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Determination of six chemotherapeutic agents in municipal wastewater using online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry

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HIGHLIGHTS

- We analyzed six chemotherapy agents in wastewaters.
- We used 1 ml injections and an 11 min SPE-LC-MS/MS.
- Limits of detection ranged from 4 to 20 ng L^{-1}.
- Cyclophosphamide and methotrexate were found in wastewater at 17–60 ng L^{-1}.

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ABSTRACT

Due to the increased consumption of chemotherapeutic agents, their high toxicity, carcinogenicity, their occurrence in the aquatic environment must be properly evaluated. An analytical method based on online solid-phase extraction coupled to liquid chromatography-tandem mass spectrometry was developed and validated. A 1 mL injection volume was used to quantify six of the most widely used cytotoxic drugs (cyclophosphamide, gemcitabine, ifosfamide, methotrexate, irinotecan and epirubicin) in municipal wastewater. The method was validated using standard additions. The validation results in wastewater influent had coefficients of determination (R^2) between 0.983 and 0.998 and intra-day precision ranging from 7 to 13% (expressed as relative standard deviation %RSD), and from 9 to 23% for inter-day precision. Limits of detection ranged from 4 to 20 ng L^{-1} while recovery values were greater than 70% except for gemcitabine, which is the most hydrophilic compound in the selected group and had a recovery of 47%. Matrix effects were interpreted by signal suppression and ranged from 55 to 118% with cyclophosphamide having the highest value. Two of the target anticancer drugs (cyclophosphamide and methotrexate) were detected and quantified in wastewater (effluent and influent) and ranged from 13 to 60 ng L^{-1}. The proposed method thus allows proper monitoring of potential environmental releases of chemotherapy agents.

1. Introduction

In recent years, an increased awareness about the occurrence of pharmaceutical compounds in the environment was observed and these substances have been identified as contaminants of emerging concern (Daughton and Ternes, 1999). Several classes of pharmaceuticals have been detected in wastewater treatment plant (WWTP) effluents, surface waters and groundwater as well as in drinking water (Kümmerer, 2008; Thomas, 2002). The high polarity and water solubility of the majority of these compounds make them less likely to be degraded or removed during the WWTP processes and thus more likely to reach the aquatic environment.

The number of cancer patients has increased during the last years with the Canadian Cancer Society reporting that the occurrence of many cancer types has increased by 2 to 7% per year in the decade spanning 1998–2007 (CCS, 2012). The higher incidence of cancer increases anticancer drug consumption as chemotherapy is one of the most commonly used treatment options (Shewach and Kuchta, 2009). The development of cancer chemotherapy began in the 1940s and involved the use of alkylating agents which opened the door for the development of a large number of anticancer drugs (Shewach and Kuchta, 2009). Chemotherapeutic agents, also called cytotoxic or antineoplastic agents are a group of compounds used to prevent or disrupt cell division. They are mainly used in hospitals and are administered for outpatients and inpatients (Allwood et al., 2002). According to the International Agency for Research on Cancer (IARC, 2011), anticancer drugs are toxic and some of them are carcinogenic as is the case of cyclophosphamide

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(CP) (Praga et al., 2005). Chemotherapy agents represent one of the most toxic compounds used as a medication. As such, their presence in the aquatic environment could have a significant impact on human and ecological health. It is therefore important to develop analytical methods which allow their detection at low nanogram-per-liter concentrations. For this study, a group of six cytotoxic agents (cyclophosphamide, ifosfamide, methotrexate, gemcitabine, epirubicin and irinotecan) were chosen. Several factors were considered in making this choice. First, the selected compounds are considered highly toxic and carcinogenic (Belfroid et al., 1993). Second, these compounds are among the most used in chemotherapy protocols in the hospitals of the province of Quebec (Table 1).[1] Third, the presence of some of these compounds in the aquatic environment has already been demonstrated (Garcia-Ac et al., 2009a; Kümmerer, 2008; Martín et al., 2011; Nussbaumer et al., 2011). Later work will certainly require a proper evaluation of the potential risks to human health for drug residues in drinking water or for biota exposed in receiving surface waters and in soils used for land-applied biosolids.

Cyclophosphamide (CP) is one of the most widely used drugs in cancer treatment since it was introduced in the 1950s (Thurston, 2007). Its structural isomer, ifosfamide (IF), has emerged as an important alkylating agent since the 1970s (Belfroid et al., 1993). They have both been found in hospital effluent samples by GC-MS analysis preceded by off-line SPE at concentrations of 146 ng L^{-1} and 24 ng L^{-1} respectively (Steger-Hartmann et al., 1996). The cytotoxic agents CP and IF were also quantified in biological samples (Sottani et al., 2008). Given the importance of methotrexate (MTX), several studies have been conducted for its detection and quantification in different matrices (mostly biological for medical purposes). This was related in an earlier review that described more than 70 studies between 1975 and 2000 (Rubino, 2001). In a study realized in China (Yin et al., 2010), MTX was quantified in hospital effluents at concentrations between 2 and 19 ng L^{-1}. Gemcitabine (GCA) is a cytotoxic nucleoside that has been used in chemotherapy in the last decade (Seo et al., 2007) and was quantified at concentrations varying from 2.4 ng L^{-1} to 9.3 ng L^{-1} in several water matrices (Martín et al., 2011). GCA has also been quantified in wipe samples used to monitor surface contamination in drug preparation and administration rooms at hospitals in Italy (Sottani et al., 2007). Few studies have been published for epirubicin (EPI). The published method in Italy for the quantification of EPI in urine samples based on off-line SPE and LC-MS/MS, revealed an LOD of 40 ng L^{-1} (Sottani et al., 2004). Furthermore, EPI has been quantified over a month in the effluent wastewater of the Vienna University Hospital (Austria) and reported concentrations of EPI vary between 100 ng L^{-1} and 1400 ng L^{-1} (Mahnik et al., 2006). Irinotecan (CPT-11) was studied but not detected in water as shown in the study carried out in Spain (Martín et al., 2011) and method limits of detection (MDL) were between 0.9 and 1.1 ng L^{-1} in different water matrices. Another study was performed for the determination of CPT-11 in human blood using LC combined with fluorescence detection (de Jong et al., 2003). Recently, several methods have included the use of online pre-concentration methods coupled to LC-MS/MS for the analysis of endocrine disrupters (López de Alda and Barceló, 2001), pharmaceutical compounds and pesticides in water matrices (Bones et al., 2006; Segura et al., 2007; Viglino et al., 2008). Both methods of SPE (off-line and online) follow the same steps and are governed by the same principles. The difference is that contrary to online SPE, the sample extraction steps in off-line SPE are completely independent of the chromatographic separation and quantitation. Published literature reviews (Chen et al., 2009; Hennion, 1999; Oliferova et al., 2006) confirm that online SPE is one of the most robust and promising techniques for the rapid extraction and preconcentration of pharmaceuticals in environmental matrices. In fact, online SPE coupled to LC allows high sensitivity and performance while showing good reproducibility (Oliferova et al., 2006). Additionally, standard additions, usually considered lengthy and arduous when combined with labor-intensive manual SPE, can be applied more easily when used with online SPE. Online SPE also lowers sample volume, limits sample loss (especially volatiles and semi-volatiles) and reduces the possibility of contamination when comparing traditional off-line SPE methods that require several manipulation steps and many more human resources. However, a key problem of online SPE remains achieving optimal recovery without affecting the coupling to liquid chromatography and atmospheric pressure ionization sources.

Table 1
Consumption of chemotherapeutic agents in Montreal's hospitals (Personal communication – President, advisory committee of pharmacists – Sigmasanté hospital group purchasing-2010).

Compounds	% mass	Kg/M people
5-Fluorouracil	45.85	21.10
Cyclophosphamide	12.45	5.72
Gemcitabine	11.12	5.11
Ifosfamide	5.00	2.30
Methotrexate	3.44	1.58
Carboplatin	2.95	1.36
Irinotecan	1.09	0.50
Epirubicin	0.26	0.12

The development of an online SPE-LC-MS/MS was challenging for the following reasons: i) the target analytes have a wide range of octanol–water partition coefficient values (a measure of hydrophobicity), varying from log $K_{ow} = -1.84$ for MTX to log $K_{ow} = 4.37$ for CPT-11; ii) hydrophilic compounds such as a GCA are poorly retained by conventional reversed phase columns (such as C_{18}) and require alternative stationary phases while reverse-wise we also want to integrate some compounds having high retention and that are more difficult to quantitatively desorb; iii) unlike off-line SPE, the optimization of the nature and percentage of solvents required for desorption (from SPE column towards analytical column) must be compatible with the MS source and must lead to a quantitative desorption since a non-quantitative desorption of compounds leads to loss of analytes that remain on the SPE column, induce a decrease of the signal and greatly affects the reproducibility (Oliferova et al., 2006) and iv) using different stationary phases between the SPE and the analytical columns could increase peak broadening and require further optimizations (Hennion, 1999).

The majority of methods developed for the analysis of chemotherapeutic agents focus on only a limited number of compounds, but a single method including several cytotoxic drugs is relevant and needed to properly evaluate potential environmental and human health risks. Furthermore, all published methods were based on off-line SPE. Up to 2012, there are only three published studies on chemotherapeutic agents that used online SPE (Garcia-Ac et al., 2009a, 2009b; Kovalova et al., 2012) in which the authors included two of the target chemotherapy agent compounds. In our project, the selected chemotherapeutic agents include some very polar compounds and have different physico-chemical properties and chemical structures which make their analysis within a single method a daunting analytical and chromatographic challenge especially when one integrates automated pre-concentration. We anticipate that this approach will be helpful to provide some much needed data to properly evaluate the risks caused by the environmental releases of chemotherapy agents.

The objective of this study was to develop a sensitive, rapid and reliable analytical method using online SPE coupled to LC-MS/MS for the analysis of the six selected chemotherapeutic agents with different physico-chemical structures and properties in wastewater matrices. Online SPE optimization (nature of sorbents, breakthrough volume and loading flow rate) and method validation will be presented.

[1] Personal communication – President, advisory committee of pharmacists – Sigmasanté hospital group purchasing-2010

2. Materials and methods

2.1. Materials and chemicals

HPLC grade water (H2O), 0.1% formic acid in H_2O (0.1% FA in H_2O), acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher scientific (Fair Lawn, NJ, USA). HPLC grade formic acid (FA) (98% pure) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Cyclophosphamide (CP) (97–100%), ifosfamide (IF) (≥98%), gemcitabine (GCA) (≥98), methotrexate (MTX) (≥98%), irinotecan (CPT-11) (≥97%) and epirubicin (EPI) (≥90%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Atrazine-^{13}C (ATZ) was used as internal standard (IS) and was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The chemical structures and the physico-chemical properties for the target anticancer drugs are shown in Table 2. Stock solutions (1000 mg L^{-1}) of each compound were prepared by dissolving the corresponding pure powder in an appropriate solvent MeOH for CP, IF, GCA, CPT-11, EPI and 1% formic acid in H_2O – MeOH 3:7 for MTX. All stock solutions were stored at −20 °C.

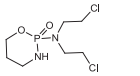
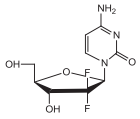
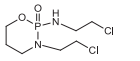
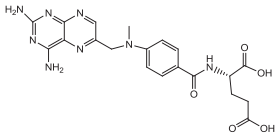
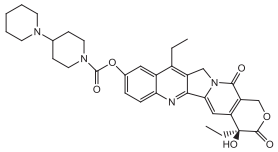
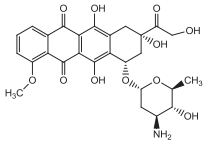
2.2. Instrumentation

LC-MS/MS analysis for wastewater samples was achieved using the Environmental Quantification (EQuan) system supplied by Thermo Fisher Scientific (Waltham MA, USA). The setup consisted of two quaternary pumps: one for sample loading into the SPE column (Accela 1200, Thermo Finnigan, San Jose, CA, USA) and the other for sample elution and separation in the analytical column (Accela 600, Thermo Finnigan, San Jose, CA, USA), a HTC Thermopal autosampler manufactured by CTC Analytics AG (Zwingen, Switzeraland) for loading samples into a 1 mL loop, two columns (an SPE online column and an analytical column for chromatographic separation) and a TSQ Quantum Ultra AM triple quadrupole mass spectrometer for detection. The online SPE column was a Hypersil Gold PFP (20 × 2.0 mm, 12 μm particle size) and the analytical column was also a Hypersil Gold PFP (100 × 2.1 mm, 3 μm) which was preceded by a guard cartridge (10 × 2.1 mm, 3 μm) of the same packing material. All columns were manufactured by Thermo Fisher Scientific. A reversed phase SPE online column made of styrene divinylbenzene copolymer Strata-X (20 × 2.0 mm, 28 μm) from Phenomenex was also used and compared to the PFP column during the optimization of the breakthrough volume.

2.3. Sample collection and preparation

Three primary advanced wastewater treatment plants (WWTPs A, B and C) in the Montreal area (Quebec, Canada) were sampled, collecting samples of the influent and effluent waters, when possible. Furthermore, two WWTPs (A and B) were sampled on two occasions to evaluate variability. The treatment in place in those WWTP is achieved in three steps: i) a screening operation is used to remove solid particles of more than 25 mm contained in wastewater; ii) a second step consists in removing abrasive materials, sand and other heavy particles that can damage mechanical equipment and iii) physico-chemical treatment is carried out in order to eliminate suspended particles and reduce the amount of phosphorus. It consists in the addition of a coagulant to accelerate the agglomeration and decanting of suspended solids. All deposited materials in the bottom of the settling tank are then removed as sludge and floating materials on the surface are removed as scum. In WWTP-B an additional UV disinfection step is performed before discharging water in the river; no chlorine is used in the three plants.

Table 2
Physicochemical properties and molecular structures of the target chemotherapeutic agents.

Compounds	Structures	MW/g.mol^{-1}	pKa	LogK$_{ow}$
Cylophosphamide $C_7H_{15}Cl_2N_2O_2P$ (CP)		261.086	2.84[1] 4.5–6.5[2]	0.6[3]
Gemcitabine $C_9H_{11}F_2N_3O_4$ (GCA)		263.198	3.6[4]	−1.24[5]
Ifosfamide $C_7H_{15}Cl_2N_2O_2P$ (IF)		261.086	1.54[1]	0.86[3]
Methotrexate $C_{20}H_{22}N_8O_5$ (MTX)		454.439	4.7[3] 4.8–5.4[2]	−1.85[3]
Irinotecan $C_{33}H_{38}N_4O_6$ (CPT-11)		586.678	N.A	4.37[6]
Epirubicin $C_{27}H_{29}NO_{11}$ (EPI)		543.519	8.08[7] 7.7[1]	1.85[3]

MW: Molecular weight
[1] (Sottani et al., 2008).
[2] (Wang et al., 2009b).
[3] (SRC, 2011).
[4] (Wang et al., 2009a).
[5] (Arias et al., 2010).
[6] (Yang et al., 2006).
[7] (Li and Huang, 2004).

Influent and effluent samples were collected in clean amber glass bottles and were immediately transported to the laboratory. In WWTP-A, grab samples of both influents and effluents were collected together (without delaying the effluent for the residence time within the plant) on two different days, while in WWTP-B a grab sample and a 24-h composite sample were taken the same day. For WWTP-C only an effluent grab sample was collected. All samples were kept at 4 °C for less than 24 h before preparation and analysis. No significant degradation for the target compounds has been reported so far during this storage time (Halling-Sørensen et al., 1998; Kiffmeyer et al., 1998). In order to remove suspended particles and avoid clogging the columns (dirty matrix), samples (0.5 L) were vacuum-filtered in three steps to allow fast filtration: first through 8.0 μm, then 3.0 μm and finally through 0.45 μm pore size, nitrocellulose membranes. This was necessary to efficiently filter the samples since the high content of suspended solids caused clogging that prevented the filtration with 0.45 μm directly. Samples were acidified by adding 0.1% FA and were then spiked with the IS to a final concentration of 150 ng L^{-1}. The samples were then transferred to 10 mL amber glass vials for online SPE-LC-MS/MS analysis.

2.4. Online SPE and LC parameters

The online SPE process takes place in three steps. First the 1 mL loop was overfilled with 2 mL of the sample using the 2.5 mL autosampler syringe. A volume exceeding the maximum capacity of the loop was used in order to reduce the dilution effect of the sample inside the loop due to the presence of the wash solution used between each injection. A 2 mL fill up ensures the elimination of the wash solution and the presence of only the sample inside the loop. After the loop was filled, the 1 mL of sample was introduced into the SPE column (Hypersil Gold PFP) with the load pump using the conditioning solution (0.1% FA in H_2O) at a flow rate of 1000 μL min^{-1}. Once the sample was loaded onto the SPE column, the divert valve of the column switching array was actuated at 1.15 min and the target analytes were back-flushed into the analytical column where the separation is achieved using the LC mobile phase A (0.1% FA in H_2O) and B (0.1% FA in ACN:MeOH, 50:50 (v/v) delivered by the analytical pump at a flow rate of 350 μL min^{-1}. The back-flushing helps to avoid peak broadening (Hennion, 1999) and allows the elution of strongly retained analytes at the head of the column. The chromatographic separation was achieved at 45 °C in order to improve the peak shape and also reduce the total run time. The elution started at 30% of eluent B followed by a linear gradient to 80% of B in 7 min which was maintained for 0.5 min. The column was then equilibrated during 3.4 min at the initial elution conditions. In order to eliminate carry over, the wash of the syringe and the injection valve was programmed before each injection, first with an organic solution of 0.1% FA in ACN:MeOH, 50:50 (v/v) and then with 0.1% FA in H_2O. All the online SPE procedures are fully automated and the total run time per sample was 11 min. All chromatographic conditions are summarized in Table S1 (Supplementary material).

2.5. Mass spectrometry parameters

In order to optimize all the MS parameters, standard solutions (1 mg L^{-1}) of each analyte and the IS were infused directly into the mass spectrometer. Electrospray ionization (ESI) was performed in the positive mode using a spray voltage of 4.5 kV; the sheath gas (N_2) was set to 50 arbitrary units and the auxiliary gas (N_2) to 35 arbitrary units. The ion transfer tube temperature was set to 400 °C and the skimmer offset to 0 V. The TSQ Quantum ultra AM triple quadrupole was operated in selected reaction monitoring (SRM) mode at unit resolution (FWHM: 0.7 u). Collision energies were optimized to get the maximum intensity for each SRM. For each compound two SRM transitions were selected but only the most intense (SRM#1) was used for quantification. Individual parameters for each analyte and IS are summarized in Table S2 (Supplementary material). The ratios of the 2 SRM transitions were also evaluated to prevent false positives.

2.6. Method validation

Method validation was achieved using two types of calibration, first an internal calibration done in distilled-deionized water (dd-H_2O); then we carried out a calibration using IS and standard additions in wastewater influent (raw sewage). Calibration solutions of the target compounds of 0, 15, 25, 50, 75, 100, 150 and 300 ng L^{-1} were prepared by dilution of a mixed working solution of 500 μg L^{-1}. Formic acid (0.1%, v/v) and IS (150 ng L^{-1}) were added to all samples. The IS was used to reduce method variability and thus improve the precision of the method. The use of atrazine as an internal standard is justified by the lack of standards labeled with stable isotopes that could fit this set of cytotoxic agents. Moreover, good results for atrazine were reported for a multi-residue method (Garcia-Ac et al., 2009b) containing two of the cytotoxic agents targeted in this project. While the standard additions method was used to compensate for matrix effects and to determine the limits of detection, quantification and precision values according to the International Conference on Harmonization of Technical requirements for Registration of Pharmaceuticals for Human Use (ICH, 2012). The limits of detection (LOD) and the limits of quantification (LOQ) were calculated using the following equations:

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} \quad (2)$$

where σ is the standard error of the intercept and S is the slope of the standard additions calibration curve.

Intra-day and inter-day precisions were determined for a concentration of 80 ng L^{-1} of cytotoxic compounds spiked in dd-H_2O and wastewater influent. Intra-day precision was calculated as the relative standard deviation in percentage (RSD %) of the peak area ratio of the cytotoxic compounds to the IS (n = 5). The inter-day precision was determined by combining the results of this process over three consecutive days (n = 10).

Matrix effects (n = 5) were determined by comparing mean peak areas of the cytotoxic compounds spiked (80 ng L^{-1}) in dd-H_2O ($A_{dd\text{-}H2O}$) with those of the cytotoxic compounds spiked in wastewater influent samples (Aww_s) after correcting for the peak area of the standard in the unspiked matrix (Aww_{ns}) and were reported as percentages:

$$Recovery(\%) = \left(\frac{A_{wws} - A_{wwns}}{A_{dd-H2O}}\right) \times 100\% \quad (3)$$

A value of 100% indicates that there is no absolute matrix effect. While values below 100% indicate ion suppression and values above 100% represent ion enhancement (Salvador et al., 2007).

Recoveries were determined from the same procedure as matrix effects, the results were expressed using the equation below:

$$Recovery(\%) = \left(\frac{C_{sm} - C_m}{C_s}\right) \times 100\% \quad (4)$$

Where C_{sm} is the measured concentration of the analyte in the spiked matrices, C_m is the original concentration of the analyte in the matrices and C_s is the concentration spiked in the matrices.

2.7. Breakthrough volume estimation

In order to rapidly compare the two online SPE columns, their breakthrough volumes were estimated using a graphical extrapolation

method, described elsewhere (Garcia-Ac et al., 2009b). Briefly, 25 μL injections of a 500 ng L^{-1} standard mix solution were used and the analytes were eluted isocratically from the online SPE column (for this setup no divert valve was used and the online SPE column is connected directly to the ion source). Different combinations of solvents A (0.1% FA. in H_2O) and B (0.1% FA MeOH) were tested in triplicate starting from 10% B and increased in steps of 10% up to 90% B. The retention times of each analyte in the different isocratic elution conditions were then used to calculate the logarithm of the capacity factors ($log\ k'$), which were then plotted as a function of the volume fraction (φ) of MeOH in the mobile phase (Fig. S1). The y-axis intercept of this graph is used to calculate k'_W, the capacity factor of the analyte when the mobile phase is 100% aqueous (Garcia-Ac et al., 2009b). Finally the estimated breakthrough volume (V'_b) is calculated using the following equation:

$$V_b^{'} = V_m\left(k_W^{'} + 1\right) \quad (5)$$

where V_m is the void volume of the column.

3. Results and discussion

3.1. LC optimization

The first step before elaborating a full scale online SPE-LC-MS/MS method is to insure the LC-MS/MS is validated on its own. With this objective, a chromatographic separation taking 11 min (Fig. 1) for the six cytotoxic drugs was achieved using a PFP column which allowed baseline separation for all analytes except for the structural isomers CP and IF that could not be separated. These two compounds differ only on the position of the two chloroethyl groups which in CP are bonded to the same nitrogen atom while in IF they are bonded to different nitrogen atoms (Table 2). This structural difference results in different fragmentation patterns which allow us to differentiate them by MS/MS. As co-elutants, these two compounds should compete for the charge on the ESI droplets, however no signal suppression was observed. Two online SPE columns were tested with the analytical PFP column to provide the best combination of recovery values, breakthrough volumes and peak shapes. It was first attempted to use a Strata-X SPE column given its better breakthrough volume results (Table 3) but it was later decided to use the PFP SPE online column instead, due to peak broadening observed for the target compounds with the Strata-X SPE column (e.g. CPT-11 had a width at 10% of the peak height (w_{10}) of 0.57 min). Peak broadening was possibly caused by the incompatibility between the two stationary phases (Oliferova et al., 2006). The online PFP SPE column coupled with the PFP analytical column resulted in a much improved chromatography, with relatively narrower (w_{10} was between 0.21 and 0.24 min) and symmetrical peaks (asymmetry factor was between 1.16 and 1.55 for all target analytes). Several tests (results not shown) were conducted for the optimization of the mobile phase used for desorption and chromatographic separation. We found that a solution of ACN:MeOH, 50:50 (v/v) was the best mix which gave adequate desorption for most of the compounds and good separation. To increase the ionization in ESI(+) and improve peak shapes, we added 0.1% of formic acid to the mobile phases A and B.

It was observed that in the majority of published methods for the analysis of chemotherapeutic agents in water matrices, C_{18} analytical columns were the most frequently used (Jie et al., 2010; Kiffmeyer

Table 3
Estimated breakthrough volumes for the two SPE columns tested.

Compounds	Columns					
	Hypersil Gold PFP			Strata-X		
	V'_b (mL)	Error (%)	R^2	V'_b (mL)	Error (%)	R^2
GCA	0.3	5	0.9823	0.3	6	0.8021
MTX	2.8	32	0.9784	2.0	40	0.9444
IF	1.4	20	0.9923	35.5	93	0.9773
CP	2	22	0.9926	68.7	98	0.9807
CPT-11	334	142	0.9807	17.8	51	0.9974
EPI	800	27	0.9995	NA	NA	NA

NA: Not available.

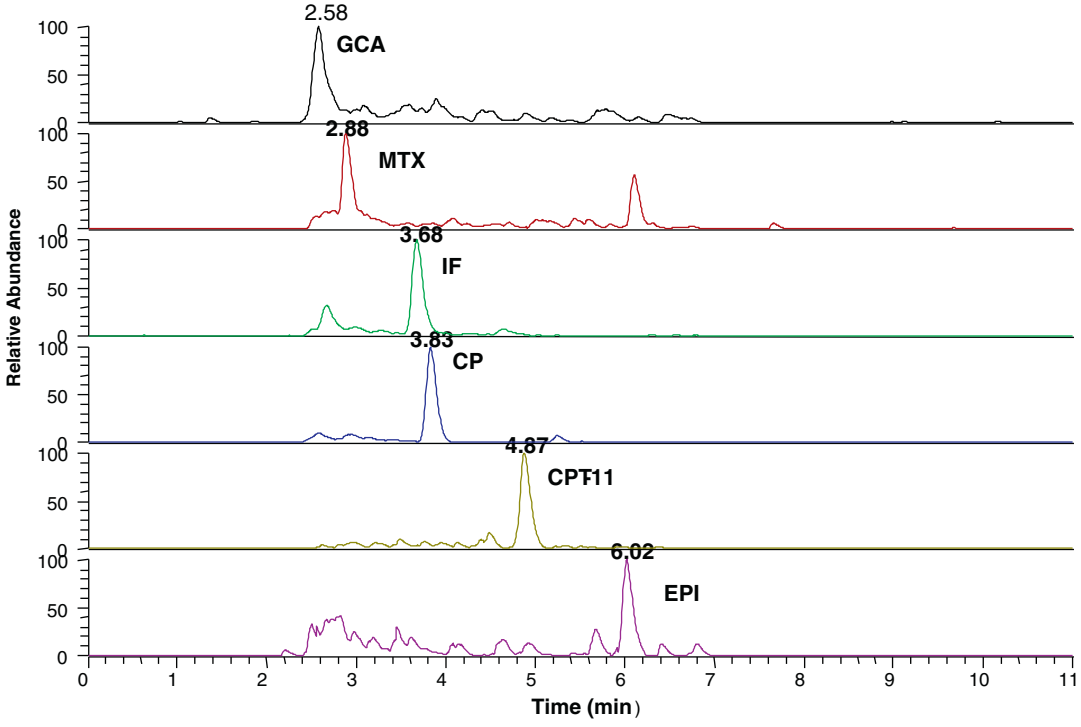


Fig. 1. LC-MS/MS chromatogram in SRM mode of the selected compounds spiked at 75 ng L^{-1} in influent wastewater.

et al., 1998; Mahnik et al., 2006; Martín et al., 2011). In order to improve the retention of the more hydrophilic compound (GCA), a PFP analytical column was used, given that this column provides alternative selectivity, particularly for halogenated and substituted aromatics when compared to C_{18}. This could be explained by the presence of the carbon–fluorine bond on the PFP which is more polar than the carbon–hydrogen bond and thus enhances the retention of the analytes by dipole–dipole interactions and the formation of hydrogen bonds. Furthermore, the presence of the aromatic rings on the PFP phase results in π–π interactions with the aromatic rings of the analytes, further increasing their retention. The selectivity of the PFP column can also be observed in the order of elution of CPT-11 and EPI, which was reversed if compared to a column such as C_{18} since the CPT-11 has the greatest hydrophobicity.

3.2. Online SPE optimization

To enable the best sensitivity of the target compounds, two parameters were optimized during the pre-concentration step: the breakthrough volume and load flow rate.

3.2.1. Breakthrough volume estimation

The determination of the estimated breakthrough volume (V'_b) allows us to calculate the maximum volume of sample that could be percolated through the online SPE column without any loss of compounds. Therefore, V'_b is helpful to find the optimal sample volume required to reach the highest sensitivity. Breakthrough volume results shown in Table 3 indicate that overall, the Strata-X online SPE column gave higher V'_b values than online PFP column, however the errors were also higher (>50%). The retention was so strong in the Strata-X online SPE column that for EPI, the breakthrough volume estimation was impossible to perform due to the lack of enough experimental points (peaks were observed only with 60–90% of MeOH in the mobile phase). This was mainly due to the high retention of this online SPE column which means that the maximum % MeOH in the mobile phase required to elute most of the analytes is above 50%. Extrapolation to the y-axis induces a considerable amount of uncertainty compared to V'_b values obtained with the PFP column, which had lower retention capacity. Unfortunately, when combining the Strata-X online SPE column with the PFP analytical column, broad peaks were observed for the target compounds which suggested that these columns were not compatible. Thus, it was decided to use the PFP online SPE column instead, because when coupled with the analytical PFP column it resulted in better peak shape and no significant peak broadening was observed. Table 3 also shows that injection volumes > 3 mL should not be used with the PFP online SPE column since significant losses were observed for GCA, MTX, IF and CP. Therefore we decided to use injections of 1 mL in order to have optimal sensitivity for most of the compounds and limit losses of GCA.

To summarize the results, Strata-X appears to be a better online SPE column for the target analytes; however this column did not provide an adequate peak shape (severe peak broadening) when coupled online with the PFP analytical column. The PFP online SPE column seems to be a good option since no breakthrough should be observed for 1 mL injections for the target analytes, except for GCA.

3.2.2. Load flow rate

The second parameter to consider was the load flow rate. In order to evaluate the flow rate that yields the highest signal, an experiment was performed by varying the flow rate (500 μL min^{-1}, 800 μL min^{-1}, 1000 μL $min^{-1}$and 1500 μL min^{-1}) while keeping the load volume (1 mL) and the concentration (500 ng L^{-1}) constant, then measuring the peak areas for each compound. Results shown in Fig. 2 indicate that at higher flow rates, the analyte response increases significantly for all the compounds, with the exception of GCA whose response decreases, leading to non quantitative results.

The results for GCA could be related to the fact that it is the most hydrophilic compound of the list with a $LogK_{ow}$ of −1.24 and the first compound to elute, therefore it partitions preferentially on the aqueous phase and needs more time to interact with the online SPE column (which is why it is better retained at lower flow rates). MTX has a $LogK_{ow}$ of −1.85, but the PFP column does offer more selectivity towards compounds with polar groups on an aromatic ring. The $logK_{ow}$ is an important characteristic to evaluate the behavior of various chemical compounds but certainly not sufficient to fully explain how they will react. Previous authors (Liška et al., 1989; Segura et al., 2007) interpreted similar lack of retention to the deconditioning of the stationary phase. The authors argued that high amounts of H_2O at low flow rates in the SPE column could cause a collapse of the C_{18} chains resulting in low retention. However, excess water to decondition the stationary phase is present not only at low flow rates but also at higher flow rates. Also if chain collapse was an issue,

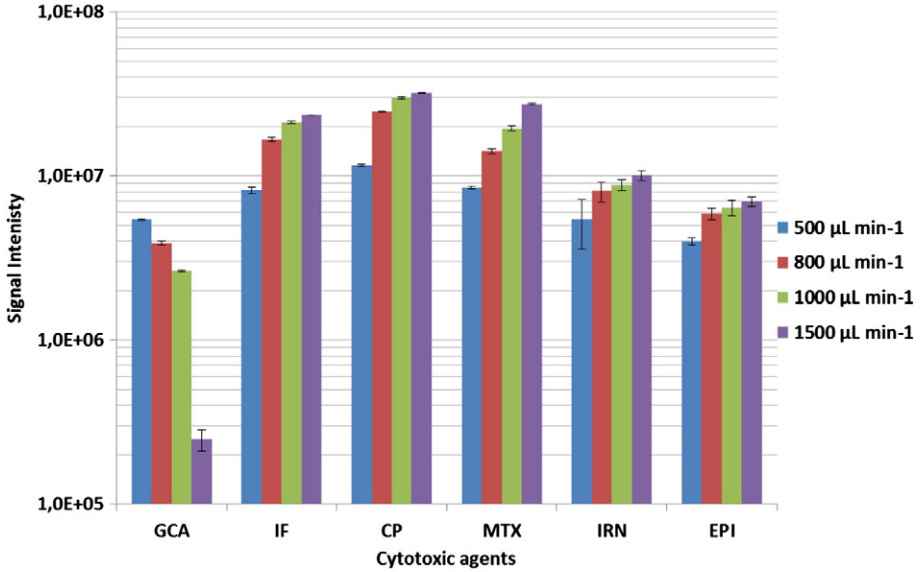


Fig. 2. Experimental determination of the effect of variation of the flow rate on analysis signal response area achieved with 0.5 ng of the cytotoxic agents (n = 3) in dd-H_2O. Error bars represent standard deviations of triplicate analysis.

Table 4
Analytical performances of the SPE-LC-ESI-MS/MS method.

Affluent wastewater								
Compounds	R^2	Detection Range	%RSD	%RSD	LOD	LOQ	%R	%ME
		ng L^{-1}	Intra-Day (n = 5)	Inter-Day (n = 10)	ng L^{-1}	ng L^{-1}	(n = 5)	(n = 5)
GCA	0.983	60–300	13	23	20	60	47 ± 22	55 ± 10
MTX	0.990	36–300	11	12	12	36	73 ± 11	118 ± 12
IF	0.997	14–300	9	17	4	14	82 ± 9	80 ± 14
CP	0.998	13–300	7	9	4	13	85 ± 16	117 ± 16
CPT-11	0.997	19–300	11	10	6	19	90 ± 10	65 ± 9
EPI	0.984	54–300	11	18	18	54	73 ± 14	78 ± 23

%R: Recovery, %ME: Matrix effects.

it would be an issue for all compounds and not just one of them. GCA would not be properly retained on the SPE cartridge if there was phase collapse especially since it is the least likely to be well retained.

GCA is the compound with the lowest affinity towards the stationary phase and increased flow rate accelerates its desorption which in turn leads to a decrease in signal. It was decided to use 1000 μL min^{-1} as load flow rate for all subsequent analysis since it was good compromise for all compounds.

3.3. Method validation

The optimized method was validated using wastewater influent (raw sewage). Standard additions were selected for the calibration method in order to minimize or eliminate matrix effects and to perform the quantification. Also an IS was used to correct for the signal distortion due to the matrix. Validation results are summarized in Table 4. It can be observed that the coefficients of determination (R^2) for the calibration curves were within an acceptable range for all the compounds (0.983–0.998). Intra-day precision (n = 5) expressed in terms of RSD % was in the range of 7 to 13% and the inter-day precision (n = 10) was less than 20% except for GCA which resulted in a value of 23%. The limits of detection (LOD) and limits of quantification (LOQ) ranged from 4 to 20 ng L^{-1} and from 13 to 60 ng L^{-1} respectively (with GCA showing the highest LOD and LOQ). The values obtained are comparable to those in the previously published method (Garcia-Ac et al., 2009a) using LC-MS/MS, online SPE and 1 mL of sample, reported limits of detection for CP and MTX of 5 and 11 ng L^{-1} in influent wastewater. Other authors (Jie et al., 2010) reported (LOD) of 2.5 ng L^{-1} for IF in wastewater influent using LC-MS/MS and off-line SPE and using 500 mL of sample. In another study performed in Spain using LC-MS-MS, off-line SPE and 250 mL of sample in the analysis of wastewater influent, the authors reported lower (MDL) concentrations, with 3.8 ng L^{-1} for EPI, 1.4 ng L^{-1} for GCA and 1.1 ng L^{-1} for CPT-11 (Martín et al., 2011).

Recovery values (Table 4) achieved for the majority of analytes were greater than 70%, except for GCA with a value of 47%. This could result from its high hydrophilic character which leads to poor initial retention on an SPE column such as PFP. It is known that ESI often leads to higher matrix effects compared to other sources due to co-eluting matrix constituents (Matuszewski et al., 1998; Schuhmacher et al., 2003). The evaluation of matrix effects is also subjected to the complexity of the samples (Garcia-Ac et al., 2009a; Martín et al., 2011) and is compound-dependent. The use of stable isotope labeled drugs as IS and standard additions have been highly recommended to compensate for matrix effects (Kloepfer et al., 2005). Otherwise, results showed (Table 4) that matrix effects obtained ranged from 55 to 118%. Signal suppression was observed for GCA (55%), CPT-11 (65%), EPI (78%) and IF (80%) while signal enhancement was observed for MTX and CP (118% and 117%, respectively).

3.4. Analysis of wastewater influent and effluent

The developed method was evaluated for the analysis of nine wastewater samples (influent and effluent) taken from three (WWTPs) in the Montreal (Quebec, Canada) area and in which primary advanced treatment is performed. Results are summarized in Table 5. Two out of six (CP and MTX) cytotoxic drugs studied were detected (Fig. 3); concentration levels of CP ranged from 17 to 22 ng L^{-1}, and as expected, there was no significant difference between concentration levels in influent and effluent samples of WWTP-A. This is in agreement with the results of previous studies (Kiffmeyer et al., 1998; Steger-Hartmann et al., 1997; Steger-Hartmann et al., 1996) in which no degradation of CP was observed. Surprisingly, the concentration of CP was similar despite different weather conditions (recorded at the station) during the first (rainfall = 7 mm) and second (rainfall = 23 mm) sampling days. It becomes more difficult to explain the similarities between the two results for CP, one option could be a variation between consumption on the sampling days or possibly an artifact from variations due to time of sampling (samples were collected in the mornings and the release pattern from morning urine can strongly impact concentration of pharmaceuticals). MTX was found in most of samples analyzed at concentrations levels between 13 and 60 ng L^{-1}. No significant difference was observed between influent and effluent for the relatively short residence time of the plant (<3 h), while some authors have reported the biodegradation of MTX within seven days (Kiffmeyer et al., 1998). A possible explanation may be the type of wastewater treatment conditions (physicochemical) with a low residence time in the treatment plant that are not long enough to allow the degradation of MTX. The concentration was different between the first and the second sampling days, it would be surprising that this would reflect a change in consumption of the drug but rather the result of an increased dilution of

Table 5
Cytotoxic agent concentrations (ng L^{-1}) measured in triplicates in different influents and effluents.

Compounds	WWTP-A		WWTP-A (3 weeks after)		WWTP-B		WWTP-B (1 day after)		WWTP-C
	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Eff.
GCA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
MTX	60 ± 4	53 ± 15	*20 ± 4*	*13 ± 3*	*17 ± 3*	*20 ± 5*	*20 ± 5*	*22 ± 9*	<LOD
IF	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CP	17 ± 3	21 ± 4	22 ± 2	18 ± 1	<LOD	<LOD	<LOD	<LOD	<LOD
CPT-11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EPI	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Values in italics were >LOD but <LOQ. Values identified as <LOD are below the detection limit and not detected.

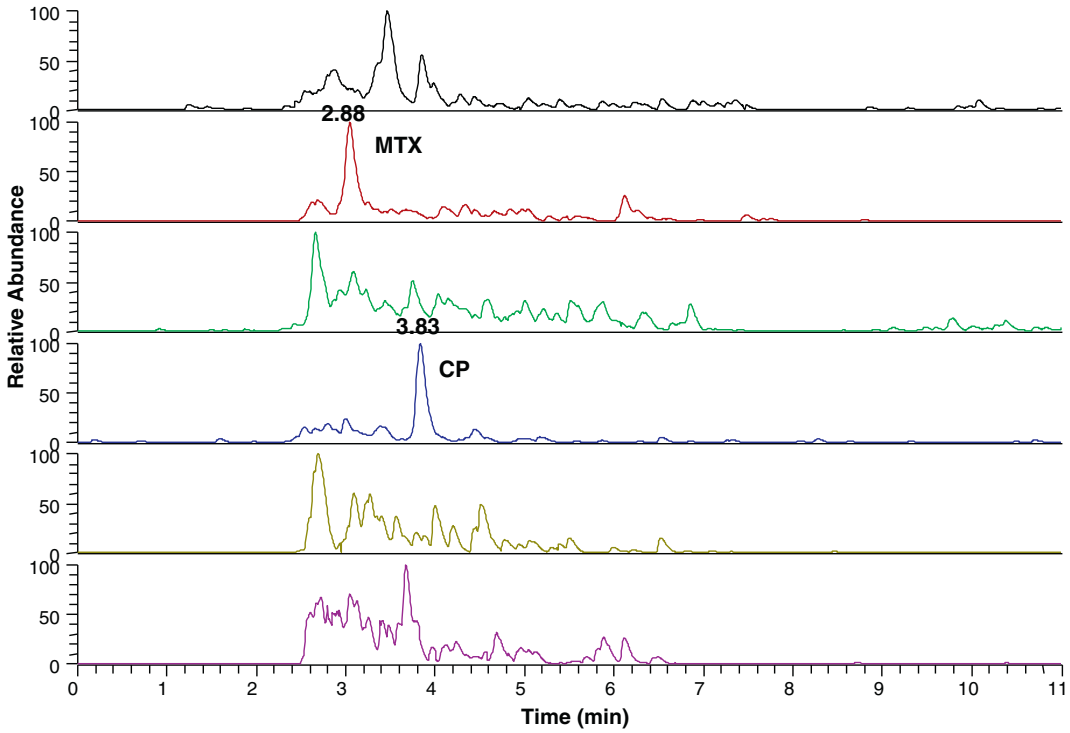


Fig. 3. LC-MS/MS chromatogram in SRM mode of the detected compounds (not spiked) in a field-collected sample (WWTP-A wastewater effluent wastewater).

the compounds when subjected to higher rainfall. In future work it would seem prudent to integrate some standard sanitary tracers such as caffeine or sucralose to help normalize the rainfall/dilution factor (Batchu et al., 2013; Sauvé et al., 2012).

Concentrations of the two chemotherapeutic agents found in aquatic environment in several countries are different because of variable number of patients requiring administration of those drugs, the amount of the drug administered which may vary from patient to patient and also the climate and amount of water consumed which could contribute to the dilution of the drug in the wastewater system. CP was detected in influent and effluent wastewater in Zurich (Switzerland) (Buerge et al., 2006) at concentration of 11 and 10 ng L^{-1} respectively. CP was also quantified in influent wastewaters in Germany (Steger-Hartmann et al., 1997) in concentration ranging from 7 to 143 ng L^{-1}. Other authors (Castiglioni et al., 2005) reported a concentration of 12.5 ng L^{-1} in effluent wastewater in Italy. MTX was also quantified ranging from 1.6 to 18 ng L^{-1} in influent wastewater in China (Jie et al., 2010).

4. Conclusion

Chemotherapeutic agents are contaminants of emerging concerns that we feel should be better monitored in environmental waters given their cytotoxicity and carcinogenic character. A sensitive, rapid and completely automated method was developed for their quantification in wastewater, which includes six chemotherapeutic agents among the most commonly used in Quebec (Canada), with a broad range of hydrophobicity and different chemical structures. Using an online SPE-LC-ESI-MS/MS setup with only 1 mL of sample, two of the six chemotherapeutic agents studied (cyclophosphamide and methotrexate) were observed in field-collected samples (influent and effluent) and quantified ranging from 13 to 60 ng L^{-1}. It was a particular challenge to combine compounds with such diverse chemistries using a single completely automated setup. Further work on chemotherapeutic agents should focus on the integration of 5-fluorouracil, the most consumed chemotherapeutic agent within the province of Quebec (Canada) and presumably elsewhere in Canada and other countries with comparable medical systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.12.050.

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