Instruction Multianalyte-IAC SENSI*Column* IAC Combi AOZDFT 3ml for LC-MS/MS

Simultaneous Extraction of Aflatoxins (A), Ochratoxins (O), Zearalenon (Z), Deoxynivalenol (D), Fumonisins (F) and T2-Toxin (T) in Food and Feedstuffs, with Simultaneous Clean-up by Multispecific Immunoaffinity Chromatography (IAC)

1. Introduction

The use of immunoaffinity columns (IAC) in the clean-up of single mycotoxins of complex food and feed extracts is well established in laboratory work.

This especially applies for the determination of mycotoxins which limits are regulated by authorities; that are aflatoxins, ochratoxins, zearalenone, deoxynivalenol, fumonisins and T2-toxin.

SENSI*Column* IAC Combi AOZDFT 3ml, the new Multianalyte-IAC, offers the user a tool to combine high purification potential of IAC, that means less matrix effects in apparatus even for difficult matrices, with multianalyte function of LC-MS/MS.

The most important mycotoxins, which are regulated by authorities, can be determined in parallel in one single run, that comprises extraction - enrichment - LC-MS/MS-measurement.

Maximum levels set by European Commission /2006[1] are met for all commodity samples. The same IAC protocol can be used for all commodities (see **2.2.1.**), except for baby food where a bigger aliquot of sample extract should be used because of very low maximum levels of aflatoxin and ochratoxin in that commodity (see **2.2.2.**).

Present instruction comprises:

- Simultaneous extraction of feed and food samples showed by rice flour (“easy matrix”), muesli and chocolate (“difficult matrix”) and baby food
- Enrichment with Multianalyte-IAC and working range of column
- Short description of common LC-MS/MS method
- Visualization of benefit using **SENSI*Column* IAC Combi AOZDFT 3ml** prior mass spectrometric determination

Full performance of **SENSI*Column* IAC Combi AOZDFT 3ml** column can only be guaranteed if pronounced criteria of solvent tolerance, analyte elution and working range of column are obeyed.

2. Procedure

2.1. Extraction

2.1.1. Example of "easy" matrix:

5g of rice flour, well homogenized by *Grindomix 200* mill, are extracted with 40ml of methanol/acetonitrile/water (25/25/50, v/v/v) by shaking (e.g. horizontal shaker *HS 501 D of IKA company*).

2.1.2. Example of "difficult" matrix:

For muesli same extraction procedure as for rice flour can be applied.

2.1.3. Example of baby food:

Baby food is currently being validated.

2.2. Enrichment

Regarding manner of commodity, the enrichment procedure by IAC has to be splitted.

All commodities except baby food can be cleaned up by applying 0.1 gram equivalents extract per IAC (see 2.2.1).

Baby food should be processed with 1 gram equivalent (see 2.2.2) to enable accurate determination of very low maximum levels of aflatoxin and ochratoxin even by sensitive mass spectrometers.

Organic solvent content of diluted extract to be applied on the column may not exceed 15%!

2.2.1. Extracts of „easy“ and „difficult“ matrices:

0.8ml extract (see **2.1.1.**, **2.1.2.**, containing AOZDFT-quantity of 0.1g commodity) is diluted with 10ml PBS and placed on top the **SENSI*Column* IAC Combi AOZDFT** column in an appropriate reservoir.

The rate of flow through the affinity gel is 1 to 3ml/min. Be aware of major air bubbles that eventually reside inside the gel or between gel and luer outlet of the column that hinder consistent flow and the necessary exchange of matter.

If extraction and enrichment procedure is carried out with given quantities, resulting organic solvent content (= 2% acetonitrile and 2% methanol) in PBS diluted extract does not affect recovery performance of **SENSI*Column* IAC Combi AOZDFT** column.

If bigger extract volumes shall be analyzed per column, the volume of the diluting PBS should be appropriately enlarged to ensure resulting organic solvent contents of acetonitrile and methanol do not exceed 15% very much in the solution to be applied on the column.

2.2.2. Extracts of baby food

E.g. 8ml extract (see **2.1.3.**, containing AOZDFT-quantity of 1g commodity) is diluted with 20ml PBS and placed on top the **SENSI*Column* IAC Combi AOZDFT** column in an appropriate reservoir...

Extraction and enrichment procedure are currently being validated.

2.3. Wash

After sample has passed the gel completely, the gel is washed by 5ml of water. After wash process is finished, remaining liquid residues in the gel are removed by applying either slight over- or underpressure.

2.4. Elution

The sample reservoir on top of the **SENSI*Column* IAC Combi AOZDFT 3ml**-column is removed and an appropriate vial to collect elute is placed below affinity column. Bond mycotoxins (AOZDFT) are eluted by using 3 x 1ml of methanol/acetic aicd (98/2, v/v) mixture.

Elution procedure in three successive seps!

To ensure complete elution of analytes from the gel, following elution conditions should be obeyed:

1. Flow rate of elution solvent through column should not exceed 1 ml/min.
2. The elution solvent is applied in 3 portions:

   First a volume of 1ml is applied on top of the gel; after shortly applying slight pressure, normally the column starts to elute by force of gravity.

   When it has passed, the next portion of 1ml is applied, when half of the quantity has passed, elution is stopped and the elution solvent is allowed to go inside gel particles for 30 seconds.

   After that the third portion is applied in the same manner like the second portion. Remaining liquid residues in the gel are removed by applying slight pressure.

Unified elution fractions are carefully evaporated to dryness by stream of nitrogen in heating block of 50°C temperature.

The residue is redissolved in 0.2ml of acetonitrile/0.1mM ammonium acetate buffer (30/70, v/v) (= LC-eluent).

3. Column characteristics

3.1. Specificity of SENSI*Column* IAC Combi AOZDFT 3ml -column

Aflatoxins (AFL) (= AFB1, AFB2, AFG1 und AFG2),

Ochratoxin (OTA),
Zearalenon (ZON),
Deoxynivalenol (DON),
Fumonisins (FUM) (= FB1, FB2),
T2-Toxin (T2, HT2).

3.2. Working range of SENSI*Column* IAC Combi AOZDFT 3ml -column

Within working range of column, recovery rates of 85-110%[a] of each mycotoxin given under 3.1. are valid.

Working ranges of the single mycotoxins!

Mycotoxin	Working Range IAC	Commodity[c]	Regulated Limits[d]
Aflatoxine (AFL)	0.02[b] - 3ng	(0.2 – 30µg/kg)	0.1[f] – 15µg/kg[g]
Ochratoxin (OTA)	0.02[b] - 2ng	(0.2 – 20µg/kg)	0.5 – 10µg/kg
Zearalenon (ZON)	0.2[b] - 20ng	(2 – 200µg/kg)	20 – 100µg/kg
Deoxynivalenol (DON)	1[b] - 350ng	(10 – 3500µg/kg)	200 – 1750µg/kg
Fumonisin (FUM)	0.2[b]- 400ng	(2 – 4000µg/kg[e])	200 – 2000µg/kg[e]
T2-Toxin (T2, HT2)	0.2[b]– 400ng	(2 – 4000µg/kg)	50 – 1000µg/kg

[a] Recovery rates are guaranteed as long as solvent contents in PBS diluted extract do not exceed 10% methanol and 5% acetonitrile.
[b] Minimum limit of working range even in this sub Nano gram dimension is only determined by sensitivity of detection system, not by affinity of IAC. Given values are typical for a common LC-MS/MS apparatus. An example is given below (see **4.2.**).
[c] Ranges of commodities contents in brackets which can be determined if 1/10 gram equivalents of extract are analyzed per column.
[d] Ranges of maximum levels according authority regulation.
[e] Sum of fumonisins B1 und B2.
[f] Only for aflatoxin B1; remaining aflatoxins have higher maximum levels.
[g] Sum of aflatoxins B1, B2, G1 und G2

3.3. Column Capacities[h] of SENSI*Column* IAC Combi AOZDFT 3ml –column

Mycotoxin	Column Capacity
Aflatoxins (AFL)	500ng
Ochratoxin (OTA)	3500ng
Zearalenon (ZON)	3400ng
Deoxynivalenol (DON)	2500ng
Fumonisins (FUM)	6000ng

T2-Toxin (T2, HT2)	5100ng

(h) An excess of aflatoxins (AFL), ochratoxin A, zearalenon, deoxynivalenol, fumonisins (FUM) and T2-Toxin, thus, 5µg each of AFL, OTA, ZON and DON and 10µg each of FUM und T2 in 2ml PBS is incubated with the column for 5 minutes. After that the column is washed with 2ml PBS. The fraction containing the unbond analytes is analyzed. The difference between spiked quantity of analytes and non bonded analytes equals maximum column capacity.

3.4. Crossreactivities(i) of SENSI*Column* IAC Combi AOZDFT 3ml -column

Mycotoxin	**Crossreactivity**
Aflatoxin B1	100%
Aflatoxin B2	86%
Aflatoxin G1	101%
Aflatoxin G2	82%
Aflatoxin M1	81%
Ochratoxin A	100%
Ochratoxin B	103%
Zearalenon	100%
α-Zearalanol	99%
β-Zearalanol	94%
α-Zearalenol	97%
β-Zearalenol	86%
Deoxynivalenol	100%
Nivalenol	31%
15-Acetyl-deoxynivalenol	33%
3-Acetyl-deoxynivalenol	<1%
Fumonisin B1	100%
Fumonisin B2	103%
T2-Toxin	100%
HT2	118%

(i) Relative recovery rates, when having the half of the bonding sites occupied. Thus, a half-saturating quantity of species of each mycotoxin group (= ½ * column capacities) is analyzed per column.

4. LC-MS/MS Method

Examples of method and apparatus are given. Both might serve as comparison.

4.1. LC conditions

HPLC-device: Agilent 1200 Series with binary pump, Column: Macherey-Nagel EC 150/3 Nucleodur Sphinx RP, 3µm, Length: 150 mm, ID: 3 mm.

Modul 1 (Aflatoxins, Ochratoxin A, Fumonisins): Mobile phase A: 0.1mM ammonium acetate / acetonitrile / acetic acid (84.9%/15%/0.1% v/v/v); mobile phase B: acetonitrile; gradient: 0min A 100%; 1min A 100%; 24min A 37.5%; 25min A 100%; flow rate: 0.4ml/min; time of analysis: 32min; injector volume: 100, 25 or 1µl, depending on which section (low/middle/high) of working range of IAC (see **3.2.**) is going to be measured.

Modul 2 (ZON, DON, HT-2, T-2): Mobile phase A: 0.1mM ammonium acetate / acetonitrile (80%/20% v/v); mobile phase B: acetonitrile; gradient: 0min A 100%; 1min A 100%; 24min A 37.5%; 25min A 100%; flow rate: 0.4ml/min; time of analysis: 32min; injector volume: 100 or 10µl , depending on which section (low/middle/high) of working range of IAC (see **3.2.**) is going to be measured.

4.2. MS/MS conditions

Apparatus: AB Sciex API 4000 LC-MS/MS system with electrospray (Turbo-V)-ionization (ESI).

Polarities:
Modul 1 (Aflatoxins, Ochratoxin A, Fumonisins): all ESI-(+).
Modul 2 (DON, ZON, HT-2, T-2): DON/ZON: ESI-(-); HT-2/T-2: ESI-(+).

Linearity parameters of the AB Sciex API 4000 LC-MS/MS system

Mycotoxin	**SRM-Transition**		**Maximum Values**	**Linearity**
	Quantifier	**Qualifier**		
Aflatoxin B1	313,2/241,0	313,2/213,4	0,2-30 µg/kg	0,9986
[$^{13}C_{17}$]Aflatoxin B1	329,9/255,1			
Aflatoxin B2	315,0/259,0	315,0/287,2	0,2-30 µg/kg	0,9992
Aflatoxin G1	329,1/243,1	329,1/215,2	0,2-30 µg/kg	0,9994
Aflatoxin G2	331,1/313,2	331,1/217,0	0,2-30 µg/kg	0,9964
Ochratoxin A	404,0/239,0	404,0/221,0	0,5-20 µg/kg	0,9953
[$^{13}C_{20}$]OTA	409,0/239,0			
Zearalenon	317,1/131,0	317,1/175,0	0,5-200 µg/kg	0,9993
[$^{13}C_{18}$]ZON	319,0/205,0			
Deoxynivalenol	355,0/265,0	355,0/295,0	10-3500 µg/kg	0,9993
[$^{13}C_{15}$]DON	370,4/279,1			
Fumonisin B1	722,6/334,3	722,6/352,3	0,5-2000 µg/kg	0,9998
[$^{13}C_{34}$]FB1	756,8/374,6			
Fumonisin B2	706,6/336,5	706,6/318,3	0,5-2000 µg/kg	0,9983
[$^{13}C_{34}$]FB2	470,6/358,4			
T2-Toxin	484,2/215,2	484,2/185,1	0,5-2000 µg/kg	0,9991
[$^{13}C_{24}$]T-2	508,4/229,2			
HT2-Toxin	442,3/263,2	442,3/215,2	2-2000 µg/kg	0,9997
[$^{13}C_{24}$]HT-2	508,4/229,2			

5. Benefit

The advantage of using **SENSI*Column* IAC Combi AOZDFT 3ml** column prior to LC-MS/MS instead of using dispersive solid extraction (DSPE)[2] or even direct injection afore, is shown by the comparison of the corresponding Extracted Ion Chromatograms (EIC) (see **5.1.**).

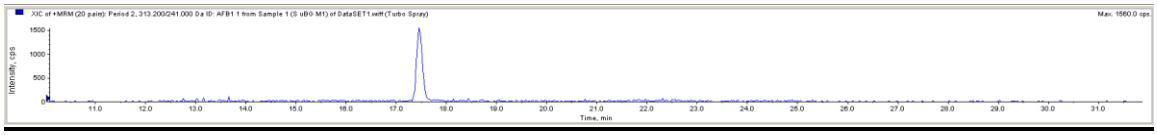
Please consider that DSPE or other SPE-methods are only able to retard certain groups of mycotoxins. **In contrast to that SENSI*Column* IAC Combi AOZDFT 3ml column retards all 11 mycotoxins in one run!**

Following figures show Quantifier-Extracted Ion Chromatograms of standard, IAC purified extract, DSPE extract and raw extract in the example of muesli measured by AB Sciex API 4000 apparatus.

5.1. Quantifier- Extracted Ion Chromatograms (EIC´s) of Aflatoxin B1, Ochratoxin A, DON and HT2 (Selection) depending on method of purification prior to LC-MS/MS

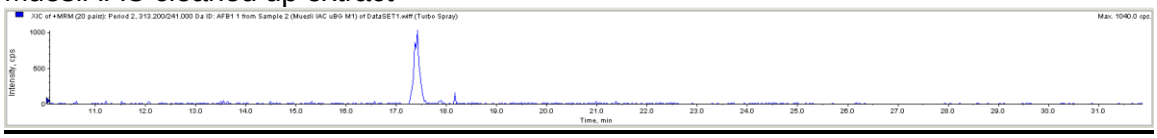
Aflatoxin B1

Standard



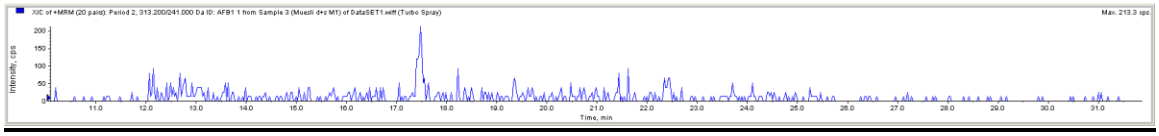
S/N: ca. 15:1

Muesli IAC cleaned up extract



S/N: ca. 15:1

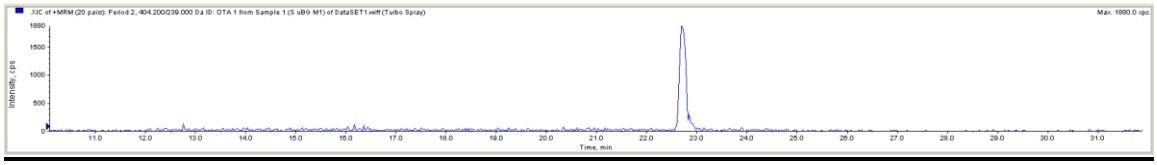
Muesli raw extract



S/N: ca. 4:1

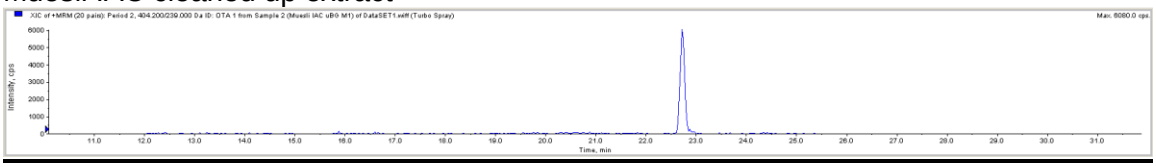
Ochratoxin A

Standard



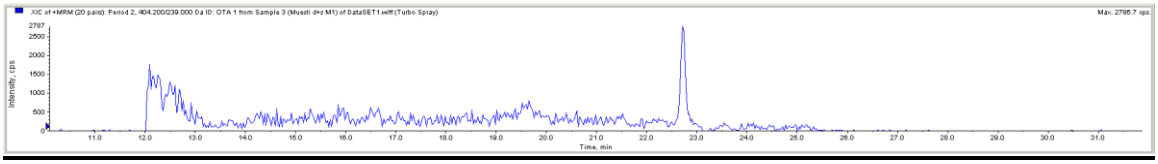
S/N: ca. 18:1

Muesli IAC cleaned up extract



S/N: ca. 60:1

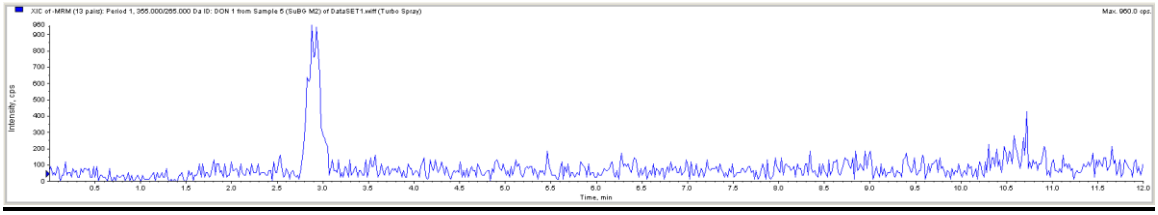
Muesli raw extract



S/N: ca. 6:1

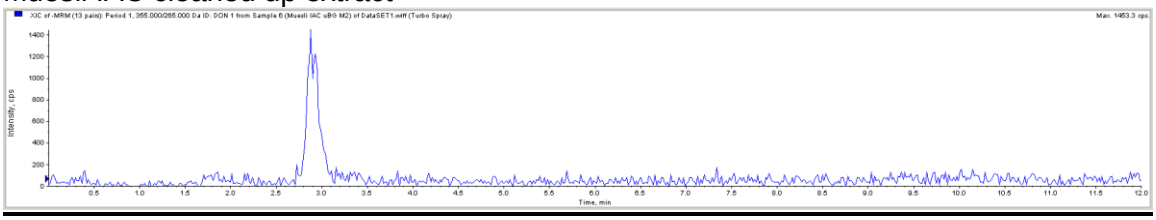
Deoxynivalenol

Standard



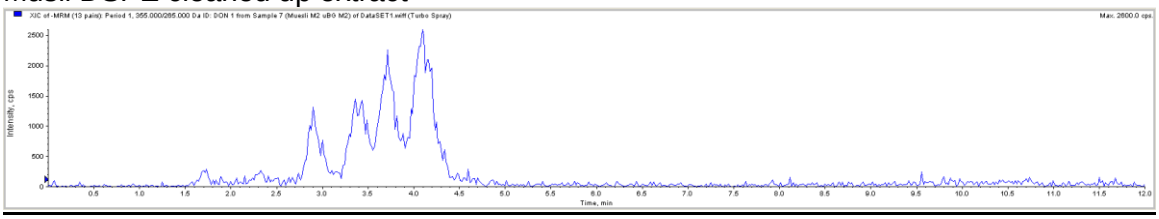
S/N: ca. 10:1

Muesli IAC cleaned up extract



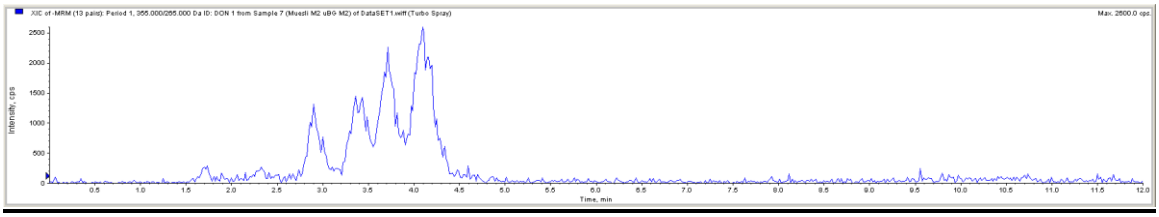
S/N: ca. 8:1

Müsli DSPE cleaned up extract



S/N: ca. 5:1

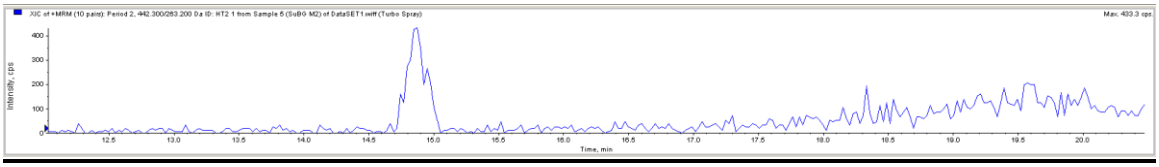
Muesli raw extract



S/N: ca. 5:1

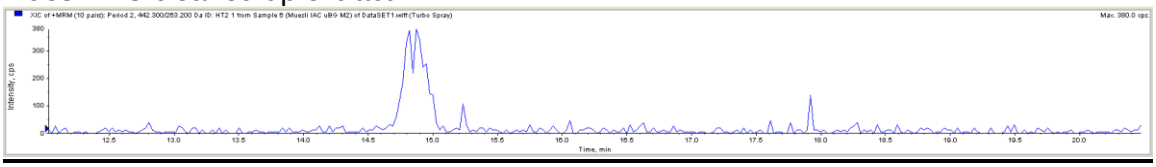
HT-2

Standard



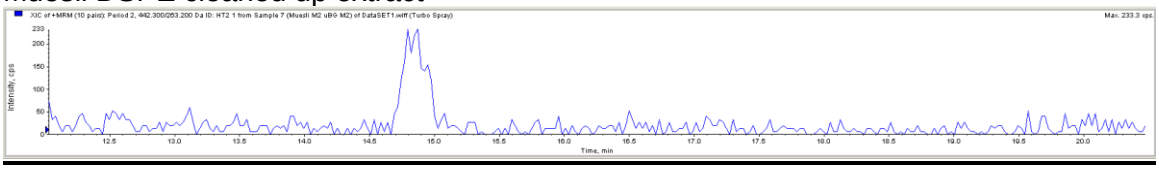
S/N: ca. 20:1

Muesli IAC cleaned up extract



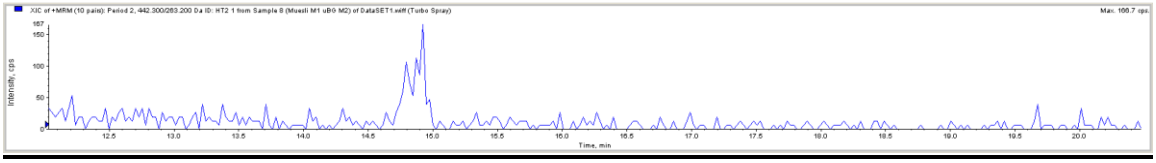
S/N: ca. 10:1

Muesli DSPE cleaned up extract



S/N: ca. 5:1

Muesli raw extract



S/N: ca. 3:1

6. Material

10mM PBS-Buffer, pH 7,4 (=PBS):

0,25 g KH_2PO_4 1,45 g K_2HPO_4 8,76 g NaCl	Dissolve salts in 1 L deionized water. If necessary, adjust pH at 7,4 (± 0.2) with 1M NaOH or 1M HCl

Ammonium acetate-Buffer:

5mM ammonium acetate:	Dissolve 0,385 g ammonium acetate in 1 L deionized water
0,1mM ammonium acetate:	Dilute 20 mL 5mM ammonium acetate with deionized water to 1 L

Elution Solvent:

Methanol/Acetic Acid (98/2, v/v):	Mix 2 mL acetic acid and 98 mL methanol

LC-Eluent:

Acetonitrile / 0,1mM ammonium acetate (30/70, v/v):	Mix 850mL 0,1mM ammonium acetate, 150mL acetonitrile (LC/MS-Grade) and 1mL acetic acid (LC/MS-Grade) and degas in ultrasonic bath

Chemicals:

- Acetonitrile, LC/MS grade
- Methanol, LC/MS grade
- Acetic acid, LC/MS grade
- Deionized water
- Dipotassium hydrogen phosphate, >98 %
- Potassium dihydrogen phosphate, >98 %
- Sodium chloride
- Ammonium acetate, LC/MS grade

Gas:

- Nitrogen for the evaporation of IAC-eluates

Consumables:

- **SENSI*Column* IAC Combi AOZDFT 3ml**

Standards:	Stable Isotope Labeled Standards:
Aflatoxin B1, Fa. Coring[(j)], Cat. No. 3009114s (1 mL, c = 2 µg/mL)	[13C17]Aflatoxin B1, Fa. Coring, Cat. No. 3009188 (1,2 mL, c = 0,5 µg/mL)
Aflatoxin B2, Fa. Coring, Cat. No. 3009115s (1 mL, c = 0,5 µg/mL)	
Aflatoxin G1, Fa. Coring, Cat. No. 3009116s (1 mL, c = 2 µg/mL)	
Aflatoxin G2, Fa. Coring, Cat. No. 3009117s (1 mL, c = 0,5 µg/mL)	
Ochratoxin A, Fa. Coring, Cat. No. 3009125s (1 mL, c = 100 µg/mL in ACN)	[13C20]Ochratoxin A, Fa. Coring, Cat. No. 3009144 (1,2 mL, c = 25 µg/mL in ACN)
Zearalenon, Fa. Coring, Cat. No. 3009128 (5 mL, c = 100 µg/mL in ACN)	[13C18]Zearalenon, Fa. Coring, Cat. No. 3009177 (1,2 mL, c = 25 µg/mL in ACN)
Deoxynivalenol, Fa. Coring, Cat. No. 3009124 (5 mL, c = 100 µg/mL in ACN)	[13C15]Deoxynivalenol, Fa. Coring, Cat. No. 3009102 (1,2 mL, c = 25 µg/mL in ACN)
Fumonisin Mix, Fa. Coring, Cat. No. 3009123s (1 mL, c (FB1) = 50 µg/mL, c (FB2) = 50 µg/mL in ACN/Wasser 1/1)	[13C34]Fumonisin B1, Fa. Coring, Cat. No. 3009143 (1,2 mL, c = 25 µg/mL in ACN) [13C34]Fumonisin B2, Fa. Coring, Cat. No. 3009149 (1,2 mL, c = 10 µg/mL in ACN)
T-2, Fa. Coring, Cat. No. 3009099s (1 mL, c = 100 µg/mL in ACN)	[13C24]T-2, Fa. Coring, Cat. No. 3009109 (1,2 mL, c = 25 µg/mL in ACN
HT-2, Fa. Coring, Cat. No. 3009091s (1 mL, c = 100 µg/mL in ACN)	[13C22]HT-2, Fa. Coring, Cat. No. 3009146 (1,2 mL, c = 25 µg/mL in ACN)

(j) Fa. Coring in Gernsheim, Germany

7. Bibliography:

[1] COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, *OJ* **L 364**, 20.12.2006, p. 5

[2] Trebstein, A.; Lauber, U.; Humpf, H.-U. (2009): Analysis of Fusarium toxins via HPLC-MS/MS multimethods: matrix effects and strategies for compensation; *Mycotoxin Research*; **25**, p. 201-213