Comparison of Online SPE Analysis and Direct Injection of Trace Level Estrogens in Drinking Water with the Agilent 6460 and Agilent 6490 Triple Quadrupole LC/MS Systems

The Agilent 1200 Infinity Series Online SPE Solution for the Highest Sensitivity

Application Note

Environmental

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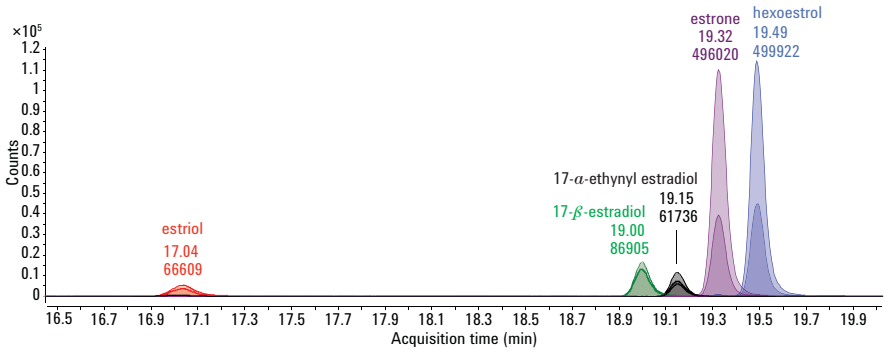
Abstract

This Application Note demonstrates the enhanced sensitivity of the Agilent 1200 Infinity Series Online SPE Solution compared to direct inject methods on a routine and a high end mass spectrometer. The sensitive analysis of estrogens in drinking water is shown with four different methods performed on an Agilent 6460 Triple Quadrupole LC/MS System and an Agilent 6490 Triple Quadrupole LC/MS System, both coupled to a 1200 Infinity Series Online SPE Solution.

The Agilent 1290 Infinity Flexible Cube, which is the heart of the 1200 Infinity Series Online SPE Solution, offers a highly flexible setup and supports direct injection and online SPE all in one module. With the option of a high volume injection kit (4.5 mL), it is possible to be sensitive as low as sub-1 ng/L with a routine mass spectrometer. With a high end mass spectrometer, detection limits of 0.02 ng/L were observed.



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Introduction

Worldwide natural and synthetic estrogens can be found in environmental water due to high pharmaceutical consumption and excretion. Estrogens, such as estrone (E1), 17-β-estradiol (E2), estriol (E3) and the synthetic 17-α-ethinylestradiol (EE2) are believed to play a major role in the interference with the hormonal system of exposed water organisms, such as fish. The potential consequences to humans remain still uncertain[1,2]. In Europe, the Water Framework Directive (WFD, 2000/60/EC)[3] has defined a list of priority pollutants that have damaging effects on the environment. Two estrogens proposed to be included in this list of priority substances are: 17-β-estradiol and 17-α-ethinylestradiol. The proposed annual average concentrations in surface waters are 0.08 (E2) and 0.007 ng/L (EE2). If these estrogens are included in this list, very sensitive methods are needed for the analysis and detection of hormones, especially estrogens, in environmental water.

For the sensitive analysis of estrogens in environmental matrixes, an extraction and purification step is often necessary[1]. In most cases, an online solid phase extraction (SPE) method is the simplest approach to achieve this goal. The 1200 Infinity Series Online SPE Solution reduces time and labor and enhances sample throughput because the whole analysis can be done with one HPLC instrument without offline sample preparation. Even with a routine mass spectrometer, high sensitivity can be achieved, as low as sub-1 ng/L, with a high volume injection (4.5 mL) coupling with different mass spectrometer.

In this Application Note, estrogen-related compounds (natural and synthetic) are analyzed and quantified with an online solid-phase extraction enrichment process on two different sensitive triple quadrupole mass spectrometers, a 6460 and a 6490 Triple Quadrupole LC/MS System. The results were compared to the direct inject methods.

Experimental and Instrumentation

All experiments were carried out on an Agilent 1200 Infinity Online SPE Solution comprising:

- Agilent 1260 Infinity Binary Pump G1312B and LAN card G1369C
- Agilent 1260 Infinity Standard Autosampler G1329B with 900 µL head (G1313-60007) and an Agilent 1290 Infinity Thermostat G1330B
- Agilent 1290 Infinity Flexible Cube G4227A with two 2-position/10-port valves
    - Agilent 1200 Infinity Online SPE starter kit: G4742A (one 2-position/10-port valve, 600 bar)
    - Agilent 1200 Infinity Online SPE direct injection kit: G4744A (one 2-position/10-port valve, 600 bar)
- External valve drive G1170A with 2-position/6-port valve G4231A and a 5-mL loop (p/n G2260-68711)
- Agilent 1290 Infinity Thermostatted Column Compartment G1316C
- Agilent G6460A Triple Quadrupole LC/MS System with Agilent Jet Stream Technology
- Agilent G6490A Triple Quadrupole LC/MS System with Agilent Jet Stream Technology and iFunnel Technology

Trapping columns

- 2 × Guard Column Hardware Kit (p/n 820999-901) (as part of G4742A)
- Agilent ZORBAX Eclipse Plus C18, 4.6 × 12.5 mm, 5 µm (p/n 820950-936)

Software

- Agilent MassHunter data acquisition for triple quadruple mass spectrometer, Version 06.00
- Agilent MassHunter Optimizer Software, Version 06.00
- Agilent MassHunter Source and iFunnel Optimizer Software, Version 06.00
- Agilent MassHunter Qualitative Software, Version 06.00
- Agilent MassHunter Quantitative Software, Version 06.00

The LC/MS method was developed based on the Application Note 5991-2473EN[6].

Table 1. HPLC Method for direct injection and online SPE.

	Direct inject	Online SPE
Injection volume	250 µL	4,500 µL
Enrichment cartridges	–	Agilent ZORBAX Eclipse Plus C18, 4.6 × 12.5 mm, 5 µm (p/n 820950-936) 12.5 mm, 5 µm (p/n 820950-936)
Column temperature	30 °C	30 °C
Column	Agilent Poroshell 120 Phenyl Hexyl 2.1 × 50 mm, 2.7 µm (p/n 699775-912)	Agilent Poroshell 120 Phenyl Hexyl 3.0 × 50 mm, 2.7 µm (p/n 699975-312)
Mobile Phase	A: water + 1 mM ammonium fluoride B: methanol : acetonitrile (65:35)	A: water + 1 mM ammonium fluoride B: methanol : acetonitrile (65:35)
Gradient	0–0.5 minutes – 10 % B, 0.5–5.5 minutes 10–100 % B, 5.5–11 minutes 100 % B	0–10.6 minutes – 10 % B, 10.6–20 minutes 10–100 % B, 20–24 minutes 100 % B
Flow rate	0.6 mL/min	0.6 mL/min

System configuration and principle of operation

This Application Note describes the Agilent 1200 Infinity Series Online SPE Solution, which is based on the 1290 Infinity Flexible Cube with an additional external valve drive next to the 1290 Infinity Flexible Cube for the use of a high volume injection loop (5 mL). For more information about the plumbing and flow profile, see the Technical Overview 5991-3173EN[4].

The Flexible Cube itself hosts two 2-position/10-port valves for the opportunity of direct injection methods and online SPE methods with alternate use of two trapping columns[5]. Additionally, with the solvent selection valve in the 1290 Infinity Flexible Cube, up to three solvents can be used to load and clean the SPE cartridges. For the analysis of estrogens, a RP C-18 guard column is perfectly suited as a SPE phase and is useable for a wide range of concentration, repeatability, and accuracy[1].

Figure 1 presents a schematic overview of the HPLC and online SPE method.

Finally, the system was coupled to an Agilent 6460 and 6490 Triple Quadrupole LC/MS System for detection and enhanced sensitivity measurements.

The online SPE procedure consisted of three steps (Figure 1):

1. Loading and enriching the sample on the SPE cartridge (SPE 1)
2. Elution of SPE 1 with the gradient flow
3. Rinsing and reconditioning SPE 2 (from the previous run)

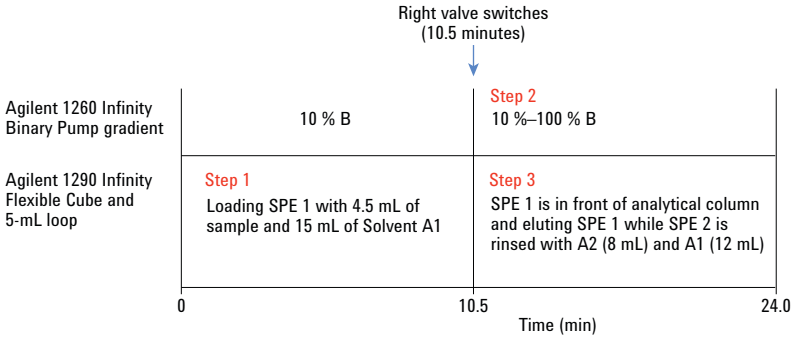

Figure 1. Schematic of the online SPE method which includes the Agilent 1290 Infinity Flexible Cube as a loading pump and an Agilent 1260 Infinity Binary Pump to run the gradient and elute the analytes from the cartridges.

Agilent 1290 Infinity Flexible Cube

- Right and left valve: 2-position/10-port QuickChange valve head
- Solvent selection valve: solvent A1: water, solvent A2: acetonitrile

To draw 4.5 mL into the 5-mL loop, an injector program has to be defined (Table 3)[4].

Samples were measured with 6460 and 6490 Triple Quadrupole LC/MS Systems in negative mode. The source parameters for the Agilent Jet Stream were optimized with the Agilent MassHunter Source and iFunnel Optimizer (Table 4).

The Multiple Reaction Monitoring (MRM) triple quadrupole MS method was developed with Agilent MassHunter Optimizer Software. Direct injections were used for all estrogens standards (100 ng/µL). For every compound, two MRM transitions were optimized regarding fragmentor voltage, cell accelerator voltage, and collision energy. Table 5 shows the suite of five estrogens.

Table 2. Piston pump timetable in the Agilent 1290 Infinity Flexible Cube for the online SPE method.

Time	Function	Parameter
0	Pump for volume	Pump 15 mL, flow: 1.5 mL/min Channel A1 (water)
10.5	Right valve change position	Increase valve position (switch valve)
10.6	Pump for time	Pump 8 mL, flow: 1.5 mL/min Channel A2 (ACN)
16	Pump for time	Pump 12 mL, flow: 1.5 mL/min Channel A1 (water)

Table 3. Injector program.

Injector program to load 4.5 mL
Repeat four times:
1. Draw 900 µL (draw speed 900 µL/min, eject speed 900 µL/min)
2. Needlewash
3. Eject to seat
4. End repeat
5. Draw 900 µL
6. Inject

Table 4. Ion source parameters for Agilent Jet Stream negative ionization for the Agilent 6460 Triple Quadrupole LC/MS System and Agilent 6490 Triple Quadrupole LC/MS System.

Parameters	Agilent 6460 Triple Quadrupole LC/MS System	Agilent 6490 Triple Quadrupole LC/MS System
Nozzle	500 V	0 V
Capillary	3,000 V	3,500 V
Sheath gas temperature	400 °C	375 °C
Sheath gas flow	12 L/min	12 L/min
Drying gas temperature	350 °C	125 °C
Drying gas flow	12 L/min	16 L/min
Nebulizer	45 psi	40 psi
Δ EMV	600 V	300 V

Table 5. Five estrogens are listed with their mass, formula, precursors and adducts, product ions, and polarity.

Steroid	Mass (g/mol)	Formula	Precursor ion (*m/z*)	Product ion 1 and 2 (*m/z*)	Polarity
Estrone (natural estrogen)	270.3	$C_{18}H_{22}O_2$	269.1	145.2/142.9	negative
17-β-Estradiol (natural estrogen)	273.3	$C_{18}H_{24}O_2$	271.2	183.2/145.1	negative
17-α-Ethinylestradiol (synthetic estrogen)	296.4	$C_{20}H_{24}O_2$	295.4	145/143	negative
Estriol (natural estrogen)	288.3	$C_{18}H_{24}O_3$	287.3	143/171.2	negative
Hexoestrol (synthetic estrogen)	270	$C_{18}H_{22}O_2$	269.15	134/119.2	negative

Samples

Tap water was spiked in different calibration levels with estrogens.

Seven calibration standards of estrogens for the direct injection on the 6460 Triple Quadrupole LC/MS System, ranging from 2.5 to 200 ng/L (2.5, 5, 10, 25, 50, 100, and 200 ng/L) each, were prepared by appropriate dilution of the stock solution (200 ng/L). A seven-level calibration curve for the online SPE method on the 6460 Triple Quadrupole LC/MS System was prepared in the range of 0.1–50 ng/L (0.1, 0.5, 1, 2, 5, 10, 25, and 50 ng/L).

A seven-level calibration curve was done for the high sensitivity 6490 Triple Quadrupole LC/MS System and direct injection, which was in the range from 0.5–50 ng/L (0.5, 1, 2.5, 5, 10, 25, and 50 ng/L). For the online SPE method, the calibration curve was in the range of 0.05–25 ng/L (0.05, 0.1, 0.5, 1, 2.5, 5, 10, and 25 ng/L).

Chemicals

All solvents used were LC/MS grade. Acetonitrile and methanol were purchased from Merck, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). Ammonium fluoride was purchased from Sigma Aldrich, St. Louis, USA.

All estrogen standards were purchased from Dr. Ehrenstorfer GmbH, Germany at a concentration of 100 ng/µL in acetonitrile.

Results and Discussion

Linearity, limit of detection (LOD), and limit of quantification (LOQ)

The online SPE as well as the direct inject methods show excellent linearity for all estrogens in different concentration ranges with the 6460 and 6490 Triple Quadrupole LC/MS System.

For the 6460 Triple Quadrupole LC/MS System, the linearity was measured in spiked drinking water for the direct inject method in the range of 2.5 to 200 ng/L and for the online SPE method in the range of 0.1 to 50 ng/L. In Figure 2, the calibration curves of 17-β-estradiol and 17-α-ethinylestradiol are shown for the online SPE method.

The direct inject and online SPE methods were transferred from a 6460 Triple Quadrupole LC/MS System to a higher sensitive triple quadrupole. The 6490 Triple Quadrupole LC/MS System includes iFunnel technology for ultra-trace analysis and highest sensitivity.

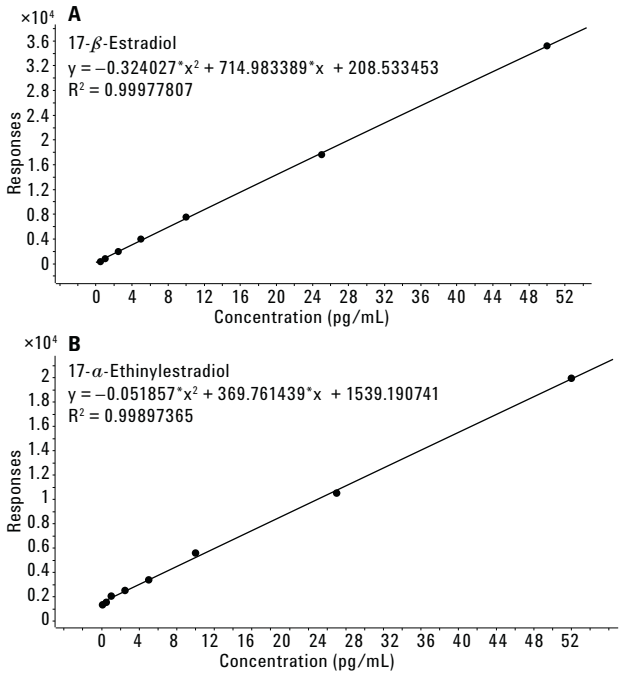

Figure 2. Calibration curve (1–50 ng/L) for 17-β-estradiol (A) and 17-α-ethinylestradiol (0.1–50 ng/L) (B) with R^2 0.999, measured with an Agilent 6460 Triple Quadrupole LC/MS System and online SPE.

Calibration curves were prepared in drinking water in the range of 0.05–25 ng/L (8 level) for all estrogens. Figure 3 shows the calibration curves of estrone, hexoestrol, and estriol with a good linearity over a wide range of calibration.

Table 6 summarizes R^2 for both direct inject and online SPE methods performed on the 6460 and 6490 Triple Quadrupole LC/MS System in different concentration ranges.

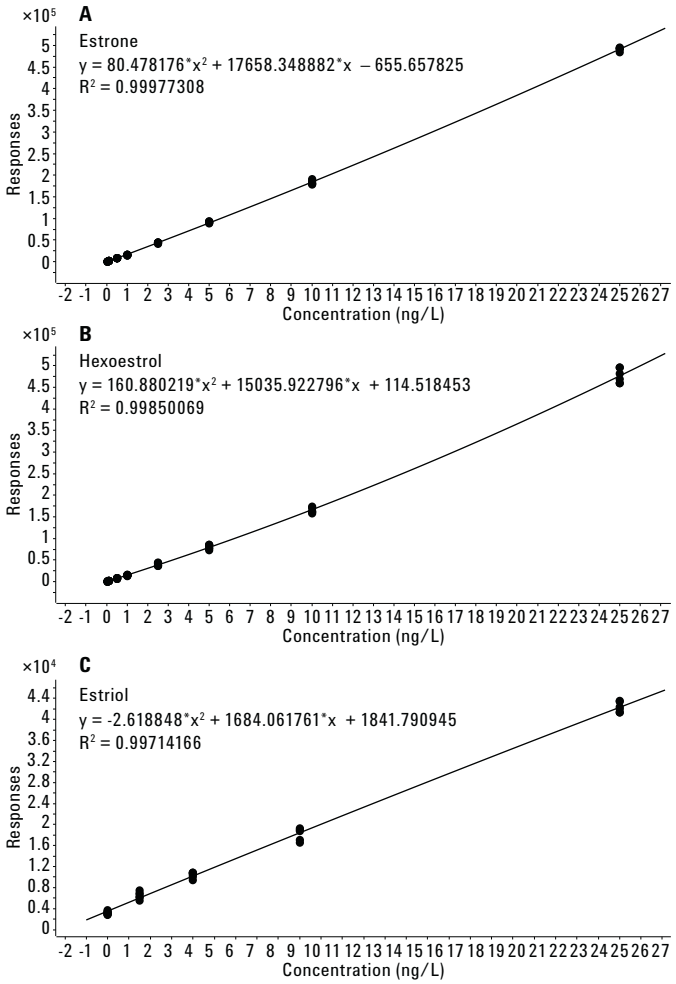

Figure 3. Calibration curve (0.05–25 ng/L) for estrone (A), hexoestrol (B), and estriol (C) (1–25 ng/L) with $R^2 < 0.997$, measured with a Agilent 6490 Triple Quadrupole LC/MS System and an Agilent 1200 Infinity Online SPE.

Table 6. R^2 for direct inject and online SPE methods on two different triple quadrupoles.

	Agilent 6460 Triple Quadrupole LC/MS System		Agilent 6490 Triple Quadrupole LC/MS System	
	Direct inject R^2 (2.5–200 ng/L)	Online SPE R^2 (0.1–50 ng/L)	Direct inject R^2 (0.5–50 ng/L)	Online SPE R^2 (0.05–25 ng/L)
Estriol	0.999	0.999	0.998	0.996
17-α-Ethinylestradiol	0.999	0.998	0.997	0.998
17-β-Estradiol	0.999	0.999	0.998	0.999
Estrone	0.999	0.999	0.999	0.999
Hexoestrol	0.997	0.999	0.999	0.998

Table 7 shows a comparison and summary of direct injections and online SPE methods on two triple quadrupoles. For all methods, the LOD, signal-to-noise ratio (S/N) > 3 and LOQ, S/N > 10 were determined.

Table 7 demonstrates a significant increase in sensitivity between direct inject methods and online SPE methods on both detectors. A further increase in sensitivity also could be observed between 6460 and 6490 Triple Quadrupole LC/MS Systems. In general, a gain of sensitivity was determined with a factor of 2 to 10 between the direct inject method compared to the online SPE method. The 17-α-ethinylestradiol particularly increased with the online SPE enrichment process. A factor of 50 for the LOD was determined with the 6460 Triple Quadrupole LC/MS System, from 5 ng/L direct inject to 0.1 ng/L online SPE (Figure 4).

The lowest detection limits were observed with the 6490 Triple Quadrupole LC/MS System and online SPE enrichment. Up to 0.02 ng/L can be measured for estrone and hexoestrol in spiked drinking water. Figure 5 shows the LOQ for estrone.

Generally, the difference in sensitivity between the 6460 and 6490 Triple Quadrupole LC/MS Systems is approximately a factor of 2 to 10.

Table 7. LOD and LOQ for direct inject and online SPE methods on an Agilent 6460 Triple Quadrupole LC/MS System and an Agilent 6490 Triple Quadrupole LC/MS System.

	6460 Triple Quadrupole LC/MS System				6490 Triple Quadrupole LC/MS System			
	Direct inject		Online SPE		Direct inject		Online SPE	
	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)
estriol	10	25	5	10	5	10	1	2.5
17-α-ethinylestradiol	5	10	0.1	2	1	2.5	0.1	0.5
17-β-estradiol	2	5	1	2.5	1	2.5	0.2	0.5
estrone	1	2.5	0.2	1	0.1	0.5	0.02	0.05
hexoestrol	1	2.5	0.2	0.5	0.1	0.5	0.02	0.05

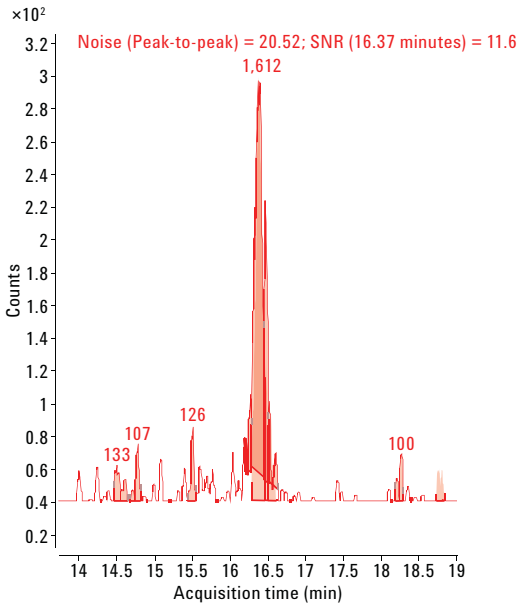

Figure 4. 17-α-ethinylestradiol at a concentration of 0.1 ng/L with online SPE injection measured with an Agilent 6460 Triple Quadrupole LC/MS System.

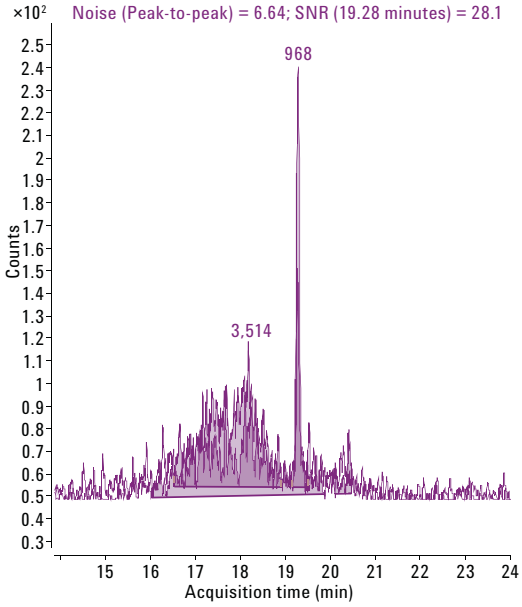

Figure 5. Estrone at a concentration of 0.05 ng/L in drinking water with online SPE injection measured with an Agilent 6490 Triple Quadrupole LC/MS System.

Area precision data of LOD, LOQ, and RT

For the online SPE method, replicative measurements were performed (n = 6) for the determination of LOD, S/N > 3 and LOQ, S/N > 10 regarding the area precision data on the 6460 as well as the 6490 Triple Quadrupole LC/MS System.

Table 8 shows that the area RSD for all compounds and each Triple Quadrupole LC/MS System was usually < 15 % and the average area RSD was approximately 10 %.

The RSD for retention time (RT) precision for all online SPE runs and two alternatively used cartridges (n = 336) was 0.1 %.

Carryover for online SPE

For the 1200 Infinity Series Online SPE coupled to the 6460 Triple Quadrupole LC/MS System, carryover was observed only for 17-α-ethinylestradiol in the blank injection after the highest standard of 50 ng/L estrogens. The blank injection was determined to be 1 % with a S/N ratio of 1.6.

For the 6490 Triple Quadrupole LC/MS System, no carry over was determined for any compound.

Table 8. Area precision data for LOD and LOQ for all compounds and online SPE methods on the Agilent 6460 and Agilent 6490 Triple Quadrupole LC/MS Systems.

	Agilent 6460 Triple Quadrupole LC/MS System		Agilent 6490 Triple Quadrupole LC/MS System	
	% RSD LOD	% RSD LOQ	% RSD LOD	% RSD LOQ
Estriol	8.31	8.35	13.07	10.29
17-α-Ethinylestradiol	2.31	11.72	19.30	18.27
17-β-Estradiol	5.11	7.91	11.51	11.68
Estrone	12.12	6.40	9.70	9.91
Hexoestrol	14.86	11.61	12.36	11.21

Conclusion

For the sensitive analysis of estrogens in water, the Agilent 1200 Infinity Series Online SPE Solution offers a flexible and cost-effective solution. Direct injections, online SPE, and high volume injections (up to 5 mL) with the online SPE can be done with one instrument. Coupled to different detectors, such as an Agilent 6460 Triple Quadrupole LC/MS System or an Agilent 6490 Triple Quadrupole LC/MS System, improved sensitivity can be achieved.

For the analysis of estrogens in drinking water, LOD down to 0.02 ng/L was determined. Excellent linearity was observed for all compounds for a wide range of concentrations on both MS detectors. It was observed that the 6490 Triple Quadrupole LC/MS System compared to the 6460 Triple Quadrupole LC/MS System was up to 10 times more sensitive.

Carryover was determined only for 17-α-ethinylestradiol on the 6460 Triple Quadrupole LC/MS System. The blank injection was determined to be 1 % with a S/N ratio of 1.6.

The results of this work shows that the 1200 Infinity Series Online SPE Solution is perfectly suited for the analysis of estrogens in water. Because of the high flexibility of the system, it is a good option for medium throughput laboratories that want more sensitive results for their water analysis and need a flexible solution for different applications.

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