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Research Article

Food Chemical Contaminants

Development and Validation of a Modified QuEChERS protocol Coupled to Liquid Chromatography–Tandem Mass Spectrometry for the Rapid and Accurate Determination of Acrylamide in Cereal-Based Baby Foods

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Abstract

Background: With the widespread consumption by children of cereal-based baby food, acrylamide contamination is a prevalent risk that may have carcinogenic consequences.

Objective: This study aims to develop and validate a modified QuEChERS protocol (quick, easy, cheap, effective, rugged, and safe) without solvent exchange, followed by rapid separation and accurate determination of acrylamide in cereal-based baby foods using reversed-phase (RP)-LC–MS/MS.

Methods: Samples were extracted using a modified QuEChERS protocol of the AOAC version and cleaned up with basic alumina. Separation was performed on a Phenomenex® Kinetex C18 column (100 Å × 3.5 µm × 4.6 mm × 150 mm) using a gradient elution program with a mobile phase of 10 mM ammonium formate–methanol. Determinations were conducted using electrospray ionization (ESI)-MS/MS in positive-ion mode.

Results: Basic alumina yielded clean extracts, resulting in acceptable recovery percentages and a tolerable matrix effect (ME) <5%. This allowed extraction without a solvent exchange step. Efficient separation was achieved at a retention time (t_R) of 3.39 ± 0.05 min employing an RP-C18 column with core-shell properties in a relatively short analysis run time of only 5 min. Trueness, precision, LOD, LOQ, linearity range, and R^2 results were 92.5–104.6%, RSD ≤12.2%, 5 µg/kg, 20 µg/kg, 4.0–1000.0 µg/kg, and > 0.9999, respectively. The test method applicability was demonstrated by proficiency testing (PT) and 50 real samples of cereal-based baby foods. Most of the tested samples were in violation of acrylamide's established European Union benchmark (40 µg/kg).

Conclusion: Acetate-buffered QuEChERS protocol in conjunction with optimized amounts of basic alumina was confirmed as an efficient extraction protocol for acrylamide from cereal-based baby foods resulting in optimal method performance. Successful selection of the RP-C18 column is key for selective separation for acrylamide in a relatively short analysis run time.

Highlights: The modified AOAC QuEChERS protocol with a dispersive solid phase extraction (d-SPE) of basic alumina assisted in reducing the ME to tolerable levels while maintaining acceptable method performance. The use of an RP-C18 column with core-shell properties enabled a rapid and accurate acrylamide determination.

Acrylamide is a common food processing contaminant caused by the interaction of an amino acid (i.e., asparagine) and monosaccharides at temperatures above 120°C (1). This chemical process occurs through the Maillard reaction (2) and is present in several foods, such as chips, French fries, roasted and instant coffee, biscuits, and cereal-based baby food (3). Acrylamide has a low molecular weight and polar properties, and can pose health risks. These risks most commonly include renal and hepatic function disorders, and neurotoxic, genotoxic, and reproductive toxic effects (4).

Since it is a recognized carcinogen in rodents, acrylamide has been classified as a potential human carcinogen (5). Due to limited data on the toxic effects of acrylamide in children under three years old, European Union (EU) regulation (2017/2158/EC) established a benchmark level of 40 µg/kg in various baby foods, including processed cereal-based baby foods, excluding biscuits and rusks (6). The rapid growth pattern of infants in the first years of life increases their need for complementary foods, which is why cereal-based baby food constitutes an important source of nutrition for children around the world. The manufacturing process of these products involves various heat treatments such as hydrolysis and spray-drying (3). The infants and children's high consumption of these products may be a significant source of daily exposure to acrylamide (7).

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In continuous context, acrylamide analysis is critical for ensuring baby food safety and accurately assessing intake in this vulnerable high-risk group. However, owing to the physicochemical characteristics of acrylamide, previously reported analytical methods exhibited a set of limitations. Michalak et al. (3) have extracted acrylamide from ready-to-eat and instant baby foods, and cakes and candy bars using methanol–water and clean-up with Oasis HLB cartridges, followed by determination using ion-pair reversed-phase (RP)-HPLC coupled with a photodiode array detector set at 200 nm. Similarly, Lambert et al. (8) conducted a solid phase extraction (SPE) method using hydrophilic–lipophilic balance (HLB) cartridges in infant and toddler foods using LC–MS/MS. Both methods are quite long and require additional clean-up techniques that have common drawbacks such as cost, reproducibility, recovery issues, and limited method throughput. Although Tolgyesi and Sharma (9) presented a dilute and shoot extraction method, a quite long extraction of up to 60 min coupled to hydrophilic interaction liquid chromatography (HILIC)–tandem mass spectrometry for determining acrylamide in gingerbread samples using a TSKgel Amide-80 column was proposed.

Recently published protocols reported extraction procedures based on the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method and a subsequent determination of acrylamide with RP-LC–MS/MS (5,10–12). Although the conventional QuEChERS protocol is widely applied for various analyses, mandatory modifications are still essential to account for differences in analytes and food matrixes especially when using different SPE techniques (13). From this standpoint, appropriate selection for SPE sorbents is of critical importance for efficient extraction of polar compounds from food matrixes based on cereals. Hence, normal-phase SPE techniques employing alumina, silica, and Florisil are commonly applied because of their adsorption capacities (14). On the other hand, ion-exchange sorbents such as primary secondary amine (PSA) provide separations based on electrostatic attraction of charged functional groups of analytes to oppositely charged functional groups on the sorbent (13).

The meticulous selection of chromatographic columns for polar compounds like acrylamide is a critical factor in their efficient separation from challenging matrixes (15). Normal-phase chromatographic columns are commonly employed for effective separation of polar substances, although their limited range of applications makes their use less widespread (16). Hence, HILIC, which is a variant of normal-phase-based column, has been described for acrylamide analysis, outperforming its common disadvantages (9,16). Nonetheless, due to HILIC's reliance on acetonitrile as a mobile phase, any alternate mobile phases could present a number of problems during positive electrospray ionization (16,17). A Hypercarb chromatographic column was recently used for acrylamide analysis due to its remarkable retention of extremely polar analytes, pH stability from 0 to 14, and resistance to aggressive mobile phases (18). On the other hand, polar analytes are not well retained on RP columns; several C18 columns have been reported for acrylamide's separation (3,10,12). Hence, further investigations are still necessary for successful chromatographic separation with C18 columns.

The current study aims to develop and validate a rapid and accurate testing method for acrylamide in various cereal-based baby foods employing a modified QuEChERS extraction protocol without solvent exchange coupled with LC–ESI-MS/MS. It also aims to provide appropriate selection of a RP chromatographic column to achieve optimal acrylamide separation with the best possible peak characteristics for accurate quantitation results. According to EU validation requirements, selectivity and recovery percentages, repeatability, and within-laboratory reproducibility, LOD, and LOQ were tested for the proposed method of analysis. The validated test method was also applied to real commercial samples and proficiency testing (PT) samples to confirm test result reliability.

Experimental

Apparatus

(**a**) ExionLC™ system (Applied Biosystems/Sciex, USA) connected to Sciex Qtrap 6500+ tandem mass spectrometer (Applied Biosystems/Sciex, USA).

(**b**) Milli-Q Integral 5 water purification system (Millipore, USA).

(**c**) SPEX™ Sample Prep 2010 Geno/Grinder™, versatile high-throughput plant and animal tissue homogenizer, with speed from 500 to 1750 strokes/min (SPEX Sample Prep, USA).

(**d**) Centrifuge Z 446 K with relative centrifugal force (RCF) of 14532 (16022 × *g*) for 10 × 50 mL (HERMLE Labortechnik GmbH, Germany).

Reagents and Standard Solutions

(**a**) HPLC grade acetonitrile (MeCN) and methanol (MeOH) with purity ≥ 99%.

(**b**) Deionized water was obtained from a Milli-Q Integral 5 water purification system (Millipore), with a resistivity of 18.2 MΩ×cm (at 25°C) and a total organic carbon (TOC) value below 5 parts per billion (ppb).

(**c**) Magnesium sulfate, anhydrous powder (≥99.5%), and sodium chloride, high purity grade, powder, (≥99.5%) were obtained from Sigma Aldrich (St. Louis, USA).

(**d**) PSA-bonded silica, bulk packing. Average pore size 70 Å and particle size 50 μm, pKa 10.1, was purchased from Supelco (St. Louis, USA).

(**e**) Formic acid (98%), sodium acetate anhydrous (≥99.0%), sodium hydroxide (≥98%), sodium citrate dehydrated, citric acid, sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate were of analytical grade and obtained from Sigma Aldrich.

(**f**) Basic alumina (basic aluminum oxide), high purity grade, powder, average pore size 58 Å and pH 9.5 ± 0.5 in water was purchased from Supelco.

(**g**) Florisil (activated magnesium silicate), high purity grade and 60–100 mesh, obtained from Sigma Aldrich.

(**h**) Silica gel, high purity grade, average pore size 60 Å, 70–230 mesh, and 63–200 μm, obtained from Sigma Aldrich.

(**i**) Standard solutions: a stock solution of 10 mg/L was prepared by dissolving 1 mg acrylamide standard in 100 mL MeCN. The working standard solution of the studied compound was prepared by diluting a suitable aliquot of the stock solutions with MeCN to a concentration level of 1 mg/L, which was used to spike baby food samples for recovery testing. A set of calibration standard solutions 4.0–1000.0 ng/mL was prepared in MeCN. Stock standards and working solutions were stored at −20±2°C and 4–8°C, respectively, away from direct light.

Sampling

Fifty samples of various commercial cereal-based baby foods for infants and young children were purchased from the domestic market. Wheat was the primary ingredient in cereal-based baby

foods. Furthermore, different sugar sources, such as follow-on baby food containing dried fruit and/or natural fruit powder derived from bananas, apples, pears, or raspberries, and/or milk, chocolate, and honey, were used in some cases. The studied sample varieties were cereals with honey (18%), chocolate (22%), fruits (18%), and milk (20%), while 22% of the samples were cereals without milk. All products were flour powder, except for those based on honey and chocolate, which were ready-to-eat flakes. All purchased commodities were present in sachets made of aluminum-coated foil inside cardboard packages (see Supplemental Table S1). The cereal-based baby foods were ground by a blender to homogenize them and the samples were kept in amber glass bottles with Teflon-lined caps at the ambient room temperature 23 ± 2°C until analysis.

Sample Preparation

A modified procedure based on an AOAC INTERNATIONAL QuEChERS protocol was carried out (19), in which an aliquot of 1.0 g ground sample was weighed in a 50 mL polypropylene centrifuge tube. For spiked samples, an appropriate volume of the acrylamide working standard solution was added to a final concentration of 50 µg/kg. The spiked samples were vortex mixed for 30 s and stored away from light at room temperature for 10 min to allow the studied compound to interact with the sample components. Deionized water (10.0 mL) was added to each tested sample, followed by vortex mixing for 1 min., till complete homogeneity was attained. MeCN (10.0 mL) was then added, and vigorously shaken by a mechanical shaker for 1 min at 900 revolutions per min (rpm). Afterward, 6.0 g $MgSO_4$ and 1.5 g sodium acetate were added. The whole mixture was vigorously shaken for 1 min., using a mechanical shaker at 900 rpm. Complete phase-out separation was obtained by centrifugation at 9300 × *g* for 5 min, under cooling conditions. An aliquot of 2.0 mL from the obtained supernatant was transferred to a 15.0 mL plastic tube containing 25.0 mg basic alumina (pH 9.5 ± 0.5). Samples were vigorously shaken and centrifuged for 5 min at 9300 × *g*, under cooling conditions. The obtained supernatants were then filtered through a 0.20 µm polytetrafluoroethylene (PTFE) Acrodisc into a 2 mL amber glass vial, and then directly injected into the LC–MS/MS system.

LC–MS/MS Analysis

Analysis was carried out using an ExionLC system (Applied Biosystems) connected to an API 6500 plus tandem mass spectrometer (Applied Biosystems). A Phenomenex® Kinetex C18 (100 Å × 3.5 µm × 4.6 mm × 150 mm) obtained from Phenomenex (Torrance, CA, USA) was used to perform chromatographic separations. The column compartment and sample tray were maintained at 40°C and 4–8°C, respectively. Separations were assessed in terms of sensitivity and peak characteristics (i.e., symmetry and responses). An additional two columns from various suppliers, of different particle sizes and or shape (Phenomenex Synergi polar-RP C18 [80 Å × 4 µm × 4.6 mm × 250 mm] and ThermoScientific® Acclaim C18 [3 µm × 3 mm × 100 mm]), were selected and tested for efficient separation testing using spiked samples and pure standards solutions at appropriate concentrations.

Separations were carried out using a multi-step gradient elution program at a flow rate of 0.3 mL/min. The mobile phase composition was: (A) 10 mM ammonium formate buffer in MeOH–water (1 + 9, v/v), pH 5.5 ± 0.05, and (B) MeOH. To achieve a run time with enough data points for the studied compound over 8 min, the gradient was as follows 0.0–2.0 min (90–10% B), 2.1–4.5 min (20–80% B), and 4.6–8.0 min (90–10% B). The injection volume was 2 µL and detection was achieved using electrospray ionization (ESI) in positive-ion mode with the following conditions: curtain gas 20.0 psi, collision activated dissociation, 8.0 (arbitrary units), ion spray voltage 4500 V, temperature 400°C, nebulizer gas 50.0 psi, and heater gas 60 psi. The main MS parameters used for acrylamide determination were a declustering potential of 51.0 V, and collision energy of 15 V. The acquisition method was carried out in multiple reaction monitoring (MRM) mode, with one MRM used for quantification (quantifier peak) and the other for confirmation (qualifier peaks). The parent and quantifier ions were *m/z* 72.1 and 55, respectively, while the qualifier ions were *m/z* 54 and 44.

Calibration and Validation

A working standard solution of acrylamide was prepared in MeCN at a concentration level of 1 µg/mL and used to prepare a set of eight calibration standard solutions (4.0–1000.0 ng/mL) by serial dilution using MeCN (4.0, 10.0, 50.0, 100.0, 300.0, 500.0, 700.0, and 1000.0 ng/mL). All calibration standards were analyzed as described using the LC–MS/MS system. Calibration curves were constructed by plotting concentration (ng/mL) versus instrument response and R^2 was estimated. Similarly, a matrix-matched calibration curve was also constructed for matrix effect (ME) assessment.

An in-house validation was performed according to Eurachem 2014 validation requirements (20), in which selectivity and mean recovery percentages (trueness), precision (repeatability and within-laboratory reproducibility), linearity, LOD, and LOQ were tested. The ruggedness of the assay was verified on an ongoing basis through its use for routine analysis of processed cereal-based baby food samples. Analyst® software version 1.6.2 (Applied Biosystems/Sciex) was used for data processing and calculations.

Blank samples were spiked at three levels, 20.0, 40.0, and 80.0 µg/kg. At each level (six replicates) the analysis was performed to measure trueness expressed as average percentage recovery ± RSD. At the same levels, precision values were also measured and expressed as CV% for both repeatability and within-laboratory reproducibility, except that the within-laboratory reproducibility was performed on three successive days at 40.0 µg/kg (six replicates, $n = 18$). Acrylamide selectivity testing was carried out by investigating the elution region for potential interferences by comparing blank samples with spiked ones at the LOQ level. Various blank processed cereal-based baby food samples of different compositions and acrylamide-spiked samples at 20.0 µg/kg were extracted using the described analysis method and determined by LC–MS/MS. A practical estimation for LOD and LOQ was carried out through further dilution of the standard solution to the lowest possible concentrations for accurate identification and quantitation. Signals that were 3 times the observed noise, were considered the LOD. On the other hand, the concentration at a level equal to 10 times the noise is used as the LOQ, taking into account that the result should be at or well below the acrylamide EU benchmark.

Results and Discussion

LC–MS/MS Optimization

Initially, the MS/MS operational parameters were monitored and recorded for the studied compound on both solvent-based and matrix-matched standards. Furthermore, operational settings were fine-tuned to achieve the highest sensitivity possible. Stable

ion signals with marked sensitivity improvements were obtained in the positive-ion mode and an acrylamide MRM method was constructed. Hence, three transitions that emerged from a parent ion of *m/z* 72.1 were selected. The quantifier ion is *m/z* 55, and the remaining ions of *m/z* 54 and 44 were used as qualifiers for further confirmation as per EU guidelines for confirmatory methods (20).

Efficient acrylamide separation is primarily dependent on the pH adjustments as well as the chromatographic column types, which is why various mobile phase compositions with pH ranging from 4 to 5.5 were tested on the three aforementioned columns using mixtures of 1, 5, and 10 mM ammonium formate in water–MeOH or –MeCN, 9 + 1, respectively. Given the polarity of the tested analyte, a multi-step linear gradient elution program rather than an isocratic elution program was required to minimize the interfering components arising from the studied matrixes.

The elution power of MeCN was immensely greater than MeOH leading to quite a short analysis time, but with many interferences noticed in the final obtained chromatograms. Gradual pH increments from 4 to 5.5 resulted in efficient separation and sensitivity improvement upon using the Kinetex C18 column in a total analysis run time of 8.0 min.

It deserves to be mentioned that a high molar concentration of 10 mM ammonium formate played a critical role in the sensitivities obtained. This is because of the high buffer capacities obtained which enhance the pH stability of the various extracted samples that have variable composition, leading to minimal fluctuation during the MS/MS ionization process. Further, high sensitivity was achieved using the Kinetex C18 column at t_R of 3.39 min, in which a composition of 20.0% of the mobile phase A is used (equivalent to 2 mM ammonium formate). This is because the low molar concentration at the elution time of the studied analyte helped reduce the background noise, with marked sensitivity obtained.

Furthermore, this improved result could be attributed to the Kinetex C18 column's core-shell properties, which aided in the best possible peak characteristics, resulting in accurate quantitation (21). On the other hand, a marked poor separation and/or sensitivity was obtained for the rest of the columns studied (i.e., Acclaim and Synergi).

As shown in Table 1, and in agreement with Bertuzzi et al. (10) and De Paola et al. (12), comparable sensitivities and a total analysis run time of approximately 10.0 min were also reported upon application of C18 columns such as X-Select HSS T3 (10), and Gemini (12). Besides, a similar performance to the Zorbax Eclipse XDB C8 column previously employed by Petrarca et al. (11) has been obtained.

A Synergi Hydro-RP C18 column, on the other hand, resulted in quite a long analysis run time of 30.0 min with comparatively low obtained sensitivity (3). Because of the exceptional retention of very polar analytes and pH stability from 0 to 14, Lambert et al. (8) have employed the Hypercarb column for efficient acrylamide separation. Nevertheless, an extremely long analysis run time of 20.0 min was reported. In terms of sensitivity and analysis run time length, the tested column (Kinetex C18) produced comparable findings to the HILIC TSKgel Amide-80 column (9).

In agreement with previous publications (10–12), the given column's size and the applied mobile phase flow rate (Table 1) resulted in a similar solvent consumption of around 2.0 mL during the entire run time. Contrary to Lambert et al. (8), Michalak et al. (3), and Tolgyesi and Sharma (9), the proposed test saved substantially more solvents.

To investigate the much suitable injection volume for optimal sensitivity along with efficient separation, various injection volumes of 1, 2, and 5 µL were tested. The obtained results revealed that 2 µL injection volume is the most appropriate quantity with a limited introduction to interferences while convenient sensitivity is kept. Hence, optimal separation and sensitivity were accomplished in 5 min using the Phenomenex® Kinetex C18 column and a gradient elution program as described earlier using a mobile phase composition of MeOH and 10 mM ammonium formate (1:9 v/v), pH 5.5.

Sample Size Optimization

In terms of acceptable recovery percentages and precision values expressed as CV% in conjunction with the low introduction of interferences, the sample size is crucial in efficient sample preparation. Thus, sample sizes of 0.5, 1, and 2 g were spiked at 50 µg/kg (triplicates, each), and the obtained results were assessed based on the established criteria. For all studied matrices, a 1 g sample size was found to be optimal in providing acceptable recoveries while keeping interferences to a bare minimum, with CV% < 12. On the other hand, a sample size of 0.5 g produced irreproducible results in terms of CV%, while a sample size of 2 g produced a huge number of interferences that hampered peak identification and determination. Afterward, extracting the acrylamide contents from the studied matrices was carried out, utilizing the optimized sample size of 1 g using the optimum procedures as will be described below.

Extraction Optimization

Non-buffered spiked samples at spiking level of 50 µg/kg were extracted using MeCN, followed by vigorous shaking using a mechanical shaker at a time interval of 10 to 60 min, aiming to provide streamlined analysis protocol. Extraction procedures yielded an extremely high mean percentage recovery of 300%, with a CV% of 40 whatever the extraction time is. This overestimated result could be primarily attributed to the high extractability power of MeCN, which leads to the introduction of high amounts of interferences that affect the final results. Therefore, various extraction solvents such as MeOH and ethyl acetate, and mixtures of MeCN/H2O and acidified MeCN/H2O with 1.0% formic acid at a respective ratio of 50:50 v/v, 70:30 v/v, and 80:20 v/v were employed to test the extraction efficiency. Both MeOH and ethyl acetate have provided no tangible difference relative to MeCN in terms of percentage recovery and precision.

In agreement with Tolgyesi and Sharma (9), convenient recoveries were successfully achieved, irrespective of the mixing ratio of MeCN–water. However, the negative peak and the strong enhancement ME (> +50.0%) obtained have severely affected the method's reproducibility. As a result, a limited scope of application to the studied matrixes was obtained owing to the complex and variable sample composition. Despite the fact that the obtained negative peak was successfully controlled via the addition of appropriate volumes of 0.1 N sodium hydroxide to the final extracts of the studied commodities, a peak split was observed owing to the keto-enol tautomerism phenomenon.

In light of the obtained results, sample processing employing various buffer systems, including both the AOAC *Official Method* **2007.01** (19) and the CEN standard method EN 15662 (22) with a QuEChERS protocol is mandatory. Briefly, the official AOAC Method calls for acetate buffer while the EN method calls for citrate buffer. Likewise, a buffered procedure based on a 0.1 M phosphate system, with a pH ∼ 5.8 was also tested for efficient acrylamide extraction from cereal-based baby food. The latter

Table 1. Outputs of acrylamide analysis methods versus the proposed assay protocol in various food commodities, including cereal-based baby food

P.O.C	Current Study	De Paola et al. (2017) (12)	Petrarca et al. (2017) (11)	Bertuzzi et al (2017) (10)	Lambert et al. (2018) (8)	Michalak et al. (2013) (3)	Tolgyesi and Sharma (2020) (9)
Commodity tested	Cereals-based baby food	Dried fruits and edible seeds	Fruit, cereal and milk-based baby foods	Roasted coffee, barley, potato crisps	Cereal-based baby foods	Cereal-based baby food	Gingerbread and other food
Determination technique	LC-ESI-MS/MS	LC-ESI-MS/MS	LC-ESI-MS/MS	LC-ESI-MS/MS	LC-ESI-MS/MS	RP-HPLC-DAD	HILIC-MS/MS
Sample processing							
Extraction protocol	Modified QuEChERS (AOAC version)	QuEChERS (MgSO4 + NaCl)/PSA	QuEChERS Principals	QuEChERS Principals	SPE	SPE	Dilute-and-Shoot
Extraction solvent	MeCN	MeCN	MeCN	MeCN with 0.2% FA	H_2O	80% MeOH in H_2O	A Mix. of acidified aqueous MeCN
Extraction time (min)	10.0	25.0	45.0	70.0	30.0	70.0	60.0
Solvent exchange (Vacuum or N_2 stream Evaporation)	No	Yes (with H_2O), N_2 stream Evaporation	Yes (acidified H_2O with FA), N_2 stream Evaporation	Yes (with MeCN: FA), N_2 stream Evaporation	Yes (with MeOH), N_2 stream Evaporation	No	No
Cleanup procedure and applied sorbent amounts	d-SPE (100 mg basic alumina)	d-SPE (25 mg PSA)	d-SPE (350 mg PSA) + SPE (100 mg Bond Elut SCX)	d-SPE (150 mg Al_2O_3) + SPE (60 mg OASIS HLB)	SPE (200 mg Oasis HLB)	SPE (200 mg Oasis HLB)	Not Applicable for all tested commodities except coffee* SPE for coffee (60 mg Strata-XL-C SPE)*
Separation							
Mobile Phase Composition	10-mM Ammonium formate/MeOH (9:1 v/v) and MeOH	99.5% of 0.1% FA in H_2O and 0.5% of 0.1% FA in MeOH	MeCN and 0.01% FA in H_2O	0.01% FA in MeCN and 0.01% FA in H_2O	0.2% FA in H_2O/MeOH and MeOH	5-mM sodium of 1-heptanesulfonate in H_2O/MeCN (97:3, v/v)	5% of 1-mM ammonium formate and 95% of MeCN
Elution program	Gradient	Isocratic	Gradient	Gradient	Gradient	Isocratic	Isocratic
Flow rate (mL/min)	0.30	0.25	0.20	0.20	0.20	1.0	0.80
Chromatographic Column	C18 Core-shell Phenomenex Kinetex column (100 Å × 3.5µm × 4.6 mm × 150 mm)	C18 Gemini column (25 cm × 2 mm i.d. × 5 µm particle size × 110 Å pore size)	C8 Zorbax Eclipse XDB column (150 mm × 2.1 mm i.d., 5 µm particle size)	C18 X-Select HSS T3 column (2.5 µm particle size, 150 × 2.1 mm i.d.)	a Hypercarb column (50 mm × 2.1 mm i.d., 5 µm particle size) Equipped with a Hypercarb guard column (10 mm × 2.1 mm i.d., 5 µm particle size)	C18 Synergi Hydro-RP (80 Å × 4 mm × 250 × 4.6 mm)	HILIC TSKgel Amide-80 column
Total run time (min)	8.0	7.0	10.0	10.0	20.0	30.0	8.0
Validation Parameters Requirements							
Linear dynamic range (ng/mL)	4.0–1000.0	1.0–500.0	10.0–300.0	1.0–250.0	0.10–200.0	5.0–20 000.0	N/A
R^2	0.9999	0.999	0.9934	N/A	N/A	0.9997	0.998
LOQ (µg/kg)	20.0	5.0	20.0	22.0	18.0	N/A	20.0
LOD (µg/kg)	5.0	2.0	10.0	9.0	7.0	10.0	8.0
RSD_{WLR}	12.2%	20.0%	8.0%	N/A	4.5%	5.0%	5.4%
Recovery%	92.5–104.6%	61–82%	107–110%	80–84%	95%	106%	101%–105%
Quantitation approach	SBC	SBC	MMC, IS	SBC, IS	SBC, IS	SBC	SBC, IS
ME%	<5% (Tolerable)	N/A	–48.0% (Intermediate suppression)	<5.0% (Tolerable)	N/A	N/A	–10.0% (Tolerable) for all tested commodities except coffee –34.0% (Intermediate suppression) for coffee

one significantly improved the recoveries and the RSD results, but peak broadening associated with relatively intermediate enhancement of MEs (> +20%, ≤ +50%) was obtained. On the other hand, both QuEChERS versions resulted in improved peak shape, while the CEN method yielded recoveries around 60% (Figure 1). It should be noted that the AOAC method yielded a relatively higher recovery than the CEN method, but with similar intermediate matrix enhancement. This performance for the studied compound in a variety of matrixes might be due to acrylamide's weak base properties and the strong ion strength of the acetate-buffered method. For accurate quantitation results, diminishing the ME to the bare minimum is of crucial importance. Further sample clean-up testing using various SPE sorbents would be beneficial as will be described below.

SPE Optimization and Matrix Effect Study

Further optimization for the sample processing efficiency using the AOAC version of QuEChERS protocol was performed. This was carried out by testing the relationship between various amounts of sorbent and the method performance improvement. Among the tested sorbents were basic alumina, PSA, Florisil, and silica gel in amounts of 1X, 2X, 4X, and 6X where X = 25 mg. Cereal-based baby food samples of various compositions were extracted and subsequently cleaned up through dispersive solid-phase extraction (d-SPE) employing the studied sorbents in their respective amounts. At the amounts of 1X to 6X, all studied sorbents yielded an average recovery of > 40% and ≤ 80%, except basic alumina at 4X and 6X, acceptable recoveries in the range of 80–120% were obtained. On the other hand, PSA yielded recoveries exceeding 120% when amounts of 6X were applied. It is worth noting that the poor recoveries obtained after 1X application of the PSA sorbent were consistent with what De Paola et al. (12) reported (Table 1). The results indicate that basic alumina is capable of providing higher acceptable recoveries at the studied amounts of 4X and 6X. Figure 1 demonstrates the relationship between the obtained recovery percentage of acrylamide and the amount of d-SPE sorbent used.

High recoveries along with controlled MEs are the key factors in considering whether the method is performing well for accurate and reliable quantitation results. As previously reported (23), an ME of ±20% is considered tolerable, while effects of > ±20% and ≤ ±50%, and > ±50% are intermediate and strong signal suppression/enhancement, respectively. As shown in Figure 2, the obtained results revealed that at amounts of 4X, all studied sorbents yielded a tolerable ME. At 1X and 2X, the ME varied between tolerable to intermediate suppression. In addition, at amounts of 6X, all sorbents also provided tolerable MEs except for silica gel which resulted in intermediate enhancements.

Considering the obtained ME profiles for the studied sorbents, perfectly tolerable effects were successfully achieved with basic alumina at whatever amount applied. Nevertheless, basic alumina as well as the remaining tested sorbents in an amount of 4X are deemed optimal for getting tolerable ME below +10%. On the contrary, silica gel resulted in signal suppression and enhancement of intermediate effect at 1X and 6X, respectively. This demonstrates an insufficient clean-up procedure for acrylamide in cereal-based baby foods by silica gel sorbent. So, whatever effects (suppression or enhancement) were observed, they could be attributed to differences in matrix composition and interactions obtained with varying amounts of applied silica gel. Unless higher amounts of 4X and 6X Florisil sorbent are used, signal intermediate suppression will be noticed. Similarly, intermediate suppression was observed with PSA applications, especially at the proposed amounts by the conventional QuEChERS (i.e., 1X = 25 mg). However, an application of 350 mg PSA (equivalent to 14 X) and 100 mg Bond Elut SCX sorbents by Petrarca et al. (11) also resulted in an ME of intermediate signal suppression of −48.0% (Table 1).

Further studies (3,8), on the other hand, have applied SPE using a 200 mg Oasis HLB cartridge for cereal-based baby food analysis, but no data have been presented for the obtained MEs. Nevertheless, acceptable recoveries were achieved. Similarly, Tolgyesi and Sharma (9) demonstrated satisfactory recoveries but with intermediate signal suppression of −34.0% when

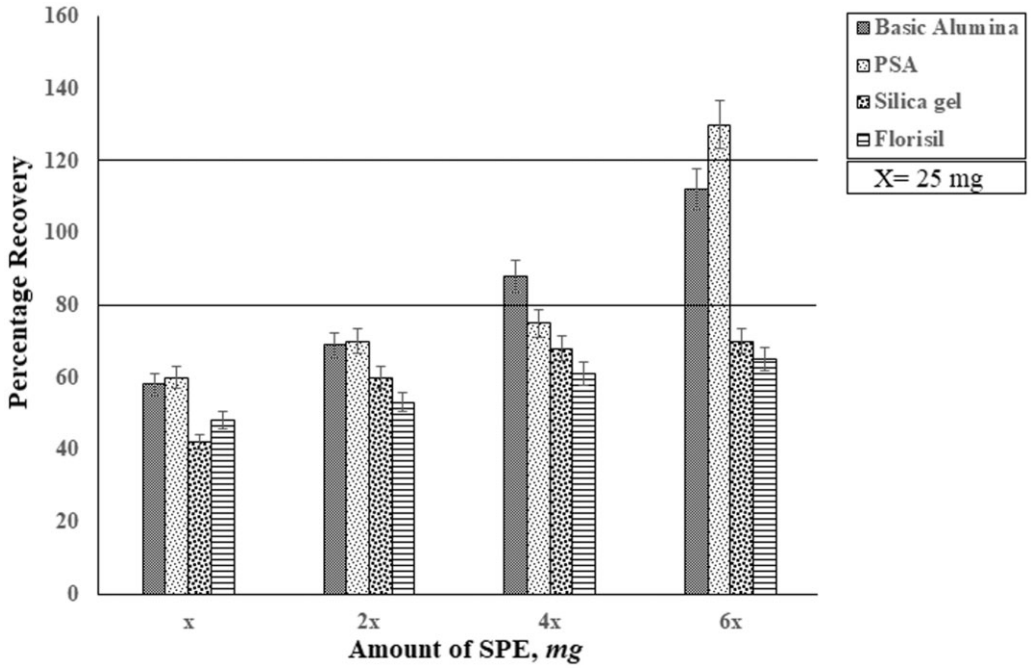

Figure 1. Relationship between the obtained recovery percentages of acrylamide and the employed amounts of d-SPE sorbents.

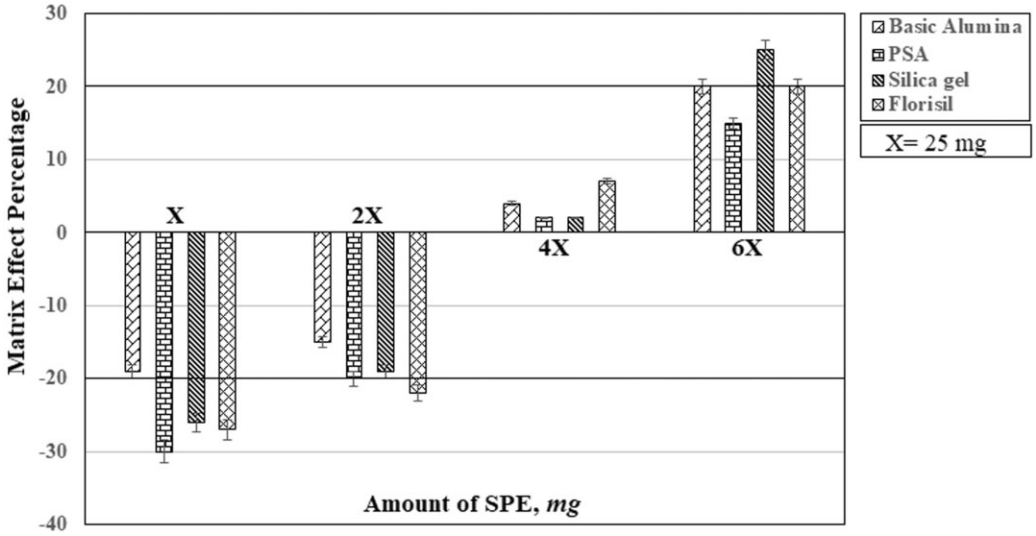

Figure 2. Matrix effect profile of acrylamide in cereal-based baby foods owing to the application of different d-SPE sorbents in amounts of 1X, 2X, 4X and 6X (X = 25 mg).

utilizing SPE for coffee analysis with a 60 mg Strata-XL-C cartridge (Table 1).

Overall, from the demonstrated results, the basic alumina optimized amount at 4X was found to be the most appropriate sorbent for achieving acceptable recoveries in the range of 80–120%, with tolerable MEs <5%. This was in agreement with Bertuzzi et al. (10), who developed an extraction protocol for roasted coffee, barley, and potato crisp samples employing 150 mg basic alumina (equivalent to 6X) in combination with additional SPE using 60 mg OASIS HLB (Table 1).

This modified QuEChERS protocol helped improve the recoveries as well as the MEs for a wide range of the studied commodities. This is because of the extensive removal of possible interferences in a pH environment suitable for acrylamide extraction and stability, leading to accurate quantitation results. The overall mean recovery was in the range of 92.5–104.6%, with a CV% ≤12.2. Furthermore, as will be discussed later, our proposed assay exhibited validation results that met the Eurachem 2014 validation requirements and were consistent with previously reported extraction processes (3,8–12), regardless of the SPE sorbent used in the established methods (Table 1).

Until now, only a small body of literature has applied the QuEChERS protocol to extract acrylamide from foods. As shown in Table 1, Petrarca et al. (11) and Bertuzzi et al. (10) applied QuEChERS protocol principles to the extraction solvent and clean-up sorbent. In both studies, the tested commodities were extracted using acidified MeCN and MeCN, respectively. Further to d-SPE employing sorbents such as PSA and Al_2O_3, additional SPE clean-up procedures were implemented using Bond Elut SCX and OASIS HLB cartridges. Therefore, a pre-concentration step through solvent exchange employing a gentle N_2 stream for evaporation was found mandatory. This has resulted in a long period of extraction extending from 45 to 70 min, as proposed by Petrarca et al. (11) and Bertuzzi et al. (10). On the other hand, however, both Petrarca et al. (11) and Bertuzzi et al. (10) performed several clean-up procedures, and some drawbacks were observed concerning the method performance. In Petrarca et al. (11), an intermediate ME of −48% was presented. This resulted in employing a quantitation approach based on matrix-matched calibration and an internal standard in order to correct for any possible fluctuation in the obtained results. Similarly, Bertuzzi et al. (10) have proposed a solvent-based calibration with an internal standard and demonstrated a tolerable ME of 5% and acceptable recoveries in the range of 80–84%.

Another study performed by De Paola et al. (12) presented an application of the QuEChERS protocol for extracting acrylamide from dried fruits and edible seeds using MeCN, followed by d-SPE using PSA. The overall extraction time, including the solvent exchange step, was 25.0 min. It was demonstrated that the obtained recoveries were better with QuEChERS pouches containing $MgSO_4$ + NaCl than with pouches with acetate buffer. The reported results were quite satisfactory, falling in the range of 61 to 82% with an RSD ≤20%. It should be noted that no data was presented for the obtained ME%; however, extremely low values for the obtained LOD and LOQ of 2.0 µg/kg and 5.0 µg/kg, respectively, were reported. In addition, all reported results were quantitated using solvent-based calibration, without internal standard application.

Bearing in mind the core benefits of the conventional QuEChERS protocol, our proposed assay protocol (i.e., modified AOAC QuEChERS) successfully extracted acrylamide from cereal-based baby foods in only 10 min with optimized amounts of d-SPE sorbent, namely basic alumina. Additional SPE procedures followed by a solvent exchange step were found unnecessary as low values of LOD and LOQ were achieved, as will be described later. Furthermore, the obtained tolerable MEs of <5% along with the efficient extractions achieved and expressed as recovery results of approximately 100%, were the basis for deciding to perform quantitation using solvent-based calibration without internal standard application.

Method Validation

(**a**) *Selectivity*.—Selectivity testing results revealed that the obtained chromatograms were devoid of any potential interference that would affect accurate identification and determination (Figure 3).

(**b**) *Linearity and working range*.—A set of eight calibration levels for acrylamide over a concentration range of 4.0 to 1000.0 ng/mL was successfully achieved using a linear

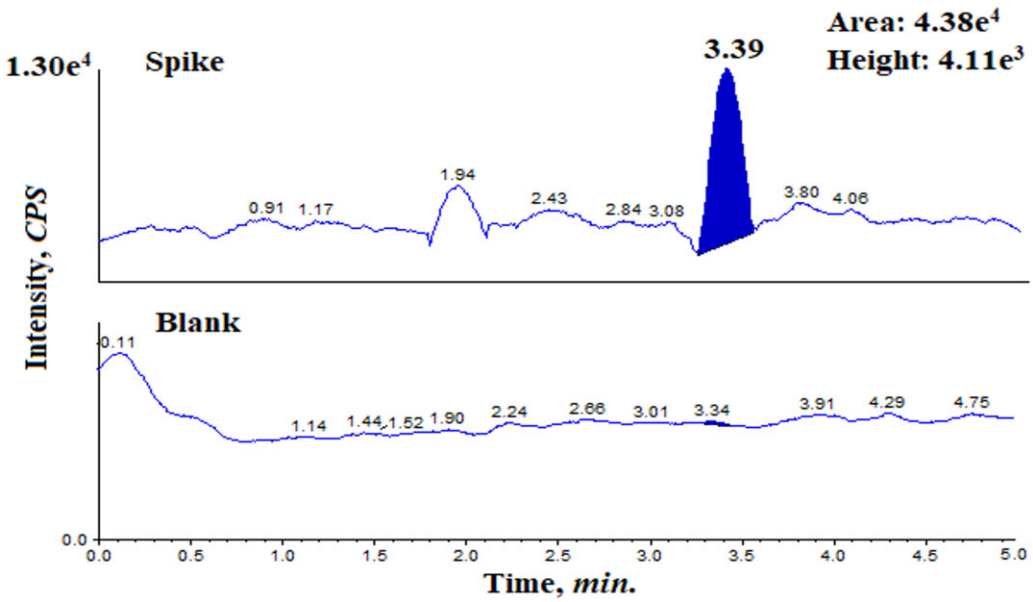

Figure 3. Typical chromatogram for acrylamide in a spiked sample at the LOQ level in comparison to a blank sample.

regression equation with a coefficient of determination (R^2) of 0.9999 for the accurate quantitation of real, spiked, and PT samples. Therefore, samples with high acrylamide concentration levels can be directly calculated using this wide-range calibration curve without further dilution. In addition, and as per Eurachem 2014 guidelines (20), the method linearity was also tested using the calibration curve method at three validation spiking levels of 20, 40, and 80 μg/kg (six replicates, each). The obtained results confirmed the method's linearity at the performed validation levels, with an R^2 of 0.9990. Table 2 presents the validation parameters, including trueness and precision values, and linearity and dynamic working range.

(**c**) *LOD and LOQ*.—The method LOD and LOQ results were 5.0 and 20.0 μg/kg, respectively. The results of LOD and LOQ were well below acrylamide's EU benchmark (Table 2).

(**d**) *Trueness and precision*.—The trueness and precision of the proposed assay protocol were measured at 20.0, 40.0, and 80.0 μg/kg (six replicates, each). The obtained results indicated acceptable method performance for the studied analyte over the studied validation levels. Average recoveries were in the range of 92.5 to 104.6%, with RSDs ranging from ±2.3 to 8.5. For both repeatability and within-laboratory reproducibility, the obtained CV results were 8.2 to 11.0%, and 12.2%, respectively. These results were in agreement with the requirements of EU guidelines (20) regarding the CV% for repeated analysis of spiked or incurred samples. The expanded uncertainty (at a 95% confidence level) was found to be less than 30% (Table 2).

Applications

(**a**) *Real samples*.—The applicability of the proposed assay protocol was investigated by analyzing 50 cereal-based baby foods to measure acrylamide levels. The obtained results for acrylamide concentration in whole samples were in the range of 13.0–256.0 μg/kg (see Supplemental Table S1). As presented in Supplemental Figure S1, the average acrylamide concentrations were 200.0 ± 60.0, 130.0 ± 10.0, 120.0 ± 70.0, 80.0 ± 50.0, and 150.0 ± 30.0 μg/kg for cereals with honey, chocolate, fruits, and milk, and cereals without milk, respectively. The majority of the processed cereal-based baby foods tested showed results exceeding the benchmark level of acrylamide (40 μg/kg) as recommended by EU Commission Regulation 2017/2158 (6). Cereal flakes with honey were the most contaminated by acrylamide, at a level ranging from 200.0 to 256.0 μg/kg. Similarly, it has been reported by Boyaci-Gunduz et al. (24) that cereals with honey resulted in more acrylamide in the final product. In contrast, cereals with milk were the least contaminated with acrylamide at levels below the LOQ. The obtained results confirm the applicability of the validated assay protocol for acrylamide testing in processed cereal-based baby foods.

Further, since processed cereal-based foods constitute the basis of complementary feeding from the age of 4 to 6 months, it would be helpful to put the obtained levels in perspective with previously published protocols. In this regard, a recent paper reported by Boyaci-Gunduz et al. (24) summarized the findings of acrylamide levels in processed cereal-based baby foods; Michalak et al. (3) and Mojska et al. (25) extracted acrylamide from ready-to-eat baby foods and their results ranged from 10.8 to 15.7 and 11.43 to 52.06 μg/kg, respectively. Also, Mojska et al. (25) reported an acrylamide level ranging from 65.0 to 296.0 μg/kg for powdered cereal-based baby foods. This indicates that the current levels obtained by the proposed assay protocol surpass the reported concentrations by Michalak et al. (3), and Mojska et al. (25) for ready-to-eat products, while powdered forms provided comparable results.

Another study conducted by Lambert et al. (8) investigated acrylamide in cereal-based foods for infants and toddlers. The obtained findings ranged between 0.53 and 99.0 μg/kg. On the other hand, Elias et al. (26) reported acrylamide concentrations in processed cereal-based foods for infants at levels below 30.0 to 353.0 μg/kg. The latter study demonstrated relatively high levels in comparison to the current method; however, both our and the former's reported results are well below the Elias et al. (26) acrylamide level.

Table 2. Percentage recoveries, precision values, LOD, LOQ, linear dynamic range, coefficient of determination (R^2), linear regression equation, and measurement uncertainty (MU) obtained for the studied acrylamide in processed cereal-based baby food

Fortification level, μg/kg	Average Percentage Recovery ± RSD	Repeatability (n = 6 replicates, each)		Within-Laboratory Reproducibility at 40 μg/kg (n = 18 replicates)		LOD, μg/kg	LOQ, μg/kg	Linear Dynamic Range ng/mL	Linear Regression Equation	R^2	MU%
		Mean Conc., μg/kg	CV%	Mean Conc., μg/kg	CV%						
20	104.6 ± 2.3	20.9	11.0	43.3	12.2	5.0	20.0	4.0–1000.0	$y = 3.29e^{+003}$ x	0.9999	30.0
40	92.5 ± 3.1	37.0	8.2								
80	95.1 ± 8.5	76.1	11.2								

In conclusion, the acrylamide safety level should be a high priority for processed cereal-based baby foods.

(**b**) *Proficiency testing (PT) samples*.—Further confirmation of the proposed method's applicability and extraction efficiency was carried out using a PT sample of instant coffee (round 30117, August 2021) as per the food analysis performance assessment scheme (FAPAS) PT program. The average results of three successively analyzed samples were expressed as μg/kg ± SD and evaluated based on z-scores, in which values of $|z| \leq 2$ are considered satisfactory. The obtained results of the three replicates were 762.0, 788.0, and 790.0 μg/kg. The reported average result was 780.0 ± 12.7, and the calculated z-score was 1.7. The minimal data variability obtained indicates that either individual or the average results of the PT samples lie within the acceptable z-scores limits. These satisfactory results confirm the applicability of the validated assay protocol for acrylamide testing in processed foods. Supplemental Table S2 summarizes the PT results of acrylamide testing in instant coffee samples using the validated assay protocol.

Conclusions

The development of a modified AOAC version of the QuEChERS protocol employing basic alumina as a d-SPE sorbent has ensured efficient acrylamide extraction from cereal-based baby foods without additional solvent exchange steps. The use of the right amount of basic alumina sorbent resulted in a dramatic ME reduction to tolerable levels, leading to accurate quantitation results. Appropriate selection of an RP-C18 column with core-shell properties facilitates rapid and accurate acrylamide determination by the ESI-MS/MS system in a relatively short analysis run time of only 5 min. The proposed analysis protocol has proved practical via its application to real domestic samples of cereal-based baby food as well as to a PT sample. Relative to the previous methods, the validated assay protocol is more sensitive in terms of LOD and LOQ, meeting the EU-defined acrylamide standard. This work will aid in the regular oversight of acrylamide in domestic samples by national regulatory agencies, thereby protecting newborns and young children.

CRediT Author Statement

Abdelrahman M. Marzouk: Methodology, Validation, Writing—Original Draft. Amr H. Shendy: Conceptualization, Writing—review & editing, Formal analysis. AlaaEldean Fathy Ahmed Aboelhassan: Methodology. Ahmed M. Gomaa: Conceptualization, Supervision, Review. M. F. El-Shahat: Conceptualization, Supervision, Review. CRediT roles are: Conceptualization; Formal analysis; Methodology; Software; Supervision; Validation; Supervision; Review; Roles/Writing—original draft; Writing—review & editing.

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Conflict of Interest

The authors declare thatthey are not aware of any financial or interpersonal issues that might have appeared to have an impact on the study that was the subject of this work.

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Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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