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Determination of patulin in apple juice by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

An HPLC–MS–MS method with selected reaction monitoring (SRM) for the determination of patulin in apple juice samples is described. Mass spectrometric detection was accomplished following atmospheric pressure chemical ionization (APCI) in both positive and negative ion modes. Collision induced dissociation (CID) of the protonated molecular ion led initially to the loss of H_2O (fragment m/z 137). At higher energies CO is lost from both the protonated parent molecule (fragment m/z 127) and the dehydrated molecular ion (fragment m/z 109). In contrast, CID of the deprotonated molecular ion led initially to the fragment at m/z 109 corresponding to the loss of either CO_2 or acetaldehyde, followed at higher CID energy by the loss of H_2O (fragment m/z 135) and CO (fragment m/z 125) from the deprotonated molecular ion. Detection in the negative ion mode proved superior and a linear response was observed over the injected range from 6 to 200 ng patulin. Apple juice samples spiked with patulin between 10 and 135 μg/l were analyzed following liquid–liquid extraction with ethyl acetate and clean up with sodium carbonate. Utilizing reversed-phase HPLC with acetonitrile–water (10:90) at 0.5 ml/min, levels down to 10 μg/l were readily quantified and a detection limit of 4 μg/l was attainable at a signal-to-noise (S/N) ratio of 4. The MS data for the spiked samples compared well to the UV data and when plotted against each other displayed a correlation coefficient (R) of 0.99.

Keywords: Liquid chromatography–mass spectrometry; Apple juice; Patulin; Mycotoxins

1. Introduction

Patulin (4-hydroxy-4*H*-furo[3,2-c]pyran-2[6*H*]-one) is a toxic secondary metabolite produced by a wide range of fungi including several *Penicillium* and *Aspergillus* species [1]. *Penicillium expansum* is the most common post-harvest invader of apples that causes ''blue mold rot'' during storage [2,3]. Hence, the major dietary exposure of humans to patulin is from the consumption of apple juice and apple juice products made from affected fruit. The contamination of apples with patulin is normally associated with the areas of spoiled tissue and although removing rotten tissue from the fruit can reduce patulin levels, penetration nevertheless occurs up to approximately 1 cm into the surrounding healthy tissue [4]. Therefore the presence and extent of residual patulin contamination in apple products can serve as an

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indicator of the quality of fruit used in their production since an appreciable concentration of the mycotoxin remains in the product after processing [5]. While this genotoxic, water-soluble β-unsaturated lactone has no reproductive or teratogenic effects, it does however show embryotoxicity accompanied by maternal toxicity [6]. Owing to its toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) has established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 μg/kg body weight/day [6]. Due to concern for human health, especially amongst children, health authorities in many countries regard patulin contamination of foods as a problem and regulate patulin in juice at levels ranging between 20 and 50 μg/l [7]. The World Health Organization recommends to limit its contents in foods to 50 μg/kg [8,9].

In view of the recognized adverse effects caused by this mycotoxin and the need for regulatory control, monitoring its level in apple juices and other food products made from apples, is important to evaluate the risk due to human consumption of these products. For these reasons, several analytical methods have been developed for detecting and determining patulin in apple products. These include methods based on thin layer chromatography (TLC) [10,11], gas chromatography (GC) [12–14], and high-performance liquid chromatography (HPLC) [15–17]. Reversed-phase HPLC, coupled with UV detection has been found to be most suitable since the toxin is relatively polar and exhibits a strong absorption spectrum. The LC method described by Brause et al. [18], was recently validated and adopted first action by AOAC International (Method 995.10) [19]. However, the lowest limit examined was 20 μg/l in a spiked test sample and 31 μg/l in a naturally contaminated sample.

In a quest to lower the detection limits of this mycotoxin numerous improvements have been made in both the extraction and detection methods used for patulin as indicated in a recent review by Shephard and Leggott [20]. Recently a solid-phase extraction method was reported for patulin determination in apple juice and unfiltered apple juice in the USA [21]. Here, a portion of the test sample was passed through a macroporous co-polymer made from a balanced ratio of the lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidine followed by washing with 1% sodium bicarbonate and then with 1% acetic acid. Patulin was eluted with 2% acetonitrile in anhydrous ethyl ether and determined by reversed-phase HPLC with UV detection at 276 nm. Recoveries ranged from 93 to 104% in test samples spiked at 20–100 μg/l. The detection limit of this method was reported to be about 5 μg/l. The shortfall of UV detection however, arises when poor resolution is observed between patulin and other co-extracted interfering substances especially 5-hydroxymethyl-2-furaldehyde (hydroxymethylfurfural, 5-HMF) thus requiring modification of mobile phases.

Stable isotope dilution assays using ^{13}C-labeled patulin as internal standard for mass spectrometric detection were also reported [22]. This method, however, used HRGC–HRMS and allowed for the determination of patulin in samples requiring more extensive clean-up procedures than currently employed. A detection limit down to 12 ng/l was attainable for the trimethylsilyl derivative, which is not possible with currently available low resolution benchtop systems. Furthermore, a technique known as ''diphasic dialysis'', which was initially introduced as a means of extracting patulin from apple juice [23–26], has subsequently been combined with in situ acylation followed by GC–MS [27]. Here apple juice samples spiked with 4-*N*,*N*-dimethylaminopyridine were dialyzed using methane chloride and acetic anhydride inside a dialysis tube and patulin was derivatized into its acetate and directly determined using GC–MS with selective ion monitoring mode. With this technique the mean recoveries ranged from 50 to 92% with the limit of detection being 1 μg/l. The shortfall of this technique is that dialysis extends over a 24-h period and is thus too time consuming if monitoring needs to be done routinely during a manufacturing process.

Recent advances in LC–MS technology have shown this technique to have wide applications in food analysis [28–30], including the analysis of mycotoxins [31–33]. HPLC–MS technology eliminates the need for sample derivatization and moreover, has the potential to yield both structural and molecular mass information. Furthermore, MS is a technique of high intrinsic specificity and MS–MS provides additional selectivity thus allowing for

increased sensitivities as a result of decreased background noise. In a continuing effort to develop a less time-consuming, more selective and sensitive analytical method for detecting patulin in apple juice, we now report on a method developed in our laboratory employing HPLC–MS–MS utilizing atmospheric pressure chemical ionization (APCI) and ion trap technology.

2. Experimental

2.1. Chemicals and solvents

Pure patulin was purchased from Sigma Chemicals (St. Louis, MO, USA). Acetonitrile, methanol, ethyl acetate (HPLC grade) and sodium sulphate were obtained from BDH chemicals (Poole, UK) while formic acid (analytical grade), ammonium acetate (98% min), ammonia solution (25% min) and sodium carbonate was obtained from Merck (Darmstadt, Germany). Water for HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Standard solutions of patulin were prepared in acetonitrile–water (10:90) and stored at 4°C.

2.2. Laboratory sample details

Apple-juice concentrate free from patulin was obtained from a local South African production facility and diluted to 12° Brix. From this, analytical samples were spiked at 10, 40, 80, 110 and 135 μg/l respectively, using a reference standard of patulin, whose concentration was determined by UV spectroscopy at 275 nm, against a solvent blank, using the molar mass and molar extinction coefficient (ε) reported by the Association of Official Analytical Chemists [34].

2.3. Extraction and clean-up from analytical portions

The method of Brause et al. [18] was used for the extraction of patulin. In brief, spiked apple juice (5 ml) was extracted with ethyl acetate (2×10 ml) and the combined organic extracts cleaned up by extraction with sodium carbonate solution (2 ml, 1.4% m/v). The ethyl acetate extract was subsequently dried over anhydrous sodium sulphate (1 g) and the filtrate reduced in volume under reduced pressure at 40°C. The extract was redissolved in minimum solvent, placed into a vial and evaporated to dryness at 40°C under a constant flow of nitrogen. With this method of extraction and clean up, recoveries averaged 96% from apple juice. Analytical results reported in this study were not corrected for recovery.

2.4. HPLC conditions

HPLC analysis was carried out using a SpectraSERIES P2000 pump equipped with an AS 1000 autosampler and a UV 1000 variable wavelength UV detector (all from Thermo Separation Products Inc, Riviera Beach, FL, USA). The purified apple juice extracts (test solutions) were reconstituted in mobile phase (200 μl), filtered through a 0.45 μm syringe filter (Millipore, Yonezawa, Japan) and injected (20 μl) onto the column. Patulin was separated isocratically on a 150×4.6 mm I.D. Luna C_{18} reversed-phase column (Phenomenex, Torrance, CA, USA) packed with 5 μm ODS-2. The mobile phase was prepared using acetonitrile–water (10:90) and pumped at a flow-rate of 0.5 ml/min. On-line UV detection at 276 nm was performed prior to MS detection.

2.4.1. Mass spectrometry

Both positive and negative ion atmospheric pressure chemical ionization–mass spectrometry (APCI–MS) was performed using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA). The MS parameters were optimized by direct infusion of 5 μg/ml patulin standard at 3 μl/min into the source. The APCI vaporizer and mass spectrometer capillary temperatures were maintained at 450°C and 150°C, while the source current and capillary voltage were maintained at 5 μA and 7 V or −4 V for either the positive ion or negative ion mode respectively. The sheath and auxiliary gas flows were maintained at 80 and 10 units respectively. During tuning, the mass spectrometer was initially programmed to perform a full scan from m/z 100 to m/z 160 in order to observe either the protonated (m/z 155) or deprotonated (m/z 153) molecular ion. The final analytical determination of patulin, however, was

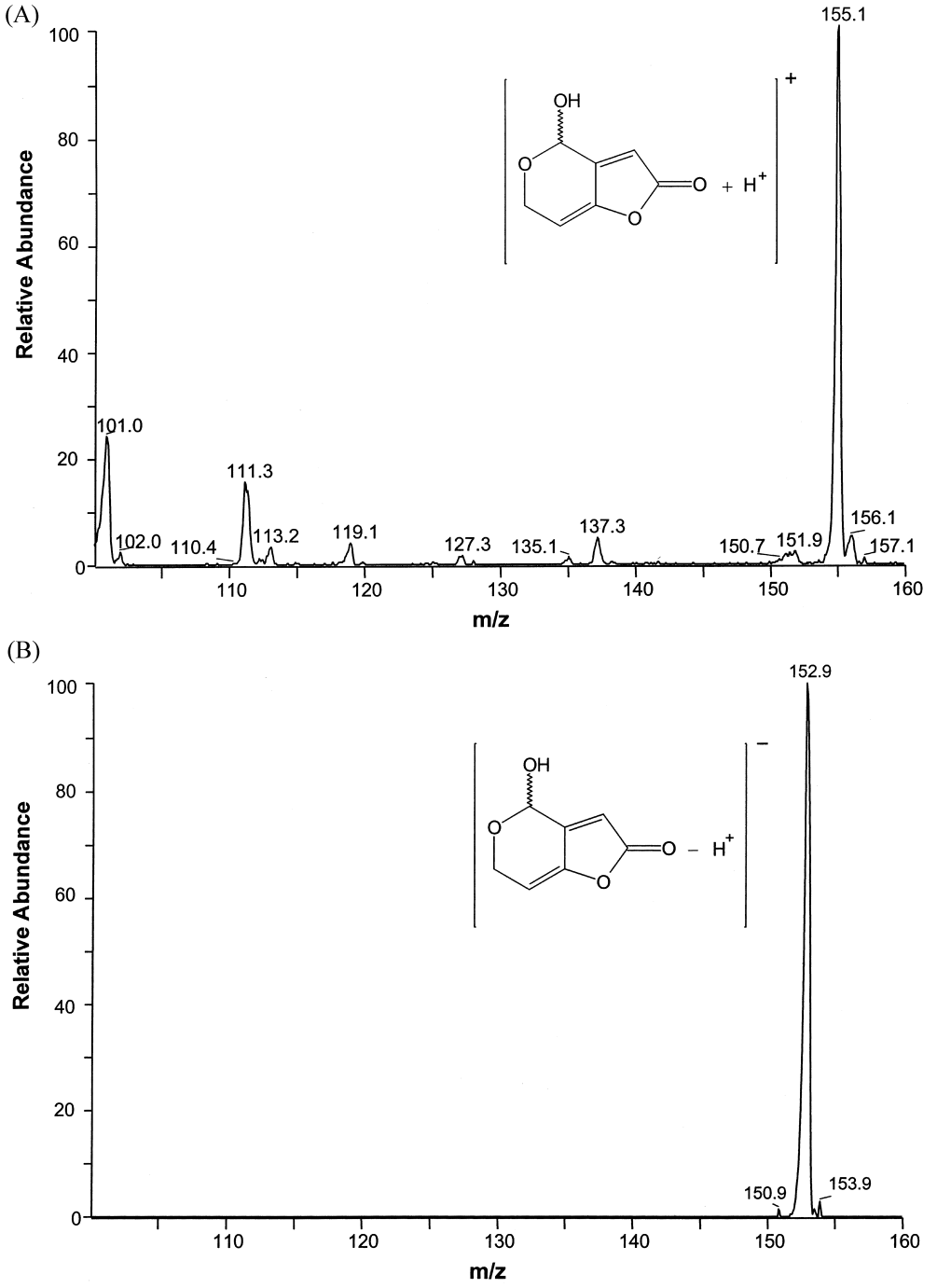

Fig. 1. Mass spectrum of patulin showing (A) the protonated molecular ion $[M+H]^+$ at m/z 155 and (B) the deprotonated molecular ion $[M-H]^-$ at m/z 153.

Table 1
Fragment ions observed for patulin under positive and negative ion CID MS–MS experiments on the ion trap mass spectrometer

Fragmentation of patulin	m/z of fragment ions observed	Interpretation
MS–MS of $[M+H]^+$ (m/z 155)	137	$[M+H-H_2O]^+$
	127	$[M+H-CO]^+$
	109	$[M+H-H_2O]^+$
MS–MS of $[M-H]^-$ (m/z 153)	135	$[M-H-H_2O]^-$
	125	$[M-H-CO]^-$
	109	$[M-H-CO_2]^-$ or $[M-H-C_2H_4O]^-$

done in the MS–MS mode by selected reaction monitoring (SRM) of the product ions resulting from collision-induced dissociation (15% collision energy) of the deprotonated molecular ion. The resulting product ions at m/z 135, 125 and 109 were monitored using an isolation width of 2 amu for each ion. Quantitation was achieved by comparing the peak areas of patulin with the corresponding calibration plot of the standards.

2.4.2. Detection limit

A series of patulin standards ranging from 0.3 to 10 μg/ml was injected in order to determine the on-column instrumental detection limit. The linearity of the calibration plot was determined over the designated calibration range. The precision of the measurement of patulin was readily determined by performing triplicate injections of each standard solution under identical experimental conditions.

3. Results and Discussion

3.1. MS tuning

The mass spectrometer was tuned in both the positive and negative ion modes in order to assess the intensity and stability of the ion signal. Full scans were performed over the range m/z 100 to m/z 160 and data acquired in the ''profile'' mode. Both the protonated and deprotonated molecular ion signals at m/z 155 and m/z 153 respectively (Fig. 1A and B) were of almost equal intensity, however the protonated molecular ion was observed against a noisy background. The tuning solution was subsequently modified using ammonium acetate and ammonium hydroxide in order to improve the degree of ionization. Ammonium acetate however failed to improve the ion signal in both the positive and negative ion modes. The addition of ammonium hydroxide in the negative ion mode, on the other hand, simply increased the level of background noise to a greater extent than observed for the protonated molecular ion in Fig. 1A. This could be attributed to the instability of patulin in alkali solution. Tuning with patulin dissolved in acetonitrile–water (20:80) in the negative ion mode produced a clear background hence no modifiers were added in subsequent investigations. It is well known that negative ion polarity mode sometimes generates less chemical noise than does the positive mode, thereby improving sensitivity. In addition, patulin, being a low molecular mass compound, is difficult to detect amongst intense background signals, hence making negative ion detection appropriate.

3.2. Collision induced dissociation

Since APCI is a relatively soft ionization technique, mass spectra generated following HPLC–MS consist almost only of the pseudomolecular ion and, consequently, give little structural information. However, during the time that the ions are resident in the ion trap, it is possible to increase the specificity for detection by selectively exciting ions of specific mass-to-charge ratios. This two-stage process (MS–MS) produces diagnostic product ion spectra, which are characteristic of the structural moieties present in the analyte. The fragmentation of patulin was subsequently studied in both positive and negative ion modes by sequentially increasing the collision energy while continuously infusing 5 μg/ml of patulin standard into the source. The fragmentation of patulin yielded product ions corresponding to the

loss of water, carbon monoxide, carbon dioxide or acetaldehyde (Table 1). The collision energy that was required for the dissociation of patulin in the positive ion mode was far greater (30%) than that required in the negative ion mode (15%). Previously, studies on the fragmentation of ^{13}C-labeled patulin [35] following electron impact (EI) and negative electrospray ionization (ESI) mass spectrometry have also produced a similar fragmentation pattern although the molecular mass was shifted by two mass units due to the incorporation of two labeled carbon atoms. In this previous study, it was proposed that the ion at m/z 111 that corresponds to m/z 109 in our study be attributed to the loss of acetaldehyde (C_2H_4O) or carbon dioxide. It is difficult to differentiate between these two products on a low resolution instrument since both have the same nominal molecular mass and can be produced as a result of patulin fragmentation. In the negative ion mode at 10% collision energy, loss of either carbon dioxide or acetaldehyde was observed as well as the fragment corresponding to the loss of water. Upon increasing the collision energy to 15%, complete fragmentation of the parent molecule was observed together with the appearance of the fragment corresponding to the loss of carbon monoxide. Hence based on these results it is postulated that the fragmentation of patulin following negative ion APCI occurs firstly by the loss of CO_2 or acetaldehyde then followed by the loss of water and finally, loss of CO. In contrast, fragmentation in the positive ion mode yielded the dehydrated molecular ion first, followed by a second fragment due to the loss of CO from the protonated parent molecule. The third fragment appeared at much higher collision energy (30%) due to loss of CO from the dehydrated molecule. Irrespective of the fragmentation pathway, it is quite evident that the resulting spectra revealed a number of fragment ions that can serve as diagnostic indicators for patulin confirmation in apple juice samples.

3.3. HPLC–MS–MS

After having identified the fragment ions and

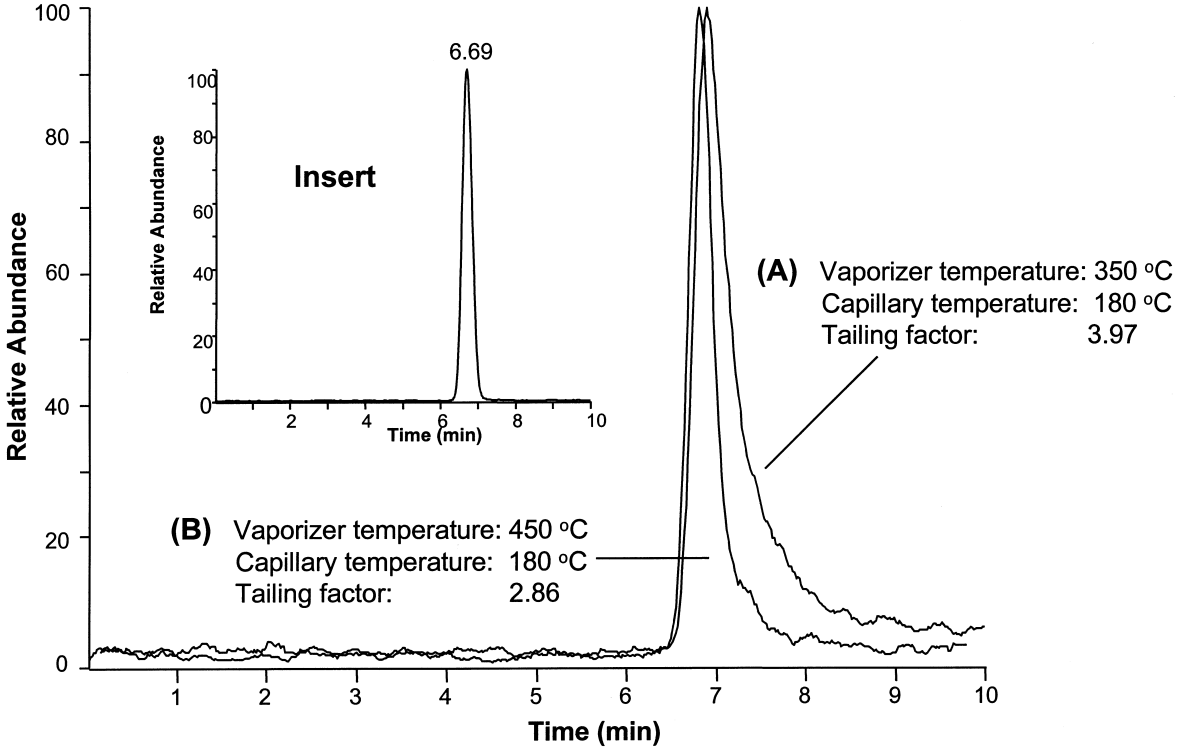

Fig. 2. Mass chromatograms of the protonated molecular ion of patulin showing the effect of vaporization temperature on peak shape. (A) 350°C, (B) 450°C. **Insert:** HPLC–UV chromatogram of patulin at 276 nm corresponding to the mass chromatogram A.

obtaining fingerprint mass spectra following collision induced dissociation (CID) for both protonated and deprotonated molecular ions of patulin, a standard solution was injected to inspect the quality of the mass chromatograms in both positive and negative ion modes. It is important to note that the number of ions arriving at the detector system is dependent on the vaporization efficiency and ion sampling efficiency into the vacuum. This efficiency can be affected by the vaporization temperature, hence it is important to determine whether this temperature is adequate for efficient vaporization of patulin. Unlike the HPLC–UV chromatogram, which was obtained on-line prior to MS, the mass chromatogram displayed excessive tailing for patulin in the positive ion mode (Fig. 2A) when the vaporizer temperature was set at 350°C. Increasing the temperature to 450°C however, reduced the tailing factor considerably from 3.97 to 2.86 (Fig. 2B). It was unlikely that tailing resulted from the column as the narrow symmetrical peak observed in the UV chromatogram (Fig. 2 insert) indicated that the HPLC conditions were suitable. At a lower vaporization temperature, the low rate of vapor formation might have had a retarding effect on the rate of ion formation resulting in a lower concentration of the ions reaching the detector as the droplet diameter was reduced during evaporation. This is significant when highly aqueous mobile phases are used as was the case in this study. Thus we associated this tailing to vapor lag. We believe that the low rate of vapor formation and its lag from the vaporizer tube to the formation of ions before entering the heated capillary might have resulted in peak tailing of the mass chromatogram. The flash vaporizer temperature was increased to 450°C to improve the rate of vaporization and desolvation. This resulted in improved peak shape as observed in Fig. 2B. In the negative ion mode, a tailing factor of 1.24 was observed, once again indicating that negative ion APCI was more suitable

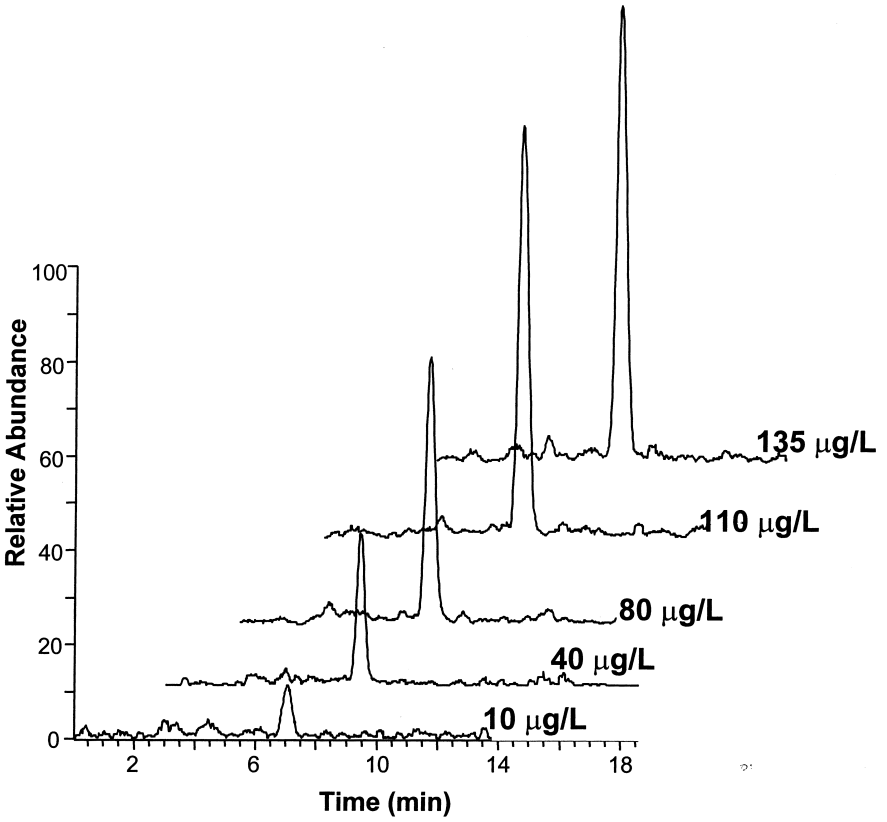

Fig. 3. Product ion mass chromatograms of apple juice samples spiked with patulin at different levels.

for patulin analysis and was thus selected as the mode of choice for further experiments. This change in the behavior between the protonated and deprotonated molecular ions can be attributed to their changes in enthalpy since enthalpy is the driving force that determines their change in state at very high temperatures.

3.4. Linearity and range

In order to determine the linearity of response versus the injected amount of analyte, different concentrations ranging from 10 to 0.3 μg/ml were injected and the daughter ions (m/z 135, 125 and 109) of the deprotonated molecular ion monitored. The repeatability of the measurements was readily determined by performing triplicate injections under identical conditions. The percent relative standard deviation (RSD) varied from 0.47% (10 μg/ml) to 7.71% (0.3 μg/ml). The minimum quantity of the pure standard injected was 6 ng at a signal-to-noise (S/N) ratio of 7.5. A linear fit with a coefficient of determination (R^2) of 0.99 was observed for the MS signal within the experimental range.

3.5. Analysis of spiked samples

To determine the applicability of this technique on natural samples, patulin-free apple juice was obtained from a local commercial production facility and spiked at 10, 40, 80, 110 and 135 μg/l, respectively. Following extraction, the samples were analyzed in the negative ion mode with SRM. Fig. 3 displays the product ion chromatograms of patulin spiked at different levels while Fig. 4 indicates the specificity of detection by showing the product ion mass spectrum of patulin spiked at 10 μg/l. The signal-to-noise ratio at this level was 10. The UV chromatogram on the other hand displayed patulin amongst other impurities (Fig. 5). Of these, 5-HMF, which is a cyclic aldehyde formed by dehydration when hexoses are heated, is the largest impurity. If the composition of the chromatographic mobile phase is not carefully selected, then poor resolution from these co-extracted interfering substances especially 5-HMF can interfere with quantitation. Mass spectrometry on the other hand averts this problem due to the high specificity of the technique. The repeatability of the injections (based on the average

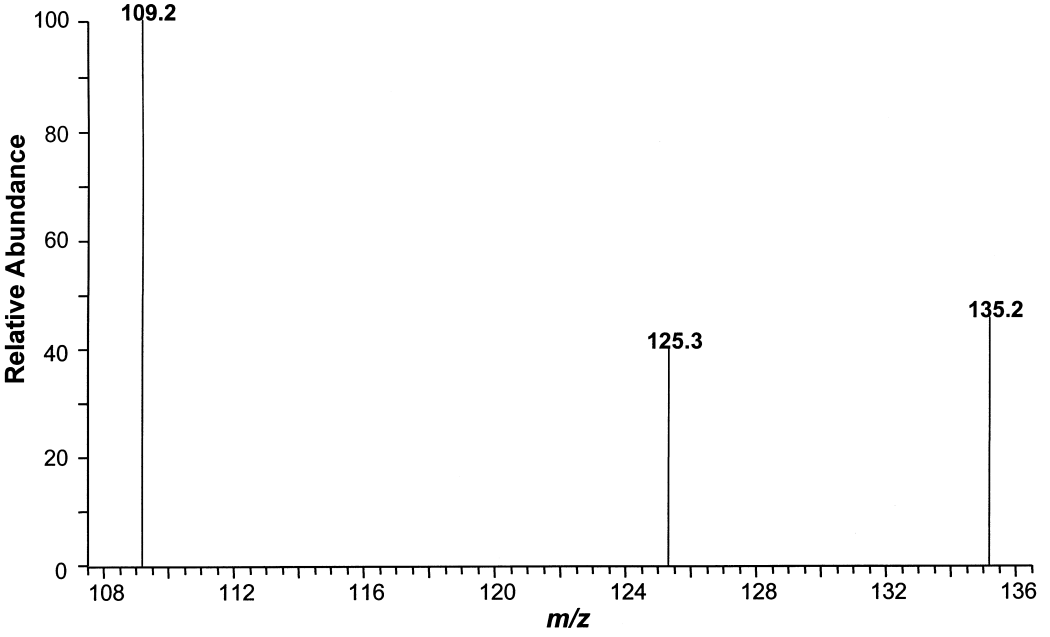

Fig. 4. Product ion mass spectrum of patulin following CID and SRM present at 10 μg/l in apple juice. For experimental conditions, see Sections 2.3 and 2.4.

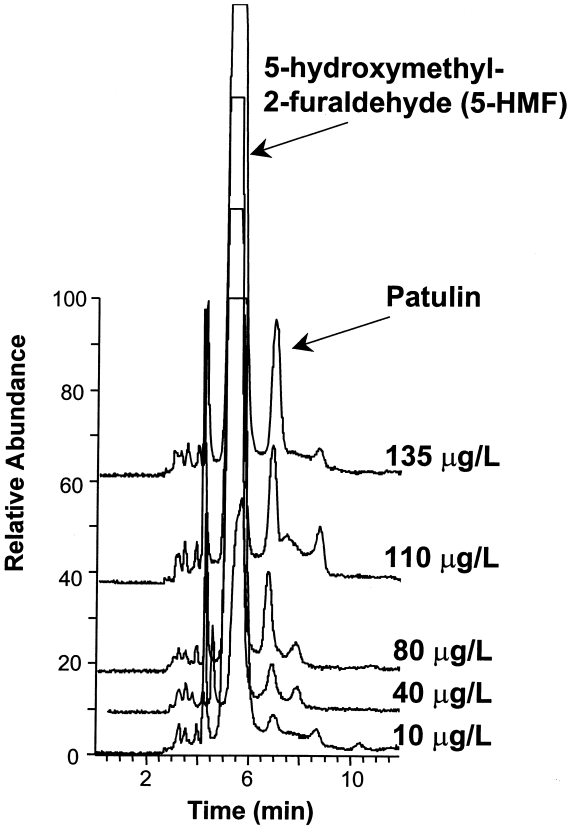

Fig. 5. HPLC–UV chromatogram of spiked apple juice samples obtained at 276 nm showing the interferences that might hinder the accurate quantitation of patulin at low levels.

of three) showed a RSD of 1.20 and 0.72% at the 135 and 10 μg/l levels respectively. A calibration plot indicated a linear response with a coefficient of determination (R^2) of 0.98 and a detection limit of 4 μg/l patulin in spiked juice ($S/N=4$) was observed. One of the limitations of ion trap technology is possible variation in the population of ions in the trap during measurement. This, in turn, is known to influence the analyte signal and affect quantitation. Thus, it has been suggested in a recent report [36] that use of an internal standard such as stable isotope-labeled patulin can compensate for any variation in the signal. However in this study, in order to evaluate the agreement between UV and MS data, patulin concentration was calculated using both detection techniques. The quantitative data were plotted against each other and compared well ($R=$ 0.99). The high correlation between the two detection modes demonstrated the absence of interferences and signal variation.

4. Conclusion

This study demonstrated the applicability of HPLC–APCI–MS–MS technique as a new tool for analyzing patulin in apple juice. The highly specific nature of the data obtained with SRM increased the sensitivity of the technique and coupled with an extraction method capable of yielding high recoveries, levels down to 10 μg/l were easily quantified and a detection limit of 4 μg/l in spiked apple juice samples was demonstrated.

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