D-JRP15-FED-AMR-WP4.1

Protocol for Antibiotics Quantification

Version 1
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A. Preliminary considerations

Sample collection for further quantification of antibiotics in **WP4** is described in the corresponding sampling protocols of each analysed matrix and/or compartment (see **annex of D-JRP15-FED-AMR-WP2.1).** Likewise, the sample identifiers should be consulted in the corresponding sampling protocols.

B. Sample preparation

1. Water samples

To the 100 mL of water sample, the IS will be added, the solution will be mixed, and left to incubate at room temperature in a dark place for 15 min. Then 6 mL of 0.5M sodium acetate, pH 5.6, and 15 µL of HFBA will be added and shaken by hand briefly for 5 min. Strata-X SPE cartridges will be conditioned sequentially with 5 mL of methanol, 5 mL of water, and 5 mL of 0.05M HFBA. Subsequently, the water samples will be loaded into the SPE cartridges with 50 mL reservoir at a flow no faster than 1 drop/5 s. Then the cartridges will be vacuum-dried for 5 min at a pressure ranging from 12 mmHg to 18 mmHg. The analytes will be eluted twice adding 3 mL of acetonitrile and 0.05M HFBA mixture (9:1, v/v). The eluates will be collected in 10 mL glass tubes and evaporated to dryness under a stream of nitrogen at 45 ± 5°C. Finally, the residues will be dissolved in 500 µL of 0.025% HFBA and filtered through 0.22 µm PVDF syringe filters into LC vials.

2. Soil samples

To 5 ± 00.1 g of soil sample the IS solution will be added before the extraction and the samples will be mixed and left to incubate at 4 °C in a dark place for 30 min. After adding 10 mL of acetonitrile, 0.5 mL of 0,1M $Na_2EDTA \times 2H_2O$ 0.5 mL of citric acid, pH 4.0, and 100 µL of 1M sodium acetate, pH 5.6, the samples will be homogenised with a vortex mixer for 2 min. Then the samples will be placed in an ultrasonic bath for 45 min, centrifuged at 4600 × g at 5 °C for 15 min, and the supernatants will be loaded into an Oasis HLB cartridges which was without any preconditioning, the cartridges serving as a filter. The filtered supernatants will be collected in glass tubes and evaporated to dryness under a stream of nitrogen at 45 ± 5°C. Finally, the residues will be dissolved in 500 µL of 0.025% HFBA and filtered through 0.22 µm PVDF syringe filters into LC vials.

3. Faeces/Manure samples

Sample preparation technique involved addition of Na_2EDTA and extraction with solution of formic acid in acetonitrile/water, and next with solution of ammonia in acetonitrile/water followed by filtration by SPE column, further evaporation and reconstitution before LC injection.

Table 1. description of the antimicrobials tested in the different matrices.

Group	Analytes	Matrices		
		Water	Manure/faeces	Soil
		LOQ [µg/L or kg]		
tetracyclines				
	chlortetracycline	1.0	50.0	5.0-10.0
	tetracycline			
	oxytetracycline			
	doxycycline			
sulfonamides				
	sulfamerazine	1.0	50.0	5.0- 10.0
	sulfamethazine			
	sulfamethoxazole			
	sulfadimethoxine			
	sulfathiazole			
	sulfamonomethoxine			
	sulfadiazine			
fluoroquinolones				
	danofloxacin	1.0	50.0	5.0-20.0
	difloxacin			
	enrofloxacin			
	ciprofloxacin			
	marbofloxacin			
	norfloxacin			
	nalidix acid			
	oxolinic acid			
	flumequine			
	sarafloxacin			
macrolides				
	tylosin	1.0-5.0	50.0	10.0-20.0
	erythromycin			
	spiramycin			
	tilmicosin			
	josamycin			
	tulathromycin			

C. LC-MS/MS analysis

The LC–MS/MS analysis will be performed on the Agilent 1200 HPLC system (Agilent Technologies, Germany) with an automatic degasser, a binary pump and an autosampler connected to the Sciex API 4000 triple quadrupole mass spectrometer (Sciex, Canada) or on the UHPLC/HPLC Shimadzu Nexera X2 (Shi-madzu, Japan) system connected to the QTRAP®4500/QTRAP®5500 triplequadrupole mass spectrometer (Sciex, Framingham the USA).

1. API 4000 + Agilent 1200 HPLC

The chromatographic separation will be performed on the Luna C18 (2) 100A column (50 × 3.0 mm, particle size 3 µm, (Phenomenex)

Temperature: 30 °C
Flow rate of mobile phase: 400 µl/ min
Injection volume: 30 µl

Table 1. Mobile phase gradient programm for the Agilent 1200 HPLC system

Time (min)	ACN (%) A	0,025% HFBA (%) B
0	15	85
1,00	40	60
3,00	60	40
4,00	95	5
7,01	15	85
13,00	15	85

2. QTRAP 4500/5500 + Nexera X2 UHPLC

The chromatographic separation will be performed on the ZORBAX SB-C18 column (50 mm × 2.1 mm × 1.8 µm) (Agilent)

Temperature	35 °C
The flow rate of mobile phase	700 µl/min
Injection volume	5 µl

Table 2. Mobile phase gradient programm for the Nexera X2 UHPLC system:

Time (min)	ACN (%) A	0,025% HFBA (%) B
0	10	90
4,00	80	20
5,30	80	20
5,31	10	90
7,00	10	90

D. References

Gbylik-Sikorska Małgorzata, Posyniak Andrzej, Śniegocki Tomasz**,** Żmudzki Jan. Liquid chromatography-tandem mass spectrometry multiclass method for the determination of antibiotics residues in water samples from water supply systems in food-producing animal farms. 2015 Chemosphere 119:8-15.