

Liquid chromatography coupled to tandem mass spectrometry for the residue determination of Ethylenethiourea (ETU) and Propylenethiourea (PTU) in water

Cristina Ripollés, Juan V. Sancho, Francisco J. López and Félix Hernández *

Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat, E-12071

Castellón, Spain. *Tel.:* +34 964 387366. *Fax:* +34 964 387368.

* Author for correspondence. E-mail: felix.hernandez@qfa.uji.es

ABSTRACT

Ethylenethiourea (ETU) and propylenethiourea (PTU) are the main degradation products of dithiocarbamates fungicides, which are widely used in agriculture from several years ago. Their determination in water at low concentrations (e.g. sub-ppb levels) is highly problematic due to their polar character and low molecular size. In the present study, two analytical methodologies have been developed and compared for the selective and sensitive determination of ETU and PTU in various types of waters. Both approaches are based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with electrospray ionization, using triple quadrupole analyzer. Whereas the first methodology used an on-line solid-phase extraction (SPE) step in order to reach the adequate sensitivity, the second one avoided sample treatment and was based on direct injection into an ultra high performance liquid chromatography (UHPLC)-MS/MS system, making use of a new-generation instrument in order to reach sub-ppb analyte levels in water.

Strong matrix effects (typically leading to signal enhancement) were observed for most of the evaluated waters, especially when applying the on-line SPE method, surely due to the higher amount of sample injected into the system. The use of the own analyte (ETU-d₄) as isotope-labelled internal standard (ILIS) allowed to compensate these effects and to achieve an accurate ETU quantification at low concentrations. Moreover, three simultaneous transitions, operating in selected reaction monitoring mode, were acquired for both ETU and ETU-d₄. This fact together with the evaluation of their relative intensity ratios assured the reliable identification of the analyte in the water samples.

The two optimized methodologies were validated by analysis of six different samples (two drinking water, two groundwater and two surface water), spiked at two levels (0.1 and 1.0 µg/

L), and analyzed each in quintuplicate. Satisfactory accuracy and precision, with recoveries ranging from 73 to 104% and RSDs lower than 20%, were obtained for ETU. Limits of detection for ETU were found to be 0.058 µg/L and 0.027 µg/L with direct injection and with the on-line methodology, respectively. No satisfactory recoveries were obtained, in general, for PTU despite using its own deuterium-labelled molecule for matrix effects correction. Notable differences in the chemical behavior between PTU and PTU-d₆ were observed, which lead to significant variation in their chromatographic retention time and ionization efficiency. Thus, no satisfactory correction of matrix effects could be reached illustrating that the use of deuterated ILIS can be problematic in some particular cases. Despite the poor correction, a semi-quantitative analysis would be feasible for PTU at sub-ppb levels in water.

To the best of our knowledge, this is the first article reporting the use of LC-MS/MS for the trace level determination of these problematic analytes in water.

Keywords

Ethylenethiourea (ETU); Propylenethiourea (PTU); on-line trace enrichment; LC-MS/MS, UHPLC; matrix effects

1. INTRODUCTION

Dithiocarbamates (DTC) have been widely used as fungicides in agriculture in different types of crops. Besides, they are also used as antioxidants and vulcanization accelerators in the rubber industry [1]. Particularly, ethylenebisdithiocarbamates (EBDCs) are among the most commonly employed organic fungicides in current agricultural practice. EBDCs have low water solubility, low short-term toxicity and degrade rapidly in presence of moisture and/or oxygen, whereas their metabolites are more polar and usually have some hazardous effects to humans. Ethylenethiourea (ETU) is the major degradation product of several EBDCs, like maneb, mancozeb, zineb and metiram [2-4]. In addition, ETU can appear as an impurity of commercial EBDCs formulations, during product storage [3,5] and also when foods containing these fungicides are cooked [6].

This compound has been evaluated by IARC (International Agency for Research on Cancer) in several occasions [4,7,8]. Initially, it was reported to cause carcinogenic and teratogenic effects to humans. However, it was finally concluded that there was not enough evidence for its carcinogenicity in humans, and it was only considered as animal carcinogen [4]. ETU is highly soluble in water (20000 mg/L at 30°C, $\log K_{ow}$ -0.66) [4,9] due to its elevated polarity and it is quite stable to hydrolysis. However, it can be degraded in presence of dissolved oxygen and sensitizers by photolysis [10,11] or by soil microorganisms [12] via ethyleneurea (EU). Regarding its behavior in the soil-water environment, ETU is generally weakly absorbed onto the soil. So, due to its notable mobility in wet soil it can easily leach to subsurface soils, where it is slowly degraded compared with surface ones, increasing the risk of groundwater contamination [9,11,13].

Another compound related to dithiocarbamates is Propylenethiourea (PTU), the main metabolite of the propylenebis(dithiocarbamate) (PBDC) propineb. As for other EBDCs, propineb is practically insoluble in water and has low acute toxicity. Nevertheless, PTU is suspected to cause various harmful effects, since it is proved to be a potential carcinogenic to animals [3]. Similarly to ETU, PTU is highly soluble in water (95000 mg/L at 20°C, $\log K_{ow}$ -0.26 at 22°C, [14,15]) and relatively stable to hydrolysis and photolysis, although it can be photodegraded in aqueous solutions in presence of humic acids [3,15].

Consequently, suitable analytical methods are needed to detect and quantify ETU and PTU in environmental waters, as they are both toxicologically relevant and can become a serious environmental concern. Most analytical methods are focused on food [16-23] and urine [24-31], and are based on the use of liquid chromatography (LC) with conventional detectors (UV, amperometric detection) [16-18,21,24-26], or coupled to mass spectrometry (MS) [19,20,22,23,27-31]. Both, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), have been used in LC-MS methods. However, there are only few reports regarding environmental samples, such as airborne [32] or soil [33], and are based on LC-UV analysis. As regards water samples, most analytical methods for ETU and PTU have used gas chromatography (GC) for their determination. The US Environmental Protection Agency (EPA) reported a method for determining ETU in water by GC-NPD (nitrogen phosphorus detector) with previous analyte extraction by using a diatomaceous earth column [34] and a limit of detection (LOD) of 2.7 µg/L. PTU was employed as surrogate standard (SS). Van der Poll *et al.* [35] developed a GC method with alkali flame ionization detection and confirmation of positive samples by MS, which was optimized for groundwater. Recoveries around 70% and a LOD of 0.1 µg/L were achieved. Another proposed methodology used extractive derivatization of ETU from surface waters, prior to its

analysis by GC-ECD (electron capture detector) [36], with LOD of 0.05 µg/L. Recently, a study about the application of Mancozeb and the consequently accumulation of ETU in soil and water (surface, subsurface and groundwater) has been reported [37]. Sample analyses were performed by GC-ECD, after extracting ETU from water with a two-step derivatization and liquid-liquid partitioning.

Taking into account the high solubility in water and elevated polarity of ETU and PTU, using LC seems better choice than GC for their determination in water samples, similarly to other “difficult” polar contaminants [38-40]. In addition, the on-line coupling SPE-LC in combination with MS/MS can be easily made, improving sensitivity and analysis time, with little sample manipulation [40,41].

Only a few papers have reported LC-based methods for determining ETU in water. In 1991, Hogendoorn *et al.* [42] proposed a column-switching (LC-LC) methodology followed by ultraviolet (UV) detection for groundwater samples. A LOD of 0.1 µg/L was achieved by applying a previous preconcentration by liquid-liquid extraction (LLE). The same year, the determination of ETU in crops and groundwater by LC with pulsed amperometric detection (PAD) was reported [43], reaching a LOD around 5 µg/L. An alternative approach was the voltammetric detection of ETU in natural and drinking waters, based on its adsorptive deposition in a mercury drop electrode, with an improved LOD (1.4 µg/L) [44].

Data reported in the literature show that ETU can reach high concentration levels in the aquatic environment. Thus, concentrations from 0.1 to 6 µg/L have been found in groundwater [35,36,42], reaching up to 53 µg/L in some cases [35]. Levels between 10 – 30 µg/L have been reported in rivers and lakes [44]. Similar concentrations have been found in surface waters (22.5 µg/L), being lower in sub-surface waters (4.3 µg/L) [37].

The aim of this work was to develop rapid and sensitive methodology for determining ETU and PTU in water using LC-MS/MS with triple quadrupole (QqQ) analyzer. On-line SPE-LC and direct injection of the water sample into UHPLC-MS/MS have been evaluated. Two isotope-labelled internal standards (ETU-d₄ and PTU-d₆) were tested for correction of the strong matrix effects observed in most of the water samples. The acquisition of three (ETU) or four (PTU) MS/MS transitions allowed to confirm the identity of the analytes detected in the samples.

2. EXPERIMENTAL

2.1. Reagents and Chemicals

Ethylenethiourea (ETU) (CAS number 96-45-7) and Propylthiourea (PTU) (CAS number 2122-19-2) reference standards (both 99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labelled internal standards (ILIS) (ETU-d₄, ≥99%) and (PTU-d₆, ≥99%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Medical Isotopes, Inc. (Pelham, USA), respectively. Reagent-grade formic acid (>98%), ammonium acetate (NH₄Ac) (98%), HPLC-grade acetonitrile (ACN), HPLC-grade methanol (MeOH), were supplied by Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA).

Individual stock standard solutions were prepared by dissolving the pure compounds in MeOH, obtaining a final concentration of 500 mg/L, and were stored in a freezer at < -18 °C. Intermediate individual standard solutions of 50 mg/L were prepared from the stock solutions by diluting with MeOH. Working mix solutions of ETU and PTU were prepared at various concentrations by diluting the intermediate solutions with HPLC-grade water. ILIS

working mix solutions at 500 µg/L and 50 µg/L were obtained from dilution of the 50 mg/L standards diluting with HPLC water. The intermediate and working standards were stored in a fridge at 4 °C.

2.2. Instrumentation

For on-line trace enrichment analysis, an Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (TQD) (Waters Micromass, Manchester, UK). This system was equipped with a Waters 2777 Sample Manager, with a loop of 1 mL (Waters), and an Agilent 1100 binary pump (Palo Alto, USA) used to conditioning and washing the SPE cartridge. Two different interfaces were tested: orthogonal Z-spray-electrospray (ESI) and atmospheric pressure chemical ionization probe (Ion Sabre APCI). Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N₂ LC-MS (Claind, Teknokroma, Barcelona, Spain). The determination of analytes was finally performed using ESI source in the positive mode. The cone gas and desolvation gas flows were optimized at 60 L/h and 600 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of approximately 2×10^{-3} mbar in the collision cell. Electrospray needle capillary voltage was fixed at 3.5 kV. Desolvation and source temperature were set to 350 °C and 120 °C, respectively. Dwell times of 50 ms/transition were selected. Temperature column was set to 40 °C. Masslynx v 4.1 (Waters) software was used to process the quantitative data obtained from calibration standards and from water samples.

For direct injection analyses, an Acquity UPLC system coupled to a Xevo TQ-S (triple quadrupole analyzer) mass spectrometer (Waters) was employed, equipped with electrospray

ionization source (ESI). The desolvation gas flow rate was set to 1200 L/h at a temperature of 650 °C; the cone gas flow rate was fixed at 250 L/h and the source temperature at 150 °C; the capillary voltage was optimized at 3.5 kV. Dwell times of 25 ms/transition were chosen. The column was kept at 45 °C.

Two conventional HPLC analytical columns were tested for chromatographic separation: Atlantis T3 C₁₈ (100 x 4.6 mm, 5 µm) and Atlantis T3 C₁₈ (100 x 2.1 mm, 5 µm) for the SPE-LC-MS/MS approach. Another two UPLC columns were also tested: Acquity UPLC HSS T3 C₁₈ (100 x 2.1 mm, 1.8 µm) and Acquity UPLC HSS T3 C₁₈ (50 x 2.1 mm, 1.8 µm) for the direct injection into the UHPLC-MS/MS system. All chromatographic columns were from Waters.

Cartridges employed for on-line SPE experiments were Oasis HLB (20 x 2.1 mm, 25 µm) (Waters). 0.20 µm Nylon filters were purchased from Scharlab.

2.3. Analytical procedure

2.3.1 On-line SPE-LC-MS/MS (TQD instrument)

10 mL water sample was taken, and 20 µL of the ILIS working solution (500 µg/L) were added, obtaining ILIS final concentration of 1 µg/L. Then, 1 mL aliquot of sample was directly injected into the SPE-LC(ESI)MS/MS system using an Oasis HLB cartridge (20 x 2.1 mm, 25 µm) for preconcentration, and an Atlantis T3 C₁₈ column (100 x 4.6 mm, 5 µm) for the chromatographic separation. The on-line SPE-LC procedure was performed as follows: firstly, the SPE cartridge was conditioned with water at a flow rate of 1 mL/min for 3 min. Then, 1 mL sample aliquot was preconcentrated into the cartridge with water at 1 mL/

min (transference time: 65 s). It was subsequently transferred in backflush mode to the analytical column, starting the LC elution, which consisted on applying a binary water/methanol gradient, where the methanol percentage was changed linearly as follows: 0 min, 5%; 1.5 min 5%; 11 min, 90%; 11.1 min, 5%. The flow rate was kept at 0.3 mL/min and the analysis run time was 12 min. A solvent delay of 6 min was selected to minimize source contamination. Standards used for quantification were also subjected to the same on-line preconcentration applied to the samples, including the addition of ILIS before the on-line SPE step.

2.3.2 Direct injection: UHPLC-MS/MS (Xevo TQ-S instrument)

10 mL water sample was filtered through 0.20 μm nylon filter and 20 μL of the 500 $\mu\text{g/L}$ ILIS working solution (ILIS final concentration 1 $\mu\text{g/L}$) were added. Afterwards, 10 μL were directly injected into the UHPLC(ESI)MS/MS system, performing the chromatographic separation on an Acquity UPLC HSS T3 C₁₈ (50 x 2.1 mm, 1.8 μm) column. A binary water / methanol gradient elution was applied at a flow rate of 0.3 mL/min, changing linearly the percentage of methanol as follows: 0 min, 5%; 1 min 5%; 3 min, 90%; 3.1 min, 5%. The chromatographic run time was 4 min. Calibration was carried out from standards prepared by adding 980 μL of the corresponding standard mix into a 2 mL-vial containing 20 μL of the 50 $\mu\text{g/L}$ ILIS solution.

2.4. Validation study

For both methods, validation was performed following European SANCO guidelines recommendations [45]. Method linearity was studied by analyzing standard solutions in triplicate at seven concentrations, ranging from 0.05 to 50 $\mu\text{g/L}$. Satisfactory linearity was

assumed when the correlation coefficient (r) was higher than 0.99, based on relative responses (analyte peak area/ILIS peak area), and the residuals lower than 30%. Accuracy (expressed as recovery, in %) and precision (repeatability, expressed as relative standard deviation, in %) were evaluated by analyzing six water samples (two groundwater, GW; two surface water, SW; two drinking water, DW) spiked at two concentration levels each: 0.1 and 1.0 $\mu\text{g/L}$. All recovery experiments were carried out in quintuplicate for each type of water sample. Quantification in both methods was performed by internal standard calibration with standards in the range 0.05 - 10 $\mu\text{g/L}$ for the low level and 0.05 - 25 $\mu\text{g/L}$ for the high level, using relative responses.

The limit of quantification (LOQ) objective was established as the lowest concentration level for which the method was fully validated with satisfactory results (recoveries between 70 and 120% and $\text{RSD} \leq 20\%$). The limit of detection (LOD) was estimated as the lowest concentration that the analytical procedure can reliably differentiate from background levels, and it was calculated for a signal-to-noise ratio of three from the chromatograms of samples spiked at the lowest concentration assayed, i.e. 0.1 $\mu\text{g/L}$.

LC-MS/MS analyses were performed by acquiring three (for ETU and ETU-d₄) or four (for PTU and PTU-d₆) simultaneous SRM transitions per compound, the most sensitive being used for quantification (Q) and the rest for confirmation (q). Confirmation of the identity of the compound was carried out by accomplishment of the Q/q ratios and retention time (RT) with acceptable deviations [46].

3. RESULTS AND DISCUSSION

3.1. MS and MS/MS optimization

For MS optimization when employing the TQD instrument, the ionization efficiency of two interfaces, ESI and APCI, was investigated. Full-scan and MS/MS spectra of analytes were obtained from infusion of 1.0 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 $\mu\text{L}/\text{min}$ for ESI and 40 $\mu\text{L}/\text{min}$ for APCI, both in positive ionization mode. Positive ESI and APCI full-scan mass spectra of ETU and PTU at the optimized cone voltage are shown in **Figure 1** (medium, bottom). In contrast to APCI experiments, both compounds showed a low abundance of sodium adducts $[\text{M}+\text{Na}]^+$ when using ESI interface. The effect of the addition of HCOOH (0.01%) and NH_4Ac 5mM into the infusion vial was checked in order to decrease $[\text{M}+\text{Na}]^+$ peak intensity in ESI. However, these additives did not lower the sodium adduct formation, but they led to a slightly decrease in the intensity of the $[\text{M}+\text{H}]^+$ ion. The most abundant peak with both sources corresponded to the $[\text{M}+\text{H}]^+$ ion, which was selected as the precursor ion.

Contrarily to other studies [19,22], in our instruments the abundance of $[\text{M}+\text{H}]^+$ ions increased around 10-fold when using ESI instead of APCI as ionization source for both analytes (**Figure 1**). So, ESI was the interface preferred as it provided the highest signal. Selecting $[\text{M}+\text{H}]^+$ ion as precursor, three product ions were obtained for ETU and ETU- d_4 , and four for PTU and PTU- d_6 . **Figure 1** top shows the product ion spectra for m/z 103.0 (ETU), at 20 eV, and for m/z 117.1 (PTU), at 15 eV. The optimized MS/MS conditions can be seen in **Table 1**. Dwell times of 50 ms/transition were chosen. Among the SRM transitions selected, the most sensitive was employed for quantification (Q) and the rest for confirmation (q). Q/q theoretical ratios were obtained as an average from injection of reference standards at six different concentrations between 0.1 and 50 $\mu\text{g}/\text{L}$.

3.2. LC optimization

Although better results regarding ionization efficiency were obtained using ESI instead of APCI interface, both sources were also tested during the chromatographic optimization.

For ESI experiments, the four chromatographic columns mentioned in the instrumentation section were evaluated. Regarding mobile phase, mixtures water:MeOH or water:ACN, with different amounts of NH_4Ac and HCOOH , were tested. The addition of these additives did not increase again peaks intensity. The use of MeOH led to more intense and narrower peaks compared to ACN. The best sensitivity and peak shape were achieved with water/MeOH as mobile phase and an Atlantis T3 C_{18} (100 x 4.6 mm, 5 μm) column. With an optimal flow rate of 0.3 mL/min, the chromatographic run time was 7 min. The selected injection volume was 50 μL , since higher volumes resulted in broad peaks, mainly for ETU and ETU- d_4 . It seems rare that improved signal intensity (5/10-fold higher) was obtained with a 4.6 mm i.d column instead of a 2.1 mm i.d one, as the selected flow rate should be more adequate for the later.

Notable matrix effects (signal enhancement) were observed for ETU and PTU in some waters tested in this optimization process, although they were successfully corrected by ETU- d_4 . However, the use of PTU- d_6 as ILIS was not so effective, as will be discussed later. Despite obtaining in general good results for ETU, the aimed sensitivity for environmental waters could not be reached by direct injection in the TQD instrument. Our objective was to develop a reliable method for quantification at 0.1 $\mu\text{g/L}$, which is the maximum concentration

allowed for pesticides as well as their metabolites and degradation products in drinking water [47].

Only one analytical column, Atlantis T3 C₁₈ (100 x 4.6 mm, 5 µm), was employed for APCI experiments because it was the most suitable for working at optimal APCI flow rates (around 1 mL/min), thanks to its wider internal diameter. The best results were obtained by using water / MeOH at 0.8 mL/min and an injection volume of 100 µL. As the sensitivity reached for all compounds was approximately 10-fold lower than that achieved with ESI, APCI was discarded for subsequent experiments.

3.3. SPE-LC-MS/MS optimization

In order to reach the required sensitivity, an on-line SPE preconcentration step was applied. Oasis HLB (20 x 2.1 mm, 25 µm) cartridge for preconcentration and Atlantis T3 C₁₈ (100 x 4.6 mm, 5 µm) column for LC separation were employed in the SPE-LC procedure. Two different loop volumes were considered (500 and 1000 µL) for sample loading, concluding that preconcentrating 1000 µL (1 mL) water sample was more appropriate to satisfy sensitivity requirements.

The transference time from the loop to the cartridge (with water at 1 mL/min) was set to 65 s. There were almost no extra washing time (~ 5 s) in order to avoid analyte losses during washing, due to their high polarity, and at the same time to minimize peak broadening (especially for ETU). Elution from the cartridge into analytical column and chromatographic separation was based on the mobile phase water / methanol selected for the direct injection into the TQD system, as it gave the best results in terms of sensitivity and peak shape. Different gradients were evaluated and final conditions involve a total analysis time of 12

min, applying a solvent delay of 6 min (see experimental section). Strong matrix effects (apparent recoveries up to 500% when compared with aqueous standards also subjected to on-line trace enrichment) were observed for both compounds, possibly because the injection volume was 20-fold higher than in the direct injection method (1 mL vs 50 μ L, respectively). Although a slight increase in sensitivity was observed, it was lower than expected, surely due to breakthrough of these polar analytes in the HLB cartridge. Despite the strong matrix effect, the correction with their own deuterium-labelled standard seemed suitable for ETU, but not for PTU. So, in order to minimize these effects and to achieve an accurate quantification of PTU, a more sensitive system (Xevo TQ-S) was investigated, where the injection of much lower sample volume still allowed obtaining a satisfactory signal-to-noise ratio with reasonable matrix effect.

3.4. UHPLC-MS/MS (direct injection)

Firstly, a re-optimization of the MS/MS conditions was required, since an improved QqQ analyzer (Xevo TQ-S) was employed for direct injection experiments, with a redesigned source ESI. Precursor and product ions chosen were the same (as expected) to the previously selected for TQD; collision energies were similar, but cone voltages were around 15 or 20 V higher than when using TQD analyzer. Dwell times were lower for TQ-S method (25ms/transition), since this mass spectrometer allows a fast data acquisition without significant losses in signal intensity and/or quality. The MS/MS parameters finally selected are shown in **Table 2**. As indicated in Section 3.1, the most abundant transition was employed for quantification (Q) and the other ones for confirmation (q) purposes. Slight changes in the ion ratios were observed compared with TQD data, especially for PTU.

Regarding LC separation, two Acquity UPLC HSS T3 C₁₈ columns (100 x 2.1 mm, 1.8 μm and 50 x 2.1 mm, 1.8 μm) were tested. For the first one, peak shape and sensitivity got worse, especially for PTU, which presented wider chromatographic peaks with this longer column. So, the shorter UPLC column was chosen, with an optimized mobile phase consisting of water / MeOH at 0.3 mL/min (see Section 2.3.2) and an analytical run time of 4 min. Higher flow rates (0.4 and 0.5 mL/min) were discarded owing to significant losses in signal intensity. The small particle size of this column (1.8 μm) led to higher chromatographic resolution as compared to TQD method, rendering narrower peaks and shorter analysis time. Moreover, water samples were filtered prior to injection into the UHPLC-MS/MS system using 0.20 μm Nylon filters (Scharlab) in order to remove particles that could deteriorate the analytical column. With an injection volume of 10 μL, reasonable matrix effects were observed in the samples tested for ETU, lower than using the SPE-LC approach. **Table 3** shows the recoveries obtained without ILIS correction for ETU and PTU with both proposed methods. Again, the use of PTU-d₆ did not allow satisfactory correction of the severe matrix effects (mainly signal enhancement) observed for PTU. These undesired results, similar to those obtained by applying the SPE-LC methodology, were possibly caused by the different physicochemical properties between PTU and PTU-d₆, which also resulted in excessive variation in their retention times (differences between analyte and ILIS of 0.61 min with the on-line method, and of 0.18 min with UHPLC direct injection). Thus, the ionization behaviour of both compounds might not be the same, and they might be affected in a different degree by the sample matrix. This poor quantification could not be solved by increasing the flow rate either, in order to improve their coelution.

Figure 2 shows the LC(ESI)MS/MS chromatograms (Q and q_1 transitions) for 1 $\mu\text{g/L}$ reference standards analyzed by direct injection in the TQD and TQ-S systems, and after on-line SPE-LC in the TQD system.

3.5. Method validation

From the results shown in previous sections, the direct injection into the UHPLC system (Xevo TQ-S) was found the most suitable approach, given the good sensitivity obtained with high chromatographic resolution and short analysis time. However, we validated the two approaches tested in order to have analytical methodology available for these compounds, depending on the instrument used.

Both methods showed satisfactory linearity from 0.05 to 50 $\mu\text{g/L}$ for ETU and PTU, with residuals below 30% and correlation coefficients greater than 0.99. For evaluating precision and accuracy, six water samples of different type (two drinking water, two surface water and two groundwater, previously analyzed to prove they were blank samples, were spiked at two levels each (0.1 and 1.0 $\mu\text{g/L}$) and analyzed in quintuplicate with the two developed methodologies (n=5).

The results obtained in method validation for ETU are summarized in **Table 4**. With regard to the on-line approach, appropriate accuracy (recoveries from 73 to 83%) and precision (RSDs < 20%) were obtained for ETU at the two spiked levels after correction with its own ILIS. As expected, the use of ETU- d_4 improved method accuracy, compensating the elevated matrix effects which normally led to signal enhancements (up to 400%). Similar matrix effects were also observed for PTU. However, it was not possible to achieve satisfactory accuracy by using its own ILIS PTU- d_6 , and recoveries varied between 50 and

170%. As previously stated, the reason of the poor correction seemed to be the difference in the chemical behaviour between PTU and PTU-d₆ (i.e. retention time and different ionization behaviour). This fact can be observed in **Figure 3 (a,b)**, where significant changes in retention time are observed for PTU and PTU-d₆. In addition, their relative areas differed notably for aqueous standard (0.33) and surface water spiked at the same concentration (0.19). As can be seen, the method was satisfactorily validated in all the samples at 0.1 µg/L level, which was the LOQ objective for this analyte in the present work. LOD was estimated in 0.027 µg/L, and it was obtained from the chromatograms of samples spiked at 0.1 µg/L.

Regarding the direct injection method (UHPLC-MS/MS), satisfactory accuracy and precision was obtained for ETU, with recoveries in the range of 81-104% and excellent RSDs (below 10% at 0.1 µg/L and below 5% at 1 µg/L). Even without preconcentration, ETU could be satisfactorily determined at the 0.1 µg/L level, i.e. the LOQ objective of method (**Table 4**). The LOD was set up at 0.058 µg/L, which was in the same range of that obtained with the on-line preconcentration approach with the TQD instrument. Considering the possibility of injecting a higher sample volume into the UHPLC-MS/MS system, the detection limit might surely be improved if required. **Figure 4** shows the LC(ESI)MS/MS chromatograms for the direct injection into the UHPLC-MS/MS TQ-S system at 0.1 µg/L level.

As mentioned above, high signal enhancements were observed for PTU in all the waters analyzed (see **Table 3**). Despite all our efforts, the satisfactory correction of matrix effects was unfeasible for PTU in all samples tested, obtaining corrected recoveries between 90 up to 300%. In addition, the quantification transition (Q) presented abundant background noise at the 0.1 µg/L level in several of the water samples. Therefore, alternative transitions were used for quantification and for confirmation (117.0>99.9 and 117.0>40.9, respectively),

using the corresponding transitions of PTU-d₆ for ILIS correction (123.0>106.1 and 123.0>46.0) (**Fig. 4**).

In this work, our first choice to perform correct quantification was the use of analyte isotopically labelled standards, since this is the most common and efficient approach to solve matrix effects. However, it must be taken into account that deuterium-labelled compounds may show different behavior than the analyte and satisfactory results are not always assured. In some particular cases, like that of PTU shown here, significant changes in their retention times and ionization efficiencies can be observed, as well as in extraction recoveries. Some authors recommend the use of minimal labelling to avoid these undesired effects, mainly when employing deuterated analytes, for which these problems are more significant [48,49].

Most of papers about LC(ESI)MS/MS determination of ETU and PTU in urine samples employ matrix-matched standards calibration to compensate matrix effects [24,27-31]. However, this option seems not much suitable in environmental analysis due to the diverse composition of the samples, a fact corroborated by the very distinct matrix effects observed in the water samples tested in this work during the validation study (**Table 3**). Other option might be sample dilution, but this would decrease method sensitivity, hampering the quantification and confirmation of the analytes at low concentration levels. Taking in mind that sub-ppb levels should be accurately determined in water, a notable dilution of the samples seems not the best option.

In view of results obtained, only a semi-quantitative analysis can be proposed for PTU, using either on-line SPE-LC or direct injection depending on the sensitivity of the triple quadrupole instrumentation available. On the contrary, ETU can be satisfactorily determined at sub-ppb levels in quite different types of water samples using both approaches. The use of

an internal standard with low number of deuterium atoms will be considered for PTU in our future research.

The two analytical methodologies developed in this work show better [34,35,42-44] or similar [36,37] sensitivity than previous methods reported for ETU in waters. In addition, higher selectivity is expected when using LC-MS/MS under SRM mode. However, the main advantage resulting from the use of LC-MS/MS is the less sample manipulation required. Just the direct injection of the water sample or an automated on-line SPE-LC is sufficient to efficiently monitoring these compounds in the aquatic environment. Not less important is the confirmation of the compounds detected, which is feasible in this work by acquiring several MS/MS transitions, this being a major advantage in comparison with other previous methods based on GC-ECD, GC-NPD, LC-UV or LC-amperometric detection.

4. CONCLUSIONS

A sensitive and rapid method has been developed for ETU and PTU determination at sub-ppb levels in water, based on direct injection by using an UHPLC-MS/MS system with a QqQ analyzer and isotope-dilution analysis. Besides, an alternative on-line SPE-LC-MS/MS methodology has been proposed making use of a less sensitive instrument, achieving the same LOQ objective (0.1 µg/L). The UHPLC method based on direct sample injection in a last-generation LC-MS/MS QqQ instrument was preferred over the on-line approach, because it minimizes the total analysis time as well as increases resolution and renders a high analytical throughput, ideal for routine analysis. Both methodologies show greater or similar sensitivity than methods reported until now for ETU in waters. The main advantages resulting from the use of LC-MS/MS are the less sample manipulation required (direct injection of the water sample or automated SPE-LC), high selectivity associated to MS/MS under SRM mode and the reliable confirmation of positives by acquiring several MS/MS transitions without the need of additional analysis.

The method applicability to different types of water was confirmed by the analysis of six water samples (DW, GW, SW) during the validation process, with satisfactory results for ETU. Regarding PTU, the high matrix effects observed for both methodologies could not be fully compensated, even using its own deuterated analyte as ILIS, illustrating the potential difficulties when employing perdeuterated-labelled standard for correction.

ACKNOWLEDGEMENTS

This work has been developed under financial support of the Ministry of Education and Science, Spain (CTM2006-06417). The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence PROMETEO/2009/054. C. Ripollés is very grateful to Ministry of Education and Science for her predoctoral grant.

REFERENCES

- [1] M-A. Kaniwa, J. Chromatogr. A 405 (1987) 263
- [2] <http://www.epa.gov/oppsrrd1/cumulative/dithiocarb.pdf> (accessed date: 25th May 2011)
- [3] World Health Organization (WHO). Environmental Health Criteria 78. Dithiocarbamate pesticides, ethylenethiourea and propylenethiourea: a general introduction. Geneva, 1988.
- [4] International Agency for Research on Cancer, IARC Vol. 79 (2001), p. 659. Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Thyrotropic Agents.
- [5] W.R. Bontoyan and J.B Looker, J. Agr. Food. Chem. 21 (1973) 338
- [6] R. R. Watts, R. W. Storherr and J. H. Onley, Bull. Environ. Contam. Toxicol. 12 (1974) 224
- [7] IARC. Vol. 7 (1974), p 45. Some Anti-Thyroid and Related Substances, Nitrofurans and Industrial Chemicals.
- [8] IARC Suppl. 7 (1987), p 207. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs. Volumes 1 to 42.
- [9] “Environmental fate of Ethylenethiourea” Sue Xu (2000). Environmental Monitoring & Pest Management. Department of Pesticide Regulation. California. <http://www.cdpr.ca.gov/docs/emon/pubs/fatememo/etu.pdf> (accessed date: 28th March 2012)
- [10] P.A. Cruickshank and H.C. Jarrow, J. Agr. Food. Chem. 21 (1973) 333
- [11] O.S. Jacobsen and R. Bossi, FEMS Microbiol. Rev. 20 (1997) 539
- [12] E-S. Hwang, J.N. Cash and M.J. Zabik, J. Agr. Food. Chem. 51 (2003) 1341

- [13] H. Johannesen, A.B. Nielsen, A. Helweg and I.S. Fomsgaard, *Sci. Total Environ.* 191 (1996) 271
- [14] “Octanol/water partition coefficient of Propylenethiourea (metabolite of Propineb)”. J. Krohn (1989). Bayer AG, Leverkusen, Germany Bayer CropScience AG, Report.
- [15] European Commission. Review report for the active substance propineb (26 February 2003). http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1-34_en.pdf
Appendix I and II. (accessed date: 28th March 2012)
- [16] M. Maruyama, *Fresen. J. Anal. Chem.* 348 (1994) 324
- [17] S. Kontou, D. Tsipi, V. Oreopoulou and C. Tzia, *J. Agr. Food. Chem.* 49 (2001) 1090
- [18] R.M. Garcinuño, P. Fernández-Hernando and C. Cámara, *J. Chromatogr. A* 1043 (2004) 225
- [19] C. Blasco, G. Font and Y. Picó, *J. Chromatogr. A* 1028 (2004) 267
- [20] L. Tölgyesi, P. Kele and K. Torkos, *Chromatographia* 71 (2010) 75
- [21] R.M. Garcinuño, L. Ramos, P. Fernández-Hernando and C. Cámara, *J. Chromatogr. A* 1041 (2004) 35
- [22] J.R. Startin, S.J. Hird and M.D. Sykes, *Food Addit. Contam.* 22 (2005) 245
- [23] G. Özhan and B. Alpertunga, *Food Addit. Contam. B* 25 (2008) 961
- [24] I. Debbarh and N. Moore, *J. Anal. Toxicol.* 26 (2002) 216
- [25] C. Saieva, C. Aprea, R. Tumino, G. Masala, S. Salvini, G. Frasca, M.C. Giurdanella, I. Zanna, A. Decarli, G. Sciarra and D. Palli, *Sci. Total Environ.* 332 (2004) 71

- [26] S. El Balkhi, P. Sandouk and M. Galliot-Guilley, *J. Anal. Toxicol.* 29 (2005) 229
- [27] C. Sottani, M. Bettinelli, M.L. Fiorentino and C. Minoia, *Rapid Commun. Mass Spectrom.* 17 (2003) 2253
- [28] C.H. Lindh, M. Littorin, G. Johannesson and B.A.G. Jönsson, *Rapid Commun. Mass Spectrom.* 22 (2008) 2273
- [29] M.A. Montesano, A.O. Olsson, P. Kuklennyik, L.L. Needham, A. Bradman and D.B. Barr, *J. Expo. Sci. Env. Epid.* 17 (2007) 321
- [30] K. Jones, K. Patel, J. Cocker, R. Bevan and L. Levy, *J. Chromatogr. B* 878 (2010) 2563
- [31] N.K. Jayatilaka, M.A. Montesano, R.D. Whitehead, S.J. Schloth, L.L. Needman and D.B. Barr, *Arch. Environ. Con. Tox.* 61 (2011) 59
- [32] E.A. Kazos, C.D. Stalikas, C.G. Nanos and C.N. Konidari, *Chemosphere* (2007) 2104
- [33] M. C. Domínguez, G. Peñuela and M.T. Flórez, *Rev. Fac. Univ. Antioquia.* 49 (2009) 42
- [34] <http://www.caslab.com/EPA-Methods/PDF/509.pdf> (accessed date: 6th July 2011)
- [35] J.M. van der Poll, G.G. Versluis-de Haan and O. de Wilde, *J. Chromatogr.* 643 (1993) 163
- [36] H.D. Meiring and A.P.J.M de Jong, *J. Chromatogr. A* 683 (1994) 157
- [37] V. Geissen, F.Q. Ramos, P.J. Bastidas.Bastidas, G. Díaz-González, R. Bello-Mendoza, E. Huerta-Lwanga and L.E. Ruiz-Suárez, *Bull. Environ. Contam. Toxicol.* 85 (2010) 407
- [38] J.M. Marín, O.J. Pozo, J.V. Sancho, E. Pitarch, F.J. López and F. Hernández, *J. Mass Spectrom.* 41 (2006) 1041

- [39] C. Ripollés, E. Pitarch, J.V. Sancho, F.J. López and F. Hernández, *Anal. Chim. Acta* 702 (2011) 62
- [40] C. Ripollés, J.M. Marín, J.V. Sancho, F.J. López and F. Hernández, *Rapid Commun. Mass Spectrom.* 23 (2009) 1841
- [41] F. Hernández, J.V. Sancho, O.J. Pozo, A. Lara and E. Pitarch, *J. Chromatogr. A* 939 (2001) 1
- [42] E.A. Hogendoorn, P. van Zoonen and U.A.T. Brinkman, *Chromatographia* 31 (1991) 285
- [43] D.R. Doerge and A.B.K. Yee, *J. Chromatogr.* 586 (1991) 158
- [44] L.M. de Carvalho, P.C. do Nascimento, D. Bohrer and L. Del-Fabro, *Electroanal.* 16 (2004) 1508
- [45] Method validation and quality control procedures for pesticide residue analysis in food and feed. Document No. SANCO/10684/2009
- [46] European Union Decision 2002/657/EC, *Off. J. Eur. Commun.* August 12 (2002) L221, 8
- [47] Council Directive 98/83/EC, *Off. J. Eur. Commun.* November 3 (1998) L330, 32
- [48] A. González-Atuña, P. Rodríguez-González, G. Centineo and J.I. García Alonso, *Analyst* 135 (2010) 953
- [49] E. Stokvis, H. Rosing and J.H. Beijnen, *Rapid Commun. Mass Spectrom.* 19 (2005) 401

Table 1. MS/MS optimized conditions for ETU, PTU and their analytes ILIS using TQD analyzer.

Compound	RT (min)	Precursor ion (<i>m/z</i>)	Cone voltage (V)	Product ion (<i>m/z</i>)	Collision energy (eV)	Q/q (*) ratio
ETU	7.39	103.0 (C ₃ H ₇ N ₂ S ⁺)	25	(-CHSN) 44.0 (Q)	15	9.4
				(-NH ₃) 86.0 (q ₁)	15	
				(-C ₂ H ₅ N) 60.0 (q ₂)	20	
PTU	8.46	117.1 (C ₄ H ₉ N ₂ S ⁺)	25	(-CHSN) 58.1 (Q)	15	4.4
				(-CH ₄ N ₂ S) 41.0 (q ₁)	20	
				(-C ₂ H ₇ N) 72.0 (q ₂)	15	
ETU-d ₄	7.35	107.0 (C ₃ H ₃ D ₄ N ₂ S ⁺)	30	(-CHSN) 48.0 (Q)	15	10.8
				(-NH ₃) 90.0 (q ₁)	20	
				(-C ₂ H ₃ D ₂ N) 62.0 (q ₂)	25	
PTU-d ₆	7.85	123.1 (C ₄ H ₃ D ₆ N ₂ S ⁺)	30	(-CHSN) 64.1 (Q)	20	9.8
				(-C ₂ H ₃ D ₄ N) 74.0 (q ₁)		
				(-CH ₃ DN ₂ S) 46.1 (q ₂)		
				(-NH ₃) 106.1 (q ₃)		

(Q) Quantification transition, (q) confirmation transition.

* Average value for six standard solutions, at different concentrations, between 0.1 and 50 µg/L

Table 2. MS/MS optimized conditions for ETU, PTU and their analytes ILIS using Xevo TQ-S analyzer.

Compound	RT (min)	Precursor ion (<i>m/z</i>)	Cone voltage (V)	Product ion (<i>m/z</i>)	Collision energy (eV)	Q/q (*) ratio
ETU	0.61	103.0	40	43.9 (Q)	15	
				85.9 (q ₁)	15	8.0
				59.9 (q ₂)	25	43.6
PTU	0.90	117.0	50	57.9 (Q)		
				99.9 (q ₁)	15	9.6
				40.9 (q ₂)		12.2
ETU-d ₄	0.61	107.0	50	71.9 (q ₃)		14.0
				48.0 (Q)	10	
				90.1 (q ₁)	15	
PTU-d ₆	0.73	123.0	50	62.0 (q ₂)	20	
				64.0 (Q)	15	
				106.1 (q ₁)	15	
				73.9 (q ₂)	15	
				46.0 (q ₃)	10	

(Q) Quantification transition, (q) confirmation transition.

* Average value for six standard solutions, at different concentrations, between 0.1 and 50 µg/L

Table 3. Matrix effects observed in different types of water samples. Average recoveries and RSDs (in brackets) without applying ILIS correction for six samples spiked at two concentration levels (n=5).

Sample	On-Line (TQD)				Direct Inj. (TQ-S)			
	0.1 µg/L		1 µg/L		0.1 µg/L		1 µg/L	
	ETU	PTU	ETU	PTU	ETU	PTU	ETU	PTU
DW1	383 (6)	130 (8)	133 (5)	63 (13)	75 (2)	300 (6)	30 (1)	529 (5)
DW2	282 (2)	145 (17)	367 (10)	345 (4)	120 (3)	241 (10)	99 (4)	517 (4)
SW1	352 (7)	175 (2)	38 (12)	300 (6)	129 (4)	317 (12)	96 (2)	498 (7)
SW2	307 (5)	167 (14)	107 (7)	244 (16)	132 (5)	270 (19)	123 (2)	507 (10)
GW1	269 (4)	212 (16)	70 (5)	377 (18)	73 (2)	232 (25)	28 (4)	144 (7)
GW2	266 (3)	31 (9)	69 (6)	498 (16)	80 (4)	144 (19)	31 (2)	236 (9)

Table 4. Average recoveries and relative standard deviations (RSDs) for ETU in six different water samples spiked at two concentration levels (n=5) (ILIS correction with ETU-d₄)

Sample	On-Line (TQD)				Direct Inj. (TQ-S)			
	0.1 µg/L		1 µg/L		0.1 µg/L		1 µg/L	
	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD
DW1	79	6	76	4	90	6	104	4
DW2	73	11	79	13	87	5	99	5
SW1	80	8	73	5	89	7	98	3
SW2	78	19	77	10	81	10	100	2
GW1	79	19	74	9	85	7	104	3
GW2	83	9	73	8	87	6	103	4

FIGURE CAPTIONS

Figure 1. Positive APCI full-scan mass spectra of ETU and PTU at a cone voltage of 25 V (bottom). Positive ESI full-scan mass spectra of ETU and PTU at a cone voltage of 25 V (medium). Product ion spectra (ESI) for m/z 103.0 (ETU) at 20 eV and m/z 117.1 (PTU) at 15 eV (top).

Figure 2. ETU and PTU LC(ESI)MS/MS chromatograms for 1 $\mu\text{g/L}$ reference standard analyzed by (a) direct injection (50 μL) into the TQD system (b) on-line SPE (1 mL) into the TQD (c) direct injection (10 μL) into the TQ-S.

Q and q_1 transitions, as well as Q/q_1 ratio, are shown for each analyte.

Figure 3. ETU, PTU, ETU- d_4 and PTU- d_6 LC(ESI)MS/MS chromatograms for (a) reference standard of 0.1 mg/L (b) SW1 sample spiked at 0.1 mg/L and (c) SW1 sample (blank), all them analyzed with the on-line method. The concentration of the two ILIS was 1.0 mg/L in all standards and samples.

Figure 4. (a) Reference standard of 0.1 mg/L (b) DW1 sample spiked at 0.1 mg/L and (c) DW1 sample (blank), all them analyzed with the direct injection method (TQ-S). The concentration of the two ILIS was 1.0 mg/L in all standards and samples.

ESI

ESI

ESI

ESI

APCI

APCI

ETU

PTU

Figure 1

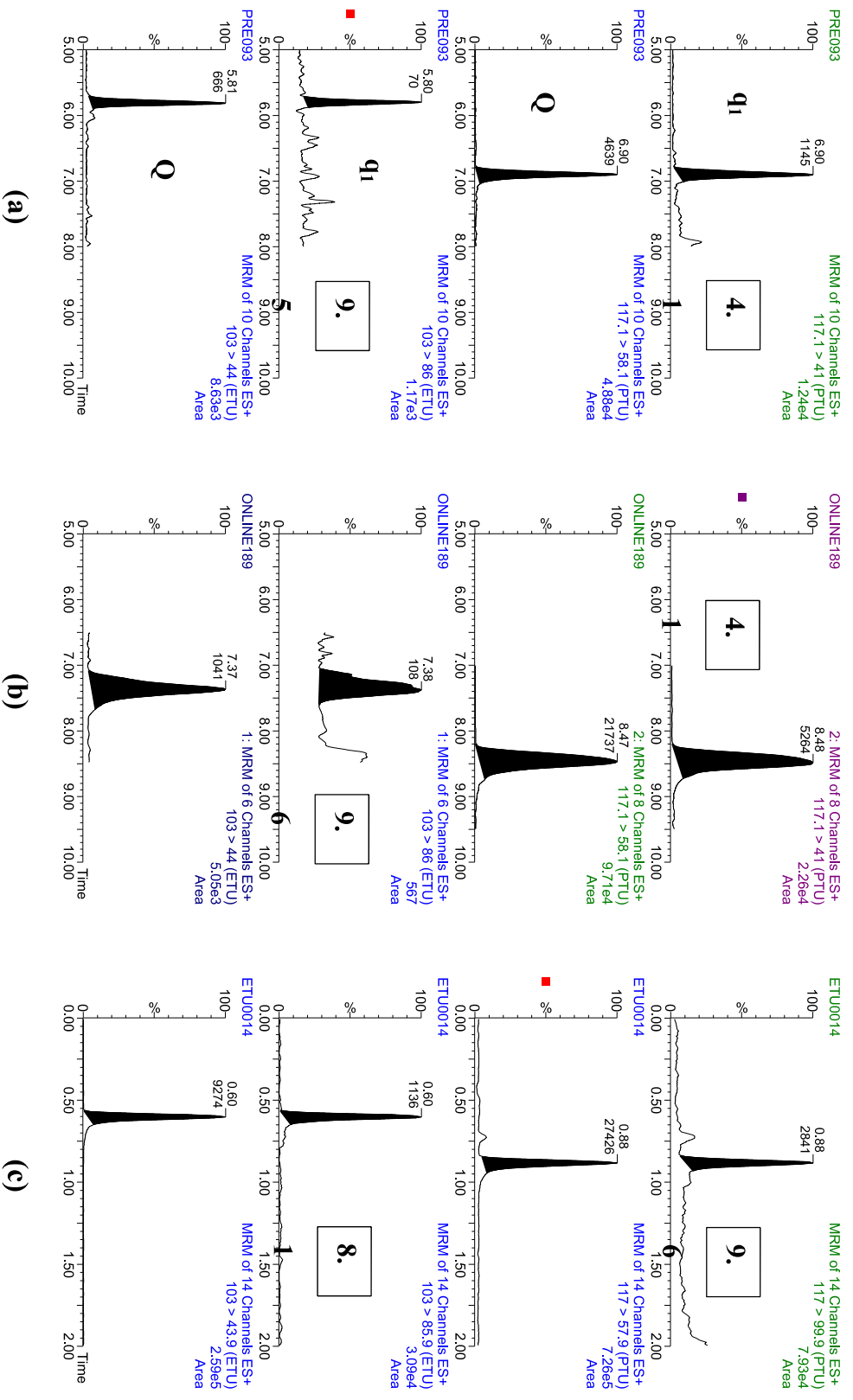


Figure 2

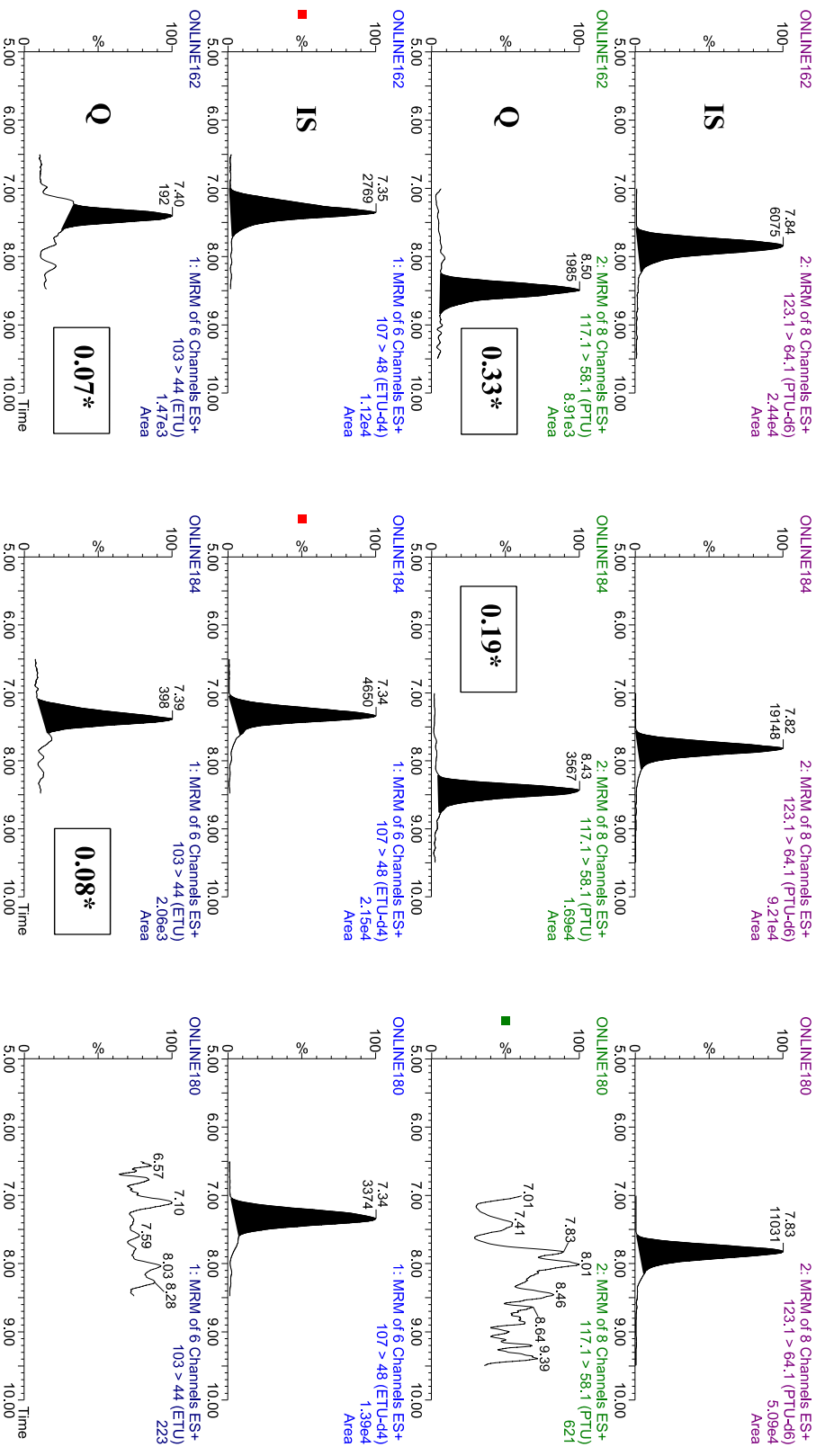


Figure 3

* Relative response between PTU and PTU-d₆

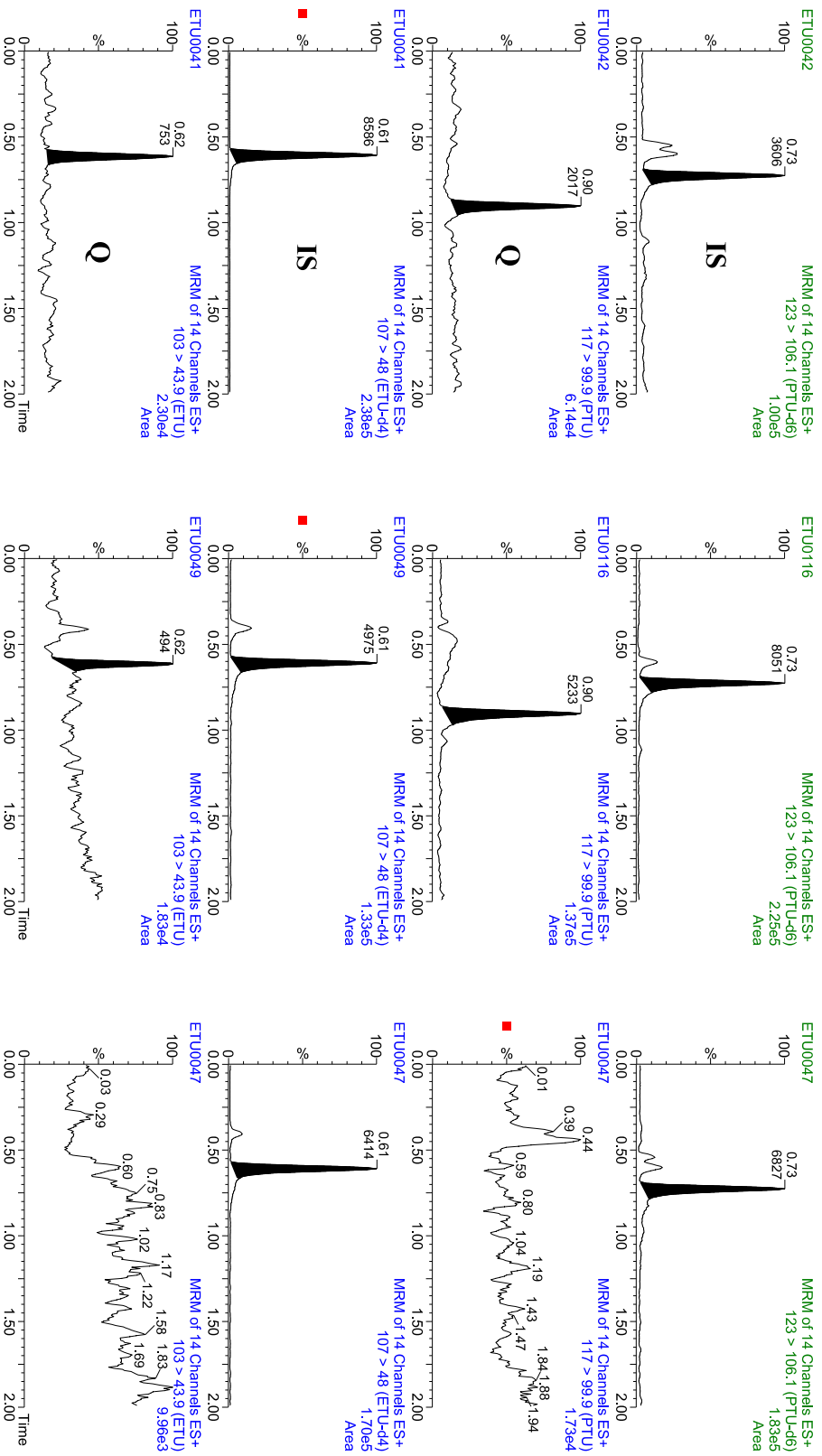


Figure 4