

# Reliable Determination of Sulfonamides in Water Using UHPLC Tandem Mass Spectrometry



## Authors

Xiulan Zhang<sup>1</sup>, Chaofei Zhu<sup>1</sup>,  
Jing Guo<sup>1</sup>, Meiling Lu<sup>2</sup>,  
Cuiling Wu<sup>2</sup>, Wei Du<sup>2</sup>,  
Wenlong Yang<sup>1</sup>, Liang Dong<sup>1</sup>,  
and Yeru Huang<sup>1</sup>

<sup>1</sup> National Research Center  
for Environmental Analysis  
and Measurement,  
Beijing 100029, China

<sup>2</sup> Agilent Technologies  
(China) Co. Limited,  
Beijing 100121, China

## Abstract

Antibiotics have been found to contaminate environmental water bodies widely. This application note describes a reliable method for determination of one class of antibiotics, sulfonamides, in water by coupling solid-phase extraction (SPE) with liquid-phase chromatography-tandem quadrupole mass spectrometry (LC/MS/MS). The LC/MS/MS method described delivered excellent linearity of quantitation for 19 compounds in water over the range of 0.5 to 100 µg/L, with linear regression coefficients higher than 0.998. Limit of quantitation (LOQ) values for the method (S/N = 10) were estimated at the several ppt level in water for all compounds. Spiking recovery test in pure water at the level of 20, 200, and 400 ng/L demonstrated that majority of recovery values ranged from 70% to 96% with RSD (n = 6) below 15%. The spiking recoveries in surface water at 20 ng/L ranged from 80% to 90% with RSD lower than 20%. These results demonstrate that the developed method can provide sensitive, accurate, and reliable analysis for real surface water samples.

## Introduction

It is estimated that over 100,000 tons of antibiotics have been used by humans and livestock worldwide annually, and there is increasing concern about the fate of these substances in the environment as the majority of the antibiotic is excreted into the environment without effective treatment.<sup>1</sup> Recent research observed that the level of some specific antibiotics in water in some Asian countries can even be as high as 450 ug/L.<sup>1</sup> Such contamination may induce antibiotic resistance, interrupt the balance of the microenvironment, and further affect the ecosystem. A number of surveys have shown that sulfonamides are one of the major classes of antibiotics present in water bodies in China.<sup>2,3</sup> To improve public awareness and risk assessment, a reliable and robust method is needed for routine monitoring of this group of antibiotics in water. US EPA 1694 method for group 1 analytes describes a method involving nine sulfonamides and one related substance.<sup>4</sup> In China, more sulfonamides have been used and frequently reported in the environment water matrixes.<sup>2,3</sup> Hence, regulation method development has been focused on the specific group of commonly reported sulfonamides and related substance in China. This study demonstrates a robust method for the simultaneous determination of 18 common sulfonamides and one co-existent substance in water using offline SPE and LC/MS/MS, meeting the criteria for regulation usage.

## Experimental

### Chemicals and reagent

Total 19 compounds including 18 sulfonamides and one commonly coexistent substance (trimethoprim) were selected for monitoring. In addition, four isotopic labeled internal standards (IS1-IS4) were included, with three of them (IS1-IS3) as surrogate internal standards which were added before

sample preparation for quantitation usage; and the remaining one (IS4) was added in the last step of sample preparation for evaluating the absolute recovery. The detailed compound information was shown in Table 1. LC/MS grade methanol (Merck) and Milli-Q water were used to prepare mobile phase, while other reagents such as formic acid, ammonia, and ammonium acetate are HPLC grade from Sigma-Aldrich.

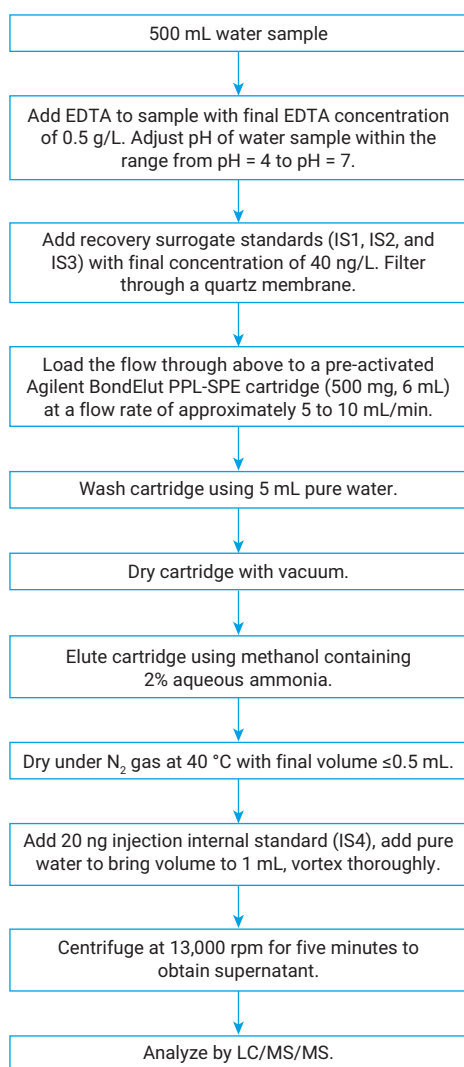
### Instrument conditions

LC Conditions			
Agilent 1290 Infinity II UHPLC System	• 1290 Infinity II binary pump with degasser • 1290 Infinity II autosampler with needle seat backflush function • Column temperature compartment		
Column	Agilent InfinityLab Poroshell C18, 2.1 × 150 mm, 2.7 μm		
Mobile Phases	A) 0.2% Acetic acid in water B) Methanol		
Flow Rate	0.30 mL/min		
Column Temperature	35 °C		
Injection Volume	2.0 μL		
Post Time	3 minutes		
Gradient	Time (min)	%A	%B
	0	90	10
	8	60	40
	12	35	65
	13	5	95
	16	5	95
MS Conditions			
MS	Agilent 6470 LC/MS/MS and Agilent Ultivo LC/MS/MS		
Ion Source	Agilent Jet Stream (AJS)		
Ionization Mode	Positive		
Capillary Voltage	3,500 V		
Nozzle Voltage	500 V		
Nebulizer Gas (N <sub>2</sub> ) Pressure	30 psi		
Drying Gas (N <sub>2</sub> ) Temperature	325 °C		
Drying Gas Flow Rate	6 L/min		
Sheath Gas (N <sub>2</sub> ) Temperature	350 °C		
Sheath Gas Flow Rate	11 L/min		
Acquisition Mode	MRM		

## Method

### Sample preparation procedure

The blank water sample was pure bottled water from a local market, and the surface water was collected from a local city river. The volume of sample can range from 200 to 1000 mL, and 500 mL was used in the described method here. The sample was prepared according to the following flowchart diagram.



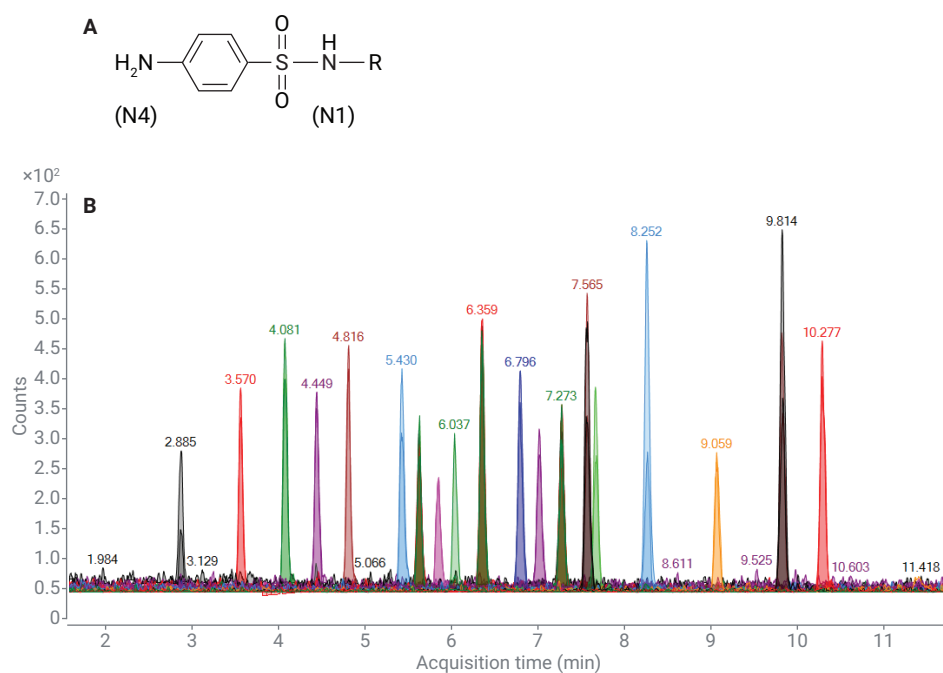
## Results and discussion

### Optimization of LC/MS/MS conditions

Sulfonamides contain a common structure (shown in Figure 1A), and can easily be protonated under acidic conditions. Hence, positive ionization and an acidic mobile phase were selected for optimizing the MS/MS acquisition parameters using an Agilent 6470 LC/MS/MS, with the resultant parameters listed in Table 1. Methanol/acidified water can provide baseline separation for both groups of isomers, but acetonitrile/acidified water showed poor resolution of one pair of isomers (sulfamethoxypyridazine and sulfameter). Therefore,

methanol/acidified water was selected as the binary mobile phase. Both Agilent Eclipse Plus and Poroshell C18 columns provided baseline separation for the isomers. Here, a Poroshell column was used due to its relatively low backpressure. The gradient elution was further adjusted to ensure that the analytes eluted out of the column in short time with good separation (Figure 1B).

An additional four minutes of flush time was used for the removal of highly retained matrix components. The same MRM transitions and other parameters are also applicable to the Agilent Ultivo LC/MS/MS system.



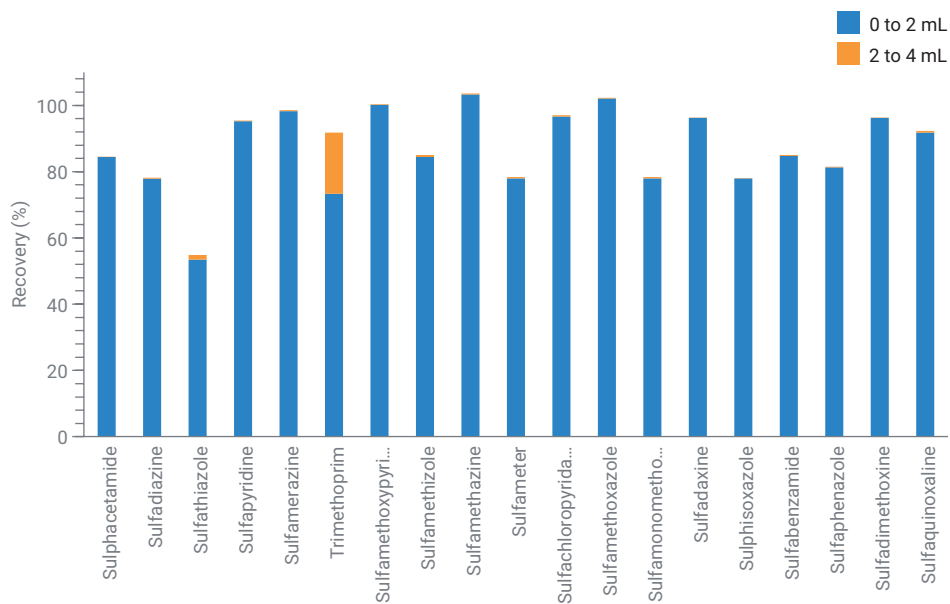
**Figure 1.** (A) The common structure of sulfonamides. (B) The typical overlapped MRM chromatograms for sulfonamides at the lowest calibration concentration of each analyte (0.5  $\mu\text{g/L}$ ) on an Agilent 6470 LC/MS/MS.

**Table 1.** Optimized parameters for MRM acquisition of each analyte.

No.	Name	RT (min)	Precursor m/z	Frag. Vol. (V)	Quantitation Ion		Qualification Ion		IS Label
					m/z	CE (V)	m/z	CE (V)	
1	Sulphacetamide	2.88	215.2	65	156	7	108	20	IS1
2	Sulfadiazine	3.57	251.3	100	156	16	92	32	IS1
3	Sulfathiazole	4.081	256	100	156.1	14	65.2	56	IS1
4	Sulfapyridine	4.444	250.3	110	91.9	32	156	16	IS1
5	Sulfamerazine	4.811	265.3	110	92	32	65.2	58	IS2
6	Trimethoprim	5.430	291.3	120	230.1	26	261	28	IS2
7	Sulfamethoxyipyridazine	5.629	281.3	100	156	16	92.2	32	IS2
8	Sulfamethizole	5.844	271.3	90	92.1	28	65.1	56	IS2
9	Sulfamethazine	6.037	279.3	100	65.2	64	92.1	32	IS2
10	Sulfameter	6.354	281.3	110	156	16	92.2	34	IS2
11	Sulfachloropyridazine	6.796	285	100	156	14	92	36	IS2
12	Sulfamethoxazole	7.017	254.3	100	65.2	54	156	16	IS2
13	Sulfamonomethoxine	7.273	281.3	70	156	18	92.2	34	IS2
14	Sulfadaxine	7.564	311.4	130	156	18	92	34	IS2
15	Sulphisoxazole	7.666	268.3	100	155.9	12	92.1	30	IS2
16	Sulfabenzamide	8.257	277.2	80	156	12	108	28	IS3
17	Sulfaphenazole	9.064	315.4	130	65	78	92	43	IS3
18	Sulfadimethoxine	9.824	311.4	130	156	22	92	38	IS3
19	Sulfaquinolaxine	10.277	301.4	110	156.1	16	92.1	36	IS3
IS1	C <sub>13</sub> -Sulfapyridine	4.444	256	110	162	17	-	-	-
IS2	C <sub>13</sub> -Trimethoprim	5.415	294	120	230	38	-	-	-
IS3	C <sub>13</sub> -Sulfadimethoxine	9.824	317	130	162	21	-	-	-
IS4	<sup>13</sup> C <sub>6</sub> -Sulfamethizole	5.844	277	90	2	28	-	-	-

### Optimization of sample preparation procedure

An Agilent Bond Elut PPL SPE cartridge was selected for enriching and cleaning the sulfonamides from water. Initially, pure water without pH adjustment was loaded to the PPL cartridge, and methanol containing 2% ammonia was selected to elute the analytes from the cartridge. Figure 2 shows the analysis of the collected analytes during the first 2-mL elution and the second 2-mL elution. All analytes exhibit total elution recovery higher than 75%, with the exception of sulfathiazole, which shows a total recovery of 54.3%. Considering the pKa of sulfathiazole is approximately 7.0, the pH of the sample was then examined. As shown in Figure 3, with 20 ng/L of the loaded analytes, all sulfonamides were recovered at



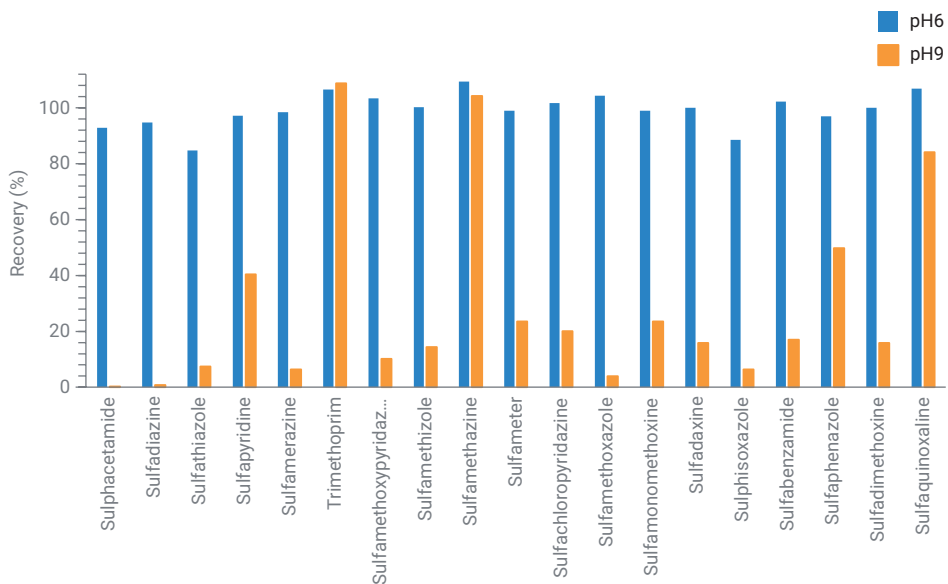
**Figure 2.** Recovery of each analyte from the SPE cartridge during the first 2 mL elution and the following 2 mL (2 to 4 mL) elution.

>80% with a pH = 6.0, while pH = 9.0 led to lower recovery. Further detailed examination of pH suggested that pH ranged from 4 to 7 all led to satisfactory recovery. Hence, the water samples need to adjust to the range from pH = 4 to pH = 7 before loading to cartridge for enrichment and cleanup.

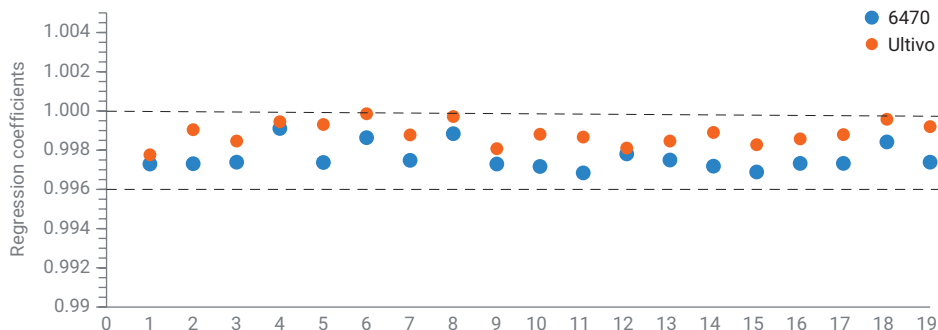
### Method linearity and sensitivity

Sulfonamides at 0.5, 1, 2, 5, 10, 20, 50, 100, 200 µg/L in methanol were used to evaluate the linear dynamic range of the method. Isotopically labeled internal standard dilution method was applied for calibration to diminish the quantitation bias due to the matrix interference effect. Figure 4 shows that the regression coefficients for all studied sulfonamides demonstrated excellent linearity with regression coefficients ( $R^2$ ) higher than 0.996. The sensitivity as signal-to-noise ratio (S/N) was estimated based on the extracted MRM chromatograms obtained at the lowest calibration level of 0.5 µg/L (Figure 1B).

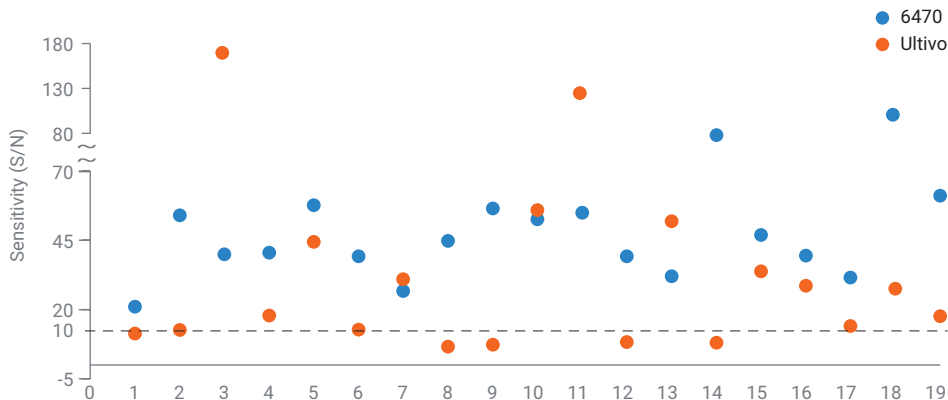
It was observed that S/N for each sulfonamide was greater than 10 when using a 6470 LC/MS/MS, ranging from 21 to 101 (Figure 5). For the Ultivo LC/MS/MS, four analytes showed S/N values slightly lower than 10, while all other S/Ns were higher than 10, though the majority exhibit lower S/Ns compared to the 6470 LC/MS/MS (Figure 5). Considering the enrichment factor of 500, it is estimated that as low as 1 ng/L and 2 ng/L in 500 mL of water can reliably be detected after effective enrichment when using a 6470 LC/MS/MS and an Ultivo LC/MS/MS for determination, respectively.



**Figure 3.** Comparison of the recovery for the enriching and cleanup procedure when the pH of water samples was maintained at 6 and 9 during loading, respectively.



**Figure 4.** Linear regression coefficient ( $R^2$ ) for sulfonamides with concentration ranging from 0.5 to 200 µg/L in water using either an Agilent 6470 LC/MS/MS or an Agilent Ultivo LC/MS/MS. **Note:** Analyte numbers (X-axis) follow the order of elution shown in Figure 1B, and the corresponding name is shown in Table 1.



**Figure 5.** Sensitivity (S/N) of each sulfonamide at the lowest calibration level (0.5 µg/L). **Note:** Analyte numbers (X-axis) follow the order of elution shown in Figure 1B and the corresponding names are shown in Table 1; the noise (N) of a chromatogram peak is based on the peak-to-peak algorithm.

### Method accuracy and precision

Method accuracy and precision were evaluated by examining the recovery of 10, 100, and 200 ng of sulfonamides spiked into 500 mL of water (corresponding concentrations are 20, 200, and 400 ng/L). Figure 6 shows that the majority of the recovery values were within 70.5% to 96.4% with the exception of the lowest value of 56.3% for sulphisoxazole at the spiking level of 20 ng/L and the other three values ranging from 63.0% to 69.7%. The RSD for all analytes were within 0.3 to 12.3%. The results demonstrate the accuracy and reliability of the method.

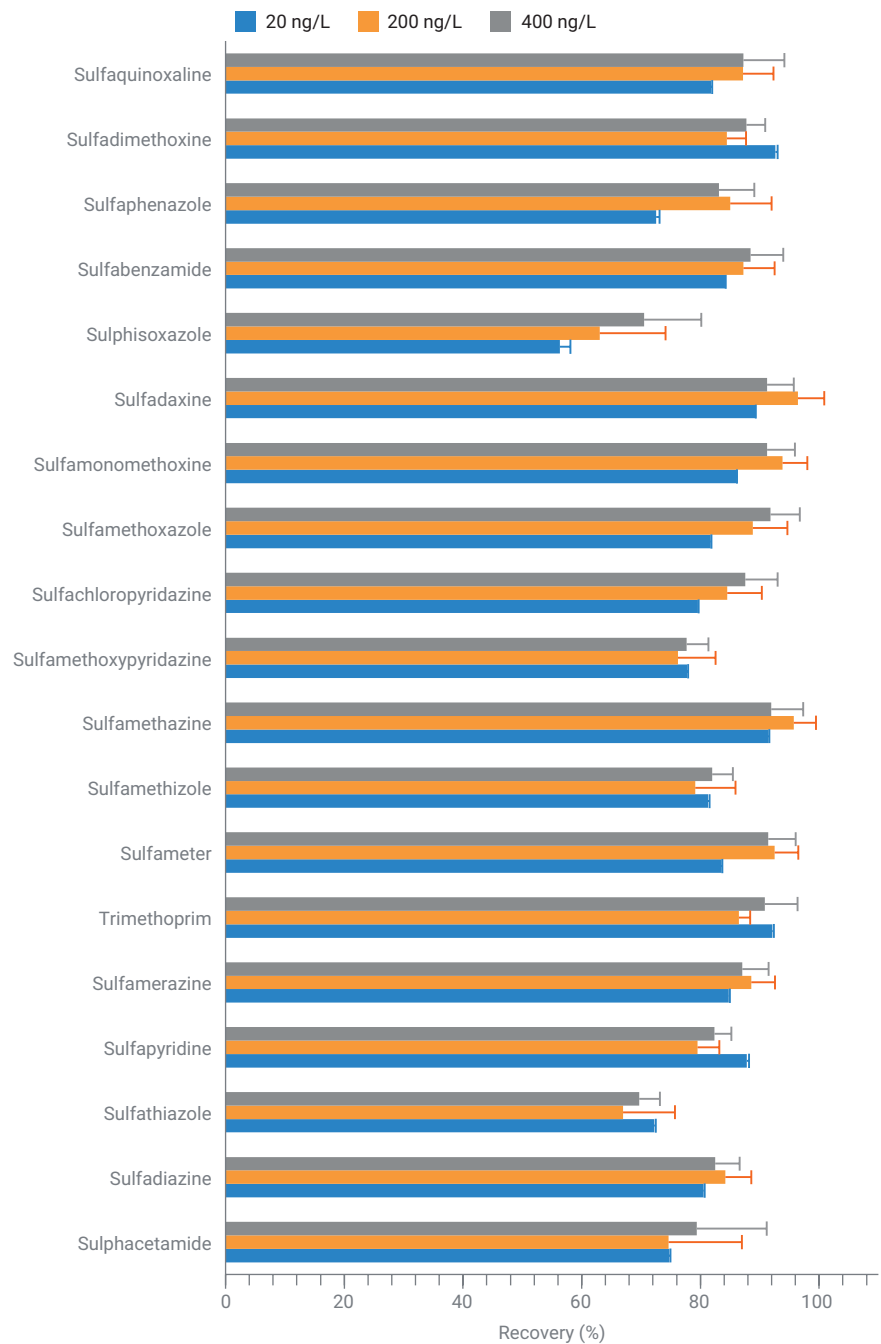


Figure 6. Recoveries for the sulfonamides spiked into pure water at three levels (20, 200, 400 ng/L).

The method was also evaluated using surface water (collected from a local river) by spiking 10 ng of sulfonamides into 500 mL of water (corresponding to 20 ng/L in original sample). The spiked samples were subjected the same procedure shown in the Method section, and the recovery and precision were evaluated. Table 2 shows that the spiking recoveries for all 19 analytes ranged from 80% to 90%, with RSD below 20% (n = 4), indicating that the method is also applicable to surface water matrices.

## Conclusion

This study demonstrates an SPE-UHPLC/MS/MS method for the simultaneous determination of trace amounts of 19 sulfonamide antibiotics in water. This method has excellent linearity, high sensitivity, and high accuracy and precision whether using an Agilent 6470 LC/MS/MS or an Agilent Ultivo LC/MS/MS, meeting the criteria specified in China GB/T 27417-2017 on conformity assessment-guidance on validation and verification of chemical analysis method.<sup>5</sup> Further evaluation of the method in environmental water matrices also demonstrates that the method can be applied for reliable routine monitoring of sulfonamides in surface water.

**Table 2.** The recovery and precision of analytes spiked in the surface water matrix at the level of 20 ng/L (N = 4).

No	Name	Average Recovery (%)	RSD (%)
1	Sulphacetamide	83.1	18.9
2	Sulfadiazine	85.4	15.4
3	Sulfathiazole	85.6	16.4
4	Sulfapyridine	88.1	17.5
5	Sulfamerazine	84.5	14.7
6	Trimethoprim	89.5	16.9
7	Sulfamethoxyipyridazine	82.4	15.6
8	Sulfamethizole	82.9	14.7
9	Sulfamethazine	83.3	16.2
10	Sulfameter	81.9	15.5
11	Sulfachloropyridazine	81.7	14.9
12	Sulfamethoxazole	80.2	11.6
13	Sulfamonomethoxine	82.0	14.6
14	Sulfadaxine	83.1	15.1
15	Sulphisoxazole	81.8	13.3
16	Sulfabenzamide	81.6	12.9
17	Sulfaphenazole	80.3	15.4
18	Sulfadimethoxine	80.8	16.9
19	Sulfaquinoxaline	80.7	16.5

## References

1. Danner, M.-C. *et al.* Antibiotic Pollution in Surface Fresh Waters: Occurrence and Effects. *Sci. Total Environ.* **2019**, 644, 793–804.
2. Zhang, Q.-Q. *et al.* Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. *Environ. Sci. Technol.* **2015**, 49, 6772–82.
3. Wang, D. *et al.* Pharmaceutical and Personal Care Products in the Surface Water of China: A Review (in Chinese). *Chin. Sci. Bull. (Chin.)* **2014**, 59, 743–751, doi: 10.1360/972013-370.
4. USEPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS, **2007**.
5. China GB/T 27417-2017: Conformity Assessment-Guidance on Validation and Verification of Chemical Analysis Method, **2017**.

[www.agilent.com/chem](http://www.agilent.com/chem)

This information is subject to change without notice.