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Biotic transformation products of sulfonamides in environmental water samples: High-resolution mass spectrometry-based tentative identification by a suspect screening approach

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ABSTRACT

The presence of pharmaceuticals in the aquatic environment is mainly due to their release from the effluents of the wastewater treatment plants (WWTPs), which are unable to completely remove them and their transformation products (TPs). Sulfonamides (SAs) are a synthetic antibacterial class used for the treatment of both human and animal infections; they have often been reported in surface water, thus contributing to the antibiotic resistance emergency. Monitoring SA TPs should be important as well because they could still exert some pharmaceutical activity; however, many TPs are still unknown since several transformation processes are possible (e. g. human and animal metabolism, WWTP activities, environmental factors etc.). In this work, three of the most used SAs, i.e., sulfamethoxazole (SMX), sulfapyridine (SPY), and sulfadiazine (SDZ), were incubated for 20 days in a batch reactor with activated sludge under controlled conditions. Then, the water sample was extracted and analyzed by ultra-high performance liquid chromatography-high resolution mass spectrometry in the data dependent acquisition (DDA) mode. Starting from the literature data, the possible transformation pathways were studied, and for each SA, a list of TPs was hypothesized and used for the identification. The raw data files were processed with Compound Discoverer, and 44 TPs (18, 13, and 13 TPs for SMX, SPY, and SDZ, respectively), including multiple TPs, were manually validated. To overcome the limitation of the DDA, the identified TPs were used in an inclusion list to analyze WWTP samples by a suspect screening approach. In this way, 4 SMX TPs and 5 SPY TPs were tentatively identified together with their parent compounds. Among these TPs, 5 of 9 were acetylated forms, in agreement with previous literature reporting that acetylation is the predominant SA transformation.

1. Introduction

The ubiquitous presence in the aquatic environment of anthropogenic chemicals, even if at low concentration levels (ng L^{-1}), is a well-known and alarming phenomenon. These contaminants belong to several commercial and chemical classes, e.g. pharmaceuticals, hormones, personal care products, flame retardants, plasticizers, perfluoroalkyl substances, and antibiotic resistance genes [1], and can cause different adverse effects on humans, biota, and ecosystems.

Due to their intrinsic activity towards organisms and their widespread use, one of the most investigated contaminant categories are antibiotics, which could contribute to the antibiotic resistance emergency [1,2]. The main routes by which antibiotics enter the water bodies are municipal wastewater effluents, and hospital and industrial discharges, because the wastewater treatment plants (WWTPs) are not able to completely remove them and their transformation products (TPs) [3,4]; also, for antibiotics widely used in animal farming, the application of manure on agriculture soils could be a significant source [5]. TPs, which can be generated by biological activities (e. g. microorganisms and human metabolism), as well as by chemical reactions and environmental

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factors (temperature, UV radiation, etc.), are cause for concern because they could still exert adverse effects on humans and other living organisms.

Sulfonamides (SAs) are synthetic antibacterial drugs used to treat both human and animal infectious. In particular, SAs are widely used in veterinary medicine, due to their low cost and high efficacy against some common bacterial diseases; in 2018, they were the third most sold veterinary antibiotic class in Europe [5].

SAs can undergo several transformation pathways, which are further affected by environmental factors, and for these reasons both parent compounds and their TPs have been detected in surface water as well as in the WWTP effluents [5], at concentration levels from ng L^{-1} up to μg L^{-1}. Acetylation is one of the most studied transformations and it can stem from the metabolism of organisms or SA degradation in municipal WWTPs or in the environment [6]; furthermore, an acetylated SA could transfer back to its parent SA [5]. However, many TPs are still unknown, and the impact on the environment and human health of the known TPs is still unclear since ecotoxicity data on single TPs are lacking [5].

In the literature, there are some studies [7–9] investigating the simulated degradation of SAs, but they rarely can fully represent the environment, and thus provide an overview of the potential TPs. On the other hand, most of the studies on the occurrence of SA TPs are targeted to commercially available standards [6,10–14]. Therefore, both the incomplete knowledge of possible TPs and their very low (but still potentially harmful for human health) concentration levels in water samples hinder a reliable assessment of the environmental contamination.

Environmental matrices, such as WWTP influents and effluents, contain several thousands of substances both of natural and anthropogenic origin, among which the compounds of interest are present at trace levels; therefore, the monitoring of antibiotics and their TPs requires some sample pre-treatment before analysis. The most widely employed strategy for contaminant enrichment from aqueous samples is still solid-phase extraction (SPE) [15], and hydrophilic-hydrophobic polymers are generally the preferred sorbents [14–19] for their ability to retain molecules in a wide polarity range and operate without pH limitations. The copolymer commercially known as Oasis HLB is the most reported in the literature for the suspect and non-target screening analysis of water samples [20]; nonetheless, for specific compound classes, other sorbents could be more selective.

The untargeted analysis relying on high-resolution mass spectrometry (HRMS), together with the development of suitable workflows for spectral features handling and interpretation, is the most popular choice [1]. Compound identification is tentatively obtained by matching with mass spectral libraries, such as mzCloud (https://www.mzcloud.org/, HighChem LLC, Slovakia) and the various MassBank versions (http://www.massbank.jp/, MassBank consortium). Although these databases are continuously implemented with new validated HR tandem mass spectra by international collaborative studies, they are far from being complete, especially for TPs, whose presence is only suspected (and often the authentic standards are not commercially available) or even unknown [21].

To obtain more information on SA TP formation and environmental fate, three of the most common SAs, namely sulfapyridine (SPY), sulfadiazine (SDZ), and sulfamethoxazole (SMX, which is the SA most used in human medicine and the most investigated in the environment), were incubated with activated sludge in a batch reactor under controlled conditions to allow the formation of TPs by microorganisms. After 20 days, the experiment was stopped, and the water sample was subjected to SPE. Two sorbents, i.e., Oasis HLB and graphitized carbon black (GCB), were tested. The ultra-high performance liquid chromatography-HRMS analysis was carried out in untargeted detection mode, whereas the software-assisted tentative identification of the most abundant SA TPs was performed following a suspect screening workflow based on the literature data. Finally, the *m/z* values of the identified TPs were used to create an inclusion list for the HRMS-based suspect screening of WWTP effluent samples.

2. Materials and methods

2.1. Chemicals, and materials

Analytical standards of SPY, SDZ, SMX, sulfamerazine (SMR), sulfisoxazole (SIX), sulfamethizole (SMT), sulfamethazine (SMZ), N1-acetylsulfamethoxazole (Ac-SMX) and 4-amino-N-(4-hydroxy-5-methoxy-2-pyrimidinyl)benzenesulfonamide (OHOMe-SDZ), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents and all other reagents used for sample preparation were provided by VWR International (Milan, Italy); LC-MS grade acetonitrile (ACN), water, and formic acid (FA) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Oasis HLB cartridges (200 mg, 6 mL) were purchased from Waters (Milford, MA, USA). GCB cartridges were assembled using 250 mg Supelclean ENVI-Carb bulk material, 6 mL polypropylene tubes, and two polypropylene frits (Merck Life Science, Darmstadt, Germany). Cellulose acetate Whatman® GD/X syringe filters, 25 mm, 0.45 μm were purchased from Sigma-Aldrich.

Standard stock solutions of each analyte were prepared in ACN with a concentration of 1.0 mg mL^{-1}, except for OHOMe-SDZ which was dissolved in H_2O/ACN (50:50, *v/v*) (Table S1). Analyte individual and mixture working solutions were prepared by diluting aliquots of the stock solutions with the proper solvent amount, stored at − 20 °C, and renewed weekly.

2.2. Biological experiment in the batch reactor

Activated sludge was collected and put in a batch reactor that was kept aerated to ensure aerobic conditions to generate SA TPs. The experiment lasted 20 days and was run in a fed-batch reactor having a working volume of 0.70 L. The reactor was stirred by a mechanical impeller, aerated with air pumps connected to ceramic diffusers, and maintained at room temperature and pH 7 (Fig. S1). Activated sludge collected from a full-scale municipal WWTP was used as inoculum. Before starting the experiment, the aerobic sludge was settled, and the liquid phase was replaced with a mineral medium to remove all the residual organics therein contained. This operation was repeated three times. The composition of the used mineral medium, which contained a phosphate buffer (pH 7), was described elsewhere [22]. At the beginning of the experiment, the concentration of microorganisms in the reactor (measured as volatile suspended solids, VSS) was 3.27 ± 0.11 g L^{-1}. Collectively, the applied reactor operating conditions resembled those occurring at a conventional municipal WWTP (i.e., the activated sludge process). The batchwise mode of operation was applied to favor the possible accumulation of SA TPs and thus enhance the likelihood of their analytical detection.

A mixture of the three antibiotics was initially supplied to the microbial culture in powder form to obtain a final concentration of 10 mg L^{-1} each. Sodium acetate was also initially supplied as a carbon source to sustain the microbial activity and was periodically readded whenever oxygen uptake rate (OUR) measurements suggested it was depleted. In correspondence to each addition, the concentration of acetate supplied to the reactor was around 300 mg L^{-1}, except for the first two spikes whereby a lower concentration (<150 mg L^{-1}) was applied. Throughout the experimentation, OUR ranged from values as high as 130 mg O_2 L^{-1} h^{-1}, which are typical of non-limited biological activity when an excess carbon source is available to microorganisms, to values below 20 mg O_2 L^{-1} h^{-1}, which correspond to the endogenous metabolism when the carbon source is completely depleted [23]. This, along with the observed trend of acetate concentration in the reactor, which was completely depleted after each addition (Fig. S2), suggested the absence of inhibitory effects of the supplied antibiotics on microbial activity. Furthermore, the cumulative acetate consumption was linked to microbial

growth which, by the end of the experiment, accounted for 4.40 ± 0.03 gVSS L^{-1} . VSS were measured according to Standard Methods [24]. Acetate quantification was carried out on filtered (0.45 μm porosity) liquid samples injected into a gas-chromatograph (Agilent 8860 GC) equipped with a flame-ionization detector.

At the end of the incubation period, the sample was collected, centrifuged at 7500g for 30 min, filtered with cellulose acetate syringe filters, and stored at 4 °C until extraction.

2.3. SPE optimization and sample preparation

SPE was performed using two different adsorbent materials, namely Oasis HLB and GCB. The three sample typologies used for the optimization step were well water, water collected from the batch reactor before SA spiking, and a pooled WWTP sample.

2.3.1. Oasis HLB SPE procedure

The Oasis HLB cartridge was conditioned sequentially with 5 mL of methanol and 5 mL of 10^{-4} mol L^{-1} HCl. Then, 100 mL of filtrated water sample was adjusted to pH 4 with HCl and loaded onto the cartridge at ca. 2 mL min^{-1} flow rate. The cartridge was washed with 5 mL of ultrapure water and vacuum dried for 15 min; elution was carried out with 10 mL of methanol 0.1% FA. The sample was evaporated to dryness and reconstituted in 100 μL of H_2O/ACN (80:20, *v/v*), then centrifugated before the UHPLC-MS/MS analysis.

2.3.2. GCB SPE procedure

The GCB cartridge was conditioned sequentially with 5 mL of CH_2Cl_2/MeOH (80:20, *v/v*) with 0.02 mol L^{-1} TFA, 5 mL of MeOH with 0.02 mol L^{-1} TFA, and 10 mL of 0.1 mol L^{-1} HCl. Then, 100 mL of filtrated water sample was adjusted to pH 2 with HCl and loaded onto the cartridge at ca. 5 mL min^{-1} flow- rate. The cartridge was washed with 5 mL of 0.01 mol L^{-1} HCl and 0.5 mL of MeOH; after that, it was vacuum dried for 10 min. The elution was carried out with 10 mL of CH_2Cl_2/MeOH (80:20, *v/v*) with 0.02 mol L^{-1} TFA. The sample was evaporated to dryness and reconstituted in 100 μL of H_2O/ACN (80/20, *v/v*), then centrifugated before the UHPLC-MS/MS analysis.

2.3.3. Recovery studies

Recovery (RE) studies were carried out to compare the two SPE protocols. Different water samples were used for these experiments: well water, water collected from the batch reactor (25 mL, obtained after centrifugation and filtration as described in subsection 2.2, diluted to 100 mL), and a pool of wastewater (previously filtered on a filter paper) from four WWTPs. Water samples were spiked with a standard mix before (set 1) and after (set 2) extraction at three different concentration levels, namely 5, 50, and 250 μg L^{-1}. For each analyte, RE (%) was evaluated by the ratio between the peak area in the sample spiked before and after the extraction procedure, according to the following equation:

$$RE(\%) = \frac{Area(set1)}{Area(set2)} \times 100$$

2.3.4. Preparation of WWTP samples

The optimized extraction using GCB was applied to four effluent samples provided from four different WWTPs surrounding the city of Milan (Italy), i.e., Assago, Bresso, Pero, and San Giuliano Milanese Est (see Supplementary Material). For each extraction, 100 mL of wastewater were filtered on a filter paper under vacuum and diluted to the final volume of 250 mL. The samples were extracted by SPE on GCB as described in Section 2.3.2.

2.4. Instrumental analysis

2.4.1. UHPLC-MS/MS targeted analysis

For RE studies, an Ultimate 3000 UHPLC system connected via a heated ESI source to a TSQ Vantage™ triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used. The UHPLC system consisted of a binary pump equipped with a degasser, a thermostated microwell plate autosampler, set at 14 °C, and a thermostated column oven. The chromatographic column was a Kinetex F5 (150 × 2.1 mm i.d., 1.7 μm particle size) equipped with a Security guard F5 (2.1 mm i.d), both from Phenomenex (Torrance, California, USA). The column was thermostated at 25 °C and the mobile phase consisted of (A) H_2O with 0.1% (*v/v*) FA, and (B) ACN with 0.1% (*v/v*) FA; the flow rate was set at 300 μL min^{-1}. After a 4 min-isocratic step at 10% B, B was increased to 40% in 8 min, kept constant for 2 min, then increased to 70% in 6 min, and kept constant for 5 min. After the gradient, B was brought to 99% in 3 min and kept constant for 2 min to rinse the column; finally, B was brought back to 10% and the column was equilibrated for 8 min. The injection volume was 10 μL.

The ESI source parameters were spray voltage 3.0 kV, capillary temperature 270 °C, and pressures of sheath gas, ion sweep gas, and auxiliary gas 30, 0, and 25 (arbitrary units), respectively.

For each analytical standard, multiple reaction monitoring (MRM) acquisition parameters were optimized in positive ion mode by directly infusing a 10 ng μL^{-1} solution in the mass spectrometer. (Table S2). For both quadrupoles, default resolution setting of 0.7 u at full width at half maximum (FWHM) was used. The UHPLC-MS/MS system was managed by Xcalibur software (v.2.1, Thermo Fisher Scientific).

2.4.2. UHPLC-HRMS analysis

Samples were purified by the GCB SPE procedure and analyzed using a Vanquish UHPLC system equipped with a binary pump, a thermostated column compartment, and an autosampler (kept at 14 °C), coupled via a heated ESI source to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The chromatographic column, the mobile phase, and the source parameters were the same as reported in the previous subsection for RE studies.

Distinct UHPLC-HRMS runs were carried out for positive and negative ESI modes. MS acquisition parameters were the same as previously reported for untargeted analysis, including resolution settings of 35,000 and 17,500 (FWHM, @200 *m/z*) for MS and MS/MS experiments, respectively [25].

For each sample, two technical replicates (UHPLC-HRMS runs) were performed, followed by an injection of a blank sample (H_2O/ACN, 80:20, *v/v*) to check for possible carry-over.

Two different analyses were carried out with this system. First, after the GCB SPE procedure, the aqueous phase of the activated sludge was analyzed in the untargeted (i.e., without any inclusion list) data dependent acquisition (DDA) mode to identify the hypothesized TPs of the three selected standards. Then, the identified compounds were inserted in an inclusion list (Table S3) that was implemented on the suspect screening MS method for the WWTP sample analysis. Raw data files were acquired by Xcalibur software (version 3.1, Thermo Fisher Scientific).

2.5. Transformation products prediction and compound identification

After preliminary processing, compound identifications were manually validated using a dedicated workflow and the Expected Compounds tool of Compound Discoverer v. 3.2 (Thermo Scientific) (Fig. S3). Spectra annotation and tentative compound identification were achieved using the tandem mass spectrum, retention time, accurate mass within a mass tolerance of ± 5 ppm, and a minimum peak intensity of 100,000. Briefly, the software extracted the *m/z* features from the raw data files, grouped them, and aligned them among the different runs; then, the features with no associated tandem mass spectrum were ruled out. TPs were matched by enabling transformations, both selected from the default ones and by the manual implementation (Table S4). The manually implemented transformation reactions of SAs were selected from the literature considering either microbial activity or natural

environmental processes under aerobic conditions [7–9,11–13,26]. For each parent compound, a maximum of three modifications was considered. Dehydration (−2 hydrogen atoms, −1 oxygen atom), desaturation (−2 hydrogen atoms), hydration (+2 hydrogen atoms, +1 oxygen atom), oxidation (+1 oxygen atom) and reduction (+2 hydrogen atoms) were chosen among the default phase I transformations; acetylation (+2 carbon atoms, +2 hydrogen atoms, +1 oxygen atom), glucoside conjugation (+6 carbon atoms, +10 hydrogen atoms, +5 oxygen atoms), glucuronide conjugation (+6 carbon atoms, +8 hydrogen atoms, +6 oxygen atoms), and methylation (+1 carbon atom, +2 hydrogen atoms) were selected among the default phase II transformations; besides 4-ipso-hydroxylation (−1 hydrogen atom, −1 nitrogen atom, +1 oxygen atom), chlorination (−1 hydrogen atom, +1 chlorine atom), deamination (−1 hydrogen atom, −1 nitrogen atom), desulfonation (−2 oxygen atoms, −1 sulfur atom), formylation (+1 carbon atom, +1 oxygen atom), loss of aniline (−6 hydrogen atoms, −6 carbon atoms, −1 nitrogen atom, +1 oxygen atom), loss of sulfanilic acid (−5 hydrogen atoms, −6 carbon atoms, −1 nitrogen atom, −2 oxygen atoms, −1 sulfur atom), methyl oxidation to carboxyl (−2 hydrogen atoms, +2 oxygen atoms), methylene oxidation to carbonyl (−2 hydrogen atoms, +1 oxygen atom), nitration (−2 hydrogen atoms, +2 oxygen atoms), and nitrosylation (−2 hydrogen atoms, +1 oxygen atom), that are all phase I transformations, were manually implemented.

3. Results and discussion

3.1. Extraction protocol optimization and recovery studies

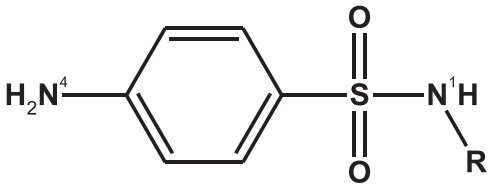
The goal of the optimization process was to set up the analytical method for the simultaneous analysis of SAs and their TPs. Sorbent choice was necessary because SAs and their TPs can have a wide range of polarity and structural differences. Two different sorbent materials were tested for this purpose, i.e., GCB and Oasis HLB, using a mixture of 10 compounds chosen from the SA class and the related TPs, for which analytical standards were commercially available. Oasis HLB is a hydrophilic/hydrophobic polymeric material very popular in environmental applications because of the sorption affinity for diverse compounds in a wide polarity range and the stability from pH 0–14. GCB is a non-porous carbon material made up of interconnected and layered graphitic sheets; the peculiar structure allows different kinds of interactions, both hydrophobic and polar ones, with a wide range of analytes and can, therefore, be used for both polar and non-polar compound extraction and clean-up [25].

The method was optimized using well water, water collected from the batch reactor (25 mL diluted to 100 mL), and a pool of wastewater from four WWTPs. The results (Table 1) showed that both sorbents yielded high recoveries, consistent with the literature, but GCB gave better results at lower concentrations, which is mandatory in environmental studies due to the complexity of the matrix and the extremely low abundance of TPs. The better RE of the GCB sorbent could be explained by the different pH of the extraction procedures because SAs have pK_2 values around 2.5 [13]. Therefore, the GCB sorbent was selected for sample preparation.

3.2. UHPLC-HRMS analysis of SA TPs from the batch reactor experiment

For this study, three of the most used and investigated SAs were selected, namely SDZ, SPY and SMX [27]. For the determination of their TPs, a two-step UHPLC-MS/MS acquisition strategy was used. At first, untargeted MS detection with a TOP 5 DDA mode was performed in both ion polarity modes to maximize the detected compounds and improve the identification of the compounds of interest. The compounds annotated at this stage were then used to create an inclusion list (Table S3) for suspect screening investigation of SAs and their TPs, so to improve the sensitivity of analysis for the WWTP samples where compounds are present at trace level. An aliquot of water sample collected from the batch reactor before adding the three SAs was analyzed using the inclusion list to verify the absence of the identified compounds.

Spectra annotation was done using a specific workflow within the Compound Discoverer software (Fig. S4). The parent structure was implemented in the method and all the selected transformation reactions (Table S4) were considered with the aim of finding as many TPs as possible. The use of this type of workflow is possible because SAs have different structures but they are also a homologous family of compounds (Fig. 1) for which the available reaction sites are predictable. The reaction sites are the N^4 or N^1, the benzene ring, and the R moiety. All of



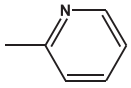
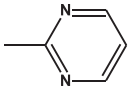
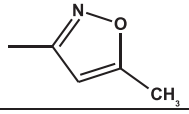
SULFONAMIDE	R	Log K_{ow}
Sulfapyridine (SPY)		0.35
Sulfadiazine (SDZ)		-0.09
Sulfamethoxazole (SMX)		0.89

Fig. 1. Sulfonamide generic structure and the three compounds selected for the batch reactor experiment. Log K_{ow} values were obtained from [27].

Table 1
Recoveries (RE%) and relative standard deviations (n = 3) obtained by extracting with Oasis HLB and GCB sorbents different water samples fortified at 250, 50, and 5 μg L^{-1} of a SA standard mixture.

Compound	RE% (RSD)									
	Well water (250 μg L^{-1})		Batch water (250 μg L^{-1})		Wastewater (250 μg L^{-1})		Wastewater (50 μg L^{-1})		Wastewater (5 μg L^{-1})	
	HLB	GCB	HLB	GCB	HLB	GCB	HLB	GCB	HLB	GCB
SPY	79 (8)	95 (4)	104 (3)	98 (4)	113 (9)	63 (6)	68 (4)	78 (6)	43 (17)	107 (9)
SDZ	73 (2)	90 (4)	79 (9)	98 (2)	113 (4)	56 (4)	55 (12)	82 (5)	17 (21)	81 (11)
SMX	74 (9)	66 (2)	96 (4)	99 (1)	110 (3)	70 (11)	77 (3)	100 (6)	77 (9)	120 (15)
SMR	85 (11)	93 (6)	65 (14)	103 (2)	111 (9)	80 (8)	58 (7)	70 (7)	72 (6)	106 (4)
SIX	78 (4)	61 (13)	80 (3)	97 (7)	107 (5)	93 (5)	54 (13)	81 (10)	77 (12)	87 (6)
SMT	90 (7)	59 (5)	83 (7)	94 (5)	101 (6)	89 (2)	52 (16)	74 (3)	55 (10)	69 (9)
SMZ	79 (6)	83 (6)	72 (7)	99 (7)	108 (11)	86 (9)	59 (6)	77 (7)	65 (8)	73 (5)
Ac-SMX	73 (7)	83 (1)	67 (10)	101 (1)	95 (4)	81 (3)	54 (11)	100 (3)	92 (8)	92 (6)
OHOMe-SDZ	106 (4)	57 (3)	82 (6)	94 (5)	110 (7)	86 (3)	61 (4)	96 (2)	85 (13)	86 (10)

them are potential sites subject to transformations and therefore they can be included in a suspect screening type of identification strategy, which allows for potentially annotating all the compounds of interest, given the structure of the parent compound and the type of transformation.

All the expected compounds obtained from the automated software processing were finally manually validated to improve the annotation confidence [21]. The manual identification step was based on the three characteristic product ions common to the SA class, i.e., *m/z* 92.0495, 108.0444, and 156.0114 in the positive ion mode (Fig. 2) [11,28]. These product ions are diagnostic for the entire SA class, whereas product ions which are characteristic of the heterocyclic R moiety allow for discriminating the single SAs. The R moiety-specific fragments, in positive ion mode, are *m/z* 99.0558, 160.0874, and 188.0823 for SMX (Fig. 2); *m/z* 94.0530, 110.0480, and 184.0869 for SPY; and *m/z* 96.0562, and 158.0024 for SDZ. Starting from this fragmentation information, by knowing the potential transformation reactions, the product ions for the related TPs can be predicted and searched in the experimental spectra.

Following this approach, in the batch reactor experiment, 18, 13, and 13 TPs were identified for SMX, SPY, and SDZ, respectively. The lists of these identified compounds are reported in Table 2 for SMX, Table 3 for SPY, and Table 4 for SDZ. The corresponding tandem mass spectra of parent compounds and their TPs are reported in Fig. S5-S22 for SMX, Fig. S23-S35 for SPY, and Fig. S36-S48 for SDZ.

The transformation reactions were selected to include the ones reported in the literature and provide a workflow suitable for the identification of the known and most probable TPs of SAs. Acetyl-SMX has been detected several times in the literature because it is excreted from organisms under antibiotic therapy or produced by aerobic bacteria; therefore, it can easily contaminate the environment. That is the reason why acetylation is the prevalent transformation reaction reported in studies on the products of metabolism present in surface water. The same goes for Acetyl-SPY and Acetyl-SDZ. The acetyl-SA characteristic fragments are *m/z* 134.0605 and 198.0224 in positive ion mode [6–8, 11,26,29].

Deamination is also a typical transformation that can be found in the literature [11,30] and has been detected for all the considered SAs by its characteristic fragments, namely *m/z* 77.0384, 93.0699, and 141.0006 in positive ion mode. Moreover, formylation reaction has been observed for all three SAs and confirmed by the diagnostic fragments at *m/z* 120.0443, 136.0394, and 184.0063 in positive ion mode. The same reasoning was used to match the spectra of other TPs, i.e.: 4-hydroxy-SMX, 4-ipso-X-dihydroxy-SMX, 4-nitroso-SMX, and N^4-hydroxy-acetyl-SMX [11]. The discrimination between N^4-hydroxy-acetyl-SMX and N^4-acetyl-hydroxy-SMX was not possible by the different retention times by using the Kinetex F5 column; in fact, even if the hydroxyl terminal group is expected to increase the polarity more than the acetyl group on the same position (calculated logP of 0.1175 and 0.3795, respectively ((source: ChemDraw)), the combined effect of the multiple interactions with the stationary phase it is not easily predictable. On the other hand, it was not possible to discriminate between the two hydroxylated forms of SMX and SPY occurred on the homolog structure moieties, because there are no references for identifying the site of hydroxyl group insertion. Two hydroxylated forms of SDZ [6,7] were identified at two different retention times, probably due to the different positions of the hydroxyl group insertion on the pyrimidine ring.

The N^4-methylated form of all the three SAs was detected with a retention time longer than the parent compound due to the reduction of the polarity by the methylation reaction. The structure was confirmed by the presence of the three characteristic product ions in positive ion mode at *m/z* 106.0651, 122.0601, and 170.0271; the presence of all methylated forms means that methylation reaction can be related to bacterial activity [31].

Methylation reactions occurred also on the R moiety; in fact, an R-methylated form was detected for all three SAs; moreover, other transformation reactions (such as acetylation and hydroxylation) took place directly on the methylated-SA TP.

Glucoside conjugation is frequently reported and was confirmed for all three SAs [11].

Apart from TPs hypothesized from the literature, the study revealed that multiple TPs could also be detected, i.e., N^4-methyl-N^4-acetyl-SMX, N^4-methyl-N^4-acetyl-SPY, N^4-methyl-N^4-acetyl-SDZ, N^4-hydroxy-acetyl-N^4-methyl-SDZ, methyl-N^4-acetyl-hydroxy-SPY, and methyl-N^4-hydroxy-acetyl-SPY. The discrimination of the two latter compounds was not possible based on the retention time, as previously described for N^4-hydroxy-acetyl-SMX and N^4-acetyl-hydroxy-SMX. The possibility that multiple transformations occur on the same compound has led to the identification of hydroxy-N^4-acetyl-SPY, N^4-hydroxy-acetyl-SDZ, and N^4-acetyl-N^4-hydroxy-SDZ. Also in this latter case of the two SDZ compounds, discrimination was not possible.

For all three compounds analyzed, a chlorinated form was also detected probably due to the presence of chloride in the mineral medium used during the incubation period.

Also, unexpected compounds could be detected by the developed approach. TP 256 had the typical SA diagnostic product ions, which allows assuming that the characteristic SA structure remained intact.

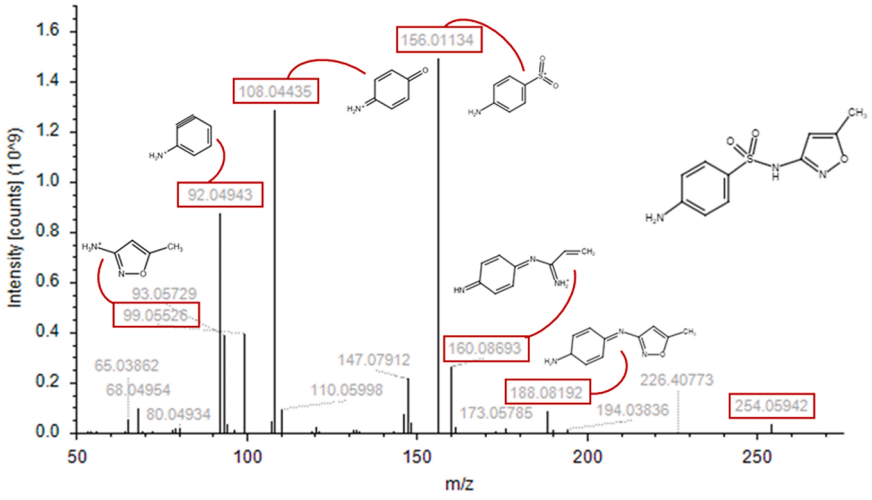


Fig. 2. Tandem mass spectrum of SMX obtained in positive ion mode. The structures of the main diagnostics fragments for both the entire SA class and SMX are reported.

Table 2
Identified compounds related to SMX transformation pathways. Tandem mass spectra and structures are reported in Supplementary Material, Fig. S5-S22.

Compound	RT (*min*)	Formula	Molecular weight (u)	Adduct ion	Experimental *m/z*	Δppm	Main product ions
SMX	10.4	$C_{10}H_{11}N_3O_3S$	253.0521	$[M+H]^+$	254.0593	0.0	92.0494, 108.0443, 156.0113, 99.0552, 160.0869, 188.0819
4-hydroxy-SMX	10.9	$C_{10}H_{10}N_2O_4S$	254.0361	$[M+H]^+$	255.0437	1.6	93.0573, 109.0478, 156.9954, 99.0553, 160.087, 188.0817
4-ipso-X-diyhroxy-SMX	6.2	$C_{10}H_{10}N_2O_5S$	270.031	$[M-H]^-$	269.0236	-0.7	109.0293, 124.0165, 172.9905, 96.0325, 159.9941, 188.0587
4-nitroso-SMX	10.5	$C_{10}H_9N_3O_4S$	267.0313	$[M-H]^-$	266.0244	1.1	122.0246, 168.0120, 185.0385, 82.0294
Acetyl-SMX	11.0	$C_{12}H_{13}N_3O_4S$	295.0626	$[M+H]^+$	296.0705	2.4	93.0573, 108.0444, 134.0601, 198.022, 99.0553, 160.087, 188.0818
Chlorinated-SMX	12.8	$C_{10}H_{10}ClN_3O_3S$	287.0131	$[M+H]^+$	288.0207	1.4	127.0184, 142.0055, 189.9724, 99.0552
Desamino-SMX	12.6	$C_{10}H_{10}N_2O_3S$	238.0412	$[M+H]^+$	239.0487	1.3	77.0384, 93.0699, 141.0006, 97.0396, 159.068
Formyl-SMX	10.6	$C_{11}H_{11}N_3O_4S$	281.047	$[M+H]^+$	282.0546	1.4	120.0444, 136.0394, 184.0063, 99.0553, 160.0869, 188.0818
Methyl-SMX (R group)	10.4	$C_{11}H_{13}N_3O_3S$	267.0677	$[M+H]^+$	268.0749	0.0	92.0494, 108.0442, 156.0112, 113.0708
N^4-hydroxy-acetyl-SMX / N^4-acetyl-hydroxy-SMX	6.7	$C_{12}H_{13}N_3O_5S$	311.0575	$[M+H]^+$	312.0649	0.6	150.0548, 166.0498, 108.0443, 214.0168, 99.0553
N^4-acetyl-hydroxy-SMX / N^4-hydroxy-acetyl-SMX	10.6	$C_{12}H_{13}N_3O_5S$	311.0575	$[M+H]^+$	312.0653	1.9	150.0551, 151.063, 108.0444, 166.0499, 214.0169, 99.0553
N^4-methyl-N^4-acetyl-SMX	11.9	$C_{13}H_{15}N_3O_4S$	309.07833	$[M+H]^+$	310.0855	0.0	148.0753, 164.0704, 212.0373, 99.0552
N^4-hydroxy-SMX H-hydroxy-SMX	9.5	$C_{10}H_{11}N_3O_4S$	269.047	$[M+H]^+$	270.0544	0.7	108.0444, 109.0523, 124.0394, 172.0064, 99.0553
N^4-glucoside-SMX	7.3	$C_{16}H_{20}N_3O_8S$	415.1049	$[M+H]^+$	416.1125	1.0	93.0573, 108.0444, 156.0115, 99.0553, 252.0869, 254.0601
N^4-methyl-SMX	12.4	$C_{11}H_{13}N_3O_3S$	267.0677	$[M+H]^+$	268.0754	1.9	106.0651, 122.06, 170.0270, 99.0552, 160.0869
TP 256	2.8	$C_{13}H_{13}N_3O_3S$	255.0677	$[M+H]^+$	256.0752	1.2	92.0494, 108.0444, 156.0114, 101.0710
TP 274	2.0	$C_{10}H_{15}N_3O_4S$	273.07833	$[M+H]^+$	274.0856	0.3	92.0494, 108.0444, 156.0114, 119.0815
TP 284	1.7	$C_{10}H_9N_3O_5S$	283.0262	$[M+H]^+$	284.0338	1.4	92.0495, 108.0443, 156.0116, 129.0544, 238.0701

Table 3
Identified compounds related to SPY transformation pathways. Tandem mass spectra and structures are reported in Supplementary Material, Fig. S23-S35.

Compound	RT (*min*)	Formula	Molecular weight (*u*)	Adduct ion	Experimental *m/z*	Δppm	Main product ions
SPY	4.7	$C_{11}H_{11}N_3O_2S$	249.0571	$[M+H]^+$	250.0643	0.0	92.0494, 108.0443, 156.0113, 94.0524, 110.0601, 184.087
Acetyl-SPY	7.3	$C_{13}H_{13}N_3O_3S$	291.0677	$[M+H]^+$	292.0755	2.1	134.0601, 108.0443, 94.0525, 184.0869, 198.022
Methyl-N^4-acetyl-hydroxy-SPY / Methyl-N^4-hydroxy -acetyl- SPY	9.7	$C_{14}H_{15}N_3O_4S$	321.0783	$[M+H]^+$	322.0859	1.2	164.0706, 180.0655, 228.0324
Methyl-N^4-hydroxy -acetyl- SPY / Methyl-N^4-acetyl-hydroxy-SPY	8.7	$C_{14}H_{15}N_3O_4S$	321.0783	$[M+H]^+$	322.086	1.6	164.0707, 180.0655, 228.0327
H-hydroxy-SPY N^4-hydroxy-SPY	3.4	$C_{11}H_{11}N_3O_3S$	265.0521	$[M+H]^+$	266.0595	0.8	95.0604, 108.0444, 124.0394, 157.0066, 172.0064
Desamino-SPY	8.7	$C_{11}H_{10}N_2O_2S$	234.0463	$[M+H]^+$	235.0538	1.3	77.0384, 94.0525, 141.0005
Formyl-SPY	5.9	$C_{12}H_{11}N_3O_3S$	277.0521	$[M+H]^+$	278.0596	1.1	95.0604, 120.0444, 136.0394, 157.0068, 184.0064
N^4-glucoside-SPY	2.1	$C_{17}H_{21}N_3O_7S$	411.11	$[M+H]^+$	412.1175	0.7	92.0490, 108.0444, 156.0115, 254.1022, 318.0646
Hydroxy-N^4-acetyl-SPY	7.3	$C_{13}H_{13}N_3O_4S$	307.0626	$[M+H^+$	308.0703	1.6	95.0603, 150.0550, 157.0067, 166.0498, 214.0168
Methyl-SPY (R group)	6.5	$C_{12}H_{13}N_3O_2S$	263.0728	$[M+H]^+$	264.0804	1.5	92.0493, 108.0443, 156.0113, 109.076, 198.1025
N^4-methyl-SPY	9.3	$C_{12}H_{13}N_3O_2S$	263.0728	$[M+H]^+$	264.0804	1.5	106.0651, 122.06, 170.027
Chlorinated-SPY	9.4	$C_{11}H_{10}ClN_3O_2S$	283.0182	$[M+H]^+$	284.0258	1.4	127.0185, 142.0055, 189.9723
N^4-methyl-N^4-acetyl-SPY	7.8	$C_{14}H_{15}N_3O_3S$	305.0834	$[M+H]^+$	306.0908	0.7	148.0756, 164.0704, 212.0373, 184.087

The transformation took place on the R moiety, with the opening of the isoxazole ring by disruption of the N-O bond (4-amino-N-(1-amino-3-oxobut-1-*en*-1-yl)benzenesulfonamide). Similarly, TP 284 presented the SA diagnostics product ions, so the transformation was expected to occur on the R moiety and involved the oxidation of the methyl group on the isoxazole moiety to a carboxyl group. This type of transformation could be an oxidation reaction by bacteria in the activated sludge [9]. These structures have been described in previous studies [7].

TP 274 was never reported before and observing the main product ions it can be assumed that the transformation took place on the R moiety. After the disruption of the N-O bond of the isoxazole, hydroxylation on the ring occurred, and it can be associated with the *m/z* 119.0815 product ion (Fig. 3).

3.3. Suspect screening analysis of WWTP samples

As mentioned before, all the identified TPs were collected in an inclusion list that was implemented in the MS method for the application

Table 4
Identified compounds related to SDZ transformation pathways. Tandem mass spectra and structures are reported in Supplementary Material, Fig. S36-S48.

Compound	RT (*min*)	Formula	Molecular weight (*u*)	Adduct ion	Experimental *m/z*	Δppm	Main product ions
SDZ	4.1	$C_{10}H_{10}N_4O_2S$	250.0524	$[M+H]^+$	251.0594	-0.8	92.0494, 96.0556, 108.0444, 156.0114, 158.002
Acetyl-SDZ	6.8	$C_{12}H_{12}N_4O_3S$	292.063	$[M+H]^+$	293.0705	1.0	96.0555, 134.06, 150.055, 198.022
Formyl-SDZ	5.1	$C_{11}H_{10}N_4O_3S$	278.0473	$[M+H]^+$	279.0548	1.1	96.0556, 120.0445, 136.0394, 158.0022, 184.0065
Desamino-SDZ	8.2	$C_{10}H_9N_3O_2S$	235.0415	$[M+H]^+$	236.0489	0.8	77.0384, 95.049, 141.0005, 158.0017
X-hydroxy-SDZ (R group)	5.4	$C_{10}H_{10}N_4O_3S$	266.0473	$[M+H]^+$	267.0546	0.4	92.0495, 108.0444, 156.0114, 112.0506, 173.9971
X-hydroxy-SDZ (R group)	3.1	$C_{10}H_{10}N_4O_3S$	266.0473	$[M+H]^+$	267.0547	0.7	92.0494, 108.0443, 156.0113, 112.0505, 173.997
N^4-hydroxy-acetyl-N^4-methyl-SDZ	9.3	$C_{13}H_{14}N_4O_4S$	322.0735	$[M+H]^+$	323.0813	1.9	164.0707, 180.0656, 228.0327, 96.0556
N^4-hydroxy-acetyl-SDZ N^4-acetyl-N^4-hydroxy-SDZ	6.5	$C_{12}H_{12}N_4O_4S$	308.0579	$[M+H]^+$	309.0658	2.3	150.0551, 151.0617, 108.0444, 214.0169
N^4-glucoside-SDZ	1.9	$C_{16}H_{20}N_4O_7S$	412.1052	$[M+H]^+$	413.1133	2.2	93.0573, 108.0444, 156.0114, 252.0854, 254.1022
N^4-methyl-SDZ	8.9	$C_{11}H_{12}N_4O_2S$	264.0680	$[M+H]^+$	265.0758	2.3	106.0651, 122.06, 170.027
Methyl-SDZ (R group)	5.4	$C_{11}H_{12}N_4O_2S$	264.0680	$[M+H]^+$	265.0757	1.9	92.0495, 108.0444, 156.0114, 110.0713, 173.0715, 199.098
Chlorinated-SDZ	9.4	$C_{10}H_9ClN_4O_2S$	284.0134	$[M+H]^+$	285.0209	1.1	127.0185, 142.0055, 189.9724
N^4-methyl-N^4-acetyl-SDZ	7.8	$C_{13}H_{14}N_4O_3S$	306.0786	$[M+H]^+$	307.0863	1.6	148.0757, 164.0715, 212.0376

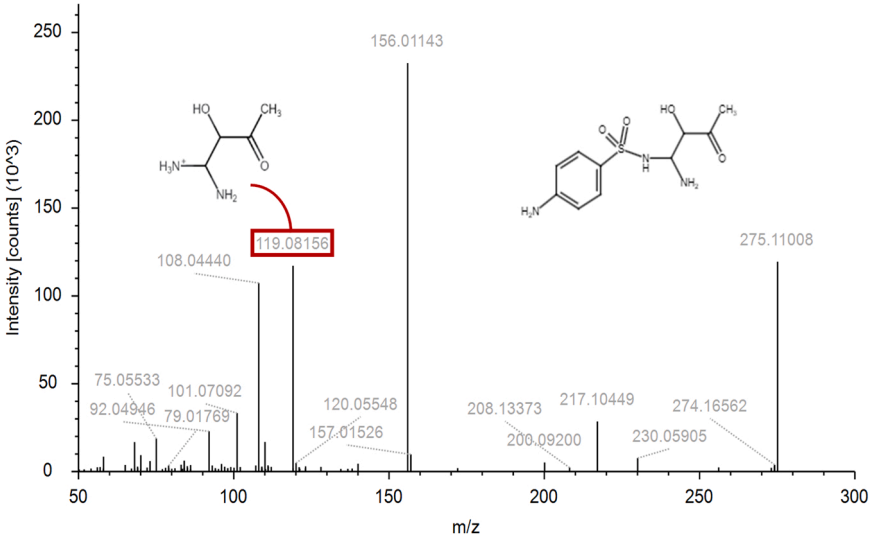


Fig. 3. Tandem mass spectrum and relative structure of TP 274 obtained in positive ion mode.

Table 5
Identified TPs in WWTP samples. Tandem mass spectra are reported in Supplementary Material, Fig. S49-S59.

Compound	RT (*min*)	Formula	Molecular weight (*u*)	Adduct ion	Experimental *m/z*	Δppm	Main product ions
SMX	10.4	$C_{10}H_{11}N_3O_3S$	253.0521	$[M+H]^+$	254.0585	-3.1	92.0491, 108.044, 156.0108, 99.0549, 160.0864, 188.0811
SPY	4.6	$C_{11}H_{11}N_3O_2S$	249.0571	$[M+H]^+$	250.0637	-2.4	92.0491, 108.044, 156.0108, 94.0523, 110.0597, 184.0863
Acetyl-SMX	11.1	$C_{12}H_{13}N_3O_4S$	295.0626	$[M+H]^+$	296.0694	-1.4	93.0332, 108.0441, 134.0597, 198.0215, 99.055, 160.0865, 188.0814
Acetyl-SPY	7.3	$C_{13}H_{13}N_3O_3S$	291.0677	$[M+H]^+$	292.0745	-1.4	134.0596, 108.044, 94.0522, 184.0864, 198.0214
Desamino-SMX	12.6	$C_{10}H_{10}N_2O_3S$	238.0412	$[M+H]^+$	239.0482	-0.8	77.0382, 93.0698, 141.0001, 97.0394, 159.0674
Desamino-SPY	8.8	$C_{11}H_{10}N_2O_2S$	234.0463	$[M+H]^+$	235.0529	-2.6	77.0382, 94.0523, 141.0001
Formyl-SMX	10.6	$C_{11}H_{11}N_3O_4S$	281.047	$[M+H]^+$	282.0536	-2.1	120.0442, 136.0388, 184.0056, 99.0548
Hydroxy-*N*4-acetyl-SPY	7.4	$C_{13}H_{13}N_3O_4S$	307.0626	$[M+H]^+$	308.0697	-0.3	95.0602, 150.0547, 157.0064, 166.0496, 214.0164
Methyl-*N*4-acetyl-hydroxy-SPY	9.7	$C_{14}H_{15}N_3O_4S$	321.0783	$[M+H]^+$	322.085	-1.6	164.0702, 180.0651, 228.0321
*N*4-hydroxy-acetyl-SPY	4.7	$C_{13}H_{13}N_3O_4S$	307.0626	$[M+H]^+$	308.0698	0.0	95.0600, 150.0548, 166.05, 214.0167
4-hydroxy-SMX	11.0	$C_{10}H_{10}N_2O_4S$	254.0361	$[M-H]^-$	253.0286	-1.2	93.0341, 109.0656, 156.9958

to the environmental samples. The inclusion list allowed also to obtain the tandem mass spectra of the less abundant TPs, overcoming the top 5 DDA acquisition limitations, without the employment of the more complex scheduled targeted MS/MS (also referred to as parallel reaction monitoring, PRM). The option "peak other" was used to include in the MS/MS scans other ions than those in the inclusion list, thus not discarding possible unexpected TPs. The obtained raw data files were processed with a dedicated workflow (Fig. S4) on Compound Discoverer implemented by the mass list of the 44 TPs identified in the batch reactor experiment.

In the WWTP samples, four SMX TPs and five SPY TPs were tentatively identified together with their parent compounds (Table 5 and Fig. S49-S59). Among these nine TPs, one acetylated and one hydroxylated forms of SPY were also tentatively identified at a different retention time than that of the reactor simulated experiment.

Among the identified TPs, 5 of 9 were acetylated forms, in agreement with previous literature reporting that acetylation is the predominant SA transformation [6].

Likely, the discrepancy between the number of identified TPs in the batch reactor experiment and in the WWTP samples is due to the different starting concentration levels of the parent compounds and the different transformation pathways occurring in the environment, including the abiotic ones.

4. Conclusion

In this work, an analytical workflow for SAs and their TPs monitoring has been developed; as these analytes are diverse but also possess a similar core structure, the method allows to search for an entire class of compounds by combining the untargeted MS analysis with the suspect screening identification approach. By knowing the potential sites of derivatization, and collecting knowledge of the possible transformation reactions, it was possible to search the experimental MS/MS data for the resulting compounds. The approach was applied to three main SAs and studied in simulated laboratory conditions. The advantage of this approach is that precursor compounds and their TPs are present in a considerable amount and can be detected by the automated software processing with Compound Discoverer and manually validated. The results of this study provided that of all the potential compounds, 18 for SMX and 13 for both SPY and SDZ were detected in the batch reactor simulation. These compounds were then used to generate an inclusion list for MS/MS acquisition, so to bypass the sensitivity problems often observed in the environmental analysis of these compounds. The method was finally tested on WWTP samples and confirmed that acetylated products are indeed the most common TPs observed in these samples. Also, compounds such as TP 256 and TP 284, as well as TPs that were never reported before (e.g. TP 274), could be tentatively identified by the developed approach.

Applying this analytical strategy to other relatively homogeneous classes of contaminants will open a way to improve the knowledge of TPs by an automated and systematic screening of potential compounds. Manual validation is necessary only at the end of the analytical workflow, with optimization of knowledge over analysis time.

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CRediT authorship contribution statement

Carmela Maria Montone: Investigation, Validation. **Benedetta Giannelli Moneta:** Investigation, Formal analysis. **Sara Elsa Aita:** Investigation, Formal analysis. **Anna Laura Capriotti:** Formal analysis. **Andrea Cerrato:** Methodology. **Aldo Laganà:** Conceptualization, Supervision. **Angela Marchetti:** Formal analysis. **Susy Piovesana:** Validation, Writing – review & editing. **Marianna Villano:** Conceptualization. **Chiara Cavaliere:** Conceptualization, Resources, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115292.

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