Determination of Acrylamide in Bread by Gas Chromatography – Tandem Mass Spectrometry

Received for publication, May 22, 2014
Accepted, June 20, 2014

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Abstract

In this paper are presented results of the experiments to develop a GC/MS/MS method to determine acrylamide in bread.

Method is based on sample extraction in water, at acidic pH, purification with Carrez solutions and centrifugation. The obtained extract is derivatized with potassium bromide and the saturated bromine-water solution. Dibromurated derivative, 2,3-dibromopropionamide (2,3-DBPA) is extracted with ethyl acetate:hexane (4:1, v/v) and then concentrated, purified and eluted with acetone. The obtained extract is concentrated and residue of 2,3-DBPA is re-dissolved with ethyl acetate and triethylamine and converted in monobromurated derivate, 2-bromopropenamide (2-BPA), stable compound at GC/MS/MS analysis. Separation, detection, identification and quantification of 2-BPA are achieved by GC/MS/MS, on capillary column based on polyethylene-glycol, 30 m x 0.25 mm x 0.25 µm, by using internal standard method. Triple quadrupole mass spectrometer is operated positive electron impact ionization mode (EI+), and selected mode of acquisition "Selected Reaction Monitoring - SRM" and monitored transitions from a precursor ion to a product ion. In order to quantify there were used transitions m/z 151 → 70 (for 2-BPA) and m/z 154 → 73 (for 2-BPA-13C3), respectively.

There were assessed and estimated some of the performance characteristics of the method: linearity, working range, linearity range, accuracy, precision and sensitivity.

Keywords: acrylamide, GC/MS/MS, performance characteristics, derivatization, bread

1. Introduction

In April 2002, scientists from the National Food Administration in Sweden, together with those of the University of the Stockholm have raised fears nationwide, finding that people consume acrylamide through their diet, the consumption of common foods, such bread, biscuits, chips, coffee, etc., at much higher levels than allowed dose in drinking water. Swedish results were quickly confirmed by working groups consisted of experts from the World Health Organization, Food and Agriculture Organization, United Nations Organization, National Center for Food Safety and Technology, etc. They identified and listed research priorities and potential risks of acrylamide in food on human health (1).

Acrylamide has been classified by the International Agency for Research on Cancer (IARC) as ‘probably carcinogenic for humans’ (2) and recognized by the European Union (EU) Scientific Committee on Food (3) as a genotoxic carcinogen.

Acrylamide, AA (CH2=CHCONH2, CAS 79-0601) is a chemical process contaminant, that is formed when foods are subjected to processes by roasting, baking at temperatures above 120°C, the result of chemical reactions between a series of specific amino acids (e.g. asparagine) and compounds with carbonyl groups (e.g. glucose, fructose, maltose). This reaction, known as the Maillard reaction, gives to foods, on the one hand, the corresponding
colour and flavour, and, on the other hand, leads to the formation of the undesired compound acrylamide(4, 5).

AA is acrylic acid amide, with structure and chemical formula (figures 1 and 2):

![Figure 1. Structural formula of acrylamide](image1)

![Figure 2. Chemical formula of acrylamide](image2)

C₃H₅ON

**Figure 1.** Structural formula of acrylamide  **Figure 2.** Chemical formula of acrylamide

AA is an organic compound which is solid, stable at room temperature with a melting point of 84.5°C, high boiling point (136°C) and high solubility in water (2155 g/L at 30°C), in alcohols (in methanol, 1550 g/L, in ethanol, 860 g/L) and other polar organic solvents (in acetonitrile, 396 g/L, in ethyl acetate, 126 g/L and in acetone, 63 g/L). Acrylamide is stable at acidic pH and unstable at basic pH, sensitivity at light.

Content of acrylamide in food is variable, depending on raw material, manufacturing recipe, processing conditions, etc., with values of 20 – 40 μg/kg in bread to 100 – 3770 μg/kg in potato chips(6).

In recent years, at international level there were developed numerous methods for quantification of acrylamide in food. Classical approaches for determination of acrylamide are based on HPLC-MS/MS or GC-(CI/EI)MS chromatographic techniques, with and without derivatization(7, 8, 9, 10, 11, 12). Complexity of food matrices and toxicity of this process contaminant require development of some analysis methods, sensitive and selective for proper separation, detection and quantification in food.

Considering these, research of present paper were focused, on one side, on the development of sensitive GC/MS/MS method, for determination of acrylamide level in bread, and on the other side, on the evaluation of some performance characteristics of the developed method, in order to further validation.

2. Materials and methods

2.1. Food matrices

In order to determine acrylamide in bread there were used soft bread and toast bread samples, purchased from the retailers on the market in Bucharest and Romania, and also wheat bread, made within the Pilot Experiments Plant for Cereals and Flours Processing of INCDBA – IBA Bucharest.

2.2. Reagents and materials

There were used standard solutions of native acrylamide, min.99% purity, of concentration 1000 μg/ml in methanol (1000 ULTRA SCIENTIFIC Analytical Solution), standard solutions of labelled acrylamide (1,2,3-13C), min.99% purity (+100 ppm hydroquinone) of concentration 1000 μg/ml in methanol (Cambridge Isotope Laboratories, Inc.) and reagents of chromatographic purity: florisil 60 - 100 mesh, anhydrous sodium sulfate, triethylamine, n–hexane, acetone, ethyl acetate; 48% hydrobromic acid, potassium bromide, bromine(min. 99.5%), sodium thiosulfate pentahydrate, acetic acid (glacial) 100%, potassium hexacyanoferrate(II) trihydrate(Carrez I), zinc sulfate heptahydrate(Carrez II).

In experiments there were used the following materials: clear glass vials, 250 μl, fused insert, target snap–lt 11 mm crimp/snap, polypropylene tubes for centrifuge with screw-cap of 50 mL, Pasteur pipette, microfilters of regenerated cellulose of 0.2 μm (Spartan 13RC) with Ø 17 mm, Eppendorf Reference micropipettes tips, glass wool, etc.
2.3. **Sample preparation** The steps of method for determination of acrylamide in bread were: sample preparation, acrylamide extraction and derivatization, dibromo derivative extraction, extract concentration and purification, and GC/MS/MS analysis, presented in figure 3.

**Figure 3.** Diagramm with steps of the method for determination of acrylamide in bread by GC/MS/MS.
2.4 Parameters and conditions GC/MS/MS of method for determination of acrylamide in bread

Acrylamide was quantified in bread by gas chromatography tandem mass spectrometry after derivatisation according to Pittet et al. (7) method with modifications, using electron impact ionization mode, positive (EI+). "Selected Reaction Monitoring - SRM" mode of acquisition. Acrylamide quantification was performed by the internal standard method. The calibration curve was constructed by plotting the area ratio $A_{aaN}/A_{aaM}$ against $C_{aaN}$, where $A_{aaN}$ is the area of unlabelled acrylamide (2-bromopropenamide, 2-BPA), as mass trace $m/z$ 70 and $A_{aaM}$ is the area of labelled acrylamide (2-bromopropenamide, 2-BPA-$^{13}$C$_3$), as mass trace $m/z$ 73. $C_{aaN}$ is concentration of 2-BPA. The calibration curve was prepared in the range of 5 – 640 µg/kg. In the first step, the precursor ions with $m/z$ of 151 and 154 were derived from 2-BPA and 2-BPA-$^{13}$C$_3$, respectively. Their collisions gave rise to daughter ions with $m/z$ of 70 (from the ions with $m/z$ of 151) and 73 (from the ions with $m/z$ of 154). The calculation of acrylamide concentration in the tested samples was based on the ratio of the surface areas under the peaks corresponding to the ions with $m/z$ of 70 and $m/z$ of 73.

- Separation: *capillary column* based on polyethylene - glycol: Trace GOLD, TG-WAXMS, 30 m (length) x 0.25 mm (inside diameter) x 0.25 µm (film thickness) (Thermo)
- Identification: acrylamide internal standard, labelled with 1,2,3-$^{13}$C of 99% purity
- Carrier gas: Helium (purity 99.9995%), flow rate of 1.6 mL/min.
- Injection volume: 1 µl
- Injection mode: split, split ratio of 10:1
- Temperature on injector: 220°C
- Temperature programme on the oven: 65°C for 1 min, then programmed at 15°C/min to 170°C, 5°C/min to 200°C, followed 40°C/minute to 240°C, and held for 5 min at 240°C
- The GC-MS/MS interface transfer line was held at 230°C
- Ionization mode: electron impact positive (EI+)
- Electron energy: 70 eV (EI mode)
- Mode of acquisition: "Selected Reaction Monitoring - SRM"
- Characteristic fragmentation transitions: *Parent mass*: $m/z$ 151 (for 2-BPA) and $m/z$ 154 (for 2-BPA-$^{13}$C) and *Product mass*: $m/z$ 70 (for 2-BPA) and $m/z$ 73 (for 2-BPA-$^{13}$C)
- Collision gas: Argon
- Collision gas pressure (Q2 CID Gas): 1 mTorr

3. Results and discussions

3.1. Linearity of the method for determination of acrylamide in bread was verified by the method of least squares (linear regression method), choosing calibration with internal standard. It was achieved a calibration curve, on real bread samples, with 8 calibration levels (each in three replicates), in the concentrations range 5 – 640 µg/kg.
Figure 4. Calibration curve to determine acrylamide in bread by GC/MS/MS, in the concentrations range 5 – 640 µg/kg

Parameters of linear regression curve in the range 5 – 640 µg/kg (figure 4) obtained are:

- Linear regression equation: \[ Y = 0.178526 + 0.00973442X \]
- Correlation coefficient: \( R = 0.9997 \)
- Regression coefficient: \( R^2 = 0.9998 \)
- Slope: \( b = 0.00973442 \)
- Intercept (intersection with the axis OY): \( a = 0.178526 \)

3.2. **Working range** is the range between inferior and superior concentrations of the interest analyte in the test sample for which it was demonstrated that the procedure has a proper level of linearity. Taking into consideration the acrylamide concentrations range, the calibration curve was carried out, working range of the method for determination of acrylamide in bread, by GC/MS/MS, was 1.67 – 640 µg/kg. The inferior limit of the working range is represented by the limit of detection, which was 1.67 µg/kg under the method conditions.

In the range 1.67 µg/kg – 640 µg/kg the proposed chromatographic method presented linearity between response factor and the analyte concentration in sample, characterised by regression coefficient, \( R^2 = 1 \).

3.3. In order to establish **accuracy (recovery)**, in the case of method for determination of acrylamide, by GC/MS/MS, bread samples (different assortments), at which acrylamide concentration was lower than 5 µg/kg, were spiked with acrylamide working solution I and II, with concentration of 10 mg/l and 1 mg/l, respectively, in the following concentrations: 0 µg/kg; 5 µg/kg; 10 µg/kg; 20 µg/kg; 40 µg/kg; 80 µg/kg; 160 µg/kg; 320 µg/kg; 640 µg/kg. Each sample was achieved in 4 - 10 parallel samples. These samples were processed according to the work protocol of method, there were run, processed and quantified with Xcalibur Programme software, on the calibration curve 5 – 640 µg/kg and were calculated: recovery for each sample; average recovery for each contamination level. After the results processing, there were obtained the following:

- Average recovery after correction of analyte lack with internal standard, for matrix with acrylamide content lower than 10 µg/kg was 101.59% (88.69 – 114.49%), being under the acceptance criteria -40% to +20% provided by CAC/GL 16-1993(13), Guide VICH GL49 (14).
- Average recovery after correction of analyte lack with internal standard, for matrix with acrylamide content between 10 µg/kg ÷ 100 µg/kg was 100.42% (99.27 – 101.22%), being under the acceptance criteria -30% to +10% provided by CAC/GL 16-1993(13), Guide VICH GL49 (14).
- Average recovery after correction of analyte lack with internal standard, for matrix with acrylamide content higher than 100 µg/kg was 99.43% (98.19 – 100.13%), being under the acceptance criteria -20% to +10% provided by CAC/GL 16-1993(13), Guide VICH GL49 (14).

3.4. Within this study, **precision** was assessed by: injection repeatability (system precision), analysis repeatability (method precision) and intermediate precision (intra-determination precision).

3.4.1. In order to verify the **injection repeatability** and the **equipment precision**, within the laboratory there were performed consequent injections (min. 5 injections) of some bread samples, in a short time (in the same day). There were calculated with Microsoft Excel Programme software, the following statistical parameters: **mean value** for acrylamide concentration determined by GC/MS/MS; **standard deviation**, under repeatability conditions, SD(r), µg/kg; **relative standard deviation** under repeatability conditions, RSD(r), %.

The obtained results are presented in table 1.
Table 1. Statistical parameters for repeatability (instrument precision), in the case of method for determination of acrylamide in bread by GC/MS/MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample name</th>
<th>No. consequent injections (n)</th>
<th>Mean value C(µg/kg)</th>
<th>SD(r) (µg/kg)</th>
<th>RSD(r) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Toast bread</td>
<td>10</td>
<td>60.150</td>
<td>1.182</td>
<td>1.965</td>
</tr>
<tr>
<td>2.</td>
<td>Intermediate bread, lot 1, direct procedure, spiked 150 µg/kg</td>
<td>6</td>
<td>177.587</td>
<td>2.858</td>
<td>1.609</td>
</tr>
<tr>
<td>3.</td>
<td>Bread whole wheat, lot 1, spiked 160 µg/kg</td>
<td>5</td>
<td>285.044</td>
<td>1.749</td>
<td>0.614</td>
</tr>
</tbody>
</table>

From Table 1 it is seen that the value of the relative standard deviation, RSD(r), obtained was lower than 2% and it can be said that at determination of acrylamide in bread by GC/MS/MS, system was precise.

3.4.2. To verify precision of the method, within the laboratory were analysed parallel bread samples, identical, in the same laboratory, by the same operator, using the same equipment and in a short time (the same day). Parallel bread samples were processed, analyzed, and calculated the statistical parameters from Table 2.

According to the obtained data, for acrylamide concentration lower than 10 µg/kg: RSD(r) was 6.861%. An acceptance criterion for RSD(r), according to CAC/GL 16-1993 (13) for this concentration range is 30% and according to Guide VICH GL49 (14) is 25%.

For acrylamide concentration between 10 µg/kg ÷ 100 µg/kg: RSD(r) was 2.006 – 3.375%. An acceptance criterion for RSD(r), according to CAC/GL 16-1993 (13) for this concentration range is 20%, and according to Guide VICH GL49 (14) is 15%. Values obtained in the laboratory conditions were lower than 4%.

For acrylamide concentration higher than 100 µg/kg: RSD(r) was between 1.411 – 2.467%. An acceptance criterion for RSD(r), according to CAC/GL 16-1993 (13) for this concentration range is 15% and according to Guide VICH GL49 (14) is 10%. Values obtained in the laboratory conditions were lower than 3%.

3.4.3. In the performed study it was evaluated also the intermediate precision. The independent results were obtained using the same procedure, on the identical samples, separately extracted each one by the same method, in the same laboratory, by different operators, using the same equipment, in different days. By intermediate precision it is verified if, in the same laboratory, using the same method, in different days, results are precise. There were calculated with Microsoft Excel Programme software, statistical parameters: mean value, standard deviation, SD(R) and relative standard deviation, RSD(R), under conditions of reproducibility for the acrylamide concentration determined.

Relative standard deviation obtained under study of the intermediate precision, in the laboratory conditions, was:

- For an acrylamide concentration lower than 10 µg/kg, it was obtained RSD(R) lower than 14%, the acceptance criterion for RSD (R) in this range is 32% (14).
- For an acrylamide concentration between 10 µg/kg ÷ 100 µg/kg, it was obtained RSD(R) = 2.663 – 9.452%, the acceptance criterion for RSD(R) in this range is 23% according to Guide VICH GL49 (14) and according to Commission Decision 657/2002 (15) is 20%.
- For an acrylamide concentration higher than 100 µg/kg, it was obtained RSD(R) = 1.964 – 3.925%, the acceptance criterion for RSD(R) in this range is 16% according to Guide VICH GL49 (14) and according to Commission Decision 657/2002 (15) is 15%.
Table 2. Statistical parameters for repeatability (method precision), in case of method for determination of acrylamide in bread

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample name</th>
<th>No. parallel samples (n)</th>
<th>C (µg/kg)</th>
<th>SD(r) (µg/kg)</th>
<th>RSD(r) (%)</th>
<th>Repeatability limit, r (µg/kg)</th>
<th>Confidence interval (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White wheat bread, spiked 10 µg/kg</td>
<td>4</td>
<td>8.872</td>
<td>0.609</td>
<td>6.861</td>
<td>1.705</td>
<td>5.048 ± 0.47</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate bread – direct procedure, lot 1</td>
<td>5</td>
<td>21.893</td>
<td>0.739</td>
<td>3.375</td>
<td>2.069</td>
<td>12.882 ± 1.21</td>
</tr>
<tr>
<td>3</td>
<td>Black wheat bread with sesame</td>
<td>4</td>
<td>53.813</td>
<td>1.079</td>
<td>2.006</td>
<td>3.021</td>
<td>32.887 ± 3.09</td>
</tr>
<tr>
<td>4</td>
<td>Bread whole wheat, lot 1</td>
<td>5</td>
<td>129.469</td>
<td>2.740</td>
<td>2.116</td>
<td>7.672</td>
<td>76.360 ± 7.25</td>
</tr>
<tr>
<td>5</td>
<td>Bread whole wheat, lot 1, spiked 40 µg/kg</td>
<td>5</td>
<td>165.154</td>
<td>2.331</td>
<td>1.411</td>
<td>6.527</td>
<td>97.407 ± 9.25</td>
</tr>
<tr>
<td>6</td>
<td>Bread whole wheat, lot 1, spiked 80 µg/kg</td>
<td>5</td>
<td>205.630</td>
<td>5.073</td>
<td>2.467</td>
<td>14.204</td>
<td>121.279 ± 11.52</td>
</tr>
</tbody>
</table>

*concentration is given by read concentration at apparatus (C) and is calculated depending on moisture of fresh bread and of dried bread crumb

Table 3. Statistical parameters achieved in the case of LOQ establishment

<table>
<thead>
<tr>
<th>Concentration spiked (µg/kg)</th>
<th>No. samples (n)</th>
<th>Mean value (µg/kg)</th>
<th>SD(r) (µg/kg)</th>
<th>RSD(r) (%)</th>
<th>Mean recovery (%)</th>
<th>Acceptance criterion for LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>5.685</td>
<td>0.392</td>
<td>6.903</td>
<td>142.117</td>
<td>Failure criterion</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5.012</td>
<td>0.622</td>
<td>12.419</td>
<td>167.057</td>
<td>Failure criterion</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>3.037</td>
<td>0.414</td>
<td>13.625</td>
<td>151.850</td>
<td>Failure criterion</td>
</tr>
</tbody>
</table>

3.5. Sensitivity of method was assessed by establishing of limit of detection (LOD) and limit of quantification (LOQ). LOQ is defined as being the lower quantity or concentration which can be determined with an acceptable level of repeatability and accuracy. In order to assess LOQ of method for determination of acrylamide in bread by GC/MS/MS, bread samples, in which acrylamide concentration was lower than 5 µg/kg, have been spiked with acrylamide work solutions II and III of 1 mg/l, 0.1 mg/l, respectively, in concentrations of: 10 µg/kg; 5 µg/kg; 4 µg/kg; 3 µg/kg; 2 µg/kg. For each level there were achieved 5 – 7 parallel samples and were calculated: relative standard deviation for repeatability, RSD(r), and recovery factor. The concentration at which the criteria were met simultaneously: relative standard deviation, RSD(r) lower than 20% and recovery factor in the range 75 – 125%, was declared LOQ. LOD was considered as being LOQ/3, at a signal/noise (S/N) >3.

The results were summarized in table 3. From the results, it was estimated that LOQ and LOD of the method for determination of acrylamide in bread by GC/MS/MS, were: LOQ = 5 µg/kg and LOD = LOQ/3 = 1.67 µg/kg; for value of 5 µg/kg (LOQ) there were met simultaneously those 2 criteria of acceptability, RSD(r) = 5.897%, recovery factor Rec. = 113.34%.

4. Conclusions

Within the Chromatography Laboratory of INCDBA-IBA Bucharest it was developed a method for determination of acrylamide in bread by GC/MS/MS and there were assessed the performance parameters of the method: linearity, working range, linearity range, accuracy (recovery), precision (system precision, method precision, intermediate precision) and sensitivity (LOD and LOQ).
Proposed method had good sensitivity, LOD =1.67 μg/kg and LOQ =5 μg/kg, and good precision, for a concentration lower than 10 μg/kg, coefficients of variation of \textit{repeatability} have been lower than 7%; for a concentration between 10 μg/kg \div 100 μg/kg, coefficients of variation of \textit{repeatability} have been lower than 4%; coefficients of variation of \textit{intermediate precision} for a concentration lower than 10 μg/kg, have been lower than 14%, and for a concentration in the range 10 μg/kg \div 100 μg/kg, coefficients of variation have been lower than 10%.

Mean recoveries obtained by spiking of some sorts of bread, with acrylamide solution, in the range 5 – 640 μg/kg, were between 99.43% - 101.59%.

Introduction into laboratory practice of a performance method GC/MS/MS, for determination of acrylamide in bread contributes to assessment of acrylamide presence in food on the Romanian market and warns against the risk of consumption of food which contain acrylamide.

\textbf{Acknowledgements}

This study was supported by the Ministry of National Education – State Authority for Scientific Research, Technological Development and Innovation, by Nucleu Programme PN 12 48, contract 48 N/2012.

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