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Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography–mass spectrometry

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Abstract

New LC–MS (full scan) and LC–MS–MS (selected ion reaction monitoring) methods for the simultaneous determination of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A, and other *Penicillium* derived mycotoxins in food and feed samples are described. The methodologies involve sample extraction with acetonitrile–water, defatting with hexane and quantification using LC–MS with atmospheric pressure chemical ionisation or LC–MS–MS. Detector responses, for each of the methods and mycotoxins, were found to be linear over the range 10–1000 ng of mycotoxin/g of extracted food mixture material. The mean recoveries ($n=3$ to 6) of the mycotoxins from spiked food mixture samples determined using MS and MS–MS detection were 87–116 and 91–112%, respectively, for mycophenolic acid, 104–109 and 91–112%, respectively, for griseofulvin, 70–85 and 75–110%, respectively, for roquefortine C, 94–109 and 81–116%, respectively, for chaetoglobosin B, 110–115 and 90–106%, respectively, for verruculogen and 78–97 and 99–108%, respectively, for penitrem A. RSDs varied from 5.6% at the 1000 ng/g level to 23.1% at the 10 ng/g level. The limits of detection for the mycotoxins using MS and MS–MS were 70 and 10 ng/g, respectively, for mycophenolic acid, 10 and 5 ng/g, respectively, for griseofulvin, 50 and 20 ng/g, respectively, for roquefortine C, 25 and 20 ng/g, respectively, for chaetoglobosin B, 25 and 20 ng/g, respectively, for verruculogen and 10 and 5 ng/g, respectively, for penitrem A.

Keywords: *Penicillium*; Food analysis; Vegetables; Fruits; Cheese; Meat; Mycotoxins

1. Introduction

Mycotoxins from *Penicillium* species can be a serious contamination problem in poorly stored foods and feeds. The toxins are naturally produced by a variety of *Penicillium* species, which can be formed rapidly during transportation and storage. Because of the toxicity of many *Penicillium* mycotoxins [1], it is desirable that a rapid and reliable analytical procedure is available to monitor mycotoxin levels in foodstuffs. Several methods for analysing *Penicillium* mycotoxins have been developed. Due to the high polarity of many of the mycotoxins, high-performance liquid chromatography (HPLC) has been the preferred separation technique. When combined with UV or fluorescence detection, many of the important, commonly encountered, *Penicillium* toxins can been determined [2–7]. These methods,

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however, often include time consuming clean up steps and seldom more than one or two mycotoxins can be determined in the same analysis. Multi-toxin methods have also been developed [8–15], but these methods often suffer from low sensitivity or specificity and are mainly applicable to simple matrices such as fungal isolates grown on agar or rice.

The combination of LC and mass spectrometry (MS) simplifies the development of analytical methods for polar mycotoxins. With electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces, mainly MH^+ ions are formed. Unequivocal identification of target mycotoxins can be achieved, using single quadrupole or ion trap LC–MS systems by monitoring the diagnostic ion fragmentation patterns obtained by collision induced dissociation (CID). Higher selectivity and sensitivity can, however, be achieved using LC–MS–MS, especially when analysing samples which exhibit complex interferences. LC–MS–MS methods can discriminate different analytes by combinations of chromatographic, parent ion (MS) and product ion(s) (MS–MS) data.

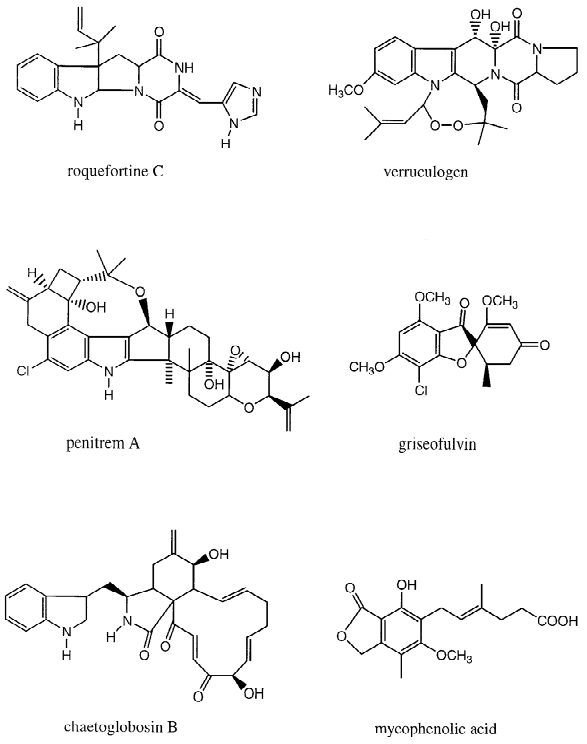

Fig. 1. Chemical structures of some *Penicillium* mycotoxins.

The concentrations of *Penicillium* mycotoxins in both food and feed can span from zero up to high μg/kg or mg/kg levels in cases of mouldy feed intoxication of animals. Levels depend on the species of fungi, growth conditions and storage time. The toxicity of *Penicillium* mycotoxins necessitates low detection limits. For example, in the European Union (EU), the legally acceptable concentration of ochratoxin A in grain for human consumption is set at 5 ng/g [16].

This paper reports the development of a rapid and highly selective LC–MS and LC–MS–MS methods for the simultaneous determination of roquefortine C, griseofulvin, mycophenolic acid, ochratoxin A, verruculogen, chaetoglobosin B, penitrem A, citrinin, rubratoxin B, cyclopiazonic acid, PR-toxin, patulin and penicillic acid. Detailed validation of the methodologies was performed using roquefortine C, griseofulvin, mycophenolic acid, verruculogen, chaetoglobosin B and penitrem A (Fig. 1).

2. Experimental

2.1. Chemicals

Methanol, ethyl acetate, dichloromethane, acetonitrile, hexane, ammonium acetate, ammonium formate, acetic acid and formic acid were HPLC or analytical-reagent grade and obtained from Rathburn (Walkerburn, UK). Deuterated T-2 toxin, roquefortine C, griseofulvin, mycophenolic acid, citrinin, rubratoxin B, cyclopiazonic acid, PR-toxin, patulin and penicillic acid, ochratoxin A, verruculogen, chaetoglobosin B and penitrem A were purchased from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

A blank food mixture (1 kg) was produced from bread (300 g), cooked rice (200 g), cooked pasta (200 g), cooked potatoes (150 g), vegetables (30 g), fruits (30 g), cheese (10 g), salami (10 g), used filter

coffee (50 g) and minced meat (50 g). All components were fresh from a local grocery store. Water (100 ml) was added, the mixture was homogenised using a H2 Handy mixer (Wodschow, Brøndby, Denmark), and the homogenised material was freeze–dried.

2.3. Fortification

Ten-gram portions of the blank food mixture were spiked with 100, 500, 1000, 5000, 10 000 ng ($n=3$) of each of roquefortine C, griseofulvin, mycophenolic acid, verruculogen, chaetoglobosin B and penitrem A, to give concentrations of 10, 50, 100, 500 and 1000 ng of each toxin/g sample.

2.4. Extraction and clean up

Freeze–dried food mixture samples (10 g) were extracted for 1 h on an automatic shaker using a mixture of acetonitrile–water containing 0.1% formic acid (9:1, v/v) (60 ml) and hexane (50 ml). After filtration (Schleicher and Schuell 520 B folded filters), a portion of the acetonitrile extract (6 ml) was evaporated to dryness at 60 °C under a gentle stream of nitrogen. The residue was dissolved in methanol (350 μl), 0.1% acetic acid (150 μl) was added and the resulting solution was filtered using a Spin-X micro centrifuge 0.2 μm nylon filter (Costar, Corning, NY, USA). Filtrates were analysed using the LC–MS and LC–MS–MS methods described below.

2.5. LC–MS and LC–MS–MS conditions

Liquid chromatography was performed on a Symmetry C_{18} column (5 μm, 150×4.6 mm) (Waters, Milford, MA, USA), using a Model P4000 pump and a Model AS3000 autosampler (TSP, San Jose, CA, USA). Separation was achieved using gradient elution starting with methanol–water (40:60, v/v, both containing 0.05 *M* ammonium acetate), rising to methanol–water (95:5, v/v) over 15 min. Isocratic elution with 95% methanol was maintained for 10 min before the eluent was switched to 40% methanol. The HPLC system was coupled to a LCQ ion trap mass spectrometer operating with an ESI or an APCI interface (Finningan MAT, San Jose, CA, USA). The MS system was operated in either MS full scan mode or MS–MS selected ion reaction monitoring (SRM) mode. The ion injection time was set to 300 ms with a total of three micro-scans. For APCI, a vaporisation temperature of 350 °C, a sheath gas rate of 25 units nitrogen (approximately 250 ml/min), an auxiliary gas rate of 5 units nitrogen (approximately 50 ml/min), a corona discharge voltage of 4.5 V and a heated capillary temperature of 200 °C were used. Typical ESI parameters were a spray voltage of 4 kV, a sheath gas rate of 70 units nitrogen (approximately 700 ml/min) and an auxiliary gas rate of 10 units nitrogen (approximately 100 ml/min). Ionisation and MS–MS collision energy settings were optimised while continuously infusing (syringe pump) 10 μg/ml of each the toxins, dissolved in methanol, at a flow-rate of 5 μl/min. The values giving the highest sensitivity for roquefortine C were typically in the following ranges; capillary voltage of 30 to 40 V and a tube lens offset of 0 to 10 V. Optimisation of ionisation parameters was performed immediately prior to the commencement of analyses. Quantification was performed using external calibration curves. Eight levels corresponding to 5, 10, 25, 50, 100, 250, 500, 1000 ng/g in the food mixture were prepared in methanol–water (60:40, v/v) and in cleaned up blank food mixture extracts. Deuterated T-2 toxin was used as internal standard and an amount corresponding to a concentration of 100 ng/g of food sample was added to all sample extracts prior to the evaporation stage. Calibration curves for each compound were constructed by plotting the amount (ng/g) vs. the relative area of the analyte (=area of analyte/area of internal standard).

3. Results and discussion

3.1. LC–MS analysis

An investigation of the positive and negative ion mass spectral characteristics of a series of *Penicillium* mycotoxins including, roquefortine C, griseo-

fulvin, mycophenolic acid, ochratoxin A, verruculogen, chaetoglobosin B, penitrem A, citrinin, rubratoxin B, cyclopiazonic acid, PR-toxin, patulin and penicillic acid, lead to the development and subsequent optimisation and validation of LC–MS and LC–MS–MS methodologies for the determination of *Penicillium* mycotoxins in a mixed food matrix. The ionisation efficiency, and hence sensitivity, for most of the mycotoxins were found to be interface dependent, at least for the mobile phase conditions that were investigated.

The use of an APCI inlet resulted mainly in the expected MH^+ ions of target species. However mycophenolic acid afforded an $(M+18)^+$ ion while chaetoglobosin B afforded a $(M+17)^+$ ion (attributable to MNH_4^+ and MOH^+ ions, respectively), in addition to the expected MH^+ ions. Some of the toxins showed strong fragmentation arising from the loss of a water molecule; i.e., $(MH-H_2O)^+$ ions. This is likely to be a consequence, at least partly, of thermally induced dehydration of the vaporising mycotoxin during its passage through the APCI evaporator stage, which was maintained at 350 °C.

In the positive ion APCI-MS mode, verruculogen (MH^+, m/z 512) predominantly afforded an m/z 494 ion corresponding to loss of H_2O. The mass spectrum of penitrem A (MH^+ m/z 634) also included a strong m/z 616 fragment ion, (ca. 33% of the MH^+ ion intensity) attributable to water loss from the MH^+ ion. This tendency, to produce more than one type of ion, lowered the sensitivity of the MS–MS detection methodology, which in the MS–MS stage detected ions derived from the MH^+ ion. Similarly, the tendency of mycophenolic acid and chaetoglobosin B to form higher mass M_2H^+ or M_2Na^+ addition (cluster) ions reduced the MH^+ ion currents of these compounds to 50–60% of the total ion current. The greater the tendency to form higher mass addition ions and/or lower mass fragment ions, the greater the extent to which the sensitivity of the MS–MS methodology was attenuated (i.e., the higher the detection limit).

The use of an ESI inlet, combined with positive ion detection, led to increased levels of MNa^+, MNH_4^+ (with ammonia buffer), MK^+, M_2H^+, M_2Na^+ and M_2K^+ ions together with the expected MH^+ ions. In the negative ion ESI mode, $M+COO^-$ and $M+CH_3COO^-$ ions were often seen in addition to $M-H^-$ ions. The relative levels of these ions varied with the choice of buffer, the amounts of salts in the sample matrix and residual memory effects arising from the gradual desorption of cations or anions from MS surfaces. The use of the ESI inlet also increased uncertainty in quantitative analyses, due to increased variability in ion current characteristics.

Comparison of results from positive and negative ion ESI, and APCI showed that penitrem A gave 10–20-times better ionisation with positive ion APCI compared to positive ion ESI, while the reverse was observed for ochratoxin A and cyclopiazonic acid. Roquefortine C, griseofulvin, citrinin, chaetoglobosin B and verruculogen had approximately the same sensitivity with both interfaces in the positive ion mode. Patulin and penicillic acid had very low ionisation with both the ESI and the APCI interfaces, while PR-toxin suffered from poor chromatography.

The use of formic acid or ammonium formate buffer solutions (in place of ammonium acetate) did not result in any significant differences in mass spectral sensitivity (ion currents). The retention times of ochratoxin A and mycophenolic acid however, varied greatly with pH. The use of formic acid or ammonium formate did not alter the tendency to form elimination, addition, or cluster ions, in addition to MH^+ ions. Rubratoxin B has been reported to undergo structural change in the presence of water [17]. Evidence for structural change was also observed in this investigation. However, none of the ions observed in positive ion ESI- or APCI-MS spectra could be attributed to modified forms of rubratoxin B. Because of these difficulties, and its relatively low importance, rubratoxin B was excluded from the methodology.

The chromatographic peaks of other *Penicillium* mycotoxins were mostly well resolved (Fig. 2), provided a prolonged methanol–water gradient was used. After each LC analysis it was necessary to wash the LC column with 95% methanol for 10 min, in order to wash out non-polar components (routinely present in food extracts) from the HPLC column, and to suppress the tendency for the baseline (background) signal to rise during the course of a series of analyses. The recoveries for ochratoxin A, citrinin

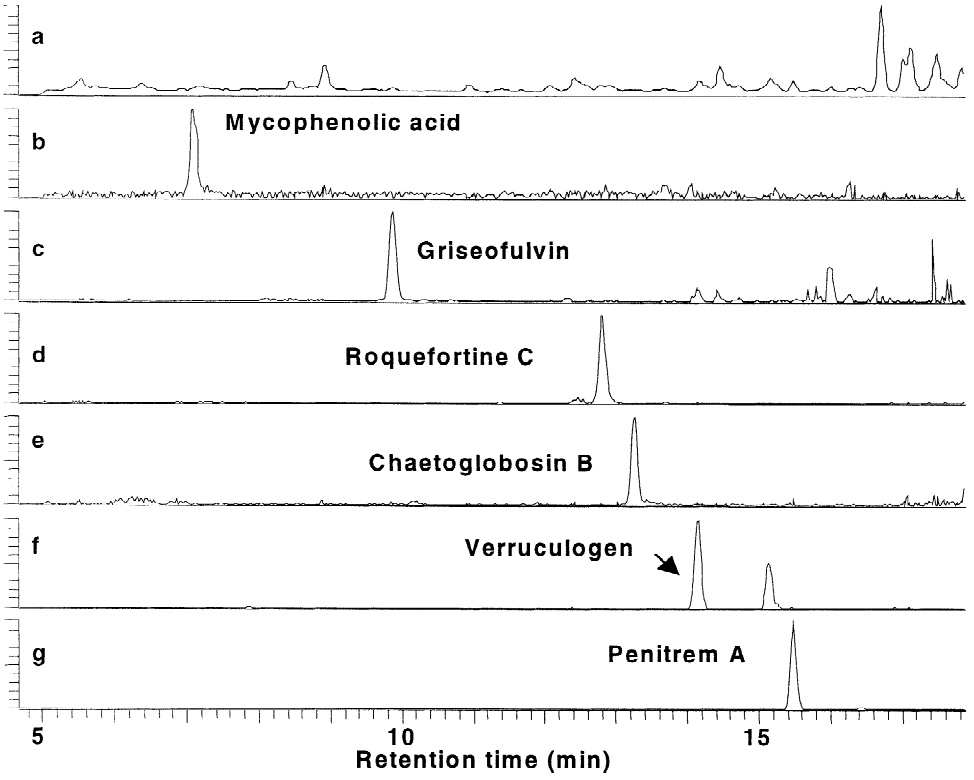

Fig. 2. Total and single ion LC–MS chromatograms of a spiked (100 ng/g) food mixture. Upper to lower traces: (a) TIC trace of the food mixture extract, (b) mycophenolic acid (m/z 321), (c) griseofulvin (m/z 325), (d) roquefortine C (m/z 390), (e) chaetoglobosin B (m/z 456), (f) verruculogen (m/z 494) and (g) penitrem A (m/z 634).

and cyclopiazonic acid from the sample matrix with the chosen conditions were low (10–30%), hence they could not be reliably quantified using the developed method.

The peak heights and areas determined for the mycotoxins were found to be dependent on the sample matrix. Calibration curves (profiles) determined for penitrem A in the MS–MS mode for standard solutions in a methanol–water mixture and for equivalent levels of penitrem A in a food mixture extract are presented in Fig. 3. Peak height and peak area reductions of up to 40% were observed in the latter matrices. Components present in these matrices appear to suppress the ionisation of mycotoxin species. Mol et al. [18] have previously reported similar changes (reductions due to ionisation suppression) in the slope and linearity due to matrix effects. A 12% decrease in the slope of the calibration curve for daminozide in apple leaves was observed when standard solutions were spiked into a sample matrix, compared to standards prepared in water. Similarly, Ito and Tsukada [19] found 13–

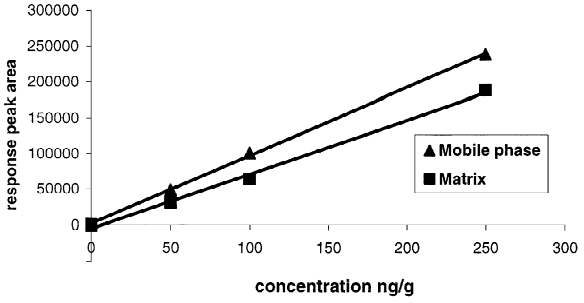

Fig. 3. MS–MS-SRM calibration curves determined for penitrem A in (▲) methanol–water (mobile phase) and (■) food mixture extracts (matrix standards) spiked with penitrem A.

Table 1
MS–MS method events, ions, and relative collision energies

Scan segment	Scan event	Mycotoxin	MS, MH^+ (m/z)	MS–MS-SRM (m/z)	Relative collision energy (%)
1		Mycophenolic acid	321	353	30
2		Griseofulvin	355	285	30
3		D_3-T-2 toxin (I.S.[a])	487	308	25
4	1	Roquefortine C	390	322	35
4	2	Verruculogen	494	410	35
4	3	Chaetoglobosin C	529	511	30
5		Penitrem A	634	558	30

[a] I.S.=Internal standard.

33% reductions in detector response when spiking diarrhetic shellfish poisoning (DST) into a shellfish matrix, compared to that obtained for standards prepared in methanol. The lesser reduction (12%) observed by Mol et al. [18], compared to the 20–40% reductions observed in this investigation, or by Ito and Tsukada [19], may be a consequence of the different extents to which the respective sets of extracts had been cleaned-up (defatted, etc.) prior to analyses. The ion suppression effects are also likely to be analyte and instrument dependent. These observations show that quantification should be performed using standards prepared in a matrix similar to the sample matrix.

Operation of a single quadrupole MS in the selected ion monitoring (SIM) mode results in an appreciable gain in sensitivity compared to the total ion current (TIC) mode. Since an ion trap MS does not possess the same ability to rapidly switch from one ion to another ion, there is little to be gained from SIM operation of an ion trap system. In this investigation, LC–MS data were acquired in the TIC mode, since this afforded additional mass spectral information. In particular, it allows for the detection during routine analyses of toxins other than the targeted *Penicillium* mycotoxins.

Unlike a single quadrupole MS, an ion trap MS can also be operated in MS–MS mode. This is a highly specific mode of detection, in which only a diagnostic fragment ion(s) derived from a selected parent ion (e.g., an MH^+ ion) is detected. This technique is often referred to as selected ion reaction monitoring (SRM). A significant limitation of MS–MS-SRM methods is that they can only be used to detect chosen analytes. On the other hand MS–MS operation offers better detection limits, especially for samples, which have only been subjected to limited clean up prior to analyses.

Deuterated T-2 toxin was selected as internal standard (I.S.). T-2 toxin possesses a polarity and a chromatographic retention time on LC columns similar to that of target *Penicillium* mycotoxins. A further consideration was that deuterated T-2 toxin would not occur naturally in any of the food mixture

Table 2
Limit of detection determined for standard solutions (ng/ml) and spiked food mixtures (ng/g) using the MS (full scan) and MS–MS (SRM) methodologies

	Standard solutions		Spiked food mixture extracts	
	MS (full scan)	MS–MS (SRM)	MS (full scan)	MS–MS (SRM)
Mycophenolic acid	10	5	70	10
Griseofulvin	5	1	10	5
Roquefortine C	5	5	50	20
Chaetoglobosin B	5	5	25	20
Verruculogen	5	5	25	20
Penitrem A	5	1	10	5

Table 3
Recovery of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A from spiked food mixtures determined using MS (full scan) detection

Level (ng/g)	n	Mycophenolic acid		Griseofulvin		Roquefortine C		Chaetoglobosin B		Verruculogen		Penitrem A	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10	6	–	–	104	9.9	–	–	–	–	–	–	78	12.8
50	3	88	16.5	116	2.7	77	15.4	98	9.8	112	5.8	97	8.2
100	6	87	8.7	115	10.8	83	10.0	94	4.5	109	6.9	86	10.4
500	6	102	8.4	119	6.8	85	1.9	109	7.3	115	5.6	93	5.4
1000	3	116	3.1	112	2.0	70	1.6	102	2.6	110	5.1	97	5.7

extracts or other samples to which the methodology was applied.

3.2. Method validation

The LC–MS method was validated using a scan mass range from m/z 300 to 700. Fig. 2 shows a LC–MS profile of a 100 ng/g spiked food mixture sample, acquired using TIC detection. Diagnostic ion responses (mainly MH^+ ions) were used to calculate mycotoxin levels. The LC–MS–MS method was validated using the ions and relative collision energies presented in Table 1. This method had lower detection and quantification limits than the full scan MS method (Table 2). The average recovery and repeatability of both of the detection methods were determined using fortified food mixture samples (Tables 3 and 4).

The recovery of deuterated T-2 toxin (I.S.) from the fortified samples, when spiked at 100 ng/g, was 93% (RSD 4.7% for 24 replicates). Instrument repeatability was determined for 10 replicate injections, using matrix assisted standard containing 100 ng/g of each of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A. The relative standard deviation (RSDs) of the peak areas determined for mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A using the MS method were 6.9, 4.8, 12.5, 5.7, 8.9 and 3.5%, respectively, compared to 2.9, 5.8, 12.5, 5.1, 8.9 and 2.5%, respectively, for the MS–MS method.

Calibration curves were determined using matrix assisted standard solutions for each of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A, using eight concentrations corresponding to levels of 5–1000 ng/g of the mycotoxins. There was a linear relationship between the peak area determined in LC–MS analyses and the concentration of each toxin over the range from 10–1000 to 75–1000 ng/g, depending on the toxin. The squared correlation coefficients (R^2)

Table 4
Recoveries of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A from spiked food mixtures determined using MS–MS (SRM) detection

Level (ng/g)	n	Mycophenolic acid		Griseofulvin		Roquefortine C		Chaetoglobosin B		Verruculogen		Penitrem A	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10	6	112	14.5	91	8.1	110	23.5	81	23.1	90	16.5	108	8.1
50	3	111	9.5	99	10.1	75	9.6	102	10.9	106	8.8	102	8.2
100	6	91	7.7	110	7.2	90	9.5	105	7.5	93	7.9	106	8.8
500	6	98	8.7	104	2.8	88	9.0	116	8.5	106	8.6	99	8.3
1000	3	101	7.8	112	0.6	93	5.7	99	5.6	101	6.3	105	6.3

for the eight-point calibration curves determined for the six mycotoxins were in the range of 0.985–0.990. In the LC–MS–MS mode there was a linear relationship between the peak areas determined for each of the mycotoxins over the range from 5–1000 to 20–1000 ng/g, depending on the mycotoxin. The squared correlation coefficients (R^2) for the eight-point calibration curves determined for the six mycotoxins were in the range of 0.980–0.999.

The limit of detections (LODs), based on a signal-to-noise ratio (S/N) of 3/1 for the MS (full scan) and MS–MS (SRM) methodologies are given in Table 3. LC–MS–MS detection was highly specific, and except for chaetoglobosin B, there was no background noise in the MS–MS profiles. In the MS–MS mode the LODs for standard solutions varied from 1 to 5 ng/g depending on the mass spectral ionisation characteristics of individual mycotoxins. The higher LODs (5–20 ng/g) determined for the six mycotoxins using spiked food samples can be attributed to matrix effects, which act to suppress mass spectral ion current.

The comparatively high MS (full scan) LOD determined for mycophenolic acid in spiked food mixture samples (70 ng/g) was primarily a consequence of weak ionisation, while that of roquefortine C (50 ng/g) was mainly attributable to a high background ion current. Since, in the MS–MS method, roquefortine C, verruculogen and cheatoglobosin were measured simultaneously, it was anticipated that their MS–MS LODs would be higher (20 ng/g in spiked food mixture) than those of mycophenolic acid, griseofulvin and penitrem A (10, 5 and 5 ng/g, respectively). On some occasions it was possible to detect <1 ng/g of penitrem A and griseofulvin using the MS–MS protocol, but at this level quantification was insecure and difficult due to the limited number of data points contributing to the detected signal.

The mean recoveries ($n=3$ to 6) of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A from spiked food mixture samples, determined using MS (full scan) detection, were in the range of 70–115% for concentrations of 10–1000 ng/g. RSDs varied from 1.6 to 16.5%. In general the lower the spike level, the greater the RSD.

The mean recoveries ($n=3$ to 6) of mycophenolic acid, griseofulvin, roquefortine, chaetoglobosin B, verruculogen and penitrem A from spiked food mixture samples, determined using MS–MS detection, were in the range 75–120% for concentrations of 10–1000 ng/g while RSDs varied from 5.6% at the 1000 ng/g level to 23.1% at the 10 ng/g level.

4. Conclusions

The methodologies reported here enable the determination of mycophenolic acid, griseofulvin, roquefortine, chaetoglobosin B, verruculogen, penitrem A and other *Penicillium* mycotoxins in food and feed, down to the same level (5 ng/g) as EU food safety regulations define for ochratoxin A in grain [16]. Full scan MS detection limits for mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen and penitrem A were 2–3.5 times higher (typically in the range 10–70 ng/g) than was the case for MS–MS detection (typically in the range 5–20 ng/g).

The LC–MS and LC–MS–MS methodologies utilised in this investigation are increasingly being applied to the analyses of other mycotoxins and it can be anticipated that because of their selectivity and sensitivity (especially in MS–MS mode) they will progressively replace conventional LC methods using UV or fluorescence detection.

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