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Identification and determination of chlorinated azoles in sludge using liquid chromatography quadrupole time-of-flight and triple quadrupole mass spectrometry platforms

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ABSTRACT

Four antimycotic drugs (tioconazole, TCZ; sertaconazole, STZ; fenticonazole, FTZ and itraconazole, ITZ) and the fungicide imazalil (IMZ) are determined in sludge from sewage treatment plants (STPs) following a bottom-up analytical strategy. First, sludge extracts, obtained under different sample preparation conditions, were analyzed by liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometry (MS). A non-target search strategy, combined with the use of the chlorine mass filter, permitted to detect several chlorinated pollutants including the above referred azoles, which either had not been previously reported (TCZ, STZ, FTZ and ITZ), or scarcely investigated (IMZ), in this environmental compartment. Then, the sample preparation procedure was validated using standards of these compounds and their sensitive and selective determination was performed by LC–MS/MS, based on a QqQ system. Under final working conditions, quantitative extraction yields were attained with negligible changes in ionization efficiencies between sample extracts and standards; therefore, the above compounds were quantified against authentic standard solutions, with absolute recoveries in the range from 75 to 124%, achieving a limit of quantification of 2 ng g^{-1}. Analysis of sludge from 10 municipal STPs demonstrated the ubiquity of the identified chlorinated azoles with average concentrations from 31 ng g^{-1}, for IMZ, to more than 200 ng g^{-1}, for ITZ.

1. Introduction

Azoles (imidazoles and triazoles) are high production volume chemicals with domestic, industrial, agriculture and pharmaceutical applications [1]. From an environmental perspective, azolic drugs (antimycotics) and azolic phytochemicals (fungicides) are concerning pollutants because of their toxicity, persistence and the risk of behaving as endocrine disrupters [2]. The ubiquity of certain antimycotics, employed in pharmaceutical preparations (such as clotrimazole, miconazole and ketoconazole) and in personal care compounds (case of climbazole), has been confirmed in sewage water, sludge and soil amended sludge samples [3–5]. Azolic fungicides have also been found in different compartments of the aquatic environment [6–9].

Due to (1) development of resistant fungal strains [10], and (2) recent applications of antimycotics in combined therapies for the treatment of some cancers [11], new azolic compounds are being continuously developed. Therefore, there is a constant need to update and to validate the existing analytical methods for these new compounds to finally investigate their potential environmental occurrence. Alternatively, non-target screening methodologies, based on the analysis of high resolution mass spectrometry (HRMS) data [12–14], will permit the detection and identification of new azolic pollutants in environmental samples. Then, the existing analytical procedures have to be finely tuned and validated just for those azoles found in the environment.

Most of medium and high lipophilic azolic compounds incorporate one or several chlorine atoms in their structures. Thus, the chlorine mass filter tool (based on the characteristic isotopic profile of chlorine and mass defect between ^{35}Cl and ^{37}Cl isotopes) can be used to sieve them, together with any other chlorinated compound, from the myriad of signals existing in liquid chromatography (LC) HRMS records from complex environmental samples [15,16].

Sludge is recognized as the main reservoir of lipophilic antimycotics in municipal sewage water treatment plants (STPs). Compounds such as climbazole, clotrimazole, miconazole and keto-

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conazole have been measured in this matrix at concentrations in the high ng per g, even μg per g [17–19]; whilst, their levels in sewage water remain in the very low ng per litre [20,21]. Therefore, despite its complexity, sludge is regarded as the most suitable compartment to search for new lipophilic antimycotic compounds introduced in the aquatic environment through municipal sewers.

In this study, we assess the performance of a bottom-up analytical strategy for the identification of novel chlorinated and lipophilic compounds in freeze-dried sludge, with particular attention to azolic species. In a first injection, sample extracts, obtained from a pooled mixture of sludge, were analyzed by LC-HRMS. Chlorinated species were searched using automated routines applied to accurate mass scan spectral data. After obtaining the most probable empirical formulae, their accurate product ion scan spectra are recorded in a further injection. Compounds identification was based on MS and MS/MS database comparison, previous literature reports and final confirmation against authentic standards. This methodology revealed the presence of five unusual chlorinated azolic compounds in a pooled sludge sample. In a second step, after adjusting sample preparation conditions, their sensitive and selective determination was carried out by LC–MS/MS using a triple quadrupole (QqQ) instrument in the multiple reaction monitoring (MRM) mode.

2. Experimental

2.1. Samples and sample preparation

The non-target search of chlorinated pollutants was carried out using a pool of freeze-dried sludge, corresponding to three STPs, obtained in 2014. Sample fractions (0.5 g) were dispersed on 2 g of C_{18} and load into a matrix solid-phase dispersion (MSPD) polypropylene syringe containing Florisil and PSA sorbents (0.5 g each) for retention of polar species and fatty acids. Extractions were performed with 20 mL of methanol passed through packed syringe by applying slight vacuum. In order to reduce the complexity of this extract, it was further load in a SCX cartridge (1 g) to fractionate neutral and acid species (not retained in the SCX sorbent) from basic ones, which were eluted with 20 mL of methanol:NH_3 (2%). A second series of experiments were carried out by passing the primary extract from the MSPD syringe through a SAX cartridge (1 g) to fractionate neutral and basic compounds from the acid ones (further eluted with methanol: formic acid, 99:1). Analytes fractionation has been reported to be useful to reduce signal attenuation problems in the ESI source during analysis of complex environmental samples [20].

Extractions were carried out in triplicate. Extracts were concentrated to 5 mL before LC-HRMS analysis using a quadrupole time-of-flight (QTOF) MS instrument. Each extract was injected (5 μL) in ESI (+) and ESI (−) modes, considering different modifiers (ammonium acetate 5 mM or formic acid 0.1%) in the mobile phase.

After adjusting MSPD extraction and clean-up conditions, a group of five chlorinated azolic compounds was quantified in new samples of sludge by LC-ESI(+)-MS/MS using a triple quadrupole instrument.

2.2. Material, standards and solvents

Polypropylene syringes (15 mL volume) and frits for MSPD extractions were purchased from International Sorbent Technology (Mid Glamorgan, UK). Dispersant (C_{18}) and clean-up sorbents (Florisil and PSA) were from Aldrich (Milwaukee, WI, USA). The ionic exchange clean-up cartridges (SCX and SAX, 1 g) were provided by Agilent (Santa Clara, CA, USA).

Methanol, HPLC-grade; glacial formic acid and ammonia (25% solution in methanol) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system by Millipore (Billerica, MA, USA).

Standards of chlorinated compounds, tentatively identified in sludge samples, were acquired from Aldrich and TCI Europe (Zwijndrecht, Belgium). The chemical structures of imazalil (IMZ), sertaconazole (STZ), tioconazole (TCZ), fenticonazole (FTZ) and itraconazole (ITZ) are provided as Supplementary information, Fig. S1. Deuterated miconazole (MCZ-d_5 nitrate salt, CAS number 1216653-51-8) was obtained from Toronto Research Chemicals (North York, ON, Canada). This compound was used as internal surrogate (IS), through extraction and clean-up steps, during the quantitative determination of the above compounds in sludge.

2.3. LC-HRMS screening of chlorinated compounds

The HRMS chromatograms for the extracts from a pooled sludge matrix were acquired using a LC-ESI-QTOF-MS instrument. The LC was an Agilent 1200 Series consisting of a binary high pressure mixing pump, an autosampler and an oven for the LC column. The QTOF was an Agilent 6520 model, equipped with a Dual-Spray ESI source.

LC separations were carried out in a Zorbax Eclipse XDB C_{18} column (100 mm × 2.1 mm, 3.5 μm) from Agilent. The column was connected to a C_{18} guard cartridge (4 mm × 2 mm) provided by Phenomenex (Torrance, CA, USA). Methanol (B) and water (A), either containing formic acid (0.1%) or ammonium acetate (5 mM), were used as mobile phases at a flow of 0.2 mL min^{-1}. Whatever the employed modifier, the following gradient was used: 5% B (0–2 min), 100% B (30–35 min), 5% B (36–44 min). The column temperature was maintained at 30 °C and extracts were injected (5 μL volume aliquots of methanolic extracts) in ESI (+) and ESI (−) modes.

The fragmentor voltage was always set at 150 V and MS spectra were acquired in the range between 70 and 1700 m/z units, with the TOF analyzer operated in the 2 GHz extended dynamic range resolution mode. Search of chlorinated species was carried out by sequential application of the *Find by Molecular Feature* and *Formula Generation* functions, which are included in the Mass Hunter software used to control the LC-QTOF-MS instrument. The first function searches and combines ions, with the same retention time, compatible with a given empirical formula. In addition to pseudomolecular ($[M+H]^+$ or $[M-H]^-$) ions, this function considers also the most common adducts ($[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+Cl]^-$ and $[M+COOH]^-$) formed in ESI-type sources. The search was limited to species with single and double charge. The output provided by the *Find by Molecular Feature* function was limited to the 500 most intense molecular entities. Then, the *Formula Generation* function was applied to filter those containing, at least, one atom of chlorine [15]. Other elements considered to propose the most probable empirical formula of each feature were S, P, N, F and Br, in addition to C, H and O. Mass accuracy and isotopic profile were used to calculate the normalized scores (0–100) corresponding to the empirical formulae proposed for the *Molecular Feature Extracted* (MFE) spectra.

Molecular features with normalized scores about 75% were submitted to collision induced dissociation (CID) experiments (their precursor ions were isolated in the Q MS analyzer and fragmented by collision with N_2 molecules) and their accurate product ion spectra compared to those existing in databases, such as Metlin (https://metlin.scripps.edu) and Massbank (http://massbank.ufz.de/MassBank). Finally, the identities of candidate compounds were confirmed against pure standards.