Mycotoxins in maize silage - detection of toxins and toxicological aspects

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PhD thesis

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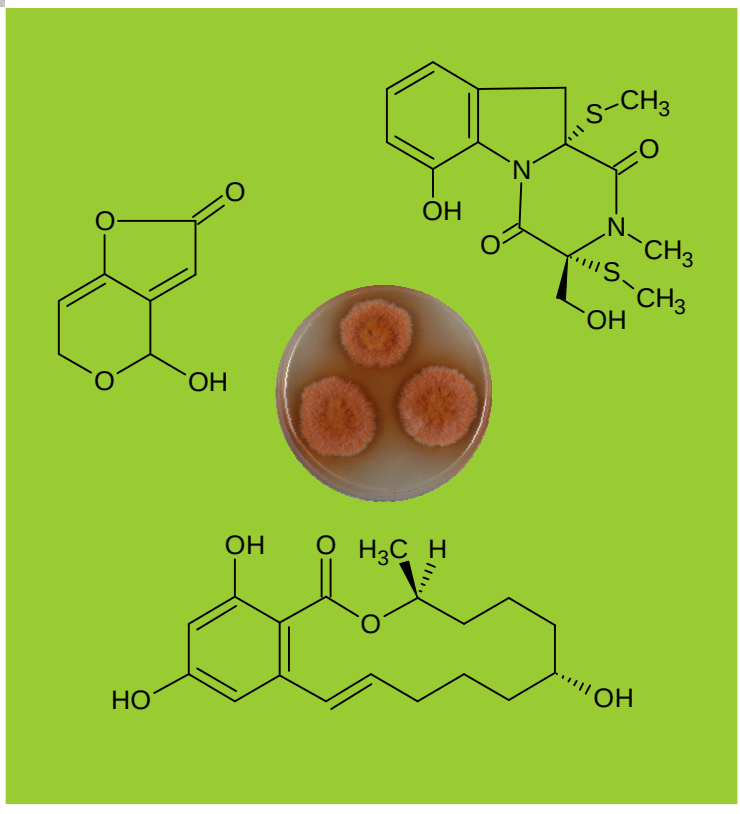

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PREFACE

The Danish production of maize silage for dairy cattle feed has increased exponentially since the 1990's. At the same time, an increase of illness among cows has been observed. This issue has raised a concern regarding mycotoxins in maize silage. In order to examine the problem a joint project "Mycotoxin carry-over from maize silage via cattle into dairy products" was initiated in 2005. National experts from the Technical University of Denmark, Aarhus University, Danish Agricultural Advisory Service, the Danish Cattle Federation and the Danish Plant Directorate have participated. The purpose has been to ascertain whether mycotoxins in maize silage may cause illness and ill-thrift in dairy cattle and whether mycotoxins in the feed can be transferred to the cattle's blood and milk.

This PhD thesis "Mycotoxins in maize silage - detection of toxins and toxicological aspects" is intended to fulfil the requirement for the PhD degree at the Technical University of Denmark (DTU). The work has been performed in parallel to and in collaboration with two fellow (now finished) PhD students: The thesis by Jens L. Sørensen (2009) focused on "Preharvest fungi and their mycotoxins in maize" and Ida M. L. D. Storm (2009) covered "Post-harvest fungal spoilage of maize silage". The Danish Directorate for Food, Fisheries and Agri Business (Grant: FFS05-3) is greatly acknowledged for funding the project.

The thesis at hand gives an overview of the most important mycotoxins in Danish maize silage and evaluates the cytotoxic significance of the most common fungi. The experiments were conducted at DTU at three different departments; the cytotoxicity experiments were conducted at the Department of Toxicology and Risk Assessment, National Food Institute (FOOD), the chemical analyses were mostly carried out at the Department of Food Chemistry, FOOD and the microbiological work was done at the Center for Microbial Biotechnology, Department of Systems Biology (Biosys).

I would like to thank my three supervisors Peter H. Rasmussen (FOOD), Mona-Lise Binderup (Cowi a/s) and Thomas O. Larsen (Biosys) for their invaluable guidance and our fruitful discussions during my study.

Huge thanks also go to Ida M. L. D. Storm and Jens L. Sørensen for their cooperation and for supplying me with fungi and silage samples. Vivian Jørgensen (FOOD), Faranak Ghorbani (FOOD) and Hanne Jakobsen (Biosys) are greatly appreciated for their invaluable help in the lab. I am also grateful to Kristian F. Nielsen (Biosys) for introducing me to high resolution mass spectrometry, and to Jørn Smedsgaard (FOOD) for giving inspiring input for method development. Ulf Thrane, Jens C. Frisvad and Birgitte Andersen from Biosys are thanked for their advices on the metabolic profiles of *Fusarium*, *Penicillium* and *Alternaria*, respectively. All other colleagues at Biosys and FOOD are thanked for making DTU such a nice place to work. My gratitude goes to my former study mates Lone Bækgaard and Lise R. Holt for reading and commenting the thesis.

Last but not least I would like to thank my dear husband and family for their endless support and enormous patience.

Rie Romme Rasmussen
Soeborg, May 2010

ABSTRACT

Since the 1990's the Danish production of maize silage for dairy cattle feed has increased exponentially. In the same period farmers experienced an increase in health problems in their herds. It raised the concern that mycotoxins in silage could be implicated in unexplained cases of diseases and death observed at Danish dairy farms. The mycotoxins can be produced either by pre-harvest fungi infecting maize while it is growing in the fields or by post-harvest fungi spoiling silage during storage.

To test for cytotoxic compounds in fungal agar extracts, a resazurin assay with Caco-2 cells was employed in this study. The genera *Alternaria, Aspergillus, Byssochlamys, Fusarium, Monascus* and *Penicillium*, which often are spoiling maize and maize silage, were all able to produce cytotoxic metabolites on various semi synthetic growth media. PR-toxin was an important cell toxic metabolite from the storage fungi *P. roqueforti* whereas andrastin A, roquefortine C and mycophenolic acid, which *P. roqueforti* also produced in maize silage, were not particularly cell toxic. The presence of other cytotoxic principles than zearalenone, deoxynivalenol, nivalenol from *F. graminearum*, citrinin from *M. ruber* and gliotoxin from *A. fumigatus* in agar extracts were recognised but these metabolites were not identified. The cytotoxicity of extracts from maize silage without fungal growth was too high to identify maize silages contaminated with mycotoxins. Only heavily *B. nivea* infected maize silage containing mycophenlic acid (~50 mg/kg), byssochlamic acid and other metabolites was found more cytotoxic than uninfected silage.

For detection of secondary metabolites from the most important toxigenic fungal species a new liquid chromatography tandem mass spectrometry (LC-MS/MS) multi-mycotoxin method has been developed. The method was successfully validated for determination of 27 analytes and included metabolites from the pre-harvest *Fusarium* and *Alternaria* species, which are relevant in Danish climatic conditions and the post-harvest fungi *Penicillium roqueforti*, *P. paneum, Byssochlamys nivea, Monascus ruber* and *Aspergillus fumigatus*. The simple pH buffered sample extraction was inspired by a very fast and simple method for analysis of multiple pesticide residues known as QuEChERS. Therefore, with this method it will probably be possible to combine mycotoxin and pesticide analyses.

The LC-MS/MS method was applied to 99 Danish fresh and ensiled maize silages. The samples were mostly contaminated with mycotoxins from pre-harvest fungi including alternariol and alternariol monomethyl ether from *Alternaria* and deoxynivalenol, enniatin B, nivalenol and zearalenone from *Fusarium.* However, none of the samples exceeded the recommended levels, which exist for deoxynivalenol and zearalenone in the European Union. From post-harvest fungi the secondary metabolites andrastin A, citreoisocoumarin, marcfortine A, marcfortine B, mycophenolic acid, roquefortine A and C were detected in the 99 samples, but only in low concentrations and at low frequency. On the other side, maize silage 'hot-spots' with visible fungal growth of post-harvest fungi contained substantial levels of e.g. PR-toxin from *Penicillium roqueforti*, marcfortines from *P. paneum*, mycophenolic acid and byssochlamic acid from *Byssochlamys nivea*, citrinin from *Monascus ruber* and fumigaclavines, fumitremorgin C and gliotoxin from *Aspergillus fumigatus*.

Overall, this PhD project has shown that animals feeding on well-fermented maize silage are exposed to low levels of mycotoxins. Mycotoxins and antibiotics were present in considerable amount in maize silage with visible fungal growth. For that reason it can not be excluded that animals feeding on heavily spoiled silage in some cases may be negatively affected. The field and storage fungi are also able to produce several other cytotoxic compounds besides the mycotoxins, which was included in the monitoring. However, the low mycotoxin levels detected in Danish maize silage stacks, do not indicate that mycotoxins in maize silage have caused the general health problems observed at Danish dairy cattle farms.

SAMMENDRAG

Siden 1990'erne er den danske produktion af majsensilage til fodring af malkekvæg steget eksponentielt. I samme periode oplevede landmænd en stigning i helbredsproblemer i deres besætninger. Det gav bekymring om, hvorvidt mykotoksiner i ensilage kunne være involveret i uforklarlige tilfælde af sygdomme og dødsfald observeret på danske malkekvægsbesætninger. Mykotoksinerne kan produceres af marksvampe, som vokser på majsplanterne i marken eller af lagersvampe, der kan vokse inde i ensilagestakken.

For at undersøge cellegiftigheden af stoffer i svampe agar-ekstrakter blev der i dette studie anvendt et resazurin testsystem med Caco-2 celler. Slægterne *Alternaria, Aspergillus, Byssochlamys, Fusarium, Monascus og Penicillium*, der ofte fordærver majs og majsensilage, var alle i stand til at producere cellegiftige metabolitter på diverse semisyntetiske substrater. PR-toxin var en væsentlig cellegiftig metabolit fra *P. roqueforti*, mens andrastin A, roquefortin C og mykofenolsyre, som også blev dannet af *P. roqueforti* i majsensilage, ikke var specielt cellegiftige. Tilstedeværelsen af andre cellegiftige stoffer udover zearalenone, deoxynivalenol, nivalenol fra *F. graminearum*, citrinin fra *M. ruber* og gliotoxin fra *A. fumigatus* i agar-ekstrakter blev erkendt, men metabolitterne blev ikke identificeret. Cellegiftigheden af majsensilage ekstrakter uden svampevækst var for høj til at identificere majsensilage forurenet med mykotoksiner. Kun majsensilage svært inficeret med *B. nivea*, der indeholdt mykofenolsyre (~50 mg/kg), byssochlamic syre og andre metabolitter, kunne identificeres som mere cellegiftig end uinficeret ensilage.

Til påvisning af flere sekundære metabolitter fra de vigtigste mykotoksinproducerede svampearter blev en ny væske kromatografisk dobbelt massespektroskopisk (LC-MS/MS) metode udviklet. Metoden blev tilfredsstillende valideret mht. påvisning af 27 analytter og inkluderede metabolitter fra de marksvampearterne *Fusarium* og *Alternaria*, der er relevante under danske forhold og lagersvampene *Penicillium roqueforti, P. paneum, Byssochlamys nivea, Monascus ruber* og *Aspergillus fumigatus*. Den simple pH bufferede prøve ekstraktion var inspireret af en meget hurtig og simple metode til analyse af flerfoldige pesticidrester kendt som QuEChERS. Derfor vil det sandsynligvis være muligt at kombinere mykotoksin- og pesticidanalyser med denne metode.

LC-MS/MS metoden er blevet anvendt på 99 danske prøver af frisk og ensileret majs. Majsensilagen var primært forurenet med mykotoksiner fra marksvampe, herunder alternariol og alternariol monometyl æter fra *Alternaria* samt deoxynivalenol, enniatin B, nivalenol og zearalenone fra *Fusarium.* Ingen af prøverne overskred dog de anbefalede grænseværdier, som eksisterer for deoxynivalenol og zearalenone i den Europæiske Union. Fra lagersvampe blev svampemetaboliterne andrastin A, citreoisocoumarin, marcfortin A, marcfortin B, mykofenolsyre og roquefortin A og C påvist, men deres koncentration og hyppighed var dog lav i de 99 prøver. Derimod indeholdt majsensilage 'hot-spot' med synlig vækst af lagersvampe større niveauer af f.eks. PR-toksin fra *Penicillium roqueforti*, marcfortiner fra *P. paneum*, mykofenolsyre og byssochlamic syre fra *Byssochlamys nivea*, citrinin fra *Monascus ruber* og fumigaclaviner, fumitremorgin C og gliotoxin fra *Aspergillus fumigatus*.

Alt i alt har dette PhD projekt vist, at dyr der fodres med godt fermenteret majsensilage udsættes for lave niveauer af mykotoksiner. Mykotoksiner og antibiotika var til stede i betydeligt omfang i majsensilage med synlig svampevækst. Derfor kan det ikke udelukkes at dyr, der fodres med synligt svampeinficeret ensilage, i visse tilfælde muligvis kan blive påvirket negativt. Mark- og lagersvampene er også i stand til at producere flere andre cellegiftige forbindelser ud over de mykotoksiner, som er blevet inkluderet i overvågningen. Imidlertid indikerer de lave mykotoksin niveaer påvist i danske ensilagestakke ikke, at mykotoksiner i majsensilage har forårsaget de generelle sundhedsproblemer, som har været observeret på danske gårde med malkekvæg.

LIST OF PUBLICATIONS

Paper I: Storm IMLD, Sørensen JL., **Rasmussen RR**, Nielsen KF, Thrane U (2008) Mycotoxins in silage. Stewart Postharvest Review 6(4). DOI 10.2212/spr.2008.6.4

Paper II: **Rasmussen RR**, Storm IMLD, Rasmussen PH, Smedsgaard J, Nielsen KF (2010) Multi-mycotoxin analysis of maize silage by LC-MS/MS. Analytical and Bioanalytical Chemistry 397: 765–776, DOI 10.1007/s00216-010-3545-7.

Paper III: **Rasmussen RR**, Rasmussen PH, Larsen TO, Bladt TT, Binderup ML (xxxx) *In vitro* toxicity of fungi spoiling maize silage. Sumitted to Food and Chemical Toxicology.

Paper IV: Storm IMLD, **Rasmussen RR**, Kristensen NB, Rasmussen PH (xxxx) Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in maize silage. Submitted to Journal of Agricultural and Food Chemistry.

TABLE OF CONTENTS

ABBREVIATIONS

Caco-2	Human intestinal epithelial cell line
CYA	Czapek yeast extract agar
DM	Dry matter
ESI^-	Negative electro spray ionisation
ESI^+	Positive electro spray ionisation
HPLC	High-performance liquid chromatograph
IBT	Fungal isolate in the culture Collection at Systems Biology-DTU, Denmark
IC_{50}	Median inhibitory concentration
ip	Intraperitoneal
iv	Intravenous
LC-DAD-HR-MS	Liquid chromatograph with diode array detection and a time-of-flight mass spectrometer
LC-MS/MS	Liquid chromatograph with a triple quadrupole mass spectrometer
LD_{50}	Median lethal dose
LOD	Limit of Detection
na	Not analysed
nd	Not detected
PDA	Potato dextrose agar
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
S9	9000 g postmitochondrial supernatant fraction of liver homogenate
SA	Silage agar
SOP	Standard operating procedure
SPE	Solid phase extraction
UDP	Uridine diphosphate
YE	Yeast extract agar
YES	Yeast extract sucrose agar

AIM

The overall aim of this PhD project has been to evaluate the mycotoxin hazard for cattle feeding on maize silage. The experimental work included *in vitro* cell toxicity testing and chemical analysis. The specific aims were:

- to determine the presence of mycotoxins in Danish maize silage
- to relate the cytotoxicity of fungal agar extracts to the presence of well-known mycotoxins
- to relate the cytotoxicity of mouldy maize silage to the presence of secondary fungal metabolites
- to identify the most cytotoxic metabolite from the fungi *Penicillium roqueforti*

THESIS STRUCTURE

The thesis is structured as follows: Chapter 1 describes the background for the study and the present knowledge on mycotoxins in silage is reviewed in chapter 2 (**I**). Chapter 3 presents a new LC-MS/MS method for detection of fungal contaminants (**II**). *In vitro* cytotoxicity tests of fungal agar extracts and maize silage (**III**) are presented in chapter 4. The occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silages stacks (**IV**) and hot-spots are presented in chapter 5. Chapter 6 contains a general discussion followed by a conclusion drawn in chapter 7. Finally, a brief overview of the knowledge gained in the joint project on maize silage (2005-2010) and perspectives are described in chapter 8. In appendices, the genotoxic screenings of few fungal agar extracts can be found as well as a detailed standard operating procedure (SOP) supplementing the information on the *in vitro* cytotoxicity method developed (**III)**. The SOP is only relevant when analyses are performed.

1. BACKGROUND

1.1 Maize silage

Farmers all over the world produce maize silage to feed dairy cows (Wilkinson and Toivonen, 2003). Maize silage is produced when chopped maize plants are compressed and packed airtight. The conversation of fresh maize into maize silage is facilitated by many naturally occurring enzymatic and microbiological processes. Because of a lactic acid fermentation of maize sugars into organic acids sealed silage is stored under anaerobic and acidic conditions. The popularity of maize silage in modern farming systems has increased because of the low production costs and high nutritional value (Wilkinson and Toivonen, 2003; Zebeli et al., 2009). Maize silage may constitute 50–75% of the daily diet (Driehuis et al., 2008b) for a dairy cow consuming ~25 kg/day of dry matter (DM) (Eastridge, 2006). Maize for green fodder covered 6% (159,000 hectare) of the Danish cultivated area in 2008 and in total 6,255 million kg maize silage was produced representing a value of approximately 200 million EUR (Statistics Denmark, 2010). The Danish maize silage production is enough to feed ~410,000 dairy cows eating silage all year around, if the maize plants are harvested at the recommended 30% DM content (Jensen et al., 2005). During the summer, approx. 50% of the Danish dairy cows are turned to pasture (Danish Cattle Federation, 2007), whereas maize and grass silage mixed with concentrates are the most common feeds during the winter time. However, big herds with loose housing systems tend to be kept in stables all year around and these animals typically receive silage continuously (Barrett, 2004).

In the past 20 years the production of Danish maize silage for dairy cattle feed has increased more than 700%. In the same period as the maize silage usage has increased, major changes in Danish dairy production have taken place. The number of dairy cattle farms has been reduced by 77% and the average heard size has increased 3-fold from 1990 to 2008. Farms with more than 100 dairy cows have increased from 2% of all farms to 52% (Figure 1.1) during this period (Statistics Denmark, 2010).

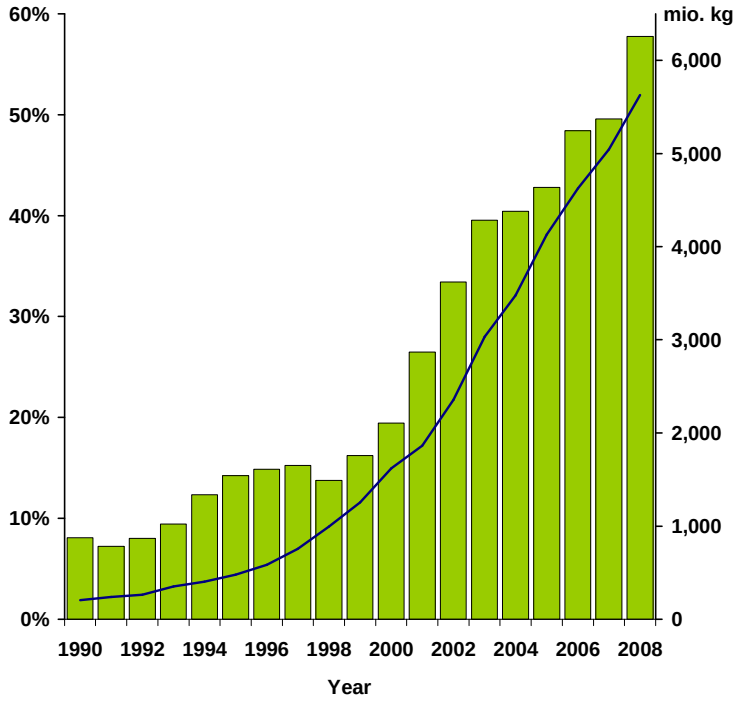

Figure 1.1. Danish production (bars) of maize for silage and percentage of dairy farms with more than 100 cows (line) from 1990 to 2008 (Statistics Denmark, 2010).

Unfortunately the mortality risk for Danish dairy cows has doubled from 2 % in 1990 to approximately 4 % in 2001 (Thomsen, 2005). The reason for death among dairy cows is often not known. Thomsen (2005) found a lower mortality risk in organic herds compared to conventional herds. The risk was also low in herds grazing during the summer, whereas an increase in mortality rate was seen when the herd size was increased. Increased morbidity and mortality is especially a problem with the so called loser-cows, which are unable to perform as good as the other cows in the herd, and therefore decrease the milk production and the animal welfare and give an extra workload.

Cases of ill-thrift, disease and death in livestock have been related to the presence of mycotoxins in silage (Cole et al., 1977b; Seglar, 1997; Boysen et al., 2000; Driehuis and Elferink, 2000; Sumarah et al., 2005; O'Brien et al., 2006) and the issue is much debated (Oldenburg, 1991; Scudamore and Livesey, 1998; Wilkinson, 1999; Driehuis et al., 2008a; Fink-Gremmels, 2008a, 2008b; Miller, 2008).

1.2 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by filamentous fungi. The fungi produce a number of different compounds when given optimal growth conditions. Strictly speaking, mycotoxins are only those secondary fungal metabolites causing diseases in vertebrate animals when introduced by natural route (Samson et al., 2002). The secondary fungal metabolites include besides mycotoxins also antibiotics and other outward-directed compounds (Frisvad et al., 2008), but specific metabolites are produced only by a limited number of fungal species.

1.2.1 Pre- and postharvest fungi

On Danish maize and silage the most important toxigenic filamentous fungi include the pre-harvest *Fusarium* and *Alternaria* species (Sørensen, 2009) and post-harvest spoilage of silage by *Penicillium roqueforti*, *Penicillium paneum*, *Byssochlamys nivea*, *Monascus ruber* and *Aspergillus fumigatus* (Storm, 2009). Attempts to control *Fusarium* infections are difficult using fungicides pre-harvest and stimulation of mycotoxin production is seen in some cases, particularly in sub-optimal fungal growth conditions and at low fungicide doses (Jennings et al., 2000; Magan et al., 2002). Planting resistant maize hybrids is the most effective way to control *Fusarium* infection (D'Mello et al., 1999), though control of insects damaging kernels can also reduce severity of the fungal infection. Also crop rotation and tillage may sometimes reduce the occurrence of infection by reducing fungal levels in the soil (Lipps et al., 1998; Mansfield et al., 2005). To prevent postharvest spoilage of silage, the most important factor is omission of oxygen, and then whole-season storage of maize silage is normally not a problem (Storm, 2009), as a well-managed stack (pH=4, <1-2% O_2 and >20% CO_2) is a very hostile growth environment for microorganisms (Forristal et al., 1999; Weinberg and Ashbell, 1994). Recently, Danish results have suggested that the risk of fungal spoilage of well-fermented maize silage can be limited by keeping stacks well sealed for more than seven months before opening (Storm, 2009).

Several mycotoxins and other secondary fungal metabolites have been detected in maize silage (Müller and Amend, 1997; Garon et al., 2006; Richard et al., 2007; Driehuis, 2008b; Mansfield et al., 2008; Sørensen et al., 2008; **II**). From the field fungi the flowing mycotoxins have been identified; aflatoxin B_1, alternariol, alternariol monomethyl ether, beauvericin, deoxynivalenol, 15-acetyl-deoxynivalenol, enniatin B and B_1, fumonisin B_1, nivalenol and zearalenone. The metabolites detected from storage fungi include, andrastin A, citreoisocoumarin, citrinin, cyclopiazonic acid, fumigaclavine A, gliotoxin, marcfortine A and B, mycophenolic acid, patulin, PR-toxin, roquefortine A and C. However all filamentous fungi spoiling maize and silage are capable of producing many other secondary metabolites (**III**), which have not been targeted in maize silage.

1.2.2 Toxicological aspects

Mycotoxins can elicit carcinogenic, mutagenic, neurotoxic, hepatotoxic, nephrotoxic, oestrogenic, immunosuppressive, antimicrobial or acute toxic effects and a compound may have a whole range of toxic effects. Some of the toxic effects elicited by secondary fungal metabolites detected in silage have been listed in Table 1.1. The toxicity of the carcinogenic aflatoxin B_1 is thoroughly described, whereas other compounds like beauvericin and enniatins have received less attention. PR-toxin, T-2 toxin and fumitremorgin A have been associated with acute toxicity, whereas no adverse effects have been described for andrastin A. The toxicity of a mycotoxin will vary with the route of administration, sex and animal species. Absorption, distribution, metabolism and excretion are important for the toxin hazard. The general symptoms of mycotoxicosis include loss of appetite, poor weight gain, feed refusal, diarrhoea, bleeding, kidney, liver or lung damages and birth defects (Scudamore and Livesey, 1998). Most of the toxic effects are documented in mice, rats or other mono-gastric animals. The conditions for farm animals are different than when single compounds are tested in high

Table 1.1 Some toxic effects of selected secondary fungal metabolites detected in silage.

Compound	**LD_{50}**	**Animal** (route)	**Cytotoxicity**	**Acute toxic**	**Antibiotic**	**Immunosuppressive**	**Neurotoxic**	**Carcinogenic**	**Hepatotoxic**	**Genotoxic**	**Oestrogenic**	**Nephrotoxic**	**Maternal toxicity**	**Insecticidal**	**Anthelmintic**	**References**
Aflatoxin B_1	1.2	rat (ip)	X					X	X	X						Hanigan and Laishes, 1984; Scudamore and Livesey, 1998
Alternariols	nd		X							X						Pfeiffer et al., 2007
Andrastin A	nd															Cole and Cox, 1981
Beauvericin	100	mouse (oral)	X		X									X		Uhlig et al., 2005; Omura et al. 1991; Jestoi, 2008
Byssochlamic acid	> 2,500	mouse (oral)							X							King et al., 1972; Gedek, 1971 as cited in Houbraken et al., 2006
Citrinin	35	mouse			X							X				Cole and Cox, 1981; Wang, 2004
Deoxynivalenol	46-78	mouse (oral)	X			X										SCF, 1999; **III**
Enniatins	nd		X		X									X		Uhlig et al., 2005; Jestoi 2008
Fumigaclavine C	150	cockerel (oral)														Cole et al., 1977a
Fumitremorgin A	0.185	mouse (iv)		X			X									Yamazaki et al., 1986
Fumitremorgin B	5.4	mouse (iv)		X			X									Yamazaki et al., 1986
Fumitremorgin C	>25	mouse														Garimella et al., 2005
Fumonisin B_1	nd*							(X)	X			X				SCF, 2000a
Gliotoxin	50	mouse	X			X										Cole and Cox, 1981; **III**
Marcfortine A	nd													X	X	Zinser et al., 2002
Mycophenolic acid	550-2,500	mouse			X	X										Cole and Cox, 1981
Nivalenol	38.9	mice (oral)				X										Ryu et al., 1988; SCF, 2000c
Patulin	5	mouse (ip)	X		X	(X)				X			X			Majerus and Kapp, 2002; **III**
PR-toxin	5.8	mouse (ip)	X	X						X						Chen et al., 1982; Aujard et al., 1979; **III**
Roquefortine A	340	mouse (ip)														Ohmomo (1975) as cited in Scudamore and Livesey, 1998
Roquefortine C	20	mouse (ip)			X		X									Kopp and Rehm, 1979; Ohmomo (1982) as cited in Scudamore and Livesey, 1998; Arnold et al., 1978
T-2 toxin	5-10	rodent (oral)	X	X		X										Cole and Cox, 1981; SCF, 2001; **III**
Zearalenone	> 4,000-20,000	rodent (oral)									X		X			Cole and Cox, 1981; SCF, 2000b

(LD_{50}) 50% lethal dose; mg/kg body weight, (ip) intraperitoneal, (iv) intravenous. (nd) has not been determined, (*) low acute toxicity.

concentration in short term trails. Animals feeding on silage can be exposed to several mycotoxins at the same time and the diagnosis of mycotoxicoses can be difficult because other diseases may give similar symptoms. A chronic exposure to low levels of mycotoxins can give non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances (Morgavi and Riley, 2007; Fink-Gremmels, 2008b). A simultaneous exposure to multiple toxins may elicit synergism; hence give a stronger effect than the sum of effects from the single toxins (Bouslimi et al., 2008). However, very few studies have until now addressed the combined effects. For example, the immunotoxic T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol appear to cause similar effects at the biochemical and cellular level, but so far an *in vivo* synergism, which would call for additional caution, has not been observed (SCF, 2002).

Dairy cows

Mycotoxin contamination caused by fungi can affect animal health (Korosteleva et al., 2009) and productivity (Fink-Gremmels, 2008b), but acute intoxications causing death are rare (Yiannikouris and Jouany, 2002). Compared to other animals, ruminants are more robust to many mycotoxins (EFSA, 2004a, 2004c, 2005) partly due to biotranformations by the rumen microorganisms (He et al., 1992). The rumen microbiota are able to inactivate and degrade some of the mycotoxins, whereas other mycotoxins can be metabolised to even more potent compounds in the rumen. For example, ochratoxin A is extensively degraded to the less toxic ochratoxin α (EFSA, 2004b), fumonisin B_1 is unaffected in the rumen (EFSA, 2005) whereas zearalenone is metabolised to α-zearalenol having stronger oestrogenic effects (EFSA, 2004c).

Cows metabolise deoxynivalenol extensively to generally less toxic metabolites mainly by de-epoxidation and glucuronidation (Figure 1.2 and 1.3) (JECFA 2001). The transformation of deoxynivalenol to the de-epoxy metabolite is facilitated by microorganisms present e.g. in the rumen (He et al., 1992), whereas the glucuronides are important conjugates formed in the liver and then excreted from the body (Prelusky et al., 1984; Rajakarier et al., 2006; Wu et al., 2007).

For the majority of mycotoxins, the interactions between rumen microorganisms and the fungal secondary metabolites are unknown. Antimicrobial fungal metabolites such as patulin (Tapia et al., 2002), mycophenolic acid (Bentley, 2000), citrinin (Wang, 2004) and roquefortine C (Kopp and Rehm, 1979) may negatively effect the rumen microorganisms (Tapia et al., 2002). An impaired rumen function causes severe metabolic disorders that can reduce the feed utilization (Chiquette, 2009) and may increase the mycotoxin uptake (Fink-Gremmels, 2008a). The consumption of fungal metabolites with antibiotic properties may substantially increase the animals' susceptibility to infectious diseases. Especially high-yielding dairy cows may be more susceptible to diseases caused by mycotoxins due to higher level of stress (Jouany and Diaz, 2005). Because of animal welfare, maize based feed for cattle is recommended to have maximum levels of the following *Fusarium* toxins; zearalenone (2,000 μg/kg), deoxynivalenol (8,000 μg/kg) ochratoxin A (250 μg/kg) and fumonisins as the sum of B_1 and B_2 (60,000 μg/kg) (European Commission, 2006). Maize silage can also contain high levels of e.g. post-harvest fungal metabolites (**III**) whose presence has not been regulated, though they can affect animals.

Young calves, which have been dosed with crude agar extracts of *A. fumigatus* with fumigaclavines, tremorgens and other metabolites, experienced severe diarrhoea, irritability, loss of appetite, serious enteritis and interstitial changes in the lungs (Cole et al,. 1977b). The acute toxicity of roquefortine seems to be low in ruminants since no clinical signs of intoxication could be recognized in sheep feed roquefortine equivalent to concentrations up to 25 mg/kg silage over a period of 16 to 18 days (Tüller et al., 1998). However, Häggblom (1990) has related diseases in a dairy herd with 25 mg/kg roquefortine C present in feed infected with *P. roqueforti*. These toxicological data are contradictory and may illustrate how difficult it is to link farm observations to the action of a single toxin. PR-toxin and patulin were not detected in the feed from the farm but other factors may have contributed. Driehuis et al., (2008a) has

evaluated the presence of 20 mycotoxins in maize silage and feed concentrates and these were found to be too low to elicit individual actions in dairy cows.

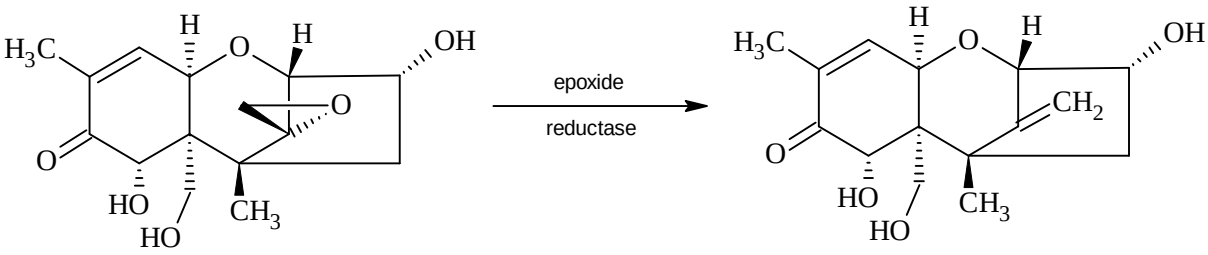

Figure 1.2. De-epoxidation reaction illustrating the microbial transformation of deoxynivalenol (DON) into the de-epoxylated form DOM-1 (after JECFA 2001).

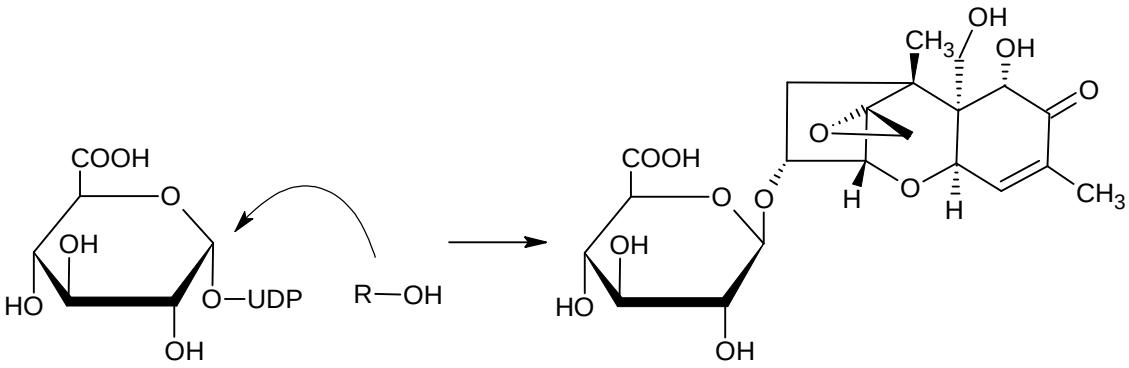

Figure 1.3. Glucuronidation of one of the OH-groups in deoxynivalenol (R-OH) forming a β-D-glucuronide after reaction with the reactant uridine diphosphate (UDP)-glucuronic acid (after Rajakarier et al., 2006, Wu et al., 2007; Richard, 2010).

Humans

Currently aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone, T-2 and HT-2 toxins and deoxynivalenol causes most significant health concern for humans (Egmond et al., 2007). To protect the consumer national regulation of specific mycotoxins in various commodities has been established in around 100 countries (FAO, 2004).

The transfer of mycotoxins in maize silage via cows to dairy and meat products is a concern for humans (Miller 2008, Fink-Gremmels 2008a). For human safety, the genotoxic carcinogen aflatoxin B_1 is regulated to maximum 5 µg/kg in complete feeding stuffs for dairy animals (European Commission, 2003) as its metabolite is transferred to milk (IARC 1993). Carry over rates of deoxynivalenol, zearalenone, ochratoxin A and fumonisins from feed to milk are known to be much lower than aflatoxin, and for these four toxins, the European Food Safety Authority (EFSA, 2004a, 2004b, 2004c; EFSA, 2005) has evaluated that the human exposure through milk to be insignificant compared to other sources such as grain. In contrast, the carry-over rates into milk of the alternariols from *Alternaria* and PR-toxin (Miller, 2008) from *P. roqueforti* are not known, although PR-toxin and alternariols have mutagenic properties *in vitro* (Levin et al., 1982; Pfeiffer et al., 2007), which indicates a possible carcinogenic effect in humans (SCF 2000a). Carry-over of carcinogenic residues would be of outmost concern for humans.

1.2.3 In vitro assays

In vitro testing systems are strong screening tools for identification of biological and toxicological activities and as to identify samples for which chemical analyses are relevant (Gutleb et al., 2002), and they provide a fast and cheap tool for screening of toxic compounds compared to animal studies. Toxicity of compounds can then be studied in different bioassay such as cell cultures of yeast, mammals or bacteria. The *in vitro* assays can bring important information about e.g. the biochemical mechanism, general toxicity and possible carcinogenic effects (Oda et al., 1985; McCann et al. 1975; Ames et al 1973; Cetin and Bullerman, 2005).

A crucial point is the cell type applied to *in vitro* assays. The effect elicited by a particular mycotoxin may arise from its ability to bind to cellular receptors and/or penetrate cell membranes (Cetin and Bullerman 2005). Compared to animal studies, *in vitro* assays may give very different results due to lack of an integrated organism response. *In vitro* test can e.g. not detect toxicity acting on the central nervous system (Gad 2000). Furthermore, several chemicals are activated *in vivo* to more toxic metabolites. To simulate *in vivo* conditions extracts with metabolic activity, e.g. liver extract, can be added to the *in vitro* assay. S9 is a 9000 g postmitochondrial supernatant fraction of liver homogenate, which provides both microsomal and cytosolic enzymes (Gad 2000). Knowledge on the distribution of a toxin within the animal tissue, the mechanism of toxicity and the need for metabolic activation is important for choosing cell type and setup of the test system. Preferably a range of different endpoints and cell types should be used when performing toxic screenings and an evaluation of the correlation between *in vitro* assays and *in vivo* studies is needed to verify the relevance of the *in vitro* assays for animal and human health risks..

Cytotoxicity

Cytotoxicity assays, can to some extent, be used as a screening test for acute toxicity in animals and humans (Binderup et al., 2002). The cytotoxicity of some mycotoxins has been shown to correlate with *in vivo* toxicity: Aflatoxin B_1 for example acted preferentially on hepatocytes, T-2 toxin, a well known *in vivo* immunsuppressor showed lymphotrophic effects, whereas citrinin, a mycotoxin with known renal toxicity had no effects on hepatocytes and lymphocytes (Robbana-Barnat et al., 1989 cited in Gutleb et al., 2002).

In vitro cytotoxicity can be determined by different endpoints e.g. cell viability (membrane leakage, dye exclusion or uptake), cell proliferation (DNA synthesis), cell functions (mitochondria metabolism) or cell and culture morphology. The sensitivity of an assay will, among others, depend on endpoint and cell type. The human intestinal epithelial cell line, Caco-2, is widely used and well validated (Videmann et al., 2008). Mitochondria metabolism of dye by viable Caco-2 cells *in vitro* can determine the general cytotoxicity with similar sensitivity as other cell lines (Cetin and Bullerman, 2005). Caco-2 cells can metabolise some toxins such as zearalenone and alternariols (Videmann et al., 2008, Burkhardt et al., 2009), but may not detect indirect toxicity activated in the liver.

Genotoxicity

Testing the mutagenic activity in bacterial systems is accepted as an initial step in the evaluation of the carcinogenic potential of chemicals (McMahon et al 1979). The Ames-test developed in the early 1970s (McCann et al., 1975; Ames et al., 1973) is used world-wide and widely accepted identifying substances, which produce genetic damage that leads to gene mutations (Mortelmans and Zeiger 2000). The umu-test developed in the early 1980s (Oda et al., 1985) is another well-validated method (ISO 2000), which determines genotoxic activity by activation of the SOS-repair system induced by DNA damage.

2. REVIEW - MYCOTOXINS IN SILAGE

Paper I





Photo: Storm IMLD (cows) and Sørensen JL (fusarium infected maize stem)

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Mycotoxins in silage

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Abstract
Purpose of review: This paper reviews the present knowledge on mycotoxins in silage, focusing on grass and maize silage. This includes the occurrence of filamentous fungi pre- and postharvest, possible and confirmed mycotoxins in silage, toxicological concerns and means to prevent the problem.
Findings: Preharvest contamination of grass and maize by *Fusarium*, *Aspergillus* and *Alternaria* can lead to contamination of silage. Well known mycotoxins deoxynivalenol (DON), zearalenone (ZEA), fumonisins and aflatoxins have been detected in silages but concentrations seldom exceed regulatory limits. It also appears that DON, ZEA and fumonisins are degraded in silage, but exact mechanisms are unknown. Postharvest spoilage is dominated by *Penicillium roqueforti*, *Aspergillus fumigatus* and Zygomycetes. Both *P. roqueforti* and *Asp. fumigatus* produce a wide range of secondary metabolites, some of them confirmed mycotoxins, others with antimicrobial or immunosuppressive effects. Some fungal metabolites have been detected in silage but many have not been looked for. Evidence for acute toxicosis caused by contaminated silage is rare. Mycotoxins in silage are more often associated with less specific symptoms like ill-thrift or decreasing yield. This may be caused by long-term exposure to the complex mixture of secondary metabolites that silage can contain. Mycotoxins with antimicrobial effects may also affect ruminant digestion. To prevent postharvest spoilage of silage the most important factor is omission of oxygen. Additives can improve certain silage properties but they are not conclusively an advantage and cannot replace good silage management.
Directions for future research: The effects of long-term exposure and of complex mixtures of bioactive fungal compounds are subjects of interest. Especially high-yielding livestock may be subject to sub-acute symptoms under these conditions. There is also a need for analytical methods with specificity and accuracy to determine many of the less known mycotoxins and secondary metabolites in silage as well as possible unknown compounds.

Keywords: silage; grass; maize; mycotoxins; preharvest; postharvest

Abbreviations

DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
ELEM	Equine Leukoencephalomalacia
FB1	Fumonisin B_1
LC-MS	Liquid Chromatography–Mass Spectrometry
NIV	Nivalenol
PPE	Porcine Pulmonary Oedema Syndrome
ZEA	Zearalenone
ZOL	Zearalenol

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Introduction

Ensiling is used worldwide as a simple and effective way to preserve forage for livestock, using a natural lactic acid fermentation of the feedstuffs which is acidified and can subsequently be stored for long periods without degrading. In modern agriculture, large amounts of silage are consumed each day all year round with dairy cows eating up to 40 kg/day. In Western Europe the total area of land harvested for silage has remained stable at around 15 million hectares since 1990 and worldwide the silage production has tended to increase from 1989–2000 [1]. The use of especially maize silage has increased over the last few decades with the availability of short season maize varieties suitable for temperate climates [1, 2]. In Denmark the production has increased by 500% from 1990 to 2007 [3] and maize silage is very widely used for cattle in both dairy and meat production.

The most common silage crops are grass and maize, but many other products like whole-crop barley, alfalfa, clover, sugar-beet tops, and residues from sugar production can be preserved as silage [4**]. A thorough review of silage making from crops to nutritive value is covered by McDonald *et al.* [4**]. In all cases the product is harvested, cut in suitable sizes and packed tightly in either silos, stacks or bales, and sealed to avoid oxygen infiltration. Residual enzymatic activity of the plant and microbial respiration of the carbohydrates released by chopping quickly depletes the small amount of O_2 in the stack and raises the concentration of CO_2. One hour after ensilage, O_2 levels in the range from 1 to 2% and CO_2 from 20 to 90% were recorded in baled silage [5]. This selects for the proliferation of natural lactic acid bacteria, whose numbers increase from below 10^2–10^5 CFU/g on plants in the field to 10^9–10^{10} CFU/g in silage that is only a few days old [4**, 6]. They ferment sugars to primarily lactic acid and acetic acid, lowering pH to ~4 or less. Clamp and baled grass silages have been reported to have an average pH of 4.0 and 4.8, respectively [5]. The combination of low O_2 concentration, high CO_2 concentration and a low pH makes silage a very hostile environment for spoilage organisms including bacteria, yeasts and filamentous fungi.

Nevertheless, growth of filamentous fungi is frequently observed in silage. This constitutes a loss of nutritive value for the farmer and, much worse, a risk for contamination with mycotoxins. Toxins in the feed may constitute a health risk for animals and there is also the risk for carry-over to humans via milk and meat [7*]. In the last 30 years, cases of ill-thrift, disease and death in livestock have been related to the presence of mycotoxins in silage [8*–12] and the issue is much debated [2, 7*, 13–18**]. Infestation and subsequent mycotoxin production may take place both pre- and postharvest and silage can thus be contaminated with both well known *Fusarium* toxins like deoxynivalenol (DON) and zearalenone (ZEA), as well as less known secondary metabolites from species of *Penicillium* and other fungi.

This article reviews the present knowledge on mycotoxins in silage including pre- and postharvest contaminants, toxicological issues and means for preventing the problem. The focus is on grass and maize silage for cattle as these are considered the economically most important use of silage crops.

Toxigenic field fungi

The three most important toxigenic genera occurring preharvest in cereals and maize are *Aspergillus*, *Fusarium* and *Alternaria* (Table 1). *Alternaria* and *Fusarium* are often categorised as field fungi whereas some species of *Aspergillus* can occur both pre- and postharvest. The occurrence of these fungi is influenced by several factors, including agricultural practices (crop rotation, crop variety, fertilisation and cultivation methods) and climatic conditions (temperature and moisture).

Small-spored *Alternaria* are common pathogens of small grains and maize with *Alt. alternata, Alt. arborescens, Alt. infectoria* and *Alt. tenuissima* as the predominant species [19]. *Alt. alternata* may not be as common as the literature indicates, as it is often mis-identified. Of these species *Alt. infectoria* is the only one with a known sexual stage (*Lewia*). The infections often occur in the late growth season as black spots on the host plants.

The two predominant toxigenic field *Aspergillus* species are *Asp. flavus* and *Asp. parasiticus*. These two species are mainly found in warm arid, semi-arid and tropical regions and cause huge problems in the Midwestern corn belt in the USA [20]. They can infect growing maize and produce mycotoxins preharvest but may apparently also survive the ensiling process, as findings of *Asp. flavus* in silages have been reported [21–23*].

Species of the anamorphic genus *Fusarium* are destructive pathogens responsible for several diseases including red/pink ear rot of maize and head blights of wheat. In areas with temperate climate, *F. avenaceum, F. culmorum* and *F. graminearum* (teleomorph: *Gibberella zeae*) are the predominant species, whereas the members of the *Liseola* section *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (teleomorph: *Gib. moniliformis*) dominate in warmer parts of the world [24].

Several additional producers of bioactive secondary metabolites are often associated with cereal and maize including species of *Epicoccum, Cladosporium, Diplodia* and *Phoma.* The natural occurrence of mycotoxins produced by these genera in food and feeds has not been studied yet and an estimation of their importance is therefore not possible. *Diplodia* toxins have however been suggested as the primary cause in an Argentinean case where 10 heifers died from eating mouldy maize infected with *Diplodia maydis* [25]. Attention should therefore be given in the future to mycotoxins produced by other genera than *Aspergillus* and *Fusarium*.

Several species of the sexual genus *Epichloë* (anamorph: *Neotyphodium*) are endophytes of some varieties of pooid

Table 1. The most common species of *Fusarium, Alternaria* and *Aspergillus* in preharvest silage crops, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage
Fusarium culmorum, *F. cerealis* and	Culmorin	
F. graminearum	Deoxynivalenol	+
	3- or 15-Acetyl deoxynivalenol	+
	Nivalenol	+
	Fusarenone-X	
	Fusarins	
	Zearalenones	+
	2-Acetylquinazolinone	
	Aurofusarin, Rubrofusarin, Butenolide, Chrysogine	
F. proliferatum, F. subglutinans and	Beauvericin	+
F. verticillioides	Fumonisins	+
	Fusaproliferin (*F. pro*. and *F. sub*.)	
	Fusapyrone (*F. pro*), Fusaric acid	
	Moniliformin (*F. pro.* and *F. sub.*)	
	Naphthoquinone pigments	
F. poae and *F. sporotrichioides*	Aurofusarin	
	Beauvericin	+
	Chrysogine (*F. sporotrichioides*)	
	Culmorin	
	Scirpentriol	+
	Monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol	
	Enniatins	+
	Fusarenone-X (*F. poae*)	
	T-2 toxin	
	HT-2 toxin	+
	Neosolaniol	
	Nivalenol (*F. poae*)	+
F. avenaceum and *F. tricinctum*	2-Amino-14,16-dimethyloctadecan-3-ol (*F. ave*.)	
	Acuminatopyrone (*F. ave*.), Antibiotic Y, Aurofusarin	
	Beauvericin	+
	Butenolide	
	Chlamydosporols	
	Chrysogine	
	Enniatins	+
	Fusarins, Gibepyrone A, Moniliformin, Visoltricin (*F. tric*)	
F. equiseti	Nivalenol	+
	Scirpentriol, monoacetoxyscirpentriol	+
	Diacetoxyscirpentriol, Equisetin, Fusarenone-X	
	Fusarochromanone, Chrysogine	
Alternaria alternata, Alt. arborescens	AAL-toxins (*Alt. arborescens*)	+[a]
and *Alt. tenuissima*	Alternariols, Altertoxins, Tentoxin, Tenuazonic acid	
Alternaria infectoria	Infectopyrones, Novae-zelandins	
Aspergillus flavus and Asp. parasiticus	Aflatoxin B_1 and B_2	+
	Aflatoxin G_1 and G_2 (*A. parasiticus*)	
	Aspergillic acid	
	Cyclopiazonic Acid	+
	Kojic acid	
	Sterigmatocystins	+
	Versicolorin and precursors, 3-Nitropropionic acid	
	Aflavinine, Aflatrem	

[a]Needs reconfirmation

Figure 1. Ball of maize silage infected with *Penicillium roqueforti*, which was observed in the middle of a well managed silage stack.

grasses [26]. *Epichloë* species can infect plants through wounds or stigmata and by seed-transmission whereas the asexual *Neotyphodium* species are only seed-transmitted. The endophytes colonise host plants systematically without causing disease symptoms. Several bioactive alkaloids can be produced by the endophytes during the infections, which are beneficial to the host plant as they can be active against feeding insects or herbivores. Other fitness improvements that may be attributed to the symbiosis between host plants and endophytes include growth stimulation and enhanced drought tolerance [27]. Some neurotoxic alkaloids have been implicated in livestock toxicosis, including ergot alkaloids [28] and lolitrems [29*]. For reviews on this subject see [16, 18**] The recognised toxicosis events occurred when livestock had been feeding on *Epichloë* or *Neotyphodium* infected grass, but so far nothing is known about their occurrence and stability in grass silage.

Postharvest contamination

The ensiling process eliminates most fungi from the field [30*, 31]. There are however other species of filamentous fungi that are able to tolerate both organic acids, carbon dioxide and the low availability of oxygen (Table 2).

The most commonly found filamentous fungi in silage are *Penicillium roqueforti* and the closely related *P. paneum* [22, 32*, 33–35]. *P. roqueforti* has its pH optimum between pH 4 and 5 [36], tolerates high levels of CO_2 [37] as well as the different organic acids commonly found in silage [22, 38]. The optimum temperature is 25°C but *P. roqueforti* may grow at 5°C [38]. Thus it is able to grow in silage all year round, even in temperate climates. *P. roqueforti* also sporulates heavily and spores are almost always present even in healthy looking maize silage [Storm IMLD, unpublished]. Growth of *P. roqueforti* and *P. paneum* is often seen in silage either in layers, on the surface or as lumps as big as 40 cm in diameter in the middle of stacks (Figure 1). The colour is green often in grey or blue shades and *P. roqueforti* and *P. paneum* cannot be differentiated visually on the silage.

Other very common fungi are various species of *Mucor* and *Rhizopus* (class Zygomycetes), which have been isolated from all types of silage [21–23*, 31, 32*–34, 40*]. They grow rapidly especially in partly aerated outer layers of silage. The rapid growth of these species may obscure the growth of other less vigorous species during cultivation and identification in the laboratory.

Aspergillus fumigatus has also been isolated from silages all over the world, both in warm [21, 41] and temperate [22, 23*, 33, 34, 40*] climates. It has a high temperature optimum and tolerates temperatures up to 55°C [42] and can therefore often be observed near degraded outer layers of silage stacks where the microbial heat from degradation has selected for heat-tolerant species.

Other species often encountered are *Monascus ruber* [23*, 33, 40*, 43] and *Byssochlamys nivea* [22, 23*, 44]. *M. ruber* often produces red pigments and can be seen as lumps both near surfaces and in central parts of silage stacks. *B. nivea* and the anamorphic form *Paecilomyces niveus* produce white colonies in silage. *B. nivea* can survive acidic and anaerobic conditions and the ascospores are heat-resistant, as illustrated by the fact that it is an important contaminant of canned fruit and fruit juices [42].

Fusarium spp. have been isolated from silage in several cases [21, 23*, 40*]. Fusaria are generally not capable of surviving the ensiling process. Only *F. oxysporum* is known to survive under acidic and anoxic conditions [42]. Mansfield and Kuldau [30*] registered several species of *Fusarium* in fresh maize but none after ensiling. The survival of spores or recolonisation after opening may explain findings of Fusaria in silage.

Classic mycological determination of mycobiota by dilution and plating may unfortunately not reflect the actual growth of filamentous fungi in field and silage. This is a classic mycological dilemma already mentioned in a review of silage mycology by Pelhate [31]. The use of suitable media and incubation in modified atmosphere may give a more representative picture of the actual mycobiota in silage, but standardised procedures need to be developed. Even so heavily sporulating species like *P. roqueforti* may be overestimated. Silage cannot be considered a homogenous medium either. Within a stack or bale there are many ecological niches. For instance *P. roqueforti* is often observed as layers at a depth of 20–80 cm [Storm IMLD, unpublished, 33] where the O_2 concentration is too low for most spoilage organisms. In the outer layers *P. roqueforti* has been out competed by yeasts, bacteria and other filamentous fungi. Molecular biological techniques can in theory reveal the presence of all fungi in

Table 2. The most common fungal postharvest contaminants of silage, some known secondary metabolites and secondary metabolites confirmed in silage.

Species	Secondary metabolites	Detected in silage[a]	Reference
Penicillium roqueforti[b]	Agroclavine	+	[8]
	Eremofortin C		
	Mycophenolic acid	+, 1.3, 35, 117	[2, 8, 34, Nielsen KF, unpublished]
	PR-toxin	+	[Nielsen KF, unpublished]
	PR-amide and PR-imine		
P. roqueforti	Roquefortine A, D, 16-OH-roquefortine	+	[8]
and *P. paneum*[b]	Roquefortine C	+, 5.7, 36, 50	[8, 35, 82, Nielsen KF, unpublished]
	Andrastin A, B and C	+	[8, Nielsen KF, unpublished]
	Citreoisocoumarin	+	[8]
	Orsellinic acid		
	Festuclavine	+	[8]
P. paneum[b]	Marcfortine A	+	[8]
	Marcfortine B and C		
	Patulin	1.2, 40	[44, 82]
	Gentisic acid		
Aspergillus fumigatus[c]	Gliotoxin	0.878	[23*]
	bis-dethio-bis(methylthio)-gliotoxin	+	[Nielsen KF, unpublished]
	Fumigatins		
	Trypacidins		
	Sphingofungins		
	Pseurotins		
	Helvolic Acid		
	Fumagillins		
	Fumigaclavines		
	Fumitremorgines		
	Diketopioperazines		
	Fumiquinazolines		
Byssochlamys nivea/	Patulin	1.2, 40	[44, 82]
Paecilomyces niveus	Byssochlamic acid		
	Mycophenolic acid	+, 1.3, 35, 117	[8, 34, 82, Nielsen KF, unpublished]
Monascus ruber	Citrinin	0.037, 0.064, 0.25	[23*, 40*, 43]
	Monacolins	65	[43]
	Pigments, eg, ankaflavin		
	Monascopyridines		
Zygomycetes	May cause zygomycosis especially in immunocompromised animals		[76]
Geotrichum candidum	May reduce palatability of silage		[31]

[a]+: Metabolite detected in silage samples. Numbers state maximum concentrations in mg/kg where quantitative determination has been performed.
[b]Based on [8, 103*]
[c]226 Extrolites registered by Frisvad *et al.* [75*]

silage. Mansfield and Kuldau [30*] compared a DNA-sequence based technique with plating on malt-yeast sucrose agar (MYSA) and Nash medium (NASH) and found a much greater abundance of species with the molecular technique. Again dormant spores can give misleading results and the quantity of DNA cannot be correlated with the amount of mycotoxins.

Mycotoxins and other secondary metabolites

The above mentioned fungi are known to produce a wide range of mycotoxins and other secondary metabolites. But the production of these is very substrate dependent and not all may be present in silage. The complex microbial ecosystem of silage can also account for degradation and binding of such compounds.

Preharvest

Of the *Fusarium* derived mycotoxins, the trichothecenes are sesquiterpenes and are produced by various species of *Fusarium*. The compounds are divided into type A and B trichothecenes. Type A trichothecenes (mainly diacetoxyscirpenol [DAS], T-2 toxin and deacetylated analogues of these) are mainly produced by *F. poae*, *F. sporotrichioides* and *F. langsethiae* and are considered more toxic than type B trichothecenes (mainly DON and nivalenol [NIV], fusarenone-X, 3- and 15-acetyl-DON as well as acetylated and deacetylated analogues of these), which are primarily produced by *F. cerealis*, *F. culmorum* and *F. graminearum* [45]. Trichothecenes have a variety of toxic effects like vomiting (DON), reduced feed uptake and immuno-suppression as the most pronounced [46]. DON is usually the predominant trichothecene in crops and is therefore also the best studied. ZEA and α- and β-zearalenol (α- and β-ZOL) are estrogenic compounds mainly produced by the trichothecene type B producing *Fusarium* species [45]. In a survey of mycotoxins in various Dutch silage types, DON and ZEA were almost completely absent in grass silage, while they were highly abundant in maize silage [47*], despite the absence of the producing organisms postharvest [30*].

Fumonisins are sphinganine analogues with carcinogenic properties [48] and are primarily produced by *F. proliferatum* and *F. verticillioides* [45]. These species are mainly present in tropical and subtropical areas and fumonisin contaminations of preharvest crops are therefore higher in these areas. There are several groups of fumonisins with several members, but fumonisin B_1 (FB1) is the predominant and best studied analogue.

DON and FB_1 were shown to be less stable than ZEA in a lab scale experiment with ensiled maize [49*]. The maximum toxin degradation observed for DON, FB_1 and ZEA was 100%, 92% and 53%, respectively [49*]. The experiments also showed that storage time and dry matter content are more important than temperature. In a study of fresh and ensiled maize, DON levels were reduced by 57% in 3–6 month old silage stacks [50]. These observations suggest a substantial degradation of DON during ensiling, which is a fate that the other trichothecenes are likely to share. Some removal of field produced mycotoxins can be attributed to lactic acid bacteria. *In vitro* studies suggest that binding of DON, ZEA and FB1 is the major mode of action for lactic acid bacteria [51].

Plants are able to reduce the toxicity of mycotoxins formed in the fields for example by conjugation of mycotoxins to polar substances such as sugars, amino acids or sulphate. Natural occurring glucoside conjugates of ZEA [52] and deoxynivenol [53] have been detected. The conjugated forms will not be detected by standard methods designed for the precursor mycotoxins as they may be harder to extract and have altered chromatography. This means that the actual amount of mycotoxins may be underestimated due to masked conjugated mycotoxins.

Species of *Fusarium* can produce several other types of mycotoxins in cereals and maize preharvest, including moniliformin, fusaproliferin, beauvericin and enniatins, but very little is known about their stability in silage. The predominant enniatin analogue, enniatin B, was detected at levels up to 218 ng/g in 3-month-old maize silage stacks, while the related beauvericin occurred less frequently and at levels up to 63 ng/g. Enniatin levels in 3, 7 and 11 month old silage were not different from each other but were all lower than in freshly harvested maize [54]. This suggests that some of the enniatins were degraded within the first 3 months. In another study of preharvest maize, moniliformin was only produced in insignificant low ppb levels [55].

The four most frequently occurring *Alternaria* species in cereals and maize are *Alt. arborescens, Alt. alternata, Alt. tenuissima* and *Alt. infectoria,* which are able to produce a wide range of compounds with disputed toxicity. *Alt. arborescens, Alt. alternata* and *Alt. tenuissima* can produce alternariols, altertoxins, altenuene and tenuazonic acid [56], but there are only few reports on the natural occurrence of these compounds in small grain cereals preharvest, summarised in [57]. *Alt. infectoria* can produce infectopyrones and novaezelandins [58], but their natural occurrence has not been studied. One paper [59] also reports finding the *Alternaria* mycotoxins AAL-toxin A and B in silage. Liquid chromatography–mass spectrometry (LC-MS) with only one SIM ion (not very specific in such dirty matrix) was used to substantiate this very interesting finding, and since only one isolate (tomato pathogen *Alt. arborescens,* syn. *Alt. alternata* f. sp. *lycopersici*) in the world until now has been found to produce AAL toxins, the findings of AAL toxins in silages seems unlikely and needs proper validation.

With *Aspergillus flavus* and *Asp. parasiticus* present in crops and silage, aflatoxins may be produced. These are the most important group of mycotoxin produced by this organism, and mainly includes the B_1, B_2, G_1 and G_2 analogues, which are all produced by *Asp. parasiticus*, whereas *Asp. flavus* can

only produce B_1 and B_2 [60]. Aflatoxins are the most carcinogenic of known secondary metabolites and their occurrence in silage can be of great concern to human health as they can be transformed by cattle to hydroxylated derivates (aflatoxins M_1 and M_2), which can be found in meat and milk products. Other mycotoxins from *A. flavus* are cyclopiazonic acid and 3-nitropropionic acids. Aflatoxin B_1 has been detected in silage in some surveys while others have looked for it with negative results (Table 3)

Postharvest

P. roqueforti and *P. paneum* are the most widespread species of filamentous fungi in silages and they have on several occasions been associated with ill-thrift and disease in cattle herds [8, 10, 11]. As seen in Table 2 they produce a wide range of secondary metabolites *in vitro* and many of them have also been detected in silage.

The roquefortines are very ubiquitous and have therefore been suspected to be involved in toxicoses [61]. Data on neurotoxicity [62] and antibiotic properties [63] are published but no acute toxicity and a low transfer to organs and tissue were observed in feeding experiments with sheep [64]. PR-toxin (only produced by *P. roqueforti*) on the other hand has acute toxic effects in rats and mice [62, 65] but its fate in ruminants is unknown. Another known toxin, patulin, is produced by *P. paneum* as well as *B. nivea*. Patulin damages the kidneys and the gastro-intestinal tract functions in rats [66] and may reduce male fertility [67]. It has antibiotic properties [68] and is immunosuppressive at high doses [69, 70]. It does however form adducts with S-containing amino acids [71, 72] and may therefore not be bio-available in ruminants. Another commonly encountered metabolite is mycophenolic acid, which is produced by both *P. roqueforti* and *B. nivea*. It is antibiotic and immunosuppressant [73, 74]. The andrastins and marcfortines have not been tested in higher animals. The clavines are similar to alkaloids produced by *Neotyphodium* endophytes in Fescue grass preharvest and may thus result in similar symptoms, however ergovaline is considered the most important toxin involved in Fescue toxicosis. In a recent survey by Driehuis *et al.* [47*] roquefortine C was reported only in 1 of 120 grass silages and none of 140 maize silages. Mycophenolic acid was not found in any samples. Sampling of the silages was however conducted only 1–2 months after harvest and the stacks were still completely sealed so growth of postharvest contaminants was unlikely.

The widespread presence of *Asp. fumigatus* in silage naturally calls for concern. It is a known producer of more than 200 secondary metabolites [75*], including the potent gliotoxin, and may cause invasive infections in animals (Aspergillosis) [76]. Many of the metabolites are known to have antimicrobial, antifungal or antiprotozoan effects [75*] and may thus affect the microbiota of the rumen. Others, like gliotoxin, are immunosuppressive [77]. Silage samples contaminated with *Asp. fumigatus* have been analysed for gliotoxin only, which is produced in highest amount on substrates with a low C/N ratio. Gliotoxin may therefore not be a very good marker for presence of *Asp. fumigatus* toxins in silage. In *Monascus ruber* infected silage, citrinin has been detected. Citrinin is nephrotoxic [78], while the monacolins produced by the same species have no toxic effects and are used as cholesterol-lowering drugs.

Some Zygomycetes can, via endophytic bacteria, produce several bioactive secondary metabolites [79, 80], but the distribution of toxigenic isolates is not well examined. The fast growth of Zygomycetes may spoil large amount of silage very rapidly. Furthermore some species are known to cause invasive infections, Zygomycosis [76], especially in immuno-compromised individuals.

Toxicology

Mycotoxins in silage can affect animal health and productivity [18**]. Exposure of humans via transfer of mycotoxins to food (eg, milk) is also of concern [2, 18**]. The mycotoxins contaminating silage can induce carcinogenic, estrogenic or immunosuppressive effects. Feed refusal, birth defects, kidney, liver or lung damages, etc have also been observed in clinical trials [17], but acute intoxications causing death are rare [81]. Animals feeding on silage may be exposed to a mixture of mycotoxins [23*, 40*, 47*, 82] and chronic exposure to low levels of mycotoxins may result in non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances [18**, 83]. The intoxication of animals under field conditions does not always match the concentration of specific toxins [18**]. A cocktail of toxins can give a stronger effect than the single toxins alone [78]. Furthermore, not all toxins in silage are described in literature since new secondary fungal metabolites are still discovered [84, 85].

A review of animal disease outbreaks due to *Fusarium* toxin contaminated feed has been given by Morgavi and Riley [83]. Clear signs of exposure to a specific toxin are rare under field conditions; for DON feed refusal has been reported in cattle, pigs and chickens. Fumonisins can induce brain lesions in horses - equine leucoencephalomalacia (ELEM) and lung damage in pigs - porcine pulmonary oedema syndrome (PPE) [83]. Mouldy maize silage infected with *P. roqueforti* produced loss of appetite, disturbance of rumen activity and gut inflammation in dairy cows [86]. Kristensen *et al.* [87] however did not see any significant effects on milk yield or rumen pH in a feeding experiment where cows were fed alternating rations, including a ration with DON-contaminated maize silage and one with *Penicillium* contaminated maize silage. There were a few changes in the ruminal fermentation pattern that were significant.

Ruminants are often less susceptible to intoxication than other animal species. For instance they show lower responsiveness to DON, ZEA and fumonisins than pigs do [88–90]. The rumen microbiota can inactivate and degrade some mycotoxins, but not all types. For example, ochratoxin A is extensively degraded to the less toxic ochratoxin α [91], whereas ZEA is metabolised to the even more potent α -ZOL

Table 3. Confirmed examples of maize silage contaminated with *Fusarium*, *Aspergillus* and *Alternaria* toxins.

Mycotoxin	Country	Concentration (µg/kg)[a]		Reference
		Mean	Range	
Deoxynivalenol	Argentina		30–870	[104]
	France	160		[23*]
	France	204		[23*]
	Germany	2,919	?–3,944	[105]
	The Netherlands	651	nd–3,142	[47*]
	USA	600	nd–3,700	[50]
15-Acetyldeoxynivalenol	Germany	59	?–127	[105]
	The Netherlands	45	nd–1,013	[47*]
Nivalenol	Germany	1,612	?–2,809	[105]
HT-2 toxin	Germany	18	?–26	[105]
Scirpentriol	Germany	25	nd–124	[105]
Monoacetylscirpentriol	Germany	20	nd–49	[105]
Zearalenone	Argentina		nd–350	[104]
	France		<20	[23*]
	Germany	432	?–1,790	[105]
	The Netherlands	92	nd–943	[47*]
α-Zearalenol	Germany	3	nd–15	[105]
β-Zearalenol	Germany	23	nd–116	[105]
Fumonisin B_1	Argentina		340–2,490	[104]
	The Netherlands	463	nd–26,200	[47*]
	USA	2,020	nd–10,100	[59]
	USA	590	nd–1,824	[106]
Fumonisin B_2	The Netherlands	130	nd–7,800	[47*]
	USA	980	nd–20,300	[59]
	USA	66	nd–276	[106]
Fumonisin B_3	USA	29	nd –161	[106]
Enniatin B	Denmark	73	nd–218	[54]
Enniatin B_1	Denmark	10	nd–48	[54]
Beauvericin	Denmark	8	nd–63	[54]
Aflatoxin B_1	Argentina		nd–176	[104]
	Italy		nd–<4	[107]
	Mexico		500–5,000	[108]
	Brazil	nd		[109]
	USA	nd		[110]

[a]nd: not detected.

[89]. FB1 largely passes the forestomach in ruminants [90]. Animals with impaired rumen fermentation are expected to metabolise toxins less effectively. Patulin is an example of a mycotoxin with antibacterial properties that can disturb the rumen fermentation [92]. Keese *et al.* [93] have also detected alterations in the ruminal fermentation pattern when cows were fed a ration containing 5.3 mg/kg DM of DON. High-yielding dairy cows may be more susceptible to diseases caused by mycotoxins, maybe due to a higher level of stress [94].

Milk can be contaminated with the carcinogenic metabolite aflatoxin M_1 [95], when lactating animals are exposed to the mycotoxin aflatoxin B_1 in feedstuffs. Up to 6% of the administered dose of aflatoxin is excreted in the milk [96]. Carry over rates of DON, ZEA, ochratoxin A, and fumonisins from feed to milk are much lower than aflatoxin. Hence humans are not significantly exposed to these four toxins through milk [88–91]. The carry-over rates from feed to milk of *P. paneum* and *P. roqueforti* toxins, eg, PR-toxin, roquefortines or festuclavine are not known [2].

Many countries have regulatory limits for mycotoxins in feed. Maximum acceptable levels of DON (0.9–12 mg/kg feed), ZEA (0.1–3 mg/kg), ochratoxin A (0.05– 0.25 mg/kg) and fumonisins (5–60 mg/kg) in feed material have been set by the European Union. These values are toxin, feed-type, and animal dependent, and address animal welfare, as the exposure of humans through animal products is low [97]. Maximum levels of aflatoxin B_1 (0.005–0.02 mg/kg) in feed is regulated based on human safety as it is a genotoxic carcinogen [98]. The lowest value in feed applies to dairy cattle due to carry-over in milk. As seen in Table 3 mycotoxin levels in silage rarely exceed the existing regulatory limits.

Preventive agricultural practices

In order to minimise the risk of fungal spoilage and mycotoxin contamination of silage, farmers can implement different strategic and practical approaches.

Preharvest infection of crops cannot be eliminated. Incidents and concentrations of preharvest toxins are very dependent on weather conditions, and models to predict the spread of plant pathogens have been developed [99]. In a survey by Mansfield *et al.* [50] agronomic practices had no effect on incidence of DON, but the concentrations were significantly higher in no till-systems than in mixed till and mouldboard till systems.

To avoid spoilage of silage in silos and bales there are several practical approaches to consider. Proper chopping, thorough compaction and sealing are very important factors for limiting the oxygen supply, which is of utmost importance. O'Brien *et al.* [32*] found that visible damage to the polythene film of baled grass silage was the only bale production and storage characteristic that significantly predisposed bales to increased fungal spoilage. Furthermore, a positive correlation was observed between polythene film damage and dry-matter content [100] most likely because dry and stiff stems are more likely to puncture the film. For silage in stacks and silos, the compaction is very important both for the quick achievement of anaerobic conditions and for minimisation of O_2 infiltration from the cutting front. Therefore particle size must not be too big as this hinders compaction. Special equipment for cutting silage rather than grabbing it from the stack may also minimise O_2 infiltration. Proportionating silage stacks to the rate of use may also help, as low rate of use has been associated with spoiled silage [33]. Optimal dry-matter content of the crop is also important for the initiation and course of the silage fermentation. Significant negative correlation between dry matter content and concentration of lactic, acetic, propionic and butyric acid was observed [32*].

In order to affect the fermentation process, silage additives can be added during silage making. These may be acids intended to restrict growth of undesirable organisms from the start, fermentable sugars (eg, molasses) to stimulate production of organic acids or biological inoculants to increase the concentration of desired microorganisms in silage. Biological additives are the most popular type worldwide but may be used in combination with the other types [1]. Biological inoculants are however not always successful and there are both advantages and disadvantages to them [101].

Conclusion

Silage can contain a wide range of mycotoxins and other secondary metabolites originating from preharvest infection of crops or from postharvest infection in silos, stacks and bales. This has been associated with ill-thrift and disease in cattle, but the evidence for acute intoxication caused by contaminated silage is rare. Many of the filamentous fungi associated with silage are however producers of antimicrobial and immunosuppressive compounds. It is possible that complex mixtures of these may result in sub-acute symptoms, ie, impaired rumen function or increased susceptibility to infections. This subject calls for further investigation.

The mycobiota of silage has been examined in several cases around the world, and the results are fairly consistent with *P. roqueforti* and *Asp. fumigatus* as some of the most abundant species. An often encountered group of filamentous fungi is the Zygomycetes but the possible effects of these have not been examined. The interplay between filamentous fungi, bacteria and yeasts is also an issue of interest, which may be able to explain the occurrence of filamentous fungi in the middle of otherwise well-preserved and managed silages.

Many of the secondary metabolites produced by known contaminants of silage have not been analysed for in silage. It is thus possible that there are so far undetected metabolites playing a role in intoxications with silage. The list of possible contaminants is very long and silage is an extremely difficult matrix since it is full of organic acids, sugars, chlorophyll and numerous other small molecules, of which many cannot

be easily removed by, eg, reversed phase solid phase extraction. Very few methods in silage have been published so there is a need for high specificity methods like LC-MS/MS with at least two transitions or daughter ion scans.

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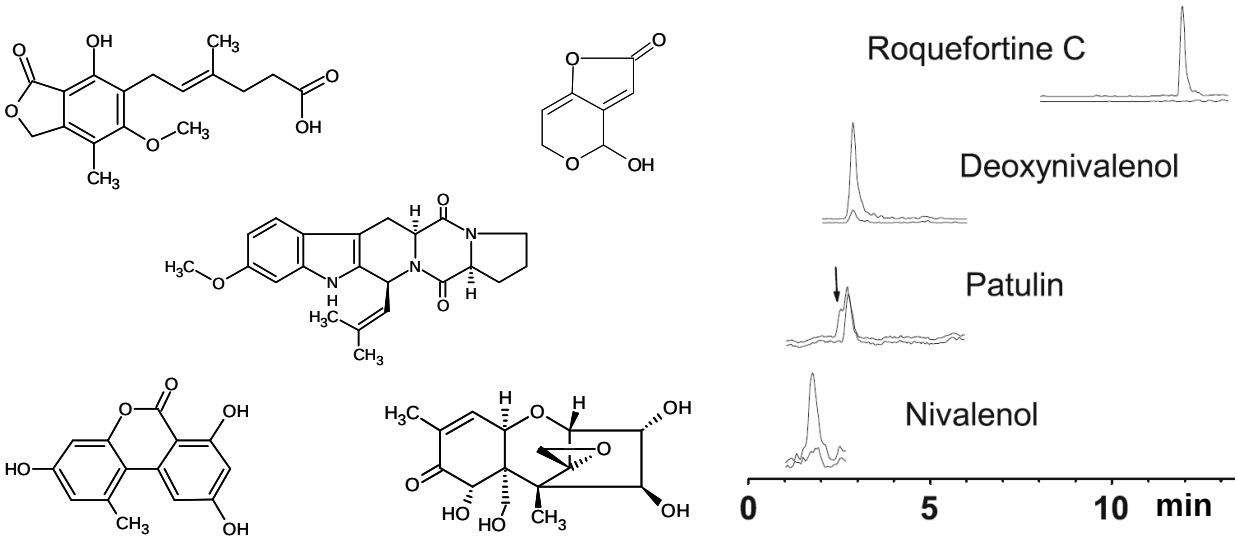
3. MULTI-MYCOTOXIN ANALYSIS OF MAIZE SILAGE BY LC-MS/MS

Paper II



Compound	ESI	MS-MS	REC_{mean}	RSD_r	RSD_{IR}	LOD
		M » D1, D2	%	%	%	ug/kg
Mycophenlic acid	-	319 » 191, 179	90	11	13	7
Zearalenone	-	317 » 131, 175	90	12	16	9
Alternariol	-	257 » 215, 147	78	9	14	10
Cyclopiazonic acid	+	337 » 196, 182	63	22	35	15
Enniatin B	+	657 » 314, 527	60	21	24	24
Deoxynivalenol	-	341 » 265, 295	83	17	18	739
Marcfortine B	+	464 » 436, 419	61	9	9	-
...						
Citrinin	+	251 » 233, 191	Unstable LC-MS-MS sensitivity			
Fumonisin B1	+	723 » 334, 528	6	13	18	544

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ORIGINAL PAPER

Multi-mycotoxin analysis of maize silage by LC-MS/MS

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Abstract This paper describes a method for determination of 27 mycotoxins and other secondary metabolites in maize silage. The method focuses on analytes which are known to be produced by common maize and maize-silage contaminants. A simple pH-buffered sample extraction was developed on the basis of a very fast and simple method for analysis of multiple pesticide residues in food known as QuEChERS. The buffering effectively ensured a stable pH in samples of both well-ensiled maize (pH<4) and of hot spots with fungal infection (pH>7). No further clean-up was performed before analysis using liquid chromatography–tandem mass spectrometry. The method was successfully validated for determination of eight analytes qualitatively and 19 quantitatively. Matrix-matched calibration standards were used giving recoveries ranging from 37% to 201% with the majority between 60% and 115%. Repeatability (5–27% RSD_r) and intra-laboratory reproducibility (7–35% RSD_{IR}) was determined. The limit of detection (LOD) for the quantitatively validated analytes ranged from 1 to 739 μg kg^{-1}. Validation results for citrinin, fumonisin B_1 and fumonisin B_2 were unsatisfying. The method was applied to 20 selected silage samples and alternariol monomethyl ether, andrastin A, alternariol, citreoisocoumarin, deoxynivalenol, enniatin B, fumigaclavine A, gliotoxin, marcfortine A and B, mycophenolic acid, nivalenol, roquefortine A and C and zearalenone were detected.

Keywords Mycotoxins · Maize · Silage · LC-MS/MS · Validation · QuEChERS

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Introduction

Maize silage is contaminated with a wide variety of pre- and post-harvest fungi, which may lead to undesired production of mycotoxins and other secondary metabolites [1]. The intake of mycotoxins may affect animal health and productivity [2]. Transfer of various mycotoxins from feed to milk and meat is also of concern [3, 4]. In modern dairy farming systems, dairy cows are consuming up to 40 kg/day, with maize silage constituting 50–75% of the diet [5].

Pre-harvest fungal contaminants of maize plants under Danish conditions include mainly *Fusarium* and *Alternaria* species [6] whereas post-harvest contaminants of maize silage include *Penicillium roqueforti*, *Penicillium paneum*, *Byssochlamys nivea* and *Aspergillus fumigatus* [7]. These species are in culture capable of producing a range of chemically very diverse compounds (Fig. 1), ranging from: (1) small polar but neutral compounds like patulin; (2) acidic compounds like mycophenolic acid and hydroxylbenzoic acids; (3) basic compounds like roquefortines and marcfortines; and (4) large apolar compounds like penitrems and enniatins [1].

Due to the chemical differences of the fungal metabolites, multi-mycotoxin methods with no sample clean-up are needed. Such methods have been reviewed by Zöllner and Mayer-Helm [8] and Krska et al. [9]. These multi-methods mainly include regulated toxins in food and feed, e.g. aflatoxins B_1, B_2, G_1, G_2, M_1, ochratoxin A, patulin,

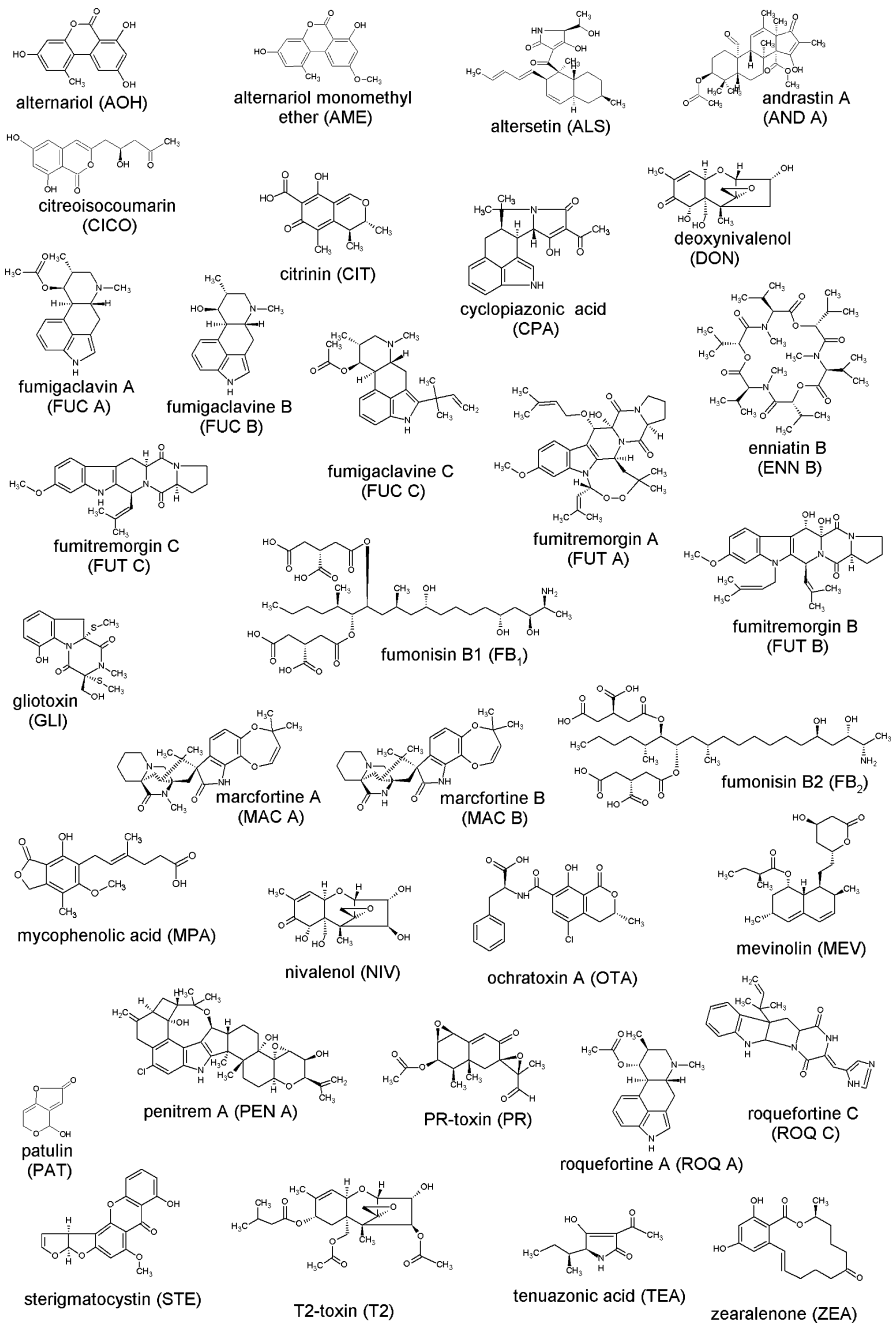

Fig. 1 Structural differences and various functional groups of the compounds tested and their names and abbreviations

deoxynivalenol, zearalenone, fumonisins B_1 and B_2, T-2 and HT-2 toxin [10]. Most of the multi-methods use LC-MS/MS on triple quadrupole systems, although LC-TOF-MS and LC-Orbitrap based methods are also looking promising [11].

Altogether, few multi-mycotoxin methods have been fully validated in silage [12, 13]. Ideally, a method for the screening of maize silage samples should include the whole secondary metabolic potential of the pre- and post-harvest contaminants with an emphasis on the mycotoxins. Driehuis et al. [12] measured 20 analytes of which five are post-harvest compounds (ochratoxin A, mycophenolic acid, penicillic acid, roquefortine C, sterigmatocystin), but none of these are associated with *A. fumigatus* or *P. paneum*.

Garon et al. [13] detected seven mycotoxins of which only citrinin and gliotoxin originate from common post-harvest contaminants of silage. The studies by Mansfield et al. [14] and O'Brien et al. [15] both use LC-TOF-MS, and focus on the metabolites from the penicillia, but none were validated adequately. Mansfield et al. did not include any qualifier ions nor used the high-resolution capability of the instrument, and the study of O'Brien et al. was mainly focusing on novel compounds like marcfortine A and andrastin A.

Maize silage is a complex matrix as the whole maize plant is fermented. It contains, e.g., chlorophylls and carotenoids from the leafy parts of the plant, starch, glucans from the cob and organic acids from the ensiling, and is thus much more complex than products based on the maize kernels. Since pH may vary from 3.6 in well-ensiled maize to 7–9 in fungal hotspots [16], pH must be controlled by buffers when extracting and analysing silage samples. pH changes the polarity of compounds with ionisable groups [17], thus affecting the extraction efficiency.

Trace analysis of pesticide residues in fruit, vegetables and cereals is in many ways comparable to mycotoxin analysis. An increasingly popular multi-method for pesticide detection in various matrixes is the Quick, Easy, Cheap, Effective, Rugged and Safe method, known as QuEChERS [18–20]. Briefly, the method uses acetonitrile for extraction of the analytes followed by the addition of high concentrations of $MgSO_4$ and NaCl. The salts induce a phase separation between ACN and water, keeping extremely polar contaminants in the water. Buffering can be applied to overcome pH effects of the matrix on the extraction efficiency of chargeable compounds [21].

The aim of the present study is to adapt, apply and evaluate the QuEChERS method to the extraction of multiple mycotoxins in maize silage samples. To our knowledge, it is the first publication describing the use of QuEChERS in mycotoxin analysis. A LC-MS/MS method was developed and validated for the detection of mycotoxins in the silage extracts. The method targets chemically very different metabolites from pre-harvest (*Fusarium culmorum*, *Fusarium graminearum*, *Fusarium avenaceum* and *Alternaria tenuissima*) and post-harvest (*A. fumigatus*, *Monascus ruber*, *P. roqueforti*, *P. paneum*, *B. nivea*) fungal contaminants of silage. The structures, names and abbreviation of the tested compounds are shown in Fig. 1. The method has been applied to 20 field samples of naturally contaminated maize silage.

Experimental

Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) were HPLC-grade (Rathburn, Walkerburn, Scotland, UK). NaOH, CH_3COOH, HCOOH, NH_4OH, $HCOONH_4$, CH_3COONa, $MgSO_4$ and CH_3COONH_4 were all of analytical reagent grade. Water was ultra-purified using a Millipore system (Molsheim, France).

Standards were purchased from commercial suppliers; FUT C, ENN B from Alexis Biochemicals (Farmingdale, NY, USA), AOH, AME, CIT, NIV, DON, GLI, MPA, CPA, OTA, PAT, ROQ C, T-2, TEA, ZEA, MEV, PEN A and STE all from Sigma-Aldrich (St. Louis, MO, USA). FB_1 and FB_2 were acquired from Romer Labs (Tulln, Austria). Quantitative standards of AND A and FUT A and B as well as qualitative standards of CICO, FUC A and B, MAC A and B, and PR were available for LC-MS/MS optimisation from earlier studies [22].

For the spiking experiments, a pooled fungal extract was prepared. Agar cultures of *A. tenuissima, P. roqueforti, P. paneum* and *A. fumigatus* were extracted according to Smedsgaard [23] with a few modifications. ALS was only available from fungal extract of *A. tenuissima*, but was confirmed by LC-TOF-MS and UV characteristics [24].

All mycotoxin stock solutions (25–5,485 $\mu g \cdot mL^{-1}$) were prepared in ACN and kept at −18 °C unless otherwise recommended by the manufacturer.

Sample preparation

Silage samples were frozen with liquid N_2 and homogenised in a domestic blender. Extraction was performed by a modified version of a method for multiple pesticide residues in food known as QuEChERS [21]: In a 50-mL polypropylene tube, 10.0 g sample (fresh weight) was extracted with a buffered mixture of 10 ml 1% acetic acid in ACN, 5 ml water and 1.67 g sodium acetate tri-hydrate by shaking for 1–2 min. Then, 4.0 g anhydrous $MgSO_4$ was added and the tube was shaken (1 min) to obtain phase separation. After a 10-min centrifugation (4,500×*g*), the upper ACN phase was collected. Before LC-MS/MS analysis, the samples were filtered through a 0.45 μm PFTE filter in Mini-UniPrep HPLC vial (Whatman International, Maidstone, Kent, UK).

Robustness of buffering

The effectiveness of the buffering incorporated in the method was tested with 12 portions of a silage sample naturally contaminated with *P. roqueforti*/*P. paneum* which were also spiked with pure standards at medium level according to the validation plan. The pH of six portions of silage was adjusted to pH>10 by adding 5 ml of 0.55 M NaOH in the first extraction step instead of 5 ml of water. Triplicate samples at both the natural silage pH of 4.2 and pH>10 were subjected to either a traditional extraction with a 4:1 (*v*/*v*) unbuffered mixture of ACN and water or the present method. Extracts were analysed by LC-MS/MS and pH in the surplus extracts was measured after dilution 1:4 (*v*/*v*) with water. The effect of pH on analyte response with each extraction method was evaluated with the PROC GLM procedure in SAS 9.1 (SAS Institute Inc., Cary, NC).

LC-MS/MS method

LC separation of 1 μL injected sample was performed on an Agilent 1100 series HPLC system (Agilent Technologies,

Palo Alto, CA, USA) with a Gemini C_6-Phenyl, (3 μm, 2.0× 100 mm) column equipped with a Gemini Security guard cartridge (Phenomenex, Torrance, CA, USA). Samples were analysed in two separate runs, one in positive electrospray ionisation (ESI) mode and one in negative ESI mode. Water-based HPLC eluents were prepared daily. The mobile phases were (A) ammonium formiate 0.4 mM, 0.2% formic acid in water (pH 2.5) and (B) 100% ACN for data recorded in ESI^+. In ESI^-, they were (A) 0.02% formic acid in water and (B) 100% ACN. The gradient conditions were identical. During data collection, a flow rate of 0.3 ml/min of was used: from 0 to 4 min 10% B was kept constant, then going to 100% B from 4 to 22 min. The LC-system and column was cleaned after each sample, by injecting three different blanks: (1) 20 μL 5% formic acid in ACN with 100% B at 0.5 ml/min for 8 min; (2) then 20 μL methanol and gradually changing to 10% B and 0.3 ml/min in 5 min; (3) and finally, 20 μL water maintained at 10% B and 0.3 ml/min for 7 min. This gave a total runtime of 44 min per sample. To protect the MS interface, a valve integrated with the MS instrument was used to direct the eluent into the MS instrument only from 1 to 22 min of the gradient. The auto sampler and column temperature was 25 °C.

A Quattro Ultima triple quadrupole MS without the high-collision hexapole (Waters, Manchester, UK) with Masslynx v. 4.1 software was used for data collection and processing. The MS was tuned to symmetrical peak shapes with a peak width of 0.5 mass unit at half peak height. The capillary voltage was 3.0 kV. The source and desolvation temperatures were 120 °C and 400 °C, respectively. The cone gas flow was 80 l h^{-1} and the desolvation gas flow was 530 l h^{-1}. Argon was used as collision gas at ~2.5×10^{-3} mbar and the electron multiplier voltage applied was 650 V. Fragment ion spectra were recorded from 15–50 V in both polarities and promising selective fragment ions tested and optimised along with the cone voltage in the multiple-reaction monitoring (MRM) mode. Analyte specific detection parameters are listed in Table 1. Inter channel delay was 0.02 s and the dwell times were optimised for the individual transitions and in the range 100–500 ms.

The response was calculated as the chromatographic peak area for all compounds, except for PAT where height were used. A linear calibration curve was obtained by plotting the response of the analyte against the concentration (c) weighted $1/c$. The spiking levels were toxin-specific and were intended to be near the expected detection limit.

Validation set-up

A total of three series were performed by two different technicians on three separate days. Each series included three blind samples, three replicates of samples spiked quantitatively at low, medium and high level and three replicates of samples spiked with a fixed volume of fungal mixture. The spiking levels for each analyte are described in Table 2. Three samples from one well-ensiled Danish maize silage stack with low toxin content were pooled and used as blank and for spiking. The blank silage had traces of 5 ppb ENN B, which was determined using standard addition. The matrix-matched calibration curve of the quantitative standards included six concentration levels and a blank matrix extract. The six levels were obtained by serial dilution with at dilution factor of 128 from the highest to the lowest level. One matrix-matched fungal standard equal to the fungal spike level was also included. Standards were analysed twice; in the beginning and at the end of each sequence. From the results obtained, the relative standard deviation under repeatability conditions (RSD_r), intra-laboratory reproducibility conditions (RSD_{IR}), and recovery (Rec.) was calculated for each compound according to ISO guidelines [25]. RSD_r and RSD_{IR} represent the variation between repeated extractions and analysis within days and between days, respectively. We accepted results from spiking levels when the RSD_{IR} was up to 35%. For compounds quantitatively available, the limit of detection (LOD) was determined as three times the standard deviation at intra-laboratory conditions (SD_{IR}) divided by the recovery, both based on results from the lowest accepted spike level.

Signal suppression and enhancement (SSE) due to matrix compounds was evaluated as the slope of a standard curve in pure ACN divided by the slope of a standard curve in blind matrix extract ($\alpha_{ACN}/\alpha_{matrix}$).

Sample analysis

Samples were extracted and analysed according to the method described above. A matrix-matched calibration curve was produced and included in each sample series. The blank silage extract for the calibration curve was produced together with the sample extracts on the basis of aliquots of the blank silage used for validation. The standards were distributed randomly over the entire sequence and used for quantification in the present series.

Sample data was processed by Quanlynx and subjected to (a) visual inspection of un-smoothed chromatogrammes for low concentration samples to determine whether peaks were above a signal to noise of 3:1 (b) visual inspection of the automatic integrations, with manual modifications to consistent peak width if necessary.

Results and discussion

Extraction and clean-up

The application of the adapted QuEChERS method to mycotoxin extraction was successful. Comparing to the

Table 1 Parameters for the mass spectrometric detection of analytes including analyte abbreviation (abbr.), retention time (RT), cone voltage, precursor ions, product ions and collision energy

	Analyte	Abbr.	RT (min)	Cone (V)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision (eV)
ESI^-	Alternariol	AOH	15.0	35	257	215, 147	25, 30
	Alternariol monomethyl ether	AME	16.7	30	271	256, 228	22, 30
	Altersetin	ALS	20.0	30	398	354, 310	22, 23
	Andrastin A	AND A	17.8	50	485	425, 453	35, 30
	Citreoisocoumarin	CICO	12.3	35	277	219, 191	20, 27
	Deoxynivalenol	DON	2.9	15	341	265, 295	10, 10
	Gliotoxin	GLI	13.6	15	325	261, 243	10, 15
	Mycophenolic acid	MPA	15.6	35	319	191, 179	25, 20
	Nivalenol	NIV	1.8	18	357	281, 311	15, 10
	Ochratoxin A	OTA	17.8	28	402	211, 167	30, 35
	Patulin	PAT	2.5	15	153	109, 81	8, 8
	Penitrem A	PEN A	19.5	50	632	546, 294	30, 50
	Roquefortine C	ROQ C	11.9	35	388	190, 318	30, 30
	Tenuazonic acid	TEA	13.4	30	196	112, 139	25, 18
	Zearalenone	ZEA	17.0	30	317	131, 175	30, 25
ESI^+	Citrinin	CIT	16.4	22	251	233, 191	20, 25
	Cyclopiazonic acid	CPA	18.0	40	337	196, 182	30, 25
	Enniatin B	ENN B	19.9	30	657	314, 527	37, 25
	Fumigaclavine A	FUC A	7.1	30	299	208, 239	28, 18
	Fumigaclavine B	FUC B	2.1	30	257	192, 167	30, 27
	Fumigaclavine C	FUC C	12.2	40	367	238, 307	30, 20
	Fumitremorgin A	FUT A	19.8	15	602	460, 498	15, 15
	Fumitremorgin C	FUT C	15.5	30	380	324, 212	20, 35
	Fumonisin B_1	FB_1	12.6	40	723	334, 528	38, 30
	Fumonisin B_2	FB_2	13.4	40	707	336, 318	35, 35
	Marcfortine A	MAC A	12.0	40	478	419, 450	35, 25
	Marcfortine B	MAC B	11.7	20	464	436, 419	22, 30
	Mevinolin	MEV	18.5	40	405	225, 173	20, 23
	Mycophenolic acid	MPA	15.5	20	321	207, 159	20, 40
	Ochratoxin A	OTA	17.2	20	404	358, 341	15, 20
	PR-toxin	PR	15.8	15	321	261, 279	10, 15
	Roquefortine A	ROQ A	10.0	25	299	239, 197	18, 25
	Roquefortine C	ROQ C	12.6	25	390	322, 334	22, 30
	Sterigmatocystin	STE	17.3	40	325	281, 301	35, 28
	T-2 toxin	T-2	15.9	30	484	215, 305	20, 20

The first product ion listed is the quantifier and the second is the qualifier. The analyte specifications are sorted by electrospray ionisation mode

extraction methods employed in [12, 26], these methods employ ACN (or MeOH) with 10–20% (*v*/*v*) water, while the present method has 33% (*v*/*v*) mix of water and ACN. This should allow for better extraction of the more polar analytes. With the induction of phase separation the extraction of less polar compounds is facilitated. According to [18], the ACN phase holds approximately 8% of water. The high concentration of salt in the water phase forces the polar analytes into the less polar ACN. In the case of varying water content in silage samples, the phase separation should also result in a more stable polarity in the extract. This is of relevance as fungal hot spots in silage are much wetter than non-infected silage due to the microbial activity.

The buffering incorporated in the method was very effective. In spiked silage samples adjusted to pH>10 and subsequently subjected to our buffered modified QuEChERS extraction, the pH of the ACN phase (diluted 1:4 *v*/*v* with water) was 4.3. In the same silage, at its natural pH of 4.2, the pH of the ACN phase was 3.7. When the same silage samples were subjected to traditional extraction

Table 2 Results of the validation for 27 analytes spiked in blind maize silage, including the accepted spike levels (concentrations or 'fungal' for qualitatively spiked analytes), average recovery (avg. rec.), no. of spiked samples accepted for the validation (n_{total}), repeatability (RSD_r), reproducibility (RSD_{IR}) and limit of detection (LOD)

Analyte		Spike levels (μg·kg^{-1})	n_{total}	Avg. Rec. (%)	RSD_r (%)	RSD_{IR} (%)	LOD (μg·kg^{-1})
ESI$^-$	Alternariol	20, 40, 80	27	78	9	14	10
	Alternariol monomethyl ether	20, 40, 80	27	79	5	10	6
	Altersetin	fungal	9	91	14	14	–
	Andrastin A[ab]	fungal	9	122	8	12	1
	Citreoisocoumarin	fungal	9	84	7	7	–
	Deoxynivalenol[c]	1399, 2797	18	83	17	18	739
	Gliotoxin	200, 400, 800	27	85	13	13	71
	Mycophenolic acid	20, 40, 80	27	90	11	13	7
	Nivalenol[c]	200, 400, 800	27	68	13	15	122
	Ochratoxin A	40, 80	18	71	8	9	10
	Patulin	700, 1400, 2800	27	100	17	17	371
	Penitrem A	20, 40, 80	27	107	6	12	8
	Roquefortine C[ab]	fungal, 200, 400, 800	27	205	9	25	158
	Tenuazonic acid[c]	fungal, 202, 404	27	37	20	20	121
	Zearalenone	20, 40, 80	27	90	12	16	9
ESI$^+$	Cyclopiazonic acid[ac]	20, 40, 80	18	63	22	35	15
	Enniatin B	25, 45, 85	27	60	21	24	24
	Fumigaclavine A	fungal	9	93	12	21	–
	Fumigaclavine C[abc]	fungal	9	176	11	13	–
	Fumitremorgin A[c]	100, 200	18	93	18	23	76
	Marcfortine A	fungal	9	63	12	16	–
	Marcfortine B	fungal	9	61	9	9	–
	Mevinolin[c]	40, 80	18	68	25	27	25
	PR-toxin	Fungal	9	56	27	32	–
	Roquefortine A	Fungal	9	103	13	32	–
	Sterigmatocystin	20, 40, 80	27	72	9	9	8
	T-2 toxin[c]	125, 250, 500	27	55	17	26	96

LOD was not calculated for the qualitatively spiked analytes

[a] Data from day 2 omitted due to high day-to-day variation in recovery

[b] Fungal spike (n=3) on day 4 included

[c] Ion ratio out of the expected range in many samples

with an unbuffered mixture of ACN and water the corresponding pH values of the extract were 10.4 and 4.4. The substantial difference in pH had effects on the analysis with both extraction methods. For AOH, CPA, FUC A, FUC C, PEN A and ZEA a significant difference in LC-MS/MS response was observed between the low- and high-pH samples extracted with the unbuffered mixture of ACN and water, with P values of 0.001, 0.029, 0.003, 0.047, 0.002 and 0.001, respectively. For these analytes, no significant difference in response was observed with the buffered QuEChERS method. The response of the analytes AND A, CICO, OTA and ROQ C differed significantly between the two pH values for both extraction methods (P values ranging from 0.047 to <0.001), while the responses for FUT A and STE only differed significantly between pH values when extracted according to the QuEChERS method (P=0.047 and 0.027, respectively).

It is possible that the extraction of field samples can be improved by performing a longer initial extraction without any salts. However, Lehotay et al. [21] did not experience any negative effect of combining the extraction and partitioning steps into one procedure. Both pesticides and fungal metabolites may be present inside and outside the plant depending on application methods and infection biology, respectively. Therefore, some analytes may be less accessible to extraction than others. Hence, the very short extraction time (<2 min) may pose a problem which is not addressed in validation with spiked samples. Optimisation

of extraction time should therefore be done with naturally infected samples. However, several metabolites were detected when the present method was applied to naturally contaminated samples (Table 3).

The amount of sodium acetate or sodium chloride in the QuEChERS method is known to influence the extraction of both analytes and matrix compounds [18, 21]. Fine-tuning of this concentration has not been done but might improve the balance between analytes and interferences in the extract.

The dispersive solid-phase extraction (SPE) with primary–secondary amine (PSA) employed in the QuEChERS methods for pesticides [18, 21], was not used for mycotoxins. PSA binds organic acids, which in our case would be MPA, OTA and CIT as well as TEA and CPA, which also have acidic properties. It was therefore chosen not to employ this clean-up procedure.

In the initial steps of the multi-method development, SPE was tested for clean-up of silage extract. Both C_{18}, polymeric (Strata X) and mixed mode columns (Strata X-C, Oasis MAX) were tested without satisfactory results. In the application of reverse-phase SPE (C_{18}) only few matrix components could be removed to fit all compounds. When also taking advantage of the functional groups on the molecules several analytes were not retained very well and the procedure was very time-consuming. Even in combined extracts from the SPE clean-up, large quantities of matrix were still present. In some cases, regulation of pH in the extracts in order to optimise SPE retention also led to phase separation of the extract, which interferes with the SPE separation. SPE clean-up did therefore not constitute an improvement.

LC-MS-MS method

The compounds were MS-tuned (Table 1) in their most sensitive ESI mode (+/−). However, for MPA, OTA, ROQ C data have been collected in both modes. The ESI^- was preferred for these compounds due to better recoveries and lower RSDs and LODs. The better sensitivity of these analytes in ESI^- than in ESI^+ was unexpected, especially since OTA and ROQ C in solvent have been found to be much greater than tenfold more sensitive in ESI^+ than in ESI^- on a Micromass LCT and an almost identical Quattro Ultima triple quadrupole MS (in this case equipped with a high-collision hexapole).

From LC-MS/MS runs of single standards, it was concluded that the compounds in the method did not interfere with each other in the measuring range. At AME concentrations much higher than the validated measuring range, some interference with ZEA was observed. This was possible due to co-elution and because AME formed a small amount of the adduct $[M+HCOO]^-$ having the same mass (*m/z* 317) as the ZEA $[M-H]^-$ precursor. The ion ratio (quantifier/qualifier) of the interfering daughter ions from AME (2.3) was however different from the ratio of ZEA (0.8). Unique ion-transitions were preferred for the MS/MS method. Daughter ions resulting from water or adduct loss were avoided when possible.

The evaluation of SSE due to matrix compounds showed a large variation in the matrix effect between analytes. Results for the quantitatively validated analytes are presented in Table 4. The signal for MEV is highly enhanced by the silage extract while AOH and ROQ C signals are

Table 3 Summary statistics for the fungal secondary metabolites detected in ten samples of visibly unspoiled silage and ten samples of hot spots with visible fungal growth (nd=not detected)

Analyte		Unspoiled silage (n=10)				Fungal hot spots (n=10)			
		n_{pos}	Concentration (μg·kg^{-1})			n_{pos}	Concentration (μg·kg^{-1})		
			$mean_{pos}$	min_{pos}	max_{pos}		$mean_{pos}$	min_{pos}	max_{pos}
Quantitative	NIV	0	nd	nd	nd	2	140	138	142
	DON	0	nd	nd	nd	2	990	888	1,092
	ROQ C	1	189	189	189	3	11,826	51	33,662
	GLI	0	nd	nd	nd	2	594	282	906
	AOH	1	24	24	24	1	236	236	236
	MPA	1	52	52	52	6	507	10	1,646
	AME	0	nd	nd	nd	1	51	51	51
	ZEA	4	99	10	311	4	71	19	156
	AND A	7	159	11	691	6	2,400	8	8,811
	ENN B	4	44	25	63	3	93	37	200
Qualitative	FUC A	0				1			
	ROQ A	4				3			
	MAC B	0				1			
	MAC A	1				3			
	CICO	5				5			

n_{pos} number of positive samples within the ten samples in each category, $mean_{pos}$, min_{pos}, max_{pos} average, minimum and maximum values of the positive samples

Table 4 Approximate analyte specific signal suppression and enhancement effects (SSE) tested for unspoiled maize silage

Analyte	SSE (%)
AME	67
AND A	89
AOH	48
CPA	79
DON	62
ENN B	89
FUT A	115
GLI	86
MEV	177
NIV	78
PAT	97
PEN A 1	07
ROQ C	48
STE	115
T-2	82
ZEA	75

suppressed. The use of matrix-matched calibration standards compensated for the matrix effects. For TEA, the sensitivity in pure ACN standards was insufficient for evaluation of SSE. MPA and OTA also showed a highly concentration-dependent matrix effect.

The post-run cleaning procedure with injections of formic acid in ACN, MeOH and water was necessary to prevent matrix build-up on the column. Without the procedure, unstable RTs and rapid decreases in sensitivity were observed after just eight injections of silage extract. To continually monitor a potential matrix accumulation on the chromatographic column, a blank ACN sample was included after nine matrix samples injected. These steps gave reliable and stable MS/MS signals throughout a sequence. To achieve lower RSD in the MS analysis, shorter sequences (<24 h) and correction by internal standards for the individual compounds should be applied.

To ensure proper formation of ammonium adducts (T-2 and ENN B) and to obtain better chromatography of the pH-dependent compounds (e.g. ROQ A, CPA, CIT) eluent A used in ESI^+ was added ammonia and formic acid. Eluent A for ESI^- had only low formic acid content and application of ammonium formiate buffer resulted in a significant signal suppression of the early eluting compounds. This was not accepted as NIV, DON, PAT and GLI already were expected to have high detection limits. It was therefore decided to analyse samples in two separate runs, even though the instrument can switch between the two modes. Switching between positive and negative ionisation requires extra time for data collection. This can, in practice, cause troubles for quantification due to few data points across the peaks and short dwell times when several compounds co-elute [26]. By the use of separate retention time windows for the two ionisation modes, Berthiller et al. [27] simultaneously determined several mycotoxins in a single run. However, because of co-elution and sensitivity drop using common eluent A, this approach was not applicable to our method and instead we accepted a longer instrument time (2×44 min) for every sample.

Method performance

For compounds with little matrix interference, the calculation of LOD on the basis of SD_{IR} at lowest accepted spike level gave a higher and probably more realistic detection limit than if based on noise in blind maize silage. The maximum content in feed of 2,000 μg kg^{-1} ZEA, 8,000 μg kg^{-1} DON and 250 μg kg^{-1} OTA recommended by the European Commission [28] can easily can be determined with the current LODs, also when accounting for the differences in dry matter content in the EC recommendation and the present method.

Previous examinations of mycotoxins in maize silages have detected ZEA and DON [12], ENN B [29] and PAT, MPA, CPA and ROQ C [14]. The average toxin concentrations (range in parentheses) in these studies were: ZEA 174 μg kg^{-1} (25–943), DON 854 μg kg^{-1} (250–3,142), ENN B 73 μg kg^{-1} (24–218), PAT 80 μg kg^{-1} (10–1,210), MPA 160 μg kg^{-1} (20–1,300), CPA 120 μg kg^{-1} (20–1,430) and ROC C 380 μg kg^{-1} (10–5,710). LOD of the current method (corrected for a dry matter content of 35% where appropriate) for ZEA, ENN B and MPA were at level with or below the reported concentration levels. However, for DON, PAT, CPA and ROQ C monitoring of silage with our method is known only to identify part of the samples with the toxins present, as the current LODs are higher than some the of reported contents. Still, it is relevant to measure the frequency of these analytes in more contaminated samples.

Precision and recovery

Compounds with accepted validation results are included in Table 2. Some average recoveries were outside the preferred range of 70% to 110% [30] and still accepted in this multi-method. Optimal extraction and detection of all analytes are not always achievable when several compounds are targeted [26, 27]. We accepted a RSD_{IR} up to 35%, though <22–23% is normally preferred in the 100 ppb range [30–32]. A high RSD_{IR} results in a large uncertainty range, when reporting results. In the application of the method, the detection of the compounds is just as important as a very narrow concentration range in reporting the result. The method was developed for research purposes, not for official food and feed control.

The European Commission [33] has specific criteria for analytical methods applied in foodstuffs (not feed) for a few

toxins. Enniatin B, OTA and zearalenone comply with all the specific criteria. The recovery of T-2 (55%) is a little lower than the demanded range (60–130%) and the RSD_r of PAT (17%) is slightly higher than the 15% accepted. The average recoveries of 6% FB_1 and 13% FB_2 were far below the accepted 70–110% range [33]. Methods focusing only on these water-soluble toxins by using immunoaffinity columns have approximately 100% recoveries [34]. However, acceptable recoveries of FB_1 and FB_2 were also achievable when acidified solvent was used to extract multiple toxins from breadcrumb matrix [26]. The validation of CIT was unsatisfying due to LC-MS/MS instrument day-to-day variations. Results from day 1 showed that CIT is extracted with the QuEChERS method, as spiking at 200 μg kg^{-1}gave 65% mean recovery with 23% RSD_r (n=3). However, on days 2 and 3, a decrease in sensitivity for CIT during the sequence gave unacceptable standard curves and recoveries. Applying LC-MS Garon et al. [13] were able to validate CIT in SPE cleaned-up silage extracts using a HPLC gradient with ACN and acidified water (0.5% acetic acid, pH 3) as mobile phases. This indicates that removal of more matrix components or the use of more acidic eluents than in the present method could be important for proper detection of CIT.

The recoveries were within the same range for low, medium, high and fungal spike and for days 1, 2 and 3 for most compounds. Concentration dependence was only seen for TEA as the recoveries were 52%, 30% and 28% for fungal, 200 μg kg^{-1} and 404 μg kg^{-1} respectively. The fungal spike of TEA was much higher than the other two levels near the LOD. On the second validation day the analytes AND A, CPA, FUC C and ROQ C showed unacceptably high day-to-day variations in recovery. These data were omitted (see footnote to Table 2) and instead an additional fungal spike was carried out. High reproducibility was observed for; ROQ A (81%, 138%, 90%), PR (55%, 44%, 69%) and CPA (50%, omitted, 76%) here expressed as the day-to-day variation in mean recovery. It is recommended always to include spiked control samples to evaluate the recovery of the analysis series.

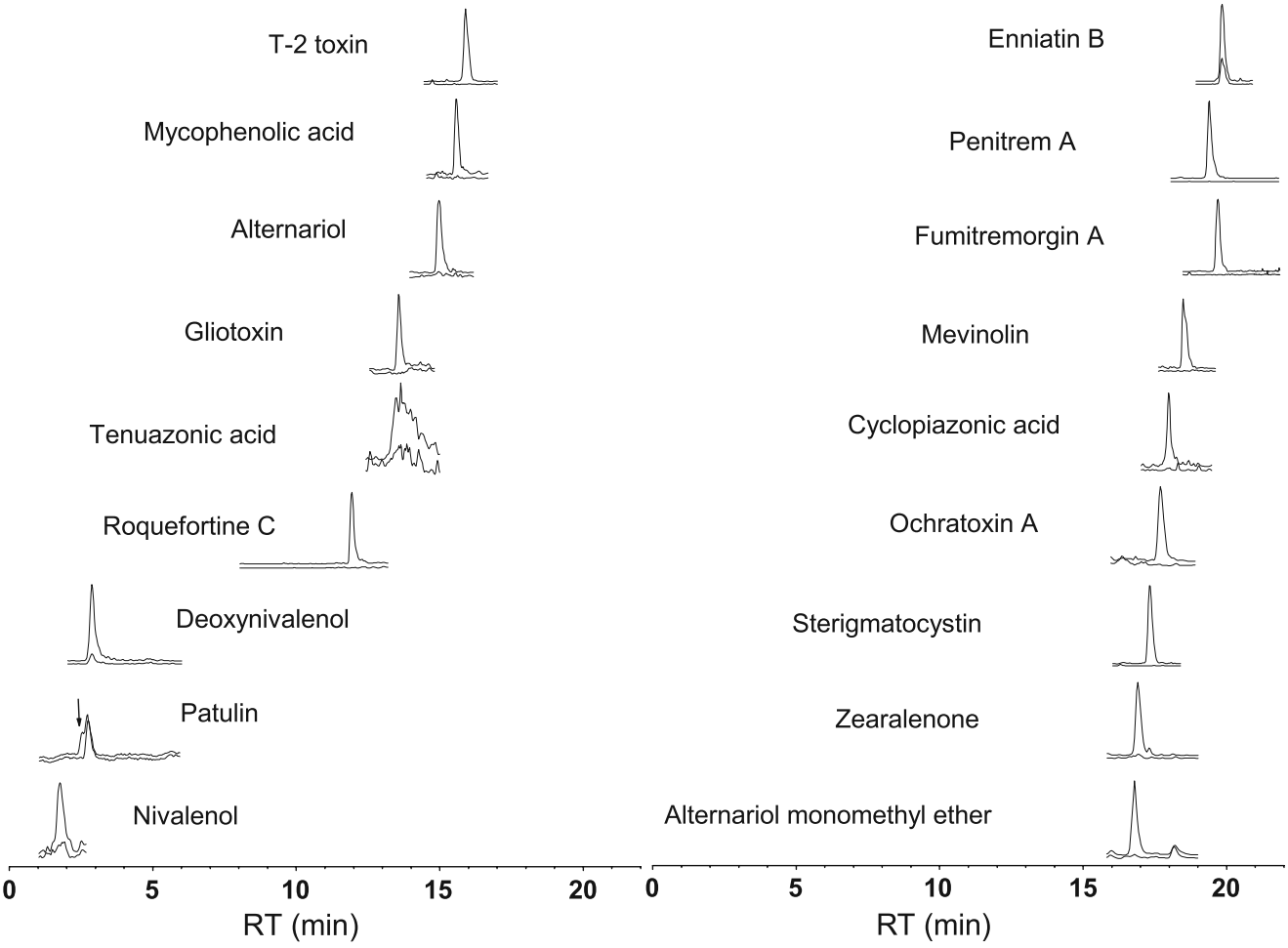

Fig. 2 The relative abundances of the MRM chromatogram traces for the quantitative ions of the mycotoxins in blank maize silage and spiked at the lowest accepted level with quantitative standards

The LC-MS/MS method detects FUC B and FUT C, but they were not present in sufficient amount in the fungal extract to be validated. Their extraction is expected to be like the compounds with structural similarities (FUC A and FUT A, respectively). Similarities in validation data are observed for the isomers ROQ A and FUC A and for the closely related AOH and AME (–OH/–CH_3 group).

Identification criteria

Ideally, identification of a compound should fulfil certain criteria: a retention time (RT) tolerance of 3%, a signal to noise ratio of at least three and similar relative abundances of the diagnostic ions as for spiked samples. At spike levels close to LOD, some ion ratios varied more than recommended in [30] (see footnote to Table 2).

Quantitative ions from mycotoxins spiked at the lowest accepted level (Fig. 2) and silage spiked with the fungal mix (Fig. 3) have been compared to the signal of blank silage. The blank silage was selected among available maize silage samples to have a low natural content of mycotoxins. The smooth chromatograms of the quantitative ions show a low noise for most compounds. Figures 2 and 3 also visualise the broad peak of TEA and FUM A and matrix interference on the quantitative ion of PAT. To overcome a large closely eluting matrix interference for the quantitative ion of PAT with *m/z* 109 (Fig. 2), the peak height was used as response variable instead of peak area. No matrix interference was observed for the less sensitive product ion (*m/z* 81). Using height instead of area enabled automatic integration and ensured acceptance of ion ratio (±20% of standards) for PAT in all spiked samples. NIV and DON were identified by their RTs only. The ion ratios in the matrix-matched standards could not be verified for the major part of the spiked NIV and DON samples due to the qualifier's low sensitivity and interference of matrix.

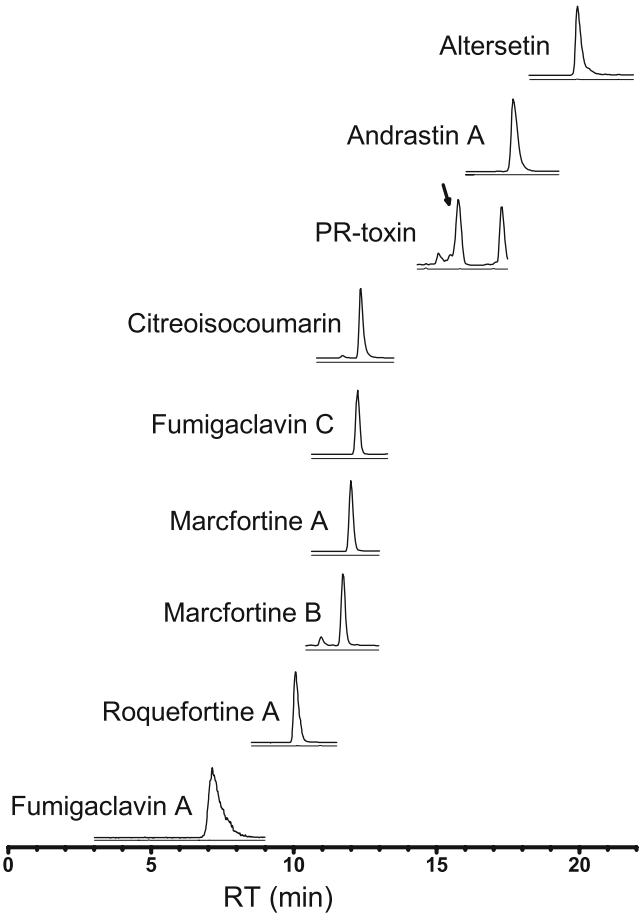

Fig. 3 The relative abundances of the MRM chromatogram traces for the quantitative ions of the mycotoxins in blank maize silage and silage spiked with a mixture of fungal extracts

Field samples

The method was applied to 20 naturally contaminated samples of maize silage collected at Danish dairy farms (Table 3). Ten samples were visibly un-mouldy samples extracted with a silage drill while ten were hot spots with visible fungal growth collected from the cutting face of the silages. The mycobiota of the selected samples was determined previously [7; unpublished data] and the hot spots selected to represent a range of the most common post-harvest contaminants of silage: *P. roqueforti*, *P. paneum*, *A. fumigatus*, *B. nivea* and *M. ruber* [1]. Reported are compounds which were above the LOD in either the unspoiled silage or in the fungal hot spots and met the identification criteria. The *P. roqueforti*/*P. paneum* metabolites AND A and CICO were very common in both hot spots and visibly uninfected silages. Likewise, ROQ A was detected in both types of samples, while MPA and ROQ C were most common in hot spots with maximum concentrations (±95% confidence interval) of 1,646 (±460) µg kg^{-1} and 37 (±18) mg kg^{-1}, respectively. The high concentrations of MPA, ROQ C and AND A in some hot spots are consistent with observation in grass silage by O'Brien et al. [15]. ROQ C was generally present in samples infected with *P. roqueforti* in accordance with Auerbach et al. [35]. AND A could be a good marker for *Penicillum* spoilage during storage because of the low detection limit and its detection in the majority of the unspoiled silages. The maximum concentration of MPA was in a hot-spot infected by *B. nivea*. This fungus is known to produce MPA [36] and this result shows that it is also capable of producing it in silage. The two hot spots with growth of *A. fumigatus* contained GLI in concentrations up to 906 (±245) µg kg^{-1}. FUC A, another known *A. fumigatus* metabolite, was also detected in one of these samples. ZEA, NIV, DON and ENN B are toxins from *Fusarium* species infecting maize pre-harvest [6]. They have also been detected with the current method.

The concentrations were near the limit of detection and much below the maximum contents in feed recommended by the European Commission [28].

Conclusion

A new method for detection of 27 fungal secondary metabolites in maize silage was developed and successfully validated. Nineteen of the analytes can be detected quantitatively and eight qualitatively with recoveries from 37 to 201%, LODs from 1 to 739 μg kg^{-1} and reproducibilities from 7 to 35%. The pH-buffered extraction method ensured the same extraction conditions for fungal hot spots (pH>7) and normal silage (pH~4). Applied to 20 Danish maize silage samples, the following mycotoxins and other fungal secondary metabolites were detected: AME, AND A, AOH, CICO, DON, ENN B, FUC A, GLI, MAC A, MAC B, MPA, NIV, ROQ A, ROQ C and ZEA, representing metabolites from common fungal pre- and post-harvest contaminants of maize silage. With this application of the QuEChERS method to mycotoxin analysis, it may in the future be possible to combine mycotoxin and pesticide analysis.

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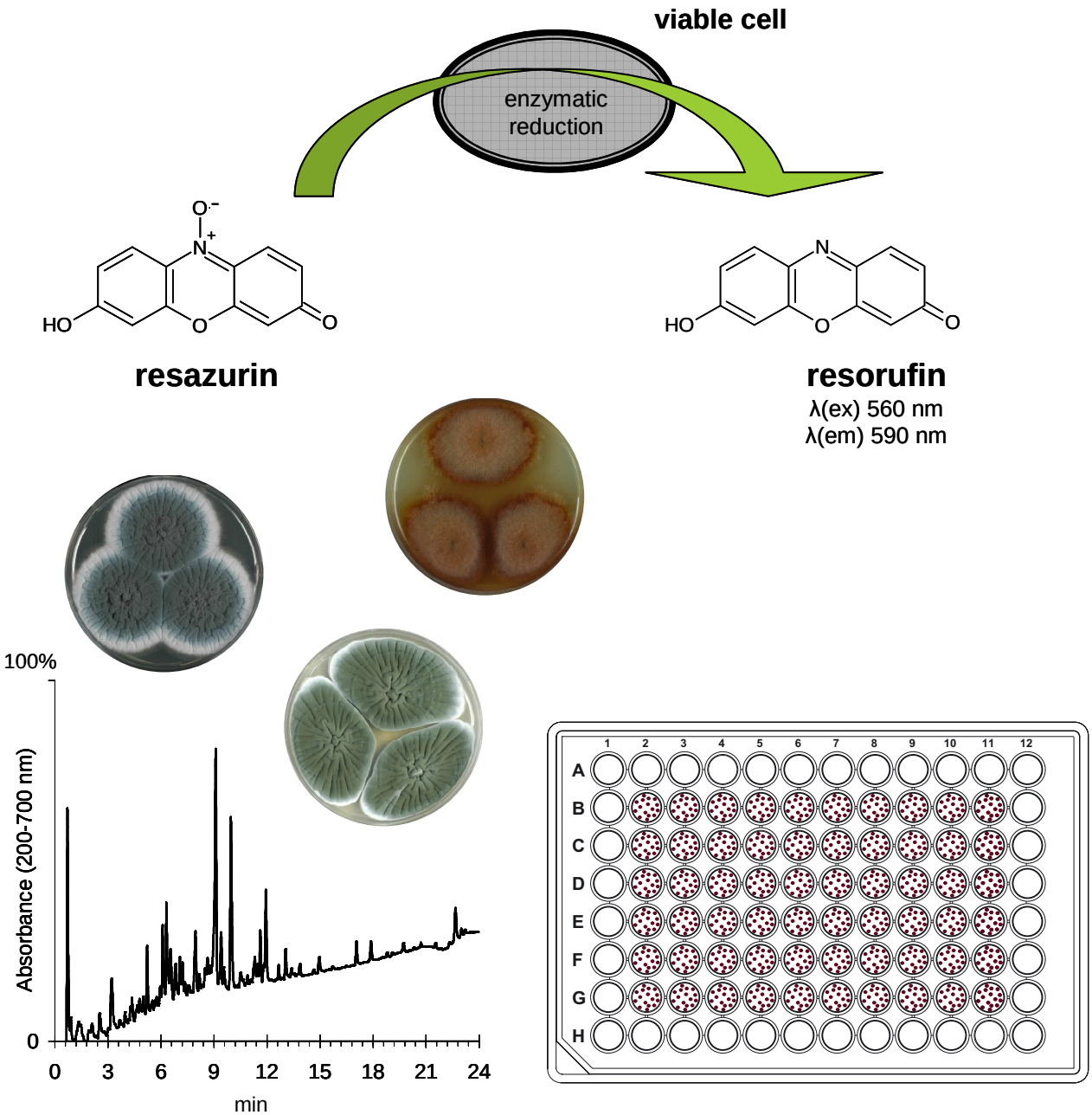
4. *IN VITRO* CYTOTOXICITY OF FUNGI SPOILING MAIZE SILAGE

Paper III



In vitro cytotoxicity of fungi spoiling maize silage

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Running title

Cytotoxicity of fungi spoiling maize silage

Abbreviations

CYA	czapek yeast extract agar
DAD	diode array detection
HPLC	high-performance liquid chromatograph
IBT	fungal isolate in the culture Collection at Systems Biology-DTU, Denmark
IC_{50}	50% inhibition concentration
LC-DAD-HR-MS	HPLC with a time-of-flight mass spectrometer
LC-MS/MS	HPLC with a triple quadrupole mass spectrometer
PDA	potato dextrose agar
SA	silage agar
YE	yeast extract agar
YES	yeast extract sucrose agar

Abstract

Penicillium roqueforti, *P. paneum*, *Monascus ruber*, *Alternaria tenuissima*, *F. graminearum*, *F. avenaceum*, *Byssochlamys nivea* and *Aspergillus fumigatus* have previously been identified as major fungal contaminants of Danish maize silage. In the present study their metabolite production and *in vitro* cytotoxicity have been determined for fungal agar and silage extracts. All 8 fungal species significantly affected Caco-2 cell viability in the resazurin assay, with large variations for each species and growth medium. The 50% inhibition concentrations (IC_{50}) of the major *P. roqueforti* metabolites roquefortine C (48 μg/mL), andrastin A (>50 μg/mL), mycophenolic acid (>100 μg/mL) and 1-hydroxyeremophil-7(11),9(10)-dien-8-one (>280 μg/mL) were high. Fractionating of agar extracts identified PR-toxin as an important cytotoxic *P. roqueforti* metabolite, also detectable in maize silage. The strongly cytotoxic *B. nivea* and *P. paneum* agar extracts contained patulin above the IC_{50} of 0.6 μg/mL, however inoculated onto maize silage *B. nivea* and *P. paneum* did not produce patulin (>371 μg/kg). Still *B. nivea* infected maize silage with mycophenolic acid (~50 mg/kg), byssochlamic acid and other metabolites present, was cytotoxic. In contrast hot-spots of *P. roqueforti*, *P. paneum M. ruber* and *A. fumigatus* were not more cytotoxic than uninfected silage.

1. Introduction

Farmers all over the world produce maize silage to feed dairy cows (Wilkinson and Toivonen, 2003). Maize silage may constitute 50–75% of the diet (Driehuis et al., 2008b) for a dairy cow consuming approximately 26 kg dry matter/day (Eastridge, 2006). Maize plants are converted into maize silage as a result of many naturally occurring enzymatic and microbiological processes taking place when chopped plant material is compressed and packed airtight. A natural lactic acid fermentation of maize sugars into organic acids enables anaerobic and acidic storage of the maize silage. Long-term storage is possible (Storm et al., in press) as a well-managed maize silage stack is a very hostile growth environment to most microorganisms (Weinberg and Ashbell, 1994; Forristal et al., 1999). Nevertheless specific filamentous fungi are known to spoil maize plants in the field or silage during the storage period. The fungi are able to produce many secondary metabolites including mycotoxins. Mycotoxin exposure can affect dairy cows health (Korosteleva et al., 2009) and productivity (Fink-Gremmels, 2008b). The most important toxigenic genera associated with maize and silage are *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium* and *Monascus* (Pelhate, 1977; Storm et al., 2008).

The fungal metabolite production depends on fungal species (Frisvad et al., 2008), isolate (O'Brien et al., 2006; Andersen et al., 2008; Frisvad et al., 2009), growth medium and environmental factors (Frank, 1998; Furtado et al., 2002). The pre-harvest secondary fungal metabolites in maize silage includes; alternariol, alternariol monomethyl ether, beauvericin, deoxynivalenol, 15-acetyl-deoxynivalenol, enniatin B and B1, fumonisin B_1, nivalenol and zearalenone. From post-harvest spoilage of maize silage aflatoxin B1, andrastin A, citreoisocoumarin, citrinin, cyclopiazonic acid, fumigaclavine A, gliotoxin, marcfortine A and B, mycophenolic acid, patulin, PR-toxin, roquefortine A and C have been detected (Müller and Amend, 1997; Garon et al., 2006; Richard et al., 2007; Driehuis et al., 2008b; Mansfield et al., 2008; Sørensen et al., 2008; Rasmussen et al., 2010). Many of those metabolites are mycotoxins, which can elicit carcinogenic, mutagenic, neurotoxic, hepatotoxic, nephrotoxic, oestrogenic, immunosuppressive, antimicrobial (Scudamore and Livesey, 1998) or acute toxic effects (Chen et al., 1982). The symptoms identified in animal trails include feed refusal, kidney, liver or lung damages, birth defects, abortion and death (Scudamore and Livesey, 1998). A chronic exposure to low levels of mycotoxins typically gives non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances (Morgavi and Riley, 2007; Fink-Gremmels, 2008b). To protect animal health some countries have recommendations for deoxynivalenol, ochratoxin A, fumonisins, zearalenone content in feed (European Commission, 2006). The transfer of toxins to dairy and meat products is a potential risk for humans (Miller 2008; Fink-Gremmels 2008a) and the regulation on aflatoxins B_1 in feed (European Commission, 2003) owes to transfer of the carcinogenic aflatoxin M_1 metabolite to milk (IARC, 1993).

Compared to other animals ruminants are more robust to many mycotoxins (EFSA, 2004a, 2004c, 2005), partly due to biotranformations by the rumen microorganisms (He et al., 1992). The rumen microbiota inactivates and degrades some mycotoxins, but not all types whereas others are metabolised to the even more potent compounds in the rumen. For example, ochratoxin A is extensively degraded to the less toxic ochratoxin α (EFSA, 2004b), fumonisin B_1 is unaffected in the rumen (EFSA, 2005) whereas zearalenone is metabolised to α-zearalenol which has stronger oestrogenic effect (EFSA, 2004c). Antimicrobial fungal metabolites such as patulin (Tapia et al., 2002), mycophenolic acid (Bentley, 2000), citrinin (Wang, 2004) and roquefortine C (Kopp and Rehm, 1979) can affect rumen microorganisms (Tapia et al., 2002). An impaired rumen function cause severe metabolic disorders, which can reduce feed utilization (Chiquette, 2009) and may increase the mycotoxin uptake (Fink-

Gremmels, 2008a). Cases of ill-thrift, disease and death in livestock have been related to the presence of mycotoxins in silage (Cole et al., 1977; Seglar, 1997; Boysen et al., 2000; Driehuis and Elferink, 2000; Sumarah et al., 2005; O'Brien et al., 2006) and the issue is much debated (Oldenburg, 1991; Scudamore and Livesey, 1998; Wilkinson, 1999; Fink-Gremmels, 2008a, 2008b; Miller, 2008). Especially high-yielding dairy cows may be susceptible to diseases caused by mycotoxins, due to a high level of stress (Jouany and Diaz, 2005) but acute intoxications causing death are rare (Yiannikouris and Jouany, 2002). Actually was the occurrence of 20 mycotoxins in feedstuffs for dairy cows low compared to the effect concentrations of the individual toxins in a maize silage based diet based (Driehuis et al., 2008a). However fungi are capable of producing many bioactives (Samson et al., 2002) and simultaneous exposure to several toxins could elicit synergism (Bouslimi et al., 2008).

In vitro testing systems are a good screening tool for toxicological effects (Gutleb et al., 2002). Cell cultures of yeast, mammalian cells or bacteria are typically applied. Compared to animal studies *in vitro* assays are fast and cheap, though they may indeed give different results than animal studies, due to lack of an integrated organism response (Gad, 2000). Cytotoxicity assays can to some extent be used as a screening test for acute toxicity in animals and humans (Binderup et al., 2002). The human intestinal epithelial cell line (Caco-2) is widely used and well validated (Videmann et al., 2008). Metabolic conversion of dye by viable Caco-2 cells *in vitro* can determine the general cytotoxicity with similar sensitivity as other cell lines (Cetin and Bullerman, 2005).

In the present study an *in vitro* cytotoxicity assay is used in combination with chemical analysis and bio-directed fractionation to identify important toxic mycotoxins in mixtures of unknown composition. For this purpose concentration-response curves were made for a range of known mycotoxins. *In vitro* cytotoxicity tests of fungal agar extracts and silage extracts have been carried out along with chemical identification using liquid chromatograph with diode array and mass spectrometry detection. The viability of Caco-2 cells was determined from their metabolic conversion of resazurin dye (Binderup et al., 2002). Filamentous fungi often isolated from Danish maize (*F. graminearum*, *F. avenaceum* and *A. tenuissima*) and maize silage (*A. fumigatus*, *M. ruber*, *P. roqueforti*, *P. paneum* and *B. nivea*) were included.

The aims were: (i) to relate the cytotoxicity of well known mycotoxins with their presence in toxic fungal agar extracts, (ii) to determine the toxicity and presence of metabolites of inoculated maize silage, (iii) to identify the most cytotoxic compound in a crude *P. roqueforti* agar extract. The present study is a part of a large Danish collaborative project aiming to determine if mycotoxins in maize silage cause disease and poor performance in dairy cattle (Kristensen et al., 2007; Sørensen, 2009; Storm, 2009).

2. Materials and methods

2.1 Chemicals

Dulbecco's Modified Eagle Medium nutrient mix (DMEM/F12, #11039-021) with HEPES (15 mM), L-glutamine (2.5 mM) and pyridoxine HCl was from GIBCO (Invitrogen, Taastrup Denmark) so was the fetal calf serum (FCS, 10106169). MEM non-essential amino acids, penicillin-streptomycin mix, L-glutamine, phosphate buffered saline, trypsin-EDTA mix, resazurin (R7017) and sucrose (Fluka, 84100) were all from Sigma-Aldrich (St. Louis, MO, USA). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$; Merck, 5886), zink sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$; Merck, 8883) and copper (II) sulfate pentahydrate $CuSO_4 \cdot 5H_2O$ (Merck, 2790) were all from Darmstadt, Germany. Czapek dox broth (Difco, 233810) contains saccharose (30 g), sodium nitrate (3.0 g), potassium phosphate (1.0 g), magnesium sulphate (0.5 g), potassium chloride (0.5 g) and ferrous sulphate (0.01 g). Potato dextrose agar (Difco, 0013-17-6) contains potato starch (4.0 g) dextrose (20.0 g) and agar (15.0 g). Difco products were from Becton, Dickinson and Company (Broendby, Denmark). Yeast extract (Biokar, A1202HA) and agar (SoBiGel) were from Biokar Diagnostics (Beauvais, France) and Bie & Berntsen (Roedovre, Denmark) respectively. Solvents were HPLC grade, chemicals were analytical grade, and the water was ultra-purified using a Millipore system (Molsheim France).

2.2 Secondary fungal metabolites

Crystalline gliotoxin, T-2 toxin, patulin, citrinin, zearalenone, mycophenolic acid (>98% purity) and deoxynivalenol (>97 % purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA) for the cytotoxic tests. Andrastin A and roquefortine C available from previous studies in our lab, and purities were confirmed prior to use. The purities of N6-formyl-roquefortin-C (>99% purity) and 1-hydroxyeremophil-7(11),9(10)-dien-8-one (>97% purity) isolated from *P. roqueforti* (IBT 28547) in this study were determined by analytical high-performance liquid chromatograph with diode array detection (HPLC-DAD) at 200-700 nm. Standard stock solutions of 0.2 to 14 mg/mL in methanol (Rathburn HPLC grade) were kept at –18 °C. For identification of fungal metabolites more than 600 authentic standards was available at DTU Biosys, (Lyngby, Denmark).

2.3 Fungal agar extracts

Strains of *P. roqueforti* (IBT 28546, 28547, 28548, 28549), *P. paneum* (IBT 28542, 28543, 28544, 28545), *M. ruber* (IBT 9655, 9658, 9664, 41178), *A. tenuissima*, *F. graminearum* (IBT 41172, 41173, 41174), *F. avenaceum* (IBT 41175, 41176, 41177, 41180), *B. nivea* (IBT 28550, 28551, 28552, 28553) and *A. fumigatus* (IBT 15720, 23720, 23737, 24699) were transferred with a three point inoculation to 9 cm Petri dishes with different media: YES (yeast extract sucrose agar), CYA (czapek yeast extract agar) and PDA (potato dextrose agar). Selected stains were also inoculated to YE (yeast extract agar) or SA (silage agar) made of 300 g finely blended maize silage and 15 g agar per 1 L medium. YE agar had the same composition as YES agar, except that sucrose was not added. An identical trace metal solution ($ZnSO_4 \cdot 7H_2O$ (1.0 g) and $CuSO_4 \cdot 5H_2O$ (0.50 g) in 100 mL water) was added to YE, YES, CYA and PDA. YES (pH = 6.4±0.1) was prepared with agar (20 g), yeast extract (20 g), sucrose (150 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), trace metal solution (1 mL) and water (885 mL). CYA (pH = 6.25±0.25) contained agar (15 g), yeast extract (5 g), czapek dox broth (35.0 g), trace metal solution (1 mL) and water (1000 mL). PDA (pH = 5.6±0.2) contained potato dextrose agar (39.0 g), trace metal solution (1 mL) and water (1000 mL). The pH was adjusted just prior to autoclaving.

Metabolites were extracted from 13-14 days old cultures incubated at 25°C in darkness, except for *A. tenuissima* which grew in alternating light. Agar plugs were cut from the colonies of several plates and extracted using a micro-scale method by Smedsgaard (1997) with a few modifications. In brief, 54 plugs (⌀6 mm = 1527 mm^2 fungal surface) were extracted twice with a 5 mL mixture of ethyl acetate, dichlormethane, methanol (3:2:1), 1% formic acid (v:v) in a 16 mL screw-cap vial by ultrasonication for 45 min each time. Both extracts were transferred to a clean vial, evaporated to dryness in a rotary vacuum concentrator (Christ, Gefriertrocknungsanlagen, Osterode am Harz), re-dissolved in 2 mL methanol, ultrasonicated and finally filtered through a 0.45 µm PFTE filter (National Scientific Company rockwood, TN, USA). All fungal strains are from the IBT collection at DTU Biosys, (Lyngby, Denmark).

2.4 Isolation of *P. roqueforti* metabolites

P. roqueforti (IBT 28547) was three point inoculated to 100 YES plates (⌀85 mm) and an extract from 10 days old culture was made. In brief, 10 plates at the time were homogenised in a stomacher (Colworth Stomacher 400 BA6021, London, UK) with 100 mL mixture of ethyl acetate, dichlormethane, methanol (3:2:1), 1% formic acid (v:v) for 1 min. and the suspension was allowed to stand for 1 hour. The supernatant was removed and a second extraction took place over night. After paper filtration (Whatman 4**,** Kent, UK) all extracts were evaporated to dryness at 32°C using a rotavapor concentrator (Büchi, Rotavapor R-134 and Büchi V-855/R-215, Flawil, Switzerland). The crude extract (4.4 g) was re-dissolved in methanol (17 mL) and stored at -20°C.

Secondary metabolites of the crude extract were fractionated by a Biotage IsoleraTM One flash purification system (Uppsala, Sweden) with a prepacked Snap Cartridge column (39 x 157 mm, C18: 100 g) from Biotage (Uppsala, Sweden) using 40 mL/min mobile phase of water/acetonitrile and gradient conditions (30-100% in 30 min). Changes in the slope of 210 nm were targeted. Further isolation was performed using Luna II C18 columns from Phenomenex (Torrance, USA) and water/acetonitrile eluent containing 50 ppm trifluoroacetic acid by targeting specific peaks or by time fractioning (45 sec). The conditions for peak and time fractioning were; 15 mL/min gradient flow 35-65% in 30 min on a 250 x 21.2 mm, 5 µm column and 5 mL/min gradient flow 25-65% in 20 min on a 250 x 10 mm, 5 µm column, respectively.

2.5 Fungal maize silage extracts

Two isolates of respectively *P. roqueforti*, *P. paneum*, *B. nivea*, *M. ruber and A. fumigatus* were inoculated onto Danish silage sampled 11 month after ensiling. Defrosted non-sterilised sub-samples of maize silage (30 g) were added 1 mL solution containing approximately $5 \cdot 10^6$ spores/mL. The closed jars were incubated for 3 weeks, 20 ^oC in water saturated air. Triplicate incubations of each isolate were pooled and stored at -20^oC until analysis. Metabolites were extracted and quantified as described by Rasmussen et al. (2010). In brief, a 10 g sample was extracted with 10 mL acetonitrile and 5 mL water buffered with 0.7% acetic acid and 1.7 g sodium acetate trihydrate. Shaking with 4.0 g anhydrous magnesium sulfate induced phase separation. After centrifugation the upper acetronitrile phase was filtrated (0.45 µm) before LC-MS-MS analysis. For cytoxic screening of samples a 5.0 mL acetronitrile sample extract was evaporated under a steam of nitrogen and redissolved in 0.8 mL methanol.

2.6 Chemical analyses

Secondary fungal metabolites have been determined by several instruments. The HPLC-DAD an Agilent 1100 (Walbronn, Germany) was operated as described by Andersen et al. (2008). In brief, sample (0.4 - 5 µL) was separated on a Luna C18 (2) column (3 µm, 2.0 x 100 mm) equipped with a guard column (both from Phenomenex, Torrance, USA) using a water-acetonitrile gradient with 50 ppm trifluoroacetic acid. The LC-DAD-HR-MS a Waters LCT time-of-flight mass spectrometer connected to an Agilent 1100 HPLC with diode array detection (Walbron, Germany) was operated similar to Larsen et al. (2007) and with 40°C column temperature using a 0.3 mL/min flow rate. In brief, sample (0.2 - 1 µL) was separated on a Luna C18 (2) (3 µm, 2.0 x 50 mm) column equipped with a guard column of the same material (both from Phenomenex, Torrance, USA) using a water-acetonitrile gradient with 20 mM formic acid. Two LC-MS/MS Quattro Ultima triple quadrupole (Waters, Manchester, UK) with Masslynx v. 4.1 software (Waters) were connected to an Agilent 1100 HPLC (Palo Alto, CA, USA). One instrument was run according to Rasmussen et al. (2010). In brief, sample (1 µL) was separated on a Gemini 3u C6-Phenyl (3 µm, 2.0 x 100 mm) column equipped with a guard column (both from Phenomenex, Torrance,USA) using a water-acetonitrile gradient. In positive electrospray ionization (ESI) eluent water was added 0.4 mM ammonium formiate and 0.2% formic acid and in negative ESI only 0.2% formic acid was added. The limit of detections were in the 1-739 µg/kg range for maize silage samples analysed by LC-MS/MS (Rasmussen et al., 2010). The other LC-MS/MS was applied to agar extracts only and run according to Sørensen et al. (2008) with a few modifications. In brief, sample (1 µL) was separated on a Gemini 3u C6-Phenyl (3 µm, 2.0 x 50 mm) using a water-acetonitrile gradient.

The secondary fungal metabolites were identified by comparing retention times, UV spectra (Frisvad and Thrane, 1987) and mass spectra (Nielsen and Smedsgaard, 2003; Larsen et al. 2007; Rasmussen et al., 2010) with analyses of standards or the Antibase database (Laatsch, 2008). The fungal metabolites without reference standards were tentatively identified from background subtracted spectra of LC-HR-MS by plotting $[M+H-H_2O]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+H+CH_3CN]^+$ and $[M+Na+CH_3CN]^+$ ions in ESI positive as well as $[M-H]^-$ and $[M+HCOO]^-$ ions in ESI negative mode (Nielsen and Smedsgaard, 2003). Identification required a peak height of minimum 3 times the noise. The co-eluting PR-toxin and mycophenolic acid having same composition ($C_{17}H_{20}O_6$) were detected in agar extracts by their different LC-HR-MS in-source fragments with m/z 279 and m/z 207, respectively (Nielsen et al., 2006). The distinct UV-max of PR-toxin (254 nm) and mycophenolic acid (210 nm) were also used to confirm the presence of PR-toxin. Secondary metabolites in fungal agar extracts have been quantified using pure standards in solvents. If no quantitatively standard was available the metabolite abundance has been calculated relative (%) to the sample with the highest content measured by UV or MS. Small polar compounds that very elute early from a reversed phase chromatography system were not observed with the analytical methods employed.

2.7 Cytotoxicity assay

The cytotoxicity of secondary fungal metabolites was evaluated using a Caco-2 cell line purchased from Aalborg University in Denmark. The Caco-2 cells can metabolise some toxins such as zearalenone, alternariol and alternariol monomethyl ether (Videmann et al., 2008, Burkhardt et al., 2009). Cells were grown in the medium DMEM/F12 added 10% foetal calf serum, 1% penicillin-streptomycin (100 units/ml and 100 µg /ml, respectively), 1% MEM nonessential amino acids and 1% L-glutamin (2 mM) in 75-cm^2 culture flasks. They were incubated at 37ºC in a humidified atmosphere of 5% CO_2, 95% air. Every 2-3 days the growth medium was changed and at 90% cell density they were split using trypsin-EDTA·4Na (0.05% and 0.02%, respectively). Only cell cultures which were tested negative for mycoplasma infection /contamination were used.

In 96-well plates with clear flat bottoms $1 \cdot 10^4$ cells/well were seeded and allowed 24 h to fasten before 48 h or 72 h toxin exposure. The viability of the Caco-2 cells was measured by adding resazurin (3.6 µg/mL). Resazurin which is the active component in Alamar Blue dye (O'Brien et al., 2000), was reduced to the fluorescent compound resofurin by mitochondria cell enzymes. The viable cells correspond to the fluorescens from the reduced form measured by fluorometry on a Victor2 Multilabel Counter (Wallac, Turku, Finland) at wavelengths 560 nm excitation and 590 nm emission both with the band width 10 nm and at 37ºC. The assay was optimized for cells density, dye concentration and dye exposure time to obtain maximum sensitivity in the linear concentration-response range. Positive controls (4 µg/ml T-2 toxin), solvent controls (1-2% methanol in medium) and blank (medium without cells) were included for all plates.

The cytotoxic concentration-responses of single fungal metabolites were tested for (µg/mL cell medium range in parentheses) andrastin A (1.6-50), citrinin (0.5-100), deoxynivalenol (0.023-100), gliotoxin (0.014-1.0), mycophenolic acid (0.046-100), patulin (0.069-50), roquefortine C (1.6-50), T-2 toxin (0.0018-4.0), zearalenone (1.6-100), 1-hydroxyeremophil-7(11),9(10)-dien-8-one (0.2-280) and N6-formyl-roquefortin-C (0.008-46). The standards were common secondary metabolites from fungi typically infecting maize; *F. graminearum*

(zearalenone, deoxynivalenol) and silage; *P. roqueforti* or *P. paneum* (andrastin A, roquefortine C, mycophenolic acid, 1-hydroxyeremophil-7(11),9(10)-dien-8-one, N6-formyl-roquefortin-C and patulin), *M. ruber* (citrinin) and *A. fumigatus* (gliotoxin). The cytotoxicity of fungal agar extracts (0.54 plug/mL cell medium), maize silage inoculations (2.6-125 µg maize silage/mL cell medium) and *P. roqueforti* fractions dissolved in methanol were tested after 48 h exposure.

Cytotoxicity was expressed as viability relative to the response of the solvent control. Results with a relative standard deviation up to 25% in replicate measurements were accepted. Each concentration was tested in 3 to 4 replicates measurements. Concentration-response curves were fitted using GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, USA). The 50% inhibition concentration (IC_{50}) is defined as the concentration giving 50% viability. In the present study this value was read on the fitted curve as the concentration corresponding to the viability midway between the top and bottom of the fitted curve. A background signal from blank samples was emitted and 0% viability was never reached.

3. Results and discussion

The cytotoxicity of pure standards (Table 1, Figure 1) has been compared to the cytoxic effects of fungal agar and silage extracts. To get an indication on whether the variations in cytotoxicity of agar extracts (Figure 2) could be explained by the fungal metabolites detected by chemical analysis (Table 2), comparisons between toxic and non-toxic extracts have been made (Figure 3). Each fungal species has been addressed separately with focus on the importance of growth medium and isolate (IBT) for production of cytotoxic metabolites. The cytotoxicity of inoculated maize silages (Figure 4) and their metabolite contents can be found in a separate section (Table 3, Figure 5 and 6).

3.1 Cytotoxic concentration-response of pure standards

Methanol used to dissolve test compounds had no effect on cell viability when added at 1-2% of growth medium. After 48 h or 72 h exposure viability of Caco-2 cells *in vitro* was determined from the cells ability to metabolise resazurin. Cell viability decreased in a concentration depended manner (Figure 1) for all the pure standards except for mycophenolic acid and N6-formyl-roquefortin-C. N6-formyl-roquefortin-C was not cytotoxic and mycophenolic acid had a bell-shaped concentration-response curve instead of the traditional S-shaped curves that were observed for patulin, gliotoxin, T-2 toxin and deoxynivalenol. Curves of 1-hydroxyeremophil-7(11),9(10)-dien-8-one, citrinin and zearalenone progressed like roquefortine C and andrastin A in Figure 1, as concentrations beyond their maximum inhibition were not tested. The weakly cytotoxic andrastin A, mycophenolic acid and 1-hydroxyeremophil-7(11),9(10)-dien-8-one reduced viability of Caco-2 cells up to 80%, 60% and 71%, respectively. The IC_{50} values (Table 1) of cytotoxic compounds ranged from 0.004 to 83 µg/mL for T-2 toxin and citrinin, respectively. The 72 h exposure tested for a few compounds gave lower IC_{50} values than the 48 h exposure. The Caco-2 resazurin assay were after 48 h exposure able to detect the general cytotoxicity with similar sensitivity as other *in vitro* cytotoxicity assays (Hanelt et al., 1994; Keblys et al., 2004; Cetin and Bullerman, 2005; Videmann et al., 2008).

After 48 h patulin and gliotoxin reduced viability to 16% and 26%, respectively at the highest concentrations tested. This low response was close to the background signal from wells without cells added and was in accordance Stec et al. (2007) and Niide et al. (2006) who also observed close to 100% cell death induced by patulin and gliotoxin *in vitro*. At the highest deoxynivalenol and T-2 toxin concentrations cells still remained 58% and 49% viability, respectively (data not shown). Calculated from the doubling time of Caco-2 cells in culture flask (41 hours) a viability of approximately 44% indicates that most cells survived but were not able to divide when exposed for 48 h. This cytostatic rather than cytotoxic effect of T-2 toxin and deoxynivalenol is in line with their ability to inhibit protein synthesis (Liao et al., 1976). Widestrand et al. (1999) have previously observed a cytostatic effect in mouse 3T3 fibroblast cells for deoxynivalenol, but not for T-2 toxin. The mycotoxins zearalenone and citrinin have high IC_{50} values in the present assay but in mammals they can exhibit estrogenic and neprotoxic effects, respectively (Bouslimi et al., 2008; Flajs and Peraica, 2009). Oestrogen-responsive cells (MCF-7) are in contrast to Caco-2 cells sensitive to very low zearalenone levels with an IC_{50} of 64 pg/ml (Welshons et al., 1990). The cytotoxicity of the *P. roqueforti* metabolites andrastin A, 1-hydroxyeremophil-7(11),9(10)-dien-8-one and N6-formyl-roquefortin-C had not been tested previously. The roquefortine related metabolite N6-formyl-roquefortin-C (Musuku et al., 1994) had no cytotoxic effect up to 46 µg/mL and was therefore less active than roquefortine C having an IC_{50} of 48 µg/mL. The low cytotoxicity of andrastin A observed in the present study are in line with high content of andrastin A in blue cheese (~20 mg/kg) eaten by humans, which also indicates low acute toxicity (Fernández-Bodega et al., 2009).

3.2 *Penicillium roqueforti*

The metabolite abundances in two fungal YES extracts were in the same range for 13 *P. roqueforti* metabolites (69-122%) for repeated experiments (isolate, growth, plug extraction, and HPLC analysis) (data not shown). It allowed comparisons between the different agars, isolates and fungal species.

The YES medium supported production of all metabolites identified from *P. roqueforti* (Table 2). The production of many *P. roqueforti* metabolites was highly substrate dependent. The major metabolites were roquefortine C, mycophenolic acid, 1-hydroxyeremophil-7(11),9(10)-dien-8-one, andrastin A and occasionally PR-toxin. Other minor metabolites included andrastin B and C andrastin D, roquefortine A, citreoisocoumarin, eremofortin C, N6-formyl-roquefortin-C, 1-hydroxyeremophil-7(11),9(10)-dien-8-one and (3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one. The metabolite profile matched Sørensen et al. (2007) and Nielsen et al. (2006) who also identified agroclavine, orsellinic acid, festudacine, roquefortine D and 16-OH roquefortine from *P. roqueforti*. On SA the metabolite production was generally low. This could indicate that only few metabolites are produced in maize silage, but this is not the case (Table 3). PR-toxin and eremofortin C were only present in the strongly cytotoxic extracts reducing viability to less than the 60% mycophenolic acid elicited. PR-toxin and eremofortin C were produced by 7 of the 10 isolates from maize silage examined in total (all data not shown). Our results were in line with O'Brien et al. (2006) who found that 77% *P. roqueforti* isolates from grass silage was able to produce PR-toxin and eremofortin C on agar. However all 21 *P. roqueforti* isolates tested by Polonelli et al. (1978) produced PR-toxin under specific growth conditions.

The cytotoxic concentration-response curves of two *P. roqueforti* YES extracts (IBT 28547 and 28549) were similar to the curves for pure standards (Figure 1). The metabolite contents are reported in Table 2. The IBT 28549 YES extract had a bell-shaped concentration-response curve similar to mycophenolic acid. The extract was tested at 0.0007-0.54 plug/mL cell medium corresponding to 0.06-44 μg/mL mycophenolic acid. Due to similar curve shapes and concentration range the toxic effects of IBT 28549 YES was mainly attributed to mycophenolic acid. Individually the concentrations of roquefortine C (1 μg/mL) and andrastin A (35 μg/mL) were too low to elicit the observed effect. PR-toxin and eremofortin C were identified in the very cytotoxic IBT 28547 YES and were not present in the moderate toxic IBT 28549 YES extract. 1-hydroxyeremophil-7(11),9(10)-dien-8-one, N6-formyl-roquefortin-C and andrastin D were slightly more abundant in the very toxic extract compared to the moderate toxic extract whereas the other metabolites identified were of higher or similar abundances. 1-hydroxyeremophil-7(11),9(10)-dien-8-one, N6-formyl-roquefortin-C and andrastin A (closely related to andrastin D) had no or very low cytotoxic effects in the Caco-2 assay. This indicated that the cytotoxicity of *P. roqueforti* were caused by PR-toxin or its precursor eremofortin C. PR-toxin strongly inhibited viability of intestinal cells (FHS 74) after 72 h exposure (IC_{50} = 0.02 μg/mL) in a AlamarBlue assay, which had similar IC_{50} values for T-2 toxin and gliotoxin as us (Purup S., unpublished data). Aujard et al. (1979) have demonstrated inhibition of DNA synthesis and liver cell viability at very low PR-toxin concentration. The high cytotoxicity of PR-toxin was in agreement with its high acute toxic effect in animals (Chen et al., 1982). Eremofortin C did opposite PR-toxin not cause death for mice dosed 10 mg/kg body weight (Moreau and Moule, 1978). Eremofortin C and PR-toxin differ only by a hydroxyl functional group and an aldehyde functional group at the C-12 position, respectively, which showed that this position was important at least for the *in vivo* toxic effect.

HPLC fractions targeting specific metabolites identified PR-toxin as the major cytotoxic compound against Caco-2 cells, while 1-hydroxyeremophil-7(11),9(10)-dien-8-one and N6-formyl-roquefortin-C isolated from *P. roqueforti* had low or no toxic effects, respectively. 1-hydroxyeremophil-7(11),9(10)-dien-8-one and N6-formyl-roquefortin-C isolated from *P. roqueforti* in this study were 14 mg (97% by HPLC-DAD) and 2.3 mg (99% by HPLC-DAD), respectively. Despite repeated fractioning using several techniques all our PR-toxin fractions had minor co-eluents. The fraction with the highest purity (81% by HPLC-DAD) indicated that the IC_{50} value of PR-toxin was in the range of 1-13 μg/mL based on the assumption that the impurities did not contribute to the weight and toxicity of the sample. The abundances of co-eluents were very low in the crude extract compared to PR-toxin. Therefore PR-toxin could be appointed as a major cytotoxic metabolite in *P. roqueforti*. However, the approximate IC_{50} value was rather high compared with literature and results from our colleagues (Aujard et al., 1979; Purup S., unpublished data). The fact that all crude extracts and fractions with traces of PR-toxin were cytotoxic indicated a very strong effect on Caco-2 cells. The existence of additional cytotoxic metabolites from *P. roqueforti* can not be excluded.

3.3 *Penicillium paneum*

The patulin concentration (> 2 μg /mL cell medium) in the two cytoxic *P. paneum* extracts can alone exhibit the strong cytotoxic effects (Figure 2). Patulin production was limited to IBT28543 grown on YES and PDA. All isolates consistently produced citreoisocoumarin, VM-55599, marcfortine A, B and C, roquefortine C, andrastin

A (Table 2). This is in accordance with Nielsen et al. (2006) who also detected orsellinic acid from *P. paneum* YES cultures, which was not detected in the present study. Individually contents of roquefortine C (up to 0.6 µg /mL) and andrastin A (up to 29 µg /mL) were too low to affect the viability of Caco-2 cells, since no cytotoxic effect was observed at these concentrations (Figure 1). Andrastin B, C and D, citreoisocoumarin, marcfortine A, B and C, N6-formyl-roquefortin-C, VM-55599 had high abundances in both toxic and non-toxic extracts and were therefore not expected to contribute to the toxic response. The *Penicillium* metabolite N6-formyl-roquefortin-C identified by Musuku et al. (1994) has for the first time shown to be produced by *P. paneum* and *P. roqueforti*. Most of the metabolites *P. paneum* and *P. roqueforti* have in common were produced in the same range, however andrastin A and roquefortine C tented to be most abundant in *P. roqueforti* extracts and citreoisocoumarin in *P. paneum* extracts.

3.3 *Byssochlamys nivea*

All agar extracts of *B. nivea* were cytotoxic (Figure 2) and contained patulin, byssochlamic acid, mycophenolic acid and several other major metabolites (Figure 3). Patulin alone could cause the observed cytotoxic effects of agar extracts (Figure 2). The patulin concentrations in the cell medium (0.5-6 µg/ml) correspond to a viability of around 70 to 20% (Figure 1), which in line with the cytotoxicity observed for the agar extracts. Highest viability (71%) had the extract (IBT 28553, PDA) with lowest patulin content. Patulin was produced in the silage derived agar medium by IBT 28552 but was not produced when the isolate was inoculated onto maize silage. Mycophenolic acid was present in the concentration range able to reduced viability of Caco-2 cells ~60%. The cytotoxicity of the natural byssochlamic acid is not known. However, its enantio isomer has moderate cytotoxic activity against HEp-2 and HepG2 cells *in vitro* (Li et al., 2007), which could indicate a possible toxic effect of the natural byssochlamic acid. The *B. nivea* metabolite byssochlamysol was not detected in any of the extracts examined in this study and has also been reported to have low cytotoxicity (Mori et al., 2003).

3.4 *Monascus ruber*

Different species distinctions for *Monascus* exist. We applied the taxonomy of Domsch et al. (2007) who recognized *M. ruber* and *M. purpureus* as one species. The *M. ruber* isolates synthesized various secondary metabolites including pigments, monacolin K and citrinin as also observed by Pattanagul et al. (2008). *Monascus* pigments have been extensively used as natural food colorants (Dufosse, 2006) in Asia for more than a millennium, though some have cytotoxic (Su et al., 2005; Knecht and Humpf, 2006) antibiotic, immunosuppressive or teratogenic effects (Martinkova et al., 1999). Most of the *M. ruber* isolates produced just few metabolites in low concentrations when grown on YES, CYA, PDA and SA (Table 2, Figure 3). Only one *M. ruber* extract (IBT 9664, PDA) with high metabolite production was cytoxic (Figure 2) and the major metabolites in this extract were besides citrinin also the pigments monacin, rubropunctatin, ankaflavin and monascorubrin. The citrinin production is influenced by the actual growth conditions (Xu et al., 2006). In the present study citrinin was produced in similar concentrations by two *M. ruber* isolates on two different media (IBT 9664 on PDA and IBT 9658 on YES) but the concentrations were too low (< 8 µg/mL cell medium) to inhibit Caco-2 cells viability below 70%. Citrinin is a hepato- and nephrotoxic compound with antibiotic activity (Wong and Koehler, 1981; Blanc et al., 1995; Flajs and Peraica, 2009), which is known to exhibit synergistic effect with another nephrotoxic mycotoxin; ochratoxin A (Bernhoft et al., 2004). Citrinin, monacin and the cholesterol-lowering agent monacolin K (Tobert, 2003) had high abundances in an extract with low cytotoxicity (IBT 9658, YES). Monacolin K was the major metabolite in most extracts with no cytotoxic effect. This corresponds well to its moderate cytotoxicity to Caco-2 cells in a MMT assay (IC_{50} = 30 µg/mL) (Lin et al., 2006), but monacolin K may affect the metabolic activity of rumen fungi (Schneweis et al., 2001). Traces of the strongly cytotoxic rubropunctamine (Knecht and Humpf, 2006) were detected in both the toxic and non toxic extracts and is therefore not regarded as important. The abundances of the identified metabolites in toxic and non toxic extracts indicate that ankaflavin, rubropunctatin and/or monascorubrin maybe in combination with citrinin may cause the cytotoxicity of *M. ruber*. In assays with the human cancer cell lines Hep G2 and A549 ankaflavin was stongly cytoxic, whereas monacin had no effect (Su et al., 2005). Ankaflavin, rubropunctatin and monascorubrin had no cytotoxic activity after 3 h exposure to hepatocytes *in vitro* using different endpoints (Martinkova et al., 1999). Therefore, it cannot be excluded that the cytotoxic effect of the cytotoxic *M. ruber* extract is due to non-identified metabolite(s), which should be further investigated.

3.5 *Aspergillus fumigatus*

Two *A. fumigatus* extracts were cytotoxic inhibiting cell viability more than 50% (Figure 2). In one extract (IBT 23737, YES) high contents of several metabolites were detected; fumigaclavine C, pseurotin A or D, fumiquinazoline D, fumitremorgin C, typacidin, methylsulochrin, verruculogen, fumigillin, fumitremorgin B and helvoic acid. Gliotoxin, a very cytoxicit *A. fumigatus* metabolite tested in this assay, was not detected (> 0.001 µg/mL) in this cytotoxic extract. This indicated that at least one other cytotoxic *A. fumigatus* metabolite was

present in the extract. This is comparable with the *in vitro* toxicity of an *A. fumigatus* extract on rumen fermentation, which could not be explained by the presence of gliotoxin alone (Morgavi et al., 2004). Fumitremorgins, verruculogen and fumigaclavines are examples of other mycotoxins from *A. fumigatus* (Samson et al., 2002), which were present in the cytotoxic extracts. The metabolite content was generally low in the other cytotoxic extract (IBT 23737, YE), which reduced viability to 44%. However 0.07 μg/mL of the cytotoxic gliotoxin was identified in the extracts, which corresponded to 25-30% viability according to the concentration-response curve (data not shown). Gliotoxin has been detected on YE, CYA (IBT 23737) and PDA (IBT 23720) media, though Frisvad et al. (2009) recommended YE for gliotoxin production due to its low C/N ratio. In a moderate cytotoxic extract (IBT 23720, PDA) the concentration of gliotoxin (0.03 μg/ml) was slightly below the IC_{50} determined it the present study, and could therefore alone be responsible for the cytotoxicity of the extract (62% viability). Until now 226 secondary metabolites have been identified from *A. fumigatus* (Frisvad et al., 2009). In the present study many *A. fumigatus* metabolites were tentatively identified from UV and MS characteristics without analysis of standards. Our identifications matched the results from Larsen et al. (2007).

3.6 *Fusarium avenaceum*

F. avenaceum extracts were moderate toxic reducing viability maximum by 50% (Figure 2). Isolates tended to be more toxic on YES than on CYA and PDA. In the most cytotoxic extracts (YES, ITB 41176 and 41777) 2-amino-14,16-dimethyloctadecan-3-ol, enniatin B and B_1 were major metabolites. They have previously been identified as the most cytotoxic compounds of *F. avenaceum* (Uhlig et al., 2005, 2006). The cytotoxic enniatins A, A_1, B and B_1 were consistently produced by all 4 isolates on all three media. In the most cytotoxic extracts the enniatins B, B_1, A_1 were present in the cell medium at 27, 13 and 6 μg/mL corresponding to levels known to be cytotoxic in the MRC-5 cells line but not in the Hep G2 using the Alamar Blue assay (Ivanova et al., 2006). The extracts with the lowest cytotoxic effect (CYA, ITB 41175 and 41780) have the highest enniatin B contents, indicating low toxicity of enniatins. Chrysogine, antibiotic Y, aurofusarin were minor metabolites present in some extracts. Employing a sensitive LC-MS-MS method Sørensen et al. (2009) identified consistently production of several minor metabolites including moniliformin, chrysogine, antibiotic Y and aurofusarin by IBT 41777 and other *F. avenaceum* isolates. Moniliformin, a small polar compound that elute early from a reversed phase chromatography system, was not detectable with the chemical methods applied in this study. Moniliformin has a low cytotoxicity against several cell lines including Caco-2 cells, which was not affected up to 100 μg/mL in a MMT assay measuring mitrocondial activity colometrically (Morrison et al., 2002; Cetin and Bullerman, 2005). Therefore, moniliformin is not expected to contribute to observed cytotoxicity. In culture *F. avenaceum* may also produce several other secondary metabolites; acuminatopyrone, butenolide, chlamydosporols, fusarins (Hershenhorn et al., 1992; Uhlig et al., 2006; Sørensen et al., 2009), which have received little toxicological attention. In line with Jestoi et al. (2008) *F. avenaceum* isolates did not produce beauvericin, as other have reported (Logrieco et al., 2002; Morrison et al., 2002). Overall the cytotoxicity of these extracts could not be attributed to the presence of specific metabolites.

3.7 *Fusarium graminearum*

YES extracts of *F. graminearum* were highly cytotoxic (Figure 2). The major metabolites zearalenone, rubrofusarin and aurofusarin were much more abundant in YES compared to CYA and PDA. Zearalenone, deoxynivalenol and nivalenol are important mycotoxins produced by *F. graminearum* (Sweeney and Dobson, 1998). Zearalenone alone could not elicit the observed cytotoxic effects (24-45% viability) of YES agar extracts as present in cell medium up to 10 μg/mL, corresponding to a viability of 82-90%. Nivalenol was not detected (<0.001 μg/mL cell medium) and only traces of deoxynivalenol (<0.004 μg/mL) far below the IC_{50} value (0.29 μg/ml) were detected in both toxic and non-toxic samples. However mixtures of deoxynivalenol and zearalenone is known to strongly reduce the viability of Caco-2 cells, almost in an additive manner (Kouadio et al., 2007). In the present study the levels of deoxynivalenol was too low to contribute unless synergistic effects occurred. It indicated that other toxic metabolites than zearalenone, deoxynivalenol and nivalenol or synergism are involved the pronounced cytotoxicity of YES extracts. Langseth et al. (1999) were also not able correlate to the cytotoxicity of *F. graminearum* agar extract and the amount of zearalenone, deoxynivalenol and nivalenol. Traces of fusarin C have been detected in the most toxic extracts, however several other secondary metabolites were also present (Figure 3). *F. graminearum* is known to produce e.g. butenolide, fusarenon X, 3-acetyldeoxynivalenol (Thrane, 1990) but they were not detectable with the methods employed.

3.8 *Alternaria tenuissima*

YES extracts of *A. tenuissima* were much more cytotoxic than PDA and CYA extracts (Figure 2) and generally had a high metabolite content (Table 2). The major secondary metabolites were alternariol monomethyl ether, alternariol and tenuazonic acid but also altertoxin I, altenuene and altersetin were detected. Minimum 9 μg/mL alternariol monomethyl ether, 3 μg/mL alternariol or 83 μg/mL tenuazonic acid were present in the cell medium

exposed to the toxic YES extracts. These levels were cytotoxic in another *in vitro* assay (Aly et al., 2008). Altersetin and tenuazonic acid were consistently produced by the four isolates. Isolate IBT 41186 lacked alternariol monomethyl ether, alternariol and altenuene production, but the YES extract were still cytotoxic, perhaps due to high tenuazonic acid content (83 μg/mL cell medium), the presence of altertoxin I and altersetin or other unidentified metabolites.

3.9 Inoculated maize silage

The uninfected maize silage inoculated with post-harvest fungi had a low initial mycotoxin content of enniatin B (36 μg/kg), fumonisin B_1, fumonisin B_2, mycophenolic acid (10 μg/kg), zearalenone (15 μg/kg). Maize silage inoculated with *B. nivea* was opposite to *P. roqueforti*, *M. ruber*, *P. paneum*, *A. fumigatus* more cytotoxic than uninfected silage. The IC_{50} of uninfected and *B. nivea* inoculated silage were (mean ± SD, of two experiments) 44±1 mg/mL and 14±4 mg/mL, respectively (Figure 4). The major *B. nivea* metabolites in the cytotoxic silage were mycophenolic acid and byssochlamic acid but also other *B. nivea* metabolites (undescribed I-III) were detected (Figure 5, Figure 6). Patulin was not detected and traces below the detection limit (371 μg/kg silage) could not alone exhibit a cytotoxic response, as the concentration in the cell medium would be too low (patulin <0.005 μg/mL, at 14 mg silage/mL). The high mycophenolic acid content in *B. nivea* inoculated silage (55 mg/kg) could explain some of the observed cytotoxicity, however mycophenolic acid has a bell-shaped dose response curve, opposed to the dose response curve for inoculated silage, which is S-shaped. At 14 mg silage/ml the viability was 50% and the mycophenolic acid concentration 0.8 μg/mL which corresponding to a viability of ~62% according to the concentration-response curve in Figure 1. Cytotoxicity matrix components maybe in combination with the other observed toxins or other fungal metabolites must have caused the S-shaped cytotoxic concentration-response of *B. nivea* inoculated silage. Maize silage is a complex matrix, with low pH (<4) and high water content. The extraction buffer used for the silage resulted in a pH of 4 in the extracts (Rasmussen et al., 2010). The low pH alone may cause cell toxicity. The toxicity of uninfected silages can vary, due to different compositions. Caution should therefore be taken when differences between samples are interpreted. Laboratory inoculation of silage with fungi allowed for comparison of the cytotoxicity of the same starting material. The matrix toxicity was too high for *in vitro* testing of uninfected silage samples with the current method. It could be calculated that more than 800 μg/kg of the strongly cytotoxic gliotoxin was needed to possible have an effect in the assay. The high concentration needed may be the reason why PR-toxin detected in *P. roqueforti* inoculated silage did not cause a significant toxic effect. Applying solid phase extraction (SPE) clean up, lowered the toxicity of the silage matrix by ~4 times (data not shown), but may also remove some toxins.

The production of secondary metabolites was shown to be highly substrate dependant. Not all secondary metabolites produced by fungi on agar were observed in silage inoculated with the same isolates (Table 3). Due to matrix interferences detection limits in maize silage are much higher than in fungal agar extracts. This has probably limited the number of metabolites detected in maize silage especially when LC-DAD-HR-MS was applied. The more selective LC-MS/MS method had lower detection limits but targeted only specific metabolites (Sørensen et al., 2008; Rasmussen et al., 2010). Secondary metabolites from *B. nivea*, *M. ruber*, *P. paneum and P. roqueforti* could be identified in the maize silage inoculations extracts. None of the *A. fumigatus* metabolites produced on agar were detected in the silage extracts. The absence of gliotoxin above the detection limit (71 μg/kg) was unexpected, as the isolates produced gliotoxin on agar and because earlier gliotoxin has been detected in maize silages up to 900 μg/kg (Richard et al., 2007). *A. fumigatus* hots-spots collected in Danish maize silage stacks contained besides gliotoxin also fumigaclavine A, B and C and fumitremorgin C (Rasmussen, unpublished; Rasmussen et al., 2010). Surprisingly patulin was not detected in maize silage inoculated with isolates of *P. paneum* and *B. nivea* that produced patulin on agar. *P. paneum* and *B. nivea* are the most common species associated with silage, which produce patulin (Storm et al., 2008). A silage stack is often inhomogeneous with differences in oxygen supply etc., which may explain why we did not observe patulin in laboratory-spoiled samples. Contradictory up to 36 mg/kg andrastin A, 55 mg/kg mycophenolic acid and 40 mg/kg roquefortine C could be detected in the laboratory inoculated maize silage (Table 3). Mansfield et al. (2008) frequently detected patulin (23%), roquefortine C (60%), and mycophenolic acid (42%) in field samples of maize and maize silage, with maximum concentrations up to 1.2 mg/kg, 5.7 mg/kg and 1.3 mg/kg dry matter, respectively. In a lab study mycophenolic acid, patulin and PR-toxin were produced in maize silage up to 3.6, 15.1, 2.2 mg/kg, respectively (Müller and Amend, 1997). In our study *B. nivea* produced much more mycophenolic acid than *P. roqueforti* in maize silage, however on agar it was the other way around. The maximum concentrations we detected in maize silage hot-spots were higher for roquefortine C and mycophenolic acid than reported in these two studies (Müller and Amend, 1997; Mansfield et al. 2008). Andrastin A, marcfortine A and B and citreoisocoumarin in silage hot-spots collected in the field has previously been reported (O'Brien et al 2006; Rasmussen et al., 2010). However production of 1-hydroxyeremophil-7(11),9(10)-dien-8-one, (3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one, andrastin B, C and D and marcfortine C in silage is reported for the first time. *M. ruber* (IBT 9664) produced citrinin in silage, which could be qualitatively detected with LC-MS-MS. *Monascus* pigments were not detected but judging from the

reddish colour of the hot spots they were present. Matrix interference and insensitive detection method (LC-DAD-HR-MS) were probably the reason why no other agar metabolites than citrinin were detectable from the two *M. ruber* isolates (IBT 9664 and 9658) inoculated to silage. Schneweis et al. (2001) have detected low citrinin levels (up to 64 μg/kg) but considerable amounts of monacolin K (up to 11,000 μg/kg) in maize silage hot-spots.

The secondary fungal metabolites (3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one, andrastin A-D and marcfortine A-C detected in silage hot-spots had low cytotoxic effects against the Caco-2 cells. This indicates that they have little toxic effect *in vivo*, but *in vitro* assays do not contain all activating and deactivating enzymes present the mammalian *in vivo* condition, and some indirect acting toxins may be undetected. *In vitro* assays may also give false positive results. Of the compounds reported in silage hot-spots byssochlamic acid, citrinin, gliotoxin, patulin, PR-toxin and roquefortine C have been recognised as mycotoxins. Byssochlamic acid is toxic to mice (Raistrick and Smith, 1933). Citrinin is a neprotoxin causing kidney damages (Bouslimi et al., 2008) and gliotoxin is immunosuppressive (Niide, 2006). Patulin damage the kidney and the gastro-intestinal tract functions (Speijers et al., 1988) it may reduce male fertility (Selmanoglu, 2006) has antibiotic properties (Madhyastha et al., 1994) and is immunosuppressive at high doses (Llewellyn et al., 1998; Bondy and Pestka 2000). PR-toxin is acute toxic (Moreau and Moule, 1978) and roquefortine C has antibacterial (Kopp and Rehm, 1979) and neurotoxic (Wagener et al., 1980) properties. Though not classified as a mycotoxin mycophenolic acid may be of concern because of its antibiotic and immunosuppressive features (Bentley, 2000).

4. Conclusion

The genera *Alternaria*, *Aspergillus*, *Byssochlamys*, *Fusarium*, *Monascus*, *Penicillium* often spoiling maize and maize silage were all able to produce metabolites on agar, which were cytotoxic to Caco-2 cells in the resazurin assay measuring cell viability. The IC_{50} values of seven mycotoxins ranged from 0.004 to 83 μg/mL for T-2 toxin and citrinin, respectively. PR-toxin was identified as a major cytotoxic metabolite of *P. roqueforti*. roquefortine C was moderate cytotoxic, whereas the *P. roqueforti* metabolites mycophenolic acid, andrastin A, 1-hydroxyeremophil-7(11),9(10)-dien-8-one and N6-formyl-roquefortin-C had low to none cytotoxic effects on Caco-2 cells. The cytotoxic *P. paneum* and *B. nivea* agar extracts contained cytotoxic levels of patulin. The presence of other cytoxic principles than zearalenone, deoxynivalenol, nivalenol from *F. graminearum*, citrinin from *M. ruber* and gliotoxin from *A. fumigatus* were recognized, but the metabolites were not identified. The *in vitro* assay used in the present study was suitable for screening of agar extracts. Its application to silage samples was limited due to high and variable cytotoxicity of the crude maize silage extracts without fungal growth.

Several secondary fungal metabolites were detected in maize silage hot-spots; including byssochlamic acid, mycophenolic acid and several undescribed metabolites from *B. nivea*, andrastin A-D, citreoiscoumarin, marcfortine A-C and roquefortine C from *P. paneum*, citrinin from *M. ruber*, and PR-toxin, 1-hydroxyeremophil-7(11),9(10)-dien-8-one, (3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one and several others from *P. roqueforti*. However none of the *A. fumigatus* metabolites produced on agar were detected in the laboratory inoculated silage hots-spots.

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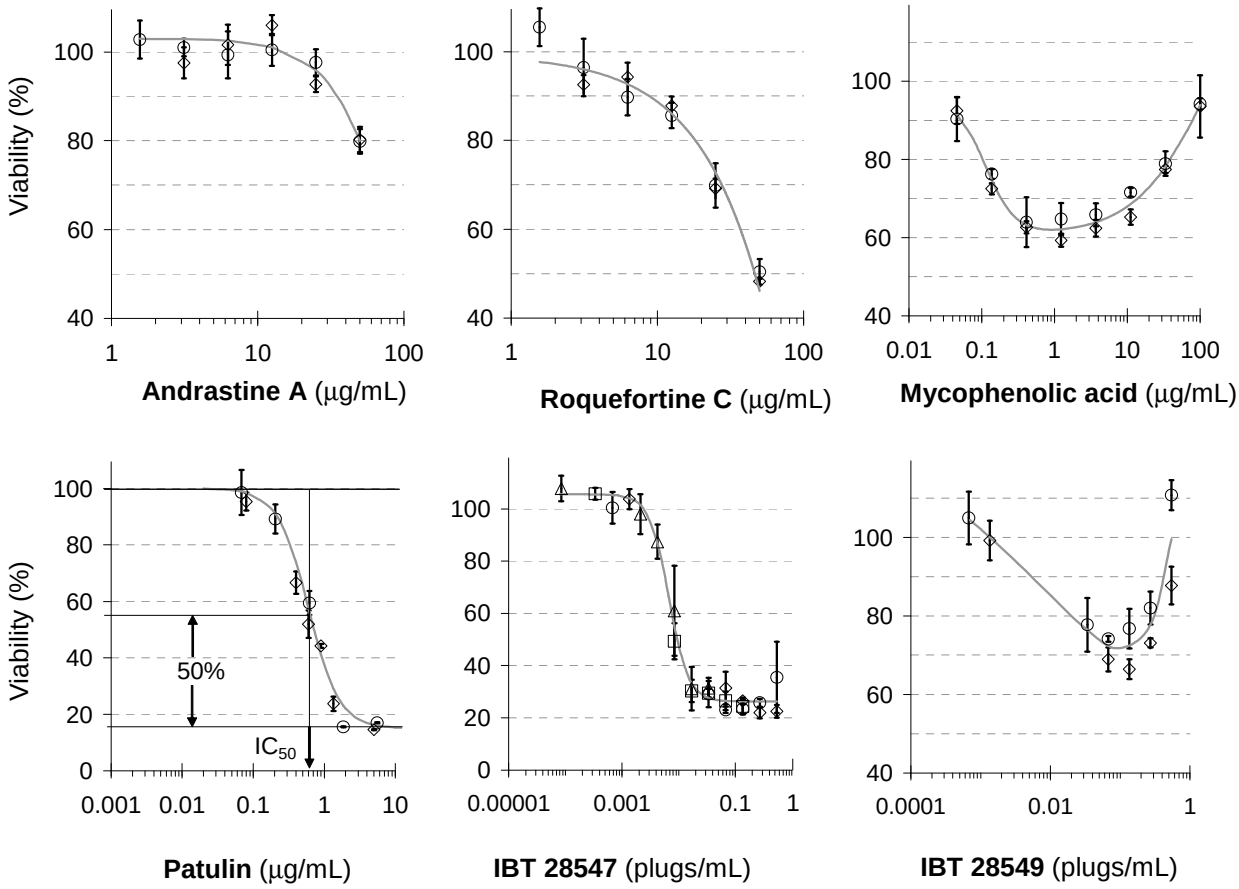

Fig. 1. Viability of Caco-2 cells after 48 h exposure to fungal metabolites and *Penicillium roqueforti* extracts at different concentrations. The fitted curves represent means ± SD of 3 replicates, measured at different days (o, ◊, Δ, □).

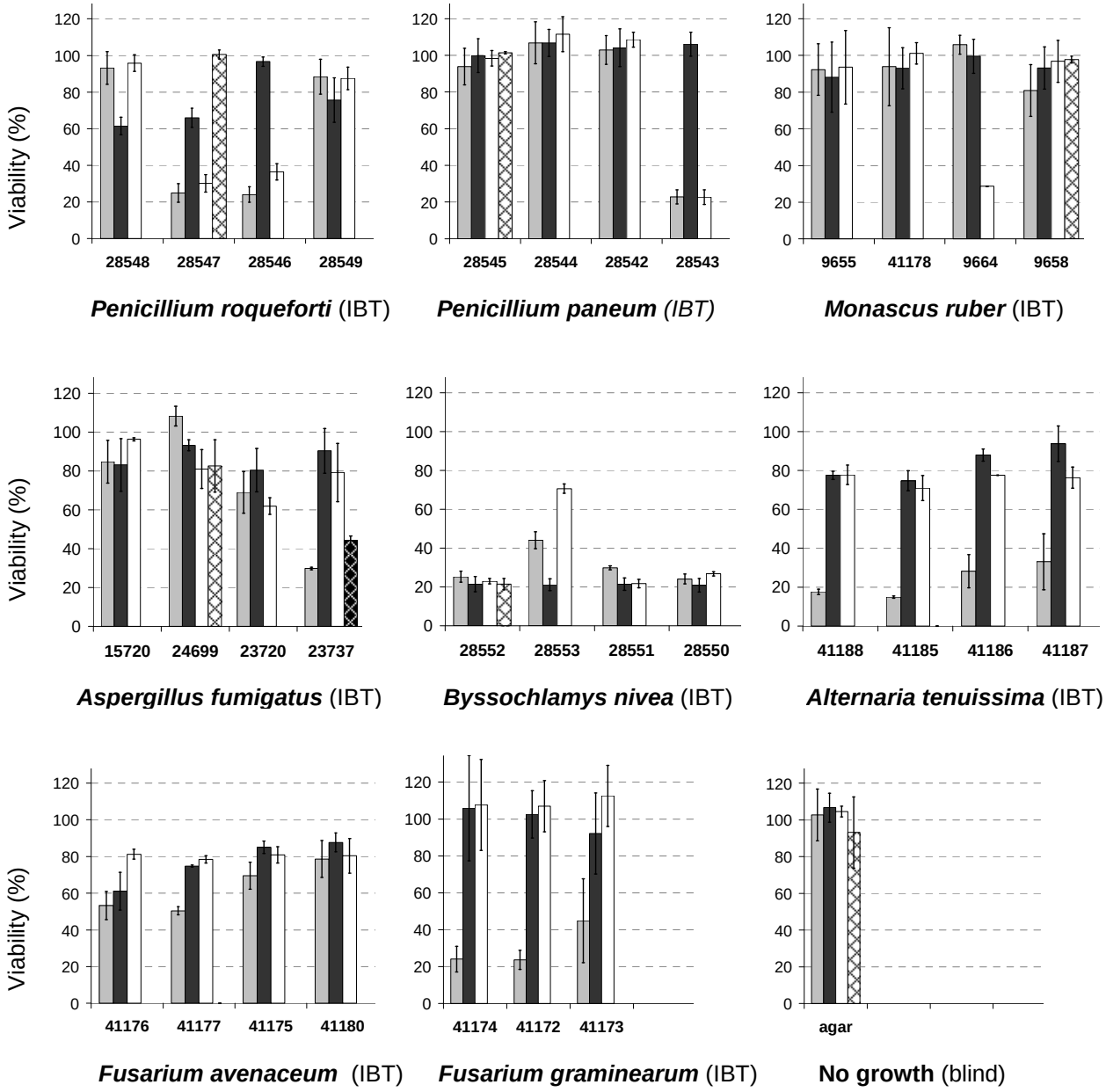

Fig. 2. Viability of Caco-2 cells after 48 h exposure to extracts of fungal cultures of ▒ YES, ■ CYA, □ PDA, ▓ YE, ▨ SA agar. Isolates from the IBT fungal collection (IBT no) was tested at one concentration (0.54 plugs/mL). YE and silage agar (SA) extracts were only tested for some isolates. Plotted are means + SD of two experiments, each having 4 replicates.

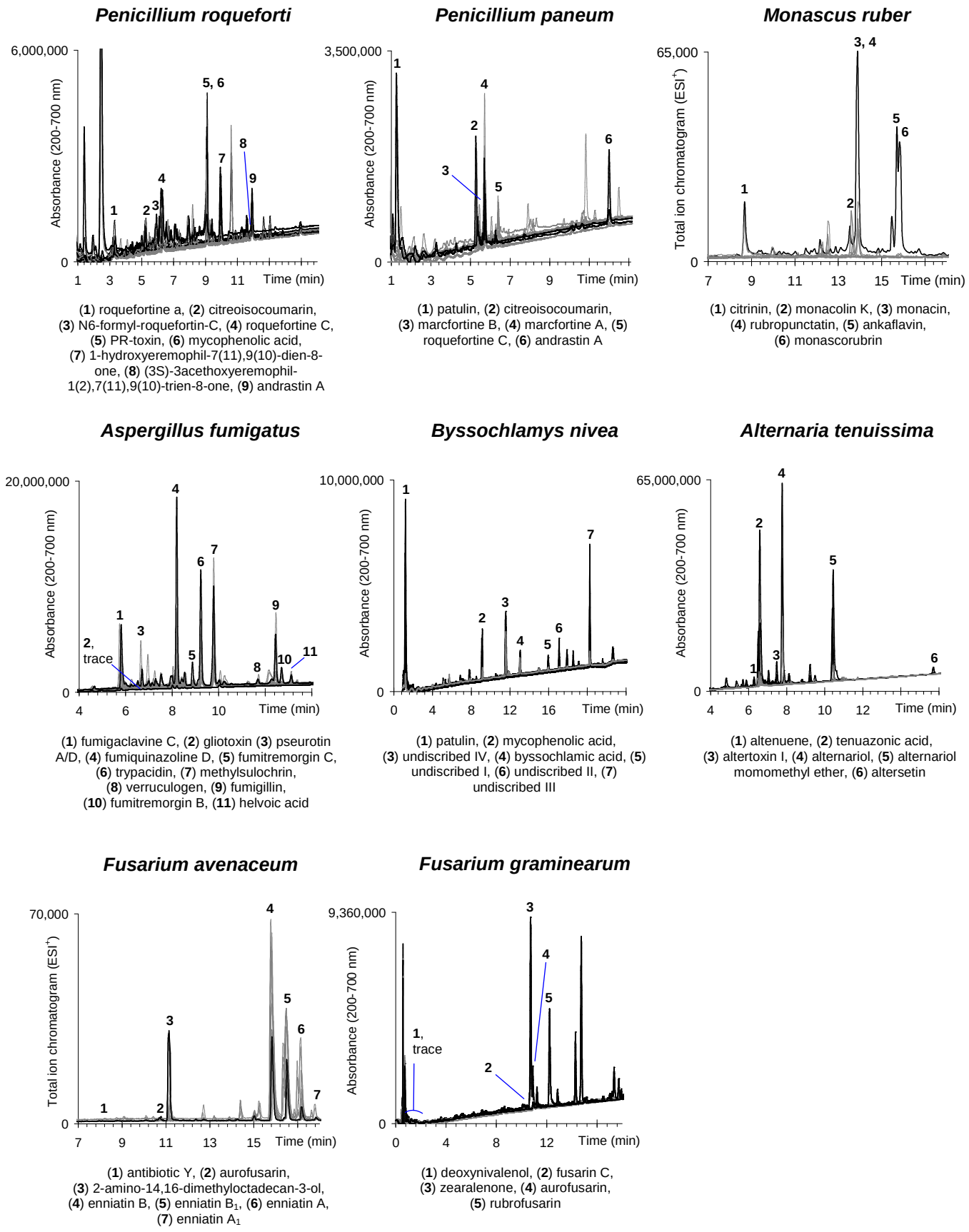

Fig. 3. Chromatograms of all fungal agar extracts plotted relatively for a specific trace and time span. Cytotoxic extracts resulting in less than 53% cell viability of Caco-2 cells has black bold lines.

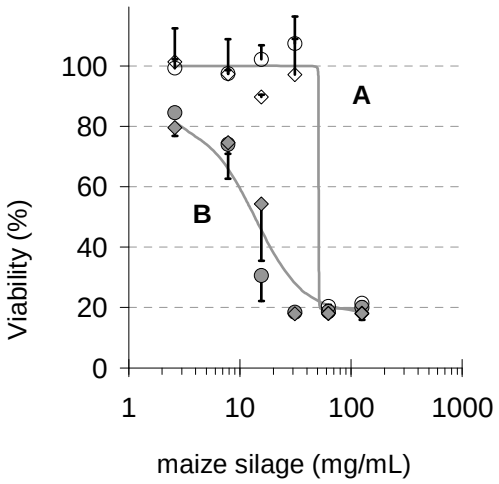

Fig. 4. Viability of Caco-2 cells after 48 h exposure to A) uninfected and B) *Byssochlamys nivea* (IBT 28551) inoculated maize silage at different concentrations. IC-50 values of 44 mg/mL and 14 mg/mL respectively. The fitted curves represent means ± SD of 3 replicates, measured at two days (o, ◊).

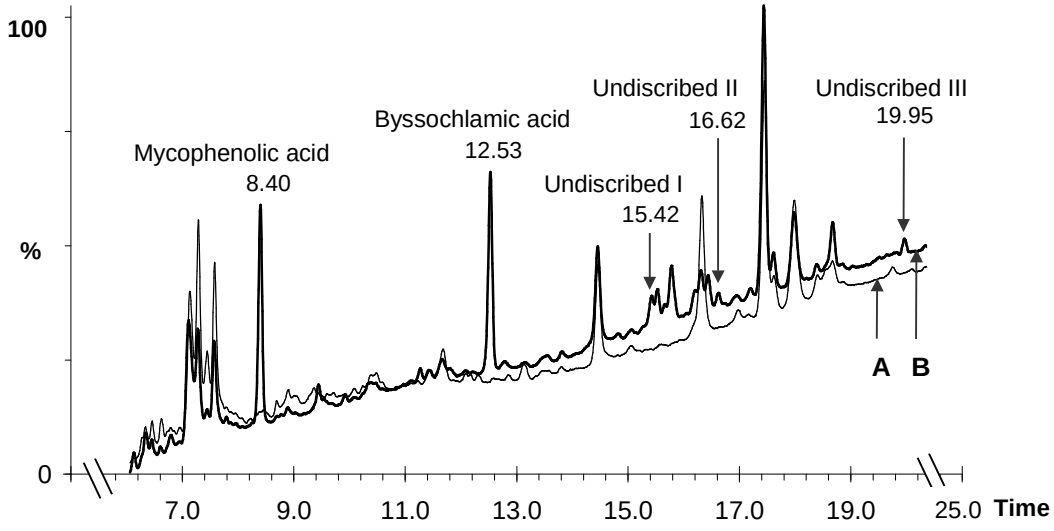

Fig. 5. Relative HPLC trace (200-700 nm, 7-20 min) from A) uninfected and B) *Byssochlamys nivea* (IBT 28551) inoculated maize silage extracts with retention times of *B. nivea* fungal metabolites.

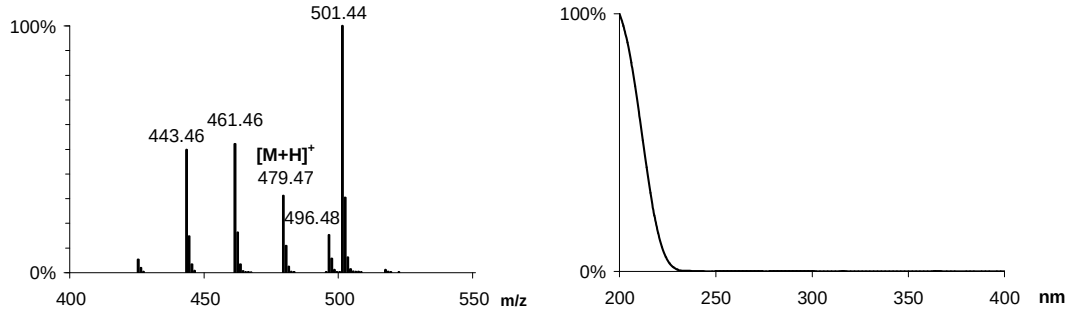

Fig. 6. ESI$^+$ and DAD spectra of the undescribed I metabolite of *B. nivea* with the molecular ion (M=478.4) and retention time index (RI) 1420.

Table 1. Cytotoxicity of fungal metabolites. The 50% inhibit concentrations (IC_{50}) means ± SD of N independent concentration-response experiments. Viability of the Caco-2 cells was determined by flourescens owing to addition of resazurin.

Caco-2 cells	**48 h exposure**				**72 h exposure**			
Metabolite		**mean IC_{50} (µg/ml)**		**SD**		**mean IC_{50} (µg/ml)**		**SD**
T-2 toxin	N=2	0.0037	+/-	0.0008				
Gliotoxin	N=2	0.035	+/-	0.003	N=2	0.034	+/-	0.001
Deoxynivalenol	N=2	0.29	+/-	0.17				
Patulin	**N=2**	0.62	+/-	0.07	N=2	0.52	+/-	0.04
Roquefortine C	N=2	48	+/-	2				
Zearalenone	N=3	58	+/-	6				
Citrinin	N=4	83	+/-	32	N=2	24	+/-	8
N6-formyl-roquefortin-C	N=2	> 46*						
Andrastin A	N=2	> 50*						
Mycophenolic acid	N=2	> 100*						
1-hydroxyeremophil-7(11),9(10)-dien-8-one	N=2	> 280*						

* Not inhibited by 50% in the tested range.

Table 2. Secondary fungal metabolites identified from fungus of the IBT collection innoculated on YES, CYA, PDA, YE or silage agar (SA). The concentrations (μg/mL cell medium) and relative abundances* of important metabolites in extracts as determined by LC-DAD-MS-HR or LC-MS-MS were compared to Caco-2 cell viability (%) in two individual experiments.

		YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	SA	YES	YES
Penicillium roqueforti	**Trace**	**IBT 28548**			**IBT 28549**			**IBT 28546**			**IBT 28547**				**IBT 28547**	**IBT 28549**
Viability +/- SD (%)		93±9%	61±5%	96±5%	88±10%	76±12%	87±6%	24±4%	97±3%	36±4%	25±5%	66±5%	30±5%	101± 2%	conc.-response**	
Andrastin A	485.3 m/z	37	21	17	48	24	15	43	25	16	33	27	15	4	34	44
Andrastin B	487.3 m/z	+++	+++	++	++++	+++	++	++++	+++	++	+++	+++	++	++	++++	++++
Andrastin C	471.3 m/z	+++	++	+	+++	++	+	++++	++	++	+++	+++	++	++	++++	++++
Andrastin D	427.3 m/z	+++	+++	++	+++	+++	++	++++	+++	++	+++	+++	++	++	++++	+++
Citreoisocoumarin	279.1 m/z	++	+	+	++	+	+	++	+	+	++	+	+	(+)	++	++
Eremofortin C	323.1 m/z	nd	nd	nd	nd	nd	nd	++++	nd	+	++	nd	(+)	nd	++++	nd
Mycophenolic acid	207.0 m/z	19	12	1	34	12	0.5	1	(+)	nd	17	8	(+)	0	19	35
N6-Formyl-roquefortin-C	418.2 m/z	+	+	(+)	+++	+	+	++++	(+)	+	+++	+	+	(+)	++++	++++
PR-toxin	279.1 m/z	nd	nd	nd	nd	nd	nd	+++	nd	+	++++	nd	+	nd	++++	nd
Roquefortine A	299.1 m/z	+++	+++	+++	++++	++++	+++	+++	(+)	+++	+++	++++	+++	+++	+++	+++
Roquefortine C	390.2 m/z	1.0	0.9	0.2	1.0	0.8	0.2	0.8	0.6	0.2	0.7	0.4	0.08	0.1	0.8	1.1
1-hydroxyeremophil-7(11),9(10)-dien-8-one	235.2 m/z	++	(+)	(+)	++	(+)	(+)	+++	(+)	+	++++	+	+	(+)	++++	++
(3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one	275.2 m/z	++++	+	(+)	+++	(+)	(+)	++	(+)	(+)	+++	(+)	(+)	(+)	+++	+++
Penicillium paneum		**IBT 28543**			**IBT 28544**			**IBT 28542**			**IBT 28545**					
Viability +/- SD (%)		23±4%	106±6%	23±4%	107±11%	107±7%	112±10%	103±8%	104±10%	109±4%	94±10%	100±9%	98±4%	101±1%		
Andrastin A	485.3 m/z	29	18	6	5	0.2	0.2	20	5	10	8	13	3	8		
Andrastin B	487.3 m/z	+++	+++	++	+	nd	nd	++++	++	+++	+++	++	+	+		
Andrastin C	471.3 m/z	+++	+++	+	+	nd	nd	++++	++	+++	+++	++	+	+		
Andrastin D	427.3 m/z	+++	++++	nd	+	nd	nd	++	++	+	+++	+++	+	++		
Citreoisocoumarin	279.1 m/z	++++	+	+++	+++	+	++	+++	(+)	+	++	(+)	+	(+)		
Marcfortine A	478.2 m/z	+++	++	+++	+++	++	++	+++	++	++	++++	+++	+++	+++	na	
Marcfortine B	464.2 m/z	+++	+++	+++	++++	+++	+++	+++	+++	+++	++++	+++	+++	+++		
Marcfortine C	448.2 m/z	+++	+++	+++	++++	+++	+++	+++	+++	+++	++++	+++	++	++		
N6-Formyl-roquefortin-C	418.2 m/z	+	+++	nd	nd	+	nd	nd	++	(+)	nd	++	nd	+		
Patulin	273 nm	3.9	nd	2.9	nd	nd	nd	nd	nd	nd	0.1	nd	nd	nd		
Roquefortine C	390.2 m/z	0.03	0.53	0.01	0.07	0.46	0.02	0.11	0.57	0.02	0.15	0.58	0.01	0.05		
VM-55599	350.0 m/z	++++	++	+++	++++	++	++++	++++	++	+++	++++	++	+++	(+)		

Table 2. continued

		YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	SA	YE	
Byssochlamys nivea	**Trace**	**IBT 28551**			**IBT 28550**			**IBT 28553**			**IBT 28552**					
Viability +/- SD (%)		30±1%	21±3%	22±2%	24±3%	21±3%	27±1%	44±4%	21±3%	71±2%	25±3%	21±4%	23±1%	21±3%		
Byssohclamic acid	331.1 m/z	++++	++	+++	++++	+	++++	++++	+	+++	++++	+	+++	+++		
Mycophenolic acid	321.0 m/z	6	4	2	6	4	2	8	4	2	7	3	1	2		
Patulin	273 nm	1.6	2.2	2.3	2.8	4.7	1.8	1.4	4.4	0.5	2.7	6.2	2.3	2.8		
Undiscribed I	501.4 m/z	++++	+++	+++	++++	++	+++	+++	++	+++	++++	++	+++	+++	na	
Undiscribed II	388.3 m/z	+++	++	+	++++	+	++	+++	+	++	++++	++	++	+		
Undiscribed III	348.3 m/z	+++	++	++	++++	++	++	+++	+	++	++++	++	+++	+		
Undiscribed IV	382.3 m/z	++++	+++	+++	++++	+	+++	++++	+	++++	++++	+	+++	+++		
Monascus ruber		**IBT 9655**			**IBT 9664**			**IBT 41178**			**IBT 9658**					
Viability +/- SD (%)		92±14%	88±19%	94±20%	106±5%	100±9%	29±0%	94±21%	93±11%	101±6%	81±14%	93±12%	97±1 1%	98±2%		
Ankaflavin	387.2 m/z	nd	nd	(+)	nd	nd	++++	nd	nd	nd	(+)	(+)	(+)	nd		
Citrinin	251.2 m/z	nd	nd	nd	nd	nd	8	nd	nd	nd	6	nd	nd	nd		
Monacolin K	422.3 m/z	+++	++	+	nd	nd	nd	++	(+)	(+)	++++	+	nd	nd		
Monascin	359.2 m/z	nd	nd	+	nd	nd	++++	nd	nd	(+)	++	(+)	+++	nd	na	
Monascorubrin	383.2 m/z	nd	nd	(+)	nd	nd	++++	nd	nd	nd	(+)	(+)	(+)	nd		
Rubropunctatamine	354.1 m/z	nd	nd	+++	nd	nd	++++	nd	nd	(+)	+	+	++++	nd		
Rubropunctatin	355.2 m/z	nd	nd	++	nd	nd	++++	(+)	nd	(+)	+	(+)	++	nd		
Aspergillus fumigatus		**IBT 15720**			**IBT 23737**			**IBT 23720**			**IBT 24699**				**IBT 23737**	
Viability +/- SD (%)		85±11%	83±14%	96±1%	30±1%	90±11%	79±15%	69±11%	80±11%	62±4%	108±5%	93±3%	81±10%	83%±14%	44%±2%	
Fumagillin	459.3 m/z	+++	(+)	(+)	+++	++	+	++	(+)	(+)	++++	+	(+)	(+)	nd	
Fumigaclavine C	367.2 m/z	nd	nd	nd	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	
Fumiquinazoline D	444.2 m/z	++	++	+	++++	+++	+	++	++	(+)	+++	++	(+)	+	(+)	
Fumitremorgin B	462.2 m/z	++++	+	(+)	++++	+	(+)	+++	+++	nd	nd	nd	nd	nd	nd	
Fumitremorgin C	380.2 m/z	++	(+)	++++	++++	+++	++	(+)	(+)	nd	nd	nd	nd	nd	+	
Gliotoxin	268 nm or 325>261 m/z	nd	nd	nd	nd,<0.001	0.001	nd,<0.001	nd	0.002	0.03	nd	nd	nd	nd	0.07	na
Helvolic acid	509.2 m/z	++	+	(+)	++++	+++	++	++++	+++	+	+++	+	+	(+)	nd	
Methyl-Sulochrin	209.1 m/z	+++	+	+	++++	+++	+	+++	+++	++	++++	+++	++	+++	nd	
Pseurotin A / D	334.1 m/z	++	nd	+	+++	(+)	+	+++	(+)	+	++++	++++	++	+	(+)	
Trypacidin	288 nm	+++	(+)	++	++++	+	+++	+++	++	+++	++	+	+++	+++	(+)	
Verruculogen	494.2 m/z	++	+++	(+)	+++	++++	(+)	+	+	nd	nd	nd	nd	nd	(+)	

Table 2. continued

		YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	YES	CYA	PDA	SA	
Fusarium avenaceum	**Trace**	**IBT41175**			**IBT41176**			**IBT41177**			**IBT41180**				
Viability +/- SD (%)		70±7%	85±3%	81±5%	53±8%	61±10%	81±3%	50±2%	75±1%	78±2%	79±10%	88±5%	80±9%		
2-amino-14,16-dimethyloctadecan-3-ol	355.3 m/z	++	+	nd	++++	nd	++	++++	nd	++	+	nd	nd		
Antibiotic Y	382.0 m/z	+++	nd	nd	nd	nd	nd	nd	nd	nd	++++	nd	nd		
Aurofusarin	571.1 m/z	+++	++	++	+++	++	+	+++	+	+	nd*	nd*	nd*		
Beauvericin	801.5 > 244.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
Chrysogine	191.1 m/z	nd	+	++++	nd	nd	+	nd	nd	+	+	nd	++	na	na
Enniatin A	699.5>211 m/z	nd	0.2	0.1	0.1	0.1	1.2	0.1	0.2	1.1	0.1	0.2	0.4		
Enniatin A1	685.4>210.4 m/z	0.2	2.1	1.3	0.7	0.7	6.1	0.9	0.9	5.6	0.5	2.1	3.6		
Enniatin B	657.4>196.3 m/z	4.9	27.3	14.3	2.8	2.4	9.3	3.7	2.5	8.7	4.2	27.1	21.0		
Enniatin B1	671.6>211 m/z	1.3	10.9	6.7	2.5	1.9	10.9	3.0	2.1	10.0	2.4	9.8	13.1		
Fusarium graminarum		**IBT 41174**			**IBT 41172**			**IBT 41173**							
Viability +/- SD (%)		24±7%	106±29%	108±25%	24±5%	103±13%	107±14%	45%±23%	92±22%	113±16%					
Aurofusarin	571.1 m/z	++++	++	+	++++	++	+	++++	++	++					
Deoxynivalenol	341.2 or 341>265 m/z	nd	<0.004	<0.004	<0.004	<0.004	<0.004	nd	<0.004	<0.004					
Fusarin C	430.2 m/z	(+)	nd	nd	(+)	nd	nd	(+)	nd	nd		na			na
Nivalenol	357 m/z	nd	nd	nd	nd	nd	nd	nd	nd	nd					
Rubrofusarin	273 m/z	++++	(+)	+	++++	+	+	+++	(+)	(+)					
Zearalenone	236 nm	6	0.07	0.08	6	0.1	0.06	10	0.2	0.1					
Alternaria tenuissima		**IBT41185**			**IBT41186**			**IBT41187**			**IBT41188**				
Viability +/- SD (%)		15±1%	75±5%	71±6%	28±9%	88±3%	78±0%	33±14%	94±9%	76±5%	18±1%	77±2%	78±5%		
Altenuene	293.1 m/z	nd	nd	(+)	nd	nd	nd	++++	nd	+	+++	nd	++		
Alternariol	257>214.9 m/z	3	0.01	0.08	nd	nd	nd	46	0.02	15	29	0.04	2		
Alternariol monomethyl ether	271.1>255.8 m/z	9	0.01	0.88	nd	nd	nd	29	0.003	7	29	0.01	0.6	na	na
Altersetin	400.25 m/z	+	(+)	nd	++	(+)	(+)	++	(+)	(+)	++++	(+)	(+)		
Altertoxin I	260 m/z	++++	nd	nd	+++	nd	nd	(+)	nd	nd	++	nd	nd		
Tenuazonic acid	196.1>111.7 m/z	139	16	19	83	9	16	27	8	9	49	11	11		

*The relative abundance was related to the extract with the highest content; ++++, denotes high (70-100% of the most abundant metabolite); +++ medium (25-70%); ++ low (10-25%); + very low (2-10%); (+) trace (<2%) present in the extract.
**Cytotoxic concentration-response tested (see Figure 1).

Table 3. Detected secondary fungal metabolites in maize silage inoculated with spores from fungi (ITB number). Metabolites were identified (x) by LC-DAD-MS-HR or quantified (mg/kg) by LC-MS-MS*.

			B. nivea		*P. roqueforti*		*P. paneum*		*M. ruber*	
Metabolite	**Trace**	**IBT**	28551*	28552	28547*	28546	28543*	28545	9658	9664*
B. nivea, undiscribed I	501.4 m/z		x	x						
B. nivea, undiscribed II	388.3 m/z		x	x						
B. nivea, undiscribed III	448.3 m/z		x	x						
Byssohclamic acid	252 nm		x	x						
Mycophenolic acid	319>191 m/z		55	25	2	0.2				
PR-toxin	321>261 m/z				x	x				
1-hydroxyeremophil-7(11),9(10)-dien-8-one	235.2 m/z				x	x				
(3*S*)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one	275.2 m/z				x	x				
Roquefortine A	299>239 m/z				x	x				
Roquefortine C	388>190 m/z				34	31	40	16		
Andrastin A	485>425 m/z				13	18	36	18		
Andrastin B	487.3 m/z				nd	x	x	nd		
Andrastin C	471.3 m/z				x	x	x	x		
Andrastin D	427.3 m/z				nd	x	x	nd		
Citreoisocoumarin	277>219 m/z				x	x	x	x		
Marcfortine A	478>419 m/z						x	x		
Marcfortine B	464>436 m/z						x	x		
Marcfortine C	448.2 m/z						x	x		
Patulin	153>109 m/z		nd< 0.37	nd< 0.37			nd< 0.37	nd< 0.37		
Penitrem A	632>546 m/z						nd< 0.008	nd< 0.008		
Citrinin	251>233 m/z								nd	x

nd; not detected, limit of detection for LC-MS/MS analyses are given (<).
* *In vitro* cytotoxicity tested.

5. FUNGAL METABOLITES IN DANISH MAIZE SILAGE

In this chapter the mycotoxin occurrence and content of Danish maize silage stacks and hots-spots are reported.

5.1 Hot-spots

Given the right growth conditions fungi are able to produce many different secondary metabolites (**III**). To investigate which compounds post-harvest fungi produce on maize silage, hot-spots samples with visible fungal growth were analysed. The hot-spots were either collected from Danish stacks or produced under laboratory conditions when post-harvest isolates were inoculated onto Danish maize silage and incubated for 3 weeks at 20°C in water saturated air. Samples were extracted by a modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, which employs a pH buffered acetonitrile and water extraction combined with a phase-separation (**II**). A high concentration of salts induced the phase separation of acetonitrile and water extraction solvents, which also forced the polar analytes into the less polar acetonitrile phase. The acetonitrile phase of sample extracts were analysed by LC-MS/MS or by liquid chromatograph with diode array detection and a time-of-flight mass spectrometer (LC-DAD-HR-MS) as described in chapter 3 (**II**) and 4 (**III**), respectively. The LC-MS/MS method identified specific metabolites and was able to detect lower levels than the LC-DAD-HR-MS detection method. Secondary fungal metabolites identified by LC-DAD-HR-MS in agar extracts were used as reference for detection of the compounds in the dirty maize silage extracts. Only the lab-inoculated samples were analysed by LC-DAD-HR-MS and the data analyses targeted metabolites produced on agar by the specific fungi. In agar extracts were the tentative identifications of secondary fungal metabolites without reference standards accomplished by comparing retention times, UV spectra and mass spectra with literature (Frisvad and Thrane, 1987; Nielsen and Smedsgaard, 2003; Larsen et al. 2007) and databases (ACD, 2008).

Table 5.1 presents the post-harvest metabolites detected in hots-spots collected from silage stacks and in laboratory inoculated samples for the different fungi involved. Ida MLD Storm performed the identification of fungal species according to Samson et al. (2002). *B. nivea, P. roqueforti, P. paneum, A. fumigatus* and *M. ruber* produced one to several fungal metabolites in Danish maize silage; however only a part of the fungal metabolites produced on agar could be observed in silage inoculated with the same isolates. There was mostly a high correlation between metabolites detected in laboratory and field samples, but the levels detected were different. The hot-spots increased the chance to detect post-harvest fungal metabolites and made it possible to link major metabolites to the presence of a specific fungus. Of the metabolites detected in Danish hot-spots only roquefortine C, PR-toxin, gliotoxin, fumigaclavine A and B and citrinin are currently categorised as mycotoxins *sensu stricto* (Samson et al., 2002). The high concentration of several *Penicillium* metabolites found in visible mouldy samples were in accordance with O'Brien et al. (2006), who in addition to us also detected 16-hydroxyroquefortine C, agroclavine, festuclavine, roquefortine B and D in grass silage. The *Penicillium* metabolites 1-hydroxyeremophil-7(11),9(10)-dien-8-one and (3*S*)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one identified in agar culture by Sørensen et al., (2007) have not been detected in silage before.

PR-toxin was present in the two lab-inoculated samples but not in the two field samples infected with *P. roqueforti*. Müller and Amend (1997) have previously detected PR-toxin in laboratory inoculated maize silage. Whether the absence of PR-toxin in the *P. roqueforti* hot-spot from the field is caused by environmental factors not favouring toxin production, non-toxigenic isolate or degradation is not known. In the present study both non-toxigenic isolates and fast degradation of PR-toxin have been observed. The degradation was in line with Müller and Amend (1997) who detected the half-life of PR-toxin to 25 days when dissolved in ethanol and stored at -18°C.

Table 5.1. Secondary fungal metabolites in hot-spots collected in the field (*in-situ*) or in maize silage inoculated with spores in the laboratory (lab). Metabolites were qualitatively (x) or quantitatively (mg/kg) determined by LC-MS-MS or LC-DAD-HR-MS and were reported for single samples infected by different fungal isolates. Summarised data collected for paper **II** and **III**.

	A. fumigatus		***M. ruber***		***B. nivea***		***P. roqueforti***	***P. paneum***	***P. paneum & P. roqueforti***
Metabolite	lab	*in-situ*	lab	*in-situ*	lab	*in-situ*	lab	lab	*in-situ*
Citrinin			x	nd					
			nd	nd					
Fumigaclavine A	nd	x							
	nd	x							
Fumigaclavine B	nd	nd							
	nd	x							
Fumigaclavine C	nd	x							
	nd	x							
Fumitremorgin C	nd	nd							
	nd	x							
Gliotoxin	nd	0.3							
	nd	0.9							
B. nivea metabolites					x	na			
					x	na			
Byssohclamic acid					x	na			
					x	na			
Mycophenolic acid					25	0.3	0.2		0.3
					55	1.6	2		0.4
PR-toxin							x		nd
							x		nd
1-hydroxyeremophil-7(11),9(10)-dien-8-one*							x		na
							x		na
(3*S*)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one*							x		na
							x		na
Andrastin A							13	18	5
							18	36	9
Andrastin B							nd	x	na
							x	nd	na
Andrastin C							x	x	na
							x	x	na
Andrastin D							nd	x	na
							x	nd	na
Citreoisocoumarin							x	x	x
							x	x	x
Roquefortine A							x	nd	x
							x	nd	x
Roquefortine C							31	16	1.8
							34	40	34
Marcfortine A								x	x
								x	nd
Marcfortine B								x	x
								x	nd
Marcfortine C								x	na
								x	na

nd = not analysed, nd = not detected, x = detected,

Patulin was not detected above the LOD (0.37 mg/kg) in the hot-spots although the maize silage was inoculated with *B. nivea* and *P. paneum* isolates, which produced patulin on agar. This may be due to the instability of patulin (O'Brien et al., 2006), the differences between field and the laboratory conditions or the agar favouring patulin formation. Müller and Amend (1997) and detected patulin in laboratory inoculated silage and it has also been detected in field samples with an inadequately validated method (Mansfield et al., 2008).

Gliotoxin was detected in two field samples but was not produced in silage inoculated with a gliotoxin producing strain, which indicate important differences between laboratory and field conditions in the present study. The gliotoxin concentration detected in our study was in line with Richard et al. (2007)

who detected 878 µg/kg in one of two maize silages analysed, contradictory Pereyra et al. (2008) detected extremely high gliotoxin levels (5,100 – 6,500 µg/kg) in all 90 maize silages from Argentina. Detection of citrinin in maize silage inoculated with a citrinin producing *M. ruber* isolate is in accordance with Schneweis et al. (2001) who found between 2.4 and 64.2 µg/kg citrinin in 10 of 135 mouldy maize silage samples collected in the field. The detection of byssochlamic acid and several others undescribed metabolites from *B. nivea* in maize silage had not been reported before. Fumigaclavine A, B and C and fumitremorgin C were detected in maize silage in the current study, but besides gliotoxin *A. fumigatus* metabolites have rarely been identified in mouldy feeds (Scudamore and Livesey, 1998).

The analysis of maize silage hot-spots showed that several post-harvest fungal metabolites may be present in high concentrations when fungal growth occurs.

5.2 Maize silage stacks

A monitoring study was carried out to determine the mycotoxin content in Danish maize silage. In total 99 samples were collected from 2007 to 2009 from different parts of Denmark without targeting the visible mouldy spots. After sample preparation the maize silage were extracted by the modified QuEChERS method and the extracts were analysed by LC-MS/MS as described in chapter 3 (**II**). The validated multi-method covers 27 mycotoxins and other secondary metabolites from the most common fungal species isolated from silage and maize in Danish climate conditions (Table 5.2) except the post-harvest fungi *Zygomycetes* and *Geotrichum candidum* and the pre-harvest fungi *Epicoccum* and *Phoma* (Nicolaisen et al., 2009; Sørensen, 2009; Storm, 2009). The method validation gave a realistic picture of the method performance. Before analysing samples it is important to assure the sensitivity of the instrument. If facing problems, factors such as column condition, cone impurities and collision gas pressure should be controlled first. Run in optimal conditions (e.g. short series, clean cone and stable room temperature) the instrument could sometimes detect lower levels of toxins than the LOD reported. Analytes present below the established (LOD) were reported as 'trace' only when the ion-ratio and retention time criteria were met.

The buffered QuEChERS extraction ensured a pH of 4 for both hot-spots and uninfected silages, which minimized the pH influence on the extraction of these two types of samples. As the procedure did not remove all matrix constituents, it is recommended to protect the MS interface by directing the eluent into the MS instrument only when data are recorded. The LC-system and column were cleaned with injections of formic acid, methanol and water in between each sample to maintain instrument sensitivity. Nevertheless for citrinin the validation data turned out unsatisfying because of varying response within a sequence. Fumonisin B_1 and B_2 had unacceptably low recovery rates (6 and 13%, respectively) probably due to poor extraction as they contain 4 carboxylic acid groups, which make them extremely polar at pH values above approximately 4 (ACD/Labs, 2008). Acceptable recoveries of fumonisin B_1 and B_2 have been achieved for breadcrumb matrix when an un-buffered acidified acetronitrile and water mixture is applied (Sulyok et al., 2007). Still the buffered QuEChERS extraction was preferred for multiple-toxin determination in the current study due to variable pH of the silage samples.

Table 5.2 Metabolites included in the LC-MS/MS method and the maize or silage associated fungi that produce them**.** Based on **I,** Visconti and Bruno (1994), Wang et al. (2004), Samson et al. (2002), Frisvad et al. (2006), Nicolaisen et al. (2009).

Analyte	Pre-harvest fungi	Post-harvest fungi
Alternariol Alternariol monomethyl ether Tenuazonic acid	*Alternaria tenuissima* *A. alternata* *A. arborescens*	
Altersetin	*A. tenuissima*	
Andrastin A Citreoisocoumarin Roquefortine A and C		*Penicillium paneum* *P. roqueforti*
Citrinin* Mevinolin**		*Monascus ruber*
Cyclopiazonic acid Sterigmatocystin	*Aspergillus flavus*	
Deoxynivalenol Zearalenone	*Fusarium culmorum* *F. graminearum* *F. cerealis*	
Enniatin B	*F. avenaceum,* *F. poae* *F. sporotrichioides* *F. tricinctum*	
Fumigaclavine A, B and C Fumitremorgin A and C Gliotoxin		*Aspergillus fumigatus*
Fumonisin B_1 and B_2*	*F. subglutinans* *F. verticillioides* *F. proliferatum*	
Marcfortine A and B		*P.paneum*
Mycophenolic acid		*B. nivea* *P. roqueforti*
Nivalenol	*F. culmorum* *F. graminearum* *F. equiseti* *F. cerealis*	
Ochratoxin A**		*A. ochraceus* *P. verrucosum*
Patulin		*B. nivea* *P. paneum*
Penitrem A****		*P. carneum* *P. crustosum*
PR-toxin		*P. roqueforti*
T-2 toxin	*F. poae* *F. sporotrichioides*	

* was not successfully validated

* *Mevinolin is also known as lovastatin, Monacolin K, and mevacor.

**** Recommendation for maximum levels of ochratoxin A in animal feed exists, but the fungi is not likely to grown in silage but in stored cereals.

**** Penitrem A have be associated to animal mycotoxicoses (Rundberget and Wilkins, 2002), but fungi are not among filamentous commonly isolated from maize or silage.

5.2.1 Occurrence of pre-and post-harvest mycotoxins and other secondary metabolites in maize silage

Paper IV

Photo: Storm IMLD

Occurrence of pre-and post-harvest mycotoxins and other secondary metabolites in maize silage

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ABSTRACT

With a recently developed liquid chromatography–tandem mass spectrometry method 99 samples of whole-crop maize, both ensiled and un-ensiled, were analysed for their contents of 27 mycotoxins and other secondary fungal metabolites. The method comprises metabolites from the majority of common pre- and post-harvest fungi associated with maize silage. Sixty-one samples contained one or more of the 27 analytes in detectable concentrations. The most common mycotoxins were zearalenone and enniatin B, which were found in 34 and 28% of samples, respectively. Other common fungal metabolites were nivalenol (16%) and the *Penicillium* metabolite andrastin A (15%). None of the samples contained mycotoxins above the recommended maximum concentrations, which exist for *Fusarium* toxins in the European Union. Thirty-one of the 99 samples contained more than one analyte with two samples containing as much as seven different analytes. This emphasizes the need for thorough examination of chronic exposure and possible synergistic effects when livestock is exposed to mixtures of mycotoxins.

KEYWORDS

Maize silage, mycotoxins, dairy cattle, LC-MS/MS

INTRODUCTION

Contamination of animal feed with mycotoxins is of concern for both farmers and consumers of animal products. Maize silage is a widely used feed product for cattle around the world, particularly in dairy production *(1)*. It is used year round and a dairy cow may consume 25 kg dry matter per day *(2)*. Maize silage may be contaminated with various fungal metabolites both pre- and post harvest. Common pre-harvest contaminants are species of *Fusarium*, *Alternaria* and *Aspergillus* while post-harvest infection is most often caused by *Penicillium roqueforti, P. paneum*, Zygomycetes, *Aspergillus fumigatus*, *Byssochlamys nivea* and a few other fungi *(3)*.

Mycotoxin contamination caused by fungi can affect animal health *(4)* and productivity *(5)*. The general symptoms of mycotoxicosis include loss of appetite, poor weight gain, feed refusal, diarrhoea, bleeding, birth defects, and kidney, liver or lung damages *(6)*. Acute intoxications of animals are rare *(7)* but it is important to know the exposure of animals since a chronic exposure to low levels of mycotoxins can give non-specific symptoms such as impaired immune system and increased infections or metabolic and hormonal imbalances *(5, 8)*. Moreover, little is known about possible synergistic effects of mycotoxins and the diagnosis of mycotoxicoses can be difficult because other diseases may give similar symptoms *(5)*.
Carry-over of mycotoxins and their metabolites to edible animal products such as milk and meat is a potential risk for the consumers. For human safety, the genotoxic carcinogen aflatoxin B1 is regulated to maximum 5 µg/kg in complete feeding stuffs for dairy animals *(9)* as its metabolite is transferred to milk *(10)*. Aflatoxins have been most extensively regulated, but also the trichothecenes deoxynivalenol, diacetoxyscirpenol, T-2 toxin and HT-2 toxin; the fumonisins B1, B2, and B3; the ergot alkaloids; ochratoxin A and zearalenone have been

regulated in feed by some countries *(11)*. In an attempt to protect animal welfare, maize based feed products are recommended not to exceed 2 mg/kg zearalenone, 8 mg/kg deoxynivalenol, 0.25 mg/kg ochratoxin A and 60 mg/kg fumonisins (sum of B_1 and B_2) *(12)*.

The fungi spoiling maize and maize silage are able to produce a vide range of secondary metabolites on different substrates *(13)*. Previous studies of mycotoxins in maize silage and whole-crop maize for silage have detected various fungal metabolites of pre- and postharvest origin *(14-24)*. The study by Driehuis *et al.* from the Netherlands *(17)* was the most comprehensive on maize silage covering 100 samples which were analysed for 20 different mycotoxins including aflatoxins, deoxynivalenol, zearalenone, and ochratoxin A, but only few compounds produced by common post-harvest silage contaminants. This study showed that the *Fusarium* toxins deoxynivalenol and zearalenone were commonly present in levels below the maximum recommended concentrations. Mycophenolic acid and roquefortine C produced by the post-harvest silage contaminant *P. roqueforti* were not detected. This may be because the silage samples were taken in October and November where maize silages are only a few weeks old, thus reducing the possibility of encountering post-harvest toxins. However, maize silage can also contain high levels of post-harvest fungal metabolites in areas with visible fungal growth *(13)* whose presence is only sparsely examined and not regulated.

With a newly developed method we are capable of determining 27 mycotoxins and other fungal secondary metabolites in maize silage samples *(20)*. It is specifically developed and validated for maize silage and detects metabolites from most of the common fungal contaminants of silage, both pre- and post-harvest *(3)*. It is therefore uniquely able to give an estimate of the overall exposure to mycotoxins through maize silage. This study describes the occurrence of alternariol (AOH), alternariol monomethyl ether (AME), altersetin (ALS), cyclopiazonic acid (CPA), deoxynivalenol (DON), enniatin B (ENN B), nivalenol (NIV), sterigmatocystin (STE), T-2 toxin (T2), tenuazonic acid (TEA), and zearalenone (ZEA), all associated with the field mycobiota, and andrastin A (AND A), citreoisocoumarine (CICO), fumigaclavine A (FUC A), fumigaclavine C (FUC C), fumitremorgin A (FUT A), gliotoxin (GLI), marcfortine A (MAC A), marcfortine B (MAC B), mevinolin (MEV), mycophenolic acid (MPA), ochratoxin A (OTA), patulin (PAT), penitrem A (PEN A), PR toxin (PR), roquefortine A (ROQ A), roquefortine C (ROQ C) from the storage fungi in 99 samples of maize silage and whole-crop maize for silage.

MATERIALS AND METHODS

Sample collection and preparation

Ninety-nine samples of maize silage or freshly harvested maize intended for silage were gathered. The samples were collected from 2007 to 2009 thus incorporating maize from the growth seasons 2006, 2007 and 2008. Samples were collected from different parts of Denmark. Sample 1-21 were collected by the Danish Plant Directorate from randomly selected farmers. Grab samples were collected from the cutting face of the silage stack or silo to form a composite sample. Sample 22-82 were silage samples collected at randomly selected dairy farms in Jutland. Twenty samples were collected in 2007 *(25)* and 41 in 2009 *(26)*. All samples were collected in full depth with a silage drill approximately 1 meter behind the cutting face of

the silage stack. Sample 83-99 were field samples of whole fresh maize plants taken at field level from all over Denmark and consisted of different maize cultivars. The samples were harvested in October 2007 and 2008 by personnel from the Danish Agricultural Advisory Service either by hand or by forage harvester.

Samples were homogenized and comminuted by two different methods. Samples 22-62 and 81-97 were freeze dried and milled. From all other samples a portion of approximately 150 g was frozen by pouring liquid nitrogen over it. As soon as the nitrogen was evaporated the samples were homogenized in a small domestic blender to a fine powder. All samples were stored at -20^{o}C until extraction and analysis.

Extraction

A fast and simple pH-buffered extraction was performed according to *(20)*. The method employs extraction with acetonitrile and water combined with phase-separation induced by addition of $MgSO_4$, a principle known as QuEChERS *(27)*. The method was developed for non-dried silage samples with a dry matter (DM) content of approximately 35 kg DM·kg^{-1} of which 10.0 g of fresh weight silage is extracted. A minor modification was included for the analysis of freeze dried samples in the present study where 3.5 g of dried sample was used together with 6.5 ml of water, thus totalling 10.0 g. Several different combinations were tested, with this mixture resulting in approximately the same extraction conditions as for the non-dried samples.

Sample analysis

The extracts were analysed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as described in *(20)* with the limits of detection (LOD) presented in Table 1.

The 99 samples were analysed in 6 separate series on separate days. Each series included 15-20 silage sample extracts, a 6-level matrix matched standard curve of quantitative standards and 1 level of matrix matched qualitative standards. One blank silage sample spiked with 6 mycotoxins (DON, NIV, GLI, PAT, ROQ C, and T-2) was also included in each series as well as one aliquot of the same old extract of a spiked sample. One control sample consisting of a combination of naturally contaminated silage samples known to contain AND A, DON, GLI, MPA, NIV, ROQ C and ZEA was also included in each series.

Data analysis

All results are reported without correction for recovery. For comparison with guidance values a dry matter content of 0.35 kg dry matter·kg silage^{-1} was assumed.

All analytical series were compared to the validation results for the method with regards to recovery and relative standard deviation under intra-laboratory reproducibility conditions (RSD_{IR}) for the spiked samples. The RSD_{IR} was also calculated on the basis of the results from the naturally contaminated control sample in each series.

Comparison of preparation method, sampling year and sample type (fresh maize vs. silage) was conducted by a homoscedastic two-tailed Students T-test *(28)*. A significance level of $P \leq 0.05$ was applied.

RESULTS AND DISCUSSION

Method performance

Evaluation of the method recoveries for DON, GLI, NIV and PAT in the spiked samples (n=6) showed a good agreement with the recoveries determined during method validation *(20)* as the average recoveries were 91, 79, 67 and 93%, respectively. For ROQ C and T2 the recoveries were 110 and 114%, respectively, which in itself is acceptable but not comparable to the previous validation results of 205 and 55%, respectively. The current sample clean-up was performed with more routine and analysed in shorter series than the validation, which may have caused the better average recoveries.

For ROQ C there was a significant ($P<0.001$) difference between the recoveries from fresh extract of a spiked sample and the recoveries from old extract of a spiked sample, which had been stored at -20^{o}C for 1-3 months. Recoveries were on average 62% in the old extracts compared to 110% in fresh extracts, indicating a degradation of ROQ C in extracts during storage. It is therefore recommended not to store sample extracts more than a few days before analysis.

For all six analytes in the spiked samples the relative standard deviation (RSD) between samples in the 6 series was comparable to RSD_{IR} from the method validation *(20)*. This was also the case for the eight analytes quantitatively detected in the control samples. However, for DON one of the control samples showed a concentration twice as high as the average of the other five control samples. This erroneously high result was omitted from the calculation of RSD. The method results in some co-eluting matrix compounds early in the chromatogram interfering with the most polar analytes. However, at concentrations near or above the guideline values the interference was negligible.

Mycotoxins in maize and maize silage

Out of the 99 analysed samples 61 contained one or more of the detectable analytes in concentrations above LOD. Summary statistics for the findings of each of the analytes are presented in Table 2 and a list of all positive results is available as supplementary material. Ten samples contained trace amounts of analytes below the LOD. The most common mycotoxins were ZEA and ENN B, which were found in 34% and 28% of the samples, respectively. Other common analytes were NIV (16%) and AND A (15 %). Of the post-harvest metabolites AND A was the most common followed by ROQ A, MAC A and CICO. These are all produced by *P. roqueforti* or *P. paneum (29)*. The abundance of and sensitivity for AND A makes it a good marker for the presence of these species in silage. There was one single finding of CICO in a fresh maize sample. This may be explained by the presence of *P. roqueforti/P. paneum* also prior to ensiling, as shown by *(30)*, or originate from other fungi e.g. Phoma *(31)*. Low occurrence of the *P. roqueforti/P. paneum* metabolites MPA and ROQ C were in line with *(17)* who did not detect these toxins in 60 maize silages sampled from sealed stacks. *P. roqueforti*

and *P. paneum* have been associated with ill-thrift and disease in cattle herds *(3)*. However, no direct effects were observed at high doses of MPA and ROQ C in two sheep studies *(32, 33)* and no adverse effects have been described for AND A *(34)*.

None of the secondary metabolites from *Aspergillus fumigatus* (GLI, FUT A, FUC A, FUC C) were detected in the present study. *Asp. fumigatus* is commonly isolated from silages in both warm and temperate climates *(3)* including Danish maize silage *(35)*. GLI has been detected in silage by *(16, 20, 36)*. The absence in this survey therefore indicates that the mycotoxin production of this fungus is limited under Danish conditions, even though the fungus is generally present. PAT and CPA were not detected in the present study either. The high occurrence of PAT, CPA, MPA and ROQ C observed by *(18)* could indicate regional differences or poor silage management, but because of the non-selective LC-MS method applied and because the recovery was tested high above the LOD, the risk of false positive results is considered high in that study. Absence of CPA was expected as *Asp. flavus* is mainly a problem in warmer climates than the Danish *(3)*. Likewise, the producers of aflatoxin B_1 are not relevant under Danish climatic conditions *(3, 31)* and aflatoxin B_1 was not included in the applied detection method.

Alternaria toxins are produced pre-harvest in maize *(22)*, but the presence of AOH and AME in maize silage is only recently described *(20)*. Their occurrence and concentrations in the present study were low. Seven samples contained at least trace amounts of these analytes and the co-occurrence of these compounds is a good marker for pre-harvest infection with *Alternaria*. The toxicity of alternariols is not well examined *(37)*. *In vitro* experiments show that alternariols have DNA strand-breaking activities *(38)*. *Alternaria* toxins have also been associated with human esophageal cancer in China *(39)*.

The concentrations of the *Fusarium* toxins ZEA, DON and ENN B detected in the present study were similar to results from previous studies *(17, 19)*. Concentrations and occurrence of ZEA and NIV were higher in a German study *(23)*. *In vitro* data suggest biological activity of enniatins but there is a clear lack of animal studies and more data is needed to evaluate their toxicity *(40)*. DON or ZEA are known to have immunosuppressive effects and oestrogenic effects, respectively *(41, 42)*. None of the analysed samples contained DON or ZEA in concentrations above the guidance values set for individual feeding products by the European Commission *(12)*. However, three samples contained DON and three samples ZEA in values above the guidance values of 5000 and 500 $\mu g \cdot kg^{-1}$, respectively, which exist for complete feedstuffs to dairy cattle. Two of these samples (# 9 and #99) were the same thus having high levels of both DON and ZEA. With silage constituting up to 50-75% of the daily feed ration to dairy cattle *(17)* such high concentrations should be taken into consideration.

The high frequency of DON observed by *(17)* compared to the current study is attributed to their 3-times lower LOD. DON is part of the trichothecene group, which comprise numerous fungal metabolites of which e.g. NIV, scirpentriol, 15-monoacetoxyscirpenol, HT-2 toxin, T2 and diacetoxyscirpenol (DAS) have been associated with maize and silage *(23)*. T2 and DAS appear to be most potent in animal studies *(12)*, however the T2 concentration and occurrence was low and DAS was not determined in the present study. The general toxicity

and immunotoxicity are considered to be the most critical effects of several trichothecenes *(43)*. Continuous exposure to low levels of immunosuppressive toxins may increase an animals susceptibility to infectious diseases. Several mycotoxins detected in Danish maize silage have immunosuppressive effects. Besides the trichothecenes DON and NIV it includes GLI and MPA at high doses *(34, 41, 45)*. Unfortunately, long-term *in vivo* studies evaluating the immunosuppressive effects of mycotoxins are sparse *(46)*.

Thirty-one of the total of 99 analysed samples contained more than one analyte with two samples containing as much as seven analytes (Figure 1). Sample 9 contained the following toxins (concentrations ($\mu g \cdot kg^{-1}$) in brackets where applicable): AME (8.8), AOH (12), ALS, DON (2974), ENN B (85), NIV (758) and ZEA (209) thus showing infection with both the *Fusarium* and *Alternaria* pre-harvest species. Sample 27 contained AND A (521), CICO, MAC A, MAC B, MPA (34), ROQ A and ROQ C (158) all known to be produced by the common post-harvest contaminants *P. roqueforti* and *P. paneum*. Except for the trichothecenes (SCF, 2002) very little is known about the *in vivo* toxicological effects of multiple mycotoxins and the possible synergistic effects of such mixtures should therefore be examined.

Sample type, preparation and year

Table 2 compares the findings of mycotoxins in fresh whole-crop maize samples collected prior to ensiling vs. the findings in ensiled maize. All the detected toxins were observed in ensiled maize while the fresh samples only contained the pre-harvest toxins ENN B, ZEA, NIV, DON and AME. Of the ensiled maize samples 74% were collected when silages were approximately 6 months old. Maize silage has been shown to contain the highest amounts of fungal propagules 5-7 months after ensilage *(35)* making it the most likely time to detect various post-harvest mycotoxins. For DON, ENN B and ZEA the concentrations as well as the percentage of positive samples was higher in the fresh maize samples than the ensiled but only for ENN B was the difference significant (P=0.009). A difference in ENN B concentrations between fresh and ensiled maize is in accordance with the findings of *(19)*. Similarly, *(23)* found a higher abundance and higher concentrations of T-2, HT-2, T-2 tetraol and T-2 triol in maize plants than in maize silage.

The different methods of preparing silage for analysis (freeze drying and milling vs. blending frozen silage) did not seem to affect the results. The average concentrations for the two groups of samples are not significantly different (Table 3). The sample preparation method and sample type (fresh maize samples vs. ensiled maize) are confounded as all the 17 fresh maize samples had been freeze dried while the 82 ensiled samples where distributed equally on the two preparation methods. The direct comparison of results is based on an assumption of 35% dry matter content in silage. In Denmark the 2008 average dry matter content for maize silage was 33.5 % with 10 and 90% quantiles of 29.3 and 37.7%, respectively *(47)*, showing that this assumption is valid under Danish conditions. Correction for dry matter content can be implemented if the value is determined.

The samples were collected from 2007 to 2009 thus representing maize grown in 2006, 2007 and 2008. No significant differences were found between the concentrations of mycotoxins each year (Table 4).

On the basis of the present study it therefore seems unlikely that Danish maize silage could be the direct cause of acute intoxications in dairy cattle. None of the regulated toxins were detected in concentrations above the guideline values recommended by the European Commission. This does not exclude the possibility of occasional incidences of high contamination levels. Variations in climate, crop cultivars and agricultural practices may also affect the mycobiota associated with maize and maize silage and thereby the possible mycotoxins. The present study also shows that contamination with low levels of multiple secondary metabolites is common. Feed rations with maize silage may therefore contain complex mixtures of fungal secondary metabolites with unknown biological activity. The possible synergistic effects and effects of long-term exposure to such mixtures are not known and further research in this subject is recommended.

ABBREVIATIONS

RSD_{IR} the relative standard deviation under intra-laboratory reproducibility conditions
QuEChERS Quick, Easy, Cheap, Effective, Rugged, Safe. Multi-method developed for analysis of pesticide residues in fruit and vegetables.
LOD Limit of detection

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Supporting information available: Table of all findings of mycotoxins and other fungal secondary metabolites in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure 1: Distribution of the 99 maize silage samples according to the number of analytes detected in each sample.

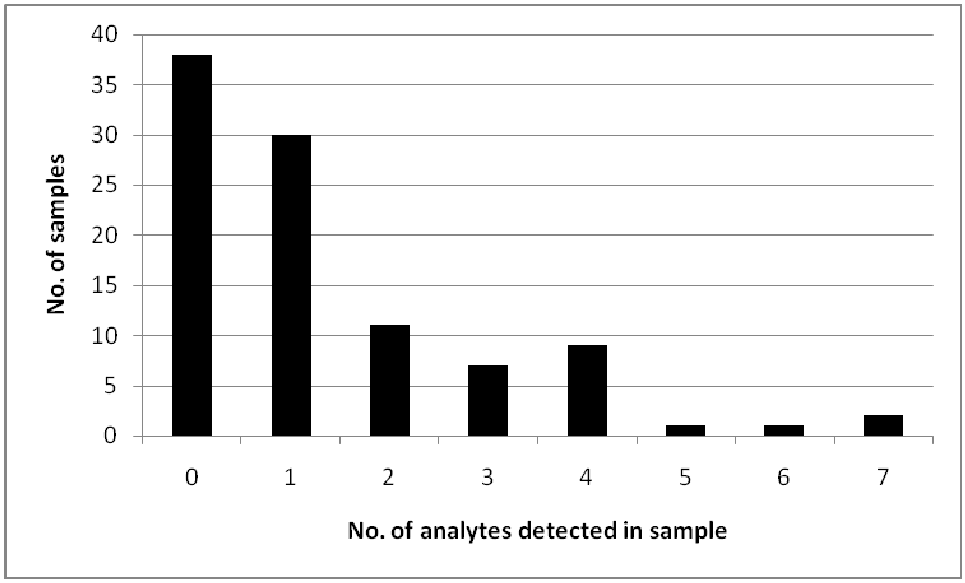

Table 1: Mycotoxins and other secondary fungal metabolites included in the present study, their abbreviations and limits of detection (LOD) for the quantitatively determined analytes.

	Analyte	Abbreviation	LOD (µg·kg^{-1})
Quantitative	Alternariol	AOH	10
	Alternariol momomethyl ether	AME	6
	Andrastin A	AND A	1
	Cyclopiazonic acid	CPA	15
	Deoxynivalenol	DON	739
	Enniatin B	ENN B	24
	Fumitremorgin A	FUT A	76
	Gliotoxin	GLI	71
	Mevinolin	MEV	25
	Mycophenolic acid	MPA	7
	Nivalenol	NIV	122
	Ochratoxin A	OTA	10
	Patulin	PAT	371
	Penitrem A	PEN A	8
	Roquefortine C	ROQ C	158
	Sterigmatocystin	STE	8
	T-2 toxin	T2	96
	Tenuazonic acid	TEA	121
	Zearalenone	ZEA	9
Qualitative	Altersetin	ALS	-
	Citreoisocoumarin	CICO	-
	Fumigaclavine A	FUC A	-
	Fumigaclavine C	FUC C	-
	Marcfortine A	MAC A	-
	Marcfortine B	MAC B	-
	PR-toxin	PR	-
	Roquefortine A	ROQ A	-

Table 2: Summary statistics on the contents of mycotoxins detected in fresh whole-crop maize samples, ensiled maize samples and all 99 samples together. The number of samples with concentrations above LOD (n_{pos}) are included for both quantitatively and qualitatively determined compounds. For quantitatively determined compounds average concentration of positive samples (avg_{pos}) with standard error of mean (SEM) and maximum concentrations (max) are presented in $\mu g \cdot kg^{-1}$ fresh weight.

		Fresh maize (n=17)			Ensiled maize (n=82)			Total (n=99)		
	Compound	n_{pos}	avg_{pos}	max	n_{pos}	avg_{pos}	max	n_{pos}	avg_{pos}	max
Quantitative	Alternariol monomethyl ether	1	11	11	2	8.0(0.8)	8.8	3	8.8	11
	Andrastin A				15	169(54)	691	15	169	691
	Alternariol				2	18(6.1)	24	2	18	24
	Deoxynivalenol	2	2369(293)	2662	5	1629(365)	2974	7	1841	2974
	Enniatin B	8	128(40)[a]	365	20	53(6.7)[b]	152	28	75	365
	Mycophenolic acid				2	43(9.3)	52	2	43	52
	Nivalenol	5	255(37)	351	11	266(53)	758	16	263	758
	Roquefortine C				2	173(15)	189	2	173	189
	Zearalenone	11	83(59)	666	23	66(15)	311	34	71	666
Qualitati	Citreoisocoumarin	1			7			8		
	Marcfortine A				6			6		
	Marcfortine B				1			1		
	Roquefortine A				9			9		

[a, b] Group means with different superscript letters differ significantly from each other (P≤0.05)

Table 3: Comparison of the detected mycotoxins in freeze dried samples vs. non-dried samples. The summary statistics include number of samples with mycotoxin concentrations above LOD (n_{pos}), average concentration of positive samples (avg_{pos}) with standard error of mean (SEM) and maximum concentrations (max) in $\mu g \cdot kg^{-1}$ (fresh weight)

		Freeze dried (n=58)			Non-dried (n=41)		
	Compound	n_{pos}	avg_{pos} (SEM)	max	n_{pos}	avg_{pos} (SEM)	max
Quantitative	Alternariol monomethyl ether	1	11	11	2	8.0(0.9)	8.8
	Andrastin A	8	177(66)	521	7	159(92)	691
	Alternariol				2	18(6.1)	24
	Deoxynivalenol	2	2369(293)	2662	5	1629(365)	2974
	Enniatin B	15	87(24)	365	13	61(9.3)	152
	Mycophenolic acid	1	34	34	1	52	52
	Nivalenol	13	235(22)	351	3	382(188)	758
	Roquefortine C	1	158	158	1	189	189
	Zearalenone	22	71(30)	666	12	72(27)	311
Qualitative	Citreoisocoumarin	3			5		
	Marcfortine A	5			1		
	Marcfortine B	1					
	Roquefortine A	5			4		

Table 4: Summary statistics on the contents of mycotoxins detected in all 99 samples divided on sampling year. The number of samples with concentrations above LOD (n_{pos}) are included for both quantitatively and qualitatively determined compounds, while average concentration of positive samples (avg_{pos}) with standard error of means (SEM) in brackets and maximum concentrations (max) in $\mu g \cdot kg^{-1}$ (fresh weight) are presented for quantitatively determined compounds.

		2007 (n=30)			2008 (n=8)			2009 (n=61)		
	Compound	n_{pos}	avg_{pos} (SEM)	max	n_{pos}	avg_{pos} (SEM)	max	n_{pos}	avg_{pos} (SEM)	max
Quantitative	Alternariol monomethyl ether				1	11	11	2	8.0(0.9)	8.8
	Andrastin A	7	159(92)	691				8	177(66)	521
	Alternariol	1	24	24				1	12	12
	Deoxynivalenol	4	1500(264)	2076	1	2662	2662	2	2110(863)	2974
	Enniatin B	15	94(23)	365	1	159	159	12	44(5)	85
	Mycophenolic acid	1	52	52				1	34	34
	Nivalenol	5	223(36)	351	1	325	325	10	276(58)	758
	Roquefortine C	1	189	189				1	158	158
	Zearalenone	13	51(23)	311	7	122(91)	666	14	65(16)	209
Qualitati	Citreoisocoumarin	6						2		
	Marcfortine A	1						5		
	Marcfortine B							1		
	Roquefortine A	4						5		

Supplementary material

Tabel S1: Table of all samples with concentrations of the analysed mycotoxins above limit of detection. Concentrations are given as $\mu g \cdot kg^{-1}$ while a + indicates detection of a qualitatively determined mycotoxin. tr indicates detection of trace amounts below limit of detection.

Sample no.	ALS	AME	ANDA	AOH	CICO	DON	ENNB	MACA	MACB	MPA	NIV	ROQA	ROQC	ZEA	STE
3							47								
6														tr	
7		7.1				1247	47				225				
9	tr	8.8		12		2974	85				758			209	
12							25								
13														13	
15						tr								38	
17							44								
22							36				293			32	
23			27					+						19	
24							29							125	
25														130	
27			521		+			+	+	34		+	158		
28			276					+			210	+			
29			139								309	+		64	
31			344		+			+				+	tr		
32			17												
33							29								
35							tr								
37							29							87	
42							65							22	
45			8.2					+					tr		
46		tr		tr										113	
49											211				
50														26	
52											129				
53														9.3	
54											185				
55		tr		tr											
56											122				
57		tr		tr			49				324			24	
60			80									+	tr		
61							38								
63						1777	70				165			55	
64			136		+							+	189		
65			11												
66						1257								38	
67			691		+			+				+			
69			15		+		54							45	
70							25								
71			56				35					+			
72														10	
74							59							8.5	
75						892	86							84	
77			187	24	+		63			52				311	
78														31	
80							152							17	
82			21		+							+			
83					+	2076					351			10	
84							33				254			19	
85							28				179				
87							32								
88														12	
89							129								
90							168								
91							114							19	

92							365				168				
93														90	
94														29	
95														18	
96														16	
97														11	
98														25	
99		11		tr		2662	159				325			666	tr

6. DISCUSSION

*The present studies (**paper I**, **II, III** and **IV**) have contributed to the current knowledge about the mycotoxin hazard the cattle faces when they are feeding on maize silage. This chapter contains a general discussion. For more detailed discussion see the individual papers.*

6.1 Maize silage sampling and extraction

The inhomogeneous nature of silage stacks challenges the sampling method. A silage stack may contain hundred tons but only a few kilograms can be sampled. Storm (2009) demonstrated that the number of filamentous fungi varied significantly between samples from the same stack. Values based on one or a few full depths samples from a whole silage stack must be interpreted with great care, as intra-stack standard deviations were high. This is especially a problem for the post-harvest contaminants since the field fungi metabolites may be more evenly distributed after maize plants have been chopped and packed.

Maize silage is a difficult matrix to analyse because many different constituents can interfere with the analysis of mycotoxins. It contains e.g. chlorophyls and carotenoids from the leafy parts of the plant, starch and glucans from the cob and organic acids from the ensiling. In this study, the QuEChERS method was adapted to extract many different mycotoxins (**II**). QuEChERS is a popular extraction and clean-up procedure applied in multi-methods for pesticide analysis (Anastassiades et al., 2003; Lehotay et al., 2005; Lehotay, 2007) and with the current buffered QuEChERS method, it may in the future be possible to combine mycotoxin and pesticide analyses. Only 10 grams fresh weight maize silage were extracted for chemical analysis in our method (**II**) and it was therefore important to homogenise the sampled silage thoroughly. A suitable method was to add liquid nitrogen and simply blend the fresh samples then the frozen pieces were turned into flour.

6.2 Chemical detection

Detection of analytes was accomplished by LC-MS/MS or LC-DAD-HR-MS and thereby follows the recent trends in mycotoxin analysis (Shephard et al., 2010). The LC-DAD-HR-MS, which recorded full scan data, has successfully detected several secondary metabolites in mouldy silage extracts. Detection was limited by matrix interferences, which was more pronounced for DAD and single MS detection than for the selective MS/MS detection. In the current study comparisons of retention time, spectra of DAD, ESI^+ and ESI^- with standards or fungal agar extracts enabled confident identification of analytes also in dirty matrixes. Mansfield et al. (2008) applied a LC-HR-MS instrument for determination of patulin, mycophenolic acid, cyclopiazonic acid, roquefortine C but did not include any qualifier ions nor used the high-resolution capacity of the instrument. Because of the non-selective LC-MS method applied and because the recovery was tested high above the LOD, the risk of false positive results is considered high in that study.

The LC-MS/MS multi-method was successfully validated for the determination of 27 mycotoxins and other fungal metabolites in silage extracted by the adapted QuEChERS method (**II).** Analysis of certified reference material could improve the validation; unfortunately such references do not exist for mycotoxins in maize silage. Matrix matched standards with non-mouldy silage was used for quantification. Different maize silage matrices and alterations due to fungal infection could influence quantification, which was not taken into account in this study. However, eventual smaller differences in matrix effects should be compared to the interval for reporting results since a high reproducibility

(RSD_{IR}) leads to a large 95% confidence interval. As an example, the mycophenolic acid concentration in a hot-spot was 1646 (±460) µg/kg with the reporting range in brackets.

Two different HPLC eluents were applied in positive and negative electrospray ionization (ESI) mode of the LC-MS/MS method. To ensure proper formation of ammonium adducts (T-2 toxin and enniatin B) and to obtain better chromatography of the pH dependent compounds (e.g. roquefortine A, cyclopiazonic acid and citrinin) the water-based HPLC eluent was added ammonium and extra formic acid when recorded in ESI^+. The MS instrument is able to switch between modes, which could save instrument time. However, using the same eluents for ESI^+ and ESI^- compromised the sensitivity of some analytes. In practice switching may also cause trouble for quantification because of few data-points across the peak and short dwell time when several compounds are co-eluting (Sulyok et al., 2007).

Interference with co-eluting matrix constituents and mycotoxins has been observed for the LC-MS/MS method. At a very high concentration, alternariol monomethyl ether formed a small amount of the adduct $[M+HCOO]^-$, which interfered with the mother ion $[M-H]^-$ of zearalenone. It was possible because they had the same mass (m/z 317) and co-eluted. Fortunately the ion-ratio of the interfering daughter ions from alternariol monomethyl ether was different from the ratio of zearalenone. Occasionally interference with matrix constituents can be very problematic due to the heterogeneous properties of silage. Major matrix interference occurred primarily for the early eluting compounds such as patulin (Figure 2 in **II**). When insecure if matrix interference might be mistaken for a mycotoxin, the sample can be added a little mycotoxin solution and be re-analysed. Co-elution confirms the presence of the mycotoxin and could preferably be tested using different chromatographic conditions.

6.3 *In vitro* cytotoxicity detection

In vitro cytotoxicity assays have been applied widely to mycotoxins and fungal agar extracts (Gutleb et al., 2002). The resazurin assay with Caco-2 cells was proofed suitable for screening of agar extracts. The common fungal species infecting maize in the field and during the storage of silage produced numerous secondary metabolites in agar cultures and were able to elicit a cytotoxic response (**III**). Bio-guided fractioning identified PR-toxin as a major toxic principle of the *P. roqueforti*. Identification of cytotoxic principles from fungi using bioassay-guided fractionation has previously been demonstrated (Uhlig et al., 2005).

It was the purpose of the *in vitro* studies to get an indication of whether the variations in cytotoxicity could be explained solely by the mycotoxins tested as pure standards, or if other toxic principals appeared to be present. Uhlig et al. (2006) applied multiple regression analysis to determine cytotoxic principles from a fungal species by testing the cytotoxicity in several assays. Several fungal isolates were tested to draw the conclusion. In the current study few isolates of several fungal species grown on different agars were screened in one assay. Without applying advanced statistics this approach allowed comparisons between fungal species and to tentatively eliminate toxic effects of specific compounds, when metabolite profiles of toxic and non-toxic extracts were compared.

The application of the Caco-2 assay to silage samples was limited due to high and variable cytotoxicity of the crude maize silage extracts without fungal growth. The QuEChERS extraction buffer applied to samples resulted in a pH of 4 in the extracts (**II**). Unbuffered extracts also had a high toxicity maybe due to the low pH of uninfected maize silage samples. The low pH alone may cause cell toxicity, but other matrix components have probably also contributed to the toxicity. Widestrand et al. (2003) applied an *in vitro* assay for rapid and sensitive screening of *Fusarium* toxins in cereal samples. They first applied a thorough clean-up step using MycoSep columns for purification of specific toxins. In the current study the chemical detection methods of known toxins were superior to the *in vitro* assay in regard to time and

sensitivity. For the discovery of new bioactives *in vitro* assays are indispensable. To produce high quality data it was necessary to obtain single cell suspension before distributing the cells in the many wells. If not the relative standard deviation of the repetitions will be too high for any conclusion. To obtain high sensitivity it is necessary to optimise the number of seeded cells and dye concentration and exposure as described by Nakayama et al. (1997). Careful evaluation of *in vitro* methods should be carried out during experimental work, data processing and data presentation. The Caco-2 assay was sensitive to most mycotoxins tested and the actual standard operating procedure applied can be found in appendix B. Ideally several assays should have been applied to the agar extracts since the *in vitro* cytotoxicity assay measured general toxicity and did not target e.g. oestrogenic and mutagenic effects as other assays do (Welshons et al., 1990; Appendix A).

6.4 Mycotoxin occurrences

The secondary fungal metabolites determined in Danish maize silage are fungal metabolites produced by fungi commonly isolated from Danish maize and silage.

In the monitoring study (**IV**) of whole-crop maize both ensiled and un-ensiled samples were analysed with the recently developed LC-MS/MS method (**II**). Sixty-one of the 99 samples contained one or more of the 27 analytes in detectable concentrations. Most common were the *Fusarium* toxins zearalenone and enniatin B, which were found in 34 and 28% of samples, respectively. Other common fungal metabolites were nivalenol (16%) also originating from *Fusarium* and the *Penicillium* metabolite andrastin A (15%). Alternariols, roquefortines, marcfortines, citreoisocoumarin, mycophenolic acid and deoxynivalenol were detected occasionally in Danish maize silage stacks and maize for silage making.

Previous examinations of maize silages stacks have detected zearalenone, deoxynivalenol (Driehuis et al., 2008b), beauvericin, enniatins B and B_1 (Sørensen et al., 2008), fumonisins (Kim et al., 2008), patulin, mycophenolic acid, cyclopiazonic acid, roquefortine C (Mansfield et al., 2008) and gliotoxin (Pereyra et al., 2008). The average toxin concentrations (frequency; µg/kg range in parentheses) in these studies were 174 µg/kg zearalenone (49%; 25 - 943), 854 µg/kg deoxynivalenol (72%; 250 - 3142), 73 µg/kg enniatin B (95%; 24 - 218), 10 µg/kg enniatin B_1 (40%; 26 - 48), 8 µg/kg beauvericin (25%; 13 - 63), 707 µg/kg fumonisins (97%; 25-2204), 80 µg/kg patulin (23%; 10 - 1210), 160 µg/kg mycophenolic acid (42% 20 - 1300), 120 µg/kg cyclopiazonic acid (37%; 20 - 1430), 380 µg/kg roquefortine C (60%; 10 - 5710) and 5130 µg/kg gliotoxin (100%; 5100 - 6500).

The concentrations of the *Fusarium* toxins zearalenone, deoxynivalenol and enniatin B detected in the present monitoring were similar to results from the Danish and Dutch study referred above (Driehuis et al., 2008b, Sørensen et al., 2008). Concentrations and occurrences of zearalenone and nivalenol were higher in a German study (Schollenberger et al., 2006), which may reflect e.g. differences climate or yearly variations. Deoxynivalenol was probably only identified in part of the samples, as the LOD was higher the content in Danish maize used for silage making (Nielsen et al., 2005; 2006; 2007; 2008).

Alternaria toxins are produced pre-harvest in maize Monbaliu et al. (2010) whereas the detection of alternariol and alternariol monomethyl ether in maize silage new. They were only present rarely and in low levels. Low occurrence of the post-harvest metabolites mycophenolic acid and roquefortine C were in line with Driehuis et al. (2008b) who did not detect these toxins in 60 maize silages sampled from sealed stacks. Gliotoxin, patulin and cyclopiazonic acid reported in the foreign studies but were not detected in the current monitoring. Gliotoxin was detected in a Danish hot-spot infected with *A. fumigatus* and may therefore occur occasionally. The *Fusarium* toxins beauvericin, enniatin B_1, HT-2 toxin, fumonisin B_1 and fumonisin B_2 might also be present in Danish silage as these toxins have been reported in the Danish maize used for silage making (Nielsen et al., 2005; 2006; 2007; 2008; Sørensen et

al., 2008), however those metabolites were not detected or included with the method applied in the monitoring.

Danish maize silage stacks were most frequently contaminated with mycotoxins from pre-harvest fungi whereas 'hot-spots' with visible fungal growth often contained high levels of mycotoxins and other secondary fungal metabolites from post-harvest fungi.

6.5 Possible consequences of mycotoxins

Fungal spoilage of maize in the field and silage during storage is characterised by biomass loss, lower feed quality and mycotoxin contamination. During fungal spoilage volatile fungal compounds are produced (Karlshøj and Larsen 2005) and since cattle are known to sort the feed according to palatability, the mouldy odour may reduce their feed consumption (Undi and Wittenberg, 1996). Therefore may fungal spoilage result in decreased feed intake and thereby decrease milk yield (Vries and Veerkamp, 2000) because of volatile compounds.

The risk of mycotoxins in maize silage depends both on the hazard and exposure. With the current monitoring of Danish maize silage and other monitoring studies (Auerbach et al., 1998; Kim et al., 2004; Driehuis et al., 2008b; Sørensen et al., 2008; Schollenberger et al., 2006) it is clear that dairy cows feeding on maize silage are continuously exposed to low levels of *Fusarium* toxins but occasionally also to other secondary fungal metabolites. Only some of the secondary metabolites detected in Danish silage have been recognised as mycotoxins able to elicit direct harm to vertebrate animals. The detected levels of the estrogenic zearalenone (SCF, 2000b) and the immunosuppressive deoxynivalenol (SCF, 1999) were below the maximum values recommended by the European Commission (2006) for *Fusarium* toxins in maize based feed. For the other mycotoxins detected, no European recommendations have been made.

Weaver et al. (1986) fed dairy cows up to 500 mg zearalenone per day during 2 consecutive oestrous cycles and concluded that zearalenone does not seem to be an important factor in dairy cow health. The zearalenone detected in Danish silages is therefore not expected to affect dairy cows. Deoxynivalenol typically has high frequency in maize silage and is a part of structural related trichothecene group, which comprise numerous bioactive fungal metabolites. The symptoms produced by trichothecenes are related to the inhibition of the protein synthesis and symptoms such as vomiting or immunosuppression may be observed (Bennett and Klich, 2003). Of the mycotoxins detected in Danish maize silage the trichothecenes deoxynivalenol and nivalenol but also gliotoxin and mycophenolic acid at high doses have immunosuppressive effects (Table 1.1). Unfortunaly, long-term *in vivo* studies evaluating immunosuppressive and combined effect are sparse (Driehuis et al., 2008a). Continuous exposure to low levels of mycotoxins with immunosuppressive effects may increase an animal's susceptibility to infectious diseases, however under normal circumstances deoxynivalenol is extensively metabolised by the cows (JECFA, 2001).

Beauvericin, citrinin, enniatins, mycophenolic acid and roquefortine C, which were present in Danish silage, all have antibiotic properties (Table 1.1) and may therefore potentially affect rumen microorganisms (Tapia et al., 2002). For example, there are indications that roquefortine C concentration can induce a shift in the rumen microflora composition at high concentrations (Tüller, 2005). However, no direct effects were observable at high doses of mycophenolic acid (300 mg /animal daily) and roquefortine C (50 mg /animal daily) in two sheep studies (Tüller, 2005; Mohr et al., 2007). It appears therefore unlikely that even consumption of few *Penicillium* hots-spots with high occurrence of these two compounds can have a direct adverse effect on the health of dairy cows. Adverse effects of andrastines, citreoisocoumarin, marcfortines, 1-hydroxyeremophil-7(11),9(10)-dien-8-one and (3*S*)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one from *Penicillum* are also not expected due to low

cytotoxicity (Aly et al., 2008; **III**). *P. roqueforti* and *P. paneum* have been associated with ill-thrift and disease in cattle herds. Mouldy maize silage infected with *P. roqueforti* produced loss of appetite, disturbance of rumen activity in dairy cows (**I**), which might be related to the odour and fungal metabolites having antibiotic properties. Intoxication might be due to PR-toxin, which is known to be very toxic in mice (Moreau and Moule, 1978; Chen et al., 1982), though PR-toxin still have not been confirmed in field samples (Nout et al., 1993; Table 5.1). Patulin was also not detected Dansih hot-spots and silages, but if present it could contribute to problems in livestock (Scudamore and Livesey, 1998).

A. fumigatus metabolites from agar extracts gave problems for young calves diarrhoea, irritability, loss of appetite, serious enteritis and interstitial changes in the lungs (Cole et al,. 1977b) and the fumigaclavine A-C, fumitremorin C and gliotoxin detected in field samples hot-spots may therefore potentially affect animals. Hereof are gliotoxin with immunosuppressive effects and fumigaclavine A and B having acute and neurotoxic properties currently categorised as mycotoxins (Table 1.1). Administration of a single 25 mg/kg fumitremorgin C dose to mice did not appear to cause any major toxicity, whereas fumitremorgin A, which was not detected in silage, caused severe tremors and death when injected intravenous in mice (Table 1.1). Depending of the intake of spoiled and actual toxin levels may experience acute toxic effects after ingesting of *A. fumigatus* hot-spots, however the toxins were not detected in Danish silage stacks sampled as a whole.

Mouldy feed containing 30-40 µg/kg citrinin may have caused mycotoxicosis at a cattle farm (Griffiths and Done 1991). This is the approximate citrinin level detected by (Schneweis et al., 2001) in maize silage hot-spots, however clinical trails do not indicate strong actute effects of citrinin (Flajs and Peraica, 2009) and its occurrence is also expected to be low. Fumonisin B_1 is unaffected in the rumen microbial (EFSA, 2005) but because of very poor absorbtion when given orally (SCF, 2000a) severe acute intoxications do not occur under farm conditions (Fink-Gremmels 2008b). Low concentrations of beauvericin and enniatins have frequently been detected in Danish maize silage (Sørensen et al., 2008). *In vitro* data suggest biological activity these compounds but there is a clear lack animal studies and more data are needed to evaluate their toxicity (Jestoi, 2008). The toxicity of alternariols is also not well examined (Frisvad et al., 2006). Though their occurrences and concentrations in Danish maize silage were low they may be relevant in relation to human exposure due to their mutagenic properties (Appendix A; Pfeiffer et al., 2007). Aflatoxin B_1 have been detected in foreign maize silage (Garon et al., 2006), but *Aspergillus flavus* and *A. Parasiticusis*, which produce this toxin, is not relevant in Danish and climate conditions (**I**; Sørensen 2009). If complete feeding stuffs for dairy cows complies with the regulatory limit of maximum 5 µg/kg (European Commission, 2003) no acute adverse health effects in dairy cattle are to be expected. (EFSA, 2004).

Co-occurrence and continuous exposure to low levels of mycotoxins may cause concerns. It has been hypothesed that mycotoxins may contribute to loss in milk yield, increased, somatic cell counts in the milk, and an increased number of animals with laminitis and other infectious diseases (Fink-Gremmels 2008b). Maize silage contained often more than one fungal metabolite (**IV**), which emphasizes the need for thorough examination of chronic exposure and possible synergistic effects when livestock is exposed to mixtures of mycotoxins. With the current knowledge it seems unlikely that the low levels and low frequency of mycotoxins in Danish silage stacks have direct adverse effect on dairy cows. Mycotoxins and antibiotics were present in considerable amount in maize silage with visible fungal growth. For that reason it cannot be excluded that dairy cows feeding on heavily spoiled silage in some cases may be negatively affected.

7. CONCLUSION

The overall aim of this PhD project was to evaluate the mycotoxin hazard for cattle feeding on maize silage. Special emphasis was placed on detection of secondary fungal metabolites in Danish maize silage. The cytotoxicity significance of secondary metabolites from common pre- and post-harvest fungi infecting maize and silage was determined.

A new LC-MS/MS method for detection of 27 fungal secondary metabolites in maize silage was developed and successfully validated. Nineteen of the analytes can be detected quantitatively and eight qualitatively with recoveries from 37 to 205% and LODs from 1 to 739 µg/kg. Only for citrinin, fumonisin B_1 and fumonisin B_2, the validation results were unsatisfying. The pH-buffered QuEChERS extraction method ensured the same extraction conditions for fungal hot spots (pH>7) and well-fermented maize silage (pH~4). As the QuEChERS extraction method originally was applied to pesticides, the current work strongly indicates that it will be possible to combine mycotoxin and pesticide analyses with this extraction method.

The LC-MS/MS method covers secondary metabolites from the most common fungal species isolated in Danish climatic conditions including the pre-harvest *Fusarium* and *Alternaria* species and the post-harvest fungi *Penicillium roqueforti*, *P. paneum*, *Byssochlamys nivea*, *Monascus ruber* and *Aspergillus fumigatus.*

When the LC-MS/MS method was applied to 99 Danish fresh and ensiled maize silage samples, the following mycotoxins and other secondary fungal metabolites were detected: Alternariol, alternariol monomethyl ether, deoxynivalenol, enniatin B, nivalenol and zearalenone originating from pre-harvest fungi and andrastin A, citreoisocoumarinm, marcfortine A, marcfortine B, mycophenolic acid and roquefortine A and C from post-harvest fungi. Fresh and ensiled maize silages were most frequently contaminated by mycotoxins from pre-harvest fungi, but the maximum recommended levels for *Fusarium* toxins in maize based feed recommended by The European Commission (2006) were not exceeded. However, few samples contained deoxynivalenol and zearalenone values above the guidance values of 5000 and 500 $\mu g \cdot kg^{-1}$, respectively, which exist for complete feedstuffs to dairy cattle.

In heavily infected maize silage several post-harvest secondary fungal metabolites were detected by LC-MS/MS or LC-DAD-HR-MS: Andrastin A-D, byssohclamic acid, citreoisocoumarin, citrinin, fumigaclavine A-C, fumitremorgin C, gliotoxin, marcfortine A-C, mycophenolic acid, PR-toxin, roquefortine A and C, (3*S*)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one and 1-hydroxyeremophil-7(11),9(10)-dien-8-one. High concentrations can be present with visible fungal growth. The maximum concentrations (±95% confidence interval) of andrastin A, gliotoxin, mycophenolic acid and roquefortine C were 36 (±10) mg /kg, 0.9 (±0.2) mg /kg, 55 (±15) mg /kg, 40 (±21) mg /kg, respectively.

A resazurin assay with Caco-2 cells has been optimised to test cytotoxicity of fungal extracts. The median inhibitory concentration (IC_{50}) of seven mycotoxins ranged from 0.004 to 83 ug/mL for T-2 toxin and citrinin, respectively.

All fungal genera (*Alternaria, Aspergillus, Byssochlamys, Fusarium, Monascus, Penicillium*), which often spoil maize and maize silage, produced cytotoxic metabolites on agar. PR-toxin was identified as a major cytotoxic metabolite of *P. roqueforti*. Roquefortine C was moderate cytotoxic, whereas the *Penicillium* metabolites mycophenolic acid, andrastin A, 1-hydroxyeremophil-7(11),9(10)-dien-8-one and N6-formyl-roquefortin-C had low to none cytotoxic effects. The cytotoxicity of *A. fumigatus* could be attributed to gliotoxin but at least one other metabolite also decreased the viability of Caco-2 cells since also an extract without gliotoxin was cytotoxic. Other cytotoxic compounds than zearalenone,

deoxynivalenol, nivalenol from *F. graminearum* and citrinin from *Monascus ruber* were also produced. The cytotoxic *P. paneum* and *B. nivea* agar extracts contained cytotoxic levels of patulin.

Heavily *B. nivea* infected maize silage containing mycophenlic acid (~50 mg/kg), byssochlamic and other metabolites than patulin, was more cytotoxic than uninfected silage. Silage samples inoculated with *P. paneum, M. ruber* or *A. fumigatus* were not significantly cytotoxic. Additionally, the cytotoxicity of a *P. roqueforti* maize silage hot-spot was also not more severe than uninfected silage, despite the presence of the strongly cytotoxic PR-toxin and other secondary fungal metabolites. Due to the high cytotoxicity of crude maize silage extracts the *in vitro* assay was unsuitable for mycotoxin screening of maize silage in general.

Overall, the present work has shown that animals feeding on well-fermented maize silage are exposed to low levels of mycotoxins. Some post-harvest secondary metabolites were present in substantial levels in hot-spots, but their frequencies and concentrations were low in Danish fresh and ensiled maize silage. Besides the mycotoxins monitored in maize silage fungi are, however, able to produce several other cytotoxic compounds and animals feeding on heavily spoiled silage may be affected by the high mycotoxin and/or antibiotics contents. Altogether the results obtained in this PhD project do not indicate that mycotoxins in maize silage have caused the general health problems observed at Danish dairy cattle farms.

8. PERSPECTIVES

In the last decade increasing industrialisation of the dairy production has resulted in a growing use of maize silage, bigger herds, less grazing and less manual attention to the individual cow (Barrett 2004; Thomsen, 2005; Statistics Denmark, 2010). Though, all the changes within dairy production are not necessarily causally related to the increase of illness among cows observed at the same time.

The current PhD project is a part of a large Danish collaborative study (2005-2010) aiming to determine if mycotoxins in maize silage cause disease and poor performance in dairy cattle and if mycotoxins in the feed are carried over into blood and milk. Today we have much more detailed information about the fungi that spoil Danish maize and silage and which mycotoxins they produce *in situ*. Based on the results collected in the joint project, it is not fully possible to exclude that mycotoxins in maize silage can cause incidents of illness and ill-thrift in Danish dairy cattle in some cases. However the low levels of pre-harvest mycotoxins and low occurrence of post-harvest contaminants in Danish maize silages indicate that mycotoxins are probably not involved in general health problems observed at Danish dairy cattle farms.

The pre-harvest contaminants of Danish maize plants included; *Fusarium, Alternaria, Epicoccum, Phoma* infects (Sørensen, 2009). The most frequent *Fusarium* species identified were; *F. graminearum* and *F. culmorum, F. avenaceum, F. verticillioides* and *F. proliferatum.* Less common were *F. subglutinans, F. tricinctum, F. equiseti, F. sporotrichioides* (Nicolaisen et al., 2009; Sørensen, 2009). The most frequent post-harvest fungi were *Penicillium roqueforti* and *P. paneum, Zygomycetes,* and *Aspergillus fumigatus*. *Byssochlamys nivea, Monascus ruber* and *Geotrichum candidum* occurred less frequently (Storm, 2009). The risk of post-harvest fungal spoilage of well-fermented maize silage could be limited by keeping stacks well sealed for more than seven months before opening (Storm, 2009).

Though *Fusarium* and *Penicillium* species are ubiquitously present, their occurrences were not always accompanied by significant mycotoxin contamination. Some post-harvest mycotoxins were present in substantial levels in hot-spots, but their frequencies and concentrations were low in Danish maize silage. Fresh and ensiled maize silages were mainly contaminated by metabolites from pre-harvest fungi, but the maximum levels for *Fusarium* toxins in feed recommended by The European Commission (2006) were not exceeded. In Danish maize used for silage making the *Fusarium* toxins deoxynivalenol, zearalenone, nivalenol, moniliformin, enniatin B and B_1 had high occurrence, whereas the occurrence of T-2 toxin, HT-2 toxin, fumonisin B_1 and fumonisin B_2 were low (Nielsen et al., 2005; 2006; 2007; 2008; Sørensen et al., 2007; 2008). However the concentration levels of the toxins were low. In the four year monitoring period (2004-2007) the recommended maximum levels for *Fusarium* toxins were only exceeded in one of 239 maize samples due to high levels of zearalenone and deoxynivalenol. The occurrence of *Fusaium* toxin was low in the monitoring period, but considerably year to year variations may occur (Rasmussen et al., 2007).

The monitoring showed that the animals feeding on silage sometimes will be exposed to several secondary fungal metabolites at the same time, which may enhance the effect of single toxins. Kristensen et al., (2007) conducted a study where four lactating dairy cows were subjected to diets containing about 50% maize silage. Treatments included one control silage and three qualities of problematic maize silage (silage with *Fusarium* toxin, *Penicillium*-infected silage, and silage with high propanol content). The milk yield, fat and protein yield did not differ between treatments.

It can not be excluded that problems could be caused by mycotoxins not included in the surveys. *F. equiseti* was e.g. frequently isolated from maize (Sørensen et al., 2007) and is able to produce scirpentriol, 15-monoacetoxyscirpenol and diacetoxyscirpenol (Hestbjerg et al., 2002). These type A trichothecenes are expected to be of minor significance (Sørensen, 2009), because they have been found

to occur in comparable levels as T-2 and HT-2 toxin in maize (Schollenberger et al., 2006). T-2 and HT-2 toxin had a low occurrence in fresh Danish maize plants but the others have not been monitored in Denmark. The possible occurrence of PR-toxin in silage requires more research. Green hot-spots collected in the joint project could be analysed to see if PR-toxin are formed in field conditions. The potential number of mycotoxins present in silage is large; however bio-guided fractioning of agar extracts using *in vitro* assays can help to identify which metabolites to target.

Carry-over of mycotoxins from feed to blood and subsequent to milk has not been tested experimentally in the joint project. However the carry-over of some *Fusarium* toxins has already been well described in literature. Human exposure through milk is considered to be low for deoxynivalenol, zearalenone, fumonisins compared to other sources such as cereal products (EFSA, 2004a, 2004c; EFSA, 2005). Instead metabolites such as alternariols and PR-toxin having mutagenic properties will probably be more relevant to target for the human exposure. The metabolism and bioavailability of mycotoxins could be examined in blood, milk and rumen fluid samples available from the dairy cows fed on mycotoxin contaminated silages (Kristensen et al., 2007). Probably after just a few modifications the developed LC-MS/MS method should be able to analyse these samples, which might bring important information on the animal and human exposure. If biotransformations of the mycotoxin occur in the rumen or liver, the degradation products have to be targeted. Decomposition of fungal agar metabolites by rumen fluid *in vitro* could hint at important metabolites to determine in rumen-fluid.

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APPENDIX A - SCREENING FOR GENOTOXICITY

Mutagenic activity in bacterial systems is accepted as an initial step in the evaluation of the carcinogenic potential of chemicals (McMahon et al., 1979). Several *in vitro* tests for genotoxicity exists (Mortelmans, 2000; Kirkland et al., 2005).

umu-test

The *umu*-test developed in the early 1980s (Oda et al., 1985) is a well-validated method (ISO, 2000), which determines genotoxic activity by activation of the SOS-repair system induced by DNA damage. The *umuC* promotor gene involved in the SOS response has been transcriptional fused with the reporter gene *lacZ* encoding β-galactosidase. The induction of *umu* gene expression in *Salmonella typhimurium* could then be measured colorimetrically, as β-galactosidase hydrolyses non-colored β-d-galactosides into a coloured cleavage product. In the current assay the fused gene *umuC*'-'*lacZ* was hosted by *Salmonella typhimurium* (TA1535) carrying the plasmid (pSK1002). The screening for genotoxic activity in 96-well plates was based on Reifferscheid et al. (1991) with few modifications. In brief: The genotoxicity was determined as hydrolyses of the *o*-nitrophenyl-β-d-galactosid into to *o*-nitrophenol. The β-d-galactosidase activity was calculated as described by Miller (1972) from the quantitative absorbance at different wavelength. This activity was expressed relative to the response of the negative control, the so-called induction rate (IR). To establish a genotoxic response two conditions should be met: An increase by 0.5 or more of the induction rate (IR≥1.5) and the observation of a concentration-response (ISO, 2000). Test both with and without introduction of S9 liver mix (S9) into the assay was carried out. Methanol used as a toxin solvent constituted 2% of the growth medium. The genotoxic concentration-responses of *A. tenuissima* (IBT 41188), *B. nivea* (IBT 28552) and *P. roqueforti* (IBT 28547) YES extract (0.004 to 0.54 plug/mL bacterial medium) were tested after 2 h exposure. Metabolites were extracted from 13-14 days old YES incubated at 25°C in darkness, except for *A. tenuissima*, which grew in alternating light. Agar plugs were cut from the colonies of several plates and extracted using a micro-scale method by Smedsgaard (1997) with a few modifications.

Results and discussion

A. tenuissima (IBT 41188) YES extract caused a significant concentration-related increase in the induction rate of the SOS repair system in two independently repeated *umu*-tests without S9 addition. The extract *A. tenuissima* also induced a genotoxic response in one experiment where S9 liver extract was introduced to the *in vitro* mutation assay (Figure A.1). *B. nivea* (IBT 28552) and *P. roqueforti* (IBT 28547) YES extracts was not identified as genotoxic tested with and without addition of S9 liver extract. The methanol applied as a toxin solvent at 2% of growth medium had no effect on bacterial cell viability and mutagenicity (data not shown).

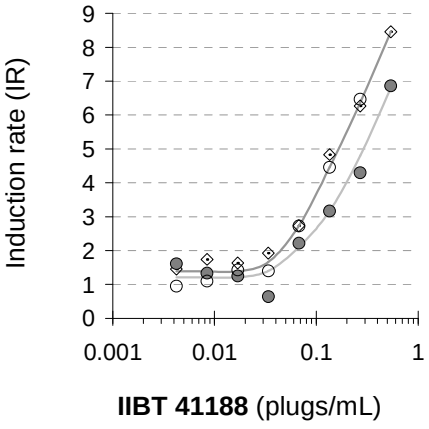

Figure A.1. Genotoxicity in the *umu*-test after 2 h exposure to *Alternaria. tenuissima* YES extract. Induction rate (IR) of umu gene expression in *Salmonella typhimurium* (TA1535/pSK) measuring *o*-nitrophenol colorimetrically tested on different days without (o, ◊) and with (●) addition of S9 liver extract. The means of 3 replicates measurements were fitted by two curves.

The content of secondary fungal metabolites have been determined for the fungal YES extracts tested for genotoxicity (Table A.1). Several *Alternaria* toxins were detectable in the YES extract of IBT41188 tested positive for genotoxic effects. The genotoxicity of *A. tenuissima,* observed in the *umu*-test, are in line with DNA strand-breaking activity of AME and AOH in mammalian cells *in vitro* (Pfeiffer et al., 2007). *Alternaria* toxins have also been associated with human esophageal cancer in China (Liu et al., 1992). The *P. roqueforti* YES extract did not induce *umu* gene expression in *Salmonella typhimurium* (TA1535/pSK), despite the presence of PR-toxin and several other fungal metabolites. Crude extracts of *P. roqueforti* were also previously tested negative in the Ames test (Schoch et al., 1984). The present results were in accordance with the *Salmonella* strain dependent mutagenicity of PR-toxin in the Ames test. Mutagenicity of PR-toxin was observed in the *Salmonella* tester stain TA97 but not in the TA1537 we applied (Levin et al., 1982). *B. nivea* metabolites patulin, mycophenolic acid and byssohclamic acid have not previously been associated with genotoxicity, which is supported by our negative results. It indicates that genotoxic metabolites are not produced by *B. nivea*. However, more extracts and different assays should be studied to confirm this.

Table A.1. Secondary fungal metabolites present in fungal YES extracts tested for genotoxicity. The concentrations (µg/mL cell medium) or presence (x) is reported for fungi isolated from silage now stored in IBT fungal collection.

Alternaria tenuissima	**IBT41188**
Altenuene	x
Alternariol	29
Alternariol monomethyl ether	29
Altersetin	x
Altertoxin I	x
Tenuazonic acid	49
Byssochlamys nivea	**IBT 28552**
Byssohclamic acid	x
Mycophenolic acid	7
Patulin	2.7
Undiscribed I, II, III and IV	x
Penicillium roqueforti	**IBT 28547**
Andrastin A	67
Andrastin B	x
Andrastin C	x
Andrastin D	x
Citreoisocoumarin	x
Eremofortin C	x
Mycophenolic acid	38
N6-Formyl-roquefortin-C	x
PR-toxin	x
Roquefortine A	x
Roquefortine C	1.6
1-hydroxyeremophil-7(11),9(10)-dien-8-one	x
(3S)-3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one	x

References - Appendix A

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APPENDIX B - SOP: CYTOTOXICITY TESTING - PROLIFERATION OF CACO-2 CELLS

SOP: Cytotoxicity testing - Proliferation of Caco-2 cells

SOP: Cytotoxicity testing - Proliferation of Caco-2 cells

Caco-2 cells maintenance

The human intestinal epithelial cell line (Caco-2) is originally received from Aalborg University in Denmark and is stored at –80 ºC. When in use cells are grown in the medium DMEM/F12 added 10% foetal calf serum, 1% penicillin-streptomycin (100 units/ml and 100 ug /ml, respectively), 1% MEM nonessential amino acids and 1% L-glutamin (2 mM) in 75-cm^2 culture flasks.

1. Seed $1 \cdot 10^6$ cells in a T-75 flasks in 30 mL growth medium.
2. Incubate at 37ºC in a humidified atmosphere of 5% CO_2, 95% air.
3. Change growth medium 2-3 days.
4. Split cells at 90% cell density (~weekly; see table 1 below) by trypsination using trypsin-EDTA·4Na (0.05% and 0.02%, respectively).

Help: Loosen cells from the flask by trypsination:

Remove the medium and rinse the flask with 2 times 4 mL PBS (Phosphate Buffer Solution).
Add 4 mL trypsin to the cells and place it 5 minutes in the incubator.
If the cells are still not detached then extent the time in the incubator and/or gently knock off the cells.
Add 11 mL medium to stop the trypsination.11

NB! It is important to have a suspension with single cells. To obtain this repeatedly passing the suspension in and out of a pipette tip can help.

Help: Counting cells by the NucleoCounter

Cell stock (100 µL) is mixed with 100 µL reagent A and vortexed. Then additional 100 uL reagent B is added to the cell suspension and vortexed.
Load the NucleoCassette with the pre-treated sample, place in NucleoCounter and press Run. The total cell count is given within ~30 seconds.

For details see:
Shah, D; Naciri, M; Clee, P; Al-Rubeai, M. (2006) *NucleoCounter-An efficient technique for the determination of cell number and viability in animal cell culture processes*, Cytotechnology 51:39–44.

NucleoCounter™ (New Brunswick Scientific Co., Inc., Edison, NJ)
NucleoCounter and NucleoCassette (New Brunswick Scientific Co., Inc., Edison, NJ, USA)

Day 1 Seeding of cells on 96-well plates

1. Use cells at ~90% confluency grown in T75 flasks (register the passage number).
2. Harvest the cells by trysination.
3. Determine the concentration of cells by counting the cells. Use a hemacytometer or as here the NucleoCounter.
4. Prepare a stock of $1 \cdot 10^5$ cells/ml by dilution with growth medium (stock A).
5. Seed 100 ul cell suspension on 96-well opaque plates with clear flat bottoms (figure 1) resulting in $1*10^4$ cells/well. NB! Cells can be seeded with a multi-channel pipette.
6. The outer wells of the plate are filled with 200 ul growth medium to minimize evaporation.
7. Incubate the cells at 37ºC in a humidified atmosphere of 5% CO_2, 95% air for 24 h.

Help: Calculation of the dilution of harvested cells to stock A

Concentration of the harvested cells = $C^{harvest}$ = unit [cells/ml]
Concentration of the dilution, stock A = $C^{stock} = 1 \cdot 10^5$ cells/ml
Volume of the harvested cells = $V^{harvest}$ = unit [ml]
Volume of medium V^{medium} = unit [ml]
Volume of stock A cellesuspension = $V^{total} = V^{harvest} + V^{medium}$
Dilution factor = $X = C^{harvest} / C^{stock\ A}$

Example:

If, V^{total} = 35 ml and $C^{harvest}$ = 15 cells/mL then the dilution factor
$X = C^{harvest} / C^{stock\ A} = 10 \cdot 10^5$ cells/ml / $1 \cdot 10^5$ cells/ml = 15.

The volumes of medium and harvested cells should be:

$V^{harvest} = V^{total} / X$ = 35 ml / 10 = 3.5 ml
$V^{medium} = V^{total} - V^{harvest}$ = 35 mL – 3.5 mL = 31.5 ml

Control:

Stock A = 3.5 mL * 10 10^5 cells/mL / 35 = $1 \cdot 10^5$ celler/ml

Day 2 Toxin exposure

1. Dissolve the test compounds in an appropriate carrier; here 100% methanol (MeOH) is used.
2. Prepare appropriate the dilutions of the test compounds. Use a sonic bath to ensure toxin solutions are dissolved, if necessary. Ensure the same concentration of the carrier in all solutions tested.
3. Add test solutions to the cells. Each toxin solution must be tested minimum in triplicates (figure 2). It is important, that the carrier is not applied in toxic concentrations; here MeOH constituted maximum 2% of the growth medium.

   Several methods are applicable:

   a. Add 4 uL toxin solution and 96 uL growth medium to the 100 uL growth medium already present.

b. Remove the medium by turning the plates up side down and add 200 uL of freshly prepared toxin-medium solution.
c. Remove the medium using a pipette and add 200 uL of freshly prepared toxin-medium solution.

Help: Experience with the different exposure methods

Method a) are good because cells are never exposed to air and less laborious. Serial dilutions with organic solvents often have extended duration compared to aqueous solutions. However method b) and c) may be easier because accurate addition of small volumes can give troubles. For experienced staff the standard derivations of the repeated measurement are approximately the same for all three methods.

4. Add the positive control, which is a compound with a cytotoxic effect, here 4 ug T-2 toxin /ml growth medium.

Help: Cells exposure to T-2 toxin at 4 ug/mL growth medium

T-2 toxin stock: 200 ug/ml MeOH

a)

Addition of 4 uL toxin stock (with 100% MeOH) to the wells
Simply add 4 uL of the T-2 toxin stock and 96 uL growth medium; 4 uL · 200 ug/ml / 200 mL in the well = 4 ug/ml. The well has 2% MeOH content as 4 ul / 200 ul · 100% = 2%.

or

b) and c)

Addition of 200 uL toxin solution (with 2% MeOH) to the wells
From the T-2 toxin stock (200 ug /ml MeOH) a solution of 4 ug/ml is prepared by mixing 1960 ul growth medium with 40 ul T-2 toxin stock. The dilution has 2% MeOH content as 40 ul / 2000 ul · 100% = 2%.

5. Add the solvent control (carrier), here MeOH. It should be added in the same concentration the test compounds.
6. After exposure the cells are again placed at 37ºC in a humidified atmosphere of 5% CO_2, 95% air.

Day 3 Incubation

1. No action.

Day 4 Indicator exposure and fluorescence measurement

1. Resazurin (0.1 mg/ml PBS) is diluted with growth medium just prior to use (2:3) (v:v). Mixing 4 mL resazurin (0.1 mg/ml) with 6 mL growth medium is sufficient for 4 plates. Protect resazurin solution from light with alu foil.
2. The wells (figure 3) are added 20 ul diluted resazurin solution. The use of a 300 uL multi-channel-pipette enables fast and accurate addition of the solution.
3. Plates wrapped in alu foil are incubated at 37ºC in a humidified atmosphere of 5% CO_2, 95% air for 5 hours.
4. The reduced form of resazurin is measured by fluorometry on a Victor2 Multilabel Counter (Wallac); Fluorescence wavelengths: λ_{ex} = 560 nm and λ_{em} 590 nm, slit 10. Temperature 37ºC. Use the programme Copy of resazurin (560 nm til 590 nm; 0.1 s, shake 2.0 s).
5. Use the fluorescence measurements generated in an excel file for each plate.
6. Check if the assay is behaving as expected: Is there an effect of toxin added in the positive control? Is the response of the solvent control in the usual range?
7. The proliferation can be calculated relative to the solvent control (figure 2, well 11E-11G). High cytotoxicity occurs for wells with low proliferation %. The maximal obtainable cytotoxic response can be calculated from the wells without cells added (figure 3, well 1B-1G, 12B-12G).
8. Evaluate also the standard derivation of the repetitions.
9. Finally remember that the experiment should be repeated on 2 separate days.

Table 1. Example of a week.

	T-75 flask*	**96-well plate****
Monday	Split cells in one flask cells and seed two new flasks	Seed cells
Tuesday	Change medium	Expose cells
Wednesday		
Thursday		Measure cells
Friday	Change medium	

*It is a good idea to have two flasks running parallel at the same time. If one flask unfortunately should be contaminated with microorganisms it is possible to continue with the other one.

** It is possible to run two experiments in one week if the cells from the second flask are seed on Tuesday, exposed Wednesday and measured Friday.

Figure 1, 2 and 3: Example of plate design for exposure of cells.

Figure 1 Seeding of cells on day 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf
B	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
C	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
D	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
E	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
F	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
G	Buf	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	**Cells**	Buf
H	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf

Buf = 200 uL growth medium
Cells = cell seeded in medium = $1*10^4$ cells/well

Figure 2 Example of design for exposure of cells on day 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf
B	Buf	**#1**	**#1**	**#1**	**#2**	**#2**	**#2**	**#3**	**#3**	**#3**	**T-2**	Buf
C	Buf	**#4**	**#4**	**#4**	**#5**	**#5**	**#5**	**#6**	**#6**	**#6**	**T-2**	Buf
D	Buf	**#7**	**#7**	**#7**	**#8**	**#8**	**#8**	**#9**	**#9**	**#9**	**T-2**	Buf
E	Buf	**#10**	**#10**	**#10**	**#11**	**#11**	**#11**	**#12**	**#12**	**#12**	**MeOH**	Buf
F	Buf	**#13**	**#13**	**#13**	**#14**	**#14**	**#14**	**#15**	**#15**	**#15**	**MeOH**	Buf
G	Buf	**#16**	**#16**	**#16**	**#17**	**#17**	**#17**	**#18**	**#18**	**#18**	**MeOH**	Buf
H	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf

#1-18 = Test solutions tested in tri-plicates, 2 %MeOH in the growth medium
T-2 = Positive control with 4 ug T-2 toxin /ml growth medium, 2% MeOH
MeOH = solvent control with 2 %MeOH in the growth medium

Figure 3 Resazurin additions of cells on day 4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf
B	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
C	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
D	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
E	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
F	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
G	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
H	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf	Buf

Res = 20 ul diluted resazurin dilution added.