Karlsruher Institut für Technologie

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Study Project

Development of Analytical Method for Determination of Selected Antibiotics of Last Resort in Aqueous Samples via LC-MS/MS

Engler-Bunte-Institut
Water Chemistry and Water Technology

Name:	Mingjiao ZHENG
Matric Number:	2082397
Supervisors:	Prof. Dr. rer. nat. Harald Horn
	M. Sc. Alexander Timm

Abstract

Linezolid and tedizolid are both antibiotics of last resort (AOLRs) that exhibit excellent treatment effects on complicated infections caused by multi-resistant bacteria. However, even at low dosages, antibiotics have raised worldwide concern by means of prolonged discharge into the aquatic environment, which is likely to induce antibiotic resistance. In this project, a rapid, sensitive and accurate Liquid Chromatography (LC) – Tandem Mass Spectrometry (MS/MS) analytical method for simultaneous determination of these two antibiotics in ultrapure water at ultra-trace level (low ng/L) was developed. Selected antibiotics and their isotopically labeled substances (ISs) linezolid-D3 and tedizolid-D3 were separated on a reversed-phase EclipsePlusC18 RRHD column (2.1 mm x 50 mm, 1.8 µm) at a flow rate of 0.4 mL/min. The mobile phase consisted of ammonia ($5*10^{-4}$%) and methanol containing 0.05% (v/v) of formic acid, which followed a determined elution gradient. Detection was achieved via an Agilent mass spectrometer, which was operated with electrospray ionization (ESI) source and quantitative determination was accomplished under dynamic Multiple Reaction Monitoring (MRM) scanning mode. Meanwhile, this method has been validated and applied to assess the concentration levels of selected AOLRs in river Rhine. The limits of detection (LODs) were 2 ng/L for linezolid and 1 ng/L for tedizolid. Calibration curves were established over the concentration range from their LODs to 5000 ng/L, with coefficients of correlations higher than 0.9993 and 0.9963 for linezolid and tedizolid respectively. Validation of this method proved that the linearity, limits of detection, precision, trueness, ruggedness were all within the acceptable limits. Therefore, the developed LC-MS/MS method for the analysis of linezolid, tedizolid in ultrapure water was accurate and promising to be applied for the determination of them in surface water at low concentration levels. In river Rhine water, neither target antibiotics nor their isotopically labeled substances were found at trace levels.

Key words: AOLR, linezolid, tedizolid, LC-MS/MS

Content

1 Introduction

Antibiotics of last resort (AOLRs) are a group of antibiotics that are employed once all other antibiotic medicines have failed to generate adequate curative effects on specific patients [1]. Linezolid and tedizolid are relatively new and prevalent AOLRs.

Linezolid is the first member in the oxazolidinone class to be tested clinically. In order to inhibit the initiation of aerobic Gram-positive bacterial protein synthesis, it binds to sites on the 23S rRNA of the 50S subunit in bacteria so that a functional 70S-initiation complex, which is an essential substance in the bacterial translation process, cannot be formed [2]. Due to its unique action mechanism, the likelihood of cross-resistance between linezolid and other classes of antibiotic is low. As one of the most effective and safe medicines that satisfies the most significant needs in healthy systems announced by the World Health Organization (WHO), it is widely utilized for the treatment of hospital- and community-acquired infections of skin and pneumonia.

Tedizolid, the biologically active moiety of tedizolid phosphate ($C_{17}H_{16}FN_6O_6P$), is generated by the reaction of tedizolid phosphate with the plasma phosphatases in vivo [3]. In vitro, it exhibits a broad spectrum and high potency against even some linezolid-resistant strains that cause Acute Bacterial Skin and Skin Structure Infection (ABSSSI) [4]. Furthermore, tedizolid's long metabolic half-life allows once per day administration. The properties of two AOLRs are listed in Tab.1, and their chemical structures are shown in Fig.1.

However, antibiotic resistance, caused by mutation, efflux pumps, altered target sites, horizontal gene transfer and so forth, has become an intractable issue globally. In clinical trials, the misuse and overuse of antibiotic medicines accelerate the natural evolution of resistant bacterial strains, thus contributing to the spread of antibiotic resistance. Resistant bacteria are capable of surviving through conventional antibiotic therapies, in that case, routine medicines become ineffective for compromised patients, possibly resulting in the emergence of uncontrollable development of diseases. In the aquatic environment, antibiotics pose a potential threat as well since continuous exposure even to low concentration may still induce faster genetic selection of more detrimental bacteria [5]. Therefore, the regulation, identification, and determination of antibiotics are of prime significance.

Nevertheless, the trace-level content, the disparate physio-chemical properties of antibiotics and the complex matrix effects in the aquatic environment make the determination of antibiotics challenging. As a consequence, highly sensitive and selective methods are demanded. Chromatographic techniques are preferable when it comes to simultaneous analysis of different antibiotics. Due to the fact that antibiotics are mostly polar, thermally labile or insufficiently volatile compounds, the direct determination of antibiotics by gas

chromatography (GC) requires pre-derivatization steps [6], which make it unpractical even though GC has high separation efficiency. Hence, liquid chromatography (LC) became the preferred technique in this project, in that it's able to separate antibiotics with simplified sample preparation process, which provides a higher sample throughput.

Among different detection techniques coupled to LC system, such as fluorescence, ultraviolet, electrical conductivity or refractive index detectors, the application of MS especially tandem MS exhibits impressively high accuracy and selectivity. As to the interface, ESI source is particularly suitable for polar and thermally unstable substances analysis. What's more, dynamic MRM mode excels at the identification of multi-classes analytes in complex matrices in the same chromatographic run by selecting specified transitions from precursor to product ions. However, to satisfy the limits of detection (LODs) required for analysis, pre-concentration steps are needed in some cases [6].

The ISs, linezolid-D3 and tedizolid-D3, are typically of great importance. First of all, they assist the confirmation of analytes by the similar retention time frame because of similar structures. Therefore, the matrix effects caused by plenty of disturbing molecules in wastewater, surface water or body fluids can be lessened to some extent. Secondly, ISs can be used for quantitative analysis. ISs are added to every sample to the same concentration and the peak area of antibiotic itself will be compared with that of the labeled compound. With the peak area ratio, the concentration of antibiotics can be calculated with higher accuracy. On the other hand, ISs are likely to be the interfering substances as well, reducing the signals produced by analytes of interest. In that case, ISs should be absent in the measurements.

The aim of the project is to develop and validate an analytical method for determining linezolid and tedizolid in ultrapure and surface water by an LC-MS/MS system. Experimental conditions have been optimized to achieve appropriate resolution, low detection limits, high accuracy, high repeatability, with which complex environmental water samples are able to be analyzed with a higher degree of confidence.

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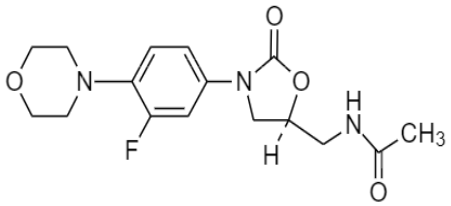

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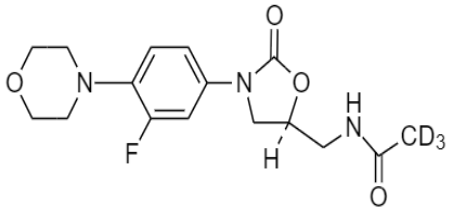

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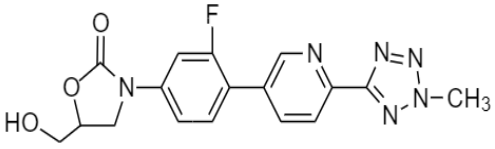

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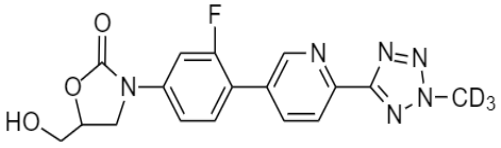


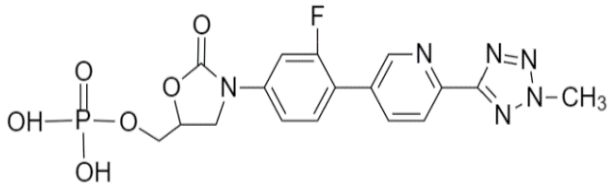
E) 

Fig. 1. Chemical structures of A) linezolid; B) linezolid-D3; C) tedizolid; D) tedizolid-D3; E) tedizolid phosphate

Tab. 1. Property of linezolid and tedizolid [2,4]

Property	Linezolid	Tedizolid
Chemical formula	$C_{16}H_{20}FN_3O_4$	$C_{17}H_{15}FN_6O_3$
Molar mass (g/mol)	337.4	370.3
Administration	intravenous/ oral	intravenous/ oral
Class	oxazolidinone	oxazolidinone
Water solubility (mg/L)	1440	608
Protein binding (%)	31	70- 90
Metabolic half-life (h)	4.5- 5.5	12
Absolute bioavailability (%)	100	91
Treatment of infections	skin and pneumonia	ABSSSI
Approved year by FDA	2000	2014

2 Experimental

2.1 Chemicals

Linezolid (analytical grade) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Tedizolid, tedizolid-D3 and linezolid-D3 standards (all analytical grade) were purchased from Toronto Research Chemicals (Canada). Ammonia (25%) and formic acid (FA, 98-100%) (both analytical grade) were supplied by Merck KGaA (Darmstadt, Germany). Acetonitrile (ACN), methanol, hydrochloric acid (HCl, 32%), ammonium acetate ($NH_4CH_3CO_2$) (all LC-MS grade) and ultrapure water were supplied by VWR International (Darmstadt, Germany). MilliQ water was generated by a Veolia Elga ultrapure water plant (High Wycombe, United Kingdom).

2.2 Instruments

The chromatographic system was composed of an Agilent 1290 Infinity II HPLC system (Agilent Technologies, USA) including a binary pump, an autosampler fitted with a 100 µL injection loop, and a reversed-phase EclipsePlusC18 RRHD

column (2.1 mm x 50 mm, 1.8 µm) (Agilent Technologies, USA) where chromatographic separation was accomplished.
The LC system was coupled to an analytically sensitive and robust Agilent 6470 Triple Quadrupole Mass Spectrometer (Agilent Technologies, USA) operated with an Agilent Jet Stream electrospray ionization (AJS-ESI) source using dynamic MRM as scanning mode. Chromatographic and mass spectrometric settings were controlled by Agilent Mass Hunter software.

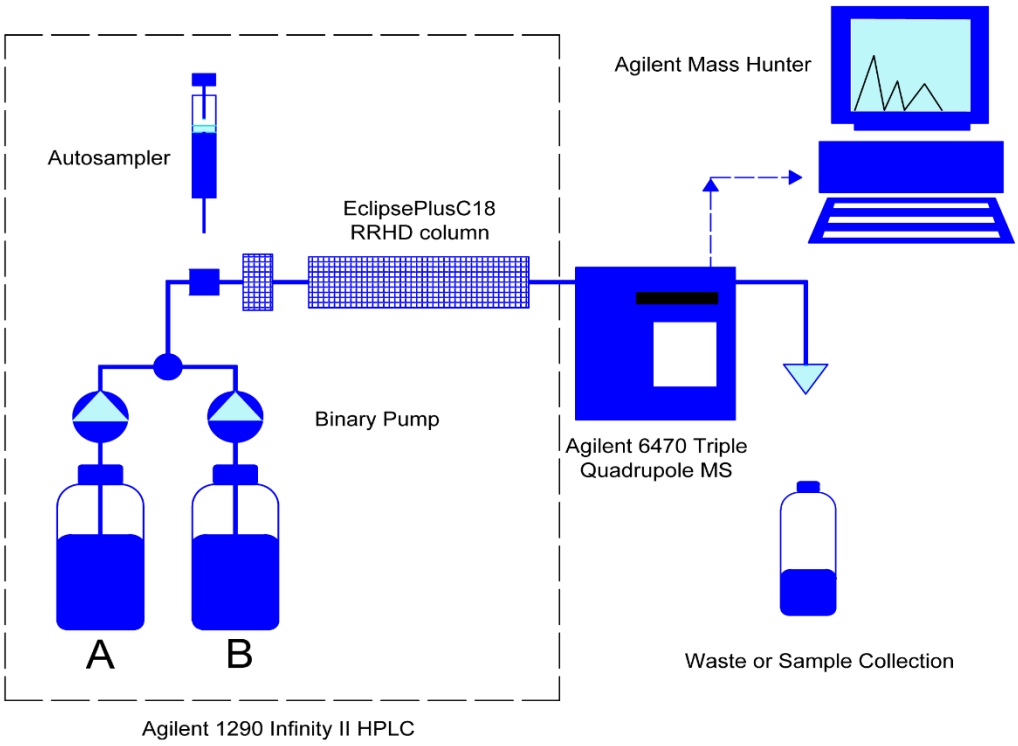


Fig. 2. Sketch of the employed LC-MS/MS system

2.3 Preparation of stock and working solutions

Stock standards of linezolid, tedizolid, linezolid-D3, and tedizolid-D3 were separately prepared to give a concentration of 1 g/L in acetonitrile.
Working samples of linezolid, tedizolid, and ISs for method development were prepared by dilution of the stock solution with ultrapure water to obtain the concentration of 1 µg/L. Standard calibration samples were also prepared by diluting the stock solution to gain 14 diverse concentration levels ranging from 0.2 ng/L to 5000 ng/L. Acidic (pH 5) and alkaline (pH 9) samples were prepared by addition of formic acid and ammonia respectively. Except for frozen samples that were stored at -15 °C before thawing, all other samples were stored in amber vials and kept in a refrigerator (4 °C) until use to avoid potential degradation.
10 µL of ISs was spiked to each sample performed by the autosampler in the

cases of the measurement with isotopically labeled substances.

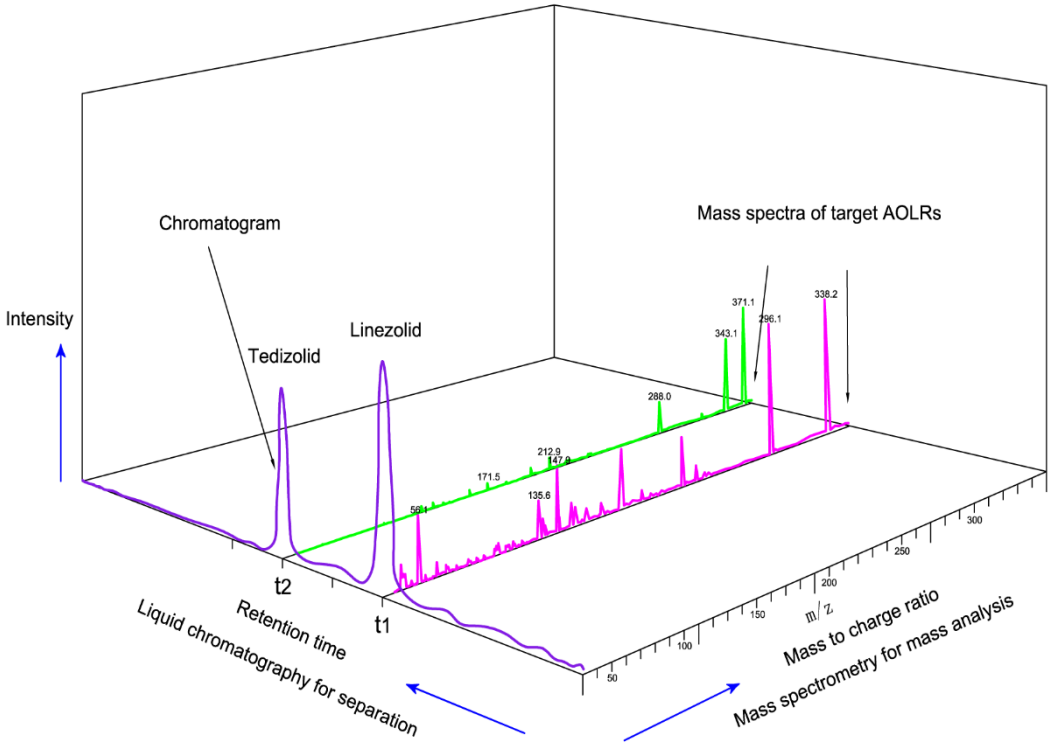


Fig. 3. Sketch of analysis principles of selected AOLRs in LC-MS/MS system

2.4 Method development

2.4.1 MS

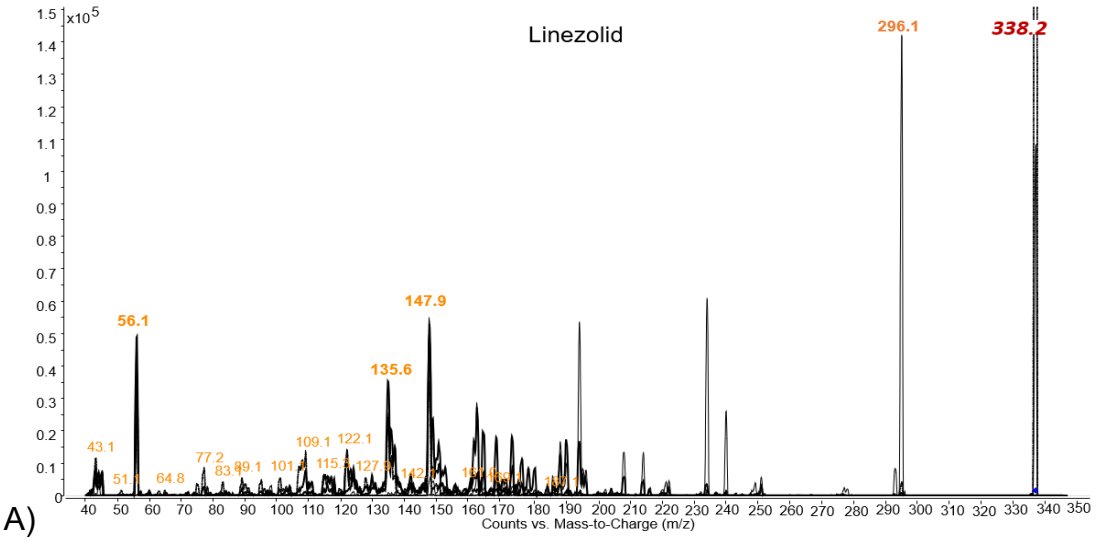
Identification of the precursor and product ions for each analyte was achieved in the full scan mode in which all ions in the range of m/z 40 to the precursor ion mass were detected. The fragmentor voltage and other full scan source parameters are listed in Tab.2. The full scan spectra of linezolid, tedizolid, and ISs as presented in Fig.4 indicated that all the precursor ions were protonated molecular ions, i.e. m/z 338.2 for linezolid, m/z 341.2 for linezolid-D3, m/z 371.1 for tedizolid, m/z 374.2 for tedizolid-D3. To improve the selectivity of MS for the analytes determination, precursor ions were collided to produce product ions. Therefore, dynamic MRM mode was selected, under which interfering fragment ions in aqueous solution were filtered out and only the most abundant product ions can be detected and quantified. To produce smoother peaks, more proper retention time and allow confirmation, only two most intensive product ions for each antibiotic were selected, which are bold in Tab.3. Nitrogen gas was used as sheath and collision gas.

The MS conditions for the detection of linezolid, tedizolid, and ISs in ultrapure water were investigated by injecting 10 µL of the analytes of interest at the concentration level of 500 µg/L and 100 µL at the concentration level of 2 µg/L

directly to the MS for tuning and optimization respectively. Then parameters will be automatically optimized by the instrument itself to obtain the highest sensitivity for quantitative analysis of the precursor and product ions of compounds. The optimal characteristic MS parameters inclusive of fragmentor voltage (V), collision energy (CE, V), polarity, and the MRM transitions for linezolid, tedizolid and IS are listed in Tab.3. MS parameters such as capillary voltage (V), dry gas flow (DGF, L/min), dry gas temperature (DGT, °C), nebulizer pressure (psi), nozzle voltage (V), sheath gas flow (SGF, L/min), sheath gas temperature (SGT, °C), were tuned and Fig.5 illustrates the respective effects of these parameters on the MS response, base on which compromised optimum operation conditions of MS were set as listed in Tab.4.

Tab. 2. Source parameters in full scan mode

Parameter	Values
Capillary voltage (V, +/-)	4000 (+), 3500 (-)
Gas flow (L/min)	8
Gas temperature (°C)	200
Nebulizer pressure (psi)	40
Vcharging (V, +/-)	500
Sheath gas flow (L/min)	12
Sheath gas temperature (°C)	300



A)

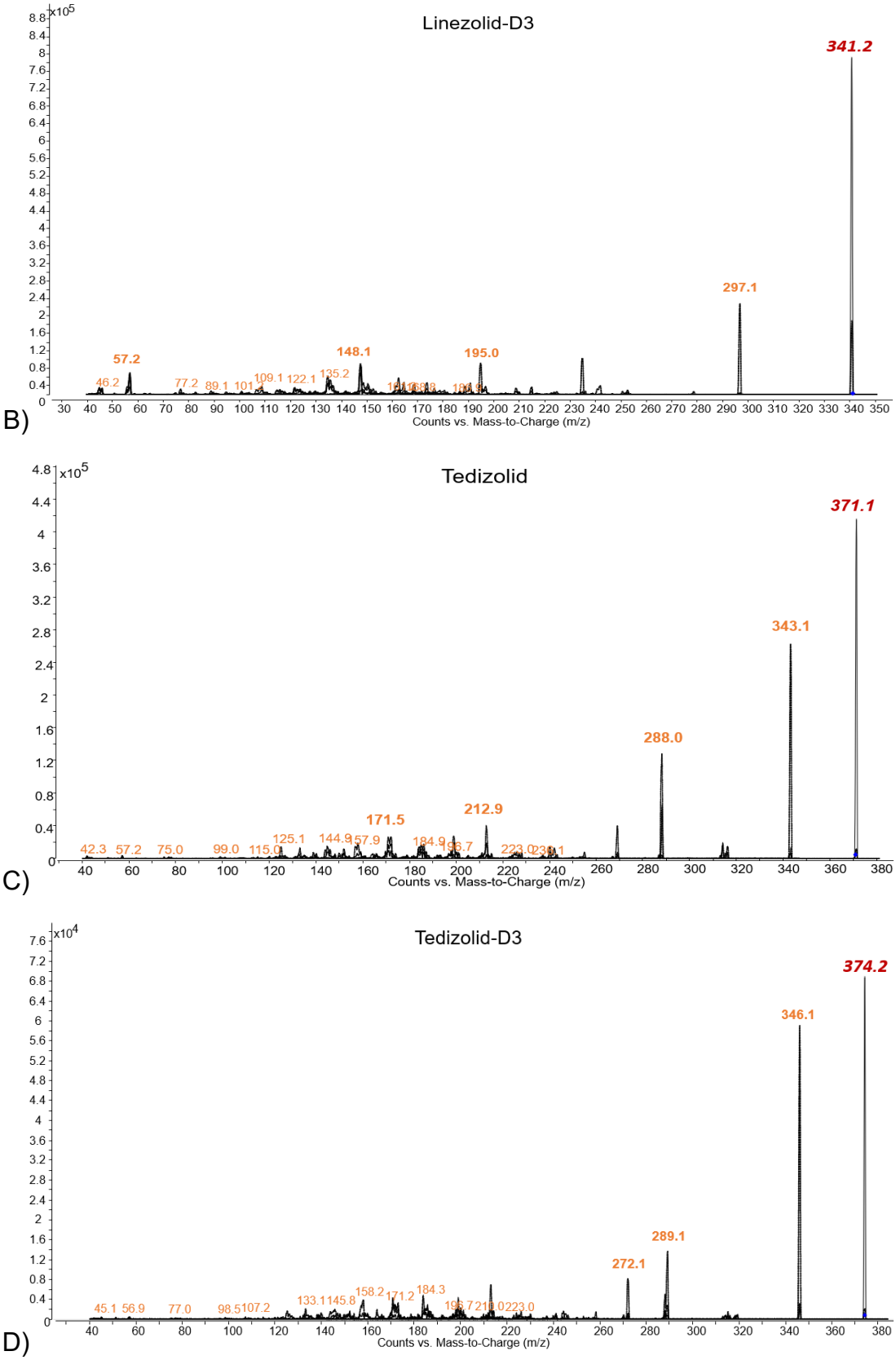


Fig. 4. Mass spectra of A) linezolid; B) linezolid-D3; C) tedizolid; D) tedizolid-D3 in full scan mode (C=2 µg/L)

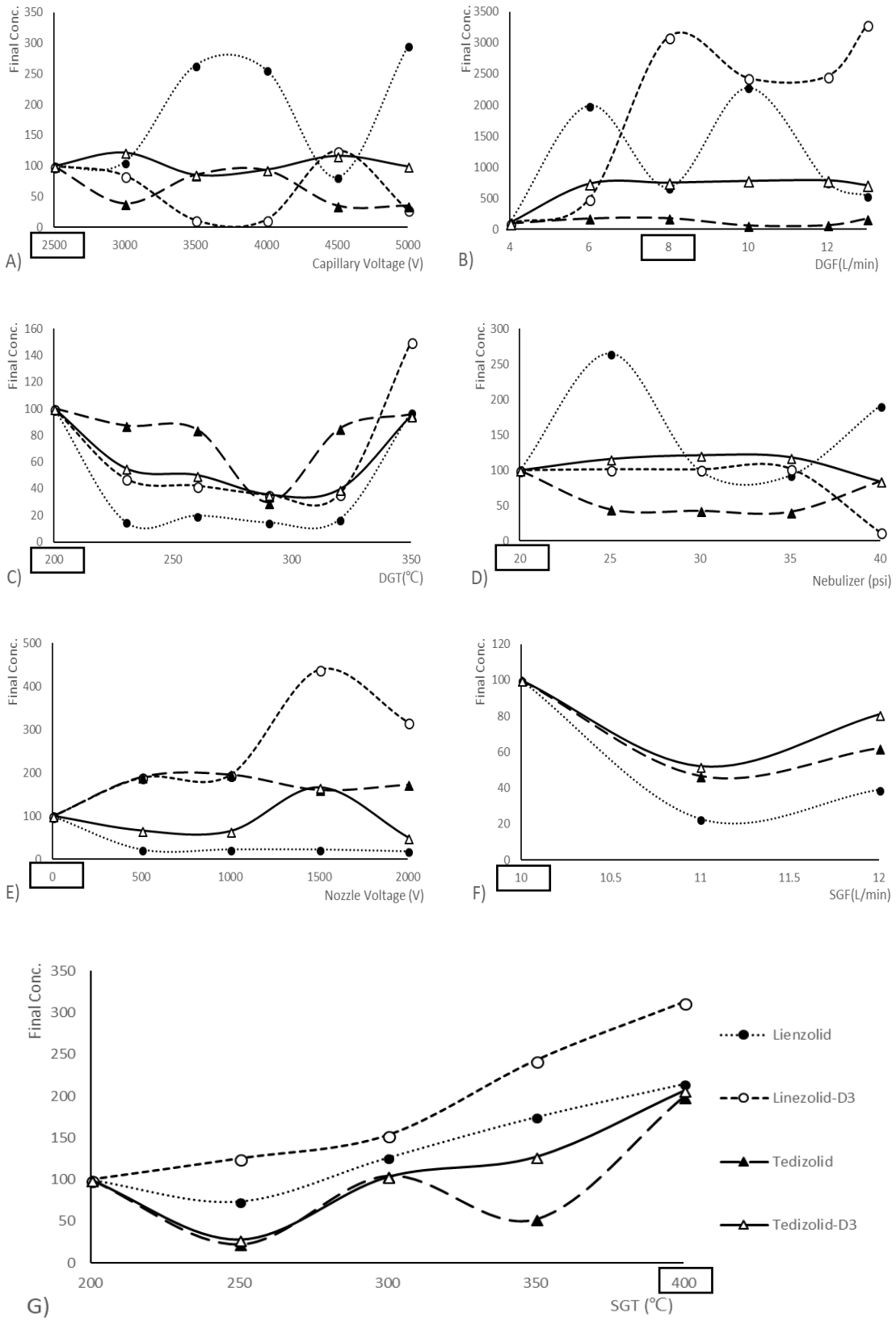


Fig. 5. Final conc. vs MS parameters of A) capillary voltage; B) DGF; C) DGT; D) nebulizer pressure; E) nozzle voltage; F) SGF; G) SGT (C=500 µg/L)

Tab. 3. MRM transition (positive mode) and conditions for analytes

Analytes	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	CE (V)
Linezolid	338.2	**296.1**	130	20
		147.9	130	48
		135.6	130	60
		56.1	130	36
Linezolid-D3	341.2	**297.1**	130	20
		195.0	130	24
		148.1	130	44
		57.2	130	36
Tedizolid	371.1	343.1	130	20
		288.0	130	32
		212.9	130	36
		171.5	130	64
Tedizolid-D3	374.2	**346.1**	145	20
		289.1	145	36
		272.1	145	36

Tab. 4. Optimized parameters by tuning

Parameter	Value
Capillary voltage (V, +/-)	2500
Dry gas flow (L/min)	8
Dry gas temperature (°C)	200
Nebulizer pressure (psi)	20
Nozzle voltage (V, +/-)	0
Sheath gas flow (L/min)	10
Sheath gas temperature (°C)	400

2.4.2 LC

LC was coupled with MS for the pre-separation of analytes of interest. Since the mobile phase composition significantly affects the peak height and shape, detention behavior of analytes on the LC column as well as the ionization efficiency, modification of the mobile phase with modifiers (e.g., formic acid, ammonia, ammonium acetate) was investigated. The column oven maintained at the temperature of 40 °C. Tab.5 lists all kinds of mobile phases applied in this project.

The optimum mobile phase was investigated using 1 µg/L of linezolid and tedizolid mixture as working solution. Following a determined gradient (Tab.6), 100 µL of working solution was introduced in LC-MS/MS system at a flow rate

of 0.4 mL/min with diverse mobile phases in sequence under previously determined optimum MS conditions. The run time was 8 minutes and 2 more minutes as post time for the return to the initial gradient.
Before starting to run with a different mobile phase, flush the tube for 4 minutes at a flow rate of 5 mL/min and wait five more minutes to wash and accommodate the column with the new type of mobile phase. Each mobile phase type was run twice and the second result was selected for comparison. The optimal mobile phase was decided by comparing the intensity, retention time, resolution and peak shape in the chromatograms. Checking the contaminant peaks with blank ultrapure water as working solution was necessary as well.

Tab. 5. Mobile phase (MP)

MP	Eluent A	Eluent B
MP1	pure H_2O	ACN + FA (0.05%)
MP2	FA (0.05%)	ACN + FA (0.05%)
MP3	ammonium acetate (1mM)	ACN + FA (0.05%)
MP4	ammonia ($5*10^{-4}$%)	ACN + FA (0.05%)
MP5	ammonia ($5*10^{-4}$%)	pure ACN
MP6	ammonia ($5*10^{-4}$%)	methanol
MP7	ammonia ($5*10^{-4}$%)	methanol + FA (0.05%)

Tab. 6. LC elution gradient

t (min)	Eluent A (%)		Eluent B (%)
0	90		10
7	0		100
8	0		100
10	90	Post time	10

Based on the comparison of MP1 to MP4, MP4 resulted in the highest intensity in the chromatogram (Fig.6). Therefore, alkaline condition (pH 8) surpassed acids and neutral water as eluent A in this case. Regarding ammonia as eluent A, MP4 to MP7 further investigated organic solutions containing acetonitrile or methanol as eluent B (Fig.7). In Fig.8, chromatograms of seven types of mobile phases were compared. M1 to M6 generated greatly different intensities for both antibiotics and MP6 showed the highest intensity, but the retention times were almost identical, that is, approximately 2.7 min and 3.2 min for linezolid and tedizolid respectively. MP7 led to similar intensity to MP6, but longer retention times were observed as 3.8 min and 4.6 min for linezolid and tedizolid respectively, which meant higher resolution.
In conclusion, MP7 consisting of ammonia ($5*10^{-4}$%) and methanol with 0.05%

(v/v) formic acid was chosen to be the working mobile phase due to the high intensity, proper retention time and adequate separation.
Impurity peaks were also observed at retention times of around 4.4 min and 6.1 min. Fig.9 illustrates the chromatograms when working solutions were analytes solution and blank pure water respectively using MP7 as the mobile phase. Indicated by the relative abundance at the same retention times, the control pure water sample was found to generate interference at 6.1 min. While the impurity peak existing at 4.4 min was unrelated to the pure water solvent.

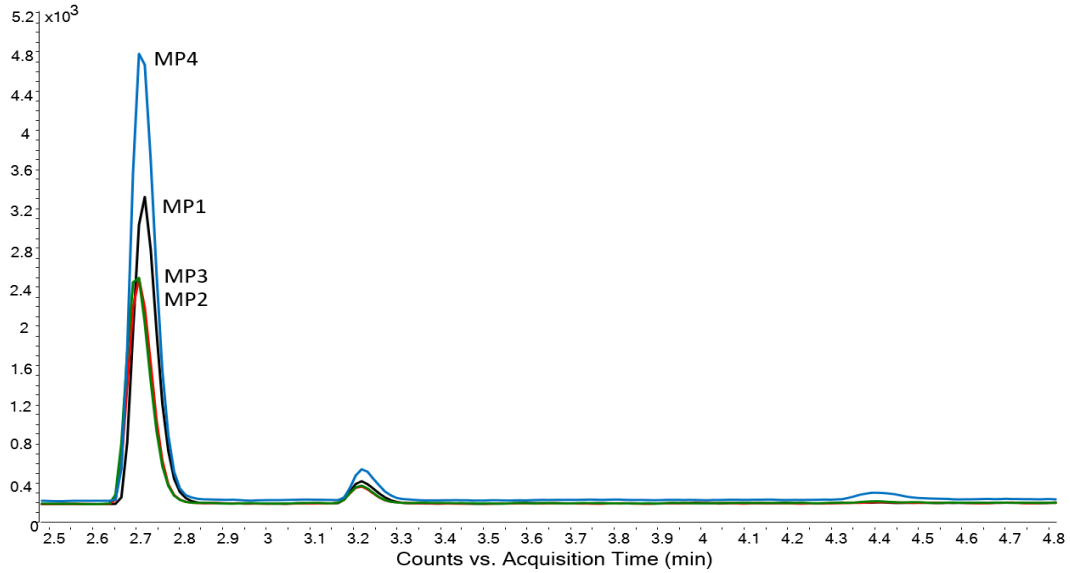


Fig. 6. Chromatograms in the case of MP1, MP2, MP3, and MP4 (C=1 µg/L)

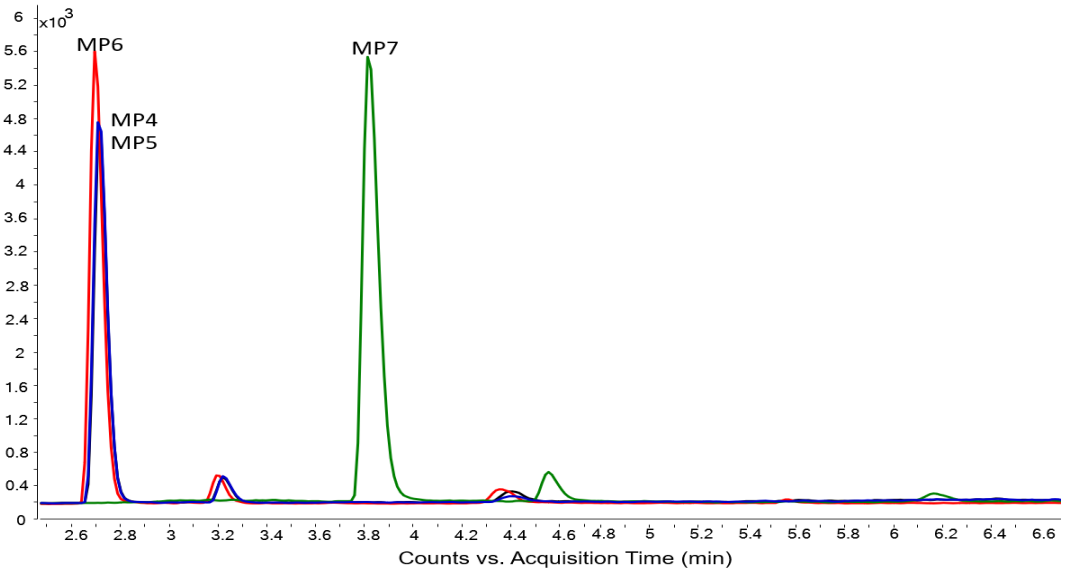


Fig. 7. Chromatograms in the case of MP4, MP5, MP6, and MP7 (C=1 µg/L)

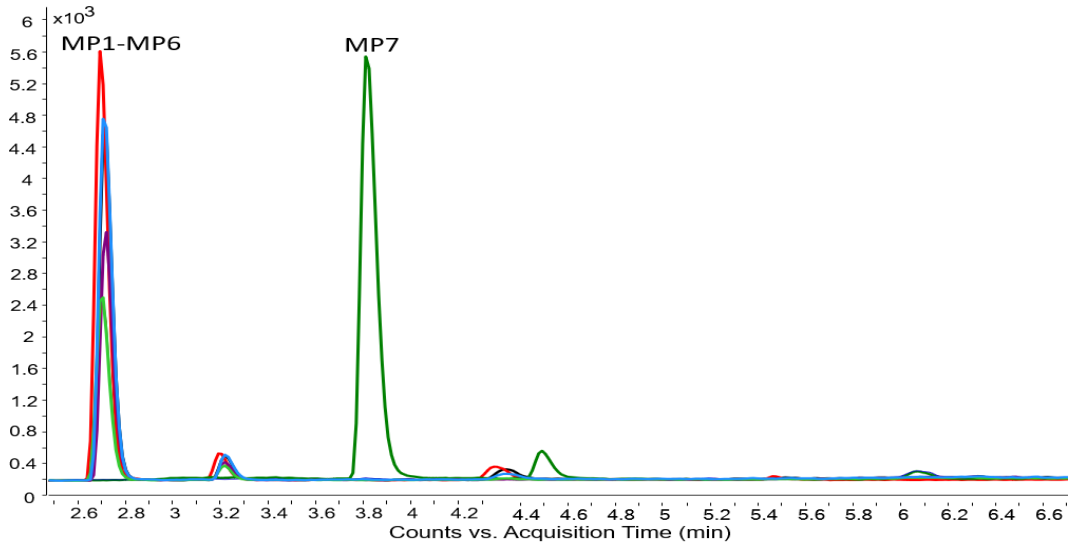


Fig. 8. Chromatograms in the case of MP1- MP7 (C=1 μg/L)

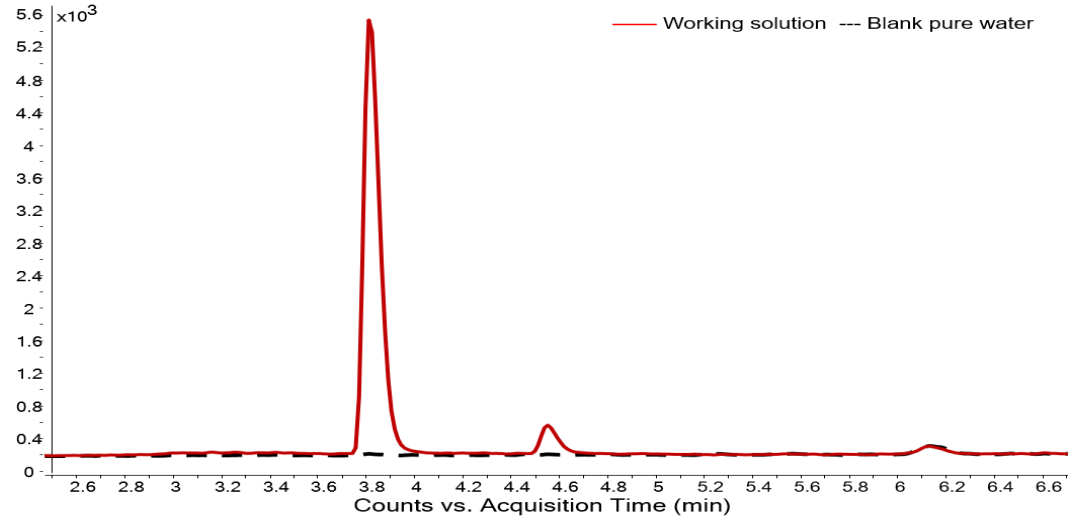


Fig. 9. Chromatograms of analytes and blank (C=1 μg/L)

2.5 Method validation

In accordance with 2002/657/EC guidelines [7], the model-dependent performance parameters in conventional approaches are recovery, repeatability, within-laboratory reproducibility, reproducibility, decision limit, detection capability, calibration curves, and ruggedness. In this project, only parts of these performance characteristics were studied.

1) The linearity between concentration and MS response for both linezolid and tedizolid was evaluated by standard calibration curves that were constructed at 14 concentration levels: 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/L, which satisfied “at least five levels” required in criteria. Linearity was assessed separately with or without ISs. Meanwhile, additional acidified calibration without ISs was also conducted at the same 14 mass concentration

levels, in which HCl (32%) presented at the molar concentration level of 0.01 mol/L.

2) Limit of detection (LOD) is usually determined according to the corresponding concentration of compound that gives rise to the signal-to-noise ratio (S/N) of 3:1 in the fortified analyte samples based on criteria. In this project, S/N ratio was calculated at each calibration concentration level to find out the least level at which the S/N ratio was larger than 3.

3) To assess the accuracy, trueness and precision were both computed. Repeatability experiments were conducted to determine spiked water samples at three concentration levels (50, 500, 5000 ng/L), five replicates for each concentration level in parallel. Trueness assesses the closeness between the mean detected value and nominal value for each level and nominal responses were regulated from calibration results. Trueness is usually expressed as bias. The minimum trueness for quantitative determination based on mass fraction is stipulated in criteria and listed in Tab.7.

Precision assesses the closeness among the five independent replicates' results obtained under pre-determined experimental conditions. The evaluation of precision is generally computed as the coefficient of variance (CV) based on Horwitz Equation described in criteria. However, for mass fraction lower than 100 μg/kg, the application of Horwitz Equation leads to unacceptable large values [7], which makes it unsuitable for trace levels that we conducted in this project. Instead, standard deviation (STD), relative standard deviation (RSD) were calculated to assess the precision.

$$\text{Trueness}(\%)=\frac{\text{mean detected value-nominal value}}{\text{nominal value}}*100$$

$$\text{RSD}(\%)=\frac{\text{STD}}{\text{mean detected value}}*100$$

Tab. 7. Minimum trueness of quantitative method in criteria [7]

Mass Fraction	Range
<= 1 μg/kg	-50% to +20%
> 1 μg/kg to 10 μg/kg	-30% to +10%
> 10 μg/kg	-20% to +10%

4) Ruggedness evaluates the susceptibility of an analytical method to the variation of the experimental conditions including storage conditions, sample preparation processes etc, during which fluctuation of outcomes is likely to be observed. Exposure of AOLRs to diverse conditions during solution preparation and storage can give rise to unexpected degradation or loss. In order to

evaluate the stability of analytes at different temperatures and pH values, frozen (-15 °C, refrigerator temperature), warm (20 °C, ambient room temperature), pH 5 and pH 9 samples were analyzed at the level of 1 µg/L, each in triplicate. Ruggedness was only analyzed in the condition of absence of ISs.
5) Carryover effects refer to any lingering effects of previous experimental conditions on the performance of a latter experiment. Blank samples were injected after the analysis of highest calibration standards (5000 ng/L) to observe carryover effects. Five runs were conducted. High carryover indicates insufficient wash of remaining analytes from the column [8].
To ensure that obtained results were reliable, quality control procedures were applied. Blank pure water samples were prepared and injected after every validation run in order to rule out any cross-contamination during the process.

2.6 Application to environmental water samples

To assess the occurrence and quantity levels of the selected antibiotics in surface water, the developed method was applied to the water sample taken from river Rhine on 23rd January, 2018. River water was collected in a clean amber glass bottle and filtered with 0.2 µm glass fiber filter immediately. Then it was five-fold diluted and kept at 4 °C in a refrigerator.
The susceptibility of LC-MS/MS systems to co-extracted matrix compounds is a troublesome problem. In complex aquatic samples, matrix effects caused by co-extracted compounds usually lead to great losses of MS sensitivity due to suppressed or enhanced ionization, thus hindering the quantification of analytes of interest [6]. In this project, matrix effects were assessed by the recovery rates of analytes in surface water. River Rhine water samples were spiked at 1 µg/L of analytes and measured three times then compared with analytes dissolved and analyzed in ultrapure water at the same level.

3 Results and discussion

3.1 Method validation results

3.1.1 Linearity

In the case of without ISs, the results showed that the standard calibration curves were linear in the range of 2 and 1 to 5000 ng/L and the correlation coefficient (R^2) were 0.9993 and 0.9963 for linezolid and tedizolid respectively. Nevertheless, when the measurements were conducted with ISs, the linearity of linezolid was slightly decreased with a loss of 0.0044 of R^2. Tedizolid was more negatively influenced by the simultaneous assessment of ISs since the R^2 was only 0.0871, which means scarce linearity between the concentration and MS response especially in higher concentration range. In conclusion, the

analyses of linezolid and tedizolid exhibited better linearity when ISs were absent.

Tab.8 lists the calibration results of linezolid and tedizolid in full concentration ranges when ISs were absent, where y represents MS response and x represents the concentration. As a matter of fact, in the lower concentration levels ranging from 0.2 to 1 ng/L, the linear correlation didn't exist for both analyte, with R^2 as 0.1401 and 0.4892 for linezolid and tedizolid respectively. On the contrary, the linearities were higher in high concentration ranges. R^2 was even 1 in the range of 100 to 5000 ng/L for linezolid. Therefore, to avoid mistakes or bias, the calibration results are not supposed to be applied to the measurement at ultra-trace levels for both antibiotics.

In acidified calibrations conducted with HCl, linezolid exhibited high linearity with R^2 of 0.9998. With regard to tedizolid, linearity was hardly observed with the presence of many greatly affected MS responses. Furthermore, acidification complicated the solution preparation process and consumed more chemicals, so it was decided to be off the table for this project.

Comparing the MS responses, ten times higher signals were observed for linezolid at nearly every concentration level than those of tedizolid.

Tab. 8. Calibration result of linezolid and tedizolid (without ISs)

AOLR	C (ng/L)	0-5000
Linezolid	y-x	y= 9.5x + 104.1
	R^2	0.9993
Tedizolid	y-x	y= 1.0x + 67.5
	R^2	0.9963

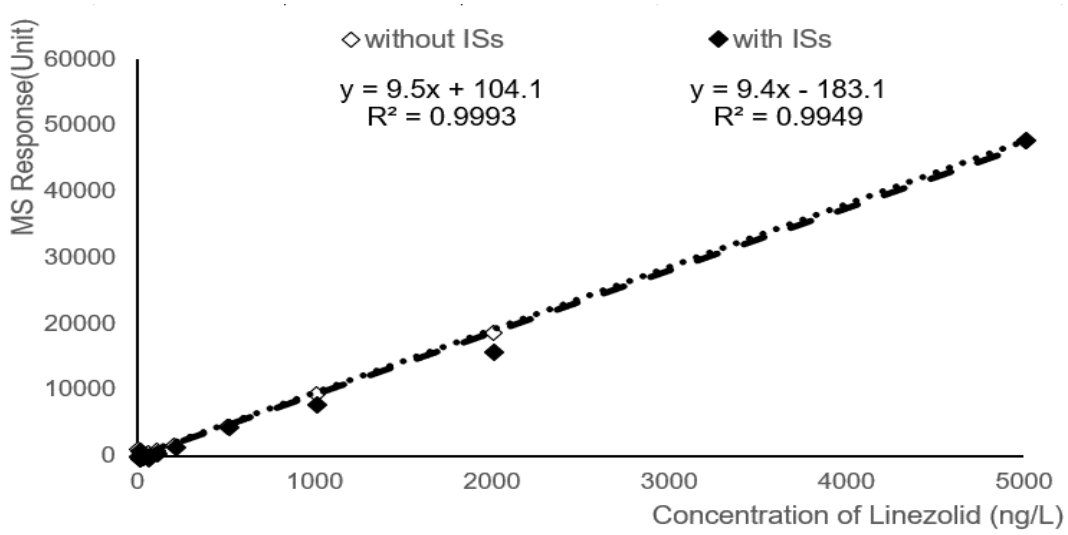


Fig. 10. Calibration curve of linezolid

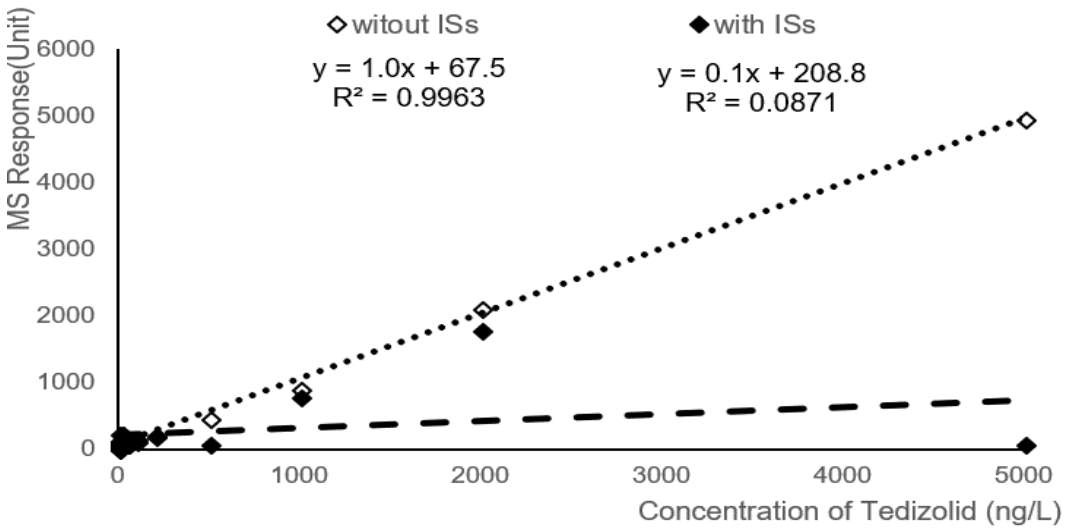


Fig. 11. Calibration curve of tedizolid

3.1.2 LODs

The result showed that the LODs were 2 ng/L for linezolid and 1ng/L for tedizolid, with S/N ratio of 5.2 and 11.4, respectively. The LODs at the ng/L level demonstrated that the proposed method was sensitive enough for the detection of these antibiotics at trace levels. Furthermore, tedizolid showed surprisingly higher sensitivity at low concentrations than linezolid.

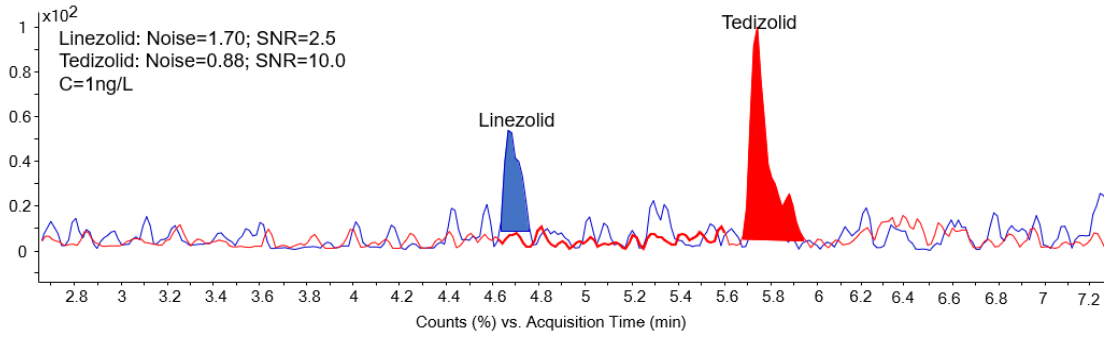


Fig. 12. Chromatogram and S/N of linezolid and tedizolid (C=1 ng/L)

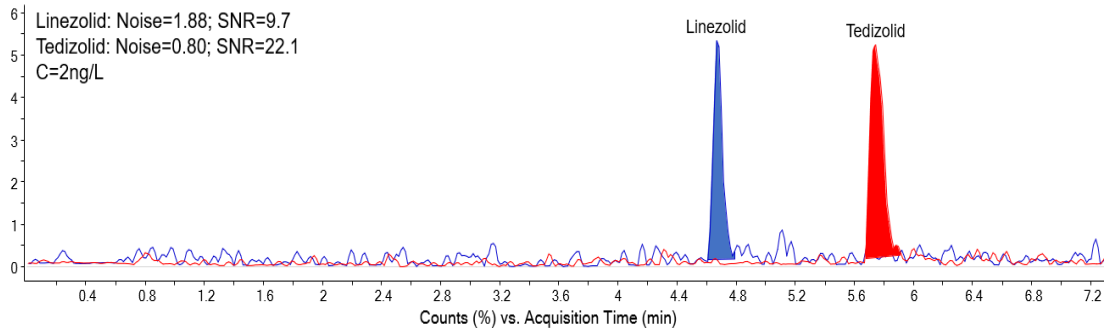


Fig. 13. Chromatogram and S/N of linezolid and tedizolid (C=2 ng/L)

Tab. 9. S/N ratio of analytes at two concentration levels

	Linezolid			Tedizolid		
C (ng/L)	Signal	Noise	S/N	Signal	Noise	S/N
1	2.5	1.7	1.5	10	0.9	11.4
2	9.7	1.9	5.2	22.1	0.8	27.6

3.1.3 Accuracy

Tab.10 presented the intra-day accuracy of the MS responses of linezolid and tedizolid at three diverse concentration levels. In general, the trueness of linezolid and tedizolid at selected concentration levels were all within ±15%, indicating the excellent closeness of agreement. But the trueness was lower in low concentration ranges due to the exist of more potential interruptions. The precision of analytes was evaluated as STD and RSD. Small RSDs (less than 3%) indicated high precision except for tedizolid at the level of 50 ng/L with RSD of 10.3%.

Tab. 10. Intra-day accuracy (n=5)

C	C=50 ng/L		C=500 ng/L		C=5000 ng/L	
AOLR	Linezolid	Tedizolid	Linezolid	Tedizolid	Linezolid	Tedizolid
Mean Resp.	540	96	4577	459	47749	5115
STD	15.8	9.9	56.0	11.8	422.0	53.3
Nomi. Resp.	632	86	4636	463	47739	4967
Trueness (%)	-14.5	10.9	-1.3	-1.0	0.0	3.0
RSD (%)	2.9	10.3	1.2	2.6	0.9	1.0

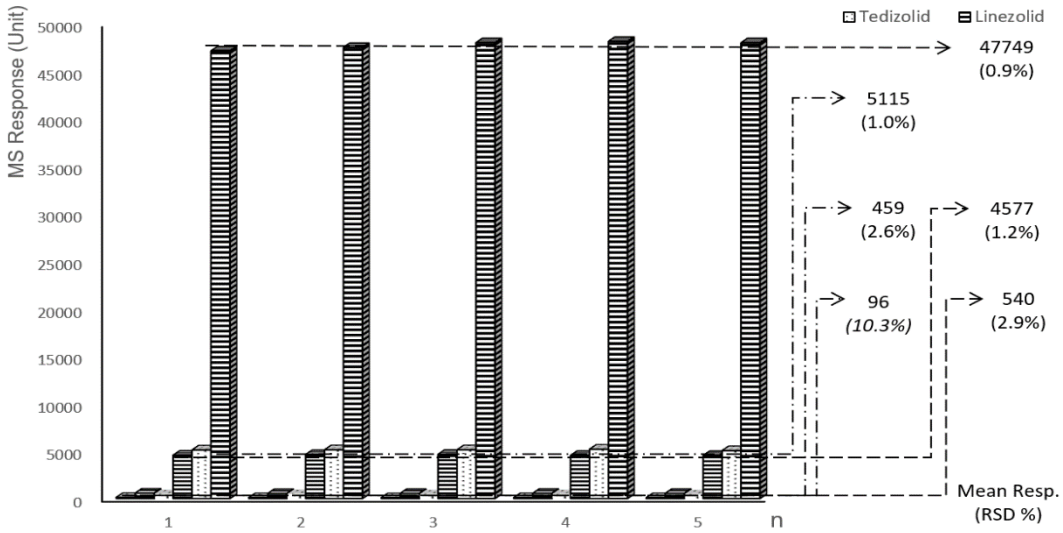


Fig. 14. MS Resp. of AOLRs at 50, 500, 5000 ng/L concentration levels

3.1.4 Ruggedness

Tab.11 and Fig.15 showed the MS responses of linezolid and tedizolid at the level of 1 μg/L when they were exposed to different storage temperatures. Judging from the trueness, in the case of frozen storage condition, linezolid and tedizolid experienced greater losses to different extents than when they were kept in ambient temperature, where tedizolid was also destroyed to a larger degree. To conclude, linezolid and tedizolid were not supposed to be stored in frozen condition and tedizolid was more susceptible to temperature.

In terms of the influence of alkalinity (Tab.12 and Fig.16), neither linezolid nor tedizolid exhibited obvious differences between the cases of pH 5 and pH 9, which implied that pH value was not a key factor that affected the analyses of analytes.

Tab. 11. MS Resp. in frozen and warm conditions (n=3, without ISs)

T(°C)	Frozen (-15 °C)		Warm (20 °C)	
AOLR	Linezolid	Tedizolid	Linezolid	Tedizolid
Mean Resp.	8823	580	9695	668
STD	63.8	21.2	27.4	21.1
Nominal Resp.	9420	910	9420	910
Trueness (%)	-6.3	-36.2	2.9	-26.6
RSD (%)	0.7	3.7	0.3	3.2

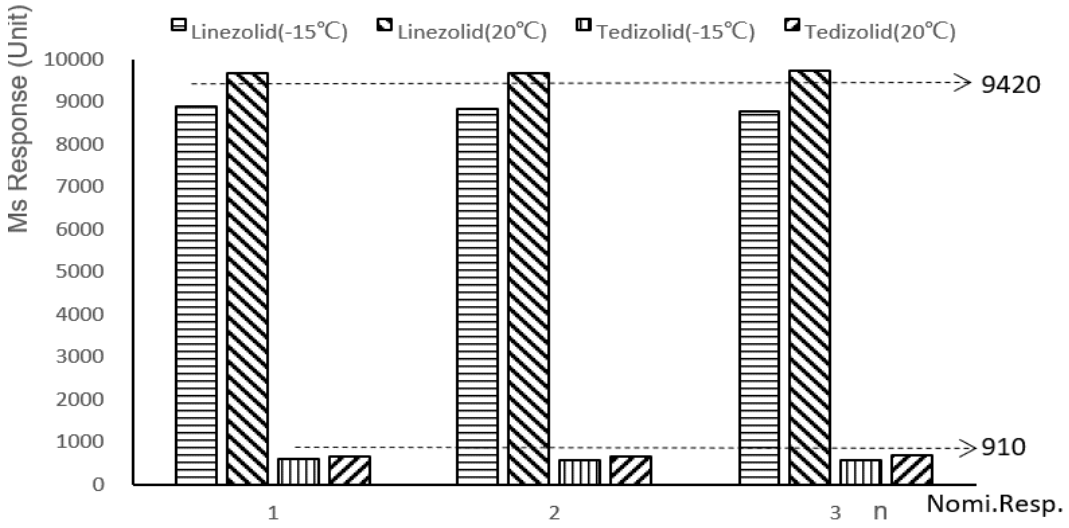


Fig. 15. MS Resp. of AOLRs in frozen and warm conditions (C=1 μg/L)

Tab. 12. MS Resp. at pH 5 and pH 9 (n=3, without ISs)

pH	5		9	
AOLR	Linezolid	Tedizolid	Linezolid	Tedizolid
Mean Resp.	10717	1137	10318	1156
Nominal Resp.	9420	910	9420	910
Trueness (%)	13.8	25.0	9.5	27.0
STD	153.6	62.4	64.6	33.0
RSD (%)	1.4	5.5	0.6	2.9

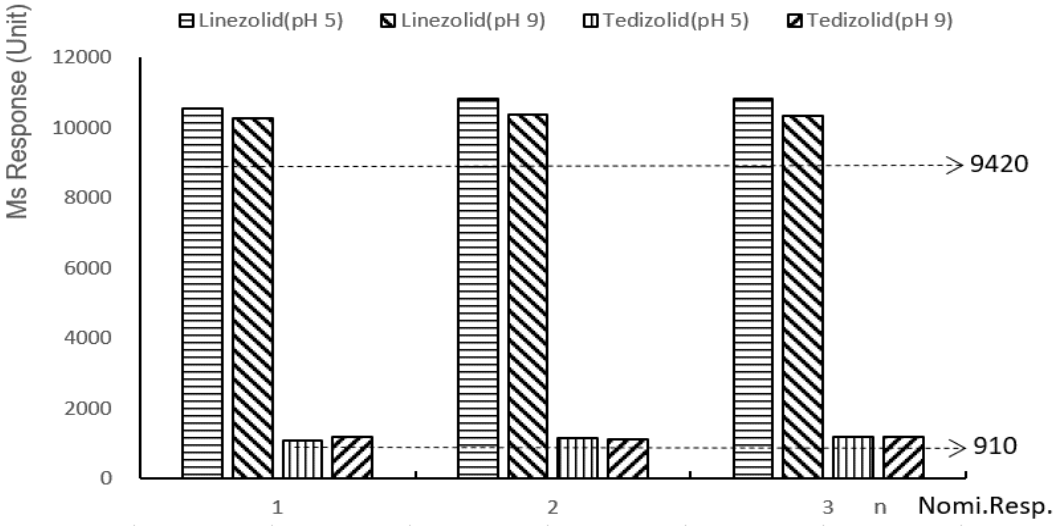


Fig. 16. MS Resp. of AOLRs in acidic and alkaline conditions (C=1 μg/L)

3.1.5 Carryover effects

Carryover effects demonstrated the mutual influence between blank ultrapure water and working solution samples containing linezolid, tedizolid (5000 ng/L) and ISs (1000 ng/L).

According to the sequent areas of analytes in the chromatograms, abnormal relation between the concentrations and peaks was observed. Therefore, this method cannot be validated with carryover effects.

3.2 Environmental water application

By means of comparing the areas in the chromatogram of pure Rhine water samples and Rhine water that was artificially spiked with analytes at 1μg/L, the concentration levels of linezolid, tedizolid and ISs were calculated and shown in Fig.17. Linezolid-D3 stayed at high concentration level with 1147 ng/L, so as tedizolid at 314 ng/L. Linezolid and tedizolid-D3 were found to be much lower, with 87 and 21 ng/L respectively. None of these analytes existed at ultra-trace levels.

According to the recovery rates, the matrix effects hardly suppressed the quantification of tedizolid but hampered linezolid analysis to some extent.

Tab.13. Application to Rhein water (n=3)

AOLR	Linezolid	Tedizolid
Mean Area	7497	660
Nominal Area	9294	667
Recovery (%)	80.7	98.9

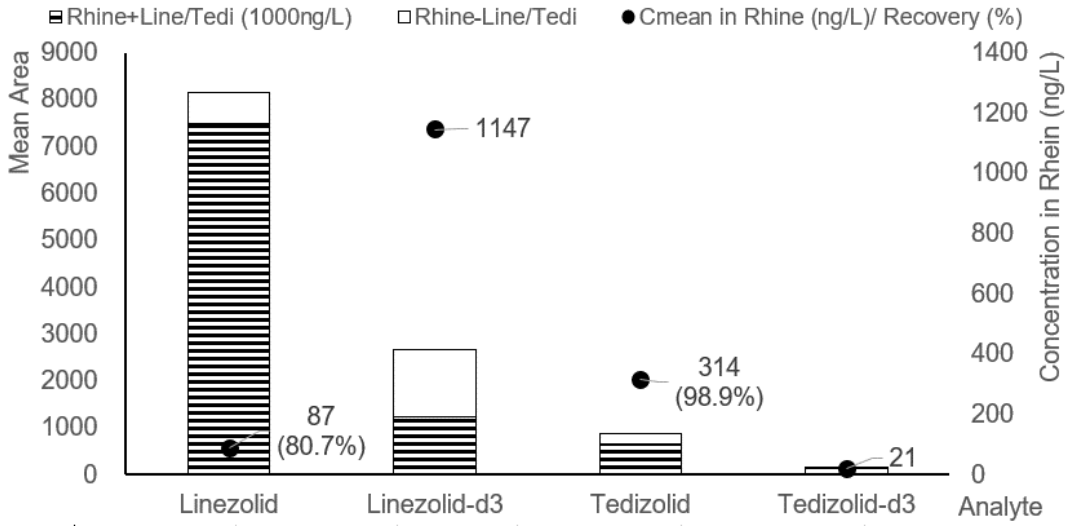


Fig. 17. The concentration of linezolid, tedizolid, and ISs in river Rhine

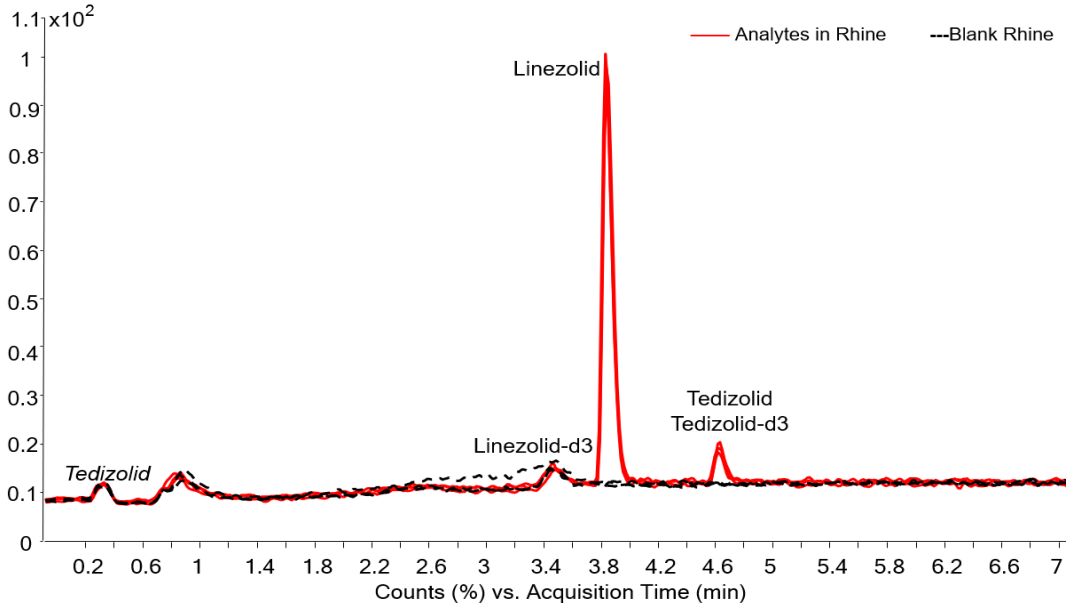


Fig. 18. Chromatogram of linezolid, tedizolid, and ISs in the spiked Rhine and blank Rhine waters (C=1 μg/L)

Tab. 14. Summary of results

Parameter		Linezolid	Tedizolid
LOD (ng/L)		2	1
R^2 (n=14)		0.9993	0.9963
RSD (%)	C=50 ng/L	2.9	10.3
	C=500 ng/L	1.2	2.6
	C=5000 ng/L	0.8	1.0
Trueness (%) (C=1 µg/L)	pH 5	13.8	25.0
	pH 9	9.5	27.0
	-15 °C	-6.3	-36.2
	20 °C	2.9	-26.5
in Rhine water	C (ng/L)	87.4	314.3
	Recovery (%)	80.7	98.9

4 Conclusion

The occurrence of antibiotics in the aquatic environment has become a matter of concern in recent years. In this project, a simple, sensitive and robust analytical method for simultaneous determination of two antibiotics of last resort, linezolid and tedizolid, in ultrapure water and environmental water matrices was developed and optimized. The method involved LC to separate target antibiotics, ESI interface to fragmentate precursor molecules, and MS to detect and quantify the product ions in dynamic MRM mode. The lowest limits of detection were 2 ng/L and 1 ng/L for linezolid and tedizolid respectively, meanwhile, linearity of two antibiotics ranged from their LODs to 5000 ng/L. In repetition experiments at three diverse concentration levels, RSDs were almost all found to be below 3%. When it comes to stability, the frozen temperature was unsuitable for storage, while the influence of alkalinity was negligible. Carryover effects cannot be validated for this method.

In most cases, linezolid generated approximately ten times larger signal than tedizolid at the same concentration level, which was probably due to its higher water solubility. Meanwhile, tedizolid exhibited worse linearity, precision, stability, especially when isotopically labeled substances were present. However, the LOD of tedizolid was lower, indicating a higher sensitivity. Nevertheless, to conclude, this method is less suitable for tedizolid than linezolid.

In the application of the method to river Rhine waters, the matrix effects only slightly suppressed the quantification linezolid analysis. It was verified that selected antibiotics and isotopically labeled substances were frequently found to be above the low ng/L range especially linezolid-D3 and tedizolid. Given the potentially high public and natural concern caused by AOLRs, there is an urgent

need, from analytical and regulatory perspectives, to continuously monitor the concentrations of these compounds and to create strategies that minimize their discharges into aqueous systems.

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