Analysis of antibiotics in aqueous and particulate phases of soil and organic residues: critical points to ensure quality of the results.

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Abstract
Veterinary and human antibiotics are emerging pollutants that can be found in organic residues and in amended soils. The analysts should be vigilant while analysing pharmaceutical compounds in such complex environmental matrices. In the frame of a method optimisation, various constraints appeared to be critical, due to the antibiotics instability, the matrix characteristics or the analytical technics. We showed that the four forms of chlortetracycline have to be quantified since equilibriums are displaced over time. Moreover attention should be paid to chlortetracycline degradation products which may be confounded with the parent compounds. Concerning the quantification performed with mass spectrometry, matrix effects due to the presence of undesirable compounds often lower the instrumental response for antibiotics, especially for matrices with a high content of organic carbon such as aqueous phase of slurry. Lastly, global extraction recoveries for sludge depend on its post-treatment (worse and very low recoveries for limed sludge) and on the antibiotics. It is thus necessary to take into account these matrix effects and extraction recoveries to ensure trueness of the results, for each matrix.

Introduction
After absorption by animals or humans, pharmaceutical compounds are excreted to a large extent as parent molecules or metabolites, and transferred to manure or wastewater. In wastewater treatment plants, pharmaceuticals can be degraded and/or removed from water through adsorption onto sludge. The storage and/or the following treatments of sludge, manure and slurry (e.g. liming, digestion, composting) can have an impact on the content and availability of pharmaceutical compounds and degradation by-products. The recycling of all these organic residues in agriculture is largely encouraged nowadays but may indirectly contribute to the dissemination of pharmaceutical compounds into the environment (soils, surface and ground waters). Even though regulations at the European levels do not include pharmaceuticals, they are considered as emerging pollutants due to their potential activity against non-target organisms and the potential risk of their transfer towards terrestrial and aquatic ecosystems. It is thus necessary to estimate their concentration in organic residues and soils. Their analyses represent a challenge, because of the trace-level concentration in complex matrices. In the frame of such method optimisation, we have been facing various constraints inherent to the contrasted physico-chemical properties of the molecules, to the matrices (liquid and particulate phases of organic samples) or to the analytical techniques (especially mass spectrometry detector coupled to liquid chromatography). Three critical points are presented, with a focus on three antibiotics: chlortetracycline, sulfamethoxazole and ofloxacin.

Material and Methods
Matrices and analytes
Three aqueous matrices (soil water, sludge and pig slurry supernatants) and two solid matrices (sludge either limed or not) were studied. Soil water was obtained by percolation of water through soil columns. Aqueous and particulate phases of sludge and slurry were separated by centrifugation. Sludge liming was performed by adding CaO to particulate phase of sludge.

Three antibiotics were studied: chlortetracycline (CTC – tetracycline – veterinary use), ofloxacin (OFL – fluoroquinolone – human use), and sulfamethoxazole (SMX – sulphonamide – human use). Analytical standards were purchased from C.I.L. (Cluzeau, France).

Extraction and analysis

Pressurized liquid extraction (PLE) was performed with ASE 200 (Dionex, France) to extract the analytes from sludge particulate phase, at 100°C – 100 bar with acetonitrile-water (95-5) during 7 min / cycle (3 cycles). Sludge samples (2 g) were mixed with tri-sodium citrate (280 mg) and celite and then placed into extraction cells. Dead volume was filled with sand. ASE extracts were evaporated to dryness with a rotavapor. They were then diluted with a mixture of acetonitrile and water pH 2.5 (1:99) and centrifuged.

Aqueous samples were adjusted to pH 2.5 with HCl and centrifuged.

Analysis of the molecules from aqueous phases and ASE extracts was carried out by online solid phase extraction (OASIS HLB cartridge, Waters, USA) coupled to ultra-performance liquid chromatography and tandem mass spectrometry (UPLC-TQD, Waters, USA). A gradient between water + 0.1% acetic acid and acetonitrile + 0.1% acetic acid was performed in 16 min on a BEH C18 column (Waters, USA). Mass spectrometric detection was performed by multiple reaction monitoring (MRM), using two transitions for each analyte.

Extraction recoveries and matrix effects determinations

To evaluate global extraction recoveries (equation 1), sludge particulate phases were spiked with a known amount of the antibiotics few hours prior to the preparation of the ASE cell.

$$Global\ extraction\ recovery\ (\%) = \left(\frac{[spiked\ sample]-[non-spiked\ sample]}{[theoretical]}\right) * 100 \quad \text{(equation 1)}$$

where [spiked sample], [non-spiked sample] and [theoretical] correspond to the concentrations of the analyte in the spiked sample and in the non-spiked sample, and the theoretical concentration of the sample after spiking, respectively.

To evaluate matrix effects (equation 2), aqueous phases and ASE extracts (non-spiked before ASE) were spiked with a known amount of the antibiotics just before SPE-UPLC-TQD analysis.

$$Matrix\ effect\ (\%) = \left(\frac{[matrix]-[blank]}{[water]} - 1\right) * 100 \quad \text{(equation 2)},$$

where [matrix], [blank] and [water] correspond to the concentrations of the analyte in the spiked sample, in the non-spiked sample and in the spiked water, respectively.

Results

Compound instability and equilibrium between compound forms

Chlortetracycline (CTC) present four chemical forms: epimers and tautomers (figure 1). In most papers of the literature, only CTC enol and more scarcely epi-CTC (ECTC) enol are considered for quantification. As the equilibriums between the four forms of CTC may be displaced, we quantified the four forms, just after sample preparation, and 6.5 and 13 hours later. Two kinds of samples were prepared, in which either CTC or ECTC were introduced. CTC enol and CTC ketone were quantified in samples in which CTC was introduced and also in samples in which ECTC was introduced. The same procedure was done for ECTC enol and ECTC ketone. Figure 2 shows the signal obtained for ECTC enol and ECTC ketone in samples in which either ECTC or CTC were introduced. This signal (area) is proportional to the compound concentration.

In the sample in which ECTC was introduced, ECTC enol decreased over time while an increase of ECTC ketone was observed. These variations mainly occurred within the 6.5 hours following sample preparation. They could be explained by a displacement of the tautomerism equilibrium in favour of the keto form. The same trend was observed for CTC enol and CTC ketone in CTC samples. Moreover ECTC enol and ketone appeared in samples in which only CTC was introduced, and CTC enol and ketone appeared in samples in which only ECTC was introduced, confirming that epimerisation was reversible and pH dependant (results not shown). In the case of chlortetracycline quantification in real samples, it is therefore necessary to quantify all the four forms, CTC enol, CTC ketone, ECTC enol and ECTC ketone – with the 479>462 mass transition – due to the displacement of the equilibriums between the forms over time, as already mentioned by Ferraz Spisso et al. [1].

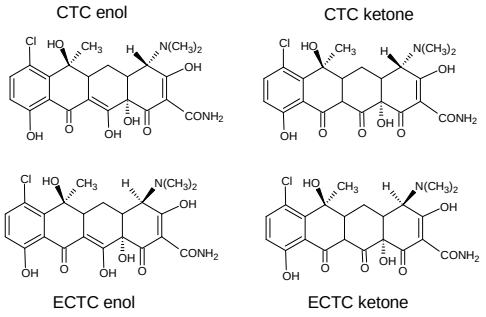

Figure 1. The four different chemical forms of CTC

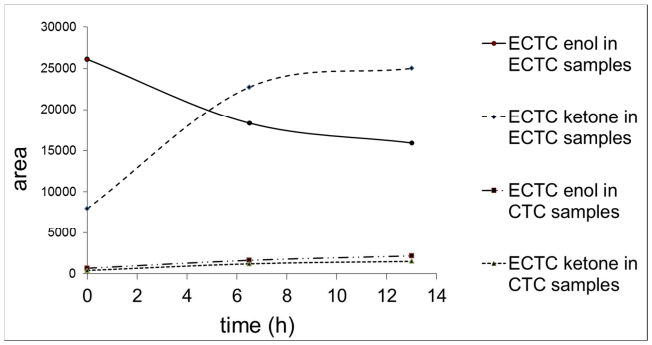

Figure 2. Areas of tautomers of ECTC in samples in which either ECTC or CTC were introduced

As tetracyclines are known to be unstable [1], we also studied the evolution of the four CTC forms during 8 days in water. Two new compounds appeared from the second day; their increase was correlated to the decrease of the four CTC forms (figure 3). These new compounds, probably isochlortetracycline and 4-epi-isochlortetracycline [1], were not detected using the specific mass transition classically performed (lower chromatograms, mass transition 479>444). Moreover, the mass transition 479>462 had to be attentively studied since the retention times of these two compounds, which generate signals superior to the native molecules ones, slightly differed from CTC ketone and ECTC enol retention times, leading to a possible confusion between compounds. Chromatograms obtained for the aqueous phase of slurry were similar to those obtained 8 days after water spiking.

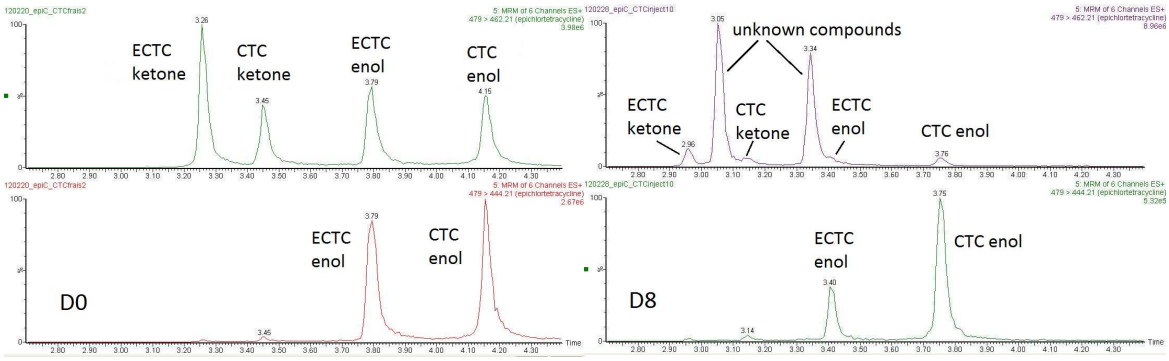

Figure 3. Chromatograms of samples containing both CTC and ECTC, after sample preparation (D0) and 8 days later (D8). The chromatograms in the bottom are obtained with MS-MS transition classically used for CTC quantification and the upper ones are obtained with another MS-MS transition

Matrix effects in mass spectrometry

Another critical point is the importance of matrix effects in mass spectrometry analysis. Indeed, undesirable molecules present in the samples can strongly modify the MS signal of the analytes, leading to wrong quantifications and increased quantitation limits. Table 1 shows matrix effects evaluated in aqueous samples (soil water, sludge and slurry supernatants) and in sludge particulate phase (either limed or not), for three antibiotics (ofloxacine-OFL, the four forms of chlortetracycline-CTCs, and sulfamethoxazole-SMX). A large range of matrix effects was observed, from -4% (low matrix effect) to -93% (very high matrix effect). Matrix effects depended on molecules and matrices, and corresponded generally to ion suppression.

The evaluation of matrix effects proved that for slurry supernatant or limed sludge extract, purification through SPE technique was not sufficient, contrarily to what is often reported [2-3]. The high content of organic carbon in slurry supernatant compared to the other aqueous phases could explain such differences of matrix effects. For these matrices, other purification methods should be tested to decrease matrix effects and therefore improve limits of quantification. Moreover such variations between matrices require a special attention while delivering analytical results of aqueous samples[1]:

[1] It is not necessary to independently evaluate matrix effects of particulate phase extracts since the calculation of the global extraction recovery takes them into account (see extraction recoveries).

internal standards (e.g. isotope labelled standards) should be added in each sample to correct matrix effects.

Table 1: matrix effects for the target compounds obtained with the online SPE-LC-MS-MS method, and total organic carbon (TOC) of the aqueous phases

Compounds	Aqueous samples			Particulate phase extracts	
	Soil water	Sludge supernatant	Slurry supernatant	Sludge	Limed sludge
OFL	-53 (1)	-28 (4)	-77 (1)	16 (4)	-69 (1)
CTCs	-25 (1)	-19 (3)	-81 (1)	-4 (9)	-57 (3)
SMX	-23 (3)	-21 (3)	-93 (0)	-19 (4)	-65 (2)
TOC (mg C/L)	45	26	9536		

SD values are given in brackets (n=3)

Extraction recoveries

Extraction recoveries are widely dependent on the initial matrix. Indeed the constituents of matrices may interact with the target analytes and therefore modify their extractability. Fluoroquinolones and tetracyclines for example are known to chelate divalent cationic ions such as calcium ions, becoming thus more retained. Moreover sample pH may exert an influence on extraction recoveries since antibiotics present some acid-base sites. The evaluation of global extraction recoveries, carried out by spiking solid matrix before extraction (equation1), takes into account both the ASE extraction performances and the mass spectromety matrix effect. Table 2 shows global extraction recoveries of antibiotics from sludge either limed (with CaO – pH=12.3) or not (pH=6.9). They were low (<30%) except for OFL in sludge. The presence of CaO did not influence global SMX extraction recoveries but dramatically decreased OFL ones. However, the large matrix effects of limed sludge (table 1) may be responsible for the low global recoveries in this matrix. Indeed the ASE extraction recovery can be calculated (equation 3) using the global extraction recoveries (table 2 – equation 1) and the matrix effects of the particulate phase extract (table 1 – equation 2).

$$ASE\ extraction\ recovery\ (\%) = \frac{global\ extraction\ recovery}{100+matrix\ effect} * 100 \qquad \text{(equation 3)}$$

Calculated ASE extraction recoveries (table 3) showed that limed sludge allowed better ASE yield for SMX (*ca* multiplied by 2) but worse for OFL (*ca* divided by 2).

Table 2: global extraction recoveries of ofloxacin and sulfamethoxazole in sludge either limed or not

Compounds	Sludge	Limed Sludge
OFL	68 (1)	10 (1)
SMX	26 (8)	20 (0)

SD values are given in brackets (n=2)

Table 3: calculated ASE extraction recoveries (from equation 2)

Compounds	Sludge	Limed Sludge
OFL	58	31
SMX	31	57

To conclude, global extraction recoveries have to be evaluated for each matrix in order to correctly quantify the analytes. The internal standard quantification method, for which particulate phase is spiked with internal standards (such as isotope labelled standards) prior to the extraction, has the advantage to take into account the global recovery (*i.e.* the extraction recovery and the matrix effect) for each sample and each analyte. However due to the ageing effects in natural samples, global extraction recoveries may not be well characterised (generally over-estimated), even though the internal standard quantification method is used.

Conclusion and perspectives

Due to the complexity of pharmaceutical products and of soil and organic matrices, precautions should be taken while conducting the analysis of pharmaceutical products, in order to ensure the quality of the results. One way to take into account MS matrix effects and extraction recoveries is the use of isotope labelled internal standards.

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References

[1] Ferraz Spisso B, Gonçalves de Araújo Júnior M.A, Alves Monteiro M, Belém Lima A.M, Ulberg Pereira M, Alves Luiz R, Wanderley da Nóbrega A, 2009. A liquid chromatography-tandem mass spectrometry confirmatory assay for the simultaneous determination of several tetracyclines in milk considering keto-enol tautomerism and epimerization phenomena. Analytica Chimica Acta 656, 72-84

[2] Buchberger W.W, 2011. Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. Journal of Chromatography A 1218, 603-618

[3] Tadeo J.L, Sánchez-brunete C, Albero B, García-Valcárcel A.I, Pérez E.A, 2012. Analysis of ermerging organic contaminants in environmental solid samples. Central European Journal of Chemistry 10, 480-520