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Influence of Matrix When Tracing Cytostatic Drugs in Urban Wastewater: A Validated SPE-LC-MS/MS-Based Method

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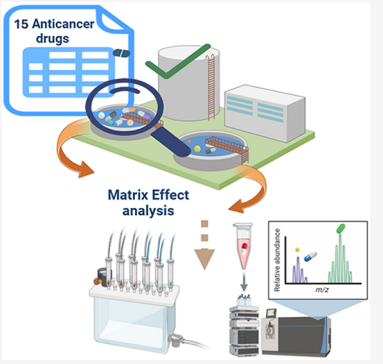
ABSTRACT: Cytostatic pharmaceuticals are not completely removed in wastewater treatment plants (WWTPs) and may affect aquatic ecosystems. Their quantification is challenging due to variations in wastewater characteristics, which influence analytical performance. An analytical procedure has been developed, based on solid-phase extraction and liquid chromatography coupled with tandem mass spectrometry, for the simultaneous quantification of 15 anticancer compounds. Influent and effluent samples from 14 Spanish WWTPs were analyzed, and 11 out of 15 target compounds were found at quantifiable levels (ng/L). These findings underscore the need for new WWTP treatments and the further development of analytical techniques capable of monitoring trace contaminants, in line with new regulatory demands. To carry out a comprehensive study of matrix influence on the analytical process, a novel physicochemical clustering approach was applied to group WWTPs, facilitating the validation of the method and widening its application to assess the load of micropollutants emitted to natural aquatic environments. Results show the influence of matrix variability on determining the concentration of cytostatics at both the influent and effluent of WWTPs. The interferences due to the matrix effect can be minimized by optimizing the dilution of the different samples.



KEYWORDS: *emerging contaminants, cancer pharmaceuticals, wastewater treatment plants, solid-phase extraction, matrix effect*

1. INTRODUCTION

Cytostatic drugs, used in cancer treatments, are classified as emerging contaminants (ECs) due to their persistence, bioactivity, and potentially toxic nature.[1] These pharmaceuticals reach wastewater mainly through excretion after patient treatment.[2] Although hospital wastewater is a significant source of persistent pollution, studies estimate that up to 75% of cytostatic residues may be excreted outside hospitals.[3,4] Hence, the significance of urban wastewater treatment plants (WWTPs), as the last barrier to minimize the release of ECs into aquatic ecosystems, has been highlighted in the new directive for urban wastewater treatment.[5] One of the noteworthy new features is stricter limits for microcontaminants, according to the European Union's zero-pollution plan. To reach this goal, the development of analytical techniques for monitoring trace ECs in complex samples is necessary.

The new directive represents a significant challenge since WWTPs are not specifically designed to remove ECs.[6,7] Therefore, these compounds are continuously released with the final effluents.[6,8] Thus far, cytostatic drugs such as tamoxifen, methotrexate, capecitabine, and ifosfamide, among others, have already been detected in European water bodies, indicating their persistence in the aquatic environment.[6,9,10]

Cytostatic compounds disrupt cellular metabolism and block cell division; consequently, given their high stability,[11] their presence in aquatic ecosystems may pose substantial ecotoxicological risks.[1,8] The state of the art shows several effects associated with these pharmaceuticals, which include mutagenicity, genotoxicity, endocrine disruption, and the development of antibiotic resistance in organisms.[12,13] Therefore, the occurrence of these compounds in water bodies may reduce the resilience of microbiotic communities and indirectly impact human health via drinking water or bioaccumulation within the food chain.[6] Their presence in surface waters and the potential for long-term ecological effects highlight the need for their removal in WWTPs to mitigate cytostatic drug pollution.[7]

Cancer is one of the foremost causes of death worldwide, with the incidence rate expected to increase from 20 million in 2022 to 27 million by 2035.[14] Cancer therapies generally include chemotherapy agents, frequently combined with other treatments, such as anti-inflammatory, antibiotic, and hormonal treatments. As a result, the global use of cytostatic drugs among other compounds will increase significantly. Between

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Table 1. Therapeutic Classes and Physicochemical Properties of Selected Anticancer Compounds[a]

anticancer compounds	code	ATC[b] classification	molecular formula	molecular mass (g/mol)	acid/basic pK_a	Log K_{ow}
5-fluorouracil	5-FLU	L01BC02 antimetabolite	$C_4H_3FN_2O_2$	130.0	7.2/−8.0	−0.89
azathioprine	AZA	L04AX01 immunosuppressant	$C_9H_7N_7O_2S$	277.3	8./2.2	0.10
bicalutamide	BIC	L02BB03 antiandrogen	$C_{18}H_{14}F_4N_2O_4S$	430.4	11.8/−4.0	2.71
capecitabine	CAP	L01BC06 antimetabolite	$C_{15}H_{22}FN_3O_6$	359.4	1.9/8.8	0.56
cyclophosphamide	CIP	L01AA01 alkylating agent	$C_7H_{15}Cl_2N_2O_2P$	261.1	2.3/11.1[27]	0.63
cyproterone acetate	CYP	G03HB01 antiandrogen	$C_{24}H_{29}ClO_4$	416.9	17.8/−5.6	3.6
doxorubicin	DOX	L01DB01 antibiotics	$C_{27}H_{29}NO_{11}{\cdot}ClH$	579.2	8/9.9	1.27
etoposide	ETO	L01CB01 plant alkaloid	$C_{29}H_{32}O_{13}$	588.2	9.8/−3.7	1.16
flutamide	FLUT	L02BB01 antiandrogen	$C_{11}H_{11}F_3N_2O_3$	276.2	12.8/−3.7	3.35
gemcitabine	GEM	L01BC05 antimetabolite	$C_9H_{11}F_2N_3O_4$	263.1	3.6[28]/11.2	−1.22
ifosfamide	IFO	L01AA06 alkylating agent	$C_7H_{15}Cl_2N_2O_2P$	261.1	<2.5/9.1[27]	0.86[28]
megestrol	MEG	L02AB01 progestogens	$C_{22}H_{30}O_3$	384.5	17.6/−4.9	3.71
methotrexate	MET	L01BA01 antimetabolite	$C_{20}H_{22}N_8O_5$	454.4	2.9/4.6/6.6[27]	−1.85
mycophenolic acid	MPA	L04AA06 inmunosupresor	$C_{17}H_{20}O_6$	320.3	3.6/−4.1	2.38
paclitaxel	PAC	L01CD01 Plant alkaloid	$C_{47}H_{51}NO_{14}$	853.9	11.9/−1.2	3.20[20]
prednisone	PRED	H02AB07 glucocorticoid	$C_{21}H_{26}O_5$	358.4	13.9[20]/−3.3	1.46[20]
tamoxifen	TAM	L02BA01 antiestrogen	$C_{26}H_{29}NO$	371.5	8.8/5.31[20]	6.35

[a]Data sources: https://pubchem.ncbi.nlm.nih.gov/; DrugBank online | database for drug and drug target info.[20,27,28] [b]ATC: anatomical therapeutic chemical (ATC).[20,27,28] https://atcddd.fhi.no/atc_ddd_index/.

2010 and 2015, the European average annual consumption of anticancer drugs through pharmacies reached 29 tons, with capecitabine accounting for 17% of this total.[15] Capecitabine is among the most widely prescribed cytostatic drugs, with an average dose of 259 μg per inhabitant per day.[8,16,17] In Spain, during this period, the estimated daily consumption of cytostatic substances was 1352 μg/inhabitant, comparable to that in other European countries. The most commonly used drug was mycophenolic acid, at 704 μg/inhabitant-day.[15]

Due to their low environmental concentrations (ng/L−μg/L) and varying chemical properties, cytostatic drugs require highly sensitive and selective analytical methods for accurate quantification. Furthermore, the removal efficiency of WWTPs is calculated by comparing the drug concentration in the wastewater influent (inlet) with that of the final effluent (outlet), which has drastically different compositions.[18] The inlet has a more complex composition and a much more variable organic content than the outlet. Therefore, suitable multiresidue analytical methods need to provide a low limit of detection (LOD) in a wide variety of complex matrices.

Solid-phase extraction (SPE) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS), most often employing electrospray ionization (ESI) in positive mode, is the most common approach for detecting trace levels of cytostatic compounds in wastewater.[9,19−21] The lowest LODs have been reported for cyclophosphamide and ifosfamide in wastewater at 0.03 ng/L.[22,23] However, robust and validated methodologies tailored to cytostatic drugs are still limited, making method development and validation essential for reliable monitoring. A key performance characteristic is the capability of the method to deal with the potentially immense number of inferences present in wastewater. At the same time, multiresidue methods, extending beyond current methods focusing on single compounds or small groups of compounds[9,24,25] are urgently needed to keep up with an increasing number of ECs.

This article presents the development of an analytical methodology based on SPE-LC-MS/MS for the quantification of 17 anticancer drugs in WWTP samples. However, only 15 were included in the final validated method. The compounds were selected based on the highest consumption rates in Spain.[15] Water samples from 14 Spanish urban WWTPs were collected and grouped according to their characteristics to assess matrix effects (MEs) and recoveries (RECs). The RECs of the target contaminants in each WWTP group were used to establish optimal conditions for accurate quantification. The validated method was finally applied to quantify the targeted compounds in influent and effluent samples collected from 14 WWTPs.

2. MATERIALS AND METHODS

2.1. Selection of Anticancer Compounds. Seventeen compounds representing different classes of drugs commonly used in oncological treatments were selected based on: (i) consumption level, according to data from oncological hospitals in Madrid; (ii) estimation of growing consumption due to increased cancer incidence;[14] and (iii) their presence in the environment, according to monitoring studies.[20,24−26] The selected compounds included 5-fluorouracil, azathioprine, bicalutamide, capecitabine, cyclophosphamide, cyproterone, doxorubicin, etoposide, flutamide, gemcitabine, ifosfamide, megestrol, methotrexate hydrate, mycophenolic acid, paclitaxel, prednisone, and tamoxifen. Table 1 shows their classification (ATC code and therapeutic function), molecular formula, and mass and main physicochemical properties (pK_a and log K_{ow}).

2.2. Chemicals and Standards. Solvents used to optimize the analytical procedure, including acetonitrile (ACN), methanol (MeOH), and ultrapure water, were obtained from Supelco (LiChrosolv hypergrade), suitable for LC-MS applications. Formic acid (FA) (purity 98−100%) and heptafluorobutyric acid (HFBA) (purity 99.5%) were purchased from Supelco and Scharlau (Barcelona, Spain), respectively. Analytical standards of the target compounds (purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, Missouri). The individual standard stock solutions of target compound at 100 mg/L were prepared in MeOH and stored at −20 °C for a maximum of six months. Working solutions of 0.5 mg/L for method optimization and quantification of target compounds were prepared by the appropriate mixture and dilution of individual stock solutions in water

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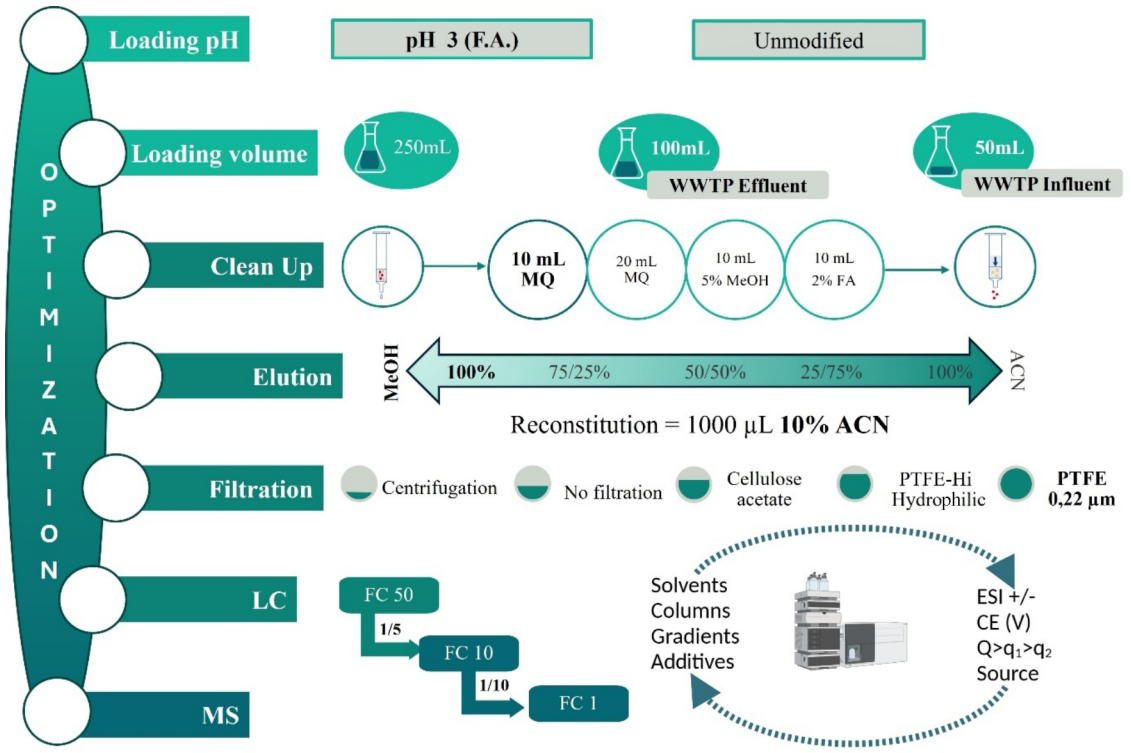

Figure 1. Optimization pathway to develop the analytical method for the targeted anticancer compounds in WWTPs samples. Selected parameters appear in bold.

2.3. Wastewater Sampling and Matrix Characterization. The WWTPs were selected considering different sizes (small WWTPs with a flow rate lower than 10 000 m^3/day, medium between 10 000 and 50 000 m^3/day, and large over 50 000 m^3/day), the use of different treatment methods (biological treatments, coagulation-flocculation, microfiltration, and ultraviolet light combined with hypochlorite, mainly), and the presence or not of hospital discharges. In October 2024, a sampling campaign was conducted to collect inlet and outlet samples from 14 Spanish WWTPs, five of which received discharges from at least one hospital. A total of 29 grab samples (14 influents and 15 effluents) were collected in polyethylene bottles and transported to the laboratory in coolers. All samples were filtered through a 2.7 μm Whatman glass microfiber filter, grade GF/A, under vacuum (Merck, Darmstadt, Germany), transferred into amber glass bottles, and stored at −20 °C until analysis.

The physicochemical characterization of the influent from each WWTP showed pH values ranging from 6.8 to 8.6, conductivity within the 365 and 2120 μS/cm range, and chemical oxygen demand (COD) between 355 and 792 mg O_2/L. Effluent samples had a pH range from 6.3 to 7.8, conductivity ranging from 168 to 1065 μS/cm, and COD between 15 and 39 mg O_2/L (Table SI 1). As expected, the inlets exhibit higher organic and inorganic content. Regarding the development of the method, the influent samples will be considered as a high-complex matrix and the effluent samples as a low-complex matrix.

2.4. Sample Treatment. In this study, the SPE protocol was optimized using wastewater samples of influent and effluent spiked at different levels (0.1 and/or 1 μg/L) of each compound to find the most efficient extraction conditions to ensure the detection and quantification of the target contaminants. Following the EPA method[29] recommendation, SPE was performed using a Waters Oasis HLB cartridge (6 cm^3, 200 mg; Waters, Milford, Massachusetts). The effect of pH (acid or sample pH), loading volumes (100 and 50 mL) and flow rates (2 and 5 mL/min), cleanup step (water, water with 5% MeOH, and water with 2% FA) and elution conditions (MeOH and ACN, 100:0 to 0: ratio) were evaluated using the same wastewater effluent (see Table SI 1, WWTPEFF00). Additionally, different filtration strategies were tested by comparing centrifugation and various syringe filters (cellulose acetate 0.45 μm, PTFE-Hi 0.22 μm, and PTFE 0.22 μm) (Figure 1).

As a result of all the optimization experiments, the final protocol was defined as follows: Wastewater samples were filtered with 0.7 μm fiberglass filters and adjusted to pH 3 with FA. Extraction of target compounds was automated through a Dionex AutoTrace 280 SPE system from Thermo Fisher Scientific (Sunnyvale, California). SPE cartridges were conditioned and equilibrated using 6 mL of MeOH and 6 mL of water acidified with 0.1% FA. Due to variations in sample complexity, 50 mL of influent (high-complex) and 100 mL of effluent (low-complex) samples were loaded onto the cartridges, followed by washing with 10 mL of water, both at 5 mL/min. Then, the cartridges were dried using a stream of N_2 for 10 min with RapidVap Vertex Model 73200 RapidVap (Labconco Corporation, Missouri). Subsequently, the analytes were eluted with four aliquots of MeOH (3 mL each) at 1 mL/min. Eluents were collected and evaporated under a gentle stream of N_2 at 35 °C. The dried extracts were reconstituted in 1 mL of 10:90 ACN/water (v/v), achieving a preconcentration factor (CF) of 100 and 50 for effluents and influents, respectively. The reconstituted sample extracts were filtered using a 0.22 μm PTFE syringe filter and stored at −20 °C until analysis by LC-MS/MS. Different dilutions of extracts were applied according to methodological criteria (decrease of

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matrix interference and linearity), yielding a specific concentration factor (CF) for each compound. Three different CFs were studied, depending on the sample matrices: CF1 (dilution 1/10 from CF10 for influents) and CF10 (dilution 1/5 from CF50 for influents and effluents). The final dilutions selected were 1/5 (CF of 10) for some compounds in the influents and all in the effluents and 1/10 (CF of 1) for the rest of the compounds in the influents.

2.5. Analysis by LC-MS/MS. Analysis was performed with an Agilent 1290 Infinity II UHPLC coupled to an Agilent triple quadrupole 6495C, using a Jet Stream ESI interface (Agilent Technologies, Palo Alto, California) operated in positive and negative ESI mode.

Chromatographic separation was performed on an InfinityLab Poroshell 120 EC-C18 column (50 mm length, 3 mm diameter, and 2.7 μm particle diameter) equipped with a guard column (UHPLC Guard) from Agilent Technologies (Santa Clara, California). For the optimization of the resolution and sensitivity of target compounds, different organic solvent combinations (MeOH and ACN), mobile phase modifiers (FA and HFBA), and flow rates (from 0.4 to 0.8 mL/min) were tested. Finally, water acidified with 0,1% FA (A) and ACN with 0,1% FA (B) were used. The gradient was set to 5% of solvent B, and it increased to 100% B within 18.5 min, was maintained at 100% B for 1.5 min, and was decreased to 5% B within 24 min at a flow of 0.6 mL/min. The injection volume was 20 μL, and the column was maintained at 40 °C[29] (Figure SI1).

A D-optimal design of experiments was conducted to optimize ESI source conditions. Five variables: gas temperature (250−280 °C), gas flow rate (14−18 L/min), nebulizer pressure (20−40 psi), sheath gas temperature (250−350 °C), and sheath gas flow rate (10−12 L/min), were examined at 12 levels (Table SI 2). The MS source settings that provided the highest average area for most of the compounds were a drying gas temperature of 280 °C with a flow of 14 L/min, a sheath gas temperature of 350 °C with a flow of 12 L/min, and a nebulizer pressure of 40 psi.

Multiple reaction monitoring (MRM) transition optimization was performed in isocratic (80% B) by flow injection analysis (FIA) using a dead volume connection between the injection valve and ESI source in positive and negative ionization modes. Precursor ions were selected from full-scan analysis (m/z 50−1000) using all analytes combined in the same solution at 1 mg/L in MeOH. Individual solutions (5 μg/L) were analyzed in MS/MS mode using different collision energies (ranging from 5 to 60 V). At least two transitions, one quantifier (Q) and one qualifier (q_1), were selected for each compound. In some cases, a second qualifier (q_2) was included to improve the selectivity of the analysis. Data was acquired in dynamic MRM (dMRM) mode, where the method was divided into 2 min time segments for each predefined MRM transition to maximize the MS duty cycle, improving detection limits compared with the traditional time-segmented MRM approach. The combination of characteristics ion transitions and retention time windows minimizes monitored MRM transitions, ensuring analytes are detected only during LC elution, optimizing MS duty cycle efficiency. Data processing was conducted using Agilent MassHunter Quantitative Analysis (version 10.1.67), and data analysis was accomplished using Excel (version 16.90) and R (R version 4.4.0).

2.6. Method Validation Process. The validation of the method was conducted according to the Commission Implementing Regulation (EU) 2021/808,[30] and following the criteria indicated in Huidobro et al.[31] The detection limits of the method were estimated by establishing the instrumental limits of quantification (LOQi) and detection (LODi). The LOQs were determined as the concentration at which the quantifier had a signal-to-noise ratio (S/N) of higher than 10, while the quantifier and qualifiers maintained the expected intensity ratios. LODs were determined as the concentration giving an S/N higher than 3 from the lowest validated level (LOQ) while the quantifier to qualifier ratio was also maintained. The precision at LOQ was determined from 5 consecutive injections ranging from 2 to 13% relative standard deviations (RSDs). Linearity was established from the corresponding LOQ level to a maximum concentration of 50 μg/L. Calibration curves were produced from five injections of each calibrant level, providing regression coefficients (R^2) above 0.99. External standards were used at two concentration ranges for each compound: from the LOQ level to 200 ng/L (for the quantification of low concentration levels) and 100 ng/L to 50 μg/L (for the quantification of high concentration levels). Method quantification limits (MQLs) were determined considering the LOQi, the preconcentration factor applied in the SPE protocol (LOQi/CF), and the achieved recoveries.

Accuracy and precision were determined by using the samples collected from the 14 urban WWTPs. The influent and effluent samples were grouped into three clusters based on the normalized input flow rate, the presence of hospital discharges, and the physicochemical properties, including COD, conductivity, and pH. Considering the greater complexity of the matrix in the influent samples, the variables of the influents were used for grouping. Only the easiest-to-measure variable was kept when normalized variables showed a high correlation ($R^2 > 0.9$). Then, the remaining variables were used to group the samples using K-means clustering directly on the normalized data. The number of clusters was determined using the elbow method. A principal component analysis (PCA) was used to illustrate the clustering (Figures SI2 and SI3). Finally, cluster-characteristic samples were made by combining equal volumes from all of the individual samples within each cluster.

RECs were assessed in grouped samples ($n = 3$) at different concentrations of the target compounds. The spiking ranges were selected according to levels reported in the literature.[6,9,20] Accordingly, the influent samples were spiked at 20 and 2 μg/L, and effluent samples were spiked at 2 and 0.2 μg/L. Percentage REC (%REC) was calculated as the difference between the response (as peak area) of the compounds in spiked samples and blank samples, compared to the response of the compound in water/ACN (90:10) (v/v) (eq 1). Precision was quantified as the %RSD. According to the following general recommendations, acceptance criteria for accuracy and precision were 70−120 and <20%, respectively.

$$\%\,\mathrm{REC} = \{[(\mathrm{concentration}_{\mathrm{extracted\ spiked\ sample}}) - (\mathrm{concentration}_{\mathrm{extracted\ sample}})] / [(\mathrm{spiked\ concentration})]\} \times 100 \quad (1)$$

ME was evaluated in each grouped sample to distinguish between inefficiencies through the sample treatment protocol and signal alterations caused by matrix interference during analysis. Two approaches were applied for determining REC variations: (i) dilutions (1/10, 1/5, and 1/2) of the extracts of spiked samples and (ii) by fortification, after SPE protocol,

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Table 2. LC-MS/MS Parameters for the Quantification of Targeted Cytostaticsatic[a,b]

compounds code	R_T (min)	precursor ion (m/z) $[M + H]^+$	product ion (m/z) Q	product ion (m/z) $q_1;q_2$	$CE_{q1;q2}$ (v)	$q_1/Q;q_2/Q$ (%)
AZA	2.99	278.0	142.1	231.9	9;13	25
MET	3.71	455.2	175.0	308.1	45;13	60
IFO	7.13	261.0	92.1	154.0;78.1	28;24;24	66;35
CIP	7.58	261.0	140.0	120.0;106.0	24;20;20	30;42
DOX	10.13	544.2	361.0	85.9	29;57	190
CAP	10.15	360.2	244.0	174.1	9;21	89
PRED	10.41	359.2	147.1	237.1;171	32;24;40	50;55
ETO	10.78	589.2	229.0	185.0	9;49	85
MPA	13.39	321.1	207.1	303.0	21;5	18
BIC	14.70	429.1[c]	255.0	185.0	13;49	67
FLUT	15.24	275.1[c]	202.1	205.0	29;21	44
TAM	15.77	371.7	72.2	129.1;70.2	28;28;50	3;8
PAC	15.84	854.3	104.9	286.1	57;13	45
CYP	16.24	417.2	357.2	279.2;147.1	16;24;28	69;75
MEG	16.30	385.3	325.2	224.1;267.2	12;32;16	100;130

[a]Retention time (RT), selected precursor and product ions (Q, q_1, q_2), collision energies (CE), and ion ratio (q/Q%). [b]Quantification transition (Q): precursor ion > product ion Q. Qualifier transitions (q): precursor ion > product ion q_1 or q_2. [c]$[M + H]^-$.

with target compounds at 10 μg/L of unspiked extracts of low-complex and high-complex samples. ME was calculated according to eq 2.

$$ME = \left(\left(\frac{area_{post\ extracted\ spiked\ sample} - area_{post\ extracted\ sample}}{area_{solvent\ standard} - area_{blank}}\right) - 1\right) \times 100 \quad (2)$$

3. RESULTS AND DISCUSSION

3.1. Sample Treatment Optimization. Given the wide variation in the chemical properties of the target pharmaceuticals, a careful optimization of the SPE procedure is essential for both analyte recovery and the reduction of interfering matrix effects in downstream analysis. The effects of different variables, such as pH, loading volume, cleanup, elution solvents, and preconcentration, were evaluated. The Oasis HLB cartridge was selected because of its high selectivity, retaining a wide range of compounds in acidic, neutral, and basic forms.[29,32]

The pH of the sample is likely to impact the SPE interactions of the selected pharmaceuticals with the cartridges, as many of them can be found as neutral, cationic, anionic, or even zwitterionic species, depending on the pH.[32] Our experiments revealed that most compounds (11 out of the drugs included in this study) showed similar %REC at both pHs (Table SI3). Other compounds such as DOX, MET, and ETO showed different behavior depending on pH conditions. Although DOX and MET increased their recoveries at pH 3 (41 and 27% of increment, respectively), ETO presented a better response without acidification of the sample (8% of REC).

To achieve a compromise in selecting the experimental conditions, a preliminary target screening analysis was conducted in samples from different WWTPs under both pH conditions (pH 6.8−8.6 (Table SI1) and pH 3) to detect the presence of the targeted compounds (DOX, MET, and ETO), and therefore, determine the most convenient conditions. As a result, these three compounds, along with TAM, were detected in our samples. Among these critical compounds, only MET and TAM were detected in most of the samples during screening. Acidification had no apparent effect on the signal intensity of MET; however, it enhanced the signal for TAM by increasing the intensity, reducing baseline fluctuations, and improving the peak shape. Consequently, and in line with a previous study,[20] sample acidification was chosen, as it provided better recovery and reduced signal interference without adversely affecting the detection of other compounds. This approach is recommended for future studies focused on the determination of compounds with similar properties.

Minimizing the analysis time is crucial from a practical standpoint. Hence, the smaller the loading volume and the higher the flow rate, the less time consumed in the sample treatment. The EPA method recommendations and previous studies suggested loading volumes ranging from 250 to 1000 mL while maintaining similar LOQs.[9,21,29,33−35] However, loading a larger volume does not necessarily result in improved recovery, particularly in complex influent matrices,[31] as observed in the recovery of MET, reported by Martin et al.[9] This study demonstrates that using 50 mL for inlet WWTP samples and 100 mL for the outlet is sufficient to reach adequate targeted compound recoveries. Reducing the loading speed from 5 to 2 mL/min did not significantly improve the analyte retention. Therefore, a 5 mL/min flow rate was selected to minimize analysis time.

Elution of analytes was tested with ACN and MeOH, with and without FA, at five combinations of ACN/MeOH including 100:0, 75:25, 50:50, 25:75, and 0:100 ratios. The best results were obtained using a 0:100 ratio and a total volume of 12 mL of eluents, which were applied in four steps of 3 mL. To improve the recovery percentage to at least 70% for all target compounds, four experiments were conducted to evaluate the optimal solvent for the cleanup stage. LC-grade water was found to effectively reduce interference from inorganic substances for CIP and IFO. Furthermore, water containing 5% MeOH or 0.1% FA did not show a reduction of interferences, achieving similar recoveries after the SPE process.

Finally, the filtration of SPE extracts before LC-MS/MS analysis was examined by using acetate, cellulose, and PTFE filters. The results revealed poor performance of acetate

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cellulose filters compared to PTFE according to the methodologies proposed by other authors.[21,31]

3.2. LC-MS/MS Optimization. A D-optimal design of experiments was performed for MS source optimization, including different ranges for drying gas temperature and flow, sheath gas temperature and flow, and nebulizer pressure. The highest pressure (40 psi) and temperature (drying gas 280 and 350 °C sheath gas) were found to provide the highest response. All analytes, except BIC and FLU, showed improved detectability in the positive ESI mode.

Development of MRM transitions for IFO and CIP was crucial since they are structural isomers, and to ensure sufficient selectivity in their detection, two qualifier transitions were selected for each of them (q_1 and q_2). Table 2 includes all of the precursor and product ions selected for MS identification.

Considering the polarities of the targeted drugs, a reversed-phase C18 column was used to achieve efficient separation in a single chromatographic run. Detection by dMRM reduces the requirement for complete baseline separation, since different chemicals rarely share both retention times and transitions. Consequently, mobile phase composition, gradient profile, and flow rate were optimized to achieve adequate chromatographic separation. ACN with 1% FA was found to provide higher resolution and show a better peak shape than MeOH for most analytes, except for 5-FLU and GEM, which elute very closely to the column's dead time (theoretically calculated at 0.75 min) and did not show good retention, resolution, and shape. These compounds present high polarity, and one of the challenges in their analysis is the weak retentions in reversed-phase chromatographic columns.[36] To improve the retention of 5-FLU and GEM, the chromatographic conditions were modified by employing HFBA as an ion-pairing agent instead of formic acid. The addition of HFBA to the mobile phase increased the retention time of GEM from 0.5 to 2.0 min but did not show good results in the case of 5-FLU. Moreover, the ionization of the rest of the compounds with HFBA was less effective compared to ionization with the addition of FA. Based on these facts, 5-FLU and GEM were discarded from the analytical methodology.

3.3. Method Validation. The WWTP influent samples were grouped by K-mean clustering to provide a smaller set of samples reflecting the large variety of existing WWTPs (Figure 2). In this attempt, conductivity, total organic carbon (TOC), and total carbon (TC) were found to be highly correlated (Figure SI2). Flow rate was chosen over equivalent inhabitants to define WWTP size, based on the rationale that it offers greater objectivity.

Clustering provided three groups. Group 1 involved medium-sized WWTPs without hospital discharges and influents with neutral pH. Group 2 included WWTPs with higher flow rates, hospital discharges, and influents with neutral to basic pH. The most heterogeneous group, group 3, included samples from one of the largest WWTPs, the smallest WWTP, and two medium-sized WWTPs, all lacking hospital discharges and with influents showing slightly basic pH.

The results of the validation are presented in Table 3. Eleven analytes showed good linearity ($R^2 \geq 0.99$) from the LOQi level to 50 000 ng/L. BIC, FLUT, TAM, CYP, and MEG showed an upper limit of linearity at 25 000 ng/L (Table RECS). The methodology of grouping WWTP samples by basin is proposed as a novel solution when assessing the load of micropollutants emitted to natural aquatic environments.

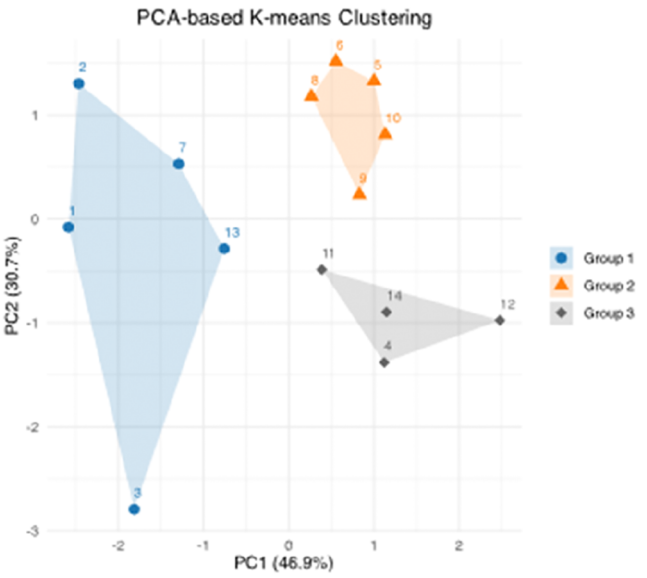

Figure 2. K-means clustering of WWTP influent samples displayed in a principal component analysis score plot. The numbers correspond to WWTP used for clustering (Table SI1).

This approach will allow us to identify hotspots, but it will also save resources.

The RECs of the analytes in the three grouped samples are illustrated in Figure 3. REC values were acceptable (70−120%) for most of the cytostatic compounds at high and low levels in influent samples, with only PAC (142%) and TAM (155%) being slightly out of range. This is in agreement with previous investigations.[37] Similarly, in effluent samples, most of the targeted compounds showed acceptable recoveries with CAP (128%) being the only exception. Most analytes exhibited RSD values below 20%, confirming the accuracy of the method across a wide range of WWTPs. Only the recoveries for CAP and TAM for influents exceeded 20% RSD. For both compounds, an accurate evaluation of the average recovery across the three groups was not feasible due to their high concentrations in certain samples, mainly from group 1 (Table SI 4).

The developed methodology allows us to successfully quantify the presence of a broad compound scope and different concentrations, even at very low concentrations. In effluent samples, MQLs for most compounds ranged from 0.3 to 9.9 ng/L, except for DOX, CYP, and PAC, which showed significantly higher MQLs (20.3, 19.6, and 44.6 ng/L, respectively). Influent samples showed a narrower range of MQLs (0.3−10.3 ng/L). The determined MQLs are similar to those previously reported,[9] although with MQL for TAM being somewhat higher in some influent samples.[24,37]

ME and recoveries can vary significantly depending on the sample type, and this is especially notable in WWTP influent samples. These samples exhibit considerable differences in composition, which can impact the reliability of the analytical results. In this case, dilution of the samples was considered a good alternative to avoid matrix interferences.

This limitation could also be solved using internal standards. Unfortunately, the unavailability of isotopically labeled standards for all compounds and their high cost hampers their use in multiresidue methods. The lack of internal standards is compensated for by validation processes, fulfilling the main SPE goal of accurate recoveries. To conduct recovery studies

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Table 3. LODi, LOQi (Instrumental Limits of Detection and Quantification), MQL, Accuracy (%REC), and Precision (%CV) for Influent and Effluent WWTPs Samples at CF10 (n = 9)[a]

				effluent samples			influent samples		
matrix				%REC ± %CV		MQL (ng/L)	%REC ± %CV		MQL (ng/L)
compounds	LODi (ng/L)	LOQi (ng/L)	linearity range (ng/L)	0.2 μg/L	2 μg/L		2 μg/L	20 μg/L	
AZA	2.8	10	10−50 000	74 ± 2	74 ± 3	1.4	91[b] ± 5	96 ± 1	1.1
BIC	1.0	10	10−25 000	126 ± 7	94 ± 1	0.3	104 ± 6	104 ± 3	0.3
CAP	1.5	10	10−50 000	128 ± 8	128 ± 6	0.4	96[b] ± 5	119 ± 29	0.5
CIP	6.0	20	20−50 000	109 ± 3	109 ± 3	1.8	98[b] ± 2	103 ± 6	2.0
CYP	30.7	150	150−25 000	77 ± 4	68 ± 5	19.6	98[b] ± 19	72 ± 10	15.3
DOX	7.8	150	150−50 000	85 ± 11	88 ± 4	20.3	73 ± 5	79 ± 8	23.7
ETO	33.5	100	100−50 000	87 ± 6	81 ± 20	11.5	78 ± 3	104 ± 3	10.3
FLUT	0.6	10	10−25 000	91 ± 2	71 ± 1	0.2	77[b] ± 7	71 ± 0	0.3
IFO	2.2	10	10−50 000	118 ± 7	117 ± 4	0.8	75 ± 4	116 ± 9	1.3
MEG	11.5	40	40−25 000	100 ± 15	70 ± 7	4.0	106[b] ± 12	72 ± 10	3.8
MET	2.3	10	10−50 000	83[c] ± 5	101[b] ± 5	0.8	102 ± 7	133 ± 5	1.0
MPA	14.3	50	50−50 000	86 ± 4	85 ± 3	5.8	80 ± 4	86 ± 3	6.3
PAC	54.7	500	500−50 000	112 ± 11	100 ± 4	44.6	142[a] ± 15	150 ± 8	35.2
PRED	18.9	100	100−50 000	95 ± 16	81 ± 3	9.9	98[a] ± 9	101 ± 1	9.7
TAM	957	3000	1000−25 000	NR[d]	NR		155[a] ± 66	79 ± 75	517.2

[a]Extended data can be found in Table SI 4. [b]Recovery of cytostatics corresponding to CF1 [c]Recovery of cytostatics corresponding to CF50. [d]NR: Recovery not evaluable.

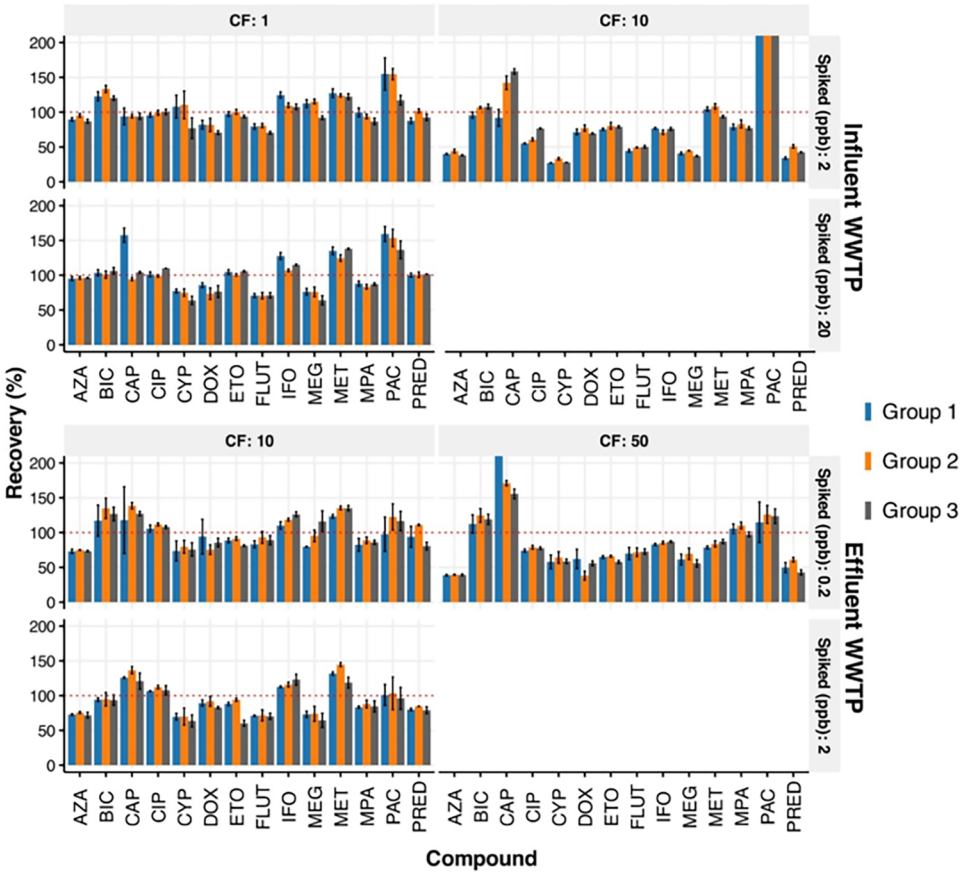

Figure 3. Recoveries of the three grouped matrices for the compounds studied (n = 3). Top: two different CFs (10, 1) from influent matrices spiked at 20 and 2 μg/L; bottom: three different CFs (50, 10) from effluent matrices spiked at 2 and 0.2 μg/L.

reaching the validation objectives, precautionary measures should be adopted, always using controls. In other words, whenever different samples with their corresponding variability are analyzed, it is recommended that recovery quality controls (QC) are introduced to ensure that the established criteria are met. These controls are performed by extracting a representative sample of the pool, spiked with a known concentration of the compounds under study. To obtain the representative sample, the grouping protocol described in Section 2.6 can be performed, or the sample with the highest

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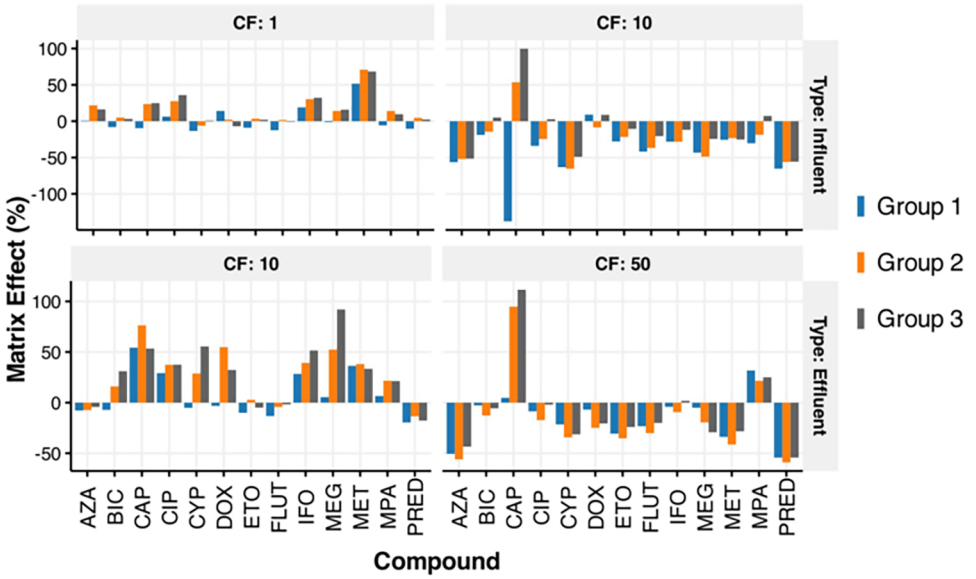

Figure 4. ME of the three grouped matrices for the compounds studied at 10 μg/L. At the top, it corresponds to ME of influent samples with CF 1 and 10; at the bottom, it corresponds to effluent samples with CF 10 and 50.

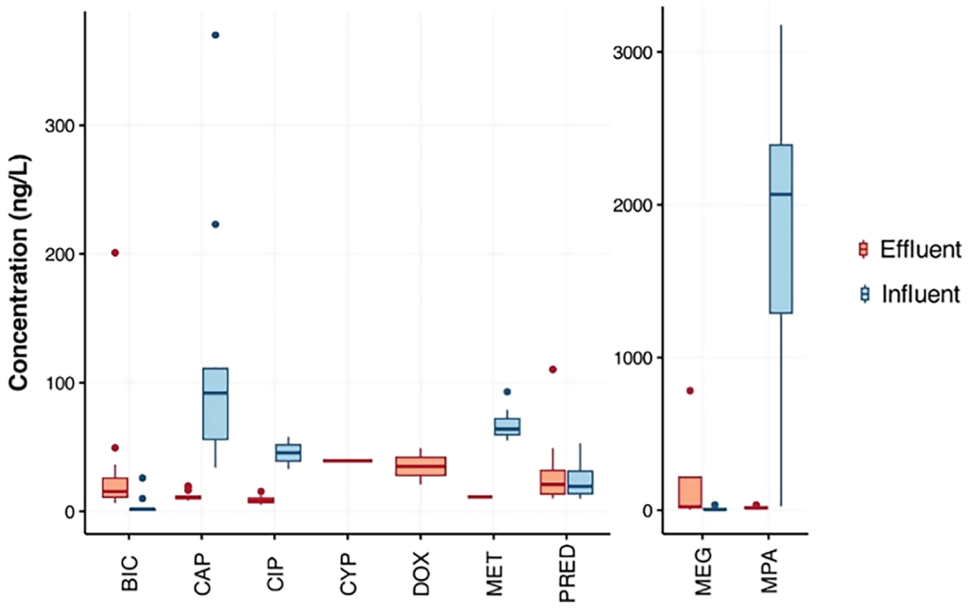

Figure 5. Range of anticancer compound concentration in WWTP samples.

interfering organic load can be selected, to consider the worst scenario.

The RECs were evaluated across the preconcentrated extracts' dilutions to minimize the ME and ensure that all analytes can be detected within the linear range. RECs for 9 analytes increased (20−70%) when using CF1 instead of CF10 in the influents (Figure 3), with AZA, CYP, MEG, and PRED showing notable improvements of more than 50%. Only DOX, ETO, and MPA showed a variation in recovery below 20% when this CF variation was applied. In the effluent samples, CF10 provided an average 30% improvement in recoveries for 10 analytes compared to that of CF50. MPA and BIC were unaffected by interferences, yielding adequate recoveries at both CFs. The recovery of CAP was greatly impacted by the matrix at the CF50. Therefore, CF1 by dilution of influent extract at CF10 and CF10 by dilution of effluent extract at CF50 were selected for the target compounds quantification (Table 3). TAM is not included in Figure 3 due to the high variability in %REC data among the three groups, with some values exceeding the *y*-axis range (Table SI4). This variability suggests that TAM would be a strong candidate for the use of an internal standard.

The ME caused by interference during analysis was also evaluated and calculated by spiking the dilutions of the extracts after SPE to 10 μg/L (Figure 4). In general, analytes showed either enhancement (positive ME) or suppression (negative ME) in the various sample groups.

Notably, the dilution of effluent samples significantly reduced signal suppression, with most analytes exhibiting signal enhancement at a 5-fold dilution (CF10). At this dilution, ME in effluents generally increased signal intensity, whereas influents experienced signal suppression. These

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findings emphasize the need to analyze specific compounds at CF1 in influents, where ME effects are substantially reduced. Furthermore, ME calculations confirmed that CAP is highly susceptible to ME, particularly in more concentrated extracts.

The lack of recoveries observed for most compounds was attributed to matrix interferences during extract analysis rather than inefficiencies in the SPE protocol. Similar patterns were observed in both influent and effluent samples, although the effect of dilution was more pronounced in the latter. Therefore, extract dilution represents a viable strategy for analyzing a wide range of compounds in complex matrices, particularly in samples with high organic loads.

3.4. Wastewater Analysis. Following the development and validation of the analytical method for the most used compounds in oncological treatments, with very broad properties, it was used to quantify the target compounds in the inlet and outlet samples from 14 WWTPs. The most frequently detected compounds, ranked by occurrence, were CAP > BIC > MPA > PRED > CIP. CAP, MET, MPA, and BIC were detected in the influents of all WWTPs (Figure 5). CAP and BIC appeared above the MQL in the effluents from 13 WWTPs, although the WWTPEFF_03 sample remained below the MQL for both compounds. MPA was quantified in 14 influents but only in five of the effluents. Notably, the concentrations of MPA were significantly reduced in effluents compared to those in influents. Among the 15 targeted compounds, five (MET, CYP, DOX, FLUT, and IFO) were detected in either the influent or the effluent. Among these, MET was exclusively found in influent samples (11 samples), while the other four substances were quantified in at least two effluents. Given that the investigated compounds are mostly polar, their removal via evaporation in the WWTP is highly unlikely.[28,38,39]

CAP and BIC were measured in almost all individual samples at ng/L levels. In general, CAP concentrations found in effluents were below 20 ng/L, while those in influents were higher, ranging from 34 to 370 ng/L, which matches the range reported by others.[39−41] Remarkably, CAP showed the highest quantities for both the influent and the effluent from WWTP_05 (28.0 and 5.7 μg/L, respectively), a medium-sized WWTP employing tertiary UV treatment. This compound is used to treat several common types of cancer, including breast and colorectal, among others.[14] Consequently, its detection over a broader range is reasonable and the applied treatment is likely insufficient to reduce CAP to undetectable levels. The greatest concentration of BIC was found in the effluent of WWTP_12 (201 ng/L), while overall values remained stable in most samples within the 1−49 ng/L range. The pK_a (11.8) and log K_{ow} (2.7) values of BIC (Table 1) imply that the solubility in the aqueous matrix is likely to be independent of pH, but BIC may also transfer into sludges.

MPA was quantified in influents from all 14 WWTPs, between 25 and 3178 ng/L, whereas in effluents, only three samples without hospital discharges (35, 16, and 14 ng/L) and one with hospital discharges (13 ng/L) showed a signal above the MQL. These findings are consistent with the consumption and removal rates reported by other authors.[40] Based on these results, it could be inferred that this compound is susceptible to water treatment processes. However, given its log K_{ow} value (>2), adsorption to sludge is likely to play a significant role, which might also occur in the case of FLUT or TAM.[38] Another compound that appears to be affected by water treatment was MET, reaching concentrations ranging from 55 to 93 ng/L across the 11 influent samples, whereas, in effluents, none of the samples exhibited a signal exceeding the LOQi. A similar pattern of MET concentration reduction between the inlet and outlet was documented by Isidori et al.,[39] supporting the assumption that levels of this drug can be significantly reduced in WWTP. The highest PRED amount was recorded in the influent at 53 ng/L (WWTPINF_12), whereas in the corresponding effluent, the PRED occurrence was below LOQi. Another 11 effluent samples gave positive results between 10 and 33 ng/L, which is slightly higher than that reported by Weizel et al.[42] in German effluents (0.2 ng/L).

Furthermore, CIP was measured at quantifiable levels in two influent samples (58 and 33 ng/L), against the 10 effluents with positive results (from 5 to 16 ng/L). For WWTP_09, a significant reduction was observed from 58 to 16 ng/L. CIP is one of the most researched compounds of those included in this study, and most of them agree on persistence after different treatments.[35,43] Considering that CIP appears to be highly affected by ME, its quantification may be hindered by the presence of organic and inorganic interferents. A similar trend was observed for MEG, since in concentrated extracted samples (CF10), the recovery was only 40%, while for CF1, a 100% recovery was achieved. This indicates that both compounds may be significantly affected, promoting CF1 as the most accurate approach for their quantification in influents. MEG was quantified in 4 influent (from 4 to 35 ng/L) and 4 effluent samples (from 4 to 782 ng/L) at similar ranges as previously reported.[20] In the case of MEG, the highest amount was detected in the influent samples from WWTPINF_02, which received hospital wastewater. However, in effluents, it was primarily detected in WWTPs with less stringent treatment processes, such as WWTP_12. According to the pK_a values listed in Table 1, some therapeutic compounds, such as ETO and MEG, are likely to be dissociated,[35] thereby increasing the knowledge about their aqueous mobility will be needed. Lastly, CYP, DOX, FLUT, and IFO were quantified in at least one effluent sample at levels similar to those reported previously.[8,9,13,42]

Although some compounds were not detectable in some individual samples, they were quantified in the grouped samples. For example, IFO was present in grouped influents 1 and 2 at concentrations of 8.0 and 7.2 ng/L, respectively, while its presence in the final grouped effluents varied from 1.3 to 6.2 ng/L over the three groups. FLUT was detected within the 0.3−10 ng/L range in all grouped samples. DOX (44.7, 117.9, and 35.7 ng/L) was also present in the three effluent groups. Lastly, TAM was detected in all samples, with higher concentrations in effluents (84.0−814.9 ng/L) than in influents (16.6−21.7 ng/L) which shows a potential hindered effect of a high concentration of organic matter. Results show the need of improved treatments because, although compounds may be individually undetectable in the WWTP, the addition of emissions can generate high levels in the same basin affecting aquatic ecosystems.[1,3,8,42] The effect of ozonation, as a tertiary advanced treatment, is observed in samples WWTP_2A and WWTP_2B, corresponding to the inlet and outlet of the treatment. Results show a significant reduction of 80% in PRED while CIP, CAP, and BIC were not affected. This agrees with results obtained by others in investigations on advanced oxidation treatments and the difficulties in agreeing on the best treatment for the removal of EC.[44]

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To summarize, the biggest challenge in the simultaneous analysis of a wide range of compounds, with very different structures and properties, in complex real matrices lies in establishing conditions enabling accurate and sensitive analysis of all of them. Although improved accuracy is expected in methods utilizing isotope dilution, the current method employed external calibration to facilitate its use in routine laboratory testing. By grouping samples from several WWTPs within a river basin, we allowed the reduction of the workload. Finally, this targeted methodology can contribute to reliable and reproducible results, facilitating decision-making in wastewater treatment processes when considering contaminants from cancer treatments.

4. CONCLUSIONS

A robust SPE−LC−MS/MS protocol was developed and validated for the simultaneous quantification of 15 cytostatic compounds in complex wastewater matrices, achieving satisfactory accuracy (73−126%) and precision (1−20%), with method quantification limits ranging from 0.3 to 517 ng/L in influents and from 0.3 to 44 ng/L in effluents. Despite poor recoveries for the most polar analytes, which were rejected from the final protocol (5FLU and GEM), the optimized workflow provided reliable separation and quantification of target compounds. Matrix effects were successfully addressed through compound-specific correction factors. Furthermore, the application of sample clustering across WWTPs within a river basin proved effective in both reducing the analytical workload and enhancing the detection of contaminants otherwise undetectable in individual samples. Overall, the proposed methodology represents a reliable and reproducible approach for monitoring cytostatic residues in wastewater, offering valuable support for assessing treatment efficiency related to antineoplastic pharmaceuticals in aquatic environments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.5c00477.

Additional experimental data: wastewater properties, MS/MS optimization details, recoveries of the target pollutants, and extended validation data, including chromatograms and correlation matrix and dendogram for wastewater clustering (PDF)

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CRediT: **Cristina Corpa** conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing - original draft, writing - review & editing; **Josep García Martínez** data curation, methodology, visualization, writing - original draft; **Isabel Lopez-Heras** data curation, formal analysis, methodology, validation, writing - original draft, writing - review & editing; **Peter Spégel** formal analysis, methodology, supervision, writing - review & editing; **M.Concepción Monte** conceptualization, formal analysis, methodology, supervision, writing - original draft, writing - review & editing; **Angeles Blanco** conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing - original draft, writing - review & editing.

Notes

The authors declare no competing financial interest.

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