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Analysis of Acrylamide, a Carcinogen Formed in Heated Foodstuffs

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Reaction products (adducts) of acrylamide with N termini of hemoglobin (Hb) are regularly observed in persons without known exposure. The average Hb adduct level measured in Swedish adults is preliminarily estimated to correspond to a daily intake approaching 100 μg of acrylamide. Because this uptake rate could be associated with a considerable cancer risk, it was considered important to identify its origin. It was hypothesized that acrylamide was formed at elevated temperatures in cooking, which was indicated in earlier studies of rats fed fried animal feed. This paper reports the analysis of acrylamide formed during heating of different human foodstuffs. Acrylamide levels in foodstuffs were analyzed by an improved gas chromatographic–mass spectrometric (GC-MS) method after bromination of acrylamide and by a new method for measurement of the underivatized acrylamide by liquid chromatography–mass spectrometry (LC-MS), using the MS/MS mode. For both methods the reproducibility, given as coefficient of variation, was ~5%, and the recovery close to 100%. For the GC-MS method the achieved detection level of acrylamide was 5 μg/kg and for the LC-MS/MS method, 10 μg/kg. The analytic values obtained with the LC-MS/MS method were 0.99 (0.95–1.04; 95% confidence interval) of the GC-MS values. The LC-MS/MS method is simpler and preferable for most routine analyses. Taken together, the various analytic data should be considered as proof of the identity of acrylamide. Studies with laboratory-heated foods revealed a temperature dependence of acrylamide formation. Moderate levels of acrylamide (5–50 μg/kg) were measured in heated protein-rich foods and higher contents (150–4000 μg/kg) in carbohydrate-rich foods, such as potato, beetroot, and also certain heated commercial potato products and crispbread. Acrylamide could not be detected in unheated control or boiled foods (<5 μg/kg). Consumption habits indicate that the acrylamide levels in the studied heated foods could lead to a daily intake of a few tens of micrograms.

KEYWORDS: Acrylamide; analysis; mass spectrometry; cooking; food; carcinogen

INTRODUCTION

In studies aimed at the identification of the causes of background carcinogenesis, acrylamide has emerged as a factor that could be associated with a considerable cancer risk (*1*). Studies of hemoglobin (Hb) adducts by mass spectrometric (MS) methods have revealed background exposures to many reactive, probably mutagenic and carcinogenic, compounds in humans (*2–4*). For instance, a "background signal", which corresponds to the Hb adduct to N-terminal valine from acrylamide, *N*-(2-carbamoylethyl)valine, has been regularly observed in unexposed control persons (*5, 6*). In connection with studies of occupational exposure to acrylamide this background level was actualized (*1, 6*). The background levels observed in Swedish adults indicate an average daily intake of acrylamide that approaches 100 μg, which could correspond to a non-negligible cancer risk to the general population (*1*). This observation called

for further studies to provide incontestable proof that the origin of the Hb adduct is acrylamide and, if so, to determine acrylamide sources and mechanisms of formation, as well as an evaluation of the associated cancer risk.

The occurrence of acrylamide in tobacco smoke (*7*), which could be observed in smokers as an increased level of the corresponding Hb adduct (*5*), indicated that acrylamide is formed during incomplete combustion or heating of organic matter. Furthermore, lower background levels of this Hb adduct were observed in wild animals when compared to humans and laboratory animals (to be published), thought to be due to intake of unheated food in wild animals. A hypothesis that acrylamide is formed in cooking was confirmed in animal experiments by verification of the identity of the acrylamide adduct in Hb by comprehensive MS/MS analysis and the demonstration that the increased adduct levels were compatible with expectation from the contents of acrylamide determined in fried feed (*8*). The present paper concerns the analysis of acrylamide in certain human foodstuffs heated in cooking or manufacturing and the development of improved methodology for the analysis of acrylamide in foodstuffs.

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EXPERIMENTAL PROCEDURES

Chemicals. ($^{13}C_3$)Acrylamide (99%) was obtained from CIL (Andover, MA); acrylamide (99+%) and *N,N*-dimethylacrylamide were obtained from Sigma-Aldrich (Stockholm, Sweden). Bromine (≥99.5%, p.a.), hydrobromic acid (48%, p.a.), potassium bromide (≥99%), sodium thiosulfate pentahydrate (≥99.5%), and sodium sulfate anhydrous (≥99%) were obtained from Merck (Darmstadt, Germany). All other solvents and chemicals used for the analysis of acrylamide were of analytical grade.

CAUTION: Acrylamide, ($^{13}C_3$)acrylamide, and *N,N*-dimethylacrylamide are hazardous and must be handled carefully.

Analytical Instruments. The quantification of acrylamide in food was performed on a Hewlett-Packard (HP) 5890 gas chromatograph (GC) coupled to an HP 5989A quadrupole mass spectrometer (MS). The routine GC column was a BPX-10 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; SGE, Ringwood, Australia); similar columns were also used. Confirmatory analyses of acrylamide were also made by liquid chromatography−tandem mass spectrometry (LC-MS/MS) with electrospray positive ionization (ESI+) using a Micromass Quattro Ultima coupled to an Agilent 1100 HPLC (1100 binary pump, 1100 micro vacuum degasser, 1100 thermostated autosampler, 1100 thermostated column compartment) with a Hypercarb column (50 × 2.1 mm, 5 μm; ThermoHypersil) together with or without a precolumn Hypercarb Guard (10 × 2 mm; ThermoHypersil).

Analysis by GC-MS of Acrylamide in Foodstuffs. The analysis of acrylamide by GC-MS was performed with a simplified version of the method previously applied for the analysis of laboratory animal feed as the sample matrix (*8*), using brominated acrylamide as the analyte (*9, 10*).

The preparation of samples for analysis involved mixing (with a Waring 700 G blender) of 10 g of sample with water (100 mL), followed by filtration (through a Sartorius glass-fiber filter; article no. 13400-90-S; Göttingen, Germany) and purification of the filtrate on a graphitized carbon black column (Carbograph 4; 7 mm × 12 mm i.d, 1 g of carbon; LARA S.r.l., Rome, Italy). The internal standard, *N,N*-dimethylacrylamide (500 μL from a stock solution in water), was added (see also comment on new internal standard below).

The samples were derivatized through bromination by using potassium bromide (7.5 g), hydrobromic acid (acidification to pH 1−3), and saturated bromine water (10 mL) according to the method of Castle et al. (*9, 10*). The sample was kept at 4 °C overnight, and the excess bromine was decomposed by adding sodium thiosulfate (1 M) as drops until the yellow color disappeared. Sodium sulfate (15 g) was added, and the solution was extracted with ethyl acetate/hexane [2 × 20 mL, 1:4 (v/v)]. The two pooled organic fractions were dried and evaporated with a rotary evaporator to ∼200 μL.

The modifications in sample preparation in the present study compared to the earlier method (*8*) are that final purification by filtration and gel permeation chromatography (GPC) has been excluded. Furthermore, during the course of the work (after initial studies on hamburger meat) the internal standard, *N,N*-dimethylacrylamide, was replaced with ($^{13}C_3$)acrylamide, to improve the accuracy and reproducibility of measurements. The new internal standard (1 mL of a stock solution of 1 μg/mL of water) was added to the samples, at the initial step, simultaneously with the water, in the workup procedure.

Two microliters of the samples was injected splitless, injector temperature of 250 °C, on the GC-MS for analysis. The temperature program for the GC was as follows: isothermal for 1 min at 65 °C, increased at a rate of 15 °C/min to 250 °C, and isothermal for 10 min. The analysis was performed using electron ionization (70 eV) and selected ion monitoring. The ions monitored for identification of the analyte, 2,3-dibromopropionamide, were [$C_3H_5{}^{81}BrNO$]$^+$ = 152 (100%), [$C_3H_5{}^{79}BrNO$]$^+$ = 150 (100%), and [$C_2H_3{}^{79}Br$]$^+$ = 106 (65−70%) using *m/z* 150 for quantification. The ions monitored for identification of the internal standard, brominated to 2,3-dibromo($^{13}C_3$)propionamide, were [$^{13}C_3H_5{}^{81}BrNO$]$^+$ = 155 (100%) and [$^{13}C_2H_3{}^{81}Br$]$^+$ = 110 (65−70%) using *m/z* 155 for quantification (a variation of ±10% in ratio between fragment ions is allowed for identification). The ions monitored for identification and quantification when using *N,N*-dimethylacryla-mide, brominated to 2,3-dibromo-*N,N*-dimethylpropionamide, as an internal standard were [$C_5H_9{}^{81}BrNO$]$^+$ = 180 and [$C_5H_9{}^{79}BrNO$]$^+$ = 178.

Quantification was performed by comparison with a calibration curve (0.5−50 μg/L water, corresponding to 5−500 μg/kg). Samples with concentrations >500 μg/kg of acrylamide were diluted up to a factor of 6 in the first step when food was mixed with water. Recovery tests were repeatedly performed by quantification of acrylamide in different (both raw and heated) foodstuffs before and after the addition of acrylamide.

Analysis by LC-MS/MS of Acrylamide in Foodstuffs. The samples were homogenized, and 100 mL of water and 1 mL of the internal standard, ($^{13}C_3$)acrylamide (1 μg/mL of water), were added to 10.0 g of the homogenized sample. The samples were centrifuged in 12 mL Pyrex glass tubes, and the particle-free supernatant was further centrifuged (at 14000 rpm for 10 min) in two Eppendorf tubes (1.5 mL/tube). An Isolute Multi-Mode SPE column (300 mg; International Sorbent Technology Ltd.), activated with acetonitrile (1 mL) and washed with water (2 + 2 mL), was used to trap nonpolar interferences by adsorption in the recombined supernatant (3 mL). The first milliliter of the filtrate was discarded and the rest passed through a syringe filter (0.45 μm; Sartorius minisart hydrophilic, article no. 17598). Five hundred microliters was ultrafiltered (Microcon YM-3, article no. 42404, Millipore) in an Eppendorf centrifuge (14000 rpm, 10 min) until 200 μL had passed through.

The samples were analyzed by LC-MS/MS (ESI+) (at ambient temperature) using the column given above, using deionized water as mobile phase, with a flow rate of 0.2 mL/min for 6.1 min (analytes recorded), washing with 80% aqueous acetonitrile (4 min at a flow rate of 0.4 mL/min), followed by reconditioning with water (0.2 mL/min, 10 min) between sample injections (20 μL). The electrospray source had the following settings (with nitrogen): capillary voltage, 3.2 kV; cone voltage, 50 V; source temperature, 125 °C; desolvation temperature, 350 °C; cone gas flow, 211 L/h; and desolvation gas flow, 653 L/h. Argon (2.5 mbar) was used as collision gas.

Acrylamide was identified by multiple reaction monitoring (MRM). The precursor ion [$M + H$]$^+$ = 72 was fragmented, and product ions [$H_2C=CH−C=NH$]$^+$ = 54 (collision energy = 16 eV) and [$H_2C=CH−C=O$]$^+$ = 55 (collision energy = 11 eV) were monitored (ratio between product ions 1:35 ± 20%). The ion *m/z* 55 was used for quantification. Monitored product ion for the internal standard was [$^{13}C_3H_3O$]$^+$ = 58 from precursor ion [$M + H$]$^+$ = 75. The collision energy was 11 eV. For all MRM transitions the dwell time was 0.3 s with the cone voltage given above. Product ion spectra of acrylamide and analyte were compared at collision energies of 10 and 20 eV, respectively.

Quantification was performed through comparison with a calibration curve with five concentrations (1−500 μg/L water, corresponding to 10−5000 μg/kg). The repeatability, recovery, and reproducibility of the LC-MS/MS method were evaluated, and the method was compared with the GC-MS method.

Laboratory Cooking. Heating under laboratory conditions by frying−searing was carried out in a thermostated iron frypan (*11*). The temperature in the frypan and in the browning zone of food during heating was controlled with a CIE model 307 digital thermometer, connected with a probe (accuracy ±2.2 °C; Clas Ohlson, Stockholm, Sweden). Heating in a microwave oven was performed with a Whirlpool model MD 121, equipped with a rotating plate, at 750 W (nominal power). Heating in the oven during controlled conditions was performed in a temperature-programmed GC oven (Hewlett-Packard model 5790A). The homogenization of potato was performed with a Philips chopper, model HR 1392.

All foodstuffs used in the cooking experiments were obtained from grocery stores. In the experiments minced or grated foodstuff formed into 9 cm diameter patties of ∼60 g portions was used, unless otherwise stated. After cooking, the samples were immediately frozen (−20 °C) prior to analysis. The samples were sent from Stockholm to Lidköping in dry ice for analysis of acrylamide content.

Influence of Temperature and Time on Acrylamide Formation during Heating of Beef by Frying. Patties of beef (commercially available as frozen hamburgers) were fried (without addition of oil) at

temperatures of 160, 180, 200, 220, and 240 °C for 3 min on each side. Two raw patties were used as controls, and two samples were prepared at each of the temperatures 160, 200, and 240 °C. In addition, the influence of heating time was studied by heating single samples of lean minced beef for 1 or 4 min on each side at 200 °C. In the first experiments the temperature was also controlled with a digital thermometer, connected with a probe.

Comparison of Different Foodstuffs with Regard to Acrylamide Formation during Heating by Frying. In one experiment the influence of protein source on the formation of acrylamide was studied in protein-rich foods. Duplicate samples of fish fillet (cod), lean beef, lean pork (one sample lost), chicken fillet, and soy flour (soaked in water) were heated. In another experiment grated potato ($n = 2$), grated beetroot ($n = 2$), and spinach ($n = 1$) were studied as examples of carbohydrate-rich foods. In both experiments the foods were heated in a frypan for 2.5 min on each side at 220 °C, without addition of oil.

Influence of Cooking Method on Acrylamide Formation. The influence of the cooking method with regard to acrylamide formation was studied by comparing heating in a frypan, boiling, and microwave heating. Lean beef ($n = 3$), minced fish (cod) ($n = 1$), grated potatoes ($n = 3$), and boiled potato mashed ($n = 2$) were heated for 2.5 min on each side in a frypan at 220 °C (without addition of oil). Patties of minced fish (cod) ($n = 2$), lean minced beef ($n = 4$), and grated potatoes ($n = 4$) were boiled (for up to 20 min). Broth from boiling of lean beef, cod, and potatoes was sampled for analysis ($n = 3 + 2 + 4$). Minced fish (cod) ($n = 2$) and grated potato ($n = 2$) were heated in a microwave oven for 3 min on both sides at 750 W (nominally). Raw potato was analyzed as control sample. (Samples of raw beef were analyzed in the first experiment, described above.)

Influence of Time on Acrylamide Formation at Microwave Heating of Potato. The influence of the duration of microwave heating on acrylamide formation in potato was studied. Potato ($\sim$150 g) was peeled, homogenized, and subdivided into smaller 30.0 g portions in Erlenmeyer flasks (250 mL). The samples were heated in a microwave oven (750 W nominally) for 50, 100, and 150 s, followed by weighing for determination of weight loss, mainly water. As a control a nonheated, homogenized portion was used.

Influence of Temperature on Acrylamide Formation in Oven-Heated Potato. Commercially available French-fried potatoes, prepared for heating in home ovens (recommended heating time at 225 °C = 25−30 min), were heated in a temperature-programmed GC oven. Fries of about equal size and length were selected to make 50.0 g samples. The samples of the frozen French fries were then heated in a glass bowl using the following temperature program for the oven: 60 °C for 1 min and then 50 °C/min until the final temperature was reached (100−220 °C). The time period of maximal heating temperature was adjusted (19−14.2 min) to obtain about the same total heating time (21 min). The samples were then cooled to 60 °C, weighed, and stored at −20 °C until analysis. The French fries heated at 180 and 200 °C were browned as normally served.

Acrylamide Contents in Food Items from Restaurants, Etc. Potato products and other foodstuffs and products were obtained from restaurants or from grocery stores, and samples were taken and analyzed (the same day as purchased) for comparison with acrylamide content in the laboratory-prepared foodstuffs. Details of the prepurchase processing were not available, and variations with respect to composition and cooking etc. method were evident. Also, for evaluation of analytical methods commercial foodstuffs were used for analysis.

RESULTS AND DISCUSSION

Methods for Analysis of Acrylamide. The analyses of acrylamide were first performed by GC-MS following bromination, and later the contents of acrylamide were confirmed, without derivatization, by LC-MS/MS. The methods using bromination of acrylamide (*9*) and GC-MS analysis are similar to the U.S. EPA method for analysis of acrylamide in water (*10*). This method has been used in our laboratory for analysis of acrylamide in water (*12*) and, after further development, for analysis of laboratory animal feed as sample matrix (*8*). In the present work the modified GC-MS method used for animal feed
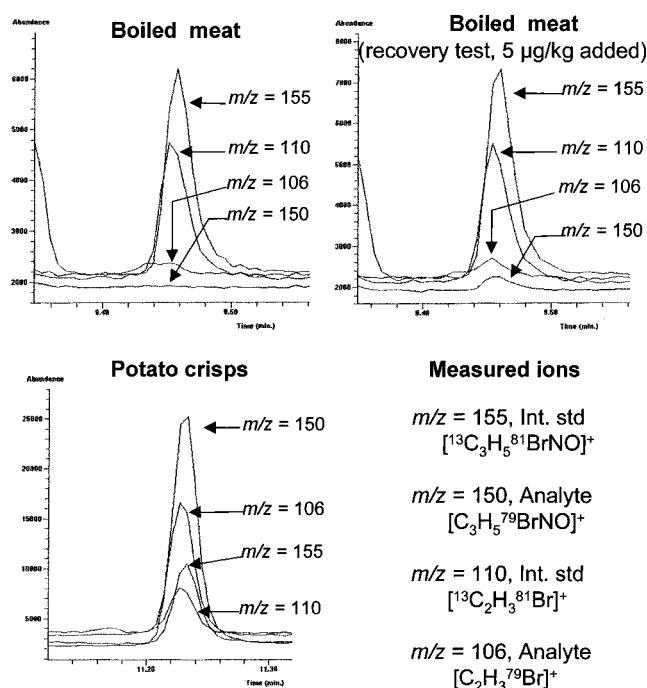

Figure 1. Analysis of acrylamide content in laboratory-boiled minced beef prior to and after addition of acrylamide and of potato crisps with high content of acrylamide; GC-MS (EI) chromatograms of four selected ions from the analyte of acrylamide (2,3-dibromopropionamide) and from the internal standard [($^{13}C_3$)acrylamide] after bromination of the samples (variation in retention times is due to use of different GC columns, PAS 1701 and BPX-10, respectively). In the figure *m/z* 152 is omitted because of contribution from the internal standard at low levels.

(*8*) was further simplified by excluding the GPC cleanup of the extract, which mainly had the aim of removing fat from the sample and preventing deposits in the ion source. The reproducibility was improved by changing the internal standard to ^{13}C-substituted acrylamide and by addition of the internal standard earlier in the workup procedure.

The calibration curve was linear in the range studied (5−500 μg/kg). In recovery tests with addition of acrylamide (5−1000 μg/kg) to different foods containing <5−>2000 μg/kg of "natural" acrylamide, the recovery with the original internal standard was 98% (SD = 26%) and with the new internal standard, 98% (SD = 7.5%). The coefficient of variation (CV) for the GC-MS method using the original internal standard is $\sim$15% in the range of 5−100 μg/kg and above this range, $\sim$10%. The reproducibility of the method was improved with the new internal standard to $\sim$5% in the analytical range. The limit of detection was 5 μg/kg. **Figure 1** illustrates analyses with the improved GC-MS method of samples with high and low contents of acrylamide (using different GC columns).

An LC-MS/MS method was developed to achieve confirmation by an independent method not involving derivatization of acrylamide (**Figure 2**). The calibration curve was linear in the range of 10−5000 μg/kg ($r^2 > 0.9999$). The repeatability, recovery, and reproducibility obtained with the method are shown in **Tables 1−3**, respectively. The repeatability (**Table 1**) of measurements was determined on samples with acrylamide contents in the range of 40−1700 μg/kg, with CV of $\sim$5% (2−9%). The recovery (**Table 2**) was determined as 99.5% (SD = 6.5%). The reproducibility (**Table 3**) was determined as CV to be in the order of 5% (2−9%). The detection level was $\sim$10 μg/kg for the samples in the present study but may vary depending on the matrix. The LC-MS/MS method was com-

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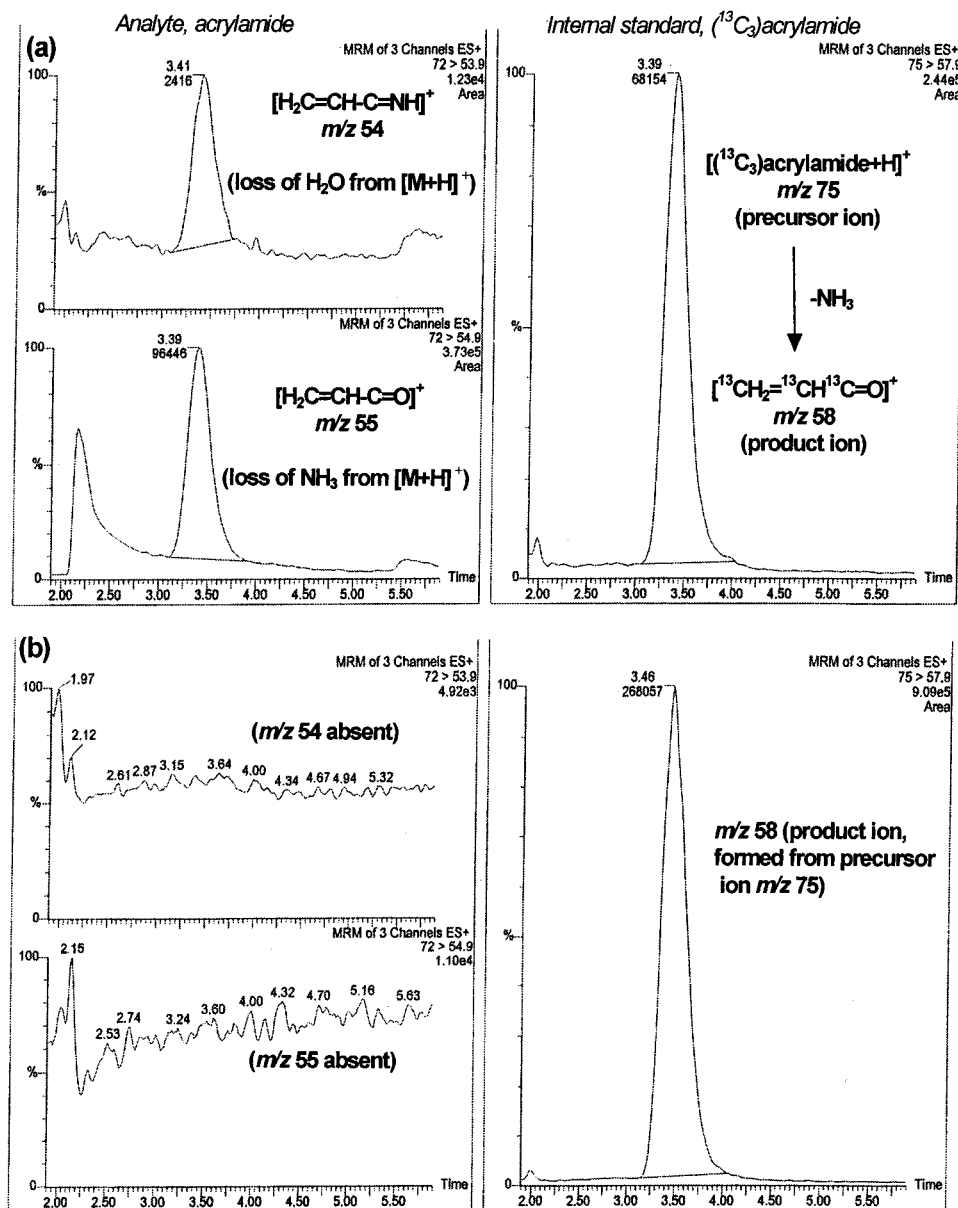*J. Agric. Food Chem.,* Vol. 50, No. 17, 2002 **5001**

Figure 2. LC-MS/MS analysis of acrylamide in French-fried potatoes (a) by monitoring the precursor ion *m/z* 72 and product ions *m/z* 55 and 54, quantified with the internal standard precursor ion *m/z* 75 and product ion *m/z* 58. (b) Analysis of a blank sample.

Table 1. Repeatability Test of the LC-MS/MS Method[a]

	sample	acrylamide (μg/kg)	mean (μg/kg)	CV (%)
I	bread	35, 33, 41, 39, 39	37	8.9
II	bread	46, 49, 51, 51, 47	49	4.7
III	French fries	446, 451, 416, 421, 388	424	6.0
IV	potato crisp	1584, 1523, 1530, 1362, 1479	1496	5.6
V	bread	1704, 1715, 1741, 1765	1731	1.6

[a] Each food sample was split into five aliquots prior to homogenization, workup, and analyses during one day and with the same laboratory staff.

Table 2. Examples of Recovery Tests of LC-MS/MS Method (Samples IV and V, Replicate Analysis)[a]

	sample	before addition (μg/kg)	added amount (μg/kg)	after addition (μg/kg)	recovery (%)
I	rye	3.3	10	13	97
II	oat	7.3	10	17	97
III	bread	37	50	93	112
IV	French fries	424	600	1036, 970	102, 91
V	potato crisps	1496	1500	2978, 2899	99, 94
VI	potato crisps	1678	1000	2716	104

[a] Percent recovery of amount acrylamide added to samples with analyzed acrylamide contents.

pared with the GC-MS method with ($^{13}C_3$)acrylamide as internal standard (**Table 4**). By orthogonal regression of log-transformed values, assuming the same CV, LC-MS/MS values were found to be 0.99 [0.95−1.04; 95% confidence interval (CI)] of the GC-MS values. The LC-MS/MS method is simpler than the GC-MS method and will be the method of choice for routine analysis of acrylamide. Both methods have been accredited (according to ISO 17025; *13*). It may be mentioned that an LC-MS/MS method for analysis of acrylamide in foodstuffs has also been developed by the Swedish National Food Administration (NFA) (*34*).

CV for duplicate samples in the complete study was up to 20% (with respect to, e.g., frying and sample workup, with analysis on the same occasion by the same method). A high

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Table 3. Reproducibility Test of the LC-MS/MS Method[a]

	sample	analysis I (μg/kg)	analysis II (μg/kg)	analysis III (μg/kg)	CV (%)
I	bread	37	41	44	8.6
II	French fries	424	434		1.6
III	potato crisps	1496	1544		2.2
IV	crispbread	1731	1564		7.2

[a] An aliquot of the food samples was taken out on different days, homogenized, and analyzed by different laboratory staff, on different occasions.

Table 4. Comparison Test of the LC-MS/MS Method versus the GC-MS Method, Both Methods Using ($^{13}C_3$)Acrylamide as Internal Standard

	sample	GC-MS (μg/kg)	LC-MS/MS (μg/kg)	difference (%)
I	hamburger	14	14	0
II	hamburger	23	23	0
III	French fries	661	684	3.5
IV	potato crisps	1538	1544	0.39
V	potato crisps	1800	1678	−6.8

stability of acrylamide in stored samples was indicated in the repeated analysis of fried potato stored for 1 year in the freezer.

Identification of Acrylamide. The identity of acrylamide is supported as follows: In the various foodstuff matrices acrylamide was determined by two different procedures for workup, chromatography, and detection as described under Experimental Procedures. Whereas the procedure for GC-MS analysis involves bromination at low pH and analysis of brominated samples at high temperatures, the procedure for analysis by LC-MS/MS is more lenient with respect to acidity and temperature. In the procedure for GC-MS analysis acrylamide is derivatized to 2,3-dibromopropionamide by bromination of the ethylenic double bond according to well-known procedures (*9, 10*). Analysis by LC-MS/MS involves direct determination of underivatized acrylamide. In both methods ($^{13}C_3$)acrylamide was used as internal standard and measured as brominated derivative or without derivatization, respectively. When applied to the same samples the two methods despite the differences in the methodology gave concordant results (cf. **Table 4**).

The analysis of acrylamide was performed on four different GC columns and two different LC columns (**Table 5**). In all separations the analytes exhibited the same retention times as the corresponding internal and external standards.

Analysis by an MS/MS method (monitoring of product ions of a precursor ion, MRM) used for the underivatized acrylamide in this study gives stronger evidence of identity than MS analysis only. The acrylamide content in potato chips was verified by recording product ion spectra in LC-MS/MS analysis. The spectra are identical for the standard and the analyte, at 10 and 20 eV, respectively (**Figure 3**). This is a strong support for the identity of the analyte. In addition, further support for the identity is that several ions are monitored for the analyte in both the GC-MS analysis and the LC-MS/MS analysis and that the relative ion abundances are the same for the analyte as for the standard. With the aid of the ^{13}C-substituted internal standard the product ion spectrum in the LC-MS/MS analysis as well as the mass spectrum in GC/MS was interpreted (see Experimental Procedures).

Studies on alternative techniques for identification will be pursued.

Quantities of Acrylamide Formed during Cooking of Different Foodstuffs. Figure 4 shows measured acrylamide

Table 5. Retention Times Obtained for 2,3-Dibromopropionamide and Acrylamide with Different Columns by the GC-MS and LC-MS/MS Methods, Respectively[a]

GC column[b]	length (m)	i.d. (mm)	film thickness (μm)	retention time[c] (min)
SE30	25	0.32	0.5	7.3
PAS1701	25	0.32	0.25	9.5
DB-1701P	30	0.32	0.25	10.0
BPX-10[d]	30	0.25	0.25	11.2

LC column	length (mm)	i.d. (mm)	chromatographic conditions	retention time[e] (min)
Shodex Rspak DE-413	150	4.6	eluent, acetic acid in water at pH 2.6; flow = 1.0 mL/min	4.0
Hypercarb[d]	50	2.1	eluent, water (without buffer); flow = 0.2 mL/min	3.5

[a] Stated retention times are approximate values. In all cases the analyte coeluted with the chosen internal standard. [b] The used chromatographic conditions were equivalent for the different columns and the same as described under Experimental Procedures. [c] The retention time of the analyte, 2,3-dibromopropionamide, and that of the brominated internal standard, ($^{13}C_3$)2,3-dibromopropionamide, were identical. [d] Column used for measurements in the present study. [e] The retention time of acrylamide and that of the internal standard, ($^{13}C_3$)acrylamide, were identical. Retention time for column without precolumn given.

levels (micrograms per kilogram of heated product) in fried hamburgers from the initial experiment with frying of hamburger meat at different temperatures. A significant dependence of acrylamide formation on temperature was demonstrated. In raw hamburgers the acrylamide content was below the detection level (5 μg/kg). A dependence of content of acrylamide on frying time was also indicated (data not shown).

In subsequent experiments different types of foodstuffs heated via cooking methods were studied with respect to the formation of acrylamide in the heated foodstuffs. The level of acrylamide was below the detection level (5 μg/kg) in boiled or raw beef and potato, in boiled fish, as well as in broth from the boiling tests. Following controlled heating, protein-rich foods exhibited acrylamide concentrations between 5 and 50 μg/kg, with lower levels in fish. In the experiments with heating of carbohydrate-rich foods, relatively higher contents of acrylamide, 150−1000 μg/kg, were measured. Following microwave-heating of fish and potato, no detectable levels of acrylamide were found in fish. Detailed data are given in **Table 6** and illustrated in **Figure 5**.

These laboratory experiments were followed by studies of acrylamide contents in commercially prepared foodstuffs purchased from restaurants or grocery stores. Analysis of the selected foods, mainly potato products, gave results compatible with those obtained after cooking under laboratory conditions (**Figure 5**; **Table 6**). French-fried potatoes and potato crisps exhibited relatively high levels of acrylamide [median values of 424 μg/kg (*n* = 5) and 1739 μg/kg (*n* = 6), respectively]. Large variations of acrylamide levels in similar foods were observed and could also be expected when prepared commercially, because of differences in heating time, etc. This was observed, particularly for French fries and potato crisps. There are also variations between samples of fried potatoes. It should be noted that the commercially fried potato pancake contained not only grated potato (as in the laboratory frying) but also is mixed with egg, milk, and flour, which might have had an influence on acrylamide formation during heating. Boiling of potatoes prior to frying seemed to reduce the formation of
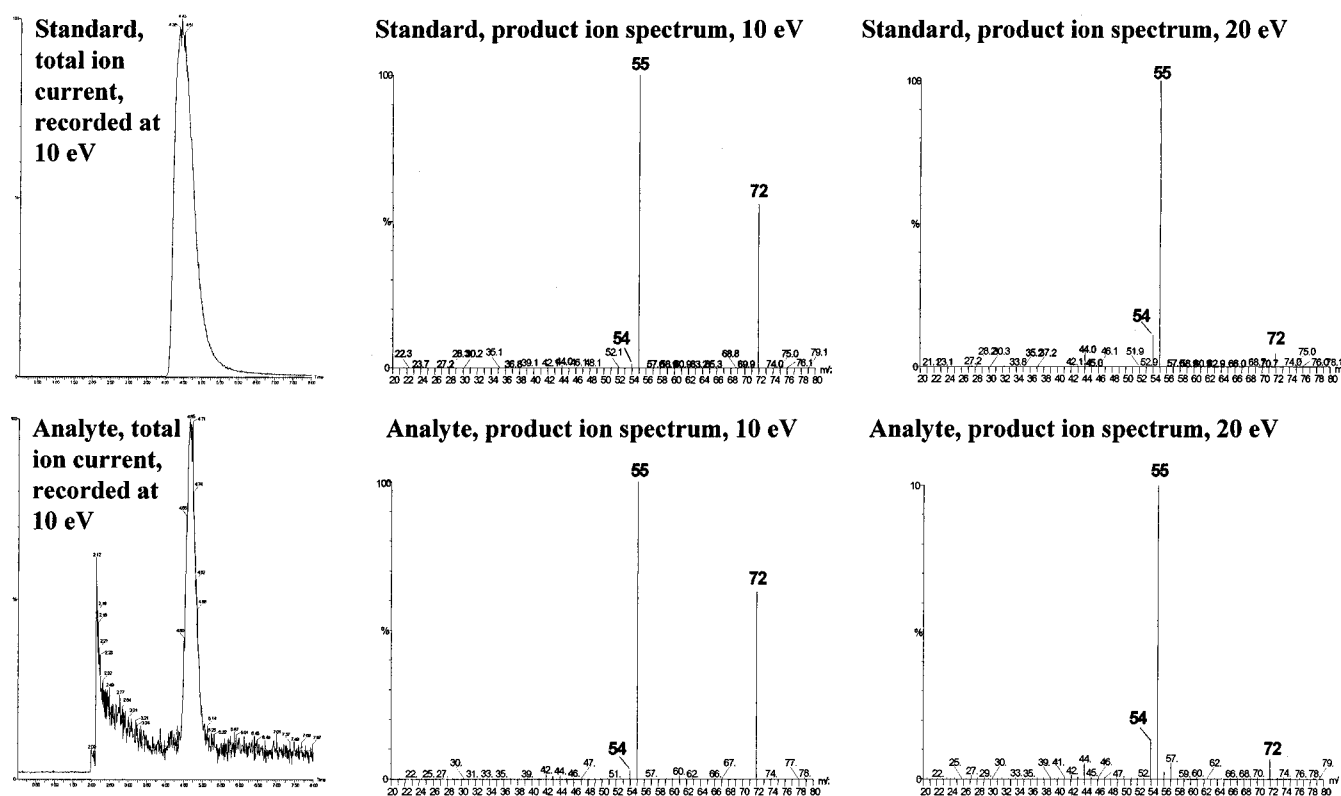
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Figure 3. LC-MS/MS (ESI+); comparison between product ion spectra from precursor ion *m/z* 72 obtained from an acrylamide standard (10 µg/mL) and an analyte (in potato chips) recorded at 10 and 20 eV collision energy and scan range of 20–80 amu.
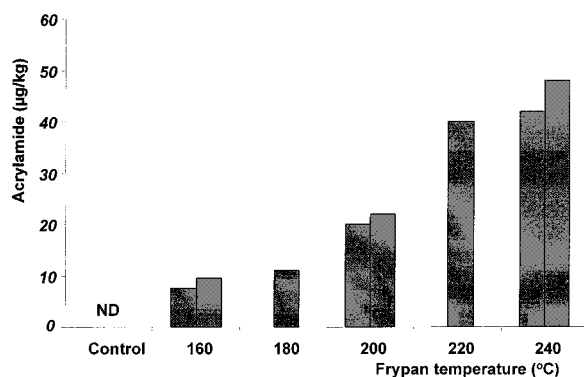

Figure 4. Acrylamide concentrations (micrograms per kilogram) in hamburgers (single and duplicate samples) dependent on frypan temperatures (3 min of frying time on each side).

acrylamide (tested in parallel experiments), an observation that will be addressed in future experiments.

Other carbohydrate-rich foods, such as crispbread (Swedish knaeckebroed), showed variable acrylamide levels (see **Table 6**, e.g., in differently prepared products of crispbread). Toasting of soft bread produced a moderate formation of acrylamide (increasing from 13 to 53 µg/kg; one trial). Acrylamide in crispbread, which is usually stored at room temperature for longer periods of time, also demonstrated that acrylamide is relatively stable in foods.

The influence of heating time on the formation of acrylamide in potatoes during heating in a microwave oven was followed in a laboratory experiment, with simultaneous measurement of weight loss (**Table 7**). When homogenized raw potato was heated for 50, 100, and 150 s, the highest levels of acrylamide were present after heating for 150 s (mean = 4400 µg/kg of heated product). A heating time of 100 s resulted in ~100 times lower levels. Measurement of weight loss showed that the

samples heated for 100 s still contained water (~40%), whereas the samples after 150 s in principle had lost all water. There is a high water content in potato (~79%) (*14*), and it appears that water retention in the potato protected against pyrolysis. Visually there are obvious differences between the samples; after 100 s, only minor parts were browned, whereas the samples cooked for 150 s were thoroughly browned with totally black areas present (not edible).

The influence of temperature on the formation of acrylamide in potato was also studied with French-fried potato heated at controlled conditions in an oven. The relatively high amount of acrylamide in the control sample (146 µg/kg) is due to heat treatment in the production (seen as weak browning). Laboratory heating for 19 min at 100 °C had no effect on the acrylamide content (see **Table 8** and **Figure 6**). A small increase (~ +30 µg/kg) in the acrylamide level measured at 120 °C indicates that the temperature needed for formation of acrylamide is >100 °C.

Aspects of Formation of Acrylamide during Heating of Foodstuffs. The hypothesis that acrylamide could be formed during the heating of food was earlier tested in animal experiments and supported through analysis of fried laboratory animal feed (*8*). A second hypothesis was that protein was involved in the formation of acrylamide, a hypothesis that seemed to be supported in our initial analyses of acrylamide levels in fried hamburger meat within the present study (**Figure 4**). However, the content of acrylamide in fried beef and other protein-rich foods was low compared to that in the fried laboratory animal feed (rich in carbohydrates) as previously studied (8). Therefore, also other types of foodstuff were tested, for example, potato. Analyses of fried potato revealed acrylamide contents at 10−100 times higher levels than in the protein-rich foods. Frying of another type of carbohydrate-rich foodstuff (e.g., beetroot, **Figure 5**) gave similar results.

Table 6. Levels of Acrylamide Analyzed in Single Samples of Different Foodstuffs (Micrograms per Kilogram of Heated Foodstuff)[a]

Laboratory-Prepared Foods				
fried food	A-1	A-2	A-5	median
beef, minced	20; 22		15; 15; 17	17
chicken, minced		16; 41		28
cod, minced	<5; <5	11		
pork, minced	52			
soymeal beef	15; 16			16
potato, grated	730; 780	447	394; 310	447
potato, boiled, mashed			201; 144	172
beetroot, grated		810; 890		850
spinach, grated		112		

microwave-heated food	A-2	median
cod	<5; <5	
potato, grated	455; 650	551

boiled food	A-2	A-4	A-5	median
potato (boiled or raw[b])	<5; <5	<5; <5	<10*[b]	
potato, broth	<5; <5	<5; <5		
beef (boiled or raw[b])	<5;[b] <5[b]	<5; <5	<5; <5	
beef, broth		<5	<5; <5	
cod	<5; <5			
cod, broth	<5; <5			

Restaurant-Prepared/Purchased Foods				
food	A-3	A-4	A-5	median[c]
hamburger			14/14*; 23/23*	18
pork (fried)	45			
"falu" sausage (fried)		<5		
French fries	732	314; 327	661/684*; 424*	424
potato pancake	167			
potato cubes	313			
potato crisps		1300; 1800/1678*	1538/1544*; 2148*; 3897*; 1496*	1739
crispbread, three types	208		37*; 1731*	208
bread, white		13;[d] 53[d]	49*	
rye			<5*	
oat			7.3*	
beer (dark and lager, three types)			<5; <5; <5	

[a] A-1–A-5 denote different analytical series. Analysis with GC-MS; analysis with LC-MS/MS marked with an asterisk (*). Acrylamide concentrations determined in the samples by both GC-MS and LC-MS/MS have been separated by a slash (/). [b] Samples before boiling. Samples of raw beef from the first experiment (**Figure 4**). [c] For repeated analysis of the same sample, separated by / mean values are used. [d] Before and after toasting.

From the results on analyses of foodstuffs heated under laboratory conditions and from the analyses of commercially available foodstuffs it can be concluded that of the studied cooking conditions, except boiling, all lead to pronounced acrylamide formation in potato.

In preliminary experiments aimed at finding cooking conditions that might reduce or prevent the formation of acrylamide, addition of oils, antioxidants, or argon atmosphere during the frying of beef was tested. These measures had minor or nonsignificant acrylamide-reducing effects (data not shown). Furthermore, it should be noted that in the present study evaporated acrylamide was not measured (boiling point estimated to be ~225 °C).

The classical cooking mutagens/carcinogens, first reported by Sugimura and his colleagues (15), consist of a number of heterocyclic amines (HCAs) (16, 17), which are characterized by participation of different amino acids and association with increasing temperature (**Figures 4** and **6**). Compared with HCAs, acrylamide (with highest contents observed in carbohydrate-rich foods), does not contain any amino acid residue and is certainly formed by other pathways, possibly via reactive three-carbon units formed in the heating of carbohydrates, for example, monosaccharides (18, 19), with, for example, ammonia, as nitrogen source. The formation of acrylamide shows similarities with the Maillard reaction.

Estimated Dietary Intake of Acrylamide. It was preliminary estimated that acrylamide in four of the investigated products together could lead to a daily intake of a few tens of micrograms of acrylamide. This estimation was also verified by using Swedish consumption statistics (20) (K. Svensson, NFA, Uppsala, Sweden, personal communication). The average level of the background Hb adduct level from acrylamide observed in earlier studies of humans with no known exposure was preliminarily estimated to result from a daily intake of ~100 μg of acrylamide by an adult person (1). It has to be recalled that these calculations of intake from the average level of background Hb adduct from acrylamide are based on parameter values in the rather complex pharmacokinetics of acrylamide (21); these values require further studies. Also, it cannot be excluded that endogenous production of acrylamide gives a certain contribution to the background level of the Hb adduct.

Estimated Health Risks Associated with Intake of Acrylamide. The present study is part of a series of investigations with the main purpose of clarifying the role in background carcinogenesis of identifiable chemical mutagens of exogenous or endogenous origin.

Disease—epidemiological investigations have been unable to either confirm or disprove human carcinogenicity of acrylamide, even in occupationally exposed cohorts (22, 23). Thus, human data useful for cancer risk estimation are not available. The magnitude of the cancer risk from acrylamide has therefore been assessed from animal experiments (two-year cancer tests with rats) (24, 25), using different linear no-threshold models.

According to WHO, a lifelong (70 years) intake of 1 μg of acrylamide per day would be associated with a lifetime cancer risk of 1×10^{-5} (26). This value is derived by using a linearized multistage model without efforts to allow for differences in metabolism between species. A scaling via dose per unit of body surface area according to the U.S. EPA (27) leads to an ~6 times higher lifetime risk, 6×10^{-5} per μg × day.

The mutagenic and carcinogenic factor in acrylamide exposure is assumed to be the epoxy metabolite, glycidamide (28–30). The cancer risk has also been estimated on the basis of the dose of glycidamide, with approximately the same result as obtained with the U.S. EPA procedure (1). With a multiplicative risk model, shown to be adaptable to the acrylamide cancer test data (31) based on the dose of this epoxide, the value for lifetime cancer risk is somewhat higher than the above value estimated according to the U.S. EPA (1).

The drinking water guideline of WHO for acrylamide is 0.5 μg/L (26), corresponding to an intake of 1 μg/day at the consumption of 2 L/day. In 2003 the recommended limit will become 5 times lower in the European Union (32).

Higher daily intakes of acrylamide may lead to neurotoxic symptoms, which, in contrast to tumors, exhibit a nonlinear dose—response. A no-effect threshold for light symptoms appears to be between 800 and 2700 μg/day corresponding to adduct levels of 300–1000 pmol/g of Hb (1, 6).

Observations on Acrylamide Contents in Heated Foods and as Hemoglobin Adducts in Consumers' Blood. Data from different studies may be used to validate the identity and levels
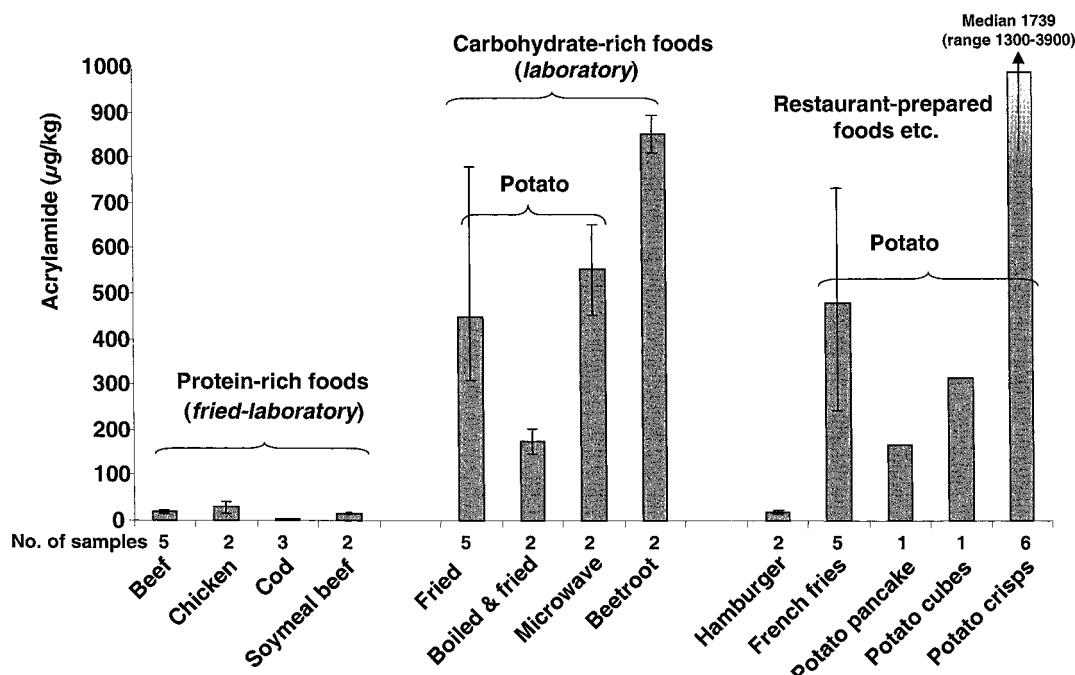
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Figure 5. Acrylamide concentrations (micrograms per kilogram) (median and range) in laboratory-heated and commercial foodstuffs (cf. **Table 5**).

Table 7. Acrylamide Concentrations and Weight Loss in Microwave-Heated Mashed Potato, Heated at a Power of 750 W, for the Heating Times Given

heating time (s)	n^a	wt loss (SE) (%)	acrylamide (SE) (μg/kg)
control	3	0	<5
50	2	27 ($\pm$1.5)	<5
100	3	61 ($\pm$0.48)	47 ($\pm$16)
150	3	77 ($\pm$2.3)	4400 ($\pm$1150)

[a] Number of experiments.

Table 8. Acrylamide Concentrations and Weight Loss in Commercially Prepared and Uncooked French-Fried Potatoes Heated in a Programmed GC Oven (Total Heating Time = 21 min)[a]

max temp (°C)	acrylamide (μg/kg)	time (min) at max temp	wt loss (%)	acrylamide, corrected values (μg/kg)
control	146	0	0	146
100	172, 174	19.0	14.9	146, 148
120	217	18.2	19.9	174
140	376	17.4	29.8	264
160	808	16.6	41.9	469
180	1965	15.8	49.0	1003
200	3479	15.0	54.3	1591
220	5051	14.2	55.0	2273

[a] Measured values were also corrected for weight loss.

Figure 6. Acrylamide concentrations (micrograms per kilogram), corrected for weight loss, in French-fried potatoes heated in a temperature-programmed oven.

of acrylamide in heated foods. Evidence for the identity of acrylamide in foodstuffs in the analyses presented in this study is summarized above. Strong supporting evidence for the identity was also obtained from the analysis of Hb adducts formed in vivo through Michael addition of acrylamide to the amino termini, valines, of Hb and detached from the protein as derivatives of *N*-(2-carbamoylethyl)valine. First, a background level of the same adduct as observed in humans with exposure to acrylamide (occupationally and by smoking) is observed in unexposed humans. The same adduct has also been observed in studies of laboratory animals treated with acrylamide and in animals with accidental exposure to acrylamide. In a previous study with rats fed fried laboratory feed, an intake of acrylamide
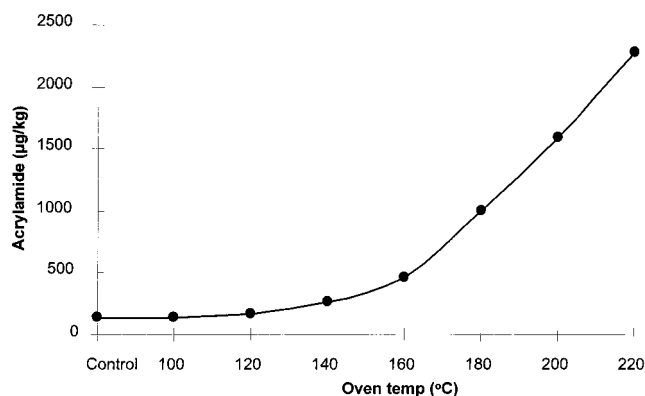
was measured as an increase of acrylamide adducts to Hb, the incremental adduct level being shown to be compatible (considering, e.g., the reactivity of acrylamide) with the content of acrylamide in the feed (*8*). The adduct structure was verified by interpretation of product ions in comparison with corresponding ions from one unsubstituted and two different isotope-substituted standards (*8*). It could further be added that the preliminarily estimated intake of acrylamide in this study is within the range expected from the background level of the adduct from acrylamide in humans. It further strengthens the proof of identity that the valine adduct, *N*-(2-carbamoyl-2-hydroxyethyl)valine, from the prime acrylamide metabolite, glycidamide, is regularly found in the same Hb samples as the acrylamide adduct and in approximately the proportion to the acrylamide adduct expected from acrylamide exposure (unpublished data).

The present work has shown that a large contribution to the background level of the Hb adduct from acrylamide in humans probably originates from acrylamide formation in cooking and food preparation. Relatively high acrylamide concentrations in certain foodstuffs were recently presented by the Swedish National Food Administration (NFA), which further explored

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our present, initial observations; the NFA results are concordant with our data (*33*).

This pilot study should be regarded as a first attempt to determine acrylamide in frequently consumed foodstuffs and also to demonstrate the importance of the cooking technique in the formation of acrylamide. The study demonstrates the need for further studies of the formation of acrylamide during cooking of foods and of the presence of this chemical in foods consumed. In future studies the determination of Hb adducts will be an important tool for measurement of the dietary intake of acrylamide.

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NOTE ADDED AFTER ASAP POSTING

The original posting made July 18, 2002, contained an error in *m/z* value on page B and omitted reference numbers on p H. These errors have been corrected in this posting.

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