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CONTRIBUTION OF METROLOGY ON DETERMINATION OF ACRYLAMIDE IN FOODSTUFFS

Catarina André [1], *Inês Delgado* [1], *Susana de Jesus* [2], Ana Ramos[2] Carlos Brandão[3], Nelson Felix [3] *Isabel Castanheira* [1]

[1] Departamento de Alimentação e Nutrição, Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P., Lisboa, Portugal
[2] Departamento de Química, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte da Caparica, Portugal
[3] Escola Superior de Hotelaria e Turismo do Estoril, Estoril, Portugal

isabel.castanheira@insa.min-saude.pt

Abstract – The aim of this study was the determination of acrylamide in food with high consumed in Portugal. Samples were bought randomly from local supermarkets and from Escola Superior de Hotelaria e Turismo do Estoril. Sample preparation involved SPE (Solid Phase Extraction). Two chromatographic methods were developed, UPLC-PDA and UPLC-MS/MS which was more suitable and quantify unequivocally acrylamide.
The acrylamide content in bread and pastries samples analyzed were above the indicative values published by EFSA.

Keywords: Acrylamide, UPLC-PDA, UPLC-MS/MS, Portuguese foodstuff, PT SCHEMES

1. INTRODUCTION

In April 2002, the National Food Administration (NFA) of Sweden and Stockholm University reported that high levels of acrylamide were found in some foods when processed at high temperatures[1]. These results were matter of concern worldwide since this compound has been classified by the International Agency for Research on Cancer (IARC) as “probably carcinogenic to humans”[2]. Besides, acrylamide is genotoxic and still be considered neurotoxic. The neurotoxicity is a problem important, since it affects the peripheral nervous system and if the exposure is high, can affect the central nervous system[3]. Acrylamide is formed in the food through the Maillard reaction that generally occurs between amino acid, especially asparagine and reducing sugars at a temperature above 120 °C[4]. However, the Maillard reaction is not only way of acrylamide formation. This contaminant may also be formed from acrolein, which is formed by glycerol [5].

Occurrence data is currently existing for at least 25 countries as reported the lasted EFSA report [6]. Since Certified Reference Materials are available and Proficiency Testing (PT) schemes are launched regularly by accredited providers, analytical data can be robust and reliable. Matrices spiked are the Quality Control tools implement during laboratory analysis. Isotopic labeled internal standards are frequently in used.

LC-MS/MS are the most reliable methods to determine acrylamide [7]. However matrix effect is the main drawback that can occur using positive electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) in positive or negative mode. This can occur due to interactions of co-eluting matrix components. Because of the interactions between matrix components with target compounds in the ion source, ion suppression of the analytes was often observed. These interferences could not be completely avoided in these methods despite the use of tandem MS due to poor retention of polar acrylamide molecule on conventional LC reversed-phase sorbents [8]. More data in different food matrices is necessary to clarify the influence of interferences in risk assessment since 100 µg of acrylamide is reported as no matter of concern.

The main objective of this work is to develop a method to determine acrylamide content in typical Portuguese food rich in reducing sugars and asparagine processed at higher temperatures (>120ºC).

2. MATERIALS AND METHODS

2.1. Sample preparation

The samples analysed included potato crisps from fresh potatoes, French fries ready-to-eat, crackers, bread, gingerbread, instant (soluble) coffee, coffee substitutes mainly based on cereals, breakfast cereals, chocolate breakfast cereals, toasted bread, and some pastries products, and two Portuguese pastries *pastel de nata* and *bolo do caco*. A total of 35 samples were analysed.

2 g of homogenised sample were accurately weighted into centrifuges tubes. 10 mL of 0.1% formic acid in water were added, shaken, vortex for 2 min and centrifuged for 15 min at 10000 rpm. Oasis HLB SPE cartridges were conditioned with 3.5 mL of methanol, equilibrated with 3.5 mL of 0.1% HCOOH. 1.5 mL of the aqueous layer was

loaded. The extract was allowed to pass through the SPE cartridge and the steps so far were discarded. 3 mL of 0.1% HCOOH, the eluant, were collected into 5 mL volumetric flask which was completed of eluant and a portion was injected.

2.2. Chromatographic method with UV detection

The chromatographic method was firstly done with ultra-violet detection on a PDA detector at 205nm. The procedure was done according to Zhang et al. [9] with some modifications. The standards elution was carried out on an UPLC BEH C18 (50 x 2.1 mm, 1.7 µm) from Waters maintained at 25 °C. The mobile phase was 0.1% formic acid (HCOOH) in water with a flow rate of 0.2 mL/min with total run time of 5 min.

2.3. UPLC-MS/MS method development

In order to determine unequivocally the analyte, mass spectrometry identification method, rather than only the retention time, was performed using direct injection of acrylamide and D_3-acrylamide (Internal standard I.S.).

Solutions with a concentration of 1 mg/kg of standard and I.S. were injected separately and MS parameters defined. The optimized parameters were: capillary voltage, cone voltage and argon collision gas pressure which gave the highest response. Other parameters such as source temperature and desolvation gas temperature, desolvation gas flow and cone gas flow were based on literature [9] and equipment requirements. Therefore, the optimized parameters are summarized in Table 1.

Table 1 – Optimized MS parameters

Capillary voltage	2.50 kV
Cone voltage	29 V
Source temperature	120 °C
Desolvation temperature	350 °C
Desolvation gas flow	800 L/hr
Cone gas flow	30 L/hr

After optimizing MS, optimization of MS/MS is required. The same standards were directly injected in the mass spectrometer. The collision gas used was argon with a collision cell gas flow set to 0.21 mL/min in order to set vacuum pressure at 3–3.5x10^{-3} mbar. Experiments regarding collision energy were performed so that intensity of parent ion was about ⅓ of daughter ion with higher intensity.

Evaluating the spectrum presented in Figure 1 collision energy was set to 14 V. Collision energies from 10 V to 20 V were also tested (spectrum not showed) but the layout of spectrum was not the best.

2.4 Traceability

Equipment used during experiments was calibrated according to approved calibration procedures and external standards traceable to national measurements standard, when available. Calibration values for the analytical balance refrigerated and thermometers were registered and corrections were made when necessary.

All volumetric glassware belongs to Class A. Certified reference material ERM –BD273 (toasted bread powder), from the IRMM was purchase from the producer. All samples were used as provided without further grinding. Moisture content was determined following the instructions for used as described in the certificates of analysis.

2.5 Quality Assurance

To garantee the laboratory performance a participation in proficiency testing programs lanched by FAPAS was cary out.

All the assays were carried out under quality assurance procedures framed by ISO/IEC 17025. SOPs (Standard Operation Procedures) for analysis, sample preparation, traceability and equipment were available.

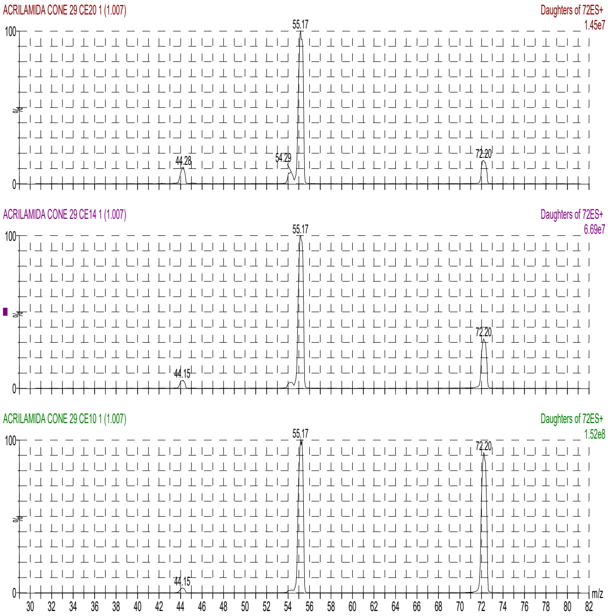

Figure 1 – MS/MS spectrum of acrylamide

The daughter ions can be explained such as following (Figure 2).

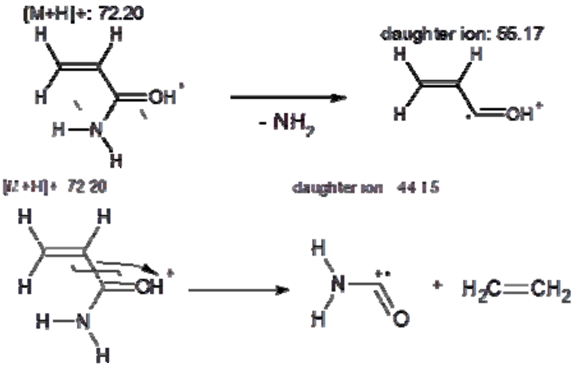

Figure 2 – Fragmentation mechanisms of acrylamide

The same was done to D3-acryalmide (Figure 3 and Figure 4).

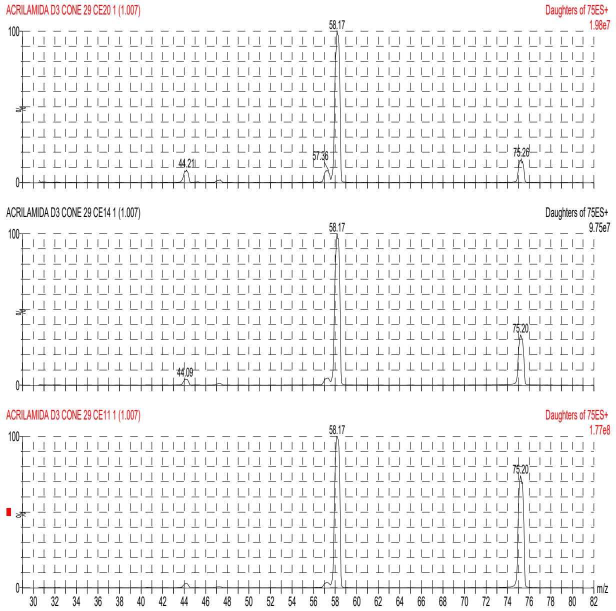

Figure 3 – MS/MS spectrum of D_3-acrylamide

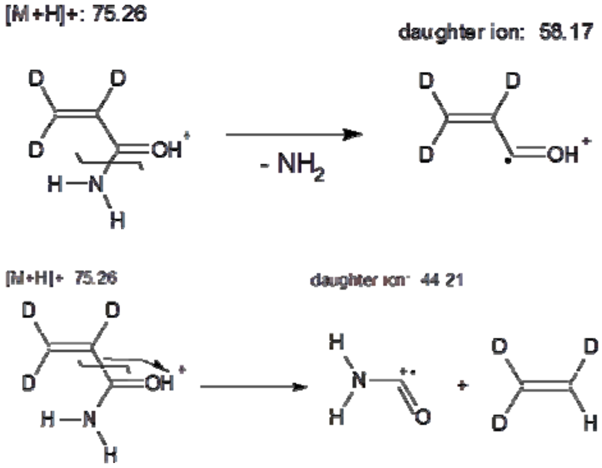

Figure 4 – Fragmentation mechanisms of D_3-acrylamide

Compound identification and quantification in LC-MS/MS is defined by correspondence in retention time and transition of mass fragments. Usually two mass fragments are considered: quantification ion (the one with major intensity) and confirmation ion (the second ion with higher intensity). Since the fragment *m/z* 44 has low intensity and is likely improbable was not considered. Thus, in this work, only two premises were considered: the retention time compared with the standard retention time and the transition 72 > 55 *m/z* for acrylamide and 75 > 58 *m/z* for D_3-acrylamide.

After MS/MS method developed just some adjustments were made to chromatographic method. Thus the run time is 3 min and the mobile phase consists in isocratic elution with 90% of 0.1% HCOOH and 10% of acetonitrile. The need of using acetonitrile is due to hydrophobic collapse of BEH columns. The column temperature was maintained at 25 °C.

3. RESULTS AND DISCUSSION

Figures of merit for method of analysis including work range, linearity, limits of detection and quantification and accuracy were determined by the use of labelled internal standard, D_3-acrylamide and by Certified Reference Material.

Recently, we participated in the acrylamide proficiency testing organized by Food Analysis Performance Assessment Scheme (FAPAS) using the optimal pretreatment and LC–MS/MS method in the present work. Compared to the level (1330 µg/kg) reported by the organization, result from our laboratory for acrylamide (1299 µg/kg) in dispatched test material with a z-score of −0.1 seemed satisfactory.

Samples were spiked with 500 µL of 1 µg/mL of D_3-acrylamide was added in the beginning of the extraction procedure in order to quantify the losses during the extraction process. The use of internal standard is also to quantify, primary in ratio, the content in samples. In Figure 5 is presented the total ion chromatogram (TIC) of acrylamide and D_3-acrylamide of a sample, in this case toasted bread. As we only have one transition TIC chromatogram is equal to the one referring to the transition 72 > 55 *m/z*.

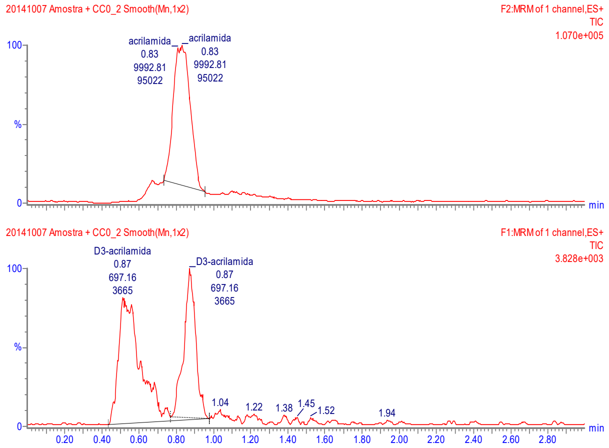

Figure 5 – Chromatogram of acrylamide and D_3-acrylamide of bread sample

Results obtained with Portuguese Foodstuffs are presented in Figure 6 and as we can observed they are lower than values published in EFSA Report [10].

Acrylamide content found in coffee was 25.02 ± 2.75µg/kg and 375.48 ± 25.70µg/kg in homemade potato crisps from fresh potatoes, respectively. The large difference between monitored value and obtained value was for coffee substitutes mainly based on cereals with 2000 µg/kg and 178.20 ± 10.83 µg/kg, respectively. In French fries ready-to-eat the values obtained in this study are from 348.33 µg/kg to 399.44 µg/kg when the indicative value is 1000 µg/kg. Gingerbread and crackers have an acrylamide content of 58.03 ± 7.92 µg/kg and 203.26 ± 12.63 µg/kg, respectively. Lastly, breakfast cereals and chocolate breakfast cereals have 187.74 ± 17.50µg/kg and 238.38 ± 11.42µg/kg when the monitored value is 300µg/kg.

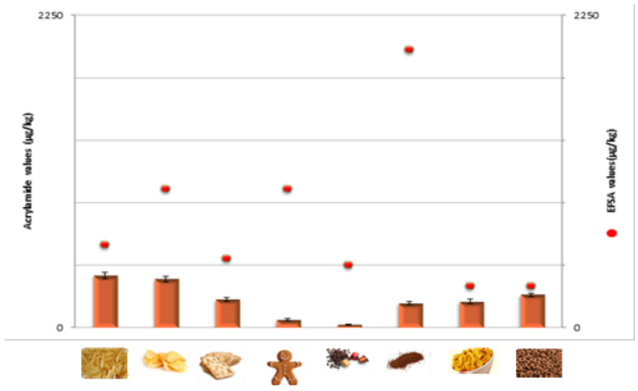

Figure 6 – Results of the samples from supermarket compared with EFSA monitoring

The result of the bread samples and some samples of pastries are presented in Table 2 .

Food	Acrylamide (µg/kg)
Baguette	137,90
Baguette integral	312,70
Bread cereals	298,93
Bread	541,96
Rye bread	625,83
Coconut pie	131,53
Orange pie	300,90
Chocolate cupcake	603,53
Almond muffin	301,58
Oat muffin	518,05
Muffin	644,28
Coconut cake	605,43
God bread	994,48
“Bôla”	310,30
Ham and cheese rolling	3742,79
Cookies with chocolate	948,24
Cookies with cacau	222,35
Toofies	110,04
Greek Cookies	354,85
Arcon Pastel	768,40
Arcon crem	912,25
“Trouxa filó”	3862,07
Arcon dough	660,88
Grass pea tart	808,98

Table 2 – Results of traditional bread and pastries

The samples are all above the indicatives values of EFSA. For baguette and for bread the indicative value is 80 µg/kg and for the others samples of bread group is 150 µg/kg.

For comparison, K. Gramby et al. [11] have results for toasted rye bread and untoasted rye bread, 27-205 µg/kg and 7-23 µg/kg respectively. Those results in comparison, with the figure 7, are lower.

From the baked samples it verifies that a sample stands out, the “trouxa filo” sample has a acrylamide value of 3862 ± 42.84 µg/kg. Then, orange and chocolate cookie cake are two of the samples have also has a high value, 1083.97 ± 18.69 mg / kg and 1057.71 ± 6.93 g / kg, respectively.

It is yet possible, see that the muffin with almond is the lowest value in comparison with the others muffins. It seems that a reduction of acrylamide occur in presence of almond. Remain unknown the compound responsible for this.

This type of product does not have any value indicative of the EFSA. These results are not very high when compared to other results obtained in other studies, as in Visvanathan et al.[12], that it was determined for bakery products and biscuits a maximum value of 3324 µg/kg.

However the results are high, being necessary, as in the case of bread, establish some strategies of reduction.

Results obtained for *Pastel de Nata* are presented in Figure 8. These values are within EFSA indicative data and are not a matter of concern because highest values were described from other bakery products.

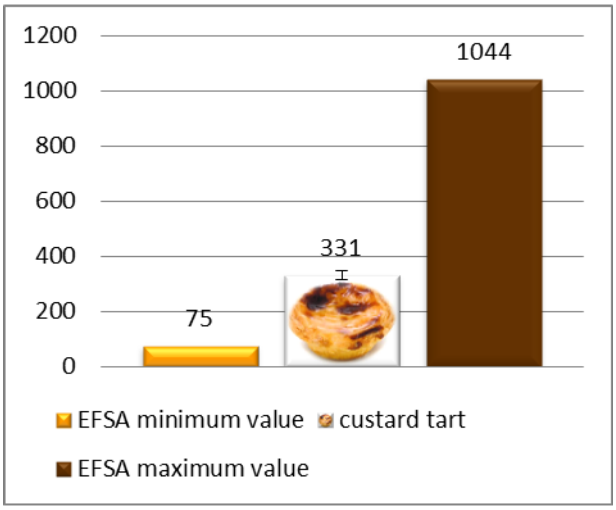

Figure 8 – Results of *pastel de nata*

Bolo do caco (Figure 9) is an artisanal product from Madeira so the manufacture is not very controlled. There are several recipes available with more sugar and others with higher eggs contents. However in our case we have studied different cooking procedures maintaining the same recipe. Four temperatures were studied and yield of acrylamide content was ranging from 300 up to 600**(µg/kg)**

Depending on the type of production the content in acrylamide differed. There is a correlation between the acrylamide formations with temperature. The acrylamide content is low when cooking process takes place at lowest temperature.

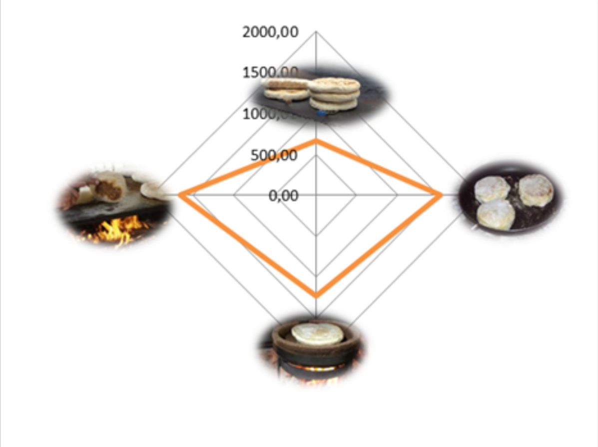

Figure 9 – Correlation between acrylamide formation and cooking process

For comparison Kim et al. [13] determined an acrylamide content of 714.2 µg/kg for biscuits while Murkovic [14] and Senyuva et al. [15] found lower values, 275 µg/kg and 389 µg/kg, respectively. There are several studies on potato chips and the values are very discrepant. Kim et al. [13] found a value of 1376.7 µg/kg similar to Becalski et al. [16] with a maximum value of 1823 µg/kg and Fernandes et al. [17] that found a maximum value of 1828.8 µg/kg. These two also describe minimum values, 50µg/kg and 186.4 µg/kg, respectively. A similar value is presented in Murkovic work [14] with an acrylamide content of 152 µg/kg. Senyuva et al. [15] determined 433 µg/kg of acrylamide content in potato chips which is alike to the value published by EFSA [6]. For coffee Murkovic [14] and Kim et al. [13] found acrylamide levels close to 200 µg/kg. These values are very different from the ones that Senyuva et al. [15] report. In this study roasted coffee values are between 12 and 29 µg/kg. Although for instant coffee the values are very different. Acrylamide level between 42 and 338µg/kg are reported.

It was developed acrylamide reduction strategies for 5 food groups, potato based snack, French fries and other cut, (deep fried) potato products, cereal/grain based products, coffee, roasted grain substitute and baby biscuits, infant cereals and baby foods other than cereal based foods[18].

For each group have been developed several strategies that can be applied in three stages.

The first corresponds to agronomy, which is important choose foods with low content of acrylamide precursors and is also important the way of using fertilizers [19][20].

The second phase, phase of the recipe, which are developed many strategies, some based on the change of pH [21] and other relatively antioxidant power of certain foods [22][23][24]. However the last strategy is not yet conclusive.

Finally, there is a processing step in which there are two main strategies that can be applied, the temperature reduction, since this is a factor which also permits the formation of clear acrylamide, and finally baking or frying time of food to fabricate the final product [25][26].

There is also an asparagine reduction strategy, in which the enzyme asparaginase is used [27] [28]. Unlike other strategies that can be applied in all food groups. However, this approach has a higher cost than the other.

*4. CO*NCLUSIONS

Acrylamide quantification remains an interesting topic in food contaminants analysis. Metrological tools and principles are likewise relevant areas to establish threshold of human toxicity. The method development in this work allows to accurately determining the content of acrylamide in foodstuffs.

When compared with conventional methods of analysis, UPLC-MS/MS method is environmental friendly, by economizing organic solvents, is low time consuming, accurate and highly sensitive, has low detection limit and is adequate to real samples under study, so it can lead to a credible contribution do EFSA. Collaborative studies in different matrices are demanding to improve method extraction and to identify the critical points of the process.

This is the first study to determine acrylamide in Portuguese foodstuff applying metrological concepts. So this work was an attempt to improve the reliability of the analytical results which are necessary to derive realistic exposures estimates of toxicological levels of acrylamide.

Finally, given the results obtained with some samples, acrylamide becomes a major contaminant being studied. Furthermore, it is essential to develop more reduction strategies and try, in the future, the avoidance of acrylamide in processed foods.

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