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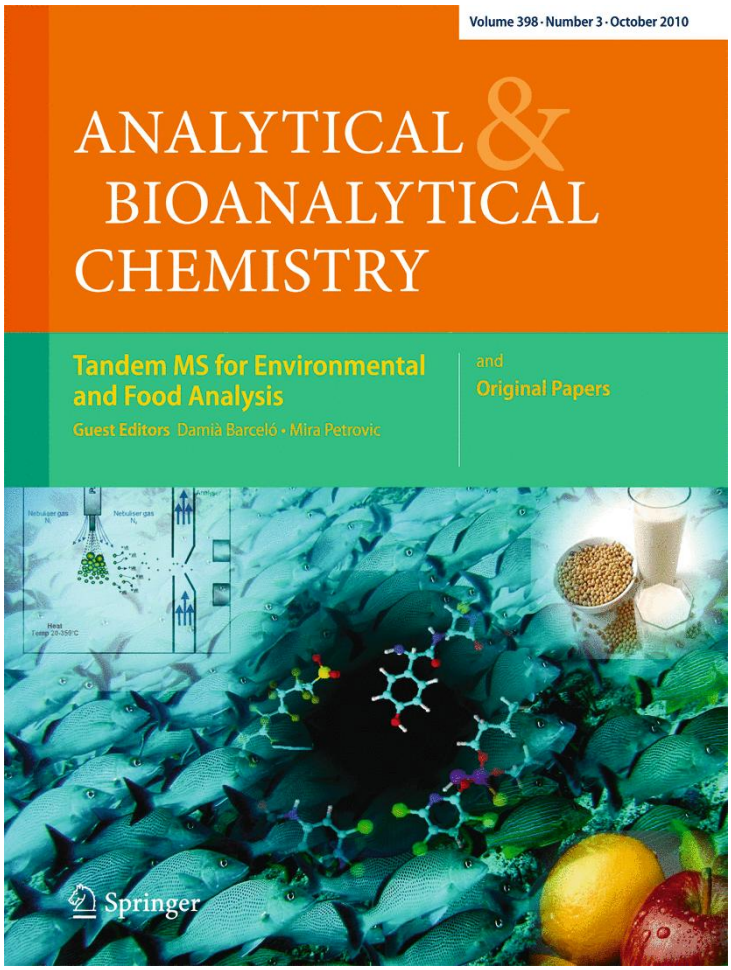

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ORIGINAL PAPER

Simultaneous determination of ochratoxin A, mycophenolic acid and fumonisin B_2 in meat products

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Abstract Here we present a method for simultaneous determination of the fungal metabolites mycophenolic acid, ochratoxin A (OTA) and fumonisin B_2 (FB_2) in meat products. Extraction was performed with water–acetonitrile, followed by acetone-induced precipitation of salts and proteins. Purification and identification of analytes was performed by mixed-mode reversed-phase anion-exchange chromatography in direct ion-exchange mode, followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) detection. Quantification was based on isotope dilution with fully ^{13}C-labelled FB_2 and OTA, and matrix-spiked calibration curves. Fermented sausages inoculated with an OTA- and FB_2-producing strain of *Aspergillus niger* were analysed, but no analytes were detected. Analysis of 22 retail products showed one Parma meat with a very high level of OTA contamination (56–158 μg/kg) that clearly exceeded the Italian regulatory limit of 1 μg/kg. This sample and uninfected control samples were subsequently reanalysed, and the high OTA content was verified by two other techniques: (i) LC–time-of-flight MS confirmed the accurate mass as well as chlorine isotope pattern; and (ii) sample methylation in methanol–BF_3 and subsequent LC-MS/MS provided indirect confirmation by detection of the OTA methyl ester. In the contaminated Parma ham, the high OTA level most likely originated from growth of *Penicillium nordicum* on the meat.

Keywords Filamentous fungi · Meat products · Mycotoxins · Isotope dilution · Mixed-mode reversed-phase anion exchange · LC-MS/MS

Introduction

Determination of mycotoxins in meat products is significantly more difficult than in cereal-based products because of the need to remove large amounts of small proteins, interfering peptides, phospholipids, and other interferences from the meat [1]. Therefore, multi-mycotoxin detection methods have not been developed for meat, since selective purification methods inevitably remove some mycotoxins.

Mycotoxins in meat products may originate from two sources: (i) carry-over from feed; or (ii) direct growth, usually on dried meat products. In pork products, ochratoxin A (OTA, Electronic supplementary material Fig. S1) is a major problem in Europe [2–5], where it originates from contaminated barley that is used to feed pigs. This leads to deposition of OTA, especially in the kidney and liver, but also in muscle tissue [6]. However, amounts rarely exceed 0.2 μg/kg, although levels up to 4 μg/kg have been reported when highly infected feed is used [2, 6–10].

The most common fungal contaminants of dried meat products are *Penicillium* species, with *P. brevicompactum*, *P. chrysogenum*, *P. solitum*, *P. palitans*, *P. nalgiovense* and *P. nordicum* being the most common [2, 11–15]. Of these, *P. nordicum* is the main health risk because of its high production of OTA [2, 5, 6, 8, 9, 16–18]. The presence of

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OTA in meat products is not yet regulated by the European Union, but a national regulation in Italy has set a maximum tolerated level of 1 μg/kg in pork and pork-derived meat products [19, 20].

Mycophenolic acid (MPA) produced by *P. brevicompactum* also poses a health problem, since it has immunosuppressive properties [21]. MPA was shown to be produced on meat products inoculated with *P. brevicompactum* [1], but retail meat products have not yet been analysed for MPA.

In warm climates such as south Europe and North Africa, *Aspergillus niger* can be isolated from air-dried meat products [22–26]. Up to 70–80% of strains of this contaminant produce fumonisins B_2, B_4 and B_6 [27–30] and, depending on the habitat, 0–40% produce OTA [29, 31–33]. *A. niger*-produced fumonisins are found naturally in green coffee [30], must [34] and vines [29]. Fumonisins B_1 and B_2 (FB_1 and FB_2) are mycotoxins with carcinogenic properties [35, 36] that have been associated with occurrence of oesophageal cancer in South Africa and China [37–40]. Prior to the recent discovery that *A. niger* produces fumonisins, these toxins were thought to be produced only by field-associated *Fusarium verticillioides* and related species. For this reason, the presence of fumonisins in meat products has never been studied, to our knowledge, even though it poses a potential health problem.

Most analytical methods for the determination of mycotoxins in meat products are based on immunoaffinity purification followed by high-performance liquid chromatography (HPLC) coupled to fluorescence detection [6, 8, 9, 17], but liquid chromatography–tandem mass spectrometry (LC-MS/MS) [41] and LC-time-of-flight (TOF) MS [1] have also been applied. Our literature review suggested that OTA, MPA and fumonisins could all be found in meat products, so we developed a method for simultaneous detection of these mycotoxins. The method used a purification strategy orthogonal to the reversed-phase LC-MS analysis [42]. Cation-exchange methods used for fumonisins [43] could not be used for OTA and MPA because functional group analysis revealed that OTA and MPA each has a single carboxylic acid and a single phenol. Fumonisins have one amine and four carboxylic acids so, theoretically, purification of all could be achieved with anion exchange. To avoid liberating phosphates when eluting with 1–2% formic acid, we elected to use a strong anion exchanger (SAX), with quaternary amine functionality [43]. We furthermore decided to test both a classic silica-based SAX column, and a reversed-phase mixed-mode anion-exchange (MAX) column, since we had previous success purifying fumonisins using mixed-mode cation exchange [43] and classic SAX [30], and isolating MPA using MAX [1, 42].

For quantification, we chose LC-MS/MS with isotope dilution since fully ^{13}C-labelled FB_2 and OTA are commercially available, and the structural similarities of OTA and MPA indicated that ^{13}C-OTA could work as an internal standard (IS) for MPA. Using this methodology, we analysed fermented sausages artificially inoculated with an FB_2- and OTA-producing *A. niger* strain, as well as a selection of retail products.

Materials and methods

Chemicals

Acetonitrile and methanol were gradient grade, from Sigma-Aldrich (St. Louis, MO, USA). Pentane, acetone, BF_3 diethyl etherate, ammonia, hydrochloric acid and acetic acid were analytical grade from Sigma-Aldrich. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). Fumonisin standards (a mixture of FB_1 and FB_2, both at 50 μg/ml), OTA (10 μg/ml), fully ^{13}C-labelled FB_2 ($^{13}C_{34}$-FB_2, 10 μg/ml) and OTA ($^{13}C_{20}$-OTA, 10 μg/ml) were purchased from Romer Labs (Tulln, Austria). Mycophenolic acid of 98% purity was from Sigma-Aldrich and dissolved in acetonitrile to a stock solution of 10.0 mg/ml. All further standard dilutions were in acetonitrile.

Extraction and purification

Whole raw meat or sausage samples (10–100 g) were homogenised in a blender and three sub-samples of 0.7 g were transferred to 15-ml Falcon tubes; one sample was analysed immediately and two were stored at −20 °C for confirmative analysis in the case of positive samples. To each sub-sample, 140 μl IS solution containing a mixture of 0.50 μg/ml ^{13}C-FB_2 and 1.00 μg/ml ^{13}C-OTA was added, followed by 4.5 ml water, 2.5 ml acetonitrile and 6 ml pentane. The tube was sealed and shaken vigorously for 1 h using a shaking table. After centrifugation for 10 min at 8,000×*g*, the upper (pentane) phase was discarded and 3.5 ml of the lower (water–acetonitrile) phase was transferred to a new tube containing 9 ml acetone. The sample was shaken briefly and after a second centrifugation for 10 min at 8,000×*g*, the upper 10 ml sample was collected. The solvent was evaporated to 1.2–1.5 ml at 45 °C under a nitrogen stream. In the case that precipitation was observed, 0.2–0.25 ml methanol was added, still keeping maximum concentration of organic solvent below 20%. Samples were transferred to 30-mg, 30-μm Oasis MAX cartridges (Waters, Milford, MA, USA) that were conditioned and equilibrated with 1 ml methanol and 1 ml water. Cartridges were sequentially washed with 1 ml 1%

ammonia in water, 1 ml methanol and 1 ml methanol/water/37% HCl (40:59:1). Elution was achieved with 2 ml methanol containing 2% (v/v) acetic acid, and the solvent was evaporated under nitrogen flow at 45 °C. Samples were re-dissolved in 200 μl acetonitrile/water (1:2) and stored at −20 °C until analysis.

Instrumental settings

LC-MS/MS was performed on an Agilent 1100 LC system (Waldbronn, Germany) equipped with a cooled autosampler, a 50 mm×2 mm inner diameter (i.d.) 3-μm Gemini C_6-Phenyl column (Phenomenex, Torrance, CA, USA) held at 40 °C and coupled to a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK).

A sample volume of 1 μl was injected and eluted at a flow rate of 0.3 ml/min using a water–acetonitrile gradient system starting from 20% acetonitrile, with a linear increase to 55% in 6 min, then to 100% in 0.5 min, followed by a holding time of 2.5 min. Water and acetonitrile were buffered with 20 mM formic acid. Detection was by a multiple reaction monitoring (MRM) in positive electrospray ionization mode with $[M + H]^+$ as the precursor ions.

Fumonisins were detected within 4 to 6 min, with transitions of *m/z* 722 → 352 and *m/z* 722 → 370 for FB_1, *m/z* 706 → 336 and *m/z* 706 → 318 for FB_2 and *m/z* 740 → 358 for ^{13}C-FB_2. For all, a collision energy of 50 eV and cone voltage of 50 V were used. MPA was measured within 5–9 min with transitions of *m/z* 321 → 159 and *m/z* 321 → 207, a collision energy of 25 eV and a cone voltage of 20 V. OTA was measured within 6–10 min with transitions of *m/z* 404 → 358 at 20 eV, *m/z* 404 → 239 at 30 eV and *m/z* 424 → 250 at 30 eV for ^{13}C-OTA (all using a cone voltage of 30 V). Other parameters were a dwell time of 100 ms, interscan time of 20 ms and interchannel delay of 20 ms. The ion source was held at 120 °C using 700 L/h as a desolvation gas with a desolvation temperature of 350 °C. MassLynx 4.1 (Micromass) was used for data collection and evaluation.

Calibration

Matrix-spiked calibration curves were prepared by adding 200 μl of a mixture of FB_1, FB_2, OTA and MPA diluted to an appropriate concentration to 0.7 g meat, for spiking levels of 30, 150, 750, 1,500 and 3,000 μg/kg for FB_1 and FB_2, and 10, 50, 250, 500 and 1,000 μg/kg for OTA and MPA. Spiked samples were kept at −20 °C for overnight or longer, until extraction. A calibration curve was constructed for each analyte by linear regression weighted by $1/X$ to describe the relationship between concentration of spiked analyte (μg/kg) and the ratio of analyte to IS peak area. A lack-of-fit test was used to determine whether the linear model was adequate to describe the data. When the intercept was not significantly different from zero in a hypothesis test, regression was redone without intercept. StatgraphicsPlus v. 4.0 (StatPoint Inc., Herndon, VA, USA) was used for linear regressions and lack-of-fit tests. The limit of detection (LOD) was set to the concentration for which both transitions were detected with a signal-to-noise ratio of 3:1, and with a ratio between the product ion peak areas of less than 35% deviation from the average ratio of standard solutions. The lower limit of quantification (LOQ) was set to the lowest concentration level for which a precision with coefficient of variation (CV) lower than 25% was obtained.

Method performance and validation

Recovery and precision were determined by spiking at the five levels used for the calibration curve in triplicate, for three individual days. Recovery of analytes was determined by the slope of a linear regression model describing the relationship between spiked analyte concentration and recovered analyte concentration, without no matrix effect correction. Recovered analyte concentration was calculated by using a standard curve prepared in acetonitrile/water (1:2) at concentrations from 0.021 to 2.1 μg/ml for FB_1 and FB_2, and 0.007 to 0.7 μg/ml for OTA and MPA (five levels with three replicates). To determine extraction and purification efficiency, a mixture of FB_1, FB_2, OTA and MPA was added to either 0.7 g meat, a water–acetonitrile–acetone extract from 0.7 g meat, or purified extract from 0.7 g meat at a spiking level corresponding to 750 μg/kg in meat for FB_1 and FB_2, and 250 μg/kg in meat for OTA and MPA.

A test for ion suppression was conducted for selected samples by post-column infusion of MPA (the best ionizing compound, monitoring *m/z* 321 → 207). A syringe pump was used to infuse MPA at a concentration of 1 μg/ml and a flow rate of 50 μl/min.

Confirmation of OTA by LC–high resolution mass spectrometry

One sample contained unexpectedly high concentrations of OTA, so we confirmed this result using LC-TOFMS on a Micromass LCT orthogonal TOFMS, as described previously [1, 44], but with a modified LC analysis using a 100×2-mm-i.d., 2.6-μm Kinetex C_{18} column (Phenomenex). A gradient of 0.400 ml/min was started with 10% acetonitrile, and increased to 20% in 0.5 min, then to 65% in 13.5 min, then 100% acetonitrile in 3 min, with holding for 3 min before reverting to 10% acetonitrile in 0.5 min and equilibrating for 9.5 min.

Confirmation of OTA by LC-MS/MS of the methyl ester formation

Further confirmation of the positive sample was obtained by converting OTA to its methyl ester [45]. Purified extracts of the positive sample, a blank and spiked samples were evaporated to dryness with nitrogen, dissolved in 90 μl methanol and 10 μl BF_3 diethyl etherate, and left at room temperature for 15 min. Samples were evaporated to dryness with nitrogen, dissolved in 100 μl acetonitrile and reanalysed by LC-MS/MS using the settings described above, except that the MRMs of methyl-ochratoxin A were 418 → 239 at 25 eV and 418 → 358 at 25 eV, both at a cone voltage at 25 V.

Fermented sausages as matrix material for calibration and validation

Raw sausages prepared from 75% pork shoulder, 21% pork fat, 3.2% NaCl, 0.006% $NaNO_2$, 0.03% sodium ascorbate, 0.3% dextrose and 0.05% starter culture (LS-25, Kiranto Foods A/S, Farum, Denmark) were stuffed in 60-mm fibre casings in a pilot plant facility, and were kindly provided by the Danish Meat Research Institute. Sausages were dried in a climate chamber (Weiss wk111 340, Weiss Umwelttechnik GmbH, Reiskirchen-Lindenstruth, Germany) at 24 °C and 95% relative humidity (RH) until fermentation was completed (48 h, pH 5.2), then left for 24 h at 20 °C, 95% RH; 24 h at 18 °C, 95% RH; 24 h at 16 °C, 95% RH; 5 days at 15 °C, 95% RH; and 8 days at 15 °C, 92%, RH. At this point the sausages had a drying loss of 20%, and were stored at 20 °C and 75% RH for 3.5 weeks to mimic the average retail period, then stored at −20 °C until analysis.

Artificial inoculation of fermented sausages

Cultures of *A. niger* IBT 28144 (CBS 101705; producer of both OTA and FB_2) on Czapek yeast autolysate agar were incubated at 25 °C for 7 days, and used to prepare a spore suspension of 5×10^5 conidia/ml in water with 0.05% Tween 80 (Merck, Hohenbrunn, Germany) and 0.05% agar (Sobigel, VWR - Bie & Berntsen A/S, Herlev, Denmark). Raw sausages ($n=5$) prepared as described above were inoculated 1 day after stuffing, by immersion in the spore suspension, followed by drying as above. Each sausage was divided into three parts: the casing, an approximately 1-cm-deep surface layer, and an approximately 2.5 to 3-cm centre sample. All samples were stored at −20 °C until analysis.

Retail meat products

From local vendors or supermarkets, we purchased 22 different products (6–12 slices), consisting of various types of fermented sausages and dry-cured hams produced in Europe. All product slices were homogenised and analysed.

Results and discussion

Purification and LC-MS/MS detection

We designed a purification procedure for the simultaneous detection of the very different fungal metabolites FB_1, FB_2, OTA and MPA, using their common characteristic of acid groups as a starting point. However, FB_1/FB_2, OTA and MPA represent three different classes of acidic compounds, so including them in a common anion-exchange protocol proved difficult. MPA did not bind efficiently (<20% recovery) to the Phenomenex Strata SAX columns we tested, consistent with a recent report [42], even when pure standards were tested at different pH values. Fumonisins, on the other hand, were easily retained on the SAX columns. However, they did not bind efficiently and reproducibly to MAX columns used in generic mode, in which targets were loaded under low-organic-acidic conditions and low pH, with the pH increased above the target pK_a for an acid-lock by ion-exchange, and subsequent high-organic wash and target elution with acidic acetonitrile or methanol. However, MAX columns used in direct ion-exchange mode gave a recovery of greater than 66% (Table 1), presumably because exclusion of competing salts improved analyte binding. To facilitate direct ion-exchange applications, analytes were extracted from meat matrices by using a polar water-acetonitrile mixture at neutral pH, which extracted analytes in deprotonated form. A defatting step with pentane was crucial for avoiding precipitation of fats during evaporation of organic solvent, and allowed the sample to be dissolved in less than 20% organic solvent

Table 1 Efficiency of extraction, purification and LC-MS/MS

	Recovery (%)			
	Extraction	MAX[a]	LC-MS/MS[b]	Total
Fumonisin B_1	105	66	93	64
Fumonisin B_2	87	88	75	50
Ochratoxin A	90	101	57	48
Mycophenolic acid	122	89	86	97

Recovery of FB_1, FB_2, OTA and MPA calculated by individual method steps. Standard additions were in triplicate into 0.7-g meat sample, a water–acetonitrile–acetone extract from 0.7 g meat, or a purified extract from 0.7 g meat, prior to LC-MS/MS. FB_1 and FB_2 were added at levels corresponding to 750 μg/kg in meat, and OTA and MPA were added at levels corresponding to 250 μg/kg in meat

[a] Mixed-mode reversed-phase anion exchange (direct mode)

[b] Blank, purified samples spiked before LC-MS/MS

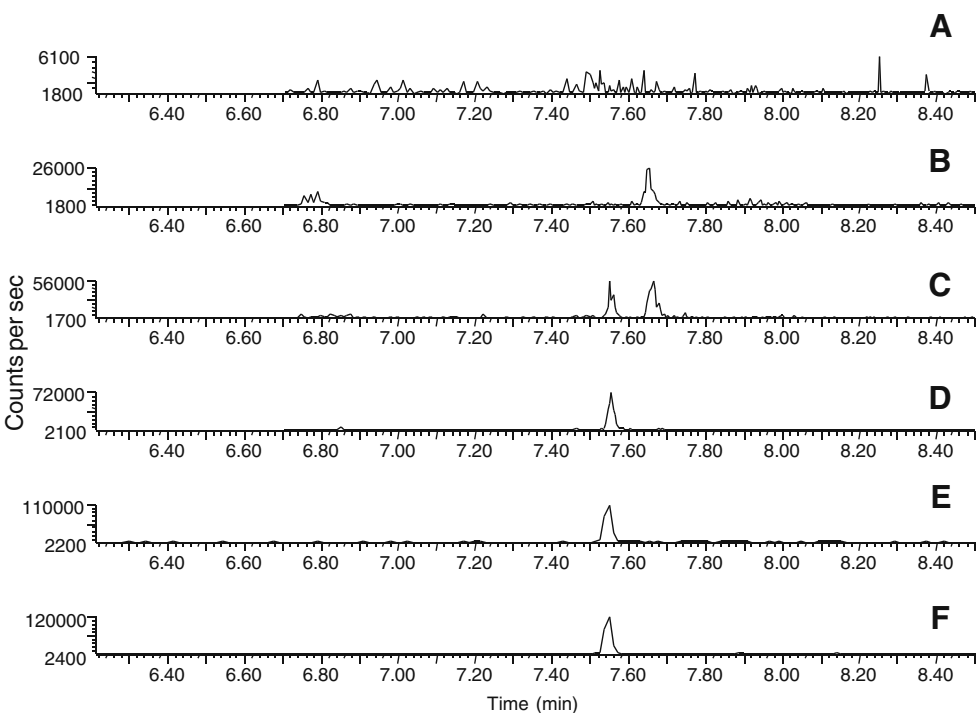

Fig. 1 LC-MS/MS MRMs of OTA showing: **a**, **b** blank sample with 404 → 239 and 404 → 358 respectively; **c**, **d** spiked sample (50 μg/kg OTA), same transitions as above; **e**, **f** naturally contaminated sample (#15), same transitions as above (113 μg/kg)

prior to Oasis MAX purification. During analysis of some very high fat-containing meat samples, double defatting with pentane was required (data not shown). After extraction, acetone-induced precipitation was required to remove much of the interfering material, presumably salts, proteins and highly polar peptides, that otherwise obstructed ion-exchange recovery of analytes, yielding less than 10% recovery.

Chromatographic parameters were optimized, resulting in retention times of 4.95±0.12 min for FB_1, 5.65±0.10 min for FB_2, 6.94±0.05 min for MPA and 7.57±0.02 min for OTA. Only one interfering peak was occasionally observed at the *m/z* 404 → 358 transition of blank samples (Fig. 1), and it was always clearly resolved from OTA, so the method was determined to be sufficiently specific for the analytes. Analytes were detected by four identification points: retention time, two MRM transitions and ion ratio, which exceeded the requirements for three identification points for mycotoxins [46, 47]. For positive detection, we used the criteria that both transitions be detected with a signal-to-noise ratio of 3:1, and the ratio between the product ion peak areas deviated less than 35% from the average ratio for standard solutions.

LOD was estimated from samples with the lowest spiked concentration (Table 2). The 6 μg/kg LOD for FB_2 was in the range obtained for methods developed for other matrices, such as corn flakes [48, 49], peanuts and figs [50] and feeds [51]. The LOD of 11 μg/kg for OTA was high compared to other published methods [2, 8, 9, 41], and about 10-fold higher than the Italian limit of 1 μg/kg OTA in meat products [41]. However, it was within the expected intervention limit of approximately 10–20 μg/kg for OTA, established on a case-to-case basis by the ministries of individual EU countries for toxic compounds in unregulated commodities. For example, the regulated levels in cereal products and raisins is 2–10 μg/kg [19, 20].

The LOD of 14 μg/kg for MPA was also higher than for our previous published method [1], however the current

Table 2 Method performance

Matrix	Recovery (%)[a]	Precision (% CV)	Accuracy (%)	LOD (μg/kg)	LLOQ (μg/kg)
Fumonisin B_1	64.0±6.5	22–31	58–95	64	150
Fumonisin B_2	50.2±1.4	5–10	73–100	6	150
Ochratoxin A	48.2±1.1	4–8	95–104	11	50
Mycophenolic acid	96.7±6.1	17–23	90–117	14	50

Data are recovery, precision, accuracy, limit of detection (LOD) and lower limit of quantification (LLOQ) for determination of FB_1, FB_2, OTA and MPA in fermented sausage

[a] Recoveries are average values ± 95% confidence intervals

methodology was both faster and more robust, as well as able to target several analytes in a very complex matrix. Also of note is that our 10-year-old Micromass Ultima triple quadrupole MS is presumably 20- to 100-fold less sensitive than the new triple quadrupole M.

Even with acetone-induced precipitation, defatting with pentane and ion exchange, some degree of ion suppression was observed during LC-MS/MS. For example, we saw a 57% recovery of OTA when it was spiked into a purified sample just prior to LC-MS/MS analysis. Ion suppression for MPA was investigated further using the classic T-piece infusion test [52] with an analyte and matrix blank sample with and without purification. As seen in Fig. 2, sample purification was critical for minimizing ion suppression, as also observed for trichothecenes in cereals [53].

To correct for loss of analytes during extraction and purification, and ion-suppression effects during LC-MS/MS, quantification was done by matrix-spiked calibration. QuEChERS methodology [54] might be valuable for reducing ion suppression, by enforcing phase separation between water and acetonitrile (low pH to avoid losing the targets), and subsequently determining if interfering compounds remain in the aqueous phase so the acetone-induced precipitation step may be omitted.

Calibration

Matrix-spiked calibration curves were generated by spiking a mixture of each of the four analytes at five different levels into samples of fermented sausages (Table 2). Correlation coefficients from 0.97 to 1.00 were obtained (Table S1), and the data were found to fit linear models by lack-of-fit tests (95% confidence level). A hypothesis test was used to determine if the intercepts were equal to 0 (95% confidence level), and when none were found to be significant, regressions were redone without intercept.

Validation

Precision within days (repeatability) and intermediate precision between days were evaluated by analysing spiked samples from three different days, in triplicate. This was done at five concentration levels covering the analytical range, and including concentrations below the expected lower limit of quantification (LLOQ). One-way ANOVA was used to calculate CV within days ($CV_{within\ days}$), between days ($CV_{between\ days}$) and total (CV_{total}) at each concentration level (Table 3). An acceptable precision (CV<25%) was obtained for FB_2, OTA and MPA at all levels, except at the lowest concentration level for each analyte (Table 3). The lower LLOQ was based on the lowest concentration level with an acceptable precision, so LLOQ was 150 μg/kg for FB_2, and 50 μg/kg for OTA and MPA. Precision was too poor for reliable results for FB_1, possibly because low recovery during MAX obscured the robust analysis of this analyte. For FB_2 and OTA, although the total recoveries were low (50.2 and 48.2% respectively), measurement precision was still satisfactory (5–10 and 4–8% CV). This indicated a fairly robust method and confirmed the strength of using isotope-labelled IS that behaves in the exactly same way as the analytes during extraction and purification, as well as during ionization and fractionation in the MS. Thus, accuracies within an acceptable range of 73–117% for FB_2, OTA and MPA were possible using isotope-labelled IS and matrix-spiked calibration.

Inoculated and retail meat products

Neither fumonisins nor OTA were detected in the inoculated meat products, even though the *A. niger* strain we used produces these mycotoxins on culture media, indicating that the *A. niger* strain was not able to produce OTA and FB_2 on fermented sausages under the conditions used in this study.

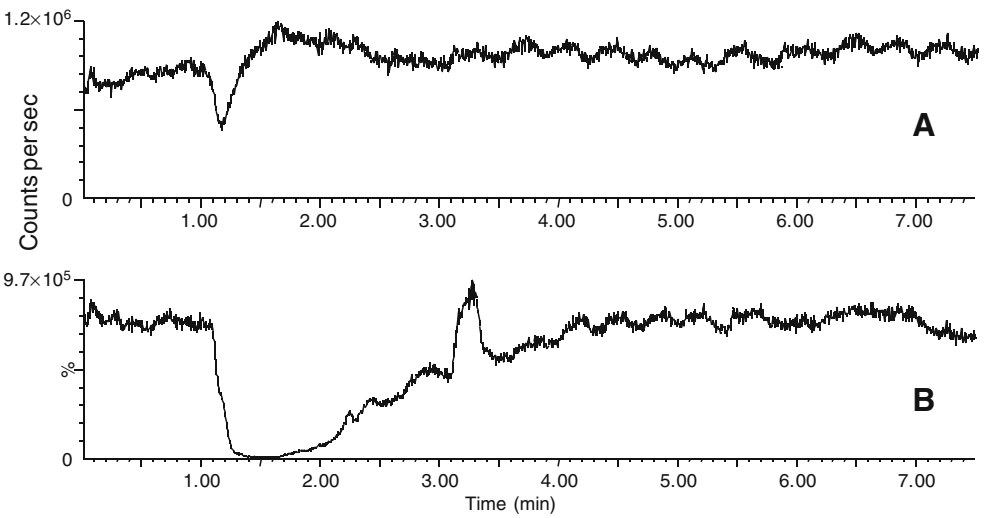

Fig. 2 Ion-suppression test using post-column T-piece infusion of MPA (1 μg/ml), which was the poorest ionizing compound at 50 μl/min, into the 0.300 ml/min gradient with continuous MRM of *m/z* 321 → 207. **a** Shows purified extract and **b** crude defatted extract. Target analytes eluted at 5.65 min (fumonisin B_2), 6.94 min (mycophenolic acid) and 7.57 min (ochratoxin A)

Table 3 Parameters for matrix-spiked calibration curves in fermented sausage

Standard	Internal standard	Calibration				
		Slope[a]	Intercept[b]	Corr. coeff.	*n*	Range (μg/kg)
Fumonisin B_1	^{13}C-FB_2	0.0032±0.0002	NS	0.97	49	30–3,000
Fumonisin B_2	^{13}C-FB_2	0.0096±0.0002	NS	1.00	49	30–3,000
Ochratoxin A	^{13}C-OTA	0.0057±0.0001	NS	1.00	49	10–1,000
Mycophenolic acid	^{13}C-OTA	0.0025±0.0001	NS	0.99	49	10–1,000

Data are slope and intercept, correlation coefficient, number of samples (*n*) and tested range for linear regression of analyte to IS peak area ratio as a function of added analyte concentration in meat, in μg/kg

NS not significant, 95% confidence level

[a] Estimates ± 95% confidence intervals

[b] A hypothesis test was used to determine if the intercept was equal to zero. When the intercept was not significantly different from zero, regression was performed without intercept

Of the 22 retail samples we analysed, one was positive for one of the analytes, measuring 56 μg/kg OTA on first analysis. The sample was an original Parma ham bought at a major high-end supermarket, and was the most expensive product we tested. The slices analysed were taken from a ham that was opened at the time the specific sample was purchased, and did not appear in any way to be contaminated. Two frozen sub-samples from this ham were analysed, and found to contain 158 and 113 μg/kg OTA (Fig. 1). Even though our method uses four identification points of retention time and two MRMs, and thus exceeds the three requirements for mycotoxins [46, 47], the high level of the results suggested confirmation was necessary. Reanalysis by an in-house, standard LC-TOFMS method was relatively straightforward [55], and verified that OTA had been detected, by accurate mass and correct ^{35}Cl/^{37}Cl ratio (Electronic supplementary material Fig. S2). We also tried to detect the biosynthetic precursors of ochratoxins B, α and β by LC-MS/MS MRM in the positive sample, but none of these could be detected (data not shown).

We also confirmed OTA detection using the classic confirmation method [45] of methylation of OTA (Electronic supplementary material Fig. S1) and redetection as the methyl ester with a different retention time and parent ion (Electronic supplementary material Fig. S3). These results also verified the previous findings.

The detected amount of OTA in the positive sample was 10- to 100-fold higher than is typically found in meat, for which levels rarely exceed 4 μg/kg [2, 6–10]. It was 10-fold higher than is usually found in samples contaminated with *Penicillium* [9]. Therefore, the OTA was likely produced by *P. nordicum* [5], emphasising the special attention that should be given to the presence of this fungus on meat products.

With an OTA concentration as high as we found in the Parma ham sample, a reasonable portion of less than 10 g per day for a week would be sufficient to reach the provisional tolerable weekly intake of 0.1 μg/kg body weight [56] for a 70-kg person. Since we found this high OTA level in an analysis of only 22 samples, this suggests that OTA-producing fungi growing on some meat products is a problem that is not uncommon.

The only limitation we found for our method was the large variation observed for the three determinations, with 109±51 μg/kg corresponding to a relative standard deviation of 47%, which is far larger than the variation observed for the spiked samples. However, the very high concentrations detected clearly indicated a surface contamination in the analysed ham. In such a case, the sample is extremely heterogeneous in OTA concentration, so small samples of highly concentrated meat particles would be expected to have large variations.

Conclusion

The methodology presented here was successfully applied to three types of fungal metabolites in meat products, which might be one of the most challenging matrices. The method was capable of detecting as low as 6 μg/kg of FB_2, 11 μg/kg OTA and 14 μg/kg MPA. Most importantly, the precision was reasonable, and in the range of 4–23% CV for the three main analytes, with an accuracy ranging from 73 to 117%. *A. niger* was found not to produce FB_2 or OTA above the LOD, under the conditions we used for production, inoculation, drying and storage of fermented sausages. OTA was detected in 1 of 22 retail products, at an unusually high average level of 109 μg/kg, presumably originating from growth of *P. nordicum*. Presence of OTA was confirmed by the accurate mass, and correct ^{35}Cl/^{37}Cl ratio, as well as by methyl ester formation. On the basis of our results, closer examination of these kinds of meat products is recommended.

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