ANALYSIS OF WASTEWATER SAMPLES FOR DETERMINATION OF MYCOTOXINS

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Mycotoxins are a large group of various structural compounds and biological exposures, and they are made by various groups of fungi. Mycotoxins are considered a global health, agriculture, and economical threat. Most mycotoxins are chemically stable, they survive storage and processing, and could even remain in processed food. Mycotoxins are metabolized by liver and/or kidney and depending on their type and their metabolized products are excreted. Wastewater based epidemiology (WBE) is a novel biomonitoring approach for tracking human exposure to mycotoxins based on the analysis of wastewater (WW) samples. For WBE applications sensitive, and selective WW sample preparation and instrumental methods were developed by several authors [1].

Within the current study the main parameters of the analysis were optimized for the determination of mycotoxins which occur in WW samples – deoxynivalenol (DON), beauvericin (BEA), enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB) and enniatin B1 (ENNB1).

13 analytical columns were compared by intensity, peak shape, sorption coefficient and number of theoretical plates (see *Table 1*).

Table 1

List of analytical columns used in the study

Nr.	Column	Size, mm	Sorption coefficient	Number of theoretical plates
1.	Luna C18 3 µm	150 x 4,6	-	-
2.	Kinetex C18 1.7 µm	50 x 3,0	7,6	411
3.	Hypercarb 5 µm (used)	100 x 2,1	18	905
4.	Kinetex C18 1.7 µm	100 x 3,0	7,3	2004
5.	Synergi Polar-RP 4 µm	150 x 3,0	7,8	1080
6.	**Kinetex PFP 1,7 µm with UHPLC filter**	**100 x 3,0**	**4,4**	**4774**
7.	Omega Polar C18 1.6 µm	100 x 1,0	18	1947
8.	Omega Polar C18 3 µm	100 x 3,0	8,8	1838
9.	Kinetex Phenyl-Hexyl 2.6 µm	100 x 2,1	6,7	958
10.	Synergi Hydro-RP 4 µm	150 x 3,0	12	1171
11.	Synergi Max-RP 4 µm	150 x 3,0	14	633
12.	Hypercarb 5 µm (used)	100 x 2,1	1.4	58
13.	**Hypercarb 5 µm (new)**	**100 x 2,1**	**45**	**804**

Hypercarb 5 µm column was selected to develop the method for the determination of DON, as it showed very good sorption coefficient, and Kinetex PFP 1.7 µm column was applied for separation of BEA, ENNA, ENNA1, ENNB and ENNB1 due to optimal resolution and the number of theoretical plates.

For the detection of mycotoxins, it is necessary to use acidified phases with the addition of salts, because the additive increases the ionization of mycotoxins.

It was observed that no analyte peaks were observed in the samples using deionized water

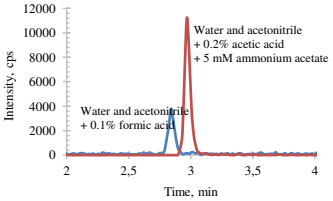

Figure 1. DON chromatogram in different phases using Kinetex PFP column

and acetonitrile. Using water and acetonitrile supplemented with 0.1% formic acid, the adducts were unstable and the samples showed poor repeatability. The phase system that solved the sensitivity and fragment stability problems was deionized water and methanol to which 0.2% acetic acid and ammonium acetate additives because the intensity and shape of the peak was satisfactory (see Figure 1).

SPE columns were used to clean-up and concentrate the samples. The SPE columns Strata C18-E, Strata X, Oasis HLB, Strata Basic Screen and Strata ABW were compared. Using the Strata ABW and Basic Screen columns, it was found that they were not suitable for DON determination because DON was not found.

100 mL of artificially contaminated deionized water was used for the method development experiment. The sample was applied in portions to the columns, one fraction of each 20 mL of sample was collected to observe analyte losses. Fractions were analysed and it was found that using Strata C18-E columns, DON leaches from the sorbent due to the addition of large sample volumes, DON losses are 53% (see Figure 2).

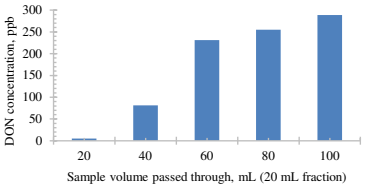

Figure 2. DON elution profile using Strata C18E columns

The absolute SPE recovery for Strata C18-E, Strata X and Oasis HLB columns was compared. 3 replicates were performed for each point, with the standard additive concentration of 5 ng/L for BEA and ENN and the standard additive concentration of 25 ng/L for DON (see *Table 2*).

Table 2

Absolute SPE recovery of mycotoxins at Milli-Q water standard addition concentration of 5 ng/L for ENN group and BEA, 25 ng/L for DON

		Strata C18-E, %	Strata X, %	Oasis HLB, %
DON		9,0	94	94
BEA		59	90	75
ENNA		67	104	92
ENNA1		71	103	82
ENNB		72	106	76
ENNB1		72	106	78
Average:	**PFP method**	**68**	**102**	**81**
	Hypercarb methode	**9,0**	**94**	**94**

The absolute recovery of DON using Strata C18-E column was found to be 9.0%. In the case of BEA and ENN, the recovery of the column was in the range of 59 - 72%. The absolute recovery of the Strata-X column with all compounds was excellent, in the range of 94 - 106%. The absolute recovery of Oasis HLB columns was similar to Strata-X columns.

The composition of different samples of WW is very variable and varied, so it was necessary to make sure that the chosen extraction is suitable for the analysis of this complex matrix. The extraction efficiency for Stata-X columns was tested by concentrating 500 mL WW sample. Tap water was used as a control (see *Table 3*).

Table 3

Strata-X column extraction efficiency

	Tap water	Watewater Nr 1	Watewater Nr 2	Watewater Nr 3
DON	97%	98%	96%	98%
BEA	56%	187%*	84%	92%
ENNA	69%	166%*	84%	97%
ENNA1	74%	104%	88%	98%
ENNB	72%	101%	89%	109%
ENNB1	82%	103%	96%	95%

* Coarse error by Dixon criterion

It was observed that in the case of Strata-X column DON with tap water is 97%, with WW matrix is 96-98%. In the case of BEA and ENN, the results with the Strata-X column are also satisfactory - the extraction efficiency was in the range of 84 - 109%.

Table 2 and Table 3 show extraction efficiencies and absolute recoveries greater than 100%, which could be explained by the presence of exogenous substances from external sources during sample preparation, such as residues released from SPE cartridges [2].

Strata-X columns were chosen for further sample preparation. Oasis HLB columns are good alternative for DON, BEA and ENN detection, but the column price is higher than Strata-X columns.

Different sample volumes were compared in our study. The larger the sample volume, the higher the signal intensity in the case of DON. 666 mL and 1000 mL were selected for comparison of BEA and ENN. It was observed that with 666 mL of sample volume, the signals were more intense than with 1000 mL, except for ENNA1. The results obtained are contrary to expectations, which could be explained by the capacity of SPE columns. An increased sample volume theoretically provides higher concentration of analytes after concentration, there are losses and larger matrix effects rather than increases in intensity. Increasing the sample volume to 1000 mL increases the signal intensity for DON and ENNA1, but decreases for BEA, ENNA, ENNB and ENNB1 due to matrix effects. For the DON analysis, the optimal volume was 500 mL of sample. Volume greater than 1000 mL strongly affected the chromatographic determination, increased the load on the SPE columns, increased the matrix effect, increased the sample preparation time.

The stability of the sample was tested by testing artificially contaminated samples after a certain time, which is stored at different temperatures. 5 L wastewater sample was used, which was initially analysed in series with a blank sample and sample with standard additive concentration of 5 ng/L. After analysis of the sample, standard addition of mycotoxins at concentration of 5 ng/L was added to the residue. The 4L sample was aliquoted into 2L and stored at -20 ℃ and + 4 ℃, respectively. The sample was retested after 1 week and after 2 weeks. During the experiment, it was shown that compounds in the sample are stable for 2 weeks when stored both in the freezer and in the refrigerator, so the analysis of the samples should be performed within 2 weeks.

The selectivity of the method was demonstrated by preparing blank matrix sample and comparing it with an analogous matrix sample supplemented with the mycotoxin standard additive at the level of the limit of quantification (LOQ). The method shows good selectivity, however, signal of natural contamination is observed in the case of DON and ENNB. During the development of the analytical procedure, natural contamination of DON and ENNB at all levels was always detected in all analysed

samples, therefore it had to be considered when continuing the development of validation.

The linearity of the method was checked by constructing calibration lines from standard solutions in the case of DON, as it is possible to use the internal standard method and matrix calibration in the case of BEA and ENN prepared according to the standard additive calibration method. In the case of the BEA and ENN groups, the concentration of mycotoxins in the samples was determined by calibrating one point for each sample (for screening purposes), which can be used because the linearity of the compounds in different matrices was demonstrated. If the concentration was determined above the first calibration point, the analysis was repeated by 5-level calibration with the standard addition method. In the case of BEA and ENN, it is not possible to use calibration with standard solutions for quantification because of the large matrix effect and the need for an internal standard to reduce it. Therefore, calibration of the standard additive method on each sample was used to reduce matrix effect errors.

The limit of quantification (LOD) was defined as the concentration in the sample with a signal-to-noise ratio ≥ 3 and was assessed by repeated analysis of blank samples with different concentrations of standard additives.

The limit of quantification (LOQ) was defined as the concentration in the sample with a signal-to-noise ratio ≥ 10 and was assessed by repeated analysis of blank samples with different concentrations of standard additives.

Data on instrumental detection limits (IDL), LOD and LOQ were collected (see *Table 4*).

Table 4

Instrumental detection limits, LOQ and LOD

Compound	IDL, ng/L	LOD, ng/L	LOQ, ng/L
Deoxynivalenol	0,18	1,9	6,4
Beauvericin	0,0025	0,039	0,13
Enniatin A	0,010	0,12	0,40
Enniatin A1	0,0089	0,14	0,47
Enniatin B	0,0055	0,044	0,15
Enniatin B1	0,017	0,13	0,43

It was concluded that the developed method is suitable for the determination of mycotoxins of the DON, BEA and ENN groups, the lowest and optimal LODs for their detection were achieved.

The relative recovery of the method was defined as the ratio of the measured area of the standard additive and the internal standard added before extraction divided by the ratio of the net measured additive to the internal standard [2]. The relative recovery was applied to the DON compound using the internal standard. The absolute recovery of the method was defined as the ratio of the concentration of the measured sample with the standard additive to the corresponding concentration of the measured standard solution [2]. The absolute recovery was applied to all combinations.

The relative recoveries of the method DON were shown in the table (see *Table 5*).

Table 5

Relative recovery of the method DON

Compound	Average relative recovery, %	Min. – max. relative recovery, %
Deoxynivalenol	102	92 – 110

It was demonstrated that the relative recovery of DON is in the range of 92 - 110%, on average 102%. The absolute recoveries of the method were shown in the table (see *Table 6*).

Table 6

Absolute recovery of the method

Compounds	Average absolute recovery, %	Min. – max. absolute recovery, %
Deoxynivalenol	105	84 – 118
Beauvericin	91	66 – 115
Enniatin A	94	63 – 126
Enniatin A_1	104	82 – 131
Enniatin B	89	59 – 113
Enniatin B_1	89	64 – 109

The experiment showed that the absolute recovery was in the range of 59 - 131% (DON 84-118%, BEA 66-115%, ENNA 63-126%, ENNA1 82-131%, ENNB 59-113% and ENNB1 64-109%).

The matrix effect (ME) was evaluated as the difference between the slope of the calibration graph performed on the matrix and the slope of the calibration graph performed on the standard solutions. 3 different wastewater samples of unknown origin were used in the experiment (see *Table 7*).

Table 7

Matrix effect for wastewater samples

	Wastewater Nr 1	Wastewater Nr 2	Wastewater Nr 3	Average value
DON	8%	–	–	**8%**
BEA	-96%	-78%	-91%	**-88%**
ENNA	-40%	-24%	-51%	**-38%**
ENNA1	-75%	-51%	-56%	**-61%**
ENNB	-65%	-49%	-70%	**-61%**
ENNB1	-67%	-38%	-64%	**-56%**

It was concluded that for all compounds in almost all samples a negative matrix effect is observed - during ionization the suppression of the ion of the compound takes place. For the DON experiment, one average sample was tested (sample obtained by combining different effluents in an average sample to obtain a representative effluent sample) and quantification was performed with standard solutions using an internal standard. The level of matrix effect varies between samples, which means that it is not correct to use matrix calibration on a single sample basis. At least one point calibration per sample must be performed to perform a correct quantification without an internal standard. If uniform samples (obtained from the same site of similar composition) are expected, it will be possible to use matrix calibration from one sample with the standard additive before performing the experiment.

The expanded measurement uncertainty (at 95% confidence interval) was assessed by repeated measurements of samples with the standard additive near the limit of quantification (quality control samples) and by calculating the relative standard deviation from the recovery of the measurement results. Expansion factor k = 2.

The estimated preliminary uncertainties for all detectable compounds ranged from 17% to 44%. To calculate the uncertainty, the quality control data were accumulated and summarized in a table (see *Table 8*).

Table 8

Uncertainty of the method

Compounds	U, %
Deoxynivalenol	17
Beauvericin	44
Enniatin A	43
Enniatin A1	36
Enniatin B	44
Enniatin B1	42

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References:

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