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OPTIMISATION AND VALIDATION OF THE ANALYTICAL PROCEDURE FOR THE DETERMINATION OF ACRYLAMIDE IN COFFEE BY LC-MS/MS WITH SPE CLEAN UP

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ABSTRACT

Background. Numerous studies have demonstrated acrylamide to be both neurotoxic and carcinogenic. At present it is widely recognised that acrylamide is mainly formed through the *Maillard* reaction from free asparagine and reducing sugars. The major sources of dietary acrylamide are potato products, processed cereals and coffee.

Objective. To optimise and validate an analytical method for determining acrylamide in coffee by liquid chromatography and tandem mass spectrometry analysis (LC/MS/MS) using SPE clean-up.

Material and methods. Analytical separation of acrylamide from roasted coffee was performed by liquid chromatography using a Hypercarb column followed by LC/MS/MS analysis, with 2,3,3–d_3 acrylamide as an internal standard. The method was based on two purification steps: the first with hexane and *Carrez* solutions in order to remove of fat and to precipitate proteins, respectively; and the second with a solid-phase extraction (SPE) column which proved to be efficient in the elimination of the main chromatographic interferences.

Results. Limit of quantification (LOQ) for measuring acrylamide in coffee was 50 µg/kg. The described method demonstrates satisfactory precision (RSD = 2.5%), repeatability (RSD = 9.2%) and accuracy (mean recovery – 97.4%).

Conclusions. Our results confirm that LC-MS/MS with SPE clean-up is selective and suitable for determination of acrylamide in coffee. Indeed, this method meets the criteria of EU Commission Recommendations (No. 2007/331/EC and No. 2010/307/EU), on the monitoring of acrylamide levels in food.

Keywords*: acrylamide, roasted coffee, clean up using SPE, LC-MS/MS*

STRESZCZENIE

Wprowadzenie. W licznych badaniach wykazano, że akryloamid jest związkiem neurotoksycznym i kancerogennym. Obecnie wiadomo, że akryloamid w żywności powstaje w wyniku reakcji *Maillarda* pomiędzy wolną asparaginą a cukrami redukującymi. Głównym źródłem akryloamidu w diecie człowieka są produkty ziemniaczane, przetwory zbożowe oraz kawa.

Cel. Optymalizacja warunków procedury analitycznej i walidacja metody oznaczania zawartości akryloamidu w różnych rodzajach kawy techniką wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS) z wykorzystaniem ekstrakcji do fazy stałej (SPE).

Materiał i metody. Akryloamid w kawie palonej oznaczono na kolumnie Hypercarb metodą chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS). Jako wzorzec wewnętrzny zastosowano 2,3,3-d_3 akryloamid. W metodzie tej zastosowano 2 stopniowy proces oczyszczania próbki: w pierwszej kolejności usunięto tłuszcz heksanem oraz strącono białka poprzez dodanie roztworów Carreza, a następnie wykorzystując ekstrakcję do fazy stałej (SPE) próbkę oczyszczono z pozostałych zanieczyszczeń, które mogłyby mieć wpływ na wynik w czasie analizy chromatograficznej.

Wyniki. Granica oznaczalności metody LC-MS/MS oznaczania akryloamidu w kawie wynosi 50 µg/kg. Opracowana metoda charakteryzuje się dobrą precyzją (RSD = 2,5%), powtarzalnością (RSD = 9,2%) oraz dokładnością (średni odzysk = 97,4%).

Wnioski. Wyniki walidacji potwierdzają selektywność i przydatność opracowanej metody do oznaczania akryloamidu w kawie. Opracowana metoda LC-MS/MS spełnia wymagania zaleceń Komisji Unii Europejskiej (2007/331/EC i 2010/307/EU) w sprawie monitorowania poziomów akryloamidu w żywności.

Słowa kluczowe*: akryloamid, kawa palona, oczyszczanie SPE, LC-MS/MS*

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INTRODUCTION

Acrylamide (H_2C=C-CO-NH_2, CAS No. 79 06 1) is a compound present in foodstuffs as a result from their being heated during preparation. It is formed as one of several products of the *Maillard* reaction between free asparagine and reducing sugars, (eg. glucose, fructose). It also arises when coffee beans are roasted.

Due to acrylamide's adverse effects, the European Commission (EC) in May 2007 gave notice that it should be monitored in foodstuffs throughout the EU during 2007-2009. This covered foodstuffs such as potato products (chips and crisps), cereals (bread, cakes, breakfast flakes and infant cereals) together with roasted coffee [3]. In January 2011 the European Commission issued recommendations based on investigations into the levels of acrylamide in food, where a 'check value' indicator was defined for a range of foods. For roasted coffee, levels not to be exceeded were set at 450 µg/kg whilst for instant coffee this was 900 µg/kg [1]. As a result it became necessary to establish suitable methods for measuring acrylamide in foodstuffs, including in roasted coffee. According to the above recommendation [3], limit of quantification (LOQ) of the analytical methods has been set at level 30 µg/kg and 50 µg/kg for bread and baby foods and for other foods, respectively. Another European Commission recommendation from 2nd June 2010 (2010/307/EU) extended the monitoring period of acrylamide in foodstuffs indefinitely, whilst also broadening the range of foodstuffs to be tested. For example, instant coffee and coffee substitutes eg. coffee grain, which are commonly used in Poland, have now been added to roasted coffee for the purposes of monitoring [2].

Acrylamide levels in foodstuffs are commonly measured by gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass spectrometric (MS) detection and quantitation (GC-MS and LC-MS) [13]. The LC-MS/MS is currently preferred because is rapid and easy to be used, however samples need to be purified, often by using SPE. GC-MS requires the formation of bromide acrylamide derivatives followed by solvent phase extraction.

Sample preparation is an essential step of vital part of such analytical procedure, irrespective of the chromatographic techniques used with the main purpose to purify and remove interfering substances. Determination of acrylamide in coffee can be fraught with difficulty because of its small particle size which must be isolated from such a complex matrix as food. Indeed, coffee is regarded as one of the more challenging food matrices to overcome [6, 12].

The presented study therefore aims to optimise conditions for acrylamide analysis in coffee using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), with particular emphasis on sample purification by SPE via carbon columns as well as performing a method validation. The presented method was developed from our earlier experience to measuring acrylamide in other foodstuff matrices by LC-MS/MS [8-10] as well as other relevant studies obtained from the literature [4-7, 11, 12].

MATERIAL AND METHODS

The test material was the *Robusta* type of ground roasted coffee which had been subjected to moderate degrees of roasting. One sample consists of two commercial packages of coffee, sampled from the same manufacturing batch. Calibration curves were prepared with the appropriate acrylamide standards added to green coffee beans which had previously been confirmed to contain no acrylamide. The half of kg of green coffee beans had been obtained from Polish coffee roasting establishments. Before analysis, individual samples were averaged by mixing and unroasted coffee beans were ground in a laboratory mill.

Reagents

Acrylamide (AA) was supplied by the Fluka Chemie GmbH (99.5+%), whereas the internal standard was bought from Cambridge Isotope Laboratories Inc. (AAd_3; 2,3,3-d_3 acrylamide; 98%). n-Hexane (HPLC grade, 99%+) and potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6x3H_2O$) were obtained from the POCh S.A. Zinc sulphate heptahydrate ($ZnSO_4x7H_20$) was purchased from Sigma-Aldrich, HPLC grade methanol (99.9%) from Rathburn Chemicals and the Mallinckrodt Baker BV supplied LC/MS grades of methanol (99.8%+), and formic acid (99%+).

Solvents

Stock solutions of AA and AAd_3, respectively, were prepared by dissolving 2.5 mg of each substance in 25 ml of deionised water followed by dilution to achieve a working concentration of 1000 µg/L. All samples were spiked with 200 µg/kg of the internal standard (AAd_3). The *Carrez* I solution was prepared by dissolving 15 g of potassium hexacyanoferrate in 100 ml water whereas the *Carrez* II solution was made up of dissolving 30 g of zinc sulphate in 100 ml of water. All stock, working solutions in amber vials, *Carrez* I and II solutions were stored at ~4ºC.

Sample preparation

The samples of 3 g of test ground roasted coffee were weight into 50 mL centrifuge bottle and spiked with 600 µL of the internal standard, (AAd_3 at a con-

centration of 1000 µg/L). Then sample was shaken with 30 ml deionised water and 10 ml hexane at room temperature for 5 minutes. Next the sample was centrifuged for 10 minutes at 10,000 rpm and organic layer was removed. The aqueous layer was incubated at 60 ºC for 30 minutes in an ultrasonic water bath. When samples had reached ambient temperature, 1 mL of the *Carrez* I solution was added, followed by agitation and then with the addition of 1 ml of *Carrez* II solution, again followed by mixing. After 10 minutes the sample was centrifuged again at 10,000 rpm for 10 minutes and the supernatant was transferred into Erlenmeyer flask for solid-phase extraction (SPE).

SPE clean-up

The Bakerbond Carbon column (1000 mg; 6 ml; Mallinckrodt Baker BV.) was conditioned with 8 ml methanol (HPLC grade) and 8 ml deionised water. Then, 10 ml of sample extract was applied to the SPE column and allowed to pass completely through the sorbent material and the column was washed with 1 ml deionised water. After 15 minutes of drying under vacuum, samples were eluted with 5 x 2 ml methanol (HPLC grade) The collected eluents were evaporated to dryness in a heating block held at around 50ºC under a gentle stream of nitrogen. The residues were dissolved in a 1 ml mixture of water and methanol (9:1, v/v) which was then passed through a PVDF(0.22 µm pore size) filter.

Calibration curve preparation

To construct matrix calibration curve six concentrations of acrylamide: 50, 100, 200, 500, 1000 and 1500 µg/L and 200 µg/L of internal standard were used. For the samples of green unroasted ground coffee (3g) were added 3 ml of each standard (including the internal standard) and then proceeding with the samples as described above. Unknown test samples were treated identically but without added acrylamide. The sample of green coffee (blank) without the addition of acrylamide were also analyzed.

Analysis of acrylamide in coffee by LC-MS/MS

Liquid chromatography was performed with the Dionex UltiMate 3000 system on a Hypercarb column (150 mm x 2.1 mm, 5 µm, Thermo Scientific) after a guard column (Hypercarb 10 mm x 2.1 mm, 5 µm, Thermo Scientific). Columns were maintained in 20 ºC during the run. Twenty microlitres of sample was injected and eluted with 0.1% formic acid in mixture of water and LC-MS grade methanol (9:1, v/v) at a flow 350 µl/min for 5 minutes [8, 9].

Mass spectrometric detection was performed on 3200 QTrap triple quadruple instrument (ABSciex, USA) using multiple reaction monitoring (MRM) in the positive ion mode. Experimental conditions were as follows: the curtain gas was nitrogen (CUR = 40), source temperature was set at 600ºC, the electrospray capillary voltage (IS) set at 5000 V and dwell time was 150 ms. The collision energy (CE) and the declustering potential (DP) are given in brackets for each monitored and specified fragment ions as follows for acrylamide: *m/z* 72.1 → 55.2 (DP – 26 V, CE – 14 V), and *m/z* 72.1 →44.1 (DP – 22 V, CE – 20 V); for deuterated acrylamide: *m/z* 75.1 →58.1 (DP – 24 V, CE – 16 V) and *m/z* 75.1 →47.1 (DP – 20 V, CE – 14 V).

The multiple degradation paterns *m/z* 72.1 →55.2 (AA) and *m/z* 75.1 →58.1 (AAd3), were used for quantification. For verification, the ions *m/z* 72.1 →44.1 (AA) and *m/z* 75.1 →47.1 (AAd_3) were used. Identification of the tested compounds was carried out by both retention time and mass spectra. Results were taken as the average between two replicate measurements performed in parallel, adjusted for recovery [8, 9].

RESULTS AND DISCUSSION

Purification of coffee samples using solid-phase extraction (SPE)

Food is a recognised as one of the most difficult matrice for performing chemical analyses due to presence of many nutritions, technological contaminants and environmental pollutants. Therefore analysis of substances that are present in foods in very small quantities requires adequate purification of the sample.

The method was based on two purification steps: the first with hexane and *Carrez* solutions in order to precipitate fats and proteins, respectively; and the second with a layered solid-phase extraction column which proved to be efficient in the elimination of the main chromatographic interferences.

The SPE used Bakerbond Carbon columns, of 6 mL volume, containing 1000 mg of carbon sorbent. This firstly permitted the test analyte to become adsorbed to the carbon and then be readily washed away when a solvent with greater affinity to acrylamide is applied, like methanol. This forms the basic SPE process. Acrylamide elution into the liquid phase was performed by washing six times with 2 mL of methanol where the eluent from each washing were collected into separate flasks followed by chromatographic analysis in order to observe how much acrylamide was present in each aliquots. Thus the amount of eluent required for complete capture of the analyte could be determined.

Table 1 shows that acrylamide was present in fractions 2-5, with 1/3 of total amount of analyte being washed out into fraction 3. No acrylamide was detected in fractions 1 or 6. Using this method, it was concluded that complete elution of acrylamide was achieved using five 2 mL of methanol.

Table 1. Acrylamide content in the fractions of the sample elution of methanol from Bakerbond Carbon (1000 mg, 6 mL)

Phase elution	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
Acrylamide content [%]	0	19.4	33.9	23.9	22.8	0

Validation of the acrylamide analysis

The aim was to determine whether the LC/MS/MS method is suitable for isolating acrylamide from coffee matrice and that it could be correctly identified. Confirmation was so achieved by analysing blank samples and coffee samples spiked with acrylamide and the deuterated internal acrylamide standard. It was concluded that the presented method enabled a satisfactory isolation of acrylamide from other matrice substances and correct identification of parent ions characteristic of acrylamide and its deuterated form; thus the method was deemed as being specific to the analyte (Figures 1 and 2). The validation process consisted of assessing the following parameters: linearity, limit of quantification (LOQ), precision, repeatability and accuracy.

Linearity of the calibration curve was estimated from analysing unroasted coffee samples spiked with various levels of acrylamide, all containing the deuterated internal standard at 200 µg/kg. The calibration curve for the determination of acrylamide in coffee was linear ($r \geq 0.998$) over the range of 50 – 1500 µg/kg (Table 2).

The LOQ was taken as being the lowest standard ie. 50 µg/kg. This level was considered sufficient for this study as measurements were performed on coffee beans but not any coffee infusion ready for consumption, where acrylamide levels are much lower. Repeatability at the 50 µg/kg level was satisfactory at RSD = 8.4% (n=11). This level met the criteria put forth in EC recommendation on the monitoring of acrylamide levels in food.

Precision and repeatability were checked in parallel samples of green unroasted coffee spiked with various levels of acrylamide corresponding to the range of calibration (for precision the RSD = 2.5% and for repeatability the RSD = 9.2%) (Table 2). Furthermore, the precision was determined by analysing 10 replicates in parallel of roasted coffee. The coefficient variation (RSD) was 0.94%. This according to the *Horwitz* coefficient demonstrates that this LC/MS/MS method for determining acrylamide fulfils the criteria for good precision (the HORRAT coefficient value is below 1) (Table 3).

Due to a unavailability of certified reference materials for coffee, together with lack of proficiency testing, accuracy of determination of acrylamide in coffee by LC/MS/MS was assessed by recovery. Samples of roasted coffee containing various levels of the tested substance were spiked with known amount of acryl-

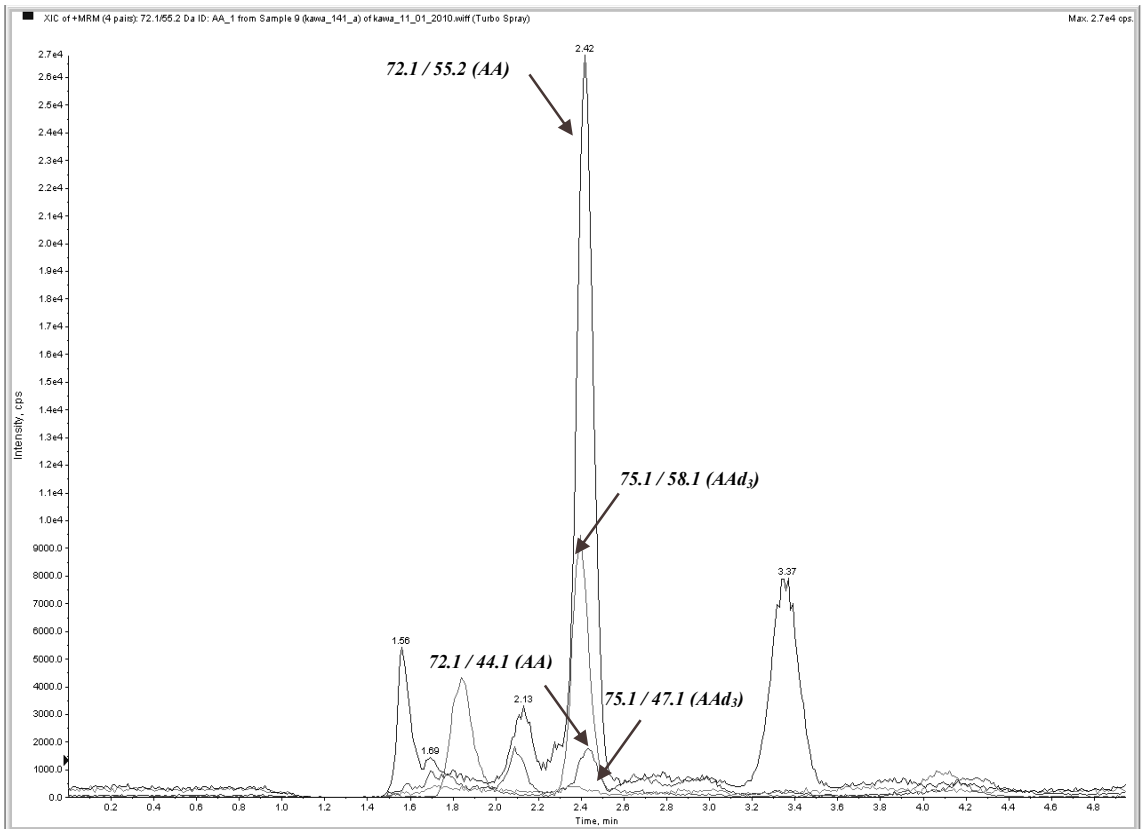

Figure 1. Chromatogram LC-MS/MS for roasted coffee

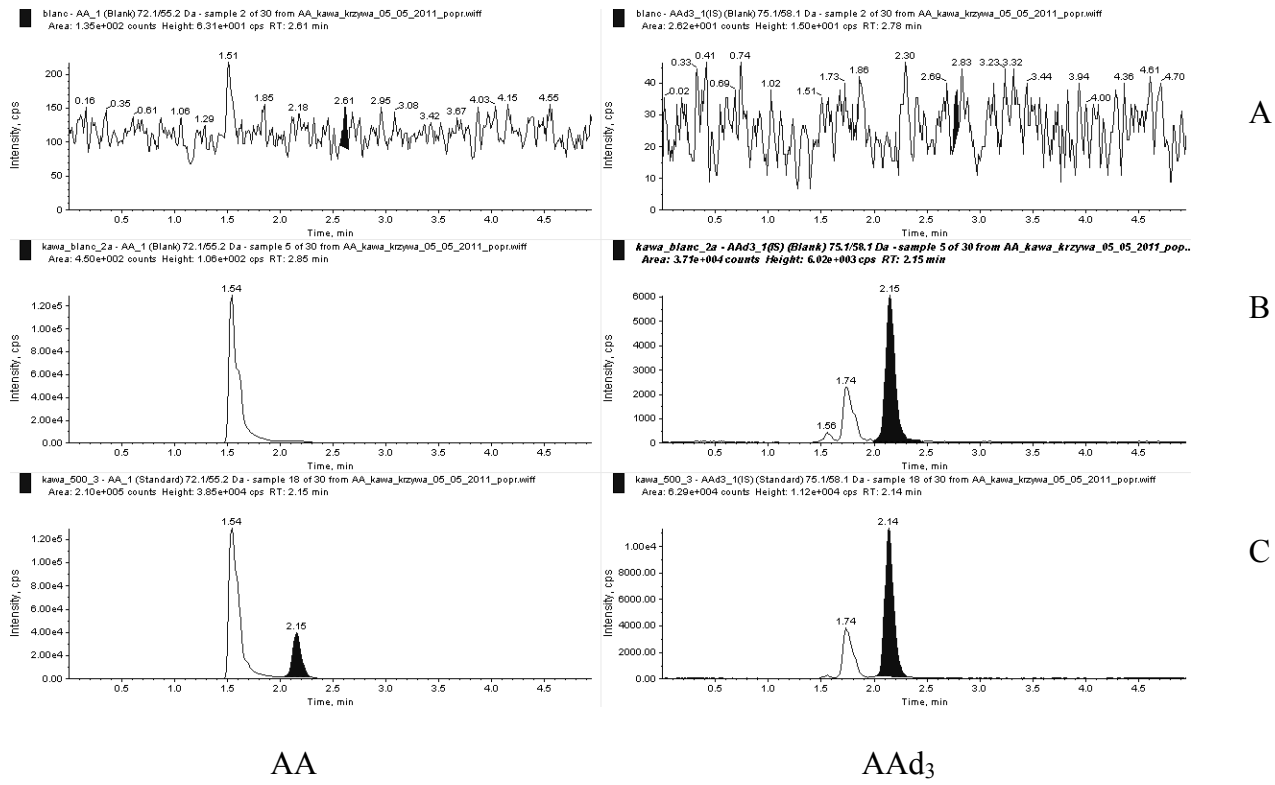

Figure 2. MRM chromatogram: A – reagent blank; B – spiked coffee sample at 200 µg/kg of internal standard (AAd_3); C – spiked coffee sample at 500 µg/kg of acrylamide (AA) and at 200 µg/kg of internal standard (AAd_3)

Table 2. Selected parameters of validated LC-MS/MS method for the acrylamide determination in the green unroasted coffee spiked acrylamide at various levels

Validation parameters	Criteria	LC-MS/MS
limit of quantification (LOQ) [µg/kg]	S/N ≥ 10	50 (S/N = 33)
linear	r ≥ 0.995	r = 0.9987
working range of the method [µg/kg]	-	50 ÷ 1500
within-day precision	RSD < 10%	RSD = 2.5% (bias ± 1.9%; n = 3)
between-day precision	RSD < 10%	RSD = 9.2% (bias ± 2.8%; n = 6)

amide. The average recovery ranged between 96.2% to 101.6% (Table 3).

The uncertainty of results of determining the acrylamide in coffee was ±8.0% as estimated by the budget uncertainty.

In summary, a precise LC-MS/MS method for determination of acrylamide in coffee using SPE (on carbon columns) was developed and the pre-treatment process was optimized. The method is selective, has good precision, repeatability and accuracy. The method is suitable for batch determination of acrylamide content in not only roasted coffee but also in other types of coffee (eg. instant or coffee substitutes) as well as in other products with roasted cocoa beans (unpublished data).

Table 3. Selected parameters of validated LC-MS/MS method for acrylamide determination in the roasted coffee

Validation parameters		LC-MS/MS
mean [µg/kg]		112
range of results [µg/kg]		111 ÷ 114
standard deviation – SD [µg/kg]		1.1
coefficient variation– RSD [%]		0.94
HORRAT coefficient		0.04
recovery at different levels of acrylamide addition [%]	50 µg/kg	98.9 (91.1 ÷ 106.0)
	112 µg/kg	97.3 (94.2 ÷ 101.6)
	500 µg/kg	95.2 (84.6 ÷ 104.0)
	1500 µg/kg	98.1 (97.1 ÷ 99.4)
uncertainty (k = 2, p < 95) [%]		± 8.0

CONCLUSIONS

1. The limit of quantification for the presented LC-MS/MS method for determination of acrylamide in coffee was 50 µg/kg which fulfils the criteria defined in EC recommendations on the monitoring of acrylamide levels in food.
2. The described method demonstrates satisfactory precision (RSD = 0.94% for roasted coffee samples, RSD = 2.5% for unroasted coffee spiked with acrylamide), repeatability (RSD = 9.2%) and accuracy (average recovery of 97.4%).
3. The method validation confirms the effectiveness of the extraction procedure (SPE) for purifying coffee samples as well as that of the LC-MS/MS method for determining acrylamide in coffee.

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