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Determination of Acrylamide in Portuguese Bread by UPLC-MS/MS: Metrological and Chemometric tools

Susana Jesus[1,2], Inês Delgado[1,3], Andreia Rego[1,2], Carlos Brandão[4], Rui Galhano dos Santos[2], João Bordado[2], Isabel Castanheira[1]

[1]*Departamento de Alimentação e Nutrição, Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P., Lisboa, Portugal*
[2]*CERENA - Centre for Natural Resources and the Environment , Universidade Técnica de Lisboa, Instituito Superior Técnico, Lisboa, Portugal*
[3]*Chemical Engineering Department, Instituto Superior Técnico, Lisboa, Portugal*
[4]*Escola Superior de Hotelaria e Turismo do Estoril, Estoril, Portugal*

ABSTRACT
Acrylamide (AA) is known for its potential health hazards and it is present in processed potatoes, coffee, and bread. Wheat is still the most important cereal for bread making, however, the interest in its substitution by other grains is growing due to the consumer demand for novels and healthy foods, like oat and rye flour. The aim of this study was to analyze the AA content of confectioned bread in fifty-four different pastries and make an association of acrylamide contents and the place of production to identify the quality of the flour used. There was a wide variability in AA levels among different breads and within different bread varieties. The median of AA values was 787 µg/kg for whole grain bread, 783 µg/kg for oat bread and 253 µg/kg in rye bread.
With the cluster analysis, it was possible to conclude that besides the factors like baking temperature-time and fermentation time which affects AA formation in bread products, several other parameters such as the formulation, flour quality and the varieties of processing techniques, among others, play a crucial role in the AA formation.

Section: RESEARCH PAPER

Keywords: acrylamide; occurrence; UPLC-MS; EFSA

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Corresponding author: isabel.castanheira@insa.min-saude.pt

1. INTRODUCTION

Acrylamide (AA) is mainly used as a monomer in the polymerization reaction of polyacrylamide. It is present in cooked foods and cigarette smoke; nevertheless, the occurrence of acrylamide in foods was only firstly reported in 2002 [1], [2].

In 1994, the International Agency for Research on Cancer (IARC) classified acrylamide as a carcinogen in animals and probably to humans (Group 2A)[1], [3]–[7]. Also, the European Commission in 2002 classified this compound as category 2 for carcinogenicity and category 2 for mutagen [8]. The harmful effect of such compound on humans was later confirmed, and nowadays the acrylamide is, in fact, considered a neurotoxic substance. In people overexposed to such compound, the peripheral nervous system can be impaired as well as the central nervous one [2], [9]. Besides that, the acrylamide metabolism is considered genotoxic [10]–[17]. After being absorbed and distributed by cells, a process of oxidation by cytochrome P450 2E1 is initiated, leading to the transformation of acrylamide in glycidamide, a genotoxic agent, which reacts with the DNA. The reactional products can originate DNA adducts that are associated with cell damage. Nevertheless, the untransformed acrylamide and its metabolites are eliminated by urine [10], [11], [15], [16], [18].

There are three pathways for the formation of acrylamide in processed foods [19]-[20]. One is the thermal decomposition of lipids which results in a significant amount of acrolein. Afterward, the oxidation of acrolein generates acrylic acid that in the presence of ammonia conducts to the formation of acrylamide. Another pathway is carnosine pyrolysis, which liberates acrylic acid. As in the case of the lipid route, the acrylic acid originates acrylamide in the presence of ammonia. Finally, the main route for acrylamide formation in foods is a consequence of the Maillard reaction which involves a free amino acid (asparagine) and reducing sugars. The reaction of these precursors results in a Schiff base which undergoes through decarboxylation to afford 5-1-oxazolidine. Subsequently, the decarboxylated product could decompose either to acrylamide, 3-oxopropanamide or 3-aminopropionamide (3-APA). The excessive sugar amount could also be responsible for the formation of acrylamide (Figure 1) [4]. M. Cengiz, C. Gündüz reported acrylamide levels of 225 μg/kg and 495 μg/kg in bread and cookies, respectively [21]. In Finland, S. Eerda, K. Hollebekkers, Hallikainen A. and K. Peltonen obtained acrylamide levels in bread and sweet biscuits of 645 μg/kg and 310 μg/kg [22], respectively, but a study in Belgium showed lower results in bread with 34 μg/kg and biscuits with 154 μg/kg [23]. Dietary acrylamide exposure of population is mainly dependent on the contaminant amounts in foodstuffs.

For the risk supervision of acrylamide, it is necessary to identify and characterize it. For non-neoplastic, Benchmark dose (BMDL10) is 0.43 mg/kg b.w. per day, but for neoplastic effects, it is 0.17 g/kg b.w. per day [25].

In this year, the European Commission published a regulation about the acrylamide mitigation measures and benchmark levels in foodstuffs [26]. This regulation establishes mitigation approaches for foodstuff, considering 3 phases, agronomical, product design and processing. However, until this date, EFSA did not set maximum limits but stated indicative values for various food groups such as cereals (Table 1) [24]. Occurrence data is required to support EFSA decisions since the data correlation between acrylamide intake and biological biomarkers of exposure become crucial to evaluate the dietary exposure: it is therefore important to accurately assess the level of acrylamide in various processed food.

To this date, in Portugal as in other European countries, occurrence studies in cereal group are scarce. Therefore, the aim of this study is to assess the levels of acrylamide in bread confectioned in several pastries and make an association of acrylamide contents with the place of production to further identify the quality of flour. These values are discussed and compared with those available in others European Countries.

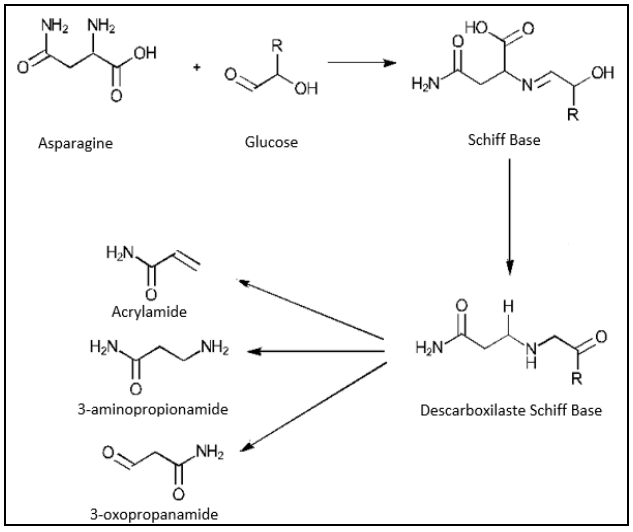

Figure 1. Acrylamide formation of the Maillard Reaction.

Table 1. Indicative values set by European Commission for acrylamide content in cereal-based foods [24].

Foodstuff	Indicative Values (μg/kg)
Soft bread	
a) Wheat based bread	80
b) Soft bread other than wheat based bread	150
Breakfast cereals	
- Bran products and whole grain cereals	400
- Wheat and rye-based products	300
- Maize, oat spelt, barley and rice based products	200
- Biscuits and wafers	500
- Crackers	500
- Crispbread	450
- Gingerbread	1000
- Products similar	500
Biscuits and rusks for infants and young children	200
Processed cereal-based foods for infants and young children	50

2. MATERIALS AND METHODS

2.1 Sampling and sample preparation

Bread varieties were collected randomly from 54 industrial producers located in the district of Lisbon. Producers were asked about dough and bread preparation. The sampling plan is present in Table 2. Samples were analyzed as pool composed of 10 breads from the same producer.

2.2 Dough and bread preparation

The bread products collected were further categorized according to the flour: rye bread, oat bread and whole grain bread (bread with wheat flour and others in minor quantity). The time and temperature of the baking process did not vary between producers, ranging from 190 ^{0}C to 200 ^{0}C. However, no information is available about either all ingredients used in recipe or the fermentation step.

2.3 Reagents, chemical standards and materials AA analysis

For this study, the following reagents were used: Acetonitrile (Merck gradient grid is liquid chromatography), formic acid (Group Carlo Erba Reagents, 99% for analysis) Methanol (Merck, hypergrade for LC-MS) and ultrapure type 1 water (captured from a Milli-Q water purification system).

The standard used was the standard of acrylamide (99%) Dr. Ehrenstorfer GmbH). For the homogenization of samples, a Knife Mill Grindomix GM 200 was used. In the extraction of the compound to be analyzed in this study, an Eppendorf

Table 2. Sampling Plan applied in this study.

Species	Nº Producer	Number of collected samples	Number of pools
Oat	23	230	23
Rye	24	240	24
Mixture	7	70	7
Total	54		

centrifuge 5804 R, a vortex shaker type stirrer, a Kline stirrer and SPE columns (BondElut plexa, 6 mL, 200 mg) were used. In the identification and quantification of acrylamide the ultra-efficient liquid chromatograph (ACQUITY UPLC) was used and the analytical column ACQUITY UPLC BEH C (18, 1.7 μm, 2.1 x 150 m) was obtained from Waters.

2.4 Acrylamide extraction

Bread was immediately prepared after the reception in the laboratory. Each sample was milled separately using a high-speed grinder (Knife mill GRINDOMIX GM), homogenized and stored in vacuum bags. 2 g of homogenized sample were weighed into a centrifuge tube, and 20 ml water/ formic acid (0.1%) was added. The sample was stirred in a vortex for 2 minutes and then in an oscillating shaker for 30 min at 70 oscillations per minute. Then, it was centrifuged at 10,000 rpm for 15 minutes.

Oasis HLB SPE cartridges (Waters) were conditioned with 3.5 ml of methanol and equilibrated with 3.5 ml of acidified water. 1.5 ml of the sample were loaded and eluted with 3 ml of acidified water to a flask of 10 ml, and the volume was completed with acidified water.

All samples were prepared and analyzed as three replicates. The results are expressed as mean and standard deviation.

2.5 LC-MS/MS analysis

Analysis was carried out in an Ultra Performance Liquid Chromatography coupled to a mass spectrometry (UPLC-MS/MS) with electrospray ionization source (ESI). The method was operated using an isocratic elution with 90% water and 10% acetonitrile at a flow rate of 0.2 ml/min. Retention and separation of acrylamide were achieved in a UPLC BEH C18 column (2.1 × 50 mm) with 3 minutes of analysis and a retention time of 0.84 minutes. The UPLC-MS/MS was operated in the positive ion electrospray at the following conditions: capillary voltage of 3 kV, cone voltage 29 V, source temperature 120 °C, desolvation gas temperature 350 °C, desolvation gas flow of 5 l/hr, cone gas flow at 30 l/hr and the pressure of the collision gas was 3×10^{-3} mbar.

2.6 Quality Assurance

The laboratory technical competence needed to carry out the acrylamide assay was provided by ISO/IEC NP 17025:2005.

Equipments used during experiments were calibrated according to approved calibration procedures and external standards traceable to national measurements standard, when available. All volumetric glassware belongs to Class A.

Certified reference material ERM –BD273 (toasted bread powder) was purchased from IRMM and used as a quality control of the method. The laboratory has participated in several PT schemes launched by accredited provider FAPAS to guarantee the laboratory performance. The performance of laboratory was expressed by the z-score, expressing the difference between the laboratory value and target value.

2.7 Statistical analysis

Statistical analysis was performed using Statistica 13 software (Statsoft Ibérica, Lisboa, Portugal). Data were expressed as mean and standard deviation. One-way analysis of variance (one-way ANOVA) was carried out in order to determine significant differences ($p<0.05$) between data.

To make an association between acrylamide content in bread and the producer's recipe the obtained data were analyzed by multivariate hierarchical cluster analysis by linkage Ward's method. The distance between samples was calculated using Euclidean Distances.

3. RESULTS AND DISCUSSION

In this study, accuracy was assessed through certified reference material (ERM-BD273 toasted bread, certified value: 425 ± 29 ng/g) where the obtained analytical values are within 95% of the confidence level of certified value. Laboratory performance was demonstrated by participation in adequate PT Schemes, T3067 (Biscuit (cookie)) and T3059 (Biscuit (cookie)). In these assays, results were rated as satisfactory in all schemes. These results demonstrated the adequacy of the analytical procedure for acrylamide quantification.

The present study focused on Portuguese bread in common commercial market. A total of 54 bread samples divided into 3 groups (oat bread, rye bread, and whole grain bread) were analyzed for acrylamide concentration.

The occurrence data is shown in Tables 3, 4, and 5. In general, the acrylamide level in bread ranged between 60 and 1273 μg/kg depending on the bread types. The acrylamide concentrations in all bread types were order from high to low as whole grain bread (786,86 μg/kg)> oat bread (783,00 μg/kg)> rye bread (252,90 μg/kg). These results revealed high variation in acrylamide amount in different bread types, suggesting that differences in the so-called bread road-map, including types and quality of raw materials, formulations, baking methods can affect the acrylamide formation in bread.

In the whole grain bread was observed a median of 786,86 μg/kg (Table 3). However, there were two samples (producer 1 and 3) in the range of 200 and 600 μg/kg and three samples higher than 900 μg/kg. Once this type of bread had a high percentage of wheat flour, the differences in acrylamide levels can be attributed to the fact that this bread may have various levels of some fractions of wheat, such as germ and bran, which contain high levels of the main precursor (asparagine) for the acrylamide formation in bread [27].

Relatively to the oat bread (Table 4), it was found a median of 783 μg/kg and a maximum level of 1258 μg/kg, results similar to the whole grain bread. Nevertheless, the producers 13, 19, 22, 23 and 51 confectioned bread with acrylamide range between 200 and 600 μg/kg. Also, breads with acrylamide amount in the range of 600 μg/kg and 900 μg/kg were observed. On the other hand, the bread from producers 16 and 53 were below the 200 μg/kg level of this contaminant. The variation among results may be justified through the differences in oat flour brands because the composition of acrylamide

Table 3. Acrylamide levels in whole grain bread collected in several bakeries.

Producer	Acrylamide (μg/kg)
1	328.78 ± 3.14
2	786.86 ± 2.24
3	247.50 ± 18.02
4	1159.66 ± 5.38
5	1235.80 ± 52.30
6	741.18 ± 35.26
7	1149.82 ± 3.32

Table 4. Acrylamide levels in oat bread collected in several bakeries.

Producer	Acrylamide (μg/kg)
13	269.40 ± 5.22
14	1182.42 ± 14.06
15	834.50 ± 18.48
16	59.94 ± 4.02
17	807.88 ± 2.06
18	715.66 ± 2.30
19	344.62 ± 12.44
20	937.32 ± 15.12
21	1105.38 ± 23.56
22	546.18 ± 2.98
23	579.96 ± 7.60
24	1273.10 ± 28.92
25	783.38 ± 17.30
26	1208.28 ± 48.20
27	851.10 ± 5.92
28	1168.96 ± 69.48
29	843.38 ± 10.26
30	723.56 ± 9.26
31	1259.00 ± 11.66
51	387.82 ± 26.88
52	195.42 ± 4.76
53	119.70 ± 4.14
54	749.58 ± 47.10

Table 5. Acrylamide levels in rye bread collected in several bakeries.

Producer	Acrylamide (μg/kg)
8	536.08 ± 44.48
9	638.34 ± 23.54
10	505.98 ± 2.28
11	712.96 ± 65.94
12	645.94 ± 25.72
32	749.58 ± 47.10
33	325.96 ± 6.22
34	209.54 ± 14.18
35	751.28 ± 35.36
36	168.32 ± 12.92
37	319.38 ± 10.16
38	368.44 ± 9.20
39	205.44 ± 18.36
40	252.82 ± 13.58
41	278.30 ± 20.28
42	210.72 ± 13.16
43	216.22 ± 17.24
44	161.98 ± 6.78
45	252.96 ± 21.30
46	204.84 ± 16.54
47	201.46 ± 13.14
48	160.38 ± 12.70
49	176.86 ± 16.56
50	159.72 ± 5.80

precursors in flours are influenced by cultivars, year to year variations, harvest period, climate and soil composition [28].

In Table 5, the rye bread had a median of 252,90 μg/kg and a maximum of 751 μg/kg. Most samples were below 600 μg/kg, even so, acrylamide levels lower than 200 μg/kg were achieved by producers 36, 44, 48, 49, 50. These differences between acrylamide levels in rye bread can indicate that there is a difference in the asparagine content among the varieties of rye flours used by the producers.

As mentioned earlier, the acrylamide indicative value set by the European Commission for soft bread other than wheat-based bread was 150 μg/kg [24]. Some of the oat and rye bread results were around this value, namely from producers 53, 16, 50, 49, 48, 44 and 36.

In 2009, EFSA reported a maximum level of acrylamide in bread group around 2430 μg/kg and in 2011 a maximum value of 2565 μg/kg and 910 μg/kg for bread non-specified and soft bred, respectively [29], [30]. The results obtained were lower than the occurrence levels of acrylamide reported by EFSA. More recently, based on analytical results from a total of 24 European Countries and six associations, EFSA reported maximum levels of acrylamide in unspecified soft bread around 141 μg/kg, which is similar to bread samples analyzed from the producers 16, 53, 50, 48, 49, 36 and 44 [25]. Nevertheless, EFSA reported occurrence values for pumpernickel (rye bread) with a maximum of 245 μg/kg [25]. The majority of bread analyzed, 13 out of 24, contained lower levels of acrylamide which may be explained by the difference between rye bread recipe in Portugal (flour mixture) and the pumpernickel (rye flour).

In the literature, Meghavarnam et al evaluated bread samples from the local market in India, obtaining a range between 1210 – 1365 μg/kg [31]. Similar results were achieved by European Commission in 2007 in bread and toast samples, 1987 μg/kg [32]. All the bread samples collected and analyzed in this work were below the maximum value of Meghavarnam et al and Wenzl studies.

Furthermore, the study published by Krishnakumar and Visvanathan reported a high variability of acrylamide concentration in bread group, <10 – 3200 μg/kg [2]. Also, in this case, the results obtained in the three types of bread were lower than 3200 μg/kg.

On the other hand, other authors had observed minor levels of acrylamide in bread. Normandi et al analyzed the acrylamide concentration in Canadian bread and found a maximum value of 107 ng/g. Also, in bread from Syria and Iran, similar results were achieved, 119 – 263 μg/kg and 166 – 290 μg/kg, respectively [33] [34]. These levels are identical to the results observed in the bakeries 3, 13, 34, 36, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52 and 53.

In a higher range, Pacetti et al obtained an acrylamide content between 102 – 594 μg/kg, which is higher than more than half of the bread samples (producers 1, 3, 8, 10, 13, 19, 22, 23, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52 and 53) [35]. Moreover, Gündüz et al analyzed bread types from Turkey and obtained a maximum of 695 μg/kg for all bread types. In this study, most of the bread samples were below this value. Gündüz et al also reported values for rye bread with a range of 209 – 624 μg/kg, which is higher than the rye bread levels shown in the present work [36].

To study the relationship between the acrylamide levels and the recipe of producers, a cluster analysis was performed.

The results of the cluster analysis (Figure 2) in the present study are derived from the tree clustering analysis.

The hierarchical tree observed in Figure 2 provides a key to understand the relation between the bread samples and the producers.

It was possible to identify the presence of 5 major clusters. The first cluster grouped the bread samples (4, 5, 7, 14, 21, 24, 26, 28 and 31) with the highest values, 1105.38 to 1273.10 μg/kg, where two types of bread, oat, and whole grain bread were included. These results can indicate that the oat bread producers of 14, 21, 24, 24, 26, 28 and 31 used flours with a similar amount of asparagine. Relatively to the whole grain bread, producers can be grouped with respect to the similarity of cooking phase. Nevertheless, the relation between these two types of bread can be explained by various reasons, such as the addition of other ingredients in the whole grain bread that influences the formation of acrylamide.

Most samples identified in the second cluster were from rye bread producers (8, 9, 10, 12, 22 and 23). In this cluster, the acrylamide content varies between 505.98 and 645.94 μg/kg. The similarity of the results observed among the rye bread producers suggests that the flour and the cooking method were identical. In the case of oat bread producers (bakeries 22 and 23) included in this cluster, the similar results can be related to the asparagine content in oat flours.

Cluster 3 included the three varieties of bread (2, 6, 11, 15, 17, 18, 20, 25, 27, 29, 30, 32, 35, 54), but mostly oat bread, where the values are among 712.96 and 937.32 μg/kg. In this cluster, the oat bread values are lower than in cluster 1, which can be related to the lower asparagine content of the varieties of oat flour. The two bakeries that produce whole grain bread should have a large amount of oat flour than wheat flour because the values are close to the oat bread.

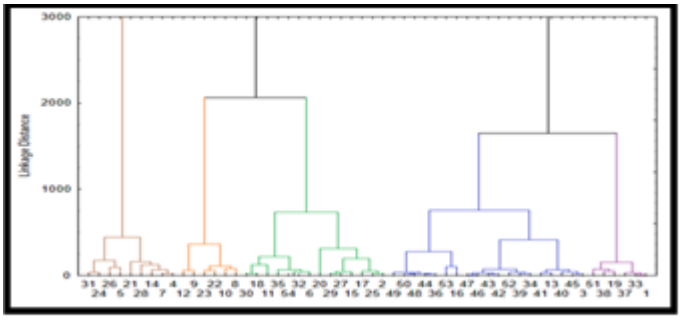

Figure 2. The relation between acrylamide formation and producer. Dendrogram grouped places in 5 clusters.

Cluster 4 was represented mostly by rye bread (3, 13, 16, 34, 36, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52 and 53). In this group, the values of acrylamide are between 59.94 and 269.40 μg/kg, which corresponds to the lowest values of acrylamide in general. To obtain the lowest value the producer may have used flour with low quantity of asparagine, but these results could also be caused by the cook confection, like the use of a high fermentation time.

The cluster 5 grouped the three varieties of bread analyzed in this study (1,19, 33, 37, 38, 51), where the rye bread producers stand out. The acrylamide levels were between 319.38 and 387.82 μg/kg, which represented the average values obtained from bread samples.

The cluster analysis grouped the samples with similar values of acrylamide, which allowed a preliminary conclusion about the association of acrylamide contents with the bread producers. However, there are many factors, such as crop management, fermentation process, that need more attention to comprehend the high variability of the results.

4. CONCLUSIONS

Food is an important source of exposure to acrylamide of the population. Thus, in this study the acrylamide content in confectioned bread in several bakeries was determined and an association of acrylamide contents with the producers was made. The applied Quality Control procedures, framed by ISO 17025 requirements, demonstrated adequacy to guarantee the reliability of the obtained data. Hierarchical cluster analysis, as applied to the data acquired from the content of acrylamide in three variants of bread, was useful. Chemometric tools allowed identifying factors that could influence the acrylamide formation.

This work is aligned with those that advocate the need for national data to estimate the real dietary exposure to acrylamide.

PUBLICATION

Part of this work was presented at 2nd IMEKOFOODS, Benevento 2016, as poster.

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