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Analytical Methods

Determination of patulin in apple and quince products by GC–MS using $^{13}C_{5-7}$ patulin as internal standard

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ABSTRACT

A reliable gas chromatographic mass spectrometric method has been validated for the determination of trace levels (<10 μgL^{-1}) of patulin in apple products and quince jam. The method was based on extraction of patulin with ethyl acetate-hexane, alkalinisation and silylation with *N,O*-bis-trimethylsilyltrifluoroacetamide with 1% of trimethylchloro-silane. The accurate determination of patulin was achieved by employing commercial $^{13}C_{5-7}$ patulin labelled as an internal standard, which allowed compensating target analyte losses and enhancement or suppression matrix effects. Limits of detection and quantification of method using real samples were 0.4 and 1.6 μgkg^{-1}, respectively. Recoveries of patulin from samples spiked at 8–50 μgkg^{-1} levels ranged between 71% and 89%. The repeatability of measurements (expressed as relative standard deviation) was lower than 16%. The method was successfully applied to the determination of patulin in apple fruit and apple products including juice, cider and baby food, and also in quince fruit and quince jam. A new PCR system for the detection of *Penicillium expansum* in samples containing highly degraded DNA was developed which permitted the detection of the mould in 2/3 of the samples containing patulin, including juices and jams.

1. Introduction

Moulds found in food raw materials enhance the possibility of spoilage during the storage, transport and processing steps, thus decreasing the production yields. Beside economic losses the presence of moulds in fruits and vegetables can represent a potential health risk to consumers due to the possible presence of mycotoxins they may produce.

Patulin is one of the several secondary metabolites produced by the fungi belonging to the genera *Penicillium*, *Aspergillus* and *Byssochlamys*. It has been demonstrated that the presence of patulin in foodstuffs, such as apples or processed apple products, may be a health concern since this mycotoxin can be responsible for severe acute (e.g. convulsions, nausea, ulceration) and chronic (e.g. carcinogenic, genotoxic, immunotoxic) hazardous effects in humans (Moake, Padilla-Zakour, & Worobo, 2005). The International Agency for Research on Cancer (IARC) has classified patulin as category 3, not classifiable as to its carcinogenicity to humans (IARC – International Agency for Research on Cancer, 1993).

Patulin is found mainly in apples and apple juices, although the presence of the compound was also described in the brown rot of other fruits such as pears and apricots. Despite the number of studies devoted to the removal of patulin from apple beverages and similar products during industrial scale production, the use of rotten apples or contaminated fruits as raw materials usually results in patulin contaminated products. Moreover the removal of the rotten part of the fruit does not ensure the complete toxin elimination, because patulin could be present in the healthy part (Beretta, Gaiaschi, Galli, & Restani, 2000).

It is therefore necessary to monitor and control patulin levels in foodstuffs in order to protect the consumer. The *Codex Alimentarius* (Codex–Codex Committee on Food Additives & Contaminants, 2003) and the Food and Drug Administration (FDA) (FDA – United States Food, 2000) have recommended a maximum level of 50 μgkg^{-1} for apple juices and their products. In the European Union the Commission Regulation (EC), NO 1881/2006 (Commission Regulation (EC), NO 1881/2006) have also established allowable limits of patulin content in fruit (apple) juice and apple juice ingredients in other beverages of 50 μgkg^{-1}, in solid apple products of 25 μgkg^{-1} and in baby food of 10 μgkg^{-1} (Mycotoxin Certification Standard, 2008).

Abbreviations: BSTFA, *N,O*-bis-trimethylsilyltrifluoroacetamide; DAD, diode array detector; dNTP, deoxynucleotide triphosphate; EI, electron ionisation; EtAc, ethyl acetate; EU, European Union; FDA, Food and Drug Administration; GC, gas chromatography; HPLC, high performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; *m/z*, mass to charge ratio; MeCN, acetonitrile; min, minutes; MS, mass spectrometry; MTBSTFA, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; n.d., not detected; PCR, polymerase chain reaction; RSD, relative standard deviation; s, seconds; SIM, selected ion monitoring; TLC, thin layer Chromatography; TMCS, trimethylchlorosilane; UV, ultraviolet.

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The development and validation of sensitive and reliable analytical methods for detecting patulin at or below the limits allowed in foodstuff are essential to meet regulatory monitoring and to protect the consumers.

Several methods have been developed in the past decades to determine patulin in apple products, the most of which are classical methods based on thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) (Moake et al., 2005; Shephard & Leggott, 2000). However, such kinds of methods are not appropriate for the analysis of patulin in complex food matrices at low $\mu g kg^{-1}$ levels. In particularly, they lack the selectivity and additional degree of analyte certainty required to confirm the presence of patulin in a complex matrix.

Recently, more selective analytical methods have been published that are based mainly on mass spectrometry (MS) as a detection technique, coupled with a chromatographic step either by liquid chromatography (LC) or gas chromatography (GC), the latter in most cases after derivation of the analyte (Marks, 2007; Rychlik & Schieberle, 1999).

The main advantage of methods based on LC-MS is the possibility to analyse patulin without derivation (Sewram, Nair, Nieuwoudt, Leggott, & Shephard, 2000; Takino, Daishima, & Nakahara, 2003). Notwithstanding the high selectivity achieved by tandem mass spectrometry recently used (with ionisation techniques, such as electrospray ionisation (EI), atmospheric pressure chemical ionisation (APCI) or atmospheric pressure photoionisation (APPI)), these instruments are still very expensive and inaccessible for most of laboratories worldwide.

The employment of GC–MS methods for the determination of free (not derivatised) patulin isolated from complex matrices is rather troublesome. In general, there are still some residual matrix co-extractives left even in purified extracts, and many of them possessing similar retention time and MS fragments common to patulin. Due to this high chemical noise, the achievement of a low limit of detection (LOD) is almost impossible. Moreover the use of an inefficient internal standard prevents the exact quantitative determination of patulin (Sforza, Dall'Asta, & Marchelli, 2006). These difficulties explain the few number of studies that reported results in patulin, employing this approach (Llovera, Viladrich, Torres, & Canela, 1999; Roach, Brause, Eisele, & Rupp, 2002).

As mentioned above, the majority of current GC–MS methods employ a derivation step (e.g. trimethylsilylation or acetylation), in order to obtain a less polar, more volatile target analyte yielding more specific ions (higher *m/z*). Moreover, the derivatives obtained are stable, with good chromatographic properties necessary for reliable patulin detection. The recent commercialisation of isotope-labelled patulin as an internal standard increased the possibility of an exact quantification of this mycotoxin in complex matrices.

Another analytical approach to the problem of patulin contamination in foods consists in the direct detection of the mycotoxin producing moulds throughout the use of DNA probes based on the PCR reaction. The presence of specific patulin producing moulds DNA sequences in processed foods is an indicator of past contamination, which greatly increases the risk of patulin presence in the final product. Marek, Annamalai and Venkitanarayanan (2003) have proposed a PCR based method using specific primers for the polygalacturonase gene of *Penicillium expansum* as a tool for the rapid screening of fruits for the presence of *P. expansum*, a major causative agent for postharvesting decay in fruits and patulin producer. However the proposed method was not assayed in food systems being tested only in DNA extracts from several mycotoxin producing moulds for method specificity.

The main objective of this study was to develop a reliable GC–MS method using commercial $^{13}C_{5-7}$ patulin as internal standard for analysis of patulin in apple and apple products, at and below limits established by EU. An additional goal was to evaluate the possible influence of the different industrial treatments (e.g. pasteurisation and high-pressure processing) and apple origin (e.g. conventional or organic agriculture) on the occurrence and levels of patulin. Another goal was to determine the levels of patulin in quince and quince jam. Also, the design of a PCR based method for the detection of *P. expansum* in processed food products was assessed.

2. Experimental

2.1. Reagents and solutions

Patulin standard solution ($100\ mg L^{-1}$ in acetonitrile) was obtained from Fluka (Neu–Ulm, Germany). Purity-corrected individual patulin stock solution ($20\ mg L^{-1}$) was prepared in acetonitrile (MeCN) and standard working solution of patulin of 60–4000 $\mu g L^{-1}$ (depending on the experiment) was prepared in MeCN and stored at −18 °C. $^{13}C_{5-7}$ patulin labelled internal standard (I.S.) solution ($100\ mg L^{-1}$ in ethyl acetate, EtAc) was obtained from Kurt–Hess–Institut für Mehl und Eiweissforschung (Garching, Germany). MeCN, EtAc and *n*-hexane were high purity grade solvents for pesticide residue analysis from Fluka (Neu–Ulm, Germany). Glacial acetic acid and sodium bicarbonate analytical grade were obtained from Sigma (West Chester, PA; USA). Ultra-high purity He (helium) for GC–MS and N_2 (nitrogen) for solvent evaporation were obtained from Gasin (Maia, Portugal). The derivation reagents *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA), BSTFA with 1% of trimethylchlorosilane (TMCS) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were obtained from Fluka.

Taq Polymerase (GoTaq Flexi), buffer solution and magnesium chloride were obtained from Promega (Madison, CA, USA). Deoxynucleotides triphosphates (dNTP) were obtained from Fermentas (Ontario, Canada) and primers synthesised by Invitrogen (Carlsbad, CA, USA).

2.2. Sampling

A total of 4 apples (*Malus communis*; variety Golden delicious) and 4 quinces (*Cydonia oblonga*; variety Portugal) fruits with different-sized brown rotten areas were randomly collected from supermarket. The percentage of the rotten area for the apple and quince fruits was classified visually. The whole samples were mixed with a blender (Moulinex, Ecully, France) to obtain a very homogeneous sample.

A total of 33 samples of retail pure apple juice, apple juice mixed with other fruits, apple purees, cider and apple-based baby foods were purchased in local supermarkets or organic food shops. These samples were tested immediately for the parameters that need to be measured fresh such as °Brix (AOAC 932.12), pH (AOAC 981.12), and titratable acidity (AOAC 942.15) (AOAC, 1990). Aliquots of each sample were distributed into 3 vials and then frozen and stored at −20 °C for less than 1 month prior to analysis.

A total of 10 samples of quince jam were purchased in local supermarkets. They were stored at room temperature (±20 °C) in the absence of light and opened only on the moment of analysis.

2.3. Sample preparation

2.3.1. Extraction

For apple and quince fruits and apple products, extracts were prepared similarly to the Marks method (2007), which entail the following steps: (1) weigh 5 g of thoroughly homogenised sample into a 50 mL fluoroethylenepropylene (FEP) centrifugation tube;

(2) add 40 μL of I.S. solution 2 mgL^{-1} ($^{13}C_{5-7}$ patulin); (3) add 0.5 g of sodium bicarbonate; (4) add 5 mL of solution EtAc: *n*-hexane (95:5 v/v); (5) shake vigorously for 1 min by hand; (6) centrifuge the tube at 5000 g for 1 min; and (7) transfer the upper layer into a 25 mL vial. Then repeat 3 times the steps 4–7; (8) add 150 μL of acetic acid to layers previously combined and (9) evaporate dryness under a stream of nitrogen.

For quince jam, a 5 g sample, 1 g of water and 125 μL of I.S. solution 2 mgL^{-1} ($^{13}C_{5-7}$ patulin) were added to the FEP tube and treated as described above for apple products.

2.4. Derivation

Three hundred micro litters of MeCN were added to the dry residue and the mixture vortexed for 30 s. Then 2 aliquots of 150 μL each were transferred into 2 silanized screw-cap vials together with 50 μL of BSTFA (with 1% TMCS). Each mixture was further vortexed for about 10 s, and heated at 75 °C for 30 min on a "Reacti-therm" heating block. After cooling, 1.5 μL aliquots were directly injected into the gas chromatograph.

2.5. GC–MS analysis

GC/MS analyses were performed on an Agilent (Little Falls, DE, USA) gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and interfaced to a MSD-5973N mass selective detector.

The GC separation was conducted with a Supelco SLB-5MS column (30 m × 0.25 mm ID × 0.25 μm film thickness) and it was preceded by a 2-m pre-column of the same inner diameter connected to the column via a press fit glass union (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas with a constant flow of 1 mL/min. The injection was made in splitless mode (pulsed pressure 40 psi, 60 s) and the injector temperature was 280 °C. The oven temperature programme was as follows: 100 °C held for 1.0 min, ramped to 180 °C at 10 °C/min, finally ramped to 280 °C at 30 °C/min and held for 12.67 min. Total run time was 25 min. The MS transfer line temperature was held at 280 °C.

EI was used at 70 eV in full scan (50–600 m/z) and selected ion monitoring (SIM) mode. The quantification was carried out in SIM mode, with the following ions:

- patulin: 226 (quantifier), 183, 211, 198, 136 (qualifiers).
- $^{13}C_{5-7}$ patulin: 233 (quantifier), 189, 203, 218 (qualifiers).

Dwell time for monitoring each ion was 30 ms allowing at least 2.53 cycles per second.

Agilent Chemstation was used for data collection/processing and GC–MS control. The Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.64, NIST, USA) was also used for identify the analytes.

2.6. PCR detection of P. expansum

Genomic DNA extractions were performed following the GenElute Plant Genomic DNA Miniprep Kit (Sigma) starting from 200 mg of sample. Apple, quince and quince jams were weighed directly whilst juices were previously centrifuged at 15000*g* in 50 mL volumes being the pellet used for DNA extraction.

Two primer systems were tested. One previously developed (Marek, Annamalai, & Venkitanarayanan, 2003) targeting a 404 bp (basepairs) fragment of the polygalacturonase gene of *P. expansum* (primers PEF and PER) and another newly designed over the same gene (GenBank Accession number AF 047713) but targeting a shorter fragment of 81 bp (forward: PESHRT1f, 5′AAA GGC AGG TTG CTC CAG TA 3′ and reverse: PESHRT1r, 5′ GAG GCC AGA CCA GTC AAA TC 3′). Primers were designed using the software Primer3 (Rozen & Skaletsky, 2000).

Amplifications were carried out in 20 μL reaction volumes containing 1.5 mM Magnesium Chloride, 200 μM each dNTP, 1 μM each primer, 1 Unit of Taq Polymerase and 1 μL of undiluted DNA template. The thermal cycler PTC100 (MJ Research, Watertown, USA) was programmed as stated in bibliography for PER/PEF primers. For PESHRT1f/PESHRT1r primer pairs amplification conditions were as follows: initial denaturation step at 92 °C for 5 min; 40 cycles at a melting temperature of 92 °C for 1 min, annealing temperature of 55 °C for 30 s, and extension temperature of 72 °C for 30 s. A final extension of 7 min at 72 °C was included.

3. Results and discussion

As shown in Section 1 few investigators have reported the use of GC to analyse the patulin content of apple products, a task for which HPLC is usually employed. However, it's higher resolution power and the possibility to use stable isotope-labelled when a mass spectrometer is used as a detector, make GC methods attractive for such monitorization. Therefore, in order to obtain lower limits of detection and quantification, we decided to improve the method recently published by Marks (2007), which was accomplished by introducing minor changes in the sample preparation step (high volume of extraction solvent) and in the derivation step (use of BSTFA added of 1% of TMCS instead of BSTFA alone) Moreover, a known amount of commercial isotopically-labelled ($^{13}C_{5-7}$) patulin was added to the samples before extraction, so as to keep control on the recoveries achieved and to keep track of possible losses occurring during the sample preparation (extraction and derivation).

3.1. Extraction

Preliminary trials with the proposed method in apple juice samples shown to be inadequate for the quantification of very low amounts of patulin. In order to increase the sensitivity of the method it was found necessary to increase the volume of extraction solvent from 5 mL to 20 mL. Patulin is unstable in a basic environment; therefore, as recommended by several authors (Marks, 2007; Moake et al., 2005) 1% acetic acid was added immediately after extraction.

In what concerns quince jam samples, characterised by their high sugar contents, 1 mL of water was added to each sample at the beginning of the extraction procedure in order to make easy the homogenisation and increase the extraction yield. Although, the presence of co-extractives on GC chromatogram was larger when compared with other products analysed, the determination of patulin was successful.

3.2. Derivation

The labour and time spent on the derivation process is usually compensated by an increase on selectivity and detectability of the analytes. In line with other studies the derivation of patulin using BSTFA by GC–MS quantification might be seen as the method of choice. Nonetheless, preliminary experiments were carried out to assess the effect of different silylating reagents on the yield and selectivity of the derivation process. A patulin solution of 8 μgL^{-1} (MeCN or EtAc) was derivatised with one of the following reagent: BSTFA, BSTFA (with 1% of TMCS), or MTBSTFA. These experiments were tested at different conditions (amount of silylating reagent, temperature and time of reaction). It was found that the type of silylating reagent used had a big influence in the yield.

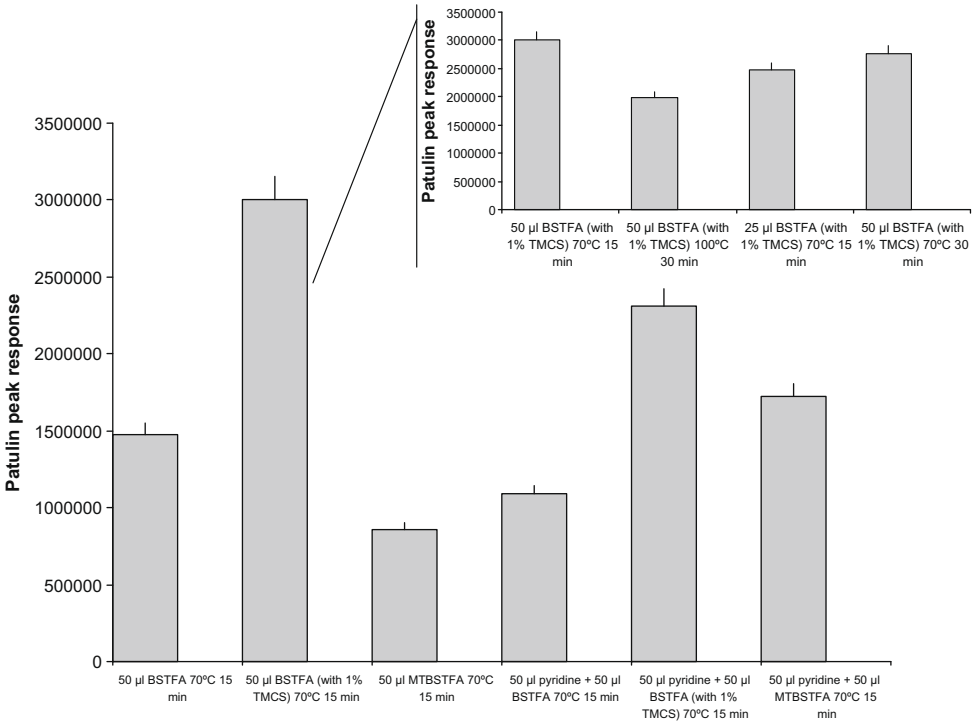

Fig. 1. Peak response of patulin solution (8 μgL^{-1}) using several silylating reagents (BSTFA, BSTFA with 1% of TMCS and MTBSTFA) at different amounts (from 25 to 50 µl), with several combinations of temperature (from 70 to 100 °C) and time of reaction (from 15 to 30 min) ($n = 2$).

The best results were obtained using BSTFA (with 1% of TMCS) as can be seen in the Fig. 1.

The derivatised solutions of apple juice and quince jam were kept at −18 °C and periodically injected over a 3-day period in order to monitor their stabilities. The analytes derivatised were stable for at least 1 month, with a RSD of 5% and 9% for the apple juice extract and quince jam extract, respectively.

3.3. Method performance

3.3.1. Selectivity

With respect to the identification capability of the method, mass spectra of the derivatisated analyte showed enough fragmentation to allow an accurate identification of the target analyte in the samples. The presence of three diagnostic ions in the SIM mode at their correct relative abundances was imposed as identification criterion. As could be expected, the $^{13}C_{5-7}$ patulin used as an internal standard, presented a retention time very close of the native analyte. Nevertheless, thanks to higher *m/z* value of isotopical ions, it can be easily mass-spectrometrically resolved.

Fig. 2 presents two chromatograms: one corresponding to an apple juice sample with 4.8 μgkg^{-1} of patulin and 16 μgkg^{-1} of $^{13}C_{5-7}$ patulin and the other to a quince jam sample with 9.7 μgkg^{-1} of patulin and 50 μgkg^{-1} of $^{13}C_{5-7}$ patulin.

3.3.2. Linearity

Experiments were carried out in order to assess the possible matrix effect on the chromatographic response. Comparing the slopes of the calibration in standard solutions with those obtained in matrix-matched standards, the method showed a slight enhancement of response for patulin. This effect, observed previously in other analytes (Cunha, Fernandes, & Oliveira, 2007), could be attributed to the presence of co-extractives in the final extract. Therefore, the linearity of the method was tested several times using matrix-matched calibration solutions (standards added to blank samples of each matrix studied as in recovery studies) prepared as described in Section 2. The range of concentrations varied between 1.6–100 μgkg^{-1}, used for apple juices, apple fruit and quince fruit analysis and 8–100 μgkg^{-1}, used for quince jam analysis, and included 8–6 points. Calibration curves were constructed by plotting the patulin/I.S. ratio obtained against the concentration of patulin. The results obtained demonstrate an excellent linearity with correlation coefficients always higher than 0.9922, for all four distinct matrices used.

3.3.3. Recovery

The recovery was determined in blank samples (one apple juice and one quince jam both free of patulin) to which 50 µl of standard solutions at 0.8 mgL^{-1}, 1.2 mgL^{-1}, 2.5 mgL^{-1} and 5 mgL^{-1} of patulin were added before extraction corresponding to final concentrations of 8, 12, 25 and 50 μgkg^{-1}, respectively. Peak areas of patulin were compared with peak areas obtained from the same samples spiked with the same levels of the patulin after the extraction procedure. I.S. was always added after the extraction step. Each test was performed six times, and the value shown is the average of six measurements. In apple juice the mean recovery values ranged from 71 to 89% and in quince jam from 74 to 87%, as can be seen in Table 1. These results provide evidence that the method achieves acceptable quantitative recoveries even for a concentration of patulin as low as 8 μgkg^{-1}.

3.3.4. Repeatability

The relative standard deviation (RSD) was calculated from six replicates of apple juice and quince jam spiked samples at four and two concentrations levels, respectively. Their values ranged from 10% to 16% (Table 1). The repeatability obtained here lies well within the commonly reported range (Commission Regulation (EC) NO, 401/2006).

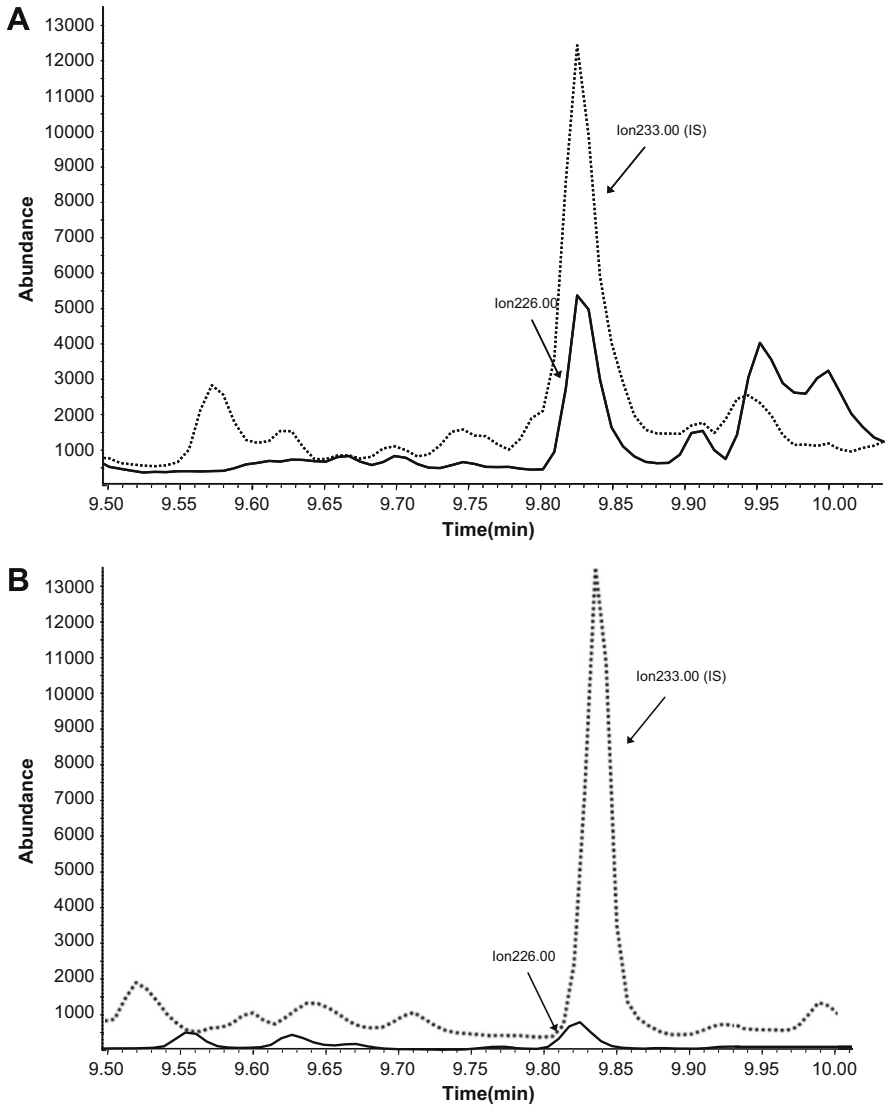

Fig. 2. Chromatogram (A) of an apple juice with 4.8 μg/kg of patulin (m/z = 226) added with 16 μg/kg of $^{13}C_{5-7}$ patulin as internal standard (m/z = 233) and chromatogram (B) of a quince jam with 9.6 μg/kg of patulin (m/z = 226) added with 50 μg/kg of $^{13}C_{5-7}$ patulin as internal standard (m/z = 233) with obtained in SIM mode.

3.3.5. Limit of detection and limit of quantification

The detection limit of the method was determined by successive analyses of chromatographic extracts of apple juice and quince jam spiked samples with decreasing amounts of the patulin until a signal-to-noise ratio 3:1 was reached (Commission Regulation (EC) NO, 401/2006). The assigned values were 0.4 $\mu g kg^{-1}$ and 1.2 $\mu g kg^{-1}$ for apple juice and quince jam, respectively. The limit of quantification was established as the lowest concentration assayed quantified with acceptable accuracy and precision (n = 6) (Commission Regulation (EC) NO, 401/2006), which was 1.6 $\mu g kg^{-1}$ for apple and 8 $\mu g kg^{-1}$ for quince jam, with a RSD of 15% and 12%, respectively. These values show that the detection and quantification limits of the method are fully compatible with the legislative requirements set by EU for patulin.

Table 1
Mean % recoveries and repeatability (% Relative Standard Deviation – RSD) obtained in spiked apple juice and quince jam samples analysed using the developed method (n = 6).

Patulin spiked (μg/kg)	Apple juice		Quince jam	
	Recovery (%)	Repeatability (% RSD)	Recovery (%)	Repeatability (% RSD)
8	71	16	-	-
12	72	10	74	14
25	89	13	-	-
50	88	11	87	14
Mean	80	13	81	14

- = not determined

3.4. Analysis of patulin in apple products and quince products

After method optimisation and the conclusion of the validation studies, the method was applied to the analysis of apples with different-sized brown rotten areas, as well as to a variety of processed apple products commercialised in Portugal (sample composition is displayed in Table 2).

As mentioned in Section 2, the percentage of rotten area in apples was estimated visually allowing their classification in four distinct categories according to the percentage of brown area: (I) about 75%, (II) about 50% (III) about 25% and (IV) not visible.

Patulin analysis in the *Golden delicious* apples revealed a correlation between the concentration of patulin and the percentage of brown rotten area. The levels of patulin in the *Golden delicious*

Table 2
Patulin levels (μg/kg mean and % RSD, $n = 2$) and other characterisation parameters (°Brix, pH and titratable acidity) in apple fruit with size rotten brown areas and apple products.

Sample	Product	Description	Treatment	°Brix	pH	Titratable acidity	Patulin μg/kg (RSD%)
1	Apple not visible brown area	Golden delicious		–[b]	–[b]	–[b]	<LOQ
2	Apple about 25% brown area	Golden delicious		-	-	-	3.2 (11)
3	Apple about 50% brown area	Golden delicious		-	-	-	11.8 (7)
4	Apple about 75% brown area	Golden delicious		-	-	-	1500.0 (7)
5	Organic juice	Apple nectar	P	10.2	3.6	5.1	2.8 (18)
6		Apple nectar	P	12.9	3.5	8.6	8.2 (6)
7		100% Apple juice	P	12.9	3.4	10.5	8.9 (9)
8		80% Apple juice and 20% bilberry	P	12.1	3.4	-	5.4 (19)
9		50% Apple juice, grape sugars and lemon juice	P	14.9	3.8	2.5	5.6 (10)
10		20% Apple juice	P	14.1	3.9	8.5	<LOQ
11	Conventional juice	100% Apple juice	P	12.4	3.6	6.0	4.5 (3)
12		100% Apple juice	P	12.4	3.6	6.9	3.3 (2)
13		100% Apple juice	P	12.0	3.6	6.2	<LOQ
14		100% Apple juice	P	12.6	3.6	6.8	3.2 (2)
15		100% Apple juice	P	13.1	3.6	7.4	12.6 (1)
16		100% Apple juice	P	12.4	3.6	6.5	7.0 (4)
17		100% Apple juice	HPP	12.7	3.9	4.5	5.5 (21)
18		100% Apple juice	HPP	13.9	3.7	7.5	<LOQ
19		100% Apple juice	HPP	14.5	3.8	6.1	4.8 (5)
20	Conventional juice	90% Apple juice 10% lemon juice	HPP	14.2	3.5	11.5	6.8 (9)
21		60% Apple juice	P	13.1	3.7	5.0	<LOQ
22		50% Apple juice	P	12.4	3.7	4.3	9.9 (20)
23		30% Apple juice	P	12.2	3.4	3.4	n.d.
24		30% Apple juice	P	13.4	5.5	3.3	<LOQ
25		16% Apple juice	P	12.6	2.8	6.5	<LOQ
26		16% Apple juice	P	5.0	3.8	6.0	n.d.
27		16% Apple juice	P	8.5	2.9	6.5	4.6 (9)
28		20% Apple juice and 10% milk	P	13.2	3.9	3.4	n.d.
29		20% Apple juice and 80% apple nectar	P	14.4	4.1	5.5	2.4 (2)
30		9% Apple juice, 26% strawberry juice and 10% apple pulp	P	5.6	3.6	-	3.2 (28)
31		8% Apple juice	P	11.5	3.3	3.9	n.d.
32		10% Apple nectar	P	11.9	3.3	3.1	<LOQ
33		–[a]	P	13.5	11.1	3.8	2.1 (18)
34	Cider	–	–[a]	13.5	8.5	3.8	n.d.
35		–	–	13.4	5.5	3.9	<LOQ
36	Baby food	75% Apple	–	13.5	11.8	3.9	n.d.
37		100% Apple	–	13.5	11.9	3.8	9.1 (14)

n.d. – not detected. *P* – pasteurisation, HPP – high pressure processing. <LOQ – lower than limit of quantification.
[a] – not referred
[b] - not determined

apples with brown rotten areas (up to 1500 $\mu g kg^{-1}$) were slightly lower than those presented by Martins, Gimeno, Martins, and Bernardo (2002) (up to 3050 $\mu g kg^{-1}$ in whole samples of Golden delicious apples with different rotten portions). These results may have different explanations namely: limited number of samples analysed, toxigenicity of the fungi the substrate composition, physical/ecological conditions (temperature, pH) or even the methodology used in the analysis.

Table 3
Patulin levels in apple products from different countries.

Country	Patulin range (μg/kg)				References
	Apples	Apple juice	Baby food	Cider	
Belgium	-	2.5–38.8	-	-	Tangni et al. (2003)
Spain	n.d.	1.5-50.9	-	n.d.	Gonzlez-Osnaya, Soriano, Molt, & Maes (2007)
Italy	-	6.4-55.4	-	-	Spadaro et al. (2007)
Greece	-	0.9-11.8	4.0-6.9	-	Moukas, Panagiotopoulou, & Markaki (2008)
Denmark	-	3.5-122.5	-	-	Murillo-Arbizu, González-Peñas, Hansen, Amézqueta, & Østergaard (2008)

- not referred. n.d.-not detected.

In what concerns the processed apple products, a total of 29 commercial apple juices (6 certified organic and 23 conventional samples), 2 samples of cider and 2 samples of baby food were analysed.

In cider samples the patulin was not detected. These results did not differ from those obtained by Stinson, Osman, Huhtanen, and Bills (1978) who proposes a possible explanation relying on the capacity of the alcoholic fermentation to eliminate patulin contamination. Some authors, however, have found patulin in cider which can be explained by the addition of apple juice to fermented cider in order to adjust sugar and flavour contents (Leggott & Shephard 2001). In one of the two baby food samples analysed, patulin was not detected whilst in the second one a 9.1 $\mu g kg^{-1}$ level was found, which was slightly below the legal limit established by EU for patulin in baby food (10 $\mu g kg^{-1}$).

In the organic apple juices the patulin levels varied from <LOQ to 8.9 $\mu g kg^{-1}$ whilst in conventional apple juice they ranged from <LOQ to 12.6 $\mu g kg^{-1}$. These results put in evidence the low degree of variation in patulin levels in both organic and conventional juices, whit no consistent overall trends being observed as it was previously referred by Ritieni (2003), Tangni et al. (2003) and Spadaro, Ciavorella, Frati, Garibaldi, and Gullino (2007). Nevertheless, some studies have shown diverging results stating that organic apple juices are more prone to contamination than the conventional ones (Piemontese, Solfrizzo, & Visconti, 2005).

In these last years the awareness of the apple industry to patulin contamination in fruit has increased the implementation of

Table 4
Patulin levels (μg/kg mean, and % RSD, $n = 2$) in quince fruit with size rotten brown areas and quince jam samples.

Sample	Product	Description	Patulin μg/kg (RSD%)
38	Quince not visible brown area	Variety Portugal	<LOQ
39	Quince about 25% brown area		4.9 (9)
40	Quince about 50% brown area		47.5 (11)
41	Quince about 75% brown area		118.3 (11)
42	Quince jam	50 g of fruit for 100 g of product	<LOQ
43		50 g of fruit and 60 g of sugar for 100 g of product	9.7 (8)
44		54 g of fruit and 42 g of sugar for 100 g of product	26.9 (7)
45		50 g of fruit and 60 g of sugar for 100 g of product	28.7 (1)
46		50 g of fruit and 65 g of sugar for 100 g of product	20.4 (14)
47		–[a]	<LOQ
48		50 g of fruit and 65 g of sugar for 100 g of product	<LOQ
49		60 g of fruit and 60 g of sugar for 100 g of product	<LOQ
50		50 g of fruit and 66 g of sugar for 100 g of product	<LOQ
51		50 g of fruit and 60 g of sugar for 100 g of product	<LOQ

<LOQ – lower than limit of quantification. RSD relative standard deviation.
[a] – not referred.

several techniques for the production of processed apple products with reduced patulin levels. However, when comparing patulin levels obtained with the utilisation of high-pressure technology with those furnished by the conventional treatments, such as pasteurisation or ultra-pasteurisation, no significant difference was observed (Table 2). Probably a higher number of samples and a wider variety of treatments would have to be assayed to evaluate the full impact of the treatments in the patulin levels. Actually, our study was hampered by the limited availability of commercially apple juices in the Portuguese market and also because the information provided by producers on the label does not mentioned in most cases the treatments used during processing.

Patulin levels detected in apple juices were similar to those reported in 2002 in Portugal and other EU countries by Majerus and Kapp (2002) and those recently referred in some publications (Table 3). On the other hand, the results of this study demonstrate that the patulin levels in organic or conventional apple juice subject to different treatments were all below the legislative requirements.

In this work four quince fruits (*C. oblonga*) with different-sized rotten brown areas were also evaluated using the same methodology applied for apple samples (Table 4). Patulin contamination levels varied from 118.3 $\mu g kg^{-1}$ (about 75% of brown area) to 4.9 $\mu g kg^{-1}$ (about 25%). Taking into consideration these high levels of contamination of the fruits we presumed that patulin could be as well found in processed quince products. Therefore, 10 samples of quince jam were analysed as mentioned above. The patulin was detected in 4 of these samples in levels ranging from 9.74 to 28.62 $\mu g kg^{-1}$. As far as we know, this is the first report on patulin presence in quince fruits and commercial quince jam. The patulin levels found in these products confirmed the importance of monitoring this contaminant in a variety of foodstuff, besides the commonly analysed apples and processed apple products.

3.5. PCR detection of P. expansum

Group representative samples were used to test the PCR methodology (4 apples and 4 quinces) with four different rotten brown areas; 10 apple juices: 10, 23, 28 (not detected or inferior to LOQ levels of patulin) and 6, 7, 9, 16, 19, 20, 22 (detected levels of patulin); 4 quince jams: 44, 45 (not detected or inferior to LOQ levels of patulin) 50, 51 (detected levels of patulin).

Using the PCR system described by Marek et al. (2003) weak amplicons of the expected size (404 bp) were obtained in two samples: juice sample 6 and the quince from category II. Accordingly, no PCR amplicons were expected in juices and jams, due to the DNA degradation occurring during industrial processes and the relatively big size of the amplicons. The lack of amplification in the extracts obtained from rotten fruits can be explained by the lack of contamination with *P. expansum* or the inefficiency of the PCR system in this samples (authors have referred that the system was not validated for fruit samples only for mould pure cultures).

In order to decrease the size of the amplicon, which theoretically would increase the chance of amplification in highly degraded DNA samples, we have designed a new PCR system targeting a short fragment of 81 bps. The PCR amplification directly in the genomic extracts resulted in strong amplicons of the expected size for four juice samples 6, 20, 19, 22; three apples (rotten category I, II and III), one quince jam (44) and one quince (rotten category I). Compared with the previously available method the one proposed permitted the detection of *P. expansum* specific sequences in three more fruit juices, in all the rotten apples, in a quince jam and in the category I rotten quince. These results are somewhat related with the patulin levels evaluated since *P.expansum* was not unequivocally detected in non-rotten apples or quinces and fruit juices or jams containing not detected levels of patulin (*i.e.* samples 10, 23, 28, 50, 51). Notwithstanding the detection of *P. Expans*um in a considerable fraction of tested samples containing patulin (2/3), in a few samples in which patulin was detected the system did not detect the mould. This could be due to either *P. expansum* was not infecting the original material being patulin produced by another different mould or the DNA was degraded to fragments of an average size under 80 bp.

Additionally, a nested PCR approach was performed by reamplifying 1 μL of the PCR products obtained using the first primer pair (PEF/PER) with the developed system (PESHRT1f/PESHRT1r). However the increase in sensitivity provided by the nested approach resulted in an increased amount of high molecular weight unspecific products, particularly in apple and quince samples. Further work is needed to optimise the nested system.

4. Conclusions

The proposed GC–MS method, even similar in its principles to that recently published, has been redesigned throughout a slightly modification of the sample extraction process in order to improve the sensitivity, allowing to achieve very good limits of detection and quantification estimated at 0.4 $\mu g kg^{-1}$ and 1.6 $\mu g kg^{-1}$, respectively. The use of commercial $^{13}C_{5-7}$ patulin as internal standard at the initial step in the work-up procedure has shown to significantly improve the precision of the measurements, allowing good recoveries and repeatability with relative standard deviations below 16%, even at low concentration levels (4 $\mu g kg^{-1}$).

The analyses of apple products commercialised in the Portuguese market showed that these are safe in regard to the presence

of patulin. The levels of patulin in quince fruits and quince jams were evaluated in the present work for the first time.

The *P. expansum* PCR detection system developed proved to be more efficient than the available methodologies for the detection of the mould in samples containing highly degraded DNA.

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