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Simultaneous determination of sulfonamides residues in treated wastewater samples by On-line solid phase extraction liquid chromatography tandem mass spectrometry: Preliminary occurrence analysis in Tunisia

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ABSTRACT: Many studies attest to the pollution of surface water, wastewater and water intended for human consumption by organic molecules including emerging drug residues at trace levels. These emerging micropollutants are mostly little or not removed by sewage treatment plants and can consequently be found in the natural environment. The occurrence of antibiotic drug Sulfonamides in influent and effluent samples from three Tunisian Wastewater Treatment Plants was evaluated. The application of optimized multi-residue method for the simultaneous quantification and confirmation of 9 commonly used antimicrobials was carried out by on-line solid phase extraction liquid chromatography tandem mass spectrometry (SPE–LC–MS/MS). Isotopically labeled compounds Sulfadimethoxine d_6 and Sulfamerazine $^{13}C_6$ were used as surrogate internal standards to compensate for possible matrix effects. The presence of 9 antimicrobials is confirmed in the influent samples. Exclusively sulfamethoxazole was detected in both influent and effluent samples of three WWTPs (WWTP1, WWTP 2 and WWTP 3) at concentrations of (1.1–5.3µg/L), (0.3–0.6 µg/L) and (0.4–0.5 µg/L), respectively. Moreover, the highest concentration of sulfamethoxazole observed i.e. 5.3 µg/L in the effluent sample is mainly due to the great size of WWTP 1 and the large volumes of wastewater inflow which is in accordance to the largest population that it serves with 400.000 inhabitant equivalents.

KEYWORDS: Antibiotics, Multi-residue method, Sulfonamides, Trimethoprim, Tunisia, Wastewater, Xenobiotics.

1 INTRODUCTION

The reuse of treated wastewater is for a country like Tunisia relevant and appropriate solution to address the lack of rainfall and harsh climate of the country. The objective is to enhance the treated wastewater in agriculture, safely and according to the needs of farmers, and expand the use of these waters to other areas, the effect of increasing the rate reuse of treated water reached 50% by at the end of 2014 instead of 30% in 2010 according to the latest report on the national state of environment issued by the Ministry of Environment [1].

In recent years, there has been a growing concern due to the assumption that various chemicals can have endocrine-disrupting effects [2]. In addition, thousands of tons of pharmacologically active substances are used each year can be found in the wastewater. The existence of xenobiotic compounds in treated wastewater is a new concern for many countries experiencing prolonged droughts implementing systems of wastewater reuse for irrigation and groundwater discharge. Consumption of medicinal products for human or veterinary use is steadily increasing in Tunisia. Incomplete metabolism of drugs consumed can lead to contamination of natural environments. Most existing conventional treatment processes applied are not designed to completely remove different classes of xenobiotics organic compounds including active pharmaceutical

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ingredients. In fact, the elimination of these pharmaceutical ingredients depends on their ability to biodegradation and adsorption to suspended solids. Many of these compounds are hydrophilic and their adsorption on sludge is limited [3]. Ones of the particularly hazardous components of this wastewater are drugs and their metabolites. Sulfonamides (SNs) are an example of such compounds. It is estimated that most of them is stable in the environment, and some are also highly toxic, or ecotoxic [4,5]. The threat caused by antimicrobial drugs is mainly based on the fact that even in trace amounts, they may generate drug resistance in microorganisms [6]. Then, the resistance genes can be transferred between different strains of bacteria, for example by conjugation [7]. Most human use of antimicrobials and other pharmaceuticals reach the aquatic environment, unchanged or transformed, mainly *via* discharge of effluents from municipal wastewater treatment plants (WWTPs). The residual concentrations of these bioactive compounds in treated effluents depend on their removal during wastewater treatment. They can potentially pose a hazard of aquatic organisms if the removal is incomplete. According to the Tunisian Ministry of Public Health, Co-trimoxazole (Bactrim®, Sulfatrim®) is the most consumed sulfonamide molecule in Tunisia (Unpublished statistical data). Co-trimoxazole is a combination of two antibiotics. Its formulation is made up of five parts sulfamethoxazole and one-part trimethoprim. This combination has a synergistic antibacterial effect. It is used to treat the simplest to the most advanced bacterial infections.

The main objective of this study was to investigate the occurrence and removal efficiency of sulfonamide antimicrobials in treated wastewater at three sewage treatment plants serving major cities in Tunisia and subsequently ascertain the degree of adaptation processing techniques used to remove these pharmacologically active substances. More specifically the detailed goals of the present study were: (i) to apply a rapid, accurate method for trace analysis of these compounds in influents and effluents wastewaters; and (ii) to evaluate the removal efficiency of the WWTPs. This paper is the first report on the occurrence of pharmaceuticals in STPs in Tunisia.

2 MATERIALS AND METHODS

2.1 DESCRIPTION OF SAMPLING SITES (WWTPS)

Wastewater samples used in this study were collected from three different Wastewater Treatment Plants Choutrana (WWTP 1), Charguia (WWTP 2) and Korba (WWTP 3) located in the northeast of Tunisia. All effluents are reused either for irrigation or groundwater replenishment. All WWTPs receives domestic and industrial inflows (Pharmaceutical Industries, Hospitals...). All the selected WWTPs operate in a similar way which consists of a conventional secondary treatment using activated sludge. Only WWTP 3 of Korba has a secondary activated sludge treatment enhanced by a tertiary treatment impoundment. The characteristics of each WWTP are listed in Table 1 where the served population and the treatmenttechnologies are included and the distribution of the WWTPs can be found in Figure 1. Influents and effluents from all WWTPs were collected as composite samples in January 2015.

Table 1. Characteristics of WWTPs

WWTP	Watershed	PE	Q (m^3/day)	Type of treatment	Degree of treatment	Origin
I	Canal El Khalij	400,000	78000	Biologic—activated sludge	Secondary	U+I
II	Canal El Khalij	195,000	60000	Biologic—activated sludge	Secondary	U+I
III	Lagoon of Korba	30,000	7500	Biologic—aerated lagoon	Tertiary	U+I

PE: Population Equivalent; U: Urban ; I: Industrial

The WWTP 1 is one of the sewerage centerpieces in Tunisia that serves a population of 400,000 (PE) and which represent the major part of the municipal wastewater derived from the capital Tunis suburb. However, WWTP 2 receives only a part of wastewater derived from the capital Tunis, the rest is transported to the WWTP 1 for treatment. Against various pressures (population growth, agricultural expansion, fertilizers, urban and industrial discharges) acting on these ecosystems, The WWTP 3 (Korba) located in the "Cap Bon" Peninsula at 100 Km east of the capital Tunis was chosen for this study given its wastewaters intended for reuse and ecological importance [8].

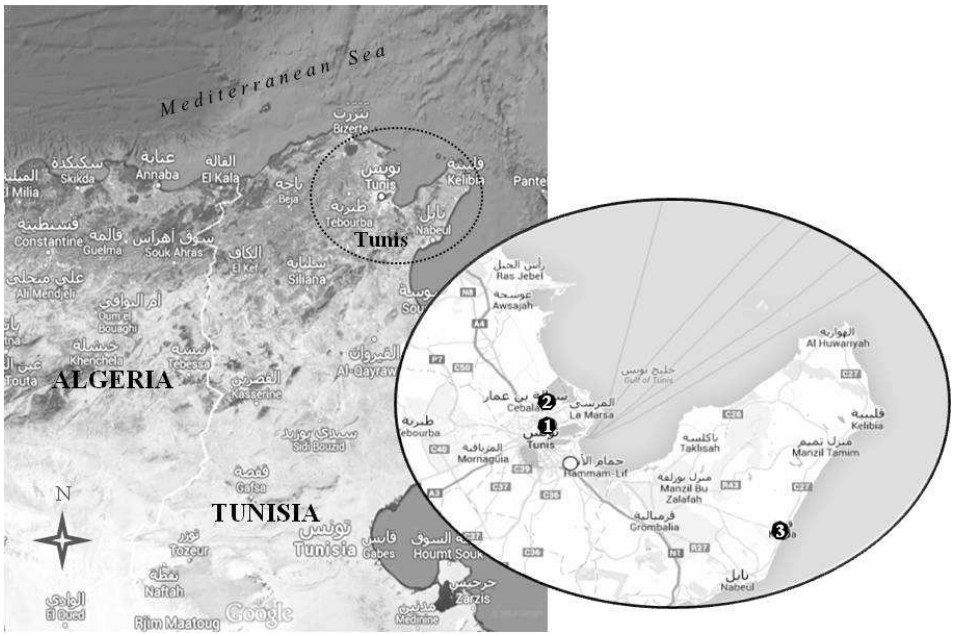

Figure 1. Locations in NE-Tunisia of the WWTPs (●) sampled.

2.2 SAMPLING

Samples were collected from the inlet and from the outlet of three wastewater treatment plant. 24-h composite samples were collected in January 2015 using a time-proportional automatic sampler (Liquiport 2010 CSP44). The time between sampling and analysis was less than 7 days. Each sample was on-line solid phase extracted and ach extracted sample was analyzed *via* chromatography.

2.3 CHEMICALS AND REAGENTS

A mixture of pharmaceutical standards used was of high purity grade (>90%). Sulfamethoxazole, sulfamoxole, sulfaquinoxaline, sulfabenzamide, sulfamonomethoxine, sulfamethazine, trimethoprim, sulfamerazine were purchased from Sigma-Aldrich (Steinheim, Germany). Isotopically labeled compounds, used as internal standards, were Sulfadimethoxine d_6 and Sulfamerazine $^{13}C_6$ obtained from Sigma- Aldrich. This mixture was prepared by appropriate dilution of individual stock solutions. Further dilutions of this mixture were prepared in methanol–water (25:75, v/v) before each analytical run and were used as working standard solutions. Internal standards used for internal calibration [9]. HPLC-grade methanol and acetonitrile were supplied by Carlo Erba (Val de Reuil, France), utra pure water from Sigma-Aldrich (Steinheim, Germany). Formic acid 99-100% was from Normapur ProLabo. Nitrogen Generator ZEFIRO - LCMS for drying > 99 % of purity was from F-DGS SAS (Evry, France).

2.4 ANALYTICAL PROCEDURE

2.4.1 SAMPLE PREPARATION

While traditional sample preparation for organic compounds using offline solid-phase extraction (SPE) has many advantages, the goal was to develop a setup for online SPE coupled with liquid chromatography (LC) and tandem mass spectrometry (MS-MS) to combat some of the disadvantages. The most urgent of the disadvantages with offline SPE was the length of time needed to work up the large number of samples typically analyzed in CITET studies. Further advantages expected with an online SPE method included the direct coupling of the extraction to the mass spectrometer to allow unattended 24/7 operation, and the storage of the extraction method within the raw data files, thereby cutting down the laboratory's administrative overhead.

The simplest approach to automation generally is a single cartridge approach [10]. The direct coupling of SPE to LC eliminates several working steps, such as evaporation, reconstitution, and injection. This results in a faster and more precise

procedure since the total enriched amount of substance is eluted directly to the liquid chromatography [11,12]. In addition, automated online SPE has the potential to reduce procedural errors.

Target sulfonamides were extracted by On-line solid phase extractor (2777 Sample Manger), was from Waters Corporation (Milford, MA, USA). The method setup used an online SPE–LC coupling with two switching valves. The online SPE system is fitted with a quaternary pump, a degasser, an autosampler, and a unit of 6 automatic valves which allow the sample to pass on an extraction cartridge, then elute the analytes by transferring them directly onto the analytical column for separation prior to detection by MS/MS. This system allows injecting the entire sample into the system. The whole procedure was controlled through Masslynx software version 4.1 (Waters Acquity).

Sample enrichment was achieved with a 2000µL sample loop on a 30 mm × 2.1 mm hydrophilic–lipophilic balanced (HLB) extraction cartridge (20 µm particle size) at sample pH. After injection, the samples are directly transferred to the analytical column using solvent gradient analysis. HPLC-grade eluents were used.

2.4.2 LC–ESI–TANDEM MS ANALYSIS

The devices used are detailed in previous works [13,14]. LC-MS/MS analysis was performed using a Waters Acquity UPLC system (Milford, MA, USA), coupled to a Micromass Quattro Premier triple quadrupole mass spectrometer detector system, equipped with a Z-spray ESI interface (Manchester, UK). Chromatographic separation was achieved with a BEH C_{18} Column (2.1* 50mm, particle size 1.7 µm), supplied by Waters corporation. The total sample run time was 12 min and the high pressure gradient for the analytical separation was achieved by changing the ratio of the elution pump. Mobile phase consisted of eluent A (Water 0.1% HCOOH) and eluent B (Acetonitril 0.1% HCOOH). The elution gradient was set at a flow rate of 0.450 mL/min.

The optimization of MS parameters (cone voltage and collision energy) was performed by flow injection analysis (FIA) for each compound. Cone voltages were selected according to the sensitivity of precursor ions, whereas collision energies were chosen to give the maximum intensity of the fragment ions obtained. The optimum collision energies and cone voltages selected for each transition are indicated in Table 2.

Table 2. MS/MS parameters for the analysis of target analytes by MRM positive ionization mode

Target Compounds	R_t window (min)	Predicted R_t (min)	R_t (min)	Precursor ion	MRM 1	CV-CE	MRM 2	CV-CE	Response Type
Sulfamerazine	0.0 to 3.60	3.30 ± 0.25	3.25	265 $[M-H]^+$	265 > 92	32-32	265 > 156	32-15	Internal*
Sulfamerazine $^{13}C_6$	0.0 to 3.60	3.30 ± 0.27	3.28	271.1 $[M-H]^+$	271.1 > 98	33-27	_	_	External
Trimethoprim	3.0 to 3.70	3.21 ± 0.15	3.23	291 $[M-H]^+$	291 > 230	39-24	291 > 123	39-27	Internal*
Sulfamethazine	3.0 to 9.00	3.4 ± 0.21	3.43	279.1 $[M-H]^+$	279.1 > 186	34-18	279.1 > 124.1	34-27	Internal*
Sulfamonomethoxine	3.0 to 9.00	3.51 ± 0.42	3.50	281.1 $[M-H]^+$	281.1 > 156	36-17	281.1 > 92	36-34	Internal**
Sulfabenzamide	3.0 to 9.00	4.02 ± 0.32	3.95	277 $[M-H]^+$	277 > 92	23-24	277 > 156	23-25	Internal*
Sulfaquinoxaline	3.0 to 9.00	3.98 ± 0.14	3.96	301.1 $[M-H]^+$	301.1 > 156	36-17	301.1 > 92	36-29	Internal**
Sulfamoxole	3.50 to 9.00	3.97 ± 0.46	3.87	268.05 $[M-H]^+$	268.05 > 156	25-15	268.05 > 113	25-18	Internal**
Sulfamethoxazole	3.50 to 9.01	3.80 ± 0.23	3.78	254.05 $[M-H]^+$	254.05 > 92	28-30	254.05 > 156	28-15	Internal**
Sulfadimethoxine	3.0 to 9.00	4.04 ± 0.24	3.98	311 $[M-H]^+$	311 > 156.1	39-19	311 > 92	39-35	Internal**
Sulfadimethoxine d_6	3.0 to 9.00	4.13 ± 0.42	3.98	317.1 $[M-H]^+$	317.1 > 162	39-21	_	_	External

CV–CE: Cone volatge and Collision energy
** Internal calibration with Sulfamerazine $^{13}C_6$; ** Internal calibration with Sulfadimethoxine d_6.*

An electrospray ionization source operated in the positive mode (ESI+) with two selected reaction monitoring transitions, the mass spectrometer was operated in the multiple-reaction monitoring mode (MRM) selecting two transitions for each compound [15]. The first one was (MRM1) for quantitation trace whereas the second one (MRM2) was used for confirmation. However, quantification was performed by the internal standard approach. Isotope-labeled internal standards (listed in table 2) used for quantitation were spiked to the samples *via* the autosampler to a concentration level of 1µg/L.

3 RESULTS AND DISCUSSION

One significant drawback in ESI MS quantitative analysis is what is known as matrix effect. It occurs because the ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement leading to erroneous results. Appropriate internal standards sulfamethoxazole d_6 and sulfamerazine $^{13}C_6$ (Isotopically labeled standards) were used for quantitation, but also to compensate these matrix effects [16].

The occurrence of antibiotic drug Sulfonamides and trimethoprim in influent and effluent samples from three Tunisian Wastewater Treatment Plants was evaluated. The data obtained from all samples analyzed are presented in Table 3.

Table 3. Detected levels of the target analytes in wastewater samples (average given in µg/L)

WWTP	WWTP I		WWTP II		WWTP III	
Target compounds	A	B	A	B	A	B
Sulfamethoxazole	1.1	5.3	0.3	0.6	0.4	0.5
Sulfamoxole	1.5	0.6	–	*bld*	*bld*	*bld*
Sulfadimethoxine	1.1	*blq*	*bld*	*bld*	–	*bld*
Sulfaquinoxaline	0.4	–	*bld*	*bld*	–	*bld*
Sulfabenzamide	0.5	–	–	*bld*	–	*bld*
Sulfamonomethoxine	0.7	0.6	*bld*	*bld*	*bld*	*bld*
Sulfamethazine	1.6	–	*bld*	*bld*	*bld*	*bld*
Trimetoprim	3.6	–	*bld*	*bld*	*bld*	*bld*
Sulfamerazine	blq	–	*bld*	–	*bld*	–

–: not detected; bld: below limit of detection; blq: below limit of quantification; (A): Inlet; (B): Outlet

From the 9 compounds studied only sulfamethoxazole, sulfamoxole and sulfamonomethoxine were actually determined and quantified both in influent and effluent samples of WWTP 1. Across the three Wastewater Treatment Plants WWTP1, WWTP 2 and WWTP 3, exclusively sulfamethoxazole was detected in both influent and effluent samples of three WWTPs at concentrations of (1.1–5.3µg/L), (0.3–0.6µg/L) and (0.4–0.5µg/L), respectively. However, the concentration of this compound in the effluent water of WWTP 1 was significantly higher than that of the influent, which can be attributed also to intense strong matrix effects in the latter, which hampered the identification of the compounds. It could also be attributed to the fact that metabolites and conjugated forms like the human metabolite N^4-acetylsulfamethoxazole and trimethoprim—frequently used as a synergist to sulfamethoxazole in the form of Co-trimoxazole (The most consumed sulfonamide drug in Tunisia, see chapter 1 Introduction), which are also present in the influent samples, may degrade and retransform into the parent compounds, being these sulfonamides released in the effluent [4,17]. This explanation can be confirmed by the occurrence of trimethoprim only in the influent sample (3.6 µg/L) for the same Wastewater Treatment Plant (WWTP 1). García-Galán et al. (2010) reported a concentration range for sulfamethoxazole of 12.4-302 ng/L in the effluents and the same compound was not detected in the most of influent samples [18]. Therefore, the concentration of this compound was found in low levels in comparison to Tunisia. These concentrations of sulmethoxazole in effluents samples were higher than those found by Göbel *et al.* (2004) where sulfamethoxazole was found in concentrations of 352 ng/L [4] and those found by Gros *et al.* (2006) where Sulfamethoxazole concentrations were blq–820 ng/L [16].

However, F-Kassinos *et al.* (2011) highlighted during the same season of sampling (Winter–January) when the consumption of this kind of antibiotic reached peak levels, a similar range of concentrations of this compound from 0.78 to 5.41 µg/L in the influent and from 0.01 to 0.78 µg/L in the effluent samples [9]. Moreover, the highest concentration of sulfamethoxazole observed i.e. 5.3 µg/L in the effluent sample is mainly due to great size of WWTP 1 and the large volumes of wastewater inflow which is in accordance to the largest population that it serves with 400.000 inhabitant equivalents.

The difference however that is observed for a number of compounds in the influent of the three UWTPs is most probably attributed to variations in the amount of sulfonamide antimicrobials use in Tunisia and consumption patterns among regions. For example, the concentrations of sulfamoxole, sulfaquinoxaline, sulfabenzamide, sulfamonomethoxine, sulfamethazine and trimethoprim were always found to be below the limit of detection in all effluents samples from WWTP 2 and WWTP 3.

The concentrations of pharmaceuticals in the urban wastewaters depend on several parameters. For example, the consumption pattern of the drugs varies from country to country and also within country. This might explain the various differences observed in respect to the concentrations determined at the WWTPs in Tunisia in comparison to others

elsewhere. Furthermore, the flow rate of the raw and treated sewage varies and so does the efficiency of the treatment process to eliminate pharmaceuticals. The concentrations can vary greatly among WWTPs and even within the same WWTP at different time periods as can be seen in the study of Tixier *et al.* (2003) [19]. Variation in the effluent loads is expected since the load is affected by the efficiency of the treatment process to eliminate pharmaceuticals. the concentration levels found in effluents show that current techniques for treatment are inefficient temporarily for the elimination of these compounds. Finally, it is worth mentioning that xenobiotics particularly antibiotics are likely to have a toxic effect at very low concentrations because of their antibacterial properties. Especially, in a very known city of Korba (WWTP 3) by its water reuse for agricultural expansion area, low concentrations of drugs may act indirectly, that is to say, by disrupting homeostasis of organisms and making them more susceptible to other environmental pollutants (pesticides, hydrocarbons, metals) or infectious agents [20].

4 CONCLUSIONS

The optimized analytical methods described in the present work, based on On-line SPE following by liquid chromatography tandem mass spectrometry, proved to be a reliable method for the simultaneous determination and quantification of 9 compounds from the same class of antibiotics (Sulfonamides and trimethoprim). The monitoring and evaluation for the first time of Tunisian samples was examined, and the applicability of the method was efficiently demonstrated by analyzing wastewater from three WWTPs. While the data are still the results of one short-term study, the presence of sulfonamides compounds is confirmed. The preliminary results show a concentration levels in influents that are in coherence with the size of the sewage treatment plant studied, plugged population to WWTP and average daily flow. Also, the determination of sulfamethoxazole and trimethoprim at higher concentration than other compounds were in accordance to the amount of antimicrobials use in Tunisia and consumption patterns among regions.

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