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Analysis of Patulin in Pear- and Apple-Based Foodstuffs by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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ABSTRACT: A liquid chromatography electrospray ionization tandem mass spectrometry method for the determination of patulin in apple- and pear-based foodstuffs was developed. The sample preparation is based on the QuEChERS procedure involving an initial extraction step with water and acetonitrile, followed by a partitioning step after the addition of magnesium sulfate and sodium chloride. The cleanup was performed by using dispersive solid-phase extraction with a mixture of magnesium sulfate, primary secondary amine sorbent, and *n*-octadecylsiloxane sorbent added together to the extract. The cleaned extract was finally evaporated and reconstituted in water prior to injection. Quantitation was performed by isotope dilution using ($^{13}C_7$)-patulin as internal standard. The method was first fully validated in three different baby food products including apple−pear juice, apple−pear puree, and infant cereals. Then the scope of application of the method was extended to pear concentrate, raw apples, apple flakes (naturally contaminated), dried apples, and yogurt. The sensitivity achieved by the method in all matrices gave limits of detection (LOD) and quantitation (LOQ) of ≤0.5 and ≤10 μg/kg, respectively, which was compliant with maximum levels settled in Commission Regulation (EC) No. 1881/2006. Method performances for all matrices also fulfilled the criteria established in the CEN/TR 16059:2010 document. Indeed, recoveries were within the 94−104% range; relative standard deviations of repeatability (RSD_r) and intermediate reproducibility (RSD_{IR}) were ≤7.5 and ≤13.0%, respectively, and trueness in an infant apple drink (FAPAS 1642) was measured at 99%.

KEYWORDS: patulin, QuEChERS, liquid chromatography tandem mass spectrometry, LC-MS/MS, isotope dilution

INTRODUCTION

Patulin is a toxic secondary metabolite produced by a number of fungal species belonging to the genera *Penicillium*, *Aspergillus*, and *Byssochlamys*. Particularly, *Penicillium expansum* is known as the main source of patulin and is commonly associated with apple rot. Patulin occurs most often in apples that have been spoiled by mold growth or in products made from spoiled apples, such as apple juice and apple puree. In the 1940s, patulin was thoroughly tested for its antibiotic properties, but its use was rapidly abandoned because of its immunotoxic, genotoxic, embryotoxic, and neurotoxic effects evidenced in animals.[1] These facts led some 50 countries to set around 160 regulatory limits for patulin, making of patulin the most regulated mycotoxin within fruits and vegetables.[2] As an example, the European Union (EU) established maximum limits (MLs) for patulin in various foodstuffs: the ML is 50 μg/kg in fruit juices and in drinks containing apple juice or derived from apples; the ML is 25 μg/kg for solid apple products, such as apple puree; and a lower ML of 10 μg/kg has been set for certain foods intended for infants and young children.[3] Consequently, there is a need for reliable analytical methods capable of determining patulin at or below the actual regulatory limits.

Numerous analytical methods devoted to patulin analysis share a common analytical strategy:[4] the basic principle is based on multiple liquid−liquid extractions with ethyl acetate (EtOAc) followed by a cleanup with a sodium carbonate (Na_2CO_3) solution and further determination by reversed phase HPLC and UV detection at 276 nm. Three main drawbacks can be highlighted: First, the several intermediate steps and the important needs of glassware render the sample preparation rather tedious and cumbersome. Second, the cleanup step by Na_2CO_3 increases the pH of the sample extract. Because patulin is unstable under alkaline conditions,[5] this step must be done as quickly as possible and can unfavorably affect recovery if not performed properly. Third, detection by UV suffers from a lack of selectivity and imposes consequently an extensive sample preparation upstream. For this purpose in some methods sharing the analytical strategy described above, a solid-phase extraction step (SPE) was even inserted after the multiple extraction steps with EtOAc to improve the method's performance.[6] In addition, in HPLC-UV methods, great care must be paid to chromatographically resolve patulin from 5-hydroxymethylfurfural and phenolic compounds, which are common interferents from apple-based products.[7] The issues concerning the intricate sample preparation to handle and the chromatographic conditions to finely optimize could be resolved using more selective detection techniques such as mass spectrometry, for example, GC-MS[8,9] or LC-MS/MS methods.[10] However, the scope of application of existing methods for patulin analysis is restricted to a few matrices, most notably apple juice. Consequently, other products from the apple industry such as dried apples[11] or apple puree were less considered despite being also matrices of high concern. Furthermore, patulin occurs not only in apples and apple-based products but also in other fruits and other foodstuffs such as vegetables and cereals. For these latter matrices, very few data have been collected, notably due to the lack of analytical methods.

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The aim of this study was to develop and validate a fast, easy, and reliable LC-MS/MS method for the quantitative determination of patulin in a wide range of matrices. For the first time, the QuEChERS (acronym of quick, easy, cheap, effective, rugged and safe) method was considered for patulin analysis. This method originally developed for pesticides,[12−14] and then extended to other contaminants such as mycotoxins,[15] was retained on the basis of the fact that patulin, other mycotoxins, and pesticides may simultaneously occur within fruits, vegetables, and cereals. For further economical and practical reasons, patulin would advantageously be included within the widely used QuEChERS procedure. Until now, patulin was almost exclusively analyzed through a single-residue method. Only one method from Christensen et al.[16] was developed for the simultaneous analysis of 33 pesticides and patulin. Nevertheless, only one matrix (apples) was considered in this method, and the limit of quantitation (LOQ) for patulin at 40 μg/kg was not compliant with the EU legislation in baby foods (ML = 10 μg/kg). Another improvement brought by our method concerns the widest scope of application considered for patulin analysis, going from raw materials to finished products. Indeed, the method was first fully validated in three baby food products including one apple−pear juice, one apple−pear puree, and one infant cereal product. The scope of application of the method was then extended to other foodstuffs including pear concentrate, raw apples, dried apples, yogurt, and apple flakes. As a regulated mycotoxin, the unambiguous identification and accurate determination of patulin were mandatory prerequisites. For this, detection by LC-MS/MS using the selected reaction monitoring (SRM)-based acquisition ensured the method's selectivity, whereas patulin was quantitated by isotope dilution using ($^{13}C_7$)-patulin as internal standard (IS). Method trueness was assessed in an infant apple drink by participating in the proficiency testing of FAPAS 1642. Finally, a minisurvey was conducted on several apple-based beverages (n = 8) and baby food purees (n = 7).

MATERIALS AND METHODS

Chemicals and Reagents. The following chemicals and reagents were obtained commercially: HPLC grade LiChrosolv water (H_2O), acetonitrile (MeCN), methanol (MeOH), and sodium chloride (NaCl) (Merck, Darmstadt, Germany); magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, Buchs, Switzerland); and a mixture of 400 mg of octadodecyl-bonded silica (C18) sorbent, 400 mg of primary−secondary amine (PSA) sorbent, and 1200 mg of $MgSO_4$ provided in 15 mL propylene tubes (Agilent Technologies, Bellefonte, PA).

Standard Solutions. Stock standard solutions of patulin (Sigma-Aldrich) at 100 μg/mL and ($^{13}C_7$)-patulin at 25 μg/mL (Biopure, Tulln, Austria), both in MeCN, were commercially available. Both standards were available in ready-to-use ampules. For both patulin and ($^{13}C_7$)-patulin, separate working standard solutions at 10 and 1 μg/mL were obtained by successive dilutions in MeCN. All stock standard and working standard solutions were stored at −18 °C and brought to room temperature before any intended use.

Samples. Three baby food products including blank samples of apple−pear juice (from concentrate 100%), apple−pear puree (pear 50% and apple 50%), and infant cereals (mainly constituted of rice and oat flours, 50%; milk, 27%; and pear and apple, 6.8%) were collected from Swiss supermarkets like other finished products of raw apples, dried apples and yogurts (containing pear and apple). Samples of pear concentrate and apple flakes (naturally contaminated) were supplied from factories based in the United States and Germany, respectively.

A minisurvey conducted on several apple-based beverages (n = 8) and baby food purees (n = 7) included common commercially available products from the Swiss market with the exception of one homemade cider.

All samples were kept at 4 °C immediately after reception either in their original packaging or in airtight containers.

Homogenization. Among available laboratory samples, three required further comminution: Five yogurts containing pieces of pear and apple and from the same batch were combined. The whole laboratory sample was comminuted as such using a domestic blender. For dried apples and raw apples, 100 g of both materials were weighed and cut into small pieces before being stored in airtight containers at −80 °C overnight. Samples were then ground, and the resulting materials were collected and weighed. A defined volume of H_2O (normally added during sample preparation) was added to assist comminution according to a published protocol.[13] For this, volumes of H_2O in the proportion of 1.5 mL/g of dried apples and 0.15 mL/g of raw apples were added. The grinder was restarted until the rehydrated samples were homogenized. The added volumes of H_2O were then taken into consideration for weighing test portions equivalent to 5 g for dried apples and 10 g for raw apples.

Sample Preparation. *First Extraction Step.* For apple−pear puree, apple−pear juice, and yogurt, a 10 ± 0.01 g test portion was weighed into a 50 mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France). For infant cereals, apple flakes, and pear concentrate, a 5 ± 0.01 g test portion was weighed and mixed with H_2O (10 mL). For dried apples and raw apples hydrated during homogenization, 12.5 ± 0.01 and 11.5 ± 0.01 g corresponding to test portions of 5 and 10 g, respectively, were weighed. All samples were fortified with 50 μL of the 1 μg/mL working standard solution of ($^{13}C_7$)-patulin (i.e., mass of IS added to any test portion is 50 ng). MeCN (10 mL) was added in all samples, and the resulting slurry was vigorously hand-mixed and placed onto an automated shaker for 5 min.

Partitioning Step. A $MgSO_4$/NaCl salt mixture (4:1, w/w) (5.0 ± 0.2 g) was added to the slurry, which was immediately and vigorously hand-shaken for a few seconds before centrifugation at room temperature at 4000g for 15 min.

Cleanup by Dispersive Solid-Phase Extraction (dSPE). The resulting MeCN-based supernatant (6 mL) was transferred to a 15 mL Falcon polypropylene tube already filled with 400 mg of PSA, 400 mg of C18, and 1200 mg of $MgSO_4$, and the tubes were vigorously hand-shaken for about 30 s. After centrifugation (4000g at room temperature for 10 min), the supernatant (1 mL) was transferred into a new 15 mL Falcon polypropylene tube filled beforehand with 10 μL of a 5% formic acid solution in water (v/v). Sample was mixed and evaporated to dryness at 40 °C under a stream of nitrogen.

Final Treatment of the Extract. The residue was reconstituted in H_2O (200 μL) before being filtered over a PTFE syringe filter, 0.2 μm, 13 mm, and collected into a HPLC amber glass vial for further LC-ESI-MS/MS analysis.

LC-ESI-MS/MS. HPLC analysis was performed on a 100 mm × 2.1 mm i.d., 3.5 μm, Eclipse C18 column (Agilent Technologies, Geneva, Switzerland) heated at 40 °C, using an Agilent 1100 binary pump system. The mobile phase was constituted by solvent A, H_2O, and solvent B, MeCN. A linear gradient program was set up with 0−2 min, 5% B; 2−5 min, 95% B; a hold at 95% B for 2 min; and a return to 5% B in 0.5 min (the HPLC column was reconditioned at 5% B for an additional 10 min). The flow rate was 0.3 mL/min, and 10 μL of the extract were injected onto the column. The HPLC flow was directed into the MS detector between 1 and 5 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX).

MS detection was performed using an 4000 QTrap (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source. MS tuning was performed in negative electrospray ionization (ESI) by syringe-infusing separately a 10 μg/mL solution of each analyte

Table 1. Transition Reactions Monitored for Patulin and ($^{13}C_7$)-Patulin and Peak Area Ratios along with their Limits of Acceptance According to the Commission Decision 2002/657/EC Document[17]

	transition reactions (m/z) used for		
	quantitation	analyte confirmation	peak area ratio ± limit (%)
patulin	153 → 109 (13)[a]	153 → 81 (18)	0.36 ± 25
($^{13}C_7$)-patulin (IS)	160 → 115 (13)	160 → 86 (18)	0.54 ± 20

[a] Collision energies in eV are given in parentheses.

(patulin and its IS at a concentration of 10 μg/mL) at a flow rate of 10 μL/min mixed with a HPLC flow made of solvents A and B (50:50, v/v; 0.3 mL/min) using a T-connector. The block source temperature was maintained at 500 °C, and the gas set values were as follows: curtain gas, 40 psi; nebulizer gas, 30 psi; turbo gas, 30 psi; collision gas, 1.2×10^{-4} psi. The entrance potential and the collision exit potential were settled at 10 and 15 V, respectively, and the electrospray capillary voltage was set at −4 kV. Quantitative analysis was performed using tandem MS in selected reaction monitoring (SRM) mode alternating two transition reactions for each compound (Table 1). Data processing was carried out using Analyst software 1.5.

Quantitation. Patulin was quantitated by means of an external calibration curve using the area $_{patulin}$/area $_{patulin\_IS}$ ratio as the y-axis and the concentration $_{patulin}$/concentration $_{patulin\_IS}$ ratio as the x-axis. Six calibration levels, each containing both labeled and unlabeled patulin and constructed in H_2O, were considered for all matrices: 0.125−1 ng of patulin was injected onto the column, whereas the concentration of patulin_IS was fixed at 0.25 ng injected onto the column.

The calibration curve was of the type (area ratio) = [(concentration ratio) × slope] + intercept. Therefore, the equation used for quantitation was

$$concentration\ (\mu g/kg) = \frac{\left(\frac{A_a}{A_{IS}}\right) - I}{S} \times \frac{m_{IS}}{m_a}$$

where A_a is the peak area of the analyte in the sample, A_{IS} is the peak area of the IS in the sample, I is the intercept of the regression line, S is the slope of the regression line, m_{IS} is the mass of IS added to the test portion in ng, and m_a is the mass of the test portion in g.

Confirmation Criteria. Patulin was considered to be positively identified when the following criteria were met simultaneously: (a) the ratio of the chromatographic retention time of patulin to that of patulin_IS, that is, the relative retention time of the patulin, corresponded to that of the averaged relative retention time of the calibration solutions within a ±2.5% tolerance; (b) the peak area ratios from the different transition reactions recorded for both unlabeled and labeled analytes were within the tolerances fixed in the Commission Decision 2002/657/EC document,[17] as shown in Table 1.

Method Validation. The method was first fully validated on three baby food products including apple−pear juice, apple−pear puree, and infant cereals. Blank matrices were spiked with patulin before extraction at three fortification levels (i.e., 5, 10, and 15 μg/kg) corresponding to 0.5, 1, and 1.5 times the most stringent ML settled at 10 μg/kg in baby food.[3] Three operators were involved in these experiments, each performing two replicates of each fortification level on two occasions. A total of n = 12 separate experiments per fortification level were thus obtained over k = 6 different days. Quantitation was performed by means of external calibration curves. Recovery, relative standard deviations of repeatability (RSD_r), and relative standard deviations of intermediate reproducibility (RSD_{IR}) were calculated from these trials according to protocol.[18] The applicability of the extraction method was then extended to pear concentrate, raw apples, apple flakes, dried apples, and yogurt. Recovery, RSD_r, and RSD_{IR} were determined as described above (3 operators, n = 12; k = 6) but at only one fortification level (10 μg/kg, except for the naturally contaminated apples flakes sample, which was analyzed as such).

The linearity of the response was tested between 0.125 and 1 ng of patulin injected onto the column, with concentration of IS fixed at 0.25 ng injected onto the column. Linearity was checked by calculating the relative standard deviation (RSD) of the average of response factors (RF, RF = y/x), which should be RSD_{RF} < 15%.[19]

Proficiency Test. The performance of the method was evaluated within the framework of one Food Analysis Performance Assessment Scheme (FAPAS) proficiency test. One infant apple juice drink (FAPAS 1642) was obtained from FAPAS (Sand Hutton, York, U.K.). Thirty participants took part in this proficiency test. Performance criteria were as follows: $|z| < 2$, satisfactory result; $2 < |z| < 3$, questionable result; $|z| > 3$, unacceptable result.

RESULTS AND DISCUSSION

LC-MS/MS. Patulin was analyzed in the negative electrospray ionization mode. Collision-induced dissociation (CID) mass spectra were then recorded at various collision energies before selection of the optimal MS/MS transition reactions (Figure 1 and Table 1) and electronic parameters. Two transition reactions were selected for patulin and ($^{13}C_7$)-patulin identification, giving a total of four identification points as recommended by the Commission Decision 2002/657/EC document[17] (Table 1). The mobile phase composition was also investigated. Interestingly, the addition of modifiers in the aqueous mobile phase (i.e., H_2O) such as formic acid (solution adjusted to pH 4) or ammonium formate (tested at a 10 mM concentration) led to a strong signal suppression. Thus, organic and aqueous mobile phases were composed by gradient grade MeCN and H_2O solutions used without any modifiers.

Method Development. The sample preparation was based on the QuEChERS procedures described in AOAC International official method 2007.01 and CEN standard method EN 15662.[13,14] Only optimized parameters will be discussed here.

Extraction Solvent. In numerous methods, EtOAc is commonly used as the extraction solvent for patulin analysis.[1] EtOAc and MeCN were tested, but MeCN was preferred because the signal produced was about twice as intense as that obtained after EtOAc extraction.

Partitioning Step. Three salt mixtures were tested: (a) 4 g of $MgSO_4$ and 1 g of NaCl; (b) 4 g of $MgSO_4$ and 1 g of sodium acetate; (c) 4 g of $MgSO_4$, 1 g of NaCl, 1 g of trisodium citrate dehydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate. Salt mixture a) was retained because it gave chromatograms free from interferent peaks and with the better S/N ratio.

Cleanup by dSPE. The salt mixture used for dSPE cleanup was composed by 1200 mg of $MgSO_4$ (used as drying agent), 400 mg of PSA (to remove sugars), and 400 mg of C18 (to remove nonpolar interferences) to provide a generic cleanup effective for

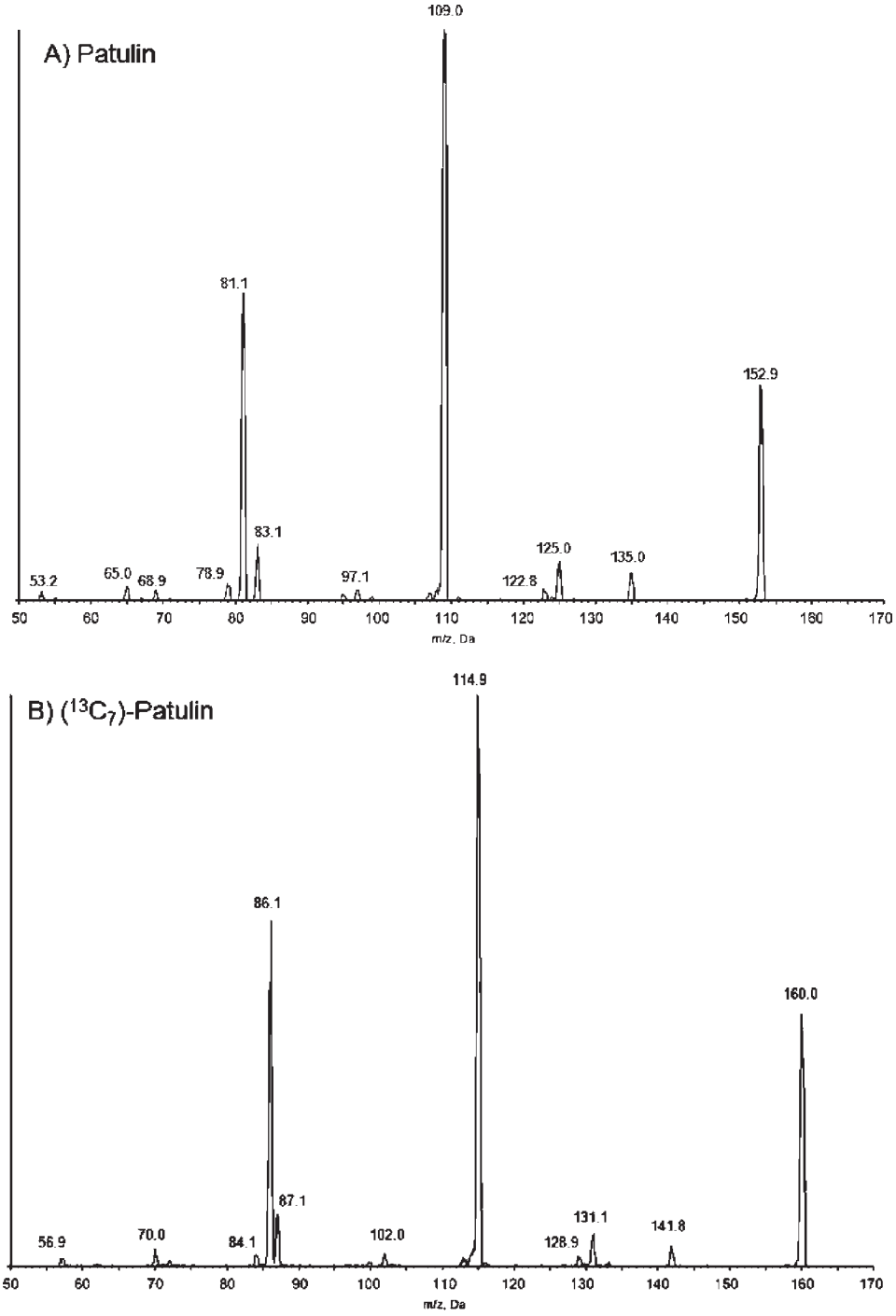

Figure 1. Product ion mass spectra of (A) patulin and (B) ($^{13}C_7$)-patulin.

a wide range of matrices. The addition of 400 mg of graphitized carbon black (GCB) to the dSPE salt mixture was also tested. Indeed, GCB was tested on the basis of its potential ability to interact with phenolic compounds, which are known as common interferents of patulin[4] in apple-based products. Nevertheless, the addition of GCB did not improve sensitivity.

Final Treatment of the Extract. Patulin is known as being unstable under alkaline conditions and after prolonged drying times.[5] Throughout the sample procedure, the stability of patulin was ensured by evaporating 1 mL of the dried MeCN extract under acidic conditions at 40 °C for no longer than 30 min. Throughout the sample procedure, the pH of the sample extracts roughly estimated by means of pH paper never exceeded 7.

Method Performance Characteristics. The limit of detection (LOD) was defined as the lowest concentration producing a chromatographic peak with a signal-to-noise ratio (S/N) ≥ 3. Despite closely depending on the cleanliness of the MS source, and thus submitted to small variations over the time, the LOD was broadly estimated within a 0.2−0.5 μg/kg range for all matrices. Figure 2 illustrates chromatograms of extracts of infant

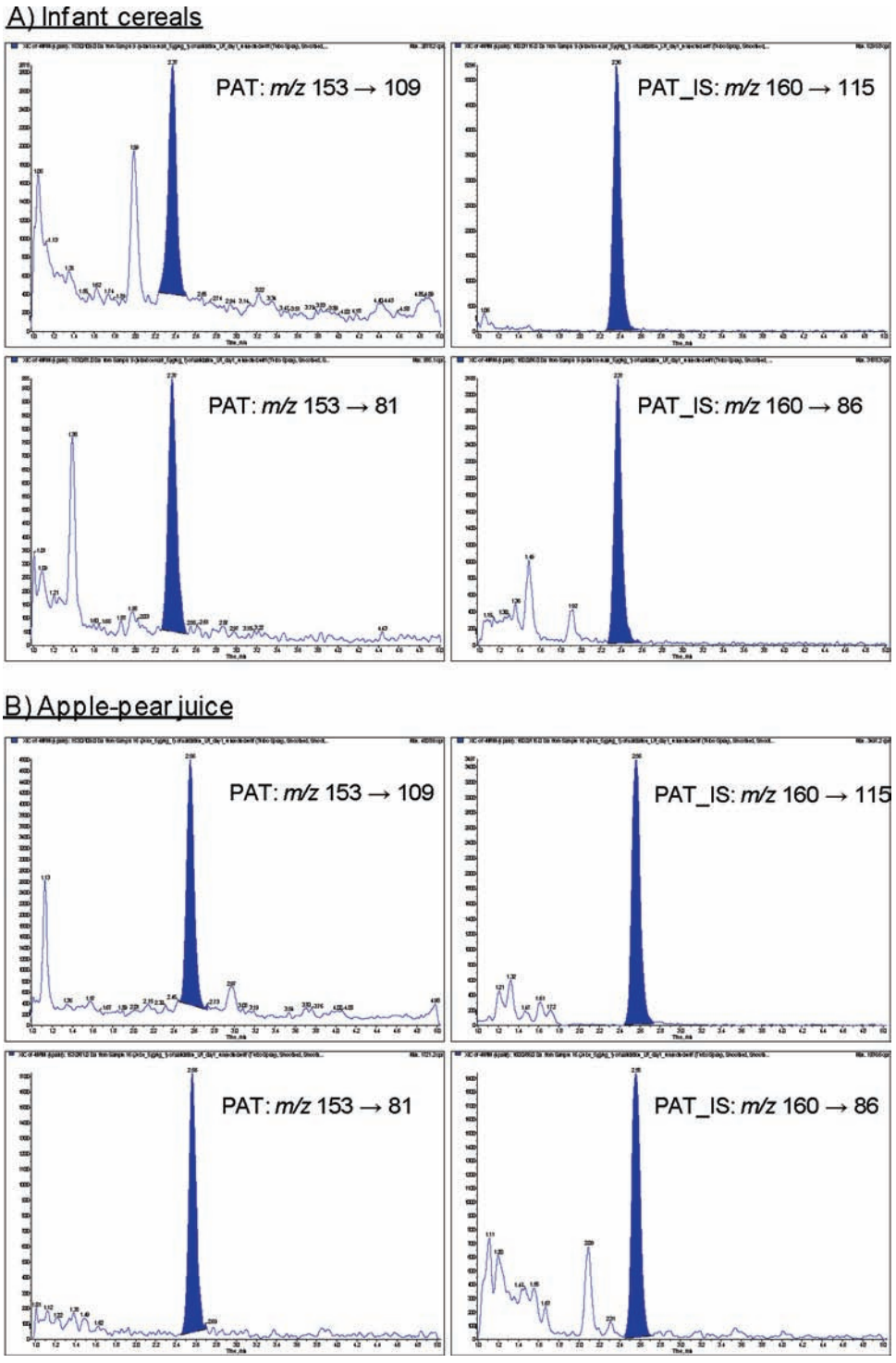

Figure 2. LC ESI-MS/MS chromatograms of patulin from extracts of (A) infant cereals and (B) apple−pear juice. Spiking levels: 5 μg/kg for patulin (PAT) and 10 μg/kg for ($^{13}C_7$)-patulin (PAT_IS).

cereals and apple−pear juice spiked with patulin at the 5 μg/mL fortification level. The limit of quantitation (LOQ) was arbitrarily defined as the lowest fortification level, that is, 5 μg/kg for apple−pear juice, apple−pear puree and infant cereals, and 10 μg/kg for pear concentrate, raw apples, dried apples, and yogurt. Recovery and precision data obtained during the validation process are summarized in Table 2. Internal standard corrected recoveries were within a 94−104% range, whereas precision data, that is, RSD_r and RSD_{IR}, fell within ranges of 1.3−7.5 and 3.3−13.0%, respectively. All of these results were compliant with the analytical requirements given in the CEN/TR 16059:2010 document.[20] The trueness of the method was also tested in an infant apple juice drink by subjecting it to the proficiency test FAPAS 1642. The assigned value of sample FAPAS 1642 was 9.64 μg/kg, whereas the patulin content calculated by the method described here was 9.50 μg/kg, giving a trueness of 99% and a z score of −0.1 (Table 3).

Table 2. Method Performance Data

matrix	fortification level (μg/kg)	recovery (%)	precision RSD_r[a] (%)	precision RSD_{IR}[b] (%)
infant cereals	5	99	3.2	11.3
	10	94	7.5	13.0
	15	95	4.6	6.5
juice	5	102	1.9	8.1
	10	97	4.8	7.1
	15	98	2.8	5.7
puree	5	104	4.0	5.8
	10	101	2.3	3.3
	15	99	2.8	3.4
pear concentrate	10	95	2.7	6.2
raw apple	10	96	3.2	3.6
dried apples	10	103	1.3	5.9
yogurt	10	96	1.5	6.6
apple flakes	55 (naturally contaminated)	c	2.9	3.8

[a] RSD_r, relative standard deviation of repeatability. [b] RSD_{IR}, relative standard deviation of intermediate reproducibility. [c] Recovery could not be assessed for apple flakes, because no reference value was available for this sample.

Table 3. Result of Proficiency Test FAPAS 1642 for the Analysis of Patulin in Infant Apple Juice Drink

	infant apple juice drink
assigned value[a] ± SD(R) (μg/kg)	9.64 ± 2.12
result (μg/kg)	9.50
z score	−0.1

[a] Assigned value was derived from the data of 30 laboratories.

To assess the method ruggedness, along with performing a minisurvey, several apple-based beverages (n = 8) and baby-food purees (n = 7) were analyzed by this method. For this survey, all samples were extracted in duplicate with one sample extracted as such (to check the presence or absence of patulin) and one sample spiked with patulin at a 10 μg/kg level (to calculate recoveries at this level and evaluate the method ruggedness). Results from this survey showed that patulin was never detected in any of the tested purees. In contrast, patulin was identified according to previous criteria[17] in seven of the eight apple-based beverages. Marìn et al. found similar results on apple-based products from the Spanish market, by detecting low levels of patulin in apple-based beverages but not in purees intended for infant consumption.[21] Quantification was only feasible in one apple juice and one homemade cider, respectively measured at 2.8 and 13.3 μg/kg. This survey indicates that the method LOQ could potentially be <5 μg/mL, at least in apple juice. In the two positive samples, the determined concentrations were below the EU legislation limit.[3] Additionally, recoveries (corrected from their basal content for positive samples) measured in these 15 samples (n = 15) fall within a 94−108% range, showing the good ruggedness of the method (Table 4).

Table 4. Occurrence of Patulin in Apple- and Pear-Based Products

matrix	unspiked samples (μg/kg)	10 μg/mL spiked samples (μg/kg)	recovery (%)
apple juice, cloudy	traces	9.61	96
apple juice, cloudy	traces	10.34	103
apple cider	not detected	9.97	100
apple drink	traces	9.54	95
apple juice	traces	10.36	104
apple juice from concentrate	2.80	9.39[a]	94
apple juice from concentrate	traces	10.43	104
homemade cider	13.28	10.24[a]	102
apple−rice puree	not detected	9.95	99
pear puree	not detected	10.37	104
apple−oat puree	not detected	10.60	106
apple puree	not detected	10.19	102
apple puree	not detected	10.64	106
pear puree	not detected	10.84	108
apple−honey puree	not detected	10.68	107

[a] Corrected value obtained by subtracting the patulin basal content to the result measured after spiking at a 10 μg/kg level.

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Notes

Safety. Patulin is supposed to cause immunotoxic, genotoxic, embryotoxic, and neurotoxic effects and should be handled with appropriate caution. The handling or preparation of standards, working solutions, and samples must be performed in a fume hood with appropriate protective attire (laboratory coat, mask, and gloves). Prior to its disposal, the contaminated glassware should be decontaminated with ammonia solution (5%) for at least 1 h.

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