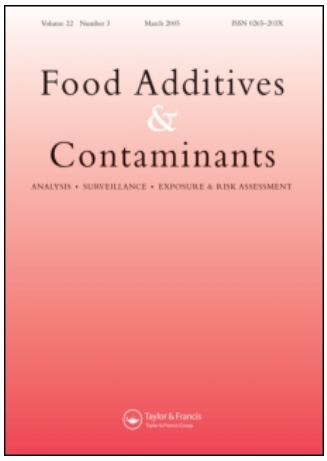

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Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry

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Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry

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Abstract
A simple and feasible method is described for analysing nine mycotoxins in cheese matrix. The method involves liquid extraction followed by high performance liquid chromatographic separation and mass spectrometric detection of the analytes, and allows the determination of aflatoxins B1, B2, G1, G2 and M1, ochratoxin A, mycophenolic acid, penicillic acid and roquefortine C simultaneously. Average recoveries of the mycotoxins from spiked samples at concentration levels of 5–200 $\mu g\,kg^{-1}$ ranged from 96–143%. Within-day relative standard deviations at these concentration levels varied from 2.3–12.1%. The limit of quantification for aflatoxin M1 was 0.6 $\mu g\,kg^{-1}$ and for the other compounds 5 $\mu g\,kg^{-1}$. The method developed was applied for analysing these mycotoxins in blue and white mould cheeses purchased from Finnish supermarkets. Roquefortine C was detected in all of the blue mould cheese samples in concentrations of 0.8–12 $mg\,kg^{-1}$. One blue cheese contained also 0.3 $mg\,kg^{-1}$ mycophenolic acid. The other investigated mycotoxins were absent in the samples.

Keywords: *LC-MS/MS, mould cheese, mycotoxins*

Introduction

Mycotoxins are toxic secondary metabolites produced by many species of fungi. The presence of these substances in dairy products can have two origins: (1) Indirect contamination, which results from lactating animals ingesting contaminated feed and (2) Direct contamination, which occurs because of intentional or accidental growth of moulds on dairy products (van Egmond 1989). Some of the fungal starters used in the cheese manufacture have been shown to produce various mycotoxins on culture media. For example, *Penicillium camemberti*, which is used in the production of surface ripened white mould cheeses (Engel and Teuber 1989), is able to produce cyclopiazonic acid (Frisvad and Filtenborg 1983). *P. roqueforti*, widely used as a starter in the blue mould cheeses (Engel and Teuber 1989), is known to synthesise roquefortine C, PR-toxin, mycophenolic acid and isofumigaclavins (Samson and Frisvad 2004). Erdogan et al. (2003) reported also penicillic acid and patulin production of *P. roqueforti* strains isolated from blue cheese. However, according to the present knowledge *P. roqueforti* do not synthesize these metabolites, but the closely related *P. carneum* and *P. paneum* do (Boysen et al. 1996, Samson and Frisvad 2004). Additionally, other fungal species may also be used in the mould cheese manufacture (Lund et al. 1998; Finoli et al. 2001; Marcellino et al. 2001). It is also likely that in natural ripening processes fungal species contribute that are not characterized.

Despite the ability of fungal starters to produce different toxins on culture media, findings in cheeses have been mainly restricted to cyclopiazonic acid in white mould cheeses (Schoch et al. 1984; Le Bars 1990) and roquefortine C and mycophenolic acid in blue cheeses (Schoch et al. 1984; López-Díaz et al. 1996; Finoli et al. 2001). Scott and Kennedy (1976) detected isofumigaclavines A and B occurring in blue cheeses together with roquefortine C. However, Schoch et al. (1984) could not find patulin, PR-toxin or penicillic acid in blue cheeses. Similarly, no PR-toxin was detected in Gorgonzola cheese in the study by Finoli et al. (2001).

Other mycobiota besides starters may be present in mould cheeses as contaminants, since various fungi are encountered in cheese factory environments (Lafont et al. 1990; Lund et al. 1995) and some of these organisms can be potential mycotoxin producers. There are not many reports indicating the occurrence of mycotoxins due to contaminating

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fungi in mould cheeses. Lafont et al. (1990) detected penicillic acid in one blue cheese sample of dubious organoleptic properties, indicating this might be because of contaminating fungi rather than actual starters. The presence of mycotoxins in dairy products is suggested to be related more to environmental factors causing mould growth than to ingestion of mouldy feed by cows (Lafont et al. 1990). However, aflatoxin M1 is sometimes found in cheese (Piva et al. 1987; El-Sayed et al. 2000) as a result of lactating animals having consumed aflatoxin B1 contaminated feed.

A number of analytical methods have been published to examine mycotoxins in a cheese matrix. Many of the methods have been developed to extract and determine toxins individually by thin layer chromatography (TLC) (Le Bars 1990; Finoli et al. 2001) or high performance liquid chromatography (HPLC) (Finoli et al. 2001; Zambonin et al. 2002) combined with UV or fluorescence detection. In some of the methods several compounds have been extracted together and analysed subsequently by TLC (Siriwardana and Lafont 1979; Schoch et al. 1984). In this kind of multi-toxin methods, where many compounds need to be extracted and analysed simultaneously, high selectivity and sensitivity is required from the method used. To achieve this, liquid chromatographic–mass spectrometric (LC-MS) or tandem mass spectrometric (LC-MS/MS) methods have been developed also for mycotoxin applications recently. Most of the methods are for grains or maize (Razzazi-Fazeli et al. 2003; Berthiller et al. 2005), and there are still very few published multi-toxin methods for more complex food matrices like cheese. Rundberget and Wilkins (2002) reported an LC-MS/MS method for analysing 13 fungal metabolites in a food mixture.

In this study, a simple method comprising a single liquid extraction and LC-MS/MS determination was developed for analysing simultaneously nine mycotoxins (see Figure 1) that may be found occurring in cheese from different sources. Ochratoxin A and aflatoxins B1, B2, G1 and G2 would most likely originate from contaminating fungi in the cheese factory or storage (Bullerman 1980). Aflatoxin M1 is excreted in milk if dairy animals have ingested feed containing aflatoxin B1 (van Egmond 1989). Mycophenolic acid and roquefortine C occur in blue cheeses due to metabolism of fungal starters (Finoli et al. 2001) and penicillic acid may be produced by starters or by contaminating fungi (Bullerman 1980; Lafont et al. 1990; Erdogan et al. 2003). The method is applied to investigate the occurrence of these mycotoxins in blue and white mould cheeses purchased from Finnish supermarkets.

Materials and methods

Samples and blank matrix

Ten blue and ten white mould cheeses and one blue-white mould cheese of different manufactures were purchased from the Finnish supermarkets in December 2003. Products with high market shares were chosen for the study, and as many Finnish products as possible were included. The samples were analysed in duplicate, and they are presented in more detail in Table I. A cheese analogue was used as a blank matrix for method validation and for matrix assisted calibration curves. The cheese powder for preparing the analogue was received from Biocentrum-DTU, Denmark.

Reagents

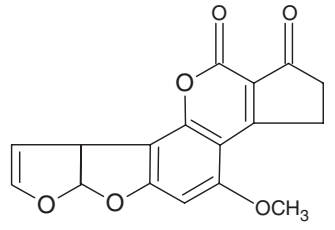
Standards for the mycotoxins were purchased from Sigma (St Louis, MO, USA) except for the standard of aflatoxin M1, which was a BCR Reference Material 423 (European Commission, Brussels, Belgium). The stock solutions were prepared in acetonitrile or methanol and further dilutions in 50% acetonitrile. Acetonitrile, methanol and hexane were of HPLC grade (J.T Baker, Deventer, Holland), and acetic acid (J.T Baker, Deventer, Holland) and formic acid (Merck, Darmstadt, Germany) of analytical grade.

Sample preparation

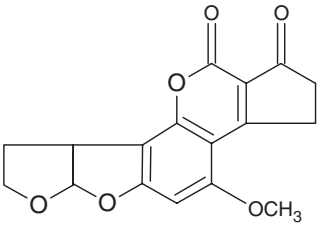
The sample preparation procedure was a slight modification of the method of Rundberget and Wilkins (2002). Ten grams of cheese were weighed and 60 ml of acetonitrile (containing 0.1% formic acid) and 50 ml of hexane were added. The sample was homogenized by Ultra Turrax homogenizer (IKA-Werke, Staufen, Germany) for five minutes and centrifuged (4000 rpm, 10 min, +10°C) (Beckman J2-21 M, Palo Alto, CA, USA). The sample was filtered through an S & S 2329 filter paper (Schleicher and Schuell, Dassal, Germany) and a 10 ml portion of the acetonitrile phase was evaporated to dryness under a stream of nitrogen at +50°C. The residue was dissolved in 0.2 ml of methanol and filtered through a 0.2 μm syringe filter (Pall Gelman Sciences, Ann Arbor, MI, USA) into an autosampler vial.

LC-MS/MS conditions

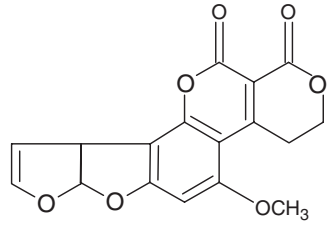
The liquid chromatography was performed with Alliance 2960 Separations Module (Waters, Milford, MA, USA), by injecting 10 μl of the sample on a Symmetry C18 column (2.1 × 100 mm, 3.5 μm) and a guard column of the same phase



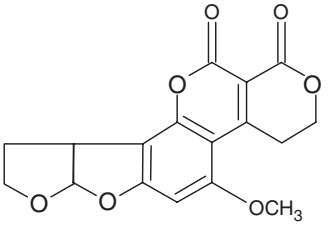
Aflatoxin B1



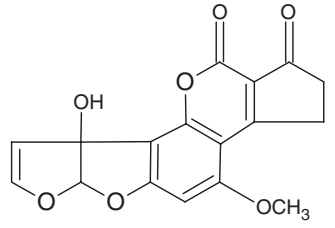
Aflatoxin B2



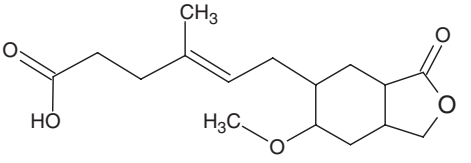
Aflatoxin G1



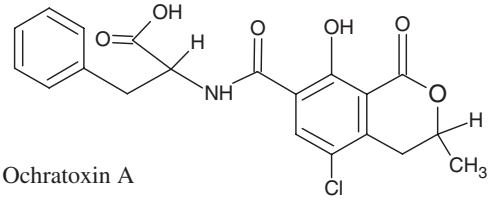
Aflatoxin G2



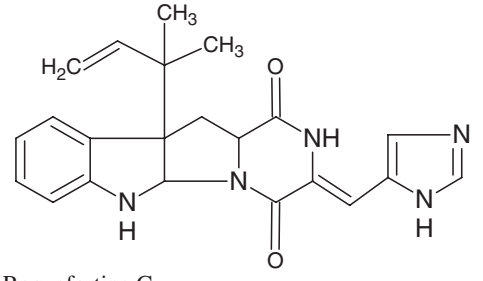
Aflatoxin M1



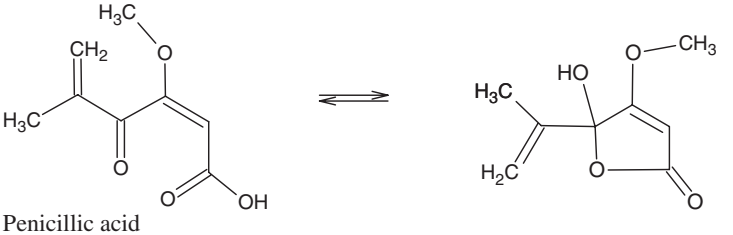
Mycophenolic acid



Ochratoxin A



Roquefortine C



Penicillic acid

Figure 1. Structures of the mycotoxins examined in the mould cheese samples.

Table I. Mould cheese samples examined and the mycotoxin concentrations determined.

Sample number	Sample type	Country of origin	Roquefortine C ($mg\,kg^{-1}$)	Mycophenolic acid ($mg\,kg^{-1}$)
1	Blue mould	Finland	1.8	Nd
2	Blue mould	Finland	2.2	Nd
3	Blue mould	Denmark[a]	5.6	nd
4	Blue mould	Denmark	4.6	nd
5	Blue mould	Denmark	2.5	nd
6	Blue mould	Germany[b]	2.0	nd
7	Blue mould	Germany[a]	1.6	nd
8	Blue mould	Germany	0.9	nd
9	Blue mould	France	12	nd
10	Blue mould	France	2.7	0.3
11	Blue–white mould	Denmark	0.8	nd
12	White mould	Finland	nd	nd
13	White mould	Denmark	nd	nd
14	White mould	Denmark	nd	nd
15	White mould	France[a]	nd	nd
16	White mould	France[a]	nd	nd
17	White mould	France	nd	nd
18	White mould	France	nd	nd
19	White mould	France	nd	nd
20	White mould	France	nd	nd
21	White mould	France	nd	nd

[a]sold under Finnish brand name; [b]sold under private label; nd = not detected.

Table II. Retention times, ions monitored and mass spectrometric parameters for the mycotoxins examined.

Mycotoxin	Retention time (min)	Molecular ion $[M+H]^+$	Quantitation ion	Confirmatory ion	Cone voltage (V)	Collision energy (eV)	Ion ratio
Penicillic acid	3.5	171	125	97	11.0	17.0	1.5
Roquefortine C	4.2	390	193	322	25.0	35.0	15.0
Aflatoxin M1	4.7	329	273	301	30.0	18.0	4.0
Aflatoxin G2	6.4	331	245	313	30.0	35.0	1.7
Aflatoxin B2	8.2	315	259	286	25.0	35.0	6.0
Aflatoxin G1	8.6	329	243	311	35.0	24.0	2.3
Aflatoxin B1	11.3	313	286	270	35.0	20.0	2.8
Mycophenolic acid	27.0	321	207	304	10.0	16.0	110.0
Ochratoxin A	31.9	404	358	239	10.0	16.0	7.4

(Waters, Milford, MA, USA). Separation was achieved using a gradient elution with the flow rate of 0.2 ml/min. The initial conditions were water–acetonitrile (75:25) (both containing 0.1% acetic acid), which were held for 16 min, then changed to water–acetonitrile (10:90) for 24 min. The column was conditioned with 25% acetonitrile for nine minutes before the next injection.

The HPLC system was coupled to a MicroMass Quattro Micro triple-quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an electrospray ionization (ESI) probe operating in the positive mode. Multiple reaction monitoring (MRM) mode was used for identification and quantification of the compounds. Protonated molecular ions $[M+H]^+$ of the analytes were fragmented within the collision cell to their product ions, which were monitored for a dwell time of 0.40 s with an inter-channel delay of 0.05 s. Parameters of the MS were optimized for the quantitation ions using standard solutions in acetonitrile containing 0.1% of acetic acid. The same parameters were used for the confirmatory ions. The best responses were recorded with capillary voltage of 3.33 kV and source and desolvation temperatures of 120 and 300°C, respectively. The retention times of the analytes, the molecular and product ions monitored and additional mass spectrometric parameters are presented in Table II.

As the sample matrix often causes suppression of the analyte response in LC-MS methods (Gilar et al. 2001), the calibrants were prepared in blank matrix. For preparing matrix assisted calibration curves, 10 g portions of blank material were spiked with each mycotoxin to achieve the concentrations of 5, 50, 100, 500 and 1000 $\mu g\,kg^{-1}$ for all the

compounds, except for aflatoxin M1 which was at concentrations of 0.5, 5, 10, 50 and 100 $\mu g\,kg^{-1}$. After spiking, the calibrants were prepared and analysed like the other samples. The linearities of the calibration curves were tested with the method of van Trijp and Roos (1991) and proved to be acceptable within the concentration range used. If a mycotoxin concentration of a sample exceeded the highest calibrant, the sample extract was diluted with the blank matrix extract to give a total volume of 10 ml.

Method performance parameters

Method performance was evaluated by determining recovery, within-day repeatability (RSD%), limit of detection (LOD) and limit of quantification (LOQ). Recoveries and repeatabilities were determined for aflatoxin M1 using spiked samples (preparation same as for the matrix assisted calibrants) at levels of 5 and 20 $\mu g\,kg^{-1}$ ($n=5$) and for the other analytes at levels of 50 and 200 $\mu g\,kg^{-1}$ ($n=5$). LOD was calculated on the basis of signal-to-noise ratio of 3:1 by using matrix assisted calibrants of the lowest concentration. LOQ was two times the value of LOD.

Results and discussion

Method performance

The method developed was simple and proved to be suitable for analysing simultaneously nine mycotoxins in cheese matrix. Especially, the sample preparation step was feasible, considering the complexity of the matrix. The samples were dissolved in methanol before the LC-MS analysis, although that probably had a negative effect on the shape of some of the peaks. Since some fat or emulsifying agents originating from the matrix were still present in the samples at the final stage, methanol was chosen as it could also dissolve the lipids and a homogenous sample could be obtained. When some other solvents closer to the mobile phase were tested, two phases were formed in the sample. The separation with LC and the detection with MS were satisfactory for all the analytes (see Figure 2). Cyclopiazonic acid was initially intended to be included in the analysis, as it has often been detected in white mould cheeses. However, the liquid chromatographic behaviour and the mass spectrometric detection of this compound were not satisfactory and no linear calibration curves or repeatable results could be obtained.

The average recoveries for aflatoxin M1 were 129% (at 5 $\mu g\,kg^{-1}$) and 96% (at 20 $\mu g\,kg^{-1}$), and the corresponding within day repeatabilities (RSDs) were 3.6 and 6.7%. The recoveries for the rest of the compounds varied from 102–143% (at 50 $\mu g\,kg^{-1}$) and from 96–119% (at 200 $\mu g\,kg^{-1}$). The RSDs at these concentration levels were 2.3–8.2% and 5.4–12.1%, respectively. The detailed results are presented in Table III.

The recoveries and the repeatabilities at both concentration levels were satisfactory for all the compounds, as the recoveries were all greater than 96% and the RSDs lower than 12.1%. Unexpectedly, the repeatability for all the analytes was better at the lower spiking level (5 or 50 $\mu g\,kg^{-1}$) compared to the higher level (20 or 200 $\mu g\,kg^{-1}$). The recoveries for most of the compounds were higher at the lower spiking level, where most of the recoveries were quite well above 100%. However, as the method was repeatable and selective, this was not considered as a problem.

The LODs calculated for the analytes were: For aflatoxin M1, mycophenolic acid and ochratoxin A 0.3 $\mu g\,kg^{-1}$, for roquefortine C 0.4 $\mu g\,kg^{-1}$, for aflatoxins B1, B2, G1 and G2 0.8 $\mu g\,kg^{-1}$ and for penicillic acid 2.0 $\mu g\,kg^{-1}$. The LOQs were 0.6 $\mu g\,kg^{-1}$, 0.8 $\mu g\,kg^{-1}$, 1.6 $\mu g\,kg^{-1}$ and 4.0 $\mu g\,kg^{-1}$, respectively. Although lower concentrations of the analytes could be quantified with the method, for practical reasons the LOQs were allocated to the lowest points of the calibration curves. Thus LOQs were 5 $\mu g\,kg^{-1}$ for all the compounds except for aflatoxin M1, for which 0.6 $\mu g\,kg^{-1}$ was used as LOQ.

Mycotoxins in cheese samples

All the blue cheeses, including the blue–white mould cheese, contained roquefortine C from 0.8–12 $mg\,kg^{-1}$. Mycophenolic acid was also found (0.3 $mg\,kg^{-1}$) in one of the blue cheeses. Other investigated mycotoxins were not detected in the samples. The mycotoxin concentrations of the cheeses are presented in Table I. The results were not corrected for recovery. A chromatogram of the cheese sample no. 10 with the peaks of roquefortine C and mycophenolic acid is presented in Figure 3.

The roquefortine C (0.8–12 $mg\,kg^{-1}$) and mycophenolic acid (0.3 $mg\,kg^{-1}$) concentrations found in the samples were mostly in accordance with the earlier studies. Schoch et al. (1984) reported roquefortine C at levels from 0.2–2.29 $mg\,kg^{-1}$ and Finoli et al. (2001) levels from 0.05–1.47 $mg\,kg^{-1}$ in various blue cheeses. Compared to these results, the concentration of 12 $mg\,kg^{-1}$ roquefortine C in one of the French blue cheeses is quite high. In the study of Zambonin et al. (2002), the estimated concentrations of mycophenolic acid were between 0.1 and 0.5 $mg\,kg^{-1}$, and Lafont et al. (1990)

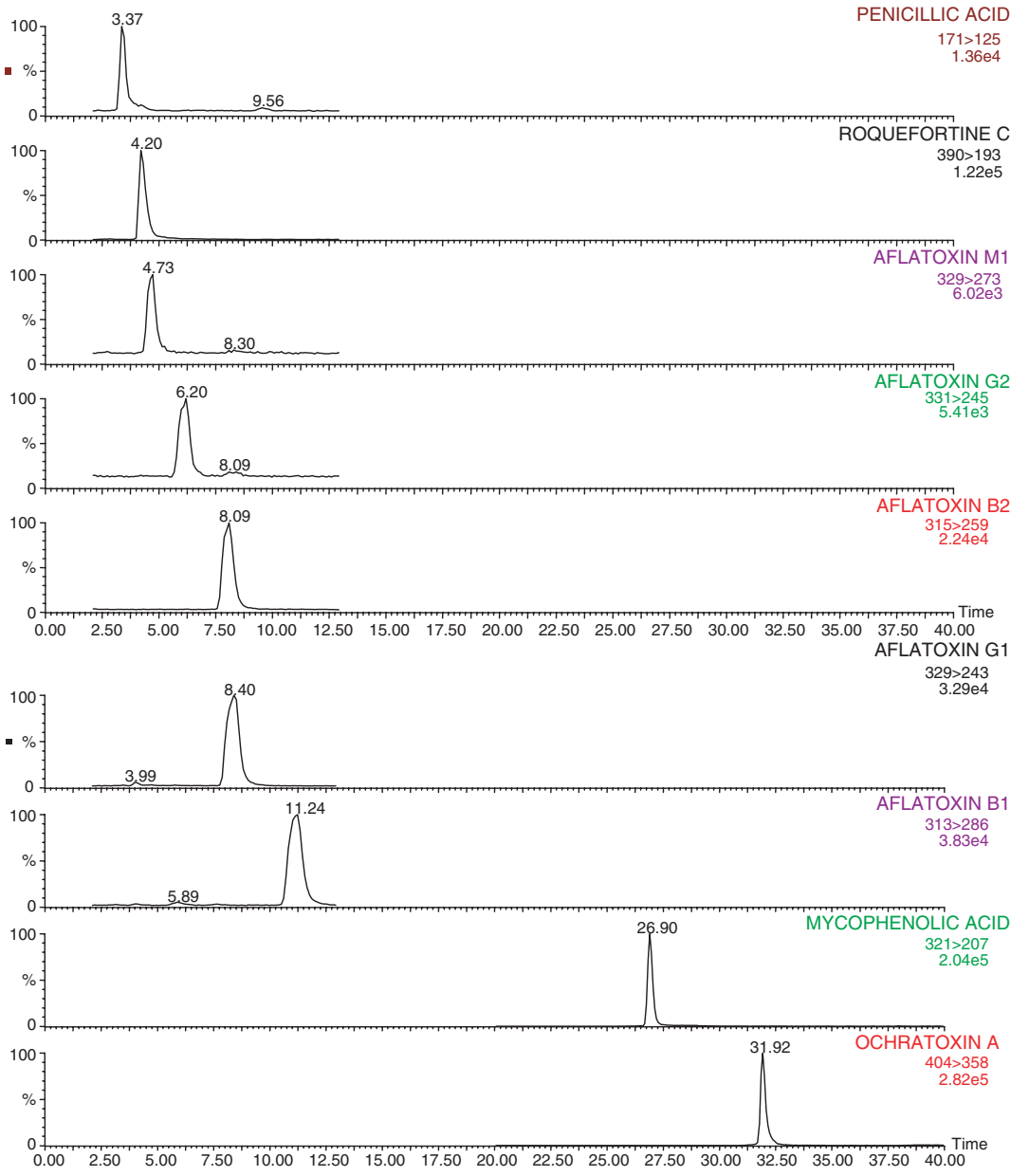


Figure 2. LC-MS/MS chromatogram of a matrix assisted calibrant 50 μg/kg (for aflatoxin M1 5 μg/kg). Peaks are from the quantitation ion transitions.

Table III. Recoveries and repeatabilities of the analytes at two spiking levels ($n = 5$).

	50 μg kg^{-1}		200 μg kg^{-1}	
Mycotoxin	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Penicillic acid	109	8.2	109	5.4
Roquefortine C	102	3.0	119	10.9
Aflatoxin M1	129[a]	3.6[a]	96[b]	6.7[b]
Aflatoxin G2	129	3.1	101	12.1
Aflatoxin B2	143	3.5	110	9.6
Aflatoxin G1	130	3.6	104	10.9
Aflatoxin B1	132	2.3	103	10.7
Mycophenolic acid	135	3.1	96	6.5
Ochratoxin A	105	2.5	100	5.9

[a]at 5 μg kg^{-1}; [b]at 20 μg kg^{-1}.

detected 0.01–15.0 mg kg^{-1} mycophenolic acid in blue cheeses. As only one blue cheese sample contained a detectable amount of mycophenolic acid, it is evident that this toxin can be avoided with the selection of the starter strain, as proposed also by Lafont et al. (1990).

The existing data indicates that the toxicities of both roquefortine C and mycophenolic acid are relatively low (Wilson 1971; Arnold et al. 1978; Kopp and Rehm 1979; Bentley 2000). Roquefortine C has been reported as a neurotoxin (Wagener et al. 1980), which is questionable as blue mould cheese has been eaten for a long time without any reports of harmful effects on humans (Bullerman 1981). On the basis of the present toxicity data and the

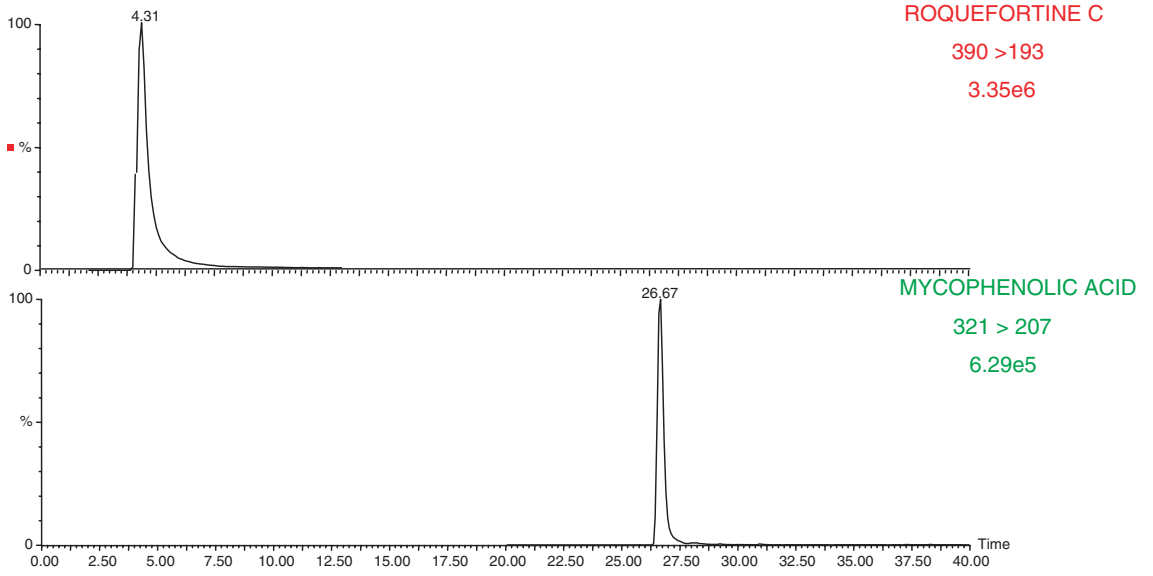


Figure 3. Chromatogram of the blue mould cheese sample number 10 showing the peaks of roquefortine C and mycophenolic acid extracted from the quantitation ion transitions.

presumed consumption of blue mould cheese, the amounts of these compounds determined in the samples are not hazardous to consumers (Teuber and Engel 1983; Finoli et al. 2001).

No other mycotoxins were found in the cheese samples. This is likely to be due to good manufacturing practises applied in the cheese production. The raw materials, the manufacturing processes and the storage conditions are all strictly controlled in order to avoid mould contamination and mycotoxin formation (Bullerman 1981; Engel and Tauber 1989). Additionally, toxigenic moulds are believed to represent only a minor proportion of the total flora in cheese and cheese factory environments (Bullerman 1980). Furthermore, cheese is not necessarily a good substrate for mycotoxin formation and some mycotoxins, like patulin, penicillic acid and PR-toxin, have been shown to be unstable in the cheese matrix (Lieu and Bullerman 1977; Scott and Kanhere 1979).

Conclusion

The LC-MS/MS method developed was suitable for analysing simultaneously several mycotoxins in a complex cheese matrix. The method performance, as evaluated by recovery and repeatability, was satisfactory for the determination of the nine toxins examined. However, the method needs further development if cyclopiazonic acid or other compounds need to be included in the analysis. Roquefortine C and mycophenolic acid were detected in the blue cheeses, but other mycotoxins were absent.

Acknowledgements

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