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A method for the determination of acrylamide in bakery products using ion trap LC-ESI-MS/MS

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Acrylamide levels of bakery products, *e.g.*, bread and bread rolls, are usually below 100 μg/kg, often even below 50 μg/kg. Therefore, usual analytical methods which have an LOQ ≥ 25 μg/kg are not sensitive enough for detailed investigations on acrylamide formation within these commodities. An improved method for trace level determination of acrylamide in bakery products was developed using ion trap LC-ESI-MS/MS. Samples were divided into crumbs and crusts to achieve an initial concentration by removing the crumbs since these are devoid of acrylamide. After sample extraction and clean-up using multimode SPE cartridges, further analyte enrichment was accomplished by solid-phase-supported liquid-liquid extraction with ethyl acetate prior to LC-MS/MS analysis. The method was evaluated using bread, bread rolls, alkali-baked bread rolls, and toast. LOQ was calculated from the confidence interval of the calibration curve and found to be 1.7 ng/mL, corresponding to 17 μg/kg of product. When crumbs and crusts were separated, an LOQ of 10.2 μg/kg of bakery product could be obtained. As demonstrated in preliminary comparative analyses, accuracy of the method met the requirements for determination of trace level acrylamide formation in bakery products. Mean recovery was 102.4% (CV 4.5%), intermediate reproducibility revealed a CV of 2.1%, and a repeatability of CV 6.0%.

Keywords: Acrylamide / Quantitation / Ion trap / ESI / Bakery products

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1 Introduction

Since its first detection in heat-treated food rich in carbohydrates [1], acrylamide has been found in many foodstuffs, with potato and cereal products as well as coffee being major sources of dietary intake, as published in the Swedish dietary intake study [2]. Animal studies revealed the neurotoxic and carcinogenic potential of acrylamide. Its chemistry, biochemistry, occurrence, metabolism, and toxicology have recently been reviewed [3, 4]. Considerable international efforts have been made to lower acrylamide contents in various food commodities [5 – 9]. For this purpose a strategic concept was agreed in Germany among the Federal Office of Consumer Protection and Food Safety (BVL), federal states, industry, and the Federal Ministry of Consumer Protection, Food and Agriculture, which is based on signal values. In this concept, foodstuffs concerned are classified in certain groups. Those foods that make up the 10% most contaminated products in each group are identified, and the lowest acrylamide content of these upper 10% is the new signal value. If acrylamide contents are above this signal value, the state prompts food producers to take adequate actions to lower the contents. Particular attention has so far been paid to potato products like French fries and potato chips, because their contents are comparatively high and the formation of acrylamide is mainly related to reducing sugars, thus facilitating studies to reduce concentrations. Consumption of French fries and potato chips is much lower compared to bakery products like bread and bread rolls. For example, in Germany, the per capita consumption was 86.6 kg in 2003, which is equivalent to an average daily intake of about 237 g [10]. Due to customary consumption habits, bakery products contribute about 25% of the total acrylamide intake via the diet [2, 11]. In contrast to potato products, there is still a lack of information on factors affecting acrylamide formation in bread and other bakery wares [5, 12, 13]. In such products acrylamide formation probably not only depends on the available amounts of the known precursors asparagine and reducing sugars, but also on enzymatic degradation of starch and proteins during

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Abbreviations: CID, collision induced dissociation; **IT**, ion trap

dough preparation, and on alkali treatment of the dough, respectively. For more detailed studies, therefore, sensitive methods for acrylamide determination are a prerequisite.

So far, two main approaches for the analysis of acrylamide in food have been suggested. One is based on GC-MS, the other on LC-MS/MS [14]. For GC-MS determination, acrylamide is measured directly or after derivatization into 2,3-dibromopropionamide [14]. LC-MS methods using derivatization of acrylamide with 2-mercaptobenzoic acid have also been reported [15]. These methods provide reliable results, but are tedious and time consuming. Therefore, LC-MS/MS methods using triple quadrupole mass spectrometers have become more popular. Whereas IT mass spectrometers are superior in multistage mass analysis, they have been considered less reliable in quantitative analyses compared to quadrupole instruments due to possible oversaturation of the trap [16]. Therefore, the applicability of an IT instrument for acrylamide analysis should be investigated in this study. Most previously published LC-MS/MS methods, with only few exceptions [17, 18], show an LOQ of about 30 µg acrylamide/kg of sample material. Because usual acrylamide contents of bread and bread rolls are close to this level, methods comprising a concentration step during clean-up, thus improving analytical sensitivity, are required [15].

For this purpose, a solid-phase-supported liquid-liquid extraction with ethyl acetate was performed without tedious derivatization. In addition, the sensitivity of the method for the determination of low level acrylamide contents in bakery products should be evaluated using bread rolls, toast, wheat mix bread, and alkali-baked bread rolls, a specialty of southern Germany comparable to pretzels. Furthermore, comparative analyses should be performed for comparison of the proposed method with the approach used by the CVUA (Chemisches und Veterinäruntersuchungsamt) Stuttgart employing a triple quadrupole mass spectrometer [19].

2 Materials and methods

2.1 Sample material

Bread rolls, alkali-baked bread rolls, wheat mix bread (750 g), and toast were purchased from a local bakery in August 2004. Toast was prepared in a toaster (Severin Elektrogeräte, Sundern, Germany) for 2 min at medium heat until a golden-brown color was obtained. Losses of acrylamide during storage of the samples at −25°C prior to analysis were not observed.

2.2 Chemicals and materials

Acrylamide (99%) was purchased from ICN Biomedicals (Eschwege, Germany), 2,3,3-D_3-labeled acrylamide (98%) was from Cambridge Isotope Laboratories (Andover, MA). Formic acid, ACN (both gradient grade), potassium hexacyanoferrate (Carrez I) and zinc acetate (Carrez II) were provided by VWR (Darmstadt, Germany). Deionized water was used throughout. SPE cartridges (Isolute Multimode, 1000 mg) were obtained from IST (Hengoed, Mid Glamorgan, UK). Chem Elut cartridges for solid-phase-supported liquid-liquid extraction were purchased from Varian (Darmstadt, Germany).

2.3 Sample preparation

The sample preparation was based on a method published by Gutsche et al. [19]. Initially, all samples with the exception of toast were manually divided into crumbs and crusts. Because acrylamide is only formed at temperatures above 100°C during baking [10], crumbs were picked out, and only crusts were used for analysis [12]. The crusts of three breads and eight bread rolls, respectively, were combined, weighed, and dried for 24 h in a drying chamber at 30°C. Dried samples were weighed again to determine yield and moisture content and ground to a fine powder using a Grindomix GM 200 laboratory mill (Retsch, Haan, Germany). To avoid thermal degradation of acrylamide, particular attention was paid to the temperature which did not exceed 40°C.

An aliquot of 10 g of each sample powder was transferred into a 250 mL Erlenmeyer flask. After addition of 100 mL water and 500 µL of internal standard (methanolic solution of deuterium-labeled acrylamide, 20 µg/mL), the sample was extracted in an ultrasonic bath for 10 min, which was adjusted to 40°C to assure constant extraction conditions. During extraction, the flasks were mixed for 5 s every 2 min. Subsequently, 1 mL Carrez I (0.4 mol/L) and Carrez II (1.25 mol/L) solutions were added, the extracts were thoroughly shaken and filtered through a $595^1/_2$ filter paper (Schleicher and Schuell, Dasseln, Germany).

For removal of nonpolar compounds, SPE was performed using Isolute Multimode cartridges activated with 10 mL methanol and subsequently rinsed with 10 mL water. To remove excessive water, air was passed through the column for 20 s. Cartridges were loaded with 25 mL of the extract and the eluate was collected.

Preliminary tests using liquid-liquid extraction with ethyl acetate in a separating funnel revealed emulsion formation as a major problem during sample preparation. Therefore, solid-phase-supported liquid-liquid extraction was performed using Chem Elut cartridges filled with Hydromatrix, a high-purity, inert diatomaceous earth. Aliquots of 20 mL of the SPE eluate were loaded on the cartridges and eluted after 5 min using 100 mL ethyl acetate. The eluate was collected in a pointed flask and the volume was reduced

to about 2 mL on a rotary evaporator at 30°C and 100 mbar. The remaining solvent was removed by a gentle stream of nitrogen to avoid analyte loss. The residues obtained were resuspended in 1 mL water and filtered through a 0.45-µm syringe filter into a 1-mL pointed amber glass autosampler vial for LC-MS/MS analysis.

2.4 LC-MS/MS analysis

LC-MS/MS analysis was carried out using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1379A degasser, a model G1312A binary gradient pump, a model G1313A autosampler, a model G1316A column oven, and a model G1315A diode array detector. The LC system was coupled on-line to a Bruker (Bremen, Germany) Esquire 3000+ IT mass spectrometer fitted with an ESI source. Data acquisition and processing was performed using Esquire Control software. The separation was carried out with a Hypercarb column (100 mm × 2.1 mm × 5 µm) (Thermo Hypersil, Dreieich, Germany), equipped with a C_{18} guard column (4.0 × 3.0 mm) (Phenomenex, Torrance, CA, USA) operated at 30°C. Detailed LC-MS/MS conditions including tune parameters are given in Table 1. The mobile phase was 1% ACN/0.05% formic acid in water (eluent A) at a flow rate of 0.2 mL/min and the total run time was 10 min. After six sample runs the following cleaning program was applied: 100% eluent B (80% ACN/20% water) isocratically (17 min), from 100% B to 100% A (1 min), and reconditioning with 100% A (7 min). The mass spectrometer was operated in the positive ion mode. Nitrogen was used both as drying and nebulizing gas. Helium was used as collision gas for CID at a pressure of 4.0×10^{-6} mbar. Signals at *m/z* 72.3 (acrylamide) and *m/z* 75.3 (deuterium-labeled acrylamide) were isolated with a peak width of *m/z* 0.6. For the transitions *m/z* 72.3 > *m/z* 55.5 and *m/z* 75.3 > *m/z* 58.5 the fragmentation amplitude was set at 1.55 V and 1.50 V, respectively. Masses were recorded using multiple reaction monitoring (MRM). For quantitation the signals at *m/z* 55.5 (*m/z* 58.5) were used, while signals at *m/z* 44.5 (*m/z* 45.5) served for qualification.

2.5 Quantitation

Acrylamide was quantified using a linear calibration curve established with standard solutions of acrylamide dissolved in water. Concentrations were 1.2, 2.4, 3.0, 4.5, 6.0, 9.0, 12.0, 18.0, 24.0, and 36.0 µg/L. Each solution contained 100 µg/L D_3-acrylamide. The same concentration was used as an internal standard during sample preparation. Calibration standards were also subjected to liquid-liquid extraction.

Table 1. LC-MS/MS conditions for acrylamide analysis

LC	
Column	Hypercarb (100 mm × 2.1 mm × 5 µm) with C_{18} guard column (4.0 × 3.0 mm)
Column temp.	30°C
Mobile phase	(A) 1% ACN/0.05% formic acid in water, (B) 80% ACN/20% water
Injection volume	40 µL
Flow rate	0.2 mL/min
Total run time	10 min
MS	
Ionization	Electrospray
Polarity	Positive
Scan range	*m/z* 30–400
Nebulizer	Nitrogen; 30.0 psi
Dry gas	Nitrogen; 8 L/min; 365°C
Capillary exit	50.0 V
Capillary HV	2238 V
Trap drive	30.1
Skimmer	25.75 V
Octopol RF	30.0 Vpp
MS/MS	
Isolation	*m/z* 72.3, *m/z* 75.3; peak width *m/z* 0.6
Fragmentation	Cut-off/amplitude: 35/1.55 V (*m/z* 72.3); 35/1.50 V (*m/z* 75.3)

2.6 Recovery studies

Recovery studies were performed by spiking samples of powdered crusts with suitable amounts of standard stock solutions of acrylamide prior to extraction. These samples were subjected to the method described above. Recovery was determined in triplicate with double injection of each sample.

2.7 Comparative analyses

The accuracy of the method was further assessed through small-scale comparative analyses. Four samples were analyzed by each laboratory at the same time. Sample preparation and method parameters of the CVUA Stuttgart using a triple quadrupole mass spectrometer have been reported previously [19]. Calibration was extended to 300 µg/L acrylamide due to the expected high levels in gingerbread.

3 Results and discussion

3.1 Methodology

The objective of this study was to develop a sensitive and reproducible method for acrylamide determination at concentrations around 30 µg/kg using an IT mass spectrometer. As shown in an earlier study [12], more than 99% of the

acrylamide present in bread is found in the crust. Because acrylamide is not formed at temperatures below 100°C [20], trace amounts detected in bread crumbs may result from improper separation of crumbs and crusts. This assumption was confirmed by our own investigations. Crumbs and crusts of bread baked at 260°C for 70 min were very carefully separated and analyzed as described. Acrylamide was found in crusts at concentrations of 510 μg/kg, whereas it could not be detected in the crumbs. Therefore, the separation of crumbs and crusts facilitated an improved LOQ by higher concentration of the analyte.

Drying the crusts resulted in further analyte enrichment due to water loss and also eased grinding of the samples. Degradation of acrylamide during this step was not observed (data not shown). Ground samples of bakery products were extracted with water as previously described without further modification. Defatting with an organic solvent such as n-hexane was not required due to the low fat content of the samples. Extraction of acrylamide employing an ultrasonic bath was shown to be advantageous in previous studies [19]. Furthermore, Carrez precipitation and SPE clean-up using multimode sorbents are well established in

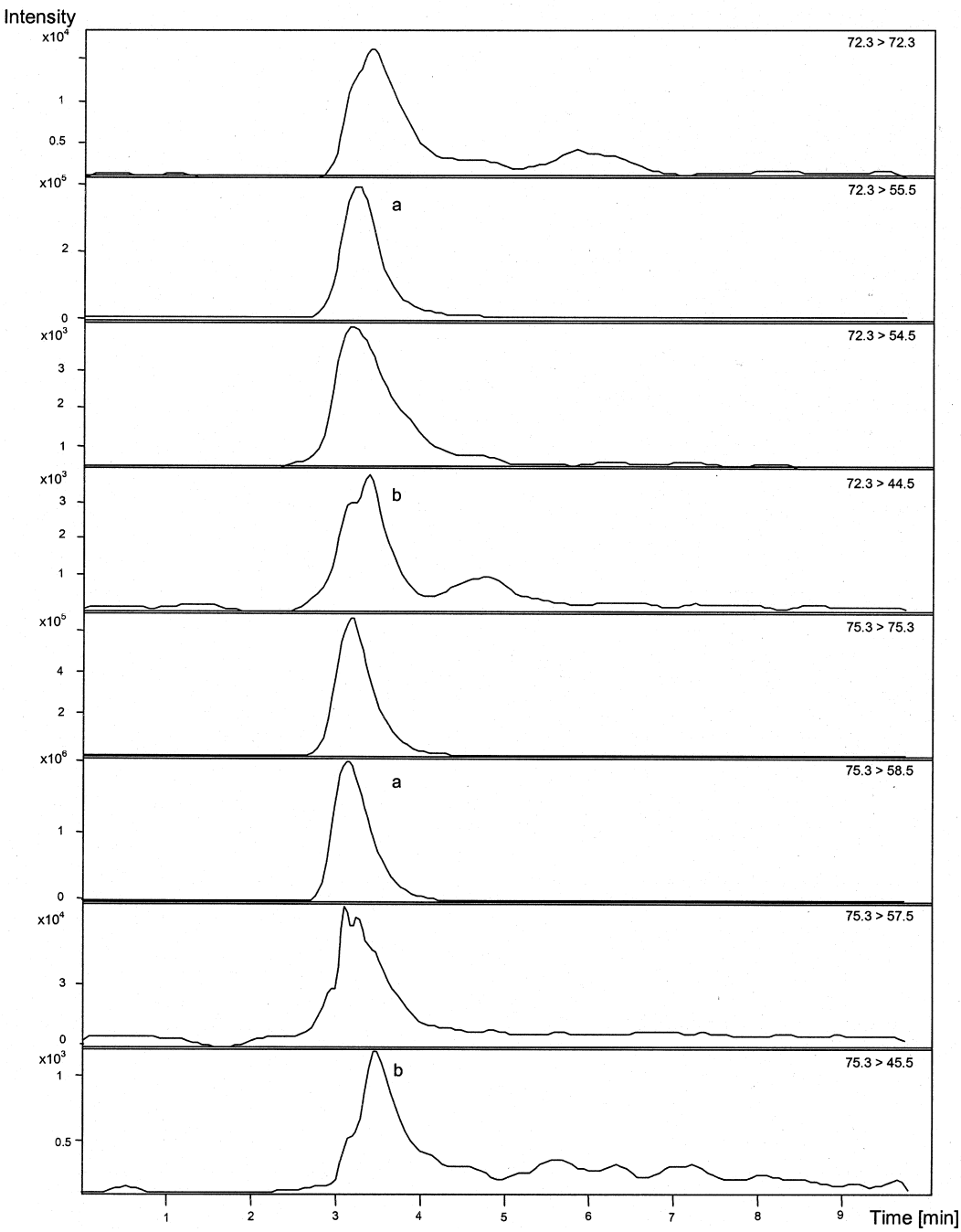

Figure 1. MRM chromatograms obtained from calibration standard of acrylamide (36 μg/L) and D_3-acrylamide (100 μg/L). (a) Signals used as quantifiers, (b) signals used as qualifiers.

acrylamide analysis [1, 21]. However, it is expected that instead of Isolute cartridges conventional C_{18} phases would also work properly with this method.

LC-MS/MS analyses were carried out using an IT mass spectrometer operated in positive ion mode. Multiple reaction monitoring of the transitions of certain precursor ions to their product ions provided a high selectivity and sensitivity. Figure 1 shows signal intensities of a model mixture of acrylamide and D_3-acrylamide. Because of their relatively high intensities, product ions at *m/z* 55.5 and *m/z* 58.5 (internal standard), resulting from a loss of NH_3, were used for quantitation. The transitions *m/z* 72.3 > *m/z* 44.5 and *m/z* 75.3 > *m/z* 45.5, respectively, indicating the loss of C_2H_4, were used to confirm peak identity. The transitions *m/z* 72.3 > *m/z* 54.5 and *m/z* 75.3 > *m/z* 57.5 (loss of H_2O) could only be found at higher concentrations of acrylamide and deuterated acrylamide, respectively, and were therefore not appropriate for peak confirmation. Using the present method, product ions at *m/z* 27 and *m/z* 29 (loss of $CONH_3$), respectively, previously reported to be good qualifiers [22], could not be detected due to the lower limit of the scan range at *m/z* 30. Additionally, the retention times of the reference compounds were used to confirm peak assignment.

To improve the LOQ, samples were concentrated using a solid-phase-supported liquid-liquid extraction. Because acrylamide solubility is considerably higher in water than in ethyl acetate, a significantly larger volume of the eluent compared to the primary sample extract had to be applied. Preliminary experiments had shown that a fivefold higher volume was adequate. Figure 2 shows the signal intensities of the product ion at *m/z* 55.5 of a toast extract spiked with 100 μg acrylamide/kg prior to sample preparation. As can clearly be seen, signal intensity was significantly higher after liquid-liquid extraction. Figure 3 presents the calibration curve for acrylamide determination with analyte concentration exhibiting a very good linearity ($r^2 > 0.99$). Surprisingly, the slope of the calibration curve obtained with the new method was higher compared to the conventional method. The peak areas of the analyte were almost doubled after concentration, whereas areas of the internal standard increased threefold, resulting in a lower peak area ratio compared to the usual method. The isotopic effect probably results from different solubilities of acrylamide and deuter-

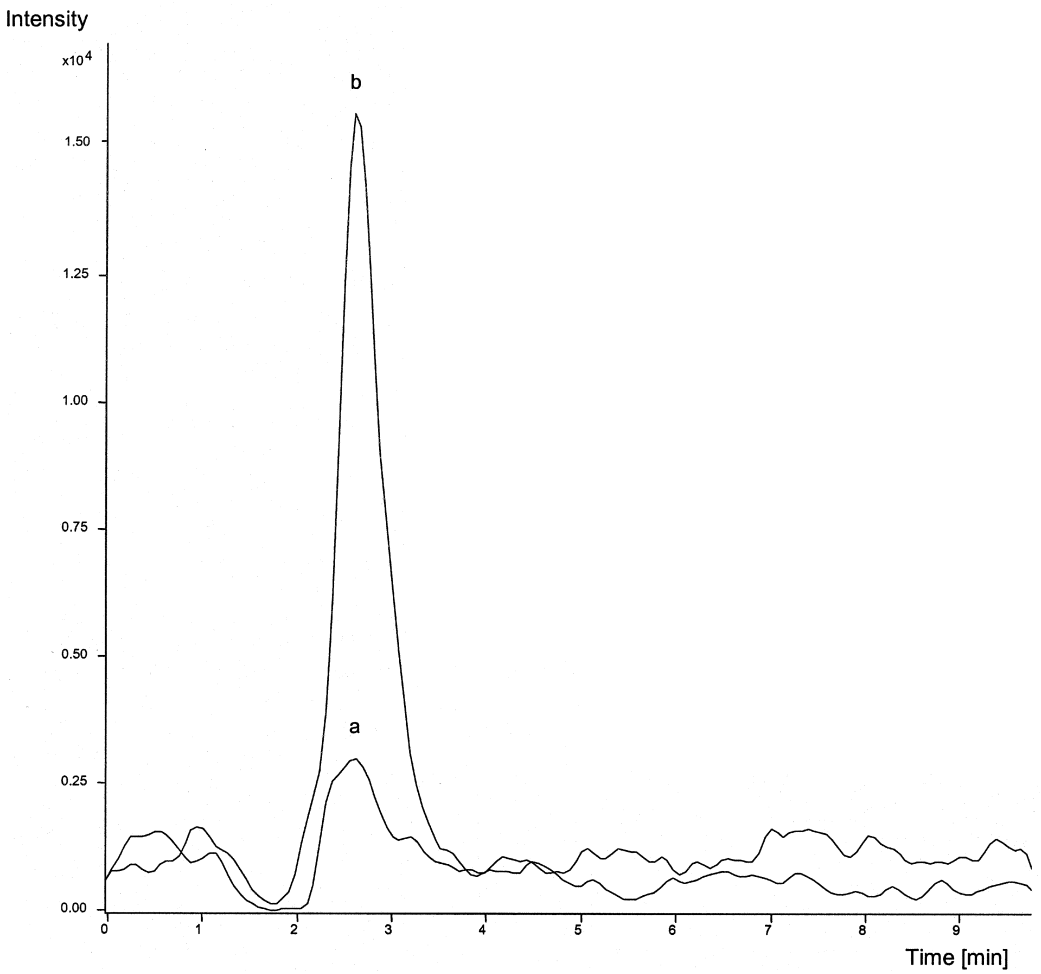

Figure 2. Extracted ion chromatograms of *m/z* 55.5 (quantifier for acrylamide) obtained from a toast sample spiked with 100 μg acrylamide/kg. (a) The sample without further concentration, (b) the sample after solid-phase-supported liquid-liquid extraction.

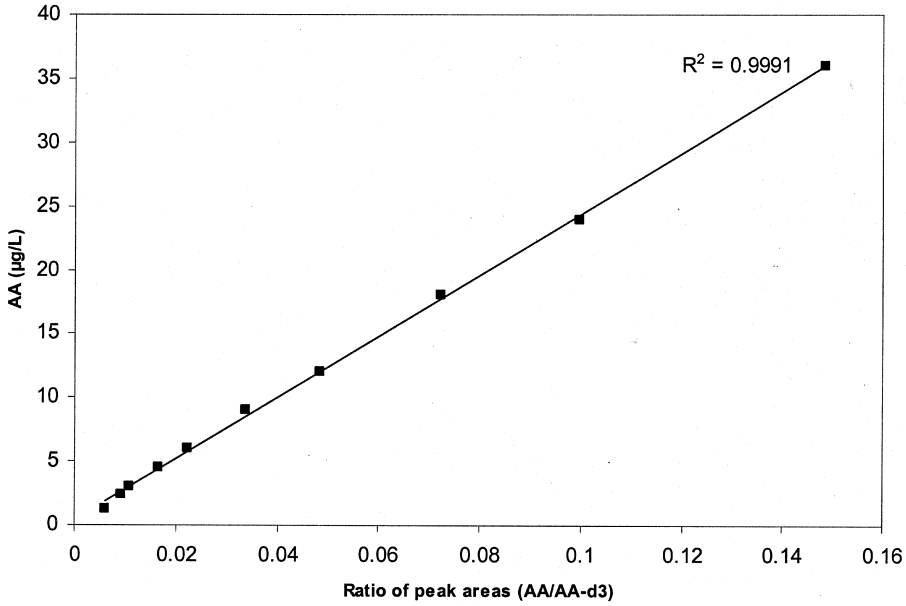

Figure 3. Calibration curve for acrylamide analysis by LC-MS/MS after a solid-phase-supported liquid-liquid extraction.

ated acrylamide in ethyl acetate. Even though all results are compensated for this effect, since both sample extracts and calibration standards are subjected to liquid-liquid extraction, this might introduce a risk of erroneous results. However, for all bread samples in the entire validation, ranging from 18 µg/kg to over 2000 µg/kg, the results were stable regarding accuracy as well as precision.

As mentioned above, IT mass spectrometers have been considered less reliable than quadrupole instruments in quantitative analysis [16]. Therefore, the mass spectrometer has to be precisely adjusted to the target ion. Especially scan range, trap drive, octopol RF, and capillary HV turned out to be critical parameters. Peak width also proved to be crucial. Setting the isolation width at *m/z* 0.6 provided optimal results. With an increased width, substances eluting shortly before acrylamide were interfering. A mobile phase containing 1% ACN/0.05% formic acid in water was chosen for LC-MS/MS analysis as described recently [19]. ACN was added to the eluent at low concentrations because improved ionization of acrylamide in the ESI source was observed, whereas addition of methanol resulted in peak broadening. Combining all these tuning steps, the IT system was found to be suitable for quantitative analysis of acrylamide even at trace levels.

3.2 Validation

The analytical method was validated for bread, bread rolls, toast, and alkali-baked bread rolls containing acrylamide at levels between 18 and 100 µg/kg. Samples were spiked with acrylamide for recovery tests at concentrations of 100–400 µg/kg. An additional test with moderately baked bread rolls spiked at concentrations between 5 and 25 µg/kg was performed to demonstrate the applicability of the method at trace levels, confirming the suitability of the method in the range of analyte concentrations usually found in bakery products (18–500 µg/kg). Although most samples used in this study could have been analyzed without concentration, the new method was applied because bakery products often show acrylamide contents lower than 25 µg/kg.

Standard curves for LC-MS/MS analyses were calculated by linear regression using the peak area ratios of the analyte product ion at *m/z* 55 and of the internal standard product ion at *m/z* 58. The calibration curve was linear in the range studied with a coefficient of variation (r^2) usually above 0.99. Detailed studies showed that linearity was also given when concentrations were increased up to 500 ng/mL. LOD and LOQ were calculated from the confidence interval of the calibration according to DIN 32645 using the program DINTEST. For all matrices investigated, LOD was found to be 0.55 ng/mL (~5.5 µg/kg) and LOQ to be 1.7 ng/mL (~17.0 µg/kg). Considering the separation of crumbs (~40% of weight) and crusts (~60% of weight), an LOQ of ~10.2 µg/kg product (LOD ~3.3 µg/kg) was achieved with the new method. Because toast cannot be separated in crumbs and crusts, the LOQ in such products is 17.0 µg/kg. Without concentration, an LOQ of ~27.0 µg/kg was obtained, which is in good agreement with recently published methods [15, 21]. Therefore, compared to the usual sample preparation, the new method provided an at least 1.6-fold improved LOQ.

The repeatability, recovery, and intermediate reproducibility obtained with the improved method according to ISO 17025 are shown in Tables 2–5. The repeatability (Table 2) was determined with the samples mentioned above. After homogenization with a laboratory mill, they were mixed in

Table 2. Repeatability test of the LC-MS/MS method

Sample	Acrylamide (μg/kg)			Mean (μg/kg)	CV (%)
Bread	72.3	67.3	80.0	73.2	8.7
Bread rolls	106.5	93.6	99.6	99.9	6.5
Alkali-baked bread rolls	53.5	56.7	53.3	54.5	3.5
Toast	91.7	100.2	91.1	94.3	5.4

Table 3. Recovery tests of the LC-MS/MS method ($n = 6$)

Sample (incurred acrylamide content, μg/kg)	Acrylamide added (μg/kg)	Average content measured (μg/kg)	Mean recovery (%)	CV (%)
Bread	100	177.4	103.8	7.5
(73.2)	200	287.4	107.1	2.8
	300	377.7	101.4	12.1
	400	515.2	110.4	8.9
Bread rolls	100	201.9	101.8	7.2
(99.9)	200	300.1	100.2	2.9
	300	403.1	101.0	6.2
	400	515.3	103.8	9.5
Alkali-baked bread	100	162.5	107.7	8.9
rolls	200	259.4	102.7	4.3
(54.5)	300	339.3	94.9	0.7
	400	443.7	97.2	2.3
Toast	100	190.9	94.4	5.3
(94.3)	200	296.0	100.9	6.1
	300	421.4	109.0	0.9
	400	501.1	101.6	2.8

Table 4. Recovery test at trace level spiking ($n = 6$)

Sample (incurred acrylamide content, μg/kg)	Acrylamide added (μg/kg)	Average content measured (μg/kg)	Mean recovery (%)	CV (%)
Bread rolls	5	23.3	93.2	5.8
(18.7)	10	28.6	99.7	8.0
	25	41.7	98.0	0.2

Table 5. Intermediate reproducibility test of the LC-MS/MS method

Sample	Acrylamide content analysis I (μg/kg)	Acrylamide content analysis II (μg/kg)	Mean (μg/kg)	CV (%)
Bread	72.2	73.3	72.8	1.1
Bread rolls	107.2	105.8	106.5	0.9
Alkali-baked bread rolls	53.8	52.7	53.3	1.5
Toast	88.0	94.2	91.1	4.8

a laboratory shaker. Extraction, sample clean-up and analysis were performed in triplicate. CV were calculated to be ~6.0% (3.5–8.7%). For the determination of recovery rates, samples were cleaned-up in triplicate and the sample solution was injected twice. Results are shown in Table 3. To verify that the method works properly at low acrylamide levels, another sample of moderately baked bread rolls was spiked at concentrations between 5 and 25 μg/kg. As can be seen from Table 4, the method shows good results even at very low analyte concentrations. Mean recovery of all samples was 102.4% (CV = 4.5%). Recovery of samples prepared without concentration of the analyte was comparable (97.8%), but CV was slightly inferior (~8.0%). Intermediate reproducibility of the method is shown in Table 5. For each matrix a sample was cleaned-up and analyzed on 2 different days by different laboratory staff. CV was determined to be in the order of 2.1% (0.9–4.8%).

Table 6. Comparative analyses of the LC-MS/MS method[a)]

Sample	Hohenheim University (μg/kg)	CVUA Stuttgart (μg/kg)
Crispbread	54 ± 2	47 ± 3
Butter cookies	179 ± 14	158 ± 5
Heated flour	2004 ± 112	1812 ± 51
Gingerbread	2381 ± 212	2177 ± 78

a) Each value represents the mean of triplicate determination

Comparative analyses by the CVUA Stuttgart using a quadrupole mass spectrometer [19] and our laboratory for comparison of the results showed excellent agreement (r^2 = 0.999). Deviation from values found by CVUA ranged from 109% to 115%. Detailed data are shown in Table 6. Stability of the method in long runs was also tested. A sample was injected 35 times (6 h runtime) with good results (CV = 3.8%).

4 Concluding remarks

In summary, the results presented in this study demonstrate that ESI-MS/MS is applicable to the quantitative analysis of acrylamide in bakery products. Oversaturation of the trap with ions has not been observed using this method. Concentration of the analyte using a solid-phase-supported liquid-liquid extraction with ethyl acetate combined with the separation and drying of crumbs allowed the quantitation of acrylamide at levels ≥10.0 μg/kg, thus resulting in an almost twofold improvement of the sensitivity compared to previously published methods. This is of particular relevance for investigating acrylamide formation at trace levels, especially in bakery wares.

Preliminary investigations with coffee showed that the method is also applicable to acrylamide analysis in more complex matrices.

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