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Improved positive electrospray ionization of patulin by adduct formation: Usefulness in liquid chromatography – tandem mass spectrometry multi-mycotoxin analysis

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Abstract

Several sensitive methods have been developed for patulin determination; however, mass spectrometric (MS) detection of this toxin in the positive electrospray ionization (ESI^+) mode is not straightforward. Furthermore, the combined determination of patulin with other mycotoxins in one single run has not been reported yet. The present paper demonstrates the formation and use of a methanol adduct of patulin in ESI^+. A study of the fragmentation pathway confirmed the authenticity of the patulin adduct, while the use of ion trap and high resolution Orbitrap mass spectrometry allowed reliable assignment of the patulin fragment ions. Exploiting the formation of the methanol adduct, patulin has been successfully included in a single run multi-mycotoxin liquid chromatography tandem mass spectrometric (LC-MS/MS) method in support of *ex vivo-in vitro* biomedical studies.

Keywords: patulin, mycotoxin, mass spectrometry, ESI, fragmentation, adduct formation

1. Introduction

Patulin is a toxic fungal metabolite mainly produced by *Penicillium* and *Aspergillus* spp. It naturally occurs in food commodities such as fruits and their processed products [1], wheat bread [2], as well as feed [3]. Recently a large amount of patulin was found in indoor settled dust [4].

It has been proven that patulin causes acute and chronic health effects in humans and animals [1, 5]. Therefore, the patulin levels in food are regulated in many countries and a proposed action level in juices is set at 50 μg/kg [6, 7].

Methods for patulin detection include thin layer chromatography (TLC) for rapid monitoring of patulin levels [8, 9], high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) [10] or diode array detection (DAD) [11], gas chromatography – mass spectrometry (GC-MS) [12, 13]. These LC and GC methods demonstrate satisfactory limits of detection (LODs), but caution has to be taken in the analysis

of fruit juices by LC-UV/DAD where interfering peaks are likely to occur [11], while a derivatization is generally required for GC-MS [14].

Liquid chromatography – tandem mass spectrometry (LC-MS/MS), being considered as the most advanced analytical technique for qualitative and quantitative mycotoxin analysis, is widely used for determination of patulin. There are several methods described allowing reliable detection of this compound in negative electrospray ionization (ESI^-) [14], atmospheric pressure chemical ionization (APCI) [15] or atmospheric pressure photoionization (APPI) [16] modes. However, methods to simultaneously analyze patulin and other toxins are scarce and require polarity switching of the MS [17]. Furthermore, in our group, attempts to achieve a useful ESI-MS signal for patulin using three different generations of triple quadrupole instruments were not successful. Therefore, in line with the approach applied by other authors dealing with multi-analyte detection of mycotoxins, patulin was omitted in previous multi-mycotoxin LC-ESI-MS/MS methods [18-20].

The present paper is focused on a closer investigation of the electrospray ionization of patulin with the goal of inclusion of this toxin in a single run multi-analyte LC-ESI^+-MS/MS method. Adduct formation of patulin appeared to be the only possibility to achieve the purpose of the study. The fragmentation behaviour of patulin (methanol adduct) under the applied conditions was thoroughly studied combining the advantageous features of ion trap and Orbitrap MS. The usefulness of our findings was demonstrated by the development of an LC-ESI^+-MS/MS method that allows a rapid analysis of patulin together with other mycotoxins. With the prospect of assessing exposure to mycotoxins through dermal route, the method was validated for fluids from transdermal penetration studies [21].

2. Experimental

2.1. Chemicals and reagents

Methanol (MeOH) and acetonitrile (ACN) both of LC-MS grade were obtained from Biosolve (Valkenswaard, The Netherlands), whereas HPLC-grade MeOH and ACN were from VWR International (Zaventem, Belgium). Ethyl acetate (EtOAc) and dichloromethane (DCM) were purchased from Acros

Organics (Geel, Belgium). Ammonia *p.a.* (min. 25 %) was provided by Vel (Leuven, Belgium). Sigma-Aldrich was the supplier of ammonium bicarbonate (NH_4HCO_3), bovine serum albumin (BSA) (RIA grade) and phosphate buffered saline (PBS). Hydrochloric acid (HCl), acetic acid (CH_3COOH), formic acid (HCOOH) and ammonium acetate (CH_3COONH_4) from Merck (Darmstadt, Germany) were used. Ultrapure water (H_2O) was produced by a Milli-Q Gradient System (Millipore, Brussels, Belgium). Oasis™ HLB 6 cc (200 mg) SPE extraction cartridges were purchased from Waters (Milford, MA, USA). Ultrafree®-MC centrifugal filter units (0.22 µm) from Millipore (Bedford, MA, USA) were used.

Standards of patulin, ochratoxins A and B, aflatoxins B1, B2, G1 and G2, citrinin, T-2 and HT-2 toxins, zearalenone, zearalanone, neosolaniol, diacetoxyscirpenol, sterigmatocystin, nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, fumonisins B1 and B2, beauvericin, alternariol monomethyl ether were purchased from Sigma-Aldrich. 3-Acetyl-deoxynivalenol was supplied as a 100 µg/mL solution in ACN. Regarding the other compounds, ready-made solid portions of commercial standards were dissolved in MeOH (in ACN:H_2O 50:50, v/v for fumonisins) to reach the concentration of 1 mg/mL. Stock solutions were stored at - 20 °C (at 4 °C for fumonisins). Tuning solutions of the mycotoxins were prepared at a concentration of 10 µg/mL. MS^n experiments and accurate mass measurements were performed on a 50 µg/mL solution of patulin in H_2O:0.2 M NH_4HCO_3 (pH10):MeOH (30:5:65, v/v/v) utilizing the ion trap and Orbitrap MS, respectively.

2.2. MS conditions

2.2.1. Ionization efficiency of patulin and multi-mycotoxin analysis

A Micromass Quattro LC triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with ESI and APCI sources and Z-spray was used during the ionization efficiency experiments and subsequent multi-mycotoxin LC-MS/MS method development. In the final method ESI was used in the positive mode. The optimized MS parameters were as follows: cone nitrogen gas flow 100 L/h, desolvation gas flow 830 L/h, capillary voltage 3.5 kV, source block

temperature 120 °C, desolvation temperature 320 °C, multiplier 700 V. The instrument was controlled by MassLynx™ 3.5 software (Micromass, Manchester, UK).

To evaluate the effects of mobile phase additives on the ionization efficiency, solutions of patulin were introduced into the MS by infusion: the solution of patulin coming from a syringe at a flow rate of 10 µL/min was mixed with the mobile phase (0.15 mL/min) through a T-shaped connector. The mobile phases (MPs) tested during the ionization efficiency experiments are listed in Table 1.

For the multi-analyte LC-MS/MS method, the selected reaction monitoring (SRM) mode of acquisition was chosen. The optimized MS parameters and the SRM transitions selected for each of the analytes are given in Supplemental Table 1.

2.2.2. Fragmentation behaviour

An LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San José, USA) equipped with an ESI source was used for the investigation of patulin fragmentation behaviour. The solution of patulin was infused into the MS as described in section 2.2.1. The set MS parameters were: source voltage 2 kV, vaporizer temperature 80 °C, capillary temperature 100 °C, sheath gas flow rate 10 arbitrary units (a.u.). Nitrogen was used as sheath gas, while helium was used as damping and collision gas in the ion trap. The maximum inject time was 200 ms and the number of microscans per scan was 3. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different collision energy (CE) levels to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

2.2.3. Accurate mass measurement

Accurate mass measurements of the patulin precursor and fragment ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with an ESI interface. The MS parameters were the following: spray voltage 4 kV, capillary temperature 150 °C, heater temperature

100 °C, sheath gas flow rate 30 a.u., auxiliary gas flow rate 5 a.u. The data were processed using the Xcalibur™ 2.1 and Exactive Tune software (Thermo Fisher Scientific). The instrument was operated in full scan positive mode with a resolution of 100,000 FWHM (Full Width at Half Maximum). The maximum injection time was 200 ms and the number of microscans per scan was 1.

Each full scan was followed by a same-polarity "all ion fragmentation" higher energy collisional dissociation (HCD) scan. The patulin solution was infused into the Orbitrap MS as described in section 2.2.1.

2.3. LC conditions

For the introduction of the patulin tuning solution into the ion trap and Orbitrap MS, an Accela™ High Speed LC (UHPLC) (Thermo Fisher Scientific) was used.

For the multi-mycotoxin analysis, the chromatographic separation was achieved on a Waters Alliance 2595 XE HPLC (Waters, Milford, MA, USA) using an XBridge™ C18 column (3.5 µm, 150 mm x 2.1 mm i.d.) (Waters). The mobile phase consisted of H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (85:5:10, v/v/v) [solvent A] and H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (5:5:90, v/v/v) [solvent B]. Two different gradient elution programs were applied for the analyses of either a limited (gradient I) or an extended (gradient II) number of analytes. They were as follows: gradient I: 0-2 min: 50-75 % B, 2-7 min: 75-85 % B, 7-9.5 min: 85-90 % B, 9.5-10 min: 90-50 % B, 10-18 min: 50 % B; and gradient II: 0-4 min: 35-50 % B, 4-7.5 min: 50-60 % B, 7.5-9 min: 60-75 % B, 9-14 min: 75-85 % B, 14-16 min: 85-100 % B, 16-19 min: 100 % B, 19-20 min: 100-35 % B, 20-30 min: 35 % B. The flow rate was 0.15 mL/min. The column temperature was set at 30 °C and temperature of the autosampler was 4 °C. Twenty µL of the sample were injected.

2.4. Clean-up of transdermal samples

The samples (1 % BSA in PBS) were cleaned-up using Oasis HLB™ SPE cartridges. Firstly, the SPE cartridges were conditioned with 10 mL of DCM:MeOH (80:20, v/v) containing 50 mM HCOOH, followed by 5 mL MeOH, 20 mL H_2O containing 10 mM HCl and 10 mL H_2O. After the conditioning step, 200 µL of the sample was quantitatively brought onto the SPE cartridge and washed with 10 mL of H_2O. Elution of

mycotoxins was achieved by passing 5 mL MeOH. The eluate was collected into glass test tubes and evaporated under a gentle stream of nitrogen at 40 °C and reconstituted with 100 µL of injection solvent H_2O:0.2 M NH_4HCO_3 (pH 8.5):MeOH (69:5:26, v/v/v). The resulting solution was transferred into an Ultrafree®-MC centrifugal device and centrifuged for 5 min at 14000 g prior to LC-MS/MS analysis.

2.5. Evaluation of the multi-analyte LC-ESI$^+$-MS/MS method

For the multi-analyte method development and evaluation, artificially fortified 1 % BSA in PBS samples were analyzed. Sensitivity of the method was characterized by LOD and was determined in spiked samples as a concentration corresponding to a peak of the analyte with a signal-to-noise ratio (S/N) of at least 3. Linearity of the method was evaluated by triplicate analysis of spiked samples at eight different concentrations by fitting a linear model. Apparent recovery was calculated at three concentration levels as a ratio between the actual and the theoretical concentration of analyte in spiked samples. The precision was expressed as relative standard deviation (RSD) of replicate measurements.

3. Results and discussion

3.1. Optimization of patulin MS signal

A mobile phase consisting of water, methanol, acetic acid and ammonium acetate was previously used for the LC-MS/MS analysis of a wide range of mycotoxins [18, 19]. Under these conditions a very poor MS signal was obtained for patulin. Furthermore, the signal was not useful in subsequent collisionally-induced dissociation (CID) experiments. In this study, the ionization efficiency of a broader range of solvents and mobile phase additives was thoroughly investigated (Table 1).

In preliminary experiments, ESI and APCI interfaces both in positive and negative modes were examined. Comparing the full MS spectra that resulted in the highest precursor ion signal for each combination of ionization mode and solvent, ESI^+ offered the best prospect for further investigation (Fig. 1). In some previous studies a better ESI^- or $APCI^-$ signal was reported [14, 15]. However, a limited number of mobile phases was investigated. Furthermore, the formation of precursor ions other than the protonated or the deprotonated molecule was not considered.

A mobile phase consisting of methanol, water and ammonium bicarbonate buffer at pH 10 (MP7) resulted in the highest signal (Fig. 1). The most abundant ions were at *m/z* 187 and 169 (Fig. 2) which were attributed to a protonated methanol-adduct and its dehydrated product, respectively (these ions were observed only with a methanol-containing mobile phase).

The prospect of this mobile phase for ESI^+-MS/MS was more apparent when MS^2 experiments were executed using the precursor ions that gave the best full MS signal with each of the mobile phases investigated (Supplemental Fig. 1). This mobile phase was therefore selected for further optimization of the MS signal by experimental design, resulting in the MS parameter settings shown in Supplemental Table 1.

3.2. Confirmation of the methanol adduct of patulin (m/z 187)

A study of the fragmentation behaviour of the ion at *m/z* 187, performed by combining MS^n experiments (ion trap) and accurate mass measurements (Orbitrap) (Supplemental Fig. 2), resulted in the fragmentation pathway shown in Fig. 3; thereby confirming the formation of a methanol adduct of patulin.

3.3. Applicability in LC-MS/MS multi-mycotoxin analysis

The usefulness of the selected ionization approach was demonstrated by the development of an LC-MS/MS multi-analyte method for the simultaneous determination of patulin and other mycotoxins. XBridge C18 stationary phase was chosen, as it has previously shown excellent pH stability [22]. The SRM ESI^+ chromatograms obtained by analyzing a mixture of 23 mycotoxins are shown in Supplemental Fig. 3. Although a baseline separation was not achieved for all adjacent peaks, this was not considered as a problem due to different SRM transitions.

With the prospect of analyzing mycotoxins in the framework of an *ex vivo-in vitro* skin penetration study, the method was refined to encompass seven relevant mycotoxins with toxicological and/or botanical importance, namely patulin, fumonisin B1, ochratoxin A, aflatoxin B1, citrinin, zearalenone and T-2 toxin. An SPE clean-up procedure using Oasis™ HLB cartridges was optimized, resulting in recoveries in the range from 67 % (citrinin) to 95 % (T-2 toxin).

Evaluation of the method (Table 2) demonstrated its fit for the detection of the seven targeted mycotoxins in a model transdermal penetration study. LODs were in the range from 1 to 10 ng/mL sample. The suitability the proposed method was also checked by comparing the LODs (Supplemental Table 2) achieved for a set of 23 targeted analytes in standard solutions with those reported for existing multi-mycotoxin methods. The current method proved to be sensitive. Remarkably, the LODs of patulin (0.2 ng on column), zearalenone (0.2 ng), zearalanone (0.05 ng), neosolaniol (2 ng), HT-2 toxin (0.15 ng), deoxynivalenol (0.8 ng) and nivalenol (6 ng) in standard solution were 1.2 – 100-fold lower than those of some methods [15, 19, 23, 24]. Other studies, demonstrated, however, lower LODs for patulin (0.0125 ng on column) [25] and fumonisins (0.001-0.005 ng) [26]. Overall, the LODs achieved with the developed method met the purpose of multi-mycotoxin analysis.

The linearity of the method was supported by the high values of regression coefficients r and lack-of-fit *p*-values above 0.05. The values of the standard error (S_{yx}) were all below 5 % of the estimate, even at low concentrations of the analytes. This feature of method performance is of a high value since some researchers experienced a non-linear signal of patulin and therefore had to omit this mycotoxin from the analysis [17]. The trueness, referred to as apparent recovery [27], was evaluated by recovery experiments from spiked samples since material with certified concentrations of mycotoxins was not available for intended study. Apparent recoveries for all the analytes were from 89 to 117 % and precision at low concentration levels did not exceed 22 %. These data were considered as appropriate and in good agreement with the Commission Decision 2002/657/EC performance criteria for quantitative methods of analysis [28].

The use of a triple quadrupole (two SRM transitions monitored for each analyte) provided high selectivity and specificity. The specificity of the method was further confirmed through the analysis of blank samples that were passed through the skin obtained from different donors. For each of the SRM transitions, no peak with a signal-to-noise ratio of at least 3 was detected at the expected retention time.

Further support of the suitability of the method for its intended use resides in the application of the method in 'real-world' samples from an *in vitro* skin penetration study using diffusion cells. The method allowed to measure simultaneously and in a relatively short time seven selected model mycotoxins (including patulin) not only in the receptor compartment of the diffusion cell, but also in the donor chamber and in the skin, thereby enabling to assess the recovery of the mycotoxins in the mass balance. Description of these data can be found in separate paper [21].

4. Conclusions

This study is the first report demonstrating an intense and stable MS signal of patulin in ESI^+ mode by formation of a protonated methanol-adduct using a methanol-containing mobile phase under alkaline conditions. The use of high resolution Orbitrap MS in combination with MS^n experiments unambiguously confirmed the authenticity of this adduct and its fragmentation pattern. Under the applied conditions other mycotoxins could also be ionized and gave satisfactory MS signals, thereby allowing inclusion of patulin in multi-mycotoxin LC-MS/MS methods. The usefulness of these findings was clearly demonstrated by the development of a sensitive, linear and accurate LC-ESI^+-MS/MS method in support of transdermal penetration experiments for selected mycotoxins with prior SPE on Oasis™ HLB cartridges.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Figures

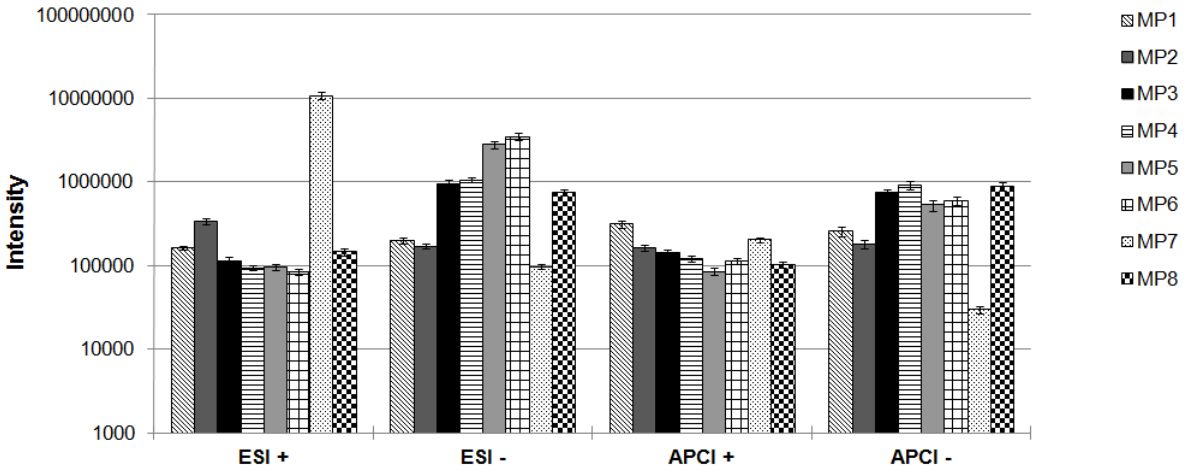

Fig. 1. Intensity of patulin MS signal using different mobile phases in ESI and APCI modes. The monitored precursor ions in ESI^+ were at *m/z* 155 (**MP1**-**MP6** and **MP8**) and *m/z* 169 (**MP7**), in ESI^- - at *m/z* 153 (**MP1**-**MP8**), in $APCI^+$ - at *m/z* 155 (**MP1**-**MP8**), in $APCI^-$ - at *m/z* 153 (**MP1**-**MP8**).

The mobile phase compositions are as described in Table 1.

For visibility reasons a logarithmic scale was applied.

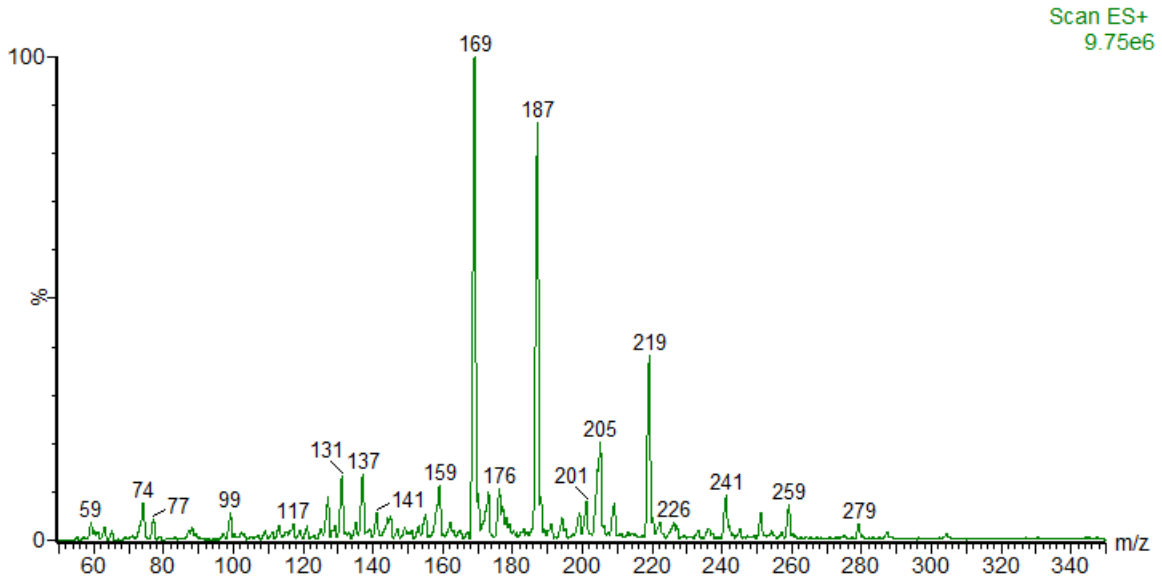

Fig. 2. A typical full MS spectrum of patulin in ESI^+ in H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (30:5:65, v/v/v).

The spectrum was acquired utilizing the triple quadrupole MS (see section 2.2.1).

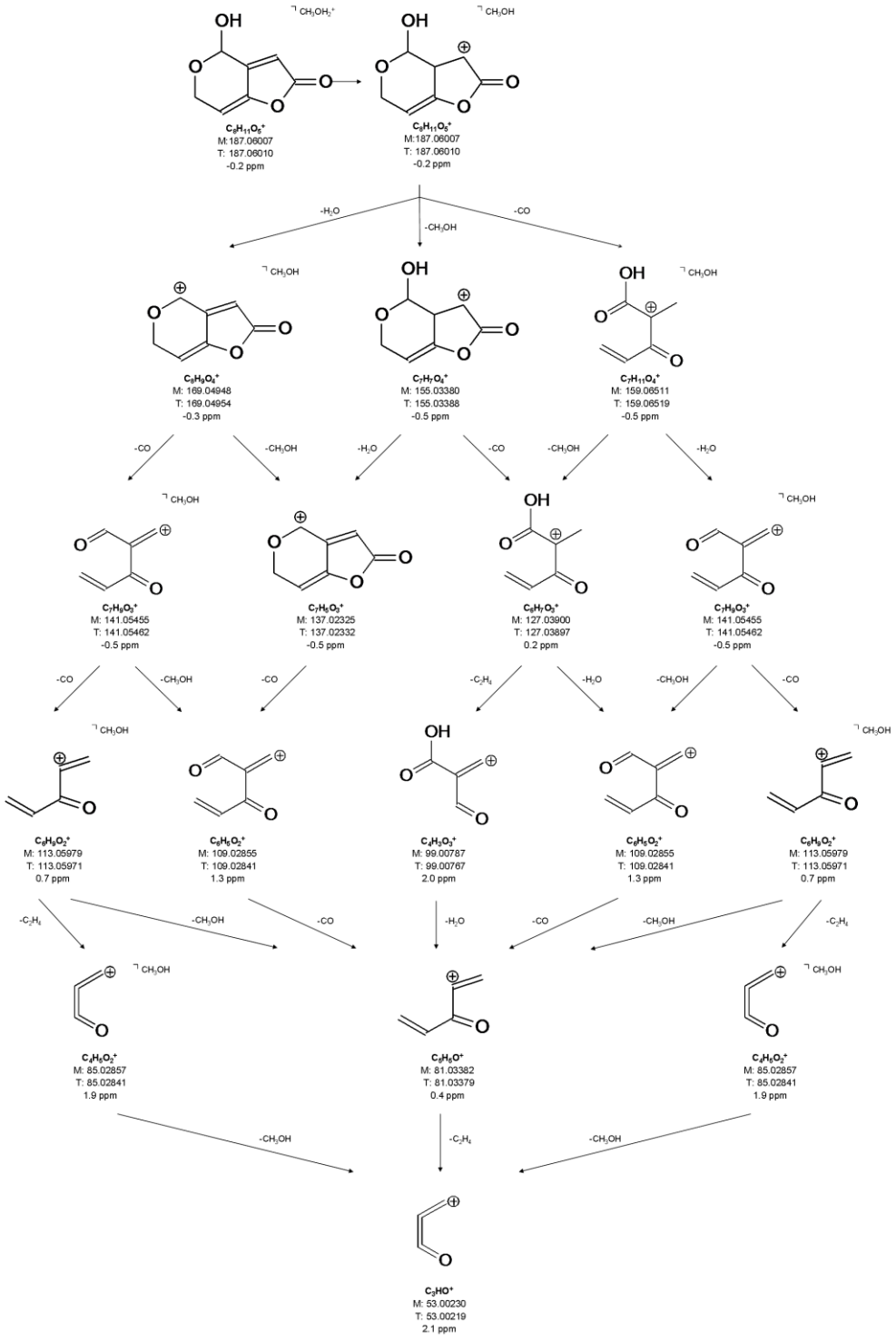

Fig. 3. Proposed fragmentation behaviour of patulin starting from its methanol adduct (*m/z* 187).

The fragment at *m/z* 159 can alternatively undergo successive losses of carbon dioxide (-44 Da), carbon oxide (-28 Da) and methanol (-32 Da) to yield an ion with *m/z* 55. This ion can also be generated on one hand from the fragment at *m/z* 99 through a loss of carbon dioxide (-44 Da); and on the other hand from

the fragment at *m/z* 155 through successive losses of carbon dioxide (-44 Da), carbon oxide (-28 Da) and ethylene (-28 Da).

The ion at *m/z* 53 can also be generated from the fragment at *m/z* 99 through a primary loss of carbon oxide (-28 Da), followed by a loss of water (-18 Da).

The assignment of fragments was supported by the Mass Frontier™ software.

Tables

Table 1. Mobile phases (MPs) used in the ionization efficiency experiments

	Mobile phase composition
MP1	H_2O:MeOH (30:70, v/v) containing 0.1% HCOOH
MP2	H_2O:ACN (30:70, v/v) containing 0.1% HCOOH
MP3	H_2O:MeOH (30:70, v/v) containing 1% CH_3COOH and 5 mM CH_3COONH_4
MP4	H_2O:ACN (30:70, v/v) containing 1% CH_3COOH and 5 mM CH_3COONH_4
MP5	H_2O:MeOH (30:70, v/v) containing 5 mM CH_3COONH_4 (pH 6.8)
MP6	H_2O:ACN (30:70, v/v) containing 5 mM CH_3COONH_4 (pH 6.8)
MP7	H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (30:5:65, v/v/v)
MP8	H_2O:0.2 M NH_4HCO_3 (pH 10):ACN (30:5:65, v/v/v)

Table 2. Evaluation of the LC-ESI$^+$-MS/MS method in support of a transdermal mycotoxin penetration study

Mycotoxin	Concentration level (ng/mL)	Apparent recovery (%)	Precision (RSD; n=6)	Linearity							
				LOD (ng/mL)	Concentration range (ng/mL)	Regression equation	r	S_{yx}	*p* -value	n_c	n_i
Patulin	Low (10)	114	22	5	10 - 250	y=0.0042x+0.0893	0.959	0.00043	0.945		
	Medium (50)	111	11								
	High (250)	104	7								
Citrinin	Low (2)	117	10	1	2 - 50	y=0.0929x+0.0162	0.997	0.00140	0.440		
	Medium (10)	93	9								
	High (50)	101	11								
Fumonisin B1	Low (20)	98	20	10	20 - 500	y=0.0031x+0.0380	0.968	0.00016	0.574		
	Medium (100)	89	16								
	High (500)	98	11								
Aflatoxin B1	Low (2)	110	21	1	2 - 50	y=0.0384x-0.0018	0.980	0.00155	0.500	8	3
	Medium (10)	104	13								
	High (50)	104	10								
Ochratoxin A	Low (10)	109	10	5	10 - 250	y=0.0088x-0.0162	0.990	0.00021	0.998		
	Medium (50)	103	14								
	High (250)	101	9								
Zearalenone	Low (20)	107	18	10	20 - 500	y=0.0025x+0.0151	0.996	0.00004	0.987		
	Medium (100)	108	7								
	High (500)	97	11								
T2-toxin	Low (5)	110	11	2.5	5 - 125	y=0.0131x+0.0018	0.994	0.00025	0.497		
	Medium (25)	96	13								
	High (125)	106	11								

n_c - number of concentration points

n_i - number of repetitions per concentration point