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Analysis of acrylamide in blood samples from adolescents in Sweden

Results from the national dietary study Riksmaten Adolescents 2016-2017

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Sammanfattning

Blodprover (600 st.) från deltagare i matvaneundersökningen *Riksmaten ungdom 2016-17* har analyserats med avseende på akrylamid och dess metabolit glycidamid. Analyserna har gjorts med masspektrometri av reaktionsprodukter med hemoglobin (Hb-addukter) som biomarkör för exponering för akrylamid. I denna rapport redovisas resultaten från dessa analyser. Skillnader i exponering mellan kön och mellan åldersgrupper (årskurs 5, årskurs 8 och årskurs 2 på gymnasiet) har undersökts samt association med bostadsort (stadsmiljö eller landsbygd) och föräldrars utbildningsnivå. Exponeringen har också undersökts i relation till uppskattat intag av akrylamid från mat, baserat på enkätsvar om matvanor från ungdomarna.

Akrylamid kunde detekteras i nästan alla prover (99 %). Medianhalten var 34 pmol/g Hb. Ingen signifikant skillnad i Hb-addukthalt av akrylamid kunde observeras mellan pojkar och flickor. Inte heller kunde någon signifikant skillnad observeras mellan åldersgrupperna för akrylamid. Vid jämförelse av Hb-addukter av glycidamid samt summan av akrylamid och glycidamid uppvisade barnen i årskurs 5 högre halter jämfört med ungdomarna i årskurs 8 och i årskurs 2 på gymnasiet. Vid jämförelse mellan bostadsort på landsbygd jämfört med i stadsmiljö observerades inga signifikanta skillnader i Hb-adduktnivåer av akrylamid eller glycidamid för någon åldersgrupp. Inte heller observerades någon association mellan Hb-adduktnivåer och föräldrarnas utbildningsnivå i någon åldersgrupp. Omkring 10 % av studenterna rapporterade att de röker, dagligen eller ibland, och dessa individer hade signifikant högre Hb-adduktnivåer av glycidamid samt av summan av akrylamid och glycidamid.

Förutom rökning utgör livsmedelskonsumtion den största källan till akrylamidexponering. De uppmätta adduktnivåerna jämfördes med uppskattat intag av akrylamid från livsmedel som är kända att innehålla akrylamid. Jämförelsen gjordes både med data från enkätsvar om generella matvanor samt från data från kostregistrering under två dagar. Ingen signifikant korrelation observerades mellan adduktnivå av akrylamid och det totala medelintaget från kostregistreringen, sannolikt för att adduktnivåerna motsvarar exponeringen som skett under de senaste ca fyra månaderna, vilket är hemoglobinets livslängd. Dock observerades signifikanta korrelationer mellan adduktnivå och registrerad konsumtion av chips och bröd för studenterna i årskurs 5. Också observerades en signifikant skillnad mellan adduktnivåer och konsumtion av chips från enkätdata om generella konsumtionsvanor.

En framtida studie om matvanor som designats med avseende på akrylamidexponering skulle ge en bättre förståelse för korrelationen mellan konsumtion och exponering, och ge en ännu bättre uppskattning om bidraget av akrylamid från mat till den totala cancerrisken.

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INTRODUCTION

In 2016-2017, the Swedish Food Agency conducted a national dietary survey to collect information on food consumption among adolescents (10-21 years), Riksmaten Adolescents 2016-17 (Moraeus et al., 2018; Warensjö Lemming et al. 2018). The data have been used to calculate nutrient and energy intakes as well as the exposure to several unwanted compounds from different foods (Livsmedelsverket, 2018; Livsmedelsverket & Naturvårdsverket, 2020). In parallel to the dietary survey, blood and urine samples were collected in a subgroup of participants for biomonitoring of several parameters. Knowledge about internal exposures to unwanted compounds enables improvement of risk assessments.

One compound that we are commonly exposed to is acrylamide (2-propenamide, CAS No. 79-06-01) which is classified as *probably carcinogenic* to humans (Group 2A) by WHO (IARC, 1994). It is an industrial chemical used in the production of polyacrylamides, primarily used as flocculants in drinking water treatment plants (EFSA, 2015). Acrylamide may also be formed through a Maillard reaction ("browning of food") between reducing sugars and certain amino acids, particularly asparagine. This reaction occurs during the baking and frying of carbohydrate rich foods, i.e., potatoes (French fries, chips/crisps), bread, biscuits, and coffee at temperatures above 120 °C and at low moisture content (Mottram et al., 2002; Tareke et al., 2002). Acrylamide is also present in cigarette smoke, and studies show that regular smokers usually have several times higher exposure to acrylamide compared to non-smoking individuals, as measured by Hb adducts (cf. Pedersen et al., 2022). Furthermore, it has been indicated that acrylamide could be formed from endogenous processes, which thus might be an additional source of exposure (Goempel et al., 2017). Acrylamide is metabolized *in vivo* to glycidamide (2,3-epoxypropanamide, CAS No. 5694-00-8) via the cytochrome P450 2E1 enzyme. Glycidamide has been shown to be genotoxic and mutagenic (EFSA, 2015). Due to the toxic properties of acrylamide and glycidamide it is of importance to monitor the human exposure to these compounds.

Biomonitoring of acrylamide and glycidamide are commonly performed in blood or in urine, but they are note monitored as such. Both acrylamide and glycidamide are electrophilic, leading to that they could bind covalently with proteins and with DNA. One such reaction is between the compounds and the N-terminal valine in hemoglobin (Hb), creating Hb adducts which are monitored in the blood (cf. Vikström et al., 2011; Carlsson et al., 2017). In urine, acrylamide and glycidamide can be monitored as mercapturic acids; N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) and N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA), respectively, where about 10-20% of acrylamide is found to be metabolized to glycidamide in humans (Fuhr et al., 2006; Hartmann et al., 2021; Fernández et al., 2022).

This report summarizes the results from analyses of acrylamide and glycidamide (as Hb adducts) in blood samples in a subset of samples (N=600) from the adolescents in the Riksmaten study. Differences in Hb adduct levels in the adolescents at different age (school grade), in different geographical regions and by sex have been investigated. Furthermore, we aimed to evaluate associations between Hb adduct levels and intake of foods known to contain acrylamide.

MATERIALS AND METHODS

Recruitment and sampling

The present investigation was performed with blood samples collected from participants of a biomonitoring subgroup of Riksmaten Adolescents 2016-17, a national dietary survey among students (10-21 years) conducted earlier by the Swedish Food Agency (Moraeus et al., 2018). In brief, 2377 students from 62 schools from all parts of Sweden were invited to participate in the biomonitoring part of the study. In total, 1105 students in grade 5, grade 8 and in the 2nd year of high school provided complete biological samples and complete dietary information. Ethical approval for the survey was obtained from the Regional Ethical Review Board in Uppsala (No. 2015/190). Written informed consent was obtained from all participants and from the legal guardians for individuals younger than 16 years. The blood samples were stored at -80°C at the Swedish Food Agency after the collection.

In the present study, 200 blood samples per age group (per school grade) (600 samples in total), equally distributed between sex, were selected for analysis of acrylamide and glycidamide (as Hb adducts) from the biomonitoring study of the Riksmaten study. The rational for the selection was to enable an equal distribution between sex and grade in school, and about an equal distribution between geographical locations of the schools in Sweden (from north to south). The characteristics of the participants are included in **Table 2**. A fraction of 750 µL per whole blood sample was transferred to plastic test tubes and stored at -80°C at the Swedish Food Agency before distribution to the laboratory for chemical analysis.

Assessment of diet

The participants recorded their consumption of food and beverages retrospectively on two non-consecutive days using a validated web-based 24-h dietary recall method (Moreaus et al 2018; Lindroos et al 2019). As a complement, web-questionnaires were used to estimate consumption of selected foods over the previous 12 months.

Chemical analysis

The blood samples (N=600) where analyzed at the Department of Environmental Science at Stockholm University. The analytical method is described in Appendix 1.

Estimation of daily acrylamide intake

Based on the dietary recall data (two days of registration), mean consumption per day (g/day) of food groups relevant for acrylamide intake was calculated for the participating adolescents. The daily intake of acrylamide was further estimated by combining consumption data with occurrence data on acrylamide in the different food items, either from analyses by the Swedish Food Agency during 2022, or from reported data in the scientific opinion on acrylamide by EFSA (2015). For acrylamide occurrence data in different food items, see **Table 1**.

Table 1. *Acrylamide mean occurrence level (µg/kg) in different food categories used for daily intake calculations. Occurrence data are either derived from analyses of food items at the Swedish Food Agency during 2022 or from reported data used for exposure assessment (Appendix D) in the scientific opinion on acrylamide by EFSA, 2015.*

Food category	**Mean acrylamide concentration (µg/kg food)**	**Occurrence data origin**	**Mean consumption from all age groups (g/day)[b]**
Bakery and cakes	271	EFSA, 2015	57 (N=244)
Bread (soft)	38	SLV, 2022	118 (N=564)
Cereals	76	SLV, 2022	27 (N=220)
Chocolate/cocoa products	73	EFSA, 2015	34 (N=120)
Coffee	13[a]	SLV, 2022	208 (N=61)
Crispbread	145	SLV, 2022	14 (N=140)
Crisps	341	SLV, 2022	66 (N=106)
Dried fruit	89	EFSA, 2015	14 (N=25)
French fries and fried potatoes	300	SLV, 2022	53 (N=293)

[a] Estimated level in ready-to-drink coffee from occurrence level in coffee powder divided by the factor 18.6 (dilution factor based on a recipe where 50 g of coffee powder is used with one liter of water when brewing, and where more water than acrylamide is stick to the filter paper (as observed from measurements). Oral communication with Johan Rosén, Swedish Food Agency). [b] Mean data from all age groups (5th grade, 8th grade, 2nd grade) where consumption was recalled during two days. N represents the number of consumers of each food category.

Calculations and statistical analysis

Statistical analyses were performed using the software package STATA, version 15 (StataCorp, College Station, TX, U.S.). The level of acrylamide and glycidamide Hb adducts, separately and as the sum, were described by the number of participants and percentages with levels above LOQ, means and standard deviations, medians, 5th percentile, 95th percentile, and minimum and maximum levels. All statistical analyses were run on data above LOQ, assuming normal distribution. Differences in levels between place of residence, i.e. urban and rural municipalities, parents´ education (elementary school, high school and university), school grades and sex were investigated using Student´s t-test and analysis of variance (ANOVA), one covariate at a time. Also, differences in levels of acrylamide Hb adducts and the sum of acrylamide and glycidamide Hb adducts between different levels of consumption (consumption frequency groups) of fried potatoes, crisps, bread (crisp bread and soft bread, with and without white bread) and coffee were investigated using ANOVA. The Bonferroni post hoc test was used to investigate the significance level between groups when a significance was observed using ANOVA. In addition, correlations between estimated daily acrylamide intake and blood levels of acrylamide and glycidamide Hb adducts were investigated (Pearson correlation coefficients).

RESULTS AND DISCUSSION

Characteristics of the cohort

The characteristics of the included groups of adolescents are shown in **Table 2**. The levels of the Hb adducts of acrylamide, glycidamide and the sum of acrylamide and glycidamide for the different age groups are presented in **Table 3**. A widespread exposure to acrylamide was observed, with a quantified frequency of almost 100% (>LOQ). The median level of acrylamide was 34 pmol/g Hb, which is comparable with other studies (summarized by e.g. Pedersen et al., 2022).

The quantification frequency in the samples for the glycidamide analyte was lower (76%) than for acrylamide. This is to a large extent due to difficulties with the analytical method for the detection of the glycidamide adduct analyte (a low signal to noise ratio) in the present study (see description of analytical method in Appendix 1). Adduct levels from both acrylamide and glycidamide are expected to be about the same level (acrylamide exposure leads to about four times lower internal dose of glycidamide than of acrylamide but glycidamide reacts about four times faster (per dose unit) with the N-terminal valine compared to acrylamide (Vikström et al., 2011)).

Due to interindividual differences in metabolic capacity to glycidamide (Pellè et al., 2018), the sum of acrylamide and glycidamide adduct levels have been discussed to be a good measure of the total acrylamide internal dose (Wilson et al., 2009). Therefore, the statistical analyses have been performed both on acrylamide and glycidamide Hb adducts separately, and on the sum of the adduct levels, further discussed below.

Table 2. *Characteristics of the adolescents in 5th grade, 8th grade and 2nd grade of high school.*

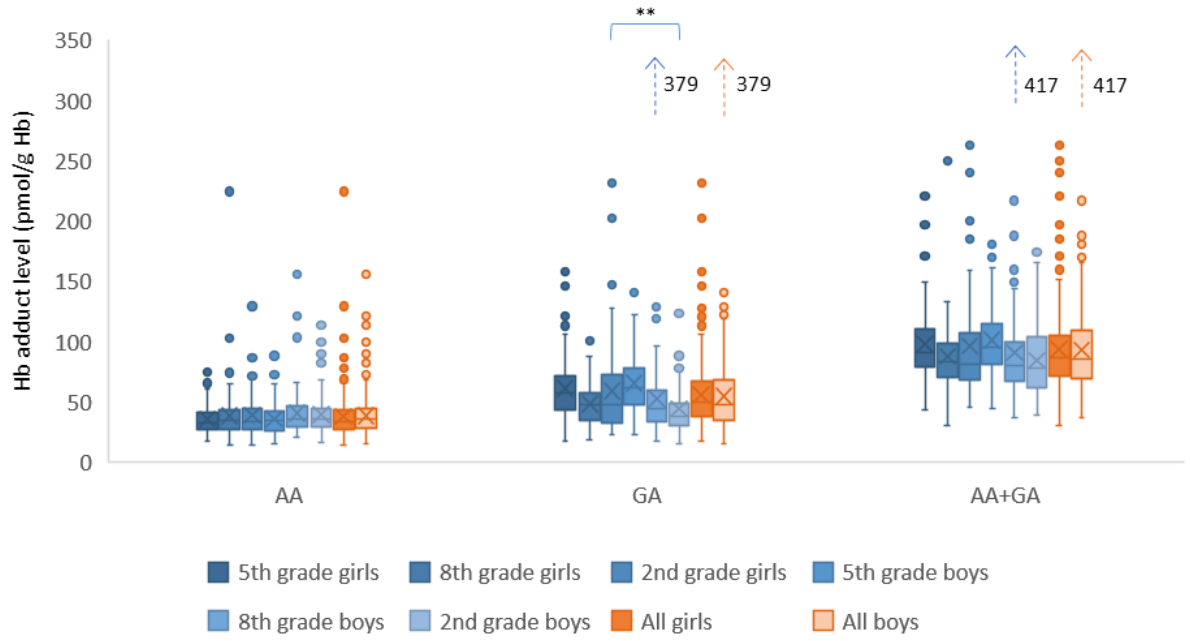
	5th grade (N=201)	**8th grade (N=201)**	**2nd grade (N=198)**
Girls (N)	100	100	100
Boys (N)	101	100	99
Median age (years) [min-max], P05-P95	11.5 [10.8-13.1], 11.0-12.3	14.6 [13.3-15.7], 13.9-15.1	17.8 [16.8-21.0], 17.0-19.2
Median weight (kg) [min-max], P05-P95	43.3 [25.9-81.6], 30.8-60.5	57.5 [37.7-107.1], 42.6-79.1	69.2 [45.1-124.4], 51.0-92.9
Smokers[a] (N)	no information	10	48 (acrylamide data available for 46)
Municipality, urban/rural (N)	142/59	150/50	122/77
Parental education level, elementary school/high school/university/no answer (N)	9/62/125/5	6/56/131/7	10/75/102/12

[a] Individuals were sorted as smokers if they had answered "Yes, occasionally" or "Yes, daily" in the survey. No information is available for any other tobacco use.

Sex difference

No statistical differences could be observed when comparing the levels of acrylamide (AA) Hb adducts between the girls and boys in any grade (**Table 4**, **Figure 1**). Nor could any statistical differences be observed when comparing the glycidamide (GA) Hb adduct levels between sexes in 5th and 8th grade or the sum of the Hb adducts (AA+GA) in any grade. For the students in 2nd grade of high school a statistical significant difference was observed, where higher levels were observed for the girls compared to the boys for GA Hb adducts (p=0.0038). No statistical differences in Hb adduct levels (AA, GA or AA+GA) between boys and girls could be observed when comparing all grades together.

***Figure 1**. Level of Hb adducts (quartiles, mean (×), median (−), min/max) of acrylamide (AA), glycidamide (GA) and the sum of the adducts (AA+GA) in relation to sex at different ages; 12 years (5th grade), 15 years (8th grade) and 18 years (2nd grade of high school). The stars (**) indicate p<0.01 at the 95% significance level.*



Age difference

For the level of acrylamide Hb adducts (AA) no significant differences were observed between any age groups (school grades). However, a significant difference in the level of glycidamide Hb adducts (GA) and the sum of adducts (AA+GA) could be observed (**Table 4**, **Figure 2**), where the youngest students (5th grade) had higher levels of both GA and AA+GA compared to the students in 8th grade (p=0.001, GA; 0=0.037, AA+GA) and of GA compared to the students in 2nd grade of high school (p=0.002). No significant difference was observed for the Hb adduct levels of GA or the sum (AA+GA) between the students in 8th grade and in 2nd grade of high school.

Table 3. *Blood levels of Hb adducts of acrylamide, glycidamide, and the sum of the adducts (AA-Hb + GA-Hb) among all students (smokers and non-smokers) in 5th gradse (12 years), 8th grade (15 years) and 2nd grade of high school (18 years). Statistics is summarized only for quantified (>LOQ) levels.*

		Acrylamide Hb adducts (pmol/g Hb)			**Glycidamide Hb adducts (pmol/g Hb)**			**AA-Hb + GA-Hb (pmol/g Hb)**		
		Mean ± sd (% detected)	**Median [min-max]**	**P05-P95**	**Mean ± sd (% detected)**	**Median [min-max]**	**P05-P95**	**Mean ± sd (% detected)**	**Median [min-max]**	**P05-P95**
All grades (N=600)	All	38.0±17.9 (99%)	34.4 [14.0-224.6]	20.3-65.2	55.6±30.9 (76%)	49.1 [14.9-379.0]	24.4-106.8	93.6±36.3 (76%)	86.4 [30.2-417.1]	53.7-159.6
	♀	38.0±19.0 (100%)	33.4 [14.0-224.6]	20.7-65.9	56.2±29.1 (72%)	50.0 [17.9-231.7]	25.1-113.2	94.0±35.6 (72%)	86.5 [30.2-263.0]	54.0-159.6
	♂	38.0±16.8 (99%)	34.8 [14.4-155.6]	19.8-64.5	55.1±32.6 (79%)	47.9 [14.9-379.0]	23.0-106.8	93.0±37.1 (79%)	86.1 [37.0-417.1]	49.5-159.7
5th grade (N=201)	All	36.1 ±13.2 (99%)	33.4 [14.9-91.4]	19.8-64.5	63.7±25.3 (79%)	59.6 [17.9-158.0]	32.5-113.2	99.8±29.3 (79%)	94.0 [43.3-220.5]	64.3-161.01
	♀	36.2±12.5 (99%)	32.9 [17.7-74.5]	21.8-65.6	61.2±26.1 (70%)	57.1 [17.9-158.0]	32.5-113.2	98.2±31.8 (70%)	91.0 [43.3-220.5]	61.1-170.7
	♂	36.0 ±13.8 (99%)	34.4 [14.9-91.4]	19.1-63.2	65.6±24.7 (88%)	61.3 [23.0-144.4]	34.8-111.2	101.1±27.4 (88%)	94.9 [44.8-180.9]	64.8-159.9
8th grade (N=201)	All	38.5±21.4 (99%)	33.7 [14.4-224.6]	20.6-64.2	50.8±33.0 (76%)	45.6 [17.7-379.0]	24.9-84.4	89.9±40.4 (76%)	82.8 [30.2-417.1]	54.0-143.6
	♀	38.8±23.4 (99%)	35.0 [14.6-224.6]	20.3-64.7	48.4±17.0 (73%)	47.7 [19.1-100.5]	23.0-78.8	87.6±28.7 (73%)	83.7 [30.2-249.6]	53.7-127.2
	♂	38.2±19.4 (98%)	33.2 [14.4-155.6]	20.6-64.2	52.8±42.6 (79%)	44.2 [17.7-379.0]	25.7-94.1	91.3±48.9 (79%)	80.4 [37.0-417.1]	53.3-160.0
2nd grade (N=198)	All	39.4±18.1 (99%)	35.2 [14.0-131.9]	20.1-72.3	51.7±32.7 (71%)	42.7 [14.9-231.7]	22.7-123.7	90.6±38.0 (71%)	80.8 [39.0-263.0]	47.2-159.6
	♀	39.1±19.5 (100%)	33.7 [14.0-131.9]	20.0-72.3	59.0±38.8 (72%)	48.3 [23.3-231.7]	24.4-128.0	96.2±44.0 (72%)	81.2 [45.4-263.0]	51.3-190.5
	♂	39.8±16.7 (99%)	36.4 [16.6-114.0]	20.1-82.7	44.4±23.2 (71%)	38.3 [14.9-127.4]	18.7-88.4	84.8±29.9 (71%)	78.4 [39.0-173.9]	44.3-133.2

♀: girls, ♂: boys, sd: standard deviation.

***Figure 2**. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for acrylamide (AA), glycidamide (GA) and the sum of the adducts (AA+GA) in different school grades; 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years). The stars (**) indicate p<0.01 at the 95% significance level.*

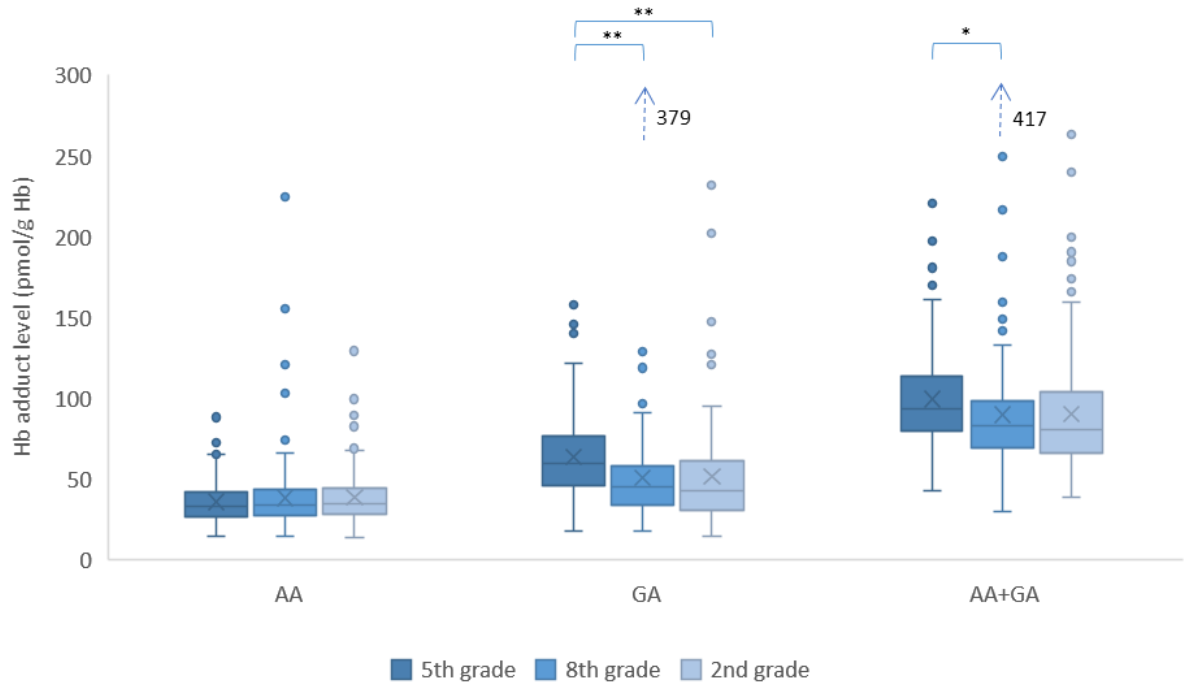


***Table 4.** Level of significance (p value, from Student´s t-test or ANOVA) between age (school grade), sex, place of residence (rural versus urban) and parental education level, and the level of Hb adducts (pmol/g Hb) of acrylamide (AA), glycidamide or the sum of acrylamide and glycidamide Hb adducts (AA+GA).*

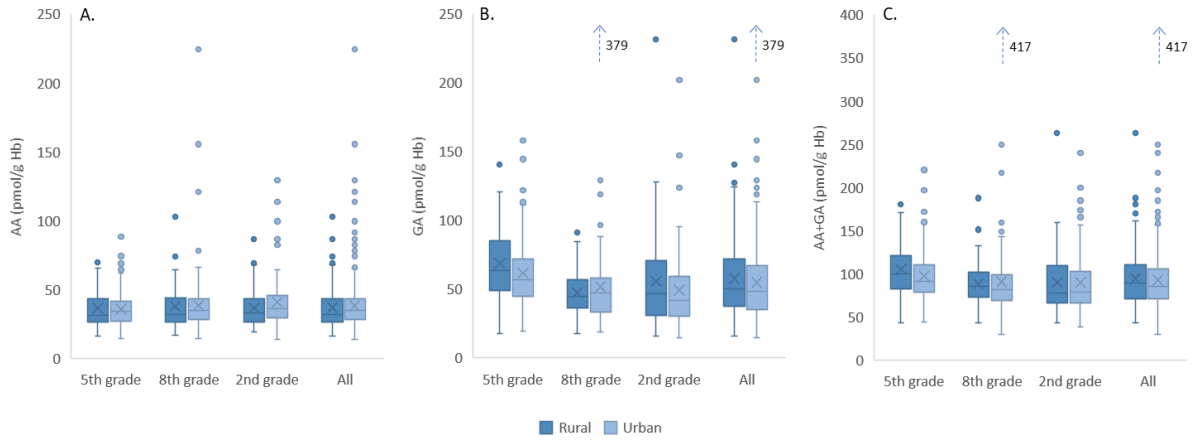
	Age (school grade)			**Sex**			**Place of residence[a] (rural versus urban)**			**Parental education level[b]**		
	AA	**GA**	**AA+ GA**	**AA**	**GA**	**AA+ GA**	**AA**	**GA**	**AA+ GA**	**AA**	**GA**	**AA+ GA**
All	0.1561	**0.0002**	**0.0227**	0.9711	0.7254	0.7856	0.5750	0.7153	0.9739	0.7440	0.5026	0.5487
5th (12 yrs)	n/a	n/a	n/a	0.9058	0.2869	0.5473	0.6906	0.1249	0.1453	0.9623	0.2589	0.2826
8th (15 yrs)	n/a	n/a	n/a	0.84161	0.3931	0.5128	0.8496	0.5034	0.5875	0.2965	0.9702	0.7827
2nd (18 yrs)[c]	n/a	n/a	n/a	0.7836	**0.0077**	0.0741	0.1124	0.2733	0.8312	0.4395	0.3505	0.8405

[a] Rural or urban, [b] Elementary school, high school, or university, [c] 2nd grade of high school.

Differences between rural and urban municipalities

No significant difference was observed for Hb adduct levels of either acrylamide, glycidamide or the sum of Hb adducts (AA+GA) between rural and urban municipalities for the students in any grade. All data are summarized in **Table 4** and in **Figure 3**.

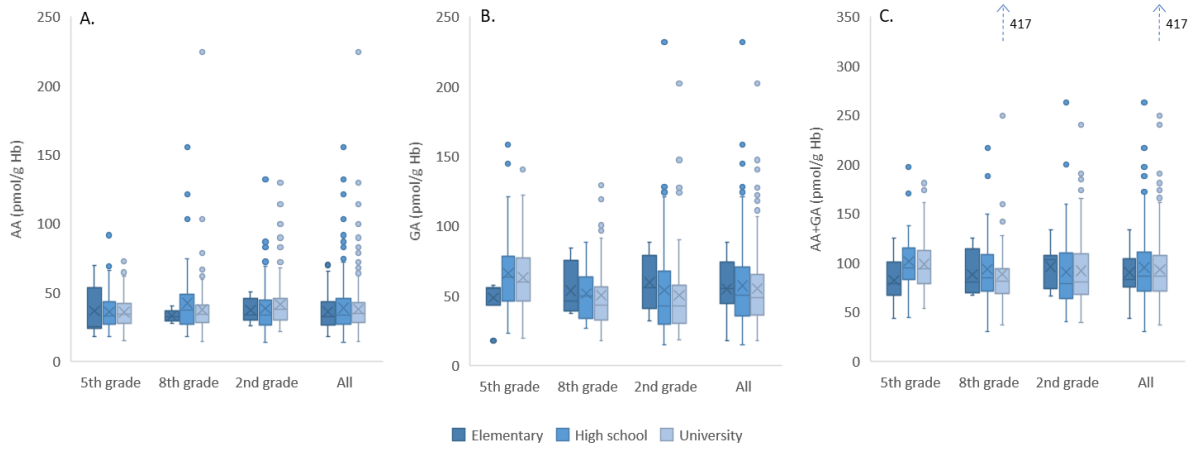
Figure 3*. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), B. glycidamide (GA) and C. the sum of acrylamide and glycidamide (AA+GA) in relation to place of residence (rural versus urban) for students at 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*



Parental education level

No significant differences in Hb adduct levels of acrylamide, glycidamide or the sum of Hb adducts were observed to be dependent on the level of education of the parents (elementary school, high school and university), see **Table 4** and **Figure 4**.

Figure 4*. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), B. glycidamide (GA) and C. the sum of acrylamide and glycidamide (AA+GA) in relation to the level of parents' education for students at 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*



Cigarette smoking

Cigarette smoke is a known exposure source for acrylamide and related Hb adducts (Bergmark, 1997), and therefore the exposure levels were compared between smokers and non-smokers.

The number of smoking students in the present study are about 10%, with in total 8 students saying that they smoke daily (no students in 8th grade, 8 students in 2nd grade of high school) and 50 students saying that they smoke occasionally (10 students in 8th grade and 40 students in 2nd grade of high school). The students in the 5th grade did not get any questions in the survey about smoking habits, and therefore the results are only based on the responses from students in 8th grade and in 2nd grade of high school (**Table 5**). No significant difference was observed between the non-smoking and smoking students when looking at all grades together. The same was true when looking specifically at the students in 8th grade. However, for the students in 2nd grade of high school a significant difference was observed between smokers and non-smokers for GA (p=0.0204), but not for AA or the sum of Hb adducts (**Figure 5**). No significant differences in Hb adduct levels were observed between sexes (smokers) in any grade. Comparing the daily smokers with occasionally smokers no significant difference was observed for AA Hb adduct levels (34.7 and 38.8 pmol/g Hb, respectively), but for GA Hb adduct levels (93.6 and 51.8 pmol/g Hb, respectively) and AA+GA Hb adduct levels (128.3 and 87.7 pmol/g, respectively).

***Figure 5**. A. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), B. glycidamide (GA) and C. the sum of acrylamide and glycidamide (AA+GA) in relation to cigarette smoking for students at 8th grade (15 years) and 2nd grade of high school (18 years). The star (*) indicate p<0.05 at the 95% significance level.*

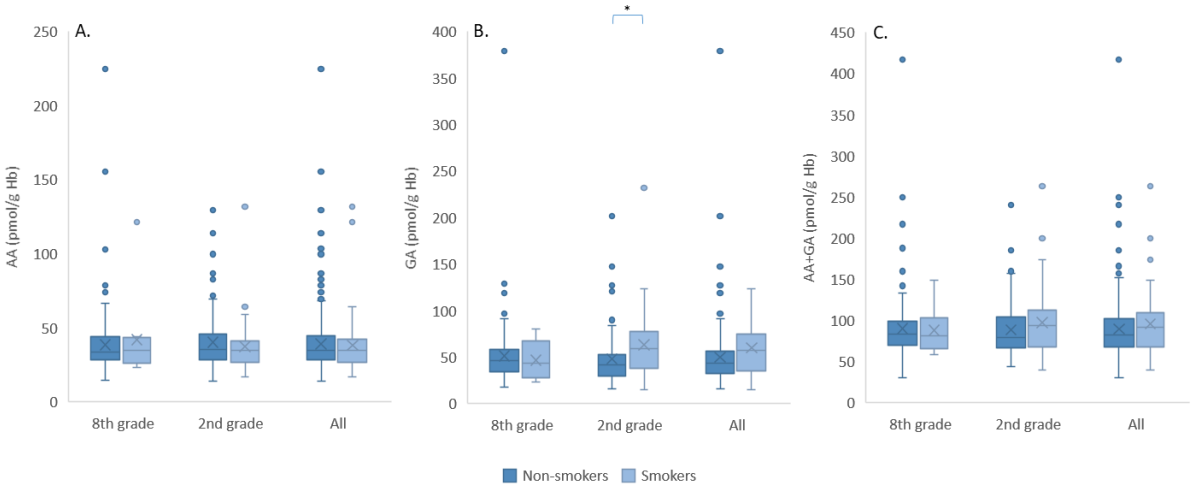


Table 5. *Level of Hb adducts of acrylamide (AA), glycidamide (GA) and the sum of acrylamide and glycidamide (AA+GA) in smoking and non-smoking students in 8th grade (15 years) and in 2nd grade of high school (18 years).*

		Acrylamide Hb adducts (pmol/g Hb) Mean± sd (N)		**Glycidamide Hb adducts (pmol/g Hb) Mean± sd (N)**		**AA-Hb + GA-Hb (pmol/g Hb) Mean± sd (N)**	
		Non-smokers	**Smokers**	**Non-smokers**	**Smokers**	**Non-smokers**	**Smokers**
8th and 2nd grade	All	39.1±19.8 (339)	38.2±19.9 (56)	49.8±32.0 (253)	51.2±32.8 (41)	89.1±38.5 (253)	95.6±43.4 (41)
	♀	39.0±21.7 (169)	38.6±20.5 (29)	51.8±27.3 (123)	64.2±42.7 (21)	90.3±34.8 (123)	101.0±48.8 (21)
	♂	39.2±17.9 (170)	37.7±19.6 (27)	47.9±36.1 (129)	55.5±28.3 (20)	88.4±41.8 (129)	90.0±37.2 (20)
8th grade	All	38.3±21,0 (188)	41.9±29.0 (10)	51.1±33.6 (144)	49.5±21.1 (8)	90.0±41.0 (144)	88.0±29.4 (8)
	♀	39.1±24.1 (92)	35.8±7.56 (7)	49.0±16.9 (68)	41.4±18.7 (5)	88.6±29.2 (68)	74.1±15.8 (5)
	♂	37.7±17.6 (96)	51.5±46.9 (4)	53.0±43.5 (76)	55.0±26.1 (3)	91.2±49.3 (76)	111.1±35.1 (3)
2nd grade	All	40.0±18.3 (151)	37.4±17.6 (46)	48.2±30.1 (108)	63.2±38.5 (33)	88.5±35.0 (108)	97.5±46.3 (33)
	♀	38.9±18.4 (77)	39.6±23.8 (21)	55.4±36.1 (55)	71.4±45.9 (16)	92.4±40.8 (55)	109.4±52.8 (16)
	♂	41.2±18.1 (74)	35.4±10.4 (24)	40.8±19.9 (53)	55.6±29.4 (17)	84.4±27.5 (53)	86.2±37.3 (17)

Data for students who did not give any answer on smoking habits (N=6 in 8th grade, N=3 in 2nd grade) were included in the non-smoker category.

Associations with acrylamide intake and food consumption

Estimated acrylamide intake

Acrylamide intake was estimated from the mean daily consumption of different food categories relevant for acrylamide exposure, using occurrence data presented in **Table 1** from the dietary recall data (two days of registration). The mean estimated daily intake of acrylamide per person from the whole studied population, 28 µg/day (**Table 6**) correlates well with a previous estimation of the mean daily intake per person for Swedish adults (31 µg/day), calculated from occurrence data in foods at the Swedish market at that time (Svensson et al., 2003). The estimated mean intake in the present study is also in line with the intake in the general adult population in Europe, estimated by EFSA in 2015 (35 µg/day, corresponding to 0.5 µg/kg/day assuming 70 kg body weight).

Table 6. *Estimated acrylamide daily intake (µg/day) among students in 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*

	Mean ± sd	**Median [min-max]**	**P05-P95**
Present study			
All students (N=600)	27.8 ±22.5	22.1 [0-127.8]	3.0-71.7
5th grade (N=201)	28.4 ± 22.7	23.3 [0.9-124.9]	3.0-69.2
8th grade (N=200)	26.0 ± 20.5	21.1 [0-118.9]	2.2-64.6
2nd grade (N=199)	29.1 ± 24.1	22.1 [0-127.8]	3.3-77.9
Previous studies			
Svensson et al., 2003	31	27	9.1-62
EFSA, 2015	35 (28-42)[a,b]	-	70 (56-91) [a,c]

[a] Calculated by assuming 70 kg bodyweight, [b] Median (min-max) for adults of the mean across different surveys, [c] Median (min-max) for adults of P95 of the mean across different surveys (where n>60).

Correlations between adduct levels and estimated intake

The estimated acrylamide intake was further compared to the acrylamide Hb adduct levels and the sum of Hb adducts of acrylamide and glycidamide. The comparisons were made both for the total estimated acrylamide intake as well as for crisps, fried potatoes, bread and coffee separately. The observed correlations, expressed as Pearson correlation coefficients, between Hb adduct levels and intake are presented in **Table 7**. No significant correlations were observed between total intake of acrylamide and measured Hb adduct levels, which may be explained by continuous exposure to acrylamide from more foods than was investigated in this study. However, some significant correlations could be observed between acrylamide Hb adduct levels and consumption of crisps and bread for the students in 5th grade

and for bread and coffee for the students in 2nd grade of high school. In general, using the sum of acrylamide and glycidamide Hb adducts instead of acrylamide Hb adducts alone in the correlation to the different registered food categories resulted in significant correlations in more categories. Adjusting for body weight did not improve the significance levels of the correlations.

Hb adduct levels reflects the exposure during the last four months (the lifetime of hemoglobin). This may be an explanation to the lack of significant correlations between the measured acrylamide adduct levels and the calculated total daily acrylamide intakes based on registered food consumption for two non-consecutive days during one week. Food frequency questions, which reflect more general food consumption over a longer time-period, may therefore be useful in explaining the measured adduct levels.

Table 7. *Pearson correlation coefficient (r) between estimated daily acrylamide intake, calculated from dietary registrations (food diary), and measured acrylamide Hb adduct (AA) or the sum of acrylamide and glycidamide Hb adducts (AA+GA). The intake was correlated as µg/day or as adjusted for body weight, µg/kg bw per day (in brackets).*

Food (number of consumers)	**All students**		**5th grade (12 years)**		**8th grade (15 years)**		**2nd grade of high school (18 years)**	
	AA	**AA+GA**	**AA**	**AA+GA**	**AA**	**AA+GA**	**AA**	**AA+GA**
Total intake[a] (N=600)	0.0155 (-0.0199)	0.0425 (0.9057***)	0.0225 (-0.0095)	0.0163 (0.0145)	0.0600 (0.0399)	0.0924 (0.1189)	-0.0331 (-0.0448)	-0.0494 (0.0878)
Crisps (N=106)	0.0451 (-0.0294)	0.1463*** (0.6029***)	-0.3195*** (-0.3491***)	-0.0510 (-0.0994)	0.1880 (0.1522)	0.1578 (0.2119*)	0.0501 (0.0362)	-0.0941 (0.1297)
Fried potatoes (N=328)	0.0543 (0.0301)	0.6732*** (-0.0333)	0.0644 (0.0563)	0.0239 (0.0315)	0.0127 (0.0437)	0.0043 (0.0201)	0.0981 (0.0703)	0.1295* (0.1755**)
Bread (N=577)	-0.0163 (-0.0307)	0.1538*** (0.2104***)	0.1027* (0.0851*)	0.0821* (0.0383)	0.0289 (-0.0048)	0.2786*** (0.2753***)	-0.1912*** (-0.1666***)	-0.2119*** (-0.1630***)
Coffee (N=61)	-0.1248 (-0.0943)	-0.0442 (0.9572***)	n/a	n/a	-0.2061 (-0.1569)	-0.3243** (-0.4727***)	-0.2886* (-0.2939*)	-0.0746 (0.0651)

[a] Total intake of acrylamide per day, estimated from registered consumption of potatoes products, bread, cereals, dried fruit, coffee, chocolate/cocoa and cookies/cakes during two days.

Food questionnaire data

The acrylamide Hb adduct levels (AA) and the sum of acrylamide and glycidamide Hb adducts (AA+GA) were compared to data from the food frequency/propensity questions in the web-questionnaire, i.e., consumption frequencies of foods known to be major dietary sources for acrylamide exposure, i.e. crisps, fried potatoes and bread, over the last 12 months (**Table 8**).

Table 8. *Number of consumers with different consumption frequencies of crisps, fried potatoes and bread, extracted from questionnaire data for students in 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*

Potatoes					**Bread (crisp bread and soft bread)**		
Consumption frequency	**Grade**	**Crisps**	**Fried potatoes**		**Consumption frequency**	**Grade**	**Bread**
Never	5th	7	3[a]		**0-2 slices/week**	5th	28
	8th	11	2[a]			8th	24
	2nd	11	1[a]			2nd	40
≤1 time per month or 1 time per month	5th	34	45		**3-10 slices/week**	5th	75
	8th	41	38			8th	117
	2nd	53	39			2nd	85
1 time per week to 2-3 times per month	5th	131	116		**11-20 slices/week**	5th	80
	8th	117	127			8th	44
	2nd	115	126			2nd	56
2-3 times per week to 6 times per week/ every day	5th	27	35		**>20 slices/week**	5th	16
	8th	31	32[b]			8th	14
	2nd	19[b]	31[b]			2nd	17

[a] The "Never" category is included in "≤1 time per month" category due to few consumers. [b] No "Every day".

Potato consumption

For crisps, no significant differences could be observed for acrylamide Hb adduct levels or the sum of acrylamide or glycidamide Hb adducts at different consumption frequencies, measured as occasions per week or per months, when looking at all individuals at the same time (**Table 9**, **Figure 6**). Students in the 5th grade showed significant differences between consumption frequency groups for the acrylamide Hb adducts. No association between consumption of crisps and Hb adduct levels was observed for students in 8th grade or in the 2nd year of high school.

For fried potatoes, no significant difference in Hb adduct levels was observed between different consumption frequency groups in any school grade (**Table 9**, **Figure 7**).

Figure 6*. A. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), and B. the sum of acrylamide and glycidamide (AA+GA) at different crisps consumption frequencies for students at 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years). The star (*) indicate p<0.05 at the 95% significance level.*

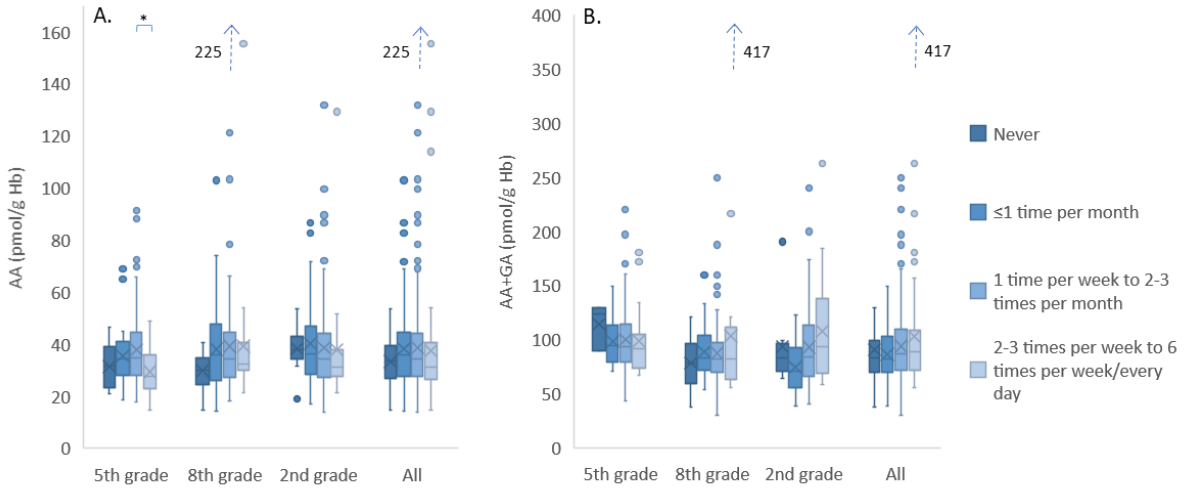
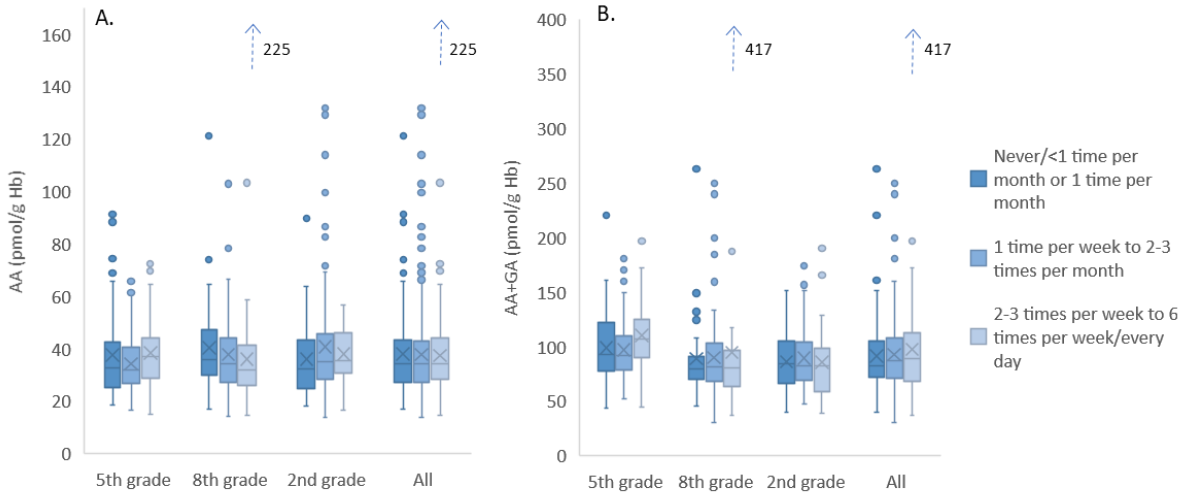


Figure 7*. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), and B. the sum of acrylamide and glycidamide (AA+GA) at different fried potatoes consumption frequencies for students in 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*



Bread consumption

For bread, no significant differences in Hb adduct levels were observed between any consumption frequency groups (number of bread slices per week) at any school grade (**Table 9**, **Figure 8**). The results were not affected when white soft bread, which is known to contain less acrylamide (if not toasted), was excluded in the statistical analysis (data not shown).

***Figure 8**. Level of Hb adducts (quartiles, mean (×), median (−), min/max) for A. acrylamide (AA), and B. the sum of acrylamide and glycidamide (AA+GA at different bread consumption frequencies for students in 5th grade (12 years), 8th grade (15 years) and 2nd grade of high school (18 years).*

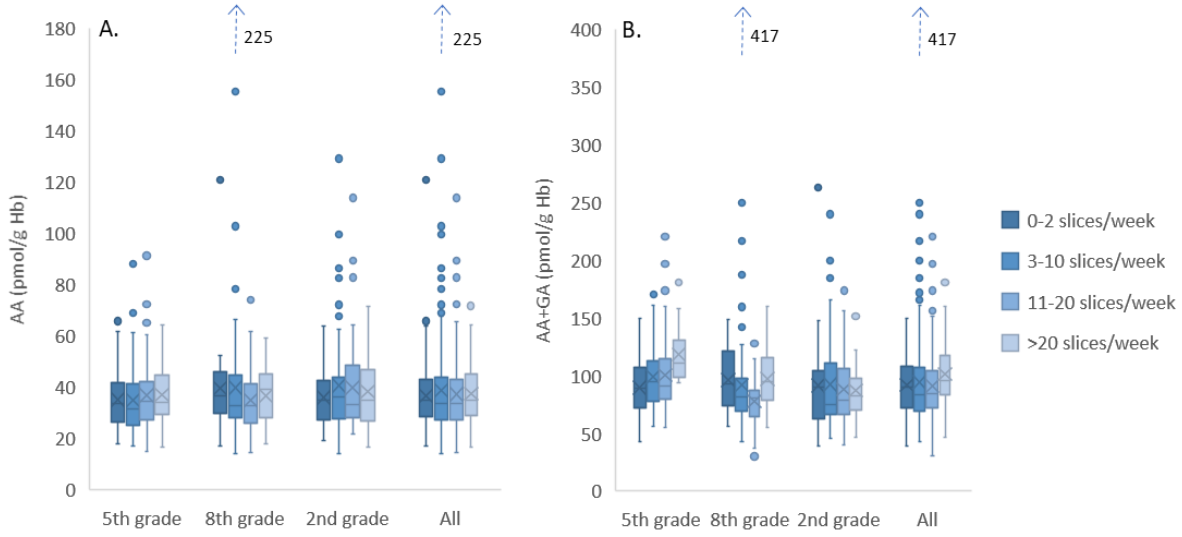


***Table 9.** Level of significance (p value) between different consumption frequency groups for crisps, fried potatoes and bread (crisp bread and soft bread) from food questionnaires, and the level of Hb adducts of acrylamide (AA) or the sum of acrylamide and glycidamide (AA+GA).*

	Crisps		**Fried potatoes**		**Bread**	
	AA	**AA+GA**	**AA**	**AA+GA**	**AA**	**AA+GA**
All students	0.6999	0.1663	0.3358	0.9952	0.1514	0.3698
5th grade[a]	**0.0137**	0.8448	0.1546	0.1970	0.4415	0.7203
8th grade[b]	0.3933	0.3705	0.4066	0.6974	0.6266	0.2728
2nd grade[c]	0.9693	0.0770	0.3807	0.9496	0.6069	0.9448

[a] 12 years, [b] 15 years, [c] 2nd grade of high school (18 years).

CONCLUSIONS

This study shows that the exposure to acrylamide, measured as hemoglobin (Hb) adducts, is widespread among Swedish adolescents, with a large variation in the blood levels of Hb adducts. Fried potatoes, crisps and bread are known major sources of exposure to acrylamide, and the consumption of these food categories was common among the studied students.

Although the dietary surveys were not designed to study acrylamide exposure specifically, associations between blood levels of Hb adducts and food consumption could be observed. A future dietary survey aiming at studying acrylamide exposure, such as e.g., a duplicate diet study in combination with biomonitoring, would be helpful to investigate true correlations, and to get a better estimation of the contribution of acrylamide from food to the overall cancer risk.

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APPENDIX 1

Determination of acrylamide and glycidamide in whole blood by HPLC-MS/MS

Department of Environmental Science, Stockholm University

General principles

Analysis was performed with the FI*R*E procedure. The principle of the method is the Edman degradation, a method developed from Pehr Edman for sequencing proteins from the N-terminal by using phenyl isothiocyanate and cycling the pH (Edman & Henschen, 1975). A modified Edman degradation method was developed by the research group at Stockholm University to measure adducts to the N-terminal valine in Hb as biomarkers of exposure to electrophiles. Initially the method used pentafluorophenyl isothiocyanate and analysis by GC-MS, and used samples of isolated globin (Törnqvist et al., 1986). Later, a method for LC-MS and whole blood was developed by using fluorescein isothiocyanate (FITC), which is called the FI*R*E procedure and it is the method used in the present study, presented in Figure A1 (von Stedingk et al., 2010).

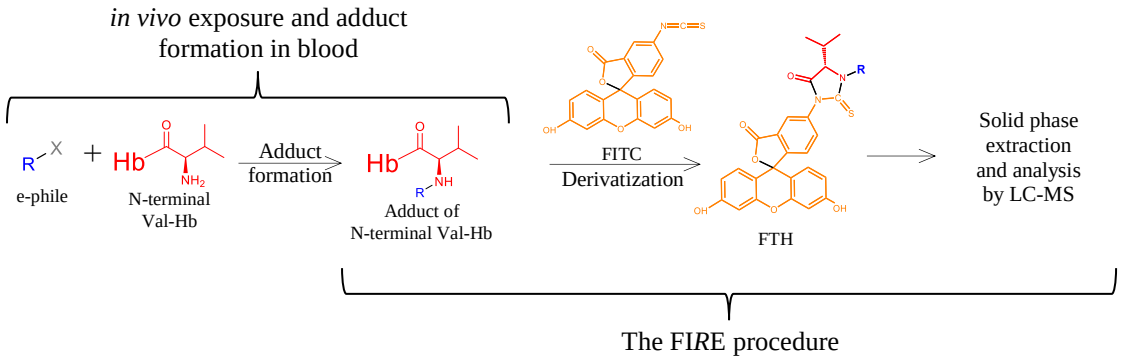

Figure A1. *Illustration of principal of adduct formation and the utility of the FIRE procedure for measuring exposure via the N-terminal Val of Hb as biomarker of exposure to electrophiles. E-phile is the electrophile that react with the N-terminal Valine, FITC is the reagent fluorescein isothiocyanate, and FTH is the resulting fluorescein thiohydantoin analytes.*

Chemicals

Internal standards

Internal standards of two types were used. Isotopically labelled (d_7 valine) FTHs for acrylamide (AA) and glycidamide (GA) adducts, and AAd_3 modified Hb in erythrocyte solution. The d_7 mixture used was at the concentration of 0.1 pmol/µL. All samples were spiked with 2 pmol during workup (20 µL × 0.1 pmol/µL). The AAd_3 incubated erythrocyte internal standard was earlier produced by incubation of human blood with AAd_3, followed by washing of erythrocytes and dilution to appropriate level for analysis. The actual concentration of this reference was diluted so that the added amount would produce analyte response at a level similar to the one in the lowest level quality control sample.

Calibration curve samples

The calibration curve (CC) was made with peptides for the valine (Val) adduct of acrylamide (N-(carbamoyl-ethyl)-Val-Leu-anilide; CAS:282725-67-1) and glycidamide (N-[(RS)-2-carbamoyl-2-hydroxy-ethyl]-Val-Leu-anilide; CAS: 1926163-77-0). A stock solution was prepared to contain approximately the same concentration between the two peptides (ca 82 pmol/µL=µM) and was further diluted according to Table A1. To create the samples for the calibration curve, 20 µL of each concentration was added to "Quality Control (QC) blood" of the same concentration. For each concentration level, three replicates were produced.

Table A1. dilution table for the concentration used in the analysis

	concentration of peptides (pmol/µl)
dil2	0.186
dil3	0.155
dil4	0.120
dil5	0.092
dil6	0.061
dil7	0.032
dil8	0.005

Methods

Sample preparation

Samples were randomly divided in batches of 96 deep well plates (DWP) containing nine Quality Control (QC) samples (triplicates at three different levels of AA) and up to 87 study samples from the students. Two different types of samples were prepared for each individual blood sample, one for the FI*R*E procedure and one for the sodium lauryl sulfate (SLS) method for determining the Hb concentration.

The FIRE procedure

The FI*R*E procedure was used for the analysis of the N-terminal Val adducts in Hb as fluorescein thiohydantoins (FTH) the by LC-MS (von Stedingk et al., 2010). In the present study, some modifications to the procedure were made to allow optimization of the workflow for 96 DWP.

To 150 µL whole blood samples, 20 µL 0.1 µM of isotopically substituted internal standards of AA(d_7)Val FTH and GA (d_7)Val FTH were added. Thereafter 100 µL of a solution containing AA(d_3)-modified Hb in 0.15 M phosphate buffer (disodium hydrogen phosphate:sodium dihydrogen phosphate; 9:1; pH ca 7.8) was added. Additionally, 100 µL water was added to the samples before

mixing in a shaker for one min at 1500 rpm. The samples were derivatized for 16 h at 750 rpm and 37°C with 3 mg FITC in 18 µL dimethyl sulfoxide.

After derivatization, 50 µL methanol was added to the samples and then mixed for one min at 1500 rpm. The samples were then transferred to a protein precipitation filter plate (Isolute PPT+, Biotage, Sweden) that was pre-loaded with 1.5 mL acetonitrile as crash solvent. The filtrate of the samples was collected in a 2 mL DWP and to that an amount of 15 µL 1 M NH_4OH was added prior loading on Oasis MAX 96-well (Waters, Sweden) solid phase extraction plate that was preconditioned with 0.01 M NH_4OH. The samples were then washed with 1 mL acetonitrile, 1 mL water and 1 mL 0.5 % cyanoacetic acid in water. The samples were then eluted with 0.8 mL 0.25 % cyanoacetic acid in 80 % acetonitrile in 1 mL 96 DWP collection plates, followed by overnight evaporation in a vacuum centrifuge. Prior LC-MS analysis, the samples were reconstituted in 100 µL 40% acetonitrile in water.

Analysis of the samples by LC-MS/MS was performed on an instrument consisting of Waters Acquity H-class UPLC coupled to a Xevo TQS-µ MS (Waters Corporation, Milford, MA, USA). The analytical column used was an ACE Excel UHPLC C18-PFP column (100 mm x 2.1 mm, 1.7 µm). Elution proceeded at 0.35 mL/min and the column temperature was kept at 40 °C. Mobile phases composed of A) 10% acetonitrile in water with 0.1% formic acid, and B) 90% acetonitrile in water with 0.1% formic acid.

Sodium lauryl sulfate method for determining hemoglobin concentration

To measure the concentration of Hb in the samples, the SLS method for determining Hb concentration, developed by Oshiro et al., 1982, was used with modifications. In short, an SLS stock solution was made by dissolving 3.0 g of SLS in 50 mL water containing 1 mL 1 M disodium hydrogen phosphate solution and 0.5 mL 1 M sodium dihydrogen phosphate solution. The SLS stock solution was then diluted 100x in water and the resulting SLS working solution was used to dilute 10 µL of whole blood cohort samples 151x in a 2 mL deep well plate. The SLS blood samples were then mixed and 300 µL were transferred in a microplate, where the optical density of the samples was measured at 538 nm in a SpectraMax iD3 microplate reader (Molecular devices, USA). To estimate the Hb concentration of the cohort samples, erythrocyte solutions of known Hb concentration were run at the same occasion.

Quantification

Calculation of adduct level

To calculate adduct levels in the samples firstly the relative response of the analyte from the FI*R*E procedure was normalised in the samples by dividing the area response of the analyte to the area response of the internal standard. Thereafter, the response was corrected for batch deviations to the

batch of the calibration curve (see also the section on Calculation of the batch correction factors). Then the batch corrected relative response of the analyte was used and the concentration of the analyte in the sample was calculated by dividing it with the calibration curve slope, resulting in the amount of analyte in the sample (pmol/sample). Lastly, the total mass of hemoglobin in g (estimated from the SLS Hb concentration method) was used to calculate the adduct level in pmol/ g Hb.

Calculation of the batch correction factors

To account for batch variability, correction factors were applied for different batches. This batch correction factor was calculated according to the following formula:

$$batch\ correction\ factor\ for\ in\ batch\ x = \frac{\left(\frac{mean\ x_{QC\ low}}{mean\ CC_{QC\ low}} + \frac{mean\ x_{QC\ medium}}{mean\ CC_{QC\ medium}} + \frac{mean\ x_{QC\ high}}{mean\ CC_{QC\ high}}\right)}{n_{QC\ levels}}$$

Where x, refers to a batch number; mean, refers to the relative response mean of samples on the same QC level; n refers to the number of different QC levels.

Evaluation of the method

Precision

The intraday precision of the analysis, measured as the mean coefficient of variation per batch of the uncorrected relative response of the AA analyte on the three QC levels was on average 13 %.

The interday precision of the analysis, measured as the coefficient of variation of the uncorrected relative response of the AA analyte was measured to be, QC low=21 %; QC medium=17 %; QC high=21 %)

Limits of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were not determined for this method. This is largely due to the lack of a representative blank matrix (blood sample with no analyte present). All blood samples of tested human origin had background adduct levels. This means that the preferred method for estimating LOD/LOQ by spiking small amounts in the same matrix to measure LOD/LOQ was not feasible. Animal blood was considered as an alternative matrix. However, preliminary tests of comparison of animal blood (ovine) with human blood spiked with the peptide standards revealed that the slope (spike-response curve) varied substantially between species (data not shown), and therefore the use of ovine blood as the matrix alternative was deemed improper, even though a background AA adduct level in ovine blood not being detectable with the current instrumentation.

The instrumental method detection limit was determined by repeated injections of FTH standard for AA at a response levels very close to LOD (S/N=3.5-7.4) and calculation of the instrumental LOD according to the following equation:

$$\text{instrumental LOD} = \frac{(t_{crit}) \times (RSD\%\ of\ injections) \times (amount\ STD\ injected)}{100\%}$$

Where LOD, refers to the limit of detection; t_{crit}, refers to the critical t distribution critical value (at 99% level); STD, refers to standard; RSD refers to the relative standard deviation in percent.

See also Agilent Technologies (2011), which was the source of the formula used for the calculation of the instrumental LOD.

The instrumental LOD is calculated to be 0.3 fmol on column of AA-Val-FTH by this method. Assuming this is from a sample with initial Hb concentration of 140 mg/ml (the mean Hb concentration observed in all samples), then the adduct level would equate to 0.5 pmol/g Hb.

However, this measurement should be viewed with caution as the FTH standard lacks the matrix effects and background noise from the matrix found in real samples.

Another way for estimating roughly the limit of quantification for the method is by considering the mean S/N of the analyte in the samples with the 10 lowest adduct levels. The mean S/N is 54 and the mean adduct level is 16 pmol/g Hb. LOQ (S/N=10) is equal to 3 pmol/g Hb when extrapolating from the relationship (16÷54×10). However, this value should also be viewed with caution. In reality, there is no direct relationship between adduct level and S/N.

References for analytical method

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