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Analytical Methods

Application of an LC–MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds

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ABSTRACT

An existing LC–MS/MS method for multi-mycotoxin determination was extended by further 19 analytes and was applied for a semi-quantitative screening of 87 mouldy food samples from private households, including bread, fruits, vegetables, cheeses, nuts and jam. In the 247 investigated sub-samples, 49 different analytes were identified, some of which were never reported before to occur in naturally contaminated food. Enniatins and ergot alkaloids occurred in all samples of (dark) bread/pastries at low μg/kg-levels. From the remaining analytes, chanoclavine, emodin, mycophenolic acid and roquefortine C were found most frequently. Regulated mycotoxins occurred less often, but the corresponding concentrations exceeded the regulatory limits up to a factor of 1000 in case of patulin. Moreover, considerable mycotoxin concentrations were observed in some sub-samples taken from non-mouldy spots of the investigated samples. Thus, it was concluded that it is not safe to remove the mouldy part and consume the remainder.

1. Introduction

Moulds are able to infect and grow on all types of food. This is usually accompanied by changes of the texture, smell and taste of the infected foodstuff due to excretion of enzymes and volatile compounds by the fungus. In some cases, these changes are desired (e.g. use of non-toxigenic strains of *Penicillium roqueforti* for the production of blue mould cheese), but most of the time, fungal infection leads to food spoilage such as off-flavours, discoloration, rotting and disintegration of the food structure (Filtenborg, Frisvad, & Thrane, 1996).

The most important aspect involved in spoilage of food is, however, the formation of mycotoxins. Although approximately 400 compounds are currently recognised as mycotoxins, only few of them are addressed by food legislation. Most of the existing analytical methods likewise focus on these regulated toxins, i.e. trichothecenes, aflatoxins, zearalenone, ochratoxin A, fumonisins and patulin. In contrast, most publications dealing with other mycotoxins produced by fungi involved in food spoilage seem to derive from the field of mycology rather than from food analysis. A typical example consists of the use of the metabolic profile of crude fungal extracts to support findings from taxonomy for the differentiation of fungal species (Nielsen & Smedsgaard, 2003).

These findings are of limited relevance concerning the occurrence of mycotoxins in naturally infected food: most mycological investigations address fungal strains that have been isolated e.g. from food and have subsequently been cultivated on synthetic culture media. However, the qualitative and quantitative mycotoxin profile, which a mould produces on a food commodity depends on the ecological and processing parameters of the particular foodstuff (Filtenborg et al., 1996) and can therefore be expected to be different from synthetic media. In addition, most of the analytical methods that are used in mycological studies such as TLC (Filtenborg et al., 1996; Freire, Kozakiewics, & Paterson, 2000; Overy, Seifert, Savard, & Frisvad, 2003) or HPLC–DAD (Andersen, Smedsgaard, & Frisvad, 2004; Andersen & Frisvad, 2004; Larsen, Gareis, & Frisvad, 2002) are sufficiently selective for the determination of fungal extracts or for single target analysis (including a dedicated procedure for sample preparation) in foodstuffs, but are incapable of dealing with a large number of analytes in complicated food matrices. Modern methods such as HPLC coupled to (tandem-) mass spectrometry offer higher selectivity, which enables multi-analyte determination without dedicated sample clean-up in principle. However, even for those methods, multi-mycotoxin analysis in food is a real analytical challenge, as it would be advantageous to work without any clean-up and analyze raw extracts instead in order not to adulterate the mycotoxin pattern by sample preparation.

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The drawback of such a "dilute and shoot"-approach is that signal suppression due to matrix effects is far more likely to occur when crude extracts are analyzed. The existing method that covers hundreds of fungal metabolites is used rather for qualitative screening of fungal metabolites (Nielsen & Smedsgaard, 2003; Nielsen, Sumarah, Frisvad, & Miller, 2006) than for quantitative analysis, which is partially a result of the lack of availability of suitable standards. On the other hand, quantitative data on metabolites involved in food spoilage is usually restricted to a selected set of analytes that are amenable to the chosen clean-up procedure (e.g. Kokkonen, Jestoi, & Rizzo, 2005).

Despite these difficulties, there is certainly a need for fast and comprehensive methods for the analysis of toxic metabolites produced by toxigenic strains of food colonising fungi, as a simple visual inspection of food is not sufficient to exclude health hazards. It was shown that toxin concentration and visible infection may not correlate in every case (Rundberget, Skaar, & Flaoyen, 2004), and mycotoxins can be present in commodities without being able to detect fungi associated with the toxins and vice versa (Freire et al., 2000). In addition, the use of any mouldy material in the processing of food may contribute to the mycotoxin level in the final product by carry over (Filtenborg et al., 1996), e.g. use of mouldy tomatoes for the production of ketchup (Andersen & Frisvad, 2004). In such cases, the mouldy material cannot be seen in the final product. A recent report by our group (Sulyok, Berthiller, Krska, & Schuhmacher, 2006) has shown that the use of mass spectrometers of the latest generation enables a quantitative determination of mycotoxins in crude food extracts, provided that the extraction efficiencies as well as matrix effects are sufficiently characterised for all investigated analyte/matrix combinations. Only recently, we have extended the range of analytes covered by our method and have provided preliminary data on the mycotoxin pattern in mouldy food samples (Sulyok, Krska, & Schuhmacher, 2007).

In the present work, the method was further extended and applied for a semi-quantitative screening of 247 sub-samples taken from mouldy and non-mouldy spots of 87 food samples from private households in Austria. It was our goal to determine the mycotoxin pattern (including toxic metabolites that have not been reported yet to occur in naturally contaminated food), that is produced by moulds spontaneously infecting food, stored under typical conditions at the end consumer. These results might be used to identify toxins as marker substances for food spoilage and to evaluate the relevance of the related toxin concentrations for the end consumer. Furthermore, the distribution of the toxins between mouldy and non-mouldy parts of the same sample has been studied. Some of the toxins detected were only present in the moulded part of the samples whereas others were excreted by the fungus into the surrounding food tissue. There is certainly a practical relevance of this issue, as it is still common practice in case of some food products to remove the mouldy parts and to consume the remainder.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Mycotoxin standards were dissolved in acetonitrile and were purchased from different sources: Brefeldin A, cytochalasins A, B, C, D, J and H, HC-toxin, kojic acid, and 3-nitropropionic acid were purchased from Sigma (Vienna, Austria), penicillic acid and roquefortine C were obtained from Iris Biotech GmbH (Marktredwitz, Germany), AAL TA toxin was a gift from Prof. David Gilchrist (University of California, Davis, United States), Enniatin B3 and 2-amino-14,16-dimethyloctadecan-3-ol were a gift from Dr. Silvio Uhlig (National Veterinary Institute, Oslo, Norway), certified reference solutions of T2-tetraol and T2-triol were received from Biopure Referenzsubstanzen GmbH (Tulln, Austria). Alpha zearalenol-4-glucoside and beta zearalenol-4-glucoside were synthesised in our laboratory from zearalenone using a genetically modified yeast strain expressing a glucosyl-transferase, followed by reduction of the resulting zearalenone-4-glucoside with sodium borohydride (Krenn et al., 2007). For details concerning the other 87 toxins see Sulyok et al. (2007).

2.2. Samples

Eighty-seven spontaneously moulded foodstuffs (including 19 breads/pastries, 20 fruits, 14 vegetables, 6 cheeses, 5 jams, 6 nuts and 17 others), which had been provided by staff members of our institute, were sampled for mycotoxins in this study. Few of the samples were completely covered by mould, whereas most samples exhibited one or several – sometimes differently coloured – mouldy spots. The latter samples can be considered to be realistic in private household practice.

After visual inspection of each sample, several sub-samples per individual sample were prepared by cutting mouldy and (if available) non-mouldy spots of the individual sample using a scalpel. (Note that the term "non-mouldy" is used throughout the manuscript although we are aware that fungal mycelium may have also been present in those parts of the samples, which did not exhibit visible fungal infection. There was no fixed distance between the sampled mouldy and non-mouldy spots; one sub-sample was usually taken from the maximum distance from the fungal infection.). The surface area of the sub-samples was approximately 1 cm^2 and their thickness ranged between 0.5 and 1 cm in order to make sure that the main part of the sampled volume consisted of food matrix.

The final set of 247 sub-samples included 68 originating from bread/rolls (49 with mouldy spots/19 spots without visible infection), 49 from fruits (30/19), 34 from vegetables (22/12), 14 from cheeses (10/4), 13 from nuts (10/3), 12 from jams (6/6) and 57 from other foodstuffs (34/23).

2.3. Sample preparation and estimation of matrix effects

Extraction was carried out using a mixture of acetonitrile/water/acetic acid 79 + 20 + 1 (v + v + v), with ratios between 3 and 16 mL solvent/g sample depending on the texture of the sample. After extraction, the samples were centrifuged, diluted 1 + 1 and injected as described in detail by Sulyok et al. (2007). For the estimation of matrix effects, raw extracts of sample spots without visible fungal infections were fortified using a multi-analyte standard on one concentration level, diluted and analyzed and the corresponding peak areas were compared to a standard prepared and diluted in neat solvent.

2.4. LC–MS/MS parameters

Detection and quantification was performed with a QTrap 4000 LC–MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic and mass spectrometric parameters of 87 of the investigated analytes are described by Sulyok et al. (2007). MS and MS/MS parameters of the additional 19 analytes were optimised by infusion of standard solutions into the mass spectrometer. For a detailed list of these compounds and the related parameters see Table

Table 1
LC–MS/MS parameters of the 19 additional analytes.

Analyte	t_R[a] (min)	*m/z* precursor ion	DP[b] (V)	*m/z* product ions[c]	Rel. int.[d]	CE[e] (V)[c]	CXP[f] (V)[c]	P[g]	Dwell time (ms)	LOD (μg/kg)
Kojic acid	3.22	143.1 $[M+H]^+$	56	113.2/69.2	3.95	31/23	10/10	+1	100/25	160
T2-tetraol	5.79	316.2 $[MNH_4]^+$	31	215.3/281.4	0.67	13/13	16/8	+1	100/25	70
Penicillic acid	9.30	171.2 $[M+H]^+$	46	125.2/97.1	0.38	17/23	8/16	+3	60/20	20
AAL TA-toxin	11.96	522.3 $[M+H]^+$	71	328.5/292.4	1.13	35/41	20/16	+5	45/15	12
T2-triol	12.51	400.2 $[M+NH_4]^+$	41	215.2/281.3	0.34	17/13	12/16	+5	45/15	40
Roquefortine C	12.54	390.2 $[M+H]^+$	61	193.2/322.3	0.43	39/29	10/18	+5	45/15	4
Cytochalasin J	13.21	452.2 $[M+H]^+$	31	434.5/416.5	0.94	13/21	12/12	+6	100/25	5
Cytochalasin D	13.48	525.2 $[M+NH_4]^+$	31	430.5/490.5	0.36	23/17	12/14	+6	100/25	4
Brefeldin A	13.49	281.0 $[M+H]^+$	36	245.3/263.3	0.72	11/9	14/14	+6	100/25	60
Cytochalasin B	13.75	480.2 $[M+H]^+$	51	462.5/444.5	0.33	23/23	10/12	+7	50/20	10
Cytochalasin H	13.76	494.2 $[M+H]^+$	26	434.5/416.5	1.35	11/19	12/12	+7	50/20	30
Cytochalasin C	13.98	525.2 $[M+NH_4]^+$	31	430.5/490.5	0.36	23/17	12/6	+7	50/20	2
Cytochalasin A	14.97	478.2 $[M+H]^+$	71	460.5/120.2	0.38	23/39	12/8	+8	60/25	30
Enniatin B3	15.39	629.4 $[M+NH_4]^+$	46	196.3/214.3	0.60	41/41	10/12	+9	60/20	0.04
2-Amino-14,16-di-methyloctadecan-3-ol	16.00	314.3 $[M+H]^+$	41	296.5/125.2	0.02	25/25	18/6	+9	50/20	20
3-Nitropropionic acid	2.93	118.0 $[M-H]^-$	−35	46.0	–	−16	−3	−1	100	25
HC-toxin	10.79	435.2 $[M-H]^-$	−95	184.0/113.1	0.21	−36/−52	−13/−3	−2	100/100	12
β-ZOL-glucosid	11.95	541.3 $[M+Ac]^-$	−28	319.1/481.1	0.56	−32/−14	−15/−11	−3	100/100	1
α-ZOL-glucosid	12.92	541.3 $[M+Ac]^-$	−28	319.1/481.1	0.48	−32/−14	−15/−11	−3	100/100	0.8

[a] Retention time.
[b] Declustering potential.
[c] Values are given in the order quantifier ion/qualifier ion.
[d] Intensity of the qualifier transition/intensity of the quantifier transition.
[e] Collision energy.
[f] Cell exit potential.
[g] Retention time period.

1. Quantification was performed in the Selected Reaction Monitoring (SRM) mode. Limits of detection were calculated from the signal to noise ratios (LOD = 3 * S/N) of the respective SRM chromatograms as derived from the analysis of liquid standards. Enhanced Product Ion scans for identity confirmation were acquired using the third quadrupole as linear ion trap applying a dynamic fill time, whereas other parameters such as the declustering potential and the collision energy were set to the optimised values that had been determined for the respective analytes.

3. Results and discussion

3.1. Evaluation of the extended HPLC–MS/MS method

As our institute is continuously expanding the multi-mycotoxin method, we applied the latest version to this experiment, as to get as much information on contamination as possible. As our approach is based on the analysis of diluted crude extracts without further sample pretreatment, the inclusion of additional analytes does not pose any problems as long as they are compatible with the chromatographic conditions and as long as they are extractable (at least to a partial, reproducible extent) using the acidified acetonitrile/water mixture that is applied in our method. However, it must be kept in mind that these conditions are a compromise resulting from the chemically diverse set of analytes and may be far from optimal for some compounds. For example, the extension of the existing method by the highly polar substances 3-nitropropionic acid and kojic acid resulted in small retention factors of 0.97 and 1.16, respectively, of these two compounds on the applied C_{18}-based column. Although a full method validation for the additional analytes has not been performed yet, preliminary results indicate that the method performance parameters will probably be not significantly different compared to the set of 87 substances: Limits of detection ranged from 0.04 for enniatin B3 to 160 μg/kg for kojic acid (see Table 1). The linearity of the detector signals of each of the 19 analytes has been confirmed over a concentration range of at least 2 orders of magnitude. Analyte losses due to incomplete extraction and/or matrix effects have been investigated in maize and were comparable to our initial method (Sulyok et al., 2006; data not shown).

Since the goal of this work was to provide information on the occurrence of toxic fungal metabolites in mouldy food and a rough estimate of the related concentrations, a detailed investigation on the extraction efficiencies and matrix effects in the different matrices was considered to go beyond the scope of this work. Judging from the results of the detailed investigation on extraction efficiencies and matrix effects of multi-mycotoxin and multi-residue analysis in various matrices (Spanjer, Rensen, & Scholten, 2008; Mol et al., 2008), analyte losses due to incomplete extraction exceed a factor of 2 only for a few analyte/matrix combinations if an (acidified) acetonitrile/water mixture is used as extraction solvent. At the same time, in these two reports matrix effects exceeded a factor of three only in few cases, but since this is an instrument-dependent parameter, we verified this finding by spiking various raw extracts of non-mouldy spots. The suppression of the analytical signal exceeded a factor of 2 in the spiked raw extracts only in 12% of all investigated analyte/matrix combinations (ergopeptides and the *Alternaria* toxins generally being the most critical analytes in that aspect) and exceeded a factor of 10 in only 5 out of the 2412 investigated analyte/matrix combinations (avenacein Y, tentoxin and HC-toxin in apricots, HC-toxin in treenuts and tomatoes). Both, incomplete extraction and matrix effects lead to an underestimation of the actual concentration in the mouldy samples. However, this combined error caused by matrix effects and incomplete extraction is in the worst case a factor of 2–3 for most analytes and (with the few exceptions stated above) a factor of approximately 6 for the ergopeptides and citrinin (that exhibited a rather low extraction efficiency of 30% in the model matrix that was investigated by Sulyok et al., 2007), which we consider acceptable for meeting our goals.

3.2. Overview of frequency and concentrations of mycotoxins in the investigated samples

Forty-nine different analytes were identified in the set of 247 sub-samples of the 87 investigated food samples. Their frequencies

and the corresponding median and maximum concentrations as well as the type of contaminated food commodities are given in Table 2; a distribution of the measured concentrations is given in Figs. 1 and 2. In three fruits (orange, plum, apricot) and three vegetables (two cucumbers, one tomato), none of the investigated toxins was found despite the presence of moulds.

The reported frequency of the different toxins is of limited value for the evaluation of their relevance, as the frequency ranking is biased by the differences in the LODs of the analytes and by the different number of sub-samples that were analyzed for each type of matrix. This is particularly true for the enniatins that were detected in all bread and pastry samples at concentrations in the low μg/kg-range, which were partially below the LOD of some of the other analytes. Enniatin contamination of grains in the μg/kg range was already reported by other workers e.g. by Jestoi et al. (2004), and our results confirm recent findings by Noser, Schmutz, Schmid, and Schneider (2007) regarding contamination of flour and bread. Generally, the enniatin concentrations did not vary significantly between mouldy and non-mouldy parts of bread and pastry samples (one example is depicted in Fig. 3), which indicates a more or less homogeneous distribution in the sample deriving from contamination of the raw product grain. We also found enniatins in a cranberry and a date sample at concentrations of several hundred μg/kg. Both samples were heavily infected by a grey mould and the cranberry sample also contained the *Fusarium avenaceum* metabolite avenacein Y. While those two samples would have probably not been consumed due to their visible infection, bread and pastries seem to be a potential source for enniatin uptake. However, to our knowledge it has not been investigated so far whether an uptake of such low concentrations of enniatins has consequences for the human health. A recent study concluded that due to their cytotoxic effects observed in human cell lines at the micromolar level and their lipophilicity that could cause bioaccumulation in animal and human tissue, enniatins might represent a potential hazard (Dornetshuber et al., 2007).

Table 2
Overview of the number and type of contaminated samples and of the corresponding mycotoxin concentrations.

Analyte	n[a]	N[b]	Median conc. (μg/kg)	Max. conc. (μg/kg)	Type and number of contaminated sub-samples
Enniatin B	100	41	2.2	950	Bread (68), nut (6), fruit (5), vegetables (5), jam (5), other (11)
Enniatin B1	96	38	23	3600	Bread (67), fruit (6), vegetables (5), jam (4), nut (3), other (11)
Emodin	88	35	7.5	2900	Bread (44), fruit (13), vegetables (7), jam (3), nut (2), other (19)
Enniatin A1	86	35	1.5	1300	Bread (61), fruit (5), nut (5), vegetables (3), jam (1), other (11)
Enniatin A	77	28	0.23	1000	Bread (60), fruit (5), vegetables (2), nut (1), other (9)
Mycophenolic acid	77	34	820	78,000	Bread (34), vegetables (7), cheese (5), fruit (4), nut (2), jam (5), other (20)
Roquefortine C	76	33	120	84,000	Bread (30), fruit (14), vegetables (12), nut (5), cheese (2), jam (1), other (12)
Chanoclavine	67	33	31	1500	Bread (40), vegetables (7), cheese (6), fruit (5), nut (4), jam (1), other (4)
Ergometrinine	57	18	7.6	160	Bread (52), cheese (4), vegetables (1),
Ergosinine	56	16	1.2	6.9	Bread (56)
Ergosine	56	16	1.4	11	Bread (56)
Ergotaminine	51	15	1.8	10	Bread (51)
Ergocryptine	50	15	3.5	22	Bread (50)
Ergotamine	50	16	3.5	23	Bread (50)
Ergometrine	50	15	3.0	75	Bread (52), cheese (3), vegetables (1)
Ergocristinine	49	15	2.3	14	Bread (48), vegetables (1)
Ergocristine	49	14	3.5	18	Bread (50)
Ergocryptinine	49	15	1.8	6.6	Bread (50), vegetables (1)
Ergocorninine	48	15	2.1	12	Bread (50), vegetables (1)
Ergocornine	47	13	2.7	16	Bread (50)
Deoxynivalenol	43	14	62	350	Bread (42), nut (1)
Tentoxin	41	15	1.8	9.3	Bread (35), fruit (1), vegetables (1), other (4)
Citrinin	41	20	320	3,400,000	Bread (14), fruit (13), vegetables (5), nut (1), other (8)
Festuclavine	31	17	52	15,000	Bread (21), cheese (3), vegetables (2), nut (2), fruit (1), jam (1), other (1)
Meleagrin	22	12	140	1,000,000	Vegetables (6), bread (5), fruit (2), nut (1), other (8)
Beauvericin	21	11	2.9	30,000	Vegetables (5), jam (2), bread (1), fruit (1), other (12)
Agroclavine	17	9	41	5400	Bread (12), cheese (3), vegetables (1), other (1)
Patulin	16	7	30000	80,000	Fruit (11), nut (1), other (4)
Alternariol	16	10	50	1800	Fruit (9), vegetables (3), bread (1), jam (1), other (2)
Alternariolmethyl-ether	14	9	9.4	650	Fruit (6), bread (2), nut (2), jam (1), other (3)
Chatoglobosin A	14	7	2100	210,000	Fruit (9), nut (2), other (3)
Ochratoxin A	12	7	260	17,000	Bread (2), vegetables (2), cheese (1), other (7)
Penitrem A	12	10	1900	7700	Bread (5), vegetables (3), fruit (2), other (2)
Deoxynivalenol-3-glucoside	9	6	24	99	Bread (9)
3-Nitropropionic acid	9	4	420	460,000	Vegetables (8), other (1)
Paxillin	9	8	69	700	Bread (3), fruit (2), vegetables (1), nut (1), other (2)
Sulochrin	9	3	170	1000	Fruit (3), other (6)
Sterigmatocystin	9	5	17	3000	Bread (4), cheese (2), vegetables (1), other (2)
Cytochalasin B	9	3	88	6000	Bread (5), other (4)
Ergine	8	3	3.4	18	Bread (8)
Fumonisin B2	6	2	120	29,000	Vegetable (4), bread (2)
Moniliformin	4	1	180000	230,000	Vegetable (4)
Fumonisin B3	4	1	310	450	Vegetable (4)
Fumonisin B1	4	1	2300	8500	Vegetable (4)
Avenacein Y	4	3	160	28,000	Fruit (2), bread (1), vegetables (1),
Elymoclavine	3	1	31	59	Bread (3)
Kojic acid	3	2	3600	5100	Fruit (2), vegetable (1)
Zearalenone	2	2		640	Bread (1), other (1)
Zearalenone-4-glucoside	1	1		62,000	Other (1)

[a] Number of positive sub-samples.
[b] Number of positive samples; in total, 87 mouldy food samples were investigated and were divided into 247 mouldy and non-mouldy sub-samples.

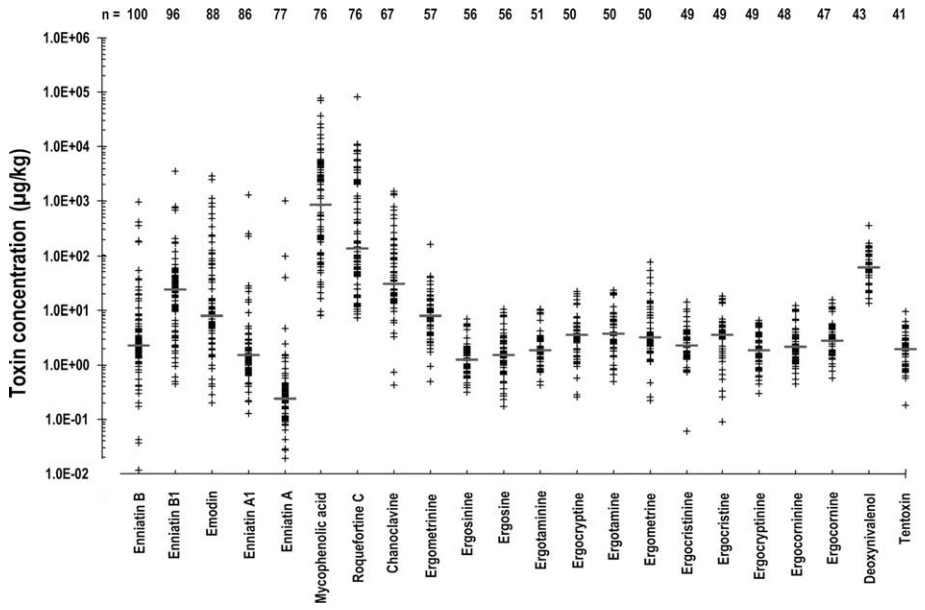

Fig. 1. Ranges of the observed mycotoxin concentrations in the investigated sub-samples (*n* = number of positive sub-samples).

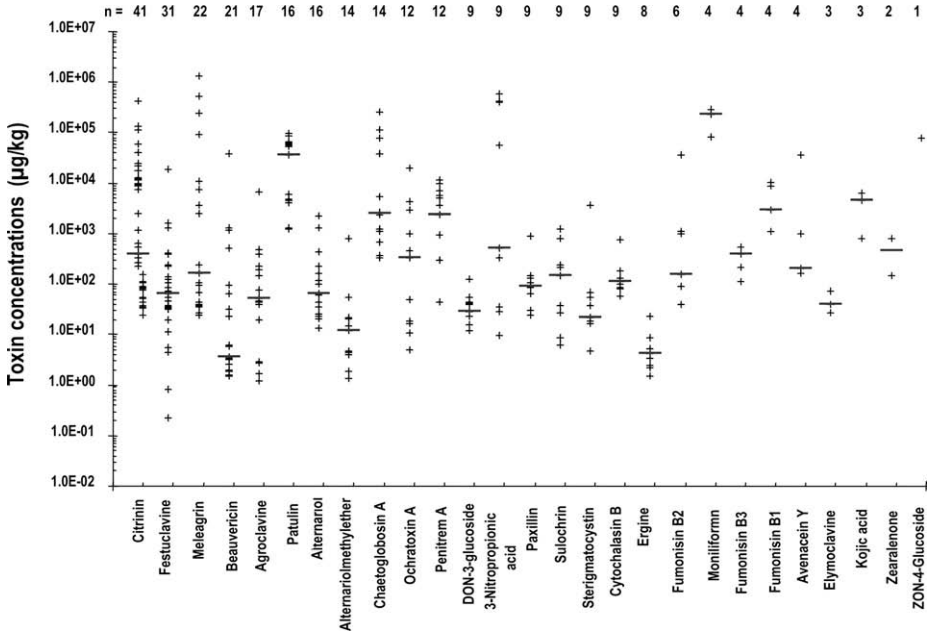

Fig. 2. Ranges of the observed mycotoxin concentrations in the investigated sub-samples (continued).

In a similar manner, dark and wholemeal bread and pastries were contaminated with each of the five ergopeptides investigated and with ergometrine (and with the corresponding epimers) at the low µg/kg-level, whereas the white bread samples and related products did not contain these toxins. Again, a homogenous distribution of the toxins between mouldy and non-mouldy spots was observed (see Fig. 3), confirming that the contamination originated from contaminated raw grain products. In contrast to the enniatins, the symptoms of ergot alkaloid intoxications are well known, yet there are no limits for their maximum allowed concentration in food. The highest concentration determined in this study was 200 µg/kg (sum of all ergot alkaloids) in a sample of dark bread. This is below the proposed guideline limit of 400–500 µg/kg for ergot alkaloids in cereals for human consumption, which has recently been discussed by Lampen and Klaffke (2006). However, as matrix effects were most pronounced for this substance class (see previous section), this limit was possibly exceeded in some samples. A potential health hazard deriving from continuous uptake of low levels of ergot alkaloids can therefore not be completely ruled out and as a consequence, further studies on their occurrence in grain products are required.

Beside enniatins and the ergot alkaloids, the compounds found most frequently were chanoclavine, emodin, mycophenolic acid and roquefortine C and their occurrence was less focused on bread and pastry samples. The occurrence of high concentrations of the latter two compounds in mouldy food and cheese is well described (Kokkonen et al., 2005; Rundberget et al., 2004). These two substances seem to be suitable markers for the infection of food by *Penicillium* species, as we observed concentrations of >100 µg/kg of other toxins without the co-occurrence of roquefortine C or

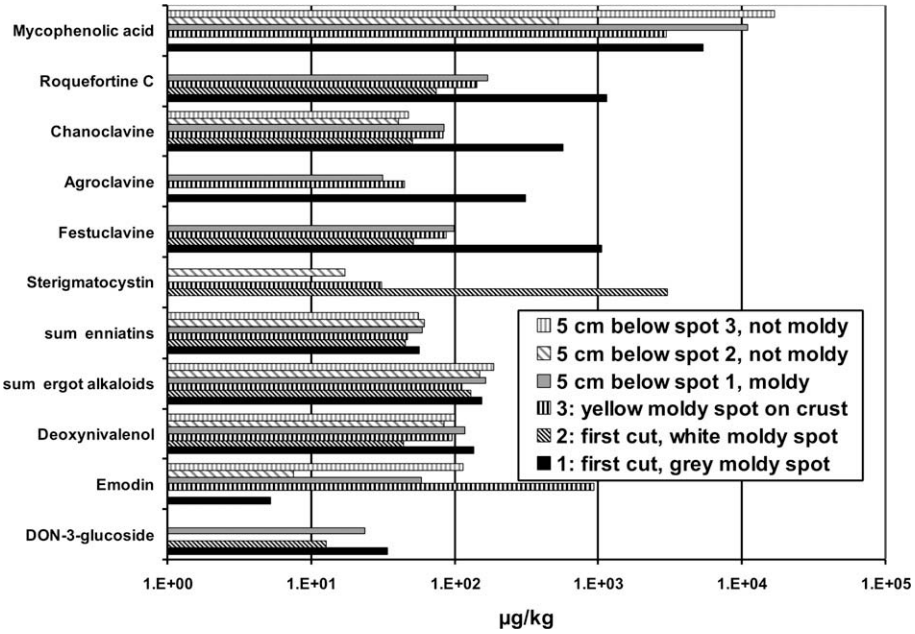

Fig. 3. Mycotoxin concentrations in different mouldy and non-mouldy sub-samples of a mouldy dark bread.

mycophenolic acid only in 6 of the 87 investigated samples. The high rate of positive findings of roquefortine C is not very surprising, as this secondary metabolite can be produced by almost the half of all species within the *Penicillium* subgenus *Penicillium*, which includes all five species that are considered to be the most important concerning infection of foodstuff (*P. carneum*, *P. expansum*, *P. griseofulvum*, *P. paneum*, *P. sclerotigenum*; Frisvad, Smedsgaard, Larsen, & Samson, 2004). At the same time, mycophenolic acid and roqueortine C exhibited concentrations >1000 µg/kg in 38 and 24 sub-samples, respectively, which certainly poses a hazard to the human and animal health: mycophenolic acid is a potent immunosuppressive compound used for several applications. Moreover, several cases of intoxication of animals have been ascribed to roquefortine C, although the actual toxicity of this compound may be comparatively low (Nielsen et al., 2006). For all these reasons, we think that roquefortine C and mycophenolic acid are among the most relevant toxic fungal metabolites in context of mouldy food and therefore advocate their inclusion in the list of target substances in multi-mycotoxin methods.

As considers emodin and chanoclavine, the observed concentrations were generally lower in comparison to roquefortine C and mycophenolic acid and exceeded 100 µg/kg only in 15 (emodin) and 16 sub-samples (chanoclavine), respectively. High concentrations of chanoclavine (>1000 µg/kg) have been found in two sub-samples of two different bread samples and in one sub-sample of apple puree. Nevertheless, this metabolite has to our knowledge not been reported yet in the context of naturally contaminated food. The fact that chanoclavine can be produced by several *Penicillium* species (Frisvad et al., 2004) in addition to *Claviceps* species might explain its higher rate of occurrence in comparison to the other ergot alkaloids. Contamination of nuts by emodin has been reported in context with infection by *Aspergillus ochraceus* (Overy et al., 2003), but this genotoxic compound is also an endogenous metabolite of vegetables and herbs (Müller et al., 1999). In the latter study, emodin concentrations of up to 700 µg/kg were found in dry beans, but the authors concluded that the mutagenicity of such substances taken up with vegetables is more than compensated by protective effects deriving from other plant constituents. It can therefore not be ruled out that in some cases, low concentrations of emodin that we found in vegetables or fruits did not originate from fungal infection. However, the highest concentrations of emodin were found in sub-samples of dark bread (2900 µg/kg) and sausage (780 µg/kg) that were infected by yellow moulds and the compound was not homogeneously distributed between the sub-samples in theses cases (see Fig. 3).

Among the mycotoxins, for which regulated concentrations exist, deoxynivalenol occurred most frequently (43 sub-samples of 13 different samples). Similar to the enniatins, bread was almost exclusively affected and there was no significant difference in deoxynivalenol concentrations between mouldy and non-mouldy spots of the same food sample. The concentrations in all the 43 positive sub-samples were below the EU-regulated value for bread and pastries, which is 500 µg/kg (Commission Regulation 1881/2006/EC). In contrast to that, patulin, ochratoxin A, fumonisins B1 and B2 and zearalenone occurred less often, but they were above the corresponding maximum permitted levels in all sub-samples. In some cases, the discrepancy between these limits and the actual concentrations was more than three orders of magnitude, e.g. 48,000 and 43,000 µg/kg patulin, respectively, were found in two sub-samples of the same apple sample, which is far above the limit of 25 µg/kg set for solid apple products. In the directive 1881/2006/EC, these limits are not explicitly stated for most of the food matrices that we found to be contaminated, e.g. fumonisins in garlic (1000–9000 µg/kg as sum of fumonisin B1 and B2), ochratoxin A in crème fraiche (300–16,000 µg/kg) and bread rolls (300–1600 and 2000 µg/kg, respectively; these levels are much higher than the concentrations of up to 14 µg/kg that have been found in naturally contaminated jam samples by Ruhland, Engelhardt, & Wallnöfer, 1998), patulin in other fruits (up to 80,000 µg/kg in cranberry) and spreading (1100 µg/kg).

Concerning the remaining toxins, some of them have already been detected in naturally contaminated food by other researchers and generally, our findings are in agreement with those results. Tentoxin has been previously identified in mouldy tomatoes (Andersen & Frisvad, 2004), whereas in our study, it occurred mainly in bread samples at concentrations below 10 µg/kg. Citrinin was reported to occur at the mg/kg level in rotten spots of apples (Martins, Gimeno, Martins, & Bernardo, 2002). In our study, different samples of bread and fruits and all five sub-samples of the investigated sample of crème fraiche were contaminated at the mg/kg level with two sub-samples of two

different bread samples exceeding 100,000 μg/kg. Traces of beauvericin have been found in Finnish grain samples (Jestoi et al., 2004), whereas we identified this compound at the μg/kg level in several types of food, with concentrations of up to 30,000 μg/kg occurring in sub-samples of one garlic and one onion sample. Alternariol and alternariolmethlyether were reported to occur at the mg/kg level in olives (Visconti, Logrieco, & Bottalico, 1986) and in fruits (Logrieco, Visconti, & Bottalico, 1990) and these mutagenic metabolites were also identified in mouldy tomatoes (Andersen & Frisvad, 2004). We identified these two substances in several fruits (strawberry, cranberry, pear, blackberry) and in paprika and tomato, but the concentration of alternariol and alternariolmethylether exceeded 100 μg/kg only in 5 and 1 sample, respectively. Chaetoglobosins were identified in several naturally infected fruits (Andersen et al., 2004) and chestnuts (Overy et al., 2003), which agrees very well with the contaminated samples in our study. We observed remarkably high concentrations >30,000 μg/kg of this toxic, cytotoxic and teratogenic compound in five sub-samples of three different samples (chestnut, cranberry and pear), which is of high relevance for the human health as the fungal infection of mouldy chestnuts was sometimes not visible on the outer peel but rather in the cavity inside the nut. Several mg/kg of Penitrem A were found in food waste (Rundberget et al., 2004), which is similar to the concentrations we found in several sub-samples of bread, vegetables and cake. Such doses are probably toxicologically relevant, as penitrem A has been made responsible for human intoxication (Lewis, Donoghue, Hocking, Cook, & Granger, 2005), although that finding was based on the identification of the penitrem producer *Penicillium crustosum* without the determination of the toxin in the contaminated food. As deoxynivalenol-3-glucoside has been previously identified in naturally contaminated wheat (Berthiller et al., 2005), our observation of its occurrence in some sub-samples of bread indicates that the glucoside bond is not completely cleaved during the baking process. 3-nitropropionic acid was identified as etiologic agent of intoxications in China after consumption of mouldy sugar canes (Ming, 1995). We found this compound in high concentrations of 44,000–460,000 μg/kg in mouldy garlic, whereas in other samples, the contamination was less severe (<500 μg/kg). The genotoxic and tremorgenic metabolite paxilline has been identified in mouldy tomatoes (Andersen & Frisvad, 2004). In our study, sub-mg/kg concentrations were found in eight different samples, including bread, banana, tomato, apple puree, chestnut and apricot cake. The aflatoxin precursor sterigmatocystin has been reported to occur at the μg/kg level in hard cheese (Northolt, van Egmond, Soentoro, & Deijll, 1980), which was confirmed by our results: Several sub-samples of dark bread were contaminated in addition to cheese and bread roll, with one sub-sample exhibiting a concentration of 3000 μg/kg. The occurrence of moniliformin in the mg/kg-range has so far been associated with grains infected by *Fusarium* species (Jestoi et al., 2004). Therefore, the concentrations of up to 200,000 μg/kg that we found in all sub-samples of a clove of mouldy garlic are somewhat surprising, although the co-presence of fumonisins and beauvericin indicates an infection by a *Fusarium* species. Zearalenone-4-glucoside was detected in naturally contaminated wheat at levels ranging from 17 to 104 μg/kg (Schneweis, Meyer, Engelhardt, & Bauer, 2002). We found 62,000 μg/kg of this compound in one sub-sample of mouldy rice. Both the inhomogeneous distribution of the glucoside within this sample as well as its 100-fold excess compared to the concentration of zearalenone in the corresponding sub-sample cannot be reasonably explained, but its positive identification has been confirmed by an Enhanced Product Ion Scan.

3.3. Fungal metabolites detected for the first time in naturally contaminated food

The remaining fungal metabolites (festuclavine, agroclavine, elymoclavine, agroclavine, meleagrin, avenacein Y, kojic acid, cytochalasin B and sulochrin) have to our knowledge not been identified yet in naturally contaminated food that has been spontaneously spoiled during storage at the end consumer. Some of them were shown to be produced by fungal strains that had been isolated from mouldy food or were found in foodstuffs that had previously been inoculated with the respective moulds. Festuclavine and agroclavine were produced by different strains of *Penicillium* species isolated from silage (Nielsen et al., 2006). Similar to chanoclavine and unlike the ergopeptides, the inhomogeneous distribution of these compounds within the sub-samples of the contaminated samples confirms that this contamination originates from infection of the processed food by *Penicillium* species rather than from contamination of the raw cereals. Assuming a similar toxicity of these compounds in comparison to the ergopeptides (due to the common tetracyclic ergoline structure), the clavines seem to pose a greater potential health hazard since the observed median and maximum concentrations exceed those of the ergopeptides by one and two orders of magnitude, respectively (see Table 2), whereas the frequencies a well as the related concentrations of elymoclavine and ergine were much lower. The antibiotic metabolite meleagrin was found to be produced by *Penicillum atramentosum* on cheese agar (Larsen et al., 2002). We observed high concentrations in the range between 73,000–1,100,000 μg/kg in the sub-samples of the investigated garlic sample, whereas concentrations in the mg/kg range were found in five other sub-samples of four samples (apple puree, pesto, dark bread, crème fraiche). In case of apple puree and crème fraiche, the corresponding sub-samples were not taken from the food itself but rather from mouldy spots located on the inner container wall in which the food has been stored. Avenacein Y has been shown to be produced by strains of *Fusarium avenaceum* on autoclaved rice (Uhlig, Jestoi, Knutsen, & Heier, 2006). In contrast to that, we observed large concentrations in sub-samples of a cranberry (28,000 μg/kg) and of a tomato (790 μg/kg). Kojic acid has been described to be produced by various *Aspergillus* strains isolated from Egyptian silages (El-Shanawany, Eman Mostafa, & Barakat, 2005). We found 4000 and 5000 μg/kg of this compound on two sub-samples of the investigated strawberry sample, but these concentrations seem to be of no concern in view of the use of this metabolite as food additive and skin-whitening agent in cosmetics.

3.4. Toxin distribution within the samples

Non-mouldy sample spots were included in the analysis in order to investigate the distribution of the toxins within the sub-samples of a given sample and thus to determine, whether the contamination derived from the contamination of the ingredients (e.g. grain) which had been used for the production of the food (which should be reflected by a homogeneous distribution of the toxin between mouldy and non-mouldy spots) or from the infecting fungi. Even in the latter case, it cannot be expected that the toxin is exclusively located at mouldy spots: on the one side, the mycelium of the fungus may be considerably larger than suggested by its visual appearance. On the other side, some secondary metabolites are excreted by the fungus (Filtenborg et al., 1996) e.g. to fight against bacterial or other fungal competitors, which implies in the present case that at least the part of the foodstuff surrounding the mycelium is contaminated.

Fig. 3 presents the concentrations of the toxins that were identified in four mouldy and two non-mouldy sub-samples of one sample of dark bread. The distribution of deoxynivalenol, peptide ergot alkaloids and enniatins is more or less homogeneous, as these fungal metabolites were obviously produced due to fungal infection of the grain in the field. The distribution of the other analytes was more inhomogeneous: chanoclavine was present in all sub-samples with an increased concentration in mouldy spot 1,

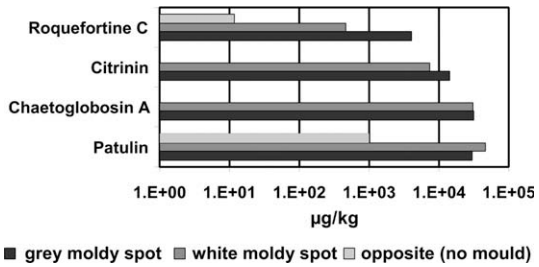

Fig. 4. Mycotoxin concentrations in different mouldy and non-mouldy sub-samples of a mouldy pear.

roquefortine C and festuclavine were present in all mouldy but not in the non-mouldy sub-samples, agroclavine and deoxynivalenol-3-glucoside occurred in some of the mouldy but not in the non-mouldy sub-samples (the concentration of the latter compound was close to the respective LOD). Emodin, sterigmatocystin and mycophenolic acid were found in some mouldy as well as in some non-mouldy sub-samples. This suggests that these three substances as well as chanoclavine were released by the fungus into the surrounding food matrix. Interestingly, the observed concentrations of mycophenolic acid were higher at the non-mouldy spots than in the mouldy parts of the sample. Another example is presented in Fig. 4, which shows the concentrations of the toxins that were identified in two mouldy and in one non-mouldy spot of the investigated pear sample. Whereas chaetoglobosin A and citrinin were exclusively present in the sub-samples taken from the mouldy spots, considerable amounts of patulin and traces of roquefortine C were found in the non-mouldy sub-sample. It shall be noted that for patulin, the observed concentration of 1000 µg/kg is still a factor of 40 above the legislative levels set for solid apple products. However, in some other samples the distribution of the toxin concentration correlated very well with the fungal infection (no mycotoxin was detected in the non-mouldy sub-samples). In view of this, a more systematic study seems to be required (e.g. including isolation and identification of the related fungi as well as sampling of a larger number of sub-samples per sample taken from standardised distances from the mouldy spots, as mycotoxin concentration was shown to decrease with increasing distance, Rychlik & Schieberle, 2001) in order to draw general conclusions. It might be speculated that the diffusion of toxic fungal metabolites into the surrounding foodstuff depends on the toxin itself (e.g. its water solubility), on the texture of the food matrix (in compact foods which have a high water content such as fruits the diffusion of toxins can be relatively fast leaving no part uncontaminated (Filtenborg et al., 1996; Rychlik & Schieberle, 2001) and on the physiological state of the fungus (e.g. presence of competitors, degree of availability of carbon and nitrogen). Nevertheless, we think on the basis of the preliminary data presented in this work that it is not safe to remove the visibly infected part of food and consume the remainder or feed it to animals.

4. Conclusions

The presented results clearly show the benefits of our multi-mycotoxin method, as 49 different fungal metabolites were identified in the 87 investigated samples. Background concentrations in the µg/kg-range in (dark) bread were revealed as well as huge concentrations in the 1,000,000 µg/kg-range of some toxins at mouldy spots. In addition, mycotoxins such as festuclavine, agroclavine, elymoclavine, meleagrin, avenacein Y, kojic acid, cytochalasin B and sulochrin, which have not been reported so far in context with food samples spoiling during storage at the end consumer, have been described. Due to their frequency and comparatively large concentrations, roquefortine C and mycophenolic acid seem to be suitable marker substances for *Penicillium* species, which obviously dominated the samples investigated in this study.

As concerns the relevance for a typical consumer, we think that the occurrence of enniatins and ergot alkaloids at µg/kg-levels in bread samples raises the biggest concern, as this contamination seems to originate from contaminated grain and can therefore not be avoided through a careful visual inspection of the related foodstuff. However, too little is known about the toxicological impact of such low concentrations (especially in case of the enniatins) and therefore, further studies on their toxicity as well as on their occurrence are required. Considering the other toxins, the observed concentrations certainly represent a hazard for the human and animal health, but most of these samples would have probably never been consumed, as the fungal infection was clearly visible (there are exceptions though, such as the mould growing in the interior cavity of the chestnut). We definitely advise against removing the mouldy part of a food and consuming the remainder, as in some cases, diffusion of the toxins from the mouldy spots into the surrounding food matrix was observed. The rate of diffusion might be very small in some foodstuffs, but in view of the very large concentrations that were found at some mouldy "hot spots", the occurrence of toxicologically relevant concentrations in other parts of the food product cannot be excluded.

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