

Simultaneous determination of triazines and their main transformation products in surface and urban wastewater by ultra-high-pressure liquid chromatography-tandem mass spectrometry

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Abstract

This work describes the optimization, validation and application to real samples of an ultra-high-pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method for the quantification and confirmation of 11 compounds (atrazine, simazine, terbutylazine, terbumeton, terbutryn and their main transformation products) in surface and wastewater samples. Most of these analytes are included in the list of priority substances in the framework on European Water Policy. The application of this method to water samples reveals that the most relevant transformation products (TPs) should be incorporated into current analytical methods (which are focused mainly on the determination of unchanged compounds), to obtain a more realistic knowledge on water quality regarding pesticide contamination. TPs are generally more polar and mobile than the parents and they can be transported to the aquatic environment more rapidly than their precursors. Additionally, they can present some degree of toxicity and in fact TPs are also included within the legislation on drinking water as pesticide derivatives.

To efficiently combine UHPLC with MS/MS, a fast-acquisition triple quadrupole mass analyzer was used. Working in selected reaction monitoring mode, up to three simultaneous transitions per compound were acquired allowing a reliable identification at ng/L levels. The method developed includes a pre-concentration step based on solid-phase extraction (OASIS HLB cartridges). Satisfactory recoveries (70-120%) and relative standard deviations (<20%) were obtained for all compounds in different water samples types spiked at two concentration levels (0.025 and 0.1 µg/L). The optimized method was found to have excellent sensitivity with instrumental detection limits as low as 50 fg.

In addition, the influences of the matrix constituents on ionization efficiency and extraction recovery have been studied in different types of Italian and Spanish surface and urban wastewater. Signal suppressions were observed for all compounds, especially for influent wastewater. The use of isotope-labelled internal standards was found to be the best approach to assure an accurate quantification in all matrix samples.

Keywords: Pesticide transformation products; ultra-high-pressure liquid chromatography; tandem mass spectrometry; matrix effects; surface and wastewater; triazines.

1. Introduction

One of the most important problems related to water resources concerns the presence in surface and drinking water of a wide variety of organic micropollutants. Many of these substances, generated by the main human activities, are characterized as being toxic and hazardous for both aquatic system and human health. Wastewater treatment plants (WWTPs), especially those serving both urban and industrial areas, consistently receive significant loads of these compounds. Therefore, it is important to evaluate the impact of micropollutants discharged with treated effluents and sewers overflows on the receiving waterbody. The problem arises from the fact that WWTPs remove them to a certain (limited) extent, but river flows are in most cases too small to dilute the residual loads discharged and concentrations in the receiving waters increase, often much over the quality objectives defined for the river water quality.

Groups of compounds present in water, specially in agricultural areas, include triazine herbicides, which are generally included in monitoring programs as a result of their widespread presence and inclusion in the Water Framework Directive (2000/60/EC) [1]. This Directive was amended by the Decision 2455/2001/EC [2], which published a list of 33 priority substances, including several pesticides, selected on the basis of the risk to or via the aquatic environment. In 2000, the Spanish legislation fixed quality objectives of 1 $\mu\text{g/L}$ for the concentration of simazine (SIMA), atrazine (ATRA) and terbuthylazine (TBZNE) in continental surface waters (SW) [3] whilst in 2009 the Italian legislation established specific limit for each of these compounds at 1, 0.6 and 0.5 $\mu\text{g/L}$ respectively [4]. Regarding to drinking water, the European authorities fixed quality standards of 0.1 $\mu\text{g/L}$ for pesticide individual concentration and 0.5 $\mu\text{g/L}$ for the sum of all pesticides [5].

Triazines are used worldwide as selective pre and post emergence herbicides for the control of both grasses and broadleaf weeds in many agricultural crops as well as for non-agricultural purposes such as soil sterilization and road maintenance [6]. Due to their higher mobility in the soil-water environment, triazines may be found in both ground and surface water [7, 8]. In water and soil, parent molecules are subjected to degradation processes such as photolysis, oxidation, hydrolysis, and biodegradation, leading to dealkylation of the amine groups, dechlorination, and subsequent hydroxylation [9, 10]. The main transformation products (TPs) in ground and surface waters via biotic mechanism are the dealkylated chloro metabolites, such as deisopropyl-atrazine (DIA), desethyl-atrazine (DEA), desethyl-terbuthylazine (DETbzne) and desethyl-terbumeton (DETer) [11]. Hydroxy-atrazine (HA), hydroxy-simazine (HS) and hydroxy-terbuthylazine (HTbzne) are the major abiotic degradation product in water and soil. Not much information is available on

environmental impact of triazine TPs, which can be also toxic [12] and are normally more polar than parent compounds. Due to their high mobility in the soil-water environment, TPs can reach water bodies more easily. The presence of metabolites when investigating the effect of herbicide application and its influence on the aquatic environment is relevant and the impact due to herbicides tends to be underestimated when samples are analyzed for the parent compounds only [13, 14]. Therefore, the most relevant TPs should be incorporated into current analytical methods to obtain a more realistic knowledge of water quality regarding pesticide contamination [15, 16].

Due to the prevailing of combined sewer systems, most runoff water, carrying pollutants from non point sources, is collected and reaches WWTPs, so that non point loads enter the receptors as point loads [17]. Conventional (biological) wastewater treatment processes are considered as ineffective in reducing the concentrations of triazine compounds with removal efficiencies consistently below 40 % [18] or even considered as no biodegradable [19]. However, other advanced processes, such as ozonation, have been reported to produce abundant triazine degradates [20]. Therefore, in practical situations, the presence of TPs has to be taken into account. Besides, primary waters contaminated by pesticides, may already contain TPs, which can be consequently found in effluent wastewater.

Most of analytical methods for residues of pesticides and their TPs in water are based on gas chromatography and/or liquid chromatography coupled to mass spectrometry. In recent years the application of tandem mass spectrometry in LC-MS/MS based methods has given and increased selectivity and sensitivity, minimizing or even removing many interferences when working in Selected Reaction Monitoring (SRM) mode making this technique highly suitable for polar pesticides and TPs in aqueous matrices [21-25]. Thus, several LC-MS/MS methods have been reported for the determination of triazines. The most recent papers have reported methods using ultra-high-pressure liquid chromatography (UHPLC) coupled to MS/MS for ultra fast separations and sensitive determination of these compounds [26-29]. UHPLC provides higher peak capacity, greater resolution, increased sensitivity and high speed of analysis by the use of stationary phases of particle size (<2 μm) smaller than conventional HPLC. To reach an efficiently combination UHPLC with MS/MS, fast-acquisition triple quadrupole mass analyzers must be used. It can allow the acquisition of more than two transitions to obtain reliable identifications, without resolution or sensitivity losses [29]. Therefore, UHPLC-MS/MS can offer not only good sensitivity but also high confidence on confirmation of compounds detected in samples, allowing to easily reach more than 3-4 identification points (IPs), as established in EU guidelines [30, 31].

According to European legislation on drinking water [5], pesticide limits of quantification (LOQs) of 0.025 µg/L, four times lower than the maximum allowed (0.1 µg/L), are required in the analytical methods applied. Usually, a SPE pre-concentration step is applied to reach the low concentration levels according to the present regulations. In spite of SPE methods are typically fast, efficient and widely applied, matrix interferences can also be pre-concentrated in SPE cartridges resulting sometimes in analyte ionization enhancement or suppression. This undesirable matrix effects can be considered as one of the main LC-MS/MS drawbacks, mainly when an electrospray ionization source (ESI) is used [32]. To ensure an accurate quantification of analytes, different approaches are applicable in order to compensate for the matrix effects [33-39]. A simple method is the dilution of sample [35]. However, this is limited by the limits of detection required for the target compounds. The use of matrix-matched standards for calibration [36], is not much useful in environmental samples, as their composition vary in a broad range, and obtaining a blank of similar composition to sample is not easy. The standard additions method can provide accurate results [37], but in practice, it needs both a time-consuming sample preparation and evaluation of the obtained results and therefore is not much suitable for fast routine analysis. The use of appropriate internal standards is one of the best approaches to compensate for matrix effects, especially when using analyte isotope labelled internal standard (ILIS), as one expects that the internal standard is affected by matrix effects in the same way than the analyte [29, 38, 39]. Apart from the high cost, the main drawback of using an internal standard is that one isotope standard is, in principle, required for each analyte and that stable isotope standards are not generally available for all compounds to be analyzed. When ILIS is not available, other compounds eluting at similar retention times or being structurally analogues could be tested, but no satisfactory data are always assured [29]

Normally, TPs are not included (or only a few are included) in multiresidue methods for several reasons: many of them are still not well known; the number of potential analytes to be investigated in water would increase drastically; the commercial availability of reference standards is rather limited; their higher polarity in relation to the parent compound makes extraction/pre-concentration more critical than for parents in the usual SPE approaches [22]. This work is focused on the development of a rapid, selective and sensitive analytical method for quantification and confirmation of triazine herbicides commonly used in Italy and in the Mediterranean coast of Spain, as well as of their main TPs. Analytical methodology developed is based on solid-phase extraction (OASIS HLB cartridges, 0.2 g) followed by UHPLC-MS/MS determination. The method has been validated for 11 compounds (ATRA, SIMA, TBZNE, terbumeton (TER), terbutryn (TBTYN) and 6 of their main TPs) in surface and wastewater samples. It has been applied to the

determination of these herbicides in small volumes of surface waters and polluted WWTP aqueous samples (Italian and Spanish 24-h composite influents – IWW - and effluents - EWW). Three SRM transitions were acquired for each analyte to give more confidence in the identification of the analytes in these complex matrices. In addition, the influence of matrix constituents on ionization efficiency and SPE extraction recovery has been studied in the types of water samples analyzed, testing different ILIS to assure the accurate quantification in all matrix samples.

2. Experimental

2.1. Reagents and materials

Pesticide and TP reference standards (desisopropylatrazine (DIA), desethylatrazine (DEA), 2-hydroxy-atrazine (HA), desethylterbutometon (DETer), simazine (SIMA), desethylterbutylazine (DEtbzne), 2-hydroxy-terbutylazine (Htbzne), atrazine (ATRA), terbutometon (TER), terbutylazine (TBZNE) and terbutryn (TBTYN)) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions were prepared dissolving 50 mg, accurately weighted, in 100 mL of acetone obtaining a final concentration of 500 mg/L. From these solutions of triazines an intermediate solution of around 50 mg/L was prepared in methanol. Mixed working solutions used for spiking water samples and for preparation of the aqueous calibration standards were prepared from intermediate solutions at different concentrations by appropriate dilution with HPLC-grade water.

Isotopically labelled compounds used were [$^2\text{H}_6$]dimethoate (dimethoate- d_6), [$^2\text{H}_5$]terbutylazine (terbutylazine- d_5), and [$^2\text{H}_6$]thiabendazole (thiabendazole- d_6) obtained from Dr. Ehrenstorfer. A mix of all isotopically labelled compounds at 100 $\mu\text{g/L}$ was prepared by dilution of individual stock solutions of 1 mg/L in methanol. Further dilutions of this mix were prepared.

To prepare calibration curves, working mix solutions of pesticides and isotopically labelled compounds were prepared in acetonitrile:water (10:90, v/v). In order to prevent photochemical degradations, standard solutions and sample extracts, were stored in brown glass vials at 4°C.

HPLC-grade methanol, HPLC-grade acetonitrile and acetone for residue analysis were purchased from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millipore, Bedford, MA, USA). Formic acid (HCOOH , content > 98%) was supplied by Scharlau. Cartridges used for SPE optimization were Oasis HLB (200 mg, 6 mL) and MCX (150 mg, 6 mL) from Waters (Milford, MA, USA). Oasis SPE polymer cartridges are built of a balanced mixture of hydrophilic and lipophilic (HLB) monomers, whilst Oasis MCX is a strong cation-exchange mixed mode polymeric

sorbent built upon HLB copolymers. A VAC ELUT SPS 20 (Varian, Palo Alto, CA, USA) was used to simultaneously process up to 20 SPE cartridges.

2.2. Ultra-high-pressure liquid chromatography

LC separations were performed in an Acquity UPLC system (Waters), using an Acquity UPLC HSS T3 column, 1.8 μm , 100 mm \times 2.1 mm I.D. (Waters). The column was kept at 40°C and the sample manager was maintained at 5°C. Mobile phase was composed of a Solvent A (water) and a Solvent B (methanol) at a constant flow rate of 0.3 mL/min. The gradient was programmed to increase the amount of methanol from an initial 5% to 95% in 6 min, returning to the initial conditions (5% A) in 0.1 min. This condition was maintained until 7 min. The sample volume injected in UHPLC system was 20 μL .

2.3. Mass spectrometry

TQD tandem mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters) was used for UHPLC analysis. Typical interface conditions were optimised for maximum intensity of precursor ion as follows: the cone gas and desolvation (drying gas) N_2 flows were set at 60 L/h flow and 1000 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Carbueros Metalicos, Valencia, Spain) with a pressure of 2×10^{-3} mbar in the T-Wave cell. Capillary voltages of 3.5 kV were used in the positive ionization mode. The desolvation temperature was set to 500°C and the source temperature to 120°C. Dwell times of 0.01 s/scan were selected.

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

2.4. Sample preparation

Water samples were centrifuged at 4500 rpm for 5 min, only when suspended particulate matter was observed. 100 mL of surface water (or ten and twenty-fold diluted effluent and influent wastewater respectively) containing 1 mL of concentrated HCOOH , were taken and 100 μL of I.S. mixture solution was added, giving a final concentration of 0.05 $\mu\text{g/L}$ for each ILIS.

Oasis HLB (200 mg) cartridges were conditioned with 5 mL of methanol, 5 mL of acetone, 5 mL of methanol and 5 mL of acidified water (1% HCOOH). After the conditioning step, aliquots of 100 mL of aqueous sample were passed through the cartridges by gravity. Then, sorbents were dried under vacuum in a manifold system for 40 min and analytes were subsequently eluted with 5 mL acetone. The extract was evaporated to dryness under a gentle nitrogen stream (40°C) and finally reconstituted with 1 mL acetonitrile–water (10:90, v/v). The final pre-concentration factor was 100. Analyses were performed by injecting 20 μL of the final extract in the UHPLC–MS/MS system.

2.5. Validation studies

The performance characteristics of the method were established by a validation procedure following the spirit of SANCO guidelines [31]. Linearity was estimated by analyzing calibration standards in acetonitrile–water (10:90, v/v) in triplicate at seven concentration levels, ranging from 1 to 100 µg/L. Satisfactory linearity, using weighed (1/X) least squares regression, was assumed when the correlation coefficient (r) was higher than 0.99 based on analyte peak areas measurements, and the residuals lower than 30%.

Accuracy (expressed as recovery, in %) and precision (repeatability expressed as relative standard deviation, in %) were evaluated by analyzing surface water samples spiked at two levels each (0.025 and 0.1 µg/L). In the case of influent and effluent wastewaters, these levels were twenty and tenfold higher, as they were diluted with HPLC water before sample treatment and analyses. All experiments were performed in quintuplicate ($n = 5$).

The limit of quantification (LOQ) was estimated for a signal-to-noise ratio of ten from the chromatograms of SW, IWW and EWW samples spiked at the lowest concentration level, using the confirmation transition q_1 . The instrumental limit of detection (LOD) was estimated for a signal-to-noise ratio of three from the SRM chromatograms (using the quantification transition Q) of low-concentration standards, ranging from 0.01 to 0.1 µg/L.

2.6. Application to real samples

The method was applied to 31 water samples (SW, IWW and EWW) collected in different sites of Italy and Spain. Samples were stored in the dark at $<-18^{\circ}\text{C}$ in plastic (high density polyethylene) containers until analysis.

SW samples were collected in 11 sampling sites along 2009 in Spain (June) and Italy (September). Spanish SW samples were collected from Ebro river (sample 1) and Ebro delta (2), Pego-Oliva marsh (3), Almenara pond (4), Massalavés Verd river (5) and Borriana Clot (6). The Ebro is the largest river in terms of volume in Spain, 928 km length, with a drainage basin of 85.550 km². The Ebro delta is one of the largest wetland areas (320 km²) in the western Mediterranean region. The rest of locations are humid zones near the Mediterranean Sea (Valencia region), with great interest from touristic and ecological point of view.

In Italy, the 5 samples were collected from the urbanized area north of Milan. Samples 7 and 8 were collected from Lura stream basin, the former in a urban area after EWW discharges and sewage spills, the latter in an agricultural and residential landscape. Sample 9 was collected from Gorgonella stream (draining

a urban catchment), sample 10 from Seveso river (draining a forested catchment), sample 11 from Livescia stream (draining a golf course area).

Municipal and industrial IWW and EWW Italian samples were collected in two sampling campaigns, April and June 2009, from two WWTPs in Northern Italy (Como Province). Triazines fluxes in the sewers were calculated using measurements of the real 24 h influent flows on the sampling days. The concerned plants, Alto Lura (AL-WWTP) and Alto Seveso (AS-WWTP), receive approx. 7.000 m³/day of domestic wastewater from the major neighbouring residential districts and approx. 18.000 m³/day of industrial wastewater, chiefly deriving from textile dyeing industry. As the sewer networks are combined, the WWTPs also treat part of the local runoff water, carrying urban pollutants, such as atmospheric deposition and traffic emissions deposited on the road surface, and non-point loads from cultivated and non cultivated lands. Wastewaters carried by the collectors undergo pre-treatments (screening, sand and oil removal) and are fed, with return flow, to the denitrification reactor where nitrogen is removed as gaseous N₂. Denitrification is followed by biological oxidation and nitrification (activated sludge reactor) and, finally, by secondary settling. Polishing is performed by tertiary treatments (sand filtration and ozonation), especially for removing residual suspended solids, colour and organic micro-contaminants.

Spanish wastewater samples were also collected in April and June 2009 from three WWTPs (Castelló de la Plana (CS), Burriana (BU) and Benicassim (BE)) of the province of Castelló (Eastern Spain), which receive approx. 44.024, 16.805 and 8.250 m³/day, respectively, of urban and industrial wastewater. Physical and biological treatments applied in these WWTPs are similar to Italian, except from ozonation. In all cases, wastewater samples were 24-h composite (influent and effluent).

In every sequence of analysis, water sample SPE extracts were injected by duplicate between two calibration curves (from 1 to 100 µg/L). In addition, two quality controls (QCs) were analysed together with each batch of samples. QCs consisted of a “blank” water (previously analyzed) fortified at two different levels, 0.025 µg/L and 0.1 µg/L. QC recoveries for every analyte were considered satisfactory in the range 70–120%, thus assuring the quality of the analysis.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q₁ and q₂) transitions and comparing them with ion-ratios obtained from a reference standard. To consider a finding as an actual positive, the experimental Q/q_i ratios should fit with those of reference standards with maximum deviations ranging from 20 to 50% depending on the relative intensities, in the line of EU Decision 2002/657/EC [30].

3. Results and discussion

3.1. MS/MS optimization

In order to optimize full-scan MS and MS/MS spectra of parent pesticides and TPs, infusion experiments were performed using the built-in syringe pump, directly connected to the interface. To this aim, individual standard solutions at 1 mg/L in methanol/water (50:50, v/v) were infused at a flow rate of 10 $\mu\text{L}/\text{min}$.

All analytes were measured in positive ionization mode presenting an abundant $[\text{M}+\text{H}]^+$, which was selected as precursor ion. The presence of halogenated atoms (Cl) in several compounds (ATRA, SIMA, TBZNE and DETbzne) allowed us to use two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl isotopes, respectively) which generated abundant product ions. Thus, the confirmation of these compounds in samples would be feasible at similar levels. Non-specific transitions, e.g. loss of water, were avoided as possible in order to minimize the risk of false positives [33].

With the aim that the method could be also used for confirmatory purposes, the acquisition of at least two specific transitions for each compound is required. As TQD is a fast-acquisition triple quadrupole mass analyzer that allows decreasing dwell times and ionization mode switching time, without apparent sensitivity losses, this gave us the possibility of acquiring up to three SRM transitions per compound at 10ms dwell time. Acquiring three SRM transitions, and using two different precursor ions for several compounds, increased the reliability in the identification process and led to a high number of IPs [30].

Optimum MS source and analyzer conditions for SRM determination of each compound are listed in Table 1. Average Q/q ratios were estimated from the seven calibration standards (concentrations between 1-100 ng/mL) used during validation study.

3.2. LC optimization

In spite of the great selectivity provided by triple quadrupole analyzers, an efficient chromatographic separation can be necessary to avoid or minimize undesirable matrix effects. Besides, an adequate mobile phase selection can also be important to enhance the detector response.

In this paper, methanol and acetonitrile, with different HCOOH and NH_4Ac contents, were tested as organic solvents during chromatographic optimization searching for a compromise between peak shape and sensitivity. All compounds presented better peak shape and ionization yield when methanol without additives was used as organic modifier possibly due to its protic character.

3.3. SPE recoveries

In order to evaluate the efficiency of SPE process, two sorbents were tested: Oasis HLB and Oasis MCX. To this aim, 100 mL of Milli-Q water spiked at a concentration of 0.05 µg/L were loaded onto the cartridges by gravity (triplicate analysis). Marked differences were observed in the performance of the two stationary phases tested, both in the recovery of analytes and in the reproducibility of the results.

OASIS HLB cartridges were chosen due to their ability to retain both non polar and polar compounds, obtaining the highest percentage recoveries (99–121%) and the lowest relative standard deviations (ranging from 6 to 9 %) in comparison to MCX sorbents (recoveries ranging from 30 to 109% with relative standard deviations in the range 5–28%).

Efficiency and robustness on analytes pre-concentration process were tested in SW, IWW and EWW samples. Influent and effluent samples were 20 or 10 times diluted, respectively, previously to SPE pre-concentration to decrease their high organic matter content and viscosity. Response obtained for samples spiked before SPE step (X) (e.g. typically at 0.05 µg/L level) and for sample extracts spiked after SPE step (Y) (e.g. 5 µg/L) were compared ($n = 3$). The ratio $(X/Y \times 100)$ was taken as SPE absolute recovery [39]. “Blank” samples, without spiking, were also processed to subtract the levels of target compounds that might be present in the samples.

Satisfactory recoveries were normally obtained for all compounds (overall range for all compounds and matrices (68-121%)) and the use of ILIS as surrogates was not strictly necessary to correct losses in the SPE step.

3.4. Matrix effect

Preliminary experiments were performed on surface and wastewater samples by spiking SPE extracts in order to evaluate signal suppression or enhancement due to co-eluting matrix constituents also present in the sample extracts. Thus, SPE blank extracts for each type of sample were spiked at 5 µg/L of each individual pesticide and labelled I.S. used (equivalent to 0.05 µg/L in sample) and matrix effects were evaluated for each compound calculating the absolute (without internal standard correction) and relative (with internal standard correction) responses in comparison to those of reference standards in solvent at 5 µg/L [39].

As can be seen in Fig. 1, matrix effects were not much noticeable in SW samples. Only for DIA and DEA, a remarkable signal suppression was found, whilst tolerable enhancement was revealed for TER and TBTYN. However, the effect of the sample matrix was more evident in wastewater samples, especially for

IWW where notable signal suppression was observed for almost all compounds (22-63%) with the exception of TER and TBTYN. In addition to these compounds, also HTbzne showed a similar behaviour (84%) in effluent wastewater samples.

Several approaches are typically applied to deal with matrix effects in quantitative LC-MS/MS analysis: improvement of the sample pre-treatment (clean-up) and/or the chromatographic separation, matrix-matched standards calibration, sample dilution, or the use of stable-isotopically labelled internal standards, the latest being widely accepted to be the most satisfactory approach. The ideal situation would be to have each analyte corrected by its own isotope-labelled molecule, but this problematic is multi-residue analysis due to the commercial unavailability of reference standards for several compounds (e.g. some TPs) and the high cost of acquiring a large number of isotope labelled reference standards. An option normally applied within the environmental field, the use of only a few ILIS [29], has been explored in this work for correction of matrix effects (3 labelled compounds were tested for analytes correction). Analytes were divided into three groups as a function of their retention time with the objective of performing correction with the ILIS of the nearest retention time. As expected (Fig. 2), satisfactory corrections were observed when terbuthylazine-d₅ was used for correction of TBZNE in all water samples tested. The results show how the use of the labelled molecule allowed to compensate the strong matrix effect obtained for IWW and EWW to a correct value around 100% allowing a right quantification of this compound. However, in spite of belonging to the same chemical class, when terbuthylazine-d₅ was used to correct for matrix effects of other triazines and TPS, unsatisfactory results were obtained in several cases (Fig. 2a). Thus, undesirable enhancements were observed for TER and specially TBTYN in all samples, making the use of this ILIS unadvisable. Despite TER was almost co-eluting with TBZNE, matrix effect for all samples tested increased from around 88% (WW) and 117% (SW), without ILIS correction, up to around 130% when using this analogue ILIS. On the contrary, for compounds eluting rather separate to terbuthylazine-d₅, like DETer, SIMA, DETbzne, HTbzne and ATRA, matrix effects were notably corrected in all samples.

Matrix suppression for DIA was not correctly compensated by this ILIS in none of the samples, whilst DEA and HA were properly corrected in only EWW samples. In order to compensate matrix effect of the first three eluting TPs (DIA, DEA and HA), other ILIS were tested: dimethoate-d₆ and thiabendazole-d₆. In this case, the use of ILIS structurally different from triazines but eluting at similar retention time was found a satisfactory approach for quantification (Fig. 2b). Dimethoate-d₆ was able to correct matrix effect on DIA, compensating the suppression from around 25% (WW) and 50% (SW) to a correct value around 90%. For

the other two compounds, thiabenzadole-d₆ was found more suitable, allowing a right quantification (around 100%).

Therefore, in order to compensate for errors associated to matrix effect, with the exception of TER and TBTYN, which were not affected significantly, each compound was corrected by ILIS as follows: dimethoate-d₆ (DIA); thiabenzadole-d₆ (DEA and HA); terbuthylazine-d₅ (for the rest of compounds) (Table 2).

3.5. Method validation

The whole analytical procedure was satisfactorily validated for linearity, precision, accuracy, sensitivity and specificity, in different type of water samples (SW, IWW and EWW) spiked with the compounds investigated in this work. "Blank" samples were previously analyzed and positive findings were subtracted from the spiked samples.

The linearity of the method was evaluated by linear regression analysis at seven concentrations. A seven-point calibration curve, in the range from 1 to 100 µg/L, was generated by injecting (in triplicate) mixed standard solutions with a fixed amount of the mixed internal standard solution. Good linearity was achieved for all analytes with correlation coefficients greater than 0.99.

Precision and accuracy were evaluated by spiking "blank" water samples at two concentration levels (0.025 and 0.1 µg/L), and analyzing five replicates of each spiked sample. It is worth to mention that three SRM transitions could be acquired also at low level for all analytes, making the reporting data highly confident from a quantitative point of view. As Table 2 shows, recoveries (between 70 and 120%) and precision (< 20%) were satisfactory for all compounds at both fortification levels. TER and TBTYN could be quantified without ILIS correction with acceptable recoveries (81-113%) and precision (3-14%).

The excellent sensitivity of the method is illustrated by instrumental LODs, which were in the range from 0.03 to 0.78 pg (Table 2). Regarding LOQs values, it must be taken into account that they were estimated from the most sensitive confirmation transition (q1). This means, that analyse could be quantified (using Q transition) but also confirmed at the same level (using q1 transition). As table 2 shows, analytes could be quantified and their identity confirmed in a reliable way at levels as low as 0.9 ng/L in SW, 6 ng/L in IWW and 3 ng/L in EWW.

The specificity of the method was evaluated by analysis of several "blank" water samples. No interfering peaks were observed at the retention times of the analytes. However, a few positives were found in the "blank" samples (e.g. TBZNE in SW) as a result of the wide use of these herbicides in the study area.

3.6. Monitoring pesticides and TPs in environmental and wastewater samples

The developed method was applied to 31 water samples (11 SW, 10 IWW and 10 EWW) collected in different sites of Italy and Spain in April, June and September, 2009. The results of the analysis are summarized in Table 3 (SW) and 4 (WW). All selected compounds were detected at least once, and one of them (TBZNE) was detected in all the samples (at concentrations higher than 0.025 µg/L in around 23% of the water analyzed). Other herbicides like TBTYN and SIMA were also frequently detected, as well as the TPs HTbzne, DETbzne, DIA and DETer. At least three compounds were present in every sample, but in only a few cases the levels were above 0.1 µg/L. These cases were dominated (>90%) by TPs (HTbzne).

Regarding SW, samples from Spain showed a higher number of positive findings (~90%) than from Italy (~50%). The highest level found (0.787 µg/L) corresponded to a TP (HTbzne) in Ebro delta. TBZNE and its dealkylated and hydroxy TPs (DETbzne, DIA and HTbzne) were found in almost all analyzed samples. It is interesting to point out, when comparing positive findings for unchanged triazines and for their TPs, that TPs levels were higher than the parent herbicides ones, in several analyzed samples, highlighting the interest of their inclusion in multiresidual methods for monitoring. Such situation was observed for ATRA, TBZNE and TER and their metabolites. Figure 3 shows SRM chromatograms for two SW samples (Ebro and Verd rivers) positives for ATRA, TBZNE and their main TPs. The high sensitivity of the method allowed the reliable confirmation of positive findings at very low concentration levels. For example, the three SRM transitions acquired for ATRA and TBZNE were reliably used for their identification at 0.009 µg/L. It is interesting to emphasize the lower concentration found for ATRA and TBZNE in comparison to their TPs, DEA (0.080 µg/L), HA (0.095 µg/L), HTbzne (0.112 µg/L) and DETbzne (0.080 µg/L). This fact illustrates how the effect of herbicide application and its influence on the environment is underestimated when samples are analyzed for the parent compounds only.

In relation to WW samples, TBZNE and HTbzne were found in all samples analyzed, and TBTYN and DETer in more than 70%. For these compounds, the highest levels found were in IWW at 0.21 µg/L. As expected, concentrations of parent herbicides in EWW were usually lower than in IWW. However, an increase was observed for TPs levels in EWW samples, probably related to the processes involved in the WWTP (Table 2). This fact could not be confirmed in the case of TBTYN as no specific TP was selected. Figure 4 shows illustrative chromatograms for IWW and EWW.

In spite that DIA or any of their precursors (ATRA, SIMA and TBzne) were not normally present in the Italian IWW, we found significant levels of DIA in the related EWW samples. Although this fact needs to be confirmed by further data, it is likely that some release of the removed and/or transformed compounds

from sewage sludge or from exhausted activated carbon occurs within the treatment plants. Other possibility for DIA findings in EWW could be the presence in the influent of other precursor triazines like cyanazine or sebutylazine, not included in the developed multiresidual method.

Conclusions

This work describes the development and validation of a multi-residue UHPLC-MS/MS method for quantification and confirmation of 11 triazine-related compounds (ATRA, SIMA, TBZNE, TER and TBTYN and their main transformation products) in surface and wastewater samples at the ng/L level. Losses associated to SPE process and matrix effects of signal suppression or enhancement due to co-eluting matrix constituents have been carefully evaluated. With the exception of TER and TBTYN, which did not require matrix effects correction, satisfactory results were obtained when using ILIS in all water samples tested. The overall analytical method has been validated in surface, influent and effluent wastewater from 0.025 to 2 µg/L, obtaining satisfactory recoveries and precision. Confirmation of the analyte identity was granted by acquiring 3 SRM transitions and the accomplishment of the ion ratio deviations in all analyte/matrix combinations, even at concentrations below 0.025 µg/L. The results obtained in this work after application of the method to real samples show the interest of including the most relevant TPs into current analytical methods to obtain a more realistic knowledge on water quality regarding pesticide contamination, as concentrations levels of TPs and detection frequency were normally higher than for parent herbicides.

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References

1. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a Framework for Community Action in the field of water policy. Official Journal of the European Union L327, 22nd December 2000.
2. Decision 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy. Official Journal of the European Union L331, 15th December 2001
3. RD 995/2000, 2 June 2000. BOE 147, 20th June 2000, p. 21558.
4. DM 56/2009, 14 April 2009. Suppl. Ord. G. U. R. I 124, 30th May 2009
5. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Union L330, 5th December 1998, p. 32
6. Barceló D (1991) *Analyst* 116:681-689
7. Hildebrandt A, Guillamón M, Lacorte S, Tauler R, Barceló D (2008) *Water Res* 42:3315-3326
8. Palma P, Kuster M, Alvarenga P, Palma VL, Fernandes RM, Soares AMVM, López de Alda MJ, Barceló D, Barbosa IR (2009) *Environ Int* 35:545-551
9. Jiang H, Adams CD, Koffskey W (2005) *J Chromatogr A* 1064:219-226
10. Sabik H, Jeannot R, Rondeau B (2000) *J Chromatogr A* 885:217-236
11. Thurman EM, Meyer MT, Mills MS, Zimmerman LR, Perry CA, Goolsby DA (1994) *Environ Sci Technol* 28:2267-2277
12. Gasser L, Fenner K, Scheringer M (2007) *Environ Sci Technol* 41: 2445-2451
13. Panchin SY, Carter DS, Bayless ER (2000) *Environ Sci Technol* 34:2131-2137
14. Hernández F, Ibáñez M, Pozo OJ, Sancho JV (2008) *Mass Spectrom* 43:173-184
15. Guzzella L, Pozzoni F, Giuliano G (2006) *Environ Pollution* 142:344-353
16. Hernández F, Marín JM, Pozo OJ, Sancho JV, López, FJ, Morell I (2008) *Int J Environ Anal Chem* 88:409-424
17. Nitschke L, Schüssler W (1998) *Chemosphere* 36:35-41
18. Meakins NC, Bubb JM, Lester JN (1994) *Chemosphere* 28:1611-1622
19. Lapertot M, Pulgarin C (2006) *Chemosphere* 65:682-690
20. Ikehata K, El-Din MG, (2005) *Ozone-Sci. Eng.* 27:173-202
21. Sancho JV, Pozo OJ, Hernández F (2004) *Analyst* 129:38-44
22. Marín JM, Sancho JV, Pozo OJ, López FJ, Hernández F (2006) *J Chromatogr A* 1133:204-214

23. Greulich, K, Alder L (2008) *Anal Bioanal Chem* 367:183-197
24. Carvalho JJ, Jerónimo PCA, Gonçalves C, Alpendurada MF (2008) *Anal Bioanal Chem* 392:955-968
25. Borba da Cunha AC, López de Alda M, Barceló D, Pizzolato TM, Dos Santos JHZ (2004) *Anal Bioanal Chem* 378:940-954
26. Mezcua M, A. Agüera A, Lliberia LJ, Cortés MA, Bagó B, Fernández-Alba AR (2006) *J Chromatogr A* 1109:222-227
27. , Sancho JV, McMillan D, Rao R, Hernández F (2008) *Trends Anal Chem* 27:481-489
28. Gervais G, Brosillon S, Laplanche A, Helen C (2008) *J Chromatogr A* 1202:163-172
29. Marín JM, Garcia-Lor E, Sancho JV, López FJ, Hernández F (2009) *J Chromatogr A* 1216:1410-1420
30. European Union Decision 2002/657/EC, Off. J. Eur. Comm., L221 (12 August 2002) 8
31. European Union Decision DG-SANCO, Method validation and quality control procedures for pesticides residue analysis in food and feed, NO. SANCO/2007/3131, Brussels, 31 October 2007
32. Taylor PJ (2005) *Clin Biochem* 38:328–334
33. , Hernández F, Niessen WMA (2006) *Trends Anal Chem* 25:1030-1042.
34. Delatour T (2004) *Anal Bioanal Chem* 380:515-523
35. Pitarch E, Marín JM, López FJ, Hogendoorn E, Hernández F (2007) *Int J Environ Anal Chem* 87: 237-248
36. Kang J, Hick LA, Price WE (2007) *Rapid Commun Mass Spectrom* 21:4065-4072
37. Ito S, Tsukada K (2002) *J Chromatogr A* 943:39–46
38. Hernández F, Sancho JV, Pozo OJ (2005) *Anal Bioanal Chem* 382:934-946
39. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) *Anal Chem* 75:3019-3030

Table 1

MS/MS optimized conditions for selected compounds. For labelled internal standards, only the quantification transition was acquired.

Compound	Rt (min)	Precursor ion (m/z)	Cone (V)	Col. Ener. (eV)	Product ion (m/z) ^a	Q/q ratio (RSD%) ^b
DIA	3.54	174.1	30	20	96.1	
				25	68.0	0.9 (3)
				20	132.1	1.9 (3)
DEA	3.86	188.1	20	20	146.1	
				25	104.0	2.4 (3)
				25	110.1	6.3 (3)
HA	3.94	198.2	35	20	156.2	
				35	86.1	1.2 (5)
				25	69.0	1.8 (5)
DETer	4.26	198.2	30	15	142.1	
				25	86.1	2.3 (5)
				30	57.1	3.8 (10)
SIMA	4.26	202.1	45	20	132.1	
		204.1		20	134.1	3.1 (5)
		204.1		25	96.1	3.2 (11)
DETBzne	4.29	202.1	30	20	146.1	
		204.1		15	148.1	3.3 (7)
		202.1		35	79.1	4.5 (6)
HTBzne	4.12	212.2	30	15	156.2	
				30	86.1	2.6 (3)
				25	97.1	5.3 (7)
ATRA	4.58	216.3	45	20	174.3	
		216.3		25	96.0	1.7 (5)
		218.3		20	176.3	3.6 (4)
TER	4.98	226.1	45	20	170.1	
				30	75.1	6.7 (5)
				25	128.1	21 (7)
TBZNE	5.02	230.1	45	15	174.1	
		232.1		15	176.1	2.9 (3)
		230.1		30	96.1	4.6 (5)
TBTYN	5.39	242.1	45	25	91.1	
				35	71.1	0.9 (3)
				25	158.1	3.8(7)
Dimethoate-d ₆	3,66	236.0	40	10	205.0	
Thiabendazole-d ₆	4,05	208.2	55	25	180.2	
Tbzne-d ₅	5,0	235.1	45	15	179.1	

^a The first transition (top) was used for quantification and the second and third transitions (bottom) were used for confirmation.

^b n=7

Abbreviations: Rt : retention time; Col. Ener.: collision energy; DIA (desisopropylazine); DEA (desethylatrazine); HA (2-hydroxy-atrazine); DETer (desethylterbumeton); SIMA (simazine); DETbzne (desethylterbuthylazine); 2-OH-tbzne (2-hydroxy-terbuthylazine); ATRA (atrazine); TER (terbumeton); TBZNE (terbuthylazine); TBTYN (terbutryn).

Table 2 Method validation for surface water (SW), influent (IWW) and effluent (EWW) wastewaters. Recovery (%) and relative standard deviation (RSD, %) for five replicates, instrumental limit of detection (LOD) and estimated limit of quantification (LOQ).

Compound	Rt (min)	LOD (pg)	SW				IWW				EWW				ILIS used			
			LOQ (ng/L)	0.025 µg/L (n=5)		0.1 µg/L (n=5)		LOQ (ng/L)	0.5 µg/L (n=5)		2.0 µg/L (n=5)		LOQ (ng/L)	0.25 µg/L (n=5)		1.0 µg/L (n=5)		
				Rec (%)	RSD (%)	Rec (%)	RSD (%)		Rec (%)	RSD (%)	Rec (%)	RSD (%)		Rec (%)		RSD (%)	Rec (%)	RSD (%)
DIA	3.54	0.29	6	77	5	83	4	20	77	14	80	9	8.0	77	7	73	9	Dimethoate-d ₆
DEA	3.86	0.12	5.9	83	7	92	6	8.7	70	9	82	11	14	72	8	73	6	Thiabendazol-d ₆
HA	3.94	0.78	1.4	98	6	88	6	22	109	9	112	4	9.0	88	14	70	12	Thiabendazol-d ₆
DETer	4.26	0.07	1.3	93	8	101	3	29	93	4	109	10	19	72	6	76	7	Tbzne-d ₅
SIMA	4.26	2.60	20	118	9	116	17	150	108	8	101	6	58	106	19	102	11	Tbzne-d ₅
DETBzne	4.29	0.19	2.3	100	12	106	4	25	101	10	111	8	20	101	14	85	12	Tbzne-d ₅
HTbzne	4.15	0.03	1.4	79	13	89	12	14	71	8	83	13	12	71	8	87	9	Tbzne-d ₅
ATRA	4.58	0.24	3.9	96	5	99	4	27	121	6	116	10	12	101	11	89	11	Tbzne-d ₅
TER	4.98	0.05	0.9	90	8	100	8	6.0	86	6	81	5	3.0	95	8	105	14	-
TBZNE	5.02	0.15	2.0	89	4	104	7	23	108	5	91	5	12	103	3	101	3	Tbzne-d ₅
TBTYN	5.39	0.25	1.3	97	6	104	8	22	83	5	99	3	11	102	3	113	6	-

Abbreviations: Rec (Recovery). DIA (desisopropylazine); DEA (desethylatrazine); HA (2-hydroxy-atrazine); DETer (desethylterbumeton); SIMA (simazine); DETbzne (desethylterbutylazine); 2-OH-tbzne (2-hydroxy-terbutylazine); ATRA (atrazine); TER (terbumeton); TBZNE (terbutylazine); TBTYN (terbutryn).

Table 3 Concentrations of triazines and their TPs in surface water from 14 sampling sites located in Italy (urbanized area north of Milan) and Spain (Mediterranean Valencian area). Samples were collected in June and September, 2009.

Compound	SW ($\mu\text{g/L}$)										
	June						September				
	1	2	3	4	5	6	7	8	9	10	11
DIA	0.017	0.008	d	0.009	0.010	0.015	-	0.018	d	d	d
DEA	0.080	d	-	0.009	0.009	0.020	-	-	-	-	-
HA	0.095	0.151	0.038	d	d	-	-	-	d	-	-
DETer	0.006	0.005	0.003	0.025	0.042	0.014	-	-	-	-	-
SIMA	0.038	d	-	0.028	0.026	0.160	d	d	-	0.025	-
DETBzne	0.008	0.016	d	0.070	0.080	0.040	0.016	0.008	0.015	0.013	
HTbzne	0.105	0.787	0.317	0.087	0.112	0.068	-	-	d	-	-
ATRA	0.009	d	-	d	0.004	-	d	d	d	-	-
TER	0.009	d	0.002	0.002	0.009	d	-	-	-	d	-
TBZNE	0.026	0.004	0.003	0.002	0.009	0.053	0.008	0.009	0.026	0.005	0.015
TBTYN	0.003	0.008	0.002	-	-	0.030	0.003	0.003	-	0.022	0.004

d: detected at concentration level <LOQ. -: not detected.

Abbreviations: 1: Ebro river; 2: Ebro Delta; 3: Pego-Oliva marsh; 4: Almenara; 5: Verd river; 6: Clot Burriana; 7: Lurate; 8: Guanzate; 9: Gorgonella; 10:Seveso; 11: Livescia.

DIA (desisopropylazine); DEA (desethylatrazine); HA (2-hydroxy-atrazine); DETer (desethylterbumeton); SIMA (simazine); DETbzne (desethylterbuthylazine); 2-OH-tbzne (2-hydroxy-terbuthylazine); ATRA (atrazine); TER (terbumeton); TBZNE (terbuthylazine); TBTYN (terbutryn).

Table 4 Concentration of triazines and their metabolites in 24-h composite influent and effluent wastewater samples of five WWTPs placed in Spain and Italy. during two sampling campaigns in April and June, 2009.

Compound	IWW ($\mu\text{g/L}$)										EWW ($\mu\text{g/L}$)									
	April					June					April					June				
	CS	BU	BE	AL	AS	CS	BU	BE	AL	AS	CS	BU	BE	AL	AS	CS	BU	BE	AL	AS
DIA	-	-	-	-	-	-	-	-	-	-	-	-	-	0.070	0.035	-	0.035	-	0.230	0.195
DEA	-	-	-	-	-	-	-	-	-	-	-	d	-	-	-	-	0.025	-	-	-
HA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DETer	d	d	0.030	-	-	d	d	0.030	-	-	d	0.024	0.041	d	d	d	0.020	0.044	-	-
SIMA	-	-	-	-	-	-	d	-	-	-	-	d	-	-	-	-	d	-	-	-
DEtbzne	-	-	-	-	-	-	0.050	-	-	-	d	0.025	d	d	d	-	d	d	d	d
HTbzne	0.110	0.140	0.210	0.050	0.060	0.080	0.180	0.170	0.050	0.020	0.063	0.076	0.060	0.057	0.073	0.050	0.076	0.084	0.055	d
ATRA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TER	-	d	d	-	-	-	0.060	d	-	-	-	d	d	-	-	-	d	d	-	-
TBZNE	d	d	d	d	d	d	0.210	d	d	d	0.027	0.060	0.016	d	0.020	d	0.029	0.015	d	d
TBTYN	0.061	0.052	0.040	0.041	d	0.030	0.082	0.043	d	-	0.012	0.046	0.028	-	-	0.019	0.015	0.015	d	d

d: detected at concentration level <LOQ. - : not detected

Abbreviations: WWTPs: CS (Castellon); Bu (Burriana); BE (Benicassim); AL (Alto Lura); AS (Alto Seveso).

DIA (desisopropylazine); DEA (desethylatrazine); HA (2-hydroxy-atrazine); DETer (desethylterbumeton); SIMA (simazine); DETbzne (desethylterbuthylazine); 2-OH-tbzne (2-hydroxy-terbuthylazine); ATRA (atrazine); TER (terbumeton); TBZNE (terbuthylazine); TBTYN (terbutryn).

FIGURE CAPTIONS

Fig. 1. Matrix effect for all selected analytes at 0.05 µg/L level in surface water (SW), influent (IWW) and effluent (EWW) wastewaters.

Abbreviations: See tables 1-4.

Fig 2. Matrix effects in different water samples (SW, IWW and EWW) with and without correction of internal standards. (a) All compounds corrected by terbuthylazine-d₅. (b) Correction with the nearest retention time labelled analyte. DIA corrected by dimethoate-d₆; DEA and 2-OH-atrazine corrected by thiabenzadol-d₆ and all the other compounds by terbuthylazine-d₅

Abbreviations: See Tables 1-4.

Fig 3. Selected UHPLC-MS/MS chromatograms for two SW samples from the Ebro and Verd Rivers. Concentrations (a) atrazine 0.009 µg/L. (b) DEA 0.080 µg/L (c) 2-OH-atrazine 0.095 µg/L. (d) terbuthylazine 0.009 µg/L. (e) 2-OH-terbuthylazine 0.112 µg/L. (f) desethylterbuthylazine 0.080 µg/L. (Q) quantification transition; (q₁) and (q₂) confirmation transition. Experimental Q/q ratios are shown in the boxes (for comparison, see theoretical Q/q ratios in Table 1).

Fig 4. Selected UHPLC-MS/MS chromatograms for Burriana IWW and EWW samples collected in June, 2009. (Quantification transition). *Abbreviations:* See Tables 1-4.

* Estimated concentrations from Q transition: 0.090 µg/L in IWW, 0.030 µg/L in EWW.

Figure 1.

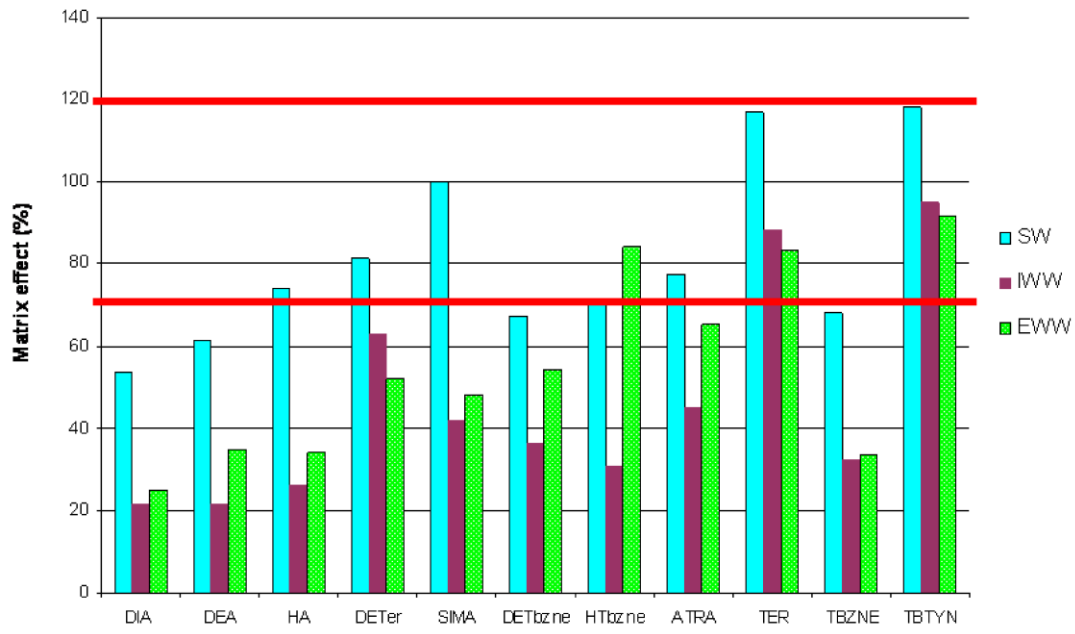


Figure 2.

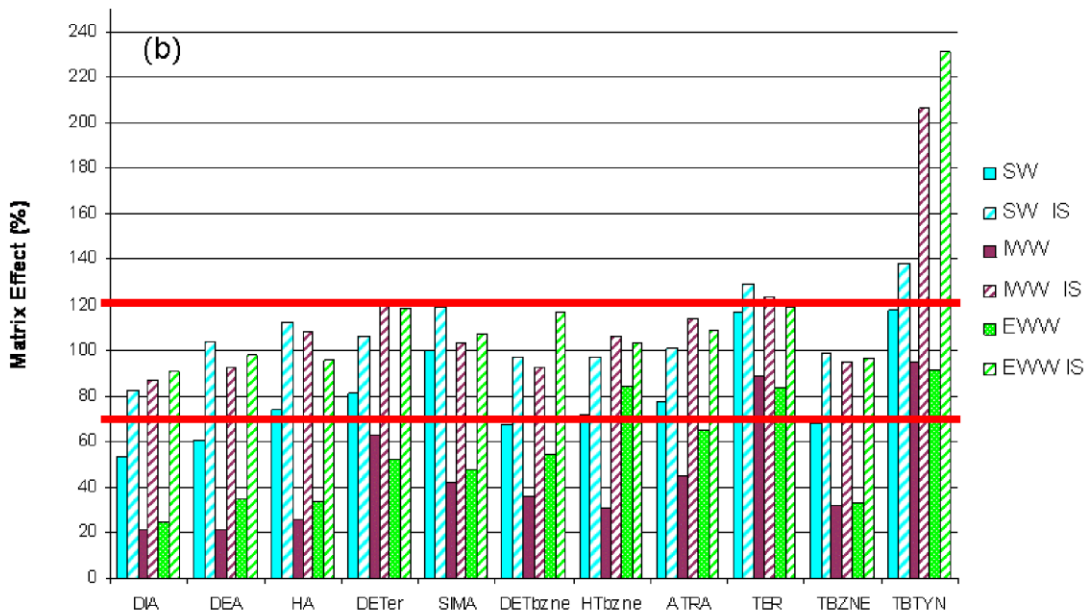
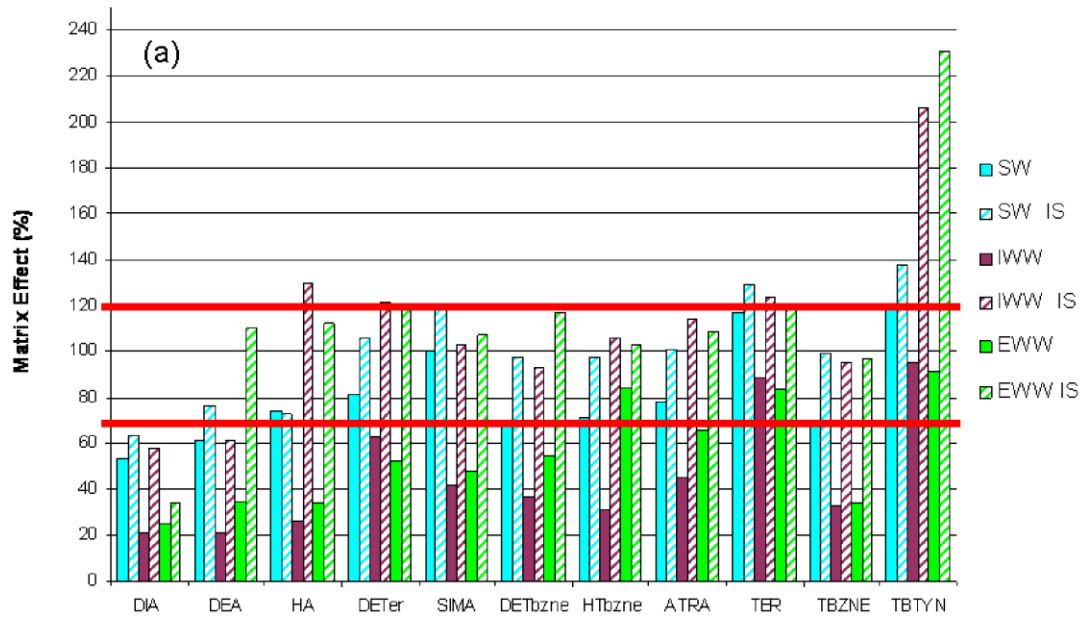


Figure 3

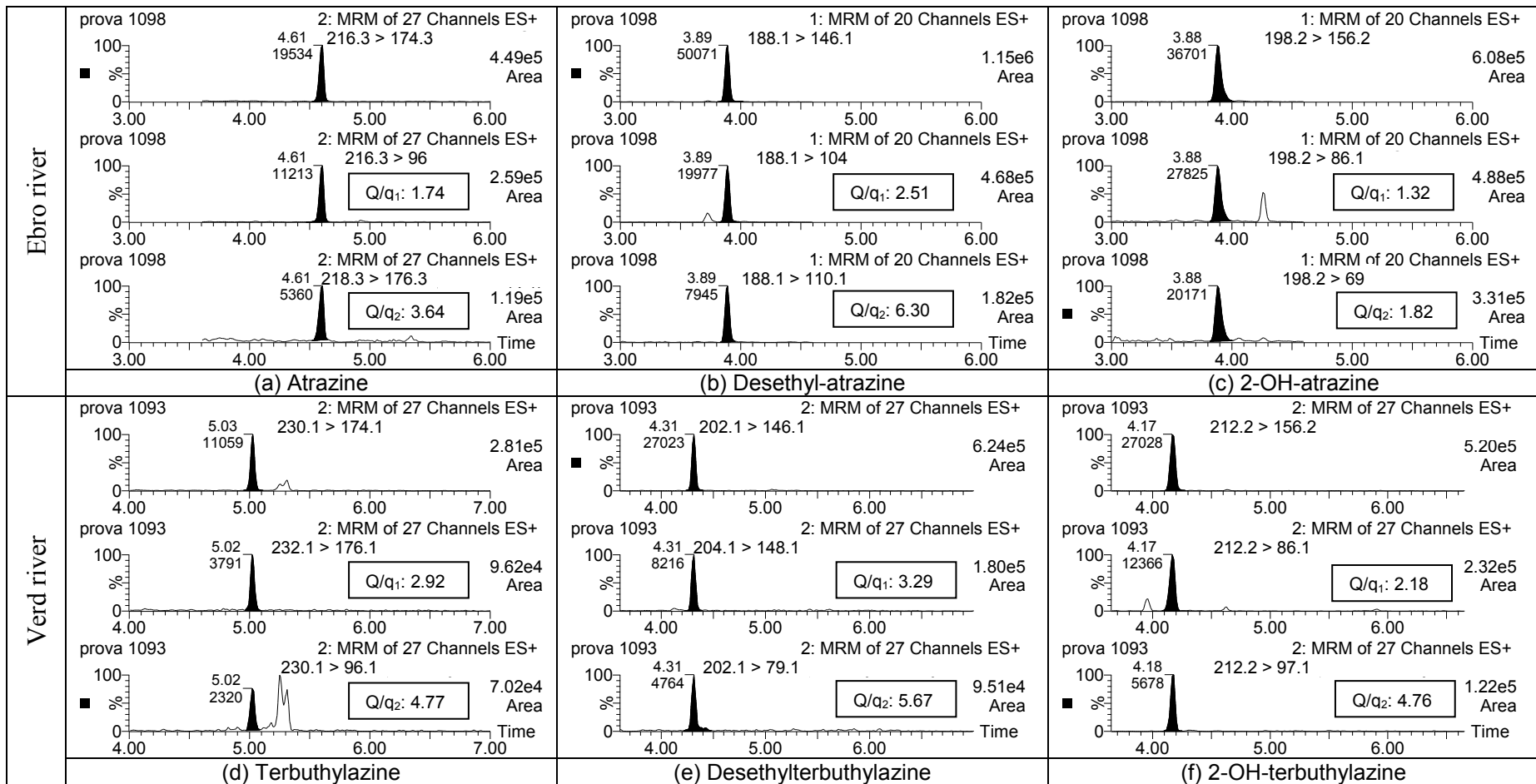


Figure 4

