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Article

Application of an Accurate and Validated Method for Identification and Quantification of Acrylamide in Bread, Biscuits and Other Bakery Products Using GC-MS/MS System

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A gas chromatography tandem mass spectrometry has been developed and validated for the separation, detection, identification and quantification of acrylamide in bread, biscuits and similar products. The method showed good precision with values lower than 6%. A good sensitivity was achieved for bread with 2.41 and 7.23 μg kg^{-1} limit of detection (LOD) and limit of quantification (LOQ), respectively, while for biscuits, LOD and LOQ were 4.63 and 13.89 μg kg^{-1}, respectively. Accuracy obtained through the bias of 2 certified reference materials ("crisp bread - ERM®-BD272" and "rusk - ERM®-BD274") gave a value below 1.68-2.52%. The method was applied by analyzing 49 types of bread, biscuits and other similar products. The results showed different levels of acrylamide in bread (values ranged between 7.6 and 165.6 μg kg^{-1}), biscuits (between LOD and 2405.0 μg kg^{-1}), sandwich biscuits with cream (112.6-570.4 μg kg^{-1}), biscuits for infants and young children (between LOD and 801.7 μg kg^{-1}), gingerbread (349.5-955.5 μg kg^{-1}) and crackers (347.8-366.1 μg kg^{-1}).

Keywords: acrylamide (AA), validation, bread, biscuits, GC-MS/MS

Introduction

Acrylamide (AA) is a chemical that can be formed in some foods during certain types of high-temperature cooking. The neurotoxicity of AA in humans is well established from occupational and accidental exposures, and experimental studies have shown reproductive, genotoxic and carcinogenic effects in animals. AA has been classified as probably carcinogenic to humans by the International Agency for Research on Cancer (IARC).[1] The possible public health risks from dietary exposure to AA attracted concern by public authorities all over the world.[2]

Based on the expert committee evaluation of FAO/WHO, 1 μg kg^{-1} body weight (bw) day^{-1} is considered as an average exposure to AA while 4 μg kg^{-1} bw day^{-1} is considered as high exposure to AA.[3] The mean dietary exposure range to AA is 0.2-1.0 μg kg^{-1} bw day^{-1} for the general adult population while 95th-percentile range is 0.6-1.8 μg kg^{-1} bw day^{-1}.[4] In 2013, the U.S. Food and Drug Administration (FDA)[5] issued draft guidance to help the food industry to reduce the amount of AA in certain foods.

Commission Recommendation 2013/647/EU established, based on the monitoring results received from the Member States during the period 2007-2012, the "indicative values" for AA in different foods. The "indicative values" are not safety thresholds, but their aim is to indicate the need for an investigation in the case the values are exceeded and to take appropriate measures to control the formation of AA.[6]

Taking into account that the presence of AA in food products could increase the risk of cancer in consumers of all ages,[7,8] an internationally priority is to develop reliable analysis method to quantify low concentrations of AA in foods (dozens of ppb) and to find solutions to reduce this contaminant. Worldwide, in recent years several methods have been developed for the determination of AA from food products.[9-11]

Although in the last decade were published a large number of analytical methods based on gas chromatography (GC)[12-17] and liquid chromatography (LC) coupled with mass spectrometry (MS; MS/MS),[18-22] the methods are mainly used by private laboratories and official control authority for routine analysis of AA in food products. Last publications apparently confirm the validity of LC and GC techniques, which enables to guarantee the determination

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of AA with quantification limits from 30 to 50 μg kg^{-1} for HPLC-MS, 4 to 30 μg kg^{-1} for GC-MS and LC-MS/MS. Commission recommendation of 2 June 2010, states that for ensuring comparability of analytical results, methods that can achieve a limit of quantification (LOQ) of 30 μg kg^{-1} for bread and foods for infants and young children and 50 μg kg^{-1} for potato products, other cereal products, coffee and other products should be chosen.[23]

The aim of this study is to present the results obtained on internal validation of GC-MS/MS method for determination of AA in bread, biscuits and other similar products with LOQ below the above recommended values. Moreover, assessing the level of AA in some bakery products from the Romanian market was performed.

Experimental

Sampling

Seventeen types of bread and similar products, 18 types of biscuits, 5 types of biscuits for infants and young children, 4 types of sandwich biscuits, 2 crackers and 3 gingerbread were studied. Samples were either purchased in local market (various types produced in different countries) or produced on a pilot plant of the National Research & Development Institute for Food Bioresources (Romania).

Chemicals and reagents

Native acrylamide (min. 99% purity, concentration 1000 mg L^{-1} in methanol) (AA) was purchased from Ultra Scientific (N. Kingstown, Rhode Island, USA), internal standard of labeled acrylamide (1,2,3-^{13}C labelled AA) (IS), min. 99% purity (+100 ppm hydroquinone) of concentration of 1000 mg L^{-1} in methanol was from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All other reagents used during the validation of the method for determination of AA were of chromatographic purity from Merck (Darmstadt, Germany), LGC Promochem GmbH (Wesel, Germany) and Scharlau (Sentmenat, Spain). Ultrapure water was obtained through a PURELAB Option-S7 and PURELAB Ultra Ionic system (Elga Labwater, High Wycombe, UK).

Carrez I and II solutions were prepared by dissolving 28.8 g of potassium hexacyanoferrate and 57.6 g of zinc sulfate in 100 mL of water, respectively.

Helium as mobile phase (min. 99.9995%) and argon (min. 99.9995%) as collision gas were used. Samples concentration was achieved under a stream of nitrogen (min. 99.9995%).

Reference test materials ERM®-BD272 crispbread (AA content of 980 ± 90 μg kg^{-1}) and ERM®-BD274 rusk (AA content of 74 ± 7 μg kg^{-1}) were certified at the BAM Federal Institute for Materials Research and Testing (Berlin, Germany) to verify the accuracy of the method.

Preparation of stock and working solutions

Stock solution of AA (100 mg L^{-1}) and IS (100 mg L^{-1}) were prepared in amber vials by dissolving in ultrapure water. Working solutions I, II and III (10, 1 and 0.1 mg L^{-1} of AA, respectively) and working solution I (10 mg L^{-1}) of IS were prepared by diluting the stock solution with ultrapure water. All stock and working solutions were kept in a refrigerator at 4 °C.

Sample preparation

The procedure of sample preparation for AA quantification in bread, biscuits and other bakery products was according to previous study[13-15] with some modifications[24,25] regarding sample preparation (sample preparation for soft bread, sample weight taken for analysis, quantities and volumes for reagents) and AA analysis by GC-MS/MS in selected reaction monitoring (SRM) acquisition mode, respectively. Briefly, the steps were as follow:

Drying, milling and weighting

Only fresh bread samples were dried in an oven at 90 °C, for 120 min, while biscuits and other similar products were used as they were. The samples were fine milled using a Retch GM 200 mill (Germany) and an ultra centrifugal ZM 200 mill (Germany). In a centrifuge vial of 50 mL, 3 g of "bread and other similar products" and 1.5 g from "biscuits and other similar products" category were weighed for AA analysis, respectively.

AA extraction in water

Over the sample weighed, working solution I of the IS of 10 mg L^{-1} (110 μL) and 30 mL ultrapure water at 60 °C were added. Extraction in water at pH 4-5 was achieved by addition of 20-40 μL of glacial acetic acid, followed by deproteinization with 400 μL Carrez I and II solutions and centrifugation (6000 × g) at 5 °C, for 30 min.

AA derivatization

The supernatant obtained was derivatized with 7.5 g KBr, 40-100 μL HBr (pH 1-3), 10 mL saturated bromine-water solution (around 1.6%) on a shaking water bath below 4 °C, for at least 2 h. After the end of the derivatization reaction, bromine in excess was removed by adding around 1-2.5 mL of 1 mol L^{-1} sodium thiosulfate, until the yellow color disappeared.

Extraction of dibromo derivative of AA 2,3-dibromopropanal (2,3-DBPA)

It was achieved with 70 mL mixture of ethyl acetate and hexane (4:1, v/v).

2,3-DBPA

The concentration was achieved in a first step using a vacuum evaporation system (Rotovapor R-210, BUCHI Labortechnik AG, Germany) till 2 mL, followed in a second step by dryness under a stream of nitrogen.

2,3-DBPA purification and concentration

The residue dissolved in 50 mL hexane was purified on a glass column filled with activated Florisil and calcinated sodium sulfate, previously conditioned with 20 mL hexane. The 2,3-DBPA derivative was eluted with acetone, then concentrated till dryness and the residue was redissolved in 400 µL ethyl acetate and 40 µL triethylamine. The final solution was filtered through a 0.2 µm regenerated cellulose microfilter (17 mm diameter, Spartan 13RC, Whatman GmbH, Dassel, Germany) directly in a vial and analyzed by GC-MS/MS in SRM mode.

Preparation of the calibration solutions

A blank and 7 calibration levels in the range 0.1-7.5 mg L^{-1} were prepared in flasks of 250 mL. In each flask, the following were added: 100 mL ultrapure water, 110 µL IS of 10 mg L^{-1} and working solution II/I of AA (1 mg L^{-1} *per* 10 mg L^{-1}) according to the calibration level. These solutions were derivatized in the same manner as the derivatization steps described for bread, biscuits and other similar samples, without purification and the final residue was redissolved in 1000 µL ethyl acetate and 100 µL triethylamine.

GC-MS/MS-SRM analysis

The calibration solutions and the derivatized sample extracts were analyzed using a gas chromatograph (TRACE GC ULTRA) coupled with triple quadrupole mass spectrometer (TSQ Quantum XLS) from Thermo Fisher Scientific (USA). The analysis was performed in the electron impact positive ionization mode (EI^+); acquisition mode: "selected reaction monitoring-SRM" and ion scanning mode: "product".

The determination was carried out with a capillary column based on polyethylene-glycol (30 m × 0.25 mm internal diameter; 0.25 µm) (TraceGOLD™ TG-WaxMS, Thermo Fisher Scientific, USA). The mobile phase was helium with a constant flow rate of 1.6 mL min^{-1}. 1 µL sample was injected in a Right PTV type injector using TriPlus AS autosampler (Thermo Fisher Scientific, USA), in a split mode with a split ratio of 1:10 and injector temperature of 220 °C. Under these conditions, the retention time of AA and of the IS was of 10.65 ± 0.5 min. AA and IS were identified by the ion fragments corresponding to the derivatized ions, 2-BPA (2-bromopropenamide) and 2-BP($^{13}C_3$)A. The fragmentation of the precursor ions with *m/z* 151 and 154 was achieved with argon (1 mTorr), leading to the formation of product ions (daughter) with *m/z* 70 (2-BPA) and 73 (2-BP($^{13}C_3$)A), being used for quantification. The calculation of the AA concentration in the test samples was based on the ratio of the peak area corresponding to the product ions, with *m/z* 70 and 73 for 2-BPA and 2-BP($^{12}C_3$)A, respectively.

For bread samples and other similar products, AA concentration was calculated based on equation 1, while for biscuits and other similar products using equation 2.

$$C_{calc.} = [440 \times C \times (100 - U_1)]/[w \times (100 - U_2)]\ \mu g\ kg^{-1} \quad (1)$$

$$C_{calc.} = C \times 440/w\ \mu g\ kg^{-1} \quad (2)$$

where C is the 2-BPA concentration measured by the instrument in the food sample (mg L^{-1}), $C_{calc.}$ is the 2-BPA concentration calculated for the food sample (µg kg^{-1}), U_1 is the moisture content of the bread sample before drying (%), U_2 is the moisture content of the bread sample after drying (%), w is the weight of the sample (g), 440 is the final volume of the sample extract (µL).

Validation procedure

The method was validated according to the guides and recommendations for methods validation.[26-31] The following performance parameters were assessed:

Calibration curve and linearity

Calibration curve and linearity were verified by the method of least squares in the range 0.1-7.5 mg L^{-1}, choosing the method of the internal standard. The calibration curve was obtained based on derivatized standard solutions and plotting the ratio of the peak area of 2-BPA and 2-BP(^{13}C)A against the concentration of 2-BPA. The calibration model was considered correct if relative standard deviation (RSD) in repeatability conditions was within the limits of ± 15% for all the levels investigated. The correlation was assessed to be linear for a value greater than 0.99 for the correlation coefficient (R).

Linearity range

In order to establish higher values for the linearity range, the samples were spiked with working solution I of

native AA (10 mg L^{-1}) at different levels of concentration: 40; 75; 80; 150; 225 and 300 μg kg^{-1} (bread and other similar products) and 250; 500; 750; 1000 and 2000 μg kg^{-1} (biscuits and other similar products). The ratio of the peak area of 2-BPA and 2-BP(^{13}C)A against the concentration of 2-BPA from the food samples studied was plotted. The correlation was assessed to be linear for a value greater than 0.99 for the correlation coefficient (R).

Sensitivity

Sensitivity was characterized by limit of detection (LOD) and limit of quantification (LOQ). Assessment of LOD and LOQ for AA analysis in bread and similar products by GC-MS/MS were performed applying IUPAC approach, while ICH approach was used for biscuits and other similar products. The criteria imposed for LOQ were: precision (expressed as relative standard deviation, RSD ≤ 20%) and accuracy (expressed as recovery in the range 80-120%).

Selectivity

Selectivity was verified by observing the possible interference given by AA in bread, biscuits and other similar products.[32] Specificity was achieved by SRM detection.

Precision

Precision was determined by repeatability (injection and analysis repeatability), inter-laboratory reproducibility and intermediate precision. Injection repeatability was achieved by carrying out between 9 and 10 consecutive injections in a short period of time (the same day). Analysis repeatability (intra-day) was determined by 5-6 repeated analyses of the same sample, in the same day by the same analyst and under the same experimental conditions. Inter-laboratory reproducibility (inter-day) was achieved through Food Analysis Performance Assessment Scheme (FAPAS)[33] of the Central Science Laboratory (FERA). Intermediate precision (inter-day) was carried out by repeated analyses of the same sample in 3 different days by the same analyst and under the same experimental conditions. Repeatability and intermediate precision was expressed as RSD (%), while inter-laboratory reproducibility as z-score.

Accuracy

The accuracy of the method was evaluated with recovery and bias measurements for 2 reference test materials. The recovery percentage (R%) of the method was established from minimum 6 repeated analysis of the same sample fortified with working solution II of AA at different concentrations.

Robustness

To assess the robustness of the method developed in the laboratory, the following parameters were varied: volume of hexane used in the purification of the extracts, derivatization time and mobile phase flow. It was considered that the method is robust for the parameters investigated, whether obtained under repeatability conditions RSD or reproducibility was below 2%.

Measurement uncertainty

The uncertainty sources were identified and analyzed and the uncertainty budget was estimated, according to the reference documents.[34,35] Expanded uncertainty (U_Δ) was calculated by multiplying the combined standard uncertainty (U_c) with a coverage factor (k = 2) for a confidence level of 95%.

Method application

The validated and developed method was applied by analyzing different products presented in Tables 1-8. The AA level was also studied in different batches of the same brand (3 batches × 2 types of breads; 2 batches × 5 types of biscuits).

Statistical analysis

AA content was expressed as mean ± standard deviation. The differences among sample groups were analyzed by one-way ANOVA (analysis of variance) followed by Tukey's test, $p < 0.05$ was considered statistically significant.

Regarding validation parameters, average for concentration values, C_{calc} (μg kg^{-1}), standard deviation in repeatability SD(r) and reproducibility conditions SD(R) in μg kg^{-1} as well as relative standard deviation in repeatability RSD(r) and reproducibility conditions RSD(R) in % were calculated using Microsoft Excel. The repeatability and reproducibility limits in μg kg^{-1} were also considered, where the ratio between repeatability and reproducibility is 2.8 × SD(r)/SD(R) for a confidence level of 95%.

Results and Discussion

An overview of the test samples together with their coding and AA content is given in Tables 1-8.

Calibration curve and linearity

Linearity was investigated with the aid of a regression line with 7 calibration levels by the method of least squares.

Table 1. Bread and similar products produced in the pilot plant

	Main ingredients	AA[a] / (μg kg^{-1})
Pan bread	white wheat flour type 550, type 2200	19.5 ± 0.5 a
White bread	white wheat flour type 550	11.3 ± 0.4 b
Bread stick	white wheat flour, dark wheat flour	26.2 ± 1.1 c
Bread stick with olive	white wheat flour	47.9 ± 1.5 d
Bread stick with nut	white wheat flour	41.6 ± 1.6 e
Bread stick with onion	white wheat flour	87.3 ± 1.8 f

[a]AA: acrylamide; data shown as mean ± SD (n = 3) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$).

The calibration curve for the determination of AA in bread, biscuits and other similar products by GC-MS/MS analysis (y = 0.8613x + 0.0332) was linear over the range of 0.1-7.5 mg L^{-1}. R was between 0.9996-1, which proves a high linearity degree. The results showed that RSD(r) for all the levels of concentration studied ranged between 0.1 and 7.8%, being within the limits of ± 15%.

The linearity range was verified according to ISO[36] for bread, biscuits and other similar products, respectively. The linearity range of the method for AA analysis was in the range of: 7.23-312.88 μg kg^{-1} (R > 0.998) for bread and similar products (n = 23) and 17.59-2191.41 μg kg^{-1} (R > 0.999) for biscuits and similar products (n = 42), respectively, n denotes the number of the analyzed samples. The results obtained are in line with a recent study,[37] which reported an LC-MS/MS method for AA determination in different types of breads, with a linear range of up to 750 μg kg^{-1} food and a determination coefficient of 0.999.

Sensitivity

For bread and similar products, LOD and LOQ obtained were 2.41 and 7.23 μg kg^{-1}, respectively. For biscuits and similar products, LOD and LOQ were: 4.63 and 13.89 μg kg^{-1}, respectively. LOQ values fulfilled the two criteria (RSD < 10% and recovery between 93.68 and 102.93%).

LOQ value for the present method was below 30 μg kg^{-1} which fulfils the criteria defined in European Commission recommendation on the monitoring of AA levels in food.[23] Also, the results obtained are in accordance with a previous GC-MS study, which reported LOD and LOQ values of 2 and 5 μg kg^{-1}, respectively for bread samples.[14]

The working range reached from 2.41-2191.41 μg kg^{-1} AA in a food sample.

Selectivity

Selectivity was demonstrated by chromatographic separation of AA in the presence of other components from the food matrix (bread, biscuits and similar products).

For specificity, SRM detection and the use of IS method lead to a specific analysis. The retention time of AA within laboratory condition for the samples was almost identical with the retention time of the IS, which fits in the

Table 2. Bread and similar products (origin: retail store, produced in Romania)

	Sample, producer code	Main ingredients	AA[a] / (μg kg^{-1})
White bread	60/R	white wheat flour	7.6 ± 0.3 a
Round sliced bread	31/M	whole wheat flour, dark wheat flour	61.3 ± 0.7 b
Whole toasted bread	32/M	whole wheat flour, dark wheat flour, malt flour different batches	115.2 ± 0.3 c
	33/M		49.7 ± 0.3 d
	34/M		57.2 ± 0.4 e
White bread with sage seeds	35/M	white wheat flour	28.0 ± 0.5 f
Hypoglucidic sliced bread	36/M	white wheat flour, bran, dark wheat flour	104.5 ± 0.3 g
Whole wheat toasted bread	37/T	whole wheat flour, fermented wheat flour, soy flour	165.6 ± 1.3 h
Whole wheat bread	38/T	whole wheat flour, fermented wheat flour, soy flour different batches	56.9 ± 0.3 i
	39/T		62.2 ± 0.1 b
	47/T		79.8 ± 0.2 j
German bread with seeds	40/T	dark wheat flour, rye flour, wheat bran, wheat malt flour	52.2 ± 0.4 k
Transylvanian dark bread	41/U	dark wheat flour	21.3 ± 0.1 l
Bread with dark flour and bran	42/V	dark and white wheat flour, wheat bran, malt flour	37.4 ± 0.1 m
Whole toasted bread	43/S	dark and white wheat flour, rye flour, soy flour, malt flour, rice flour	46.0 ± 0.3 n

[a]AA: acrylamide; data shown as mean ± SD (n = 3) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$).

tolerance of ± 0.5% imposed by Commission Decision (2002/657/CE).[30] For AA quantification, ion *m/z* 70 (2-BPA) and ion *m/z* 73 for IS (2-BP($^{12}C_3$)A) were used.

The values obtained for injection and analysis repeatability, intermediate precision are presented in Table 9.

RSD(r) was between 0.71 and 3.30% for injection repeatability and between 0.44 and 4.90% for repeatability analysis, respectively. The RSD(R) intermediate precision was between 0.35 and 5.83%. The applied method showed a good precision, RSD < 10% for AA analysis in bread, biscuits and similar products and the results were within the limits set by the following regulations: VICH GL49,[28] CAC/GL 16,[29] EC.[30]

The AA result for crispbread sample from the FAPAS proficiency test (laboratory No. 24) showed a value of 193.77 μg kg^{-1} (z-score of 0.2), which fulfilled the acceptability criteria from the organization.[33]

Method accuracy

The bias obtained for the two reference materials were: 1.68% (n = 39) for ERM®-BD272 crispbread and 2.52% (n = 30) for ERM®-BD274 rusk, respectively. The described method demonstrated a satisfactory accuracy, between 99.59 and 102.28% for bread and similar products and between 95.44 and 104.63% for biscuits and similar products.

Table 3. Biscuits samples produced in the pilot plant

	Main ingredients	AA[a] / (μg kg^{-1})
Biscuit from rice flour	RF[b], fat, sugar, eggs	31.3 ± 0.6 a
Biscuit from white wheat flour	WF[c], fat, sugar, eggs	152.0 ± 2.9 b
Biscuit from white wheat and oat flour	WF[c] 75%, OF 25%, fat, sugar, eggs	148.9 ± 2.7 b
Biscuit from white wheat and oat flour	WF[c] 50%, OF 50%, fat, sugar, eggs	359.0 ± 4.2 c
Biscuit from whole oat flour	OF[d] 100%, fat, sugar, eggs	346.3 ± 2.3 d
Biscuit from oat and bran flour	OF[d] 70%, oat bran 30%, fat, sugar, eggs	< LOQ[e] e

[a]AA: acrylamide; data shown as mean ± SD (n = 3) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$); [b]RF: rice flour; [c]WF: wheat flour; [d]OF: oat flour; [e]LOQ: limit of quantification.

Table 4. Biscuits samples (origin: retail store, produced in different countries)

	Sample, producer code, country	Main ingredients	AA[a] / (μg kg^{-1})
Biscuit from oat	50/E/Turkey	OF[b], WF[c], WWF[d], fat, sugar, eggs	702.2 ± 0.7 a
Biscuit with wheat bran	51/B/Romania	WF[c], wheat bran, sugar, fat, barley malt extract, wheat fiber	329.7 ± 2.7 b
Digestive biscuit with cinnamon	52a/M/Romania	WWF[b], fats, sugar, wheat bran, malt extract different batches	1213.6 ± 6.7 c
	52b/M/Romania		1665.2 ± 17.9 d
Digestive biscuit	53/E/Turkey	WWF[d], fats, sugar, egg, barley malt extract, wheat bran, milk powder	487.7 ± 3.2 e
Biscuit with salt	56/H/Romania	WF[c] flour, oil, barley malt extract, egg, salt	355.9 ± 0.7 f
Biscuit from oat flour and fruit jam (fasting)	57/C/Moldavia	WF[c], OF[b], sugar, fat, fruit jam	263.1 ± 2.8 g
Biscuit from whole oat and fruits, without gluten (fasting)	69/A/Scotland	OF[b], palm oil, raisins, sugar, Tapioca starch	1774.2 ± 13.1 h
Biscuit from BIO whole oat	70/P/Germany	OF[b], WF[c], sugar, fat, egg powder, maize starch	46.8 ± 1.2 i
Biscuit with oat	71/Z/Romania	WF[c], OF[b], sugar, palm oil, wheat starch	1220.7 ± 17.7 j
Digestive biscuit with cereals, raisins, orange and lemon	72a/M/Romania	cereals, raisins, orange and lemon different batches	2305.3 ± 2.7[e] k
	72b/M/Romania		2405.0 ± 1.4[e] l
Biscuit with whole cereals and cranberry red, vitamins and minerals	73a/H/Romania	whole cereals and cranberry red, vitamins, minerals different batches	702.3 ± 13.5 a
	73b/H/Romania		788.3 ± 6.8 m
Biscuit with whole cereals, nuts and honey, vitamins and minerals	74a/H/Romania	whole cereals, nuts and honey, vitamins, minerals different batches	448.6 ± 2.5 n
	74b/H/Romania		242.7 ± 2.7 o

[a]AA: acrylamide; data shown as mean ± SD (n = 3) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$); [b]OF: oat flour; [c]WF: wheat flour; [d]WWF: whole wheat flour; [e]the AA levels were higher than the maximum value of the working range, but in order to quantify it, the samples weight taken for analysis was lower (0.75 g instead of 1.5 g).

Table 5. Biscuits for infants and young children (origin: retail store, produced in different countries)

	Sample, producer code, country	Main ingredient	AA[a] / (μg kg^{-1})
Biscuit with vitamins and minerals	89/I/Romania	WF[b], fats, sugar, maize starch, egg powder, milk powder, vitamins	801.7 ± 8.2 a
Biscuit with apple for children	91/J/Switzerland	WF[b], WWF[c], apple juice, apple extract	69.7 ± 1.9 b
First biscuit of the child	92/J/Switzerland	WF[b], sugar, wheat starch, milk powder, vitamin B1	< LOQ[d] c
Biscuit enriched with 5 vitamins, calcium and iron	93/K/Italy	WF[b], sugar, milk fat, wheat starch, honey, vitamins, minerals	117.7 ± 2.8 d
Biscuit enriched with 5 vitamins, calcium and iron for young children	94/K/Italy	WF[b], sugar, oil, milk powder, rice malt extract, vitamins, minerals	37.8 ± 0.3 e

[a]AA: acrylamide; data shown as mean ± SD (n = 6) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$); [b]WF: wheat flour; [c]WWF: whole wheat flour; [d]LOQ: limit of quantification.

Table 6. Sandwich biscuits with cream filling (origin: retail store)

	Sample, producer code, country	Main ingredients	AA[a] / (μg kg^{-1})
Biscuit with cocoa cream	80/L/Romania	WF[b], fat, sugar	527.2 ± 5.8 a
Biscuit with honey and milk cream	81/M/Romania	WF[b], fat, sugar, honey, powder milk, starch	570.4 ± 7.2 b
Biscuit with sour cream	82/B/Romania	WF[b], sugar, fat, cocoa powder, milk powder, cream powder	112.6 ± 3.1 c
Vanilla biscuit with cocoa cream	76a/I/Romania	WF[b], sugar, palm oil, maize starch, cocoa different batches	243.7 ± 5.8 d
	76b/I/Romania		177.8 ± 6.0 e

[a]AA: acrylamide; data shown as mean ± SD (n = 5) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$); [b]WF: wheat flour.

Table 7. Crackers samples (origin: retail store)

	Sample, producer code, country	Main ingredients	AA[a] / (μg kg^{-1})
Cheese cracker	3437/O/Romania	WF[b], oil, cheese powder, pea flour, corn starch	347.8 ± 4.3 a
Caraway cracker	3438/O/Romania	WF[b], oil, caraway, cheese powder, pea flour	366.1 ± 21.3 a

[a]AA: acrylamide; data shown as mean ± SD (n = 6) for the amount of AA; values with the same letter denote no significant difference ($p > 0.05$) between samples; [b]WF: wheat flour.

Table 8. Gingerbread samples (origin: retail store)

	Sample, producer code, country	Main ingredients	AA[a] / (μg kg^{-1})
Gingerbread	58/N/Romania	WF[b], rye flour, sugar, honey	955.5 ± 2.9 a
Gingerbread	59/C/Moldavia	WF[b], sugar, sorbitol	588.8 ± 16.8 b
Gingerbread	90/C/Moldavia	WF[b], sugar, sorbitol	349.5 ± 3.9 c

[a]AA: acrylamide; data shown as mean ± SD (n = 3) for the amount of AA; means followed by different letters are significantly different ($p < 0.05$); [b]WF: wheat flour.

Robustness

The small variations in the volume of hexane used in the purification of the extracts, derivatization time and mobile phase flow are not susceptible to variations in the area corrected. Standard deviation was between 1.79 and 2.07%.

Measurement uncertainty

The uncertainty of results for AA determination in bread, biscuits and similar products by GC-MS/MS was ± 21% as estimated by budget uncertainty.

Table 9. Performance parameters of the method

Performance parameter		Sample				
		Bread and similar products	Biscuit	Sandwich biscuit	Cracker	Gingerbread
		Precision				
Injection repeatability	AA / (mg L^{-1})	0.040 (n = 9)	0.210-5.658 (n = 10)	0.611-1.960 (n = 9-10)	NC	NC
	RSD(r) / %	3.30	0.71-1.55	1.14-1.47	NC	NC
Analysis repeatability (intra-day)	AA / (μg kg^{-1})	7.58-157.10 (n = 5-6)	30.91-1778.97 (n = 6)	113.97-1065.47 (n = 5-6)	347.78 (n = 6)	608.62-1318.48 (n = 5-6)
	RSD(r) / %	0.53-3.33	0.74-4.90	0.95-1.55	1.24	0.44-2.93
Intermediate precision (inter-day)	AA / (μg kg^{-1})	19.50-73.62 (n = 3-4)	37.60-1221.29 (n = 3)	113.16-576.69 (n = 2-3)	NC	349.70-954.49 (n = 2-3)
	RSD(R) / %	1.17-5.83	0.94-5.34	1.53-3.84	NC	0.35-1.76

AA: acrylamide; NC: not calculated; RSD(r): relative standard deviation in repeatability; RSD(R): relative standard deviation reproducibility conditions.

Method application

As it can be noticed from Tables 1-8, the results for the AA content in the different food samples studied, varied greatly not only from one type of product to another one, but also in the same type of product coming from different brands or the same product with different batches. From the 49 types of bread, biscuits, crackers and gingerbread analyzed coming from 20 different suppliers, 16 of them (4 bread samples, 11 biscuits and sandwich biscuits and 1 biscuit for infants and young children) had an AA level higher than the "indicative values" provided in the Commission Recommendation (EC 2013).[6] The indicative values were for soft bread obtained from wheat flour were set at a value of 80 μg kg^{-1}, biscuits for infants and young children at 200 μg kg^{-1}, biscuits and crackers at 500 μg kg^{-1} and gingerbread at 1000 μg kg^{-1}, respectively.

Bread products manufactured in the pilot plant under controlled conditions (Table 1) do not exceed the indicative values, except the bread sample prepared with onion. This could be explained by the fact that onion contributes to AA formation. It was shown[38] that onion among other ingredients used for seasoning increased the AA content. Analysis of the AA content in the same type of food from the market (the same receipt and manufacturing process according to the producer declaration) with different batches showed differences in the AA content. For example, for 3 batches of bread (code 32, 33 and 34) coming from the same producer, M, the AA level showed significant differences, while for another 3 batches of bread (code 38, 39 and 47, from producer T), the AA levels showed similar values (Table 2). This could be explained by the technological conditions (temperature, time) which could vary or by variation in the quality of the raw materials.

Adult chronic dietary exposure estimated by EFSA[39] was between 0.4 and 0.9 μg kg^{-1} bw *per* day. Considering than in Romania, the bread intake for an adult weighing 80 kg is 300 g *per* day and based on the average level of AA obtained in this study for the breads from the market (62.99 μg kg^{-1}, Table 2), it can be concluded that bread contributes to a chronic dietary exposure of 0.9 μg kg^{-1} bw *per* day with about 26%. The same procedure was applied for biscuits and similar products. Thus, for a daily intake of 5.8 g and an average for AA obtained for these products (456.01 μg kg^{-1}, Tables 3-8), the biscuits contributes to a chronic dietary exposure of 0.9 μg kg^{-1} bw *per* day with about 3.7%.

The AA content obtained for bakery products are in accordance with the data presented in literature (Table 10).

Conclusions

The proposed GC-MS/MS method corresponds to the validation parameters imposed and it was applied with good results to determine AA content in bread, biscuits and other similar products. The LOD and LOQ values were 2.41 and 7.23 μg kg^{-1} for bread and similar products, while for biscuits and similar products were 4.63 and 13.89 μg kg^{-1}, respectively. This study assesses the presence of AA in foods found on the Romanian market. Taking into account the consumption of bakery products among adults and children, establishment of measures is necessary in order to protect consumer health. In this respect, an important measure is to control in order to avoid the sale of foods with high levels of AA.

Considering all of the above data for method performance and proficiency test, the GC-MS/MS method and sample pretreatment employed in the present work can be regarded as being sensible, precise and robust.

Table 10. Survey of AA content in bakery products

Sample	AA / (μg kg^{-1})	Reference
Bread and similar products	< 10-3.200	40
	446	41
	126-274	42
	228-257	43
	49-235	44
	7.4-175	45
	87	46
	75	47
	42	39
	11.3-87.3 (pilot plant)	this study
	7.6-165.6 (market)	this study
Biscuit and similar products	18-3324	40
	83-423	46
	350	41
	313-317	42
	204-284	44
	231	39
	237	47
	131-163	43
	< 4.63-359.0 (pilot plant)	this study
	< 4.63-2405.0 (market)	this study
Gingerbread	910	48
	501	49
	437	44
	407	39
	387	47
	300	43
	349.5 63-955.5 (market)	this study

AA: acrylamide.

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