Analysis of Four β-Lactam Antibiotics in Water Using an Agilent 1290 Infinity II LC and an Agilent 6470 Triple Quadrupole LC/MS with Direct Injection and Online SPE

Application Note

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Abstract

β-lactam antibiotics are some of the most commonly used drugs in human and veterinary medicine, which may lead to the presence of these substances and their metabolites in the aquatic environment. The effect and relevance of these antibiotics in terms of water quality and antibiotic resistance are still largely unknown, and require highly sensitive and robust detection methods. This application note describes a sensitive method for the quantification of four β-lactam antibiotics in the low ng/L range in water using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 Triple Quadrupole LC/MS system using an Agilent Jet Stream electrospray ionization source. The hardware setup for direct injection and online SPE with delay volume reduction allows for increased analytical sensitivity, with detection limits in the sub-ng/L range.

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Introduction

Due to the common use of β-lactam antibiotics in veterinary and human therapy, and the potential of the development and spreading of resistant bacteria, these substances have become an important topic for environmental research [1]. In Germany, β-lactam antibiotics, such as penicillins, aminopenicillins, and cephalosporins are the most used antibiotics in human medicine. With a yearly consumption of 199 tons, β-lactam antibiotics are the second most used antibiotics in veterinary medicine [2]. The concentrations in environmental samples are very low, usually in the lower ng/L range. A study realized by the German Federal Committee for Safety of Chemicals found that the concentrations of β-lactam antibiotics in wastewater treatment plant effluents are below the detection limits. The highest measured concentrations for penicillin V were 13 ng/L, and for piperacillin 40 ng/L [3]. This application note describes a sensitive method for the determination of the four β-lactam antibiotics ampicillin, amoxicillin, penicillin V, and piperacillin at trace levels in water. These compounds are representative for β-lactams, and there is scientific interest in their environmental and ecotoxicological fate.

The system setup allowed for an automated online solid phase extraction (SPE) of up to 1.5 mL of water samples, as well as the direct injection of 100 µL with delay volume reduction without any hardware modification. This was accomplished using an:

- Agilent 1290 Infinity II UHPLC system
- Agilent 1290 Infinity Flexible Cube
- Additional external universal valve drive with a six-position 2-port valve
- Additional Agilent 1260 Infinity II isocratic pump for sample loading

The UHPLC system was coupled to the highly analytically sensitive and robust Agilent 6470 Triple Quadrupole LC/MS system equipped with an Agilent Jet Stream technology (AJS) ionization source. Both modes, online SPE and direct injection, were compared and characterized based on precision, linearity, limits of detection (LODs); in addition, online SPE was characterized based on recovery.

Experimental

Chemicals

Amoxicillin trihydrate, ampicillin, piperacillin sodium, and penicillin V potassium (all analytical grade) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Ammonia (25 %) and formic acid (98–100 %) (both analytical grade) were supplied by Merck KGaA (Darmstadt, Germany). Acetonitrile (LC/MS grade) was supplied by VWR International (Darmstadt, Germany). Ultrapure water was generated by a Veolia Elga ultrapure water plant (High Wycombe, United Kingdom). All analytes were dissolved in ultrapure water.

Instrument configuration and flow path

This method was developed using an Agilent 1290 Infinity II UHPLC system consisting of:

- Agilent 1290 Infinity II Multisampler (G7167B) equipped with a 100 µL analytical head and a 100-µL sample Loop-flex
- Agilent 1290 Infinity Flexible Cube (G4227A) with a second valve drive (G4227A#058) installed. The 1290 Infinity Flexible Cube was equipped with:
    - G4742A Online SPE Starter Kit
    - G4744B Online SPE Direct Inject Kit, 1300 bar
    - Agilent 1260 Infinity II Isocratic Pump (G7110B)
    - Agilent 1290 Infinity Valve Drive (G1170A) equipped with a 2-position 6-port valve (G4231C) with a 1.4 mL seat extension loop (G1313-87308) and a by-pass capillary (stainless steel, 0.12 × 50 mm, p/n 5067-4636) installed

The UHPLC system was coupled to an Agilent 6470 Triple Quadrupole LC/MS system using an AJS ionization source.

Figure 1 shows a graphic representation of the valve connections for online SPE (A) and direct injection with delay volume reduction (B). For direct injection analysis, the system is operated in by-pass mode at both the external valve and the 1290 Infinity Flexible Cube. Consequently, injection volumes from 1 to 100 µL can be injected with the lowest possible delay volume with this configuration.

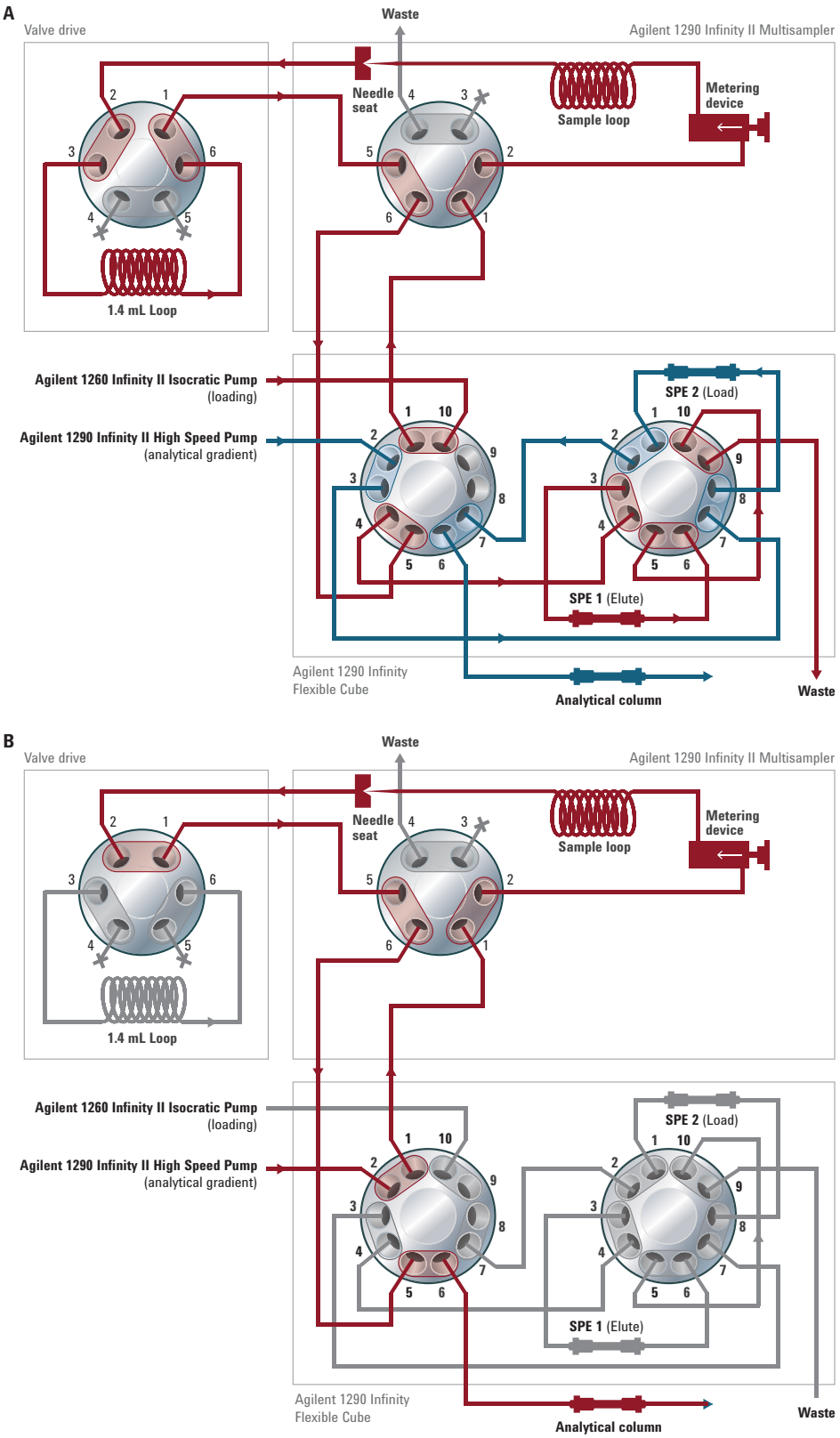

Figure 1. Schematic overview of the valve connections for online SPE (A) and direct injection with reduced delay volume (B).

For online SPE, the 2-position 10-port valve in the 1290 Infinity Flexible Cube is switched to connect the loading pump to the 1290 Infinity II Multisampler, and enables the alternate loading of the SPE cartridges. In addition, the external 2-position 6-port valve is switched to include the 1.4-mL sample loop in the flow path. The 1.4-mL loop can be filled by repetitive draw-and-eject cycles resulting in a maximum injection volume of 1.5 mL. When using larger seat extension loops, this volume can be extended. Subsequently, the sample is loaded onto the SPE cartridges using either the pump of the 1290 Infinity Flexible Cube or, as described in this method setup, with an additional 1260 Infinity II Isocratic Pump.

This setup permits the direct switching between direct injection and online SPE enrichment in the Agilent MassHunter software. It also results in minimal delay volumes during direct injection without hardware modifications such as reconnecting capillaries or sample loops. Thus, signals of direct injection and online SPE can be directly compared.

UHPLC/MS/MS parameters

A gradient between an aqueous phase with slightly basic pH (mobile phase A, pH 8) and acidic acetonitrile (mobile phase B) enables a baseline separation of all analytes within 12 minutes on a fully endcapped Agilent ZORBAX EclipsePlus C-18 column. The detection of piperacillin was made in positive ionization mode, while negative ion mode was used for amoxicillin, ampicillin, and penicillin V due to better response and lower matrix effects. Tables 1 and 2 list the UHPLC conditions for measurements with direct injection and the gradient program. Tables 3 and 4 show the MS conditions. Transitions and conditions for the antibiotics were optimized using Agilent MassHunter Optimizer software with flow injections of individual standard solutions for each compound.

Table 1. UHPLC Conditions for Direct Injection

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C-18, 50 × 2.1 mm, 1.8 µm particle size (p/n 959757-902)
Temperature	40 °C
Injection volume	100 µL
Flow rate	0.4 L/min
Run time	12 minutes
Post time	2 minutes
Mobile phase	A) Ultrapure water containing 27 nM ammonia (pH 8) B) Acetonitrile containing 13 µM formic acid

Table 2. UHPLC Gradient Program

Gradient	Time (min)	%A	%B
	0	95	5
	2	95	5
	3	75	25
	9	62	38
	9.1	0	100
	12	0	100
Post time	2	95	5

Table 3. Agilent G6470A Triple Quadrupole LC/MS Parameters

Parameter	Value
Ionization mode	AJS with fast-polarity switching
Scan type	Dynamic MRM
Gas temperature	200 °C
Gas flow	6 L/min
Nebulizer pressure	20 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Capillary voltage	5,000 V (for positive and negative ions)
Nozzle voltage	Positive: 1,000 V; negative: 1,500 V
Cycle time	500 ms
Resolution	Unit (MS1), Unit (MS2)

Table 4. MRM Transitions and Conditions

Compound	Polarity	Precursor	FragV	Product	CE	CAV
Amoxicillin	negative	364.1	125	319.9	4	4
				222.9	8	4
				206.4	20	4
Ampicillin	negative	348.1	90	304.1	4	4
				206.9	8	4
				74.0	36	4
Penicillin V	negative	349.1	90	207.9	4	4
				113.9	16	4
				92.2	32	4
Piperacillin	positive	518.2	150	159.9	8	4
				142.2	20	4
				114.9	72	4

Online SPE

Table 5 shows the conditions for online SPE analysis. The 1.5-mL sample was transferred to the Agilent Bond Elut PLRP-S Online SPE cartridge with a flow of ultrapure water delivered by the isocratic pump. Table 6 shows the timetable for the loading pump and the solvent selection valve of the 1290 Infinity Flexible Cube. After 2 minutes, the loading of the sample was completed and the cartridge was switched to the flow path of the analytical pump. The analytes were eluted from the cartridge in backflush mode to the column and the detector using the same gradient program shown in Table 2. The two SPE cartridges were alternated while one cartridge was eluted, the other SPE cartridge was conditioned using acetonitrile and water delivered by the isocratic pump, while connected to the solvent selection valve of the 1290 Infinity Flexible Cube.

Results and Discussion

After optimizing the mobile phase conditions and gradient program, baseline separation and good peak shapes could be achieved for direct injection within a runtime of 12 minutes. Ammonia strongly enhanced the ionization of all compounds, while acidic acetonitrile was required to produce sharp and symmetric peak shapes for the less polar analytes piperacillin and penicillin V. Figure 2 shows the chromatographic separation of the four β-lactam antibiotics with direct injection of 100 µL. LODs were calculated based on a signal-to-noise ratio (S/N) of 3:1. Table 7 shows the calculated LODs for direct injection, ranging from 1 to 5 ng/L, depending on the compound.

Table 5. Autosampler Conditions for Online SPE

Parameter	Value
Injection volume	1.5 mL (15 × 100 µL)
Draw speed	100 µL/min
Eject speed	400 µL/min
Time after draw	1.2 seconds
Flush out factor	5
Loading solvent	Ultrapure water
Online SPE cartridges	2x Agilent Bond Elut PLRP-S, 2.1 × 12.5 mm (p/n 5982-1271)

Table 6. Prerun Isocratic Flow Conditions for SPE Loading

Time	Flow	Flush eluent	Right valve
0	0.1	Water	
0.1	1.5	Water	
2	1.5	Water	Next position
2.1	0.1	Water	
2.5	1.5	Acetonitrile	
5.5	1.5	Water	
8.5	1.5	Water	
9	0	–	

Table 7. Validation Parameters LOD, Linearity, Precision, and Recovery of Direct Injection

	LOD (ng/L)	R^2 (n = 15)	RSD (%) (n = 12, c = 1 µg/L)	Peak width (s) (c = 100 ng/L)
Amoxicillin	5	0.997	1.8	2.9
Ampicillin	1	0.999	1.5	10
Piperacillin	2	0.999	1.3	6.4
Penicillin V	1	0.999	1.8	7.9

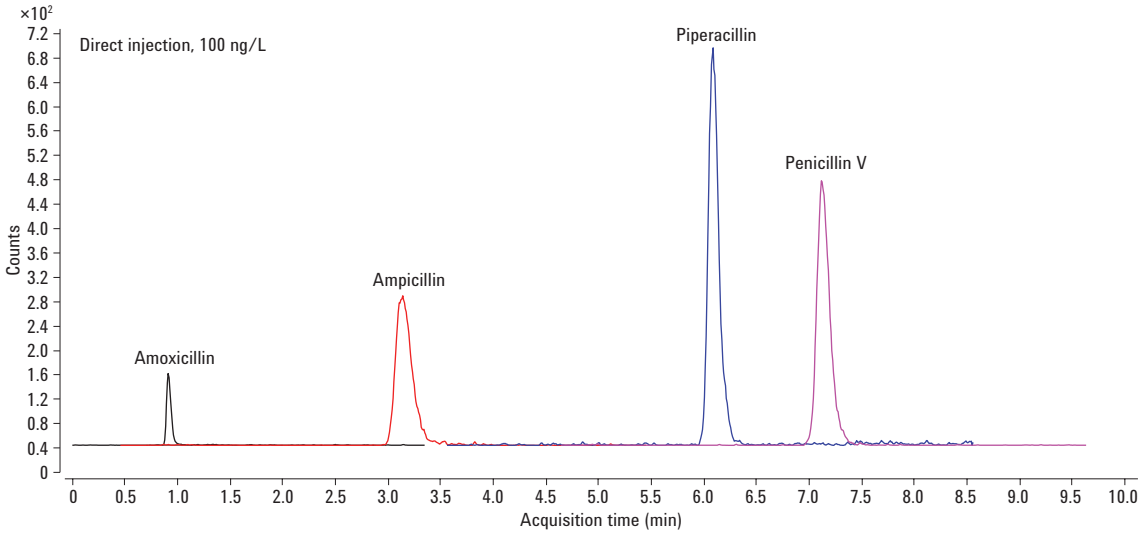

Figure 2. Chromatogram showing baseline separation of four β-lactam antibiotics with direct injection (injection volume = 100 µL), c = 100 ng/L.

The same water sample was injected using the online SPE mode to enrich the analytes and reach lower LODs. Figure 3 shows a comparison of the direct injection of 100 µL of a water sample and the online SPE of 1.5 mL of the same sample. Due to the reduction of the delay volume in the direct injection analysis, retention times in direct injection and online SPE were similar. With the online SPE method, the retention times for amoxicillin and ampicillin were longer. This can be explained by the 2-minute delayed start of the gradient program and the additional interaction of these compounds with the SPE material, which is more polar compared to the endcapped C18 material of the analytical column. Piperacillin and penicillin V elute earlier with the online SPE method. One reason for that is the direct connection of the analytical pump to the online SPE cartridges, resulting in a reduced void volume.

The recoveries for ampicillin, piperacillin, and penicillin V were between 51.7 and 94.1 %, indicating sufficient retention of these three analytes on the SPE material. The less polar analytes, piperacillin and penicillin V, show higher recoveries compared to the more polar analytes. Only 6.5 % of amoxicillin is retained compared to the direct injection method, and the low recovery is accompanied with peak broadening. As a result, amoxicillin was removed from the online SPE method. LODs for the online SPE method were between 0.2 and 0.5 ng/L based on a S/N of 3:1. Due to incomplete recoveries, increased chemical noise, and broadening chromatographic peaks (as compared to direct injections), the LODs obtained for SPE were higher than anticipated for a 15-fold increase in injection volume. Figure 4 shows the chromatograms of ampicillin, piperacillin, and penicillin V at 500 pg/L spiked into a water sample.

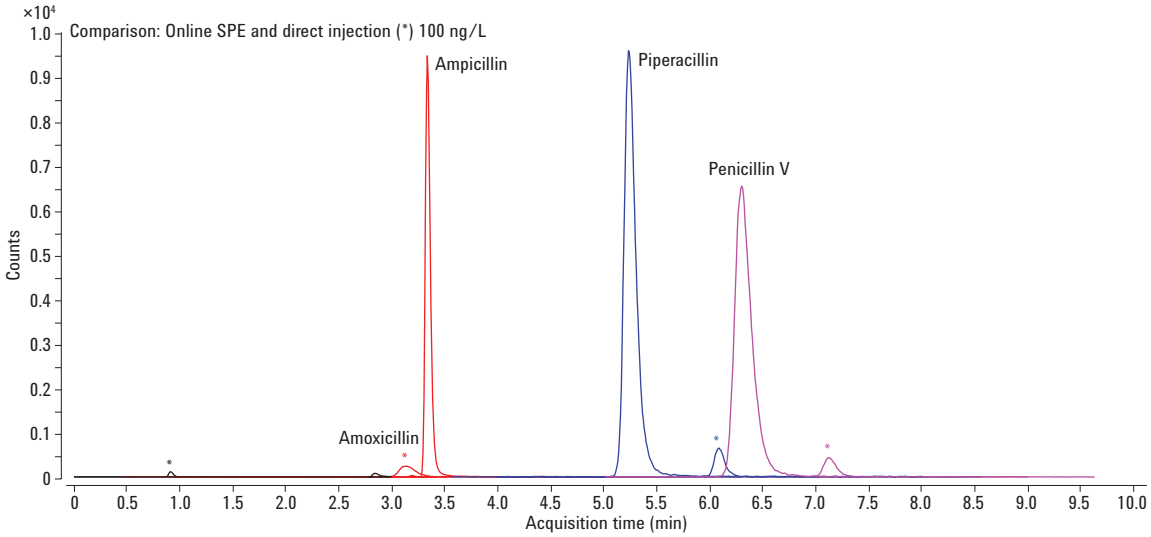

Figure 3. Comparison of online SPE with a sample volume of 1.5 mL and direct injection of 100 µL of a water sample spiked to a concentration of 100 ng/L; the asterisks indicate the direct injection.

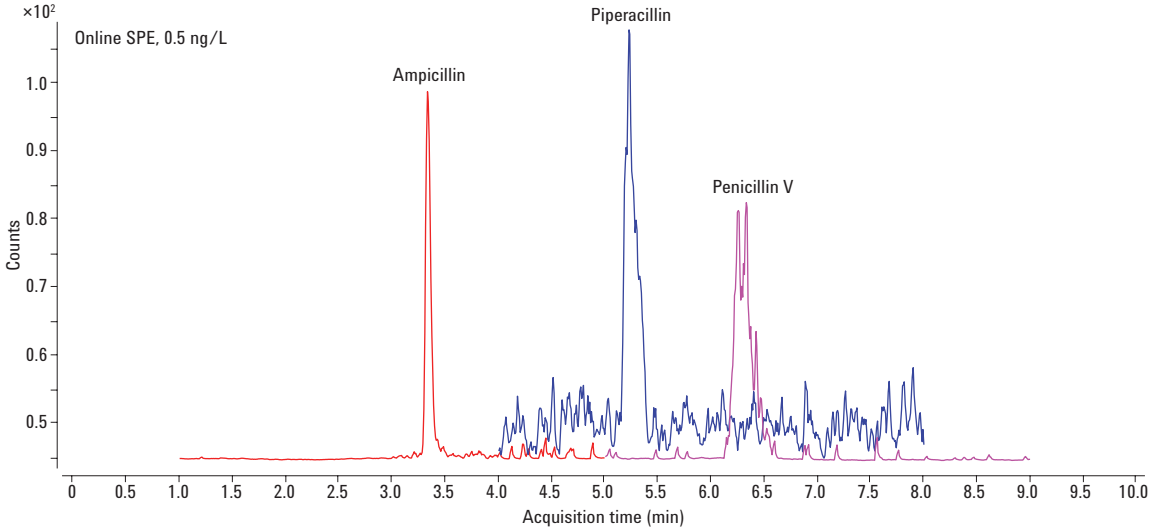

Figure 4. Chromatogram showing three β-lactam antibiotics analyzed with online SPE, concentration 0.5 ng/L.

Increased enrichment of amoxicillin was obtained when using acidified water for loading the SPE cartridges. However, this resulted in decreased signals for the less polar analytes by up to a factor of two.

Calibration for the four β-lactam anitbiotics with direct injection displayed excellent precision and linearity, up to a concentration of 20 ug/L. Relative standard deviations (RSDs) for replicate injections (n = 12) of a water sample spiked to 1 µg/L were below 2 % for all compounds. Linearity for the online SPE method was slightly lower than for direct injection, with correlation coefficients (R^2) between 0.97 and 0.99. Precision of the online SPE method was calculated based on replicate injections (n = 5) of a water sample spiked to 5 ng/L. RSDs between 1.9 and 5.3 were observed. The lower precision of the online SPE method can be explained by the lower spike concentration, as well as by the additional enrichment step. Tables 7 and 8 show selected parameters for the method characterization of direct injection and online SPE.

Table 8. Validation Parameters LOD, Linearity, Precision, and Recovery of Online SPE (Enrichment Included)

	LOD (ng/L)	R^2 (n = 8)	RSD (%) (n = 5, c = 5 ng/L)	Peak width (s) (c = 100 ng/L)	Recovery (%) (n = 5, c = 100 ng/L)
Amoxicillin	–	–	–	–	6.5
Ampicillin	0.2	0.980	5.3	3.2	51.7
Piperacillin	0.5	0.989	3.7	6.7	94.1
Penicillin V	0.2	0.969	1.9	10.6	83.9

Conclusion

A method for the quantification of four β-lactam antibiotics in the low ng/L-range by UHPLC/MS/MS was developed. This method takes full advantage of the low delay volumes of the Agilent 1290 Infinity II UHPLC system, and the increased ionization efficiency of the AJS ionization source, coupled to the highly sensitive and robust Agilent 6470 Triple Quadrupole LC/MS system. With a direct aqueous injection of 100 µL, precision was better than 2 %, and linearity from the low ng/L range up to 20 µg/L was observed. The performance of the method allows the analysis of β-lactam antibiotics in water samples in the low ng/L concentration range, which is typical for wastewater effluent and surface water samples [4].

Adding external valves to the system configuration allows switching between direct aqueous injection and online SPE with delay volume reduction directly from the Agilent MassHunter software, without any hardware modification. Online SPE of 1.5 mL of the water sample resulted in even lower LODs of 0.2 to 0.5 ng/L. Recoveries ranged between 51.7 and 94.1 % for ampicillin, piperacillin, and penicillin V. Due to the low recovery (6.5 %), the most hydrophilic compound amoxicillin was excluded from the online SPE method. Further optimization of the loading conditions and the SPE material would be required to include this compound in the online SPE method.

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