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A New LC/MS-Method for the Quantitation of Acrylamide Based on a Stable Isotope Dilution Assay and Derivatization with 2-Mercaptobenzoic Acid. Comparison with Two GC/MS Methods

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Acrylamide (AA) was found to form a stable thioether in reasonable yields (45−50%) when reacted with 2-mercaptobenzoic acid at 20 °C for 3 h. On the basis of this finding and using [$^{13}C_3$]-acrylamide as the internal standard, a sensitive and selective new stable isotope dilution analysis for AA quantitation in food samples was developed based on single stage LC/MS. Comparison of the quantitative results obtained by applying the new method to potato chips, crispbread or butter cookies with data obtained by two stable isotope dilution analysis, using direct measurement of AA by GC/MS, but differing in the workup procedure, revealed detection limits in the same order of magnitude (6.6 μg/kg). Quantitative data obtained by application of the three methods on the same samples of potato chips or cookies, respectively, were also in very good agreement. Quantitation of AA in crispbreads treated with an amylase/protease mixture did not show increased AA levels, thereby indicating that inclusion of AA in starch/protein gels is not very probable during breadmaking.

KEYWORDS: Acrylamide; [$^2H_3$]-acrylamide; [$^{13}C_3$]-acrylamide; food; stable isotope dilution analysis; derivatization; 2-mercaptobenzoic acid; GC/MS; LC/MS

INTRODUCTION

Acrylamide (AA) is an important industrial chemical, and mainly the contamination of drinking water, or of plants grown hydroponically, has been the driving force in the past to develop methods for the quantitation of this probable human carcinogen (*1*−*3*). Furthermore, AA is also a long known constituent of tobacco smoke (*4*, *5*).

Among the first reports on low concentrations of AA in certain foods is a study on sugar, reporting amounts of about 5−12 μg/kg (*6*). About three years ago, Tareke et al. (*7*) were the first to show that a thermal treatment of animal feed led to the generation of significant amounts of acrylamide, thereby raising the question whether the amide may be a cooking carcinogen. Interestingly, this result did not cause much response in the literature. However, two years later, when this assumption was confirmed by the same group (*8*) for a significant number of processed foods, the food industry, governmental institutions, and research groups at universities immediately focused their interest on this topic.

Because of its high solubility in water and its high reactivity (*9*), and also, because of the lack of a chromophor, AA is not easy to detect. To increase the selectivity, and also, the sensitivity in GC analysis, bromination of the double bond in combination with GC/electron capture detection has, therefore, been applied previously (*1*). The "bromination" method was improved later on, using either methacrylamide or *N*,*N*-dimethylacrylamide as the internal standards (*2*), a method which has been used until recently (*7*, *8*, *10*, *11*). Several groups also described methods to quantify AA by direct GC/MS measurements without bromination (*12*, *13*).

Stable isotope dilution assays (stable isotope dilution analysis), in which an isotopomer of the analyte is used as the internal standard, are among the most reliable methods for quantitation of compounds, which are labile and/or reactive. The use of 2,3,3-[$^2H_3$]-acrylamide ([$^2H_3$]-AA) as an appropriate internal standard has been reported for cigarette smoke already in 1990 (*5*). [$^2H_3$]-AA has also been used in very recent publications aimed at quantifying AA in foods (*14*−*20*). Because the deuterium labeling is positioned at the double bond in [$^2H_3$]-AA, thereby making a deuterium/protium exchange possible during workup, [$^{13}C_1$]-acrylamide (*21*) and [$^{13}C_3$]-acrylamide, which became available very recently, are now preferably used (*8*, *19*).

In stable isotope dilution assays, the differentiation between the analyte and the internal standard is done by recording the respective molecular masses and/or mass fragments. Due to the low molecular weight of acrylamide (71 g/mol), and therefore, also due to its resulting low mass fragment ions, background interference may impede the analysis. Better confirmation of the analyte and selectivity can be achieved with a three-stage mass spectrometer by monitoring more than one characteristic mass transition (*8*, *11*, *19*, *20*).

Although reliable data have been published, up to now, only stable isotope dilution assays using direct analysis of AA have

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been applied. So, except the bromination procedure, which is quite time-consuming, no method is available that involves the derivatization of AA prior to MS analysis, thereby increasing the selectivity, in particular, when single-stage GC/MS detection is used. Such methods involving such a derivatization of AA prior to MS analysis could also be useful (e.g., as reference method to approve data obtained by multi-stage LC/MS). Furthermore, triple quadrupole mass spectrometers are quite expensive, and according to our own results, the cheaper single stage LC mass spectrometers are not sensitive enough for the direct analysis of AA.

The purpose of the following investigation was, therefore, (i) to study possibilities for AA derivatization to obtain derivatives, which can sensitively be determined by single-stage LC/MS, and (ii) to develop a stable isotope dilution assay based on the derivatization procedure. This concept has recently been successfully applied by our group in the analysis of the mycotoxin patulin (*22*). Because in most publications, only one method has been applied in AA quantitation, results determined by using the same batch of foods, but using different methods, should also be compared.

MATERIALS AND METHODS

Food Samples. Samples of crispbread, potato chips, sweet cookies, butter cookies, and rusk were purchased in a local supermarket.

Chemicals. [$^{13}C_3$]- (99%) and [$^2H_3$]-acrylamide (98%) were obtained from CIL (Andover, MA); acrylamide (99.9%) was obtained from VWR International (formerly Merck, Darmstadt, Germany); 2-mercaptobenzoic acid, methyl-2-mercaptobenzoate, 4-mercaptobenzoic acid, and 4-bromothiophenol were obtained from Sigma-Aldrich (Steinheim, Germany). All other solvents and chemicals used were of analytical grade. CAUTION: Acrylamide as well as [$^{13}C_3$]- and [$^2H_3$]-acrylamide are hazardous and must be handled carefully.

Acrylamide Quantitation by Stable Isotope Dilution Assays. *Derivatization with 2-Mercaptobenzoic Acid/Analysis by LC/MS (Method I).* After additon of the internal standard ([$^{13}C_3$]-acrylamide, 5.0 μg in 1 mL of ethanol) and water (80 mL), the sample (10 g) was homogenized and extracted using a homogenizer (Ultra-Turrax, Jahnke and Kunkel, Oberstaufen, Germany) for 2 min followed by ultrasound treatment for 10 min. The homogenate was centrifuged at 5000 rpm for 10 min at 10 °C, the supernatant (about 20 mL) was either filtered (glass filter, pore size 3) or centrifuged twice, and the solution obtained was subsequently defatted twice by extraction with *n*-hexane (total volume: 60 mL). The extract was adjusted to pH 8 ± 0.3 by adding aqueous sodium hydroxide (1 mol/L). After addition of 1 mL of a solution of 2-mercaptobenzoic acid in aqueous sodium hydroxide (154 mg in 10 mL of 1 mol/L NaOH) for derivatization (pH was increased to about 10), the reaction mixture was stirred in the dark for 3 h. The excess of the reagent was then removed by treatment with lead (II) acetate (5 mL of a saturated solution in water), and after centrifugation at 5000 rpm for 10 min, the supernatant was acidified to pH 1.5 ± 0.3 using hydrochloric acid (5 mol/L), and finally extracted with ethyl acetate (3 × 30 mL). The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed at about 20 kPa and 40 °C. For LC/MS analysis, the residue was dissolved in methanol (250 μL).

LC/MS spectra were recorded by means of a mass spectrometer (LCQ-MS; Finnigan MAT, Bremen, Germany) coupled to a spectra series high-performance liquid chromatograph (Thermo Separation Products, San Jose, CA) equipped with a Luna Phenyl-Hexyl HPLC column (250 × 4.6 mm i.d., 5 μm, Phenomenex, Aschaffenburg, Germany). A 20-μL portion of the sample was separated at a flow rate of 0.8 mL/min using acetonitrile and acetic acid (1 g/L water) mixtures as a gradient. A linear gradient was programmed within 15 min from 30 + 70 to 60 + 40 (H_2O/diluted acetic acid; by vol). The mass spectrometer was operated in the positive electrospray mode (ESI) with a spray needle voltage of 5 kV and a spray current of 80 μA. The temperature of the capillary was 200 °C, and the capillary voltage was 16 V. The sheath and auxiliary gas nitrogen nebulized the effluent with

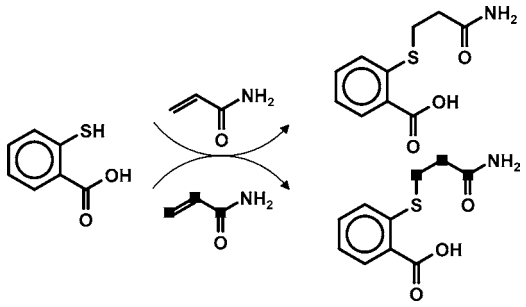

Figure 1. Derivatization procedure applied for conversion of acrylamide and [$^{13}C_3$]-acrylamide into stable thioethers prior to the stable isotope dilution assay in combination with LC/MS (method I).

flows of 80 and 20 arb, respectively. The ion trap operated at a helium pressure of 1.3×10^{-4} kPa.

For quantitation, the mass traces *m*/*z* 226 and *m*/*z* 229 for the unlabeled and labeled thioethers formed in the derivatization procedure (cf. **Figure 1**), were monitored. For calibration, defined mixtures of AA and [$^{13}C_3$]-AA (1 + 10 to 10 + 1) were analyzed, and the response factor was calculated as described recently (*23*).

Workup Procedure for GC/MS-Analysis (Method II). After additon of the internal standard ([$^2H_3$]-acrylamide, 5.0 μg in 1 mL of ethanol) in a mixture of acetonitrile/water (150 mL, 85 + 15, by vol), the sample (10 g) was homogenized by means of an Ultra-Turrax (Jahnke and Kunkel, Oberstaufen, Germany; 2 min) followed by ultrasound treatment for 30 min. After precipitation of proteins by Carrez-clarification (5 mL of a solution of 150 g $K_4[Fe(CN)_6] \times 3H_2O$ per liter of water (Carrez I) and 5 mL of a solution of 230 g Zn acetate $\times 2H_2O$ per liter of water (Carrez II), the suspension was centrifuged at 4000 rpm for 10 min at 10 °C. The supernatant was defatted with *n*-hexane (total volume: 60 mL) and concentrated to 15 mL at about 2 MPa and 40 °C. The extract was then applied to an Extrelut NT 20 column (VWR, Darmstadt, Germany), and after an equilibration time of 15 min, elution was performed with ethyl acetate (100 mL). The solvent was dried over anhydrous sodium sulfate and concentrated to 1.0 mL at about 20 kPa and 40 °C. This extract was used for GC/MS. This was performed by means of a Varian 3800 gas chromatograph (Varian, Darmstadt, Germany) using an FFAP-capillary (free fatty acid phase; 30 m × 0.32 mm i.d. fused silica capillary, 0.25 μm; J & W Scientific, Agilent, Waldbronn, Germany). The samples (2 μL) were applied by the cold on-column technique at 60 °C. After 2 min, the temperature was raised at 10 °C/min to 240 °C and finally held for 10 min isothermally. The flow of the carrier gas helium was 2.5 mL/min. Mass spectrometry was performed using an ion trap detector Saturn 2000 GC/MS (Varian, Darmstadt, Germany) running in the chemical ionization (CI) mode with methanol as the reactant gas using an ionization energy of 70 eV. The concentrations were calculated from the selected ions using the following equation:

$$C = \frac{A_{AA} \times m_{[^2H_3]-AA} \times Rf}{A_{[^2H_3]-AA}}$$

where A_{AA} is the area of unlabeled acrylamide as mass trace *m*/*z* 72, $A_{[^2H_3]-AA}$ is the area of [$^2H_3$]-labeled acrylamide as mass trace *m*/*z* 75, $m_{[^2H_3]-AA}$ is the amount of added [$^2H_3$]-labeled acrylamide, and Rf is the response factor determined by analysis of defined mixtures of AA and [$^2H_3$]-AA.

Alternative Workup for GC/MS-Analysis (Method III). The analysis of acrylamide was performed according to Biedermann et al. (*12*) using a modified workup procedure: Samples (25 g) were homogenized with water (75 g) using an Ultra-Turrax (Jahnke and Kunkel, Oberstaufen, Germany, 2 min) followed by ultrasound treatment for 15 min. After addition of the internal standard ([$^2H_3$]-acrylamide, 2.5 μg in 1 mL of ethanol) to an aliquot (25 mL) of the homogenate, this was mixed with 75 mL of 1-propanol using the Ultra-Turrax (2 min) followed by ultrasound treatment for 15 min. The supernatant was transferred into a glass vessel (100 mL) and centrifuged at 5000 rpm for 10 min at 10 °C. The supernatant was transferred to a 100-mL point flask, 10 droplets

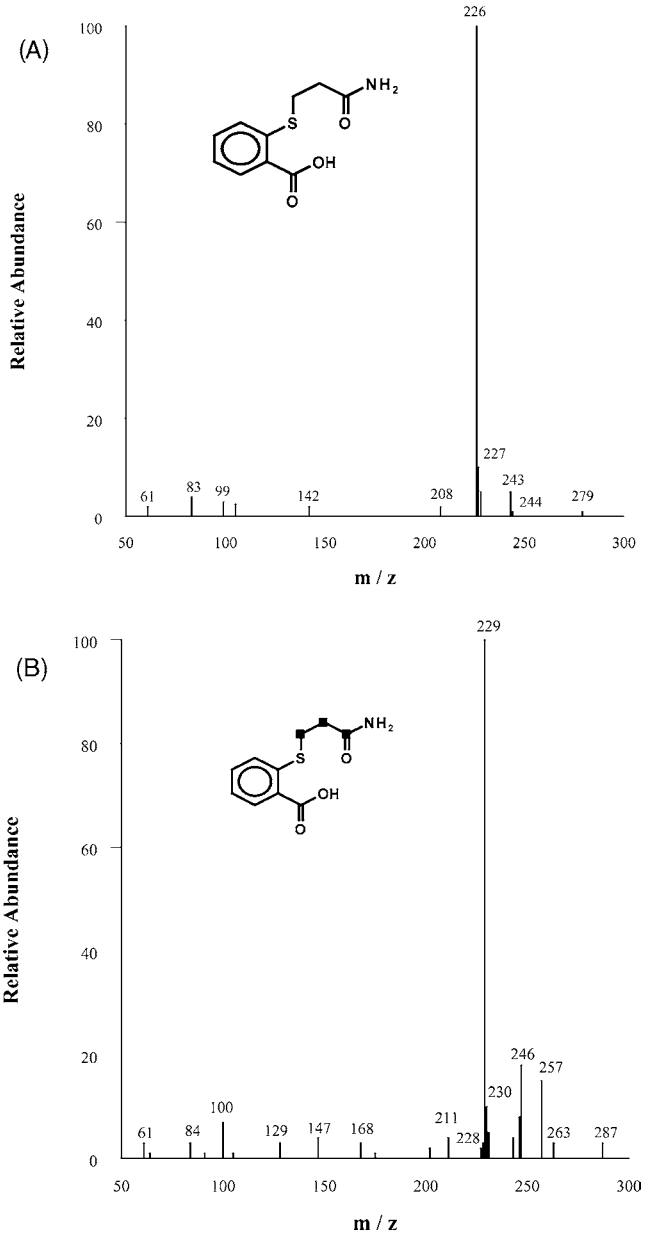

Figure 2. Full scan mass spectrum (ESI positive) of the reaction product obtained from acrylamide and 2-mercaptobenzoic acid (A) and of [$^{13}C_3$]-acrylamide and 2-mercaptobenzoic acid (B).

of sunflower oil were added, and the solvent was removed at about 8 kPa and 50 °C. The residue was extracted with acetonitrile (2 mL) by ultrasound treatment and finally defatted with *n*-hexane (2 × 5 mL). After drying over anhydrous Na_2SO_4, the acetonitrile phase was used for GC/MS analysis. The GC/MS analysis was performed as given above for method II.

Estimation of the Detection Limit (LoD) and the Quantitation Limit (LoQ). To acrylamide-free rice starch, the following amounts of acrylamide were added: 5.2, 13.0, 26.0, and 52.0 μg/kg for GC/MS- and 10.4, 26.0, 52.0, and 104.0 μg/kg for LC/MS-analysis. Each sample was analyzed in triplicate. LoD and LoQ were calculated according to the method of Hädrich and Vogelgesang (*24*): LoD is the addition value referring to the 95% confidence limit of the calibration line at the zero addition level. LoQ is the addition level that lowers the 95% confidence limit of the addition level at LoD.

Enzymatic Digestion. Powdered crispbread samples (10 g) were homogenized and extracted by means of an Ultra-Turrax (Jahnke and Kunkel, Oberstaufen; Germany; 2 min) followed by ultrasound treatment for 30 min. After addition of either a protease (3 mg; type XIV from bacteria, EC 3.4.24.41, Sigma, Deisenhofen, Germany) or an

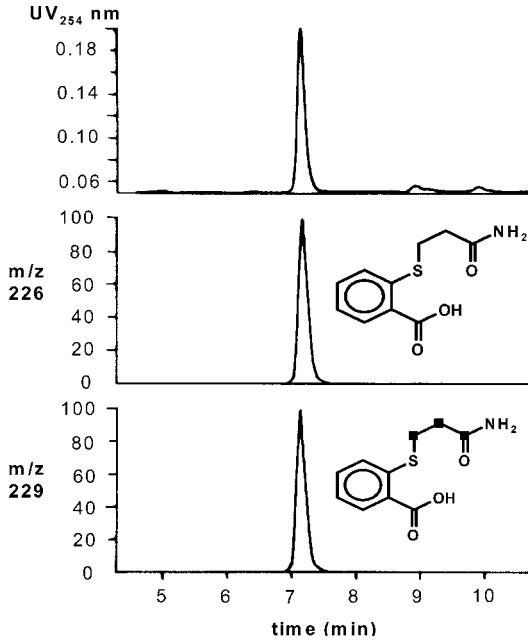

Figure 3. Mass chromatograms obtained for a model mixture of acrylamide and [$^{13}C_3$]-acrylamide (1 + 1 by weight).

α-amylase (3 mg from *Bacillus subtilis*, EC 3.2.1.1, VWR, Darmstadt, Germany) incubation was performed for 5 h at 37 °C. Additionally, a control experiment without enzymes added was performed in the same way. After enzymatic digestion, the acrylamide content was determined by method I.

RESULTS AND DISCUSSION

It is well-known that nucleophilic compounds, such as thiols, are able to react with the double bond of acrylamide (*9*). This gave us the idea to use this reactivity vs thiols in the generation of stable derivatives for the quantitation of AA using a single stage LC mass spectrometer, because direct analysis showed only a very unsatisfactory detection limit of about 1 mg/kg with the LC/MS system (data not shown).

In preliminary trials, several thiols were reacted with acrylamide, and the derivatives formed were analyzed. As a result, 2-mercaptobenzoic acid was found to convert acrylamide within 3 h with reasonable yields into a stable thioether, which could sensitively be detected by LC/MS. Other thiols checked (e.g., 4-mercaptobenzoic acid or 4-bromothiophenol) showed much lower reactivity (data not shown). Also methyl-2-mercaptobenzoate was a good derivatization reagent, but the standard curve showed a poorer linearity (data not shown). The positive ESI/MS full scan spectrum of the 2-mercaptobenzoic acid derivative is given in **Figure 2A**. The respective protonated molecular ion *m*/*z* 229 $[M + H]^+$ was obtained by reacting [$^{13}C_3$]-AA (**Figure 2B**) with 2-mercaptobenzoic acid.

Next, a calibration curve was generated based on results obtained for seven model mixtures containing defined amounts of AA and [$^{13}C_3$]-AA. These were worked-up as described for method I in the experimental part. The samples were analyzed by LC/MS and the intensity of the M + 1 masses *m*/*z* 226 (unlabeled derivative) and *m*/*z* 229 (labeled derivative), respectively, were recorded (**Figure 3**). From the data obtained, a calibration curve was drawn (**Figure 4**). The curve looked promising and showed a very good linearity.

To evaluate the method, first, from four model mixtures consisting of starch and water, which had been spiked with 10−100 μg of acrylamide, an LoD of 6.6 μg/kg (**Table 1**) and a LoQ of 19.6 μg/kg (**Table 1**) were calculated based on a

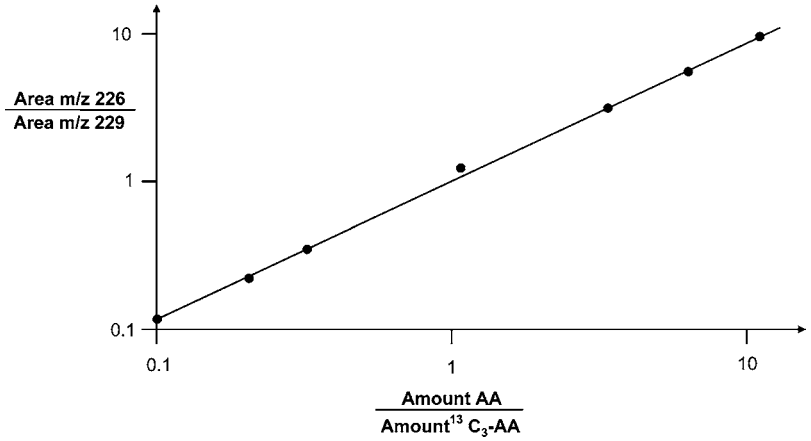

Figure 4. Calibration curve obtained by direct analysis of seven mixtures of acrylamide and [$^{13}C_3$]-acrylamide in a defined concentration ratio (method I).

Table 1. Detection and Quantitation Limits Determined for Three Methods Used in Acrylamide Quantitation

method	detection limit (μg/kg)[a]	quantitation limit (μg/kg)[a]
I	6.6	19.6
II	4.0	11.8
III	5.6	16.7

[a] Mean value of triplicates.

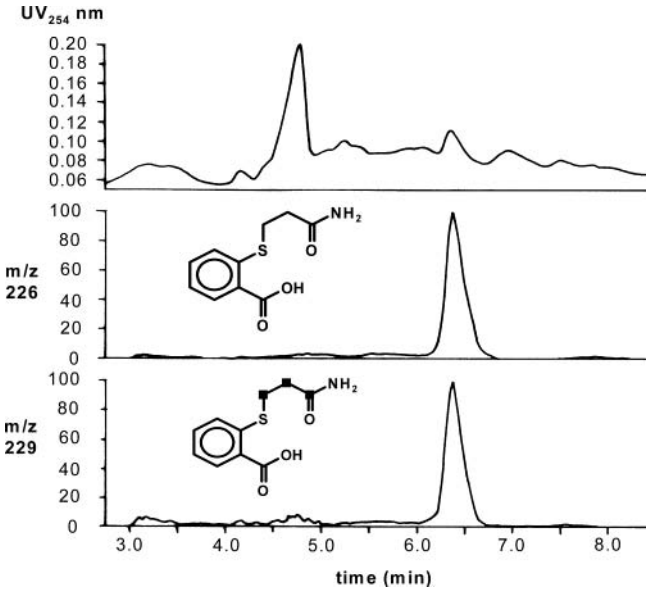

Figure 5. LC/MS chromatogram (A) and mass spectra (B) obtained in the analysis of acrylamide in a potato chip sample containing about 500 μg acrylamide.

procedure described in (*24*). The data showed that the detection limit of the new method was comparable to the two GC/MS methods, which will be discussed later. However, by derivatization, the detection limit was lowered by a factor of 1000 compared to the direct LC/MS measurement using the LC/MS in single stage mode.

In combination with a stable isotope dilution assay, the method was then used in the analysis of three different foods known to contain acrylamide. As exemplified for a potato chip sample in **Figure 5**, intense signals of the AA and [$^{13}C_3$]-AA derivative were obtained for a sample containing about 550 μg/kg of AA. The UV trace at 254 nm is given for comparison.

Table 2. Concentrations of Acrylamide in Different Food Samples as Determined by LC/MS after Derivatization with 2-Mercaptobenzoic Acid (method I)

food sample	conc. (μg/kg)[a] undoted	doted[b]	RSD (%)[d]	recovery (%)[c]
rye crispbread	517.4	1047.4	±0.5	101.9
potato chips	524.7	1107.4	±2.9	112.1
rusk	101.3	599.9	±8.7	95.9

[a] Data are mean values of triplicates for undoted samples and mean values of duplicates for spiked samples. [b] The samples were spiked with 520 μg acrylamide/kg prior to analysis. [c] Foods were spiked with a defined amount of the analyte (520 μg/kg) and the internal standard. Recoveries were calculated from the concentrations determined in the unspiked and the spiked sample. [d] RSD, relative standard deviation.

The three food samples were analyzed in triplicates showing a good standard deviation (**Table 2**). Analysis of the same samples, but spiked with 520 μg of acrylamide, also showed very good recoveries of 96−112%.

In further experiments, two GC/MS methods were established in the lab, one (method III) had already been proposed in the literature (*12*), the second was modified, in particular, in the workup procedure by using Extrelut cartridges for purification (method II). In both methods, the direct MS analysis of acrylamide and [$^2H_3$]-acrylamide was performed as exemplified for a model mixture in **Figure 6**. On the basis of the analysis of model mixtures containing starch and water, for both methods, good detection limits and quantitation limits between 4 and 17 μg/kg could be achieved (**Table 1**), which were in good agreement with the literature (*13*) and also with the derivatization procedure (method I). In **Figure 7**, a calibration curve for the determination of the detection and the quantitation limit for method II is displayed.

Three samples of rye crispbread, two samples of potato chips, and one sample of rusk were then analyzed using method II as well as method III. Analysis of AA by method II showed a relative standard deviation (RSD) between ± 0.0 and ± 2.7% for foods high in AA and an RSD of 10% for the lower concentrations found in rusk (**Table 3**). Also, good recovery rates of 106.4 and 108.4% were found for two samples spiked with acrylamide. Application of method III to the same samples revealed nearly indentical concentrations in the six foods analyzed, and also, similar standard deviations and recovery rates (**Table 4**).

For comparison of the three methods, in a further experiment, the same batch of potato chips and butter cookies was analyzed,

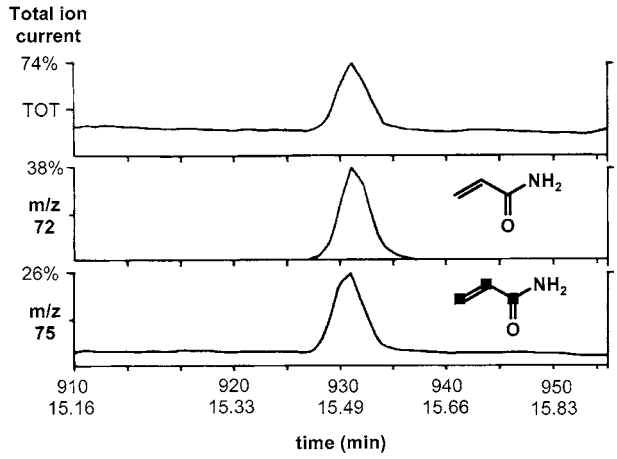

Figure 6. Mass chromatograms obtained for the ions *m/z* 75 ([$^{13}C_3$]-AA) and *m/z* 72 (AA) using GC/MS in the chemical ionization mode (food sample containing an amount of AA > 500 μg/kg).

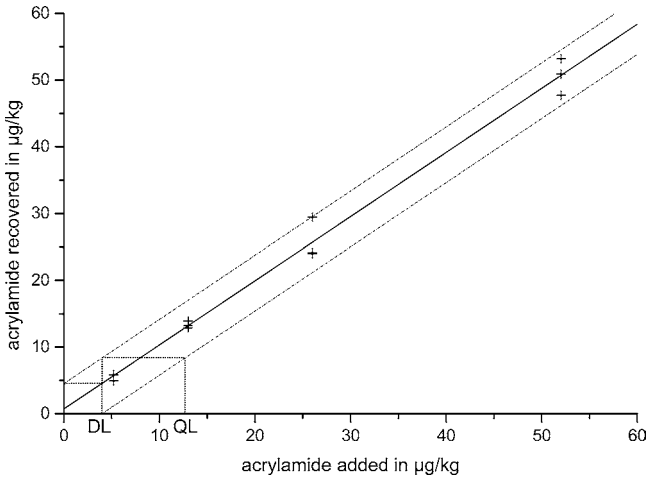

Figure 7. Determination of the LoD and the LoQ for acrylamide by applying method II on a starch/water model with solutions containing decreasing amounts of acrylamide. 95% Confidence limit (dotted lines) of the calibration curve (black line).

Table 3. Quantitation of Acrylamide in Different Food Samples Using Direct Analysis by GC/MS (method II)

	conc (μg/kg)[a]			
food sample	undoted	doted[b]	RSD (%)[d]	recovery (%)[c]
crispbread A	451.5		±1.7	
crispbread B	528.1		±2.2	
crispbread C	510.8	1063.0	±0.4/0.0	106.4
potato chips A	446.7		±2.7	
potato chips B	468.1	1031.6	±0.8/±2.7	108.4
rusk	59.2		±10.0	

[a] Data are mean values of triplicates for undoted samples and mean values of duplicates for spiked samples. [b] The samples were spiked with 520 μg acrylamid/kg prior to analysis. [c] Foods were spiked with a defined amount of the analyte (520 μg/kg) and the internal standard. Recoveries were calculated from the concentrations determined in the unspiked and the spiked sample. [d] RSD, relative standard deviation.

and the results obtained are compared in **Table 5**. For chips, the highest concentrations were measured, when the derivatization was applied, while the lowest concentrations were determined using method III. For butter cookies, the measured concentrations were nearly identical for the three methods. Compared to the two GC/MS methods, the standard deviation

Table 4. Quantitation of Acrylamide in Different Samples Using Direct Analysis by GC/MS (method III)

	conc (μg/kg)[a]			
food sample	undoted	doted[b]	RSD (%)[d]	recovery (%)[c]
crispbread A	451.0		±1.4	
crispbread B	521.5		±8.5	
crispbread C	468.1	1006.2	±3.5/±4.1	103.5
potato chips A	427.9		±1.8	
potato chips B	454.1	978.0	±0.6/±3.8	100.8
rusk	62.0		±5.4	

[a] Data are mean values of triplicates for undoted samples and mean values of duplicates for spiked samples. [b] The samples were spiked with 520 μg acrylamid/kg prior to analysis. [c] Foods were spiked with a defined amount of the analyte (520 μg/kg) and the internal standard. Recoveries were calculated from the concentrations determined in the unspiked and the spiked sample. [d] RSD, relative standard deviation.

Table 5. Comparison of Acrylamide Concentrations Determined in the Same Batch of Potato Chips and butter Cookies Using Three Different Methods

	potato chips		butter cookies	
method	conc (μg/kg)[a]	RSD (%)	conc (μg/kg)[a]	RSD
I	517.4	±0.5	157.4	±0.4
II	510.8	±0.4	159.6	±1.5
III	468.1	±3.5	158.0	±0.8

[a] Mean values of triplicates.

Table 6. Concentration of Acrylamide in Crispbread as Influenced by a Pretreatment with hydrolytic enzymes

sample	conc (μg/kg)[a]	SD (%)
control	657.5	±0.1
pretreatment with a protease	630.3	±8.3
pretreatment with an α-amylase	692.8	±1.7
pretreatment with both enzymes	645.3	±2.7

[a] Mean values of triplicates.

for the newly developed derivatization method was in the same order of magnitude (**Table 5**).

It might be possible that AA will be included in the starch/protein matrix during baking of (e.g., bread). Therefore, in another experiment, a crispbread suspension was either treated with a protease or an α-amylase or both. The AA concentration was then determined using method I. As shown in **Table 6**, no significant influence of the starch/protein digestion prior to the analysis on the amounts of AA was detected. These data suggest that no additional amounts of AA were included in the bread matrix during baking.

In summary, the results show that derivatization of acrylamide and the [$^{13}C_3$]-isotopomer with 2-mercaptobenzoic acid prior to a stable isotope dilution assay followed by LC/MS measurements in the single stage mode is a useful alternative method for its quantitation. The method helps to increase the selectivity in AA determination, in particular with single stage LC mass spectrometers. Very recently, the method was used by our lab in an international proficiency test (about 50 labs participated) resulting in a very good evaluation (*25*).

ABBREVIATIONS USED

AA, acrylamide; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; LoD, detection limit; LoQ, quantitation limit.

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