Internal Validation of the Method for Determination of Acrylamide in Bread by Gas Chromatography Tandem Mass Spectrometry

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The paper presents practical application on true samples of the performance method for determination of acrylamide in bread. The proposed method was internally validated, establishing their performance parameters: linearity, working range, linearity range, selectivity, sensitivity, accuracy, measurement uncertainty, precision, robustness and extracts stability. In-house validation data for commercial and experimental bread samples showed good precision of the method, with repeatability and reproducibility relative standard deviations below 10%, a good sensitivity, LOD = 1.67 μg/kg, LOQ = 5 μg/kg and an average recovery of acrylamide from samples spiked at levels of 5 – 640 μg/kg and 50 – 1200 μg/kg ranged between 99.10 and 101.59% after correction of analyte loss by the internal standard. Method performance was verified by using a certified reference material, ERM-BD272, Crispbread – Acrylamide, 980 ± 90 μg/kg, achieving a bias of 6.33 μg/kg (0.65%).

Keywords: acrylamide, validation, bread, GC/MS/MS

Acrylamide, AA (CH_2=CHCONH$_2$, CAS79-0601) is a chemical process contaminant, that is formed when foodstuffs are subject to processes by roasting, baking, at temperatures above 120^{0}C, being the result of the chemical reactions of a number of specific amino acids (e.g. asparagine) and compounds with carbonyl groups (e.g. glucose, fructose, maltose) [1-4].

Acrylamide content of food varies from one category to another: 343 μg/kg in cereals and cereal-based products, 477 μg/kg in potatoes and processed potato products, 509 μg/kg in coffee and coffee substitutes, green tea, 19 μg/kg meat and offal, 17μg/kg in dried fruits and vegetables, etc., depending on raw material, recipe formulation, processing conditions, etc. For most countries, foods that contribute to the intake of acrylamide, through diet, were fried potatoes (16 - 30%), potato chips (6 - 46%), coffee (13 - 39%), bakery (10 - 30%) and pastry products (10 - 20%) [5-11].

In 1994, the International Agency for Research on Cancer (IARC) classified AA as „a carcinogenic potential in humans" (Group 2A), and in 2001, the Scientific Committee on Toxicity, Ecotoxicity and Environment demonstrated health risks and toxic properties of acrylamide, neurotoxicity, genotoxicity, carcinogenity and reproductive toxicity [12-18].

Since 2002, food industry, Member States and the European Commission have made considerable efforts to establish training modes, methods to reduce the levels of acrylamide in processed foods and the development of reliable methods of analysis, to quantify small concentrations of acrylamide in foods.

The European Commission considers that it is necessary that Member States'competent authorities to carry out investigations where the level of acrylamide found in a specific food exceeds "indicative values" laid down in the Recommendation 2013/647/2013. Surveys should include hazard analysis and critical control points, HACCP, to identify the relevant processing phases, they can form acrylamide and establish appropriate measures to control them [19-20].

In the recent years, internationally, many methods have been developed for the quantification of acrylamide in food. Most classical methods are based on high performance liquid chromatography or gas chromatography. Toxicity of this contaminant requires the development of methods of analysis sensitive and selective proper for separation, detection and quantification. In this regard, have been developed methods based on liquid chromatography coupled to mass spectrometry or gas chromatography coupled to mass spectrometry, with or without compound derivatization [21-26].

Although internationally have been and are being carried out researches related to this process contaminant, with serious danger to the health of consumer, in Romania, issues related to acrylamide in food have not enjoyed the same attention from authorities in the field or food industry. So far, in Romania, research related to this contaminat have been reduced and sporadically approached, analytical [27, 35] and toxicological aspect [28].

In this context, research of the present paper were directed on internal validation of a sensitive method, GC/MS/MS, for determination of the level of acrylamide in bread.

Experimental part

Food matrices

The experiments were carried out using samples of soft bread of wheat flour and other grains, purchased from retail traders on the market in Bucharest, Romania, and also were used bread samples made within the Pilot Experiments Plant for Cereals and Flours Processing of INCDBA – IBA Bucharest.

Reagents and consumables

There were used standard solutions of native acrylamide, min. 99% purity, of concentration 1000 μg/mL in methanol (1000 ULTRA SCIENTIFIC Analytical Solution), internal standards of labelled acrylamide (1,2,3-^{13}C), min. 99%

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purity (+100 ppm hydroquinone) of concentration 1000 μg/mL in methanol (Cambridge Isotope Laboratories, Inc.). All reagents used were of chromatographic purity, from Merck (Darmstadt, Germany), LGC Promochem GmbH (Wesel, Germany) and Scharlau (Spain). The water used was obtained from a purification system, PURELAB Option S7/15 and PURELAB Ultra Ionic.

Helium was the mobile phase used, min. 99.9995% (5.0) purity. The used collision gas, argon, was of min. 99.9995% (5.0) (purity).

In order to achieve concentrations under nitrogen atmosphere it was used nitrogen of min. 99.9995% (5.0) purity.

In experiments were used the following materials: microvial target crimp, 350 μL insert, 11 mm red alum crimp seal, tef/sil, amber vials of 40 mL (27.5 x 95 mm), polypropylene tubes for centrifuge with screw-cap of 50 mL, Pasteur pipette, microfilters of regenerated cellulose of 0.2μm (Spartan 13RC) with ∅17 mm, Eppendorf Reference micropipettes tips, glass wool, etc.

Preparation of stock solutions and working solutions

There were preparated, stock solutions of acrylamide (100 μg/mL) and solutions of (1,2,3-^{13}C)acrylamide (100 μg/mL), in amber vials of 40 mL, by diluting 100 μL standard of acrylamide/(1,2,3-^{13}C)acrylamide with 900 μL ultrapure water. All stock solutions were stored in a refrigerator at 4^{0}C, for maximum 3 months.

From the stock solutions of acrylamide and (1,2,3-^{13}C)acrylamide there were prepared by diluting with ultrapure water, working solutions I (10 μg/mL), II (1 μg/mL) and III (0.1 μg/mL) of acrylamide and working solution I of (1,2,3-^{13}C)acrylamide (10 μg/mL).

All working solutions were stored in a refrigerator at 4^{0}C, for maximum 1 month.

Equipment

In the conducted experiments there was used equipment of the Chromatography Laboratory of INCDBA – IBA Bucharest: Analytical balance, precision ± 0.0001g, Technical balance, precision 0.01 g, Switzerland; Retsch GM 200 mill and ZM 200 ultracentrifugal mill, Germany; Vortex Heidolph Multi Reax, Germany; Ultrasonic bath, type ELMASONIC S40H, Germany; Water bath with stirring, JULABO Labortechnik GmbH, Germany; 5804R Eppendorf centrifuge with cooling, Eppendorf, Germany; Evaporation system, complete with accessories, BUCHI Labortechnik AG, Germany, etc.

GC/MS/MS system was composed of: Gas chromatograph (TRACE GC ULTRA), Mass spectrometer (TSQ Quantum XLS), Autosampler (TRIPLUS), Dell computer with Dell monitor. For instrument control, data acquisition and processing was used Xcalibur Software (Thermo Scientific).

In order to achieve the bread samples there was used equipment from the Pilot Experiments Plant for Cereals and Flours Processing of INCDBA-IBA Bucharest, specific for manufacturing technology of bakery products: oven with two overlapping hobs, with controlled temperature and time baking (Mondial Forni, Mod. 4T – 40/60 UT25 + VAP), rotative oven with proofer (Gima Forni, Mod. Forno Rotor 60-80 ELETT. 50 kW), dough mixer of low capacity (Diosna, DM 08 – 4/6) and dough mixer of 60 kg capacity (Avancini S.P.A., Mod. MIX 60 2V), manual dough divider (Vitella, Mod. SQ SA 30), long dough moulder (Kolher Group, Mod. K – FRF – 500).

Chromatographic conditions

Separation, detection, identification and quantification of monobromurated derivative of acrylamide (2-bromopropenamide, 2-BPA) are achieved by GC/MS/MS, on capillary column based on polyethylene-glycol, Trace GOLD, TG-WAXMS (Thermo), 30 m x 0.25 mm x 0.25 μm, by using the internal standard method of the labelled acrylamide (1,2,3-^{13}C), 99% purity.

Helium was used as a mobile phase, with a flow rate of 1.6 mL/min. Injections by an TRIPLUS autosampler (1 μL), were made split mode (split ratio of 10:1). Temperature on injector, 220^{0}C; temperature on interface transfer line, 230^{0}C. Column temperature was held at 65°C for 1 min, then programmed at 15^{0}C/min to 170^{0}C, 5^{0}C/min to 200^{0}C, followed by 40^{0}C/min to 240^{0}C, and held for 15 min at 240^{0}C (total run time: 30 min). Under these conditions, the retention time was 10.65 min. ± 30 s. Mass spectrometer with triple quadrupole has operated in electron impact positive ionization mode (EI$^+$); mode of acquisition: "Selected Reaction Monitoring - SRM"; ion scanning mode - "Product".

The calibration curve was constructed by plotting the ratio A_{aaN}/A_{aaM} against C_{aaN}, in the range of 50 – 1200 μg/kg and 5 – 640 μg/kg, where A_{aaN} is the area of unlabelled acrylamide, 2-BPA, as mass trace m/z 70 and A_{aaM} is the area of labelled acrylamide, 2-BP($^{13}C_3$)A, as mass trace m/z 73. C_{aaN} is the concentration of 2-BPA. In the first step, the precursor ions with m/z 151 and 154 were derived from 2-BPA and 2-BP($^{13}C_3$)A, respectivelly. Their collisions gave rise to daughter ions with m/z 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 70 (2-BPA) and 73 (2-BP($^{13}C_3$)A).

Samples preparation

Samples preparation was achieved after derivatization according to Pittet et al. (2004) [21] with modifications.

Steps of the procedure for determination of acrylamide in bread by GC/MS/MS were: sample preparation, acrylamide extraction, bromination of acrylamide to 2,3-dibromopropionamide (2,3-DBPA), extract concentration and purification. 2,3-DBPA was converted to 2-bromopropenamide (2-BPA) by dehydrobromination with triethylamine before GC/MS/MS analysis (fig. 1).

Bread samples were dried in etuve, at 90^{0}C for 120 min, coarse milled at Retch GM 200 mill and then fine milled at ZM 200 ultra centrifugal mill, through a sieve having a mesh size of 0.2μm. Of the global fine bread crumb were weighing about 3 g, in a centrifuge vial of 50 mL. In each centrifuge vial was added working solution I of the internal standard (10 μg/mL) and working solutions I (10 μg/mL) and II (1μg/mL) of acrylamide, proper to calibration level. Then it was added water in a ratio sample:water of 1:10, at about 60^{0}C, homogenousing for 15 min at Vortex stirrer. After adjusting the *p*H to 4 – 5, by adding acetic acid (glacial, 100%), deproteinisation was been made with Carrez I (28.8 g potassium hexacyanoferrate(II) trihydrate/100 mL water) and Carrez II (57.6 g zinc sulfate heptahydrate/100 mL water) and, then, samples centrifugation 30 min, at 6000 rpm, 5^{0}C. The supernatant obtained, which contains extract of acrylamide has been transferred into a conical flask with ground glass stopper (Erlenmeyer) of 250 – 300 mL. Because acrylamide is not thermal stable and has low volatility, supernatant was derivatized with calcinated potassium bromide (7.5 g), ca. 100 μL of hydrobromic acid (*p*H 1-3) and 10 mL of saturated bromine-water solution

Fig. 1. Steps of the procedure for determination of acrylamide in bread by GC/MS/MS

(ca. 1.6%). The flask was covered with aluminum foil and transferred into an ice bath with stirring, at maximum 4^0C, minimum 2 h.

After bromination reaction finished, bromine in excess was removed by adding a few drops of 1M sodium thiosulfate solution (until the yellow color disappeared). 2,3-DBPA derivative was extracted from solution with a mixture ethyl acetate: hexane (4:1, v/v), then, the organic phase was filtered into a 150 mL round-bottom flask through glass wool covered with ca. 15 g of calcinated sodium sulfate, then were evaporated to ~ 2mL into a Buchi vacuum evaporation system (Rotovapor R-210; Vacuum pump V-700; Professional vacuum controller V-855; Heating bath, B-491; Chiller – F-108) with the following parameters: water bath temperature, 40^0C, cooling agent temperature, 0^0C, hexane vaporisation pressure, 156 mbar and 95 mbar for ethyl acetate), and then to dryness under a stream of nitrogen.

Purification of the 2,3-DBPA extract was performed as:

- Column preparation - on a glass column with L = 300 mm and ∅11 mm, equipped with teflon tap, there were introduced: a plug of glass wool, 5 g of activated Florisil with 5% water, 5 g of calcinated sodium sulfate;
- Column conditioning - glass column was washed with 20 mL of hexane;
- Residue purification - the residue was trasferred quantitatively in the glass column using small aliquots taken from 50 mL of hexane, and the effluent was discarded;
- Eluation with 150 mL acetone of the extract of the 2,3-DBPA .

The eluate was evaporated under vacuum, up to a volume of about 2 mL (water bath temperature, 40^0C, cooling agent temperature, 0^0C, acetone vaporisation pressure, 239 mbar), and then to dryness under a stream of nitrogen.

The purified residue of 2,3-DBPA was redissolved in 400 µL of ethyl acetate. 2,3-DBPA was converted to 2-BPA by dehydrobromination with triethylamine, compound much more stable at GC/MS/MS analysis [23], [26]. The final extract was filtered through a 0.2 µm microfilter (filter cell acetate, Thermo Scientific) in vial autosampler. A volume of 1µL was injected into the split mode for GC/MS/MS analysis.

Performance characteristics of the developed method

The procedure for internal validation of the method assumed the evaluation and completion of performance parameters through repeated measurements and interpretation of the obtained results depending on the established acceptance criteria [29].

There were assessed the following parameters of the method: linearity, working range, linearity range, selectivity, sensitivity (LOD, LOQ), accuracy, measurement uncertainty, precision, robustness and extracts stability [30 - 34].

Linearity of the method was verified by the method of least squares (linear regression method). The calibration curve was constructed by plotting the ratio A_{aaN}/A_{aaM} against C_{aaN}, in the range of 50 – 1200 µg/kg and 5 – 640 µg/kg, where A_{aaN} is the area of unlabelled acrylamide, 2-BPA, as mass trace *m/z* 70 and A_{aaM} is the area of labelled acrylamide, 2-BP($^{13}C_3$)A as mass trace *m/z* 73. C_{aaN} is the concentration of 2-BPA. 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* 70 (2-BPA) and 73 (2-BP($^{13}C_3$)A). There were determined the following parameters: *correlation coefficient* (R); *regression coefficient* (R^2); *linear regression equation* in order to verify the method linearity.

Working range is the range between inferior and superior concentration of the interest analyte in test sample for which it has been demonstrated that procedure has a proper level of linearity. The inferior limit of the working range is the LOQ.

Linearity range is the capability of an analytical method to allow, in a predetermined range, obtaining of variable results, directly proportional with acrylamide concentration in the sample. The correlation was assessed as linear at a coefficient value higher than 0.99. In order to determine the statistical parameters of the linearity range (linear regression equation, correlation coefficient (R), regression coefficient (R^2) it was used Microsoft Excel Programme.

Selectivity, performance parameter of the analytical method, represents the ability of method to differentiate and to measure the analyte of interest in the presence of other components that are expected to be present into the food matrix. To demonstrate method selectivity, in the laboratory there were checked possible interferences given by the analyte of interest in bread samples spiked with acrylamide solution. Thus, different bread samples were spiked with acrylamide and it was analyzed the presence of interferences (signals, peaks) in the area where the compound is eluted: *white wheat bread, bread whole wheat* spiked with 0µg/kg, 80 µg/kg, 160µg/kg working

Parameters	Working range	
	50 – 1200 µg/kg (fig. 2)	**5 – 640 µg/kg**
Linear regression equation	y = 0.318254 + 0.00734236x	y = 0.178526 + 0.00973442x
Correlation coefficient	R = 0.9994	R = 0.9997
Regression coefficient	R^2 = 0.9989	R^2 = 0.9998
Slope	b = 0.00734236	b = 0.00973442
Intercept (intersection with the axis OY)	a = 0.318254	a = 0.178526

Table 1
PARAMETERS OBTAINED FROM THE CALIBRATION CURVE OF THE METHOD FOR DETERMINATION OF ACRYLAMIDE IN BREAD BY GC/MS/MS

solution I of acrylamide (10 µg/L), *intermediate bread* spiked with 0 µg/kg; 5 µg/kg; 10 µg/kg working solution III of acrylamide (0.1µg/L). For these samples there were viewed chromatograms, being monitorised specific compounds for the target compound, 2-BPA (*m/z* 70) and for internal standard, 2-BP($^{13}C_3$)A (*m/z* 73).

Sensitivity reflects the ability of the method to analyze samples with low content of the compound of interest. 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. 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.

Accuracy

In this study, *accuracy of the method* was demonstrated through:

- evaluation of the *recovery* of the analyte of interest in matrices spiked with analyte of known concentration
- evaluation of *bias,* by using a CRM

Initially, not having a CRM, it was verified the accuracy by evaluation of the recovery of analyte in enriched food matrices. Different assortments of bread samples were spiked with working solutions I (10 µg/mL), II (1 µg/mL) and III (0.1µg/mL) of acrylamide, in different 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. In each sample it was added the same amount of internal standard. These samples were processed according to the working protocol of the method, were run, processed and quantified with the Xcalibur Programme, on the calibration curve 5 – 640 µg/kg and were calculated: recovery for each sample; average recovery for each level of contamination.

Also, in the experiments achieved, accuracy was verified through the use of a CRM: ERM-BD272, Crispbread – Acrylamide, with value certified by the producer (BAM-LGC-IRMM), μ_{MRC} = 980±90 µg/kg, determining the bias of the method. Bias is defined as, the absolute value of the difference between the mean value (X) obtained in a number of measurements of the CRM, achieved into repeatability conditions within the laboratory and the certified value of the CRM (μ_{MRC}): $|X - \mu_{MRC}|$, µg/kg or $|X - \mu_{MRC}|*100/ \mu_{MRC,}$ %.

Measurement uncertainty was established by the uncertainty budget, given by: calibration curve, purity and preparation of acrylamide standards, of the internal standard, volumes from micropippeting (solutions preparation, dilutions), the mass of the weighted sample, sample moisture determined with thermobalance, method precision, method bias (CRM), instrument precision, etc.

Expanded uncertainty (U_D) was calculated by multiplying the compound standard uncertainty (U_c) with a cover factor, k=2 for a confidence level P of 95% [31]. Expanded uncertainty of the method for determination of acrylamide in bread by GC/MS/MS was estimated at 9.5%.

Precision

International Conference of Harmonization (ICH) [32] defines precision as being composed of 3 components: repeatability, reproducibility and intermediate precision**.**

Repeatability, intra-laboratory reproducibility, intermediate precision were calculated with Microsoft Excel Programme software, and the calculated statistical parameters were: mean value (X); standard deviation (SD), relative standard deviation (RSD) in repeatability/ reproducibility conditions, repeatability limit (r) and confidence interval.

Robustness

Robustness of an analytical method is defined by measuring the method capacity to remain unaffected by small variations, deliberate, of some parameters. Parameter provides an indication of the method rehabilitation during use. If measurements are susceptible to variations in terms of analytical conditions, these conditions have to be checked or have to be taken some precautionary measures.

In the laboratory, in order to assess the robustness of the method were varied the following parameters: *mobile phase flow* (1.5 mL/min, 1.6 mL/min, 1.7 mL/min) and *derivatization time* (1.5h, 2h, 4h, 24h).

Stability of extracts

Often, samples are processed in one day and then analized the next day or due to a malfunction of the instrument, samples are stored for several days, during week-end, in a refrigerator or freezer.

Stability of analyte in the sample extract has been examined in the different storage conditions of the processed sample. It was verified the stability of analyte in an extract of bread sample with concentration 5 µg/kg, 320 µg/kg, stored in the different storage conditions: room temperature, 24^{0}C (initially, after 24 h, after 48 h), in refrigerator, at 4^{0}C (initially, after 48 h).

Stability of the processed sample is judged appropriate, where the mean of the obtained concentration for specified storage conditions is correlated with the results obtained at the running of fresh sample (immediatelly after preparation), imposing themselves as a criterion of acceptance, obtaining of RSD as small (RSD <5%).

Results and discussions

Performance characteristics of the method

Linearity

In table 1 are presented the obtained parameters of linear regression curve in the range 50 – 1200 µg/kg (fig. 2) and 5 – 640 µg/kg.

The correlation coefficients (R) and regression coefficients (R^2) obtained in the laboratory conditions were: R = 0.9994 (50 - 1200 µg/kg); R= 0.9997 (5 – 640 µg/kg); R^2 = 0.9989 (50 - 1200 µg/kg); R^2 = 0.9998 (5 - 640 µg/kg).

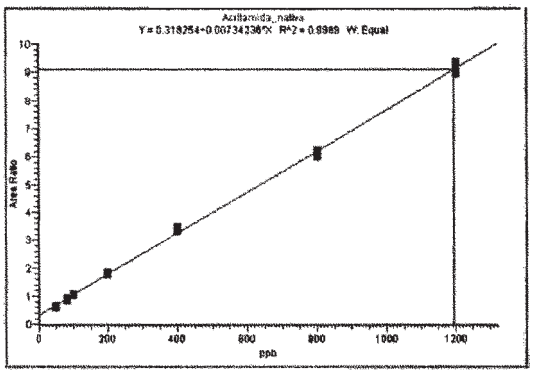

Fig. 2. Calibration curve to determine acrylamide in bread by GC/MS/MS, in the concentrations range 50 - 1200 μg/kg

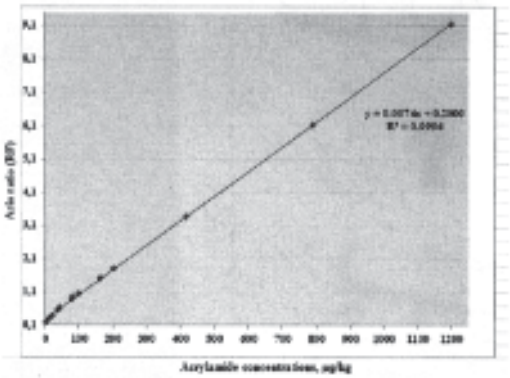

Fig. 3. Linearity range of the method for determination of acrylamide in bread, by GC/MS/MS, in the concentrations range 5μg/kg+ -1200 μg/kg

Working range

Considering the range of concentrations in acrylamide in which there were achieved the calibration curves, *working range* was 5 – 1200 μg/kg. The inferior limit of the working range is represented by the LOQ, that was 5 μg/kg.

Linearity range

Range linearity is range working. Ario Ratio was plotted against acrylamide concentration measured in the bread sample on the range 5 – 1200 μg/kg. Conclusion was that on the studied range the chromatographic method showed linearity, characterized by a correllation coefficient of data of 0.9997. Parameters obtained from the calibration curve, in *study of the linearity range* are presented in the figure 3.

Selectivity

In cromatogramms of bread matrices taken into study: *white wheat bread, whole wheat bread*, *intermediate bread,* spiked with working solutions I and III of acrylamide, in different concentrations, it was not observed presence of one or much more interferences obstructing the identification of the analyte, there were not recorded peaks which interfere with the used solvent or with other compounds present in the food matrix, in relation to the retention time of the analyte of interest, quantification was not influenced, what demonstrate that the method for determination of acrylamide by GC/MS/MS was selective.

In figure 4, 5 and 6 are presented cromatograms for samples of *intermediate bread* spiked with 0 μg/kg, 5 μg/kg and 10 μg/kg working solution III of acrylamide.

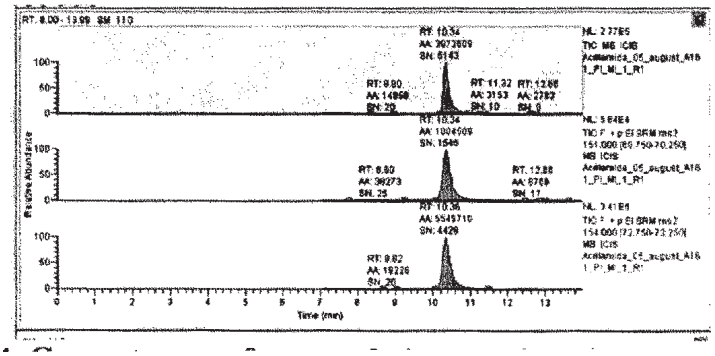

Fig. 4. Cromatogram for sample intermediate bread (0 μg/kg)

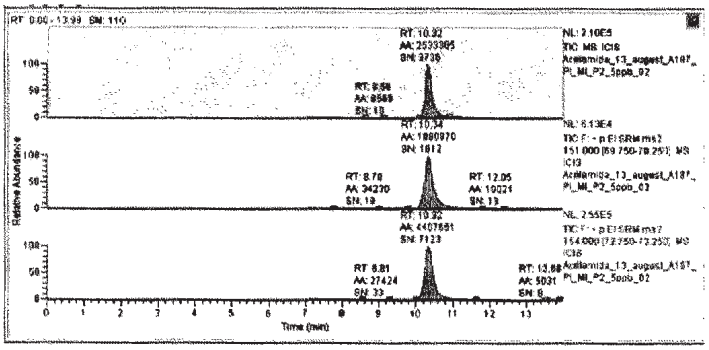

Fig. 5. Cromatogram for sample intermediate bread (5μg/kg)

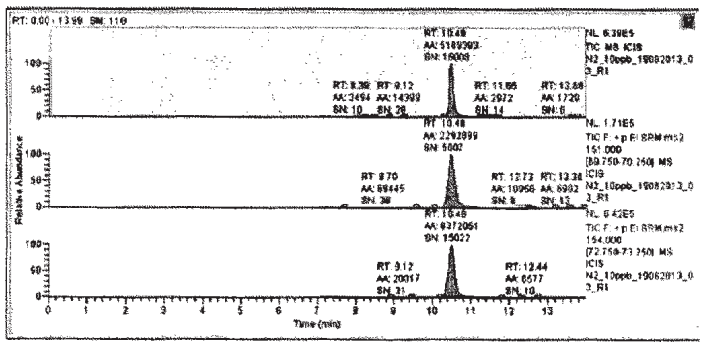

Fig. 6. Cromatogram for sample intermediate bread (10 μg/kg)

No. samples, n	Conc. AA** initially in matrix (C_i), µg/kg	Conc. of spiked AA ($C_{ad.}$), µg/kg	Mean conc. of AA found ($C_{meas.}$), µg/kg	Recovery*, %	Mean recovery, %
Concentration in acrylamide: C < 10 µg/kg					
5	< LOQ	5	5.724	114.48	**101.59**
6		10	8.869	88.69	
Concentration in acrylamide: 10 µg/kg ≤ C < 100 µg/kg					
6	< LOQ	20	20.152	100.76	**100.05**
8		40	40.049	100.12	
6		80	79.418	99.27	
Concentration in acrylamide: C ≥ 100 µg/kg					
10	126.838	40	165.646	97.02	**99.10**
10		80	206.800	99.95	
7		160	285.335	99.06	
4	< LOQ	320	317.940	99.36	
4		640	640.821	100.13	

Rec. % = $[(C_{meas.} - C_i)/C_{ad}]$ 100; **AA-acrylamide

Table 2 Mean recovery obtained by spiking bread samples

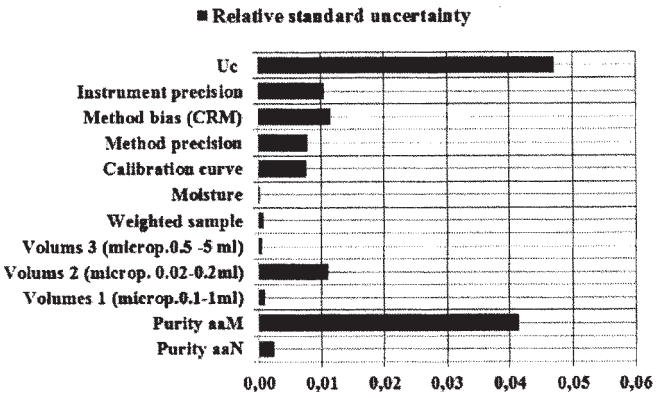

Fig. 7. Histogram of U(x)/x values at acrylamide determination in bread by GC/MS/MS

Sensitivity

From the results obtained, it was estimated that LOQ and LOD of the method were: LOQ = 5 µg/kg and LOD = LOQ/3 = 1.67 µg/kg. For the estimated value of the LOQ of 5 µg/kg there were met, simultaneosuly, criteria: relative standard deviation, RSD(r) ≤ 20%, recovery factor in the range 75 – 125% (in the laboratory conditions it was obtained RSD(r) = 5.897% and recovery of 113.34%) [35].

Accuracy

In case of the method for determination of acrylamide in bread by GC/MS/MS, average recovery obtained by spiking with acrylamide solution of bread samples, after correction of analyte lack with internal standard, was within the specified values under acceptance criteria of regulations/recommendations/guides [30, 33 -34] and was between 99.10% - 101.59%, in concentrations range 5–640 µg/kg. Results are presented in table 2.

Bias of method obtained by using CRM was of 0.65% (6.33 µg/kg). According to the Commission Directive 2002/657/EC [33], in the case of analyses repeated on CRM, deviation of the average content experimentally determined from certified value has to be ± 10%. The relative standard deviation in the laboratory conditions was 4%.

Average recovery obtained by achieving of 12 CMR samples was 100.65%.

Measurement uncertainty

Expanded uncertainty of the method for determination of acrylamide in bread by GC/MS/MS was evaluated by using of uncertainty budget and was estimated at 9.5%.

Size of uncertainty contributions is compared in a histogram (fig. 7) indicating relative standard uncertainty.

Precision

In the study of the *instrument precision* (system precision), according to the obtained results, in case of consecutive injections of bread samples, RSD(r), %, for determined concentration of acrylamide was in the range 0.614 – 1.965%. Considering the value RSD(r) ≤ 2% obtained, it can be said that in determination of acrylamide in bread, by GC/MS/MS, system was precise [35].

In the study of *method precision* there were calculated, RSD(r), %, for concentration and response factor, repeatability limit and confidence interval [35]:

- for a concentration lower than 10 µg/kg, the acceptance criterion for RSD(r), according to the Guide VICH GL49, 2011[34] is 25% and according to the CAC/GL 16-1993 [30] is 30%. The obtained values of RSD(r), in the laboratory conditions have been lower than 10%;
- for a concentration between 10 µg/kg ÷ 100 µg/kg, the acceptance criterion for RSD(r), according to the Guide VICH GL49, 2011[34] is 15% and according to the CAC/GL 16-1993 [30] is 20%. The obtained values of RSD(r), in the laboratory conditions have been lower than 3%;
- for a concentration higher than 100 µg/kg, the acceptance criterion for RSD(r), according to the Guide VICH GL49, 2011[34] is 10% and according to the CAC/GL 16-1993 [30] is 15%. The obtained values of RSD(r), in the laboratory conditions have been lower than 3%.

In the study of *intra-laboratory reproducibility* were obtained values of the statistical parameters in table 3:

- for a concentration between 10 µg/kg ÷ 100 µg/kg, the acceptance criterion for RSD(R), according to the Guide VICH GL49, 2011[34] and according to the Commission Decision 657/2002 [33] is 23%. The obtained values of RSD(R), in the laboratory conditions have been lower than 10%;
- for a concentration higher than 100 µg/kg, the acceptance criterion for RSD(R), according to the Guide VICH GL49, 2011 [34] and according to the Commission Decision 657/2002 [33] is 16%. The obtained values of RSD(R), in the laboratory conditions have been lower than 5%.

In table 4 are presented results of the study of the *intermediate precision*, using the same procedure, on the identical procedure, in the same laboratory, by the same operators, using the same equipment, in different days (3, 4, 5 days):

- for a concentration lower than 10 µg/kg, the obtained values of RSD(R), in the laboratory conditions have been lower than 14%.
- for a concentration between 10µg/kg ÷ 100 µg/kg, the obtained values of RSD(R) in the laboratory conditions have been lower than 10%.

No.	Sample name	No. samples, n	C_{meas}, µg/kg	SD(R), µg/kg	RSD(R), %
1.	White wheat bread (supplier 1)	**6** (3A+3B)*	25.395	1.350	5.314
2.	Toast bread	**6** (3A+3B)*	51.175	4.357	8.514
3.	Bread whole wheat, lot 1	**5** (3A+2B)*	127.150	3.340	2.626
4.	Bread whole wheat, lot 1, spiked 40 µg/kg	**6** (3A+3B)*	166.129	1.760	1.059
5.	Bread whole wheat, lot 1, spiked 80 µg/kg	**6** (3A+3B)*	206.876	4.155	2.008

* A – analyst A, B – analyst B

Table 3
STATISTICAL PARAMETERS FOR INTRA-LABORATORY REPRODUCIBILITY

Sample name	No. days, n	No. samples	$C_{meas.}$, µg/kg	SD(R), µg/kg	RSD(R), %
White wheat bread, spiked 10 µg/kg (supplier 3)	3	8	8.410	1.176	13.983
White wheat bread, lot 1 (supplier 2)	3	3	16.335	1.544	9.452
Black wheat bread with bran	3	4	17.287	1.344	7.774
Intermediate bread-direct procedure, lot 1	3	6	22.186	0.836	3.766
Bread with bran and graham	3	3	33.254	0.886	2.663
White wheat bread spiked 40 µg/kg (supplier 3)	3	6	40.820	1.327	3.250
Bread whole wheat	4	14	128.665	3.435	2.669
Bread whole wheat, spiked 40 µg/kg	3	11	163.192	4.649	2.849
Bread whole wheat, spiked 80 µg/kg	3	10	203.544	7.989	3.925
Bread whole wheat, spiked 160 µg/kg	5	10	285.040	8.091	2.839

Table 4
STATISTICAL PARAMETERS FOR INTERMEDIATE PRECISION

- for a concentration higher than 100 µg/kg, the obtained values of RSD(R) in the laboratory conditions have been lower than 4%.

Robustness

Bread samples were processed according to working procedure, and samples acquisition and quantification were achieved by different application of the flow of mobile phase (1.5 mL/min, 1.6 mL/min, 1.7 mL/min). There were calculated: mean value ($\overline{X}$), standard deviation (SD) and relative standard deviation (RSD) for concentrations values measured by GC/MS/MS. According to the obtained data, for those 3 variations of the flow mobile phase RSD(r) value was 2.37%.

By variation of derivatization time (1.5 h, 2 h, 4 h, 24 h), RSD, calculated for those 16 samples (4 parallel samples for each derivatization variant) was not higher than 2%.

Stability of extracts

It was concluded that in the case of extracts stored in the autosampler carusel or stored into refrigerator, for 24 h, 48 h, for concentrations of 5 µg/kg and 320 µg/kg, respectively, there were obtained variations of RSD between 2-3%, for the average response factor.

Conclusions

It was internally validated a GC/MS/MS method for determination of acrylamide in bread. There were assessed the performance parameters of the method: linearity, working range, linearity range, selectivity, sensitivity, accuracy, measurement uncertainty, precision, robustness and extract stability.

Values of the performance parameters obtained in the own laboratory conditions were lower or close to those of the speciality literature. Method had good selectivity, good linearity, low limit of detection (LOD = 1.67µg/kg) and limit of quantification (LOQ = 5 µg/kg); good precision (system precision, RSD(r) ≤ 2%; method precision, RSD(r) < 5% for a concentration higher than 10 µg/kg and RSD(r) < 10% for a concentration lower than 10µg/kg; intra-laboratory reproducibility, RSD(R) < 5% for a concentration higher than 100µg/kg and RSD(R) < 10% for a concentration between 10 µg/kg ÷ 100 µg/kg; intermediate precision, RSD(R) < 10% for a concentration higher than 10 µg/kg and RSD(R) < 14% for a concentration lower than 10 µg/kg).

Mean recoveries obtained by spiking with acrylamide solution some assortments of bread, in the range 5 - 640 µg/kg, were between 99.10% - 101.59%.

Basic foodstuffs such as bread contain acrylamide in small amounts, but still can contribute considerably to the daily diet of the Romanian consumer. Considering that the daily bread consumption in Romania for an adult of 80 kg weight is of 300 g/day and considering that bread has an average acrylamide concentration of 30 µg/kg, it can be deduced theoretically that, in our country, only bread contributes with 14% to 37.5% at maximum allowed dose, for water, in estimated daily diet [37] by FAO/WHO (0.3 – 0.8 µg acrylamide/kg body/day).

Introduction to the laboratory practice of the performance GC/MS/MS method for determination of acrylamide in bread and bakery products contributes to assess acrylamide in foodstuffs on the Romanian market and warms against consumption risk of products containing acrylamide.

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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Manuscript received: 20.06.2014