On-line solid phase extraction-liquid chromatography-tandem mass spectrometry for determination of 17 cytostatics and metabolites in waste, surface and ground water samples

Noelia Negreira, Miren López de Alda*, Damià Barceló

Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18-26, E-08034 Barcelona, Spain

Corresponding Author

* E-mail address: mlaqam@cid.csic.es (Miren López de Alda). Tel.: +34 93 4006100; fax: +34 93 2045904.

Abstract

A fully automated on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) method has been developed for the determination of 13 cytostatics and 4 metabolites in aqueous matrices, including groundwater, surface water, and raw and treated wastewater. On-line SPE is performed by loading 5 mL of water sample at pH 2 through a PLRP-s cartridge. MS/MS is performed with an electrospray (ESI) interface operating in the positive ion mode and registering two selected reaction monitoring (SRMs) transitions per compound. Quantification is carried out by the isotope dilution method using 15 different isotope-labelled compounds, specific for the target analytes, as internal standards (IS). The main advantages of the method are high sensitivity, with limits of determination in groundwater, surface water, and raw and treated wastewater below 5 ng L^{-1} for all compounds except for gemcitabine (6.9-9.3 ng L^{-1}) , temozolomide (26-50 ng L^{-1}), imatinib (80-180 ng L^{-1}) and etoposide (38-65 ng L^{-1}), repeatability, with relative standard deviations (RSDs) in most cases below 15%, and selectivity and reliability of results. The method is also fairly simple and fast, with an analysis time per sample (excluding the manual steps, i.e., sample filtration, pH adjustment, and addition of IS) of 40 min. Application of the method to influent wastewater samples collected daily during eight consecutive days from a wastewater treatment plant (WWTP) from Catalonia showed the presence of methotrexate, ifosfamide, capecitabine, tamoxifen and 6(α)-hydroxypaclitaxel but at fairly low concentrations (up to 43 ng L^{-1}).

Keywords: Cytostatics; Water; Analysis; On-line SPE; LC-MS/MS

1. Introduction

Cytostatic drugs are used in the chemotherapy of oncological patients [1]. The use of chemotherapy began in the 1940s with nitrogen mustards, which are extremely powerful alkylating agents, and antimetabolites. Since the early success of these initial treatments, a large number of additional anticancer drugs have been developed [2]. The Anatomical Therapeutic Classification (ATC) classifies them into five classes: L01A alkylating agents; L01B antimetabolites; L01C plant alkaloids and other natural products; L01D cytotoxic antibiotics and related substances; and L01X other antineoplastic agents [1,3]. These substances act by either inhibiting cell growth or directly killing cells but acting unselectively on both tumour and healthy cells [2,4-5]. Therefore, many antineoplastic agents have cytotoxic, mutagenic, carcinogenic, embryotoxic and/or teratogenic effects [5-7]. The alkylating agents chlorambucil, cyclophosphamide, etoposide, tamoxifen and melphalan have already been classified by the International Agency for Research on Cancer (IARC) as carcinogens in humans (group 1), and carmustin and cisplatin as presumable carcinogens (group 2A) [8].

Occupational exposure of health care workers to cytotoxic drugs has been studied intensively and has resulted in guidelines for the safe handling of these substances in many countries [9]. However, despite high safety standards traces of cytotoxic agents have been found in urine and blood of healthcare professionals [10-11], and monitoring studies in pharmacies and hospitals have revealed that contamination of the workplace occurs frequently [11-13]. Less attention has been paid to the effects of cytostatics on the environment where different sources like emissions from production sites, direct disposal of pharmaceuticals in households, or excretions of patients under medical treatment can contribute to its potential pollution. In fact, some cytostatics have been

48 detected in hospital wastewaters and even influent wastewaters at concentration levels
49 varying from ng L^{-1} to μg L^{-1} [1,3-4,14-19].

50 Consequently, the development of analytical methods for determination of
51 anticancer drugs is of outmost importance. Most of the analytical methods published for
52 environmental samples are limited to individual determinations of the most consumed
53 anticancer drugs: cyclophosphamide and ifosfamide [15,17]. Other authors have
54 published analytical methods for the determination of one or two cytostatic drugs [3-
55 4,16,18,20-22] or various but belonging to the same family [3,16]. However, to get a
56 wider picture of the potentially existing contamination, multi-compound methods
57 addressing the analysis of various drugs from different families are desirable. Yin et al.
58 [19,23] and Martin et al. [24] have developed two analytical methods for the
59 simultaneous determination of 9 and 14 cytostatics, respectively. Both methods used
60 solid phase extraction for preconcentration of the compounds prior to their
61 determination by LC-MS/MS. Although SPE offers considerable advantages, it requires
62 relatively large sample volumes (from 0.3 to 1 L), a moderate consumption (10 – 15
63 mL) of organic solvents for analytes desorption from the cartridge, and possibly further
64 clean-up to compensate for its limited selectivity when applied to wastewater. In recent
65 years, on-line SPE has emerged as a powerful and reliable tool for sample treatment of
66 complex environmental [25] and biota [26] matrices, since it allows reducing most
67 problems associated with off-line sample preparation, such as time-consumption,
68 contamination, procedural errors and risk of low recoveries. Conditioning, washing and
69 elution steps can be performed automatically and some systems also permit to extract
70 one sample while another one is being analysed [27]. To the best of our knowledge, on-
71 line SPE-LC-MS/MS has been only applied to the determination of two cytostatics

(cyclophosphamide and methotrexate), together with other organic contaminants, in drinking and surface water [28]; however, recoveries remained below 70% and the method was not validated in wastewater. Hence, it is important to optimize new analytical methods for the simultaneous determination of different cytotoxic agents. In addition to the parent compound, active metabolites should be included in the methods since these compounds can appear in the environment and might therefore contribute to the biotoxic and mutagenic potential effects in the environment.

In this context, the aim of this work was to develop and validate a multiresidue method based on on-line SPE-LC-MS/MS for determination of 13 cytostatics (gemcitabine, temozolomide, methotrexate, irinotecan, imatinib, ifosfamide, cyclophosphamide, erlotinib, etoposide, doxorubicin, capecitabine, tamoxifen and paclitaxel) and 4 metabolites (hydroxymethotrexate, desmethyl-hydroxytamoxifen, hydroxytamoxifen and hydroxypaclitaxel) in water samples (groundwater, surface water and wastewater). To the best of our knowledge, temozolomide, imatinib, erlotinib, capecitabine, hydroxytamoxifen, desmethyl-hydroxytamoxifen and hydroxypaclitaxel have not been included in any previously optimized method for cytostatics in environmental samples. The analysis of other compounds at the same time than those above, i.e., with the same methodology, was initially attempted but without success due to their very different physical-chemical properties. These compounds are: 5-fluorouracil, vinblastine, vincristine, vinorelbine, carboplatin and oxaliplatin.

2. Experimental

2.1. *Standards and solvents*

All solvents were of HPLC grade and all chemicals were of analytical reagent grade.

Formic acid (98-100%), hydrochloric acid (HCl, 37%), methanol and ultrapure water were purchased from Merck (Darmstadt, Germany), while dimethyl sulfoxide (>99.9%) and sodium hydroxide (98%) were acquired from Aldrich (Milwaukee, WI, USA) and Carlo-Erba (Milan, Italy), respectively.

Standards of cytostatic compounds: cyclophosphamide (CP), ifosfamide (IF), temozolomide (TMZ), methotrexate (MET), hydroxymethotrexate (OH-MET), gemcitabine hydrochloride (GEM), capecitabine (CAP), etoposide (ETP), 6(α)-hydroxypaclitaxel (OH-PAC), doxorubicin hydrochloride (DOX), imatinib mesylate (IMA), erlotinib hydrochloride (ERL), irinotecan hydrochloride trihydrate, tamoxifen citrate (TAM), endoxifen or 4-Hydroxy-N-desmethyl-tamoxifen (OH-D-TAM), and (Z)-4-hydroxytamoxifen (OH-TAM) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany), and paclitaxel (PAC) was supplied by Aldrich at the highest available purity (>99%). The isotopically labelled standards: cyclophosphamide-d_4, ifosfamide-d_4, temozolomide-d_3, methotrexate-methyl-d_3, 7-hydroxymethotrexate-d_3, gemcitabine-$^{13}C,^{15}N_2$ hydrochloride, capecitabine-d_{11}, etoposide-d_3, paclitaxel-d_5, 6α-hydroxypaclitaxel-d_5, N-desmethyl imatinib-d_8, erlotinib-d_6 hydrochloride, irinotecan-d_{10} hydrochloride, 4-hydroxy-N-desmethyl-tamoxifen-d_5, and 4-hydroxy-ethyl-tamoxifen-d_5 were purchased from Santa Cruz Biotechnology.

The selected cytostatics and metabolites are shown in Fig. 1, grouped into six families attending to their mode of action and chemical structure. The parent compounds were selected based on consumption data in the European Union (EU), and the metabolites on the basis of excretion rate and activity [7].

Individual solutions of each compound (ca. 1000 μg mL^{-1}) and a mixture of them (ca. 25 μg mL^{-1}) were prepared in dimethyl sulfoxide (DMSO) and stored in the dark at -20ºC.

Different working standard solutions were made by appropriate dilution in HPLC water and immediately analyzed by LC/MS-MS.

2.2. Safety considerations on cytostatic drugs handling

As cytostatic drugs are highly toxic compounds, their handling requires strict safety precautions in order to guarantee the best possible protection of research workers. All stock solutions were prepared under a biological safety hood with laminar airflow, and an absorbent paper was used to protect the work surfaces. All disposable material that was in contact with tested compounds was treated as hazardous waste.

2.3. Sample pre-treatment

The method was optimized using groundwater, river water, and WWTP effluent and influent. Amber glass bottles were used for sample collection. Water samples were acidified to pH 2 with HCl and were filtered through 1 μm fiberglass filters from Whatman (Fairfield, Connecticut, USA) followed by 0.45 μm nylon membrane filters from Teknokroma (Barcelona, Spain). The extraction of the samples was always carried

out within 24 hours of collection to keep microbial degradation to a minimum. When this was not possible, samples were frozen at -20 °C until analysis.

2.4. On-Line Solid-Phase Extraction

Preconcentration of the samples and chromatographic separation was performed using an automated on-line SPE–LC device Symbiosis™ Pico from Spark Holland (Emmen, The Netherlands). The base of the Symbiosis™ Pico system is a high-end HPLC system with a high performance injector that handles sample volumes from 10 µL up to 10 mL fully automated. This equipment also counts with the Alias™ autosampler that includes positive headspace pressure, extensive wash routines and 2 injection modes, off-line and on-line SPE. Off-line mode was only used in the optimization procedure to assess the recovery by comparing the peak areas obtained in the on-line analyses of spiked waters samples with those obtained from the injection of standard mixtures of the analytes in HPLC water at equivalent concentrations.

Five different 10 mm x 2 mm i.d. disposable trace enrichment cartridges were evaluated for their efficiency in the on-line SPE of cytostatics from water: the polymeric cartridge PLRP-s (crosslinked styrene-dininylbenzene polymer, 15-25 µm particle size), a Hysphere Resin GP 10 cartridge (polydivinylbenzene, 40-90 µm particle size), an Isolute C18 (octadecyl-bonded silica cartridge, end-capped, 40-90 µm), an Isolute HCX (mixed mode, cation exchange, 40-90 µm), all provided by Spark Holland, and an Oasis HLB (macroporous copolymer of divinylbenzene and *N*-vinylpyrrolidone, 30 µm particle size and 10 mm x 1 mm i-d) from Waters Corporation (Milford, Massachusetts, USA).

In the optimized procedure, preconcentration of all samples, aqueous standard solutions, and blanks is performed using PLRP-s cartridges previously conditioned with 1 mL of methanol and 1 mL of water (flow rate 5 mL min^{-1}). Loading of the sample (5 mL) and subsequent washing of the cartridge with 0.5 mL of HPLC water is performed at a flow rate of 1 mL min^{-1}. Upon completion of each SPE protocol, which takes place in the left clamp of the Symbiosis Pico, the cartridge is moved to the right clamp where the trapped analytes are eluted to the LC column with the chromatographic mobile phase. Meanwhile, a new cartridge is placed in the left clamp where preconcentration of the next sample in a sequence takes place. Therefore, SPE is carried out entirely in parallel with the LC-MS/MS run. This kind of configuration allows shortening the cycle time, which in our approach is 40 min/sample.

2.5. LC-MS/MS analysis conditions

LC-MS/MS analyses were carried out connecting in series the Symbiosis™ Pico with a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source from Applied Biosystems-Sciex (Foster City, California, USA). 4000QTrap is controlled by means of the Analyst 1.4.2 Software from Applied Biosystems-Sciex (Foster City, California, USA) and a companion software appendix for controlling the Symbiosis™ Pico from Spark Holland (Emmen, The Netherlands).

Chromatographic separation of the cytostatic drugs was performed on a reversed-phase column Purospher STAR RP-18e (125 x 2 mm, 5 µm particle size) from Merck, maintained at 25 ºC. Ultrapure water (A) and methanol (B), both containing 0.1% of formic acid, were employed as mobile phase (flow-rate 0.2 mL min^{-1}). Under final

183 optimized conditions, compounds were separated using the following gradient: 0–1 min,
184 5% B; 2 min, 20% B; 12 min, 80% B; 25–30 min, 100% B; 35–40 min, 5% B.

185 The mass spectrometer was operated using positive ESI mode under the following
186 optimized conditions: curtain gas, 10 V; source temperature, 700ºC; nitrogen collision
187 gas, high; ion spray voltage, 4000 V; ion source gases GS1 and GS2 40 and 60 V,
188 respectively. Data acquisition was performed in the selected reaction monitoring (SRM)
189 mode, recording the transitions between the precursor ion and the two most abundant
190 product ions for each target analyte. Optimized MS/MS ion transitions for each
191 compound are detailed in Table 1.

192

193 **3. Results and discussion**

194 *3.1. Optimization of on-line SPE parameters*

195 *3.1.1. Preliminary experiments*

196 In the initial steps of this study, extraction experiments were carried out in amber
197 vessels which contained 5 μg L^{-1} of spiked cytostatics and TPs (the percentage of
198 DMSO was lower than 0.5%) in HPLC water. The sample extraction volume was 5 mL
199 and after loading the sample, the cartridge was washed with 0.5 mL of HPLC water.

200 The most important parameter to be evaluated in the optimization of a new SPE
201 procedure is the type of sorbent. Its selection depends basically on the nature of the
202 matrix and the physical-chemical properties of the target analytes. Fig. 2 shows the
203 recovery percentages obtained with all five cartridges tested by on-line SPE for
204 triplicate assays. Extraction efficiencies were calculated from the peak areas obtained in

205 the on-line analysis of the water samples as percentages of the peak areas obtained in
206 the direct chromatographic injection (10 μL) of equivalent amounts of the standard
207 mixtures in HPLC water (percentage of DMSO ≤ 0.5%).

208 The GP cartridge showed poor repeatability for some compounds, the HCX and
209 the C18 cartridges yielded poor recoveries also for some compounds, and Oasis HLB
210 and PLRP-s were the preferred ones for most analytes.

211 *3.1.2. Multilevel optimization of SPE conditions*

212 The efficiency of SPE methods is affected by a considerable number of factors, which
213 are sometimes correlated. A strategy based on the use of a multi-level experimental
214 design was used to assess the effects of cartridge, sample volume and pH on the
215 performance of the SPE process, and search for the optimal extraction conditions with a
216 minimum effort and cost. Low and high values for each of these parameters are given in
217 Table 2. Previous assays showed better efficiencies using PLRP-s and Oasis HLB as
218 sorbents operating at room temperature; therefore, both cartridges were used in the
219 design. The spiked level was 5 μg L^{-1}. HCl and sodium hidroxide were used for pH
220 adjustment.

221 Peak areas obtained for each compound in the 18 extractions involved in the
222 above design were compared with those obtained from the injection of standards
223 mixtures, and they were used as variable responses. Standardized values for main
224 effects corresponding to each factor were calculated with the Statgraphics Centurion
225 XV software (Manugistics, Rockville, MD, USA). Fig. 3 shows the Pareto Charts for
226 the compounds that resulted more affected for the conditions of the design. The
227 metabolites OH-MET, OH-PAC, OH-D-TAM and OH-TAM followed the same

228 behaviour than the corresponding parent drugs MET, PAC and TAM. The length of
229 plotted bars is proportional to the change in the response of a given compound when the
230 associated factor varies from the low to the high level within the domain of the design.
231 A positive sign indicates an increase in the observed response, whereas a negative value
232 shows the opposite effect. The blue vertical lines correspond to the statistic significance
233 limit, established for a 95% confidence level. The pH of the water samples showed a
234 negative effect on the efficiency of the extraction step for almost all compounds being
235 statistically significant for MET, IMA, DOX, TAM, OH-TAM and OH-D-TAM. The
236 sample volume played a negative effect and it was statistically significant for the most
237 polar species (GEM and TMZ), which are eluted while their extraction takes place. The
238 effect of the sorbent (PLRP-s and Oasis HLB) was not statistically significant for a 95%
239 confidence level but PLRP-s is preferred for most compounds. Two-factor interactions
240 played influence on the SPE process; therefore additional experiments were carried out
241 to corroborate the result. Fig. 4 shows the results for HPLC-water at pH 2 and 6, spiked
242 at 5 $\mu g\ L^{-1}$, using the PLRP-s cartridge and 5 mL of sample in triplicate. In other series
243 of experiments 5 mL and 10 mL of HPLC water adjusted to pH 2 were compared in
244 triplicate (see Fig. 5). As it can be seen in the above Figures, some compounds (IMA,
245 TAM, OH-D-TAM and OH-TAM) were not efficiently extracted at pH 6 (Fig. 4), and 5
246 mL of sample extraction volume presented better recoveries than 10 mL (see Fig. 5).
247 The most polar compounds (GEM and TMZ) presented a very low response with both 5
248 and 10 mL. However, if the sample extraction volume is reduced, the extraction
249 efficiency of the rest of compounds becomes worse; therefore 5 mL adjusted at pH 2
250 and extracted with a PLRP-s cartridge were selected as optimal conditions for further
251 experiments.

252 The washing step was not optimized due to the high polarity of some of the target
253 compounds. Polar species can be easily eluted from the cartridge with water or if the
254 content of methanol is increased. So, 0.5 mL of water was considered to be the optimum
255 volume to wash the cartridge without losing the analytes and was therefore selected for
256 all experiments.

257 *3.2. Method performance*

258 The method performance was evaluated through estimation of the linearity,
259 repeatability, accuracy and sensitivity of the method.

260 Quantification, based on peak areas, was performed by the isotope dilution
261 method. For each target analyte, except for DOX and TAM, isotope-labelled analogues
262 were available and were thus used as IS (see section *Standards and solvents*). In the
263 absence of appropriate isotopically labelled IS for DOX and TAM their quantification
264 was performed with the closely eluting compounds erlotinib-d6 hydrochloride and 4-
265 hydroxy-ethyl-tamoxifen-d5, respectively.

266 The linearity of the method was investigated with standards prepared in HPLC
267 water at eight different concentrations, from 1 ng L^{-1} (or the limit of quantification if
268 higher) to 5000 ng L^{-1} (1, 5, 10, 50, 100, 500, 1000 and 5000 ng L^{-1}). The concentration
269 of the IS was in all cases 500 ng L^{-1}. Within the above range, both the SRM1 and the
270 SRM2 signals versus the concentration of each analyte fitted a linear model with R^2
271 values higher than 0.99 for all compounds (see Table 3).

272 The method limits of detection (LODs) and quantification (LOQs) were
273 experimentally estimated from the online analysis of spiked HPLC water (lowest level

274 included in the calibration curve) as the concentration of analyte giving a signal-to-noise
275 ratio (S/N) of 3 and 10, respectively. Table 3 shows the method LODs and limits of
276 determination (LDet, minimum concentration of a compound that can be quantified
277 (>LOQ, SRM1) and confirmed (>LOD, SRM2)). LODs were in the picogram per liter
278 range for all compounds except IMA (22 ng L^{-1}) and ETP (3.0 ng L^{-1}). These
279 comparatively higher LODs for IMA and ETP are the result of an inefficient ionization
280 in the ESI interface. Meanwhile, the limits of determination (LDets) varied between 0.3
281 and 3 ng L^{-1} for all compounds except GEM (6.9 ng L^{-1}), TMZ (21 ng L^{-1}), IMA (75 ng
282 L^{-1}) and ETP (38 ng L^{-1}). Due to the similarity of responses obtained with the two SRM
283 transitions selected for quantification and confirmation of each analyte (SRM1/SRM2
284 ratio lower than 7 for all compounds except TMZ (24.3), ETP (26.0) and OH-D-TAM
285 (15.8)), the LDets coincide with the LOQs in most instances and remain fairly low.

286 The precision of the method was evaluated for n=5 extractions of HPLC water
287 fortified at three different concentrations: 20, 500 and 5000 ng L^{-1}. Relative standard
288 deviations (RSDs) were in all cases below 15%, with the single exception of the 20%
289 RSD obtained for IMA when fortified at 500 ng L^{-1} (see Table 3). This satisfactory
290 repeatability is possible with automated procedures such as that described here where
291 manipulation of the sample is reduced to its filtration, pH adjustment, and addition of
292 IS.

293 Absolute recoveries calculated by comparing with the standard injected in off-line
294 mode were above 70% for all compounds except GEM, TMZ, IMA and ETP (see Table
295 3). Relative recoveries calculated with respect to the IS were within the margin 100 ±
296 30% for all compounds except IMA (58%).

297 The influence of matrix effects in quantitative LC-MS/MS analysis is a widely
298 observed and studied phenomenon. In order to evaluate the degree of ion suppression or
299 enhancement for each target compound, matrix effects in different water matrices
300 (groundwater (GW), surface water (SW), wastewater influent (WWI) and wastewater
301 effluent (WWE)) were evaluated by comparing the peak areas of the analytes in spiked
302 real samples (after subtracting the peak areas corresponding to the native analytes
303 present in the sample) with those obtained in spiked HPLC water. Fig. 6 shows the
304 results obtained for the samples spiked at 500 ng L^{-1} (n=5). In the case of GW, the
305 recoveries were between 86 and 119%; however, in the other, more complex matrices
306 (SW and WW) a reduced response was observed for some compounds. The reduction in
307 the efficiency of the ionization of the target species in the more complex matrix, WWI,
308 varied between 10% for MET and 73% for DOX, while GEM, OH-PAC and PAC
309 showed some signal enhancement. It is also interesting to note that the results in the SW
310 sample are fairly similar to those of the WW samples, which is due to the origin of the
311 SW sample: a highly polluted Mediterranean river localized in the NE of Spain
312 (Llobregat). On the other hand, for the purpose of evaluating the eventual correction
313 and/or minimization of matrix effects through sample dilution the aqueous matrices
314 were diluted 1:1 with HPLC water. For OH-MET, IMA, IRI and ETP, dilution of the
315 samples led to a reduction of the signal suppression by about 20%, but for most
316 compounds the problem was not solved. Therefore, the use of isotopically labelled
317 compounds for quantification is nearly indispensable in order to obtain accurate results
318 in complex matrices.

319 Table 4 shows the recoveries of the method for the four matrices at three
320 fortification levels, 20, 500 and 5000 ng L^{-1} (n=5), after correcting the responses of the

analytes with the corresponding IS. Corrected recoveries ranged from 72 ± 3% to 119 ± 5% for all compounds.

The repeatability of the method was also evaluated in the four aforementioned matrices, and the results obtained showed good repeatability, with relative standard deviations (RSDs) in most instances below 15%, even in the most complex matrix (WWI) (see Table 4).

As regards the sensitivity, Table 5 lists the method LODs and LDets in each matrix. As it can be seen, the LODs were between 0.2 and 1.6 ng L^{-1} and the LDets between 0.4 and 5.0 ng L^{-1}. The exceptions were GEM (with LODs between 0.2 and 0.7 ng L^{-1} and LDets between 6.9 and 9.3 ng L^{-1}), TMZ (with LODs between 0.8 and 1.1 ng L^{-1} and ~~LDtes~~ LDets between 26 and 50 ng L^{-1}), IMA (with LODs between 24 and 54 ng L^{-1} and ~~LDtes~~ LDets between 80 and 180 ng L^{-1}), and ETP (with LODs between 3.0 and 19.5 ng L^{-1} and ~~LDtes~~ LDets between 38 and 65 ng L^{-1}), i.e., the compounds presenting the worst SPE efficiency. Fig. 7 shows, for illustration, a chromatogram of a groundwater sample spiked with the compounds at 20 ng L^{-1}.

Overall, the method limits of determination obtained in wastewater are lower or in the same range of those reported for by other authors [15-16,23-24]. There are no method detection limits reported for TMZ, IMA, ERL, CAP, OH-TAM, OH-D-TAM and OH-PAC in environmental samples.

Finally, for positive confirmation of the presence of a compound in a sample, the LC retention of the compound in the sample must match that of the standard with a margin of ± 2%, and its SRM1/SRM2 ratio cannot deviate more than 20-50% (depending on the SRM1/SRM2 value) from the ratio in the standard [29].

3.3. Application to real water samples

As a part of the validation procedure, the method developed was applied to the analysis of the target analytes in various wastewater samples collected daily during 8 consecutive days (in April 2012) from the inlet of a WWTP located in Catalonia. Time-proportional sampling, collecting 50 mL of sample every 10 min, for a daily total sample volume of 7.2 L, was carried out with the help of an ISCO 6172 FR Stationary system (Instrumentación analítica, El Prat de Llobregat, Barcelona, Spain). Upon collection the sample was homogenized by manual agitation and an aliquot (1 L) was transferred to an amber PET bottle and transported to the laboratory. During collection and during transport the samples were maintained refrigerated at 4 °C and protected from light. Once at the laboratory the samples were filtered and subsequently stored at -20 °C until analysis.

At the time of analysis, quality control (QC) samples (HPLC water spiked with the analytes at 100 ng L^{-1}) were run in between samples. Potential contamination problems were evaluated with procedural blanks (plain HPLC water).

The results obtained (see Table 6) showed the presence of CAP and MET in all samples at concentrations between 2.1 ng L^{-1} for MET and 30 ng L^{-1} for CAP. IF and TAM were also found in several samples at concentrations up to 43 and 17 ng L^{-1}, respectively. OH-PAC was detected in only one sample at a level of 4.4 ng L^{-1}, while the rest of compounds remained below the quantification limits reported in Table 5.

IF had been previously found in wastewater samples from Germany at levels similar to those reported in the present study [15], and at considerably higher concentrations in a hospital effluent from China where IF and MET reached values around 11000 and 3000 ng L^{-1}, respectively [19]. On the other hand, to the best of the authors' knowledge, this study constitutes the first evidence of the presence of CAP and TAM in water samples.

4. Conclusions

The method developed, based on on-line SPE-LC-MS/MS, allows the simultaneous multi-analyte determination of most the target compounds at the pg or low ng L^{-1} level in GW, SW, WWE and WW. Some compounds are affected by matrix ionization effects; hence, the use of isotopically labelled compounds as IS for accurate quantification is required.. On the other hand, the performance of the SPE process entirely in parallel with the LC-MS/MS run allows to achieve analysis times per sample of only 40 min). The analysis of composite wastewater samples from the inlet of a WWTP showed the presence of only 5 of the compounds investigated (MET, IF, CAP, TAM and OH-PAC) and at fairly low concentrations (between 2.1 and 43 ng L^{-1}). However, health effects cannot be discarded. For this reason, sensitivity is of utmost importance. To the best of the authors' knowledge, this method constitutes the first multiresidue method based on on-line SPE developed for the determination of cytostatics in the aquatic environment. Moreover, temozolomide, imatinib, erlotinib, capecitabine, hydroxytamoxifen, desmethyl-hydroxytamoxifen and hydroxypaclitaxel have not been included in previously optimized methods for environmental samples.

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457 **Captions to figures**

458 **Fig. 1.** Structures and log K_{ow} of the target compounds.

459 **Fig. 2.** Comparison of the recovery percentages and corresponding standard deviations
460 obtained for the various target analytes in the replicate (n = 3) on-line SPE-LC-MS/MS
461 analysis of spiked (5 μg L^{-1}) HPLC water with different SPE cartridges (extraction
462 volume 5 mL, wash volume 0.5 mL).

463 **Fig. 3.** Standardized Pareto chart showing the main effects of cartridge, sample volume
464 and pH on the performance of the extraction step for the compounds that resulted more
465 affected by the conditions tested in the design.

466 **Fig. 4.** Influence of the sample pH on the on-line SPE efficiency (PLRP-s cartridge, 5
467 mL sample volume, wash volume 0.5 mL, n=3).

468 **Fig. 5.** Comparison of the recovery percentages and corresponding standard deviations
469 obtained for the various target analytes in the replicate (n = 3) on-line SPE-LC-MS/MS
470 analysis of 5 and 10 mL of spiked (5 μg L^{-1}) HPLC water.

471 **Fig. 6.** Matrix effects in groundwater, surface water and wastewater (effluent and
472 influent).

473 **Fig. 7.** SRM chromatograms corresponding to the analysis of a groundwater sample
474 spiked with the analytes at 20 ng L^{-1}.

Figure

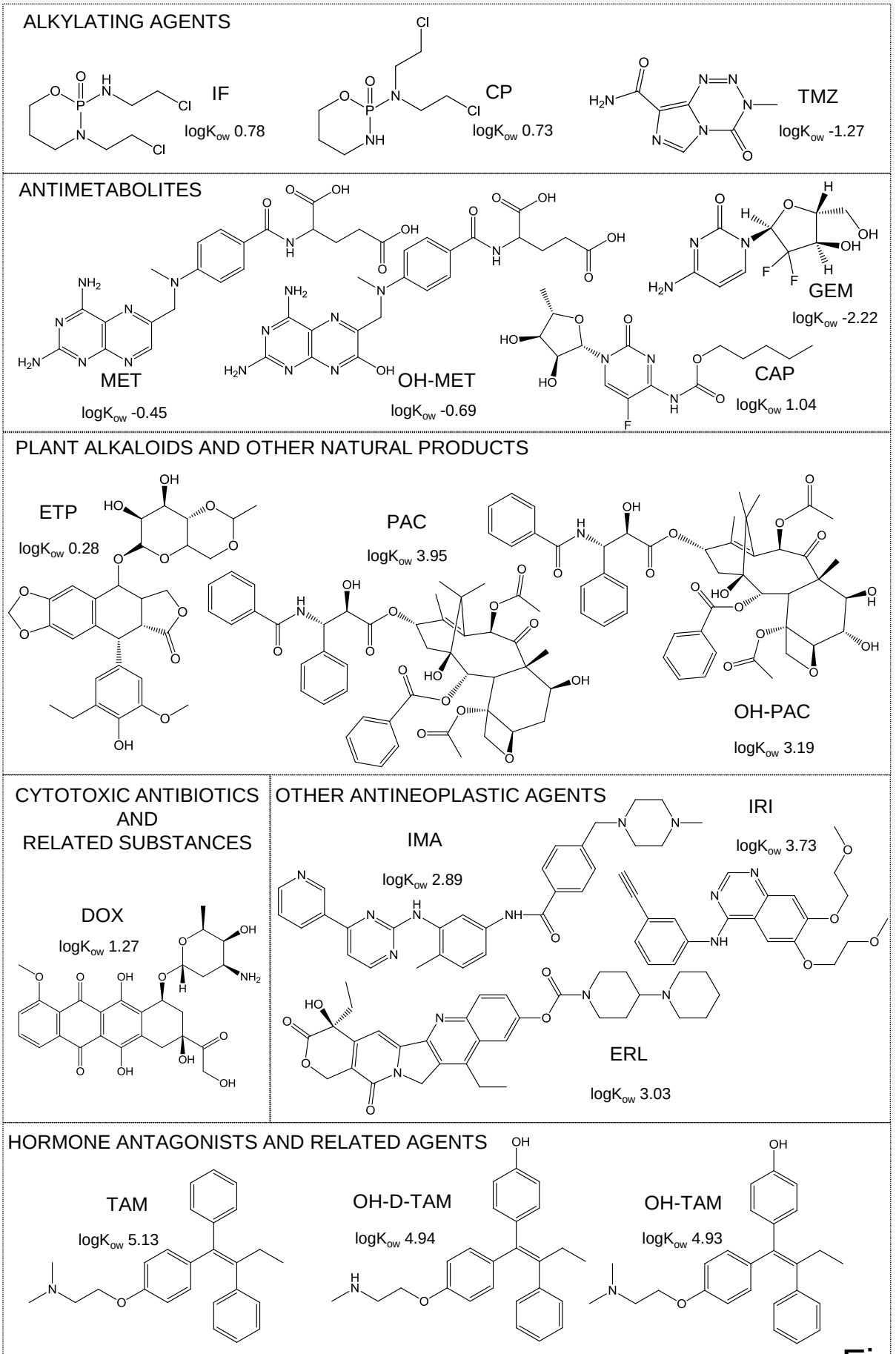
ALKYLATING AGENTS
IF
logK$_{ow}$ 0.78
CP
logK$_{ow}$ 0.73
TMZ
logK$_{ow}$ -1.27
ANTIMETABOLITES
MET
logK$_{ow}$ -0.45
OH-MET
logK$_{ow}$ -0.69
GEM
logK$_{ow}$ -2.22
CAP
logK$_{ow}$ 1.04
PLANT ALKALOIDS AND OTHER NATURAL PRODUCTS
ETP
logK$_{ow}$ 0.28
PAC
logK$_{ow}$ 3.95
OH-PAC
logK$_{ow}$ 3.19
CYTOTOXIC ANTIBIOTICS AND RELATED SUBSTANCES
DOX
logK$_{ow}$ 1.27
OTHER ANTINEOPLASTIC AGENTS
IMA
logK$_{ow}$ 2.89
IRI
logK$_{ow}$ 3.73
ERL
logK$_{ow}$ 3.03
HORMONE ANTAGONISTS AND RELATED AGENTS
TAM
logK$_{ow}$ 5.13
OH-D-TAM
logK$_{ow}$ 4.94
OH-TAM
logK$_{ow}$ 4.93

Fig. 1

Figure

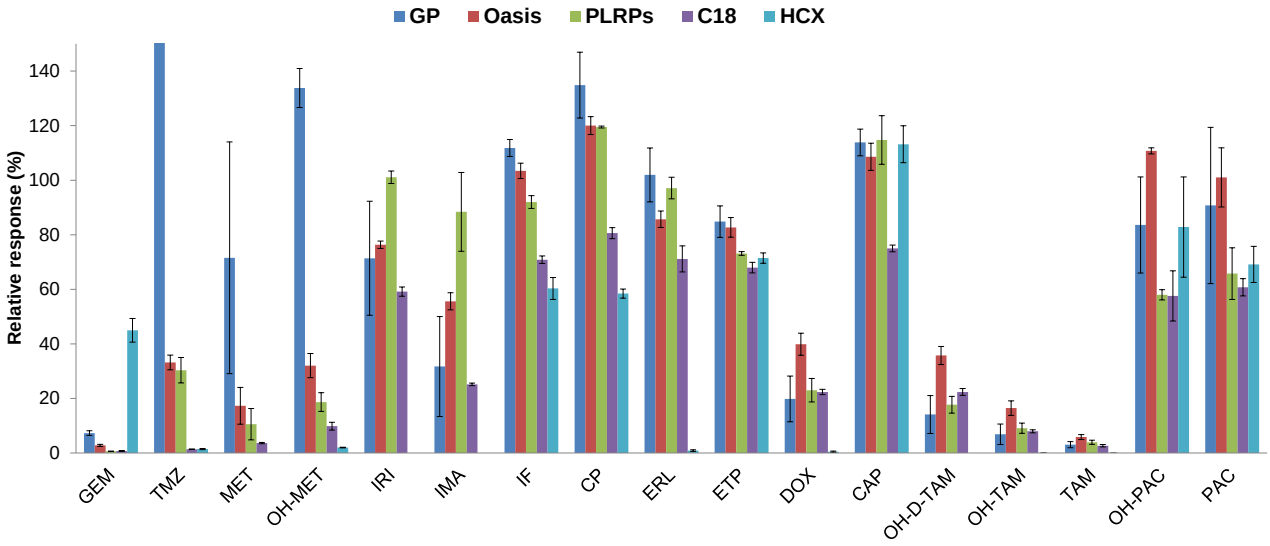
GP
Oasis
PLRPs
C18
HCX
Relative response (%)
0
20
40
60
80
100
120
140
GEM
TMZ
MET
OH-MET
IRI
IMA
IF
CP
ERL
ETP
DOX
CAP
OH-D-TAM
OH-TAM
TAM
OH-PAC
PAC

Fig. 2

Figure

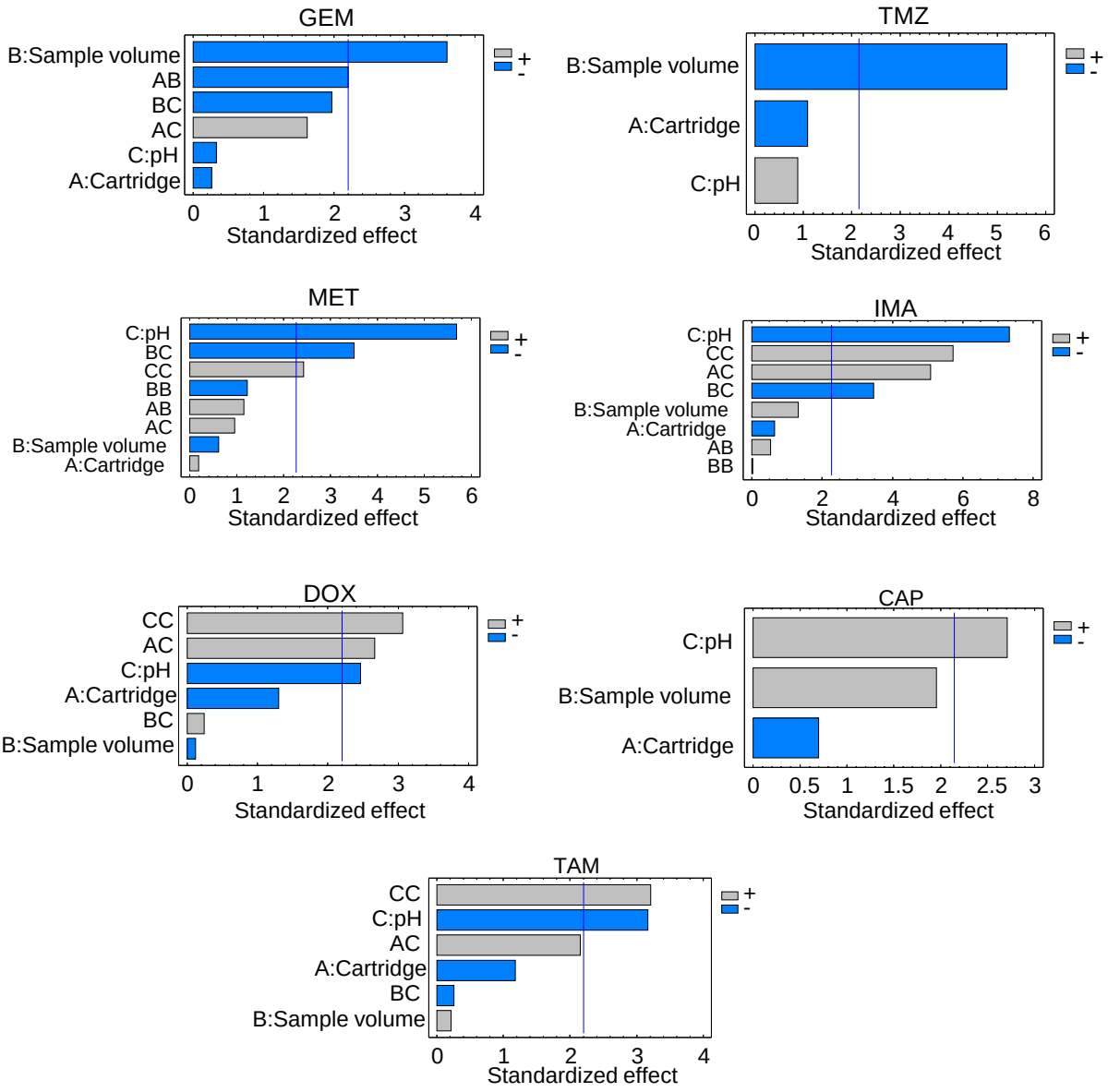
GEM
B:Sample volume
AB
BC
AC
C:pH
A:Cartridge
0 1 2 3 4
Standardized effect
+
-
TMZ
B:Sample volume
A:Cartridge
C:pH
0 1 2 3 4 5 6
Standardized effect
MET
C:pH
BC
CC
BB
AB
AC
B:Sample volume
A:Cartridge
0 1 2 3 4 5 6
Standardized effect
IMA
C:pH
CC
AC
BC
B:Sample volume
A:Cartridge
AB
BB
0 2 4 6 8
Standardized effect
DOX
CC
AC
C:pH
A:Cartridge
BC
B:Sample volume
0 1 2 3 4
Standardized effect
CAP
C:pH
B:Sample volume
A:Cartridge
0 0.5 1 1.5 2 2.5 3
Standardized effect
TAM
CC
C:pH
AC
A:Cartridge
BC
B:Sample volume
0 1 2 3 4
Standardized effect

Fig. 3

Figure

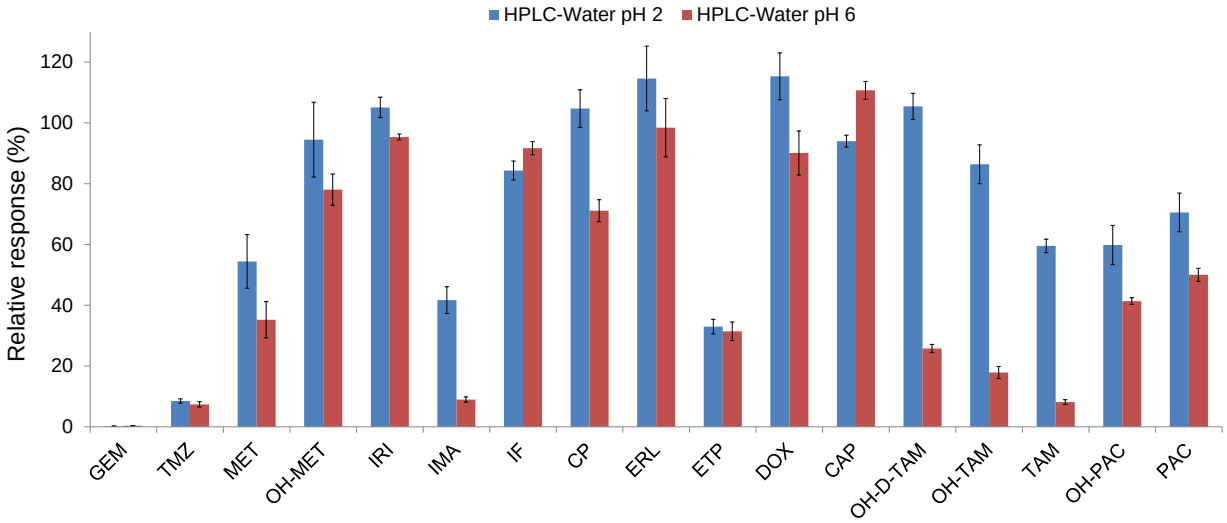
HPLC-Water pH 2
HPLC-Water pH 6
Relative response (%)
0
20
40
60
80
100
120
GEM
TMZ
MET
OH-MET
IRI
IMA
IF
CP
ERL
ETP
DOX
CAP
OH-D-TAM
OH-TAM
TAM
OH-PAC
PAC

Fig. 4

Figure

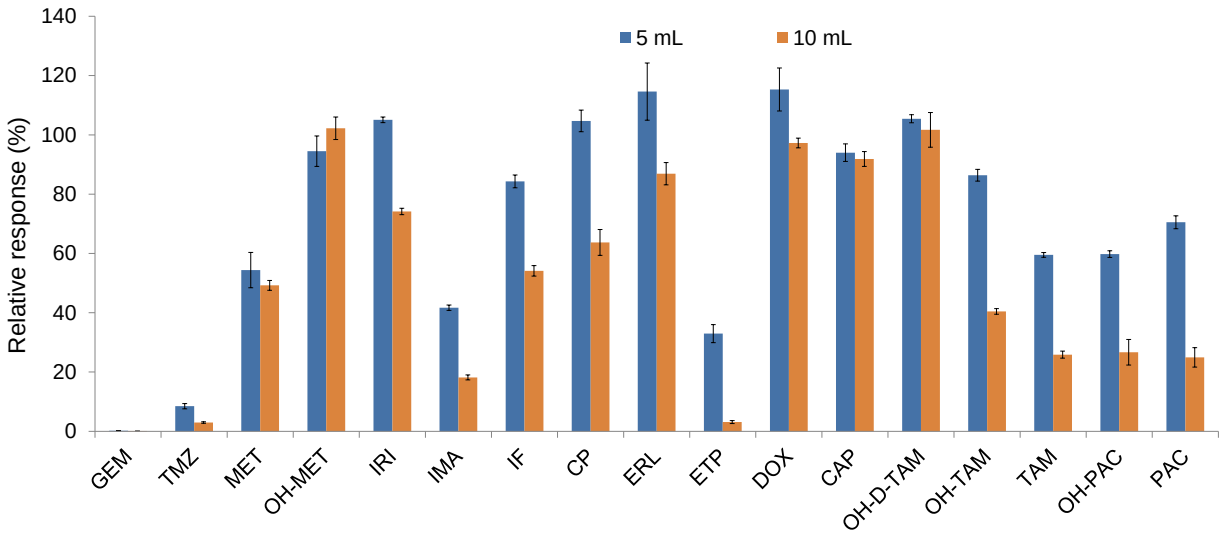
140
120
100
80
60
40
20
0
Relative response (%)
5 mL
10 mL
GEM
TMZ
MET
OH-MET
IRI
IMA
IF
CP
ERL
ETP
DOX
CAP
OH-D-TAM
OH-TAM
TAM
OH-PAC
PAC

Fig. 5

Figure

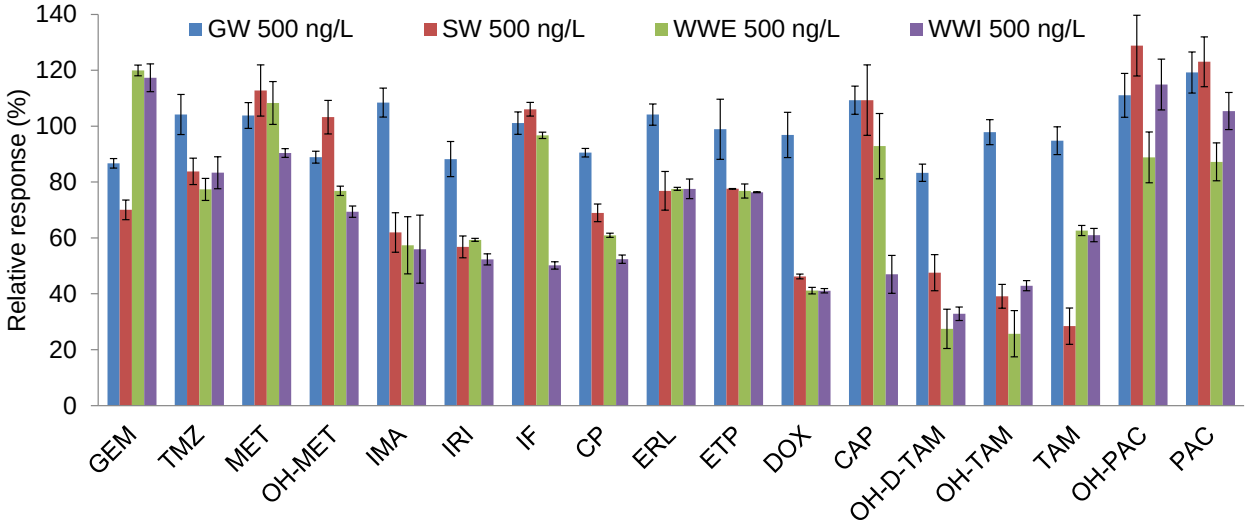
GW 500 ng/L
SW 500 ng/L
WWE 500 ng/L
WWI 500 ng/L
Relative response (%)
140
120
100
80
60
40
20
0
GEM
TMZ
MET
OH-MET
IMA
IRI
IF
CP
ERL
ETP
DOX
CAP
OH-D-TAM
OH-TAM
TAM
OH-PAC
PAC

Fig. 6

Figure

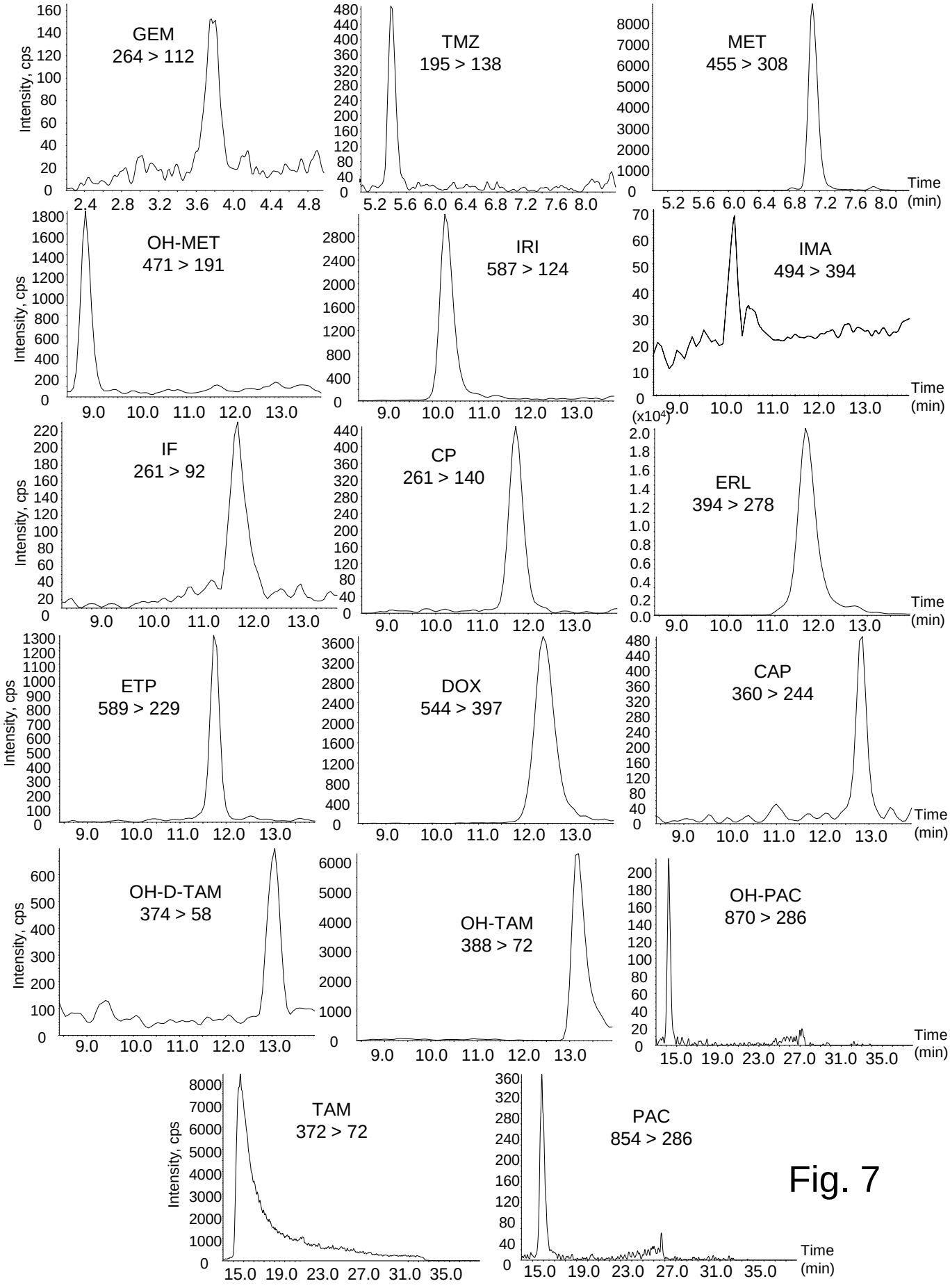
GEM
264 > 112
TMZ
195 > 138
MET
455 > 308
OH-MET
471 > 191
IRI
587 > 124
IMA
494 > 394
IF
261 > 92
CP
261 > 140
ERL
394 > 278
ETP
589 > 229
DOX
544 > 397
CAP
360 > 244
OH-D-TAM
374 > 58
OH-TAM
388 > 72
OH-PAC
870 > 286
TAM
372 > 72
PAC
854 > 286
Intensity, cps
Time (min)
(x10⁴)

Fig. 7

Tables

Table 1
Specific SRM Conditions for Determination of Cytostatics.

Compound	t_R[a] (min)	Seg[b]	DP[c] (V)	CE[d] (eV)	MS/MS transition	Corresponding IS	SRM ratio (SRM1/SRM2)
GEM	3.6	1	71	25	**264.2 > 112.0**	**267.0 > 115.0**	6.8 ± 0.9
				63	264.2 > 95.0	267.0 > 97.0	
TMZ	5.4	2	26	9	**195.0 > 138.0**	**197.8 > 138.0**	24.3 ± 2.5
				13	195.0 > 67.2	197.8 > 54.9	
MET	7.2	2	91	33	**455.2 > 308.2**	**458.2 > 311.1**	1.8 ± 0.8
				59	455.2 > 175.1	458.2 > 175.1	
OH-MET	8.7	3	36	37	**471.1 > 191.1**	**474.0 > 327.1**	1.1 ± 0.1
				15	471.1 > 324.2	474.0 >191.0	
IMA	10.9	3	116	37	**494.3 > 394.2**	**488.2 > 394.2**	2.9 ± 0.3
				35	494.3 > 217.2	488.2 > 211.1	
IRI	10.2	3	66	51	**587.4 >124.1**	**598.2 > 133.1**	1.2 ± 0.1
				57	587.4 >167.2	598.2 > 177.2	
IF	10.7	3	81	35	**261.1 > 92.0**	**266.0 > 157.0**	1.3 ± 0.2
				31	261.1 > 154.0	266.0 > 187.0	
CP	11.2	3	86	33	**261.1 > 140.0**	**264.9 > 140.0**	2.4 ± 0.2
				25	261.1 > 106.1	264.9 > 106.0	
ERL	11.2	3	81	45	**394.2 > 278.1**	**400.2 > 278.0**	1.5 ± 0.2
				33	394.2 > 336.3	400.2 > 339.1	
ETP	11.5	3	71	15	**589.0 > 229.0**	**592.3 > 229.0**	26.0 ± 2.1
				10	589.0 > 185.0	592.3 > 185.1	
DOX	12.6	3	81	17	**544.3 > 397.1**	**400.2 > 278.0**	3.4 ± 0.2
				37	544.3 > 361.0	400.2 > 339.1	
CAP	12.9	3	101	17	**360.2 > 244.2**	**371.0 > 255.2**	2.2 ± 0.2
				29	360.2 > 174.1	371.0 > 175.0	
OH-D-TAM	13.1	3	56	45	**374.1 > 58.1**	**379.1 > 58.0**	15.8 ± 1.6
				37	374.1 > 129.1	379.1 >228.1	
OH-TAM	13.2	3	91	51	**388.2 > 72.1**	**393.1 > 72.0**	6.9 ± 0.6
				89	388.2 > 44.1	393.1 > 45.0	
TAM	14.5	4	91	49	**372.3 > 72.1**	**393.1 > 72.0**	6.3 ± 0.8
				91	372.3 > 44.1	393.1 > 45.0	
OH-PAC	14.7	4	111	23	**870.5 > 286.1**	**876.4 > 291.1**	2.6 ± 0.3
				15	870.5 > 525.4	876.4 > 526.2	
PAC	15.0	4	61	95	**854.5 > 105.1**	**860.0 > 105.0**	0.7 ± 0.2
				25	854.5 > 286.2	860.0 > 291.1	

[a] Retention time (min). [b] Segment. [c] Declustering potential. [d] Collision energy.

Table 2

Experimental Domain of the Multi-level Design.

Factor	Level		
	Low (-)	Medium	High (+)
A: Cartridge	PLRP-s	-	Oasis HLB
B: Sample volume (mL)	1	5	20
C: pH	2	6	8

Table 3

Quality Control Parameters of the Analytical Method: Linear Estimation Coefficients (r^2), LOD, Limits of Determination (LDet), Repeatability (RSD), and Absolute (AR) and Relative Recoveries (RR) in HPLC Water.

Compound	Linearity r^2	LOD[a] (ng L^{-1})	LDet[b] (ng L^{-1})	Repeatability (%) n=5			AR[d] (%)	RR[e] (%)
				20[c] ng L^{-1}	500[c] ng L^{-1}	5000[c] ng L^{-1}		
GEM	0.9999	0.3	6.9	8.7	7.6	14	0.13 ± 0.02	106 ± 9
TMZ	0.9934	0.7	21	6.2	7.5	9.0	18 ± 4	110 ± 6
MET	0.9970	0.1	0.5	9.9	4.2	2.9	99 ± 12	116 ± 7
OH-MET	0.9995	0.2	0.7	8.9	6.2	1.7	111 ± 5	97 ± 7
IRI	0.9996	0.1	0.4	7.2	1.2	2.5	91 ± 2	93 ± 7
IMA	0.9945	22	75	-	20	10	54 ± 15	58 ± 9
IF	0.9996	0.2	0.8	3.8	2.9	14	119 ± 4	81 ± 3
CP	0.9998	0.1	0.4	2.3	1.7	2.3	109 ± 6	104 ± 5
ERL	0.9989	0.1	0.3	4.2	1.2	10	97 ± 1	98 ± 3
ETP	0.9981	3.0	38	14	12	15	36 ± 17	73 ± 11
DOX	0.9997	0.1	1.8	6.5	2.6	3.3	75 ± 12	86 ± 10
CAP	0.9989	0.2	1.3	7	5.2	4.0	107 ± 12	99 ± 5
OH-D-TAM	0.9986	0.3	1.8	2.7	7.5	11	91 ± 11	84 ± 12
OH-TAM	0.9991	0.06	0.5	6.4	5.8	8.3	114 ± 10	91 ± 4
TAM	0.9978	0.3	1.0	8.6	10	11	83 ± 7	96 ± 11
OH-PAC	0.9990	0.4	3.0	14	5.6	6.9	77 ± 7	96 ± 2
PAC	0.9999	0.6	3.0	15	2.2	3.6	73 ± 17	75 ± 3

[a] Limit of detection (defined as a S/N 3) of the first SRM transition. [b] Limit of determination: minimum concentration that can be quantified (>LOQ, SRM1) and confirmed (>LOD, SRM2). [c] Spiked level. [d] Calculated from the peak areas obtained in the on-line analysis of spiked (500 ng L^{-1}) HPLC water as percentages of the peak areas obtained from direct chromatographic injection (10 µL) of equivalent amounts of the standards in HPLC water (mean of the average results obtained at each concentration). [e] Relative to the associated IS. -not quantifiable

Table 4

Relative recoveries (n=5 replicates) calculated in Four Different Water Matrices (Groundwater, Surface Water, Wastewater Effluent and Wastewater Influent) spiked with the Analytes at Three Different Concentrations (20, 500, and 5000 ng L^{-1}).

Compound	Average recovery (%) ± relative standard deviation											
	GW			SW			WWE			WWI		
	[a]20 ng L^{-1}	[a]500 ng L^{-1}	[a]5000 ng L^{-1}	[a]20 ng L^{-1}	[a]500 ng L^{-1}	[a]5000 ng L^{-1}	[a]20 ng L^{-1}	[a]500 ng L^{-1}	[a]5000 ng L^{-1}	[a]20 ng L^{-1}	[a]500 ng L^{-1}	[a]5000 ng L^{-1}
GEM	116 ± 10	90 ± 1	103 ± 3	108 ± 13	115 ± 12	99 ± 4	96 ± 15	93 ± 1	104 ± 5	96 ± 2	114 ± 2	110 ± 9
TMZ	112 ± 3	86 ± 5	98 ± 5	103 ± 15	98 ± 13	97 ± 4	98 ± 12	94 ± 2	100 ± 4	95 ± 12	103 ± 4	107 ± 1
MET	90 ± 12	96 ± 4	88 ± 3	84 ± 8	103 ± 3	92 ± 2	87 ± 15	99 ± 1	102 ± 2	94 ± 14	104 ± 8	87 ± 9
OH-MET	91 ± 11	103 ± 2	96 ± 3	85 ± 25	113 ± 5	75 ± 10	88 ± 14	88 ± 1	89 ± 2	83 ± 5	99 ± 2	99 ± 4
IMA	nq	88 ± 15	77 ± 15	nq	109 ± 7	103 ± 15	nq	111 ± 6	91 ± 4	nq	108 ± 10	120 ± 11
IRI	119 ± 5	90 ± 6	96 ± 5	104 ± 7	96 ± 5	97 ± 3	82 ± 2	95 ± 2	107 ± 3	72 ± 11	88 ± 1	82 ± 2
IF	114 ± 1	100 ± 3	107 ± 8	101 ± 12	101 ± 3	89 ± 3	107 ± 13	95 ± 2	93 ± 4	92 ± 4	107 ± 1	96 ± 11
CP	115 ± 12	97 ± 2	96 ± 4	104 ± 13	100 ± 3	83 ± 4	115 ± 12	96 ± 2	93 ± 1	102 ± 11	99 ± 1	102 ± 3
ERL	110 ± 16	96 ± 2	94 ± 5	95 ± 5	109 ± 1	100 ± 4	91 ± 2	95 ± 1	108 ± 4	111 ± 12	111 ± 1	114 ± 5
ETP	104 ± 16	102 ± 17	111 ± 9	nq	98 ± 2	77 ± 12	nq	94 ± 4	76 ± 1	nq	81 ± 3	106 ± 7
DOX	72 ± 8	96 ± 8	94 ± 11	78 ± 5	74 ± 10	94 ± 1	98 ± 10	95 ± 5	101 ± 2	75 ± 4	71 ± 1	76 ± 1
CAP	84 ± 11	89 ± 3	91 ± 6	105 ± 15	105 ± 9	92 ± 5	107 ± 8	104 ± 12	104 ± 3	88 ± 8	119 ± 12	95 ± 8
OH-D-TAM	109 ± 12	100 ± 5	97 ± 7	98 ± 4	81 ± 2	74 ± 4	76 ± 5	99 ± 17	60 ± 3	79 ± 14	96 ± 7	104 ± 6
OH-TAM	108 ± 15	103 ± 5	109 ± 8	76 ± 9	84 ± 4	92 ± 6	89 ± 4	99 ± 11	80 ± 4	103 ± 12	100 ± 8	114 ± 13
TAM	110 ± 14	119 ± 16	84 ± 13	92 ± 4	92 ± 1	81 ± 9	101 ± 2	117 ± 1	105 ± 10	104 ± 11	107 ± 2	105 ± 4
OH-PAC	93 ± 22	97 ± 8	81 ± 11	113 ± 11	94 ± 9	108 ± 9	92 ± 11	94 ± 10	112 ± 4	113 ± 6	111 ± 9	93 ± 13
PAC	117 ± 10	104 ± 7	72 ± 13	99 ± 14	107 ± 7	93 ± 10	102 ± 2	99 ± 1	108 ± 6	108 ± 12	101 ± 7	113 ± 9

Abreviations: GW, groundwater; SW, surface water, WWE, wastewater effluent; WWI, wastewater influent; nq, not quantifiable.
[a]Spiked level

Table 5

LODs and LDets in Groundwater (GW), Surface water (SW), and Wastewater Effluent (WWE) and Influent (WWI).

Compound	LOD (ng L^{-1})				Ldet (ng L^{-1})			
	GW	**SW**	**WWE**	**WWI**	**GW**	**SW**	**WWE**	**WWI**
GEM	0.2	0.7	0.7	0.7	6.9	9.3	9.3	9.3
TMZ	0.8	0.9	1.0	1.1	26	50	42	50
MET	0.2	0.6	0.5	0.6	0.5	2.0	1.8	2.0
OH-MET	0.2	1.3	1.3	1.6	0.7	4.3	4.3	5.2
IRI	0.1	0.4	0.4	1.4	0.4	1.3	1.2	4.5
IMA	24	45	36	54	80	150	120	180
IF	0.3	0.6	0.6	0.6	1.0	2.0	2.0	2.0
CP	0.2	0.6	0.5	0.6	0.8	2.0	1.5	3.0
ERL	0.2	0.7	1.0	0.5	0.5	2.3	3.4	1.7
ETP	3.0	13	12	20	38	43	40	65
DOX	0.2	0.7	0.7	0.8	1.8	2.4	2.4	2.5
CAP	0.3	0.5	0.5	0.7	2.5	3.5	3.5	5.0
OH-D-TAM	0.3	1.5	1.5	1.5	1.8	5.0	5.0	5.0
OH-TAM	0.2	0.3	0.3	0.7	0.6	1.0	1.1	5.0
TAM	0.3	0.7	0.9	1.0	1.0	2.3	3.0	3.4
OH-PAC	0.6	0.9	1.1	1.1	3.0	2.9	3.6	3.6
PAC	0.5	0.9	1.2	1.3	3.0	3.1	4.0	4.4

Table 6
Levels of Detected Cytostatics in Raw Wastewater.

		Concentration (ng L^{-1})				
Code	Collection date	MET	IF	CAP	TAM	OH-PAC
1	17-18/04/2012	7.8	nq	24.7	nq	4.4
2	18-19/04/2012	2.1	43.3	20.0	4.4	nd
3	19-20/04/2012	2.4	29.7	27.0	17.2	nd
4	20-21/04/2012	20.1	13.5	9.7	nd	nd
5	21-22/04/2012	6.9	7.3	8.2	nd	nd
6	22-23/04/2012	4.1	nq	nq	4.3	nd
7	23-24/04/2012	4.8	nq	21.0	nd	nd
8	24-25/04/2012	2.2	nq	14.3	3.5	nd

nq, below LDet; nd, below LOD