Comparison of Estimated Dietary Intake of Acrylamide with
Hemoglobin Adducts of Acrylamide and Glycidamide

Thomas Bjellaas,*† Pelle T. Olesen,‡ Henrik Frandsen,‡ Margaretha Haugen,* Linn H. Stølen,* Jan E. Paulsen,* Jan Alexander,* Elsa Lundanes,† and Georg Becher*†,1

*Department of Environmental Medicine, Norwegian Institute of Public Health, Nydalen, NO-0403 Oslo, Norway; ‡Department of Chemistry, University of Oslo, PO Box 1033, Blindern, N-0315, Norway; and †National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

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In a study comprising 50 subjects, we investigated the relationship between acrylamide (AA) intake from food using food frequency questionnaires and the concentration of hemoglobin (Hb) adducts of AA and its genotoxic metabolite glycidamide (GA) as a measure of the internal exposure. A method using solid-phase extraction and liquid chromatography with negative electrospray tandem mass spectrometric (MS/MS) detection for the determination of the Hb adducts as phenylthiohydantoin derivatives in human blood was developed. The limit of quantification for AA- and GA-Hb adducts were 2 and 6 pmol/g globin, respectively, and the between-assay precision was below 25%. The estimated dietary intake of AA was (median and range) 13.5 µg/day (4.1–72.6) in nonsmokers and 18.3 µg/day (7.8–32.0) in smokers. In nonsmokers, males had a higher intake than females, 16.6 µg/day (18.6–72.6) and 12.8 µg/day (4.1–30.2), respectively. Non-smokers had a median AA and GA adduct concentration of 36.8 (range 17.9–65.5) and 18.2 (range 6.7–45.6) pmol/g globin, respectively. In smokers, the values were 165.8 (98.8–211) and 83.2 (29.1–99.0) pmol/g globin, respectively. Using multiple linear regression analysis, a significant positive correlation was found between the AA-Hb adduct concentration and the intake of chips/snacks and crisp bread. GA-Hb adduct did not correlate with consumption of any of the main food groups. Neither AA-Hb nor GA-Hb adduct concentration correlated with total dietary intake of AA as calculated from the reported food intake. Adduct concentrations did not correlate with 24 h urinary excretion of mercapturic acid metabolites of AA and GA in the same subjects reported previously.

Key Words: hemoglobin adducts; dietary exposure; acrylamide; glycidamide; food; biomarkers; safety evaluation.

Based on epidemiological analyses, it is believed that about 20–50% of all human cancers are due to dietary causes. However, only few specific carcinogens have so far been identified in food (Strickland and Groopman, 1995). Acrylamide (AA), classified by the International Agency for Research on Cancer (IARC, 1994) as possibly carcinogenic to humans (class 2A), was recently identified in food. Several studies have shown that Maillard browning reaction products derived from glucose and asparagine, being abundant in potatoes and cereals, are responsible for the formation of AA in these foods (Becalski et al., 2004; Mottram et al., 2002; Stadler et al., 2002; Yaylayan et al., 2003). In a recent review, the mean adult dietary intake of AA was on the basis of records of food consumption and AA concentrations in food items, estimated to be between 0.3 and 0.7 µg/kg bw/day, with a 1.5-fold higher intake or more among children and adolescents (Dybing et al., 2005). However, these estimates, as well as individual exposures in epidemiological studies using food frequency questionnaires (FFQs), need validation by chemical-specific biomarkers (Hagmar et al., 2005).

AA is readily absorbed and metabolized in humans mainly by two pathways: Inactivation by glutathione conjugation (Sumner et al., 1992, 1997) or bioactivation through CYP2E1-dependent epoxidation to glycidamide (GA) (Sumner et al., 1999). GA is detoxified by glutathione conjugation or hydrolyzed to glyceramide (Fennell et al., 2006). Both AA and GA bind covalently in vivo with cellular nucleophiles (i.e., AA and GA to proteins and GA to DNA) forming different types of macromolecular adducts (Doerge et al., 2005; Tornqvist et al., 2002). Hemoglobin (Hb) adducts represent the time-concentration integral of AA and GA present in the circulation during the lifetime of the erythrocytes (~125 days), and the adduct concentration is proportional to the internal dose (Bergmark, 1997; Fennell et al., 2005). AA-Hb can be used as biomarker for average AA exposure, while GA-Hb can be utilized as biomarker for the internal GA dose and the genotoxic impact.

Determination of the concentration of N-terminal-bound AA- and GA-Hb adducts was first conducted by a gas chromatography–mass spectrometry (GC-MS) method developed by Swedish researchers (Bergmark et al., 1993, 1997; Paulsson et al., 2003; Tornqvist, 1994) based on a modified Edman reaction where the resulting products are AA-Val-pentafluorophenylthiohydantoin (PFPTH) and GA-Val-PFPTH.
The GC-MS method has many merits, but it is laborious due to its many extractions steps and additional derivatization of the hydroxyl group of GA-Val-PFPTH prior to GC-MS analysis (Paulsson et al., 2003). Faster liquid chromatography–mass spectrometry (LC-MS) methods that allow simultaneous determination of AA and GA adducts have been developed based on liquid-liquid extraction on diatomaceous earth for sample clean-up (Vesper et al., 2006) or solid-phase extraction (SPE) (Fennell et al., 2003; Ospina et al., 2005). AA-Hb adduct concentrations were found to be in the range of 34–306 pmol/g globin (Vesper et al., 2006). A large variation in molar ratio between GA and AA adducts has been observed in several studies (Perez et al., 1999; Vesper et al., 2005, 2006).

The measurement of AA- and GA-Hb adducts has successfully been used to determine exposure to AA in the work environment and through smoking (Bergmark, 1997). In recent studies, AA exposure was estimated using FFQ; however, it proved difficult on an individual level to predict the AA-Hb adduct level from data on estimated dietary exposures to AA (Hagmar et al., 2005; Kutting et al., 2005). There might be several reasons for this, e.g., the design of the FFQs and large variation in AA level of food items even within the same brand. Interindividual variation in AA toxicokinetics is less likely as a cause.

To our knowledge, no studies have been reported addressing the relationship between consumption of various food items in the diet using FFQ and Hb adduct biomarkers of AA exposure. In a previous study comprising 53 persons, we examined the relationship between recent AA intake as estimated from a 24-h recall interview and the amount of AA excreted as urinary AA- and GA mercapturic acid metabolites (Bjellaas et al., 2007). In the same study, FFQ was used for recording the diet during the last year, and blood samples were collected for Hb adduct determination. The aim was to examine independent dietary predictors of Hb adduct concentration. In addition, we examined the relationship between the estimated total AA exposure and AA- and GA-Hb adduct concentrations. The relationship between Hb adducts and urinary metabolites were also examined. An analytical method using SPE and ion trap LC-tandem mass spectrometric (MS/MS) analysis was developed for AA-Hb and GA-Hb adduct determination using in-house synthesized reference standards.

**MATERIALS AND METHODS**

**Subjects**

Forty-seven nonsmoking employees at the Norwegian Institute of Public Health accepted an e-mail invitation to participate in a study. In addition, six smokers were recruited through personal invitation. In total, the study population comprised 53 participants, 20 males and 33 females (mean age of males and females were 45 ± 13 years and 41 ± 11 years, respectively). The study consisted of two parts. In the first part, the participants were asked to collect 24 h urine followed by a 24-h dietary recall for short-time exposure. Urine samples were analyzed, and the results are presented elsewhere (Bjellaas et al., 2007). The second part was performed to evaluate the long-term exposure in the same participants. All except three participants (one male, two females) completed a FFQ, filling in what their usual dietary intake had been the last year, and a blood sample was drawn. Two males and four females were smokers (14% of the study population). Median number of cigarettes per day was 14 (range 7–21). The Regional Ethics Committee and the Data Inspectorate approved the study, and all participants gave their written informed consent.

**Dietary Data**

Using a validated FFQ, all participants recorded how often they had consumed different food items during the last year. The nutrient calculations were performed with use of FoodCalc (Lauritzen, 2005) and the Norwegian food composition table (Rimestad et al., 2001). Total daily intake of AA was calculated with help of AA values found in foods analyzed by the Norwegian Food Safety Authority and National Food Administration in Sweden (Livsmedelsverket, 2002a,b; SNT 2002a,b). For foods not analyzed by Norwegian or Swedish Authorities, values were collected from the database published by European Commission (IRM/JRC, 2005). Additional information about concentration levels of AA in foods and beverages was also collected from various sources (Croft et al., 2004; Delatorre et al., 2004; FAO/WHO, 2006; Fennell and Fagt, 2004; Hoenicke and Guterman, 2005; Rouch et al., 2003; Sadd and Hamlet, 2005; Svensson et al., 2003; Taeymans et al., 2004; Tareke et al., 2002; U.S. Food and Drug Administration, 2005).

**Chemicals**

**Caution.** GA is mutagenic in Salmonella typhimurium, and piperidine is toxic. AA is neurotoxic and carcinogenic. Caution should therefore be exercised in the handling of these compounds.

Phenyl isothiocyanate (PITC, 98%), isopropyl, n-pentane, 1-propanol (> 99.9%), AA (> 99%), L-valine (99%), triethylamine (> 99%), Leu-Leu methyl ester hydrobromide (> 97%), 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl, > 99.0%), N,N,N',N'-tetramethyl-O-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uronium tetrafluoroborate (> 99.0%), methyl-tert-butyl ether (MTBE, > 99.5%), and ethyl acetate (> 99.8%) were all bought from Sigma-Aldrich (Milwaukee, WI). Methanol (> 99.9%) was bought from Fischer Scientific (Loughborough, UK). Acetonitrile (> 99.9%), dimethyldisulfide (DMSO, > 99.9%), HCl (fuming 37%), and potassium acetate (> 99.9%) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (> 98%) was obtained from Romil Ltd (Waterbeach, UK) and distilled before use. GA (> 98%) was bought from Toronto Research Chemicals Inc. (ON, Canada). L-14C]valine (9.47 GBq/mmol) was obtained from Amersham (Buckinghamshire, UK). The internal standards [D3]AA-Val-PFPTH and [D3]GA-Val-PFPTH were kind gifts from Professor M. Tornaqvist (Stockholm University, Sweden). Filtered, twice deionized, and twice UV-treated water (type I) was supplied from a TKA GenPure water purification system (TKA Water purification systems GmbH, Niederelbert, Germany). Saline solution (0.9% NaCl) was supplied by B. Braun (Melsungen, Germany).

**Synthesis of AA-Val-PFPTH and GA-Val-PFPTH Containing 0.001% [14C]Val**

The synthesis of AA-Val and GA-Val mainly followed methodology as previously described (Bergmark et al., 1993). In brief, synthesis of GA-Val was done by dissolving 1.6 mmol of L-valine in 2.0 ml H2O and subsequently adding 170 µl triethylamine, 100 µl (0.02 µmol) L-14C]valine solution, and 0.8 mmol GA. The radioactivity was used later to calculate the calibration stock solution concentrations and SPE recovery. The mixture was left to react for 24 h at room temperature under magnetic stirring. AA-Val was synthesized similarly except that 1.4 mmol of AA was added instead of GA and the mixture was left to react for 3 days. One milliliter of 1-propanol and 250 µl of PITC were added to the mixtures and left to react for 24 h at room temperature. The mixtures were diluted with 12 ml of water. The diluted reaction mixture was applied onto STRATA divinyl benzene SPE columns (500 mg, Phenomenex, Torrance, CA), washed with 5 ml of H2O, and 5 ml of 10% methanol prior to elution with 5 ml of methanol. The methanol was evaporated, and the residue was dissolved in 20% methanol and, subsequently, purified by high-performance liquid chromatography (HPLC) (1100 series LC, Agilent Technologies, Palo Alto, CA) on a Gemini RP C18 column (5 µm, 150 × 2.0 mm, Phenomenex) using an
acetonitrile gradient (0.01% formic acid). Fractions containing AA- and GA-Val-PTH were evaporated to dryness and redissolved in acetonitrile. MS analysis of the products showed the correct molecular ions [M-H\(^-\)] of m/z 304 and 320 for AA-Val-PTH and GA-Val-PTH, respectively. The radiochemical purity of the products was better than 95%. The concentrations of stock solutions were determined by scintillation counting.

**Synthesis AA-Val-Leu-Leu and GA-Val-Leu-Leu Containing 0.004% \([^{13}C]\)-Val**

In order to evaluate the efficiency of the Edman degradation reaction, AA- and GA-Val-Leu-Leu tripeptide standards were synthesized.

**Synthesis of radiolabelled tripeptide methyl ester.** The synthesis was performed as previously outlined with minor modifications (Meienhofer et al., 1979; Perez et al., 1999). In brief, l-valine (0.5 mmol) and l-[\(^{14}C\)]-valine (0.02 mmol) were reacted with FMOC-Cl (0.5 mmol). The derivatized valine was extracted with MTBE, evaporated, and redissolved in dichloromethane before Leu-Leu-methyl ester hydrobromide (0.5 mmol) was added. The organic phase was washed and evaporated. In order to decouple the FMOC from the peptide, the product was redissolved in dichloromethane containing 20% piperidine. The peptide was purified using a strong anion exchange SPE column (AG 1-X8 resin, Bio-Rad Laboratories, Hercules, CA). The peptide was eluted with 2 ml of 50% methanol and 2 ml of 100% methanol. The methanol was evaporated from the eluate, which was then applied to a STRATA divinyl benzene SPE column (500 mg, Phenomenex). The column was washed with 5 ml of water, and the compounds were eluted with 5 ml of methanol. The methanol was evaporated, and the l-Val-Leu-Leu methyl ester was dissolved in 1 ml of water.

**Synthesis of modified tripeptides.** AA-Val-Leu-Leu was synthesized by reacting 25 mmol l-Val-Leu-Leu methyl ester, dissolved in 0.5 ml water with 15 mmol AA. Triethylamine was added to the mixture (10 μl). The reaction mixture was left for 12 days. HPLC analysis showed that the methyl ester group was completely hydrolyzed in the alkylated tripeptides (results not shown). The mixture was then neutralized by adding formic acid, and the AA-Val-Leu-Leu was purified using HPLC (apparatus and solvents described in “Determination of AA and GA-Val Adducts in Hb” section). The following gradient was used with acetonitrile as solvent B: 0–2 min: 5% B, at 9 min: 10.6% B, and at 10 min: 85.6% B at a constant flow rate of 0.3 ml/min. GA-Val-Leu-Leu was made as AA-Val-Leu-Leu by substituting AA with GA. Fractions containing AA- and GA-Val-Leu-Leu from HPLC purification were evaporated to dryness and redissolved in 20% methanol as stock analytic solution. MS analysis of the products showed the correct molecular ions [M-H\(^-\)] of m/z 415 and 431 for AA-Val-Leu-Leu and GA-Val-Leu-Leu, respectively. The radiochemical purity of the products was better than 95%.

**Blood Collection and Globin Purification**

The red blood cells were separated from the plasma and ultimately stored at \(-25^\circ\text{C}\) until further treatment. The stored frozen erythrocyte fractions were prepared for determination of Hb adducts according to the modified N-alkyl Edman method described earlier (Tornqvist et al., 1986). In brief, the thawed erythrocyte sample was diluted with water, added to acidic isopropanol and centrifuged. The pellet of the cell residues was discarded, and the globin was precipitated from the supernatant by slow addition of ethyl acetate under constant agitation.

**Determination of AA and GA-Val Adducts in Hb**

**N-Alkyl Edman reaction.** In the modified Edman reaction, the toxic formamide and NaOH were substituted with 1-propanol and potassium acetate, respectively. To ensure a stable pH (pH 5.5) during the Edman reaction, potassium acetate was used instead of NaOH.

A sample of 20 mg of globin was dissolved in 950 μl 40% (vol/vol) 1-propanol in water, and 50 μl of 5.0M potassium acetate and, subsequently, 5.0 μl of PITC were added. After 18 h under rotation (15 rpm) at room temperature, the reaction mixture was transferred to a water bath for 2 h at 45°C.

**Yield of the Edman reaction.** Tripeptide standards of 29.2 nmol AA-Val-Leu-Leu or 58.5 nmol GA-Val-Leu-Leu were dissolved in 950 μl of 40% (vol/vol) 1-propanol in water along with 20 mg of globin. The Edman reaction was carried out, and the sample cleaned according to the SPE procedure described below. The washing solutions were, however, substituted by 20% methanol. The radiochemical purity of AA-Val-PTH or GA-Val-PTH was checked by HPLC with radioactivity determined in collected fractions. The yield of the Edman reaction was determined by scintillation analysis conducted on a TriCar 3100TR (Perkin-Elmer, Wellesley, MA). Hionic-Flour was used as scintillation cocktail (Perkin-Elmer).

**SPE procedure.** Internal standards (2.4 pmol [D\(_3\)]AA-Val-PTH and 1.8 pmol [D\(_3\)]GA-Val-PTH), 20 μl formic acid, and 5.0 ml H\(_2\)O were added to the Edman reaction mixture. A SPE column (Isolute 101, Biotage, Uppsala, Sweden) was conditioned with 2 ml of methanol and equilibrated with 2 ml of a conditioning solution (0.05M potassium acetate and 0.1M formic acid). The reaction mixture was applied to the SPE column. In addition, the test tube was washed with 1.0 ml of conditioning solution and applied to the SPE column. The column was washed with 3.0 ml of H\(_2\)O, 2.0 ml of 30% (vol/vol) methanol, and 2.0 ml 10% (vol/vol) acetonitrile. The column was then dried at 5 mmHg for 15 min, followed by eluting AA-Val-PTH and GA-Val-PTH with 2 \times 1.5 ml of ethyl acetate. Before the eluate was evaporated under nitrogen at room temperature, 10 μl of DMSO was added as a keeper, and the residue reconstituted in 100 μl of 80% (vol/vol) acetonitrile in H\(_2\)O.

To determine the recovery of the SPE clean-up, the Edman reaction was performed on four globin samples and subsequently added 160 nmol AA\([^{13}C]\)-Val-PTH or 44 nmol GA\([^{13}C]\)-Val-PTH. The globin samples were then subject to SPE as described above.

**LC-MS/MS analysis.** An 1100 series LC coupled to an ion trap SL mass spectrometer (Agilent Technologies) using negative electrospray ionization was used. Ten microliters were injected onto a Gemini RP C18 column (3 μm, 150 \times 2.0 mm, Phenomenex) and an acetonitrile gradient (0.01% formic acid) was used with acetonitrile as solvent B: 0 min: 5% B, 2.5 min: 5% B, 3.5 min: 35% B, 9.0 min: 68% B, 10.5 min: 100% B and 16.0 min: 100% B. The flow rate was constant at 0.2 ml/min during the chromatographic separation of AA-Val-PTH and GA-Val-PTH. The adducts were detected using multiple reaction monitoring. The precursor ions were fragmented in the ion trap, and the daughter ions were used for quantification. Following ions were recorded: m/z 304 \rightarrow 233 for AA-Val-PTH, m/z 307 \rightarrow 233 for [D\(_3\)]AA-Val-PTH, m/z 320 \rightarrow 233 and 302 for GA-Val-PTH, and m/z 323 \rightarrow 233 and 304 for [D\(_3\)]GA-Val-PTH. No loss of deuterium was detected during ionization; only fragmentation resulted in deuterium loss.

**Calibration and Method Evaluation**

Calibration solutions of AA-Val-PTH and GA-Val-PTH were made by diluting appropriate amounts of stock solutions in the range of 0.28–28.0 pmol/ml for AA-Val-PTH and 0.48–48.1 pmol/ml for GA-Val-PTH. By diluting appropriate amounts of stock solutions in the range of 0.28–28.0 pmol/ml for AA-Val-PTH and 0.48–48.1 pmol/ml for GA-Val-PTH, 10 μl were injected onto a Gemini RP C18 (3 μm, 150 \times 2.0 mm, Phenomenex) and an acetonitrile gradient (0.01% formic acid) was used with acetonitrile as solvent B: 0 min: 5% B, 2.5 min: 5% B, 3.5 min: 35% B, 9.0 min: 68% B, 10.5 min: 100% B and 16.0 min: 100% B. The flow rate was constant at 0.2 ml/min during the chromatographic separation of AA-Val-PTH and GA-Val-PTH. The adducts were detected using multiple reaction monitoring. The precursor ions were fragmented in the ion trap, and the daughter ions were used for quantification. Following ions were recorded: m/z 304 \rightarrow 233 for AA-Val-PTH, m/z 307 \rightarrow 233 for [D\(_3\)]AA-Val-PTH, m/z 320 \rightarrow 233 and 302 for GA-Val-PTH, and m/z 323 \rightarrow 233 and 304 for [D\(_3\)]GA-Val-PTH. No loss of deuterium was detected during ionization; only fragmentation resulted in deuterium loss.
Median and range were calculated for AA- and GA-Hb adducts and for AA intake because of nonnormality distribution of the data. Spearman correlation coefficient ($r_{sp}$) was used to analyze correlation between food intake and Hb adducts concentrations. To obtain normal distribution of the Hb adduct concentration data, smokers and one nonsmoking outlier were removed from the study population. Correlation between Hb adducts and age was investigated with the use of Pearson correlation coefficient ($r_{p}$) in nonsmokers. Differences in Hb adducts and dietary intake of AA between smokers and nonsmokers and between males and female were calculated by Mann-Whitney $U$-test. One sample was determined according to Grubbs test to be an outlier ($p = 0.05$). For a linear regression model, smokers and one nonsmoking outlier were removed from the calculations of Hb adduct concentrations to obtain normal distribution of the data. All multiple regression analyses were thoroughly checked for possible violations from the model assumptions during analyses. $p$ Values less than or equal to 0.05 were considered significant. All statistical analyses were performed using the SPSS version 13.0 (SPSS Inc, Chicago, IL, 2005).

### RESULTS

#### Analytical Method for Hb Adduct Determination

AA-Val-PTH and the two diastereomers of GA-Val-PTH were baseline separated in reversed-phase LC (Fig. 1). Negative ionization was used for the detection because no suitable ions were produced when GA-Val-PTH was fragmented in the positive mode. The yield of the Edman reaction was found to be 80 and 75% for AA-Val-PTH and GA-Val-PTH, respectively. The SPE recovery was calculated to be 87% (SD 3%, $n = 3$) for AA-Val-PTH and 69% (SD 6%, $n = 4$) for GA-Val-PTH. Results from the evaluation of the method are presented in Table 1.

#### Dietary Intake

The median (range) dietary intake of AA estimated from the FFQ was 13.5 µg/day (4.1–72.6) in nonsmokers and 18.3 µg/day (7.8–32.0) in smokers. The dietary intake of AA was higher in males 16.6 µg/day (18.6–72.6) compared with that of females 12.8 µg/day (4.1–30.2) ($p < 0.05$) among nonsmokers but correlated neither with age nor body weight.

#### Hb Adduct Levels

Hb adducts of AA and GA were found in all blood samples analyzed (Table 2). Smokers had a significantly higher concentration of both adducts compared with nonsmokers ($p < 0.001$ for both adducts). The molar ratios of AA and GA adducts did not differ between smokers and nonsmokers.

### Table 1

**Evaluation Data of LC-MS/MS Method**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (pmol/ml)</th>
<th>% found</th>
<th>RSD ($n = 6$)</th>
<th>Concentration (pmol/ml)</th>
<th>% found</th>
<th>RSD ($n = 6$)</th>
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<tbody>
<tr>
<td>I</td>
<td>0.28</td>
<td>108</td>
<td>38</td>
<td>0.48</td>
<td>164</td>
<td>76</td>
</tr>
<tr>
<td>II</td>
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<td>102</td>
<td>25</td>
<td>0.80</td>
<td>135</td>
<td>46</td>
</tr>
<tr>
<td>III</td>
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<td>136</td>
<td>39</td>
<td>1.60</td>
<td>105</td>
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<td>9</td>
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<td>9.34</td>
<td>96</td>
<td>7</td>
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<td>4</td>
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<td>28.0</td>
<td>100</td>
<td>17</td>
<td>48.1</td>
<td>100</td>
<td>16</td>
</tr>
</tbody>
</table>

**Accuracy as recovery (%)**

- AA-Val-PTH: Within-assay precision - Sample 1 ($n = 8$): 69% ± 13%, Sample 2 ($n = 8$): 33% ± 11%. Between-assay precision - Sample 3 ($n = 6$): 87% ± 24%, Sample 4 ($n = 6$): 44% ± 16%. SPE recovery (%) - 87% ± 4%, Edman yield (%) - 80% ± 0.1%. Limit of detection - 1 pmol/g globin, Limit of quantification - 2 pmol/g globin.

- GA-Val-PTH: Within-assay precision - Sample 1 ($n = 8$): 54% ± 16%, Sample 2 ($n = 8$): 25% ± 12%. Between-assay precision - Sample 3 ($n = 6$): 47% ± 24%, Sample 4 ($n = 6$): 21% ± 23%. SPE recovery (%) - 69% ± 9%, Edman yield (%) - 75% ± 0.6%. Limit of detection - 2 pmol/g globin, Limit of quantification - 6 pmol/g globin.

**RSD**: Relative Standard Deviation.

### Table 2

**Hb Adduct Levels of AA and GA as pmol/g Globin in Nonsmokers and Smokers**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers ($n = 44$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA adduct</td>
<td>38.4</td>
<td>11.9</td>
<td>36.8</td>
<td>(17.9–65.5)</td>
</tr>
<tr>
<td>GA adduct</td>
<td>19.6</td>
<td>7.9</td>
<td>18.2</td>
<td>(6.7–45.6)</td>
</tr>
<tr>
<td>GA/AA ratio</td>
<td>0.53</td>
<td>0.2</td>
<td>0.49</td>
<td>(0.12–1.08)</td>
</tr>
<tr>
<td>Smokers ($n = 6$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA adduct</td>
<td>154</td>
<td>46.3</td>
<td>165.8</td>
<td>(98.8–211)</td>
</tr>
<tr>
<td>GA adduct</td>
<td>76.5</td>
<td>26.3</td>
<td>83.2</td>
<td>(29.1–99.0)</td>
</tr>
<tr>
<td>GA/AA ratio</td>
<td>0.5</td>
<td>0.2</td>
<td>0.46</td>
<td>(0.29–0.98)</td>
</tr>
</tbody>
</table>

**FIG. 1.** Chromatographic separation of AA-Val-PTH, the two diastereomers of GA-Val-PTH and the deuterated internal standards.
There was no statistically significant difference in adduct concentrations between males and females. Also, adduct concentrations did not correlate with body weight. However, a negative correlation between AA-Hb concentration and age \( (r_{sp} = -0.30, p < 0.05) \) and between GA-Hb concentration and age \( (r_{sp} = -0.35, p < 0.02) \) in nonsmokers was observed (see Fig. 2). A significant negative correlation was found between the amount of AA from intake of chips/snacks and age \( (r = -0.312, p = 0.023) \).

### Dietary Determinants to Hb Adducts

Neither the AA-Hb nor the GA-Hb adduct concentrations correlated with total dietary intake of AA as calculated from the FFQ and the levels of AA in foods. However, in a multiple linear regression model adjusted for age, intake of potatoes, chips/snacks, crisp bread, and jam/preservatives were identified as independent predictors of AA-Hb adducts \( (r^2 = 0.48, p < 0.001) \) (Table 3). Similarly, chips/snacks and crisp bread were independent predictors of total Hb adduct concentration, AA-Hb + GA-Hb \( (r^2 = 0.37, p < 0.001, \text{Table 4}) \). It was not possible to find any independent predictors of GA-Hb adducts.

### Comparison of AA-Hb Adducts with AA Excretion in Urine

To examine whether there was an association between short- and long-term intake of AA, the Hb adduct levels found in this study were compared with the amounts of AA- and GA mercapturic acid metabolites excreted in urine in the same subjects presented elsewhere (Bjellaas et al., 2007). For nonsmokers, the amount of AA metabolites excreted in urine during 24 h did not correlate to AA-Hb concentration. No correlation between the ratio of GA- to AA-Hb adducts and the ratio of GA- to AA mercapturic acid metabolites in urine was found.

### DISCUSSION

The aim of this study was to investigate independent predictors to AA dietary exposure using Hb adducts as biomarkers. The first approach was to examine whether estimated AA intake calculated from the FFQ and a database of AA contents in various food items could predict exposure to AA determined by Hb adducts as a long-term biomarker. In addition, the results from this study were compared with the results found in our previous study using urinary biomarkers of AA (Bjellaas et al., 2007).

The median AA-Hb adduct level for nonsmokers found in this study \( (36.8 \text{ pmol/g globin}) \) accords well with levels previously reported \( (Bergmark, 1997; Hagmar et al., 2005; Schettgen et al., 2003) \). This was also the case for the median GA-Hb adduct level \( (18.2 \text{ pmol/g globin}) \) for nonsmokers (Paulsson et al., 2003; Schettgen et al., 2004).

The median dietary intake of AA estimated from the FFQs was 13.7 \( \mu \text{g/day} \) for nonsmokers. The estimated total AA intake from food did not correlate with Hb adduct levels, which has also been observed in a previous pilot study (Kutting et al., 2005). There might be several reasons for the poor ability to predict individual total AA intake. The quality of the FFQ could influence the accuracy to recall intake of various food items and quantity consumed over the preceding year. In addition, a large variation in AA content may exist within similar food products and even within brands (Dybing et al., 2005). Another contributor of uncertainty is the difficulty to estimate the contribution to AA intake from home-cooked foods.
Our next approach was to examine whether Hb adducts correlated with the intake of any food item or nutrient. The AA- and GA-Hb adducts were investigated separately for possible correlations to intake of various food items. Although it is well known that coffee contains AA (Andrzejewski et al., 2004; Delatour et al., 2004), it did not result in one of the independent predictors of the AA dietary exposure. In a linear regression model, adjusted for age, the intake of potato chips/snacks and crisp bread was identified as independent predictors of Hb adducts of AA. Several studies have shown that potato-based products and crisp bread may contain large quantities of AA (Amrein et al., 2003; Becalski et al., 2003, 2004; Tareke et al., 2002). In contrast, GA-Hb adducts did not correlate with the intake of any of the main food groups. In the present study, a negative correlation of AA- and GA-Hb adduct with age was found, which might be explained by a higher consumption of certain food types high in AA, such as chips/snacks in the younger population.

In our previous study, the relationship between the amount of AA excreted as urinary metabolites and recent AA intake estimated from a 24-h recall interview was investigated (Bjellaas et al., 2007). Total 24 h excretion of AA as urinary metabolites did not correlate with estimated AA intake; however, the intake of coffee, aspartic acid, and starch were found to be significant contributors to the amount of AA excreted as urinary metabolites excreted. These results are in good agreement with the findings of Mottram et al. (2002) that AA can be generated from food components during heat treatment as a result of Maillard reaction between amino acids, in particular asparagine and reducing sugars.

No correlations were found between urinary mercapturic acid metabolites and Hb adducts among nonsmokers. Urinary biomarkers are primarily dependent on the very recent dietary intake of AA (Bjellaas et al., 2005; Boettcher et al., 2006), whereas Hb adducts represent an average exposure over a period of about 4 months. Hence, the Hb adduct concentration would not be influenced by the short-term fluctuations in dietary intakes of AA as would the amount of mercapturic acid metabolites excreted in urine. However, a strong increase in dietary AA intake was recently shown in six individuals who ate potato chips once each day for 7 days (1.9 µg/kg bw/day) to increase Hb adduct concentrations (Vesper et al., 2005). The average adduct levels changed during this period from 39 pmol/g globin to 45 pmol/g globin for AA-Hb and from 26 pmol/g globin to 32 pmol/g globin for GA-Hb. In our previous study, a 10-fold increase in urinary biomarkers was observed even after a few hours after ingestion of potato chips (Bjellaas et al., 2007), illustrating that urinary biomarkers are much more sensitive to minor short-term changes in the diet in comparison to Hb adducts.

The overall median (range) molar ratios GA-Val-PTH/AA-Val-PTH found for nonsmokers and smokers in this study were very similar, 0.49 (0.12–1.08) and 0.46 (0.29–0.98), respectively. The molar ratios between GA-derived urinary mercapturic acid metabolites and AA-derived urinary mercapturic metabolites for nonsmokers and smokers in our previous study were found to be quite similar, 0.07 and 0.06, respectively (Bjellaas et al., 2007). These ratios were lower than molar ratios previous reported (Boettcher et al., 2005; Kellert et al., 2006). In addition, it was recently shown that a significant proportion of GA was converted to glyceramide in humans (Fennell et al., 2005). Thus, it would be of importance to determine glyceramide in human urine. Unfortunately, we had no method available for determination of this highly polar compound (Bjellaas et al., 2007).

Smoking has previously been shown to significantly contribute to AA exposure, which was also observed in this study (Bergmark, 1997; Hagmar et al., 2005; Schettgen et al., 2004). No significant difference in molar ratio for GA- and AA-Hb adducts in smokers and nonsmokers was found. However, a large interindividual variation in molar ratios was observed (from 0.12 to 1.08). This may be due to interindividual variation in AA metabolism caused by differences in levels and activities of CYP2E1 (Bolt et al., 2003; Lieber, 2004). Also, human CYP2E1 has previously been shown to be inhibited by nicotine and cotinine (Van Vleet et al., 2001). No significant difference in molar ratios was found in the present study, which could be due to the uncertainty of only having six smokers in the study population.

Human biomonitoring of exposure to AA and the genotoxic metabolite GA requires analytical methods with high-throughput capacity and sufficient sensitivity to measure low exposures. The method developed in this study is based partly on GC-MS and LC-MS methods previously published. The sensitivity of the method developed in this study is comparable with other developed LC-MS methods (Fennell et al., 2003; Vesper et al., 2006) and GC-MS methods (Bergmark, 1997; Paulsson et al., 2003; Perez et al., 1999; Schettgen et al., 2003), however, it is less time consuming than the traditional GC-MS methods. Vesper et al. (2006) reported a lower between-assay precision (9% for AA-Hb adducts and 11% GA-Hb adducts). The triple quadrupole MS as used by Vesper et al. (2006) generally has lower analytical variation than the ion trap MS, which likely accounts for the higher variation of our method (Table 1).
In summary, a LC-MS/MS method for the quantification of Hb adducts from AA exposure was developed. Using AA- and GA-Hb adduct concentrations as biomarkers for long-term intake, no correlation to total dietary intake of AA as estimated on the basis of a FFQ and AA contents of various food items was found. However, the food items chips/snacks and crisp bread were identified as independent predictors of the concentration of AA-Hb adducts in blood.

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