Analysis of pharmaceuticals, steroids and antibiotics using UHPLC- Orbitrap mass spectrometry with enhanced sensitivity, selectivity and minimal matrix effects

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Overview

Ultrahigh pressure liquid chromatography/tandem mass (UHPLC-MS/MS) and UHPLC-orbitrap analytical methods have been applied to the analysis of pharmaceuticals and personal care products (PPCPs) in environmental sample matrices. Special emphasis was made on difficulties associated with matrix effects in the electrospray ionization source (ESI) and strategies that can be used to alleviate this phenomenon utilizing the unique ability of a new orbitrap mass spectrometer.

Introduction

While it has been known for over 20 years that pharmaceuticals can enter the environment, it has only been in the last 10 years that we have begun to identify and quantify their presence in sewage treatment plant (STP) effluents, receiving waters, ground water, in agricultural settings (tile drains and run-off) and drinking water. Similar to modern pesticides, PPCPs represent a diverse group of biologically active chemicals that may present a risk to the environment. Using UHPLC-MS/MS with isotopically-labelled chemical analogs as internal standards has been the method of choice in PPCP analyses to achieve superior data quality and the highest selectivity and sensitivity. The use of LC-MS/MS also comes with challenges in signal suppression/enhancement due to matrix effects in the ESI, which cause quantitative analysis to be ineffective, even using the internal standard approach. In the current study, and with one of the most commonly used medication ethinylestradiol (CAS # 57-63-6) as an example, we evaluated the viability using a new UHPLC-orbitrap to alleviate/resolve this concern on the matrix effects.

Methods

Sampling, Sample Preparation and LC-MS/MS Analysis

Sample shuttles containing bottles, submission forms and detailed sampling instructions outlining sampling methodology, sample preservation requirements and quality assurance and quality control (QA/QC) measures were prepared at the Laboratory Services Branch (LaSB), Ministry of the Environment, Etobicoke, Ontario, Canada. Samples were collected from waste water treatment plant effluents and agricultural tile drain by the field staff and returned to the MOE laboratory in Etobicoke for analysis. All samples were collected in 2011.

Laboratory Services Branch (LaSB) method E3454[1] was used to analyze field samples. This method has been accredited by the Canadian Association of Laboratory Association (CALA) since 2004. Using solid phase extraction (SPE) to extract the 47 target analytes from a sample and analyzes them by LC-MS/MS (AB Sciex API-4000, Concord, Ontario) in both positive and negative ionization modes using isotopically-labelled 14 analogs of target analytes as internal standards[2]. The LC-MS/MS consisted of a a Shimadzu Prominence/20 series (Columbia, MD) LC and was interfaced to the API-4000 an ESI interface. The injection volume used was 10 mL. Data acquisition was done by *Scheduled* MRM™ and processed for all compounds in positive and negative ionization modes. Identification of target compounds were done using two specific MRM transitions to achieve an identification point (IP) of four [3]. Mobile phase and UHPLC gradient parameters used are listed in Table 1 with typical chromatographic full-width-at-half-maximum (FWHM) was 3-5 seconds.

UHPLC-High Resolution MS (HRMS) Analysis

An UHPLC-orbitrap (Thermo Fisher Scientific, Inc., Bremen, Germany) mass spectrometer system was used in this study. The UHPLC-Orbitrap consists of a Thermo Dionex 3000 series UHPLC and an Exactive® Plus orbitrap-MS with data acquired without using lock mass(es). High purity nitrogen (> 99%) obtained from a nitrogen generator was used in the electrospray ionization source. Separation was achieved using an Agilent Poroshell XB-C18 (2.7 µm, 2.1x100 mm). The injection volume used was 10 and 5 mL for the LC-MS/MS and Exactive® Plus Orbitrap detector based systems. Mobile phase and UHPLC gradient parameters used were similar to the LC (Table 1) minor gradient program changes for a faster UHPLC analytical turnaround time.

Table 1. LC and UHPLC Parameters

Column Oven Temperature	35°C		
Mobile Phase	A: 95:5/H_2O:CH_3CN, 0.5 mM $HCOONH_4$ & pH adjusted to 6.95 ± 0.03 using 3 M NH_4OH B: Acetonitrile		
Flow Rate	450 µL/min		
Gradient	**Time (min)**	**% A**	**%B**
	0	90	10
	3	35	65
	9	2	98
	12	2	98
	12.2	85	15
	15	85	15

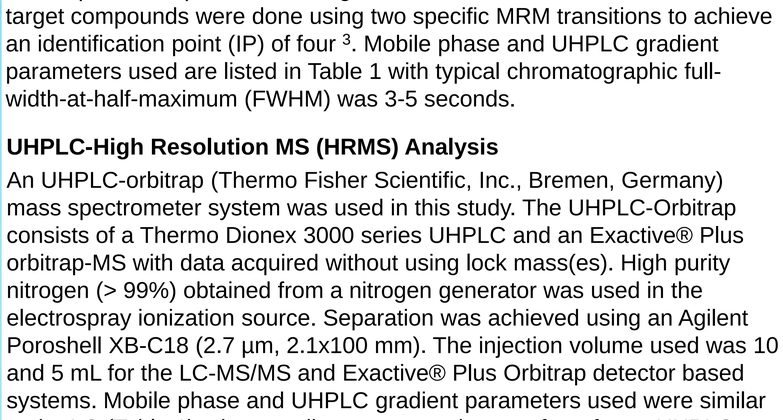

Figure 1. Analysis of PPCPs

Results

UHPLC-MS/MS Analysis and the Matrix Effects – Ethinyl Estradiol (EE2)

The analysis of EE2 is done by using $^{13}C_2$-labelled EE2 (^{13}C2-EE$_{2)}$ as internal standard and has good quality assurance data in sample matrices of drinking water and surface water origin.

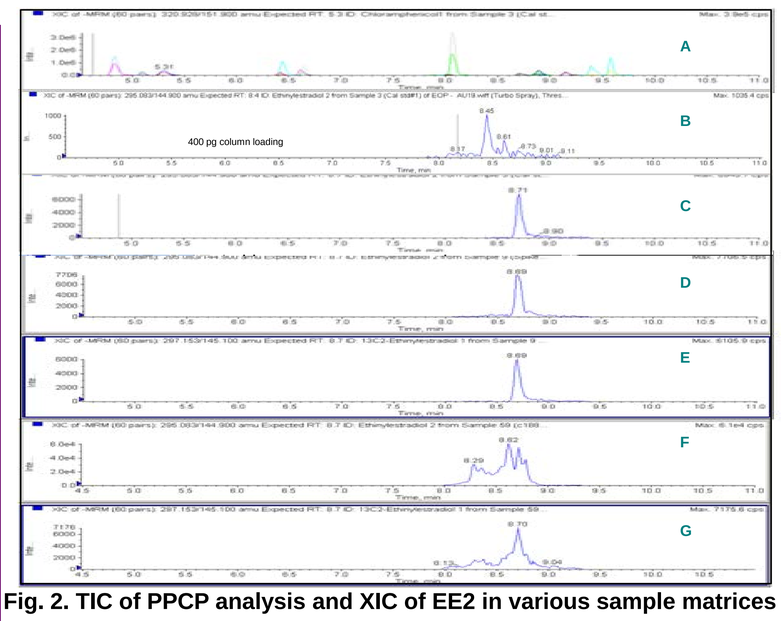

Fig. 2. TIC of PPCP analysis and XIC of EE2 in various sample matrices

Figues 2A is a typical TIC of the LC-MS/MS analysis. Figure 2B is the XIC of EE2 acquired using a column loading of 400 pg. Figure 2C and 2D were the XICs of the EE2 in solvent and that obtained from a method spike quality control (QC) sample. XIC of the ^{13}C2-EE$_2$ from QC samples is shown in 2E and has a superior signal-to-noise ratio (SNR) to that of the native EE2. In the case of waste water treatment plant effluents samples, the analysis became a challenge. As can be seen in Figure 2F and 2G, XICs of both native EE2 and ^{13}C2-EE$_2$ were non-specific, had a lot of interferences from the waste water effluents, and could not be used for either qualitative or quantitative analysis.

UHPLC-orbitrap Analysis of Ethinyl Estradiol

Figure 3 showed XICs of typical low level (200 pg on column) analysis of EE2 using UHPLC-orbitrap (top row) at the four orbitrap resolving power (RP) settings of 17.5k, 35k, 70k and 140k. Gradient elution parameters used in the UHPLC separation was adjusted to purge out contaminants at the end of the analysis and EE2 was eluted earlier at about 5.6 ± 0.2 min, establishing a secondary criterion for the identification of target compound EE2 using UHPLC retention time (RT). As can be seen the FWHM of the mass spectral peaks decreases with increasing RP. At higher RP (i.e. 7ok), one could also observe impurities in the EE2 standard. The impurity was completely separated from the EE2 peak at RP 0f 140k. Area counts decreases marginally while SNR improves with increasing RP. All XICs were done using a mass extraction window (MEW) equal to ± 0.5 x FWHM.of the mass spectral peak.

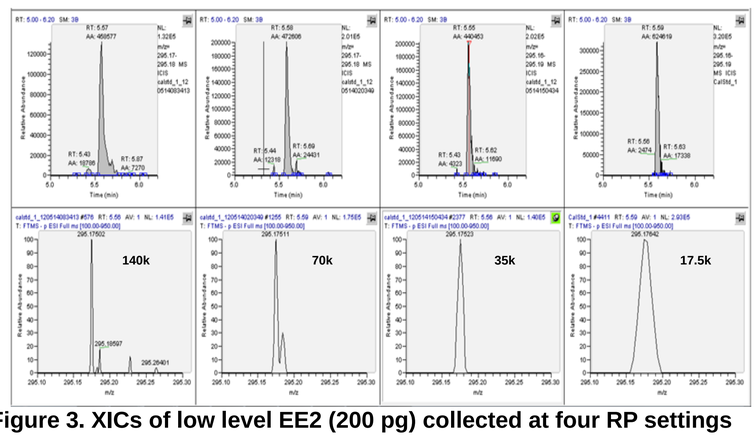

Figure 3. XICs of low level EE2 (200 pg) collected at four RP settings

Ethinyl Estradiol Analysis - Biosolids Samples and Matrix Effects

Figure 4 showed analytical results of a calibration standard and three biosolid samples collected in the field. These data were acquired at a RP of 140k. As can be seen, mass spectral peak of EE2 was completely separated from the sample matrix effects. Therefore, the identification of the EE2 can be achieved using two different analytical technologies, i.e. both chromatographic RT and accurate mass. The four XICs (top row) were separated at the baseline, allows for reliable quantitative analysis.

Relative intensities of the EE2 and two interfering mass spectral peaks (traces E, F and G) changes in different samples and were all eluted within 5.6 ± 0.4 min time frame. Because of these compounds were sharing the same MRM transition, resulting in the overlapped XIC shown in Figure 2F and 2G.

This was also demonstrated using a MEW of m/z 295.18 ± 0.05 amu, similar XICs to those obtained from the UHPLC-MS/MS based EE2 analysis can be generated from the UHPLC-orbitrap data. The result is shown in Figure 5. The combination of target peaks superimposed on a high background making the identification and quantitation difficult.

With the proper selection of MEM and accurate mass measurement using higher RP (i.e. 70k or 140k) resolution mass spectrometer, one can resolve interference co-eluting with the target compounds.

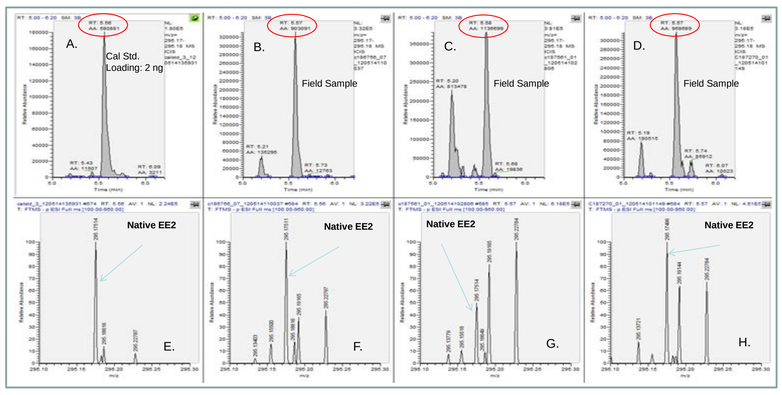

Figure 4. XICs of calibration stand level 3 (Cal. Std.) and mass spectrum of EE2 measured at RP = 140k (traces A and E) and results obtained from three different biosolid samples.

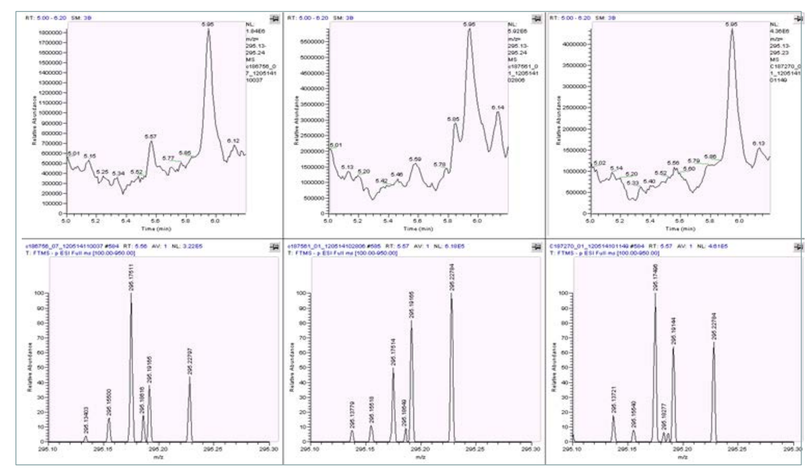

Figure 5. XICs of EE2 obtained from the three biosolid samples shown in Figure 4, using a MEW of 295.18 ± 0.05 amu.

Ethinyl Estradiol Analysis – Ionization Enhancement or an Artifacts in Process?

Shown in Figure 6 are results of two ebiosolid samples collected in the field and a calibration standard for comparison. XICs A, C and D were obtained from 140k RP data while XIC B was obtained from 70k RP data. Also note that XICs B and C were from the same sample but were derived from data collected using RP 70k and 140k. From traces F, G and H, effluents were having less interference but like biosolid but will also have high background and interference if a larger MEW were to be used.

The XIC B showed a common problem integrating an overlapped and/or unresolved mass spectral peak. As can be seen, area counts of EE2 increased from 2.35E^{+6} (XIC C) to 2.86E^{+6} because of the use of a completely resolved spectral peak (G) or a patially resolved peak (F). As intensities of interference (295.1847 and 295.1867 in spectra F and G) can vary depending on sample composition and works done at lower RP will have a higher risk including more interference in the quantitative results and be treated as “enhancement”.

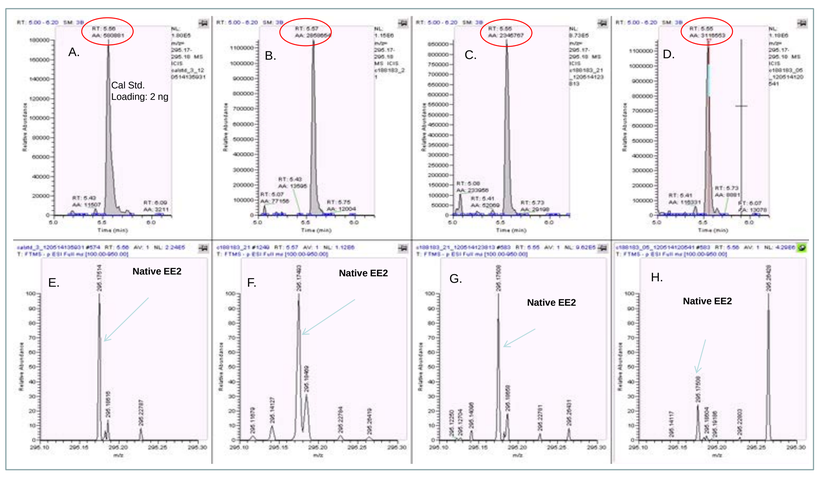

Figure 6. . XICs of calibration stand level 3 (Cal. Std.) and mass spectrum of EE2 measured at RP 140k (traces E, G and H) and RP 70k (trace B) of two effluent samples.

Conclusion

This proof-of-concept work demonstrated that matrix effects can be resolved using HRMS in the case of the EE2 analysis. Additional studies and works are needed to validate results derived from this work. These include:

- Finalize method validation works to ensure current approach is rugged for routine analysis;
- Carry out field study with quality control samples to verify that matrix effects can be separated from target compounds using HRMS;
- Current analytical process need a meticulous selection of MEW to ensure good quantitative results and can be time consuming. An automated process will be desirable.

References

1. “The Determination of Emerging Organic Pollutants in Environmental Matrices by LC/MS/MS.” LaSB Method E3454, Ontario Ministry of the Environment, 2010.
2. Chunyan Hao, Xiaoming Zhao, Shahram Tabe, and Paul Yang, Environ. Sci. Technol., 42 (11), 4068–4075, 2008.
3. Commission Decision 2007/657/EC of 12 August 2002. Implementing Council Directive (96/23/EC) concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Communities 2002, L221, 8-36.

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