations found in dark chocolate. A number of foodstuffs were freeze-dried prior to extraction to facilitate quantification of NAA and NAG (**Table 2**). However, it was also possible to detect NAA and NAG without freeze-drying (**Table 2**).

DISCUSSION

Both NAG and NAA were found at quantifiable concentrations in all foods tested (**Tables 1** and **2**) indicating that these two acetylated amino acids are common components of the human diet. This is the first published report of the quantitative concentrations of these substances in foodstuffs. Another research group has reported semiquantitative concentrations of NAG in the leaves and roots of rice and barley (*18, 19*).

The described method has a lower limit of quantification of 1 ng/mL for both NAA and NAG. With a 5 μL injection volume that amounts to 5 pg or 28.5 fmol of NAA and 26.4 fmol NAG. By comparison, the lower limit of quantification was approximately 17.5 μg/mL for NAA by NMR (*10*), 70 ng/mL ($S/N > 5$) for NAA by HPLC (*14*), 88 ng/mL NAA by HPLC (*15*), and 175 ng/mL NAA by HPLC-ESI-MS/MS (*21*). Several methods only report limits of detection, all of which are higher than the lower limit of quantification in the current article (*4, 12, 13, 16, 17, 20*).

The use of phase partitioning and UPLC-ESI-MS/MS helped minimize the influence of interfering compounds and improved the accuracy of quantifying NAA and NAG. Previous work with NMR suggested as much as 37% of the proton signal associated with NAA was derived from compounds other than NAA (*16*). A number of compounds with polarities and UV spectra similar to those observed for acetylated amino acids could potentially interfere with HPLC/UV (*13*). Others (*16*) encountered inadequate specificity with HPLC/UV when working with cerebrospinal fluid, and NAG was found to be difficult to quantify by HPLC in the presence of large amounts of NAA (*4*). Gerlo et al. (*17*) obtained poor recoveries of NAA and NAG due to the presence of multiple derivatives and coeluting compounds.

Table 1. NAA and NAG Concentrations in Soybean, Maize, and Various Foodstuffs

	NAA (μg/g)[a]		NAG (μg/g)[a]	
sample	mean	standard error	mean	standard error
soybean (93B87)	0.6991	0.0021	0.9296	0.0141
soybean (93B86)	0.6820	0.0306	0.9939	0.0196
soybean (93B15)	0.7065	0.0036	1.203	0.071
soybean (93M40)	0.3230	0.0157	0.6842	0.0344
maize (33J56)	0.03624	0.00186	0.2345	0.0022
maize (33P66)	0.04369	0.00091	0.2021	0.0022
maize (33R77)	0.05560	0.00125	0.2840	0.0074
Adzuki bean	0.3014	0.0157	1.056	0.020
soft spring wheat berries	0.03403	0.00220	0.2718	0.0091
roasted buckwheat	0.4112	0.0145	0.7305	0.0186
buckwheat groats	0.2570	0.0019	0.3542	0.0002
medium brown rice	0.04859	0.00255	1.519	0.038
pearled barley	0.05913	0.00242	0.1147	0.0111
hulled barley	0.09160	0.00102	0.2458	0.0013
stout beer	0.1375[b]	0.0031	0.2060[b]	0.0007
walnut	0.04647	0.00281	0.4219	0.0088
roasted coffee bean (Arabica)	62.78	0.39	4.965	0.114
brewed coffee (Arabica)	3.806[b]	0.099	0.2936[b]	0.0195
brewed espresso (Arabica)	15.37[b]	0.03	1.844[b]	0.034
ground coffee blend (Arabica/Robusta)	56.08	5.69	10.12	0.85
green espresso coffee bean (Arabica)	1.849	0.025	4.731	0.078
green Colombian Supremo coffee bean (Arabica)	0.2950	0.0064	1.437	0.031
green Costa Rican coffee bean (Arabica)	0.2673	0.0008	0.9890	0.0059
green House Blend coffee bean (Arabica)	0.5587	0.0344	2.155	0.489
green Sumatra coffee bean (Arabica)	0.6459	0.0720	3.227	0.515
green French/Italian coffee bean (Arabica)	0.7254	0.0205	3.688	0.254
cocoa powder	26.80	0.06	62.25	0.18
dark chocolate	4.549	0.038	10.25	0.29
green tea (dried leaves)	0.4469	0.0020	5.296	0.065
black tea (dried leaves)	0.5736	0.0191	3.625	0.146

[a] Values are the mean ± standard error of two determinations and are not adjusted for moisture content of the sample. [b] Units are μg/mL.

Table 2. NAA and NAG Concentrations in Various Foodstuffs before or after Freeze Drying

	NAA (μg/g)[a] freeze dried (measured)		NAG (μg/g)[a] freeze dried (measured)			NAA (μg/g)[b] wet weight (calculated)	NAG (μg/g)[b] wet weight (calculated)	NAA (μg/g)[a] wet weight (measured)		NAG (μg/g)[a] wet weight (measured)	
sample	mean	standard error	mean	standard error	moisture removed (%)	mean	standard error	mean	standard error	mean	standard error
baking potato (unpeeled)	0.01196	0.00129	0.8121	0.0600	71.13	0.003451	0.2344	0.001155	0.000006	0.06724	0.00531
carrot (peeled)	0.08279	0.00245	0.2870	0.0121	83.65	0.01354	0.04694	0.006891	0.000481	0.06082	0.00448
banana (peeled)	0.06281	0.00324	1.163	0.072	71.65	0.01781	0.3297	0.1041	0.0013	0.06388	0.00243
royal gala apple (unpeeled)	0.08894	0.01496	0.08773	0.00696	83.81	0.01440	0.01420	0.01478	0.00098	0.01070	0.00013
valencia orange (peeled)	0.3118	0.0100	0.4545	0.0084	85.98	0.04371	0.06371	0.06849	0.00027	0.08093	0.00282
roma tomato	0.03169	0.00066	0.1354	0.0165	93.69	0.002001	0.008550	0.002220	0.000076	0.01281	0.00011
sweet potato (unpeeled)	0.02155	0.00002	0.7926	0.0102	77.98	0.004745	0.1745	0.003794	0.000148	0.1092	0.0023
broccoli crown	0.9189	0.1591	7.910	1.189	90.24	0.08968	0.7719	0.1078	0.0040	0.9665	0.0265
green bean	3.563	0.083	1.399	0.008	90.66	0.3326	0.1306	0.3148	0.0230	0.1622	0.0009
spinach	0.5267	0.0709	21.31	2.52	91.56	0.04447	1.799	0.06725	0.00005	0.8967	0.0045
romaine lettuce heart	0.3243	0.0259	2.212	0.176	93.97	0.01955	0.1334	0.01468	0.00005	0.1495	0.0072
whole egg	6.277	0.100	0.1920	0.0041	76.36	1.484	0.04539				
egg yolk	4.641	0.032	0.2097	0.0066	50.48	2.298	0.1038				
egg white	11.52	1.20	0.1155	0.0061	87.27	1.466	0.01471				
ground pork	0.3251	0.0081	0.2609	0.0131	69.60	0.09882	0.07931				
ground beef	0.7147	0.0467	0.1805	0.0057	61.77	0.2732	0.06901				
ground chicken burgers	16.74	0.19	0.2439	0.0319	71.94	4.697	0.06844				
ground turkey burgers	23.35	0.35	0.2742	0.0177	68.29	7.405	0.08694				
sardine with bone	24.07	0.13	0.5035	0.0045	57.45	10.24	0.2142				
whole milk	0.02207	0.00294	0.09420	0.00675	87.72	0.002711	0.01157	0.003641[c]	0.000002	0.02213[c]	0.00029

[a] Values are the mean ± standard error of two determinations and are not adjusted for moisture content of the sample. [b] Wet weight values were calculated from freeze-dried sample values using moisture removed with freeze-drying. [c] Units are μg/mL.

While the current method required more steps than that described for urine analysis by HPLC-ESI-MS/MS (*21*), the greater matrix complexity and lower concentrations in the foodstuffs analyzed here justified the more complicated sample preparation and chromatography. Nevertheless, our sample preparation was less complex than required for the derivatization for GC-MS or for methods that involve chromatographic steps prior and in addition to analysis by HPLC.

The Maillard reaction may contribute to the concentrations of NAA and NAG in foods. The Maillard reaction is known to occur during coffee roasting and to result in the production of a number of compounds from the reaction of carbohydrates with

amino acids that are found in green coffee beans (*23*). Both free aspartate and free glutamate are present in green coffee beans (*23*). Highest concentrations of NAA were found in roasted coffee beans and were substantially lower in green coffee beans (**Table 1**). The Maillard reaction may also contribute to the high concentrations of NAG in cocoa powder. However, raw cocoa beans were not analyzed. In addition, no attempt was made to investigate the effect of cooking on NAA or NAG concentrations in meat or other foodstuffs. Some foods were freeze-dried to concentrate NAA and NAG (**Table 2**) to improve the chance of detecting both substances. Concentrating was beneficial in studies with urine where a number of acetylated amino acids were detected by GC-MS in concentrated urine (*22*). In contrast, only trace amounts of NAA were found in the unconcentrated urine of healthy human individuals using GC-MS (*17*). Although NAA and NAG were quantifiable in freeze-dried materials, it was possible to measure NAA and NAG in several of the same foodstuffs without first drying (**Table 2**). A comparison of NAA and NAG concentrations on a fresh weight basis calculated from freeze-dried materials and measured directly in nondried materials did not always provide equivalent results (**Table 2**). This suggests that the method was not optimal for all matrices. The method was developed for use with soybean seed and maize grain. The good recoveries of NAA and NAG for soybean and maize and the use of deuterated NAA and deuterated NAG to correct for ion enhancement or ion suppression ensure that the values reported here, if not accurate, are underestimated. Clearly, NAA and NAG were present in the foodstuffs analyzed.

Nevertheless, NAA and NAG are minor constituents relative to other substances in foods. For example, the concentrations of NAA and NAG in soybean seed (**Table 1**) are considerably less than the approximately 0.2 mg/g and 0.4 mg/g of free glutamate and aspartate, respectively, found in soybean seed (Maxwell, C., unpublished data).

While the presence of NAG in foodstuffs can be explained by its role in different biological processes (*1, 2*), the origin of NAA in foodstuffs is less clear. The N-terminus of proteins can be acetylated (*24*), and perhaps the degradation of these proteins can release free acetylated amino acids including NAA and NAG. The acetylated amino acids are not artifacts produced by the extraction method. The extraction removes protein, but peptide bonds would not be broken and therefore could not cause the release of free amino acids. Furthermore, acetylation of existing free amino acids during extraction would not happen. In the case of plants, NAA may be involved in phytohormone metabolism. For example, indole-3-acetic acid has been found conjugated with NAA as indole-3-acetylaspartic acid (*25*). NAA may also be an intermediate in the biosynthesis of other compounds such as *N*-phenylacetylaspartate, which was found in some plants but has no known function (*26*).

ACKNOWLEDGMENT

We thank Julie Eble and Karen L'Empereur of Critical Path Services, LLC for helpful discussions on the analysis of NAA and NAG by LC-ESI-MS/MS. Bryan Delaney, Russ Essner, Ian Lamb, Ray Layton, Mary Locke, and Carey Mathesius provided helpful discussions and critical review of the manuscript.

LITERATURE CITED

(1) Caldovic, L.; Tuchman, M. *N*-Acetylglutamate and its changing role through evolution. *Biochem. J.* **2003**, *372*, 279–290.

(2) Slocum, R. Genes, enzymes and regulation of arginine biosynthesis in plants. *Plant Physiol. Biochem.* **2005**, *43*, 729–745.

(3) Philip-Hollingsworth, S.; Hollingsworth, R.; Dazzo, F. *N*-Acetylglutamic acid: An extracellular *nod* signal of *Rhizobium trifolii* ANU843 that induces root hair branching and nodule-like primordia in white clover roots. *J. Biol. Chem.* **1991**, *266*, 16854–16858.

(4) Alonso, E.; García-Pérez, M.; Bueso, J.; Rubio, V. *N*-Acetyl-L-glutamate in brain: Assay, levels, and regional and subcellular distribution. *Neurochem. Res.* **1991**, *16*, 787–794.

(5) Tallan, H.; Moore, S.; Stein, W. *N*-Acetyl-L-aspartic acid in brain. *J. Biol. Chem.* **1956**, *219*, 257–264.

(6) Moffett, J.; Ross, B.; Arun, P.; Madhavarao, C.; Namboodiri, M. A. *N*-Acetylaspartate in the CNS: From neurodiagnostics to neurobiology. *Prog. Neurobiol.* **2007**, *81*, 89–131.

(7) Baslow, M. *N*-Acetylaspartate in the vertebrate brain: Metabolism and function. *Neurochem. Res.* **2003**, *28*, 941–953.

(8) D'Adamo, A.; Yatsu, F. Acetate metabolism in the nervous system. *N*-Acetyl-L-aspartic acid and the biosynthesis of brain lipids. *J. Neurochem.* **1966**, *13*, 961–965.

(9) Burri, R.; Steffen, C.; Herschkowitz, N. *N*-Acetyl-L-aspartate is a major source of acetyl groups for lipid synthesis during rat brain development. *Dev. Neurosci.* **1991**, *13*, 403–411.

(10) Engelke, U.; Liebrand-Van Sambeek, M.; De Jong, J.; Leroy, J.; Morava, E.; Smeitink, J.; Wevers, R. *N*-Acetylated metabolites in urine: Proton nuclear magnetic resonance spectroscopic study on patients with inborn errors of metabolism. *Clin. Chem* **2004**, *50*, 58–66.

(11) Baena, B.; Cifuentes, A; Barbas, C. Analysis of carboxylic acids in biological fluids by capillary electrophoresis. *Electrophoresis* **2005**, *26*, 2622–2636.

(12) Alonso, E.; Rubio, V. Determination of N-acetyl-L-glutamate using high-performance liquid chromatography. *Anal. Biochem.* **1985**, *146*, 252–259.

(13) Tavazzi, B.; Vagnozzi, R.; Di Pierro, D.; Amorini, A.; Fazzina, G.; Signoretti, S.; Marmarou, A.; Caruso, I.; Lazzarino, G. Ion-pairing high-performance liquid chromatographic method for the detection of N-acetylaspartate and N-acetylglutamate in cerebral tissue extracts. *Anal. Biochem.* **2000**, *277*, 104–108.

(14) Tavazzi, B.; Lazzarino, G.; Leone, P.; Amorini, A. M.; Bellia, F.; Janson, G. C.; Di Pietro, V.; Ceccarelli, L.; Donzelli, S.; Francis, J. S.; Giardina, B. Simultaneous high performance liquid chromatographic separation of purines, pyrimidines, N-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis of inborn errors of metabolism. *Clin. Biochem.* **2005**, *38*, 997–1008.

(15) Tranberg, M.; Stridh, M. H.; Jilderos, B.; Weber, S. G.; Sandberg, M. Reversed-phase HPLC with UV detection for the determination of N-acetylasparatate and creatine. *Anal. Biochem.* **2005**, *343*, 179–182.

(16) Faull, K. F.; Rafie, R.; Pascoe, N.; Marsh, L.; Pfefferbaum, A. *N*-Acetyl aspartic acid (NAA) and N-acetyl aspartylglutamic acid (NAAG) in human ventricular, subarachnoid, and lumabar cerebrospinal fluid. *Neurochem. Res.* **1999**, *24*, 1249–1261.

(17) Gerlo, E.; Van Coster, R.; Lissens, W.; Winckelmans, G.; De Meirleir, L.; Wevers, R. Gas chromatographic-mass spectrometric analysis of N-acetylated amino acids: The first case of aminoacylase I deficiency. *Anal. Chim. Acta* **2006**, *571*, 191–199.

(18) Roessner, U.; Patterson, J. H.; Forbes, M. G.; Fincher, G. B.; Langridge, P. An investigation of boron toxicity in barley using metabolomics. *Plant Physiol.* **2006**, *142*, 1087–1101.

(19) Jacobs, A.; Lunde, C.; Bacic, A; Tester, M.; Roessner, U. The impact of constitutive heterologous expression of a moss Na^+ transporter on the metabolomes of rice and barley. *Metabolomics* **2007**, *3*, 307–317.

(20) Ma, M.; Zhang, J.; Sugahara, K.; Ageta, T.; Nakayama, K.; Kodama, H. Simultaneous determination of N-acetylaspartic acid, N-acetylglutamic acid, and N-acetylapartylglutamic acid in whole brain of 3-mercaptopropionic acid-treated rats using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Anal. Biochem.* **1999**, *276*, 124–128.

(21) Al-Dirbashi, O.; Rashed, M.; Al-Mokhadab, M.; Al-Qahtani, K.; Al-Sayed, M.; Kurdi, W. Stable isotope dilution analysis of N-acetylaspartic acid in urine by liquid chromatography electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **2007**, *21*, 898–902.

(22) Liebich, H.; Forst, C. Urinary excretion of N-acetylamino acids. *J. chromatogr.* **1985**, *338*, 187–191.

(23) Murkovic, M.; Derler, K. Analysis of amino acids and carbohydrates in green coffee. *J. Biochem. Biophys. Methods* **2006**, *69*, 25–32.

(24) Polevoda, B.; Sherman, F. N-terminal Acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J. Mol. Biol.* **2003**, *325*, 595–622.

(25) Nordstrom, A-C.; Eliasson, L. Levels of endogenous indole-3-acetic acid and indole-3-acetylaspartic acid during adventitious root formation in pea cuttings. *Physiol. Plant.* **1991**, *82*, 599–605.

(26) Gianfagna, T.; Davies, P. N-Benzoylaspartate and N-phenylacetylaspartate from pea seeds. *Phytochemistry* **1980**, *19*, 959–961.

Received for review May 15, 2008. Revised manuscript received July 24, 2008. Accepted August 13, 2008.

JF801523C