FAST DETERMINATION OF 40 DRUGS IN WATER USING LARGE VOLUME DIRECT INJECTION LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

This work describes a rapid analytical method based on direct sample injection of water samples for the simultaneous identification/quantification of 40 emerging compounds, including pharmaceuticals and drugs of abuse. The water samples were analyzed by ultra-high-performance liquid chromatography coupled to hybrid triple quadrupole mass spectrometer (UHPLC-MS/MS QqQ). Taking profit of the increasing sensitivity of nowadays's tandem mass spectrometers, direct sample injection of large volumes has been an attractive alternative to pre-concentration steps. In this work, the developed methodology has been validated at three concentration levels (10, 100 and 1000 ng/L) in 10 different water samples of different types (5 effluent wastewaters and 5 surface waters). The majority of compounds could be satisfactory validated at these concentrations, showing good recoveries and precision. With only few exceptions, the limits of quantification (LOQs), estimated from the sample chromatogram at lowest spiked level tested, were below 3 ng/L. The method was applied to the analysis of 10 effluent wastewaters and 10 surface water samples. Venlafaxine was the compound most frequently detected (80%) in surface water, followed by acetaminophen (70%). Regarding effluent wastewater, valsartan and 4-acetyl aminoantipyrine were detected in 9 out of 10 samples analyzed. These two compounds together with 4-formyl aminoantipyrine and naproxen showed the highest concentrations (>2000 ng/L). In these cases, a dilution step was required for a correct quantification. As an additional evaluation of the method performance, the same water samples were analyzed in other laboratory by a second analytical methodology, based on on-line solid-phase-extraction coupled to LC-MS/MS (QqQ).

Keywords

Direct injection, illicit drugs of abuse, pharmaceuticals, effluent wastewater, surface water, liquid chromatography, triple quadrupole mass spectrometry.

1. INTRODUCTION

The presence of human and veterinarian pharmaceuticals, as well as illegal drugs of abuse, in environmental samples has been recognized as a potential environmental threat [1,2]. These groups of contaminants are of present concern, due to their very high biological activity, psychoactive properties and still not well known effects to the aquatic environment [1,3]. After their consumption, these compounds can be excreted as the parent compound, as metabolites or as a mix of unchanged compound plus metabolites, reaching first the wastewater treatment plants (WWTPs) and finally the aquatic environment if they are not completely removed by WWTPs. The concentrations of these compounds in the environment depend on many factors, including their consumption pattern and use, the percentage of wastewater collected and the characteristics of the processes used for wastewater treatment [4]. Recently, several works have reported the presence of drugs and metabolites in the environmental, showing concern for its unknown impact [5–7].

Current analytical methods developed for quantifying low concentration of pharmaceuticals [2,8–10] and illicit drugs [11,12] in aquatic samples, usually include pre-concentration steps, the most common being those based on solid-phase extraction (SPE). Extraction from water samples has usually been performed by off-line SPE [5,6,8,9,11], although on-line SPE-LC has also been reported as a time and cost-saving alternative thanks to its fully automation [7,13]. Large-volume injection (LVI) is an attractive approach for aqueous samples that has been applied in several works as a rapid and efficient alternative to conventional SPE [14–18]. Typically, LVI involves the direct injection of sample volumes that range from 100 to 5000 µL versus the more conventionally injected volumes of 10-20 µL [14]. The improvement in sensitivity comes from the injection of sample volumes larger than usual. LVI provides good reproducibility and low sample contamination as a consequence of the minimal sample handling. Moreover, it allows to increase sample throughput at minimal cost

compared to both off- and on-line SPE, because no SPE cartridges and solvents are needed [14]. Despite the injection of larger volumes, modern and sensitive instruments are commonly needed for final measurement, as the increase in injection volume does not compensate the pre-concentration factors normally reached by SPE. In addition, peak shape may be deteriorated for early eluting analytes when increasing injection volume despite the lower eluotropic strength of water sample. Moreover, only clean water is usually directly injected in the system otherwise matrix effects could not be properly compensated for. Although, we show that effluent wastewater might be considered clean water in our LVI approach.

Modern multi-class methods applied for the determination of polar pharmaceuticals or drugs of abuse are mostly based on liquid chromatography (LC). The use of UHPLC in combination with tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) [2,8,10,11,19–22] or ion trap (IT) analyzers [23–27], has made possible the development of faster and more sensitive methods. Moreover, the fact of working with short dwell times in new instruments, allows increasing the number of selected reaction monitoring (SRM) transitions acquired simultaneously per compound making possible not only quantification but also a reliable identification. Although LC-MS/MS is the technique of choice at present to analyze polar compounds in aquatic samples, the presence of pharmaceuticals in environmental samples has also been investigated by LC coupled to high resolution mass spectrometry (HRMS), using time-of-flight (TOF MS) [28–30] or Orbitrap analyzers [31–33]. HRMS analyzers have strong potential for large screening and for identification/elucidation purposes, but they show less sensitivity than state-of-the-art MS/MS instruments, making that LC-MS/MS are considered the optimum analyzers for quantification at trace level.

The goal of the present paper is to develop fast and sensitive analytical methodology combining the advantages of UHPLC-MS/MS with last-generation triple quadrupole and large-volume direct sample injection. Thus, a rapid method avoiding sample manipulation

(i.e. pre-concentration and clean-up) has been developed for the determination of forty highly consumed compounds, including pharmaceuticals, drugs of abuse and some veterinary drugs, in waters. The quantitative validation has been performed at three concentration levels (10, 100 and 1000 ng/L) in 5 surface water (SW) and 5 effluent wastewater (EWW) samples. Several isotopically-labelled internal standards have been tested for correction of expected matrix effects. In order to evaluate the applicability of the method, 20 water samples (10 SW and 10 EWW) were analyzed. The same samples were analyzed by another laboratory using a methodology based on on-line SPE-LC-MS/MS (QqQ).

2. EXPERIMENTAL

2.1. Reagents and chemicals

Pharmaceutical reference standards were purchased from Sigma–Aldrich (St Louis, MO, USA), LGC Promochem (London, UK), Toronto Research Chemicals (Ontario, Canada), Across Organics (Geel, Belgium), Bayer Hispania (Barcelona, Spain), Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid, Spain) and Aventis Pharma (Madrid, Spain). All reference standards presented purity higher than 93%.

Illicit drugs and metabolites studied were amphetamine, 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), cocaine, cocaethylene and benzoylecgonine. These compounds were obtained from Sigma–Aldrich (Madrid, Spain), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol, acetonitrile or as salt.

Standard stock solutions of each compound were prepared at 100 mg/L in methanol or acetonitrile. Intermediate solutions (10 mg/L) were prepared by dilution of the stock solution ten-fold with methanol. Mixed working solutions containing all analytes were prepared daily from intermediate solutions by appropriate dilution with water, and were used for preparation of the aqueous calibration standards and for spiking samples in the validation study.

Isotopically-labelled internal standards (ILIS) of omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, valsartan-d_8, carbamazepine 10,11-epoxide-d_{10} and salicylic acid-d_3 were from CDN Isotopes (Quebec, Canada); atorvastatin-d_5 from Toronto Research Chemicals and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$ were from Cambridge Isotope Laboratories (Andover, MA, USA). Deuterated drugs of abuse were purchased from Cerilliant as solutions in methanol or acetonitrile at a concentration of 100 mg/L (amphetamine-d_6, MDMA-d_5, benzoylecgonine-d_3, cocaine-d_3 and cocaethylene-d_8). A mix ILIS working solution at 100

µg/L was prepared in MeOH and used as internal standard. All solutions were stored in amber glass bottles at −20 °C.

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide (NaOH, >99%) were purchased from Scharlab (Barcelona, Spain). HPLC grade water was obtained from distilled water passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

UHPLC analysis were carried out with a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatography separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size analytical column 100 mm × 2.1 mm (Waters). The mobile phases used were A = H_2O and B = MeOH, both with 0.01% HCOOH and 1mM NH_4Ac. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 5%; 7 min, 90%; 8 min, 90%; 8.1 min, 5%; 10 min, 5%. The flow rate was 0.4 mL/min. The column was kept at 40 °C and the sample manager was maintained at 5 °C. Analysis run time was 10 min. The sample injection volume was 100 µL.

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer Xevo TQS (Waters) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operated in positive and negative ion mode. Cone gas as well as desolvation gas was nitrogen (Praxair, Valencia, Spain) set up 250 L/h and 1200 L/h, respectively. For operation in the MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) with a pressure of 4×10^{-3} mbar in the collision cell (0.15 mL/min). Other parameters optimized were: capillary voltages 3.5 kV (ESI+) and 3.0 kV (ESI-); source temperature 150 °C and desolvation temperature 650 °C. Cone voltage was selected as 10 V for all compounds, due to

no variations were observed. Dwell times were automatically selected in order to obtain enough points per peak and can be decreased down to 3 ms.

All data were acquired and processed using MassLynx v 4.1 software (Waters).

2.3. Sample preparation

All water samples were centrifuged at 4500 rpm for 5 min. 1-mL surface water or effluent wastewater was spiked at 50 ng/L with the ILIS mix. 100 µL of the sample were directly injected in the UHPLC–MS/MS system.

2.4. Validation study

Acquisition was performed in SRM mode, with the (de)protonated molecular ion of each compound chosen as precursor ion. The most abundant product ion of each target analyte was typically used for quantification and two additional product ions were used for confirmation. LC retention time was also compared with that of the reference standards (within ±2.5%) to help to confirm the compounds detected in samples. 14 compounds were quantified using their corresponding labelled analyte as internal standard and 5 compounds were quantified using an analogue IS (see **Table 2**). The remaining 21 compounds were quantified by external calibration using absolute responses.

The linearity of the method was studied by analyzing standard solutions in triplicate at eight concentrations, in the range from 1 to 2500 ng/L. Satisfactory linearity was assumed when the correlation coefficient (r) was higher than 0.99, based on relative responses (analyte peak area/ILIS peak area), except for those compounds that were quantified without ILIS (absolute response).

Method accuracy (estimated by means of analysis of spiked samples directly injected into the LC-MS/MS system) and precision (expressed as repeatability, in terms of relative standard deviation (RSD)) were evaluated in surface water and effluent wastewater, spiked at three concentrations (10, 100 and 1000 ng/L). A total of 10 different water samples were used for

the method validation (5 effluent wastewater and 5 surface water samples). Quantification was made by using calibration standards in solvent and relative or absolute responses as a function of the ILIS was used or not for matrix effects correction. Recovery values between 70% and 120%, with RSD lower than 20% were considered as satisfactory. The limit of quantification (LOQ) was estimated for a signal to noise (S/N) ratio of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. Adequate blank samples were not found for several analytes as they were present in all samples collected. In these cases, LOQ values were estimated from the chromatograms of the non-spiked "blank" samples, considering the concentration levels.

2.5. Water samples

20 water samples (10 EWWs and 10 SWs) were collected in polyethylene high-density bottles in selected sites of the Spanish Mediterranean area (Castellon and Valencia provinces). Composite EWW samples were collected from different WWTPs using primary and secondary treatment methods. Grab SW were sampled from different rivers (3), reservoirs (3) and lakes (4). All samples were taken from October to December in 2012. Samples were stored at −18 ºC until analysis. Before analysis, samples were thawed at room temperature.

2.6. On-line SPE LC-MS/MS QqQ

An alternative analytical methodology was also applied following the protocol used in routine analysis by an ISO 17025 certified laboratory in Spain (IPROMA S.L.). For LC analysis, an Agilent 1200SL binary pump was coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer system API3200QTRAP from Applied Biosystems-Sciex (Foster City, California, USA). On-line SPE was performed by using an Agilent 1200 pump and a Strata-X cartridge (2 × 20 mm, 25 μm) from Phenomenex (Torrance, CA, USA). This equipment also includes a PAL autosampler (CTC Analytics, Switzerland) for automated sample injection. The injection volume to the on-line SPE was 2 mL of water, previously centrifuged at 2500

rpm for 5 min (wastewater samples were diluted 1/20). Chromatographic separation was performed on a reversed-phase column ZORBAX Eclipse XDB-C18 (50 × 4.6 mm, 1.8 μm) from Agilent (Palo Alto, CA, USA) maintained at 40 °C. Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in methanol, respectively. The following linear gradient was used: hold at 95%A for 4.5 min, decreased to 70%A over 4.6 min, decreased to 0%A over 6.5 min and then increased to 95%A over 10.1 min, returning to the initial conditions. The flow rate was set to 600 μL/min [34].

3. RESULTS AND DISCUSSION

In this work, 35 human and veterinary pharmaceuticals and 5 drugs of abuse were selected **(Table 1)**. Eight pharmaceuticals were among the most widely consumed in Spain [35]. The rest of compounds were selected due to their reported presence in water samples and to their potential negative effect on living organisms of the aquatic environment. Moreover, 4 compounds corresponded to metabolites of pharmaceuticals: salicylic acid, metabolite of acetylsalicylic acid [36]; and 4-aminoantipyrine, 4-acetyl aminoantipyrine and 4-formyl aminoantipyrine, metabolites of dipyrone [37,38,39].

3.1. MS/MS optimization

Individual standard solutions were directly infused in the MS/MS system. The majority of the compounds (33 out of 40) were determined with ESI operating in positive ionization mode, using the protonated molecule $[M+H]^+$ as precursor ion. The 7 remaining compounds were determined in negative ionization using $[M-H]^-$ as precursor ion. The three most sensitive SRM transitions (in terms of signal-to-noise ratio) were selected for each compound. The most abundant was used for quantification (Q) whereas the other two transitions were acquired for confirmation (q_1, q_2). The only exception was salicylic acid (only one transition), and gemfibrozil and naproxen (two transitions) because of their poor fragmentation. MS/MS parameters as well as SRM transitions and retention times are listed in **Table 1**. This table also shows the average (q/Q) ratios obtained from the calibration standards. The RSDs for q/Q ratios illustrate whether these ratios might be considered to be concentration dependent or not (e.g. RSD < 15% would indicate little variation of the q/Q values over the concentration range tested, from 1 to 2500 ng/L).

Three SRM transitions were acquired per compound, whereas for ILIS, only the quantification transition was monitored. Using our fast-acquisition triple quadrupole mass analyzer, dwell times as low as 3 ms per transition could be automatically set-up allowing

satisfactory peak shape (at least 10 points-per peak) and sensitivity for all 40 compounds investigated.

3.2. UHPLC conditions

In this work, different mobile phases (acetonitrile and methanol) with different composition (HCOOH and NH_4Ac at various concentrations) were tested. The effects of pH and ionic strength of the mobile phase on the peak shape, resolution and efficiencies were evaluated by varying the buffer concentration. Finally, a gradient consisting of water (solvent A) and MeOH (solvent B) both with 1 mM ammonium acetate and 0.01% formic acid was chosen as an appropriate mobile phase.

Initially, 10 µL were injected in the system as reference conditions. In order to further improve sensitivity, injection of increasing sample volumes was performed. On the basis of the column dimensions and the particle size (in this case, 2.1 x 100 mm, 1.7 µm), the dead volume of the column was estimated to be 400 µL. The recommended injection volume should not exceed the 10% of this dead volume, this is, 40 µL. Trying to perform LVI for this system, 50 and 100 µL were tested, obtaining satisfactory chromatographic peak shape in all cases. The best sensitivity was achieved when injecting 100 µL. Hence, the injection of 100 µL was selected for further validation.

3.3. Matrix effects: quantification

The high complexity and variability of the matrices in water samples (especially in wastewaters) affected considerably the recovery values of some compounds. For almost half of the studied compounds, matrix effects resulting in ionization suppression were observed, being more important in EWW samples than in SW. Thus, acetaminophen and atorvastatin showed recoveries between 60-120% in the five SW tested, but decreased down to 27-60% in EWW. A few compounds experimented ionization enhancement due to co-eluted matrix

components, leading to recoveries >100%. This was the case of levamisol, MDMA or trimethoprim. Among the different approaches proposed in the literature to remove or compensate for the matrix effects, the use of isotopically-labeled internal standards (if available) was considered the preferred option. Fourteen compounds could be corrected with their own ILIS, as they were available to our laboratory, obtaining satisfactory figures after correction, as expected. Erythromycin, levamisol, pravastatin, sulfadiazine and venlafaxine were corrected using an analogue ILIS **(Table 2)**. The selection of analogue ILIS was mainly based on chemical structure and/or retention time similarity between analyte and ILIS, as it was expected that both were affected by similar constituents of the matrix. In particular cases, e.g. erythromycin, an ILIS eluting at different retention time and with different chemical structure (sulfamethoxazole-$^{13}C_6$) was able to perform an efficient matrix effects correction, as previously reported by Gracia-Lor et al. [8]. The rest of the analytes were quantified using absolute response as matrix effects in the ten water samples tested were not much relevant.

3.4. Method validation

Analytical characteristics of the method were evaluated in two types of water samples: five surface water and five effluent wastewater samples, spiked at three concentration levels each (10, 100 and 1000 ng/L).

The linearity of the method was studied in the range 1-2500 ng/L for all compounds. Calibration curves showed in all cases correlation coefficients greater than 0.99, and residuals lower than 25%.

Accuracy and precision were estimated from injection of different water samples spiked at the three concentrations indicated above. All the “blank” samples contained at least one or more target analytes. Thus, the samples were previously analyzed and those with lower drug

concentration were selected as “blank” samples for method validation. Concentration of target compounds found in these “blank” samples were subtracted from the spiked samples.

The results obtained for most compounds were satisfactory at the three validation levels, with recoveries between 70-120% and precision (RSD) below 20% **(Table 2)**. At the lowest level (10 ng/L) amphetamine, diclofenac, olanzapine, roxithromycin and salicylic acid could not be validated, due to their lower sensitivity. For some compounds, validation was not feasible in all the samples tested due to the high analyte concentration found in different “blank” samples (e.g. the three dipyrone metabolites or gemfibrozil). In these cases, the number of data used in validation was less than 10 (5 SW and 5 EWW) (highlighted as * or ** in **Table 2**).

The method presented satisfactory precision for most compounds with RSDs below 20% at the three fortification levels. Regarding LOQ, they were ≤3ng/L for 32 out of 40 compounds in SW. For another 5 analytes LOQs ranged from 3 to 7 ng/L, and for the remaining 3 were slightly higher, between 12-38 ng/L. In EWW, 29 compounds presented LOQs ≤3ng/L, 7 ranged from 3 to 9 ng/L and the remaining 4 were between 12-41 ng/L. According to our data, it seems that the type of water did not much affect the attainable sensitivity despite of being a direct injection method.

3.5. Analysis of water samples

To demonstrate the applicability of the method developed, 10 effluent wastewater and 10 surface water samples were analyzed. In every sequence of analysis, a calibration curve in solvent was injected at the beginning and at the end of the batch sample. Quality controls (QCs) were also included in every sequence, consisting on selected EWW and SW samples spiked with all pharmaceuticals at 100 ng/L. QC recoveries were satisfactory (in the range of 70-120%) for the majority of the compounds. However, QCs recoveries for venlafaxine (using atorvastatin-d_5 as IS) and for levamisol (using cocaethylene-d_8) were around 130%. As

it has been already reported in the literature, the use of analogues IS does not always assure an efficient matrix effects correction [40,41].

Identification of positive findings was supported by evaluation of q_1/Q and q_2/Q ratios. The finding was considered as positive when retention time and at least one experimental ion-ratio were within the established tolerances [42], when compared with a reference standard. Although the acquisition of two SRM transitions per compound together with the accordance in the retention time are normally considered sufficient for a reliable confirmation of the compound identity, in this work three transitions were acquired in order to increase the confidence of the confirmation process [40]. Using three transitions, one can minimize the possibilities of reporting false negatives when the ion ratio is not accomplished, in those cases where one of the transitions seems to be interfered. As an example, **Figure 1** shows positive findings of alprazolam, bezafibrate and sulfamethoxazole in EWW. As it can be seen, the three transitions showed a peak at the same retention time. Moreover, at least one q/Q ratio was within tolerance limits.

Tables 3 and **4** show the concentration values (ng/L) found for each compound in EWW and SW, respectively. 32 analytes were detected in the 10 EWWs analyzed, illustrating the frequent occurrence of drugs in wastewaters and the fact that many of them are not completely removed in WWTPs. Carbamazepine, used for the treatment of epilepsy and bipolar disorder, was the compound most frequently detected, appearing in all samples analyzed. This was followed by the angiotensin II antagonist valsartan and 4-acetyl aminoantipyrine (metabolite of the analgesic dipyrone), which were present in 90% of EWWs. 4-formyl aminoantipyrine (another metabolite of dypirone), the anthelmintic levamisol, the antibiotics sulfamethoxazole and trimethoprim, and the antidepressant venlafaxine appeared in 80% of EWWs. The highest concentrations corresponded to 4-acetyl aminoantipyrine (7.2 µg/L), valsartan (4.6 µg/L), 4-formyl aminoantipyrine (3.2 µg/L) and

the analgesic naproxen (1.9 µg/L). In these cases, samples were diluted and re-analyzed to fit the linear range of the method.

In relation to surface waters, up to 26 compounds were detected in the samples analyzed. All these compounds were also found in EWWs, normally at higher concentrations. Venlafaxine and acetaminophen were the compounds most frequently detected, being present in 80% and 70% of the samples, respectively. 4-acetyl, 4-formyl aminoantipyrine, cocaine and its metabolite benzoylecgonine, were present in 60% of SWs analyzed. The highest concentration corresponded to dipyrone metabolites: 4-formyl (0.72 µg/L) and 4-acetyl aminoantipyrine (0.66 µg/L).

As an illustrative example, **Figure 2** shows UHPLC-MS/MS chromatograms for *SW 4* (only the quantitative transition Q is shown), which was positive for 19 out of the 40 target compounds. Concentration data for this sample are shown in **Table 4**, where it can be seen that acetaminophen presented the highest value (480 ng/L). Four drugs of abuse (benzoylecgonine, cocaethylene, cocaine and MDMA) were also detected in the range of 7-31 ng/L. These figures reveal that licit and illicit drugs can actually reach surface waters due to the incomplete removal in WWTPs.

3.5.1. On-line SPE HPLC-ESI-MS/MS

The same 20 samples were analyzed by another laboratory that applied an analytical methodology based on on-line SPE-LC coupled to triple quadrupole mass spectrometry. With this methodology, only 25 human and veterinary pharmaceuticals and drugs of abuse were included in the target method. All of them were determined with ESI operating in positive ionization mode. For confirmation, two SRM transitions at the same retention time, and the accomplishment of the q/Q ratios were required. Regarding quantification parameters, two internal standards were used to correct possible deviations: diclofenac-$^{13}C_6$ for

pharmaceuticals and cocaine-d_3 for drugs of abuse. The linearity of the method was studied in the range 2-150 ng/L for all compounds. The method presented satisfactory accuracy and precision for all compounds, with recoveries values >85% and RSDs below 13%. Regarding LOQ, they ranged from 2 to 20 ng/L for SW and from 40-400 ng/L for EWW. The on-line SPE-LC method was implemented in this laboratory under requirements of ISO-170025 [34].

Data obtained are also shown in **Tables 3** and **4** (between brackets). Six compounds (cocaine, benzoylecgonine, diclofenac, naproxen, sulfamethoxazole and venlafaxine) were found in EWW samples, less than in the direct injection methodology (32 compounds). This was surely due to the higher LOQs obtained in the on-line procedure, due to the dilution step (1/20) applied to EWW samples prior to on-line SPE. The concentration values ranged from 0.048 to 3.1 µg/L and were in agreement with the results obtained by the direct injection approach. Regarding surface water samples, where no dilution was performed, up to 8 compounds could be detected (benzoylecgonine, cocaine, acetaminophen, clarithromycin, diclofenac, levamisol, naproxen and venlafaxine). Among them, venlafaxine was the compound most frequently detected (3 out of 10 SW analyzed), and the highest concentration found was for acetaminophen (0.65 µg/L). All concentration values obtained by this methodology were also in accordance with the results reported after direct injection analyses.

Except for the differences due to the distinct sensitivity of the two procedures, the concentrations found by both of them for the wide majority of positive samples were rather similar, supporting the applicability and reliability of our more-sensitive large-volume direct injection approach.

4. CONCLUSIONS

Analytical methodology based on UHPLC-MS/MS QqQ has been developed for the simultaneous quantification and confirmation of 40 human and veterinary pharmaceuticals and drugs of abuse in effluent wastewater and surface samples. The direct injection of water samples (100 µL), without any previous sample treatment, has been shown as an attractive approach as it avoids time-consuming sample preparation steps and reduces the amounts of solvents used. The determination of target compounds was performed in positive/negative voltage switching mode in a single chromatographic run of only 10 min. With a few exceptions, a highly reliable identification of the compounds was feasible thanks to the acquisition of three SRM transitions per compound and the accomplishment of the ion ratio and retention time deviations. Satisfactory accuracy and precision were obtained in recovery experiments at three concentration levels in two kinds of water matrices, EWW and SW, using 10 different samples to this aim. The LOQs were in most cases lower than 3 ng/L. The application of this method to 10 effluent wastewater and 10 surface samples, allowed the detection of 32 and 26 compounds, respectively. Carbamazepine was the compound most frequently detect (100%) in EWW and venlafaxine (80%) in SW samples. This methodology has been proven to be an attractive and efficient approach for rapid determination of pharmaceuticals and drugs of abuse in environmental waters, achieving low LOQs without the need for a preliminary pre-concentration step.

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FIGURE CAPTIONS

Figure 1. UHPLC-MS/MS chromatograms detected in EWW samples.

Figure 2. UHPLC-MS/MS chromatograms (Q transition) for a surface water sample (*SW 4*, see Table 4) where 19 target compounds were found. Positive/negative voltage switching mode applied within the same run.

Table 1. MS/MS optimized conditions for selected compounds.

Compound	ESI	T_R (min)	Precursor ion (*m/z*)	Q transition	C.E. (eV)	q_1 transition	C.E. (eV)	q_1/Q (RSD)	q_2 transition	C.E. (eV)	q_2/Q (RSD)
4-Acetyl aminoantipyrine	+	2.85	245.9	246 > 228	10	246 > 83	20	0.71(3)	246 > 104	20	0.38(4)
4-Aminoantipyrine	+	3.15	204.1	204 > 56	15	204 > 159	10	0.29(5)	204 > 83	15	<0.01(5)
4-Formyl aminoantipyrine	+	2.81	232.1	232 > 83	20	232 > 104	20	0.63(4)	232 > 214	10	0.63(7)
Acetaminophen	+	1.99	152.1	152 > 110	15	152 > 65	25	0.20(5)	152 > 93	20	0.26(8)
Alprazolam	+	5.77	308.9	309 > 281	25	309 > 205	25	0.11(4)	309 > 274	25	0.21(5)
Amphetamine	+	2.81	136.2	136 > 91	15	136 > 119	10	0.43(6)	136 > 65	10	0.12(15)
Atorvastatin	+	6.68	558.9	559 > 440	20	559 > 466	15	0.20(5)	559 > 292	25	0.17(4)
Benzoylecgonine	+	3.32	290.0	290 > 168	15	290 > 105	25	0.34(6)	290 > 92	25	<0.01(16)
Bezafibrate	-	6.10	359.8	360 > 274	20	360 > 154	25	0.24(5)	360 > 85	15	0.07(6)
Carbamazepine	+	5.32	236.9	237 > 194	20	237 > 192	20	0.24(5)	237 > 179	25	0.08(5)
Clarithromycin	+	6.11	590.0	590 > 158	20	590 > 116	25	0.20(13)	590 > 98	25	0.06(9)
Cocaethylene	+	4.24	318.0	318 > 196	20	318 > 82	25	0.71(4)	318 > 150	25	0.18(7)
Cocaine	+	3.74	304.1	304 > 182	15	304 > 82	25	0.56(8)	304 > 105	25	0.19(9)
Diclofenac	-	6.87	294.1	294 > 250	10	294 > 214	20	0.04(3)	294 > 178	20	<0.01(5)
Enalapril	+	4.99	376.9	377 > 234	15	377 > 117	25	0.24(4)	377 > 303	15	0.30(10)
Erythromycin	+	5.62	734.2	734 > 158	25	734 > 576	15	0.11(5)	734 > 558	15	0.03(10)
Florfenicol	-	3.32	355.7	356 > 336	10	356 > 185	20	1.00(1)	356 > 119	25	0.04(15)
Flumequine	+	5.11	261.9	262 > 244	15	262 > 202	25	0.30(9)	262 > 174	25	0.01(13)
Furaltadone	+	2.37	324.9	325 > 100	20	325 > 252	15	1.00(6)	325 > 281	10	0.77(4)
Gemfibrozil	-	7.46	248.9	249 > 121	20	249 > 127	10	0.07(10)			
Irbesartan	+	6.26	428.8	429 > 207	25	429 > 195	20	0.17(2)	429 > 180	25	0.04(4)
Levamisol	+	2.48	205.0	205 > 178	20	205 > 91	25	0.29(10)	205 > 123	25	0.43(11)
Lincomycin	+	2.89	407.0	407 > 126	20	407 > 359	15	0.07(9)	407 > 389	15	0.03(9)
Lorazepam	+	5.76	320.9	321 > 275	20	321 > 303	15	0.50(6)	321 > 229	25	0.34(8)
MDMA	+	2.90	194.0	194 > 163	10	194 > 105	20	0.34(5)	194 > 135	20	0.33(9)
Nalidixic acid	+	4.92	233.0	233 > 215	10	233 > 187	25	0.71(4)	233 > 159	25	0.19(8)
Naproxen	-	6.11	230.2	185 > 169	20	229 > 169	15	0.01(15)			
Olanzapine	+	3.25	312.9	313 > 256	20	313 > 84	20	0.56(12)	313 > 213	25	0.45(10)
Omeprazole	+	5.23	345.7	346 > 198	10	346 > 136	25	0.45(3)	346 > 151	15	0.32(4)
Oxolinic acid	+	4.24	261.9	262 > 244	15	262 > 216	25	0.13(12)	262 > 158	25	0.04(8)
Pantoprazole	+	5.18	383.9	384 > 200	10	384 > 138	25	1.10(4)	384 > 153	15	0.36(5)
Pravastatin	-	5.76	423.0	423 > 321	15	423 > 303	15	1.00(5)	423 > 101	25	0.53(11)

Roxithromycin	+	6.22	679.1	679 > 158	25	679 > 116	25	0.22(6)	679 > 98	25	0.04(16)
Salicylic acid	-	4.26	137.0	137 > 93	15						
Sulfadiazine	+	2.11	251.0	251 > 156	15	251 > 92	25	0.71(5)	251 > 108	20	0.43(2)
Sulfadoxine	+	3.44	310.9	311 > 156	15	311 > 92	25	0.42(8)	311 > 108	25	0.48(13)
Sulfamethorxazole	+	3.26	253.8	254 > 92	25	254 > 156	15	1.27(7)	254 > 108	20	0.56(6)
Trimethoprim	+	2.88	291.0	291 > 123	25	291 > 230	20	1.11(7)	291 > 261	25	0.83(5)
Valsartan	+	6.27	435.8	436 > 207	25	436 > 235	15	1.12(6)	436 > 261	15	<0.01(17)
Venlafaxine	+	4.61	278.1	278 > 58	15	278 > 260	10	0.43(5)	278 > 121	25	0.24(2)
ILIS											
Acetaminophen-d_4	+	1.89	155.9	156 > 114	15						
Amphetamine-d_6	+	2.79	141.7	142 > 93	15						
Atorvastatin-d_5	+	6.67	563.9	564 > 445	20						
Benzoylecgonine-d_3	+	3.32	293.1	293 > 171	20						
Carbamazepine 10,11-epoxide-d_{10}	+	4.47	263.0	263 > 190	25						
Cocaethylene-d_8	+	4.23	326.0	326 > 204	20						
Cocaine-d_3	+	3.74	306.9	307 > 185	20						
Diclofenac-d_4	-	6.85	299.9	300 > 256	10						
MDMA-d_5	+	2.90	199.0	199 > 1650	10						
Omeprazole-d_3	+	5.22	348.8	349 > 198	10						
Salicylic acid-d_4	-	4.26	140.7	141 > 97	15						
Sulfamethoxazole-$^{13}C_6$	+	3.27	260.0	260 >162	15						
Trimethoprim-$^{13}C_3$	+	2.87	294.1	294 >264	18						
Valsartan-d_8	+	6.24	443.9	444 > 207	15						

ES. electrospray ionization; T_R, retention time; Q quantification; q confirmation, C.E. collision energy

Table 2. Results of the method validation for effluent wastewater (EWW) and surface water (SW). Limit of quantification (LOQ), recovery (%) and relative standard deviation at the three validation levels studied.

Compound	SW (n=5)				EWW (n=5)				ILIS used for correction
	Recovery (RSD) (both in %)			LOQ (ng L-1)	Recovery (RSD) (both in %)			LOQ (ng L-1)	
	10 ng L-1	100 ng L-1	1000 ng L-1		10 ng L-1	100 ng L-1	1000 ng L-1		
4-Acetyl aminoantipyrine	59 (16)[a]	79 (19)[a]	72 (5)[a]	0.8	95[b]	69[b]	78 (14)[a]	2.0	-
4-Aminoantipyrine	95 (12)[a]	74 (14)[a]	81 (9)	0.7	110[b]	66[b]	97[b]	0.4	-
4-Formyl aminoantipyrine	72 (13)[a]	105 (12)[a]	88 (4)[a]	1.9	120[b]	68[b]	82 (18)[a]	1.7	-
Acetaminophen	103 (17)[a]	111 (10)	107 (9)	1.1	131 (3)[a]	113 (15)	118 (7)	1.5	Acetaminophen-d_4
Alprazolam	88 (18)[a]	79 (10)	78 (8)	0.3	81 (16)[a]	74 (9)	77 (11)	1.2	-
Amphetamine	-	96 (12)	78 (19)	6.3	-	110 (11)	107 (11)	12.5	Amphetamine-d_6
Atorvastatin	84 (11)	85 (7)	100 (9)	0.8	92 (14)	92 (5)	109 (2)	0.8	Atorvastatin-d_5
Benzoylecgonine	88 (18)	83 (7)	97 (10)	0.1	88 (20)[a]	80 (16)	109 (2)	0.1	Benzoylecgonine-d_3
Bezafibrate	87 (20)[a]	83 (16)	95 (12)	1.3	82 (11)[a]	102 (24)[a]	111 (13)	2.1	-
Carbamazepine	81 (19)	65 (7)	91 (8)	0.2	77 (16)[a]	75 (15)	94 (8)	1.1	Carbamazepine 10,11-epoxide-d_{10}
Clarithromycin	93 (4)[a]	97 (14)[a]	90 (9)	2.9	117[b]	73 (17)[a]	81 (16)	4.1	-
Cocaethylene	89 (12)	93 (9)	97 (9)	0.7	100 (5)	93 (5)	102 (4)	0.8	Cocaethylene-d_8
Cocaine	77 (19)[a]	69 (11)	111 (10)	1.0	70 (9)	88 (17)	116 (3)	1.1	Cocaine-d_3
Diclofenac	-	82 (14)[a]	105 (5)	6.8	-	78 (17)[a]	104 (11)	7.2	Diclofenac-d_4
Enalapril	99 (17)	88 (6)	92 (5)	0.7	109 (9)	81 (4)	94 (8)	1.8	-
Erythromycin	115 (6)	85 (15)	72 (14)	0.8	125[b]	92 (23)[a]	94 (19)	2.1	Sulfamethoxazole-$^{13}C_6$
Florfenicol	69 (17)	91 (12)	83 (15)	2.2	97 (11)	84 (14)	109 (11)	8.6	-
Flumequine	87 (14)	87 (10)	113 (5)	0.4	90 (17)	73 (14)	97 (9)	1.2	-
Furaltadone	88 (11)	87 (10)	88 (10)	0.7	88 (16)	69 (16)	80 (17)	1.4	-
Gemfibrozil	83 (15)[a]	99 (12)[a]	92 (8)	2.3	103[b]	92[b]	90 (18)	1.8	-
Irbesartan	86 (16)[a]	87 (13)[a]	97 (8)	0.2	115[b]	78 (12)[a]	99 (5)	1.0	-
Levamisol	83 (15)	95 (7)	101 (5)	0.2	87 (19)[a]	98 (16)	106 (10)	2.1	Cocaethylene-d_8
Lincomycin	84 (15)[a]	81 (17)[a]	104 (10)	0.1	88 (12)	78 (15)	75 (12)	0.4	-
Lorazepam	88 (14)[a]	82 (15)	86 (7)	3.1	109 (9)[a]	78 (20)[a]	94 (5)	4.5	-

MDMA	100 (10)	96 (6)	105 (8)	0.5	99 (18)	93 (2)	103 (8)	1.4	MDMA-d_5
Nalidixic acid	93 (14)	91 (9)	114 (6)	1.8	90 (17)	75 (13)	98 (9)	2.7	-
Naproxen	77 (18)[a]	70 (7)	80 (13)	11.7	62[b]	78[b]	85 (13)	7.3	-
Olanzapine	-	86 (1)[a]	108 (12)[a]	0.8	-	-	156 (13)	11.6	-
Omeprazole	103 (13)	89 (8)	98 (8)	0.2	118 (24)	95 (4)	102 (2)	1.1	Omeprazole-d_3
Oxolinic acid	96 (12)	83 (10)	86 (5)	1.8	98 (13)	70 (10)	80 (16)	2.9	-
Pantoprazole	93 (15)	99 (8)	103 (5)	0.1	93 (13)	81 (12)	105 (8)	0.8	-
Pravastatin	96 (14)	81 (13)	85 (13)	15.4	113[b]	82 (10)	83 (8)	16.7	Diclofenac-d_4
Roxithromycin	-	92 (4)[a]	83 (13)	5.6	-	95 (9)[a]	91 (16)	5.4	-
Salicylic acid	-	-	93 (15)	37.6	-	119[b]	84 (8)	41.1	Salicylic acid-d_4
Sulfadiazine	102 (16)	99 (9)	116 (8)	1.4	106 (19)	97 (7)	111 (12)	1.8	Sulfamethoxazole-$^{13}C_6$
Sulfadoxine	85 (12)	83 (11)	104 (7)	0.2	80 (18)	64 (12)	86 (16)	0.5	-
Sulfamethorxazole	96 (16)[a]	80 (10)	98 (11)	0.5	103[b]	83 (10)	106 (7)	0.8	Sulfamethoxazole-$^{13}C_6$
Trimethoprim	83 (14)	87 (13)	93 (10)	1.8	111[b]	81 (19)	104 (10)	2.3	Trimethoprim-$^{13}C_3$
Valsartan	74 (11)	88 (4)	98 (12)	3.8	114[b]	98 (18)[a]	89 (14)[a]	4.2	Valsartan-d_8
Venlafaxine	79 (21)[a]	78 (15)	102 (11)	0.2	111[b]	88 (19)[a]	100 (6)	1.0	Atorvastatin-d_5

[a] Validation performed for n=2-4, due to the high analyte concentration found in some “blank” samples
[b] Recovery values without RSD mean (n=1)

Table 3. Summary of the results obtained for target pharmaceuticals in EWW, applying the analytical methodology described in this article. Between brackets, the concentrations obtained using the on-line SPE-LC-MS/MS alternative method.

Compound	EWW (ng/L)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
4-Aminoantipyrine*	9	15	<LOQ	-	<LOQ	40	43	14	-	26
Acetaminophen*	-	-	-	14	-	45	<LOQ	-	8	-
Amphetamine*	-	21	-	-	29	-	-	-	-	-
Benzoylecgonine*	<LOQ	40 (48)	6	<LOQ	656 (735)	11	100 (127)	43 (54)	<LOQ	43 (50)
Clarithromycin*	-	14	26	-	34	<LOQ	27	15	-	-
Cocaethylene*	-	<LOQ	-	-	15	<LOQ	8	-	-	<LOQ
Cocaine*	9	<LOQ	-	<LOQ	72 (54)	12	-	12	8	24
Diclofenac*	-	266 (251)	884 (1115)	-	216 (313)	158 (241)	845 (1181)	300 (386)	-	212 (322)
Erythromycin*	13	55	-	-	37	18	49	14	-	25
Flumequine*	-	-	<LOQ	-	-	-	-	-	-	7
MDMA*	-	45	-	-	45	<LOQ	-	22	-	48
Nalidixic acid*	-	-	17	-	<LOQ	-	-	-	-	8
Naproxen*	-	42	32	-	1942 (3007)	-	515 (642)	<LOQ	-	357 (419)
Oxolinic acid*	-	-	-	-	-	-	-	-	-	5
Pantoprazole*	-	5	2	-	<LOQ	4	4	7	-	4
Sulfadiazine*	-	-	28	-	-	-	-	10	-	-
Sulfamethoxazole*	89	35	372 (308)	<LOQ	19	21	29	25	-	29
Trimethoprim*	15	83	9	-	75	4	13	86	-	25
Venlafaxine*	414 (366)	316 (282)	421 (389)	-	343 (457)	263 (314)	252 (265)	201 (208)	<LOQ	239 (260)
4-Acetyl aminoantipyrine	77	3032	253	-	7239	197	1357	2298	18	689
4-Formyl aminoantipyrine	860	1583	3425	-	3208	766	1898	1235	<LOQ	853
Alprazolam	14	11	17	-	<LOQ	12	13	10	-	12
Atorvastatin	-	7	-	-	16	-	-	-	-	<LOQ
Bezafibrate	-	29	-	-	87	10	35	16	-	53
Carbamazepine	112	52	119	3	135	64	149	54	2	90
Gemfibrozil	-	765	4	-	538	25	507	365	<LOQ	95
Irbesartan	-	531	<LOQ	-	506	404	799	266	<LOQ	484
Levamisol	44	311	155	-	150	163	768	178	-	497
Lincomycin	-	-	-	-	<LOQ	-	6	109	-	7
Lorazepam	-	52	-	-	109	58	81	46	-	74
Pravastatin	-	16	-	-	-	-	<LOQ	<LOQ	-	-
Valsartan	41	2864	54	-	4575	291	1457	246	13	399

*Compounds also analyzed by the on-line SPE LC-MS/MS methodology described in section

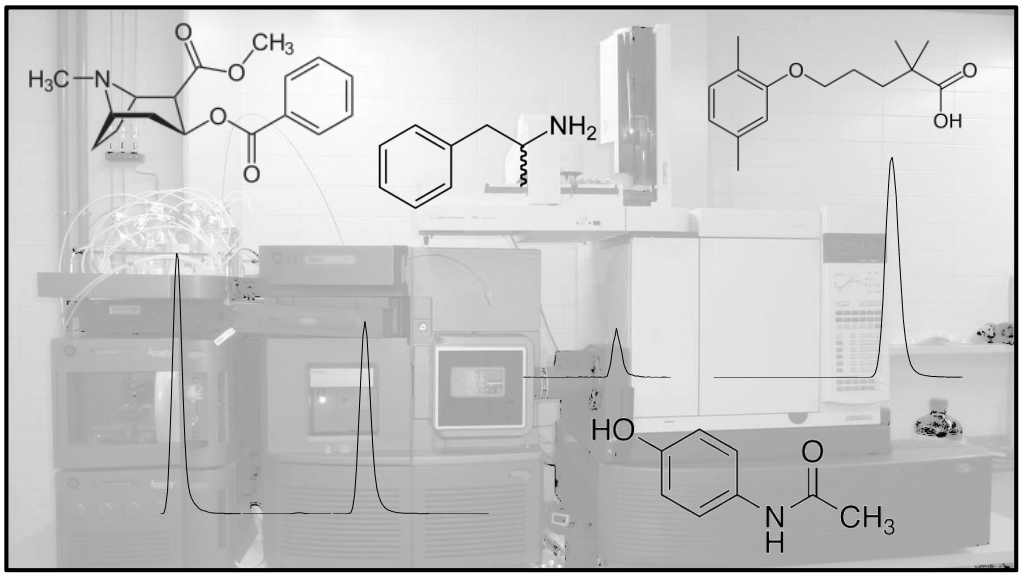
-: not detected

Table 4 Summary of the results obtained for target pharmaceuticals in SW, applying the analytical methodology described in this article. Between brackets, the concentrations obtained using the on-line SPE-LC-MS/MS alternative method.

Compound	SW (ng/L)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Acetaminophen*	-	-	-	480 (654)	9	13	13	12	32 (10)	10
Benzoylecgonine*	<LOQ	<LOQ	18 (8)	31 (23)	6	<LOQ	<LOQ	7	6	6
Clarithromycin*	-	-	11	34 (45)	-	-	-	-	-	-
Cocaethylene*	-	-	-	7	-	-	-	-	-	-
Cocaine*	8	<LOQ	8	14 (8)	-	8	8	10 (5)	-	<LOQ
Diclofenac*	-	34 (24)	135 (99)	14	-	-	-	-	-	-
Erythromycin*	-	-	10	<LOQ	-	-	-	-	-	-
Flumequine*	3	-	-	-	-	-	-	<LOQ	-	-
Levamisol*	-	4	76 (44)	5	-	-	-	-	-	-
MDMA*	-	-	15	13	-	-	-	-	-	-
Nalidixic acid*	3	-	-	<LOQ	-	-	-	4	-	-
Naproxen*	-	-	67 (56)	114 (172)	-	-	-	-	-	-
Oxolinic acid*	5	-	-	-	-	-	-	-	-	<LOQ
Pantoprazole*	-	1	-	-	-	-	-	-	-	-
Sulfamethoxazole*	-	21	25	11	13	<LOQ	-	-	-	<LOQ
Trimethoprim*	3	-	<LOQ	5	-	-	-	-	-	-
Venlafaxine*	9	244 (217)	93 (61)	30 (18)	16	10	<LOQ	<LOQ	9	16
4-Acetyl aminoantipyrine	-	<LOQ	719	182	8	9	6	<LOQ	-	21
4-Formyl aminoantipyrine	-	9	663	101	33	13	-	<LOQ	-	60
Alprazolam	-	11	8	<LOQ	-	-	-	-	-	-
Carbamazepine	<LOQ	73	22	10	7	-	2	2	-	6
Gemfibrozil	-	-	105	80	-	-	-	-	-	-
Irbesartan	-	<LOQ	5	40	-	-	-	-	-	6
Lincomycin	-	12	5	1	-	-	-	-	-	-
Lorazepam	-	13	18	<LOQ	-	-	-	-	-	44
Valsartan	-	-	13	224	-	-	-	-	-	-

*Compounds also analyzed by the on-line SPE LC-MS/MS methodology described in section

-: not detected



Graphical Abstract

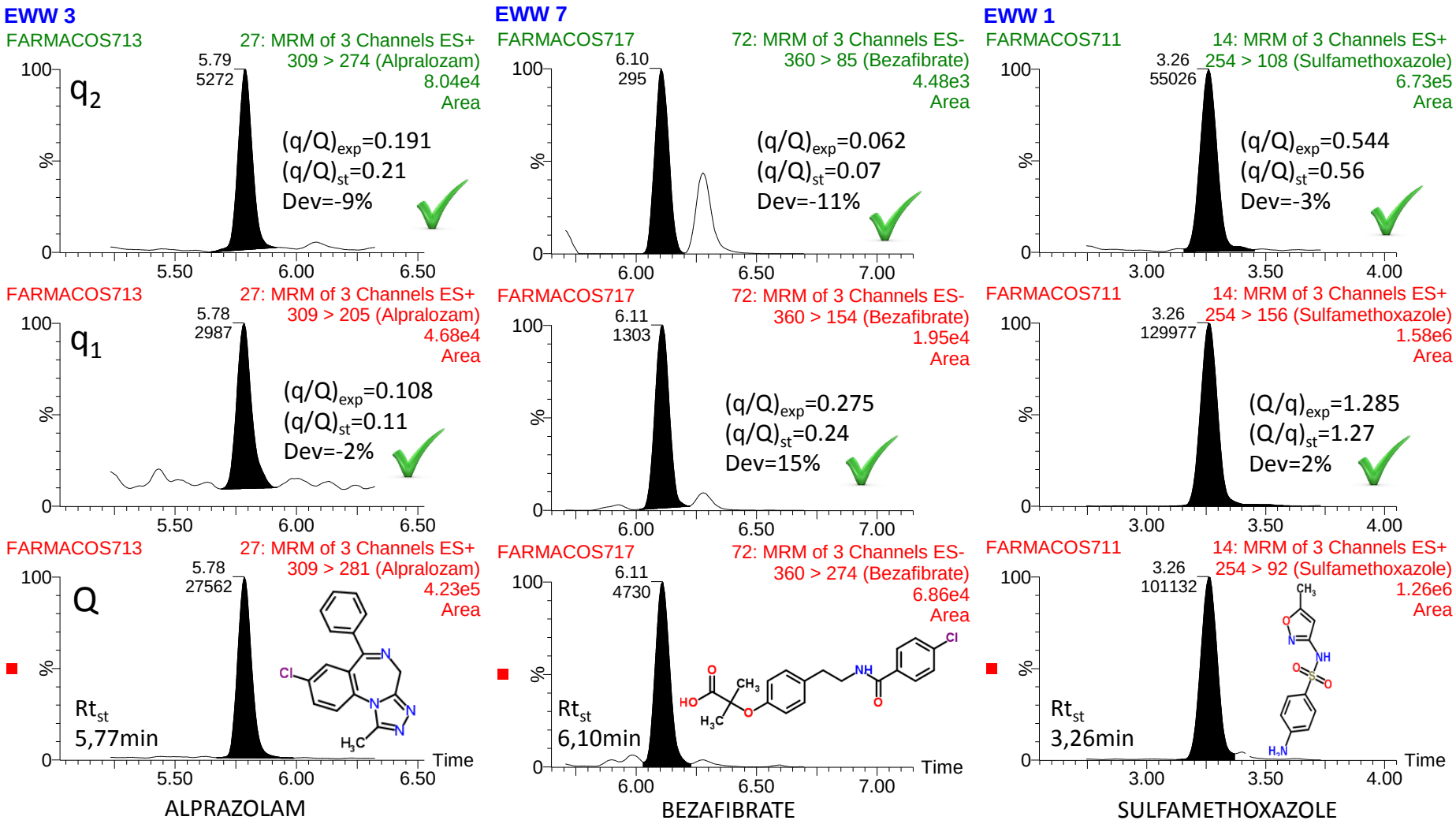

EWW 3
FARMACOS713
27: MRM of 3 Channels ES+
309 > 274 (Alpralozam)
8.04e4
Area
5.79
5272
q2
(q/Q)exp=0.191
(q/Q)st=0.21
Dev=-9%
FARMACOS713
27: MRM of 3 Channels ES+
309 > 205 (Alpralozam)
4.68e4
Area
5.78
2987
q1
(q/Q)exp=0.108
(q/Q)st=0.11
Dev=-2%
FARMACOS713
27: MRM of 3 Channels ES+
309 > 281 (Alpralozam)
4.23e5
Area
5.78
27562
Q
Rtst
5,77min
ALPRAZOLAM
EWW 7
FARMACOS717
72: MRM of 3 Channels ES-
360 > 85 (Bezafibrate)
4.48e3
Area
6.10
295
(q/Q)exp=0.062
(q/Q)st=0.07
Dev=-11%
FARMACOS717
72: MRM of 3 Channels ES-
360 > 154 (Bezafibrate)
1.95e4
Area
6.11
1303
(q/Q)exp=0.275
(q/Q)st=0.24
Dev=15%
FARMACOS717
72: MRM of 3 Channels ES-
360 > 274 (Bezafibrate)
6.86e4
Area
6.11
4730
Rtst
6,10min
BEZAFIBRATE
EWW 1
FARMACOS711
14: MRM of 3 Channels ES+
254 > 108 (Sulfamethoxazole)
6.73e5
Area
3.26
55026
(q/Q)exp=0.544
(q/Q)st=0.56
Dev=-3%
FARMACOS711
14: MRM of 3 Channels ES+
254 > 156 (Sulfamethoxazole)
1.58e6
Area
3.26
129977
(Q/q)exp=1.285
(Q/q)st=1.27
Dev=2%
FARMACOS711
14: MRM of 3 Channels ES+
254 > 92 (Sulfamethoxazole)
1.26e6
Area
3.26
101132
Rtst
3,26min
SULFAMETHOXAZOLE
Time

Figure 1

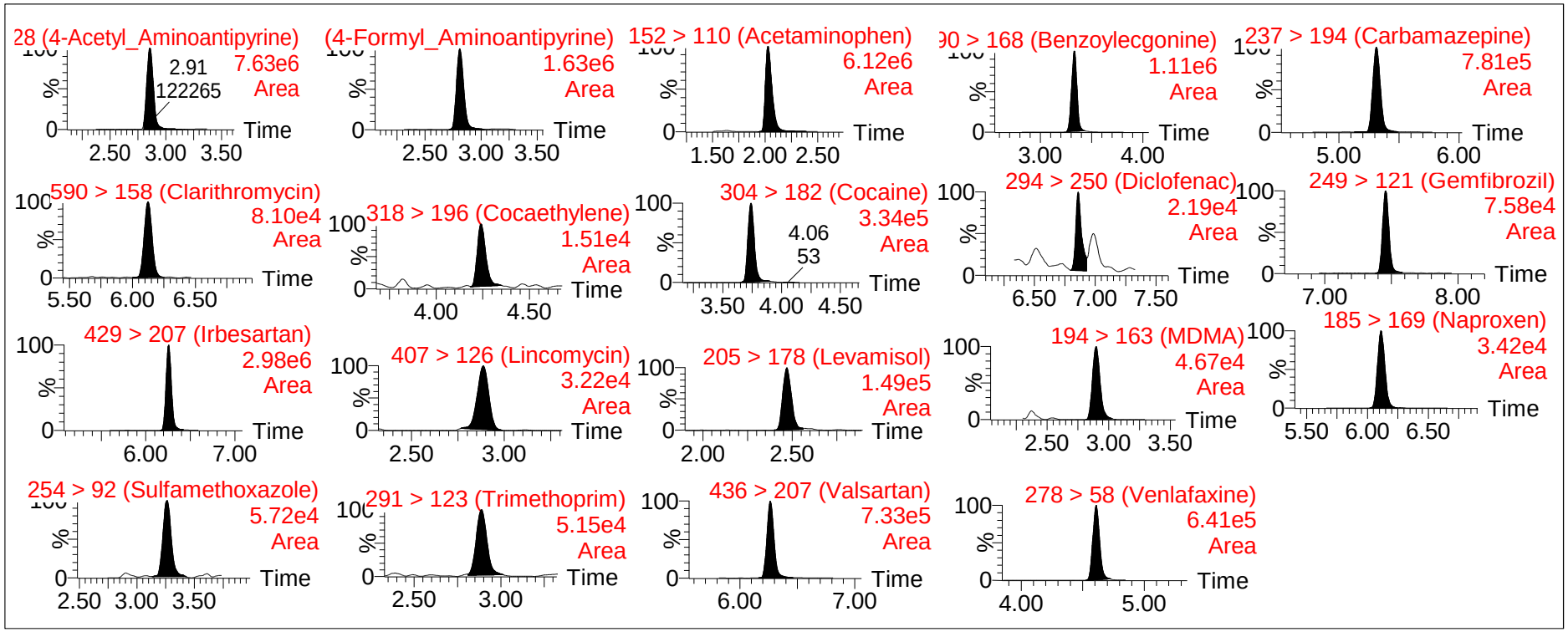

28 (4-Acetyl_Aminoantipyrine)
2.91
122265
7.63e6
Area
(4-Formyl_Aminoantipyrine)
1.63e6
Area
152 > 110 (Acetaminophen)
6.12e6
Area
90 > 168 (Benzoylecgonine)
1.11e6
Area
237 > 194 (Carbamazepine)
7.81e5
Area
590 > 158 (Clarithromycin)
8.10e4
Area
318 > 196 (Cocaethylene)
1.51e4
Area
304 > 182 (Cocaine)
4.06
53
3.34e5
Area
294 > 250 (Diclofenac)
2.19e4
Area
249 > 121 (Gemfibrozil)
7.58e4
Area
429 > 207 (Irbesartan)
2.98e6
Area
407 > 126 (Lincomycin)
3.22e4
Area
205 > 178 (Levamisol)
1.49e5
Area
194 > 163 (MDMA)
4.67e4
Area
185 > 169 (Naproxen)
3.42e4
Area
254 > 92 (Sulfamethoxazole)
5.72e4
Area
291 > 123 (Trimethoprim)
5.15e4
Area
436 > 207 (Valsartan)
7.33e5
Area
278 > 58 (Venlafaxine)
6.41e5
Area
Time
%
100
0

Figure 2