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Abstract: A multianalyte liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) method for determination of 19 cytostatics and 5 metabolites, from 6 different therapeutic families, has been developed, and the structures of the main characteristic fragment ions have been proposed. Instrumental limits of detection and quantification are in the range 0.1-10.3 and 1.0-34.3 ng mL-1, respectively. Moreover, the stability of the compounds in aqueous solution was investigated in order to establish the best conditions for preparation and storage of both calibration standards and water samples. Dimethylsulfoxide (DMSO) was selected as solvent for preparation of the stock solutions. At room temperature (25 °C), 11 of the 24 target compounds were shown to be unstable in water (percentage of organic solvent 4%), with concentration losses greater than 20% in less than 24 h. At 4 °C (typical storage temperature for water samples) all compounds, except MTIC and chlorambucil, were stable for 24 h, but the number of stable compounds decreased to 10 after 9 days. Freezing of the aqueous solutions improved considerably the stability of various compounds: after 3 months of storage at -20 °C, 10 compounds, namely, 5-fluorouracil, carboplatin, gemcitabine, temozolomide, vincristine, vinorelbine, ifosfamide, cyclophosphamide, etoposide, and capecitabine, remained stable (in contrast to only carboplatin and capecitabine at 4 °C). The addition of acid improved the stability of methotrexate and its metabolite hydroxy-methotrexate but not that of the rest of compounds. The addition of organic solvent (50% methanol or DMSO) prevented the degradation at 4 °C of the otherwise unstable compounds oxaliplatin, methotrexate, erlotinib, doxorubicin, tamoxifen, and paclitaxel. To the authors' knowledge, five of the analytes investigated have never been searched for in the aquatic environment (imatinib, 6α-hydroxypaclitaxel, endoxifen, (Z)4-Hydroxytamoxifen, and temozolomide), and for many of them the stability

data provided, and even the analytical LC-MS/MS conditions, are the first ever published.

Cover Letter

February 12nd, 2013

Dear Editor,

Please find enclosed the manuscript entitled “Simultaneous analysis of 24 cytostatics and metabolites by liquid chromatography-electrospray-tandem mass spectrometry: preparation of standards, stability, and optimum storage conditions for their determination in water”, by Noelia Negreira and col., which is submitted for publication in Talanta. In this manuscript, we describe the optimization of a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the simultaneous determination of 19 anti-cancer drugs and 5 metabolites belonging to different Anatomical Therapeutic Classification (ATC) classes, provide information on their fragmentation pattern, and study the stability of the compounds in aqueous solution for storage of water samples and standard solutions. To the best of the authors’ knowledge, five of the analytes investigated have never been searched for in the aquatic environment and for many of them the stability data provided, and even the analytical LC-MS/MS conditions, would be the first ever published.

We are looking forward to hearing from you soon.

Yours sincerely,

Dr. Maria J. López de Alda

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Novelty Statement

The number of studies describing methodologies for analysis of cytostatics is very limited. The method here described allows the simultaneous determination of 24 cytostatics and metabolites, the largest number of compounds ever measured with a single method. Moreover, to the best of the authors' knowledge, seven of the analytes investigated have never been searched for in the aquatic environment, and for many of them the analytical LC-MS/MS conditions, the fragmentation pattern, and the stability data provided, would be the first ever published.

Highligths

- 24 compounds from 6 different families were included in the same LC-MS/MS method.
- Fragmentation patterns, most often overlooked in the literature, are provided.
- Most cytostatics were degraded in water in short periods of time, even at -20ºC.
- Temperature played an important role on their degradation.
- Addition of organic solvent improved the stability in water.

Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography-electrospray-tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution

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Abstract

A multianalyte liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) method for determination of 19 cytostatics and 5 metabolites, from 6 different therapeutic families, has been developed, and the structures of the main characteristic fragment ions have been proposed. Instrumental limits of detection and quantification are in the range 0.1-10.3 and 1.0-34.3 ng mL^{-1}, respectively. Moreover, the stability of the compounds in aqueous solution was investigated in order to establish the best conditions for preparation and storage of both calibration standards and water samples. Dimethylsulfoxide (DMSO) was selected as solvent for preparation of the stock solutions. At room temperature (25 ºC), 11 of the 24 target compounds were shown to be unstable in water (percentage of organic solvent 4%), with concentration losses greater than 20% in less than 24 h. At 4 ºC (typical storage temperature for water samples) all compounds, except MTIC and chlorambucil, were stable for 24 h, but the number of stable compounds decreased to 10 after 9 days. Freezing of the aqueous solutions improved considerably the stability of various compounds: after 3 months of storage at -20 ºC, 10 compounds, namely, 5-fluorouracil, carboplatin, gemcitabine, temozolomide, vincristine, vinorelbine, ifosfamide, cyclophosphamide, etoposide, and capecitabine, remained stable (in contrast to only carboplatin and capecitabine at 4 ºC). The addition of acid improved the stability of

methotrexate and its metabolite hydroxy-methotrexate but not that of the rest of compounds. The addition of organic solvent (50% methanol or DMSO) prevented the degradation at 4 ºC of the otherwise unstable compounds oxaliplatin, methotrexate, erlotinib, doxorubicin, tamoxifen, and paclitaxel. To the authors' knowledge, five of the analytes investigated have never been searched for in the aquatic environment (imatinib, 6α-hydroxypaclitaxel, endoxifen, (Z)4-Hydroxytamoxifen, and temozolomide), and for many of them the stability data provided, and even the analytical LC-MS/MS conditions, are the first ever published.

Keywords: Cytostatics; Water samples; Stability; Electrospray; Fragmentation; Liquid chromatography-mass spectrometry

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1. Introduction

During recent years great consideration has been given to the contamination of the environment by veterinary and human pharmaceuticals. Several groups of pharmaceuticals, such as antibiotics and hormones, have been studied intensively; however, other potentially more toxic compounds, such as cytostatic agents, have received very little attention [1].

The use of cytostatics for cancer therapy has increased considerably in the last decade [2, 3], and their production has been estimated to be 5000 kg per year [4]. These substances act by either inhibiting cell growth or directly killing cells (cytotoxic) [4, 5]. The reaction mechanisms of cytostatic compounds and their mainly non-specific nature

of attack within organisms often give rise to secondary side effects and increased health risks [4]. Thus, many antineoplastic agents have cytotoxic, mutagenic, carcinogenic, embryotoxic and/or teratogenic effects [1, 4-6]. Recent studies have reported that persons working with cytostatic drugs, e.g. clinical and pharmaceutical staff, show a marginally higher frequency of DNA damage. Moreover, they can be introduced in the environment and even enter the food chain through excretion from patients under medical treatment as main source [5, 7]. In fact, some of them have been detected in hospital wastewater at concentration levels varying from ng L^{-1} to µg L^{-1} [8, 9]. 5-Fluorouracil (5-Fu), ifosfamide (IF), and cyclophosphamide (CP) have been shown to be the most abundant compounds [8, 10-12] but these are also the most commonly investigated cytostatics. A few works have addressed the study of doxorubicin (DOX), vincristine (VCN), etoposide (ETP) and methotrexate (MET) [2, 10, 13], which have been found at low concentrations [8]. Martin et al. [14] analysed some cytostatics not included in previous works, namely, paclitaxel (PAC), irinotecan (IRI), vinorelbine (VRB), and gemcitabine (GEM), but they were either not detected (PAC and IRI) or detected at low levels (lower than 10 ng L^{-1} in influent wastewater) (VRB and GEM). Recently, the application in a second-phase of the present work of a newly developed on-line solid phase extraction (SPE)-LC-MS/MS method to the analysis of 13 cytostatics and 4 metabolites in waters, has expanded the list of anti-cancer drugs measured in environmental samples to 8 new compounds (temozolomide (TMZ), imatinib (IMA), erlotinib (ERL), capecitabine (CAP), hydroxytamoxifen (OH-TAM), endoxifen or 4-hydroxy-N-desmethyl-tamoxifen (OH-D-TAM) and hydroxypaclitaxel (OH-PAC)) and has evidenced for the first time the presence of CAP and OH-PAC in influent wastewaters at levels up to 30 ng L^{-1} [15]. Carboplatin has also been detected in a hospital effluent [7] and, to the authors' knowledge, other compounds have never been studied in water samples.

On the other hand, some experimental studies [1, 16-19] have reported genotoxicity in hospital wastewater effluent samples collected both before and after treatment in

wastewater treatment plants (WWTPs), but the genotoxicity of pure compounds with ecotoxicological bioassays has been investigated in only a few occasions [16, 20].

Most cytostatics are polar compounds often having a high molecular weight, circumstances that limit the use of gas chromatography (GC) techniques for their determination since a derivatization step is usually necessary to enhance the volatility and the thermal stability of the compounds before their injection into the chromatographic system. Thus, only 5-Fu, TAM, IF and CP have been determined by GC coupled to mass spectrometry (MS) [11, 21, 22]. Liquid chromatography (LC) coupled to ultraviolet (UV) detection has been commonly employed for determination of cytostatics [4, 8, 23]. However, trace-level environmental analysis requires higher sensitivity, specificity and accuracy, which are only possible using MS detection. The application of this advanced technique to cytostatics analysis has been described in some recent works [6, 12, 14, 24-26]. However, many of them focus on the analysis of just a few classes of cytostatics, and structural information on the product ions chosen for selected reaction monitoring (SRM) determination has been often overlooked. Only Gómez-Canela et al. [27] have provided mass spectral characterization for 26 cytostatics, of which, 15 are measured in the present study and 11, namely, TMZ, 5-(3-N-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC), MET, 5-Fu, VRB, carboplatin (Carb-Pt), Oxaliplatin (Oxa-Pt) and the metabolites OH-MET, OH-PAC, OH-D-TAM and OH-TAM, are not.

The number of studies investigating and reporting the presence of cytostatics in water samples is quite short. This fact can be attributed to either a low medical use or, most probably, to the existence of degradation processes in the aqueous medium leading to the transformation of the active principles into other products, which, in turn, can be more toxic and persistent than the original ones. Little information is known in this respect, even though some biodegradation products have already been identified. For example, the biodegradation process of MET is combined with the generation of the

toxic and persistent degradation product 7-hydroxymethotrexate (OH-MET) [4], TMZ decomposes to MTIC in the DNA at pH > 7 [28], PAC to OH-PAC, and tamoxifen (TAM) to OH-TAM and OH-D-TAM. Moreover, low recoveries in the analysis of some cytostatics in aqueous samples have been reported by some authors [11, 14, 29], which, in fact, could have their origin in the possible instability of the compounds in the samples.

In this context, the objectives of this work were: (1) to explore the possibilities of LC-ESI-MS/MS for the analysis of cytostatics by optimizing a multi-analyte method for the simultaneous determination of 19 anti-cancer drugs and 5 metabolites belonging to different Anatomical Therapeutic Classification (ATC) classes; (2) to provide information on their fragmentation pattern, an aspect overlooked in the literature; and (3) to study their stability and optimum storage conditions in aqueous solution.

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%), ammonium hydroxide (25%), methanol and HPLC-water were purchased from Merck (Darmstadt, Germany), while DMSO (DMSO, >99.9%) was acquired from Aldrich (Milwaukee, WI, USA).

Analytical standards of the cytostatic compounds CAP, Car-Pt, chlorambucil (CHL), CP, DOX hydrochloride, ERL hydrochloride, ETP, 5-Fu, GEM hydrochloride, IF, IMA mesylate, MET, OH-MET, MTIC, Oxa-Pt, 6(α)-OH-PAC, TAM citrate, (Z)-4-OH-TAM, OH-D-TAM, TMZ, vinblastine (VBL) sulfate, VCN sulfate, and VRB ditartrate were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Paclitaxel (PAC) was supplied by Sigma-Aldrich at the highest available purity (>99%).

The selected cytostatics, grouped into six families attending to their mode of action and chemical structure, are shown in Table 1.

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

Different working standard solutions were made by appropriate dilution in ultrapure water, methanol, DMSO or mixtures of them, and were then 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 instruments and materials that were in contact with tested compounds were disposable and treated as a hazardous waste.

2.3. Instrumental and chromatographic conditions

Analyses were carried out using an Acquity UPLC system (Waters, Milford, MA, USA) consisting of a thermostated autosampler, a binary pump, a vacuum degasser, a thermostated column compartment and a UV-Vis programmable detector. The UPLC system was coupled to a Waters TQD triple quadrupole (QqQ) mass spectrometer equipped with an electrospray ionization (ESI) source. The whole LC–MS/MS system was controlled by MassLynx 4.1 software (Waters).

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), without modifier or containing 0.1% of formic acid, were tested as mobile phases. Under final optimized conditions, compounds were separated with the acidified mobile phase using the following gradient: 0–1 min, 5% B; 2 min, 20% B; 12 min, 80% B; 19–23 min, 100% B;

25–30 min, 5% B. The flow rate and the injection volume were set at 0.2 mL min^{-1} and 10 µL, respectively.

The mass spectrometer was operated using both positive and negative ESI modes under the following specific conditions: capillary voltage 3.0 kV, extractor voltage 3 V, source temperature 150 °C, dessolvation temperature 350 °C, cone gas flow 50 L h^{-1} and dessolvation gas flow 600 L h^{-1}. Nitrogen (>99.98%) was employed as cone and dessolvation gas.

Detection was accomplished in the selected reaction monitoring (SRM) mode using argon (>99.999%) as collision-induced dissociation (CID) gas at a pressure of 4×10^{-3} mbar in the collision cell. Selected ionization polarities and optimized MS/MS ion transitions for each compound are detailed in Table 2.

3. Results and discussion

3.1. Chromatographic analysis

In general, the use of tandem mass spectrometry detection reduces the need for baseline resolution of all analytes, because it is very rare to find molecules with the same retention time sharing the same unique MS/MS transitions. However, in many instruments the sensitivity of the MS detector decreases as the number of transitions recorded increases, and therefore a certain degree of separation is necessary in order to enable programming of the various SRM transitions into different time windows along the chromatogram.

Taking into account the different polarities of the studied cytostatics, a reversed-phase (RP) C18 column was evaluated in an attempt to achieve a suitable separation in the same chromatographic run.

Different modifiers were tested with the purpose of improving not only the response but also the peak shape of the compounds. When ammonium acetate (5 mM) was used, the competitive formation of the corresponding ammonium adducts in the ESI positive

196 mode was observed for most of compounds. This finding was in agreement with the
197 results obtained by Martin [14] & Tuerk [24] for the analysis of ETP and PAC. As most
198 of the studied compounds are better ionized in the positive mode (Table 2), the use of
199 this salt as mobile phase modifier was discarded in an attempt to maximize the
200 ionization efficiency of the protonated molecular ions $[M+H]^+$.
201 The addition of 0.1% formic acid to the mobile phase improved (approximately 40–
202 60%) the responses of most compounds, especially MET, PAC and the metabolites
203 OH-MET and OH-PAC, which are hardly observed without acid. This is consistent with
204 the fact that acidic conditions generally increase positive-ion ESI response (i.e., low pH
205 conditions favor the formation of the protonated compounds). Nevertheless, the
206 presence of this acid produced a reduction in the negative ion ESI response of 5-Fu by
207 about 25%. On the other hand, the positive ion responses of IMA and VRB slightly
208 decreased with respect to those observed in the absence of modifier, but the peak
209 shape was considerably improved. So, under optimized conditions, 0.1% formic acid
210 was added to the mobile phase.
211
212 *3.2. Optimization of MS/MS detection conditions*
213 The selection of the specific SRM conditions to achieve maximal sensitivity and
214 selectivity was performed by injection of individual standard solutions of the
215 compounds. Table 2 summarizes retention times, most intense SRM transitions,
216 ionization modes (positive or negative), cone voltages and collision energies (CE) for
217 target species.
218 Positive ionization proved to be the most sensitive ionization mode for all the studied
219 cytostatics, yielding the corresponding protonated parent ion $[M+H]^+$, except for 5-Fu.
220 The latter could only be ionized in the ESI negative mode, showing the single
221 negatively charged parent ion $[M-H]^-$.

222 Two SRM transitions were selected for each cytostatic (Table 2). The most intense
223 transition was used for quantification, while the other one was employed for
224 identification.
225
226 *3.3. Fragmentation study*
227 The use of LC-MS/MS for cytostatic analysis is growing. However, structural
228 elucidation of the product ions selected for their SRM determination is usually missing.
229 In this work, the fragmentation pattern of each cytostatic considered was investigated.
230 Fig. 1 shows the corresponding product-ion mass spectra obtained for all studied
231 compounds, with the chemical structures proposed for each of the resulting fragments.
232 The strong differences in the chemical structure between the different classes of
233 anticancer drugs cause also great differences in their fragmentation. In general the
234 cytostatics belonging to the same group follow a similar mechanism of fragmentation.
235 For the alkylating agents CP and IF, the most important fragmentation reaction under
236 the selected conditions was the cleavage of the N-P bond leading to the formation of
237 the ions *m/z* 140 and 120 for CP, and 183, 155 and 92 for IF, as it has been previously
238 reported [12]. The main fragments of CHL are at *m/z* 192, due to the loss of two
239 molecules of CH_4Cl, and at *m/z* 168, due to the loss of CH_4Cl and $C_3H_5O_2$. For MTIC
240 the most intense ions are at *m/z* 109 and 126; and for TMZ at *m/z* 138 and 82.
241 MET and its metabolite OH-MET form fragments at *m/z* 308 and 324, respectively, due
242 to the loss of *m/z* 148 ($C_5H_{10}NO_4$). The pyrimidine analogue 5-Fu breaks its ring
243 yielding fragments at *m/z* 86 ($C_2H_2N_2O_2^-$) and *m/z* 42 (CNO^-). The loss of the
244 tetrahydrofuran ring with their substituents of the GEM and CAP molecules gives
245 product ions at *m/z* 112 and 244, respectively, data not found in the literature.
246 The group of the vinca alkaloids, VBL, VCN, and VRB, with parent ions of 812, 826,
247 and 780, respectively, break in very small fragments. VBL and VRB lose, initially, an
248 acetyl group and give fragments at *m/z* 751 and 658, respectively. VCN loses a
249 molecule of H_2O (18 uma) and gives the fragment at *m/z* 807. Then, the compounds'

rings break forming ions at *m/z* 224, 138 and 323 for VBL, VCN, and VRB, respectively.

ETP fragments at m/z 229 and 185. The first one is due to the loss of the glycoside and the dimethoxyphenol rings and formation of two double bonds in the central molecule. In addition, the last molecule losses the dioxolane ring and gives the m/z 185.

The taxanes PAC and OH-PAC give two main fragments at *m/z* 569 and 286 in the case of PAC and at *m/z* 525 and 286 in the case of OH-PAC due to the breakdown of the ester into two molecules and the formation of a double bond.

DOX losses the glycoside portion and gives a product ion at *m/z* 397, and with a further loss of two water molecules leads to a product ion of *m/z* 361. The transitions from *m/z* 544 to 361 and from *m/z* 544 to 397 were used in our SRM analysis [6].

The fragments of Car-Pt and Oxa-Pt are difficult to elucidate, though some structures are proposed.

The two protein kinase inhibitors (IMA and ERL) undergo different mechanisms of fragmentation. IMA gives two fragments at *m/z* 394 and 99 due to the formation of a double bond between the unshared pair of nitrogen belonging to the piperazine ring with the piperazine ring, and the subsequent breakdown of the molecule. The fragment at *m/z* 217 corresponds to the rupture of the bond between N and C of the amide and formation of an aldehyde group. ERL fragments at *m/z* 336 and 278 are due to the loss of one and two substituents C_3H_7O remaining one and two hydroxyl groups, respectively.

The anti-estrogens TAM and their metabolites (OH-TAM and OH-D-TAM) break down in very small fragments. TAM and OH-TAM share the same product ions at *m/z* 72 and *m/z* 45, which correspond to imines.

3.4. Method performance

Fig. 2 illustrates the analysis of the target compounds, which were separated in 30 min. In order to improve the limits of detection (LOD) and quantification (LOQ), they were grouped into 11 segments according to their elution order. The total dwell time per segment was maintained at 1.2 s. The dependence between peak areas and analytes concentration was investigated with standards prepared in HPLC water (maximum percentage of DMSO 4%) at 7 different concentrations in the range from 5 to 2000 ng mL^{-1} (injection volume 15 µL). Most compounds gave a linear response in the above range, with determination coefficients (R^2) between 0.9917 and 0.9996, whereas MTIC and CHL showed R^2 values of 0.7481 and 0.9776, respectively, because they were not stable under those conditions (see Table 3). Instrumental LODs, defined for a signal to noise ratio (S/N) of 3, varied between 0.1 and 10.3 ng mL^{-1}, whereas the LOQs (S/N = 10) were in the range 1.0 - 34.3 ng mL^{-1}. The repeatability in the responses of the system was evaluated with standards at two different concentrations: 50 and 200 ng mL^{-1}. Relative standard deviations (RSDs, %) for 5 injections made in the same day ranged from 0.3 to 6.1%, if MTIC (RSD = 30.2 - 32.9%) and CHL (RSD = 20.6 – 33.3%) are excluded.

3.5. Stability of cytostatics

The fact that some compounds did not present linearity and had relatively large RSDs induced us to think that they were not stable in aqueous solution. Moreover, low recoveries in the analysis of some cytostatics in water samples have been reported by some authors, which could be also due to stability problems. In this context, one purpose of this work was to study the stability of the 24 target cytostatics and

304 metabolites in aqueous solutions and in organic solvents in order to establish the best
305 conditions for preparation and storage of both calibration solutions and water samples.
306
307 *3.5.1. Effect of the temperature*
308
309 The first aspect investigated in this respect was the short-term stability of the
310 compounds in water at different temperatures. For this purpose HPLC-water was
311 spiked with the target compounds at 1 µg mL^{-1} (percentage of DMSO 4%) and was
312 injected in the HPLC system immediately after preparation and every 30 min within the
313 next 24 hours. During this period, the solution was maintained protected from light at
314 different constant temperatures: 4, 15 and 25 ºC. The selection of these temperatures
315 was based on the following premises: 4 ºC is the temperature at which most
316 laboratories store water samples from collection to analysis; 25 ºC was selected as
317 room temperature, and it is also a temperature used in multiple laboratory exposure
318 experiments, and a temperature that can be reached in Southern European rivers in
319 summer; and 15 ºC was selected as an intermediate value between the other two,
320 expected to be close to the annual average water temperature of most European
321 rivers. Table 4 shows the percentage of each compound that remained in solution after
322 24 h at the three studied temperatures. As it can be seen, at 4 °C most compounds
323 were stable, with relative responses within the range 100 ± 20%. The only compounds
324 that showed clearly lower peak signals after 24 h, as compared to the peak signals at
325 time zero, were MTIC (12%) and CHL (20%). Meanwhile, at higher temperatures a
326 reduction of the initial peak signal with time was evident for about half of the
327 compounds investigated and, as expected, the signal decay increased with
328 temperature. At 25 °C, 12 of the 24 compounds tested, namely, 5-Fu, GEM, TMZ,
329 VCN, VBL, VRB, IF, CP, ERL, ETP, CAP, and OH-PAC, were stable (relative response
330 above 80%) for 24 h; 6 compounds, namely, Car-Pt, IMA, DOX, OH-D-TAM, OH-TAM,
331 and PAC showed some degradation, with relative responses within the margin 50 to

80%; and the remaining 6 compounds, namely, Oxa-Pt, MET, MTIC, OH-MET, TAM, CHL, were degraded to a large extent, with relative responses below 50% (in fact, the last three compounds were not detected after 24 h at both 25 and 15 °C).

In the light of these results, the stability of the compounds at 4 ºC was studied during a longer period of time by analysing sample aliquots every day during the first 9 days and after 1, 2 and 3 months of storage in the dark at this temperature. Fig. 3 shows that after 9 days the amount of 11 of the target compounds, namely, Oxa-Pt, MET, its metabolite OH-MET, VBL, IMA, ERL, DOX, TAM, its metabolite OH-D-TAM, PAC, and its metabolite OH-PAC, decreased substantially (to around 50%), MTIC and CHL disappeared completely already after 3 days, and the remaining compounds (5-Fu, Car-Pt, GEM, TMZ, VCN, VRB, IF, CP, ET, CAP, and OH-TAM) were stable (relative response above 80%). After 1 month, all the compounds that were shown to be stable for 9 days, with the exception of OH-TAM, stayed stable (see Fig. 4), whereas after 3 months only Car-Pt and CAP remain unaltered.

These results are in line with those previously published by other authors. Very recently, Tuerk et al. [24], reported recoveries of about 40 and 70% for PAC and docetaxel (another cytostatic that belongs to the family of the taxanes), respectively, in tissues used to wipe sampling areas in pharmacies, and further wetted with 1 mL phosphate buffer and stored at room temperature for 48 hours and at -18 ºC for 7 days. They studied also other cytostatics, namely, 5-Fu, GEM, MET, CP, IF, and ETP, but recovery rates were closer to 100%. Sottani et al. [6] studied the stability of CP, IF and DOX in human urine, and found that all of them were stable (concentration measured equal to the initial concentration ± 20%) for at least 8 hours at room temperature and for 15 days at -20 ºC. Chen et al. [23] found that 5-Fu and PAC were stable in samples of drug-coeluting stents prepared in 15 mL of phosphate-buffered saline (PBS) (pH 7.4; 0.05 M, 1% sodium dodecyl sulfate) for at least 24 hours at room temperature (which is in line with our results) and for 1 month at 4 ºC (when in our experiment PAC

359 decreased in concentration more than 50% after 3 days of storage at the same
360 temperature (4 ºC)).
361 Since about half of the compounds were observed to be unstable at 4 ºC after various
362 days, freezing at -20 ºC was evaluated as a possible means to store and preserve both
363 water samples and calibration solutions. For this purpose, samples of HPLC-water
364 spiked with the target compounds at 1 µg mL^{-1} and stored in the dark at -20 ºC were
365 analysed after different storage times (3 days, 9 days, and 1, 2 and 3 months) and
366 compared with a sample prepared in the same way and analysed immediately after. As
367 it can be seen in Fig. 5, freezing improved considerably the stability of various
368 compounds in water. After 3 months of storage at -20 ºC, 10 compounds, namely, 5-
369 Fu, Car-Pt, GEM, TMZ, VCN, VRB, IF, CP, ETP, and CAP, remained stable, in contrast
370 to only Car-Pt at 4 ºC. Freezing for 1 month would be suitable for the above mentioned
371 10 compounds plus Oxa-Pt and OH-D-TAM, i.e. for 12 of the 24 target compounds,
372 whereas at 4 ºC only 10 compounds were stable for 1 month. Between 1 month and 9
373 days the differences are minimal: the only compounds unstable for 1 month but stable
374 for 9 days were MET, OH-MET, and OH-TAM; hence, the number of compounds stable
375 at -20 ºC for 9 days is 15. Finally, storage at -20 ºC for 3 days would be acceptable for
376 all but 7 compounds (MTIC, IMA, ERL, DOX, OH-PAC, PAC, and CHL), which are not
377 stable either under these conditions.
378 These findings are in agreement with those of Nussbaumer et al. [30] who studied the
379 stability of some cytostatics (cytarabine, GEM, MET, ETP, CP, IF, IRI, DOX, EPI and
380 VCN) on filter papers over 3 months at three storage temperatures (-20, 4 and 25 ºC).
381 They found that at 25 ºC only IF was stable for 3 months. Concentrations of ETP, DOX,
382 EPI and VCN were already decreased to 20% after 1 week. At 4 ºC, the wiping
383 samples were stable for 1 week. After 2 months, the concentrations of DOX, EPI and
384 VCN were inferior to 50% of the initial amount, and at 3 months 50% of ETP was lost.
385 The other compounds were stable for 3 months at 4 ºC. At -20 ºC all drugs tested were
386 stable for 2 months.

Osawa et al. [31] validated a method for the determination of the contamination of the exterior surface of vials containing platinum anticancer agents (cisplatin and carboplatin) and found that stock solutions of cisplatin and carboplatin prepare in saline solution and ultra-pure water, respectively, were stable at 4 ºC for at least 3 months, which is in agreement with our results.

3.5.2.Effect of the addition of acid

The effect of addying acid to the water samples as a preservation method was subsequently investigated by using HPLC-water spiked with the analytes (same concentration as above, 1 µg mL^{-1}) and formic acid (0.1%). This solution was maintained in the dark at 4 ºC and injected every day during 9 days. Fig. 6 shows comparatively the results obtained for the various compounds in the ninth day with and without acid. As it can be seen the behaviour was very variable. Most compounds showed similar results with and without acid. MET and its metabolite OH-MET, compounds that without acid decreased in concentration to about 60 and 40%, respectively, were stable after 9 days when acid was added. MTIC decreased in concentration in both cases, but its degradation in the acidified sample was much less pronounced (60% versus nearly 100% in the non-acidified sample). In contrast, other compounds like IMA, TAM, and its metabolite OH-TAM were even less stable in the presence of acid. Therefore, the addition of acid would only be a solution for MET and its metabolite, not for the rest of compounds.

3.5.3.Effect of the addition of organic solvent

Another factor that could improve the stability of sparingly water soluble species is the addition of a miscible organic solvent. This effect was studied with methanol and DMSO. For this purpose standard solutions containing the mixture of the target

compounds at 1 µg mL^{-1} were prepared in DMSO:water 4:96, DMSO:water 50:50 and methanol:water 50:50 and were injected daily during 9 days. The peak shape got worse when the percentage of organic solvent increased but the peak areas obtained could still be used to compare and establish the best conditions for preparation and storage of standard solutions and water samples. Fig. 7 shows the results obtained after 3 and 9 days of storage at 4 ºC. This figure does not include those compounds that were observed to be stable in 4% DMSO. Moreover, the metabolites OH-TAM, OH-D-TAM, OH-MET and OH-PAC are not represented because they follow the same behaviour as their parent compounds. Hence, in Fig. 7 we can observe the behaviour of Oxa-Pt, MTIC, MET, VBL, IMA, ERL, DOX, TAM, PAC, and CHL. Oxa-Pt, MET, ERL, DOX, TAM, and PAC resulted to be stable in 50% of organic solvent (DMSO and methanol) and no major differences were observed between the third and the ninth days of storage. Meanwhile, the rest of compounds were degraded to a different extent, and MTIC and CHL were again the least stable compounds. However, the degradation increased when the percentage of organic solvent was lower (4%). Hence, it is clear that the presence of water in the solution plays an important role.

3.5.4.CHL and MTIC

Among all analytes investigated CHL and MTIC stand out clearly as the most unstable compounds.

CHL is a chemotherapy drug that has been mainly used in the treatment of chronic lymphocytic leukemia. It is a nitrogen mustard alkylating agent and can be given orally. It has also been associated with the development of other forms of cancer [4]. However, to the best of our knowledge, this compound has not been investigated in the aquatic environment and there are no data about its presence or its stability in it. Hence, this compound was studied further in more detail.

When prepared in HPLC-water, this compound was observed to completely disappear after only half an hour at room temperature, and this was accompanied by the appearance of a new chromatographic peak at a lower retention time (6.2 min). The mass spectrum of the newly formed compound obtained in the ESI positive mode exhibited a $[M+H]^+$ ion at *m/z* 269, corresponding to the loss of a chlorine atom from the CHL molecule. Its capillary voltage, collision energy and MS/MS transitions were optimized and the most intense fragment was observed at *m/z* 192, the same as CHL. The second most intense fragment was found at *m/z* 132.

In order to establish whether this product was stable and how much time was necessary for a total conversion, CHL was spiked in HPLC-water at 1 μg mL^{-1} (4% DMSO) and this solution was injected immediately after and at different time intervals during the following 8 days while maintained at different temperatures (4, 15 and 25 ºC). Under these conditions, CHL disappeared quickly and the new compound, without a chlorine atom, was formed. Between 15 and 25 ºC there were no differences, but at 4 ºC the subproduct was formed in a comparatively minor extension.

Fig. S1 (in the Supplementary Information) shows that the response of the subproduct increased with time up until the equilibrium was reached (which at 4 ºC occurred after 120 hours), remaining stable thereafter for at least 4 more days.

A similar behaviour was also observed for MTIC. This compound is a pharmacologically active hydrolysis product of TMZ [28], but our results indicate that MTIC is less stable than TMZ. Fig. S2 (Supplementary Information) shows how MTIC is quickly degraded, remaining only 1% of the compound after 3 and 4 hours at 25 and 15 ºC, respectively. The degradation at 4 ºC is slower than at the other temperatures studied and after 3 hours 30% of the compound remained in solution.

4. Conclusions

An LC-MS/MS method has been developed for the analytical determination of 19 cytotoxic drugs and 5 transformation metabolites at the low ng mL^{-1} level. Their stability

in water has been studied under different conditions (in relation with temperature, addition of acid, and addition of organic solvent) over 3 months, constituting for many of them the first study of these characteristics carried out. All compounds with the exception of Car-Pt have been shown to be degraded to a higher or lower extent when stored in aqueous solution for a certain time, which may vary from hours to months depending on the temperature (see Table 5). Hence, stock standard solutions shall be prepared in pure organic solvent and the aqueous calibration solutions shall be made up immediately before use. Since some compounds are not readily soluble in methanol (e.g. Oxa-Pt and GEM) and some may also undergo hydrolysis and transesterification in this solvent (e.g. PAC) [32], DMSO appears as a suitable solvent for preparation of stock standard solutions. DMSO presents a high capacity to dissolve substances without interacting with them and is miscible with water. Nevertheless, since DMSO has a relatively high freezing point (18.5°C) and hence it is solid at, or just below, room temperature, precaution should be taken at not injecting a pure 100% DMSO standard solution directly into the HPLC system.

On the other hand, the fact that many compounds are unstable in water may be behind the lack of studies on cytostatics in water. The inherent instability associated with some compounds makes their quantification extremely difficult in aqueous matrices and this may explain the absence of published data on validated quantitative analytical methods for the determination of many of them. Further studies should investigate the possible transformation of these compounds into other products, their potential environmental effects, and the eventual risks to humans from environmental exposure to these substances. To this end, the application of the LC-MS/MS method developed to the analysis of environmental samples may be of help, though a previous stage of pre-concentration is necessary.

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571

572 **Captions to figures**

573 **Fig. 1**. Product-ion mass spectra obtained for each of the target compounds in the ESI
574 positive mode and corresponding suggested structures.
575 **Fig. 2.** SRM chromatograms obtained from the analysis of a standard mixture in HPLC-
576 water at 100 ng mL^{-1} by LC-ESI-MS/MS.
577 **Fig. 3.** Stability data of the target cytostatic compounds and metabolites in HPLC water
578 (initial concentration 1 µg mL^{-1}) stored at 4 ºC for 9 days.
579 **Fig. 4.** Relative response of the most stable cytostatic compounds and metabolites in
580 HPLC water (initial concentration 1 µg mL^{-1}) after 1, 2, and 3 months of storage at 4 ºC.

Fig. 5. Stability data of the target cytostatic compounds and metabolites in HPLC water (initial concentration 1 µg mL^{-1}) stored at -20 ºC for different time periods up to 3 months.

Fig. 6. Effect of the addition of formic acid on the stability of the compounds in water after storage for 9 days.

Fig. 7. Effect of the addition of organic solvent on the stability of the compounds in water stored at 4 ºC for 3 and 9 days.

Table 1

Abbreviated names, molecular mass, acid-base dissociation constant (p*K*a), octanol-water partition coefficient (log *K*ow), and ATC group of the target cytostatics.

Compound (acronym)	Molecular Mass	[b]pKa	[b]log K_{OW}	Group
ALKYLATING AGENTS				
Cyclophosphamide (CP)	260.02	2.84	0.73	Nitrogen mustard analogues
Ifosfamide (IF)	260.02	1.44	0.78	Nitrogen mustard analogues
Chlorambucil (CHL)	304.21	4.82 (acidic) 4.62 (basic)	2.61	Nitrogen mustard analogues
Temozolomide (TMZ)	194.20	14.77 (acidic) -1.63 (basic)	-1.27	Other alkylating agents
[a] MTIC	168.16	10.07 (acidic) 2.23 (basic)	-1.16	Other alkylating agents
ANTIMETABOLITES				
Methotrexate (MET)	454.45	3.47 (acidic) 5.56 (basic)	-0.45	Folic acid analogues
[a] Hydroxymethotrexate (OH-MET)	470.44	3.48 (acidic) 4.99 (basic)	-0.69	Folic acid analogues
5-Fluorouracil (5-FU)	130.02	-	-0.65	Pyrimidine analogues
Gemcitabine (GEM)	299.66	11.65 (acidic) 4.26 (basic)	-2.22	Pyrimidine analogues
Capecitabine (CAP)	359.15	5.41 (acidic) 1.75 (basic)	1.04	Pyrimidine analogues
PLANT ALKALOIDS AND OTHER NATURAL PRODUCTS				
Vinblastine (VBL)	810.97	11.36 (acidic) 7.90 (basic)	5.92	Vinca alkaloids and analogues
Vincristine (VCN)	824.96	11.10 (acidic) 7.90 (basic)	5.75	Vinca alkaloids and analogues
Vinorelbine (VRB)	778.93	11.36 (acidic) 6.90 (basic)	7.08	Vinca alkaloids and analogues
Etoposide (ETP)	588.57	9.94±0.40 (acidic)	0.28	Podophyllotoxin derivatives
Paclitaxel (PAC)	853.91	11.90 (acidic) -2.19 (basic)	3.95	Taxane
[a] 6(α)-Hydroxypaclitaxel (OH-PAC)	869.91	11.90 (acidic) -2.19 (basic)	3.19	Taxane
CYTOTOXIC ANTIBIOTICS AND RELATED SUBSTANCES				
Doxorubicin (DOX)	543.52	7.35 (acidic) 8.68 (basic)	1.27	Anthracyclines and related substances
OTHER ANTINEOPLASTIC AGENTS				
Carboplatin (Car-Pt)	371.25	-	-2.19	Platinum compounds
Oxaliplatin (Oxa-Pt)	397.29	-	-1.67	Platinum compounds
Imatinib (IMA)	493.60	13.28 (acidic) 7.55 (basic)	2.89	Protein kinase inhibitors
Erlotinib (ERL)	393.44	5.32	3.03	Protein kinase inhibitors
HORMONE ANTAGONISTS AND RELATED AGENTS				
Tamoxifen (TAM)	371.51	8.69	5.13	Anti-estrogens
[a] 4-Hydroxy-N-desmethyl-tamoxifen or Endoxifen (OH-D-TAM)	373.49	9.38 (acidic) 9.34 (basic)	4.94	Anti-estrogens
[a] (*Z*)-4-Hydroxytamoxifen (OH-TAM)	387.51	10.35 (acidic) 8.70 (basic)	4.93	Anti-estrogens

[a] active metabolite

[b] Values obtained from SciFinder Scholar Database, http://www.cas.org/products/sfacad/.

Table 2

LC retention time (t_R) and selected MS/MS detection conditions for determination of the target cytostatics.

Compound	t_R (min)	Seg.[a]	Ionization	Parent ion	MS/MS Transition[b]	Cone (V)	CE (eV)[c]
5-FU	3.25	1	ESI-	$[M-H]^-$	**129.0 > 42.0**	30	15
					129.0 > 86.0	30	15
Car-Pt	3.25	2	ESI+	$[M+H]^+$	**372.0 > 355.0**	25	10
					372.0 > 294.0	25	20
GEM	3.82	3	ESI+	$[M+H]^+$	**264.0 > 112.0**	30	15
					264.0 > 95.0	30	45
Oxa-Pt	4.03	3	ESI+	[M+H]+	**398.0 > 96.0**	40	25
					398.0 > 308.0	40	20
MTIC	4.05	3	ESI+	$[M+H]^+$	**169.0 > 109.0**	10	5
					169.0 > 124.0	10	5
TMZ	4.69	4	ESI+	$[M+H]^+$	**195.0 > 138.0**	25	10
					195.0 > 82.0	25	20
MET	6.49	5	ESI+	$[M+H]^+$	**455.0 > 308.0**	20	20
					455.0 > 175.0	20	35
OH-MET	8.00	6	ESI+	$[M+H]^+$	**471.2 > 324.2**	20	10
					471.2 > 191.0	20	20
VCN	8.71	7	ESI+	$[M+H]^+$	**825.6 > 138.0**	50	40
					825.6 >807.0	50	45
VBL	9.13	8	ESI+	$[M+H]^+$	**811.6 > 751.0**	50	45
					811.6 > 224.0	50	45
VRB	9.54	8	ESI+	$[M+H]^+$	**779.6 > 658.0**	40	25
					779.6 > 323.1	40	25
IMA	9.71	8	ESI+	$[M+H]^+$	**494.3 > 394.0**	45	35
					494.3 > 99.0	45	35
IF	9.95	8	ESI+	$[M+H]^+$	**261.1 > 183.0**	35	20
					261.1 > 154.0	35	20
CP	10.44	8	ESI+	$[M+H]^+$	**261.1 > 140.0**	30	25
					261.1 > 106.0	30	20
ERL	10.53	8	ESI+	$[M+H]^+$	**394.2 > 278.0**	35	35
					394.2 > 336.1	35	25
ETP	10.60	8	ESI+	$[M+H]^+$	**589.0 > 229.0**	55	15
					589.0 > 185.0	55	40
DOX	11.39	9	ESI+	$[M+H]^+$	**544.3 > 397.0**	25	10
					544.3 > 130.0	25	15
CAP	11.93	9	ESI+	$[M+H]^+$	**360.2 > 244.1**	25	10
					360.2 > 174.0	25	20
OH-D-TAM	12.41	9	ESI+	$[M+H]^+$	**374.3 > 223.0**	35	15
					374.3 > 58.0	35	25
OH-TAM	12.43	9	ESI+	$[M+H]^+$	**388.3 > 72.0**	40	30
					388.3 > 45.0	40	35
TAM	13.74	10	ESI+	$[M+H]^+$	**372.3 > 72.0**	45	25
					372.3 > 45.0	45	30
OH-PAC	13.93	10	ESI+	$[M+H]^+$	**871.0 > 286.0**	20	10
					871.0 > 526.0	20	25
PAC	14.26	10	ESI+	$[M+H]^+$	**854.5 > 105.0**	20	40
					854.5 > 286.0	20	15
CHL	15.34	11	ESI+	$[M+H]^+$	**304.1 > 192.0**	35	25
					304.1 > 168.0	35	35

[a] Segment; [b] Values in bold correspond to the transitions used for quantification; [c] Collision energy.

Table 3

Determination coefficients (R^2), repeatability (RSD), and limits of detection (LODs) and quantification (LOQ) obtained in the analysis of the target cytostatics by LC-ESI-MS/MS.

Compound	Linearity R^2	RSD (%)		LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		50 ng mL^{-1}	200 ng mL^{-1}		
5-Fu	0.9991	5.3	5.0	5.0	16.6
Car-Pt	0.9972	4.7	4.4	2.5	8.3
GEM	0.9982	2.3	2.3	0.6	2.0
Oxa-Pt	0.9978	5.6	1.5	3.0	10.0
MTIC	0.7481	32.9	30.2	7.4	24.7
TMZ	0.9995	3.3	2.5	0.7	2.2
MET	0.9918	1.4	1.6	0.7	2.2
OH-MET	0.9983	3.3	3.1	0.9	2.9
VCN	0.9963	5.6	5.9	10.3	34.3
VBL	0.9924	6.1	1.4	7.5	25.0
VRB	0.9943	4.8	4.5	8.3	27.6
IMA	0.9917	4.8	2.4	8.0	26.7
IF	0.9989	3.7	1.2	2.3	7.7
CP	0.9978	2.0	1.3	0.1	1.7
ERL	0.9940	4.1	3.7	0.1	1.0
ETP	0.9963	4.1	5.0	9.0	30.0
DOX	0.9943	1.3	3.6	0.8	2.5
CAP	0.9996	2.1	1.8	0.6	1.9
OH-D-TAM	0.9975	4.0	2.2	3.0	10.0
OH-TAM	0.9961	1.7	1.8	1.5	5.0
TAM	0.9976	2.8	1.9	5.0	16.7
OH-PAC	0.9955	3.9	4.2	3.0	10.0
PAC	0.9955	4.1	0.3	3.2	10.5
CHL	0.9776	33.3	20.6	9.0	30.0

Table 4

Percentage of compound remaining in solution (HPLC water) after 24 hours at three different temperatures (4, 15 and 25ºC).

Compound	4ºC	15ºC	25ºC
5-Fu	101	100	89
Car-Pt	107	85	71
GEM	100	99	103
Oxa-Pt	97	54	26
MTIC	12	0.73	0.87
TMZ	98	91	88
MET	95	46	35
OH-MET	86	-	-
VCN	98	96	93
VBL	91	98	83
VRB	101	105	104
IMA	95	93	77
IF	100	103	94
CP	98	107	103
ERL	96	101	100
ETP	100	104	96
DOX	89	90	77
CAP	97	105	103
OH-D-TAM	88	87	70
OH-TAM	94	91	75
TAM	88	-	-
OH-PAC	92	84	87
PAC	89	72	78
CHL	20	-	-

- not detected

Table 5

Stability of the compounds in HPLC water (with 4% DMSO) at different temperatures.

Compound	Temperature of storage			
	25ºC	15ºC	4ºC	-20ºC
5-FU	At least 1 day	At least 1 day	1 month	3 months
Car-Pt	< 1 day	At least 1 day	3 months	3 months
GEM	At least 1 day	At least 1 day	2 months	3 months
Oxa-Pt	< 1 day	< 1 day	6 days	1 month
MTIC	< 1 day	< 1 day	< 1 day	< 1 day
TMZ	At least 1 day	At least 1 day	2 months	3 months
MET	< 1 day	< 1 day	6 days	1 month
OH-MET	< 1 day	< 1 day	2 days	1 month
VCN	At least 1 day	At least 1 day	2 months	2 months
VBL	At least 1 day	At least 1 day	7 days	1 month
VRB	At least 1 day	At least 1 day	2 months	3 months
IMA	< 1 day	At least 1 day	3 days	< 1 week
IF	At least 1 day	At least 1 day	2 months	3 months
CP	At least 1 day	At least 1 day	3 months	3 months
ERL	At least 1 day	At least 1 day	3 days	< 1 week
ETP	At least 1 day	At least 1 day	3 months	2 months
DOX	< 1 day	At least 1 day	3 days	< 1 week
CAP	At least 1 day	At least 1 day	3 months	3 months
OH-D-TAM	< 1 day	At least 1 day	7 days	1 month
OH-TAM	< 1 day	At least 1 day	9 days	1 month
TAM	< 1 day	At least 1 day	5 days	1 month
OH-PAC	At least 1 day	At least 1 day	3 days	< 1 week
PAC	< 1 day	< 1 day	3 days	< 1 week
CHL	< 1 day	< 1 day	< 1 day	< 1 day

Figure(s)

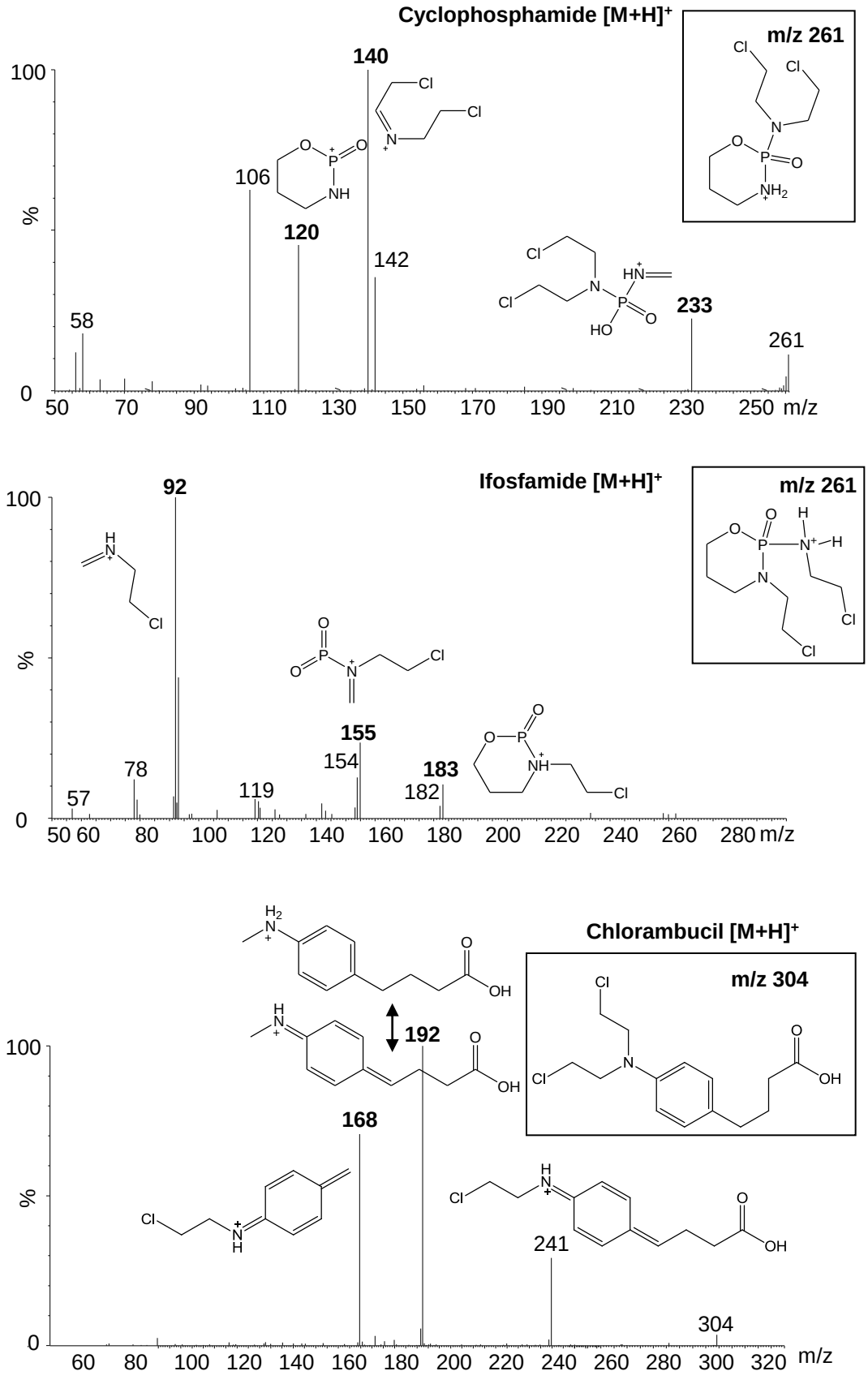
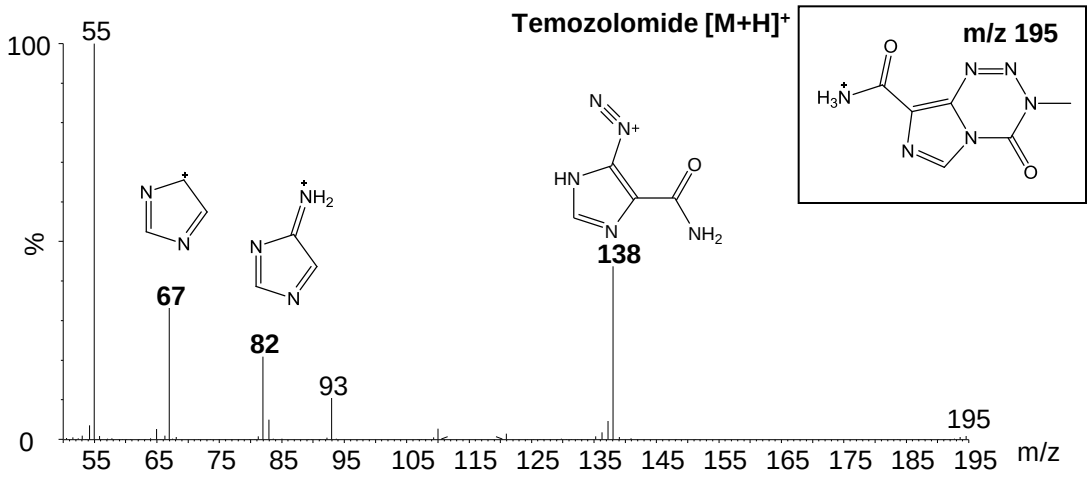
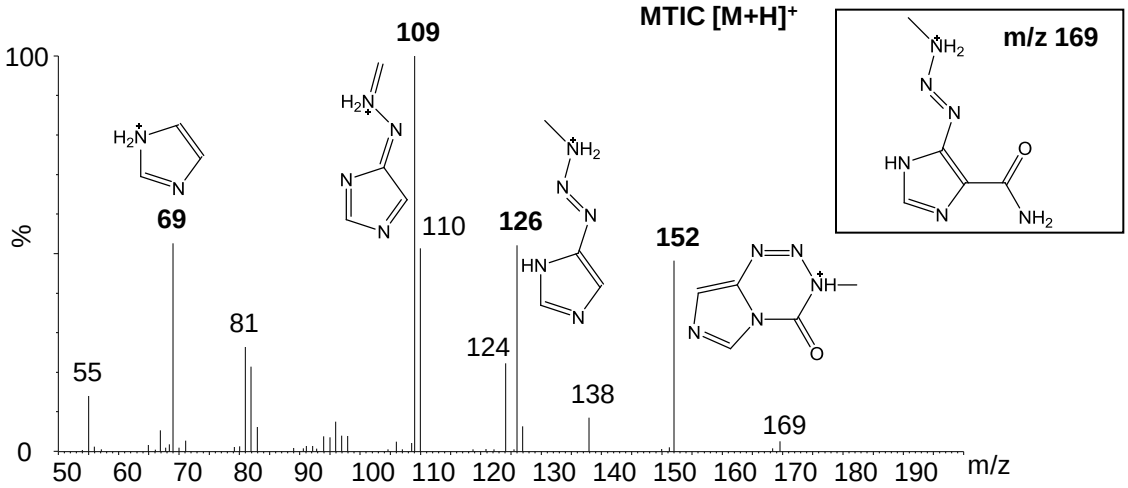
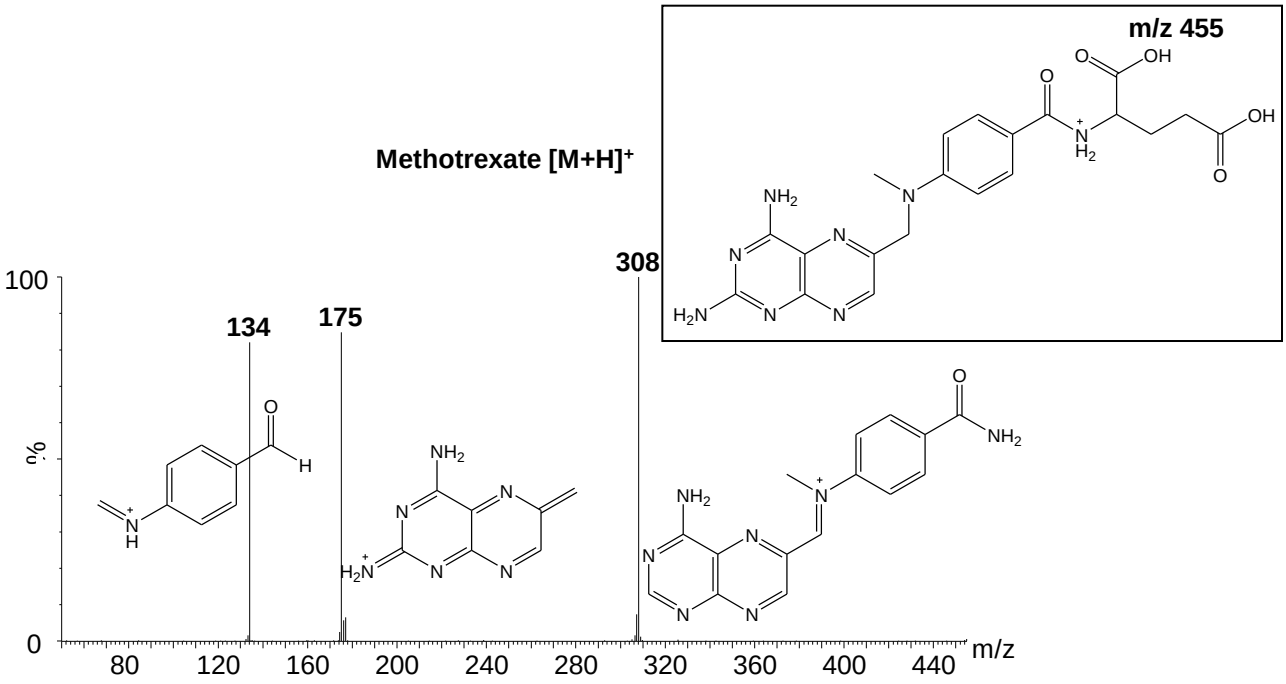
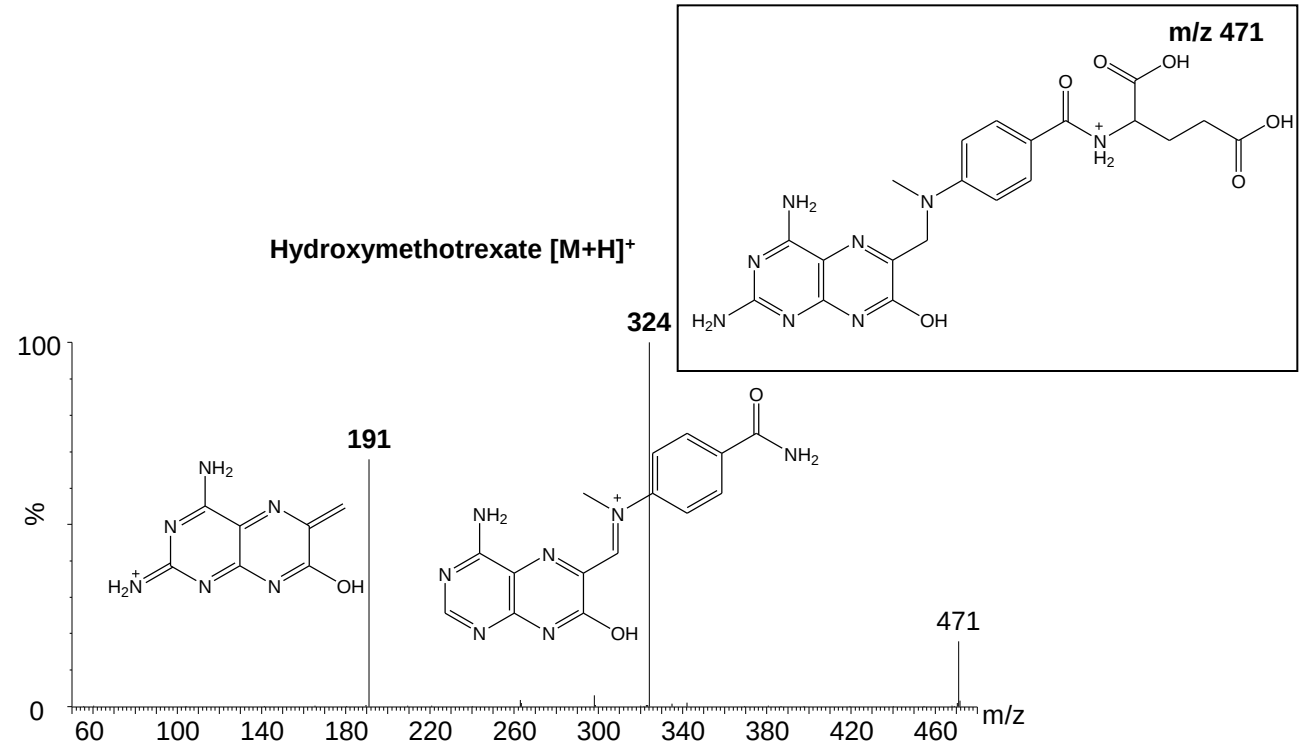


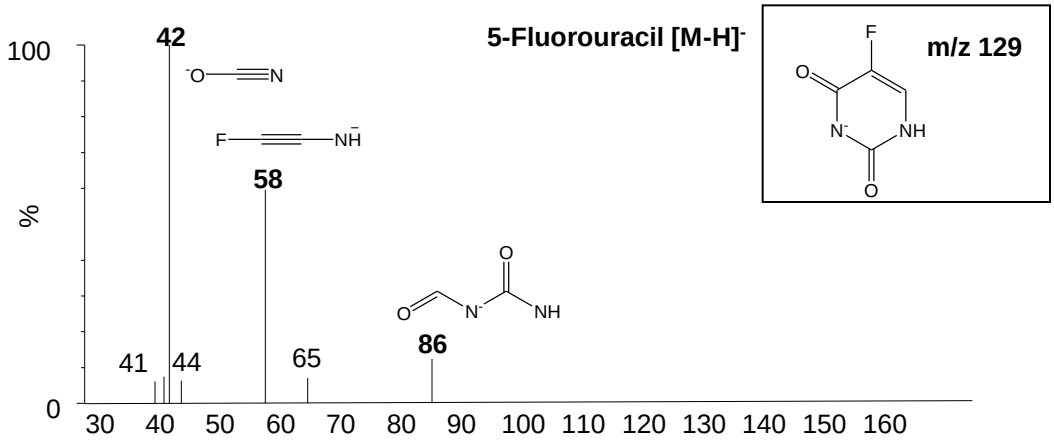
Fig. 1

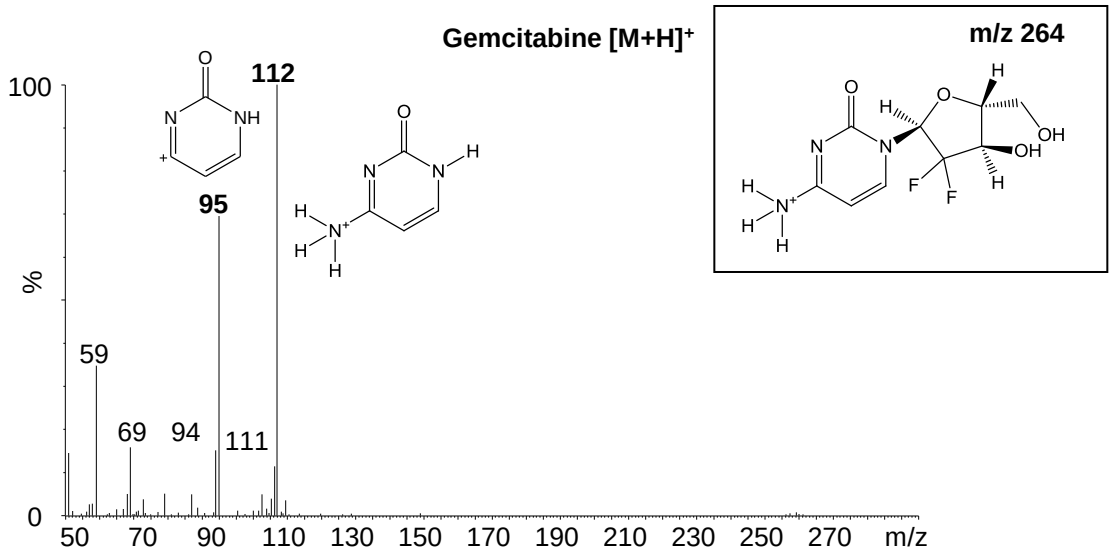

Temozolomide [M+H]+
m/z 195
100
%
0
55
67
82
93
138
195
55 65 75 85 95 105 115 125 135 145 155 165 175 185 195
m/z

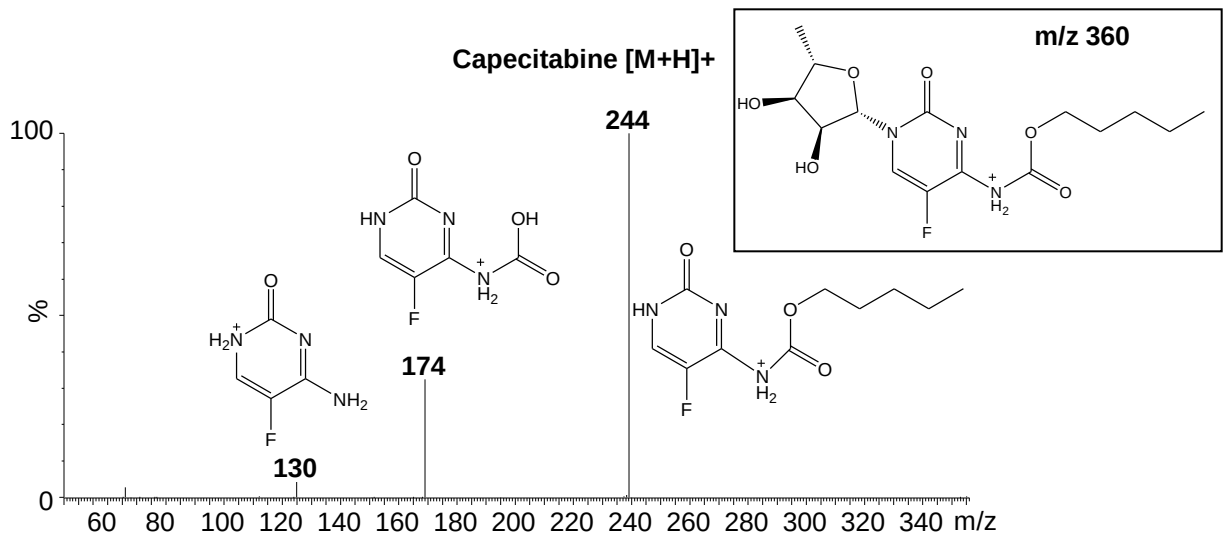

MTIC [M+H]+
m/z 169
100
%
0
109
69
110
126
152
81
124
138
169
55
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
m/z

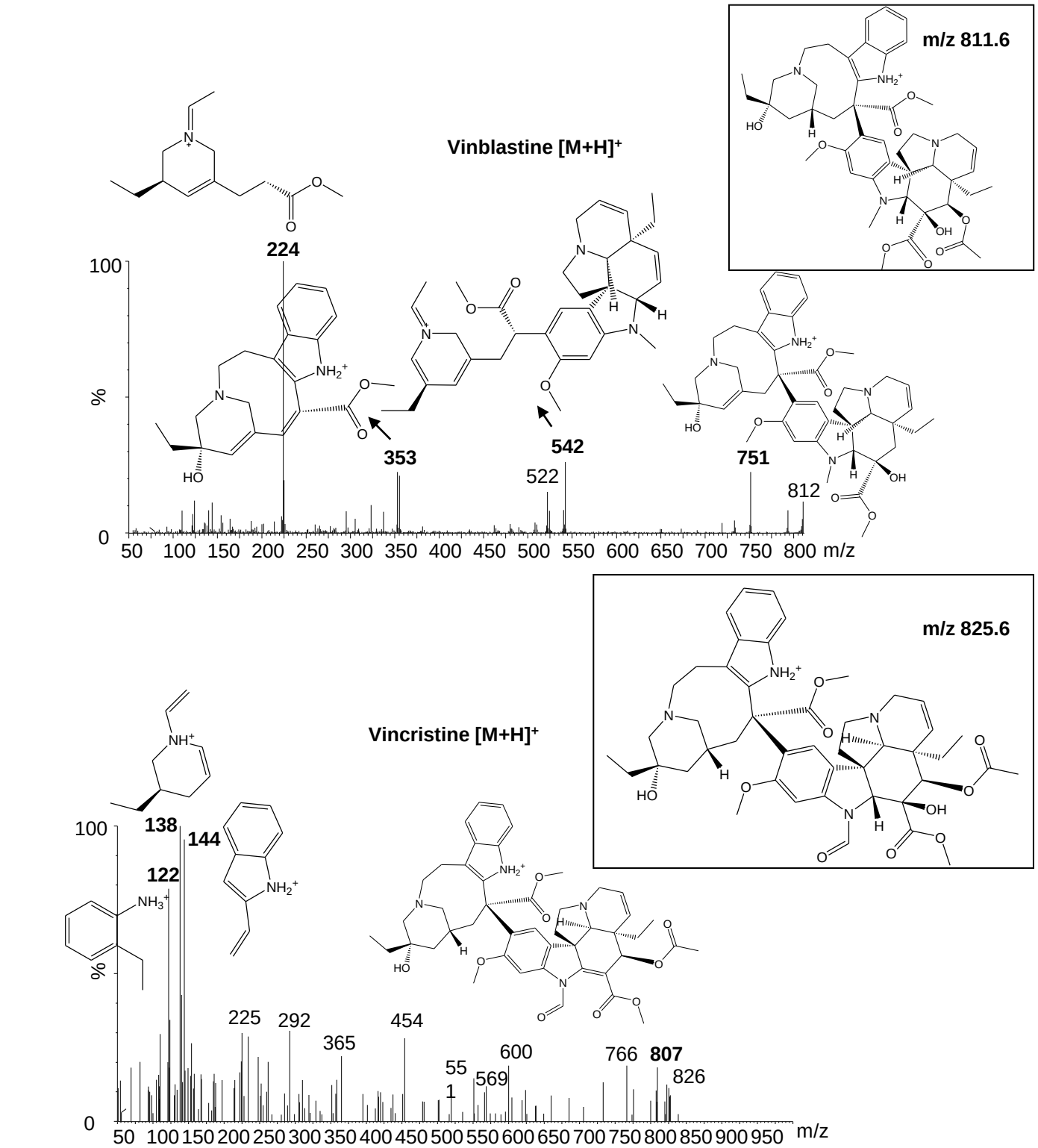

m/z 455
Methotrexate [M+H]+
308
134
175
100
%
0
80
120
160
200
240
280
320
360
400
440
m/z

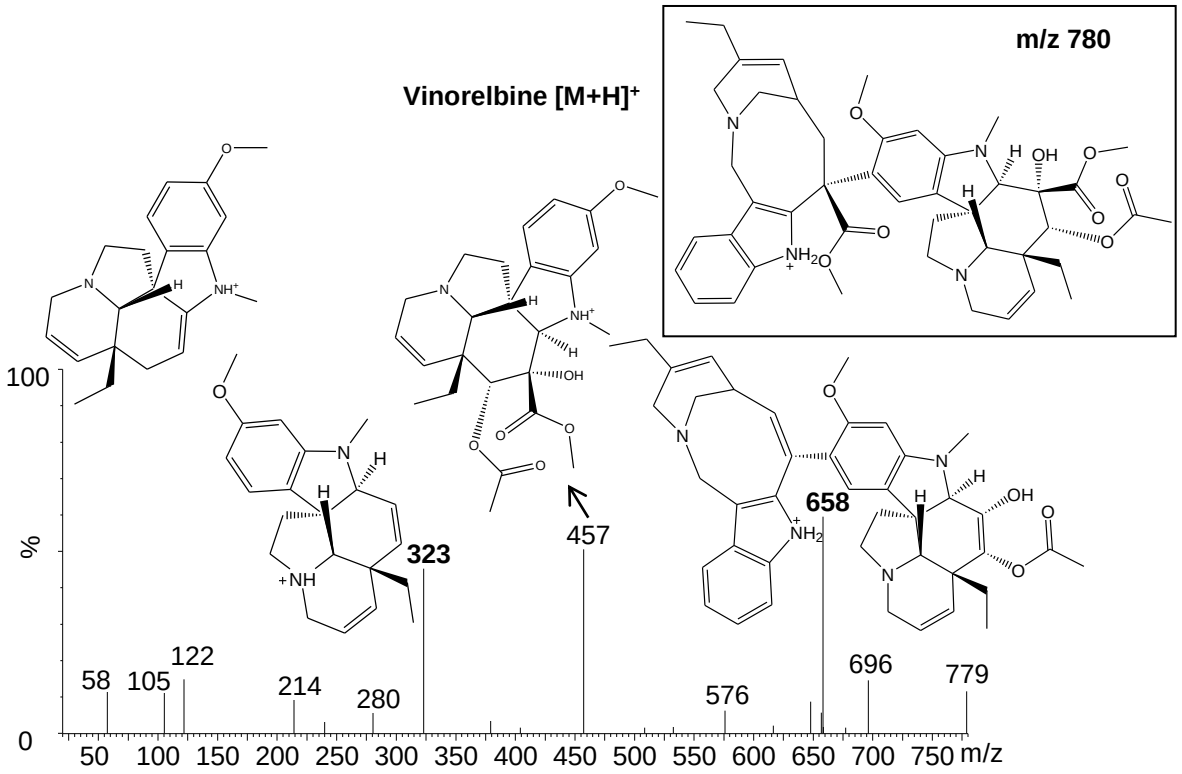

m/z 471
Hydroxymethotrexate [M+H]+
324
191
471
100
%
0
60
100
140
180
220
260
300
340
380
420
460
m/z

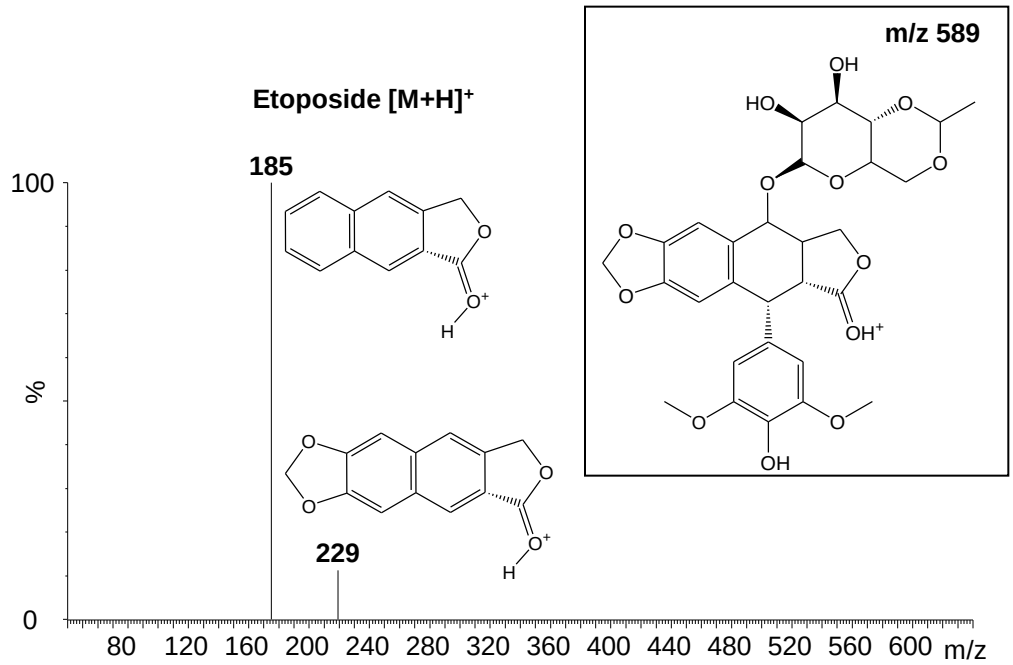

5-Fluorouracil [M-H]-
m/z 129
100
%
0
42
58
86
41
44
65
30 40 50 60 70 80 90 100 110 120 130 140 150 160

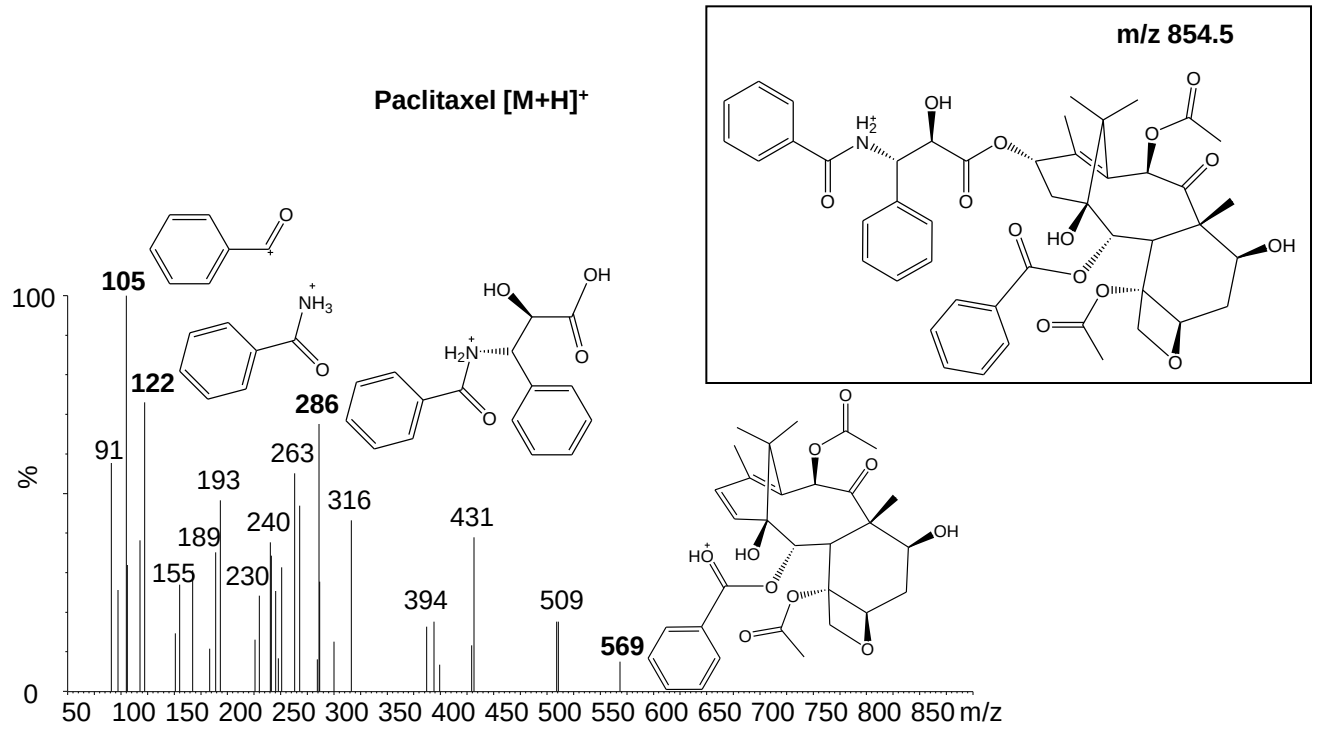

Gemcitabine [M+H]+
m/z 264
100
%
0
112
95
59
69
94
111
50 70 90 110 130 150 170 190 210 230 250 270 m/z

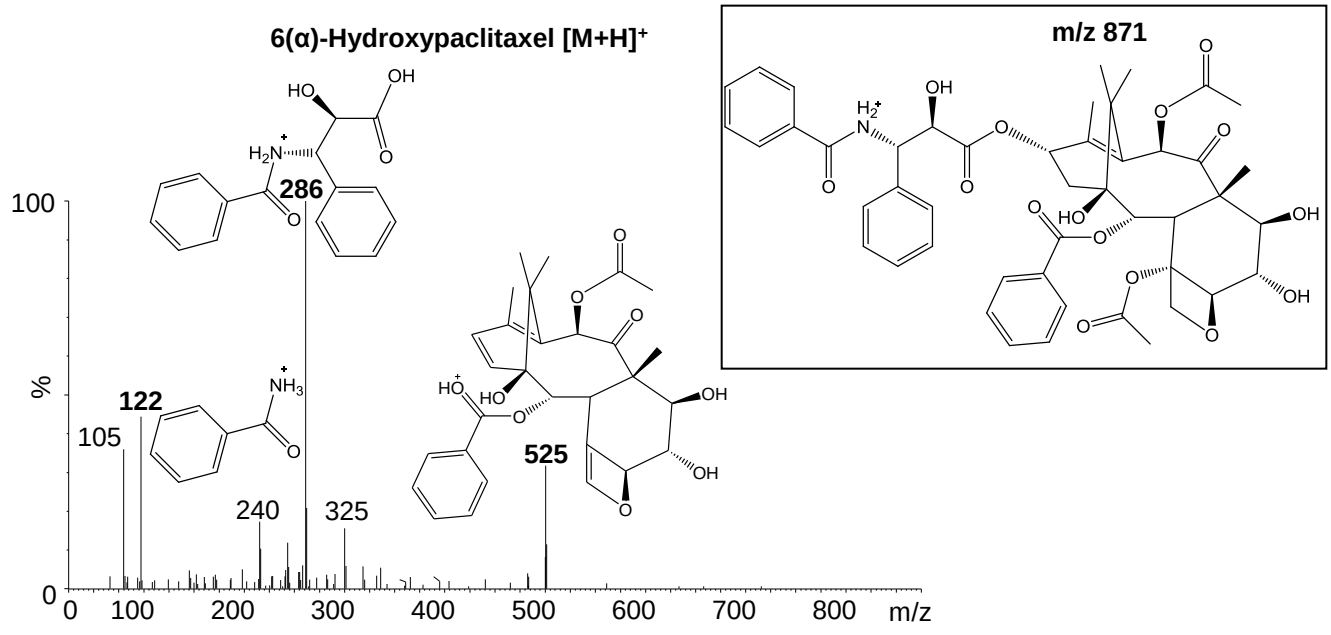

Capecitabine [M+H]+
m/z 360
100
%
0
244
174
130
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 m/z

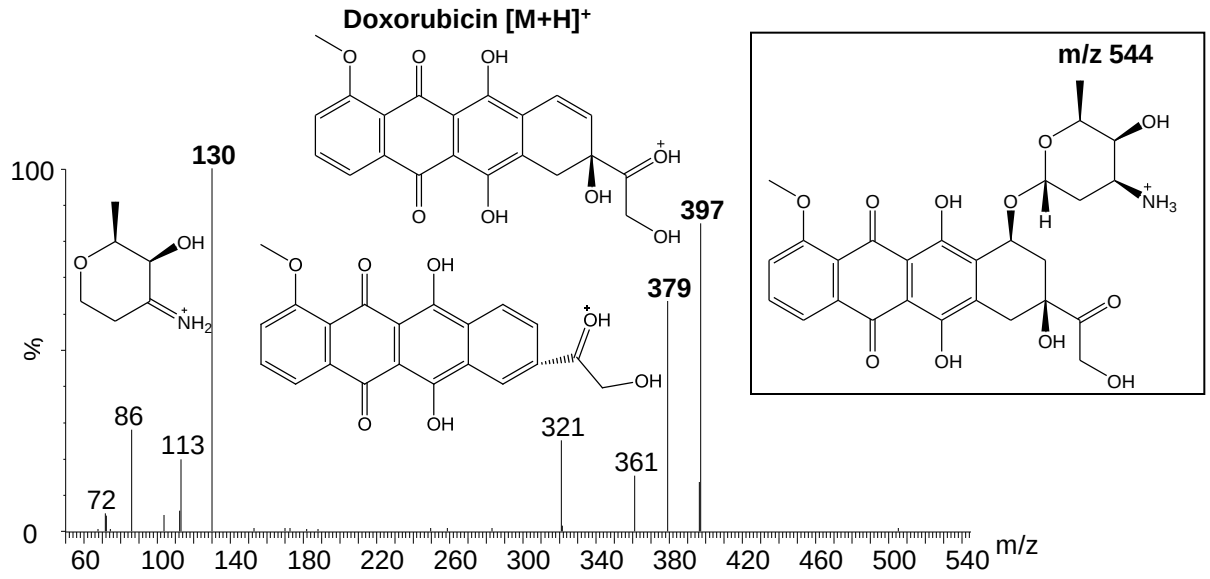

m/z 811.6
Vinblastine [M+H]+
224
353
542
751
522
812
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 m/z
m/z 825.6
Vincristine [M+H]+
138
144
122
225
292
365
454
600
766
807
826
55
1
569
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 m/z

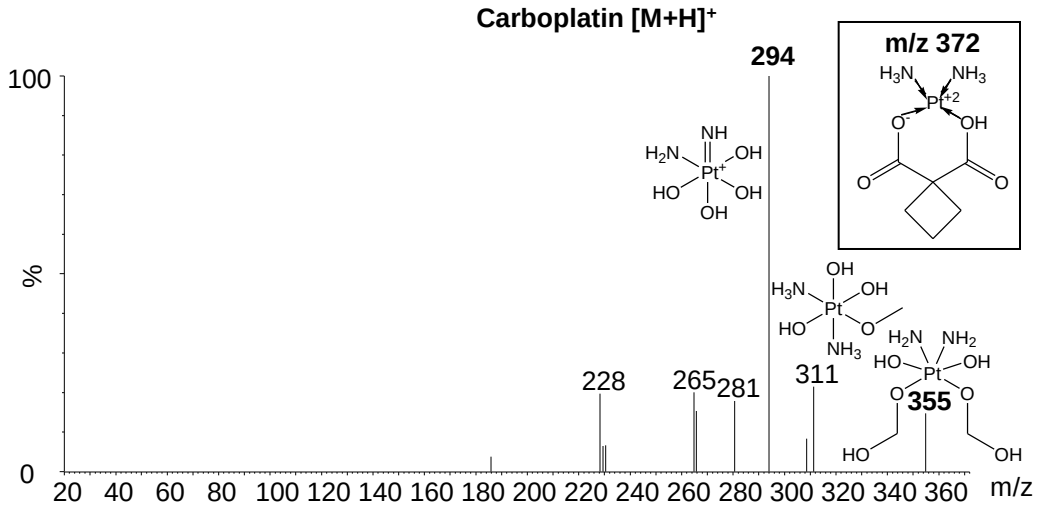

Vinorelbine [M+H]+
m/z 780
100
%
0
323
457
658
58
105
122
214
280
576
696
779
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 m/z

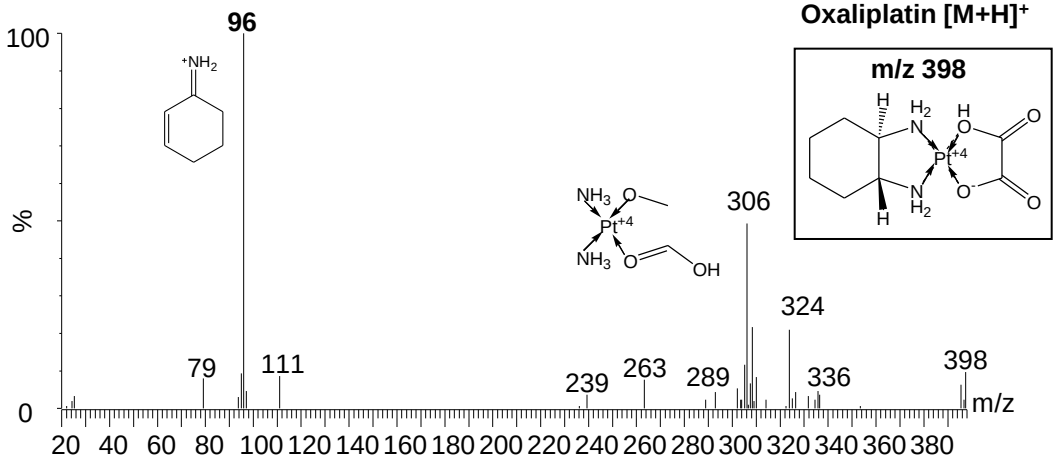

Etoposide [M+H]+
m/z 589
185
229
100
%
0
80 120 160 200 240 280 320 360 400 440 480 520 560 600 m/z

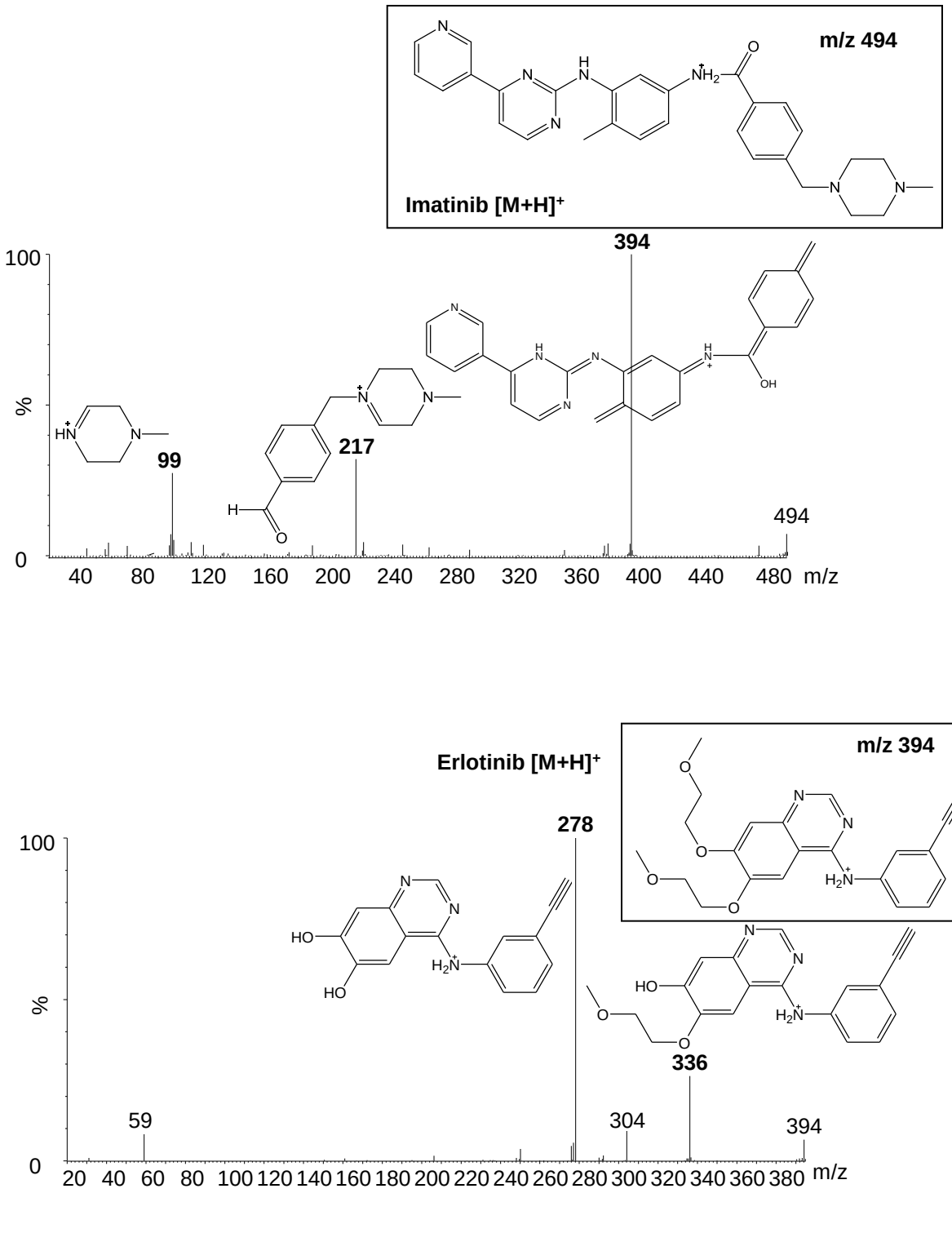

Paclitaxel [M+H]+
m/z 854.5
105
122
286
91
263
193
316
189
240
431
155
230
394
509
569
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 m/z

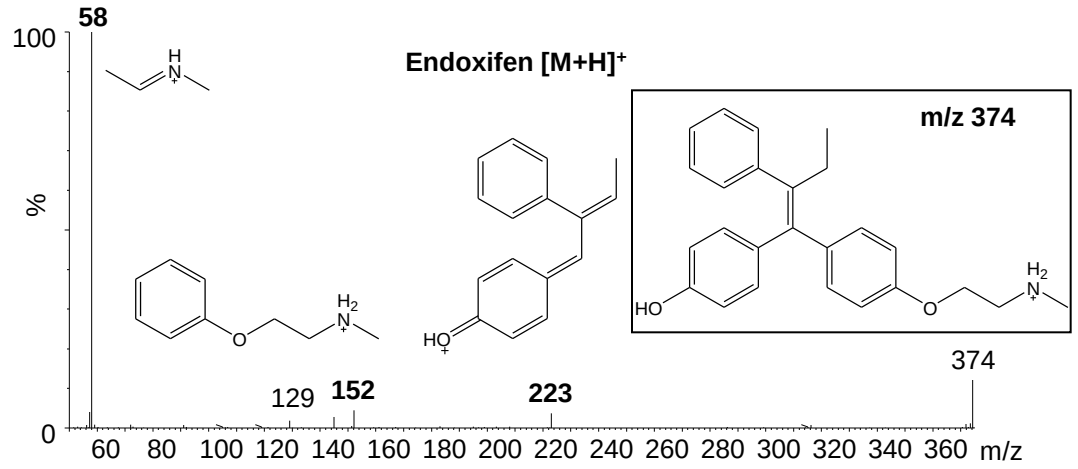

6(α)-Hydroxypaclitaxel [M+H]+
m/z 871
286
122
105
525
240
325
100
%
0
0 100 200 300 400 500 600 700 800 m/z

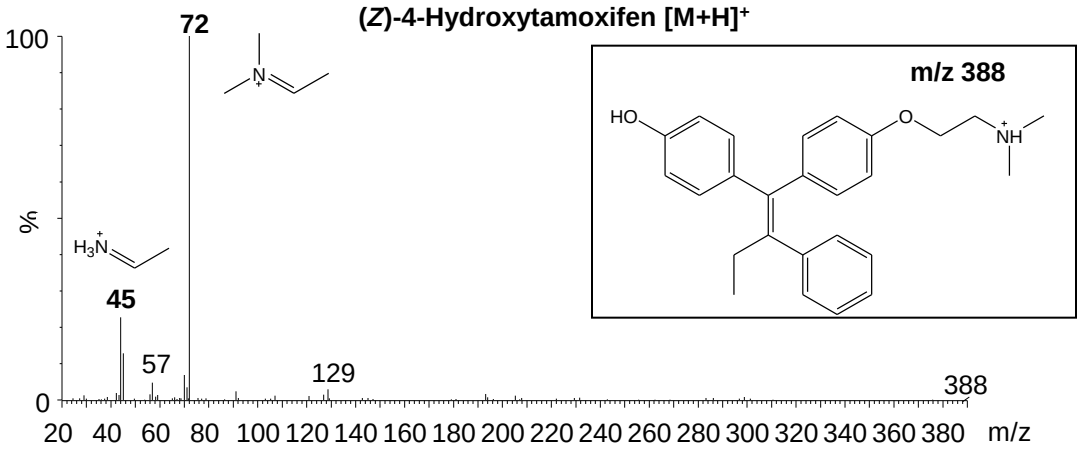

Doxorubicin [M+H]+
m/z 544
100
%
0
130
397
379
86
113
72
321
361
60
100
140
180
220
260
300
340
380
420
460
500
540
m/z

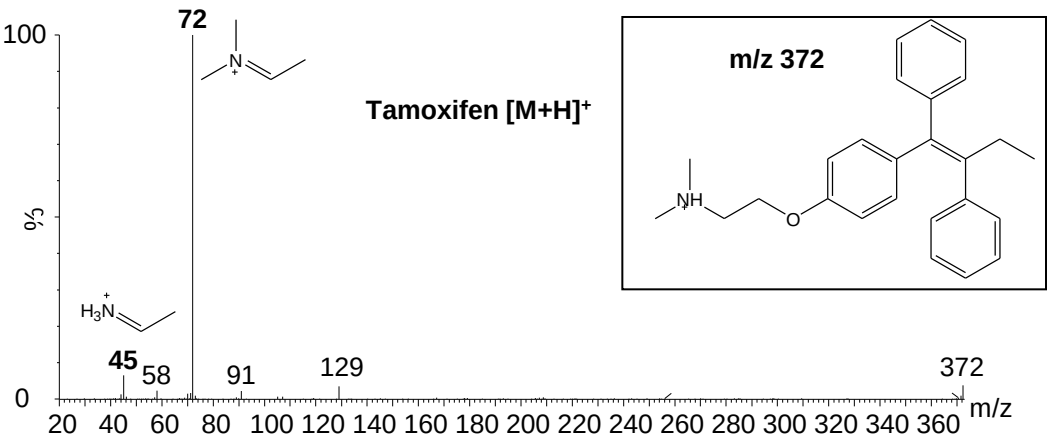

Carboplatin [M+H]+
294
m/z 372
100
%
0
228
265
281
311
355
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
m/z


Oxaliplatin [M+H]+
96
m/z 398
100
%
0
306
324
79
111
239
263
289
336
398
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
m/z


m/z 494
Imatinib [M+H]+
394
100
%
0
99
217
494
40 80 120 160 200 240 280 320 360 400 440 480 m/z
Erlotinib [M+H]+
m/z 394
278
336
59
304
394
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 m/z


Endoxifen [M+H]+
m/z 374
58
129
152
223
374
100
%
0
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 m/z


(Z)-4-Hydroxytamoxifen [M+H]+
m/z 388
72
45
57
129
388
100
%
0
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 m/z


Tamoxifen [M+H]+
m/z 372
72
45
58
91
129
372
100
%
0
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 m/z

Figure(s)

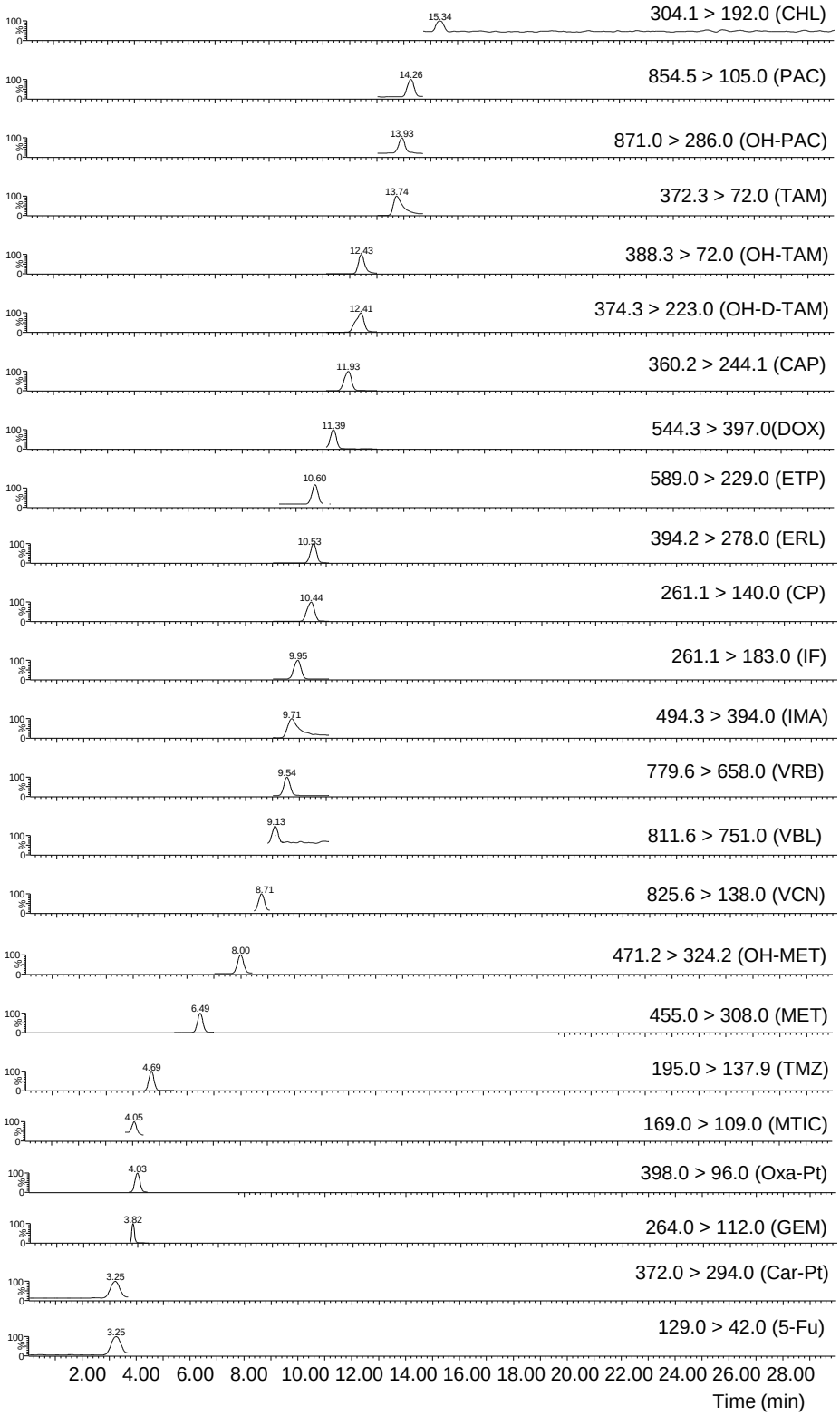


Fig. 2

Figure(s)

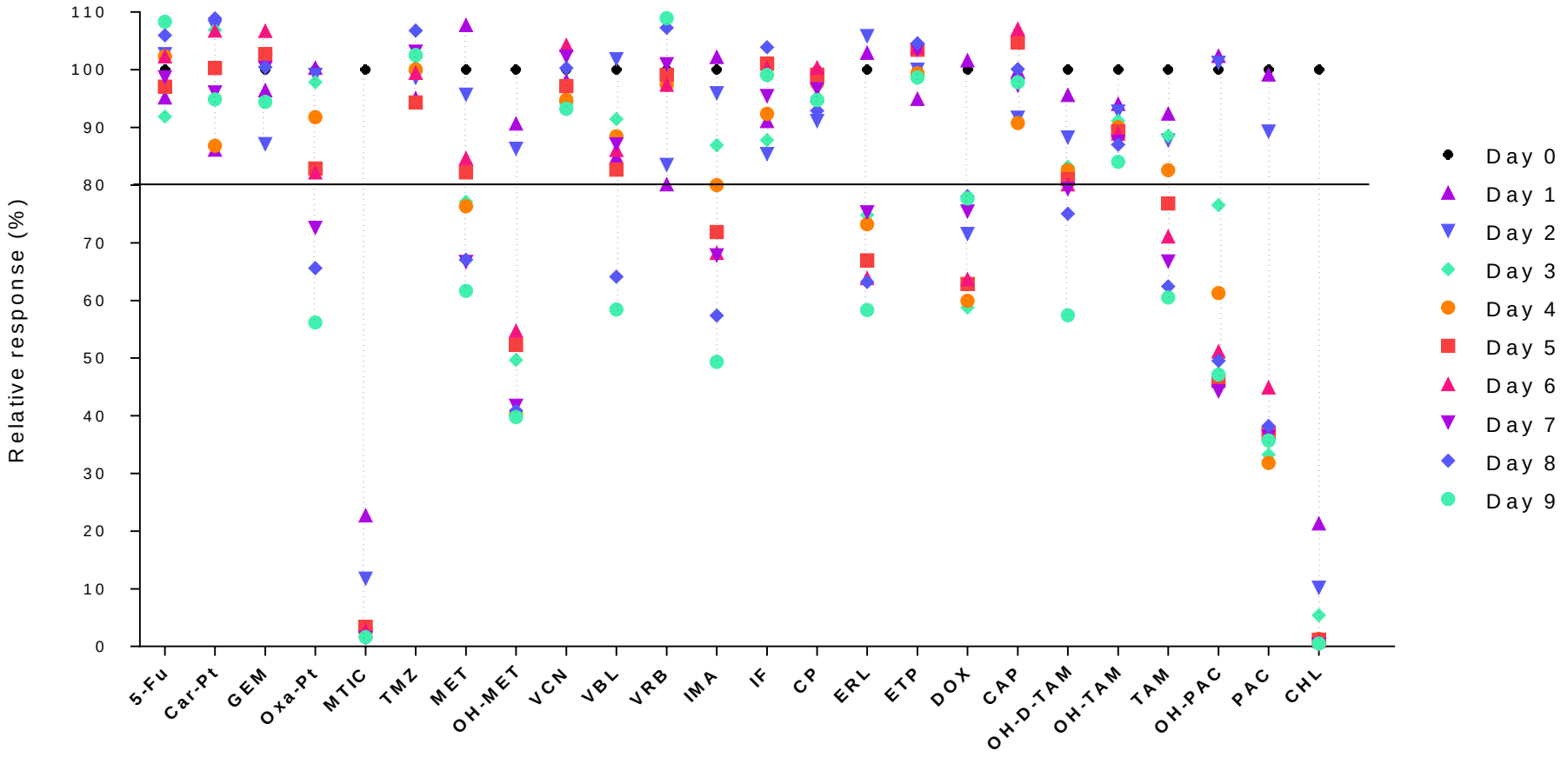


Fig. 3

Figure(s)

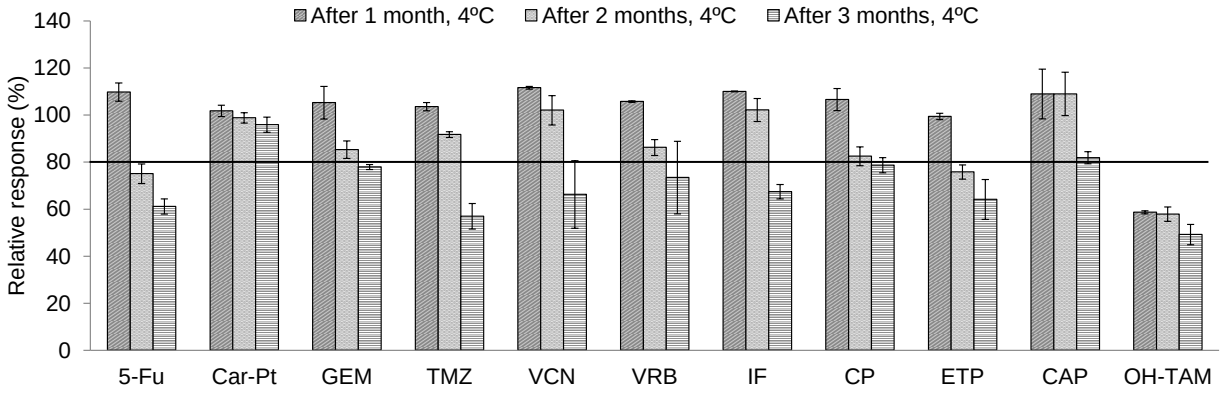


Fig. 4

Figure(s)

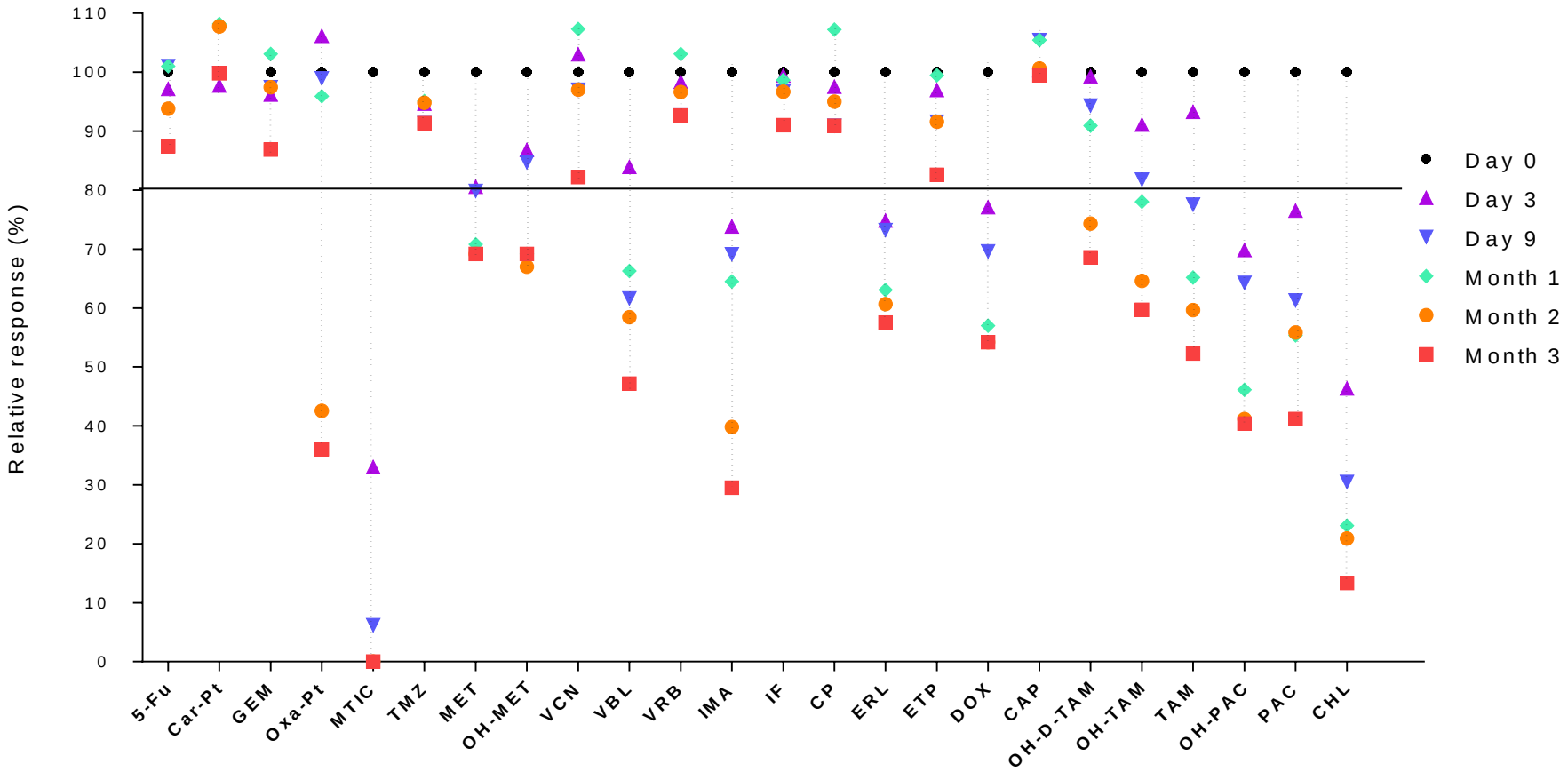


Fig. 5

Figure(s)

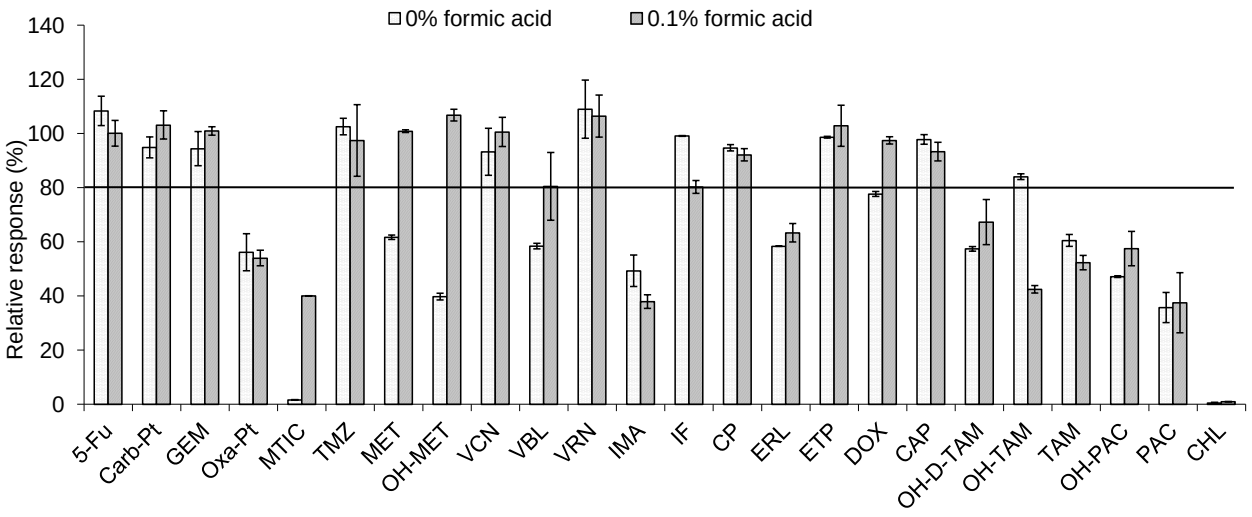


Fig. 6

Figure(s)

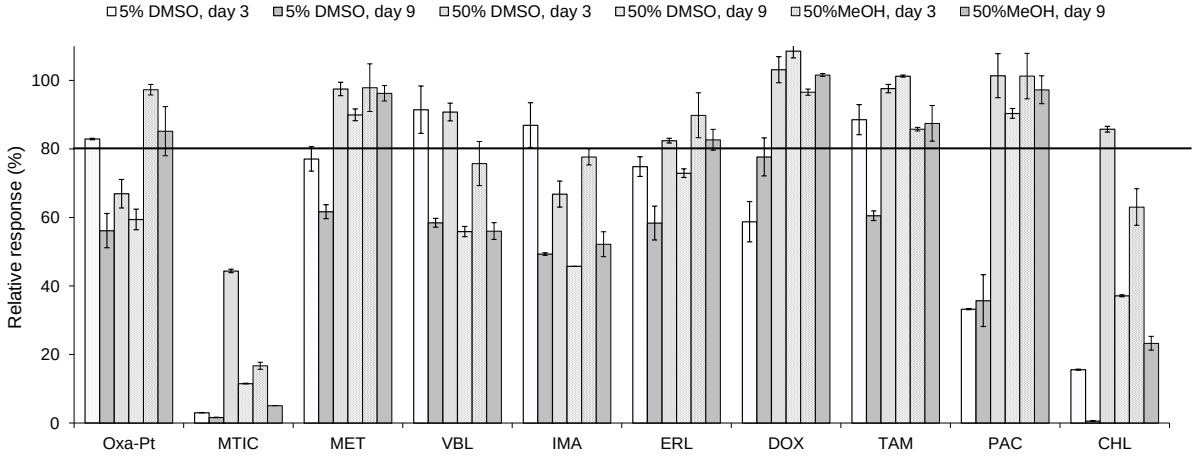


Fig. 7

***Checklist**

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• Keywords: Cytostatics; Water samples; Stability; Electrospray; Fragmentation; Liquid chromatography-mass spectrometry

• All figure captions:

Fig. 1. Product-ion mass spectra obtained for each of the target compounds in the ESI positive mode and corresponding suggested structures.

Fig. 2. SRM chromatograms obtained from the analysis of a standard mixture in HPLC-water at 100 ng mL^{-1} by LC-ESI-MS/MS.

Fig. 3. Stability data of the target cytostatic compounds and metabolites in HPLC water (initial concentration 1 µg mL^{-1}) stored at 4 ºC for 9 days.

Fig. 4. Relative response of the most stable cytostatic compounds and metabolites in HPLC water (initial concentration 1 µg mL^{-1}) after 1, 2, and 3 months of storage at 4 ºC.

Fig. 5. Stability data of the target cytostatic compounds and metabolites in HPLC water (initial concentration 1 µg mL^{-1}) stored at -20 ºC for different time periods up to 3 months.

Fig. 6. Effect of the addition of formic acid on the stability of the compounds in water after storage for 9 days.

Fig. 7. Effect of the addition of organic solvent on the stability of the compounds in water stored at 4 ºC for 3 and 9 days.

• All tables (including title, description, footnotes)

Table 1

Abbreviated names, molecular mass, acid-base dissociation constant (p*K*a), octanol-water partition coefficient (log *K*ow), and ATC group of the target cytostatics.

Table 2

LC retention time (t_R) and selected MS/MS detection condition for determination of the target cytostatics.

Table 3

Correlation coefficients (R^2), repeatability (RSD), and limits of detection (LODs) and quantification (LOQ) obtained in the analysis of the target cytostatics by LC-ESI-MS/MS.

Table 4

Percentage of compound remaining in solution (HPLC water) after 24 hours at three different temperatures (4, 15 and 25ºC).

Table 5

Stability of the compounds in HPLC water (with 4% DMSO) at different temperatures.

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

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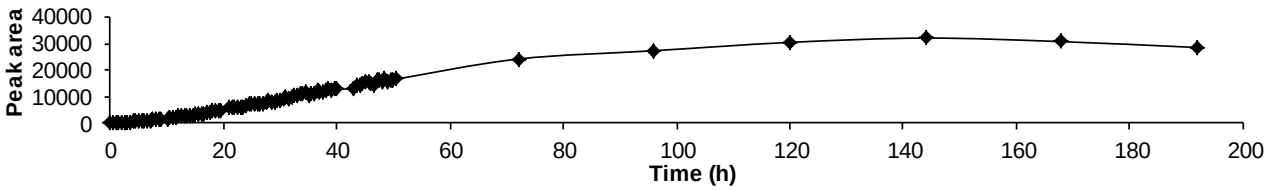
Peak area
40000
30000
20000
10000
0
0
20
40
60
80
100
120
140
160
180
200
Time (h)

Fig. S1

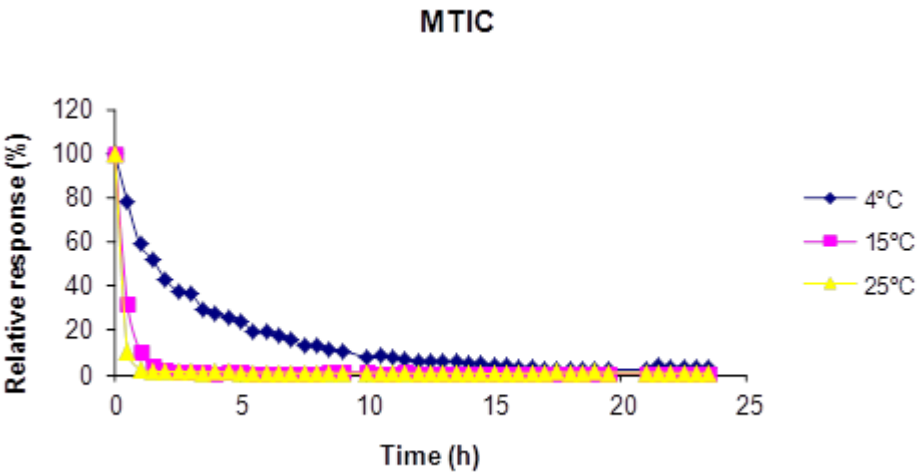
MTIC
Relative response (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
Time (h)
4°C
15°C
25°C

Fig. S2

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*Graphical Abstract

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