

1 Article

2 Determination of Acrylamide in Biscuits by High- 3 Resolution Orbitrap Mass Spectrometry: Validation 4 and Application

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9 **Abstract:** Acrylamide (AA), a molecule which potentially increases the risk of developing cancer, is
10 easily formed in food rich in carbohydrates, such as biscuits, wafers and breakfast cereals, at
11 temperatures above 120 °C. Thus, it is eminent the need to detect and quantify the AA content in
12 processed foodstuffs, in order to delineate the limits and mitigation strategies. This work reports
13 the development and validation of a high-resolution mass spectrometry-based methodology for
14 identification and quantification of AA in specific food matrices of biscuits, by using LC-MSⁿ with
15 electrospray ionization and Orbitrap as mass analyser. The developed analytical method showed
16 good repeatability (RSD_r 11.1%) and 3.55 µg kg⁻¹ and 11.8 µg kg⁻¹ as limit of detection (LOD) and
17 limit of quantification (LOQ), respectively. The choice of multiplexed targeted-SIM mode (t-SIM)
18 for AA and AA-d3 isolated ions provided enhanced detection sensitivity, as demonstrated in this
19 work. Statistical processing of data was performed in order to compare the AA levels with several
20 production parameters, such as time/cooking temperature, placement on the cooking conveyor belt,
21 color and moisture for different biscuits. The composition of the raw materials was statistically the
22 most correlated factor with the AA content when all samples are considered. The statistical
23 treatment presented herein enables an important prediction of factors influencing AA formation in
24 biscuits contributing for putting in place effective mitigation strategies.

25 **Keywords:** acrylamide, biscuits, mitigation measures, benchmark levels, contaminant

26

27 1. Introduction

28 Once Tareke et al [1] have reported acrylamide (AA) as a carcinogen formed in heated foodstuffs
29 the food industry, Member States of the European Union and the European Commission have made
30 considerable efforts to investigate AA formation pathways in order to reduce the levels of this
31 compound in processed foods. Besides being present in foods, AA has also been found in the
32 environment (due to industrial discharges), cosmetics, drinking water as well as tobacco smoke.
33 Human exposure to AA may be by ingestion, inhalation or contact with the skin [2]. Dietary exposure
34 is the most concerning, since about 40% of the food may contain this compound. Between 10 and 50%
35 of AA of the diet of pregnant women passes through the placenta and breast milk also contains this
36 toxin [3]. In the US, most exposure to AA comes from potato chips, breads, cereals, crackers and other
37 snacks [4]. In Europe, toasted bread, coffee and potatoes are the main food sources of AA [5].

38 Human exposure to AA may have toxicological effects (neurotoxicity, genotoxicity,
39 carcinogenicity and reproductive toxicity), and AA has been classified as carcinogenic by the
40 International Agency for Research on Cancer [6] in the 2nd group (probably carcinogenic in humans).
41 AA has an α,β -unsaturated carbonyl group with electrophilic reactivity, which can react with
42 nucleophilic groups of biological molecules, thus contributing to toxic effects. The reaction of AA
43 with proteins is extensive and the products of this reaction are used as biomarkers of its presence [5].
44 After entering the circulatory system, it is rapidly distributed into the tissues, passes through the
45 placenta and is transferred into breast milk. It is metabolized together with glutathione (GSH) and
46 also by epoxidation, resulting in glycidamide (GA). The formation of GA is mediated preferentially

47 by cytochrome P450, and is on the basis of neuro and genotoxicity of AA. Covalent DNA adducts of
48 GA were observed in vitro and in animal experiments and were used as biomarkers [2, 5].

49 Only the legal limit of AA for water has been established, with the value of 0.1 µg/L [7]. As a
50 large percentage of food contains this molecule, it is imperative to legislate its limits. The levels of
51 AA in foodstuffs of the Member States of the European Union were monitored between 2007 and
52 2012. Based on the results, the European Commission outlined indicative values for AA in different
53 foodstuffs [8]. These values are not safety values but rather indicative values, so that further research
54 is promoted in foods with higher AA levels and consequent reduction throughout the agronomical
55 factors, the food recipe, processing and final preparation [5]

56 The level of free asparagine in cereals has been claimed to be the major influence on the
57 formation of AA [9], since the largest pathway of AA formation involves this amino acid. The choice
58 of cereal varieties with lower levels of free asparagine is recommended, but challenging given the
59 influence of environmental conditions on their production [10].

60 Corn and rice products tend to have lower AA contents than wheat, barley, oats or rye. Products
61 with whole flours have higher levels of AA [10]. The choice of different varieties of cereals also
62 determines the development of AA: five varieties of rye with different fertilizations were used to
63 study the effect of nitrogen and sulfur on AA formation. A positive correlation was found between
64 asparagine concentration in grains and the highest levels of nitrogen used and the final concentration
65 of AA [11, 12].

66 The influence of cereal types on bread was investigated by Przygodzka and collaborators [13],
67 concluding that rye loaves form more AA in cooking, followed by spelled loaves and loaves of refined
68 flour - "white bread." In the same study, the extraction rates of the flour were compared with AA
69 formation: 100% whole flours obtained higher concentrations of AA, followed by flours with
70 extractions of 70%, indicating that "whole flours" have more AA precursors.

71 Several AA mitigation measures have been established that involve the use of the enzyme
72 asparaginase, which converts asparagine to aspartic acid, although control of adverse effects on
73 organoleptic properties is necessary [14].

74 The requirement for ultra-trace level detection of AA has led to development of several
75 analytical methods, most of which involving chromatographic separation techniques, both liquid and
76 gas chromatography. Determination of AA in food by GC-MS methods can be carried out with or
77 without derivatization. The advantage of derivatization processes is increased volatility and
78 improved selectivity. The bromination [1, 15-20], xanthidrol [21-25] and silylation [26, 27] have been
79 widely used for determining of AA in foodstuffs.

80 In recent years, the use of ultra-performance liquid chromatography (UPLC) has become more
81 popular because of its high sensitivity and selectivity, without the need for derivatization. Liquid
82 chromatography coupled to mass spectrometry have become the method of choice for the
83 determination of AA in food products, by using different mass analysers. Conventional triple
84 quadrupole (QqQ) have been for long the technique of choice by selecting the characteristic
85 transitions m/z 72→55, and 72→27. Ion trap [28-30] and TOF [28, 31-33] have also been useful for
86 quantitative analyses of AA.

87 Considering that the capabilities of high-resolution mass spectrometry (HRMS) based
88 methodologies for quantitative LC/MS analysis of AA in foodstuffs have been scarcely explored [34-
89 36] the present work aims at validating a HRMS methodology for detection and quantification of AA
90 in biscuits. The implemented procedure has been applied for investigating the impact of several
91 production parameters on the AA content in biscuits.

92 **2. Results and Discussion**

93 *2.1. Method performance*

94 Detection and quantification limits (LOD and LOQ) were estimated by using the signal-to-noise
95 method, as specified in the European Pharmacopoeia [38]. The peak-to-peak noise around the AA
96 (m/z 72.0444) retention time was measured, and subsequently, the concentration of the AA that

97 yielded a signal equal to a certain value of noise to signal ratio was estimated, by comparing
 98 measured signals from samples with known low concentrations of the AA with those of blank
 99 samples. This method allows a decrease of the signal (peak height) to be observed to the extent that
 100 the concentration is reduced through a series of dilutions, establishing the minimum concentration
 101 at which the analyte can be reliably quantified. The signal-to-noise (S/N) ratios accepted as estimates
 102 of the LOD and LOQ were 3:1 and 10:1, respectively [39]. The values found in this study are 3.55 μg
 103 kg^{-1} for LOD and 11.8 μg kg^{-1} for LOQ, as shown in Table 1.

104 **Table 1.** Repeatability, limit of detection (LOD) and limit of quantification (LOQ) of the proposed
 105 methodology.

<i>Assay</i>	<i>AA content</i> ($\mu\text{g kg}^{-1}$)	<i>Average</i> <i>AA content</i> ($\mu\text{g kg}^{-1}$)	<i>SD</i>	<i>RSD_r</i> %	<i>LOD</i> ($\mu\text{g kg}^{-1}$)	<i>LOQ</i> ($\mu\text{g kg}^{-1}$)
1	254.1					
2	277.3					
3	343.9					
4	309.8	297.9	33.1	11.1	3.55	11.8
5	269.8					
6	345.0					
7	290.9					
8	292.0					

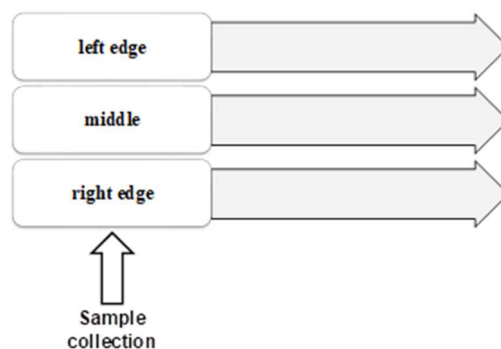
106 Commission regulation of 20 November 2017 states that the method of analysis used for the
 107 analysis of AA must comply with the following criteria: LOQ less than or equal to two fifths of the
 108 benchmark level (for benchmark level < 125 $\mu\text{g kg}^{-1}$) and less than or equal to 50 $\mu\text{g kg}^{-1}$ (for
 109 benchmark level \geq 125 $\mu\text{g kg}^{-1}$); LOD less than or equal to three tenths of LOQ [40]. According to the
 110 same regulation, benchmark level for the presence of AA in biscuits and wafers is 350 $\mu\text{g kg}^{-1}$. This
 111 means that LOD and LOQ is required to be less than or equal to 15 $\mu\text{g kg}^{-1}$ and 50 $\mu\text{g kg}^{-1}$, respectively.
 112 The method herein presented clearly meets these requirements. Moreover, in a proficiency test
 113 recently organized by the EURL-PAH, for the determination of the AA content in potato chips, the
 114 method performance LOD and LOQ were reported [41]. Twenty six laboratories guarantee the
 115 determination of AA with an average LOD of 22.5 $\mu\text{g kg}^{-1}$ and LOQ 55.8 $\mu\text{g kg}^{-1}$ by liquid
 116 chromatography (LC) coupled with mass spectrometry (MS; MS/MS). Nine laboratories participating
 117 in this proficiency test reported 15.2 $\mu\text{g kg}^{-1}$ and 36.3 $\mu\text{g kg}^{-1}$ as average LOD and LOQ, respectively,
 118 based on GC-MS methods. By using the analytical method herein reported, it is possible to increase
 119 the detectability and thus achieve lower limit of quantification, which can be particularly useful in
 120 the case of low-abundance AA matrices. The selection of the acquisition mode in the Orbitrap has a
 121 direct impact on the detection sensitivity. In a recent paper, Kaufmann demonstrated that the
 122 sensitivity of eight selected analytes is strongly increased by the use of SIM (selected ion monitoring)
 123 relatively to the FS (full scan) mode (1.5-fold increase for analytes in pure standard solutions and 2-
 124 fold increase for analytes spiked in a heavy matrix) [42]. A detailed study of the acquisition method
 125 for determination of eight synthetic hormones in animal urine concluded that reducing the scan range
 126 for Full MS (using the quadrupole) and targeted modes give higher S/N ratios and thereby better
 127 detection limits for analytes in complex matrices [43]. In fact, the targeted-SIM (t-SIM) is not more
 128 selective than full MS, but it does provide enhanced detection sensitivity. As only a small fraction of
 129 the continuously entering ion beam is sampled by the C-trap, the number of ions transmitted is
 130 greatly reduced and a much longer segment of the ion beam can be collected. Accordingly,
 131 significantly higher sensitivity can be achieved, mainly for small molecules applications, like the
 132 present case of AA.

133 The precision of the method was evaluated by measuring the repeatability (intra-day
 134 variability). The relative standard deviation was calculated for repeatability (RSD_r) by performing
 135 eight repeated analyses for samples of the same biscuit. The results showed that the RSD_r (11.1%)
 136 was less than 12% for a sample with an average AA content of 297.9 $\mu\text{g kg}^{-1}$ (Table 1). The use of

137 isotopically labeled internal standard (acrylamide-d3) is herein especially useful, as sample loss may
 138 occur during sample preparation steps prior to analysis, as it is known that the fat/water distribution
 139 of the matrix may affect the extraction and analysis.

140 2.2. Acrylamide content in biscuits

141 The optimized and validated procedure was applied to different samples of biscuits collected
 142 from the baking oven. Three sample points were considered, as depicted in Figure 1. One is in the
 143 middle of the oven and two are in the edges of the oven (left edges and right edges).



144

145 *Figure 1.* Sample collection points from the baking oven.

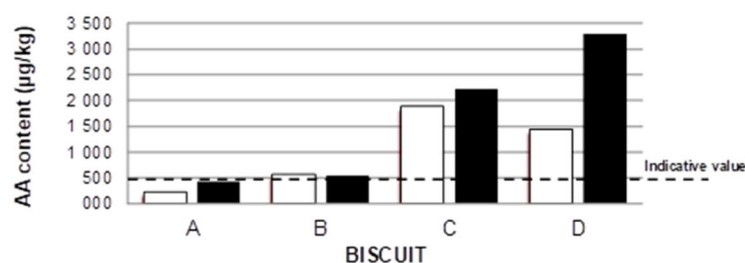
146 A huge difference was observed between samples collected from the middle and edges of the
 147 oven (Table 2). Except for biscuit B, the AA content is higher for samples taken from the middle of
 148 the oven, where the temperatures are higher. The observed increase is higher for biscuits D (from
 149 1443 up to 3303 $\mu\text{g kg}^{-1}$, corresponding to 129% increase) and A (from 216 up to 431 $\mu\text{g kg}^{-1}$,
 150 corresponding to 99% increase). Except for biscuit A, the AA content found for all the inspected
 151 biscuits was above the benchmark level referred to in the EU Commission Regulation [40]. Of more
 152 concern is the fact that biscuits C and D contain AA in concentration clearly above (average 2056 and
 153 2373 $\mu\text{g kg}^{-1}$, respectively) the indicative value reported by European Food Safe Agency (dashed line
 154 in Figure 2), confirming the pressure of establishing mitigation measures for the reduction of the
 155 presence of AA in these matrices.

156 **Table 2.** Acrylamide content ($\mu\text{g kg}^{-1}$) in biscuits collected from different points of the baking oven.

<i>Biscuit</i>	<i>Edges of the baking oven ($\mu\text{g kg}^{-1}$)</i>	<i>Middle of the baking oven ($\mu\text{g kg}^{-1}$)</i>	<i>Average ($\mu\text{g kg}^{-1}$)</i>
<i>A</i>	216	431	324 \pm 36
<i>B</i>	563	551	557 \pm 61
<i>C</i>	1881	2231	2056 \pm 226
<i>D</i>	1443	3303	2373 \pm 261

157 The current analyses are in line with the hypothesis that the raw materials are the major factors
 158 influencing the formation of AA, in particular the asparagine content of cereal flours [44-47]. The
 159 highest value obtained for biscuit D (average 2373 $\mu\text{g kg}^{-1}$) can be justified by its composition, since
 160 one of its raw materials is the wheat bran. Wheat bran is the outer part of the wheat grain, which is
 161 removed in flours such as wheat flour type 65. "Whole" flours contain wheat bran and are associated

162 with higher concentrations of asparagine, the amino acid responsible for the major pathway of AA
 163 formation. Another type of raw material that may increase the concentration of AA in biscuits is that
 164 undergoing heat treatment, such as cocoa. Cocoa, which is a raw material with thermal pretreatment
 165 and therefore prone to the formation of AA [48], is present in biscuit C explaining the high content
 166 found (average 2056 $\mu\text{g kg}^{-1}$).



167

168 **Figure 2.** Effect of the position in the oven on the acrylamide content of biscuits. The dashed line
 169 depicts the indicative level (500 $\mu\text{g/kg}$) reported by the European Food Safe Agency [8]. □ middle of
 170 the baking oven ■ edges of the baking oven.

171 2.3. Correlation between acrylamide content and biscuit colour

172 The AA content has been compared with both the colour, by measuring the Hunter Scale
 173 parameters, L, a and b in a colorimeter, as well as with the moisture content of biscuits collected in
 174 different oven positions. As can be seen in the images shown in Figure 3, the browning of the biscuit
 175 associated with the increase of the AA content is clearly observed. The Hunter colour scale
 176 parameters L (darkness) and a (red) increased during cooking confirming that a correlation exists
 177 between the AA content and the browning of the biscuit.



178

408 $\mu\text{g kg}^{-1}$

1443 $\mu\text{g kg}^{-1}$

3303 $\mu\text{g kg}^{-1}$

179 **Figure 3.** Photograph of the same lot of biscuits subject to different cooking temperatures. The
 180 different colors can be observed as well as the corresponding acrylamide levels.

181 Multivariate statistical analyses were carried out in order to define the parameters which most
 182 well correlate with the AA content (Table 3). The L parameter, which is darkness, is the first variable
 183 correlated with AA content whereas an inverse correlation was found between the moisture and the
 184 AA content. This is not surprising, since the temperature of the baking oven is expected to be
 185 negatively correlated with the final moisture content of the biscuit, showing the direct impact of
 186 temperature on the AA content. The influence of temperature on the formation of AA thus seems
 187 confirmed, as demonstrated in previous studies [13, 49, 50]. In addition, Jozinovic et al. [51] have
 188 recently shown that the moisture content and temperature during extrusion had a greater impact on

189 the formation of AA in relation to screw speed. Recent results revealed that at low temperatures used
 190 for the thermal treatment, the amount of AA formed was lower, even if the treatment duration was
 191 longer [52]. In the current work, the baking times are identical for the four biscuit types, thus it is not
 192 possible to associate them with the AA values analyzed.

193 **Table 3.** Correlation matrix between the acrylamide content and the colour and moisture of biscuits.
 194 In bold, significant values (except diagonal) at the level of significance 95%.

	<i>AA content</i>	<i>L</i>	<i>a</i>	<i>b</i>	<i>Moisture</i>
<i>AA content</i>	1,0				
<i>L</i>	0,541	1,0			
<i>a</i>	0,310	0,352	1,0		
<i>b</i>	0,276	0,864	0,663	1,0	
<i>Moisture</i>	- 0,277	0,307	0,304	0,576	1,0

195 3. Materials and Methods

196 3.1. Chemicals

197 Acrylamide ($\geq 95\%$ for HPLC) and acrylamide-d3 standard solution (500 mg/l in acetonitrile)
 198 were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (for UHPLC), ethanol (99.5%)
 199 and dichloromethane (for HPLC) were from Panreac (Barcelona, Spain). High-purity water from a
 200 Millipore Simplicity 185 water purification system (Millipore Iberian S. A., Madrid, Spain) was used
 201 for all chemical analyses and glassware washing. The solvents employed for HPLC were filtered
 202 through a Nylon filter of 0.45 μm pore size (Whatman, Clifton, USA) and degasified for 10 min in an
 203 ultrasound bath.

204 3.2. Standard solutions

205 Concentrated stock solutions of acrylamide (1 mg/mL) and acrylamide-d3 (1 mg/mL), used as
 206 internal standard, were prepared by dissolving the compounds in ethanol. Diluted standard
 207 solutions were further prepared by adding the appropriate volume of each stock solution to water.

208 3.3. Biscuit samples

209 This work has been carried out in close collaboration with a leading company at the national
 210 level and with an international dimension, whose confidentiality will be maintained for obvious
 211 reasons. Four biscuit types were supplied, collected from three different sample points in the baking
 212 oven, as depicted in Figure 1.

213 3.4. Sample preparation

214 The biscuits were grinded in a solid sample grinder (Moulinex, France) and put through a
 215 Endecott's test sieve (London, England). Approximately 1g of each ground biscuit were transferred
 216 into a 50 mL polypropylene graduated conical tube with cap. 250 ng of internal standard (acrylamide-
 217 d3) and then 15 ml of ultrapure water were added to each tube, which was placed in the ultrasonic
 218 bath for 15 min. 2 ml of dichloromethane was added to each tube, left on the rotary shaker for further
 219 20 min. The tubes were centrifuged at 5000 rpm for 15 minutes. 1500 μl of supernatant from each tube
 220 was withdrawn for extraction and purification by solid phase extraction (SPE).

221 For the SPE clean-up, the Oasis HLB SPE cartridge (Waters) was conditioned under vacuum
 222 with methanol (3.5 mL), and equilibrated with water (3.5 mL). Then, 1.5 mL of the withdrawn
 223 supernatant were loaded on the Oasis HLB SPE cartridge and allowed to pass completely through
 224 the sorbent material. The cartridge was rinsed with 500 μL of ultrapure water and samples were
 225 eluted with 1.5 mL of water.

226 For the second step of the clean-up, the Bond Elut AccuCAT SPE cartridge was conditioned
227 under vacuum with methanol (2.5 mL), and equilibrated with water (2.5 mL). Then, the cartridge was
228 loaded with the solution from the previous step and 1 mL was discarded. The remaining volume was
229 collected directly to an injection vial.

230 3.5. LC-ESI-Orbitrap

231 The samples were separated on Accela HPLC (Thermo Fischer Scientific, Bremen, Germany)
232 Electrospray *Orbitrap*, using a C18 Phenomenex Gemini (Phenomenex, USA), particle size of 3
233 microns and size 4.6 mm ID x 150 mm. The samples were eluted through a gradient of 90% solvent
234 A (0.1% HCOOH in water) and 10% solvent B (methanol) for 2 min at a flow rate of 0.4 mL / min,
235 thereafter for 18 min over 100% solvent B and 10 minutes in a 10% solvent B gradient.

236 The analysis was performed on a hybrid mass spectrometer LTQ XL *Orbitrap*TM (Thermo
237 Fischer Scientific, Bremen, Germany), controlled by LTQ Tune Plus Xcalibur 2.5.5 and 2.1.0. The
238 following ionisation (positive mode) parameters were applied: electrospray voltage 3.2 kV, capillary
239 temperature 300°C, sheath gas (N₂) 40 arbitrary units (arb), auxiliary gas (N₂) 10 (arb), and S-Lens
240 RF level at 25 (arb). The automatic gain control was used to fill the C-trap and gain accuracy in mass
241 measurements (ultimate mass accuracy mode, 1 × 10⁵ ions), the SIM maximum IT was set to 50 ms,
242 the number of micro-scans to be performed was set at three. Mass spectra were recorded in
243 multiplexed targeted-SIM mode (t-SIM) with a mass resolving power of 60,000 full width at half
244 maximum (FWHM) with a quadrupole isolation window of 1.0 Da for isolated ions (72.0444 Da for
245 acrylamide and 75.0632 Da for acrylamide-d₃).

246 3.6. Analysis of colour

247 The color of the biscuits was analyzed with the Minolta CR-410 colorimeter. The parameters
248 used were Luminosity (*L*), Red (*a*) and Yellow (*b*). The biscuits were analyzed in their form of
249 consumption (without being ground), so that the color could be considered a method of control in
250 future industrial tests and quality parameters.

251 3.7. Moisture content determination

252 The moisture level of the analyzed biscuits was assessed on the same day of the AA extraction.
253 About 5 g of ground biscuit were dried for 3 h at 100 ° C. After drying and cooling, the dry mass of
254 the biscuit was measured and the moisture content was calculated.

255 3.8. Statistical analysis

256 To measure the strength of relationship between the measured variables, the two most
257 commonly used correlation coefficients were calculated. Pearson's correlation coefficient (*r*) requires
258 both variables to be measured on an interval or ratio scale and the calculation is based on the actual
259 values. Pearson's correlation coefficient may provide the best estimate of the population correlation
260 coefficient if the data are normally distributed. This correlation has two limitations: it is neither robust
261 nor resistant. It is not robust because strong yet nonlinear relationship between the two variables may
262 not be recognized. It is not resistant since it can be extremely sensitive to one or a few outlying point
263 pars. Spearman's correlation coefficient (*ρ*) is a nonparametric statistic and so can be used when the
264 data have violated parametric assumptions and/or the distributional assumptions. Spearman's rank
265 coefficient requires data that are at least ordinal and the calculation, which is the same as for Pearson
266 correlation, is carried out on the ranks of the data. Each variable is ranked separately by putting the
267 values of the variable in order and numbering them [37]. Thus, while the Pearson correlation
268 coefficient reflects the strength of linear relationships, the Spearman rank correlation reflects the
269 strength of monotonic relationship.

270 The statistical package, *StatBox* 7.5 (Grimmer Logiciel, Paris, France) was used for all statistical
271 calculations.

272 4. Conclusions

273 A sensitive and efficient HRMS methodology, based on LC-MSⁿ with electrospray ionization
274 and *Orbitrap* as mass analyser, allowing quantification of AA for specific food matrices of biscuits
275 was presented. Combining the multiplexed targeted-SIM mode for AA and isotopically labeled
276 internal standard (acrylamide-d₃), the proposed HRMS method enables reliable and accurate
277 analyses of AA with very little influence by the matrix components. Under these conditions 3.55 µg
278 kg⁻¹ for LOD and 11.8 µg kg⁻¹ for LOQ are attainable.

279 During baking an increase in AA concentration was observed, as well as for samples taken from
280 the middle of the oven, where the temperatures are higher. Statistical processing of data shows that
281 the composition of the raw materials of the biscuits was statistically the most correlated factor with
282 the AA content. Statistical treatment shows the direct impact of temperature on the AA content as
283 well.

284 This study also reported that two types of biscuits (out of four) contain AA in concentration
285 clearly above the indicative value reported by European Food Safe Agency, confirming the pressure
286 of establishing mitigation measures for the reduction of the presence of AA in these matrices.

287 **Author Contributions:** Conceptualization, L.F.G.; methodology, C.L.F. and D.O.C.; data curation: C.L.F. and
288 L.F.G.; writing - original draft preparation, L.F.G.; writing - review and editing, all authors; supervision, L.F.G.

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