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Multi-residue Screening Method To Quantify Mycotoxins in Aqueous Environmental Samples

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Mycotoxins are naturally occurring secondary metabolites of fungi colonizing agricultural products on the field or during storage. In earlier work, we have shown that two common mycotoxins, i.e., zearalenone and deoxynivalenol, can be present at significant levels in the aquatic environment. This raises the question about the relevance of a wider range of mycotoxins in natural waters. In this investigation, we present the first validated method for analysis of some additional 30 mycotoxins in drainage, river, and wastewater treatment plant effluent water. The method includes solid-phase extraction over Oasis HLB cartridges, followed by liquid chromatography with electrospray ionization triple quadrupole mass spectrometry. Absolute method recoveries for 13 of the 33 mycotoxins were higher than 70% in wastewater treatment plant effluent (at 25 ng/L), and 27 compounds had method detection limits (MDLs) below 10 ng/L. The applicability of this method is illustrated with selected data from our ongoing monitoring campaigns. Specifically and for the first time, beauvericin and nivalenol were quantified in drainage and river water samples with mean concentrations of 6.7 and 4.3 ng/L and 6.1 and 5.9 ng/L, respectively. These compounds thus add to the complex mixture of natural and anthropogenic micropollutants in natural waters, where their ecotoxicological risk remains to be evaluated.

KEYWORDS: Micropollutants; natural toxins; natural waters; liquid chromatography; tandem mass spectrometry; trace analysis; isotope-labeled internal standard

INTRODUCTION

Mycotoxins are naturally occurring secondary metabolites of fungi colonizing a variety of cereals, fruits, vegetables, and organic soil material but can also be produced as a result of moist conditions during storage. These compounds are therefore commonly found in crops grown and stored for human or animal consumption, as well as in processed food (*1*, *2*).

More than a hundred filamentous fungi are known, e.g., *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium*, and *Penicillium* species, which produce a plethora of mycotoxins with a great structural diversity and, thus, different chemical and physical properties. Among the most prominent substance classes are *Alternaria* toxins, aflatoxins, ergot alkaloids, fumonisins, ochratoxins, resorcylic acid lactones, and trichothecenes. Mycotoxins and their associated health impact on humans and animals have been broadly investigated (*3*, *4*). Several of them (e.g., aflatoxins, ochratoxins, and fumonisins) have been ranked as the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues (*5*). Therefore, their occurrence in food and feed has been studied extensively (*6*, *7*).

In contrast, very little is known about the distribution of mycotoxins in the environment, and only a few studies have been published. Recent work (*8*−*10*) demonstrated that the common mycotoxins zearalenone and deoxynivalenol can be emitted into the aquatic environment via drainage and runoff from infested agricultural fields. The concentrations and corresponding emission rates of deoxynivalenol and zearalenone in drainage water were 20−5000 ng/L and 600 mg/ha for deoxynivalenol and up to 35 ng/L and 3 mg/ha for zearalenone (*10*). Comparable amounts of pesticides are emitted from agricultural-used fields via drainage water ranging from 3 mg/ha to 56 g/ha (*11*). Several authors (*12*, *13*) reported significant correlations between mycotoxin intake and excretions by humans. This indicates that human excretions may be yet another relevant source of mycotoxins in the aquatic environment, depending upon the removal rate in wastewater treatment plants (WWTPs). In fact, Wettstein and Bucheli (*14*) reported only partial elimination of deoxynivalenol by Swiss WWTPs. Together, runoff from agricultural fields and WWTP effluents can lead to the frequent (deoxynivalenol) and occasional (zearalenone) occurrence of mycotoxins in river water (*10*). Overall, only fragmentary information on mycotoxin emission pathways and their environmental fate and behavior is available. In comparison to deoxynivalenol and zearalenone, other mycotoxins might be produced in even higher amounts in wheat and maize (*15*) and have similar or even higher aqueous solubilities. We therefore hypothesize that not only deoxynivalenol and zearalenone, but also a larger number of mycotoxins is likely to enter the aquatic environment primarily via the two pathways indicated above.

The accurate and precise quantification of mycotoxins in the needed low nanograms per liter concentration range in natural waters requires a selective and sensitive analytical method. To achieve such low detection limits, preconcentration by solid-phase

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extraction (SPE) prior to detection is indispensable. The applicability of SPE for the simultaneous quantification of different classes of micropollutants with widely varying chemical and physical properties in various water samples has been demonstrated in earlier publications (*16*, *17*). High-performance liquid chromatography−electrospray ionization−tandem mass spectrometry (HPLC−ESI−MS/MS) has in recent years become the method of choice for separation and detection of mycotoxins in various biological matrices (*7*, *18*). Nonetheless, one drawback of this widely acknowledged technique is signal suppression, which is caused by co-extracted matrix components. Ion suppression has a profound influence on accuracy, precision, and sensitivity and leads to imprecision when quantifying low levels of micropollutants in environmental samples by HPLC−ESI−MS/MS. Hence, there are several methods to compensate for the imprecision caused by ion suppression. Commonly used methods are matrix-matched calibration or standard addition, but these methods require an extra effort for analysis in dynamic environmental systems with fast changing matrix composition (e.g., rivers). Despite the fact that ion suppression is a severe problem, it is not always accounted for in the literature (*19*, *20*). The use of isotope-labeled internal standards (ILISs) is the most powerful method to overcome matrix effects (*10*, *14*, *21*). If ILISs are available, their application is a very time-effective alternative to standard addition or matrix-matched calibration. Some of the commercially available isotope-labeled mycotoxins were used for quantification of mycotoxins in cereals (*22*, *23*), as well as in environmental trace analysis (*10*, *14*, *24*).

In this study, we describe an accurate, precise, and sensitive analytical method for 33 selected mycotoxins covering the major compound classes in various aqueous samples. The selection of mycotoxins was based on several factors, such as the frequency of occurrence in food and feed matrices, the chemical and physical properties, as well as the toxicity of the compound. The method comprises SPE followed by separation and detection with HPLC−MS/MS (±ESI) and applies seven ILISs for target analytes representing several different compound classes. To our knowledge, this is the first screening method for the quantification of mycotoxins in samples from different aqueous environments. The method is validated for Milli-Q water, drainage water, river water, and WWTP effluent, and its application is demonstrated with some initial data from different currently ongoing field studies.

MATERIALS AND METHODS

Chemicals. 3-Acetyl-deoxynivalenol,15-acetyl-deoxynivalenol, aflatoxin B_1 (≥99%), aflatoxin B_2 (≥98%), aflatoxin G_1 (≥99%), aflatoxin G_2 (≥99%), aflatoxin M_1 (≥99%), citrinin (≥99%), deoxynivalenol (≥99%), HT-2 toxin (≥99%), fumonisin B_1 (≥98%), fumonisin B_2 (≥96%), patulin (≥99%), and T-2 toxin (≥99%) were supplied by Fermentek (Jerusalem, Israel). Alternariol, alternariol monomethylether, altenuene, beauvericin, diacetoxyscirpenol (≥99%), ergocornine, fusarenon-X, neosolaniol, nivalenol, ochratoxin A, ochratoxin B, sterigmatocystin, sulochrin, tentoxin, verrucarin A, zearalenone (≥99%), α-zearalenol (≥98%), and β-zearalenol (≥95%) were supplied by Sigma (Sigma-Aldrich GmbH, Buchs, Switzerland). Fumonisin B_3 and ergocryptine were purchased from Biopure (Referenzsubstanzen GmbH, Tulln, Austria). For structures, see **Figure 1**. The six [$^{13}C_x$] ILISs $^{13}C_{17}$-3-acetyl-deoxynivalenol, $^{13}C_{15}$-deoxynivalenol, $^{13}C_{22}$-HT-2 toxin, $^{13}C_{34}$-fumonisin B_1, $^{13}C_{20}$-ochratoxin A, and $^{13}C_{24}$-T-2 toxin were obtained from Biopure (Referenzsubstanzen GmbH, Tulln, Austria). All compounds were delivered diluted in acetonitrile (MeCN), except $^{13}C_{34}$-fumonisin B_1, which was diluted in a MeCN/Milli-Q water (1:1, v/v) mixture. Concentrations of the ILISs were 25 μg/mL, except for ochratoxin A with 10 μg/mL. D_6-Zearalenone was prepared in our own laboratory as described earlier (*24*).

Methanol (MeOH, HPLC-grade) and MeCN (HPLC-grade) were purchased from Scharlau (Barcelona, Spain). Ammonium acetate (pa) and acetic acid (pa) were supplied by Fluka (Fluka AG, Buchs, Switzerland). Deionized water was further cleaned with a Milli-Q-gradient A10 water purification system from Millipore (Volketswil, Switzerland). High-purity N_2 (99.99995%) and Ar (99.99999%) were obtained from PanGas (Dagmarsellen, Switzerland).

Mycotoxin crystalline compounds were dissolved in pure MeCN, except fusarenon-X, neosolaniol, ochratoxin A, and ochratoxin B, which were dissolved in a MeCN/Milli-Q water (1:1, v/v) mixture. The individual stock solutions contained concentrations ranging from 100 to 1000 mg/L. Multi-component stock solutions were prepared in MeOH in concentrations of 100, 500, and 1000 ng/mL. The internal standard solutions were diluted with MeOH to obtain a concentration of 1 mg/L, except D_6-zearalenone with a concentration of 2 mg/L. All compounds, in either single- or multi-component MeOH stock solution, were stored at +4 °C in the dark. Aqueous calibration standards holding all mycotoxins equivalent to the concentration range of 1−100 ng/L and all seven ILISs at 50 ng/L were freshly prepared in Milli-Q water/MeOH (9:1, v/v) from the multi-component stock solutions each time a new series of samples was analyzed.

Sample Collection and Preparation. Drainage water samples were collected at our field study site at Reckenholz, Switzerland (*8*) using portable automatic flow proportional samplers (Teledyne Isco, Inc., Lincoln, NE). Surface water samples were obtained weekly and fortnightly from the monitoring program from the Canton of Zurich [Office for Waste, Water, Energy, and Air (AWEL)] and the monitoring program of the Swiss government [National Long-Term Surveillance of Swiss Rivers (NADUF)], respectively. Details have been published previously (*25*). Grab samples of WWTP effluents were collected at the Kloten/Opfikon (Zurich, Switzerland) facility (*14*).

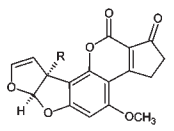
Raw water samples were filtered (glass fiber filters, pore size of 1.2 μm, Millipore, Volketswil, Switzerland) by vacuum filtration (Supelco, Bellefonte, PA), transferred to 1 L glass bottles, and stored in the dark at +4 °C until analysis within 2 weeks (storage tests showed that mycotoxins were stable over this period of time). Before SPE, the pH was adjusted between 6.6 and 7.0 by adding either ammonium acetate or acetic acid. In routine analysis, the exact volume of 1 L was spiked with 50 μL of the ILIS mixture before the storage or processing of the sample. During method validation, the ILISs were spiked as stated under the method validation parameters. The samples were shaken vigorously before further treatment.

SPE. Filtered water samples (1 L) were concentrated and purified by performing reversed-phase SPE (Oasis HLB cartridges, 6 mL, 200 mg, Waters Corporation, Milford, MA) on a 12-fold vacuum extraction box (Supelco, Bellefonte, PA). The SPE cartridges were consecutively conditioned with 5 mL of MeOH, 5 mL of Milli-Q water/MeOH (1:1, v/v), and 5 mL of Milli-Q water. Water samples (1 L) were passed through the cartridges with a maximum flow rate of 10 mL/min. Subsequently, the cartridges were washed with 5 mL of Milli-Q water. Without any additional column drying step, the analytes were eluted with 5 mL of MeOH and the aliquots were collected in conical reaction vial vessels (Supelco, Bellefonte, PA). The 5 mL MeOH aliquots were reduced to 100 μL under a gentle nitrogen gas stream at 50 °C. The extracts were reconstituted in 900 μL of Milli-Q water/MeOH (90:10, v/v) and transferred into 1.5 mL amber glass vials. The samples were stored at +4 °C and analyzed within 48 h.

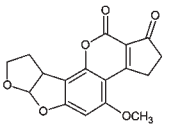
The following other SPE phases were initially tested according to the guidelines of the manufacturer for their mycotoxin extraction efficiency: Bond Elut Plexa cartridges, 6 mL, 200 mg (Varian, Inc., Santa Clara, CA) and Chromabond HR-X cartridges, 6 mL, 200 mg (Macherey-Nagel GmbH and Co. KG, Düren, Germany).

LC−MS/MS Analysis. LC−MS/MS was performed on a Varian 1200 L LC−MS instrument (Varian, Inc., Santa Clara, CA). The mycotoxins were separated using a 125 mm × 2.0 mm inner diameter, 3 μm, Pyramid C_{18} column, with a 2.1 mm × 20 mm inner diameter, 3 μm, guard column of the same material (Macherey-Nagel GmbH and Co. KG, Düren, Germany) at room temperature. Two different chromatographic runs were used for the separation of all compounds, with one in the positive and one in the negative ionization modes. The optimized LC mobile-phase gradient for the analysis of the analytes measured in negative ionization mode was as follows: 0.0 min, 0% B (100% A); 2.0 min, 0% B; 15.0 min, 100% B; 18.0 min, 100% B; 19.0 min, 0% B; and 24.0 min, 0% B. The analytes measured in the positive ionization mode were separated with the following gradient: 0.0 min, 27% B (73% A); 1.0 min, 27% B; 1.3 min,

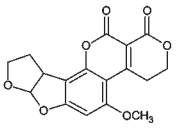
Structures of aflatoxins [AFs] including Aflatoxin B_1, Aflatoxin B_2, Aflatoxin M_1, Aflatoxin G_1, Aflatoxin G_2



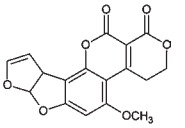
Aflatoxins	R
Aflatoxin B_1	H
Aflatoxin M_1	OH



Aflatoxin B_2

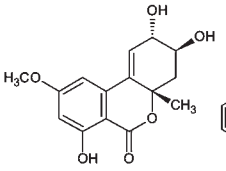


Aflatoxin G_1

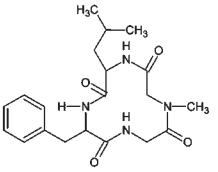


Aflatoxin G_2

Structures of Alternaria toxins:



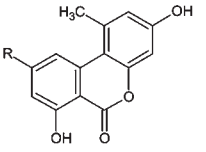
Altenuene



Tentoxin

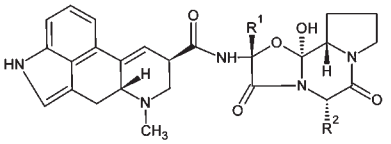
Alternariol R=OH

Alternariolmethylether R=OCH_3



Alternariol

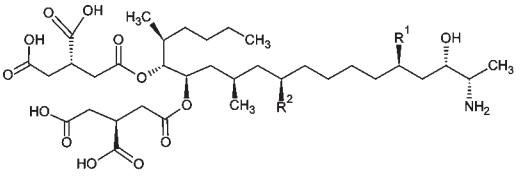
Structures of ergot alkaloids:



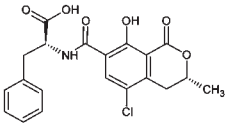
Analyte	R^1	R^2
Ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocryptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$

Structures of fumonisins:

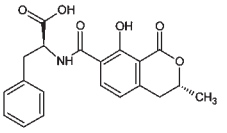
Analyte	R^1	R^2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH



Structures of ochratoxins:

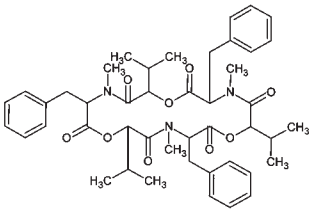


Ochratoxin A

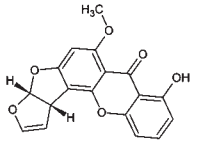


Ochratoxin B

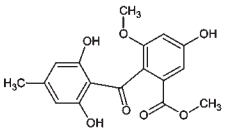
Structures of "Other" toxins:



Beauvericin



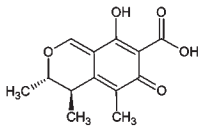
Sterigmatocystin



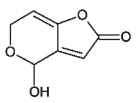
Sulochrin

Figure 1. Continued

Structures of Penicillium toxins:

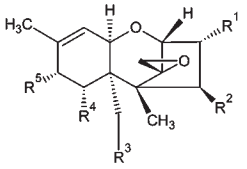


Citrinin



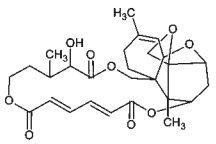
Patulin

Structures of trichothecenes:

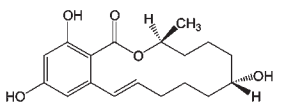


Trichothecene	R^1	R^2	R^3	R^4	R^5
Type A					
Diacetoxyscirpenol	OH	$OCOCH_3$	$OCOCH_3$	H	H
HT-2 toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
Neosolaniol	OH	$OCOCH_3$	$OCOCH_3$	H	OH
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
Type B					
Deoxynivalenol	OH	H	OH	OH	=O
Fusarenon-X	OH	$OCOCH_3$	OH	OH	=O
Nivalenol	OH	OH	OH	OH	=O
3-Acetyldeoxynivalenol	$OCOCH_3$	H	OH	OH	=O
15-Acetyldeoxynivalenol	OH	H	$OCOCH_3$	OH	=O

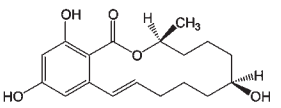
Verrucarin A



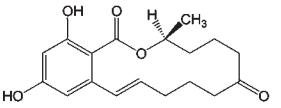
Structures of resorcyclic acid lactones:



α-zearalenol



β-zearalenol



zearalenone

Figure 1. Chemical structures of the investigated compounds.

45% B; 5.0 min, 45% B; 5.3 min, 63% B; 9.0 min, 63% B; 9.3 min, 81% B; 13.0 min, 81% B; 13.3 min, 100% B; 20.0 min, 100% B; 21.0 min, 27% B; and 24.0 min, 27% B. In both cases, eluent A consisted of Milli-Q water/MeOH/acetic acid (89:10:1, v/v/v) and eluent B consisted of Milli-Q water/MeOH/acetic acid (2:97:1, v/v/v). Both eluents were buffered with 5 mM ammonium acetate. The injection volume was 40 μL, and the mobile phase flow was 0.2 mL/min.

LC−MS interface conditions for the ionization of the acidic mycotoxins in the −ESI mode were as follows: needle voltage, −4000 V; nebulizing gas (compressed air), 3.01 bar; drying gas (N_2, 99.5%), 275 °C and 1.24 bar; and shield voltage, −600 V. The neutral mycotoxins were ionized in the +ESI mode under the following conditions: needle voltage, +4500 V; nebulizing gas (compressed air), 3.01 bar; drying gas (N_2, 99.5%), 275 °C and 1.24 bar; shield voltage, +600 V.

The analyte-dependent MS and MS/MS parameters and collision cell energies were acquired by direct infusion of standards with concentrations of 4 ppm into the MS using an external syringe pump (Pump 11, Harvard Apparatus, Holliston, MA) with a flow rate of 50 μL/min. The main fragments were identified using multiple reaction monitoring (MRM). Precursor−product ion transitions and corresponding collision cell voltages were obtained for each analyte. A second product ion was monitored for identity confirmation. The collision cell gas (Ar, 99.999%) pressure was 2.0 (±0.1) e^{-6} Torr, and the detector voltage was set to 1800 V. Dependent upon the number of ion transitions per time segment, scan times ranged from 0.1 to 0.4 s and from 0.2 to 2.4 s for acidic and neutral compounds, respectively. Scan widths were m/z 1.0 for Q1 and m/z 1.5 for Q3, and mass peak width was m/z 0.7.

Analytes without ILIS were quantified using matrix-matched calibrations. If not stated otherwise, the analytes with corresponding ILIS were quantified using the internal standard method, i.e., calibration standards in Milli-Q water containing increasing amounts of analytes and constant amounts of the seven ILISs (see above) and matrix-matched calibrations. Data processing was carried out using the Varian MS Workstation (version 6.9.2.) software.

Method Validation Parameters. *Linearity*. The linearity of the MS/MS detector was tested with Milli-Q water/MeOH (9:1, v/v) containing mycotoxins at concentrations between 0.1 ng/L and 100 μg/L, corresponding to 0.005−5 ng at the detector, respectively. The linearity for the three different environmental matrices was tested for matrix calibrations ranging from 1 to 100 ng/L.

Ion Suppression. Matrix effects during analyte ionization causing suppression or enhancement of the analyte signal were evaluated by comparing the obtained signal from injection of the same amount of analyte in Milli-Q water/MeOH (9:1, v/v) and an extracted matrix blank. For this purpose, 8 L of each matrix (Milli-Q, drainage, river, and WWTP effluent water) was filtered and concentrated on an individual SPE column following the procedure as described above. The eight obtained SPE eluates were combined and, afterward, divided into eight equal portions.

Matrix-matched calibration samples were obtained by carrying out standard addition to the final extracts to produce a concentration equivalent to those of 1 L samples containing 1, 5, 10, 25, 50, and 100 ng of each analyte. Additionally, 50 ng of ILISs was added to each portion. Calibration curves were obtained by plotting measured analyte peak areas against corresponding analyte concentration levels in pure solvent and in the extracted matrix, respectively. Linear regression was performed for each curve. The ion suppression (expressed in percentages) was quantified as 1 minus the ratio between the slope of the curve obtained for the final extracts and the slope of the curve for the pure solvent (see the Supporting Information).

Absolute and Relative SPE Recoveries and Absolute and Relative Method Recoveries. Absolute SPE recoveries were determined for all mycotoxins in Milli-Q water, testing three different extraction cartridge materials. Therefore, 1 L samples were spiked with mycotoxins prior to SPE to produce concentration levels of 10, 25, 50, and 100 ng/L. ILIS (50 μL) was added directly into the 5 mL eluates extracted from the cartridge. The matrix-matched SPE calibration curve was obtained by carrying out standard addition to the pure Milli-Q matrix SPE eluates. The absolute SPE recovery for the analytes without an ILIS was defined as the ratio of the slope obtained by adding the analyte before the extraction to the slope of the matrix-matched SPE calibration (see the Supporting Information). The absolute SPE recovery for the compounds with corresponding ILIS was defined as the ratio of the slope resulting from the measured area of the analyte added before the extraction to the measured area of the corresponding ILIS added to the SPE eluate, divided by the slope of the matrix-matched SPE calibration (see the Supporting Information).

Absolute method recoveries were determined for all mycotoxins in Milli-Q, drainage, river, and WWTP effluent water. Samples (1 L) were spiked with mycotoxins prior to SPE to produce concentration levels of 5, 25, and 100 ng/L. Five replicates were prepared for each concentration level. A total of 1 L of native matrix was tested for native mycotoxin contents and/or contamination because of the addition of ILISs. After SPE and eluate evaporation, sample residues were reconstituted in 650 μL of Milli-Q water and 50 μL of each ILIS solution was added. The matrix-matched calibration curve for the determination of absolute and relative recovery was obtained by carrying out standard addition to the final extracts to produce a concentration equivalent to those of 1 L samples containing 1, 5, 10, 25, 50, and 100 ng of each analyte. The absolute method recovery for the analytes without an ILIS was defined as the ratio of the slope obtained by adding the analyte before the extraction to the slope of the matrix-matched calibration (for details, see the Supporting Information). The absolute method recovery for the analytes for which an ILIS was available was defined as the ratio of the slope resulting from the measured area of the analyte added before the extraction to the measured area of the corresponding ILIS added after the extraction, divided by the slope of the matrix-matched calibration (see the Supporting Information).

The relative method recovery was determined only for those analytes that have an ILIS. It was defined as the ratio of the slope of the measured area of analyte and ILIS added before the extraction, divided by the slope of the matrix-matched calibration (see the Supporting Information).

Method and Instrument Precision and Method and Instrument Detection Limits. The method precision (MP) values presented in the Results and Discussion were determined as the relative standard deviation (RSD) of five replicates at the concentration level of 5 ng/L, except for those compounds exhibiting higher MDL. The instrument precision (IP) was obtained from five consecutive analyses of an individual sample.

The MDL was defined as 3 times the absolute standard deviation of the five replicates at the lowest quantifiable concentration level (*26*). The instrument detection limit (IDL) was defined as 3 times the absolute standard deviation of five standard calibration replicates at the lowest concentration level. For each sample type, 1 L of unfortified matrix, i.e., containing ILISs only, was analyzed for its native mycotoxin content and/or contamination because of the addition of ILISs. No mycotoxins were quantified in any of these control samples.

RESULTS AND DISCUSSION

Optimization of the Extraction Procedure. The mycotoxin extraction procedure was optimized with respect to their recoveries over different SPE cartridges, elution solvent, and pH value of the aqueous sample. For the extraction of multiple components covering a broad range of polarities and acid/base properties, usually polymeric, end-capped functionalized adsorber materials are applied (*27*). Two such materials, i.e., Chromabond HR-X and Bond Elut Plexa cartridges, were tested for their enrichment efficiency. Oasis HLB was additionally chosen because of its wide application range and the ability to effectively retain a large number of compounds, basic, neutral, and acidic (*16*, *28*). It is designed, in fact, to retain both hydrophilic and hydrophobic compounds with high capacity by means of both van der Waals and H-donor−H-acceptor interactions.

With a median SPE recovery of 95%, Oasis HLB exhibited the best performance for analytes measured in the negative ionization mode (see the Supporting Information). Only two compounds, alternariol monomethylether and nivalenol, showed recoveries below 30%. According to good laboratory practice, recoveries ranging from 70 to 120% are excellent recoveries (*29*). The other two tested cartridge materials showed median recoveries below 90% in the negative ionization mode. Bond Elut Plexa exhibited the best cartridge recoveries for the analytes measured in positive ionization mode (see the Supporting Information), while Oasis HLB had a median recovery of 90%. Consequently, Oasis HLB was chosen as the extraction cartridge. Because fumonisins were not eluted when using acetonitrile as the extracting solvent (*30*) and because of the advice of the supplier, 5 mL of methanol was chosen as mycotoxin eluent from the cartridge material.

No significant differences in absolute SPE recoveries were observed between pH 5 and 7. Given the fact that many surface water samples have a pH close to 8, the sample pH was further adjusted with acetic acid to lie between 6.6 and 7.0.

Chromatographic Separation and MS Detection. The reversed-phase liquid chromatography separation of the mycotoxins was optimized with respect to LC column type, mobile phase, and gradient dynamics to achieve the greatest possible selectivity and sensitivity. The Nucleodur Gravity reversed-phase C_{18} column was chosen as the stationary phase because well-resolved peak shapes were generally obtained for all analytes, despite their chemical diversity. The often difficult separation of ergot alkaloid epimers was improved by adding 1% acetic acid into the eluent solutions (*31*). Quantification problems because of intensity differences of the MRM transition of the two epimers were overcome by building the sum of the two epimer peaks. As a representative example, a chromatogram of a WWTP effluent extract spiked with 25 ng/L of each investigated compound is shown in the Supporting Information.

The mycotoxins were ionized to either their $[M - H]^-$ or $[M + H]^+$ form or corresponding ammonia and acetate adducts (**Table 1**). The polarity showing the more abundant precursor ions was selected for each analyte. A total of 13 compounds were optimized using the negative ionization mode, because several of them, like the *Alternaria* toxins, exhibited no detectable MS signal in the positive ionization mode. A total of 20 compounds were measured in the positive ionization mode, such as the aflatoxins, which exhibited no detectable MS signal in the negative ionization mode. Because of the large number of analytes, polarity switching during the chromatographic run was not possible. Therefore, both positive and negative ESI modes were used in two separate chromatographic runs to ensure stable and sensitive MS conditions for all analytes. Because of the addition of 5 mM ammonium acetate to the chromatographic eluents, some analytes (trichothecene A) formed $[M + NH_4]^+$ adducts with higher precursor signal intensities than the related $[M + H]^+$ species. The monitored product ions from these adduct precursors were analyte-specific and selected to fulfill the requirement for the confirmation of substances according to the Annex I of Directive 96/23/EC (*32*). The parameters of the optimized MS detection,

Table 1. Analytical Parameters for the Selected Mycotoxins and Their Available Corresponding ILISs

compound	retention time (min)	precursor ion (*m*/*z*)	adduct	main product ion (*m*/*z*)	main product ion collision energy (eV)	secondary product ion (*m*/*z*)	secondary product ion collision energy (eV)	capillary voltage (V)
			Aflatoxins					
aflatoxin B_1	7.52	313.0	$[M + H]^+$	285.0	20	270.0	30	65
aflatoxin B_2	6.37	315.0	$[M + H]^+$	287.1	20	259.1	30	65
aflatoxin G_1	5.44	329.0	$[M + H]^+$	243.0	28	214.7	30	60
aflatoxin G_2	4.68	331.0	$[M + H]^+$	313.1	20	245.1	30	65
aflatoxin M_1	4.61	329.0	$[M + H]^+$	273.0	20	259.0	20	65
			Alternaria Toxins					
altenuene	14.52	291.0	$[M - H]^-$	247.9	20	275.8	20	60
alternariol monomethylether	19.43	271.1	$[M - H]^-$	255.7	20	227.6	20	60
alternariol	14.61	257.0	$[M - H]^-$	212.8	20	214.8	20	70
tentoxin	16.34	413.2	$[M - H]^-$	271.0	10	140.9	10	65
			Ergot Alkaloids					
ergocornine	8.19/10.96	562.3	$[M + H]^+$	544.0	10	305.2	30	40
ergocryptine	9.57/12.34	576.3	$[M + H]^+$	558.4	10	305.2	30	50
			Fumonisins					
fumonisin B_1	12.02	722.3	$[M + H]^+$	352.3	30	334.4	50	80
$^{13}C_{34}$-fumonisin B_1	11.97	756.8	$[M + H]^+$	374.7	30	738.8	13	80
fumonisin B_{2+3}	14.57	706.3	$[M + H]^+$	336.4	40	318.4	40	75
			Ochratoxins					
ochratoxin A	13.63	404.2	$[M + H]^+$	239.2	20	358.1	10	40
$^{13}C_{20}$-ochratoxin A	13.59	424.4	$[M + H]^+$	250.0	20	377.3	10	40
ochratoxin B	11.56	370.2	$[M + H]^+$	205.0	20	324.1	11	40
			Others					
beauvericin	19.77	784.4	$[M + H]^+$	244.0	20	262.4	26	70
sterigmatocystin	14.98	325.0	$[M + H]^+$	310.1	20	281.3	40	35
sulochrin	6.89	333.2	$[M + H]^+$	208.9	10	136.2	40	25
			Penicillium					
citrinin	16.24	281.1	$[M + Ac]^-$	204.8	20	248.0	20	35
patulin	2.97	152.9	$[M - H]^-$	108.9	10	80.9	10	30
			Resorcyclic Acid Lactones					
α-zearalenol	17.88	319.4	$[M - H]^-$	275.0	20	160.0	30	60
β-zearalenol	16.81	319.1	$[M - H]^-$	275.2	20	301.1	20	60
zearalenone	18.23	317.4	$[M - H]^-$	175.0	20	131.0	25	60
D_6-zearalenone	18.19	323.4	$[M - H]^-$	134.0	30	279.0	20	60
			Trichothecene A					
diacetoxyscirpenol	7.18	384.3	$[M + NH_4]^+$	307.0	10	247.0	12	30
HT-2 toxin	10.32	442.2	$[M + NH_4]^+$	263.1	12	215.2	12	30
$^{13}C_{22}$-HT-2 toxin	10.32	464.4	$[M + NH_4]^+$	278.2	12	215.8	12	30
neosolaniol	3.05	400.2	$[M + NH_4]^+$	305.0	11	185.0	20	20
T-2 toxin	12.43	484.4	$[M + NH_4]^+$	185.1	21	215.0	17	35
$^{13}C_{24}$-T-2 toxin	12.43	508.6	$[M + NH_4]^+$	198.0	17	229.4	13	35
verrucarin A	12.44	520.1	$[M + NH_4]^+$	249.1	20	231.2	20	45
			Trichothecene B					
3-acetyl-deoxynivalenol	11.76	397.1	$[M + Ac]^-$	307.1	18	337.1	14	25
$^{13}C_{17}$-3-acetyl-deoxynivalenol	11.77	413.5	$[M + Ac]^-$	323.6	14	182.9	30	25
15-acetyl-deoxynivalenol	4.14	339.2	$[M + H]^+$	321.6	20	137.0	30	35
deoxynivalenol	5.06	355.1	$[M + Ac]^-$	295.4	10	264.8	6	50
$^{13}C_{16}$-deoxynivalenol	5.01	370.0	$[M + Ac]^-$	310.0	10	279.0	10	50
fusarenone-X	9.19	413.1	$[M + Ac]^-$	353.0	14	263.0	11	35
nivalenol	2.66	371.1	$[M + Ac]^-$	280.9	10	311.0	6	35

including capillary and collision cell voltages, and MRM transitions of each analyte are summarized in **Table 1**.

Validation of the Optimized Method. *Linearity*. MS/MS was linear for matrix solutions between 0.1 ng/L and 100 μg/L, corresponding to 5 pg to 5 μg at the detector, respectively ($0.9410 < R^2 < 0.9998$). The linearity was also tested with standard solutions ranging up to concentrations of 500 μg/L. For aflatoxin B_2, alternariol, alternariol monomethylether, citrinin, ergocornine, T-2 toxin,

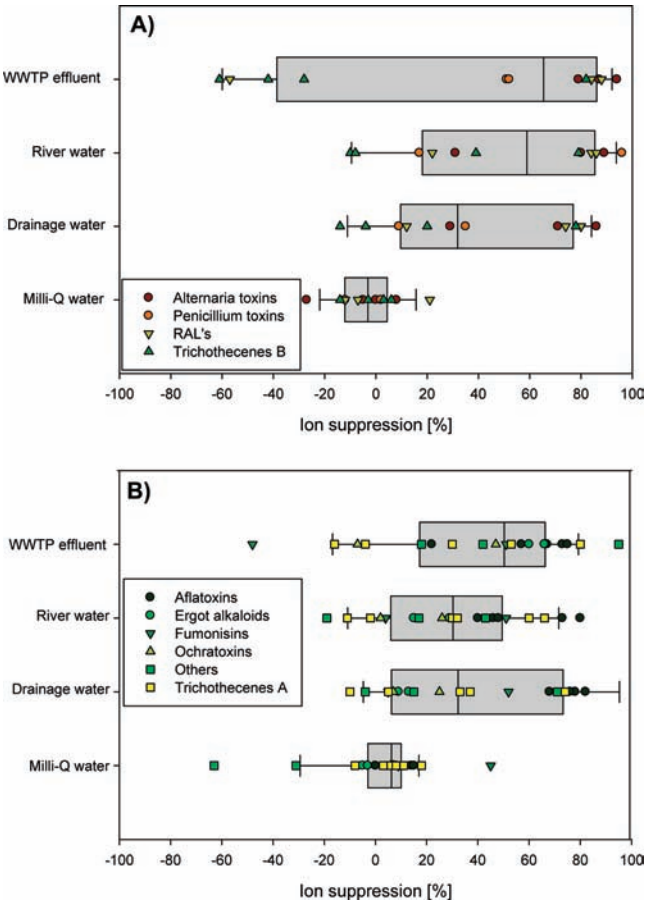


Figure 2. Ion suppression of (A) mycotoxins measured in negative ionization mode (−ESI) and (B) mycotoxins measured in positive ionization mode (+ESI). Line in box, median (50th percentile); box margins, 25th and 75th percentile; lines with whiskers, 10th and 90th percentile.

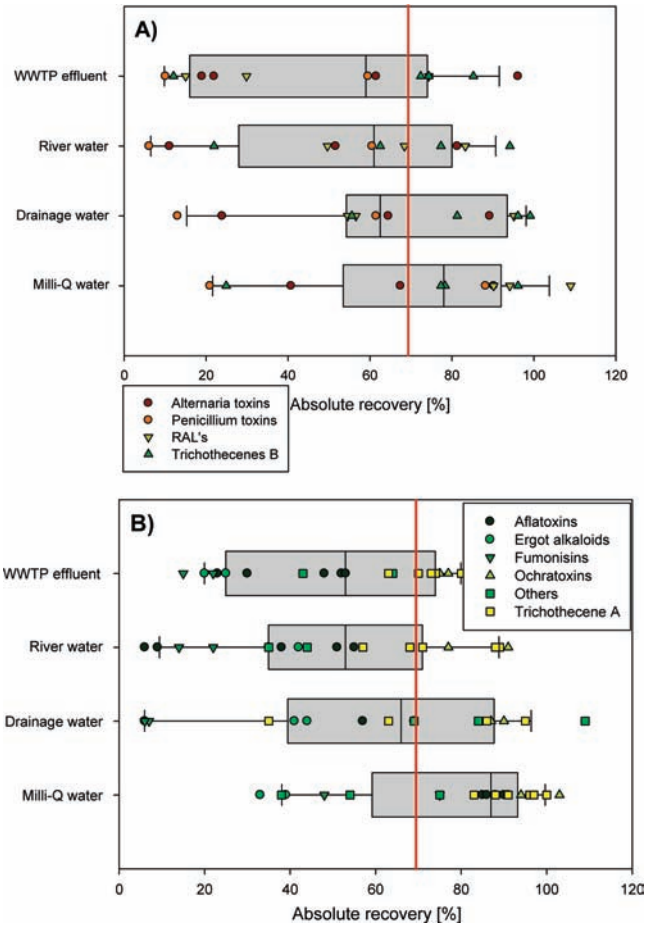


Figure 3. Absolute method recovery of (A) mycotoxins measured in negative ionization mode (−ESI) and (B) mycotoxins measured in positive ionization mode (+ESI) at the 100 ng/L concentration level in various natural waters. The red line indicates the lower end of the satisfactory recovery range (70%). Line in box, median (50th percentile); box margins, 25th and 75th percentile; lines with whiskers, 10th and 90th percentile.

tentoxin, and zearalenone, nonlinear conditions were reached in the concentration range of 400−500 μg/L. The varying aqueous matrices did not show any effects on the MS/MS linearity.

Ion Suppression. Ion suppression was quantified for all analytes in all investigated matrices and reached up to 94% (**Figure 2**). The negative values for some analytes of the class of trichothecenes A and B correspond to a signal enhancement caused by matrix interference. Generally, the extent of ion suppression depends upon the type of matrix, as shown in panels **A** and **B** of **Figure 2**. Overall, ion suppression was consistently higher in WWTP effluents than in drainage and river water. This is plausible considering the high output of organic material from WWTPs. As expected, the lowest but sometimes still considerable ion suppression was quantified in Milli-Q water. Reasons for this could be exogenous substances from external sources during sample preparation, for example residues released from the SPE cartridge or buffers (*33*). Generally, it was observed that ion suppression of the analytes was more pronounced in ESI− than ESI+ mode (**Figure 2**), which is in agreement with earlier investigations (*34*). The explicit ion suppression data quantified for all analytes in each matrix is listed in the Supporting Information.

Unfortunately, there are only four publications available with ion suppression data for mycotoxins in comparable matrices (*10*, *14*, *24*, *35*). Lagana et al. (*35*) reported atmospheric pressure chemical ionization (APCI)− ion suppression of 15−25% for different resorcyclic acid lactones in aqueous environmental samples, and Wettstein and Bucheli (*14*) reported 18, 14, and −20% APCI− ion suppression for deoxynivalenol in drainage, river, and WWTP effluent water, respectively. These numbers contrast to the ESI− ion enhancement observed in the present study (−14, −8, and −61% for drainage, river, and WWTP effluent water, respectively; see the Supporting Information) and may be explained with the different ionization methods in use. Hartmann et al. (*24*) reported ESI− ion suppression values of 33−57% in drainage, 28−54% in river, and 56−68% in WWTP effluent waters for resorcyclic acid lactones. These are somewhat lower than the data reported here (drainage, 12−80%; river, 22−86%; WWTP effluent, 57−88%; see the Supporting Information).

Absolute Method Recoveries. The absolute recoveries were determined for all selected mycotoxins in Milli-Q water, drainage water, river water, and WWTP effluent at three different concentration levels (5, 25, and 100 ng/L). All data are presented in the Supporting Information, and the results obtained with 100 ng/L are visualized in **Figure 3**. In Milli-Q water, the absolute recoveries for all 33 mycotoxins ranged from 20 to 109% at 100 ng/L (median at 76%). These recoveries are lower than the SPE recoveries presented in the Supporting Information, which is probably due to analyte losses during SPE eluate evaporation and reconstitution. At a concentration level of 25 ng/L, the absolute recoveries for all 33 mycotoxins were in the range of 17−102% (median at 70%). At the 5 ng/L concentration level, 30 of 33 compounds could be quantitated and showed absolute recoveries of 16−104%. Median absolute recoveries at 5 ng/L were 77%. The natural samples took their toll of the absolute recoveries and increasingly so for drainage, river

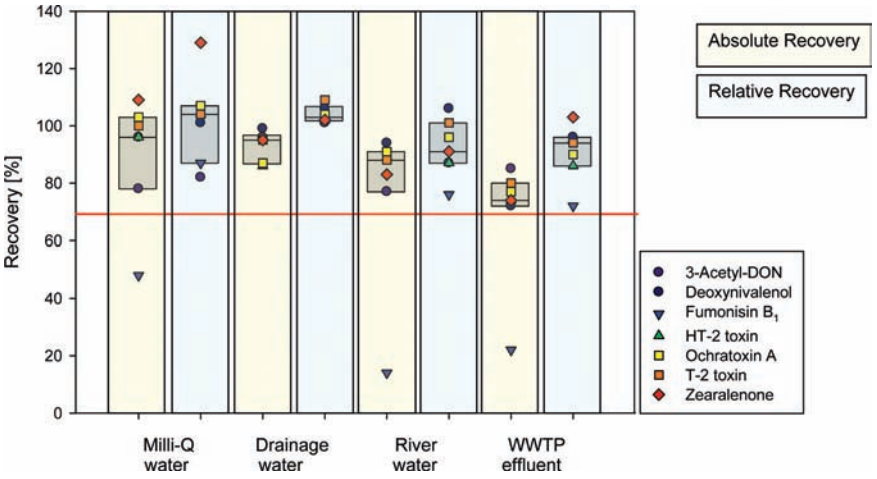


Figure 4. Absolute and relative recoveries of mycotoxins with available ILIS at a concentration of 100 ng/L in various natural waters. The red line indicates the lower end of the satisfactory recovery range (70%). Line in box, median (50th percentile); box margins, 25th and 75th percentile.

and WWTP effluent waters, i.e., matrices of increasing complex nature in terms of dissolved organic carbon content or ionic strength. For instance, at the 100 ng/L spike level, median absolute recoveries dropped from 76% in Milli-Q water to 62, 56, and 57% in drainage, river, and WWTP effluent water, respectively. Simultaneously, the number of compounds with satisfactory absolute recoveries of above 70% was reduced from 23 in Milli-Q water to 12, 10, and 13 in drainage, river, and WWTP effluent water, respectively. This trend was corroborated by and even more pronounced for the lower spike levels (see the Supporting Information). Specifically, compounds with absolute recoveries below 50% (or spike concentrations below detection limits) were 15-acetyl-deoxynivalenol, the three aflatoxins (G_1, G_2, and M_1), alternariol monomethylether, alternariol, the two ergot alkaloids (ergocornine and ergocyrptine), the two fumonisins (B_1 and B_{2+3}), nivalenol, patulin, and sulochrin α- and β-zearalenol, whereas aflatoxin B_1 and B_2, citrinin, neosolaniol, sterigmatocystin, and tentoxin exhibited values between 50 and 70%. The low absolute recoveries of some of these compounds can be explained by their acidity (e.g., nivalenol and patulin), resulting in very low extraction efficiencies (see the Supporting Information). If quantifiable, recoveries above 70% were predominantly found for 3-acetyl-deoxynivalenol, altenuene, beauvericin, diacetoxyscirpenol, deoxynivalenol, fusarenon-X, HT-2, ochratoxin A and B, T-2, verrucarin A, and zearalenone.

Whereas the absolute recoveries of deoxynivalenol presented here in drainage (99%) and river (91−104%) water were above those reported by Bucheli et al. (*10*) (85 and 87%, respectively), they were lower (33−72%) than those obtained by Wettstein and Bucheli (*14*) in WWTP effluent (91%). Lagana et al. (*35*) reported absolute method recoveries of 85−92% for different resorcyclic acid lactones in aqueous environmental samples, whereas Hartmann et al. (*24*) reported values of 73−82% in Milli-Q water, 84−103% in drainage water, 92−98% in river water, and 93−94% in WWTP effluents, respectively. These are somewhat higher than the data reported here (drainage, 54−104%; river, 49−95%; WWTP effluent, 14−80%; see the Supporting Information). Overall, it is in the nature of multi-residue methods that one has to cope with a broad range of absolute recoveries. For instance, various classes of concomitantly analyzed pharmaceuticals were recovered from river water at 17−101% (*36*); 6−98% absolute method recoveries were reported for 26 biocides, 5 UV filters, and 5 benzothiazoles in WWTP and surface waters (*17*).

Relative Method Recoveries. If available, ILISs were applied to compensate for the variability of matrix effects, which affect SPE as well as analyte ionization. The effectiveness of ILISs is illustrated by the comparison of absolute and relative recoveries of seven mycotoxins in various aqueous matrices (**Figure 4**). Their absolute recoveries of 49−109, 86−99, 14−94, and 22−85% in Milli-Q, drainage, river, and WWTP effluent water, respectively, translated into relative recoveries of 82−129, 101−109, 76−106, and 72−103% in these waters at the 100 ng/L spike level. Similarly enhanced recoveries were observed at the lower spike levels of 5 and 25 ng/L (see the Supporting Information). Hence, even in the case of some critical compounds with unsatisfactory absolute recoveries, such as fumonisin B_1, the application of a corresponding ILIS provides dependable relative recoveries, allowing for a quantitative analysis of even such problematic analytes.

Precision (Method and Instrument), Blank Levels and MDLs, and Repeatability. The values of the overall MP ranged from 2 to 28% (median at 4%), from 4 to 17% (median at 4%), from 2 to 37% (median at 4%), and from 2 to 19% (median at 4%) in Milli-Q, drainage, river, and WWTP effluent water (see the Supporting Information). Considering the structural diversity of the analytes, the precision obtained for this multi-component method is satisfactory (*31*, *36*) and confirms the robustness of the analytical method. In comparison, the IP was between 1 and 21% for all mycotoxins in all matrices. Specifically, the median number was 4, 5, 6, and 7% in Milli-Q, drainage, river, and WWTP effluent water (for details, see the Supporting Information). This indicates that most of the uncertainty is caused by sample analysis and not sample preparation.

The MDL ranged from 0.2 to 5.2 ng/L (median at 0.8 ng/L), from 0.3 to 44.9 ng/L (median at 0.9 ng/L), from 0.3 to 29 ng/L (median at 1 ng/L), and from 0.4 to 47.7 ng/L (median at 1.0 ng/L) in Milli-Q, drainage, river, and WWTP effluent water (panels **A** and **B** of **Figure 5**; for details, see the Supporting Information). The IDL ranged from 0.1 to 5.1 ng/L (median at 0.3 ng/L) obtained for standard calibration solutions. The MDLs obtained here can be compared to earlier methods specializing in the quantification of individual target analytes in natural waters. Hartmann et al. (*24*) reported MDL of 0.9−1.4, 0.5−2.1, 0.6−1.1, and 0.8−12.4 ng/L for resorcyclic acid lactones in Milli-Q, drainage, river, and WWTP effluents, respectively. Whereas the data presented here were similar in Milli-Q water (0.8−1.1 ng/L), they proved inferior in natural waters (6.0−44.9, 4.9−18.6, and 12.9−31.1 ng/L in drainage, river, and WWPT effluent water, respectively). Once more, this discrepancy is a result of accommodating various chemically diverse target analytes in one multi-residue method. However, it performed equally well in the case of deoxynivalenol (MDL of 0.8−1.3 ng/L) as previously reported (*14*) (MDL of 0.8−1.5 ng/L). Generally, the

Table 2. Concentrations (ng/L) of the Mycotoxins Found in Drainage Water, Surface Water, and WWTP Effluent Monitored from January to June 2010[a]

	drainage water				river water				WWTP effluent			
compounds	min−max (ng/L)	mean (STD) (ng/L)	number of samples	detected in *X* samples	min−max (ng/L)	mean (STD) (ng/L)	number of samples	detected in *X* samples	min−max (ng/L)	mean (STD) (ng/L)	number of samples	detected in *X* samples
beauvericin	d−10.5	6.7 (2.0)	129	39	d−52.8	4.3 (1.6)	223	77	d	d	4	4
deoxynivalenol	d−22.5	8.3 (1.9)	129	22	d−11.9	5.5 (2.6)	223	37	16.4−38.8	26.1 (3.2)	4	4
nivalenol	d−8.5	6.1 (3.3)	129	26	d−9.2	5.9 (2.7)	73	35	nd	nd	4	0
3-acetyl-deoxynivalenol	d	d	129	10	d	d	223	93	d	d	4	4
zearalenone	d	d	129	3	d	d	223	7	nd	nd	4	0

[a] d, detected but not quantifiable; nd, not detected; STD, standard deviation.

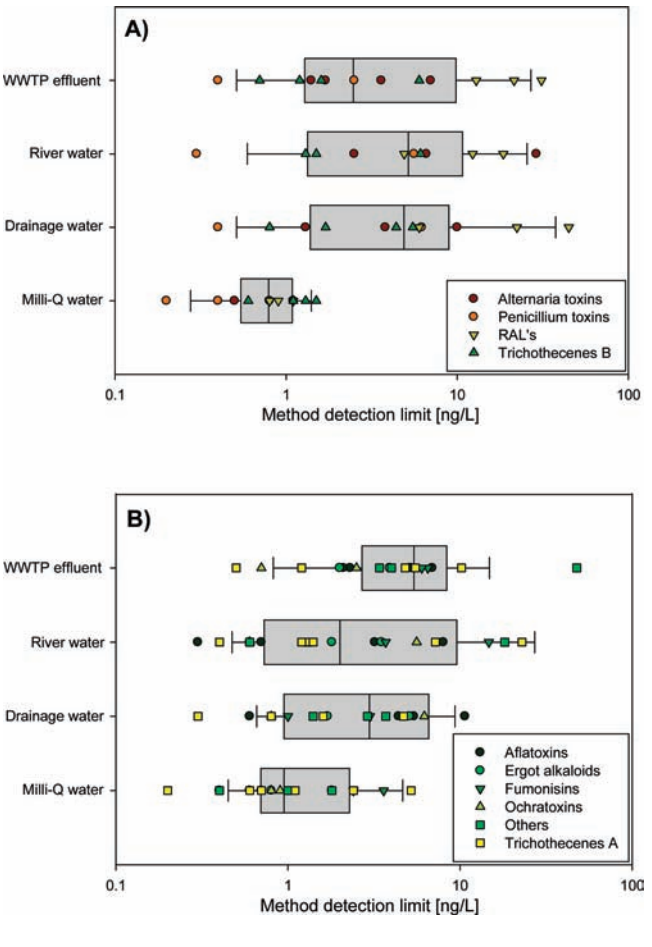


Figure 5. MDLs of (A) mycotoxins measured in negative ionization mode (−ESI) and (B) mycotoxins measured in positive ionization mode (+ESI) at low concentrations in various natural waters. Line in box, median (50th percentile); box margins, 25th and 75th percentile; lines with whiskers, 10th and 90th percentile.

MDLs obtained here are comparable to those published for multiple classes of acidic and neutral pharmaceuticals and personal care products (*16*, *36*) or even better in comparison to the application of a multi-mycotoxin method for the analysis in beer (*30*).

Environmental Application. The validated analytical multiresidue method presented here is currently applied to verify the presence of mycotoxins in natural waters. First, the method is used to study the emission of mycotoxins from a drained test field cultivated with winter wheat at Reckenholz, Zurich (Switzerland) (*8*). The wheat was artificially infected with four different mycotoxin-producing *Fusarium* species, specifically *F. graminearum*, *F. crockwellense*, *F. poae*, and *F. avenaceum*, in June 2009 and 2010. Flow-proportional drainage water samples have been collected regularly prior to and following this infection. Initial results of this still ongoing field study are summarized in **Table 2**. Deoxynivalenol was detected in 22 of 129 drainage water samples, with a maximum concentration of 22.5 ng/L. Such numbers are about 1000 times lower than during summer (*10*), because of pre-infection and early vegetation conditions during the sampling period shown here. Whereas nivalenol was detected at about the same frequency as deoxynivalenol, beauvericin was even more prevalent. Mean concentrations of nivalenol and beauvericin were 6.7 and 6.1 ng/L, respectively.

Second, seasonal WWTP effluent samples were collected at the Kloten/Opfikon (Zurich, Switzerland) facility (*14*) and studied for mycotoxin emission because of human excretion. In WWTP effluents, deoxynivalenol was detected in 4 of 4 samples, with a mean concentration of 26.1 ng/L (**Table 2**). These results are comparable to our earlier study (*14*) and proved again the applicability of this method. As for the other mycotoxins, only beauvericin was detected in WWTP effluent but was not quantifiable.

Third, weekly and fortnightly, flow-proportional surface water samples from the Canton of Zurich (AWEL) and the monitoring program of the Swiss government (NADUF) (*25*) were analyzed for mycotoxins. Deoxynivalenol was detected in Swiss river water samples with a mean concentration of 5.5 ng/L, which again is comparable to earlier data (*10*). Similar to drainage water, beauvericin and nivalenol were also detected in Swiss river waters with mean concentrations of 4.3 and 5.9 ng/L, respectively.

Whereas the origin of deoxynivalenol in river water and the relative importance of its sources were recently elucidated by ref *14*, the presence of other mycotoxins, such as nivalenol and beauvericin, need to be investigated in further detail. Both nivalenol and beauvericin are primarily field-produced mycotoxins, with a considerable aqueous solubility [log K_{ow} deoxynivalenol = −2.2, EPISuite 4.0; pK_a (beauvericin) = −1.2, (*37*)]. This makes runoff from contaminated fields a likely source, which is supported by the preliminary data presented here. Other compounds, such as 3-acetyl-deoxynivalenol and zearalenone, were detected but not quantifiable in all of the three different measured environmental matrices. Overall, the necessity to study mycotoxin occurrence, fate, and behavior in natural waters is indicated. Whether or not the presence of such compounds in the nanograms per liter up to a few micrograms per liter range poses an ecotoxicological risk in the aqueous environment remains to be investigated with corresponding effect studies.

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Supporting Information Available: Schematic overview of the method validation process (Figure S1), LC−MS/MS chromatograms from WWTP effluent extracts spiked with 25 ng/L of all mycotoxins (Figure S2), absolute cartridge recovery of mycotoxins in Milli-Q water (Figure S3), absolute SPE recoveries of mycotoxins in Milli-Q water (Table S1), ion suppression of mycotoxins in various natural water extracts (Table S2), absolute method recoveries, method precision, and instrument precision (Table S3), relative method recoveries (Table S4), and method detection limits (Table S5) of mycotoxins at low concentrations in various natural waters. This material is available free of charge via the Internet at http://pubs.acs.org.

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