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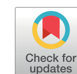
Development and validation of chromatographic methods for screening and subsequent quantification of suspected illegal antimicrobial drugs encountered on the Belgian market



Yaxin Tie[a,b], Celine Vanhee[a], Eric Deconinck[a,1], Erwin Adams[b,1,*]

[a] Scientific Direction Chemical and Physical Health Risks, Section Medicines and Healthcare Products, Sciensano, J. Wytsmansstraat 14, 1050 Brussels, Belgium

[b] KU Leuven, University of Leuven, Department of Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, O&N2, PB 923, B-3000 Leuven, Belgium

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ABSTRACT

Estimations, made by the World Health Organization (WHO), state that 10% of the medical products in low- and middle-income countries are substandard or falsified (SF). Among them, antibiotics and antimalarials are the most commonly reported since 2013. Besides the fact that falsification is a crime, the worldwide use of poor quality antimicrobials could result in treatment failures, stronger antimicrobial resistance and even the promotion of the emergence of superbugs. Therefore, simple and accurate analytical methods are necessary, which are capable to detect and quantify a wide range of antimicrobials in suspected illegal products. In this work, a screening and a quantification method using ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS^2) and diode array detection (UHPLC-DAD), respectively were developed and validated. These methods could be used for routine analysis and enable a more in-depth characterization of SF-antimicrobials.

According to their popularity as SF-antimicrobials, 31 antibiotics, 3 antibacterial agents, 1 antifungal agent and 1 beta-lactamase inhibitor, covering eleven different antibacterial classes, were selected. The UHPLC-MS^2 screening method with gradient elution is able to selectively detect these 36 compounds within 18 min (including wash and equilibration step). It was validated for sensitivity, selectivity and matrix effects. Within an analysis time of 32 min, the UHPLC-DAD method could quantify 32 compounds (4 showed insufficient UV absorbance) and resulted in sufficient selectivity, necessary since some SF-antimicrobials may include more than one antimicrobial component. This quantification method was validated for the positive hits found during screening tests of suspected illegal samples. This resulted in a validation set of 11 antimicrobials and 1 beta-lactamase inhibitor. The "total error" approach in accordance with the validation requirements of ISO-17025 was employed for the validation. 57 real-life illegal samples, seized by inspectors from the Belgium Federal Agency for Medicinal and Health Products (FAMHP), were analyzed using the two described methods. About half of them were not compliant and some samples that contained clavulanic acid showed a serious reduction in the amount of this molecule (in one sample only 14% of the claimed dosage was found). These quality issues might be attributed to either poor manufacturing, storage or transportation conditions.

1. Introduction

Despite the multitude of local and global efforts that were made during the last decade to curtail the manufacturing, distribution and supply of substandard and falsified (SF) medicines, these products are still considered a continuously growing threat to public health [1]. Estimations made by the World Health Organization (WHO) state that 10% of the medical products in low- and middle-income countries are SF. Among them, antibiotics and antimalarials are the most commonly reported since 2013 [2]. Moreover, it seems that antibiotics account for more than 25% of all SF-drugs [3]. This is likely due to the success of the antibiotic market since it has been reported that this had a revenue of USD 39.8 billion in 2015 and is even expected to grow up to USD 57.0 billion by 2024 [4]. Recent estimates claimed that the illegal antibiotic market occupied around 5% of the global antibiotic market [3]. In addition, a warning issued by WHO stated that SF-antibiotics are no

* Corresponding author.
E-mail address: Erwin.Adams@kuleuven.be (E. Adams).

[1] Equally contributing project leaders.

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longer only limited to developing countries, but are also gaining popularity in the developed ones. This phenomenon is attributed to the expansion of the internet and the ease of online purchasing. Although it is well known that more than 50% of the medicines sold by web shops not disclosing their physical identity are SF-medicines, a recent survey conducted in the United States indicated that the number of people purchasing drugs online has increased by three times in the past decade [2]. Moreover, it was reported that in total about 21,000 dose units of SF-antibiotics were seized in 12 European countries within one week in 2012 [5].

According to WHO, SF medical products may include products exclusively containing inactive ingredients, wrong ingredients or improper dosages [6]. SF-antimicrobials with insufficient dosages may result in sub-potent drug efficacy, leading to increased morbidity of patients, treatment failures and selection of drug-resistant microbial strains. SF-antimicrobials with excessive dosages or impurities, can also directly be responsible for serious side effects and even deathly outcomes, especially in children [7]. A case report, originating from the late 1990s showed that the use of SF-gentamicin, containing toxic impurities, caused around 2000 adverse incidents of the eosinophilia-myalgia syndrome and resulted also in 66 deaths in the United States [3]. The use of SF-antimicrobials worldwide may also result in stronger antimicrobial resistance and even promotes the emergence of superbugs [8]. Therefore, great efforts are currently ongoing to tackle these malignant practices in favor of public health. In this framework, not only regulatory efforts are required, but also adequate analytical methods are paramount. In literature, many validated methodologies are available for the identification and quantification of antimicrobials, but most of them are designed for one specific class of antimicrobials or are analyzed by a targeted approach [9–17]. These approaches are known to be selective and sensitive, but have an inherent bias since they will not detect molecules other than the preselected ones. Therefore, a full screening method is necessary in order to detect unexpected SF-antimicrobial drug products and possible adulterants. Moreover, the European pharmacopoeia methods for antibiotics are designed only for one specific antibiotic [18] which require different reagents and different experimental conditions. Therefore, these methods are not feasible for high throughput screening of SF-antimicrobials. Potential adulterants in falsified samples are not able to be properly detected using the European pharmacopoeia methods. Given the possible high amount of suspected antibiotics seized at a certain moment, a fast and selective analytical technique is demanded by regulatory authorities to monitor drug quality control and survey the (illegal) market.

This study aims to develop such a method capable of efficient detection and sufficient separation of suspected illegal antimicrobials frequently encountered by the Belgian controlling agency. Besides real antibiotics, the set included also a beta-lactamase inhibitor (clavulanic acid), an antifungal (griseofulvin) and some antibacterial agents (trimethoprim, sulfamethoxazole and nitrofurantoin). The best performing LC gradient was selected, further refined and validated for identification by mass spectrometry (MS) and quantification by a diode array detector (DAD). For quantification, DAD was preferred over MS because the first provides more accurate results without the need of expensive isotopically labelled reference standards. Good accuracy was required in view of acceptance limits of ± 5% for the assessment of the actives in pharmaceutical products (tablets and capsules). All collected illegal antimicrobial samples were successfully screened and quantified using the validated methods.

2. Materials and methods

2.1. Reagents and standards

2.1.1. Reagents

HPLC and MS-grade acetonitrile (ACN) and methanol were obtained from Biosolve (Valkenswaard, the Netherlands). Formic acid (ACS and Ph. Eur. reagent) was purchased from Merck (Darmstadt, Germany). Water (R = 18 MΩ cm, TOC < 4 ppb) was generated by using a milliQ-Gradient A10 system (Millipore, Billerica, MA, USA).

2.1.2. Standards and stock solutions

The reference standards of amoxicillin trihydrate, azithromycin, cefaclor, ceftazidime, cefazolin, cefuroxime axetil, clavulanate lithium, clindamycin and cloxacillin sodium were purchased from the European Directorate for the Quality of Medicines (EDQM) (Strasbourg, France). Ampicillin, cefadroxil, cefotaxime sodium, cefradine, ceftriaxone disodium salt heptahydrate, erythromycin A, tetracycline hydrochloride and roxithromycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Benzathine penicillin G tetrahydrate, cefalexin, doxycycline hyclate, lincomycin hydrochloride monohydrate, penicillin V potassium, trimethoprim and norfloxacin were bought from Sigma-Aldrich (Seelze, Germany), while ciprofloxacin and rifampicin were acquired from Sigma-Aldrich (Buchs, Switzerland). Bacitracin zinc was purchased from Alpharma (Oslo, Norway). Clarithromycin and ofloxacin were obtained from Ranbaxy (Madhya Pradesh, India). Nitrofurantoin, sulfamethoxazole and neomycin sulfate were obtained from Fagron (Waregem, Belgium). Griseofulvin was purchased from Sigma-Aldrich (Laramie, WY, USA), polymyxin B sulfate from Kela laboratoria N.V. (Hoogstraten, Belgium) and cefepime from Bristol-Myers Squibb (Syracuse, New York, USA). Gentamicin sulfate was obtained from Alcon Cusi (Barcelona, Spain).

For the sample matrix components, magnesium trisilicate was purchased from Qualiphar (Bornem, Belgium). Starch was obtained from VWR (Darmstadt, Germany) and lactose monohydrate was bought from Synergyhealth (Uitgeest, Netherlands). Mannitol, croscarmellose sodium and cellulose microcristalline were obtained from Fagron (Waregem, Belgium). Sucrose, propylparaben and methylparaben were from Sigma-Aldrich (St. Louis, MO, USA). Maltodextrin was bought from Procter & Gamble (Strombeek-Bever, Belgium).

Individual stock solutions were prepared by dissolving standards in the corresponding solvent (See Table 1) at a concentration of 1 mg/mL. Afterwards, stock solutions were divided in different aliquots and stored at −20 °C for maximum six months whilst quinolones were refreshed every two months and β-lactams renewed monthly, as recommended by Gros et al. [11]. It is noted that tetracyclines have to be stored in the dark due to photodegradation [19]. The standard mixture solution containing all antimicrobials was prepared in a mixture of methanol/H_2O (50:50, v/v) by mixing proper amounts of individual stock solutions at a concentration of 25 μg/mL. The standard mixture solution was stored at −20 °C and renewed every week.

2.2. Samples of suspected illegal antimicrobials

All illegal samples were seized and collected by inspectors from the Belgium Federal Agency for Medicines and Health Products (FAMHP) during the period 2016–2017. 57 samples were included in the study, claiming 18 different antimicrobials and 1 beta-lactamase inhibitor: amoxicillin, ampicillin, azithromycin, benzathine penicillin G, ceftriaxone, cefalexin, ciprofloxacin, doxycycline, erythromycin, griseofulvin, ofloxacin, penicillin V, roxithromycin, tetracycline (hydrochloride), nitrofurantoin, norfloxacin, sulfamethoxazole, trimethoprim and clavulanic acid. This set was further completed with 17 other antimicrobials, based on their popularity in literature as SF-antimicrobials [3,5,7]. Taken together, a total 36 compounds were included for method development, covering eleven different classes of antimicrobials (See Table 1). The collected 57 illegal samples consisted of four different formulations: capsules (46%), tablets (47%), injections (5%) and syrup (2%). It is demonstrated that counterfeit β-lactams are frequently encountered by the controlling agency according to existing criminal cases [3,7]. Conformably, two thirds of the collected counterfeit products in the present study are β-lactam antibiotics.

Table 1
List of compounds for the UHPLC-MS^2 screening method and the UHPLC-DAD quantification method. Solvent A is methanol/ H_2O mixture (50:50, v/v), solvent B is methanol and solvent C is methanol/ H_2O with 1% HCOOH mixture (20:80, v/v).

Compounds	Classification	Solvent	Wavelength (nm)
Amoxicillin*	Penicillins	A	230
Ampicillin*	Penicillins	A	230
Azithromycin[1]	Macrolides	B	
Bacitracin Zn	Polypeptides	C	
Benzathine penicillin G*	Penicillins	B	215
Cefaclor	Cephalosporins	A	
Cefadroxil	Cephalosporins	A	
Cefepime	Cephalosporins	A	
Cefotaxime	Cephalosporins	A	
Ceftazidime	Cephalosporins	A	
Ceftriaxone*	Cephalosporins	A	280
Cefuroxime axetil	Cephalosporins	B	
Cefalexin*	Cephalosporins	A	230
Cefazolin	Cephalosporins	A	
Cefradine	Cephalosporins	A	
Ciprofloxacin*	Quinolones	C	280
Clarithromycin	Macrolides	C	
Clavulanic acid*	β-lactamase inhibitor	A	230
Clindamycin	Macrolides	A	
Cloxacillin	Penicillins	A	
Doxycycline*	Tetracyclines	A	350
Erythromycin[1]	Macrolides	B	
Gentamicin[1]	Aminoglycosides	C	
Griseofulvin*	Antifungal agent	B	254
Lincomycin	Macrolides	A	
Neomycin[1]	Aminoglycosides	C	
Nitrofurantoin*	Nitrofurans	Dimethylformamide (DMF)	254
Norfloxacin*	Quinolones	C	280
Ofloxacin*	Quinolones	C	280
Penicillin V*	Penicillins	A	215
Polymyxin	Polypeptides	A	
Rifampicin	Rifamycins	B	
Roxithromycin*	Macrolides	A	215
Sulfamethoxazole*	Sulfonamides	B	254
Tetracycline (HCl)*	Tetracyclines	A	280
Trimethoprim*	Other	B	215

* Compound used for validation.
[1] Compound only included in the screening method, not included in the quantification method.

2.3. Sample preparation

2.3.1. Screening method

2.3.1.1. Validation samples. Individual stock solutions (1 mg/mL) were prepared as described in Section 2.1.2. To build up a screening library with MS data, working solutions of 0.1 mg/mL were made by diluting individual stock solutions with the corresponding solvent (See Table 1).

To verify the matrix effect of capsules and tablets, three typical matrices were used. Matrix 1 was composed of talc and lactose, matrix 2 consisted of cellulose and starch and matrix 3 was made up of mannitol and croscarmellose sodium. Sucrose, maltodextrin, methylparaben and propylparaben were tested for the matrix effect of syrup. The antimicrobials were divided into three groups according to their corresponding solvents, namely methanol, methanol/H_2O mixture (50:50, v/v) and methanol/ H_2O with 1% HCOOH mixture (20:80, v/v) (Table 1). The stock mixture solutions (1 mg/mL) of three groups were prepared respectively. 30 mg of one of the selected matrices was brought into a flask of 10.0 mL and spiked with the appropriate amounts of stock mixture solutions of antimicrobials. The content of the flask was brought up to 10.0 mL to obtain a concentration of 25 μg/mL active ingredient.

2.3.1.2. Sample treatment. The number of tablets or capsules to be pulverized was determined as follows: i) if the number of offered units is less than or equal to 3, the number to be pulverized is one, ii) if the number of offered units is more than 3, but less than or equal to 11, the number to be pulverized is half of the total unit number, iii) if the number of offered units is more than 11, the number to be pulverized is a quarter of the total unit number. The maximum number to be pulverized is 20. This way of working allows to preserve enough sample for retest or in case the inspection or the justice department does not want all of the evidence pieces to be destroyed.

An amount of 25 mg of the pulverized drug products was accurately weighed and around 20 mL of the corresponding solvent was added, followed by ultrasonication for 15 min. Next, the acquired solution was brought up to 25.0 mL, further diluted 10 times and filtered through 0.2 μm polytetrafluoroethylene (PTFE) filters before injection. A comparison between the use of filtration and centrifugation was executed to check whether some components were retained in the filters. The outcome indicated that there was no significant difference between the two debris removal techniques.

2.3.2. Quantification method

2.3.2.1. Calibration standards. The concentrations of the calibration standards are indicated in Table 2 and Table 3. Calibration standards were made by diluting the stock solution of each antimicrobial in the corresponding solvent as indicated in Table 1. The solutions were filtered through 0.2 μm PTFE filters before injection.

2.3.2.2. Validation samples. The placebo was made by mixing talc, lactose, cellulose, starch, mannitol and croscarmellose sodium in equal proportions. These compounds are the most commonly present excipients in tablets and capsules. The spiked placebo validation samples were prepared by adding a certain amount of individual stock solution to 30 mg of placebo and diluting with the corresponding solvent to 10.0 mL, in order to obtain the concentrations mentioned in Table 2. The solutions were ultrasonicated for 15 min and filtered through 0.2 μm PTFE filters before injection.

To perform a recovery study, a mimic tablet was made by mixing the labelled amount of active pharmaceutical ingredient (API) with an appropriate amount of placebo according to the art of pharmacy profession. The added amount of placebo for each sample was calculated by subtracting the labelled API content from the average weight of a tablet. This simulated drug products underwent the same preparation procedure as described in Section 2.3.2.3. Each sample was analyzed in triplicate. Calibration ranges and recovery concentrations are shown in Table 3.

2.3.2.3. Sample treatment. An amount of 30 mg of the pulverized drug products (Section 2.3.1.2) was accurately weighed and around 8 mL of the corresponding solvent was added, followed by ultrasonication for 15 min. Then, the acquired solution was brought to 10.0 mL. Further dilution was required to obtain an intermediate concentration within the calibration range. The solutions were filtered through 0.2 μm PTFE filters before injection.

2.4. Instrumental conditions

2.4.1. Screening method

The screening of all samples was performed by UHPLC-MS^2 on a Dionex UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Scientific, Sunnyvale, CA, USA) connected to an amaZon™ speed ETD mass spectrometer (Bruker Daltonics, Bremen, Germany). The chromatographic separation was carried out at 25 °C on a Waters Acquity BEH shield RP18 (150 mm × 2.1 mm, 1.7 μm) column. The flow rate was 0.3 mL/min and the injection volume was 2 μL. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in

Table 2
Concentrations of the calibration standards and the validation samples.

Compounds	Concentrations Calibration standards (µg/mL)	Validation samples (µg/mL)
Amoxicillin	10	30
	100	150
	200	300
	400	
Ampicillin	10	30
	100	150
	200	300
	400	
Benzathine penicillin G	10	30
	50	90
	100	150
	200	
Clavulanic acid	10	10
	20	30
	40	50
	60	
Ceftriaxone	150	150
	200	200
	300	300
	350	
Cefalexin	10	30
	100	150
	200	300
	400	
Ciprofloxacin	10	30
	50	90
	100	150
	150	
Doxycycline	10	30
	100	150
	200	300
	400	
Ofloxacin	10	30
	100	150
	200	300
	400	
Penicillin V	10	30
	100	150
	200	300
	400	
Roxithromycin	10	30
	100	150
	200	300
	350	
Tetracycline (HCl)	10	30
	100	150
	200	300
	400	

Table 3
Recoveries of five additionally investigated antimicrobials (SD: standard deviation).

Compounds	Concentrations Calibration standards (µg/mL)	Recovery concentrations (µg/mL)	Recovery: mean % (SD)
Griseofulvin	10, 50, 100, 200	84	99.6 (1.4)
Norfloxacin	10, 50, 100, 200	102	98.9 (1.0)
Nitrofurantoin	10, 50, 100, 200	96	98.9 (1.2)
Sulfamethoxazole	10, 50, 100, 200	119	102.9 (0.7)
Trimethoprim	10, 100, 200, 400	238	98.0 (0.2)

ACN, respectively. The chromatographic gradient started with an isocratic elution of 99% A for 1 min, followed by a linear decrease to 85% in 4 min, then the percent of mobile phase A further linearly dropped to 25% in 10 min, after which the column was rinsed by 1% A for 1.5 min. The post-gradient equilibration with 99% A was performed for 1.5 min. The total run time was 18 min for the screening method.

The mass spectrometer settings were based on those described by Vanhee *et al.* [20]: the mass spectrometer was operated in alternating positive electrospray ionization (ESI+) and negative electrospray ionization (ESI-) mode, with a spray voltage of 3.5 kV and an end plate voltage of 500 V. The nebulizer was set to 2 bar and the desolvation gas temperature was 180 °C at a flow rate of 4.0 L/min. MS spectra were obtained within a mass range of 100–1000 m/z and the smart parameter setting (SPS) was set to 475 m/z. For MS^2 precursor selection, the most intense ion was isolated above an absolute intensity of 2.500 and a 5% relative intensity threshold. The ion charge control (ICC) was set to 100,000 for ESI- and 200,000 for ESI+ with a maximum accumulation time of 200 ms. CID (Collision Induced Dissociation) was performed using helium as collision gas. The fragmentation amplitude was set to 100% using SmartFrag™ Enhanced for amplitude ramping (80–120%). The fragmentation time was set to 20 ms.

2.4.2. Quantification method

Method development, optimization, validation and application were achieved on an Aquity UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent manager, a sample manager and a DAD detector. Data integration and analysis were performed with Empower 3 Citrix software.

The optimal separation (see also Section 3.1) was obtained on a Waters Acquity BEH shield RP18 (150 mm × 2.1 mm, 1.7 µm) column, which is the same as the screening method. The column and sample temperatures were kept at 25 °C and 15 °C, respectively. The flow rate was 0.2 mL/min and the injection volume was 5 µL. The gradient was composed of 0.01% formic acid in water as mobile phase A and ACN as mobile phase B. The gradient started with an isocratic elution of 99% A for 5 min followed by a linear decrease to 85% in 10 min. Then the percent of mobile phase A further linearly dropped to 25% in 15 min, followed by returning to the initial composition in 2 min. A wavelength scan with the DAD detector was performed for all antimicrobials within the range 190–400 nm for identity confirmation. For method development and optimization, all antimicrobials were detected and monitored at 215 nm whereas the method validation and application were performed at a corresponding detection wavelength for each antimicrobial as shown in Table 1.

2.5. Method validation

2.5.1. Screening method

The screening method should avoid false positive and false negative results as much as possible. Therefore, it has to be validated in terms of sensitivity, selectivity and matrix effects. The evaluation of sensitivity is based on the measurement of the screening detection limit (SDL), defined as the lowest concentration at which an analyte can be correctly detected in at least 95% of the samples [21,22]. The selectivity ensures the method to detect the analyte of interest without interferences of other analytes and excipients. Blank samples (only matrix) were tested to validate false detects. The detection of the active compounds was verified in the presence of different matrices as described in Section 2.3.1.1. According to ''Method validation and quality control procedures for pesticide residues analysis in food and feed" (Sanco/12495/2011) [21], the validation should include at least 20 samples covering multiple matrices with a minimum of two samples per matrix. In the present study, 36 samples covering three matrices, representative for the matrix scope of the laboratory, were analyzed before and after spiking, in which the concentration was in line with a SDL of 25 µg/mL (See Section 3.3). To verify the matrix effect of syrup, the retention times of sucrose, maltodextrin, methylparaben and propylparaben were determined to check interferences [21].

2.5.2. Quantification method

Analytical method validation aims to assure that future

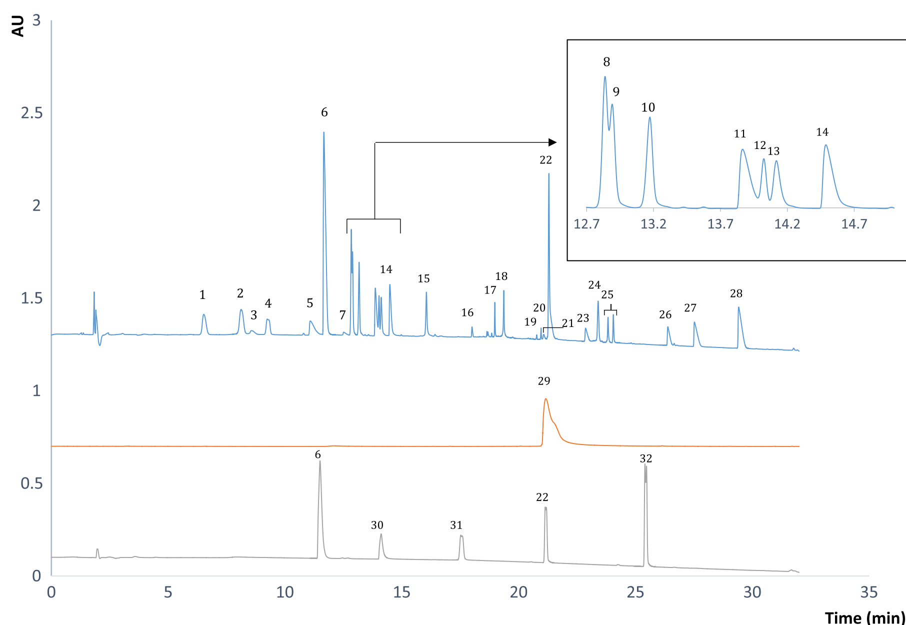

Fig. 1. Typical chromatograms obtained by the UHPLC-DAD quantification method (1 = amoxicillin, 2 = cefadroxil, 3 = lincomycin, 4 = cefepime, 5 = clavulanic acid, 6 = trimethoprim, 7 = polymyxin B1, 8 = cefalexin, 9 = ampicillin, 10 = cefaclor, 11 = ofloxacin, 12 = cefradine, 13 = ceftazidime, 14 = ciprofloxacin, 15 = tetracycline, 16 = clindamycin, 17 = bacitracin Zn, 18 = doxycycline, 19 = clarithromycin, 20 = roxithromycin, 21 = cefotaxime, 22 = sulfamethoxazole, 23 = cefazolin, 24 = rifampicin, 25 = cefuroxime axetil, 26 = benzathine penicillin G, 27 = penicillin V, 28 = cloxacillin, 29 = ceftriaxone, 30 = norfloxacin, 31 = nitrofurantoin, 32 = griseofulvin).

measurements for the content of analytes are reliable and close enough to the true value. The UHPLC-DAD quantification method was validated according to ISO-17025 via the "total error" approach by building accuracy profiles, a graphical decision-making tool [23,24]. It is a fitness-for-purpose approach, which evaluates the systematic error (trueness) as well as the random error (intermediate precision) to know the deviation between the measured result and the true value. This approach is able to compute an interval predicting the values of future measurements.

Specifically, the "total error" approach verifies whether the analytical method could provide a result that deviates from the true value within the predefined acceptability limits [$-\lambda$, λ], being for licensed pharmaceutical products [−5%, 5%]. This is carried out by the calculation of the β-expectation tolerance interval, which comprises the future results that will fall inside the acceptability limits with a given probability β [25,26]. In this study, β was set at 95% and the calculation was made at three concentration levels. The method can be regarded as accurate within the studied concentration range on the premise that the calculated β-expectation tolerance interval is located within the acceptance limits of ± 5% [25].

In practice, the quantification method was only fully validated for the 11 antibiotics (amoxicillin, ampicillin, benzathine penicillin G, ceftriaxone, cefalexin, ciprofloxacin, doxycycline, ofloxacin, penicillin V, roxithromycin, tetracycline) and 1 beta-lactamase inhibitor (clavulanic acid) that were present from the beginning and for which positive hits were obtained in the screening experiment. Validation samples at three different concentration levels were prepared daily as three independent replicates (See Table 2). The analysis of validation samples continued for at least three days depending on the compounds. The concentrations were determined by back-calculation based on the calibration lines (see Section 2.3.2.1). Based on these calculated concentrations, the linearity of the results, trueness, precision (repeatability and intermediate precision) and accuracy were assessed [27].

Since the samples of five other antimicrobials (nitrofurantoin, norfloxacin, trimethoprim, sulfamethoxazole and griseofulvin) were received at a later stage, a reduced validation of the quantification method was performed where the construction of accuracy profiles was replaced by recovery tests.

3. Results and discussion

3.1. Quantification method development and optimization

Because MS is less cost-effective than DAD, the quantification method was developed first starting with the set of 35 antimicrobials and 1 beta-lactamase inhibitor (Section 2.2). However, erythromycin, azithromycin, neomycin and gentamicin had to be excluded due to their insufficient UV absorbance in combination with the selected mobile phases. A Waters Acquity BEH shield RP18 column (150 mm × 2.1 mm, 1.7 µm) was used to start the method development, because many reports have demonstrated that it can generate a satisfactory separation among antibiotics such as quinolones, macrolides and β-lactams [28–30]. ACN was initially used as solvent for all standards.

Concerning the mobile phases, 0.01% formic acid in water and ACN were selected. ACN was chosen as organic modifier due to its low cutoff value and more stable baseline compared to methanol. The initial UHPLC gradient started with 95% aqueous phase, which was held for 5 min followed by a linear decrease to 5% in 30 min and an isocratic elution for 10 min before returning to the initial conditions in 10 min. Flow rates of 0.2 mL/min, 0.3 mL/min and 0.4 mL/min were tested as well as column temperatures of 25 °C, 30 °C and 40 °C. It was found that the flow rate of 0.2 mL/min and the column temperature of 25 °C gave the best performance in general. Since the separation among cefadroxil, lincomycin and cefepime was not optimal, the starting condition of the gradient was changed from 5% ACN to 1% ACN. However, some peak shapes were not ideal and even distorted. This problem was solved by using methanol as solvent instead of ACN. In order to shorten the analysis time, the percentage of ACN was calculated at the moment the last peak eluted. In this way, the gradient could be stopped at 75% ACN instead of 95%. Under these conditions, cefalexin and ampicillin co-eluted, but this was solved by decreasing the slope of the increase rate of ACN in the elution time frame of 5–15 min. Under the final conditions mentioned in Section 2.4.2, all 32 compounds were separated. Typical chromatograms obtained with the references of all 31 antimicrobials and the 1 beta-lactamase inhibitor are presented in Fig. 1.

For the quantification of erythromycin, azithromycin, neomycin and gentamicin, the chromatographic procedures in the respective monographs of the European Pharmacopoeia [18] can be followed.

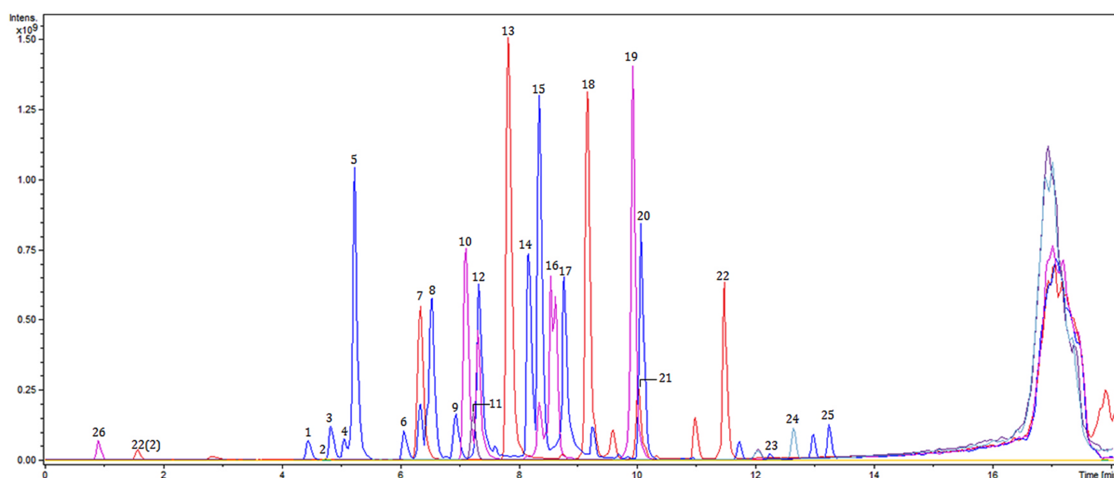

Fig. 2. Overlay of LC-MS² total ion chromatograms of 35 antimicrobials and 1 beta-lactamase inhibitor (1 = cefepime, 2 = clavulanic acid, 3 = amoxicillin, 4 = cefadroxil, 5 = lincomycin, 6 = ceftazidime, 7 = cefaclor and trimethoprim, 8 = cefalexin and ampicillin, 9 = cefradine and polymyxin B1, 10 = ofloxacin, 11 = norfloxacin, 12 = tetracycline and ciprofloxacin, 13 = azithromycin, 14 = ceftriaxone and cefotaxime, 15 = clindamycin and nitrofurantoin, 16 = bacitracin, 17 = doxycycline, 18 = erythromycin and cefazolin, 19 = clarithromycin, 20 = roxithromycin, 21 = sulfamethoxazole, 22 = penicillin G, rifampicin and cefuroxime axetil, 22(2) = benzathine (of benzathine penicillin G), 23 = penicillin V, 24 = griseofulvin, 25 = cloxacillin, 26 = gentamicin and neomycin).

Table 4
Characteristics of antimicrobials by LC-MS² (*m/z*: mass-to-charge ratio).

Name	Monoisotopic mass (Da)	Chemical formula	Retention time	LOD* (μg/mL)	Adduct	*m/z*
Amoxicillin	365.10	$C_{16}H_{19}N_3O_5S$	5.0	2.5	$[M+H]^+$	366.1
Ampicillin	349.11	$C_{16}H_{19}N_3O_4S$	6.6	1	$[M+H]^+$	350.1
Azithromycin	748.51	$C_{38}H_{72}N_2O_{12}$	8.1	1	$[M+2H]^{2+}$	375.2
Bacitracin	1421.75	$C_{66}H_{103}N_{17}O_{16}S$	8.7	1	$[M+3H]^{3+}$	475.0
Benzathine	240.16	$C_{16}H_{20}N_2$	2.0	10	$[M+H]^+$	241.0
Benzylpenicillin	334.10	$C_{16}H_{18}N_2O_4S$	11.2	0.25	$[M+H]^+$	335.1
Cefaclor	367.04	$C_{15}H_{14}ClN_3O_4S$	6.4	1	$[M+H]^+$	368.0
Cefadroxil	363.09	$C_{16}H_{17}N_3O_5S$	5.2	1	$[M+H]^+$	364.0
Cefepime	480.13	$C_{19}H_{24}N_6O_5S_2$	4.6	5	$[M+H]^+$	481.1
Cefotaxime	455.06	$C_{16}H_{17}N_5O_7S_2$	8.2	1	$[M+H]^+$	456.0
Ceftazidime	546.10	$C_{22}H_{22}N_6O_7S_2$	6.1	2.5	$[M+2H]^{2+}$	274.0
Ceftriaxone	554.05	$C_{18}H_{18}N_8O_7S_3$	7.9	25	$[M+H]^+$	555.0
Cefuroxime axetil	510.11	$C_{20}H_{22}N_4O_{10}S$	11.7/12.3	1	$[M+Na]^+$	533.1
Cefalexin	347.09	$C_{16}H_{17}N_3O_4S$	6.6	2.5	$[M+H]^+$	348.1
Cefazolin	454.03	$C_{14}H_{14}N_8O_4S_3$	9.2	5	$[M+H]^+$	455.0
Cefradine	349.11	$C_{16}H_{19}N_3O_4S$	7.1	1	$[M+H]^+$	350.1
Ciprofloxacin	331.13	$C_{17}H_{18}FN_3O_3$	7.4	0.25	$[M+H]^+$	332.1
Clarithromycin	747.48	$C_{38}H_{69}NO_{13}$	10.2	0.1	$[M+H]^+$	748.5
Clavulanic acid	199.05	$C_8H_9NO_5$	4.7	15	$[2M-H]^-$	396.8
Clindamycin	424.18	$C_{18}H_{33}ClN_2O_5S$	8.5	0.1	$[M+H]^+$	425.2
Cloxacillin	435.07	$C_{19}H_{18}ClN_3O_5S$	13.2	1	$[M+H]^+$	436.0
Doxycycline	444.15	$C_{22}H_{24}N_2O_8$	8.9	0.1	$[M+H]^+$	445.1
Erythromycin A	733.46	$C_{37}H_{67}NO_{13}$	9.5	0.5	$[M+H]^+$	734.5
Gentamicin	477.32	$C_{21}H_{43}N_5O_7$	1.0	10	$[M+H]^+$	478.3
Griseofulvin	352.07	$C_{17}H_{17}ClO_6$	12.6	1	$[M+H]^+$	353.0
Lincomycin	406.21	$C_{18}H_{34}N_2O_6S$	5.4	0.1	$[M+H]^+$	407.2
Neomycin	614.31	$C_{23}H_{46}N_6O_{13}$	0.9	10	$[M+H]^+$	615.3
Nitrofurantoin	238.03	$C_8H_6N_4O_5$	8.3	5	$[M-H]^-$	236.8
Norfloxacin	319.13	$C_{16}H_{18}FN_3O_3$	7.2	1	$[M+H]^+$	320.0
Ofloxacin	361.14	$C_{18}H_{20}FN_3O_4$	7.2	0.1	$[M+H]^+$	362.1
Penicillin V	350.09	$C_{16}H_{18}N_2O_5S$	12.3	2.5	$[2M-H]^-$	699.3
Polymyxin B1	1202.75	$C_{56}H_{98}N_{16}O_{13}$	6.9	25	$[M+3H]^{3+}$	402.0
Rifampicin	822.41	$C_{43}H_{58}N_4O_{12}$	11.8	0.25	$[M+H]^+$	823.4
Roxithromycin	836.52	$C_{41}H_{76}N_2O_{15}$	10.3	0.1	$[M+H]^+$	837.5
Sulfamethoxazole	253.05	$C_{10}H_{11}N_3O_3S$	10.2	0.5	$[M+H]^+$	253.9
Tetracycline	444.15	$C_{22}H_{24}N_2O_8$	7.5	0.1	$[M+H]^+$	445.1
Trimethoprim	290.14	$C_{14}H_{18}N_4O_3$	6.5	0.1	$[M+H]^+$	291.0

* LOD was measured in the total ion chromatogram (TIC).

3.2. Screening method development and optimization

The screening method was developed and optimized based on the quantification method. By applying the mobile phases of the quantification method (A: 0.01% formic acid in water and B: ACN), ceftriaxone (100 μg/mL) could not be detected by MS. In order to acquire sufficient ionization and higher sensitivity, the mobile phases have been modified to 0.1% formic acid in water as mobile phase A and

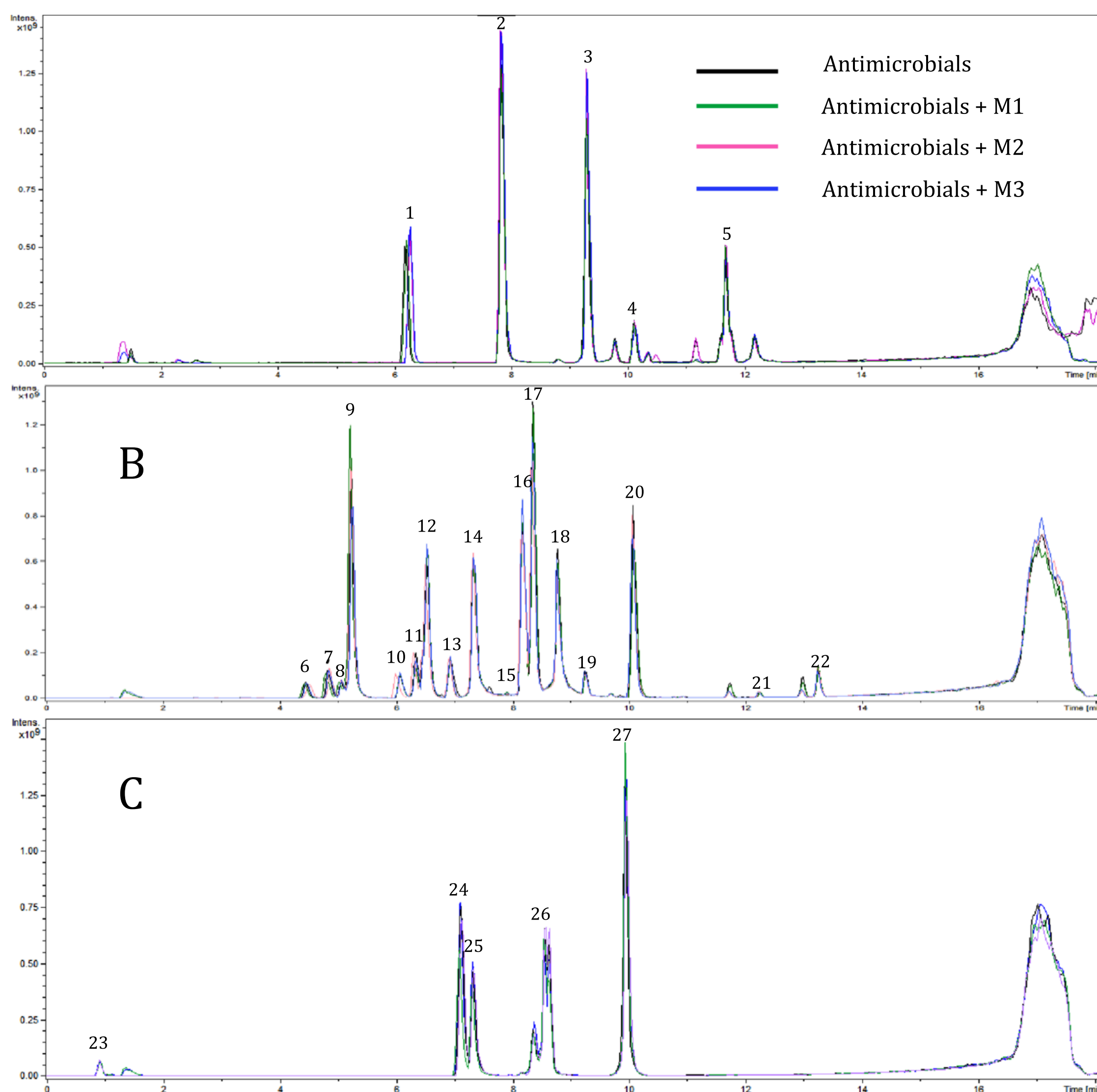

Fig. 3. Overlay of UHPLC-MS^2 total ion chromatograms in the positive mode of antimicrobials in their corresponding solvent. A: the group of compounds dissolved in methanol, B in methanol/H_2O mixture (50:50, v/v), C in methanol/H_2O with 1% HCOOH mixture (20:80, v/v) (1 = trimethoprim, 2 = azithromycin, 3 = erythromycin, 4 = sulfamethoxazole, 5 = penicillin G, cefuroxime axetil and rifampicin, 5(2) = benzathine (of benzathine penicillin G), 6 = cefepime, 7 = amoxicillin, 8 = cefadroxil, 9 = lincomycin, 10 = ceftazidime, 11 = cefaclor, 12 = cefalexin and ampicillin, 13 = cefradine and polymyxin B1, 14 = tetracycline, 15 = ceftriaxone, 16 = cefotaxime, 17 = clindamycin, 18 = doxycycline, 19 = cefazolin, 20 = roxithromycin, 21 = penicillin V, 22 = cloxacillin, 23 = gentamicin and neomycin, 24 = ofloxacin, 25 = ciprofloxacin, 26 = bacitracin, 27 = clarithromycin, clavulanic acid was monitored in the negative mode).

0.1% formic acid in ACN as mobile phase B. In this case, all 36 compounds could be detected. The injection volume was decreased from 5 µL to 2 µL due to higher sensitivity of the MS detector compared to the DAD detector. The column temperature was kept at 25 °C. To optimize the screening method, the flow rate was increased from 0.2 mL/min to 0.3 mL/min. The LC gradient of the quantification method was further refined for a shorter analysis time which should be achievable due to the additional selectivity of MS. Moreover, the elution profiles of the different antimicrobials were not altered too much so that the link was kept between the screening and the quantification method. The initial isocratic elution of 99% A lasted for 1 min instead of 5 min, then 99% A linearly decreased to 85% within 4 min and further decreased to 25% within 10 min. Subsequently, online column cleaning with 1% mobile phase A was maintained for 1.5 min. The total runtime decreased from 32 min to 18 min (including washing and re-equilibration). A typical LC-MS^2 total ion chromatogram obtained by the optimized method is shown in Fig. 2.

3.3. Validation of the screening method

3.3.1. Sensitivity

The sensitivity was evaluated based on the SDL, which was measured by the maximum value of the limit of detection (LOD) as explained in Section 3.4.2 among all compounds. The LOD values of all compounds were determined in the total ion chromatogram (TIC) and are presented in Table 4. The SDL was set at 25 µg/mL for the necessary sensitivity of the screening method.

3.3.2. Selectivity and matrix effect

The selectivity was confirmed by the retention time of each antimicrobial and their corresponding MS spectra. A 0.3 Da tolerance on the MS and the MS^2 spectra was considered acceptable. An in-house LC-MS^2 screening library was constructed by analysis of working solutions (0.1 mg/mL). The screening library comprised the theoretical monoisotopic exact mass, the mass-to-charge ratio (m/z) of the adduct with the most intense signal for each compound (shown in Table 4), the MS spectrum and the MS^2 spectrum. Compounds eluting at the same time

Table 5
Validation parameters of 17 compounds for the UHPLC-DAD quantification method (QC: quality coefficient).

Compounds	LOD (µg/mL)	LOQ (µg/mL)	Lack of fit	R^2	QC (%)
Amoxicillin	0.1	0.5	0.7445	0.9997	1.455
Ampicillin	0.1	0.4	0.7999	0.9996	1.816
Benzathine penicillin G	0.2	0.8	0.0957	0.9994	2.024
Ceftriaxone	30	100	0.1901	0.9991	1.545
Cefalexin	0.1	0.3	0.9752	0.9997	1.605
Ciprofloxacin	0.2	0.5	0.0807	0.9996	1.428
Clavulanic acid	0.2	0.6	0.1405	0.9993	1.686
Doxycycline	2.5	8.0	0.0596	0.9993	2.334
Ofloxacin	0.2	0.4	0.8658	0.9994	2.069
Penicillin V	0.5	1.5	0.2064	0.9997	1.432
Roxithromycin	2.0	7.0	0.1405	0.9998	1.259
Tetracycline (HCl)	0.5	2.0	0.0698	0.9992	2.495
Sulfamethoxazole	0.1	0.3	0.1813	0.9994	2.116
Trimethoprim	1.0	2.5	0.2232	0.9997	1.639
Nitrofurantoin	0.1	0.3	0.0557	0.9994	2.116
Norfloxacin	0.1	0.3	0.1883	0.9998	1.274
Griseofulvin	0.5	2.0	0.1017	0.9999	0.655

could be distinguished based on their different MS data. Additionally, 21 negative samples (containing only matrix components) and 36 samples (composed of matrix components spiked with the different antimicrobials) were analyzed. No false positives or false negatives could be observed, i.e. no antimicrobials were detected in the blank samples, while all targeted antimicrobials could be found in the spiked samples at a SDL level.

Overlay of total ion chromatograms of the solutions of antimicrobials and three matrices spiked with antimicrobials are shown in Fig. 3. It could be proved that no ion suppression occurred and that the matrices had a limited influence on the retention times (all shifts being less than 0.3 min). As for the matrix effect of syrup, methylparaben showed no interference with any antimicrobial. However, sucrose and maltodextrin had the same retention time as benzathine penicillin G, and the same was found for propylparaben and cloxacillin. Even though benzathine penicillin G and cloxacillin co-eluted with syrup excipients, they had different MS and MS^2 spectra. To conclude, the compound can be identified if it meets the screening criteria, which are: the shift of the retention time is less than or equal to 0.5 min (compared with the reference standard), *m/z* of the mother ion is equal to this in the in-house library (error tolerated: 0.3 Da) and the MS^2 spectrum matches at least 85% with the reference spectrum (fragment ions and their relative intensities) of the in-house library.

3.4. Validation of the quantification method

The quantification method was validated based on the screening results of positive hits of suspected illegal antimicrobials. In total, 57 suspected illegal antimicrobials were screened by applying the validated screening method. The results confirmed that amoxicillin, cefalexin, penicillin V, tetracycline, ampicillin, ciprofloxacin, ofloxacin, roxithromycin, clavulanic acid, benzathine penicillin G, ceftriaxone, doxycycline, griseofulvin, nitrofurantoin, norfloxacin, trimethoprim and sulfamethoxazole were indeed present in the respective illegal samples. Hence, the quantification method was validated for these 17 compounds (full validation for 12 and reduced validation for 5 compounds as explained in Section 2.5.2) and afterwards applied to quantify illegal antimicrobial samples. The method was validated for these 17 compounds at different wavelengths (See Table 1) selected based on the peak shapes and the absence of interference in the chromatogram.

3.4.1. Selectivity

To evaluate the method selectivity, the retention time and the UV spectrum of each compound were determined and monitored for 17 compounds. Constant retention times and selective UV spectra confirmed the method selectivity.

3.4.2. Limits of detection and quantification

LOD and the limit of quantification (LOQ) were determined according to the European Pharmacopoeia and the International Council on Harmonization (ICH) [18,31]. LOD and LOQ were obtained via experiments of serial dilutions. In general, signal-to-noise ratios of 3 and 10 are regarded as LOD and LOQ, respectively. The LOD and LOQ of these 17 compounds are given in Table 5. Ceftriaxone had the highest LOQ, 100 µg/mL, which was sufficient for its quantification.

3.4.3. Linearity of the calibration lines

Ordinary least square linear regression was used to construct the calibration curves. The linearity was evaluated through R^2 values, the quality coefficient (QC) and the p-values of the lack-of-fit (LOF) test. The latter is needed since R^2 values and QC individually are not adequate enough to confirm the linearity. After being verified by the LOF test, the limits of $R^2 \geq 0.995$ and QC $\leq 2.5\%$ can be adopted for the system suitability test [32]. Within the selected calibration ranges, all 17 compounds had R^2 values higher than 0.999, QC values below 2.5% and p-values of the LOF test above 0.05, as summarized in Table 5.

3.4.4. Linearity, trueness, precision and accuracy

Following ISO-17025, the developed method was validated by building accuracy profiles based upon the ''total error'' approach [23,24] for the first set of positive illegal samples consisting of amoxicillin, cefalexin, penicillin V, tetracycline, ampicillin, ciprofloxacin, ofloxacin, roxithromycin, clavulanic acid, benzathine penicillin G, ceftriaxone and doxycycline. The accuracy profiles of these 12 compounds (shown in Fig. 4) are plotted to assess the present and future accuracy of the method. A reduced validation was performed for the other five antimicrobials as mentioned in Section 2.5.2.

3.4.4.1. Linearity of the results. It is compulsory that the theoretical concentration is linearly linked to the measured concentration [27,33]. With the investigation of the linear relationship for the 12 compounds individually, the results suggested that the relationship was linear since the R^2 values were above 0.9999 with an equation being close to $y = x$.

3.4.4.2. Trueness. In ISO guideline 5725, the trueness is defined as the closeness of agreement between the mean of a number of test results and the actual (true) value [34]. It is an estimate of the systematic error of the method and is indicated as relative bias. For the current method, the trueness is satisfactory since the maximum absolute value of the relative bias is 3.3% (See Table 6). All the relative biases for the 12 investigated compounds are located within the error limits of [−5%, 5%].

3.4.4.3. Precision. The precision of an analytical method is an estimate of the margin of random errors. It describes the closeness of agreement between a series of repeated measurements under the prescribed conditions [25]. The precision is expressed as the relative standard deviation (RSD) and may be considered at three levels: repeatability, intermediate precision and reproducibility. In this study, we investigated the intraday precision (repeatability) and the interday precision (intermediate precision). The repeatability of the method evaluates the precision under the same operating conditions in a short time interval, which was acquired from the RSD values of the triplicate measurements at each concentration level. The intermediate precision describes the within-laboratory variations, which is normally investigated on different days using different equipment and different analysts, though only the factor of the different days was taken into account here, due to practical reasons [26].

The results are shown in Table 6. The RSD of repeatability and intermediate precision are below 1.3% and 1.7%, respectively. In

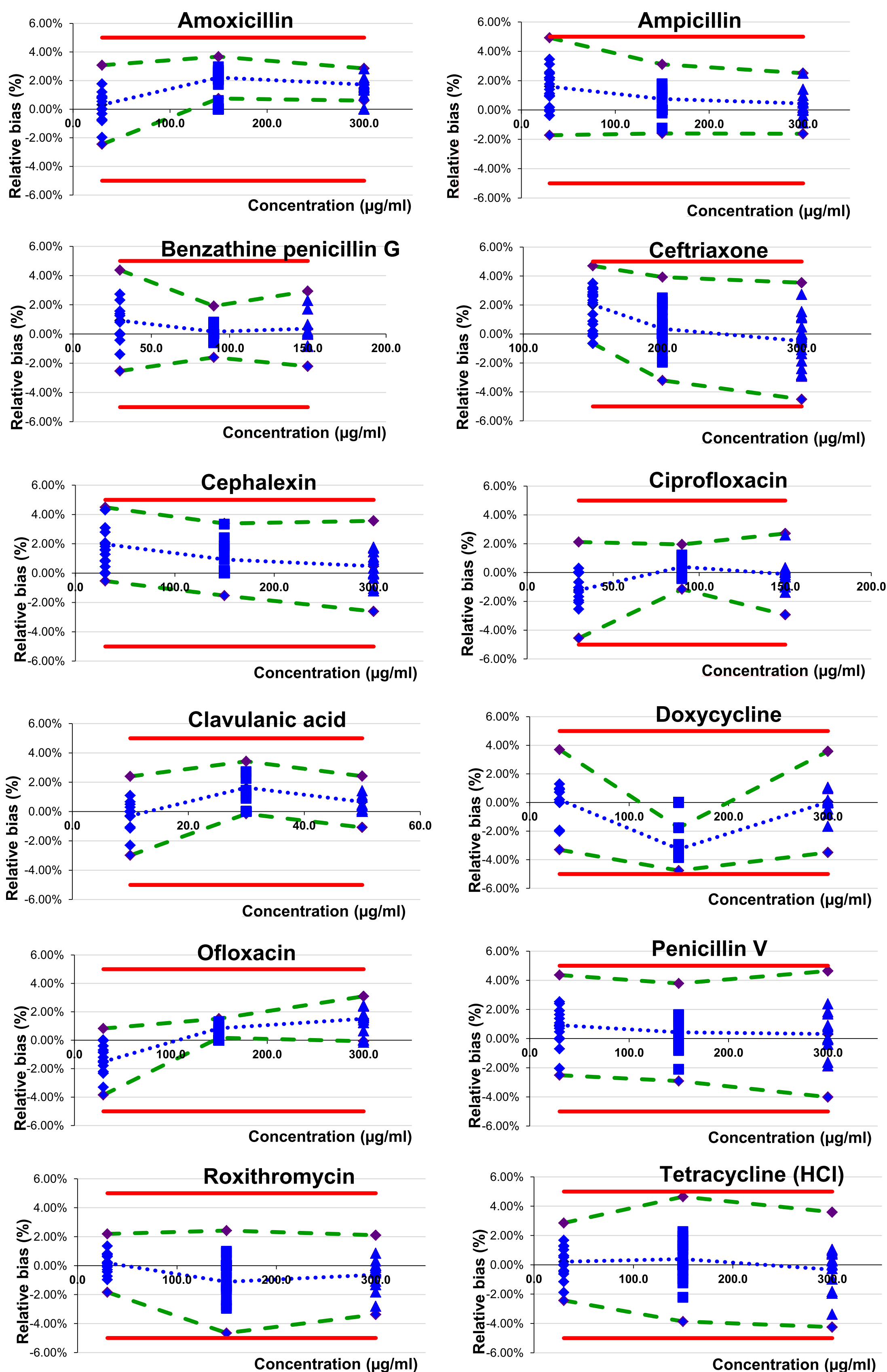

Fig. 4. Accuracy profiles of all 12 compounds with 95% β. Relative bias (dashed blue line), β-expectation tolerance limits (dashed green line), ± 5% acceptance limits (solid red line) and relative back-calculated concentrations (dots).

Table 6
Trueness, accuracy, precision and uncertainty of the quantification method.

Compounds	Trueness			Precision						Accuracy			Uncertainty		
	Relative bias (%)			Repeatability (RSD %)			Intermediate precision (RSD %)			β-expectation tolerance limits (%)			Relative expanded uncertainty (%)		
Concentration level	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Amoxicillin	0.31	2.21	1.73	0.79	0.55	0.42	1.10	0.63	0.48	[− 2.45, 3.08]	[0.75, 3.67]	[0.60, 2.85]	2.36	1.33	1.02
Ampicillin	1.60	0.76	0.44	0.50	0.47	0.50	1.21	0.91	0.83	[− 1.72, 4.93]	[− 1.60, 3.11]	[− 1.62, 2.51]	2.63	1.95	1.78
Benzathine penicillin G	0.92	0.16	0.36	1.23	0.50	1.05	1.35	0.63	1.05	[− 2.53, 4.37]	[− 1.59, 1.92]	[− 2.21, 2.93]	2.89	1.37	2.21
Ceftriaxone	2.03	0.36	− 0.49	1.26	0.75	1.10	1.30	1.46	1.73	[− 0.66, 4.71]	[− 3.21, 3.93]	[− 4.51, 3.54]	2.64	3.10	3.65
Cefalexin	1.97	0.93	0.47	1.14	1.12	0.57	1.14	1.12	1.07	[− 0.54, 4.49]	[− 1.53, 3.38]	[− 2.61, 3.56]	2.36	2.30	2.35
Ciprofloxacin	− 1.22	0.40	− 0.11	0.68	0.64	1.15	1.04	0.64	1.15	[− 4.55, 2.12]	[− 1.16, 1.96]	[− 2.93,2.72]	2.31	1.34	2.43
Clavulanic acid	− 0.29	1.63	0.66	1.04	0.68	0.30	1.08	0.72	0.51	[− 2.98, 2.40]	[− 0.16, 3.43]	[− 1.08, 2.41]	2.29	1.52	1.14
Doxycycline	0.20	− 3.26	0.04	1.21	0.61	0.58	1.31	0.61	1.01	[− 3.30, 3.69]	[− 4.75, − 1.77]	[− 3.50, 3.57]	2.84	1.29	2.27
Ofloxacin	− 1.51	0.85	1.51	0.54	0.29	0.68	0.87	0.30	0.71	[− 3.85, 0.83]	[0.17, 1.52]	[− 0.07, 3.09]	1.88	0.63	1.47
Penicillin V	0.92	0.44	0.31	0.95	0.87	0.63	1.35	1.30	1.42	[− 2.52, 4.36]	[− 2.91, 3.79]	[− 4.02, 4.64]	2.91	2.78	3.14
Roxithromycin	0.18	− 1.12	− 0.63	0.45	0.98	0.70	0.74	1.39	1.05	[− 1.84, 2.20]	[− 4.66, 2.43]	[− 3.37, 2.11]	1.61	2.99	2.26
Tetracycline (HCl)	0.21	0.39	− 0.33	0.96	0.88	0.96	1.13	1.53	1.48	[− 2.44, 2.85]	[− 3.87, 4.65]	[− 4.25, 3.60]	2.39	3.34	3.21

conclusion, the precision of the method is acceptable for all components.

3.4.4.4. Accuracy. To evaluate the accuracy of the analytical method, the β-expectation tolerance intervals were calculated based on the results of trueness and precision [24–26]. Accuracy takes the total error of the test results into consideration and predicts the future measurements. The acceptance limits of licensed pharmaceutical products are [−5%, 5%], which is the same as the manufacturing limit [24]. The accuracy profiles for the 12 selected compounds are shown in Fig. 4. The obtained accuracy profiles indicate that 95% (β) of the sample measurements in the future will be located within the bias limit of [−5%, 5%], which is acceptable for the intended use.

The measurement uncertainty describes the dispersion of the values that can be rationally ascribed to the analyte. The expanded uncertainty is determined with a 95% confidence level, representing an interval in which the unknown true value can be obtained. The relative expanded uncertainty as illustrated in Table 6 is calculated by dividing the expanded uncertainty by the corresponding concentration. The maximum value of relative expanded uncertainty was 3.7%, which is acceptable and reasonable for the analysis of counterfeit antimicrobials.

3.4.5. Reduced validation procedure

Some illegal samples were received after the validation of the quantification method was finalized already. As no major problems were arisen for the first 12 compounds, it was decided to apply a reduced validation in terms of selectivity, LOD, LOQ, linearity and recovery for sulfamethoxazole, trimethoprim, norfloxacin, nitrofurantoin and griseofulvin. LOD, LOQ and linearity data are given in Table 5. The recovery test was performed on the simulated tablet for each antimicrobial separately. The preparation procedure has been explained in Section 2.3.2.2. All recoveries were 98% or higher (Table 3), indicating that the sample treatment procedure is also suitable for these antimicrobials.

3.5. Market study

In total, 57 illegal antimicrobials were collected. They contained 23 samples of amoxicillin, 9 samples of amoxicillin with clavulanic acid and 6 samples of azithromycin. Two samples were received for ampicillin, doxycycline, tetracycline and sulfamethoxazole with trimethoprim, respectively. For the rest of the 11 antimicrobials, 1 sample of each was collected.

3.5.1. Identification of suspected illegal antimicrobials

The validated screening method was applied to the 57 seized samples. The result indicated that these 57 samples were all positive for the claimed API.

3.5.2. Quantification of the suspected illegal antimicrobials

All suspected illegal antimicrobial samples that were positive for the indicated API could be successfully quantified using the validated UHPLC-DAD method, except for erythromycin and azithromycin. As mentioned in Section 3.1, these were quantified using the methods for related substances described in the European Pharmacopoeia [18]. The quantification results of the 57 samples are shown in Table 7, in which 49% of the samples contained amounts of API out of the limits of [95%, 105%]: 46% of the 57 samples were underdosed (ranging from 14.1% to 94.4%), while 3% were overdosed. Nine out of the 23 samples of amoxicillin drug products had unqualified contents ranging from 82.5% to 94.0%. Seven out of the 9 samples containing amoxicillin and clavulanic acid were severely underdosed for clavulanic acid. These findings concur previous ones indicating that samples containing this molecule were more prone to degradation [35]. This phenomenon was attributed to the chemical nature of clavulanic acid and could be the results of non-standard manufacturing, distribution or storage

Table 7
An overview of the quantification results of 57 illegal samples.

Identified compounds		Amount claimed (mg)	Amount present (mg)	Content (%)
Amoxicillin	Sample 1	250	243	97.1
	Sample 2	250	234	93.6
	Sample 3	250	248	99.2
	Sample 4	250	242	96.6
	Sample 5	250	246	98.3
	Sample 6	250	235	94.0
	Sample 7	250	230	92.1
	Sample 8	500	482	96.3
	Sample 9	500	413	82.5
	Sample 10	500	491	98.3
	Sample 11	500	490	98.1
	Sample 12	500	442	88.4
	Sample 13	500	464	92.8
	Sample 14	500	493	98.6
	Sample 15	500	498	99.6
	Sample 16	500	479	95.8
	Sample 17	500	458	91.5
	Sample 18	500	485	97.0
	Sample 19	250	236	94.6
	Sample 20	7500	7000	93.7
	Sample 21	500	496	99.3
	Sample 22	500	480	95.9
	Sample 23	250	235	94.0
Amoxicillin/ Clavulanic acid	Sample 1	875/125	869/18	99.3/14.1
	Sample 2	875/125	800/98	91.4/78.5
	Sample 3	875/125	813/50	92.9/40.0
	Sample 4	875/125	948/80	108.3/63.7
	Sample 5	500/125	498/103	99.5/82.1
	Sample 6	500/125	434/104	86.8/83.4
	Sample 7	500/125	471/126	94.2/100.6
	Sample 8	500/125	483/127	96.6/101.8
	Sample 9	250/125	239/90	95.6/72.3
Ampicillin	Sample 1	250	224	89.8
	Sample 2	1000	784	78.4
Benzathine Penicillin G	Sample 1	1800	1795	99.7
Ceftriaxone	Sample 1	1000	1045	104.5
Cefalexin	Sample 1	500	486	97.3
Ciprofloxacin	Sample 1	500	512	102.4
Doxycycline	Sample 1	100	87	86.8
	Sample 2	100	98	98.3
Ofloxacin	Sample 1	200	189	94.4
Penicillin V	Sample 1	327	323	98.7
Roxithromycin	Sample 1	300	284	94.7
Tetracycline (HCl)	Sample 1	500	498	99.6
	Sample 2	250	244	97.4
Sulfamethoxazole/ trimethoprim	Sample 1	800/160	807/130	100.8/81.2
	Sample 2	800/160	822/129	102.7/80.6
Nitrofurantoin	Sample 1	50	55	110.9
Norfloxacin	Sample 1	400	448	112.0
Griseofulvin	Sample 1	250	249	99.7
Erythromycin[a]	Sample 1	500	403	80.5
Azithromycin[a]	Sample 1	250	260	104.0
	Sample 2	500	453	90.5
	Sample 3	500	513	102.6
	Sample 4	250	249	99.7
	Sample 5	500	511	102.2
	Sample 6	500	455	91.0

[a] Analyzed by methods described in the European Pharmacopoeia.

procedures. Two samples of ampicillin, two samples of trimethoprim, one out of the two doxycycline samples, two out of 6 samples of azithromycin, one sample of erythromycin and one sample of ofloxacin were also found to contain insufficient API. An overview report indicated that 93% (14/15) of the studies concerning SF-antimicrobials addressed similar findings of insufficient amounts of API [36]. Underdosed antimicrobials show a lower efficacy and so may aggravate illness of patients and induce bacterial resistance [7]. In such a way, a potential threat may be posed to the public health.

4. Conclusion

A suitable LC-system for analysis of counterfeit antimicrobials was developed, optimized and validated for the identification via MS and quantification via UV/DAD. In this framework, a fast UHPLC-MS^2 screening method taking 18 min was developed for the identification of 35 antimicrobials and 1 beta-lactamase inhibitor. This method has been validated in terms of selectivity, sensitivity and matrix effects. In addition, a UHPLC-DAD quantification method with an analysis time of 32 min was developed for 31 antimicrobials and 1 beta-lactamase inhibitor. Neomycin, gentamicin, erythromycin and azithromycin were excluded from the quantification method due to their insufficient UV absorbance. The quantification method was validated for samples yielding positive hits in the screening test. As a result, it was validated for 16 antimicrobials and 1 beta-lactamase inhibitor through a full or reduced validation process.

The validated screening method and quantification method have been successfully applied to 57 suspicious illegal antimicrobials seized by FAMHP. All suspected samples contained the indicated API. Whereas about half of the suspected illegal antimicrobials displayed sufficient amounts of API, some (3%) were overdosed, while others (46%) expressed insufficient amounts of API. It was noticed that suspected samples of amoxicillin with clavulanic acid had a quality issue since most of them were underdosed for the beta-lactamase inhibitor.

Taken together, the two described methods demonstrate that they are suitable for quality control of both legal and illegal antimicrobials. They are fast, not labor intensive and make it possible to analyze a wide range of antimicrobials simultaneously, enabling a more in-dept analysis of these SF-antimicrobials.

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Conflicts of interests

The authors state that there is no conflict of interest.

References

[1] World Health Organization, A study on the public heath and socioeconomic impact of substandard and falsified medical products, 2017. 〈http://www.who.int/medicines/regulation/ssffc/publications/se-study-sf/en/〉 (Accessed 6 June 2018).
[2] World Health Organization, WHO global surveillance and monitoring system for substandard and falsified medical products, Geneva. 〈http://www.who.int/medicines/regulation/ssffc/publications/GSMS_Report.pdf?Ua=1〉 (Accessed 13 July 2018).
[3] A. Delepierre, A. Gayot, A. Carpentier, Update on counterfeit antibiotics worldwide; Public health risks, Med. Mal. Infect. 42 (2012) 247–255, https://doi.org/10.1016/j.medmal.2012.04.007.
[4] Grand view research, Antibiotics market size to reach $57.0 billion by 2024, 2016. 〈https://www.grandviewresearch.com/press-release/global-antibiotic-market〉 (Accessed 17 July 2018).
[5] B.J. Venhuis, P.H.J. Keizers, R. Klausmann, I. Hegger, Operation resistance: a snapshot of falsified antibiotics and biopharmaceutical injectables in Europe, Drug Test. Anal. 8 (2016) 398–401, https://doi.org/10.1002/dta.1888.
[6] WHO, Substandard and falsified medical products. 〈http://www.who.int/mediacentre/factsheets/fs275/en/〉 (Accessed 20 July 2018).
[7] T. Kelesidis, M.E. Falagas, Substandard/counterfeit antimicrobial drugs, Clin. Microbiol. Rev. 28 (2015) 443–464, https://doi.org/10.1128/CMR.00072-14.
[8] World Health Organization, WHO global strategy for containment of antimicrobial resistance, Geneva, World Health Organization, 2001. 〈http://www.who.int/iris/handle/10665/66872〉 (Accessed 1 August 2018).
[9] E. Patyra, C. Nebot, R.E. Gavilán, A. Cepeda, K. Kwiatek, Development and validation of multi-residue and multi-class method for antibacterial substances analysis in non-target feed by liquid chromatography–tandem mass spectrometry, Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess. 35 (2018) 467–478, https://doi.org/10.1080/19440049.2017.1414961.
[10] A. Chahbouni, F.A.M. Van den Dungen, R.M. Vos, J.C.G. Den Burger, A. Sinjewel,

A.J. Wilhelm, A.I. Veldkamp, E.L. Swart, M.M. Van Weissenbruch, An UPLC–MS detection method for the quantification of five antibiotics in human plasma, Bioanalysis 7 (2015) 2321–2329, https://doi.org/10.4155/bio.15.121.

[11] M. Gros, S. Rodríguez-Mozaz, D. Barceló, Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry, J. Chromatogr. A 1292 (2013) 173–188, https://doi.org/10.1016/j.chroma.2012.12.072.

[12] L. Tong, H. Liu, C. Xie, M. Li, Quantitative analysis of antibiotics in aquifer sediments by liquid chromatography coupled to high resolution mass spectrometry, J. Chromatogr. A 1452 (2016) 58–66, https://doi.org/10.1016/j.chroma.2016.05.027.

[13] T. Van den Meersche, E. Van Pamel, C. Van Poucke, L. Herman, M. Heyndrickx, G. Rasschaert, E. Daeseleire, Development, validation and application of an ultra high performance liquid chromatographic-tandem mass spectrometric method for the simultaneous detection and quantification of five different classes of veterinary antibiotics in swine manure, J. Chromatogr. A 1429 (2016) 248–257, https://doi.org/10.1016/j.chroma.2015.12.046.

[14] İ. Kivrak, Ş. Kivrak, M. Harmandar, Development of a rapid method for the determination of antibiotic residues in honey using UPLC-ESI-MS/MS, Food Sci. Technol. 36 (2016) 90–96, https://doi.org/10.1590/1678-457X.0037.

[15] L. Chiesa, S. Panseri, E. Pasquale, R. Malandra, R. Pavlovic, F. Arioli, Validated multiclass targeted determination of antibiotics in fish with high performance liquid chromatography–bench top quadrupole orbitrap hybrid mass spectrometry, Food Chem. 258 (2018) 222–230, https://doi.org/10.1016/j.foodchem.2018.03.072.

[16] D. Shin, H.S. Kang, J. Jeong, J. Kim, W.J. Choe, K.S. Lee, G.S. Rhee, Multi-residue determination of veterinary drugs in fishery products using liquid chromatography-tandem mass spectrometry, Food Anal. Methods 11 (2018) 1815–1831, https://doi.org/10.1007/s12161-018-1179-0.

[17] E. Dubreil, S. Gautier, M.P. Fourmond, M. Bessiral, M. Gaugain, E. Verdon, D. Pessel, Validation approach for a fast and simple targeted screening method for 75 antibiotics in meat and aquaculture products using LC-MS/MS, Food Addit. Contam. - Part A Chem. Anal. Control Expo. Risk Assess. 34 (2017) 453–468, https://doi.org/10.1080/19440049.2016.1230278.

[18] European Pharmacopoeia, 9th ed., Council of Europe, Strasbourg, France, 2018.

[19] Y. Chen, C. Hu, J. Qu, M. Yang, Photodegradation of tetracycline and formation of reactive oxygen species in aqueous tetracycline solution under simulated sunlight irradiation, J. Photochem. Photobiol. A Chem. 197 (2008) 81–87, https://doi.org/10.1016/j.jphotochem.2007.12.007.

[20] C. Vanhee, E. Tuenter, A. Kamugisha, M. Canfyn, G. Moens, P. Courselle, L. Pieters, E. Deconinck, V. Exarchou, Identification and quantification methodology for the analysis of suspected illegal dietary supplements: reference standard or no reference standard, that's the question, J. Forensic Toxicol. Pharmacol. 07 (2018) 1–7, https://doi.org/10.4172/2325-9841.1000156.

[21] European Commission, Method validation and quality control procedures for pesticide residues analysis in food and feed, document No. SANCO/12495/2011, Brussels, 2012. 〈http://www.crl-pesticides.eu/library/docs/fv/SANCO12495-2011.pdf〉 (Accessed 8 August 2018).

[22] CRLs, Guidelines for the validation of screening methods for residues of veterinary medicines, Community reference laboratories residues, 2010. 〈https://ec.europa.eu/food/sites/food/files/safety/docs/cs_vet-med-residues_guideline_validation_screening_en.pdf〉 (Accessed 8 August 2018).

[23] ISO/IEC, 17025. General requirements for the competence of testing and calibration laboratories, ISO, Geneva, 2017. 〈www.iso.org〉.

[24] M. Feinberg, Validation of analytical methods based on accuracy profiles, J. Chromatogr. A 1158 (2007) 174–183, https://doi.org/10.1016/j.chroma.2007.02.021.

[25] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal - Part I, J. Pharm. Biomed. Anal. 36 (2007) 579–586, https://doi.org/10.1016/j.jpba.2004.07.027.

[26] A. Gustavo González, M. Ángeles Herrador, A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles, TrAC - Trends Anal. Chem. 26 (2007) 227–238, https://doi.org/10.1016/j.trac.2007.01.009.

[27] P.Y. Sacré, E. Deconinck, P. Chiap, J. Crommen, F. Mansion, E. Rozet, P. Courselle, J.O. De Beer, Development and validation of a ultra-high-performance liquid chromatography-UV method for the detection and quantification of erectile dysfunction drugs and some of their analogues found in counterfeit medicines, J. Chromatogr. A 1218 (2011) 6439–6447, https://doi.org/10.1016/j.chroma.2011.07.029.

[28] M. Ibáñez, C. Guerrero, J.V. Sancho, F. Hernández, Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1216 (2009) 2529–2539, https://doi.org/10.1016/j.chroma.2009.01.073.

[29] A. Junza, N. Dorival-García, A. Zafra-Gómez, D. Barrón, O. Ballesteros, J. Barbosa, A. Navalón, Multiclass method for the determination of quinolones and β-lactams, in raw cow milk using dispersive liquid-liquid microextraction and ultra high performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1356 (2014) 10–22, https://doi.org/10.1016/j.chroma.2014.06.034.

[30] X.L. Hou, Y.L. Wu, Y. Lv, X.Q. Xu, J. Zhao, T. Yang, Development and validation of an ultra high performance liquid chromatography tandem mass spectrometry method for determination of 10 cephalosporins and desacetylcefapirin in milk, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 931 (2013) 6–11, https://doi.org/10.1016/j.jchromb.2013.05.006.

[31] ICH, Q2(r1), International Council on Harmonization: Validation of Analytical Procedures: Text and Methodology, 1996.

[32] J. Van Loco, M. Elskens, C. Croux, H. Beernaert, Linearity of calibration curves: use and misuse of the correlation coefficient, Accredit. Qual. Assur. 7 (2002) 281–285, https://doi.org/10.1007/s00769-002-0487-6.

[33] B. Desmedt, V. Rogiers, P. Courselle, J.O. De Beer, K. De Paepe, E. Deconinck, Development and validation of a fast chromatographic method for screening and quantification of legal and illegal skin whitening agents, J. Pharm. Biomed. Anal. 83 (2013) 82–88, https://doi.org/10.1016/j.jpba.2013.04.020.

[34] ISO Guideline 5725, Part 1: General principles and definitions, in: Accuracy (trueness and precision) of Measurement Methods and Results, 1994. 〈www.iso.org〉.

[35] M.H. Khan, K. Hatanaka, T. Sovannarith, N. Nivanna, L.C.C. Casas, N. Yoshida, H. Tsuboi, T. Tanimoto, K. Kimura, Effects of packaging and storage conditions on the quality of amoxicillin-clavulanic acid-an analysis of Cambodian samples, BMC Pharmacol. Toxicol. 14 (2013), https://doi.org/10.1186/2050-6511-14-33.

[36] T. Almuzaini, I. Choonara, H. Sammons, Substandard and counterfeit medicines: a systematic review of the literature, BMJ Open (2013) 1–7, https://doi.org/10.1136/bmjopen-2013-002923.