MASTERARBEIT / MASTER'S THESIS

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„Development and validation of analytical methods for the determination of mycotoxins in plant based foodstuff with UHPLC-MS/MS"

verfasst von / submitted by

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Fröhlich erkannt und geschätzt, nennst das du weniger dein?

Johann Wolfgang von Goethe

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich diese Masterarbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die aus fremden Quellen direkt oder indirekt übernommenen Gedanken als solche kenntlich gemacht habe. Die Arbeit wurde bisher nicht veröffentlicht und ich erkläre mich damit einverstanden, dass die Arbeit mit Hilfe eines Plagiatserkennungsdienstes auf enthaltene Plagiate überprüft wird.

Wien, 2016

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List of Abbreviations

15-AcDON	15-acetyldeoxynivalenol
3-AcDON	3-acetyldeoxynivalenol
ACN	acetonitrile
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFLA	aflatoxins
AFM1	aflatoxin M1
AFP1	aflatoxin P1
AFQ1	aflatoxin Q1
AGES	Agentur für Gesundheit und Ernährungssicherheit
ALARA	as low as reasonable achievable
AME	alternariol-monomethylether
AOH	alternariol
AP	apurinic site
AUC	area under the curve
BBB	blood-brain barrier
BEA	beauvericin
CAV	collision cell accelerator voltage
CCK	cell counting kit
CE	collision enery
CHO	Chinese hamster ovary
CIT	citrinin
CPA	cyclopiazonic acid
CSA	chemical shift anisotropies
CSB	transcription-repair coupling factor
CX43	connexin 43

CYP	cytochrome p
DAS	diacetoxyscirpenol
DDB	DNA damage-binding protein
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DON-3-G	deoxynivalenol-3-glucoside
DSB	double-strand DNA break
EFSA	European Food Safety Authority
EMV	electron multiplier voltage
ENB	enniatin B
ERCC1	excision repair protein CC1
ESI	electrospray ionization
EXP	expiry
FAO	Food and Agriculture Organization
FAPY	formamidopyrimidine
FB1	fumonisin B1
FB2	fumonisin B2
FB3	fumonisin B3
FUM	fumonisins
FX	fusarenon X
GC	granulose cells
GGR	global genome repair
GLP	good laboratory practice
GPX2	glutathione peroxidase 2
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCK	hematopoietic cell kinase
HMGR	3-hydroxy-3-methyl-glutaryl reductase
HPLC	high-performance liquid chromatography
HT-2	HT-2-toxin

IAC	immunoaffinity column
IARC	International Agency for Research on Cancer
IC_{50}	inhibitory concentration, 50%
IFA	Interuniversitäres Department für Agrarbiotechnologie
IGF-I	insulin-like growth factor I
IL-8	interleukin 8
IP	intraperitoneal
IV	intravenous
LOD	limit of detection
LOQ	limit of quantification
LVA	Lebensmittelversuchsanstalt
MAPK	mitogen-activated protein kinase
MC2R	melanocortin 2 receptor
MMC	matrix matched calibration
ML	maximum level
MRM	multiple reaction monitoring
MS	mass spectrometry
MW	molecular weight
m/q	mass-to-charge ratio
NER	nucleotide excision repair
NIV	nivalenol
NR0	nuclear receptor subfamily 0
NRF2	nuclear factor (erythroid-derived)-like 2
NTD	neural tube defect
OTA	ochratoxin A
PAR	population attributable risk
PAT	patulin
PBCEC	primary porcine brain capillary endothelial cell
PKR	RNA-activated protein kinase
PTFE	polytetrafluorethylene membrane filter

RNA	ribonucleic acid
RNAPII	RNA polymerase II complex
ROS	reactive oxygen species
RPA	replication protein A
RR	recovery rate, or relative risk
RRHD	rapid resolution high definition
RSD	relative standard deviation
SCF	Scientific Committee on Food
SSB	single-strand DNA break
SST	sphingosine-sphinganin-transferase
STE	sterigmatocystin
T-2	T-2-toxin
TCR	transcription-coupled repair
TDI	tolerable daily intake
TFIIH	transcription factor IIH
TIC	total ion chromatogram
TNF-α	tumor necrosis factor alpha
TRI	trichodiene synthase
XP-group	xeroderma pigmentosum complementation group
ZON	zearalenone

Aim of the Thesis

The aim of the thesis was an expansion of an already implemented multi-confirmation method for the determination of mycotoxins in plant based foodstuff. First, the method was optimised and validated for the analysis in cereals and cereal products, nuts, pastries, pasta products and dried fruits.

This work is based on the existing routine multi-confirmation method “SM04” for mycotoxins of the Austrian Competence Centre of Food Safety (LVA GmbH) including the substances aflatoxin B1, B2, G1, G2, deoxynivalenol, fumonisin B1, B2, HT-2 toxin, ochratoxin A, T-2 toxin and zearalenone.

The project presents a scope extension of analytes as well as an extension of validated matrices. The following analytes are optimised and captured in the method: 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, alternariol, alternariol-monomethyl ether, beauvericin, citrinin, cyclopiazonic acid, diacetoxyscirpenol, enniatin B, fumonisin B3, fusarenon X and sterigmatocystin. Further patulin, aflatoxin M1, nivalenol and deoxynivalenol-3-glucoside were optimised but excluded from validation.

The validation was carried out for following matrices: wheat flour, maize, oat flakes, almonds, walnuts, sultanas, pastry, marble cake, and wholemeal bread. Further attempts were made with soy beans, red yeast rice, coffee, pepper, oat, rye, and spelt rice.

Due to the enhanced focus of different national (AGES) and international (EFSA) agencies on these substances and because of climate changes, resulting in an increased natural contamination of mycotoxins, the expansion of this screening method is an important challenge to ensure consumers health. The aim of this project was to achieve the limit of determination in alignment with the Commission Regulation 1881/2006/EC, combined with a fast, rugged and simple analytical method.

Introduction

Mycotoxins are secondary metabolites of moulds with low-molecular-weight and different negative mode of actions, e.g. mutagenic, carcinogenic, hepatotoxic, immunosuppressive, or estrogenic effects in mammals. [VARGA et al., 2012]

Primarily mycotoxins are produced by fungal genera like *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* genus. Concerning their chemical structure, they show a great diversity, resulting in a high variability of target organs and toxic impacts. For a mycotoxicosis, an involvement of the toxin-producing fungus is not required, therefore they are abiotic hazards with biotic origin. [MARIN et al., 2013] [MALACHOVÁ et al., 2014]

In general mycotoxin related health issues have increased over the years and therefore it was necessary to implement several regulations to control the maximum levels of these health hazard substances in food and feed. The Commission Regulation 1881/2006/EC from the European Commission includes maximum levels for specific mycotoxin-matrix combinations which are based on the evaluation of risk assessment with consideration of agriculturally achievable levels. [MALACHOVÁ et al., 2014]

According to an estimation by the Food and Agriculture Organization of the United Nations (FAO), about 25% of the cereals produced worldwide are contaminated with mycotoxins. Along the food chain of agricultural crops there are several spots where the production of mycotoxins can occur, e.g. during storage, drying, harvesting and pre-harvesting. Storage and transport conditions, handling, packaging, improper drying, poor agricultural and harvesting practices are therefore the most essential parameters of promoting fungal growth and thereby associated with an increased risk of mycotoxin production. [MARIN et al., 2013]

Classification

There are more than 31,000 different mould metabolites which are known so far and it is expected to find many more of these substances in future. For humans and animals just a small fraction of about 300-400 mycotoxins can be dangerous at naturally occurring concentrations. [BERTHILLER et al., 2007]

A selection of the most relevant groups of mycotoxins for this work is listed in *table 1*.

Table 1: Mycotoxins, mycotoxin metabolites and producing species

Mycotoxin	Acronym	Species producing
Aflatoxins	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1	*Aspergillus flavus, A. parasiticus, A. nomius*
Alternariol	AOH AME	*Alternaria alternata, A. solani*
Beauvericin	BEA	*Beauveria bassiana*
Citrinin	CIT	*Penicillium citrinum, P. verrucuosum, Monascus purpureus*
Cyclopiazonic acid	CPA	*Penicillium camemberti, P. cyclopium, P. griseofulvum*
Enniatin B	ENB	*Fusarium species*
Fumonisins	FB1 FB2 FB3	*Fusarium verticillioides, F. proliferatum*
Ochratoxin A	OTA	*Aspergillus section circumdati, A. nigri, Penicillium verrucosum, P. nordicum*
Patulin	PAT	*Penicillium expansum, Bysochlamis nívea, Aspergillus clavatus*
Sterigmatocystin	STE	*Aspergillus nidulans, A. versicolor*
Trichothecenes – type A	DAS T-2 HT-2	*Fusarium acuminatum, F. poae, F. sporotrichioides, F. langsethiae*
Trichothecenes – type B	DON DON-3-G 3-AcDON 15-AcDON NIV FX	*Fusarium graminearum, F. culmorum, F. cerealis, F. nivale*
Zearalenone	ZON	*Fusarium graminearum, F. culmorum, F. equiseti, F. cerealis, F. verticillioides*

The *Aspergillus*, *Penicillium* and *Fusarium* genera are the most important mycotoxigenic fungi which are involved in the human food chain. [SWEENEY and DOBSON, 1998]

Aspergillus

The growth of the fungal genus *Aspergillus* is toxicologically significant due to its ability to produce mycotoxins under exposure of proper conditions. This species infests living plants and stored food products which causes a food contamination all over the world. A specially increased risk is shown in the production of the hepatocarcinogenic and genotoxic aflatoxins. These polyketides are produced by *A. flavus* and *A. parasiticus* and are a high risk for consumer safety due to the extremely low tolerance levels. Further the *Aspergillus* species is responsible for the synthesis of ochratoxins, patulin and sterigmatocystin. [MOREIRA et al., 2013]

Penicillium

The *Penicillium* fungi include more than 100 different toxigenic species which positions it the biggest producer of mycotoxins compared to all other genera. Based on their toxicological effects and target systems, the *Penicillium* toxins can be divided into two groups: those affecting neurons and those affecting liver and kidney functions. The four most important mycotoxins produced by *Penicillium* species are ochratoxin, mainly produced by *P. verrucosum*, as well as citrinin, cyclopiazonic acid and patulin. [SWEENEY and DOBSON, 1998]

Fusarium

There are a large number of different toxin producing *Fusarium* moulds. The main compounds hereby is the group of trichothecenes like deoxynivalenol and its metabolites 3- and 15-acetyldeoxynivalenol as well as deoxynivalenol-3-glucoside, a masked mycotoxin derivate. Furthermore diacetoxyscirpenol, T-2 toxin, HT-2 toxin, nivalenol, fusarenon-x, zearalenone, fumonisin B1, B2, B3 and enniatin species are produced by *Fusarium* genera. [SKRBIC et al., 2011]

Occurrence of mycotoxins

The contamination by mycotoxins can occur in nearly all feed and feed raw materials through an infestation with different moulds, producing these toxic substances as secondary metabolites. This fungal contamination generally occurs during storage or directly on the field. The main influential factors hereby are environmental and improper deposit conditions. [STREIT et al., 2013]

Global Occurrence

For the BIOMIN mycotoxin survey program 2011, over 4,300 samples were collected and in total 13,854 analyses were conducted to determine the occurrence of aflatoxins, zearalenone, deoxynivalenol, fumonisins and ochratoxin A in different regions all over the world. The tested samples were raw materials like corn (33%), wheat (9%), barley (7%) and soybeans (5%) as well as finished feed (25%), silage (8%) and other feed ingredients (13%). A graphical representation of the worldwide mycotoxin contamination based on this survey is shown in *figure 1.*

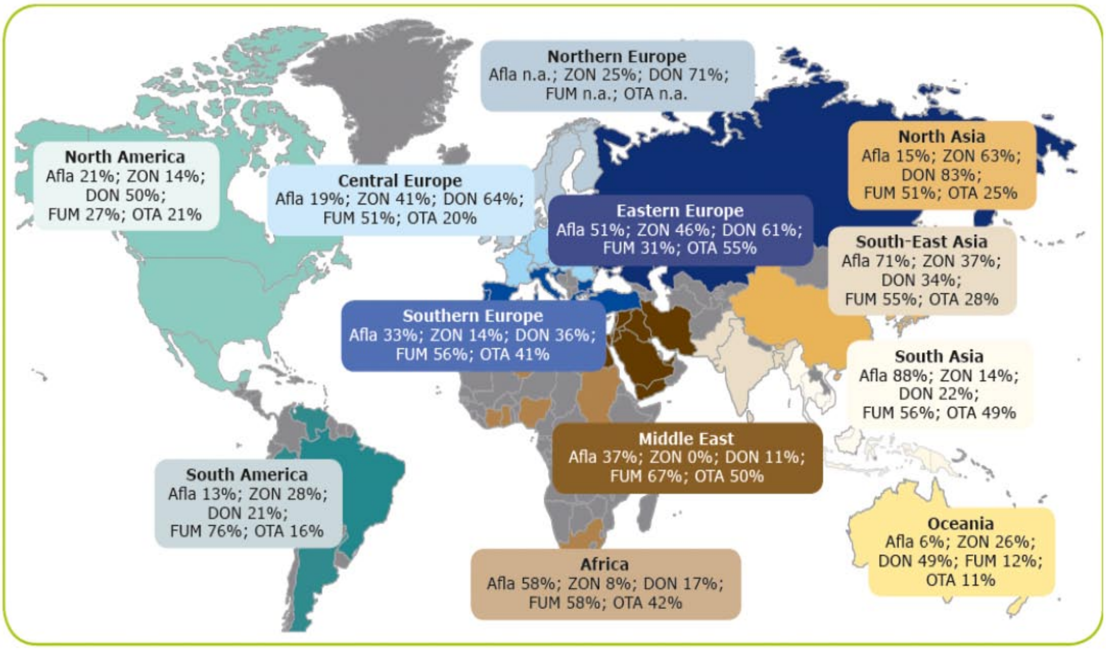


Figure 1: Mycotoxin contamination worldwide [NAEHRER 2012]

The most prevalent mycotoxins in North Asia are produced by *Fusarium* fungi like DON (83%), ZON (63%) and FUM (51%) with average amounts of 782 µg/kg, 164 µg/kg and 1,068 µg/kg, respectively. In comparison, aflatoxins (71%) are the most prevalent toxins in South-East-Asia with average contamination levels of 42 µg/kg. About a half of the analysed samples were positively tested for DON in North America with average contamination levels of 459 µg/kg. The fumonisins (76%) are the most common mycotoxins in Southern America with average amounts of 1,501 µg/kg. The field mycotoxins DON (49%) and ZON (26%) present mean contamination levels of 200 µg/kg and 100 µg/kg in Oceania. Samples were tested positive in Northern Europe for DON (71%) and ZON (25%) with levels up to 885 µg/kg and 29 µg/kg. The biggest concern in Central Europe are showed from *Fusarium* mycotoxins like DON (64%), FUM (51%) and ZON (41%) with average contamination levels of 729 µg/kg, 241 µg/kg and 49 µg/kg, whereas FUM (56%), OTA (41%) and aflatoxins (33%) occur more often in Southern Europe with average levels of 807 µg/kg, 2 µg/kg and 1 µg/kg average contamination levels. For Eastern Europe the toxins DON (61%), OTA (55%) and ZON (46%) are the most prevalent with average amounts of 189 µg/kg, 3 µg/kg and 114 µg/kg. Finally, in Africa the fumonisins (58%) and aflatoxins (58%) are the most frequent toxins with average contamination levels of 457 µg/kg and 59 µg/kg. [NAEHRER 2012]

Occurrence in Austria

Between January 2009 and July 2016, the LVA GmbH tested 1,357 mycotoxin samples from different food manufacturers and agricultural economists. These analyses were conducted with a multimycotoxin confirmation-method including DON, AFLA (B1, B2, G1, G2), FUM (B1, B2), HT-2 toxin, T-2 toxin, ZON and OTA, resulting in a total number of 14,927 analyses. The tested sample material included grains (40%) like maize, milled products (25%) like wheat flour, cereals (18%) like muesli, pastries (11%) like croissants, edible nuts (4%) like almonds and other foods (2%). The mycotoxin contamination of foodstuff on the Austrian market is shown in *figure 2.*

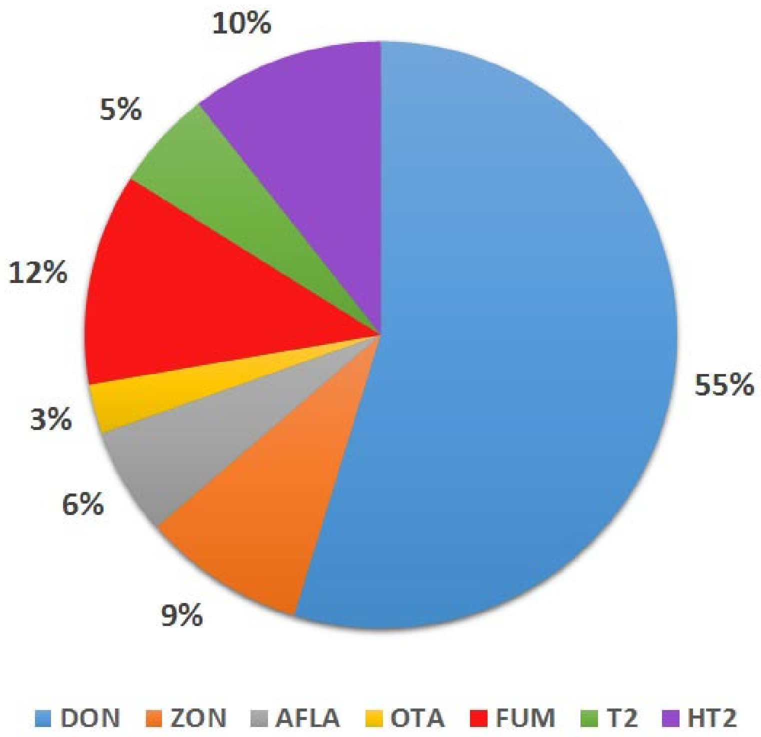


Figure 2: Mycotoxin contamination in Austria [LVA 2016]

More than a half of the sample material was positively tested on DON (55%) with average contamination levels of 321 µg/kg. This analyte is followed by FUM (12%), HT-2 toxin (10%) and ZON (9%) with average levels of 300 µg/kg, 41 µg/kg and 182 µg/kg. A minor occurrence is shown by AFLA (6%), T2 toxin (5%) and OTA (3%). The average measured concentrations are hereby at 0.8 µg/kg, 35 µg/kg and 4.6 µg/kg. A complete list with average measured levels of mycotoxins per year is shown in *table 2.*

Table 2: Mycotoxin contamination in Austria per year in µg/kg [LVA 2016]

Year	AFLA	DON	FUM	HT2	OTA	T2	ZON
2009	0.41	81.53	42.95	23.95	-	15.55	45.30
2010	0.23	238.58	79.90	147.15	5.77	44.94	202.18
2011	-	99.96	122.65	24.06	4.64	23.30	60.20
2012	0.20	82.41	109.15	42.24	-	68.63	10.68
2013	0.72	177.42	601.17	5.36	5.52	2.88	163.91
2014	0.96	649.94	623.86	47.95	3.63	59.56	649.53
2015	0.90	870.48	547.81	24.66	2.50	30.40	187.90
2016	2.39	369.11	275.93	17.94	5.88	-	137.00
Mean	**0.83**	**321.18**	**300.43**	**41.66**	**4.66**	**35.04**	**182.09**

Risk Characterization

Aflatoxins

The International Agency for Research on Cancer (IARC) rates aflatoxins as group I carcinogens. This means that these substances are showing a high carcinogenic potential against humans in very low concentrations. A consumption of aflatoxin B1 (AFB1) contaminated food leads to a metabolisation by cytochrome P450 in the liver, resulting in an AFB_1-8,9-epoxide intermediate. This epoxide can spontaneously build adducts in the DNA with guanine bases to the primary adduct AFB_1-N^7-guanine. [TAGUCHI et al., 2016]

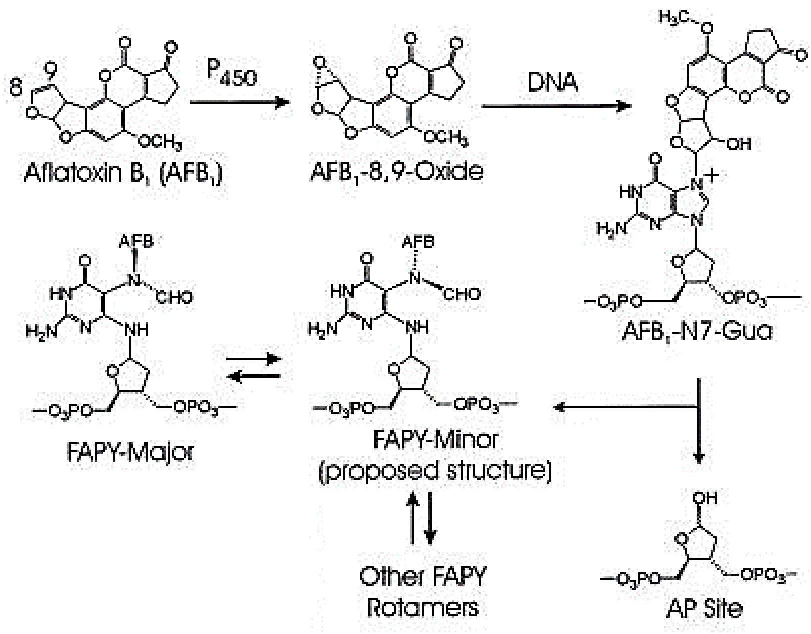


Figure 3: Synthesis of AFB1 adducts [SMELA et al., 2002]

A break-down of this adduct can form two secondary lesions, the ring-opened AFB_1-formamidopyrimidine (AFB_1-FAPY) adduct and the apurinic sites. There are two rotameric forms of the FAPY adduct itself, the FAPY major and minor. [SMELA et al., 2002]

These FAPY-adducts can further cause dangerous DNA mutations resulting in the formation of cancer. The risk for cervical cancer for instance, is six-fold higher (OR) 6.1 [95% CI = 1.4 – 25.4] with the presence of AFB_1-FAPY (1,025 pg adducts/mg DNA) compared to the control group (≤ 2.6 pg/mg DNA) in a nested case-control study (P = 0.00006). [CARVAJAL et al., 2016]

Beside cervical cancer, aflatoxins, especially AFB_1 are highly associated with the pathogenesis of hepatocellular carcinoma (HCC). In a follow-up cohort study conducted in China, 18,244 middle-aged (45-64 years) male subjects were recruited. The aim of this study was to figure out a relationship between aflatoxin exposure and liver cancer in four small geographically defined areas of Shanghai. The presence of aflatoxins was measured via urine biomarkers of AFB_1-N^7-guanine, AFB1, AFP1, AFM1, AFQ1 and AFG1. Additionally, a quantitative estimation of Shanghai market foods was performed to determine the aflatoxin exposure for the study population. After a follow-up period of 70,000 person-years, 55 cases of HCC were reported. In 50 of these cases, high levels of urinary AFB_1-N^7-guanine and AFB1 were detected and showed a significant association between the attendance of aflatoxins and the risk of HCC (RR = 59.4; 95 % CI [16.6, 212.0] after an adjustment for cigarette smoking as a confounder. [QIAN et al., 1994]

Results from a systematic review and meta-analysis including 17 studies (8 case-control studies, 8 nested case-control studies, 1 cohort study) are demonstrating a population attributable risk (PAR) of aflatoxin related HCC of 23 %. The HCC risk is higher in populations with hepatitis B (HBV). OR of HCC with 95 % CI is 73.0 [36.0 – 148.3] for combined effects of HBV and aflatoxin, from aflatoxin only 6.37 [3.74 – 10.86] and from HBV only 11.3 [6.75 – 18.9]. [LIU et al., 2012]

Analysis of the relationship between aflatoxin exposure and anthropometric status in 480 children (9 months to 5 years) in Benin and Togo detected aflatoxin-albumin adducts in 475 samples with average concentrations of 32.8 pg/mg. A continuous rise of the aflatoxin-albumin level with age up to 3 years was observed. The average level of breast fed children up to 3 years was 18.0 pg/mg; 95 % CI [15.2 – 21.3], in comparison, the mean concentration for fully weaned children was 45.6 pg/mg; 95 % CI [38.8 – 53.7] which represents a 2.5 fold higher value. A multivariable adjustment for sex, age, weaning status, socioeconomic status and agroecological zone showed a significant association with aflatoxin-albumin levels (P = 0.0001). [GONG ete al., 2002]

Therefore the removal of AFB_1-DNA damage is important to sustain a healthy mammalian complex. This self-regeneration system is called nucleotide excision repair and can be divided into the global genome repair (GGR) and the transcription-coupled repair (TCR). The difference between these subpathways is based on the mechanism of damage recognition. A screening for DNA lesions of the entire genome is made by GRR, while TCR deals more specifically with lesions that arrest RNA polymerase.

XPC-HR23B and DDB as a part of the XPE complementation group are GRR-specific elements and are regularly screening the genome for damage in mammals. The activity of TCR however is triggered by an elongation block of the RNA polymerase II complex (RNAPII). CSA and CSB are relocating the stalled RNA polymerase which makes the defect repairable. The transcription factor TFIIH opens about 30 basepairs of DNA around the damage via its helicase subunits XPB and XPD. The single-stranded binding protein RPA (replication protein A) is stabilizing the opened DNA, followed by the cleavage of the damaged strand conducted by the endonucleases ERCC1/XPF and XPG at the 3’ and 5’ borders. Finally the DNA polymerase (δ and ε) and ligase are completing the repair by filling the gap. [BEDARD and MASSEY, 2006]

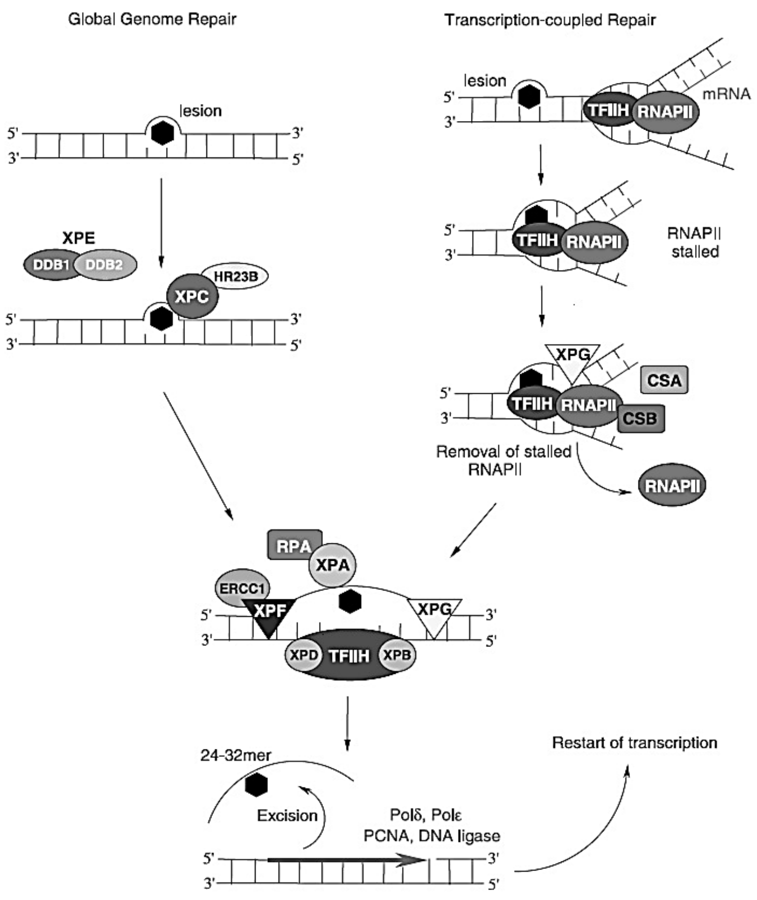


Figure 4: Mammalian nucleotide excision repair [BEDARD and MASSEY, 2006]

Fumonisins

The main representative part of these compounds is fumonisin B1 (FB1), usually occurring in cereals like wheat and especially maize. Concerning carcinogenity, there is a possible carcinogenic potential shown by all fumonisins in humans, resulting in a 2B rating from the IARC. Unfortunately, there are no human data available regarding to toxicokinetic processes. When given orally, the absorption of FB1 is poor, less than 6 % followed by a fast elimination by biliary excretion in animals like hen, cow, swine, rat and non-human primates. [SCF 2000]

Studies with Wistar rats have shown a very fast T_{max} of 1.02 h, but also a very poor absorption rate of 3.5 % after a single orally administration of 10 mg FB1/kg bw. [VOSS et al., 2007]

The absorption follows a small accumulation of these toxins in the liver and kidneys representing their primary target organs. After a fumonisin containing diet in rats after several weeks, the accumulated levels of fumonisins in kidneys were about 10 times higher than in liver. [RILEY and VOSS, 2006]

Unlike aflatoxins, ochratoxin A, citrinin, zearalenone and T-2 toxin, there is no significant permeation through the human skin of FB1 and therefore a systematic health risk after a dermal exposure of this substance seems to be safe for humans. [BOONEN et al., 2012]

The initial elimination of FB1 in rats is fast since $T_{1/2}$ is about 10-20 minutes after an intraperitoneal (ip) or intravenous (iv) administration. In a one or two compartment rat model, the elimination kinetics is consistent in accordance with an ip or iv administration of FB1. An isotopic labelled FB1 ip administration in rats resulted in a 66 % of the radioactivity in faeces and 33 % in urine. [SCF, 2000]

Fumonisins are competitive inhibitors of sphingolipid biosynthesis and metabolism. Due to their analogy, an inhibition of sphingosine-sphinganin-transferase (SST) and ceramide synthases is possible through these substances.

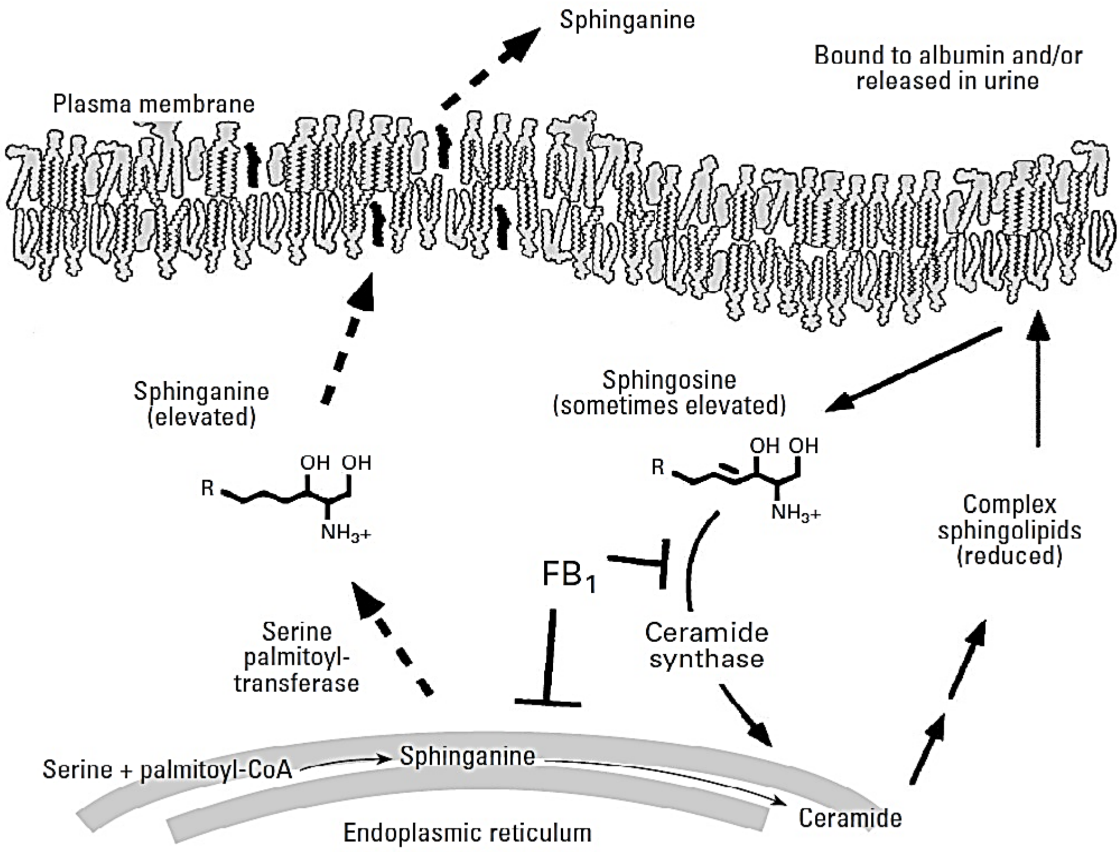


Figure 5: Inhibition of the ceramide synthase and SST by FB1 [MERRILL et al., 2001]

A schematic overview about the fumonisin mode of action is shown in *figure 5*. The inhibition of the ceramide synthase, which acylates sphingoid bases blocks the ceramide formation via two pathways. First, through the inhibition of de novo sphinganine and fatty acyl-CoA. And second, via the inhibition of the enzyme ceramidase, resulting in low ceramide concentrations. This restraint leads to an accumulation of sphinganine, sphingosine, sphinganine-1-phosphate metabolite and decreased levels of the sphingolipid complex. The increased concentration of these substances is the key reason of the FB1 toxicity. The cytotoxic sphinganine and sphingosine especially cause growth inhibitory effects. Further the imbalance of these intracellular compounds can cause an increased apoptosis which seems to be a key factor of tumor induction. [MERRILL et al., 2001]

Because of the disruption of the sphingolipid metabolism, FB1 could affect folate uptake and cause neural tube defects (NTD). Between 1990 and 1991, an exceptional high number of NTDs occurred along the Texas-Mexico border. This outbreak could have been associated with high concentrations of FB1 in corn during previous years in this region. Further, regions in South Africa and China showed similarities between high intake levels of corn and the prevalence of NTDs. [STOCKMANN-JUVALA and SAVOLAINEN, 2008]

There is a possible relationship between human esophageal cancer and the occurrence of *Fusarium verticillioides*. High levels of this mycotoxin producing mold and its secondary metabolites FB1 and FB2 are present in corn, especially in regions with a high prevalence of esophageal cancer. This allows the conclusion that high corn consumer in these regions are at higher risk to develop esophageal cancer than low corn consumer. [WILD and GONG, 2009]

In 1995, 27 villages in India were affected by a disease outbreak with symptoms like abdominal pain and diarrhea. Because of rain damage, people in this region consumed high amounts of moldy sorghum and corn, resulting in a high number of mycotoxicosis. Samples from corn and sorghum were collected and compared with unaffected households. The analysed samples showed a contamination by Fusarium and contained high concentrations of FB1. [STOCKMANN-JUVALA and SAVOLAINEN, 2008]

A risk evaluation of fumonisins was made by the Scientific Committee on Food (SCF) of the European Commission and they defined a tolerable daily intake (TDI) for FB1, FB2 and FB3 in combination or alone of 2 µg/kg bw. [SCF, 2003]

The polysaccharide glucomannan which can be extracted from the yeast *Saccharomyces cerevisiae* is able to bind mycotoxins. A treatment of fumonisin contaminated corn with glucomannan reduces the bioavailability of FB1 with a binding capacity of 67 %. [YIANNIKOURIS and JOUANY, 2002]

Type A Trichothecenes

From the family of type A trichothecenes, T-2 toxin is the most acutely toxic member and HT-2 toxin its major metabolite. Known symptoms caused by T-2 toxin are apoptosis, lethargy, diarrhea, emesis, hemorrhage, inhibition of immunity, weight loss, necrosis and death. T-2 toxin is able to bind the enzyme peptidyltransferase which is a part of 60s ribosomal subunits, resulting in an inhibition of protein synthesis. Animal studies with mice have shown apoptotic effects of T-2 toxin in the Peyer's patches, in the mesenteric lymph nodes and the thymus. The severity of lymphocyte apoptosis depends on the lymphoid tissue. [LI et al., 2011]

Further T-2 toxin and HT-2 toxin have a potential influence on the release of steroid hormone progesterone (P_4). An incubation of porcine ovarian granulose cells (GCs) with a combination of T-2 toxin (at 100 ng/ml), HT-2 toxin (at 100 ng/ml) and insulin-like growth factor-I (IGF-I) (at 1.10 and 100 ng/ml) inhibits the progesterone secretion significantly ($P < 0.05$). Whereas an incubation with (1,000 ng/ml) T-2/HT-2 toxin with IGF-I (at 1, 10 and 100 ng/ml) significantly ($P < 0.05$) stimulates the P_4 release by GCs. Results of this *in vitro* study allow the conclusion that these substances may have a major impact at the progesterone secretion and are maybe participated in the regulation process of steroidogenesis. [MARUNIAKOVA et al., 2014]

An assessment for the cytotoxic effects of T-2 and HT-2 toxin was performed on primary porcine brain capillary endothelial cells (PBCEC) as a blood-brain barrier (BBB) representative via a CCK- 8 assay. Results after an application of 1 nM – 10 µM with both mycotoxins for 24 h and 48 h are demonstrated in *figure 6.*

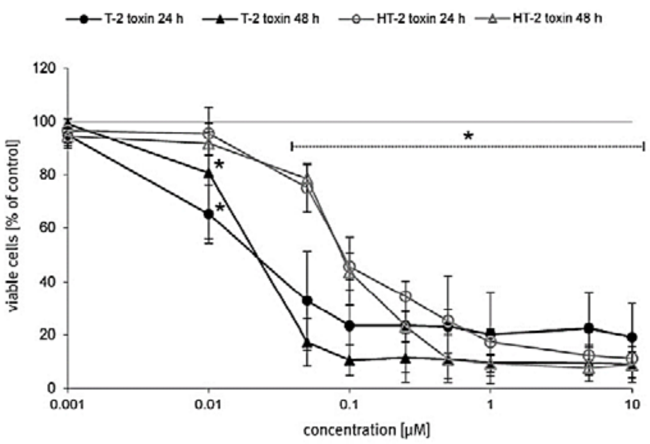


Figure 6: Effects of T2/HT2 toxin on viability PBCEC [WEIDNER et al., 2013]

After an incubation of 10 nM T-2 toxin for 24 h the cell viability dropped significantly ($P \leq 0.05$) to 65 %. The same incubation of HT-2 toxin reduced cell viability only for 4 %, without a statistically significance. An application between 50 nM and 10 µM of both substances showed the most significant ($P \leq 0.05$) reduction of cell viability compared with control cells. Results from the longer incubation period of 48 h were similar compared with the 24 h incubation. [WEIDNER et al., 2013]

The *Fusarium* toxin diacetoxyscirpenol (DAS) is also known as anguidine and responsible for mycotoxicosis in livestock. Several LD_{50} values are described in different animal toxicity studies. The intraperitoneal (ip) administration of DAS in Swiss mice lead to an LD_{50} value of 15.3 mg/kg bw, resulting in radiomimetic cellular injury and karyorrhexis in the small intestine. An orally administration of DAS in broiler chicken leads to an LD_{50} of 3.82 mg/kg bw. Observed symptoms were diarrhea, inappetance, asthenia, coma, skin lesions, necrosis in liver, gall, bladder and gut as well as decreased body weight gain and decreased feed consumption. The intravenous administration of DAS in swine lead to an LD_{50} of 0.376 mg/kg bw. For 18 h the animals showed symptoms like lethargy, emesis, posterior paresis, frequent defaecation, prostration and staggering gait until they died. In rats, the lowest LD_{50} values resulted by an ip administration and were at 0.75 mg/kg bw. In contrast, the LD_{50} value orally administered was at 7.3 mg/kg bw and intravenously administered at 1.3 mg /kg bw. For dogs and cattle, the LD_{50} level is at 0.5 mg/kg bw with effects on bone marrow and haematology.
Concerning genotoxic potential of DAS resulting from *in vitro* studies did not show an induction of sister chromatid exchanges in human lymphocytes. An ip administration of 0.5 – 1 mg/kg bw in Swiss mice leads to an increase in chromosomal abnormalities in germ cells and somatic cells. Further a reduction in mitotic activity in bone marrow was described. In germ cells, the structural abnormalities contained X-Y univalents and breaks and in bone marrow endomitosis, breaks and centromeric attenuation. Additionally, DAS showed teratogenic potential in mice when given oral doses of 1, 2, 3 or 4 mg/kg bw on gestation days 9 to 11. [PRONK et al., 2002]

Type B Trichothecenes

In principle, mycotoxins from the trichothecene family are sesquiterpene epoxide metabolites of the fungus *Fusarium,* which are able to inhibit protein synthesis in eukaryotes. The common nature of this large substance group of mycotoxins is a basic 12,13-epoxytrichothecene structure with differences in their substitution. These structural patterns are depending on the phylogenetic fungi strains and affect the cytotoxic potential. The biosynthetic pathway of the main type B trichothecenes is shown in *figure 7.*

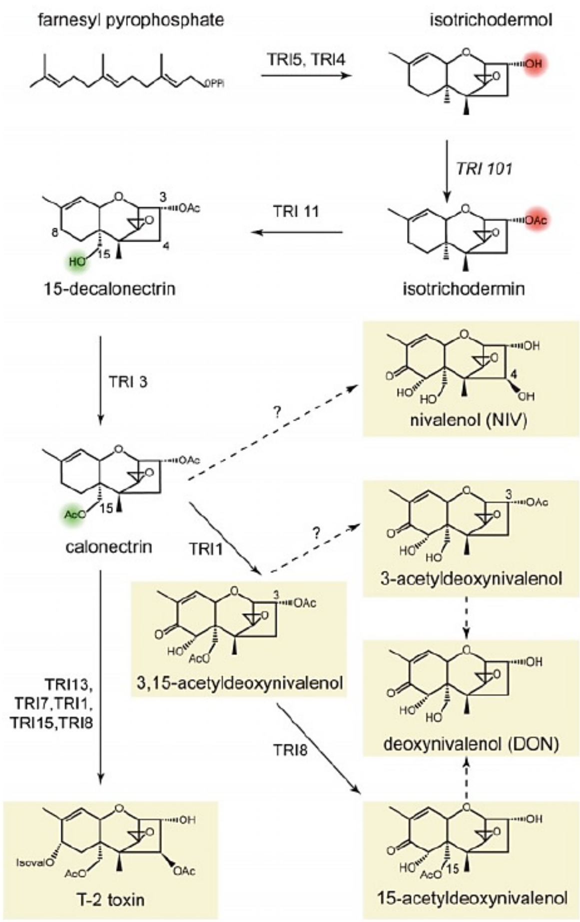


Figure 7: Biosynthetic pathway of trichothecenes [GARVEY et al., 2009]

The biosynthetic pathway starts with the formation of the core trichothecene ring through the cyclization of farnesyl pyrophosphate by the synthases Tri5 and Tri4. The acetylation of the C3 hydroxyl group through Tri101 is a selfprotection step by the fungus which reduces the toxicity of the mycotoxin by a factor of 100. The toxicological potential is unfolded through further modifications on the C4 and C15 positions by the CYP P450 monoxygenase and acetyltransferase 0 pairs Tri13/Tri7 and Tri11/Tri3. Tri1 induces an oxygenation on C8, followed by a further modification by Tri16. Finally the protecting acetyl group at C3 gets removed by Tri8. The classification of trichothecenes is often made by the C8 substitution. Type A trichothecenes, like T-2 toxin, carry an ester side chain, whereas type B trichothecenes like deoxynivalenol possess a ketone group. [GARVEY et al., 2009]

The toxicological potential of deoxynivalenol (DON) is less, compared to T-2 toxin, but very high doses (unlikely through food intake) can lead to shock-like death. Intraperitoneal administration of DON in mice leads to LD_{50} values from 49 to 70 mg/kg bw. In contrast, orally administered, the values ranged from 46 to 78 mg/kg bw. Additionally to the toxicological potential from DON itself, the relative toxicity from its major precursors 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) has become an important health issue because of their simultaneous occurrence with DON in cereal grains. The reported LD_{50} value for 3-ADON in mice after an intraperitoneal injection is 54 mg/kg bw and for 15-ADON 113 mg/kg bw. Typical clinical signs after a dietary exposure of DON in animal studies are anorexia, decreased weight gain and altered nutritional efficiency. The biggest concern in context with DON exposure and its metabolites is shown with the potential to induce apoptosis. This process is also known as the ribotoxic stress response induced by a ribosomal binding of trichothecenes which activates the mitogen-activated protein kinases (MAPKs). This molecular mechanism of DON is shown in *figure 8*.

After entering the cell, DON binds to activated ribosomes followed by a signal transduction to hematopoietic cell kinase (HCK) and RNA-activated protein kinase (PKR). The resulting phosphorylation of MAPKs induces apoptosis and activates transcription factors (TFs) resulting in chronic and immunotoxic effects. [PESTKA, 2007]

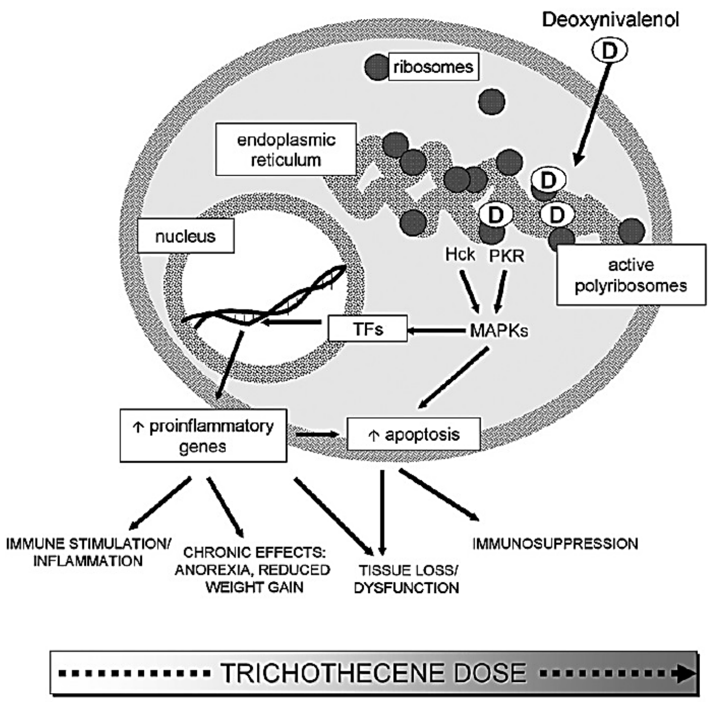


Figure 8: Toxicological mechanism of deoxynivalenol [PESTKA, 2007]

A comparison of the toxicity of deoxynivalenol and nivalenol on K562 human erythroleukemia cell line basis analysed the influence of these mycotoxins on cell viability, cell metabolism, cell proliferation and cell cycle. Concerning cell viability, a non significant decrease of 80 % after concentrations of 80 µM nivalenol and 84 µM deoxynivalenol were observed. The inhibition of cell metabolism was about four times higher through nivalenol than deoxynivalenol. Furthermore, both toxins inhibit cell proliferation with no significant difference from each other. The total cytotoxic potential of 100 % was reached after 84 µM nivalenol and 80 µM deoxynivalenol. No treatment-related alterations on cell cycle phases G_0, G_1, S, G_2 and M were observed. A result of this trial indicates that nivalenol and deoxynivalenol have major impacts on blood cells with a higher observed toxic potential by nivalenol. The cytotoxic effects are plasma membrane damage, apoptosis, necrosis and DNA damage. [MINERVINI et al., 2004]

A toxicokinetic investigation of nivalenol and its derivate 4-acetyl nivalenol (fusarenon-X) in mice was conducted to gain a better understanding of the excretion way of these mycotoxins. The five week old mice were treated orally with ^{3}H-NIV (20 µg/kg bw) and ^{3}H-FX (18 µg/kg bw). A collection of urine and feces samples was made 48 h after administration. Additionally, before and 10, 20 and 30 minutes and 1, 2, 4, 8, 12, 24 and 48 h after treatment, blood samples were taken via heart puncture as well as bile samples from the gall. The excretion of nivalenol was generally made via feces, whereas fusarenon-X was mainly excreted via urine. Fusarenon-X reached plasma peak after 30 minutes, while nivalenol reached plasma peak after 60 minutes. Furthermore a 10 times higher area under the curve (AUC) and a 5 times higher plasma peak level was observed for fusarenon-X, resulting in the assumption that the absorption of fusarenon-X via gastrointestinal tract is more efficient compared to nivalenol. Also a faster metabolization of fusarenon-X was investigated through the HPLC profile of urine and feces samples. The high oral toxicity of fusarenon-X is thus related to the fast absorption, followed by a conversion of fusarenon-X to nivalenol via liver and kidneys. [POAPOLATHEP et al., 2004]

Other Mycotoxins

The toxicological characterization of zearalenone (ZON) is based on its potential to induce oxidative stress by reducing the expression of junction proteins connexin43 (Cx43), occludin and claudin-4. Additionally, ZON decreases the expression of cytokines like interleukin-8 (IL-8), but increases the expression of gastrointestinal glutathione peroxidase (GPx2). Furthermore the Nrf2 expression is up-regulated in mRNA and protein levels via ZON. This mode of action suggests that the toxicological mechanism of ZON is made by the modulation of Nrf2 pathway resulting in an influence on inflammatory response. [LIU et al., 2014]

Monascus, *Aspergillus* and *Penicillium* are the major fungi producing the food contaminant citrinin. This mycotoxin is associated with a nephrotoxic potential with different pathways like mitochondrial dysfunction, an induction of apoptotic cell death or lipid peroxidation. Through an intensified production of micronuclei, citrinin is further responsible for genotoxic effects. The major toxic impacts are related to the enhanced formation of ROS. [PASCUAL-AHUIR et al., 2014]

Knowledge about the toxicological mechanism of alternariol (AOH) is generally based on *in vitro* and very limited *in vivo* trials. Similar to citrinin, AOH promotes the production of ROS and is able to interact with DNA topoisomerase, resulting in single (SSB) and double-strand DNA breaks (DSB). Via arresting the G_2/M-phase of the cell cycle, it also affects cell proliferation in mammalian cells. Additionally AOH enhances autophagic activity in macrophages and induces senescence, resulting in a decreased immune response to infections. [SOLHAUG et al., 2016]

Information about toxicological pathways of alternariol methyl ether (AME) is even more limited compared to alternariol. AME is associated with a cancerogenic and mutagenic potential especially with oesophageal cancer. Furthermore, damage of liver and kidneys were observed in rats feeded by *Alternaria alternate* fungi. So far no results are available concerning toxicological endpoints of AME. [OSTRY, 2008]

In rodents ochratoxin A (OTA) is associated with a renal carcinogenity. Toxic impacts to humans caused by OTA are not completely discovered so far. A microarray study in rats showed a significant reduction of Nrf2 gene expression at mRNA level in kidneys. This reduction leads to an oxidative DNA damage by an enhanced production of abasic sites confirmed by *in vitro* and *in vivo* studies. This reduced defense against oxidative stress could be a possible mechanism of its nephrotoxic and carcinogenic potential. [CAVIN et al., 2007]

Enniatin B (ENB) is a *Fusarium* mycotoxin known for an endocrine interfering activity. Investigations concerning gene transcription showed a significant influence of ENB on a various number of genes apparent through a downregulation of CYP11A, HMGR and CYP17 and an upregulation of MC2R, CYP19 and NR0B1. This gene regulation proposes that the main hazard potential of ENB is based on the endocrine toxicity. [KALAYOU et al., 2015]

In food and feed commodities, the natural co-occurrence of sterigmatocystin (STE), beauvericin (BEA) and patulin (PAT) has been verified. An investigation of the individual and combined cytotoxic effects of these mycotoxins was made on immortalized ovarian cells (CHO-K1). The half maximal inhibitory concentration (IC_{50}) values for PAT were 2.9 µM (10.7 to 2.2 µM) and for BEA and STE ranged from 25.0 to 12.5 µM after 24, 48 and 72 h. For a quantitative measurement and the creation of an interaction degree of these toxins, the isobolgram method was used. A dose dependent effect was shown in binary and tertiary combinations. Synergetic effects were shown at low fraction, while additive effects were observed at high fraction. The co-occurrence of small amounts of these three mycotoxins could enhance the cytotoxic impacts in food. [ZOUAOUI et al., 2016]

In vitro studies concerning immunotoxicity and cytotoxicity of cyclopiazonic acid (CPA) on human cells show an influence of this toxin on the activation of macrophages, resulting in a higher TNF-α secretion. [HYMERY et al., 2014]

Due to a wide toxicological potential of mycotoxins, it is essential, in order to protect public health, to keep these contaminants at levels which are toxicologically acceptable. Therefore in December 2006 the Commission of the European Communities drafted a new order of contaminants, the Regulation (EC) 1881/2006, to replace at this point in time current maximum levels. Because of the different laws of Member States and the resulting risk of distortion of competition, for some contaminants joint actions were provided to ensure market unity in consideration of proportionality. The maximum levels have to be set at reasonably achievable levels having regard to good agricultural and manufacturing practices as well as the risk related to the consumption of the food. For substances with a genotoxic potential, the maximum level has to be set by the ALARA (as low as reasonable achievable) principle. Currently 15 different mycotoxins are regulated with maximum levels; an overview is shown in *table 3*. [EUROPEAN COMMISSION, 2008]

Table 3: Maximum levles for certain contaminants in foodstuff and animal feed [EUROPEAN COMMISSION, 2008]

Toxin	Maximum levels (µg/kg)	Source
Foodstuff		
Aflatoxins		
Aflatoxin B1	0.1 – 12	groundnuts, nuts, dried fruits, cereals, spices
Sum of B1, B2, G1, G2	4 – 15	
Aflatoxin M1	0.025 – 0.050	
Citrinin	2,000	red yeast rice supplements
Deoxynivalenol	200 – 1,750	cereals, cereal products, pasta
Ergot sclerotia	500,000	cereals
Ochratoxin A	0.5 – 80	cereals, wine, coffee, juice, dried vine fruits
Patulin	10 – 50	juice, apple products
Sum of fumonisins B1, B2	200 – 4,000	maize
Sum of T-2 + HT-2	15 – 2,000	cereals and cereal products
Zearalenone	20 – 400	cereals and cereal products

Materials and Methods

Reagents

Chemicals

- **2-Propanol** – Emsure® ($CH_3CH(OH)CH_3$); Product code: 1.09634.1000; Lot: K47724234617; Exp.: 03/2021; Merck Millipore (Darmstadt, Germany)
- **Acetone** min. 99,70 % (C_3H_6O) – Product code: 83656.320; UN Nr.: 1090; Exp.: 04/2019; VWR Chemical (Fontenay-sous-Bois, France)
- **Acetonitrile** for HPLC – super gradient (H_3CCN) – Product code: 83639.320; Lot: 16F241231; Exp.: 06/2016; VWR Chemical (Fontenay-sous-Bois, France)
- **Ammonium formate** for HPLC ≥ 99.0% (HCO_2NH_4) – Product code: 17843-250G; Lot: BCBP5469V; Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Formic acid** 99-100 % (CH_2O_2) – Product code: 20318.297; Lot: 15L220510; Exp.: 12/2020; VWR Chemical (Fontenay-sous-Bois, France)
- **Methanol**, LC-MS grade (CH_3OH) – Product code: CL00.1377.1000; UN Nr.: 1230; Exp.: 04/2019; Chem-Lab NV (Zedelgem, Belgium)
- **Water** for LC/MS – Milli-Q®; Milli-Q water purification system; 0.22 µm; Lot: F4CA66816; Merck Millipore (Darmstadt, Germany)

Solvents

- **acetonitril/water/formic acid** – 79:20:1 (v/v/v)
- **water/methanol** – 70:30 (v/v)

Eluent

For both eluents á one litre volume, a final concentration of 5 mM ammonium formate is needed. The preparation was conducted as follows:

- **eluent A:** to one litre water, 1 ml of formic acid and 0.3153 g ammonium formate (63.06 g/mol) were added
- **eluent B:** to one litre methanol, 1 ml of formic acid and 0.3153 g ammonium formate (63.06 g/mol) were added

Materials

Equipment

- **Agilent Technologies LC-QQQ-MS liquid-chromatograph**
  - 1290 Infinity UHPLC
  - 6490 Triple Quadrupole Mass Spectrometer; Model: G6490A; Serial: SG1152A201; (Singapore)
  - Agilent Technologies RRHD-column Zorbax Eclipse Plus C18 2.1*100mm; 1.8 µm
  - 1290 sampler; Model: G4226A; Serial: DEBAP02121
  - 1290 Bin Pump; Model: G4220A; Serial: DEBAA02564
  - 1260 Iso Pump; Model: G1310B; Serial: DEAB903902
  - ALSTherm; Model: G1330A; Serial: DE82203645
  - 1290 TCC; Model: G1316C; Serial: DEBAC02955
  - Agilent MassHunter workstation software – Quantitative Analysis (B.07.01), Qualitative Analysis (B.07.00)
- **Centrifuge 5430**; max. speed: 17,500 min^{-1}, Serial: 5427AL013297; Eppendorf AG (Hamburg, Germany)
- **Collomix**; Type: VIBA 300; Serial: 892014; Rühr- und Mischgeräte GmbH (Gaimersheim, Germany)
- **Grindomix**; Type: GM200; Serial: 129240218G; Retsch GmbH (Haan, Germany)
- **Incubation-/inactivation bath;** Type: 1003; max. temperature 99.9°C, vol. 14 l; Nr.: 11717614 K; Gesellschaft für Labortechnik GmbH
- **Industrial high shear mixer;** Type: E.X; Nr.: 5M2451; Silverson (Chesham, England)
- **Sartorius** laboratory **scale**; max. 820 g, d = 0.01 g; Serial: ENTRIS822I – 1S; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)

- **Shaker**; max. speed: 2,500 rpm; Type: REAX control; Serial: 120402886; Heidolph (Schwabach, Germany)
- **Sonorex ultrasonic bath**; Type: RK 510 S; Serial: 327063027; Bandelin electronic (Berlin, Germany)

Accessories

- **Chromatographic caps**; bonded blue screw cap PTFE/red silicone septa; Lot: AGI 199643; Agilent Technologies
- **Chromatographic caps**; cap 9 mm red screw PTFE/RS; Lot: AGI 197640; Agilent Technologies
- **Chromatographic vials**; clear; screw top; micro sampling; Batch: GTG040116226; Agilent Technologies
- **Chromatographic vials**; screw; 2 ml; Lot: 886-04-16/001; Agilent Technologies
- **Disposable syringes**; Omnifix® Solo; capacity 5 ml; Braun Sharing Expertise
- **Eppendorf research® lus pipette**; single channel; variable; 0.5 – 10 µl; incl. epT.I.P.S®-box; middle grey
- **Glas pasteur pipettes**; disposable; approx. 150 mm; Lot: 11 NS; Brand
- **Measuring cylinder**; capacity 1,000:10 ml; In 20 °C; Glasfirn Simplex
- **Multiple dispenser**; HandyStep® electronic; single channel; variable; 1.0 µl – 50 ml; incl. PD-Tips; Brand
- **Organic bottle dispenser**; Dispensette®; analog; 5 – 50 ml; Brand
- **Pasteur pipette rubber bulb**; capacity 1 ml; Brand
- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 20 – 200 µl; incl. epT.I.P.S®-box; yellow; Eppendorf
- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 100 – 1,000 µl; incl. epT.I.P.S®-box; blue; Eppendorf
- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 0.5 – 5 ml; incl. epT.I.P.S®-sample bags; purple; Eppendorf

- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 1 – 10 µl; incl. epT.I.P.S®-sample bags; turquoise; Eppendorf
- **Polytetrafluorethylene (PTFE) membrane filter**; diameter 0.45 µm; Sartorius
- **Tube**; volume 50 ml; 114x28 mm; PP; Sarstedt
- **Miscellaneous**: beaker glass, bulkhead bottle, ground-glass stoppers, hopper, sample vials, scoop, volumetric flask, weighing boat, clean up columns

Reference substances

Calibrant Solutions

- **15-Acetyldeoxynivalenol** in acetonitrile ($C_{17}H_{24}O_6$); 101.0 µg/ml; CAS: 88337-96-6; Lot: L13374A; Exp.: 03/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **3-Acetyldeoxynivalenol** in acetonitrile ($C_{17}H_{22}O_7$); 100.4 µg/ml; CAS: 50722-38-8; Lot: L13354A; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Aflatoxin M1** in acetonitrile ($C_{17}H_{12}O_7$); 504 ng/ml; CAS: 6795-23-9; Lot: L15271M; Exp.: 06/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Aflatoxin Mix M5** in acetonitrile ($C_{17}H_{12}O_6$ - 251 ng/ml, Aflatoxin B1), ($C_{17}H_{14}O_6$ - 253 ng/ml, Aflatoxin B2), ($C_{17}H_{12}O_7$ - 253 ng/ml, Aflatoxin G1), ($C_{17}H_{14}O_7$ - 250 ng/ml, Aflatoxin G2); CAS: BRM 002022; Lot: L15503M; Exp.: 12/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Alternariol** – dried down ($C_{14}H_{10}O_5$); 100.0 µg/ml; CAS: 641-38-3; Lot: L15521A; Exp.: 12/2018; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Alternariolmethylether** – dried down ($C_{15}H_{12}O_5$); 102.3 µg/ml; CAS: 26894-49-5; Lot: L14081B; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Beauvericin** – dried down ($C_{45}H_{57}N_3O_9$); 100.1 µg/ml; CAS: 26048-05-5; Lot: L15365B; Exp.: 09/2018; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Citrinin** in acetonitrile ($C_{13}H_{14}O_5$); 100.1 µg/ml; CAS: 518-75-2; Lot: L15231C; Exp.: 11/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Cyclopiazonic Acid** in acetonitrile ($C_{20}H_{20}N_2O_3$); 100.3 µg/ml; CAS: 18172-33-3; Lot: L14133B; Exp.: 08/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)

- **Deoxynivalenol** in acetonitrile ($C_{15}H_{20}O_6$); 100.4 µg/ml; CAS: 51481-10-8; Lot: L15383C; Exp.: 03/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Deoxynivalenol-3-Glucoside** in acetonitrile ($C_{21}H_{30}O_{11}$); 50.9 µg/ml; CAS: 131180-21-7; Lot: L15281A; Exp.: 01/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Diacetoxyscirpenol** in acetonitrile ($C_{19}H_{26}O_7$); 100.3 µg/ml; CAS: 2270-40-8; Lot: L13474D; Exp.: 05/2018; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Enniatin B** – Powder ($C_{33}H_{57}N_3O_9$); 10 mg/ml; CAS: 917-13-5; Product: E5411; Sigma-Aldrich (Saint Louis, USA)
- **Fumonisin B3** in acetonitrile ($C_{34}H_{59}NO_{14}$); 50.0 µg/ml; CAS: 136379-59-4; Lot: L15281D; Exp.: 01/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Fumonisin Mix 3** in acetonitrile; ($C_{34}H_{59}NO_{15}$ - 50.2 µg/ml, Fumonisin B1), ($C_{34}H_{59}NO_{14}$ - 50.0 µg/ml, Fumonisin B2); CAS: 002006; Lot: L16071M; Exp.: 08/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Fusarenon X** in acetonitrile ($C_{17}H_{22}O_8$); 100.3 µg/ml; CAS: 23255-69-8; Lot: L13391A; Exp.: 03/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **HT-2 Toxin** in acetonitrile ($C_{22}H_{32}O_8$); 100.2 µg/ml; CAS: 26934-87-2; Lot: L15444H; Exp.: 04/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Nivalenol** in acetonitrile ($C_{15}H_{20}O_7$); 100.6 µg/ml; CAS: 23282-20-4; Lot: L15222N; Exp.: 11/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Ochratoxin A** in acetonitrile ($C_{20}H_{18}ClNO_6$); 10.05 µg/ml; CAS: 303-47-9; Lot: L15411A; Exp.: 04/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Patulin** in acetonitrile ($C_7H_6O_4$); 100.2 µg/ml; CAS: 149-29-1; Lot: L13354P; Exp.: 02/2018; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Sterigmatocystin** in acetonitrile ($C_{18}H_{12}O_6$); 50.6 µg/ml; CAS: 10048-13-2; Lot: L16021S; Exp.: 01/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **T-2 Toxin** in acetonitrile ($C_{24}H_{34}O_9$); 100.4 µg/ml; CAS: 21259-20-1; Lot: L16083A; Exp.: 08/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **Zearalenone** in acetonitrile ($C_{18}H_{22}O_5$); 100.4 µg/ml; CAS: 17924-92-4; Lot: L15383B; Exp.: 03/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)

Calibrant Mixtures

A standard straight line and various spike working solutions were prepared with a mixture of all analytes which are listed above. For the standard straight line, a stock mix-solution of all 27 substances with different concentrations from 1 – 1,000 µg/L was prepared and the individual levels were constructed in accordance to a dilution scheme. Three different solvents, acetonitrile/water/formic acid (79/20/1), water/methanol (70/30) and pure methanol were used for the purpose of research. The calibration as well as spike solutions were transferred into chromatographic vials and stored at -18 °C. For a valid calibration curve at least 3 standard-points have to be used in the defined area of L1-L7. A detailed overview of used calibration and spike volumes for the validation is attached on pages 75 and 76.

^{13}C Calibrants

- **U-[$^{13}C_{17}$]-3-Acetyldeoxynivalenol** in acetonitrile ($^{13}C_{17}H_{22}O_7$); 26.1 µg/ml; CAS: 50722-38-8; Lot: I15061A; Exp.: 08/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{17}$]-Aflatoxin B1** in acetonitrile ($^{13}C_{17}H_{12}O_6$); 0.510 µg/ml; CAS: 1217449-45-0; Lot: IR12085B; Exp.: 08/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{17}$]-Aflatoxin B2** in acetonitrile ($^{13}C_{17}H_{14}O_6$); 0.500 µg/ml; CAS: 1217470-98-8; Lot: IR11472B; Exp.: 04/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{17}$]-Aflatoxin G1** in acetonitrile ($^{13}C_{17}H_{12}O_7$); 0.507 µg/ml; CAS: 1217444-07-9; Lot: I11472D; Exp.: 11/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{17}$]-Aflatoxin G2** in acetonitrile ($^{13}C_{17}H_{14}O_7$); 0.515 µg/ml; CAS: 1217462-49-1; Lot: I12271G; Exp.: 07/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{17}$]-Aflatoxin M1** in acetonitrile ($^{13}C_{17}H_{12}O_7$); 0.502 µg/ml; CAS: 6795-23-9; Lot: I15232M; Exp.: 12/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)

- **U-[$^{13}C_{13}$]-Citrinin** in acetonitrile ($^{13}C_{13}H_{14}O_5$); 10.6 µg/ml; CAS: 518-75-2; Lot: I15125C; Exp.: 09/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{20}$]-Cyclopiazonic Acid** in acetonitrile ($^{13}C_{20}H_{20}N_2O_3$); 10.01 µg/ml; CAS: 18172-33-3; Lot: I14133A; Exp.: 08/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{15}$]-Deoxynivalenol** in acetonitrile ($^{13}C_{15}H_{20}O_6$); 25.0 µg/ml; CAS: 911392-36-4; Lot: I09274A; Exp.: 01/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{19}$]-Diacetoxyscirpenol** in acetonitrile ($^{13}C_{19}H_{26}O_7$); 25.0 µg/ml; CAS: 2270-40-8; Lot: I15323B; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{34}$]-Fumonisin B1** in acetonitrile/water ($^{13}C_{34}H_{59}NO_{15}$); 25.1 µg/ml; CAS: 116355-83-0; Lot: I15201B; Exp.: 11/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{34}$]-Fumonisin B2** in acetonitrile/water ($^{13}C_{34}H_{59}NO_{14}$); 10.01 µg/ml; CAS: 116355-84-1; Lot: I16091A; Exp.: 08/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{34}$]-Fumonisin B3** in acetonitrile/water ($^{13}C_{34}H_{59}NO_{14}$); 10.02 µg/ml; CAS: 136379-59-4; Lot: I15323F; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{22}$]-HT-2 Toxin** in acetonitrile ($^{13}C_{22}H_{32}O_8$); 25.4 µg/ml; CAS: 1486469-92-4; Lot: I10044A; Exp.: 07/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{15}$]-Nivalenol** in acetonitrile ($^{13}C_{15}H_{20}O_7$); 25.5 µg/ml; CAS: 23282-20-4; Lot: I14372N; Exp.: 10/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{20}$]-Ochratoxin A** in acetonitrile ($^{13}C_{20}H_{18}ClNO_6$); 10.08 µg/ml; CAS: 911392-42-2; Lot: I11344A; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_7$]-Patulin** in acetonitrile ($^{13}C_7H_6O_4$); 25.08 µg/ml; CAS: 149-29-1; Lot: I14462A; Exp.: 11/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{24}$]-T-2 Toxin** in acetonitrile ($^{13}C_{24}H_{34}O_9$); 25.1 µg/ml; CAS: 75-05-8; Lot: I10101C; Exp.: 02/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)

- **U-[$^{13}C_{18}$]-Sterigmatocystin** in acetonitrile ($^{13}C_{18}H_{12}O_6$); 25.4 µg/ml; CAS: 10048-13-2; Lot: I15171B; Exp.: 10/2016; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)
- **U-[$^{13}C_{18}$]-Zearalenone** in acetonitrile ($^{13}C_{18}H_{22}O_5$); 25.1 µg/ml; CAS: 911392-43-3; Lot: I10511A; Exp.: 06/2017; Romer Labs Diagnostic GmbH – Europe (Tulln, Austria)

^{13}C Calibrant Mixtures

Isotopic-labelled-internal standards are used if sample component losses or other systematic errors are expected. Internal standards are sample foreign compounds which are chemically related but not identical to the analyte. These labelled standards are added to each sample and calibration standard in a known concentration and are thus reference values. If the internal standard concentration changes, it is assumed that the analyte will change the same way. With this way it is possible to correct matrix influences by adding the internal standard at the end of the sample preparation simultaneously before injection. Furthermore, it is possible to correct both the matrix influences as well as losses through the extraction method by adding the internal standard at the beginning of the sample preparation. For this, higher amounts of labelled standards are required because the added quantity depends on the sample weight.

In this method the isotopic-labelled-internal standard mixture was injected automatically via autosampler in each calibration level and each sample to ensure equal concentrations. Therefore a ^{13}C-mix-solution of all listed internal standards was prepared. Unfortunately, the availability of internal standards was reduced to 20 substances. The ^{13}C-mix-solution was prepared in acetonitrile/water/formic acid (79/20/1), in water/methanol (70/30) and pure methanol. The solutions were transferred into chromatographic vials and stored at -18 °C. A detailed overview of used internal standard concentrations for the validation is attached at page 76.

Samples

Due to an existing accredited multi-mycotoxin method of the LVA GmbH in cereals, cereal products, nuts, pastries, pasta products and dried fruits, the priority of analytical research and optimisation steps were preferred set on these matrices. Furthermore analytical focus was set on food products which are anchored in the regulation (EC) 1881/2006. Most of the analysed matrices were retention samples from the LVA GmbH, only a few were purchased in grocery stores. After homogenization, the samples were stored in accordance with their dry content. Dry samples like cereals were stored at room temperature. In contrast, water containing samples like almonds were stored frozen at -18 °C. For analytical investigations the frozen samples were defrosted either at room temperature or at 36 °C in an incubation-/inactivation bath. In total, scientific tests were made in 16 different matrices. An overview is given in *table 4.*

Table 4: Overview of analysed samples

Sample	Origin	Sample	Origin
almonds	LVA	**coffee**	LVA
pepper	LVA	**maize**	LVA
marble cake	purchased	**oat**	LVA
oat flakes	LVA	**pastry**	LVA
red yeast rice	purchased	**rye**	LVA
soy beans	LVA	**spelt rice**	LVA
sultanas	LVA	**walnuts**	LVA
wheat flour	LVA	**wholemeal bread**	purchased

The selection of suitable retention samples was based on previous performed measurements. These former analyses were made for a multi-mycotoxin quantification including 11 analytes. Only samples with a low natural contamination, lower than the limit of quantification were chosen.

Sample homogenisation

In food analysis the sample homogenisation is essential for a quantitative determination of pesticides, nutrients and mycotoxins as well as to ensure a representative sample preparation. Very important tools are hereby laboratory mills with different designs. For a sufficient extraction of mycotoxins from the raw material, the sample has to be crushed and homogenised previously. Because of a mostly nested natural occurrence of mycotoxins, the sample amount has to be adequate to verify a contamination. Representative amounts are hereby 1 to 2 kilogram per ton of supplied products. Because of a good fat solubility of mycotoxins the grinding process has to be performed very careful to prevent an undesired release of fat into the sample material. To inhibit an adverse temperature increase and to reduce the degradation of the analytes, dry ice is added during homogenisation. Small amounts of sample material (< 2 kilogram) were shredded in a laboratory mill (Grindomix), whereas bigger amounts (> 2 kilogram) of sample material were crushed in an industrial high shear mixer. For further extraction steps the samples were transferred into appropriate synthetic boxes.

LC-MS/MS Optimisation

For the optimisation of the native standards and isotopic labelled substances, single standards for all analytes were prepared with a concentration of 100 µg/l. Instead of a column, a filter with no retention attributes was used for this purpose. Thereby, especially the duration of the optimisation methods is reduced significantly and it is possible to optimise several analytes in a short time.

At the acquisition of the mass spectrum, the detector records the ion-intensity in dependence to the mass-to-charge ratio (m/q). The resulting Gauss curves are summarized to lines, receiving a line spectrum. The graphic representation of the spectrum includes the relative ion-intensity as ordinate (y-axis) and the m/q-ratio as abscissa (x-axis). An example is shown in *figure 14* on page 43.

(dynamic) Multiple Reaction Monitoring

For this work, originally a multiple reaction monitoring procedure was applied. With this method, it is possible to determine several transitions in a fixed time limit. Hereby, the precursor ions are successively selected in the first quadrupole, fragmented in the hexapole and finally measured in the second quadrupole. This very sensitive measurement procedure enables a fast analysis of the chromatographic co-elution and increases the selectivity of the analysis. For each single optimisation step it is therefore important to adjust the first parameter, the dwell time. The dwell time, or measurement period per measurement point, is important for a sufficient admission of data points in the chromatogram. The time adjustment has to be between 1 and 2 cycles per second. For standard-optimisation steps and previous method optimisation trials the method was used in MRM-mode. After all optimisation work the method was converted into dyanamic MRM. In dynamic MRM-mode the data are only gained in a specific retention time screen. This way it is possible to reduce the impact of concurrent ions, resulting in a higher sensitivity. [AGILENT, 2011]

Scan

In the first step, the scan, the precursor ions are selected after a positive or a negative electro-spray-ionisation (ESI). In ESI-mode, the sample reaches the ionisation region via a capillary. An electromagnetic field is created at the end of the capillary to support the ionisation process. During ionisation, multiple charged ions are created and transferred into the mass spectrometer which is consisting of different analysers. An overview about different MS-elements is shown in *figure 9*.

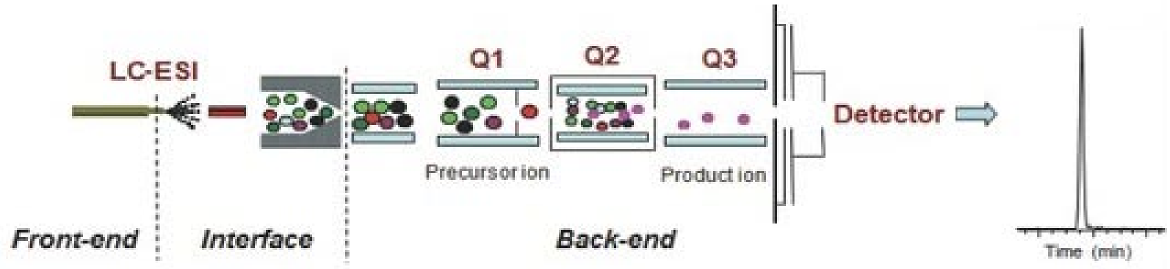


Figure 9: Electro-spray-ionisation and triple quadrupole MS [SHI et al., 2012]

In this work a triple-quadrupole MS was used, consisting of two analysers and one collision cell which are stringed together. The analysers which are used for the measurement are the first and the last quadrupole. The second part, a hexapole acts as a collision cell and fragments the precursor-ion. During the scan, the first and the second quadrupoles are permeable, so that the third quadrupole is taking over the measurement. For the scan-mode, 10 µl of the single standard was injected without any gradient. To increase the signal, the multiplier can be adjusted within a range of 3 EMV (electron multiplier voltage) and 3,000 EMV. For optimisation, the EMV was set at 300 for all subsections. Because of the iFunnel technology, it was not necessary to optimise the fragmentor. The ion-funnel technology desolvates and concentrates the ions close to the sample inlet for an efficient collection. This new structure facilitates an increased ion-transfer into the first quadrupole and is simultaneously reducing the high gas amounts. For the evaluation of the scan results, the Agilent qualitative analysis (B.07.00) software was used.

Product Ion

Product ions are resulting through the fragmentation of their precursor ions which are determined in the previous step. Hereby the first quadrupole is exclusively responsible for the m/q-ratio of the precursor ion. The charge of the ionisation depends on the ionisation of the precursor ion and can therefore be positive or negative. Generally the signal intensity is higher in ESI-positive mode, but also resulting in higher matrix effects compared to ESI-negative.

After selection of the certain m/q-ratio through the first quadrupole, the hexapole fragments the selected ions, followed by an analysis of the created fragments through the third quadrupole. Through the “product-ion-method” it is thus possible to figure out different transitions and fragments.

Furthermore the collision energy (CE) is determined for each transition. The collision energy is important for a further fragmentation of the molecules. Inconclusive identified fragments are accelerated through an electric field and are fragmented through a collision with neutral gas-molecules to get smaller identifiable fragments. To figure out the specific collision energies for each product ion, every single substance was injected eight times at different collision energy levels. The substances were tested at 5, 10, 15, 20, 25, 30, 35 and 40 V.

Collision Cell Accelerator Voltage

To reach the best possible signal intensity, it is further important to define the collision cell accelerator voltage (CAV). This parameter enables the transfer of the substance from the hexapole to the third quadrupole. Otherwise the hexapole would endlessly fragment the substance, which is similar to an ion-trap. For each fragment thus there is a specific collision cell accelerator voltage where the substance is residing long enough into the hexapole to build the corresponding transition. The collision cell accelerator voltage was tested at 1, 3, 5 and 8 V. Therefore the single standards were injected 4 times, while the collision cell accelerator voltage changes at each injection.

Retention Time

The retention time is the time which is needed for an analyte to pass the way from the injector through the column to the detector and can directly be read from the chromatogram. For the determination of the corresponding retention time of each analyte, the single-standards were measured with the specific measurement method. Thereby the substances interacted with the Zorbax Eclipse Plus C18-column under following gradient conditions:

Table 5: Adjusted gradient conditions

Time (min)	mobile Phase (A:B)
0	90:10
0.5	90:10
8	0:100
9.5	0:100
9.6	70:30
11.5	90:10

The gradient was modified to suit retention times of several substances to the dead volume. For analytical determinations, it is very important that retention properties of the analytes are adjusted on the dead volume of the HPLC-system. The dead volume describes the volume of the mobile phase which is necessary to fill cavities of the system including capillary,- injection,- column and detection volume. Those sections are responsible for an expansion of the sample droplets without a chromatographic separation event. It is important to keep the dead volume of the HPLC-system as small as possible. A comparison between the originally used gradient conditions and the adjusted gradient is further shown on the example of nivalenol in the attachment on the pages 73 and 74.

Quantifier and Qualifier

For the determination of the quantifier, the product ion with the highest signal intensity was used. All other transitions are used as qualifier. The quantifier is used for the quantification of the analyte, whereas the qualifier helps for the verification of the transition within a qualitative analysis. It is possible to change quantifier and qualifier simply during the evaluation of results to optimise and adjust these parameters. Hereby one of the qualifiers is used as quantifier while the former quantifier is used as qualifier afterwards. Despite a previous optimisation it was necessary to switch some of these parameters, due on matrix interactions.

Extraction

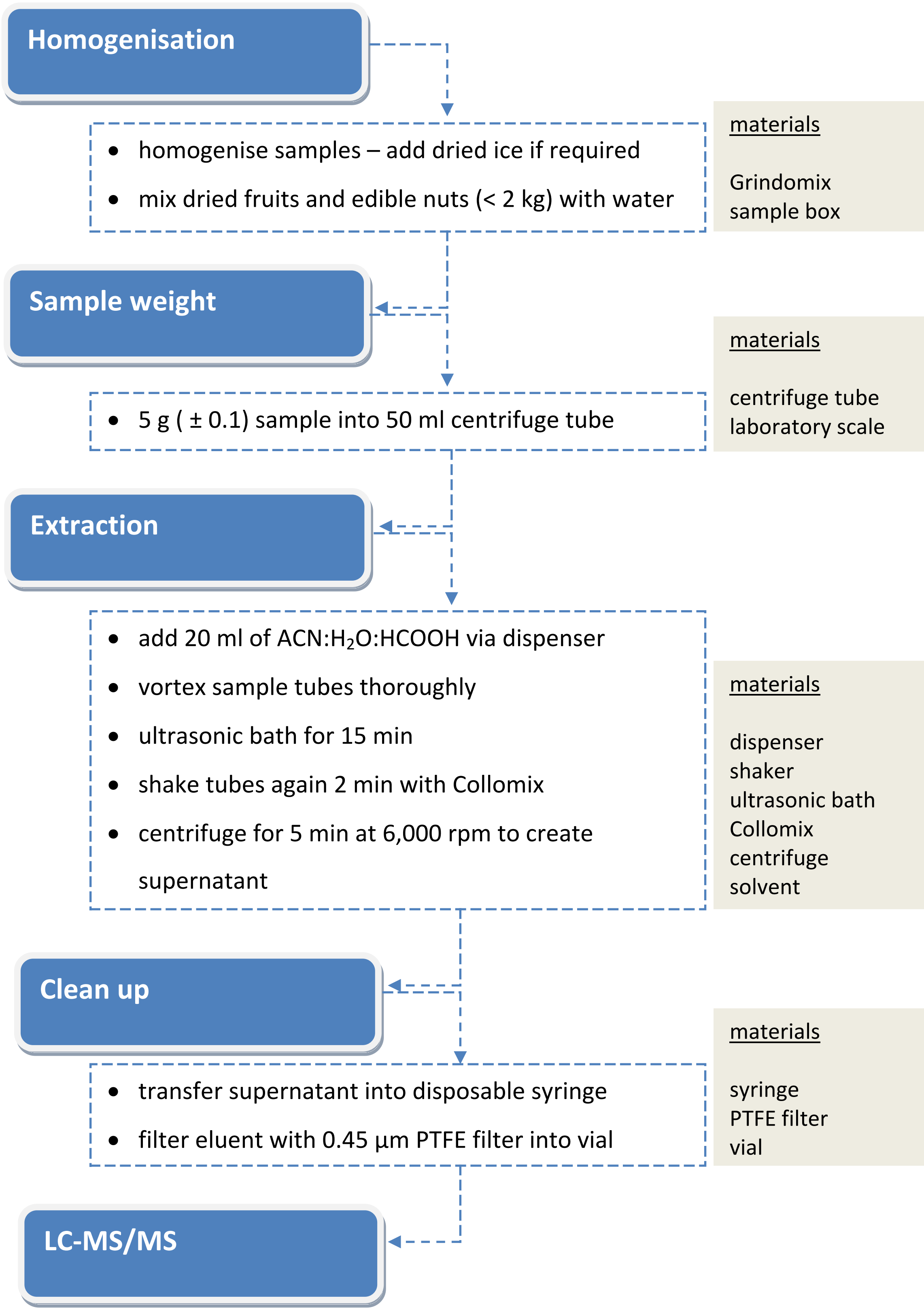
Preparation

The first step of the sample preparation includes homogenisation and sample weight. Samples of dried fruits and edible nuts, even amounts smaller than 2 kilogram have to be mixed with water. Samples which are not undergoing a batch blending are homogenised with dried ice as finely and homogenously as possible with Grindomix. 5 gram of the homogenised sample is weight into a 50 ml centrifuge tube. Consequently 20 ml of acetonitrile/water/formic acid mixture are added to the 5 gram sample with a dispenser and shaked properly. Afterwards the centrifuge tubes are put into an ultrasonic bath for 15 minutes followed by a 2 minute shaking process via Collomix. Finally the samples are put into a centrifuge for 5 minutes at 6,000 rpm.

Clean Up

A clean up step in the proper meaning of the word is not included in this method. The centrifuged samples are just transferred with a Pasteur-pipette into a disposable syringe and further filtered with a 0.45 µm polytetrafluorethylene membrane filter. This is an important step to protect the HPLC-system of undesired disturbing particles. For a single determination of e.g. deoxynivalenol, aflatoxins or ochratoxin A, there is the opportunity to use for instance MycoSep push trough columns. These columns include an adsorbent which is especially designed for each analyte and should be applied for complex matrices like coffee. Beside this very fast clean up opportunity the application of immunoaffinity columns (IAC) is very popular. The mode of action hereby is based on the principles of affinity chromatography like interactions between enzymes and substrate, receptor and ligand or antibody and antigen. Although the efficiency of this clean up possibility is undisputed, it is also a very time-consuming procedure and therefore not the method of choice in a routine laboratory. A schematic presentation of the complete sample preparation is shown in *figure 10.*

Figure 10: Sample preparation scheme of extraction method



Measurement with HPLC-MS/MS

After extraction, the analyte is transferred into the HPLC-MS/MS-system. The high pressure liquid chromatography is a very efficient technique for the separation and analysis of chemical substances. It is based on the principle of column chromatographic procedures where the separation is made through a different distribution of substances in two phases, a mobile phase (liquid) and a stationary phase (solid material or liquid). The eluent represents hereby the mobile phase, is moving along the stationary phase, a column, and is carrying substances with different speed. During this transport, the analytes are interacting with the stationary phase. Because of the universal application for polar and apolar substances, a reversed phase column was used. The interactions hereby are based on the Van-der-Waals forces.

Table 6: HPLC conditions

mobile phase	eluent A: water, 0.1% HCOOH, 5mM NH_4OOCH eluent B: methanol, 0.1% HCOOH, 5mM NH_4OOCH
column	RRHD-column Zorbax Eclipse Plus C18 2.1 * 100 mm; 1.8 µm
injection	3.8 µl extract injected with 0.2 µl internal standard solution
flow	0.35 ml/min
column temp.	40°C
runtime	11.5 min
gradient	see chapter retention time

Table 7: MS/MS conditions

gas temp.	200°C
gas flow	15 l/min
nebulizer	30 psi
sheath gas temp.	375°C
sheath gas flow	11 l/min
capillary voltage	4,000 V (pos)/3,000 V (neg)

Validation

The target of a validation is a harmonised and cheap quality assurance within the European Union. Further it is important to ensure quality and comparability of analytical results and achieve an acceptable precision. The quality of a validation is subjected to different factors like the quality of employees, a suitable analytical system, a rugged method and good laboratory practice (GLP), which is part of the quality management system.

To ensure reproducible and reliable results of an analytical method, it is important to validate the method constantly. Those results deliver evidence that the procedure serves the purpose for which it is designed.

Recovery

The systematic deviation between the mean value and the true value is defined as accuracy. To establish the accuracy it is necessary to determine the recovery rate, which represents the percentage amount of the mean value from the detected spike concentration in reference to the true value. The calculation is made by adding a known amount of an analyte concentration to the sample, followed by extraction and measurement with the selected method. Thus it is possible to assess the complete method by the recovery rate. [LEITERER, 2008]

Figure 111: Recovery rate in percent

$$RR\ [\%] = \frac{c\ (spiked\ sample) - \ c\ (matrix)}{c\ spike} * 100\%$$

RR	recovery rate
c (spiked sample)	concentration of the sample inclusive added analyte
c (matrix)	natural contamination of the sample
c spike	concentration of the added analyte

Precision and reproducibility

During an analytical determination, two kinds of errors can occur. First, after repeated measurements the results can differ among themselves. Those are so called random errors of the single measurement. The second error would be a deviation from the true value. Those deviations are better known as systematic errors and can affect the precision of the analytical method. The standard deviation from the mean value of repeated measurements delivers information about the precision of the analysis. It is a degree for the spread around the mean value and is indicated as relative standard deviation. [WELLMITZ and GLUSCHKE, 2005]

Figure 122: Relative standard deviation in percent

$$RSD\ [\%] = \frac{S}{\bar{X}} * 100\%$$

RSD	relative standard deviation
s	standard deviation
$\bar{x}$	mean value

Limit of Detection/Quantification

For the assessment of an analytical method the limit of detection (LOD) is of great importance. It represents the smallest amount of a substance which is clearly detectable in contrast to the blank and delivers information about the occurrence of an analyte. This limit is generally used for qualitative analysis. In contrast to the LOD, the limit of quantification (LOQ) is connected to a numerical data of the determined agent and delivers information about the practicability of prospective quantitative analysis. So it can be concluded that the LOQ is the smallest amount of a substance which can be quantified within a prescribed statistical safety and means that the LOQ provides a higher accuracy as the LOD. [WELLMITZ and GLUSCHKE, 2005]

Validation process

The validation was performed on 7 consecutive days for the following matrices: oat flakes, maize, wheat flour, wholemeal bread, marble cake, pastry, almonds, walnuts and sultanas. Each matrix was spiked with two different analyte concentrations. Additionally one blank sample was analysed. The spike concentrations were selected based on their maximum levels (ML) anchored in the Regulation (EC) 1881/2006. For regulated substances the low spike concentration was a tenth from the ML if analytically possible to determine. The high spike concentration was at least at the height of the ML. For non-regulated substances the spike concentrations were selected concerning to pre analytical trials and adjusted to an acceptable signal-to-noise ratio. After preliminary investigations 23 substances were included for validation. A complete list with spike-concentrations of all analytes as well as the creation of the calibration is attached.

Performance criteria

According to the regulation EC 401/2006, for a successful completion of the validation the following, in *table 8* listed performance criteria have to be fulfilled.

Table 8: Mycotoxin performance criteria [EUROPEAN COMMISSION, 2006]

Analyte	Conc. µg/kg	Recovery %	RSD %
Aflatoxins	< 1	50 – 120	Horwitz
B1, B2, G1, G2	1 – 10	70 – 120	
Citrinin	all	70 – 120	Horwitz
Deoxynivalenol	> 100 - ≤ 500	60 – 110	≤ 20
Fumonisin B1, B2	≤ 500	60 – 120	≤ 30
Ochratoxin A	≥ 1	70 – 110	≤ 20
T-2, HT-2 Toxin	15 – 250	60 – 130	≤ 30
Zearalenone	≤ 50	60 – 120	≤ 40
	> 50	70 – 120	≤ 25
Other substances *	all	70 – 120	≤ 20

* not regulated by EC 401/2006

Results and Discussion

LC-MS/MS Optimisation

Optimisation example

By scanning the single analyte, the precursor was identified through the m/q-ratio. A scan illustration example of sterigmatocystin is shown in *figure 13*.

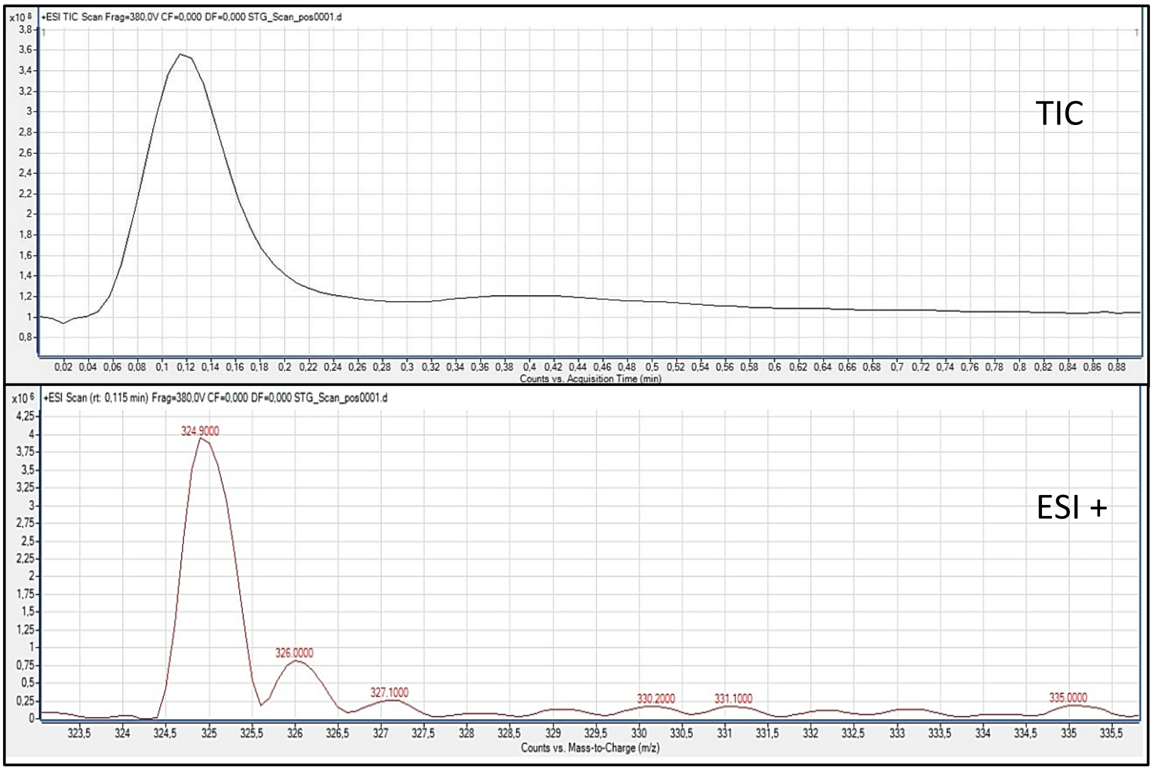


Figure 13: Scan of sterigmatocystin – TIC and ESI+

The molecular weight of sterigmatocystin is at 324.28428 g/mol. Therefore the scan was made in the range of 320 to 360, because the precursor ion was assumed in this area. The total ion chromatogram (TIC) in *figure 13* shows that the substance appears very early, after 0.12 minutes. This is because of the use of the filter instead of a column. The m/q-ratio was also detected and is at 324.9 in ESI positive mode. This value seems to be plausible because after an admission of a hydrogen atom in consideration of the molecular weight of sterigmatocystin, only this m/q-ratio comes into question.

After the determination of the precursor ion, several transitions and fragments were detected with a separate method. Additionally for each transition, the appropriate collision energy was tested. With increasing collision energy the yield of specific product-ions is raising, which means the higher the voltage, the more fragments are formed. Hereby every molecule degrades into a specific fragment, which is further degrading after applying higher voltage as shown in *figure 14*.

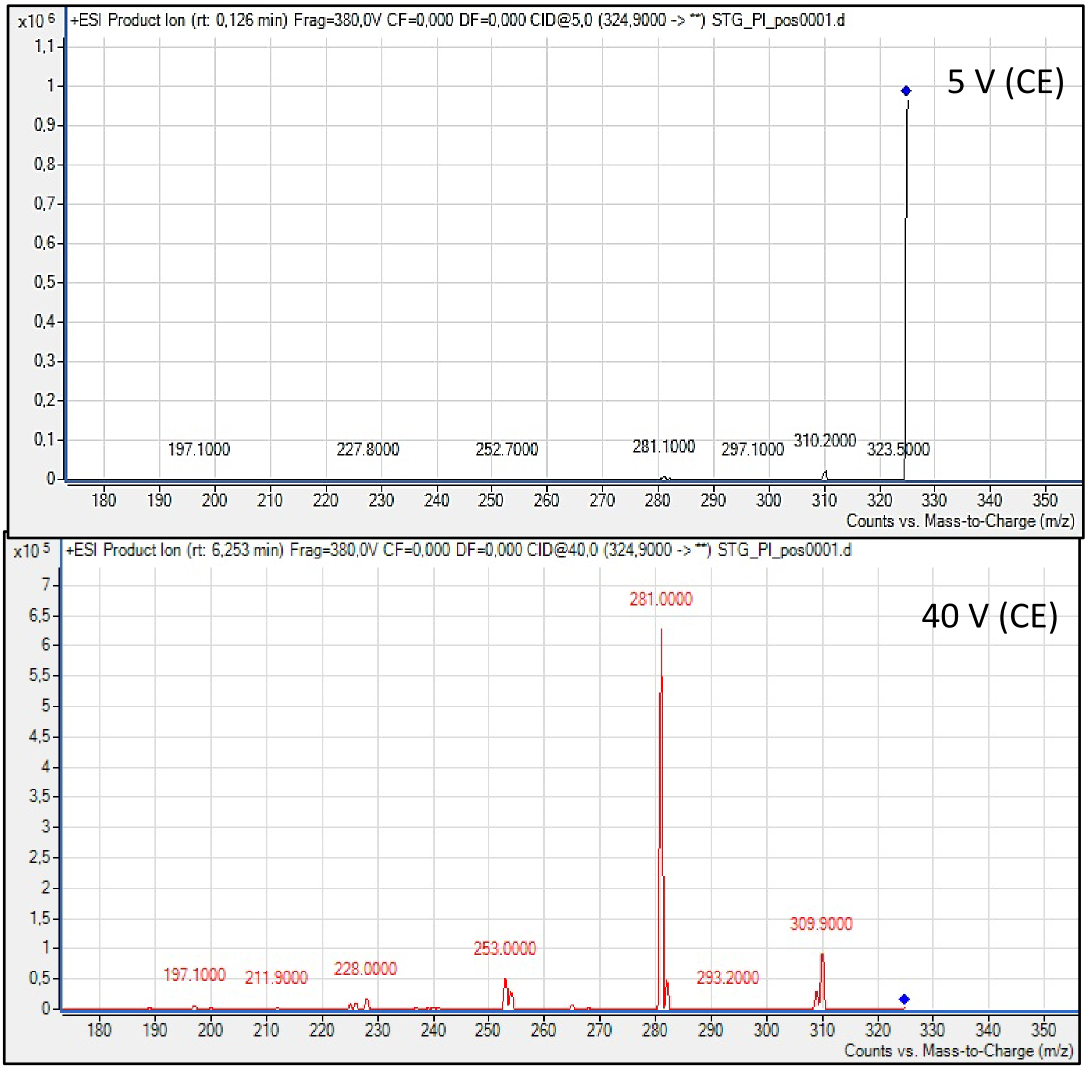


Figure 14: Fragmentation pattern of sterigmatocystin at 5 V (CE) and 40 V (CE)

In *figure 14* it can be realized that the precursor ion of 324.9 is rarely fragmented at collision energy of 5 V, whereas it is almost completely fragmented at 40 V. Within this range, all possible fragments are determined, for STE they are 310.1, 297.1 and 281.0 which are shown in *figure 15*.

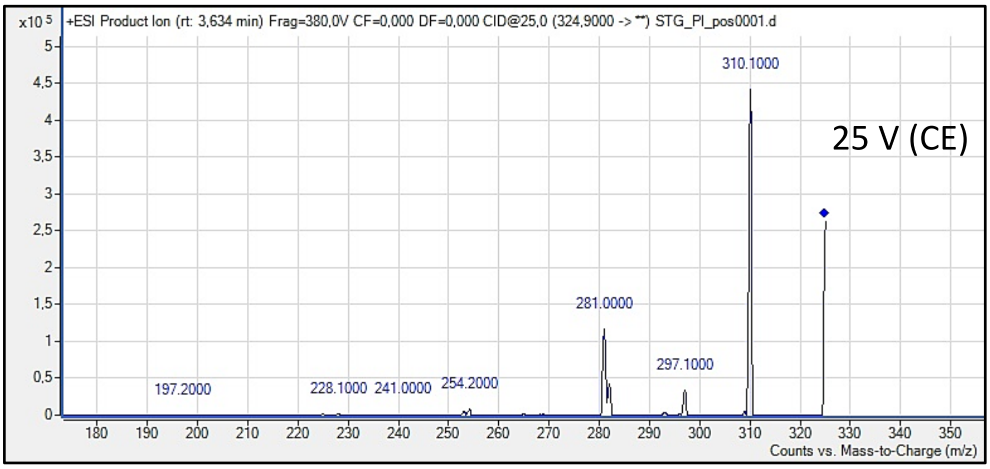


Figure 15: Fragmentation pattern of sterigmatocystin at 25 V (CE)

To figure out the optimal collision energy for each fragment a comparison of the signal intensity of each peak was made. The collision energy with the highest peak was used for the optimised method.

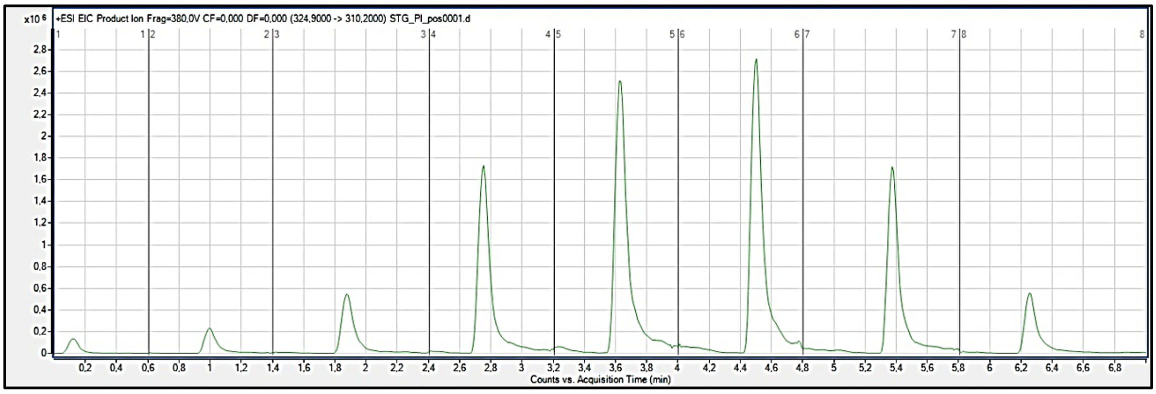


Figure 16: Peaks for the transition 324.9 → 310.1 at collision energies from 5-40 V

In *figure 16* an example of the signal intensity is shown for the transition 324.9 → 310.1 at different collision energies. The peak with the collision energy of 30 V shows the highest signal intensity and is thus used for the method. In this way collision energies for all transitions were determined.

Similar to the determination of the collision energy, for the evaluation of the collision cell accelerator voltage, peaks with the highest signal intensity were chosen for each transition. If all peaks have the same response the medium peak with a CAV of 5 V was used.

With those optimised parameter, it was possible to determine the specific retention time of each analyte. The results from the previous optimisation steps were set into a “new” multi method and a single standard of each substance was injected to determine the retention time using a C18-column.

After definition of the precursor ion, transitions, collision energy, collision cell accelerator voltage and retention time, a calibration curve including a minimum of 6 levels was made for each substance. A very important indicator how well the data fits a curve is the R^2 value. The closer this value is to 1 the better is the prediction of the outcomes and shows how well the data fits to the model. *Figure 17* shows a calibration curve of sterigmatocystin with 6 calibration levels, a slight quadratic trend is observable.

Figure 17: Calibration curve of sterigmatocystin

Complete List of selected Parameter's

An overview of all optimised substances with the described parameters is shown in *table 9*. This summary includes the molecular weight (MW), the precursor ion and its related adduct, the product ions, the collision energy (CE), the cell accelerator voltage (CAV), the polarity and retention time.

Table 9: Complete list of optimised analytes with selected parameters

Analyte	MW (g/mol)	Precursor (m/z)	Adduct	Product (m/z)[a]	CE (V)	CAV (V)	Polarity (pos/neg)	Retention (min)
13C13-CIT	263.2	264.2	$[M+H]^+$	246.2	15	1	Positive	5.80
13C15-DON	311.3	312.2	$[M+H]^+$	263.1/216	12/1	3/3	Positive	3.20
13C15-NIV	327.3	372.1	$[M+CHO_2]^-$	326.1/294.8	7/10	3/5	Negative	2.30
13C17-3AcDON	355.3	356.1	$[M+H]^+$	245.2/216.2	1/19	1/1	Positive	4.70
13C17-AFB1	329.2	330.1	$[M+H]^+$	301.1/255.3	21/40	3/3	Positive	5.70
13C17-AFB2	331.2	332.2	$[M+H]^+$	303/273.3	21/30	3/3	Positive	5.50
13C17-AFG1	345.2	346.1	$[M+H]^+$	328.3/257.3	20/25	5/5	Positive	5.20
13C17-AFG2	347.2	348.1	$[M+H]^+$	330.3/259.1	25/25	5/5	Positive	5.00
13C17-AFM1	328.2	346.1	$[M+H]^+$	317.2/288.1	20/25	1/1	Positive	5.10
13C18-STE	342.2	343.2	$[M+H]^+$	327.1/297.1	30/40	1/1	Positive	7.40
13C18-ZON	336.3	335.2	$[M-H]^-$	290	17	7	Negative	7.20
13C19-DAS	385.4	403.2	$[M+NH_4]^+$	324.3/262.2	5/10	1/1	Positive	5.80
13C20-CPA	356.3	357.2	$[M+H]^+$	210.2/191.1	25/20	1/1	Positive	7.60
13C20-OTA	423.8	424.2	$[M+H]^+$	377/250.1	10/25	3/3	Positive	7.20
13C22-HT2	446.4	464.3	$[M+NH_4]^+$	278.1	9	3	Positive	6.40
13C24-T2	490.5	508.3	$[M+NH_4]^+$	322.1/229.2	8/15	5/5	Positive	6.90
13C34-FUMB1	755.8	756.5	$[M+H]^+$	374.4	37	3	Positive	6.50
13C34-FUMB2	739.8	740.5	$[M+H]^+$	358.3/340.4	41/45	3/3	Positive	7.20
13C34-FUMB3	739.8	740.6	$[M+H]^+$	722.5	30	8	Positive	6.90
13C7-PAT	161.1	158.8	$[M-H]^-$	131/113.1	3/12	3/5	Negative	1.90

Analyte	MW (g/mol)	Precursor (m/z)	Adduct	Product (m/z)[a]	CE (V)	CAV (V)	Polarity (pos/neg)	Retention (min)
3-15-AcDON	338.3	339.0	$[M+H]^+$	261.0/279.0	10/10	3/3	positiv	4.70
AFB1	312.2	313.1	$[M+H]^+$	285.0/241.0	21/41	3/3	positiv	5.80
AFB2	314.2	315.1	$[M+H]^+$	287.0/258.9	21/29	3/3	positiv	5.60
AFG1	328.2	329.1	$[M+H]^+$	243.0/200.1	25/41	3/3	positiv	5.40
AFG2	330.2	331.1	$[M+H]^+$	313.0/245.1	21/25	3/3	positiv	5.20
AFM1	328.2	329.1	$[M+H]^+$	273.0/229.0	25/40	1/3	positiv	5.20
AOH	258.2	259.1	$[M+H]^+$	243.9/213.1	30/30	1/1	positiv	6.40
AME	272.2	273.2	$[M+H]^+$	258.0/230.0	25/30	1/1	positiv	7.40
BEA	783.9	801.4	$[M+NH_4]^+$	784.4/262.1	15/30	8/1	positiv	8.60
CIT	250.2	251.2	$[M+H]^+$	233.1/215.1	10/30	1/1	positiv	5.80
CPA	336.3	337.2	$[M+H]^+$	196.2/182.1	20/15	1/5	positiv	7.60
DON	296.3	297.1	$[M+H]^+$	249.0/203.0	4/12	3/3	positiv	3.10
DON-3-GLU	458.4	503.3	$[M+CHO_2]^-$	457.1/427.3	10/10	1/1	negativ	3.10
DAS	366.4	384.0	$[M+NH_4]^+$	307.0/247.0	5/10	1/1	positiv	5.80
ENB	639.8	657.4	$[M+NH_4]^+$	640.3/196.0	15/30	8/3	positiv	8.50
FUMB1	721.8	722.4	$[M+H]^+$	352.4/334.4	37/37	3/3	positiv	6.50
FUMB2	705.8	706.4	$[M+H]^+$	336.4/318.3	41/41	3/3	positiv	7.20
FUMB3	705.8	706.4	$[M+H]^+$	512.5/354.4	30/35	1/1	positiv	6.90
FX	354.3	355.1	$[M+H]^+$	247.1/229.2	10/15	3/1	positiv	3.90
HT2	424.4	442.2	$[M+NH_4]^+$	263.0/215.0	9/13	3/3	positiv	6.40
NIV	312.3	357.0	$[M+CHO_2]^-$	281.0/203.0	10/20	5/1	negativ	2.30
OTA	403.8	404.1	$[M+H]^+$	238.9/102.1	25/70	3/3	positiv	7.20
PAT	154.1	153.0	$[M-H]^-$	81.0/53.0	5/10	1/3	negativ	2.00
STE	324.2	324.9	$[M+H]^+$	310.0/281.0	30/40	1/1	positiv	7.40
T2	466.5	484.3	$[M+NH_4]^+$	305.0/215.1	8/9	5/5	positiv	6.90
ZON	318.3	317.1	$[M-H]^-$	272.9/130.9	17/29	7/7	negativ	7.20

Method-Optimisation-Trials

Sample weight

The first optimisation of the extraction-method was a reduction of the sample weight. This step is based on the multi-mycotoxin method of the inter-university department of agriculture (IFA) in Tulln. With a reduction from 10 g to 5 g of the sample weight while maintaining the extraction volume at 20 ml, the matrix effect should be reduced. This improvement should take a positive impact on the recovery rate in percent as well as on the chromatographic allocation of the analytes.

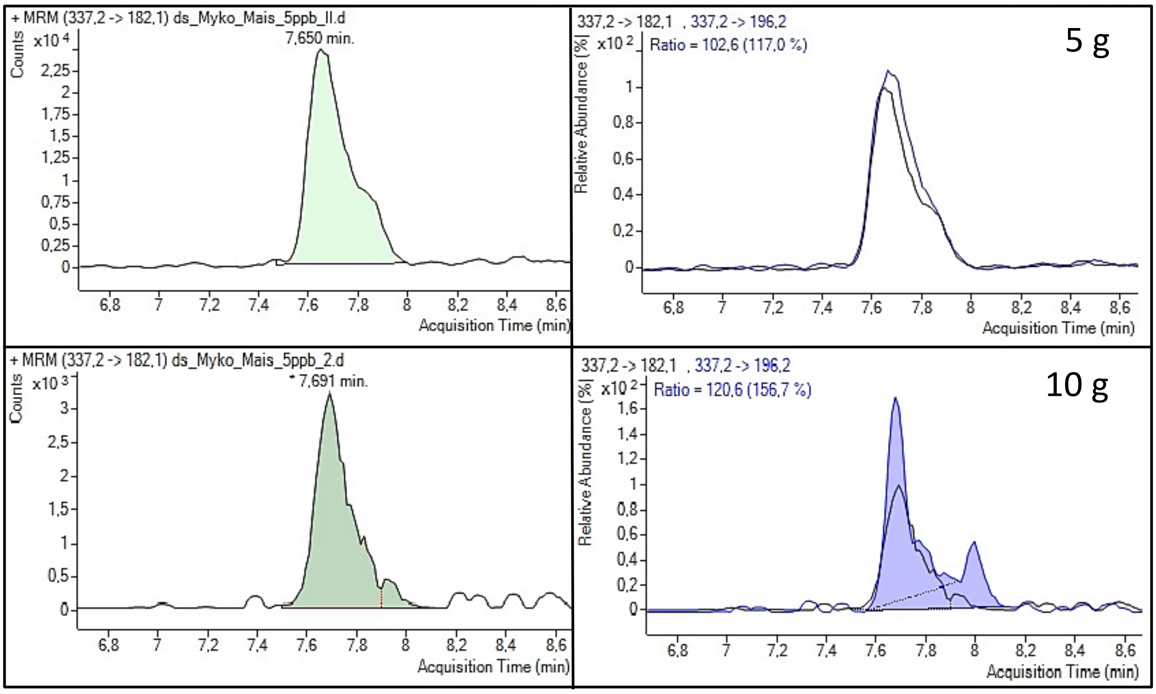


Figure 18: Chromatogram of cyclopiazonic acid in maize at 5 µg/kg

In *figure 18*, a chromatographic comparison of cyclopiazonic acid in maize is shown. Based on the peak shapes it is clearly evident that the lower sample weight helps for a better detection of the analyte. This is especially visible through a superior overlap of the qualifier with the lower weight. Further the recovery rate reaches 106 % (mean value: 5.33 µg/kg) with 5 g weight versus 36 % (mean value: 1.83 µg/kg) with 10 g weight. The samples were tested in dual approach and spiked with 5 µg/kg.

Dilutions

A further way to reduce unwanted matrix impacts is to dilute the sample extract with water. The dimension of this effect is depending on the dilution factor. In this work dilutions of 1:2, 1:5 and 1:10 were applied for the 9 matrices which were validated. A reasonable dilution can reduce the impact of overload effects and disturbing elements which are bonded in the matrix. Thereby the background noise of the chromatogram can be reduced significantly resulting in a better peak shape, which helps for the assignment of analytes and improve the recovery rate. For the further usage of the raw data, it is important to take the influence of the dilution on the measured value into account. The measured result is reduced by the value of the dilution factor and therefore the calibration curve has to be adjusted on the expected values. But the higher the dilution the lower gets the sensitivity of the instrument, whereby the use of a dilution has to be estimated according to the matrix and the losses of sensitivity. The opposite of a dilution is the concentrating. This part of the sample preparation is often used for samples of high volume to avoid analyte losses.

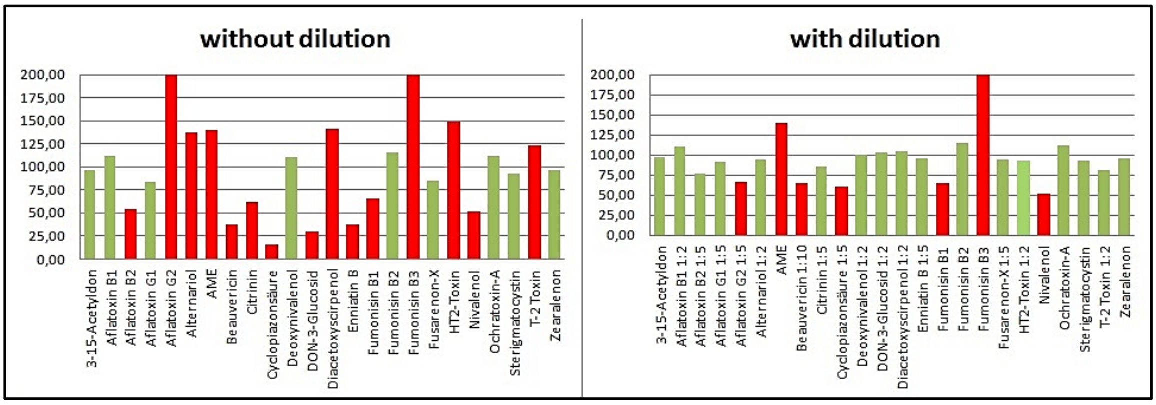


Figure 19: Recovery in percent with and without dilution for 25 analytes in marble cake

In *figure 19*, a comparison between the recovery rates in percent with and without dilutions in marble cake is shown. The results with dilutions include the recovery rate of each analyte with the optimal dilution factor. With dilution, 18 from 25 analytes are in the striven recovery rate of 70-120 % (self determined criteria – green bar) compared to 10 from 25 analytes without dilution.

Matrix Impacts

The amount and impact of interfering matrix compounds depends on the matrix itself and varies even within the same product group. The co-elution of these disturbing compounds at the same retention time as the analytes results in a high signal suppression of mycotoxins within the chromatogram. Furthermore, there is an association of negative matrix effects between the chemical attributes of the analyte or the matrix. As described in the chapter before, signal suppression through the co eluting matrix components can be reduced by dilution of the extract. Complex matrix trials with high negative impact on the signal intensity were made with coffee, pepper and red yeast rice. [GÓMEZ-RAMOS et al., 2013]

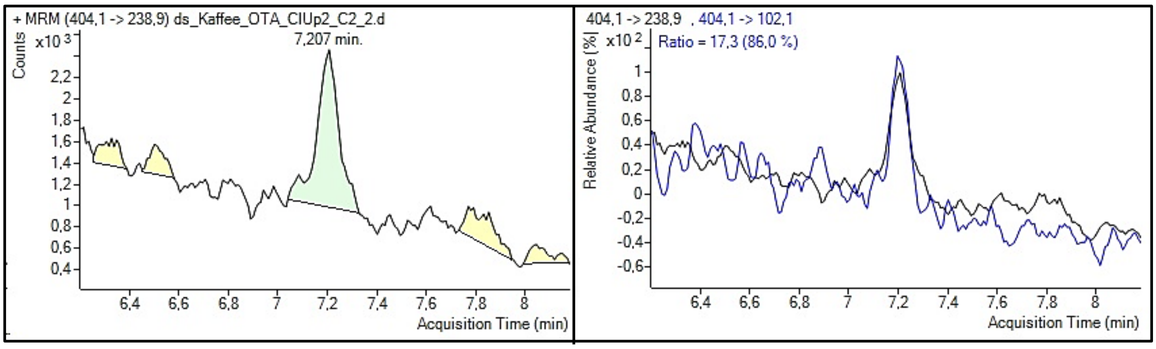


Figure 20: Chromatogram of ochratoxin A in coffee, spiked with 2.5 µg/kg

In *figure 20*, a chromatogram of ochratoxin A in coffee is shown. The sample was spiked with 2.5 µg/kg of a single standard solution. To lower disturbing matrix effects the sample weight was reduced to 2 gram. For a better clean up the samples were further treated with MycoSep® 229 Ochra push trough columns from Romer Labs. In the chromatogram on the right, showing an overlap between quantifier and qualifier, a high background noise is clearly visible, resulting in an unprecise allocation of the analyte. The recovery rate was hereby at 134 % and thus clearly above an optimal result. To achieve a better allocation of the analyte a further reduction of the sample weight could help to reduce unwanted matrix impacts. Additionally, a different clean up for instance with immunoaffinity columns could also be useful.

Similar matrix impacts are expected from spices like pepper, chili or curry. Analytical trials with ochratoxin A and aflatoxin B1, B2, G1 and G2 were carried out in pepper. Hereby the sample weight was reduced to 1 gram and a clean-up was done with MycoSep® 229 Ochra and 224 AflaZON columns to lower the potential of interfering substances.

Figure 21: Chromatogram of ochratoxin A in pepper, spiked with 5 µg/kg

Figure 21 shows a chromatogram of ochratoxin A at an expected concentration of 5 µg/kg. The recovery rate is hereby at 58 % primarily resulting by reduced signal intensity. The allocation of this analyte is further impeded by a bad qualifier ratio.

Red yeast rice is a traditional Chinese food processed by fermentation of the mold *Monascus purpureus*, which causes the typical red colour. Food supplements based on red yeast rice like angkak are currently very popular because of several positive attributed effects as maintaining a normal serum cholesterol and triglyceride level, resulting in a positive influence on coronary heart diseases. Because of this pharmacological effect this product is often seen as a medical drug instead of a food supplement, which makes a clear classification more complicate. So the compliance regarding a safe intake of this product is important in two ways. The intake should not exceed physiological dosages and the exposition with citrinin should be minimized. Citrinin is the major produced mycotoxin of this mold and is therefore regulated by the European Commission in this matrix with 2,000 µg/kg. Due to this existing regulation for citrinin, analytical trials were made with this substance in this matrix.

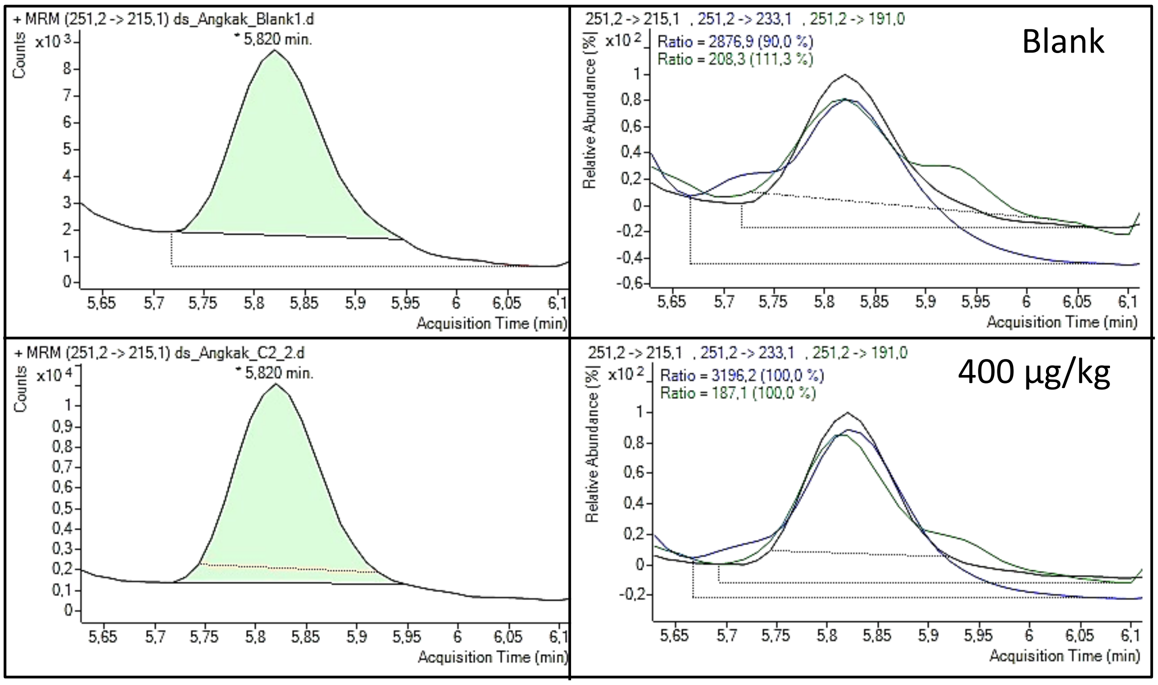


Figure 22: Chromatogram of citrinin in red yeast rice blank and spiked with 400 µg/kg

In *figure 23*, a comparison between a blank and a 400 µg/kg spiked sample of red yeast rice is shown. Hereby the internal standard was added to the sample directly after weight. In order to correct the sample preparation step additionally to the matrix correction. On the basis of the quantitative response, peak shape and the qualifiers, it is obvious that there is no significant difference between these two samples. Therefore it can be concluded that the sample material shows a natural contamination with this mycotoxin. However, a comparison of the responses of these samples shows neither a significant difference. The response of the blank sample is at 41,348 counts and the response of the spiked sample at 57,527 counts. Compared to the response of an appropriate value from the calibration in solvent, the expected response for 400 µg/kg should be located at about 5,000,000 counts. The response of the internal standard in the calibration levels shows up in the range of 19,278,169 and 24,201,012 counts compared to a response range of 197,009 and 258,420 counts in the samples, which leads to a difference by the factor 100. Based on these results the calculated value for the blank sample is at 1,092 µg/kg and for the spiked sample at 2,019 µg/kg resulting in a recovery rate of 232 %. So it can be concluded that the matrix takes a strong influence on the quantification.

Matrix Matched Calibration

For a better demonstration of matrix effects a so called matrix matched calibration is useful. In this way the extract of a processed blank sample is used for the preparation of the calibration instead of a solvent. The matrix matched calibration is therefore used for the quantification of the analyte with correction of the matrix ionization influence and makes thus the use of internal standards redundant. However, a routine application of this method is not possible because of the high labor intensity. For a clarification of different matrix influences, matrix matched calibrations were prepared for 5 matrices.

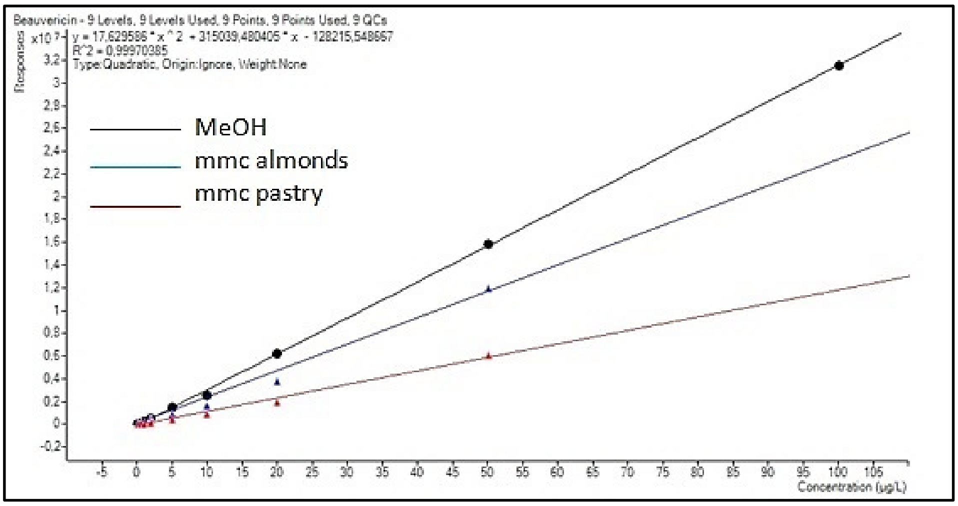


Figure 23: MMC of beauvericin in almonds and pastry

Figure 23 shows a comparison of calibration curves of beauvericin in methanol (black) and matrix matched calibrations in almonds (blue) and pastry (red). As shown, both matrices are taking a massive lowering influence on the analyte, due to a signal suppression. The recovery rate for a spiked concentration of 20 µg/kg based on the solvent calibration is at 65 % in almonds and 30 % in pastry. By comparison, the recovery rate based on the specific matrix matched calibration is at 110 % in almonds and a recovery rate of 80 % in pastry. Another matrix induced signal suppression was observed with enniatin B in wheat flour and is shown in *figure 24*.

The recovery rate for a spike concentration of 10 µg/kg is at 60 % based on methanol calibration and 93 % based on matrix matched calibration.

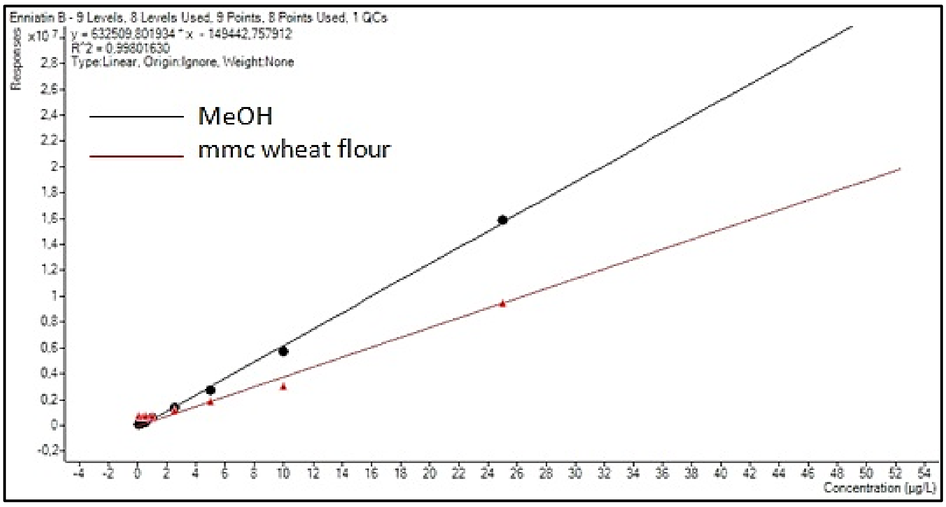


Figure 24: MMC of enniatin B in wheat flour

However, matrices can also show a raising effect on the analyte recovery, based on a signal enhancement, observed with alternariol-methylether in wholemeal bread and marble cake and is demonstrated in *figure 25*. The matrix matched calibration based recovery rate at a spike concentration of 50 µg/kg is at 90 % in marble cake against 140 % with a calibration in solvent and 97 % in wholemeal bread against 152 % to the solvent calibration.

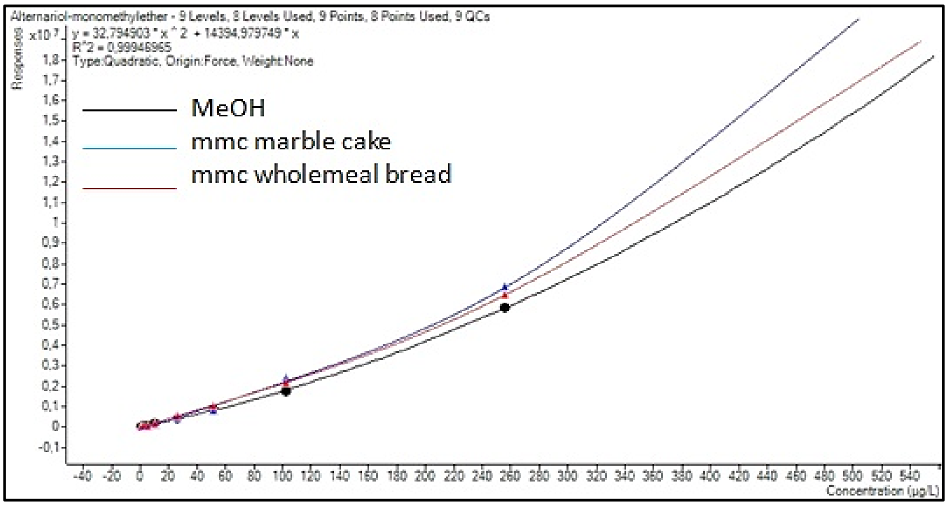


Figure 25: MMC of alternariol-methylether in wholemeal bread and marble cake

Exclusion of Analytes and Matrices

Based on analytical preliminary investigations several analytes and matrices were excluded for the validation. The exclusion implies analytes which cannot be determined in several matrices even in high concentrations. Hereby patulin, deoxynivalenol-3-glucoside and nivalenol are affected. The poor detection of these substances is probably based on the influence of the extraction method, the molecule characteristics or due to a failed optimisation. Further aflatoxin M1 was excluded because of similar properties concerning quantifier, qualifier and retention time compared to other toxins of this family. The deoxynivalenol metabolites 3- and 15-acetyldeoxynivalenol are optimised and implemented as the sum of both substances, because of identical quantifiers, qualifiers and retention times. In addition several matrices were excluded for the validation because of massive previously described matrix impacts. Hereby pepper, coffee and red yeast rice were affected and require specific clean up steps to comply with defined validation performance criteria.

Validation Results

The choice of relevant matrices for validation was based on sample amounts of the year 2015. FB3 performance criteria was adjusted on FB1 and FB2 criteria based on the EC 401/2006. AcDON performance criteria was adjusted on DON criteria.

Table 10: Validated matrices with sample amounts (2015)

matrix	samples 2015	matrix	samples 2015
almonds	103	cake (marble)	40
maize	90	oat flakes	32
pastry	37	sultanas	85
walnuts	101	wheat flour	49
bread (wholemeal)	198		

Maize

Table 11: Mycotoxin validation results in maize

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean RR in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	105	5	
Aflatoxin B1	0.21	0.95	114	13	5 B1/ 10 in sum
Aflatoxin B2	0.20	0.96	157	27	10 in sum
Aflatoxin G1	0.20	0.96	113	9	10 in sum
Aflatoxin G2	0.20	0.95	163	14	10 in sum
Alternariol	50.0	100.0	237	19	
Alternariol-methylether	20.5	51.2	156	15	
Beauvericin	10.1	20.0	29	22	
Citrinin	40.0	70.1	51	17	
Cyclopiazonic Acid	50.2	100.7	131	5	
Deoxynivalenol	20.1	50.2	82	12	1750
Diacetoxyscirpenol	10.0	50.2	107	12	
Enniatin B	5.00	10.00	46	28	
Fumonisin B1	50.9	101.8	300	58	4,000 in sum with FB2
Fumonisin B2	50.1	100.2	191	18	4,000 in sum with FB1
Fumonisin B3	50.0	100.0	159	34	
Fusarenon-X	50.2	100.3	48	54	
HT2-Toxin	5.04	10.07	106	24	200 in sum with T2
Ochratoxin-A	1.50	3.01	106	15	5
Sterigmatocystin	10.2	20.2	116	6	
T-2 Toxin	5.03	10.06	120	10	200 in sum with HT2
Zearalenone	20.0	50.1	108	9	350

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

In maize, performance criteria were successfully reached for 7 by the EU 401/2006 regulated substances AFB1, AFG1, DON, HT2, OTA, T2, ZON. Further, a RR within 70 and 120 % with a RSD lower than 20 % was reached for 4 non-regulated substances AcDONs, DAS, STE. At least one specific performance criteria was not achieved for 4 regulated AFB2, AFG2, FB1, FB2 and 8 non-regulated substances AOH, AME, BEA, CIT, CPA, ENB, FB3, FX. A possible reason for the non-achievement of these substances could be a negative impact by disturbing matrix compounds. Especially due to a high amount of carbohydrates with 64 g per 100 g maize and fat with 4 g per 100 g maize.

Wheat flour

Table 12: Mycotoxin validation results in wheat flour

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	105	9	
Aflatoxin B1	0.21	0.95	129	10	**2 B1/ 4 in sum**
Aflatoxin B2	0.20	0.96	128	13	**4 in sum**
Aflatoxin G1	0.20	0.96	140	16	**4 in sum**
Aflatoxin G2	0.20	0.95	118	17	**4 in sum**
Alternariol	50.0	100.0	163	18	
Alternariol-methylether	20.5	51.2	132	15	
Beauvericin	10.1	20.0	27	18	
Citrinin	40.0	70.1	43	10	
Cyclopiazonic Acid	50.2	100.7	128	6	
Deoxynivalenol	20.1	50.2	97	6	**750**
Diacetoxyscirpenol	10.0	50.2	109	8	
Enniatin B	5.00	10.00	59	23	
Fumonisin B1	50.9	101.8	158	29	
Fumonisin B2	50.1	100.2	183	26	
Fumonisin B3	50.0	100.0	192	37	
Fusarenon-X	50.2	100.3	72	58	
HT2-Toxin	5.04	10.07	105	24	**50 in sum with T2**
Ochratoxin-A	1.50	3.01	112	16	**3**
Sterigmatocystin	10.2	20.2	118	9	
T-2 Toxin	5.03	10.06	117	14	**50 in sum with HT2**
Zearalenone	20.0	50.1	108	11	**75**

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

In wheat flour, 5 regulated analytes AFG2, DON, HT2, T2, ZON and 4 non-regulated analytes AcDONs, DAS, STE successfully reached the performance criteria, while 4 regulated substances AFB1, AFB2, AFG1, OTA and 10 non-regulated compounds AOH, AME, BEA, CIT, ENB, FB1, FB2, FB3, FX did not reach at least one criteria. Similar matrix effects to maize can be held responsible for the non-achievement of the performance criteria. The carbohydrate amount is hereby at 67 g per 100 g wheat flour and fat at about 2 g per 100 g.

Oat Flakes

Table 13: Mycotoxin validation results in oat flakes

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	106	5	
Aflatoxin B1	0.21	0.95	132	7	2 B1 / 4 in sum
Aflatoxin B2	0.20	0.96	137	14	4 in sum
Aflatoxin G1	0.20	0.96	124	14	4 in sum
Aflatoxin G2	0.20	0.95	123	14	4 in sum
Alternariol	50.0	100.0	211	17	
Alternariol-methylether	20.5	51.2	126	14	
Beauvericin	10.1	20.0	16	15	
Citrinin	40.0	70.1	65	16	
Cyclopiazonic Acid	50.2	100.7	81	5	
Deoxynivalenol	20.1	50.2	108	8	500
Diacetoxyscirpenol	10.0	50.2	112	10	
Enniatin B	5.00	10.00	53	37	
Fumonisin B1	50.9	101.8	162	47	
Fumonisin B2	50.1	100.2	157	6	
Fumonisin B3	50.0	100.0	154	47	
Fusarenon-X	50.2	100.3	102	50	
HT2-Toxin	5.04	10.07	126	10	200 in sum with T2
Ochratoxin-A	1.50	3.01	92	13	3
Sterigmatocystin	10.2	20.2	116	5	
T-2 Toxin	5.03	10.06	119	15	200 in sum with HT2
Zearalenone	20.0	50.1	117	13	50

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

Performance criteria in oat flakes were reached by 5 regulated substances DON, HT2, OTA, T2, ZON and by 5 non-regulated substances AcDONs, CPA, DAS, STE. Whereas 4 regulated analytes AFB1, AFB2, AFG1, AFG2 and 9 non-regulated analytes AOH, AME, BEA, CIT, ENB, FB1, FB2, FB3, FX did not reach at least one criteria. Nutrients with a possible negative impact on recovery rate and reproducibility are carbohydrates with 61 % and fat with about 7 % of total share. Additionally oat flakes are very rich in dietary fibres like beta-glucan, a further potential disturbing compound.

Wholemeal Bread

Table 14: Mycotoxin validation results in wholemeal bread

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	95	9	
Aflatoxin B1	0.21	0.95	125	12	**2 B1/ 4 in sum**
Aflatoxin B2	0.20	0.96	101	13	**4 in sum**
Aflatoxin G1	0.20	0.96	116	18	**4 in sum**
Aflatoxin G2	0.20	0.95	98	22	**4 in sum**
Alternariol	50.0	100.0	220	27	
Alternariol-methylether	20.5	51.2	145	13	
Beauvericin	10.1	20.0	43	19	
Citrinin	40.0	70.1	92	19	
Cyclopiazonic Acid	50.2	100.7	114	6	
Deoxynivalenol	20.1	50.2	95	7	**500**
Diacetoxyscirpenol	10.0	50.2	110	9	
Enniatin B	5.00	10.00	74	19	
Fumonisin B1	50.9	101.8	218	72	
Fumonisin B2	50.1	100.2	179	11	
Fumonisin B3	50.0	100.0	164	41	
Fusarenon-X	50.2	100.3	59	51	
HT2-Toxin	5.04	10.07	113	10	**25 in sum with T2**
Ochratoxin-A	1.50	3.01	109	14	
Sterigmatocystin	10.2	20.2	113	7	
T-2 Toxin	5.03	10.06	110	9	**25 in sum with HT2**
Zearalenone	20.0	50.1	114	7	**50**

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

In wholemeal bread, 7 regulated substances AFB2, AFG1, AFG2, DON, HT2, T2, ZON and 8 non-regulated substances AcDONs, CIT, CPA, DAS, ENB, OTA, STE have reached their specific performance criteria and only one regulated analyte AFB1 did not reach criteria concerning recovery rate. Also 7 non-regulated substances AOH, AME, BEA, FB1, FB2, FB3, FX did not reach at least one criteria. Potential disturbing matrix components are hereby complex high molecular dietary fibre like lignin, which could have a major impact on the chromatographic determination.

Marble Cake

Table 15: Mycotoxin validation results in marble cake

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	114	6	
Aflatoxin B1	0.21	0.95	156	7	**2 B1/ 4 in sum**
Aflatoxin B2	0.20	0.96	125	16	**4 in sum**
Aflatoxin G1	0.20	0.96	133	13	**4 in sum**
Aflatoxin G2	0.20	0.95	127	14	**4 in sum**
Alternariol	50.0	100.0	177	16	
Alternariol-methylether	20.5	51.2	134	12	
Beauvericin	10.1	20.0	36	15	
Citrinin	40.0	70.1	80	13	
Cyclopiazonic Acid	50.2	100.7	141	6	
Deoxynivalenol	20.1	50.2	90	8	**500**
Diacetoxyscirpenol	10.0	50.2	120	12	
Enniatin B	5.00	10.00	63	22	
Fumonisin B1	50.9	101.8	211	78	
Fumonisin B2	50.1	100.2	180	13	
Fumonisin B3	50.0	100.0	165	23	
Fusarenon-X	50.2	100.3	70	60	
HT2-Toxin	5.04	10.07	128	18	**25 in sum with T2**
Ochratoxin-A	1.50	3.01	106	11	
Sterigmatocystin	10.2	20.2	119	7	
T-2 Toxin	5.03	10.06	130	12	**25 in sum with HT2**
Zearalenone	20.0	50.1	120	10	**50**

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

3 regulated analytes DON, HT2, T2 and 4 non-regulated substances CIT, DAS, OTA, STE have reached the performance criteria in marble cake. On the other hand 5 regulated anlytes AFB1, AFB2, AFG1, AFG2, ZON and 11 non-regulated compounds AcDONs, AOH, AME, BEA, CPA, ENB, FB1, FB2, FB3, FX exceed at least in one criteria. Hereby, the non-achievement of specific performance criteria of so many analytes is probably related to a very high amount of fat with 16 % of total share and also a high amount of carbohydrates with 52 % of total share.

Pastry

Table 16: Mycotoxin validation results in pastry

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	50.4	100.7	101	7	
Aflatoxin B1	0.21	0.95	122	8	
Aflatoxin B2	0.20	0.96	124	10	
Aflatoxin G1	0.20	0.96	129	9	
Aflatoxin G2	0.20	0.95	107	19	
Alternariol	50.0	100.0	159	21	
Alternariol-methylether	20.5	51.2	128	14	
Beauvericin	10.1	20.0	22	13	
Citrinin	40.0	70.1	96	10	
Cyclopiazonic Acid	50.2	100.7	125	5	
Deoxynivalenol	20.1	50.2	97	6	**750**
Diacetoxyscirpenol	10.0	50.2	103	12	
Enniatin B	5.00	10.00	45	21	
Fumonisin B1	50.9	101.8	184	53	
Fumonisin B2	50.1	100.2	189	16	
Fumonisin B3	50.0	100.0	189	33	
Fusarenon-X	50.2	100.3	68	56	
HT2-Toxin	5.04	10.07	116	19	
Ochratoxin-A	1.50	3.01	104	13	
Sterigmatocystin	10.2	20.2	110	9	
T-2 Toxin	5.03	10.06	107	10	
Zearalenone	20.0	50.1	108	12	

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

An existing regulation for pasta products like pastry is only made for one substance, DON, which has successfully reached its specific performance criteria. Additionally, 10 substances AcDONs, AFG2, CIT, DAS, HT2, OTA, STE, T2, ZON which are not regulated have also reached the striven targets. Furthermore, the 12 remaining non-regulated analytes AFB1, AFB2, AFG1, AOH, AME, BEA, CPA, ENB, FB1, FB2, FB3, FX did not reach at least one specific criteria. Pastry shows with 25 % fat of total share very high amounts of this macronutrient, which could be related to massive matrix impacts.

Almonds

Table 17: Mycotoxin validation results in almonds

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	150.8	301.5	83	7	
Aflatoxin B1	0.62	2.86	109	12	8 B1/ 10 in sum
Aflatoxin B2	0.61	2.88	108	5	10 in sum
Aflatoxin G1	0.61	2.88	93	14	10 in sum
Aflatoxin G2	0.60	2.84	102	17	10 in sum
Alternariol	149.7	299.4	144	15	
Alternariol-methylether	61.3	153.1	89	11	
Beauvericin	30.3	59.9	52	26	
Citrinin	119.9	209.8	51	10	
Cyclopiazonic Acid	150.2	301.5	99	6	
Deoxynivalenol	60.1	150.3	86	8	
Diacetoxyscirpenol	30.0	150.2	93	10	
Enniatin B	14.9	29.9	57	21	
Fumonisin B1	152.4	304.8	202	93	
Fumonisin B2	150.0	300.0	136	18	
Fumonisin B3	149.7	299.4	120	38	
Fusarenon-X	150.2	300.3	60	55	
HT2-Toxin	15.1	30.2	83	20	
Ochratoxin-A	4.50	9.00	81	20	
Sterigmatocystin	30.5	60.6	92	8	
T-2 Toxin	15.1	30.1	98	12	
Zearalenone	59.9	149.9	87	13	

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

Based on a mixing ratio of 1:1.5 of almonds with water, the spike concentration changed, because of a lower sample weight. All 4 regulated compounds AFB1, AFB2, AFG1, AFG2 and additionally 10 non-regulated substances AcDONs, AME, CPA, DON, DAS, HT2, STE, T2, ZON showed optimal recovery rates and relative standard deviation. 9 non-regulated analytes AOH, BEA, CIT, ENB, FB1, FB2, FB3, FX, OTA did not reach at least one target criteria. The major negative matrix impact is hereby related to a very high fat amount of 54 % of total share.

Walnuts

Table 18: Mycotoxin validation results in walnuts

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	150.8	301.5	82	9	
Aflatoxin B1	0.62	2.86	114	14	8 B1/ 10 in sum
Aflatoxin B2	0.61	2.88	96	15	10 in sum
Aflatoxin G1	0.61	2.88	90	18	10 in sum
Aflatoxin G2	0.60	2.84	89	34	10 in sum
Alternariol	149.7	299.4	134	21	
Alternariol-methylether	61.3	153.1	97	12	
Beauvericin	30.3	59.9	37	18	
Citrinin	119.9	209.8	54	18	
Cyclopiazonic Acid	150.2	301.5	99	4	
Deoxynivalenol	60.1	150.3	100	10	
Diacetoxyscirpenol	30.0	150.2	91	11	
Enniatin B	14.9	29.9	54	24	
Fumonisin B1	152.4	304.8	197	87	
Fumonisin B2	150.0	300.0	142	22	
Fumonisin B3	149.7	299.4	125	50	
Fusarenon-X	150.2	300.3	48	55	
HT2-Toxin	15.1	30.2	87	15	
Ochratoxin-A	4.50	9.00	81	17	
Sterigmatocystin	30.5	60.6	88	8	
T-2 Toxin	15.1	30.1	92	14	
Zearalenone	59.9	149.9	87	12	

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

In walnuts, the mixing ratio with water was 1:1.5 as well. Hereby 2 regulated analytes AFB2, AFG1 and 11 non-regulated analytes AcDONs, AME, CPA, DON, DAS, HT2, OTA, STE, T2, ZON have reached their specific performance criteria. Furthermore, 2 regulated compounds AFB1, AFG2 and 8 non-regulated substances AOH, BEA, CIT, ENB, FB1, FB2, FB3, FX did not reach the specific recovery rate or relative standard deviation. Similar to almonds the high fat content with about 63 % of total share is the major disturbing matrix component in walnuts.

Sultanas

Table 19: Mycotoxin validation results in sultanas

Analyte	conc. low in µg/kg	conc. high in µg/kg	mean in %	RSD in %	ML in µg/kg
3-15-Acetyldeoxynivalenol	100.7	201.4	126	6	
Aflatoxin B1	0.41	1.91	159	8	**2 B1/ 4 in sum**
Aflatoxin B2	0.40	1.92	159	9	**4 in sum**
Aflatoxin G1	0.40	1.92	92	15	**4 in sum**
Aflatoxin G2	0.40	1.90	119	14	**4 in sum**
Alternariol	100.0	200.0	199	18	
Alternariol-methylether	40.9	102.3	188	13	
Beauvericin	20.2	40.0	65	20	
Citrinin	80.1	140.1	117	14	
Cyclopiazonic Acid	100.3	201.4	182	11	
Deoxynivalenol	40.2	100.4	88	12	
Diacetoxyscirpenol	20.1	100.3	139	8	
Enniatin B	10.0	20.0	81	23	
Fumonisin B1	101.8	203.6	239	94	
Fumonisin B2	100.2	200.4	206	9	
Fumonisin B3	100.0	200.0	173	40	
Fusarenon-X	100.3	200.6	82	54	
HT2-Toxin	10.1	20.1	147	23	
Ochratoxin-A	3.01	6.01	138	14	**10**
Sterigmatocystin	20.4	40.5	149	9	
T-2 Toxin	10.1	20.1	136	11	
Zearalenone	40.0	100.1	149	11	

conc.: concentration; RR: recovery rate; RSD: relative standard deviation; ML: maximum level;
green: within performance criteria; red: exceed performance criteria (regulated); yellow: exceed performance criteria (non-regulated)

In sultanas, the mixing ratio with water is 1:1. Corresponding changes of the spike concentrations are listed in *table 19*. In this matrix, 2 regulated analytes AFG1, AFG2 and 2 non-regulated substances CIT, DON reached the performance criteria. The remaining 19 regulated and non-regulated compounds AcDONs, AFB1, AFB2, AOH, AME, BEA, CPA, DAS, ENB, FB1, FB2, FB3, FX, HT2, OTA, STE, T2, ZON did not reach at least one target criteria. The non-achievement of almost all substances is probably related to a high amount of low-molecular carbohydrates with 65 % of total share.

Validation Summary

A successful validation and thus an optimal achievement of the specific performance criteria concerning percentage recovery rate and relative standard deviation was made by 48 % of all analytes in maize, 39 % in wheat flour, 43 % in oat flakes, 65 % in wholemeal bread, 30 % in marble cake, 47 % in pastry, 61 % in almonds, 57 % in walnuts and 17 % in sultanas. On average of all matrices, 45 % of all compounds (57 % regulated substances) reached their criteria successfully.

In 93 % of all validated matrices, the 5 analytes (22 % of total agents) AOH, AME, BEA, ENB and FX did not reach especially the optimal recovery rate. A possible reason for the non-achievement is a non-availability of internal standards for these substances. This is resulting in a non-correction of matrix impacts, which significantly influences the striven targets. The most likely matrix effects are related to high amounts of fat, low and high molecular carbohydrates.

The aflatoxins B1, B2, G1 and G2 (17 % of total agents) did not reach the specifications, basically concerning percentage recovery rate in 58 % of all validated matrices. However, in most of the cases the overriding of this criterion was of limited extent. The reason for this was possibly the very low spike concentration of less than 1 µg/kg in most of the matrices. Although these substances are analytically well determinable in low concentrations, an increase of the endowed amounts may have led to better outcomes.

Unfortunately, fumonisin B1, B2 and B3 (13 % of total agents) did not accomplish a positive validation result in any matrix. A possible reason for this poor performance could be related to the calibration solvent. Prior analytical trials have shown that the reproducibility and recovery rates are more consistent with a calibration solved in ACN:H_2O:HCOOH (79:20:1), instead of methanol. Further remains to mention, that fumonisins are difficult analytes concerning repeatability of analytical determinations. For safe analytical results a specific clean up should be therefore taken into account.

Conclusion

Mycotoxins are substances of low molecular weight and are synthesized by moulds as secondary metabolites. Thus they can be classified as natural contaminants and infest food and feed under proper conditions. Related to their chemical attributes they show a wide range of toxicological mode of action, whereas a chronic intake of these compounds can lead to massive organ damages. Furthermore, even a low intake of several substances can cause an acute life-threatening situation and promote the pathogenesis of cancer.

Based on the health hazards originating from these toxins, the European Commission draft an order to regulate these compounds with maximum levels in relevant food matrices in 2006. A continuous risk-related evaluation of these substances is one of the main tasks of national and international authorities in the section of food safety.

Thus, one of the targets of laboratories specialized on food analysis should be a regular development of methods for the determination of these compounds in food and feed. Hereby the spectrum of active reagents should be adjusted on the current existing regulation from the Commission. It is further important to verify the reliability of such multi-methods by continuous validations and control of the quality by participating on proficiency tests or comparative studies.

For a successful validation of these analytes in different matrices, the passing of individual performance criteria is essential. Preliminary trials should be therefore conducted to optimise all analytical method-parameters to ensure a fast, precise and rugged method. In this work 57 % of regulated substances reached their specific performance criteria and are thus successfully validated for the corresponding matrix.

A positive validation of difficult food matrices like coffee, spices or food supplements as well as a safe analytical determination of complex analytes like fumonisins, or patulin should be made through special individual clean-up steps to ensure consumers health.

Zusammenfassung

Mykotoxine sind niedermolekulare Substanzen, die als Sekundärmetabolite von Schimmelpilzen gebildet werden. Sie gelten als natürliche Kontaminanten und können bei ungünstigen Bedingungen in Nahrungs- und Futtermitteln auftreten. Aufgrund ihrer chemischen Eigenschaften entfalten diese Verbindungen ein breites toxisches Wirkspektrum und können bei chronischem Verzehr zu massiven Organschädigungen führen. Des Weiteren kann der Verzehr von vereinzelten Substanzen in bereits geringen Mengen eine akute lebensbedrohliche Situation hervorrufen.

Aufgrund der Gefahr, die von diesen Toxinen ausgeht, wurde im Jahr 2006 von der Kommission der Europäischen Union eine Verordnung etabliert, die Höchstgehalte für diese Verbindungen in diversen Lebensmittelgruppen regelt. Die laufende risikobezogene Evaluierung dieser Substanzen stellt eine der Hauptaufgaben von nationalen und internationalen Autoritäten im Bereich der Lebensmittelsicherheit dar.

Daher sollte das Ziel eines lebensmittelanalytischen Unternehmens die laufende Weiterentwicklung von Methoden zum Nachweis dieser Substanzen darstellen. Dabei sollte das Wirkstoffspektrum an bestehende Regelungen der Verordnung angepasst werden. Die Zuverlässigkeit solcher Methoden muss mittels regelmäßiger Validierungen gesichert und durch die Teilnahme an Ringversuchen oder Vergleichsuntersuchungen qualitativ überprüft werden.

Das Erreichen von substanzspezifischen Leistungskriterien steht bei der Validierung einer Methode im Vordergrund. Voruntersuchungen sollten hierbei zur Optimierung analytischer Methodenparameter dienen, um eine schnelle, genaue und robuste Methode zu entwickeln. In dieser Arbeit haben 57 % aller geregelten Analyten die jeweiligen Leistungskriterien erfüllt und eine Validierung kann für die entsprechenden Matrizen als erfolgreich betrachtet werden.

Die Bestimmung schwer analysierbarer Substanzen wie Fumonisine, oder Patulin sollte mittels speziellen Clean-ups gesichert werden.

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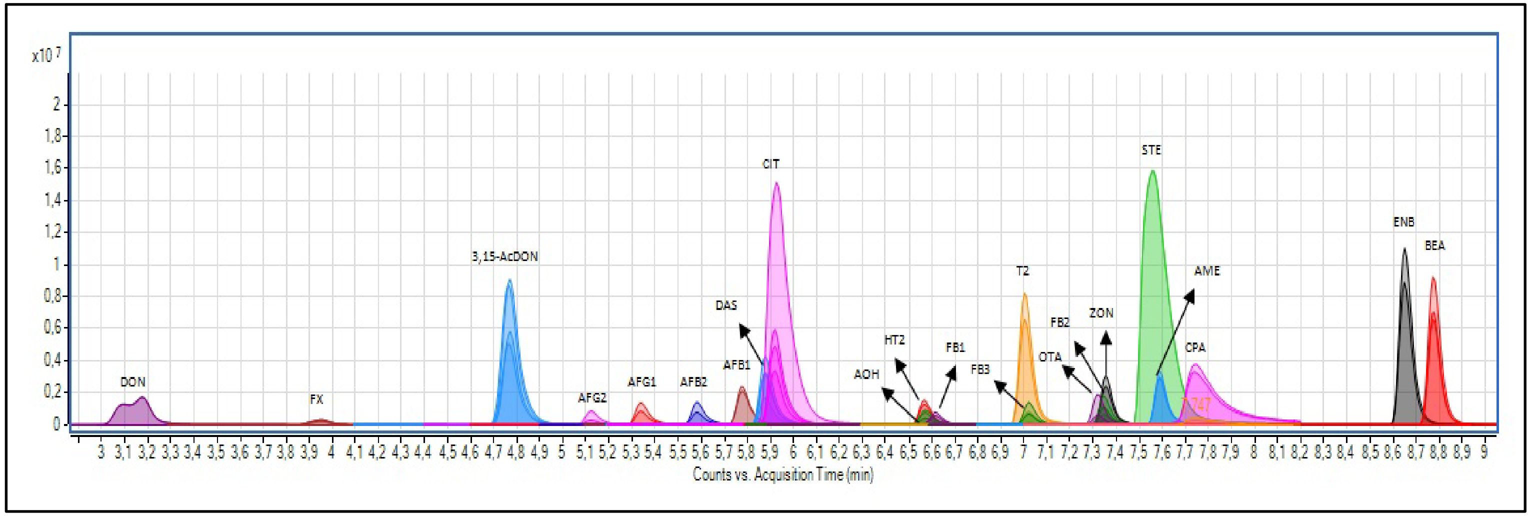
Annex

Comparison of former and optimised gradient conditions

former gradient conditions		optimised gradient conditions	
Time (min)	Ratio (A:B)	Time (min)	Ratio (A:B)
0	70:30	0	90:10
0.5	70:30	0.5	90:10
8	0:100	8	0:100
9.5	0:100	9.5	0:100
9.6	70:30	9.6	70:30
11.5	70:30	11.5	90:10

Chromatogram of 23 validated analytes with optimised gradient conditions



Retention time comparison of 100 µg/l nivalenol-standard solutions with former and optimised gradient conditions

former conditions

optimised conditions

Preparation of calibration solutions in solvent MeOH

standard solution no.	calibration solution	calibration volume (µl)	solvent volume (µl)
L7	mycotoxin working solution	1000	0
L6	mycotoxin working solution	500	500
L5	mycotoxin working solution	250	750
L4	mycotoxin working solution	100	900
L3	standard solution L6	100	900
L2	standard solution L5	100	900
L1	standard solution L2	100	900

dilution: 1:2:2:2,5:2:2:10

Preparation of the mycotoxin working solution for the seven-level standard curve in 5 ml solvent (MeOH)

3AcD	15AcD	AflaB1	AflaB2	AflaG1	AflaG2	AME	AOH	BEA	CIT	CPA	DAS	DON	ENB	FumB1	FumB2	FumB3	Fus-X	HT2	OTA	STG	T2	ZON	
250	**250**	**500**				**500**	**500**	**50**	**5**	**500**	**100**	**250**	**100**	**50**		**50**	**250**	**100**	**250**	**100**	**50**	**250**	**standard vol. in µl**
2.01	2.02	0.01	0.01	0.01	0.01	2.05	4.00	2.00	100.10	2.01	2.01	2.01	1.00	50.90	50.10	50.00	2.01	2.01	0.20	1.01	2.01	2.00	standard conc. in mg/l
100.40	101.00	1.00	1.01	1.01	1.00	204.60	400.00	20.02	100.10	201.40	40.12	100.40	20.00	509.00	501.00	500.00	100.30	40.28	10.03	20.24	20.12	100.10	working solution in µg/l

Level (µg/l)	3AcD	15AcD	AflaB1	AflaB2	AflaG1	AflaG2	AME	AOH	BEA	CIT	CPA	DAS	DON	ENB	FumB1	FumB2	FumB3	Fus-X	HT2	OTA	STG	T2	ZON
L7	100.40	101.00	1.00	1.01	1.01	1.00	204.60	400.00	20.02	100.10	201.40	40.12	100.40	20.00	509.00	501.00	500.00	100.30	40.28	10.03	20.24	20.12	100.10
L6	50.20	50.50	0.50	0.51	0.51	0.50	102.30	200.00	10.01	50.05	100.70	20.06	50.20	10.00	254.50	250.50	250.00	50.15	20.14	5.02	10.12	10.06	50.05
L5	25.10	25.25	0.25	0.25	0.25	0.25	51.15	100.00	5.01	25.03	50.35	10.03	25.10	5.00	127.25	125.25	125.00	25.08	10.07	2.51	5.06	5.03	25.03
L4	10.04	10.10	0.10	0.10	0.10	0.10	20.46	40.00	2.00	10.01	20.14	4.01	10.04	2.00	50.90	50.10	50.00	10.03	4.03	1.00	2.02	2.01	10.01
L3	5.02	5.05	0.05	0.05	0.05	0.05	10.23	20.00	1.00	5.01	10.07	2.01	5.02	1.00	25.45	25.05	25.00	5.02	2.01	0.50	1.01	1.01	5.01
L2	2.51	2.53	0.025	0.025	0.025	0.025	5.12	10.00	0.50	2.50	5.04	1.00	2.51	0.50	12.73	12.53	12.50	2.51	1.01	0.25	0.51	0.50	2.50
L1	0.25	0.25	0.0025	0.0025	0.0025	0.0025	0.51	1.00	0.05	0.25	0.50	0.10	0.25	0.05	1.27	1.25	1.25	0.25	0.10	0.03	0.05	0.05	0.25

Preparation of the ISTD-standard solution in 1 ml solvent (MeOH)

FumB1	FumB2	FumB3	AflaB1	AflaB2	AflaG1	AflaG2	DON	OTA	ZON	T2	HT2	3AcDON	CIT	DAS	CPA	STG	
25.10	10.01	10.02	0.51	0.50	0.51	0.52	25.00	10.08	25.10	25.10	25.40	25.00	10.60	25.00	10.10	25.40	native standard conc. in mg/l
80	**150**	**200**	**10**	**10**	**10**	**10**	**40**	**10**	**20**	**10**	**10**	**40**	**50**	**10**	**50**	**20**	**standard vol. in µl**
2008.00	1501.50	2004.00	5.10	5.00	5.07	5.15	1000.00	100.80	502.00	251.00	254.00	1000.00	530.00	250.00	505.00	508.00	mix standard conc. in µg/l
100.40	75.08	100.20	0.26	0.25	0.25	0.26	50.00	5.04	25.10	12.55	12.70	50.00	26.50	12.50	25.25	25.40	standard conc. in µg/l in sample/cal

0.2 µl ISTD-standard solution is automatically injected with 3.8 µl sample

Preparation of high spike-standard solution in 10 ml solvent (MeOH)

	FumB1	FumB2	FumB3	AflaB1	AflaB2	AflaG1	AflaG2	DON	OTA	ZON	T2	HT2	AOH	AME	3AcD	15AcD	CIT	DAS	Fus-X	CPA	STG	BEA	ENB
1	50.90	50.10	50.00	0.25	0.25	0.25	0.25	100.40	10.03	100.10	100.60	100.70	100.00	102.30	100.40	101.00	100.10	100.30	100.30	100.70	50.60	100.10	100.00
2	**1000**	**1000**	**1000**	**1900**	**1900**	**1900**	**1900**	**250**	**150**	**250**	**50**	**50**	**500**	**250**	**250**	**250**	**350**	**250**	**500**	**500**	**200**	**100**	**50**
3	5.09	5.01	5.00	0.05	0.05	0.05	0.05	2.51	0.15	2.50	0.50	0.50	5.00	2.56	2.51	2.53	3.50	2.51	5.02	5.04	1.01	1.00	0.50
4	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
5	101.80	100.20	100.00	0.95	0.96	0.96	0.95	50.20	3.01	50.05	10.06	10.07	100.00	51.15	50.20	50.50	70.07	50.15	100.30	100.70	20.24	20.02	10.00

1: native standard conc. in mg/l; *2:* standard vol. in µl; *3:* mix standard conc. in mg/l; *4:* spike volume in µl *5:* standard conc. in µg/l in 5 g sample;

Preparation of low spike-standard solution in 10 ml solvent (MeOH)

	FumB1	FumB2	FumB3	AflaB1	AflaB2	AflaG1	AflaG2	DON	OTA	ZON	T2	HT2	AOH	AME	3AcD	15AcD	CIT	DAS	Fus-X	CPA	STG	BEA	ENB
1	50.90	50.10	50.00	0.25	0.25	0.25	0.25	100.40	10.03	100.10	100.60	100.70	100.00	102.30	100.40	101.00	100.10	100.30	100.30	100.70	50.60	100.10	100.00
2	**500**	**500**	**500**	**400**	**400**	**400**	**400**	**100**	**75**	**100**	**25**	**25**	**250**	**100**	**125**	**125**	**200**	**50**	**250**	**250**	**100**	**50**	**25**
3	2.55	2.51	2.50	0.01	0.01	0.01	0.01	1.00	0.08	1.00	0.25	0.25	2.50	1.02	1.26	1.26	2.00	0.50	2.51	2.51	0.51	0.51	0.25
4	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
5	50.90	50.10	50.00	0.21	0.20	0.20	0.20	20.08	1.50	20.02	5.03	5.04	50.00	20.46	25.10	25.25	40.04	10.03	50.15	50.15	10.18	10.11	5.00

1: native standard conc. in mg/l; *2:* standard vol. in µl; *3:* mix standard conc. in mg/l; *4:* spike volume in µl *5:* standard conc. in µg/l in 5 g sample;

Complete validation results of maize with low spike concentrations

measured concentrations in samples										results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	1.1844	14.1607	13.5211	14.5763	14.8093	-	14.6431	13.1974	14.15	0.66	103	98	106	108	107	95	103	5
AFB1	0.21	0.0232	0.0833	0.0706	0.0538	0.0851	-	0.0894	0.0692	0.08	0.01	117	92	59	121	129	89	101	18
AFB2	0.20	0.0000	0.1071	0.1202	0.1075	0.0523	-	0.0915	0.0717	0.09	0.03	212	237	212	104	181	142	181	28
AFG1	0.20	0.0039	0.0533	0.0691	0.0581	0.0581	-	0.0680	0.0560	0.06	0.01	98	129	107	107	127	103	112	11
AFG2	0.20	0.0050	0.1068	0.0901	0.1039	0.0986	-	0.1244	0.0930	0.10	0.01	202	169	196	186	237	175	194	12
AOH	50.0	0.0000	36.4255	34.5195	25.3148	27.1436	-	24.8710	31.0006	29.88	4.88	292	276	202	218	199	248	239	16
AME	20.5	0.3629	8.4494	9.0474	7.6703	8.1945	-	8.3990	6.0895	7.98	1.02	158	169	143	153	157	112	149	13
BEA	10.1	0.2533	1.1323	1.1213	0.8694	0.9290	-	0.8789	0.5904	0.92	0.20	35	34	24	27	25	13	26	22
CIT	40.0	0.0000	6.0405	6.6566	4.5036	4.8315	-	4.8561	4.5788	5.24	0.89	60	66	45	48	49	46	52	17
CPA	50.2	0.8000	18.1066	17.0876	17.5139	18.9805	-	16.3099	17.0405	17.51	0.93	138	130	133	145	124	130	133	5
DON	20.1	3.8442	8.5515	7.1756	8.2551	8.3594	-	6.5396	7.4990	7.73	0.79	94	66	87	90	54	73	77	10
DAS	10.0	0.0000	2.5649	2.2365	2.5241	2.7888	-	2.7453	3.4678	2.72	0.41	102	89	100	111	109	138	109	15
ENB	5.00	0.1666	0.8749	0.9011	0.7127	0.6910	-	0.6474	0.3697	0.70	0.19	57	59	44	42	38	16	43	27
FB1	50.9	15.7915	29.6268	27.3282	38.7988	101.2568	-	66.8234	119.0041	63.81	38.94	109	90	180	673	401	811	377	61
FB2	50.1	2.6128	24.0582	22.1508	26.7366	31.4529	-	36.2167	27.0484	27.94	5.13	172	156	192	231	268	195	202	18
FB3	50.0	4.0866	15.1254	30.3230	13.7174	18.0554	-	36.7455	27.8520	23.64	9.34	88	209	77	112	261	190	156	40
FX	50.2	0.0000	8.4820	1.9356	4.2415	9.8616	-	2.4053	8.7016	5.94	3.49	68	15	34	79	19	69	47	59
HT2	5.04	1.2628	2.3015	3.5282	1.7203	3.0417	-	2.5229	2.1553	2.54	0.65	83	179	36	142	100	71	102	25
OTA	1.50	0.0202	0.4263	0.4465	0.4204	0.4899	-	0.3251	0.4175	0.42	0.05	108	113	106	125	81	106	107	13
STE	10.2	0.0058	2.8638	2.8875	3.1138	2.8923	-	2.9590	3.3464	3.01	0.19	113	113	122	114	116	131	118	6
T2	5.03	0.1917	1.7198	1.8629	1.5050	1.7877	-	1.9077	1.6028	1.73	0.15	122	133	104	127	136	112	122	9
ZON	20.0	0.0825	4.7501	4.9341	4.4945	5.7937	-	5.6772	5.2453	5.15	0.52	93	97	88	114	112	103	101	10

	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7
weight (g)	4.99	4.99	5.01	5.01	4.99	5.01	5.00	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of maize with high spike concentrations

measured concentrations in samples										results									
analyte	conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	100.7	1.1844	29.0857	26.7259	27.7860	28.6963	-	29.6654	25.4290	27.90	1.59	111	101	106	109	113	96	106	6
AFB1	0.95	0.0232	0.3499	0.3533	0.3198	0.3384	-	0.3089	0.2869	0.33	0.03	137	138	125	132	120	111	127	8
AFB2	0.96	0.0000	0.2387	0.3512	0.2930	0.4693	-	0.3177	0.2548	0.32	0.08	99	146	122	195	132	106	134	26
AFG1	0.96	0.0039	0.2740	0.2729	0.2978	0.2778	-	0.3015	0.2545	0.28	0.02	112	112	123	114	124	104	115	6
AFG2	0.95	0.0050	0.3706	0.2657	0.2957	0.2606	-	0.3228	0.3871	0.32	0.05	154	110	123	108	134	161	131	17
AOH	100.0	0.0000	75.2011	72.4428	45.9054	54.7296	-	46.4468	56.4986	58.54	12.61	301	290	184	219	186	226	234	22
AME	51.2	0.3629	23.2447	24.2692	20.3225	24.7252	-	16.9290	17.1029	21.10	3.51	179	187	156	191	130	131	162	17
BEA	20.0	0.2533	2.2162	2.1636	1.7779	1.8416	-	1.7961	1.0471	1.81	0.42	39	38	31	32	31	16	31	23
CIT	70.1	0.0000	10.9992	10.2960	8.1093	8.7843	-	8.3266	6.5639	8.85	1.60	63	59	46	50	48	37	51	18
CPA	100.7	0.8000	36.3650	34.4782	31.1407	32.2505	-	32.5491	32.9437	33.29	1.86	141	134	121	125	126	128	129	6
DON	50.2	3.8442	13.5088	17.7354	13.9013	13.9268	-	12.9373	16.4009	14.74	1.89	77	111	80	80	73	100	87	13
DAS	50.2	0.0000	11.9690	11.9591	12.9096	14.0270	-	14.2027	14.3920	13.24	1.12	95	95	103	112	114	115	106	8
ENB	10.00	0.1666	1.8335	1.8126	1.4409	1.3989	-	1.3561	0.6888	1.42	0.42	67	66	51	49	48	21	50	29
FB1	101.8	15.7915	39.0028	42.3224	53.8425	84.3686	-	69.5024	144.5214	72.26	39.29	91	104	150	269	211	506	222	54
FB2	100.2	2.6128	40.1746	43.0467	47.8366	64.0989	-	41.7450	48.7454	47.61	8.76	150	161	181	245	157	184	180	18
FB3	100.0	4.0866	35.5380	51.5139	44.4164	28.6739	-	63.5157	41.8945	44.26	12.23	126	190	162	98	238	151	161	28
FX	100.3	0.0000	15.9912	5.0136	12.5292	18.6973	-	4.6781	16.6344	12.26	6.08	64	20	50	75	19	66	49	50
HT2	10.07	1.2628	5.4737	3.2396	3.3418	4.9181	-	3.8804	3.4922	4.06	0.93	167	78	83	145	104	88	111	23
OTA	3.01	0.0202	0.7327	1.0649	0.6476	0.8209	-	0.8390	0.7593	0.81	0.14	95	139	84	106	109	98	105	18
STE	20.2	0.0058	5.4031	6.0446	5.4343	5.7763	-	5.6650	6.2241	5.76	0.33	107	119	107	114	112	123	114	6
T2	10.06	0.1917	3.0507	3.5966	2.9978	3.5413	-	3.0681	2.7573	3.17	0.33	114	135	112	133	115	102	118	10
ZON	50.1	0.0825	13.1937	16.1396	13.6278	14.0551	-	13.9438	15.7158	14.45	1.19	105	128	108	112	111	125	115	8

weight (g)	4.99	5.00	5.00	4.99	5.00	5.01	4.99	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)
comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well
green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of wheat flour with low spike concentrations

measured concentrations in samples										results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	0.0000	11.3019	11.0994	13.2384	13.3873	-	13.0862	12.0387	12.36	1.02	90	88	105	106	104	96	98	8
AFB1	0.21	0.0031	0.0635	0.0673	0.0885	0.0618	-	0.0714	0.0622	0.07	0.01	117	125	166	114	133	115	128	15
AFB2	0.20	0.0021	0.0632	0.0787	0.0891	0.0526	-	0.0819	0.0583	0.07	0.01	121	151	172	100	158	111	135	21
AFG1	0.20	0.0007	0.0562	0.0868	0.0778	0.0858	-	0.0730	0.0618	0.07	0.01	109	170	152	168	143	121	144	17
AFG2	0.20	0.0000	0.0580	0.0566	0.0550	0.0484	-	0.0777	0.0573	0.06	0.01	115	112	109	96	154	114	117	17
AOH	50.0	0.0000	23.2164	20.6494	29.2036	16.8749	-	17.2997	17.3711	20.77	4.81	185	165	234	135	139	139	166	23
AME	20.5	0.6225	6.6348	7.8323	6.0574	8.3377	-	6.6929	5.1227	6.78	1.17	117	141	106	151	119	88	120	17
BEA	10.1	0.0537	0.8257	0.8097	0.6357	0.6495	-	0.7660	0.5163	0.70	0.12	30	30	23	24	28	18	26	17
CIT	40.0	0.0000	4.2934	5.5454	4.1406	4.0311	-	4.3474	3.8537	4.37	0.60	43	55	41	40	44	39	44	14
CPA	50.2	2.5278	18.6584	18.0805	17.8230	19.3965	-	17.3927	18.0731	18.24	0.70	128	124	122	134	119	124	125	4
DON	20.1	1.3284	6.3981	6.4055	6.3070	6.9737	-	5.6737	5.8991	6.28	0.45	101	101	99	112	87	91	99	7
DAS	10.0	0.0105	2.6450	2.6261	2.3505	3.0178	-	2.5292	2.8643	2.67	0.24	105	104	93	120	101	114	106	9
ENB	5.00	0.3309	1.2932	1.1897	1.0210	0.9724	-	1.1069	0.6610	1.04	0.22	77	69	55	51	62	27	57	21
FB1	50.9	0.2376	12.2554	14.5393	19.1319	24.1293	-	32.3191		20.47	8.03	94	112	148	187	253		159	39
FB2	50.1	0.2219	19.8077	19.0538	38.4553	16.7184	-	22.3856	22.7750	23.20	7.80	156	150	305	131	177	180	183	34
FB3	50.0	0.0000	41.4375	17.8686	15.0047	26.2916	-	30.4951	21.3253	25.40	9.65	331	143	120	210	244	171	203	38
FX	50.2	0.0000	10.9729	2.6089	6.0878	14.8675	-	3.6797	13.7257	8.66	5.25	87	21	49	118	29	110	69	61
HT2	5.04	0.1065	1.4443	1.8197	0.5769	1.0957	-	1.8993	1.3741	1.37	0.49	106	136	37	78	143	101	100	36
OTA	1.50	0.0444	0.4667	0.6209	0.4297	0.5184	-	0.3528	0.3893	0.46	0.10	112	153	102	126	82	92	111	21
STE	10.2	0.0481	2.7883	3.1045	3.2161	2.9312	-	2.7647	3.2950	3.02	0.22	107	120	124	113	107	128	117	7
T2	5.03	0.0026	1.6520	1.7643	1.5185	1.3584	-	1.2244	1.2043	1.45	0.23	131	140	121	108	97	96	115	16
ZON	20.0	0.0000	5.2364	4.9512	4.6175	5.7982	-	5.2359	4.3202	5.03	0.52	104	99	92	116	105	86	100	10

weight (g)	5.01	5.01	5.00	5.00	5.01	5.00	4.99	4.99
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of wheat flour with high spike concentrations

measured concentrations in samples										results									
analyte	conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	100.7	0.0000	24.0019	28.7835	31.8325	29.4218	-	26.3980	29.2031	28.27	2.72	95	114	126	117	105	116	112	10
AFB1	0.95	0.0031	0.3027	0.3204	0.3349	0.3171	-	0.2927	0.3092	0.31	0.01	126	133	139	132	121	128	130	5
AFB2	0.96	0.0021	0.2915	0.2731	0.2856	0.2736	-	0.3192	0.2957	0.29	0.02	120	113	118	113	132	122	120	6
AFG1	0.96	0.0007	0.2845	0.3780	0.2796	0.3083	-	0.3380	0.3875	0.33	0.05	118	157	116	128	140	161	137	14
AFG2	0.95	0.0000	0.2465	0.2934	0.2468	0.2716	-	0.2687	0.3808	0.28	0.05	104	124	104	114	113	160	120	18
AOH	100.0	0.0000	47.8060	40.7657	38.4265	41.9801	-	32.3560	38.8898	40.04	5.05	191	163	153	168	129	156	160	13
AME	51.2	0.6225	20.7123	21.9034	16.8933	20.5605	-	18.4144	15.6634	19.02	2.44	157	166	127	156	139	118	144	13
BEA	20.0	0.0537	1.6652	1.7626	1.1696	1.3421	-	1.5651	1.0714	1.43	0.28	32	34	22	26	30	20	27	19
CIT	70.1	0.0000	7.2463	7.9508	7.7763	6.9810	-	7.3829	6.7875	7.35	0.45	41	45	44	40	42	39	42	6
CPA	100.7	2.5278	35.9823	40.7905	35.3047	32.7448	-	32.4391	33.9503	35.20	3.07	133	152	130	120	119	125	130	9
DON	50.2	1.3284	13.2481	13.1659	13.3978	13.9063	-	12.2375	13.9735	13.32	0.63	95	94	96	100	87	101	96	5
DAS	50.2	0.0105	13.5320	13.8921	12.7394	15.3578	-	14.3494	14.4931	14.06	0.90	108	111	101	122	114	116	112	6
ENB	10.00	0.3309	2.3840	2.1298	1.5803	2.1884	-	1.7716	1.1590	1.87	0.45	82	72	50	74	58	33	62	24
FB1	101.8	0.2376	30.0104	35.3909	41.6010	42.2756	-	50.5245		39.96	7.74	117	138	162	165	198		156	19
FB2	100.2	0.2219	51.4363	38.9469	35.1916	42.2243	-	54.4419	53.8307	46.01	8.28	204	155	139	168	216	214	183	18
FB3	100.0	0.0000	26.9481	47.3498	29.7284	50.1739	-	71.2928	46.3107	45.30	16.02	108	189	119	201	285	185	181	35
FX	100.3	0.0000	21.6618	6.5770	18.3070	30.8918	-	7.2954	28.4413	18.86	10.29	86	26	73	123	29	113	75	55
HT2	10.07	0.1065	2.9475	2.6199	2.6087	2.6748	-	3.5265	2.9026	2.88	0.35	113	100	99	102	136	111	110	12
OTA	3.01	0.0444	0.9509	0.9806	0.7380	0.9128	-	1.0028	0.8053	0.90	0.10	121	124	92	115	127	101	114	12
STE	20.2	0.0481	5.3736	5.9493	6.3651	6.2505	-	5.5945	7.1128	6.11	0.62	105	117	125	123	110	140	120	10
T2	10.06	0.0026	2.9903	3.0164	2.6054	3.6049	-	2.5344	3.0698	2.97	0.38	119	120	103	143	101	122	118	13
ZON	50.1	0.0000	15.1909	12.7936	12.0913	15.8401	-	13.9382	16.6017	14.41	1.77	121	102	96	127	111	133	115	12

weight (g)	5.01	5.00	5.00	5.01	5.00	5.00	5.00	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)
comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well
green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of oat flakes with low spike concentrations

		measured concentrations in samples								results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	0.0000	14.6036	14.7723	13.9180	13.6914	-	12.6196	12.2159	13.64	1.04	116	117	111	109	100	97	108	8
AFB1	0.21	0.0000	0.0744	0.0689	0.0659	0.0699	-	0.0807	0.0628	0.07	0.01	144	134	129	136	157	122	137	9
AFB2	0.20	0.0000	0.0633	0.1041	0.0510	0.0726	-	0.0829	0.0705	0.07	0.02	125	206	101	144	164	139	146	24
AFG1	0.20	0.0045	0.0619	0.0851	0.0654	0.0697	-	0.0641	0.0480	0.07	0.01	113	159	121	129	118	86	121	18
AFG2	0.20	0.0072	0.0886	0.0813	0.0591	0.0650	-	0.0788	0.0611	0.07	0.01	161	147	103	115	142	107	129	17
AOH	50.0	0.0000	36.7853	28.1728	29.1850	26.5770	-	22.2421	21.1101	27.35	5.63	294	225	234	213	178	169	219	21
AME	20.5	0.6697	8.3960	7.1434	6.1012	7.7460	-	6.7623	5.8776	7.00	0.96	151	127	106	139	119	102	124	14
BEA	10.1	0.0546	0.4739	0.5199	0.4101	0.4125	-	0.5069	0.3520	0.45	0.07	17	18	14	14	18	12	15	15
CIT	40.0	0.0000	6.7026	7.8440	6.5455	6.5426	-	7.0425	4.8327	6.58	0.99	67	78	66	65	70	48	66	15
CPA	50.2	7.2799	15.2160	14.5524	15.3067	17.9779	-	15.5007	16.4088	15.83	1.21	63	58	64	85	65	73	68	8
DON	20.1	0.0000	5.9286	5.2506	5.9393	5.9761	-	4.6441	5.9018	5.61	0.55	118	105	119	119	93	118	112	10
DAS	10.0	0.0000	2.9656	2.5788	2.4635	3.3349	-	2.7079	2.8860	2.82	0.31	118	103	98	133	108	115	113	11
ENB	5.00	0.4035	1.1317	1.5736	0.9810	0.9990	-	0.9715	0.5725	1.04	0.32	58	94	46	48	45	13	51	31
FB1	50.9	0.0394	10.5406	16.1482	15.9770	18.5139	-	31.1670		18.47	7.68	82	127	125	145	245		145	42
FB2	50.1	0.1951	21.3458	20.6032	19.4852	19.0878	-	18.4671	19.7714	19.79	1.04	169	163	154	151	146	156	157	5
FB3	50.0	1.5733	13.7201	30.0841	7.0670	13.9989	-	40.3179	20.9982	21.03	12.26	97	228	44	100	310	155	156	58
FX	50.2	0.0000	16.9318	4.6578	11.2979	20.1008	-	5.4498	17.2635	12.62	6.52	135	37	90	161	43	138	101	52
HT2	5.04	0.0706	1.3781	1.7849	1.4673	1.6215	-	1.8784	1.6856	1.64	0.19	104	136	111	123	144	128	124	12
OTA	1.50	0.0654	0.3729	0.4712		0.4653	-	0.4042	0.3264	0.41	0.06	82	108		107	90	69	91	15
STE	10.2	0.0231	2.7822	2.9373	2.8810	2.9239	-	2.8767	3.0532	2.91	0.09	108	115	113	114	112	119	113	3
T2	5.03	0.1218	2.2225	1.8151	1.5984	1.6995	-	1.3216	1.3923	1.67	0.33	167	135	118	126	95	101	124	19
ZON	20.0	0.0267	6.2517	5.2691	4.9921	5.0674	-	6.3270	5.5089	5.57	0.59	124	105	99	101	126	110	111	11

weight (g)	4.99	5.01	5.00	4.99	4.99	4.99	5.00	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of oat flakes with high spike concentrations

measured concentrations in samples										results									
analyte	**conc. high**	**Blank**	**C1_1**	**C1_2**	**C1_3**	**C1_4**	**C1_5**	**C1_6**	**C1_7**	**mean**	**SD**	**RR % C1_1**	**RR % C1_2**	**RR % C1_3**	**RR % C1_4**	**RR % C1_6**	**RR % C1_7**	**RR % mean**	**RSD %**
comment							**not usable**												
AcDON	100.7	0.0000	26.5120	25.2471	25.7107	27.3272	-	26.1023	27.1289	26.34	0.81	105	100	102	109	104	108	105	3
AFB1	0.95	0.0000	0.2999	0.2984	0.2894	0.3202	-	0.2860	0.3087	0.30	0.01	126	125	122	134	120	129	126	4
AFB2	0.96	0.0000	0.2878	0.3277	0.2989	0.3121	-	0.3041	0.3131	0.31	0.01	120	136	125	130	127	130	128	4
AFG1	0.96	0.0045	0.3079	0.3474	0.2739	0.2902	-	0.2961	0.3521	0.31	0.03	126	143	112	119	121	145	128	10
AFG2	0.95	0.0072	0.2574	0.3239	0.2733	0.2423	-	0.2900	0.3126	0.28	0.03	105	133	112	99	119	129	116	11
AOH	100.0	0.0000	63.2516	48.8805	50.4274	53.4537	-	42.8309	47.2358	51.01	6.95	253	196	202	214	171	189	204	14
AME	51.2	0.6697	20.0751	18.5982	14.0379	18.2889	-	15.8711	15.0190	16.98	2.35	151	140	105	138	119	112	128	14
BEA	20.0	0.0546	1.0296	1.0202	0.7583	0.8276	-	0.9756	0.7210	0.89	0.14	19	19	14	15	18	13	17	15
CIT	70.1	0.0000	12.5667	14.7209	10.0694	9.9116	-	9.7951	10.5973	11.28	1.98	72	84	58	57	56	60	64	18
CPA	100.7	7.2799	31.0155	31.6268	31.2106	28.9880	-	31.2273	30.7525	30.80	0.93	94	97	95	86	95	93	93	3
DON	50.2	0.0000	13.4133	11.8994	13.9038	13.7351	-	12.7577	12.4695	13.03	0.78	107	95	111	109	102	99	104	6
DAS	50.2	0.0000	14.6334	12.2508	12.3080	14.8045	-	15.0125	14.9518	13.99	1.33	116	98	98	118	120	119	112	10
ENB	10.00	0.4035	3.0333	2.3840	1.6535	1.4019	-	1.4432	0.8939	1.80	0.77	105	79	50	40	42	20	56	43
FB1	101.8	0.0394	26.7792	29.4627	29.7161	42.9055	-	55.8834	89.9241	45.78	24.27	105	116	117	168	219	353	180	53
FB2	100.2	0.1951	39.1612	37.2044	38.5012	37.6729	-	39.2447	45.4159	39.53	2.99	155	148	153	150	156	181	157	8
FB3	100.0	1.5733	24.6738	62.4201	29.6833	30.7441	-	49.4225	41.9350	39.81	14.30	92	243	113	117	191	161	153	36
FX	100.3	0.0000	31.3664	11.4873	28.8275	36.1223	-	10.0389	38.8402	26.11	12.41	125	46	115	144	40	155	104	48
HT2	10.07	0.0706	3.5452	3.2121	2.9391	3.5373	-	3.0304	3.5544	3.30	0.28	138	125	114	138	118	138	128	8
OTA	3.01	0.0654	0.6631	0.8640	0.7039	0.8610	-	0.7632	0.7598	0.77	0.08	79	106	85	106	93	92	94	11
STE	20.2	0.0231	5.6639	6.0782	5.9734	6.0167	-	5.5690	6.8582	6.03	0.46	111	120	118	118	110	135	119	8
T2	10.06	0.1218	2.7506	3.1256	2.7049	2.6705	-	3.1372	3.5517	2.99	0.35	104	119	103	101	120	136	114	12
ZON	50.1	0.0267	17.5172	14.7054	11.0418	15.3240	-	16.5720	17.0430	15.37	2.37	140	117	88	122	132	136	123	15

weight (g)	4.99	5.01	5.00	4.99	5.00	4.99	5.00	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)
comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well
green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of pastry with low spike concentrations

measured concentrations in samples										results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	0.0659	11.5947	12.6499	13.7394	11.7671	-	12.1262	11.7823	12.28	0.81	91	100	109	93	96	93	97	7
AFB1	0.21	0.0013	0.0734	0.0600	0.0608	0.0555	-	0.0621	0.0544	0.06	0.01	140	114	116	105	118	103	116	11
AFB2	0.20	0.0005	0.0633	0.0681	0.0685	0.0499	-	0.0647	0.0592	0.06	0.01	124	133	135	98	127	116	122	11
AFG1	0.20	0.0004	0.0564	0.0662	0.0738	0.0654	-	0.0591	0.0536	0.06	0.01	110	130	145	129	116	105	122	12
AFG2	0.20	0.0107	0.0775	0.0631	0.0530	0.0521	-	0.0593	0.0719	0.06	0.01	132	104	84	82	96	121	103	16
AOH	50.0	0.0000	26.7132	20.5413	23.1028	19.9053	-	15.5373	16.2569	20.34	4.20	213	164	185	159	124	130	163	21
AME	20.5	0.6859	7.6994	7.2515	6.7187	7.9210	-	5.7179	5.6471	6.83	0.98	137	128	118	141	98	97	120	14
BEA	10.1	0.0573	0.6215	0.7371	0.5425	0.5548	-	0.6107	0.4718	0.59	0.09	22	27	19	20	22	16	21	15
CIT	40.0	0.0000	9.3893	11.0454	8.7349	10.5442	-	9.1988	7.9322	9.47	1.15	94	110	87	105	92	79	95	12
CPA	50.2	2.2413	17.6587	16.5966	17.6017	17.5917	-	16.1459	17.8368	17.24	0.69	123	114	123	122	111	124	120	4
DON	20.1	2.5231	7.6419	6.8661	8.2828	8.0182	-	6.9378	7.2730	7.50	0.58	102	86	115	109	88	94	99	8
DAS	10.0	0.0000	2.0864	2.2027	2.3513	2.3572	-	2.7597	3.0744	2.47	0.37	83	88	94	94	110	122	99	15
ENB	5.00	0.2716	0.9332	0.9959	0.8574	0.7703	-	0.8243	0.4725	0.81	0.18	53	58	47	40	44	16	43	23
FB1	50.9	0.0000	10.0207	16.1738	18.8486	22.4870	-	35.9052		20.69	9.65	79	127	148	177	282		163	47
FB2	50.1	0.3283	17.6934	29.8105	19.4638	26.3906	-	23.6007	27.9081	24.14	4.80	138	235	153	208	186	220	190	20
FB3	50.0	0.0000	22.6567	32.5591	13.1944	17.8124	-	32.4053	22.8901	23.59	7.76	181	260	106	142	259	183	189	33
FX	50.2	0.0000	10.1741	2.2163	6.8764	14.5197	-	3.3548	11.8848	8.17	4.87	81	18	55	116	27	95	65	60
HT2	5.04	0.0000	1.7752	1.8898	1.7407	1.1353	-	1.7003	1.5066	1.62	0.27	141	150	139	90	135	119	129	17
OTA	1.50	0.0346	0.3888	0.5389	0.2888	0.4986	-	0.4638	0.3812	0.43	0.09	94	134	68	123	114	92	104	21
STE	10.2	0.0171	2.3944	2.6496	2.8414	2.7864	-	2.4944	3.1551	2.72	0.27	93	103	111	109	97	123	106	10
T2	5.03	0.0580	1.5981	1.5519	1.5235	1.2267	-	1.3312	1.3161	1.42	0.15	122	119	117	93	101	100	109	11
ZON	20.0	0.0000	5.7583	5.3890	4.2488	5.0711	-	5.4153	5.1874	5.18	0.51	115	107	85	101	108	103	103	10

weight (g)	5.00	5.01	5.01	4.99	5.00	5.00	5.00	5.01
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of pastry with high spike concentrations

measured concentrations in samples										results									
analyte	conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	100.7	0.0659	27.4400	24.7688	27.6952	26.0414	-	24.0683	28.8569	26.48	1.84	109	98	110	103	95	114	105	7
AFB1	0.95	0.0013	0.2920	0.3169	0.2985	0.2863	-	0.3158	0.3218	0.31	0.01	122	133	125	120	132	134	128	5
AFB2	0.96	0.0005	0.2834	0.3153	0.3499	0.2824	-	0.2952	0.2783	0.30	0.03	118	131	146	117	123	116	125	9
AFG1	0.96	0.0004	0.3102	0.3488	0.3140	0.3391	-	0.3061	0.3484	0.33	0.02	129	145	131	141	127	145	136	6
AFG2	0.95	0.0107	0.2014	0.2573	0.2714	0.2400	-	0.2782	0.3833	0.27	0.06	80	104	110	97	113	157	110	22
AOH	100.0	0.0000	52.3631	34.7005	39.7366	41.9330	-	27.6455	35.7583	38.69	8.31	209	139	159	168	111	143	155	21
AME	51.2	0.6859	19.5530	19.5637	16.9956	20.9383	-	15.8978	14.7230	17.95	2.43	148	148	128	158	119	110	135	14
BEA	20.0	0.0573	1.2024	1.3471	1.1612	1.0644	-	1.2354	1.0115	1.17	0.12	23	26	22	20	24	19	22	10
CIT	70.1	0.0000	16.6897	19.3479	17.8101	16.8228	-	16.2226	15.7058	17.10	1.31	95	111	102	96	93	90	98	8
CPA	100.7	2.2413	36.5199	38.2998	34.1799	33.6365	-	33.9720	33.9931	35.10	1.88	136	144	127	125	126	126	131	5
DON	50.2	2.5231	15.1427	13.8865	14.1929	14.5187	-	14.3246	15.0622	14.52	0.50	101	91	93	96	94	100	96	3
DAS	50.2	0.0000	13.0048	12.3185	12.2583	14.3395	-	13.7471	14.8476	13.42	1.07	104	98	98	114	110	118	107	8
ENB	10.00	0.2716	1.7235	1.7987	1.5046	1.3927	-	1.4430	0.9543	1.47	0.30	58	61	49	45	47	27	48	20
FB1	101.8	0.0000	24.6843	37.9394	44.3192	41.3466	-	53.2250	112.7634	52.38	31.01	97	149	174	162	209	443	206	59
FB2	100.2	0.3283	41.0766	47.1151	43.7156	45.4628	-	53.9904	54.4383	47.63	5.48	163	187	174	180	214	216	189	12
FB3	100.0	0.0000	59.9992	56.6811	20.1885	38.2560	-	56.4610	52.4678	47.34	15.34	240	227	81	153	226	210	189	32
FX	100.3	0.0000	21.3712	5.9763	21.1636	27.0545	-	6.6487	25.8009	18.00	9.36	85	24	85	108	27	103	72	52
HT2	10.07	0.0000	2.6390	1.8980	2.2968	2.1679	-	3.0146	3.3960	2.57	0.56	105	76	91	86	120	135	102	22
OTA	3.01	0.0346	0.8082	0.8286	0.8060	0.8825	-	0.7735	0.8059	0.82	0.04	103	106	103	113	98	103	104	4
STE	20.2	0.0171	5.2397	5.6264	6.2418	5.8573	-	5.2093	6.2521	5.74	0.46	103	111	123	115	103	123	113	8
T2	10.06	0.0580	2.9420	3.1386	2.5196	2.4535	-	2.6047	2.5878	2.71	0.27	115	123	98	95	101	101	105	10
ZON	50.1	0.0000	14.5173	13.1247	12.2829	12.4783	-	17.5559	14.9756	14.16	1.99	116	105	98	100	140	120	113	14

weight (g)	5.00	5.00	4.99	4.99	5.00	4.99	5.00	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of wholemeal bread with low spike concentrations

measured concentrations in samples										results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	3.0177	15.3844	18.4324	15.1232	15.0784	-	13.3422	13.2519	15.10	1.88	98	122	96	96	82	81	96	12
AFB1	0.21	0.0255	0.1210	0.0905	0.0710	0.0933	-	0.1021	0.0928	0.10	0.02	186	126	89	132	149	130	135	17
AFB2	0.20	0.0201	0.0734	0.0660	0.0784	0.0648	-	0.0686	0.0685	0.07	0.01	105	90	115	89	96	95	98	7
AFG1	0.20	0.0000	0.0595	0.0589	0.0702		-	0.0511	0.0511	0.06	0.01	118	116	139		101	101	115	14
AFG2	0.20	0.0876	0.1316	0.1487	0.1237	0.1336	-	0.1838	0.0998	0.14	0.03	87	120	72	91	190	24	97	20
AOH	50.0	0.0000	35.6777	33.8480	20.1568	30.8737	-	23.3125	24.5939	28.08	6.27	285	270	162	247	186	196	225	22
AME	20.5	0.8309	9.0239	8.5344	7.6592	8.4609	-	6.7027	6.5017	7.81	1.04	160	150	134	149	115	111	136	13
BEA	10.1	0.0646	1.1959	1.3642	1.1129	1.1126	-	1.1733	0.6795	1.11	0.23	45	51	42	42	44	24	41	21
CIT	40.0	0.0000	9.8955	10.7156	9.4422	8.4553	-	9.0760	5.7595	8.89	1.71	99	107	95	85	91	57	89	19
CPA	50.2	3.8703	16.8751	17.4280	16.9783	17.4561	-	15.8444	16.4991	16.85	0.61	104	108	105	109	95	100	103	4
DON	20.1	2.5361	8.4681	7.0988	7.9027	7.4561	-	6.5936	7.9063	7.57	0.67	118	91	107	98	81	107	100	9
DAS	10.0	0.0000	2.5472	2.3153	2.7317	2.6421	-	2.6794	3.2077	2.69	0.29	102	92	109	106	107	128	107	11
ENB	5.00	0.7711	1.8032	2.0960	1.6138	1.5926	-	1.8505	1.1709	1.69	0.31	82	106	68	66	86	32	73	19
FB1	50.9	0.1355	12.1911	15.2976	23.7425	23.8256	-	35.9924	80.2476	31.88	25.10	95	119	186	187	282	628	249	79
FB2	50.1	0.2552	23.9135	24.1142	18.6210	24.9563	-	27.3108	20.4136	23.22	3.16	189	190	147	198	216	161	183	14
FB3	50.0	0.0000	18.7406	15.1977	10.1970	14.9342	-	30.5099	21.0231	18.43	6.97	150	121	82	120	244	168	147	38
FX	50.2	0.0000	10.2970	2.6720	6.1806	10.6953	-	3.2624	9.4385	7.09	3.57	82	21	49	85	26	75	57	50
HT2	5.04	0.1162	1.8598	1.3515	1.5123	1.3047	-	1.5086	1.4869	1.50	0.19	139	98	111	95	111	109	110	13
OTA	1.50	0.0553	0.4486	0.4764	0.3499	0.4661	-	0.4492	0.4100	0.43	0.05	105	112	78	109	105	94	100	11
STE	10.2	0.0350	2.8399	2.6779	2.9384	2.9797	-	2.8255	2.8820	2.86	0.11	110	104	114	116	110	112	111	4
T2	5.03	0.0882	1.4707	1.6765	1.2792	1.4088	-	1.4986	1.2574	1.43	0.15	110	126	95	105	112	93	107	11
ZON	20.0	0.0000	5.7772	4.8211	4.8423	5.4893	-	5.5281	5.3308	5.30	0.39	115	96	97	110	110	106	106	7

weight (g)	4.99	5.00	5.01	4.99	4.99	4.99	5.00	5.01
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)
comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well
green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of wholemeal bread with high spike concentrations

measured concentrations in samples										results									
analyte	conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	100.7	3.0177	25.2076	29.2345	25.9367	26.7281	-	26.9400	27.1319	26.86	1.37	88	104	91	94	95	96	95	5
AFB1	0.95	0.0255	0.3091	0.3379	0.2763	0.2760	-	0.2961	0.3065	0.30	0.02	119	131	105	105	113	118	115	8
AFB2	0.96	0.0201	0.2802	0.1899	0.2791	0.2771	-	0.3514	0.2421	0.27	0.05	108	71	108	107	138	92	104	20
AFG1	0.96	0.0000	0.2501	0.2573	0.2544	0.2519	-	0.2700	0.4042	0.28	0.06	104	107	106	105	112	168	117	22
AFG2	0.95	0.0876	0.2454	0.2525	0.4468	0.3028	-	0.3247	0.3606	0.32	0.08	66	69	152	91	100	115	99	23
AOH	100.0	0.0000	75.0258	73.8196	35.7501	48.5618	-	41.4283	49.3357	53.99	16.60	300	295	143	194	166	197	216	31
AME	51.2	0.8309	21.1804	23.2825	17.9203	24.0941	-	18.0345	18.1881	20.45	2.80	159	176	134	182	135	135	153	14
BEA	20.0	0.0646	2.4772	2.7495	2.2195	2.4083	-	2.4944	1.5483	2.32	0.41	48	54	43	47	49	30	45	18
CIT	70.1	0.0000	17.5423	22.8803	16.2596	14.6396	-	15.0914	14.3802	16.80	3.20	100	131	93	84	86	82	96	19
CPA	100.7	3.8703	37.4997	39.9212	35.4784	33.7788	-	32.7505	33.0170	35.41	2.83	134	143	126	119	115	115	125	8
DON	50.2	2.5361	13.4362	12.8986	14.1074	14.8846	-	13.5949	14.6492	13.93	0.76	87	83	92	98	88	96	91	5
DAS	50.2	0.0000	15.0403	12.8824	13.2400	13.6299	-	15.4214	15.2451	14.24	1.12	120	103	106	109	123	121	114	8
ENB	10.00	0.7711	2.8117	3.2425	2.5719	2.7587	-	2.7101	1.7486	2.64	0.49	82	99	72	79	77	39	75	19
FB1	101.8	0.1355	23.8278	26.0804	33.1846	41.4343	-	54.9646	105.5531	47.51	30.62	93	102	130	162	215	413	186	64
FB2	100.2	0.2552	44.5126	49.4851	40.0769	38.7925	-	46.1954	45.5061	44.09	4.00	177	197	159	154	183	180	175	9
FB3	100.0	0.0000	26.0497	61.8804	35.7964	24.5945	-	75.2339	47.1282	45.11	20.32	104	248	143	98	301	188	180	45
FX	100.3	0.0000	18.1122	6.0335	15.3038	24.6411	-	5.6073	22.0339	15.29	8.00	72	24	61	98	22	88	61	52
HT2	10.07	0.1162	2.8959	2.9545	3.5046	3.0933	-	2.9735	2.8402	3.04	0.24	110	113	135	118	113	108	116	8
OTA	3.01	0.0553	0.9889	0.9560	0.8234	1.0948	-	1.0640	0.6857	0.94	0.15	124	120	102	138	134	84	117	17
STE	20.2	0.0350	5.7175	5.2240	5.5472	6.0643	-	5.9617	6.8823	5.90	0.57	112	103	109	119	117	135	116	10
T2	10.06	0.0882	3.2370	2.9693	2.8644	3.0186	-	2.8468	2.6683	2.93	0.19	125	115	111	117	110	102	113	7
ZON	50.1	0.0000	15.9749	15.6044	13.4047	16.3810	-	14.8970	14.8694	15.19	1.06	128	125	107	131	119	119	121	7

weight (g)	4.99	5.00	5.00	4.99	5.00	5.00	5.00	5.01
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of marble cake with low spike concentrations

measured concentrations in samples										results									
analyte	conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	50.4	0.0000	14.1591	13.7805	14.5299	13.4909	-	13.0278	12.4022	13.57	0.77	112	109	115	107	103	99	108	6
AFB1	0.21	0.0088	0.0950	0.0966	0.1028	0.1062	-	0.0922	0.0803	0.10	0.01	168	171	183	190	162	139	169	9
AFB2	0.20	0.0091	0.0503	0.0711	0.0854	0.0721	-	0.0560	0.0658	0.07	0.01	81	123	151	124	93	112	114	19
AFG1	0.20	0.0091	0.0676	0.0852	0.0580	0.0769	-	0.0827	0.0679	0.07	0.01	116	150	97	134	146	117	126	14
AFG2	0.20	0.0084	0.0765	0.0599	0.0621	0.0661	-	0.0816	0.0673	0.07	0.01	135	102	107	114	145	117	120	12
AOH	50.0	0.0000	23.4968	24.2720	26.0551	23.7709	-	16.6374	18.8013	22.17	3.63	188	194	208	190	133	151	177	16
AME	20.5	0.9010	7.6422	9.0692	7.0478	7.6584	-	6.5938	6.3294	7.39	0.98	132	160	120	132	111	106	127	13
BEA	10.1	0.0643	0.9804	1.0230	0.8597	0.9440	-	0.9813	0.6859	0.91	0.12	36	38	31	35	36	25	34	14
CIT	40.0	0.0451	7.0998	8.5893	7.2258	8.5607	-	8.6296	6.8674	7.83	0.85	70	85	72	85	86	68	78	11
CPA	50.2	1.0680	17.2970	17.2787	18.1875	18.9799	-	17.0902	18.1408	17.83	0.73	129	129	137	143	128	136	134	4
DON	20.1	0.7214	4.9151	4.7204	5.8456	5.2234	-	4.9805	5.5720	5.21	0.43	84	80	102	90	85	97	89	8
DAS	10.0	0.0000	2.4713	2.5315	2.5920	3.2508	-	3.1503	3.3561	2.89	0.40	99	101	103	130	126	134	115	14
ENB	5.00	0.2557	1.1100	1.2402	1.0135	0.9828	-	1.0252	0.6164	1.00	0.21	68	79	61	58	62	29	59	21
FB1	50.9	0.1098	11.6386	16.1878	14.6162	20.0702	-	34.3303	76.3109	28.86	24.57	91	126	114	157	269	600	226	85
FB2	50.1	0.1140	19.5021	19.6081	24.3885	22.1368	-	26.9792	23.0971	22.62	2.88	155	156	194	176	214	184	180	13
FB3	50.0	0.9231	19.5926	22.7105	21.2216	12.7098	-	28.2312	22.5158	21.16	5.06	149	174	162	94	218	173	162	24
FX	50.2	0.0000	10.2121	2.5996	3.3242	15.0001	-	3.5843	14.0998	8.14	5.68	81	21	27	120	29	113	65	70
HT2	5.04	0.0329	1.0713	2.0710	1.7186	1.6393	-	2.0954	1.8240	1.74	0.37	82	162	134	128	164	143	135	22
OTA	1.50	0.0894	0.4344	0.5659	0.3908	0.5843	-	0.4531	0.4471	0.48	0.08	92	127	80	132	97	95	104	16
STE	10.2	0.0070	2.8697	2.7354	3.2793	3.0310	-	2.6831	3.3018	2.98	0.27	112	107	129	119	105	130	117	9
T2	5.03	0.0179	2.2851	1.8376	1.4485	1.5145	-	1.5625	1.4432	1.68	0.33	180	145	114	119	123	114	132	20
ZON	20.0	0.8936	6.7448	6.2825	6.2553	6.1121	-	7.2252	7.0272	6.61	0.46	117	108	107	104	127	123	114	7

weight (g)	5.01	5.00	5.00	5.00	5.00	4.99	5.00	4.99
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)
comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well
green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of marble cake with high spike concentrations

measured concentrations in samples										results									
analyte	conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment							not usable												
AcDON	100.7	0.0000	31.8795	32.3327	27.4889	30.2580	-	30.0943	28.3210	30.06	1.91	127	128	109	120	119	112	119	6
AFB1	0.95	0.0088	0.3480	0.3462	0.3179	0.3538	-	0.3688	0.3526	0.35	0.02	142	141	130	145	151	144	142	5
AFB2	0.96	0.0091	0.2909	0.3145	0.3422	0.4171	-	0.3545	0.3077	0.34	0.05	117	127	139	170	143	124	137	13
AFG1	0.96	0.0091	0.3432	0.3406	0.3002	0.3309	-	0.3446	0.4196	0.35	0.04	139	138	121	134	139	171	140	11
AFG2	0.95	0.0084	0.2643	0.3645	0.2952	0.3827	-	0.2834	0.3804	0.33	0.05	108	150	121	158	116	157	135	16
AOH	100.0	0.0000	54.0957	51.4806	40.1812	44.2347	-	36.4228	38.2129	44.10	7.26	216	206	161	177	145	153	176	16
AME	51.2	0.9010	20.8102	21.4710	16.6178	19.4490	-	18.7660	16.6807	18.97	2.03	156	161	123	145	139	123	141	11
BEA	20.0	0.0643	2.1839	2.3760	1.8131	1.9532	-	2.1046	1.4229	1.98	0.33	42	46	35	38	41	27	38	17
CIT	70.1	0.0451	14.2216	18.6679	14.1159	13.3137	-	14.1042	12.5919	14.50	2.14	81	106	80	76	80	72	83	15
CPA	100.7	1.0680	39.0875	44.3125	37.5216	35.0454	-	37.6999	36.1166	38.30	3.26	151	172	145	135	145	139	148	9
DON	50.2	0.7214	11.1130	12.3643	12.5211	11.9889	-	11.0519	13.3957	12.07	0.90	83	93	94	90	82	101	90	7
DAS	50.2	0.0000	14.1131	14.6380	13.4977	16.6585	-	17.1418	17.3918	15.57	1.69	113	117	108	133	136	139	124	11
ENB	10.00	0.2557	2.2295	2.4499	1.8615	1.9210	-	2.0101	1.1751	1.94	0.43	79	88	64	67	70	37	67	22
FB1	101.8	0.1098	22.4282	26.0550	35.0129	42.5671	-	56.5364	116.6282	49.87	34.92	88	102	137	167	221	458	196	70
FB2	100.2	0.1140	45.4869	49.1163	51.3740	38.8320	-	36.8698	48.8712	45.09	5.95	181	196	205	155	146	195	180	13
FB3	100.0	0.4234	37.1007	50.3696	49.0850	26.5884	-	48.9895	42.7010	42.47	9.27	147	200	195	105	194	169	168	22
FX	100.3	0.0000	22.9850	7.1801	21.1422	27.9973	-	7.3596	27.3637	19.00	9.45	92	29	84	112	29	109	76	50
HT2	10.07	0.0329	2.9049	3.6160	2.3992	2.8559	-	3.5822	3.1153	3.08	0.47	114	142	94	112	141	122	121	15
OTA	3.01	0.0894	0.8590	0.9737	0.9786	0.9075	-	0.8672	0.8530	0.91	0.06	102	118	118	109	103	102	109	6
STE	20.2	0.0070	6.0340	5.6683	6.2805	6.4023	-	6.0265	6.5722	6.16	0.32	119	112	124	127	119	130	122	5
T2	10.06	0.0179	3.4451	3.2906	3.2147	2.9490	-	3.2092	3.2454	3.23	0.16	136	130	127	117	127	128	128	5
ZON	50.1	0.8936	15.6620	16.5607	13.4995	15.9104	-	19.2599	19.0489	16.66	2.19	118	125	101	120	146	145	126	13

weight (g)	5.01	5.00	5.00	4.99	4.99	4.99	5.01	5.00
dilution factor	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
solvent vol. (ml)	20							

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH)

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of almonds with low spike concentrations

analyte		conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
			measured concentrations in samples								results									
comment								not usable												
AcDON	50.4	150.7	0.5108	9.5936	10.2987	11.8667	10.2529	-	11.3673	10.9659	10.72	0.83	69	75	87	74	83	80	78	8
AFB1	0.21	0.62	0.0076	0.0873	0.0602	0.0722	0.0585	-	0.0765	0.0496	0.07	0.01	149	98	121	95	129	78	112	20
AFB2	0.20	0.61	0.0000	0.0600	0.0605	0.0615	0.0582	-	0.0570	0.0510	0.06	0.00	114	115	117	110	108	97	110	7
AFG1	0.20	0.61	0.0000	0.0358	0.0517	0.0608	0.0472	-	0.0594	0.0367	0.05	0.01	68	98	116	89	113	70	92	22
AFG2	0.20	0.60	0.0000	0.0465	0.0474	0.0645	0.0466	-		0.0649	0.05	0.01	88	90	123	89		123	103	18
AOH	50.0	149.7	0.0000	18.2057	18.3156	22.9122	19.4411	-	15.6698	16.6562	18.53	2.52	140	141	176	149	120	128	142	14
AME	20.5	61.3	0.5301	5.7671	5.3676	4.5537	5.3283	-	5.1033	3.9490	5.01	0.65	98	91	76	90	86	64	84	13
BEA	10.1	30.3	0.0000	1.3690	1.4489	1.1559	1.6317	-	1.6134	0.7311	1.32	0.34	52	55	44	62	61	28	50	26
CIT	40.0	119.9	0.0134	6.6934	5.4556	5.6555	5.6159	-	5.2815	5.4796	5.70	0.51	64	52	54	54	51	52	54	9
CPA	50.2	150.1	1.8502	15.7433	13.8307	15.0895	16.1739	-	14.8159	15.6573	15.22	0.83	106	92	102	109	99	106	102	5
DON	20.1	60.1	0.2040	5.3909	4.5275	5.3124	5.3003	-	4.4500	4.3577	4.89	0.49	99	83	98	97	81	79	90	10
DAS	10.0	30.0	0.0000	2.6534	2.1466	2.1529	2.2072	-	2.4923	2.8190	2.41	0.29	101	82	83	84	95	108	92	12
ENB	5.00	14.97	0.1665	0.9821	1.0324	0.8504	0.8990	-	0.9720	0.5278	0.88	0.18	63	67	53	56	62	28	55	21
FB1	50.9	152.4	0.2099	12.6930	10.9428	15.3523	14.4530	-	31.0105	79.8388	27.38	26.69	94	81	114	107	232	600	205	97
FB2	50.1	150.0	0.1941	15.4501	14.7809	13.9984	17.8180	-	15.0390	20.6651	16.29	2.50	117	112	106	135	114	157	123	15
FB3	50.0	149.7	3.6790	11.3684	28.2379	13.6992	19.0610	-	18.7175	19.7152	18.47	5.84	59	189	77	118	115	123	113	32
FX	50.2	150.1	0.0000	9.2703	2.0824	6.4065	12.6507	-	3.2117	11.4303	7.51	4.34	71	16	49	97	25	87	57	58
HT2	5.04	15.07	0.0000	1.0270	0.8812	0.9927	0.6661	-	0.9606	1.2592	0.96	0.19	78	67	76	51	73	96	74	20
OTA	1.50	4.50	0.0262	0.4854	0.3225	0.3020	0.3117	-	0.3178	0.2641	0.33	0.08	117	76	71	73	74	61	78	23
STE	10.2	30.5	0.0044	2.4627	2.2845	2.4045	2.3002	-	2.3904	2.8194	2.44	0.20	93	86	91	86	90	106	92	8
T2	5.03	15.06	0.0101	1.3068	1.6700	1.1879	1.3423	-	1.1528	1.0481	1.28	0.22	99	127	90	102	87	79	97	17
ZON	20.0	59.9	0.1531	4.9410	3.6216	3.8122	4.8473	-	4.9077	4.5456	4.45	0.58	92	67	70	90	91	84	82	13
factor	2.994																			
weight (g)			4.99	5.01	5.00	4.99	5.01	4.99	5.00	5.01										
dilution factor			0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09										
solvent vol. (ml)			20																	
	1.5	water ratio																		

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of almonds with high spike concentrations

measured concentrations in samples											results									
analyte		conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment								not usable												
AcDON	100.7	301.5	0.5108	23.6954	21.0187	25.1704	25.1656	-	23.0388	22.3567	23.41	1.63	88	78	94	94	86	83	87	7
AFB1	0.95	2.86	0.0076	0.2777	0.2767	0.2892	0.2713	-	0.2675	0.2576	0.27	0.01	109	108	114	106	104	101	107	4
AFB2	0.96	2.88	0.0000	0.2588	0.2777	0.2801	0.2624	-	0.2623	0.2492	0.27	0.01	103	111	112	105	105	100	106	4
AFG1	0.96	2.88	0.0000	0.2258	0.2311	0.2502	0.2506	-	0.2431	0.2190	0.24	0.01	90	92	100	100	97	88	95	6
AFG2	0.95	2.84	0.0000	0.2853	0.2371	0.2163	0.2053	-	0.3007	0.2657	0.25	0.04	115	96	88	83	121	108	102	15
AOH	100.0	299.4	0.0000	47.2559	34.6583	42.7910	36.5449	-	30.6169	34.8371	37.78	6.10	181	133	165	140	117	134	145	16
AME	51.2	153.1	0.5301	14.4490	13.9564	13.4915	13.7076	-	11.4877	11.9258	13.17	1.19	104	101	98	99	82	86	95	9
BEA	20.0	59.9	0.0000	2.9001	2.9936	2.4325	3.4194	-	3.3887	1.4921	2.77	0.72	56	57	47	65	65	29	53	26
CIT	70.1	209.8	0.0134	8.8549	9.8054	8.4509	7.2786	-	9.5620	7.8294	8.63	0.98	48	54	46	40	52	43	47	11
CPA	100.7	301.5	1.8502	30.4909	27.4875	27.2089	25.1139	-	26.5360	25.9507	27.13	1.86	109	98	97	89	94	92	96	7
DON	50.2	150.3	0.2040	11.3653	10.8973	11.6896	11.4299	-	10.0918	10.8327	11.05	0.57	85	82	88	86	76	81	83	5
DAS	50.2	150.1	0.0000	11.8254	11.4994	11.6174	13.0626	-	11.3850	13.7611	12.19	0.98	90	88	89	100	87	106	93	8
ENB	10.0	29.9	0.1665	2.0093	1.9607	1.5936	1.7032	-	1.9187	1.0226	1.70	0.37	71	69	55	59	67	33	59	22
FB1	101.8	304.8	0.2099	20.9952	35.4157	33.1190	39.1663	-	40.7573	147.3419	52.80	46.84	78	133	124	147	153	556	199	89
FB2	100.2	300.0	0.1941	35.1209	33.3971	44.2086	27.9164	-	46.6712	45.3871	38.78	7.69	134	127	169	106	178	174	148	20
FB3	100.0	299.4	3.6790	18.5544	52.3690	24.8929	24.1631	-	54.8776	44.8684	36.62	15.93	57	187	82	78	196	158	126	43
FX	100.3	300.3	0.0000	19.6567	5.2362	18.9548	24.3085	-	5.8350	24.3235	16.39	8.70	75	20	73	93	22	93	63	53
HT2	10.1	30.1	0.0000	2.9055	2.6120	2.4031	1.5292	-	2.2066	2.7588	2.40	0.49	111	100	92	58	84	105	92	21
OTA	3.01	9.01	0.0262	0.6646	0.8816	0.5370	0.7361	-	0.6720	0.6211	0.69	0.12	81	109	65	90	82	76	84	17
STE	20.2	60.6	0.0044	4.8343	4.6404	4.9512	4.6951	-	4.5351	5.7343	4.90	0.43	91	88	94	89	86	109	93	9
T2	10.1	30.1	0.0101	2.6387	2.5684	2.4895	2.4716	-	2.9660	2.5353	2.61	0.18	100	98	95	94	113	97	99	7
ZON	50.1	149.8	0.1531	12.3548	11.5772	11.7729	10.7125	-	11.4519	15.0305	12.15	1.51	93	88	89	81	87	114	92	12

factor	2.994								
weight (g)		4.99	5.01	5.00	4.99	5.01	4.99	5.01	4.99
dilution factor		0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
solvent vol. (ml)		20							
1.5	**water ratio**								

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of walnuts with low spike concentrations

measured concentrations in samples											results								
analyte		conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment								not usable												
AcDON	50.4	150.7	0.0000	10.8227	12.3518	10.0746	11.9259	-	9.3740	11.2208	10.96	1.12	**82**	**94**	**77**	**91**	**72**	**85**	**84**	**10**
AFB1	0.21	0.62	0.0072	0.0837	0.0744	0.0846	0.0548	-	0.0805	0.0460	0.07	0.02	**143**	**126**	**145**	**89**	**137**	**72**	**119**	**23**
AFB2	0.20	0.61	0.0143	0.0623	0.0759	0.0458	0.0663	-	0.0654	0.0644	0.06	0.01	**91**	**117**	**60**	**99**	**97**	**95**	**93**	**15**
AFG1	0.20	0.61	0.0032	0.0397	0.0387	0.0593	0.0579	-	0.0610	0.0477	0.05	0.01	**69**	**68**	**107**	**104**	**110**	**84**	**90**	**20**
AFG2	0.20	0.60	0.0000	0.0451	0.0069	0.0678	0.0680	-	0.0693	0.0298	0.05	0.03	**86**	**13**	**129**	**130**	**132**	**57**	**91**	**53**
AOH	50.0	149.7	0.0000	22.2551	20.4092	20.1080	19.1338	-	12.4564	14.3225	18.11	3.84	**171**	**157**	**155**	**147**	**96**	**110**	**139**	**21**
AME	20.5	61.3	0.3466	5.6736	5.2788	4.3876	5.7998	-	5.5149	4.7236	5.23	0.56	**100**	**93**	**76**	**103**	**97**	**82**	**92**	**11**
BEA	10.1	30.3	0.0573	1.1009	1.0631	0.8980	1.0798	-	1.1437	0.6790	0.99	0.18	**40**	**38**	**32**	**39**	**41**	**24**	**36**	**18**
CIT	40.0	119.9	0.0000	5.9904	5.8295	4.9696	4.4114	-	5.5403	6.9511	5.62	0.88	**57**	**56**	**48**	**42**	**53**	**67**	**54**	**16**
CPA	50.2	150.1	1.6008	14.1534	14.2852	13.7782	14.8327	-	14.7408	15.4196	14.53	0.58	**96**	**97**	**93**	**102**	**101**	**106**	**99**	**4**
DON	20.1	60.1	0.6881	5.8551	5.2840	5.8533	6.6658	-	5.8550	7.2951	6.13	0.72	**99**	**88**	**99**	**115**	**99**	**126**	**104**	**12**
DAS	10.0	30.0	0.0147	1.9751	2.1480	2.4230	2.4247	-	2.1821	2.9628	2.35	0.35	**75**	**82**	**92**	**92**	**83**	**113**	**90**	**15**
ENB	5.00	14.97	0.7569	1.6913	1.7035	1.3655	1.3928	-	1.5091	0.7930	1.41	0.33	**72**	**73**	**47**	**49**	**58**	**3**	**50**	**24**
FB1	50.9	152.4	0.1467	12.1574	12.1620	16.9052	21.7828	-	27.8678	80.2326	28.52	26.04	**90**	**91**	**127**	**164**	**210**	**603**	**214**	**91**
FB2	50.1	150.0	0.2348	18.3507	14.7196	17.1993	17.7648	-	19.1872	27.7879	19.17	4.49	**139**	**111**	**130**	**135**	**146**	**211**	**145**	**23**
FB3	50.0	149.7	0.5272	14.8418	30.6148	12.2779	6.1912	-	10.5965	18.2747	15.47	8.46	**110**	**232**	**90**	**44**	**78**	**136**	**115**	**55**
FX	50.2	150.1	0.0000	7.5979	1.4162	4.4615	9.6467	-	2.8622	9.5534	5.92	3.51	**58**	**11**	**34**	**74**	**22**	**73**	**45**	**59**
HT2	5.04	15.07	0.2236	1.8347	1.3454	1.3167	0.9145	-	1.6381	1.2293	1.38	0.32	**123**	**86**	**84**	**53**	**108**	**77**	**88**	**23**
OTA	1.50	4.50	0.0473	0.3663	0.4075	0.2372	0.3896	-	0.3678	0.4293	0.37	0.07	**81**	**92**	**49**	**88**	**82**	**97**	**82**	**18**
STE	10.2	30.5	0.0097	2.0392	2.1746	2.3284	2.3242	-	2.1482	2.5506	2.26	0.18	**76**	**82**	**88**	**87**	**81**	**96**	**85**	**8**
T2	5.03	15.06	0.0882	1.5810	1.6872	1.1990	1.2996	-	0.9450	1.1897	1.32	0.27	**114**	**122**	**85**	**93**	**66**	**84**	**94**	**21**
ZON	20.0	59.9	0.2166	4.9116	4.2004	3.7580	4.0982	-	4.8759	5.1784	4.50	0.56	**90**	**77**	**68**	**75**	**90**	**95**	**82**	**12**
factor	2.994																			
weight (g)			5.01	5.01	4.99	4.99	4.99	4.99	4.99	5.01										
dilution factor			0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09										
solvent vol. (ml)			20																	
	1.5	**water ratio**																		

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of walnuts with high spike concentrations

			measured concentrations in samples								results									
analyte		conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment								not usable												
AcDON	100.7	301.5	0.0000	19.3204	22.2656	22.1067	22.2530	-	22.0651	19.2842	21.22	1.48	74	85	84	85	84	74	81	7
AFB1	0.95	2.86	0.0072	0.2841	0.3002	0.2712	0.2862	-	0.2621	0.2627	0.28	0.02	112	118	106	113	102	103	109	5
AFB2	0.96	2.88	0.0132	0.2441	0.2982	0.2265	0.3129	-	0.2213	0.2536	0.26	0.04	92	114	85	120	83	96	98	15
AFG1	0.96	2.88	0.0032	0.2669	0.2405	0.2365	0.2182	-	0.2352	0.1643	0.23	0.03	106	95	93	86	93	64	89	15
AFG2	0.95	2.84	0.0000	0.2385	0.2451	0.1635	0.2053	-	0.2041	0.2236	0.21	0.03	97	99	66	83	82	91	86	14
AOH	100.0	299.4	0.0000	46.1959	34.0083	33.1737	30.6686	-	24.5382	32.8568	33.57	7.07	178	130	128	118	94	126	129	21
AME	51.2	153.1	0.3466	15.2844	16.4071	12.6793	14.7913	-	12.7444	11.9520	13.98	1.77	112	120	93	109	93	87	102	13
BEA	20.0	59.9	0.0573	2.2943	2.2288	1.8156	2.2436	-	2.3754	1.3917	2.06	0.38	43	42	34	42	44	26	38	18
CIT	70.1	209.8	0.0000	11.2659	13.0296	7.4513	8.7925	-	10.6312	8.5025	9.95	2.07	62	71	41	48	58	47	55	21
CPA	100.7	301.5	1.6008	28.9753	27.8281	26.7410	28.0926	-	25.4604	28.9580	27.68	1.36	105	100	96	101	91	105	100	5
DON	50.2	150.3	0.6881	14.4343	12.4535	12.7503	12.6863	-	11.7870	14.4350	13.09	1.10	105	90	92	92	85	105	95	8
DAS	50.2	150.1	0.0147	11.9529	10.9432	11.2380	12.4461	-	12.4094	12.9853	12.00	0.78	92	84	86	95	95	100	92	6
ENB	10.0	29.9	0.7569	2.8401	2.6703	2.0848	2.2847	-	2.2989	1.3432	2.25	0.52	80	73	51	59	59	23	58	23
FB1	101.8	304.8	0.1467	16.8479	26.5943	26.5743	40.9196	-	50.9443	124.3864	47.71	39.45	63	100	100	154	191	470	180	83
FB2	100.2	300.0	0.2348	30.5595	33.4128	32.7774	43.1264	-	30.4988	48.6164	36.50	7.56	116	127	125	165	116	186	139	21
FB3	100.0	299.4	0.5272	25.1606	54.8729	18.3610	23.9460	-	54.2514	38.4487	35.84	15.93	95	208	69	90	206	146	136	44
FX	100.3	300.3	0.0000	15.9966	4.2983	15.3282	19.6911	-	5.2155	17.8043	13.06	6.61	61	16	59	76	20	68	50	51
HT2	10.1	30.1	0.2236	2.6943	2.4839	2.5684	2.2969	-	2.3576	2.4308	2.47	0.14	94	86	90	79	81	84	86	6
OTA	3.01	9.01	0.0473	0.5903	0.8530	0.6189	0.7629	-	0.6828	0.5730	0.68	0.11	69	103	73	92	81	67	81	16
STE	20.2	60.6	0.0097	4.6979	4.5667	4.9808	4.3477	-	4.7454	5.3892	4.79	0.36	89	86	95	82	90	102	91	8
T2	10.1	30.1	0.0882	2.6477	2.5836	2.3615	2.3017	-	2.2299	2.5518	2.45	0.17	98	95	87	85	82	94	90	7
ZON	50.1	149.8	0.2166	11.7462	14.2133	11.2088	10.4867	-	11.7800	12.8610	12.05	1.32	89	107	85	79	89	97	91	11
factor	2.994																			
weight (g)			5.01	4.99	5.01	4.99	4.99	5.00	5.01	4.99										
dilution factor			0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09										
solvent vol. (ml)			20																	
	1.5	**water ratio**																		

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

green: within performance criteria; **red:** beyond performance criteria with regulation; **yellow:** beyond performance criteria without regulation;

Complete validation results of sultanas with low spike concentrations

measured concentrations in samples											results									
analyte		conc. low	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment								not usable												
AcDON	50.4	100.7	0.0000	12.0684	13.0200	13.6383	12.3585	-	13.7064	12.0894	12.81	0.75	108	116	122	110	122	108	114	6
AFB1	0.21	0.41	0.0068	0.0708	0.0833	0.0835	0.0765	-	0.0798	0.0627	0.08	0.01	140	167	168	152	159	123	152	11
AFB2	0.20	0.40	0.0000	0.0647	0.0765	0.0742	0.0651	-	0.0905	0.0722	0.07	0.01	144	170	165	145	201	161	164	13
AFG1	0.20	0.40	0.0043	0.0332	0.0374	0.0402	0.0429	-	0.0467	0.0349	0.04	0.01	64	74	80	86	94	68	78	13
AFG2	0.20	0.40	0.0199	0.0508	0.0798	0.0583	0.0787	-	0.0589	0.0677	0.07	0.01	69	134	85	131	87	107	102	18
AOH	50.0	100.0	0.0000	27.9786	26.2429	22.1766	26.8340	-	16.8937	18.0417	23.03	4.74	252	236	199	242	152	163	207	21
AME	20.5	40.9	1.0530	10.7457	10.0410	7.4148	10.0369	-	8.9442	7.8357	9.17	1.33	213	198	140	198	173	149	178	15
BEA	10.1	20.2	0.0544	1.6742	1.7262	1.3619	1.5714	-	1.5409	0.9171	1.47	0.30	72	74	58	68	66	38	63	20
CIT	40.0	80.1	0.0000	11.8911	13.2836	9.2295	11.1084	-	10.4538	7.4981	10.58	2.03	134	149	104	125	117	84	119	19
CPA	50.2	100.3	0.9712	24.1223	19.1049	20.4654	21.9118	-	20.8757	20.1668	21.11	1.74	208	163	175	188	178	173	181	8
DON	20.1	40.2	0.5780	4.0039	4.1733	5.2596	4.8833	-	3.9539	3.8524	4.35	0.58	77	81	105	96	75	74	85	13
DAS	10.0	20.1	0.0000	3.3840	2.8527	2.9539	3.1096	-	3.0409	3.3629	3.12	0.22	152	128	132	140	136	151	140	7
ENB	5.00	10.00	0.1636	1.2026	1.2140	1.0200	1.0033	-	1.1040	0.5991	1.02	0.23	94	95	77	76	84	39	77	22
FB1	50.9	101.8	0.1771	10.5412	12.1948	15.7611	20.8436	-	34.7527	78.4336	28.75	25.85	92	106	138	183	305	693	253	90
FB2	50.1	100.2	0.0000	24.0488	21.8929	23.5368	18.5479	-	26.6349	23.7673	23.07	2.69	216	197	211	167	239	214	207	12
FB3	50.0	100.0	0.5939	15.5104	19.2118	10.5914	15.8077	-	36.3899	22.8148	20.05	8.98	134	168	90	137	322	200	175	45
FX	50.2	100.3	0.0000	11.7640	2.9806	7.5033	13.9105	-	3.3399	12.7481	8.71	4.81	106	27	67	125	30	115	78	55
HT2	5.04	10.07	0.8483	1.9055	2.5609	2.4136	2.1931	-	2.5921	3.8576	2.59	0.67	94	153	140	120	155	270	155	26
OTA	1.50	3.01	0.1449	0.5831	0.7240	0.5851	0.6319	-	0.6745	0.4621	0.61	0.09	131	173	131	146	158	95	139	15
STE	10.2	20.4	0.0305	3.1124	3.2780	3.2033	3.3616	-	3.0557	3.9743	3.33	0.33	136	144	140	147	133	175	146	10
T2	5.03	10.06	1.0737	2.2811	2.3960	2.2385	2.8473	-	2.3963	2.2555	2.40	0.23	108	118	104	159	118	106	119	10
ZON	20.0	40.0	0.1628	6.9383	5.6605	5.4970	6.7581	-	6.4378	6.4458	6.29	0.58	152	124	120	148	141	141	138	9
factor	2																			
weight (g)			5.00	5.00	5.00	5.01	5.00	5.01	5.01	4.99										
dilution factor			0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11										
solvent vol. (ml)			20																	
	1	**water ratio**																		

conc. low: low spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

comment: due to a low system impact, run 5 was not evaluable and therefore excluded for the validation; outlier were excluded as well

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Complete validation results of sultanas with high spike concentrations

measured concentrations in samples											results									
analyte		conc. high	Blank	C1_1	C1_2	C1_3	C1_4	C1_5	C1_6	C1_7	mean	SD	RR % C1_1	RR % C1_2	RR % C1_3	RR % C1_4	RR % C1_6	RR % C1_7	RR % mean	RSD %
comment								not usable												
AcDON	100.7	201.4	0.0000	33.4657	28.9208	32.8327	28.4134	-	30.4831	31.2390	30.89	2.04	150	129	147	127	136	139	138	7
AFB1	0.95	1.91	0.0068	0.3251	0.3656	0.3875	0.3711	-	0.3650	0.3384	0.36	0.02	150	169	180	172	169	156	166	6
AFB2	0.96	1.92	0.0000	0.2941	0.3406	0.3364	0.3327	-	0.3400	0.3250	0.33	0.02	138	159	158	156	159	152	154	5
AFG1	0.96	1.92	0.0043	0.2605	0.2657	0.2451	0.2290	-	0.2104	0.1666	0.23	0.04	120	122	113	105	97	76	105	16
AFG2	0.95	1.90	0.0199	0.3155	0.2898	0.2736	0.2973	-	0.2895	0.3634	0.30	0.03	140	128	120	132	128	162	135	10
AOH	100.0	200.0	0.0000	51.9069	43.0318	41.8216	44.9954	-	32.2562	40.3140	42.39	6.40	234	193	189	203	145	181	191	15
AME	51.2	102.3	1.0530	24.5884	26.7075	21.5552	25.8355	-	21.5738	21.0102	23.55	2.47	207	225	181	218	181	175	198	11
BEA	20.0	40.0	0.0544	3.3157	3.7237	2.8754	3.4034	-	3.1329	1.9561	3.07	0.61	73	82	64	75	69	43	68	20
CIT	70.1	140.1	0.0000	19.0278	19.7842	18.8677	16.1322	-	16.7826	16.9984	17.93	1.48	122	127	121	104	108	109	115	8
CPA	100.7	201.4	0.9712	43.8267	51.9124	42.9853	38.3689	-	38.1751	36.4893	41.96	5.67	192	227	188	167	167	158	183	14
DON	50.2	100.4	0.5780	10.0348	10.0260	10.1660	12.9252	-	11.6940	10.2056	10.84	1.20	85	85	86	111	100	86	92	11
DAS	50.2	100.3	0.0000	16.5294	13.9337	14.0294	17.0293	-	16.0021	15.2408	15.46	1.29	148	125	126	153	144	137	139	8
ENB	10.0	20.0	0.1636	2.3728	2.5929	2.0054	2.0683	-	2.1448	1.2044	2.06	0.47	99	109	83	86	89	47	86	23
FB1	101.8	203.6	0.1771	18.0544	24.3430	31.5015	34.2570	-	46.0376	151.6087	50.97	50.21	79	107	139	151	203	668	224	99
FB2	100.2	200.4	0.0000	45.2840	45.9549	43.0572	41.5919	-	47.3532	50.1703	45.57	3.06	203	206	194	187	213	225	205	7
FB3	100.0	200.0	0.5939	22.4643	52.3445	28.0696	28.2476	-	45.0297	54.3593	38.42	13.83	98	232	124	125	200	242	170	36
FX	100.3	200.6	0.0000	24.8122	6.9987	19.7920	28.7190	-	6.6760	26.7061	18.95	9.84	111	31	89	129	30	120	85	52
HT2	10.1	20.1	0.8483	3.9016	3.0739	3.6393	3.7622	-	3.7873	5.4292	3.93	0.79	136	99	125	131	132	204	138	20
OTA	3.01	6.02	0.1449	1.0156	1.2046	0.9163	0.9161	-	1.2106	1.0880	1.06	0.13	130	158	116	116	160	141	137	13
STE	20.2	40.5	0.0305	6.0722	7.3372	6.7056	6.8158	-	6.7487	7.6295	6.88	0.54	134	162	149	151	150	169	152	8
T2	10.1	20.1	1.0737	4.0821	3.7796	5.0486	5.2482	-	4.3683	4.5484	4.51	0.56	135	121	178	187	148	155	154	12
ZON	50.1	100.1	0.1628	18.0923	18.9020	14.2186	16.7841	-	20.6355	19.7769	18.07	2.31	161	168	127	150	184	176	161	13
factor	2																			
weight (g)			5.00	5.00	5.01	4.99	4.99	5.01	4.99	5.01										
dilution factor			0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11										
solvent vol. (ml)			20																	
	1	water ratio																		

conc. high: high spike concentration; **Blank:** natural contaminated amount; **C1_1-7:** measured value of samples in run 1-7; **mean:** mean value of measured amounts; **SD:** standard deviation of measured values; **RR % C1_1-7:** percentage recovery rate of samples 1-7; **RR % mean:** mean value of percentage recovery rate; **RSD %:** relative standard deviation in percent; **factor:** correction factor for sample weight; **weight (g):** weight of homogenised sample taken; **dilution factor:** weight/solvent volume; **solvent vol. (ml):** amount of extraction volume (ACN:H_2O:HCOOH); **water ratio:** ratio between sample and water

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