Optimizing Detection of Steroids in Wastewaters Using the Agilent 6490 Triple Quadrupole LC/MS System with iFunnel Technology

Application Note

Environmental

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Abstract

Taking full advantage of the enhanced sensitivity of the Agilent 6490 Triple Quadrupole LC/MS System requires optimization of method parameters derived on other Triple Quadrupole platforms. Optimization of steroid analysis methods in the complex matrix of wastewater results in sensitivity as low as sub-1 ng/L (1 part per trillion).

Introduction

Pharmaceuticals are consumed in high quantities worldwide, and these amounts will continue to increase due to improving health care and longer life expectations. Administered pharmaceuticals are excreted by humans, as a parent compound or metabolites. They enter sewage treatment plants where they are not entirely removed and end up in the environment. These residual pharmaceuticals can negatively impact aquatic and terrestrial ecosystems.

A recent study by the United States Geological Survey (USGS) found chemical contaminants in 80% of the streams sampled, and steroids were one of the chemical groups most frequently detected. These steroids elicit biological responses at very low concentrations, including feminization of male fish. A seven year, whole-lake experiment in Canada demonstrated that chronic exposure of a fish species to the steroid 17 alpha-ethynyl estradiol led to a near extinction of that species from the lake [1]. There is also potential for these steroids to travel up the food chain, as well as into drinking water.

Agilent Technologies

The European Union Water Framework Directive (2000/60/EC) [2] promotes sustainable water use, including the long-term reduction of wastewater contaminant discharges to the aquatic environment, including steroids. Implementation of the Directive requires the development of sensitive, accurate, and reliable testing methods.

This application note describes the optimization of sensitive methods for steroid detection on the Agilent 6490 Triple Quadrupole LC/MS System with iFunnel Technology, as part of the Chemical Investigation Program of Directive 2000/60/EC. This instrument takes detection limits lower than ever, enabling zeptomole level sensitivity at conventional flow rates, making it an ideal choice for critical pharmaceutical applications, including the detection of steroids in the environment. While liquid chromatography/mass spectrometry (LC/MS) methods for steroid detection and quantification have been developed on earlier generation MS instruments, they must be optimized on the 6490 Triple Quadrupole LC/MS System in order to maximize sensitivity. Using the optimal parameters determined for detection of several steroid compounds, detection limits as low as sub-1 ng/L (one part per trillion) in wastewaters have been demonstrated.

Experimental

Reagents and Standards

The ethyl acetate, acetonitrile, propan-2-ol, cyclohexane, and methanol, were all HPLC grade or glass-distilled. The hydrochloric acid was 37% analytical reagent grade and ammonia solution 30%. The copper nitrate was general purpose grade reagent or better. The water was HPLC grade or Elga polished water. The styrene divinyl benzene solid phase extraction (SPE) cartridges (200 mg) were obtained from Baker. The GF/D glass microfiber filter papers were obtained from Whatman (Kent, UK)

Individual solutions of 100 mg/L of each steroid (estrone, estradiol, and ethynyl estradiol) in acetonitrile were obtained from QMX Laboratories Ltd (Thaxted, UK). A mixed calibration standard was prepared by adding each of the individual steroid solutions to methanol to a concentration of 1.0 mg/L. Working calibration standards were prepared by dilution in 90:10 water:methanol to 1, 2, 5, and 10 µg/L. These calibration standards also contained 2 µg/L of each internal standard.

Solid deuterated estrone-D4, estradiol-D5, and ethynyl estradiol-D4 were obtained from QMX Laboratories for use as internal standards. Individual 100 mg/L solutions of each internal standard were prepared in acetronitrile, then a mixed internal standard was prepared by adding each internal standard to methanol to a final concentration of 1.0 mg/L. A second mix was prepared by diluting an aliquot of the 1.0 mg/mL mix to 0.1 mg/L with methanol.

Instruments

Method optimization and analysis of wastewaters were conducted on the Agilent 1260 Infinity LC with a 100-µL sample loop, coupled to the 6490 Triple Quadrupole LC/MS System with iFunnel Technology. Postcolumn addition of 0.1% ammonia solution was accomplished using an external pump attached to a T-junction between the column and the nebulizer. The instrument conditions are listed in Table 1.

Sample Collection, Preparation and Cleanup

Samples were collected in 2-L amber glass bottles and preserved at the time of sampling with 2 mL of concentrated hydrochloric acid and 0.5 g of copper nitrate and stored at below 10 °C. Sample stability under these conditions has been tested up to 14 days after preservation. Once extraction has taken place, the resulting extracts can be stored for at least four weeks in a spark-proof refrigerator prior to analysis.

Each sample was filtered through GF/D papers prior to extraction. An aliquot of 20 µL of mixed internal standard was added to 1 L of sample. For crude sewage samples, 100 mL of sample was diluted with 900 mL of water, and 20 µL of mixed internal standard was added. The sample was then extracted with the SPE cartridge, which was first conditioned with ethyl acetate (5 mL), then methanol (5 mL), then water (3 mL). The cartridge was loaded with 250 mL of sample, then washed with 60% methanol (3 mL) followed by water (3 mL). After drying with gas for 40 minutes, the cartridge was eluted with 4 mL of ethyl acetate. The extract was evaporated under a gentle air stream, with the vial on a 45 °C heating block, and the residue redissolved in 250 µL of cyclohexane:propan-2-ol (95:5)

The extracts were cleaned up using normal phase chromatography on an Agilent 1100 series LC with fraction collection on an Agilent ZORBAX Cyano column (p/n 883952-705), 4.6 x 150 mm, 5 µm thermostated at 55 °C, using an isocratic separation, 95:5 cyclohexane:propan-2-ol at a flow rate of 1 mL/min, fraction collection time range 5.8–8.6 minutes. The extract was evaporated under a gentle air stream, with the vial on a 45 °C heating block, and the residue re-dissolved in 250 µL of methanol:water (90:10). With a final volume of 250 µL, this is equivalent to a 1000 fold concentration step. Therefore, the calibration standards are equivalent to original concentrations of 1, 2, 5, and 10 ng/L.

Table 1. LC and MS Instrument Conditions

LC Conditions			
Analytical column	Agilent C-18 Eclipse Plus, 2.1 × 50 mm, 3.5 µm (p/n 959763-902)		
Guard column	Luna C-18, 4.0 mm × 2.0 mm		
Column temperature	40 °C		
Injection volume	25 µL		
Mobile phase	A = Water		
	B = Acetonitrile		
Run time	16.0 min		
Flow rate	0.3 mL/min: 0.1 mL/min postcolumn addition of 0.1% NH_3		
Gradient program	Time (min)	% Solvent A	% Solvent B
	0	90	10
	0.5	60	40
	10.0	20	80
	10.2	0	100
	11.5	0	100
	11.6	90	10
MS Conditions			
Acquisition parameters	ESI mode, negative ionization; Dynamic MRM		
Sheath gas temperature	300 °C		
Sheath gas flow rate	11 L/min		
Gas temperature	180 °C		
Drying gas	Nitrogen, 16 L/min		
Nebulizer pressure	45 psig		
Nozzle voltage	1,500 V		
Vcap voltage	3,000 V		
Cell accelerator voltage	Varied per optimization study		
Delta EMV	Varied per optimization study		
Low and high pressure ion funnel voltage	Varied per optimization study		
Scan type dynamic MRM	Delta EMV 300 V		

Analysis Parameters

The Agilent Triple Quadrupole LC/MS System analysis parameters are shown in Table 2.

Table 2. Time Segments, Transitions, and Fragmentor Voltages used for Optimal Analysis of the Steroid Compounds

Time segment	Retention time (min)	Compound	Precursor ion	Product ion	CAV	Fragmentor voltage	Collision energy
1	8.0	Estradiol	271.0	145.0	2	380	50
1	8.0	Estradiol-d5	276.0	147.0	2	380	50
1	8.5	EthynylEstradiol	295.0	145.0	2	380	52
1	8.5	EthynylEstradiol-d4	299.0	147.0	2	380	52
1	8.8	Estrone	269.0	145.0	2	380	45
1	8.8	Estrone-d4	273.0	147.0	2	380	45

Results and Discussion

Optimization of Analysis Parameters

While methods exist for steroid analysis on previous instruments, the analysis parameters for these methods must be optimized in order to take full advantage of the unique sensitivity of the Agilent 6490 Triple Quadrupole LC/MS with iFunnel Technology (6490). In this case, the method used as a starting point was developed on the 6460 Triple Quadrupole LC/MS (6460).

Collision cell accelerator voltage (CAV) is a key parameter in optimizing production, detection, and quantitation of ions. Figure 1 illustrates the significant increase in response for detection of the three steroids (prepared as calibration standards) obtained by changing the accelerator voltage from 5 to 2 volts.

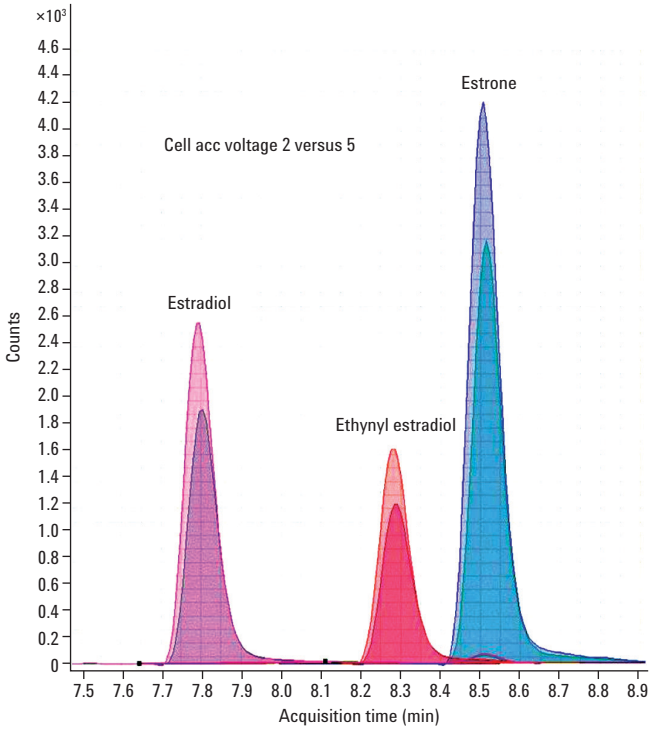

Figure 1. Effect on response of changing the cell accelerator voltage (CAV) from 5 volts (lower peaks) to 2 volts (higher peaks).

The multiplier voltage (Delta EMV) is another parameter that significantly influences response. By varying the voltage from 100 V to 400 V in 100 V increments, the response is increased by as much as a factor of 14 across the range. In excess of 400 volts, the response will still increase, but the signal-to-noise ratio can stay static or indeed decrease. The optimized value was found to be around 200–300 volts (Figure 2).

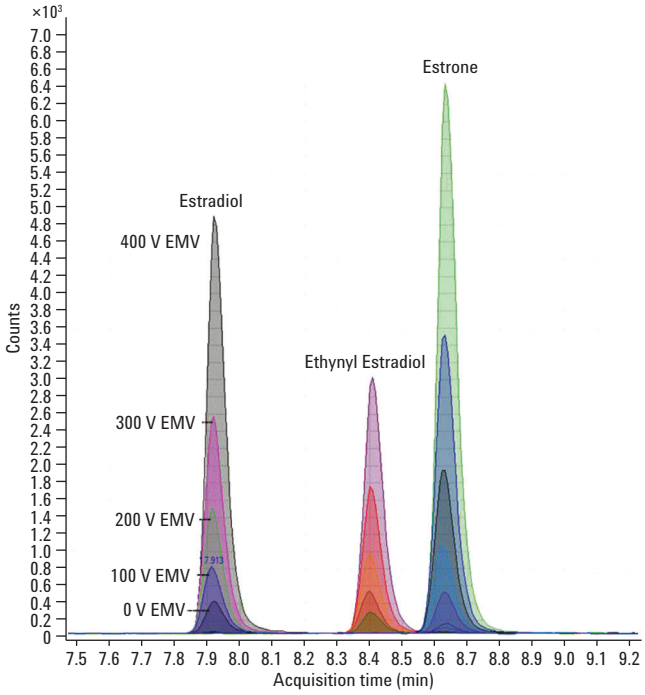

Figure 2. Significant increase in response due to increasing the multiplier voltage (Delta EMV).

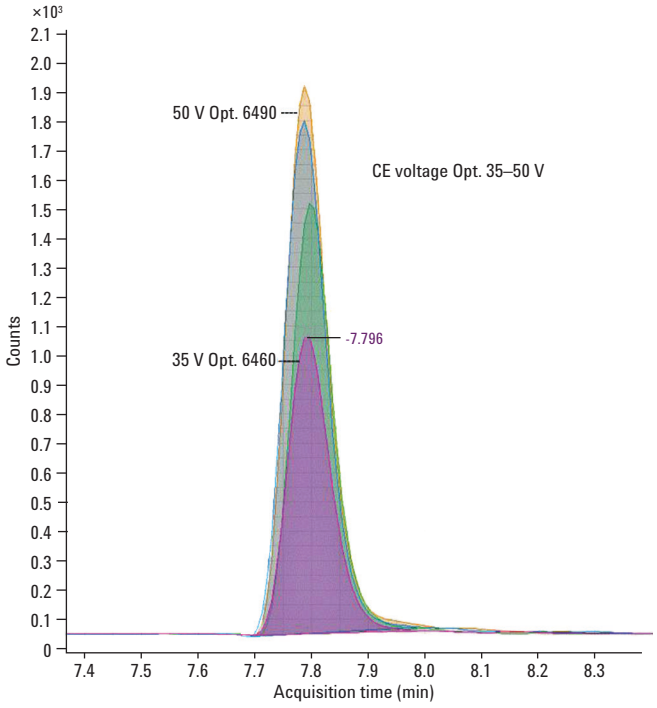

Figure 3. Optimization of collision energy (CE) on the Agilent 6490 Triple Quadrupole LC/MS System provides an increase in response for detection of estradiol of almost two fold, compared to the collision energy that was optimal on the 6460 (35 volts).

Optimization of collision energy (CE) is also critical for maximizing response. While the optimal collision energy for the 6460 method was 35 volts for estradiol, increasing the voltage in 5 volt increments on the 6490 continually increased response, with a nearly two-fold increase at 50 volts (Figure 3).

One of the keys to the enhanced sensitivity of the Agilent 6490 Triple Quadrupole LC/MS System is the proprietary Agilent iFunnel Technology, which utilizes a novel dual stage ion funnel assembly. Both the low and high pressure voltage have default settings in the tune file of the 6490. However, both voltages can be optimized to significantly increase sensitivity. For example, changing the low pressure voltage from the default of 60 volts to 100 volts can increase response by as much as 65%, as can changing the high pressure (high RF) voltage from the default of 150 volts to 160 volts (Figure 4).

Applying all of these optimized parameters to the method on the 6490 results in response that is as much as three times higher than that obtained from these steroids using the optimized parameters from the 6460 method on the 6490 (Figure 5). Applying these optimized parameters enables the detection of as little as 0.1 pg of estrone (Figure 6), estradiol, and ethynyl estradiol calibration standards on the column (10 pg/L in the sample).

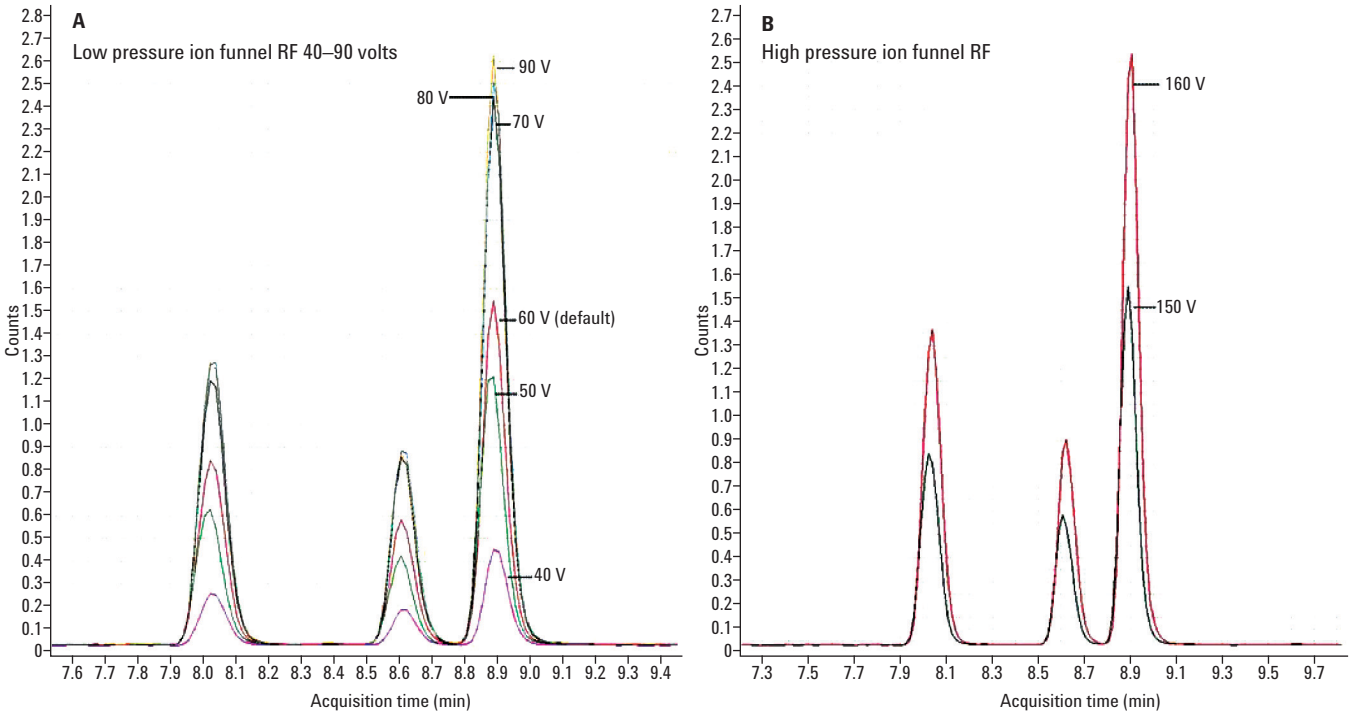

Figure 4. Optimization of both the low pressure and high pressure ion funnel voltages on the Agilent 6490 Triple Quadrupole LC/MS System provides an increase in response of as much as 65% for both settings, for the three steroids.

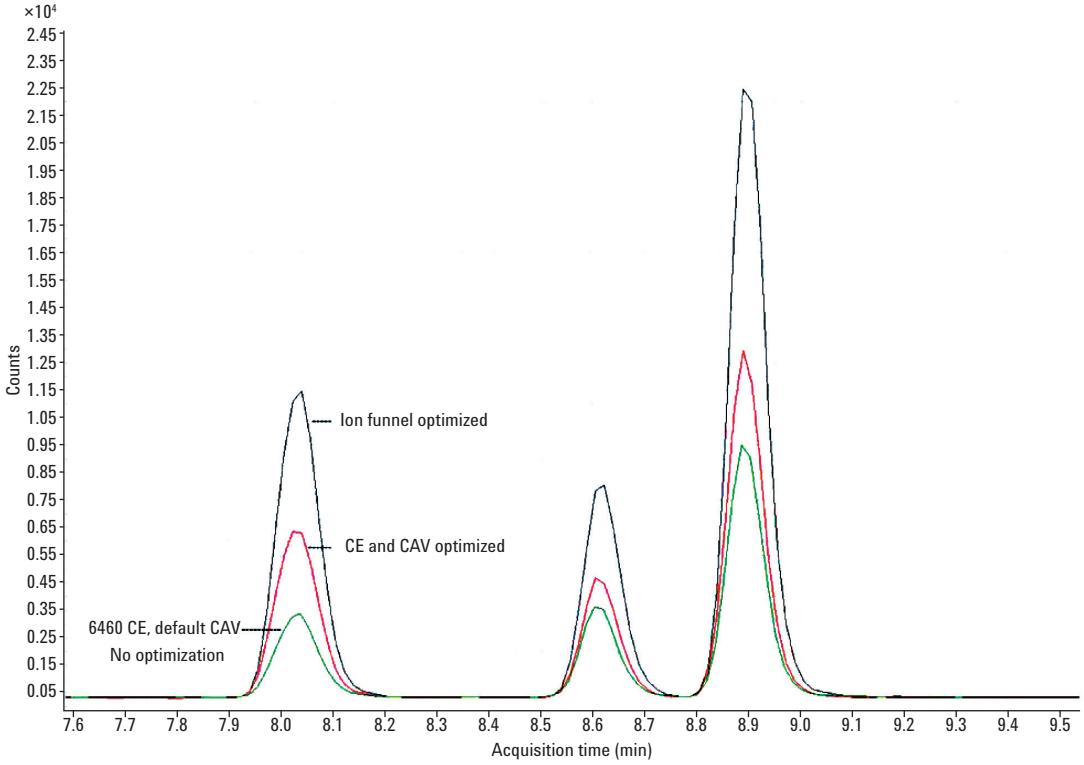

Figure 5. The cumulative effect of the various Agilent 6490 Triple Quadrupole LC/MS System optimization steps on response is shown, resulting in as much as much as three times higher response than that obtained from these steroids using the optimized parameters from the Agilent 6490 Triple Quadrupole LC/MS System method.

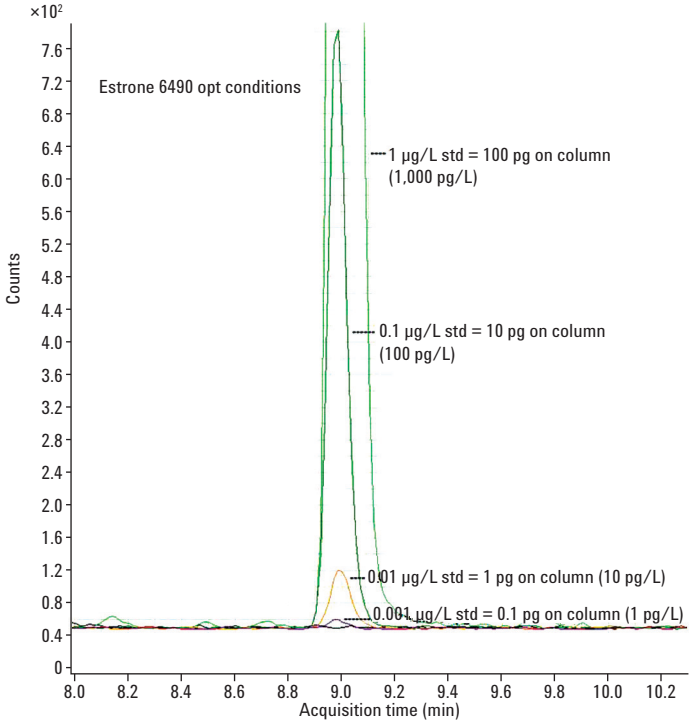

Figure 6. Detection of 1 pg to 100 pg on column of estrone calibration standard, using the optimized method parameters.

Postcolumn addition of 0.1% ammonia solution at 0.1 mL/min also gives an increase in response for the steroids when using the optimal MS parameters, in the order of 3–4 fold, compared to no addition (Figure 7).

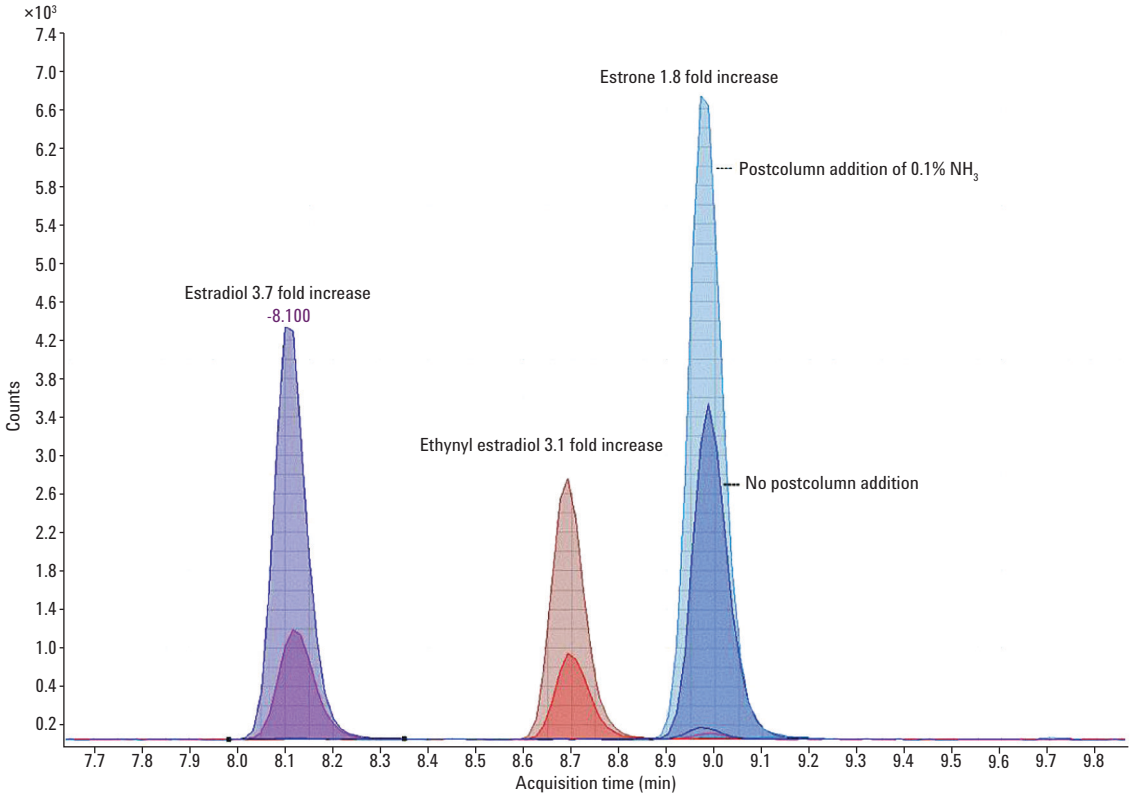

Figure 7. Postcolumn addition of 0.1% ammonia can increase response by as much as almost 4 fold, for all three steroids analyzed.

Sensitive Detection of Steroids in Sewage Effluent.

This optimized method can detect sub part per trillion (ng/L) steroids in wastewater. Figure 8 shows ethynyl estradiol at 0.91ng/L in crude sewage, as well as the excellent linearity of the calibration curve (R^2>0.9999). Estrone was easily detectable at 53.7 ng/L (Figure 9) in crude sewage, and estradiol at 44.5 ng/L (Figure 10). Both of these steroids also showed excellent linearity of quantification, with R^2 values >0.9999.

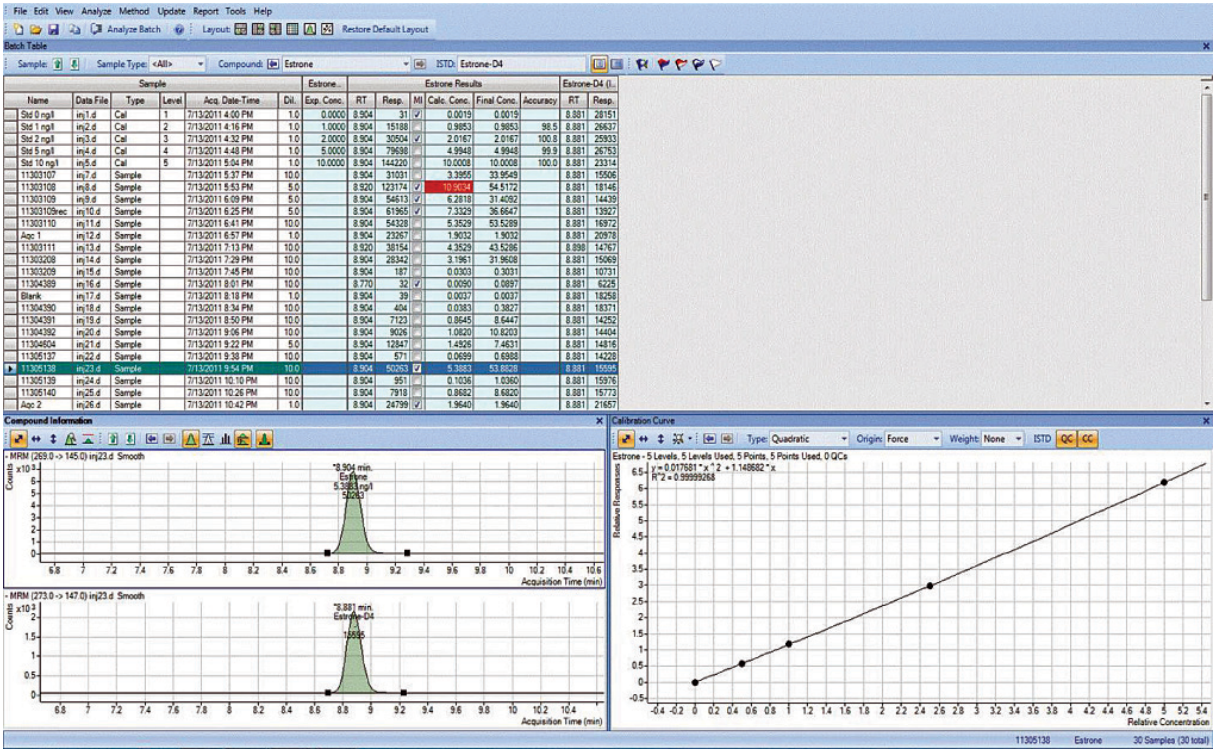

Figure 8. Analysis of 0.91 ng/L of ethynyl estradiol in crude sewage, showing the MRM chromatogram, as well as the chromatogram for the internal standard and the calibration curve with an R^2 value >0.9999.

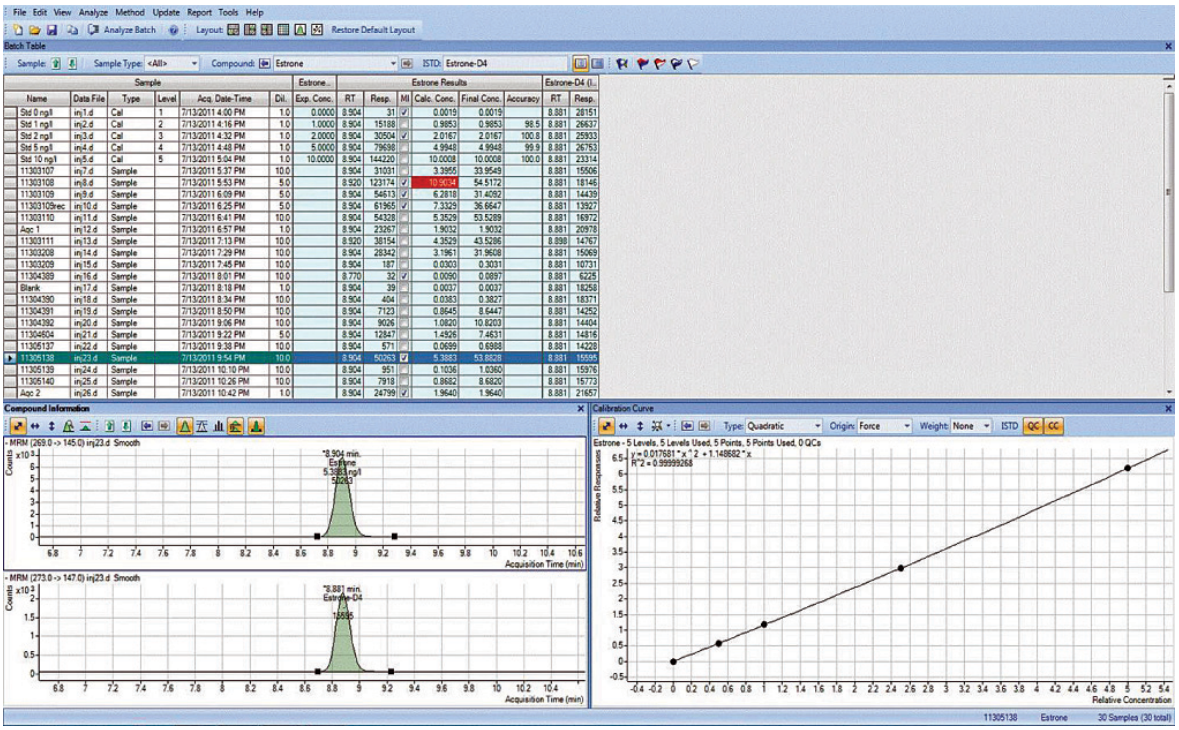

Figure 9. Analysis of 53.7 ng/L of estrone in crude sewage, showing the MRM chromatogram, as well as the chromatogram for the internal standard and the calibration curve with an R^2 value >0.9999.

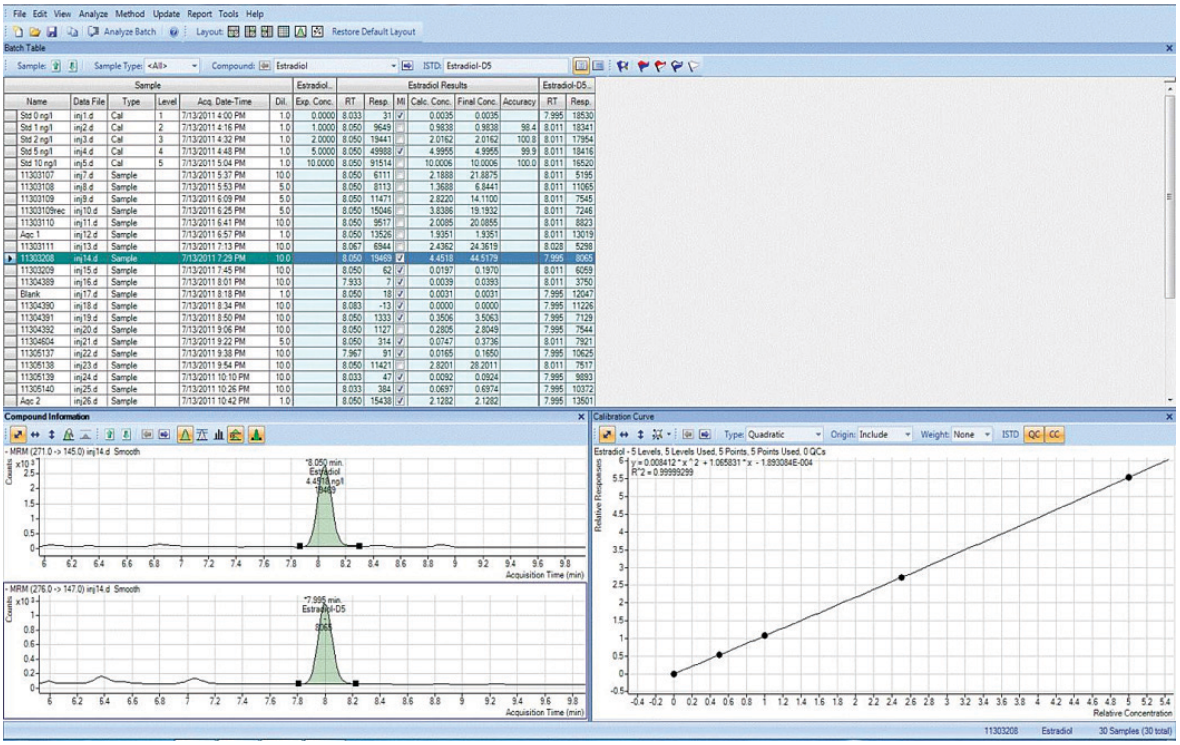

Figure 10. Analysis of 44.5 ng/L of estradiol in crude sewage showing the MRM chromatogram, as well as the chromatogram for the internal standard and the calibration curve with an R^2 value >0.9999.

Conclusions

The Agilent 6490 Triple Quadrupole LC/MS System with iFunnel Technology provides exquisite sensitivity, making it the ideal choice for applications that require the detection of minute quantities of analyte. However, as with any other instrument, methods that have been developed on earlier generation MS instruments must be optimized on the 6490 in order to maximize sensitivity. Such optimization can increase the response for detection of steroids by as much as three times versus the response obtained when using the parameters taken from the method developed on the Agilent 6460 Triple Quadrupole LC/MS System. The optimized method can provide sub ng/L detection of steroids in wastewaters, which are very complex matrices.

References

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2. Pollutants in Urban Waste Water and Sewage Sludge, http://ec.europa.eu/environment/waste/sludge/pdf/sludge_pollutants_xsum.pdf

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