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Spectroscopic techniques combined with chemometrics for fast on-site characterization of suspected illegal antimicrobials

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ABSTRACT

The threats of substandard and falsified (SF) antimicrobials, posed to public health, include serious adverse drug effects, treatment failures and even development of antimicrobial resistance. Next to these issues, it has no doubt that efficient methods for on-site screening are required to avoid that SF antimicrobials reach the patient or even infiltrate the legal supply chain. This study aims to develop a fast on-site screening method for SF antimicrobials using spectroscopic techniques (mid infrared, benchtop near infrared, portable near infrared and Raman spectroscopy) combined with chemometrics. 58 real-life illegal antimicrobials (claiming 18 different antimicrobials and one beta-lactamase inhibitor) confiscated by the Belgian Federal Agency for Medicines and Health Products (FAMHP) and 14 genuine antimicrobials were analyzed and used to build and validate models.

Two types of models were developed and validated using supervised chemometric tools. One was used for the identification of the active pharmaceutical ingredients (APIs) by applying partial least squares-discriminant analysis (PLS-DA) and another one was used for the detection of non-compliant (overdosed or underdosed) samples by applying PLS-DA, *k*-nearest neighbors (*k*-NN) and soft independent modelling by class analogy (SIMCA). The best model capable of identifying amoxicillin and clavulanic acid (co-amoxiclav), azithromycin, co-trimoxazole and amoxicillin was based on the mid-infrared spectra with a correct classification rate (ccr) of 100%. The optimal model capable of detecting non-compliant samples within the combined group of amoxicillin and co-amoxiclav via SIMCA showed a ccr for the test set of 88% (7/8) using mid infrared or benchtop near infrared spectroscopy. The best model for detecting non-compliant samples within the group of amoxicillin via SIMCA was obtained using mid-infrared or Raman spectra, resulting in a ccr of 80% for the test set (4/5) and a ccr for calibration of 100%. For the group of co-amoxiclav, the optimal models showed a ccr of 100% for the detection of non-compliant samples by applying mid-infrared, benchtop near infrared or portable near infrared spectroscopy. Taken together, the obtained models, hyphenating spectroscopic techniques and chemometrics, enable to easily identify suspected SF antimicrobials and to differentiate non-compliant samples from compliant ones.

1. Introduction

Since decades, pharmaceutical falsification is gradually attracting more and more attention worldwide. In order to simplify and generalize the term used for falsified medicines, the World Health Organization (WHO) adopted the term “substandard and falsified (SF) medical products” to replace the term “substandard/spurious/falsely-labeled/falsified/counterfeit (SSFFC)” [1]. The definition of SF medical products includes three categories: substandard, unregistered/unlicensed and falsified medicines.

It has no doubt that falsification of antimicrobials spreads drug resistance, endangers public health and leads to economic loss [2,3]. In

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2017, industry estimates have shown that illegal and falsified medical products made profits ranging from USD 163 to USD 217 billion per year [4]. Such lucrative business boosted the illegal drug trade. Indeed, the incidents of pharmaceutical crime increased by 102% over the past five years according to the Pharmaceutical Security Institute (PSI) [5]. Moreover, it has been reported that four million SF drug tablets were confiscated in Germany in 2015 [4]. To curtail the pharmaceutical crime and eliminate the detrimental effects posed by SF medical products, efforts in all aspects were made. In this context, regulations were made and implemented. The European Union executed the Falsified Medicines Directive (FMD) in 2019. According to the FMD, obligatory safety features, e.g. tamper-evident package, are prescribed for drug products and also strict rules on wholesale distributors and the import of drug substances are put in place [6].

Among these SF medical products, antimicrobials are one of the most commonly reported SF drugs [1]. According to the global surveillance and monitoring system (GSMS) of WHO, more than 40% of the reported cases were antimicrobials and of the reported antibiotics, around 90% were listed by the WHO as critically important antimicrobials [7]. These case reports of SF medical products came from all WHO regions: 42% reports were from Africa, 21% from America, 21% from Europe, 8% from the western pacific region, 6% from the Eastern Mediterranean and 2% from South-East Asia [7]. The greater the efforts to look for SF medical products, like in Western Europe and North America, the more SF samples are found. In the meantime, the dangers of SF antimicrobials have been revealed. It has been reported that in Uganda the treatment failure of bacterial meningitis was due to severely underdosed SF ceftriaxone [8]. WHO released medical product alert N° 9/2019, confirming falsified Augmentin (amoxicillin trihydrate-potassium clavulanate) found in Uganda and Kenya. These falsified versions of Augmentin contained no active pharmaceutical ingredients (APIs) [9]. The two major quality issues regarding SF antimicrobials were wrong APIs and insufficient dosage [7], which caused detrimental effects in the past [8]. Therefore, these two primary quality criteria for SF antimicrobials, i.e. correct API and dosage, are required for on-site inspection. Additionally, Belgium is a hub for medicines coming from Asia and going to Africa, due to its central position and the specialization of the Belgian airliners for the African continent. In this context, these transit medicines are at high risk of being falsified. Thus, efficient on-site methods capable of high-throughput screening of transit medicines are paramount for Belgian inspectors.

Many methods have been published for the off-site evaluation of SF antimicrobials, i.e. LC-UV and LC-MS [10–12]. It has to be admitted that off-site evaluation can provide a holistic characterization of SF antimicrobials concerning APIs, impurities, dissolution profiles and microbiological quality. However, the on-site inspection, e.g. at customs or hospitals, of SF antimicrobials is also important since the in-field monitoring of suspected illegal antimicrobials is the first line of defense. On-site inspection should be characterized with little or no sample preparation, fast measurements and easy manipulation. Spectroscopic techniques embrace these advantages and are commonly applied in the detection of falsified or adulterated medical products [13]. However, limited information can be extracted from the raw data obtained by spectroscopic techniques. In this context, chemometric methods serve as a tool to interpret and to capture more information from the spectra [14]. Studies applying chemometric-assisted spectroscopic methods for (SF) antimicrobials are rare. Lê et al. used near infrared (NIR) spectroscopy combined with chemometrics to qualitatively and quantitatively analyze amoxicillin and penicillin [15]. Rodionova et al. performed a feasibility study on antimicrobial drugs using NIR hyperspectral imaging together with chemometric analysis to identify SF antimicrobial drugs [16]. Unfortunately, detailed information was not disclosed in the article. Other studies on antimicrobials using chemometrics reside in the domain of food or wastewater analysis [17,18]. A systematic and comprehensive method using chemometric-assisted spectroscopic techniques for rapid in-field detection of SF antimicrobials in terms of APIs identification and dosage compliance was not available. The current study aims to develop such an on-site screening method enabling rapid evaluation of 18 different antimicrobials and one beta-lactamase inhibitor that are frequently encountered by Belgian controlling agencies.

58 suspected illegal antimicrobial samples confiscated by the Belgian Federal Agency for Medicines and Health Products (FAMHP) and 14 genuine samples were used in this study. All of these samples were analyzed using Fourier transform (FT)-mid infrared (MIR), FT-NIR-benchtop, NIR-handheld and Raman spectroscopy. Initially, it was examined whether aforementioned spectroscopic techniques were capable to identify the APIs present in the samples by performing unsupervised analysis. Based on the promising results obtained from unsupervised analysis, it was decided to perform supervised analysis. Partial least squares-discriminant analysis (PLS-DA) as a supervised tool was employed here in order to build a classification model for APIs.

Besides the right API, the correct dosage is also essential. Subsequently, models were built enabling the detection of non-compliant (underdosed or overdosed) samples. Three chemometric modelling techniques were tested for this purpose, i.e. PLS-DA, *k*-nearest neighbors (*k*-NN) and soft independent modelling by class analogy (SIMCA).

2. Methods and materials

2.1. Samples and standards

58 suspected illegal samples were collected by the inspectors of the FAMHP and were composed of 18 different antimicrobials and one beta-lactamase inhibitor, i.e. amoxicillin, ampicillin, azithromycin, benzathine penicillin G, ceftriaxone, cephalexin, ciprofloxacin, doxycycline, erythromycin, griseofulvin, ofloxacin, penicillin V, roxithromycin, tetracycline (hydrochloride), nitrofurantoin, norfloxacin, sulfamethoxazole, trimethoprim and clavulanic acid.

14 Genuine drug products were bought from a Belgian pharmacy, consisting of Amoxiclav (amoxicillin/clavulanic acid, 500/125 mg or 875/125 mg), amoxicillin (500 mg) and azithromycin (250 mg or 500 mg) from Sandoz (Vilvoorde, Belgium), Flemoxin Solutab (amoxicillin, 500 mg) from Astellas (Brussels, Belgium), Augmentin (amoxicillin/clavulanic acid, 500/125 mg) from GlaxoSmithKline (Wavre, Belgium), Bactrim forte (trimethoprim/sulfamethoxazole, 800/160 mg) from Roche (Brussels, Belgium) and Eusaprim forte (trimethoprim/sulfamethoxazole, 800/160 mg) from Aspen (Dublin, Ireland). Moreover, genuine drug products of Amoclane (amoxicillin/clavulanic acid, 875/125 mg), amoxicillin syrup (250 mg/5 mL), azithromycin (250 mg or 500 mg) and roxithromycin (250 mg) were from Eurogenerics (Brussels, Belgium). It has to be noted that the genuine drugs of amoxicillin syrup and roxithromycin were received in a later phase. As a result, these two genuine drugs were only analyzed by benchtop NIR and Raman spectroscopy. Overview of samples including illegal and genuine ones are described in Supplemental Table 1.

2.2. Sample preparation

For recording the spectra, all tablets were powdered and homogenized prior to measurement. The powders of capsules, injectables and syrups were measured directly.

2.3. Data acquisition

2.3.1. MIR spectroscopy

A Nicolet iS 10 FT-IR (ThermoFisher Scientific, Waltham, USA) supplied with a Smart iTR accessory and a deuterated triglycine sulfate (DTGS) detector was employed for the MIR spectra acquisition. The Smart iTR accessory was used with a single bounce diamond crystal and

was calibrated every week via a polystyrene film.

To record the spectrum, a small quantity of sample powder was placed directly on the diamond crystal. In order to ensure uniformity of the spectra, proper pressure was applied to the sample in order to obtain a homogenous surface. This pressure is part of the basic specifications of the instrument. After each measurement, the crystal was cleaned with methanol and dried in ambient air. MIR spectra were recorded from 4000 to 650 cm^{-1}. The instrument resolution was set at 4 cm^{-1} and comprised 32 co-added scans. Prior to each sample testing, a blank (air: no sample) was measured to verify the crystal for contamination and carry over according to the absorbance limits prescribed by the European Directorate for the Quality of Medicines (EDQM) [19]. A background spectrum was recorded hourly against air using the same instrumental conditions as for the samples. Spectral visualization and data export were performed using OMNIC software version 8.3 (Thermo Scientific, Madison, USA).

2.3.2. Benchtop and portable NIR spectroscopy

The sample powder was brought into a glass vial and spectra were measured using a benchtop and a portable NIR spectrometer respectively. Spectra for benchtop NIR were collected in the region of 10000–4000 cm^{-1} with a resolution of 16 cm^{-1} and an average scan of 16 using a benchtop FT-NIR Antaris MX (Thermo Fisher Scientific, Erembodegem, Belgium). A portable NIR microPHAZIR RX spectrometer (ThermoFisher Scientific, Boston, USA) with a resolution of 12 nm and co-added scans of 5 was employed to record spectra in the wavelength region from 1600 to 2400 nm.

2.3.3. Raman spectroscopy

Sample powder was placed in a specific metal holder for the measurements using a Raman Rxn2 spectrometer (Kaiser Optical Systems, Ann Arbor, MI, USA) equipped with a charge coupled device (CCD) detector. A fiber-optic cable was used to connect the Raman PhAT probe with the spectrometer. The laser wavelength was 785 nm and the resolution was 5 cm^{-1}. Raman spectra were recorded with an exposure time of 15 s in the range from 250 to 1890 cm^{-1}. Spectral data manipulation was performed using iC Raman 4.1 collection software (Kaiser OS, MI, USA).

2.4. Chemometric approaches

2.4.1. Data pretreatment

The complete spectra obtained from benchtop NIR, portable NIR and Raman were used as input for the chemometric methods. MIR spectra were tailored to the fingerprint regions from 2000 to 650 cm^{-1} prior to chemometric analysis. The MIR spectral region of 4000–2000 cm^{-1} was deleted due to the atypical characteristics of the spectra.

The raw spectral data need to be pre-processed in order to eliminate the influence of noise, fluctuations of the baseline, variations in the detector and so on. Five different approaches were selected for data pretreatment. Standard Normal Variate (SNV) was used to remove variations generated from the measurement itself, e.g. scattering effects. Moreover, the methods of the first derivative and the second derivative were applied since they correct for drift and baseline errors and magnify small differences between spectra of different samples [20]. In this context, the first or second derivative was calculated using the Savitzky-Golay method [21] with a second order polynomial and a window size of 17. Additionally, SNV followed by the first derivative or the second derivative were used as two supplementary approaches for data pretreatment. Generally, SNV and the second derivative were performed separately as pretreatment methods for unsupervised analysis and supervised analysis. The first derivative and also SNV followed by the first derivative or the second derivative were only applied for modelling dosage compliance (see Section 3.2.2).

2.4.2. Unsupervised analysis

Unsupervised analysis was performed to provide a general idea of data distribution and to detect clusters of samples that could be linked to the API in the samples. This unsupervised analysis can be seen as a data exploration step in which differences between samples are searched for, giving a rational basis to proceed to supervised modelling. In this study, Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA) were selected for this purpose.

2.4.2.1. PCA. PCA is a projection technique simplifying the interpretation of multivariate data by projecting high dimensional data onto a lower number of dimensions, defined by new latent variables called principal components (PCs). The latent variables or PCs are defined as linear combinations of the original variables, in which the loadings indicate the contribution of each variable to a given PC. The first PC describes the largest variation in the data and the second PC the largest residual variation around PC 1. PC 3 explains the highest remaining variation around the plane PC 1–PC 2. By definition all PCs are orthogonal to each other. The projections of the objects on a PC are called the scores. A plot of the scores in two or three dimensions allows to explore the (dis)similarities among the objects and is called a score plot. The loading plot allows to identify those original/manifest variables responsible for the difference among samples or clusters of samples [20].

2.4.2.2. HCA. HCA is used for clustering the objects according to their (dis)similarity and is therefore an alternative to PCA for interpreting the distribution and structure of a data set. Data clustering is depicted by a dendrogram, which is hierarchical since large clusters are split into smaller ones until each cluster only comprise one or a predefined number of objects. Different distance metrics and clustering algorithms were tested in this study to compare clustering performances.

2.4.3. Supervised analysis

Contrary to the unsupervised method, supervised analysis incorporates preliminary obtained information from the samples, e.g. drug classes of samples. In this way, supervised methods generate models to classify samples or predict information for a new object. Many techniques are developed for supervised data modelling. In this study, PLS-DA, *k*-NN and SIMCA were employed for modelling, among which PLS-DA was performed for all supervised analysis (APIs identification and dosage compliance) while *k*-NN and SIMCA were only applied for modelling the compliance of the samples (see Section 3.2.2).

2.4.3.1. Selection of training and test set. For model validation, an external test set needs to be selected. Two selection methods were applied, i.e. Kennard & Stone and Duplex [22]. These two algorithms assure that the selected test set is representative for the complete original data set, and thus is uniformly distributed within the whole data space [23]. In general 20% of the samples were assigned to the test set, used for external validation of the obtained models, and the remaining samples were allocated to the training set, used for the construction of the models.

According to the Kennard & Stone algorithm, the sample located farthest from or closest to the data mean is selected first for the test set, subsequently the second sample, situated farthest from the first one, is selected. The third selected sample is the one, most distant from the previous two selected samples. This procedure continues until the desired number of samples is obtained for the test set. The un-assigned samples are used for the training set [24].

The Duplex algorithm is performed based on Euclidean distances. All samples are selected in pairs. Initially, the two samples with the highest Euclidean distance between each other are selected and assigned to a first set. Subsequently, another two samples with the highest Euclidean distance are allocated to a second set which is the test set. This continues in an iterative way until a predefined number of samples

is obtained for the test set. The first set and the remaining, not selected samples, compose the training set [23].

2.4.3.2. PLS-DA. PLS is a supervised projection technique. Similar to PCA, the PLS latent variables, also called PLS factors, are obtained by the linear combination of manifest variables. However, the criterion of constructing latent variables in PLS requires maximal covariance with the targeted response variable(s). PLS-DA is a variant of PLS, used when the response variables are categorical [20]. In this study, PLS-DA was used to build classification models in terms of API identification and detection of non-compliant samples, respectively. A 10-fold cross validation procedure was applied here to select the optimal number of PLS factors for the different models.

2.4.3.3. k-NN. *k*-NN is a simple supervised modelling tool using nearest neighbors to classify objects. This algorithm starts with calculating the Euclidean distances (or correlation coefficients) between the unknown objects and the objects of the training set. Then *k* objects with the lowest Euclidean distance (or the highest correlation coefficients) are selected. According to the majority rule, the unknown object is attributed to the group to which the majority of the *k* nearest neighboring objects belong [20]. Since *k*-NN is performing best to solve binary problems, it was only applied to distinguish between compliant and non-compliant samples. According to the characteristics of known samples (training set), samples were divided into two classes, i.e. compliant samples and non-compliant samples. *k*-NN was applied on these known samples to build classification models allowing unknown samples to be assigned to one of the classes, based on the spectroscopic data. In this case, a 10-fold cross validation procedure was performed to select the optimal *k* value to obtain the model with highest correct classification rate (ccr) for cross-validation (see Section 3.2).

2.4.3.4. SIMCA. SIMCA is used as a supervised tool for the classification of data. Unlike previously introduced chemometric tools, SIMCA gives an emphasis on the similarity inside the class rather than the discrimination between the classes. This is also called disjoint class modelling. In this study, SIMCA uses samples with known features to construct classification models with two classes (compliant or non-compliant), which enables to assign new samples with unknown features to one of the classes. By performing SIMCA, each class is modelled separately using PCA. Once the number of PCs for a specific class is chosen, a space around the class is defined by two critical values, defining the boundaries of samples residing in that class. The two critical values are the Euclidian distance towards the SIMCA model and the Mahalanobis distance determined in the space of scores following the Hotelling T^2-distribution.

To determine the location of a new sample, the scores and loadings of the created PCA model are calculated. The new sample is classified into a certain class if it is located within the defined space around the training set of a particular class. This classification algorithm is repeated for each of the classes in the model according to the obtained PCA model for each class. Confidence limits are usually set at 95% [20]. The algorithm of SIMCA uses cross-validation for the selection of the optimal number of PCs to be included [20]. Unfortunately, cross validation results for the whole model cannot be retrieved. Therefore, only calibration errors and errors for the external test set are reported.

2.5. Software

Data calculation and modelling were performed using Matlab R2016b (The Mathworks, Natwick, MA, USA). The algorithms of PCA, HCA, Kennard & Stone, Duplex, Savitzky and Golay, PLS-DA and *k*-NN were part of the ChemoAC toolbox (Freeware, ChemoAC Consortium, Brussels, version 4.0). The SIMCA toolbox was downloaded from the Matlab Central [25].

3. Results

Spectra were recorded for all suspected illegal and genuine samples using MIR, benchtop NIR, portable NIR and Raman spectroscopy. The obtained spectral data was subjected to chemometric analysis.

3.1. Unsupervised analysis

In a first step, it was examined whether MIR, NIR and Raman techniques were capable to identify the different APIs in the samples. Unsupervised analysis was performed as exploratory analysis. PCA and HCA were selected as analysis tools and to assist in data visualization. Three dimensional score plots (PC1-PC2-PC3) were computed for all sample sets. Concerning HCA, single and Ward's methods were applied with five different distance metrics, i.e. the Euclidean distance, the standardized Euclidean distance, the city block distance, the Mahalanobis distance and the Minkowski distance. All dendrograms computed with different combinations of methods and distance metrics were compared considering the clusters based on the different APIs. In this section, all spectra were preprocessed by SNV and the second derivative separately.

3.1.1. MIR spectroscopy

MIR spectra were recorded for all suspected and genuine samples and used as input for PCA analysis. Three dimensional score plots (PC1-PC2-PC3) obtained with the different data pretreatment procedures were compared and the best score plot, obtained with SNV as preprocessing is shown in Fig. 1a. Clusters of azithromycin (contaminated with one erythromycin sample) and co-trimoxazole can clearly be distinguished, while co-amoxiclav and amoxicillin samples were clustered together. Similarly, it can also be observed in the dendrogram obtained with HCA (Ward's method with the Euclidean distance as similarity measure) on MIR data preprocessed via SNV that amoxicillin and co-amoxiclav were mixed (Fig. 1b). Additionally, it has been observed in Fig. 1b that samples of tetracycline, doxycycline and ampicillin were clustered, although the sample size was small within the respective groups. The rest of the samples (not marked in Fig. 1) were sole in their respective class of APIs.

3.1.2. Benchtop NIR spectroscopy

For the spectra obtained with the benchtop NIR, the best score plot for PCA was computed on spectra pretreated with the second derivative as shown in Fig. 2a. The groups of co-trimoxazole and tetracycline were clustered while the rest of samples were not well separated. However, better clustering was observed in the dendrogram obtained with Single-Euclidean distance and SNV as pretreatment (see Fig. 2b). An additional clustering of the azithromycin class was obtained.

3.1.3. Portable NIR spectroscopy

The best score plot for PCA was obtained on spectra preprocessed by SNV (See Fig. 3a). The best dendrogram was computed with the single algorithm and the Minkowski distance when data were pretreated with SNV (See Fig. 3b). Similar results as benchtop NIR were obtained: amoxicillin and co-amoxiclav were not separated and even mixed with ampicillin.

3.1.4. Raman spectroscopy

When co-amoxiclav, tetracycline and nitrofurantoin were screened by Raman spectroscopy, signals were saturated. For this reason, these three drugs were excluded from the Raman data set. The optimal PCA score plot (data preprocessed by SNV) shows no clear differentiation in APIs except for the group of co-trimoxazole (See Fig. 4a). The optimal dendrogram computed using HCA (single-Euclidean distance) on data pretreated with the second derivative is illustrated in Fig. 4b. It displays better clustering compared to the PCA plot and it is able to differentiate macrolide antibiotics (Fig. 4b), i.e. azithromycin, erythromycin and roxithromycin.

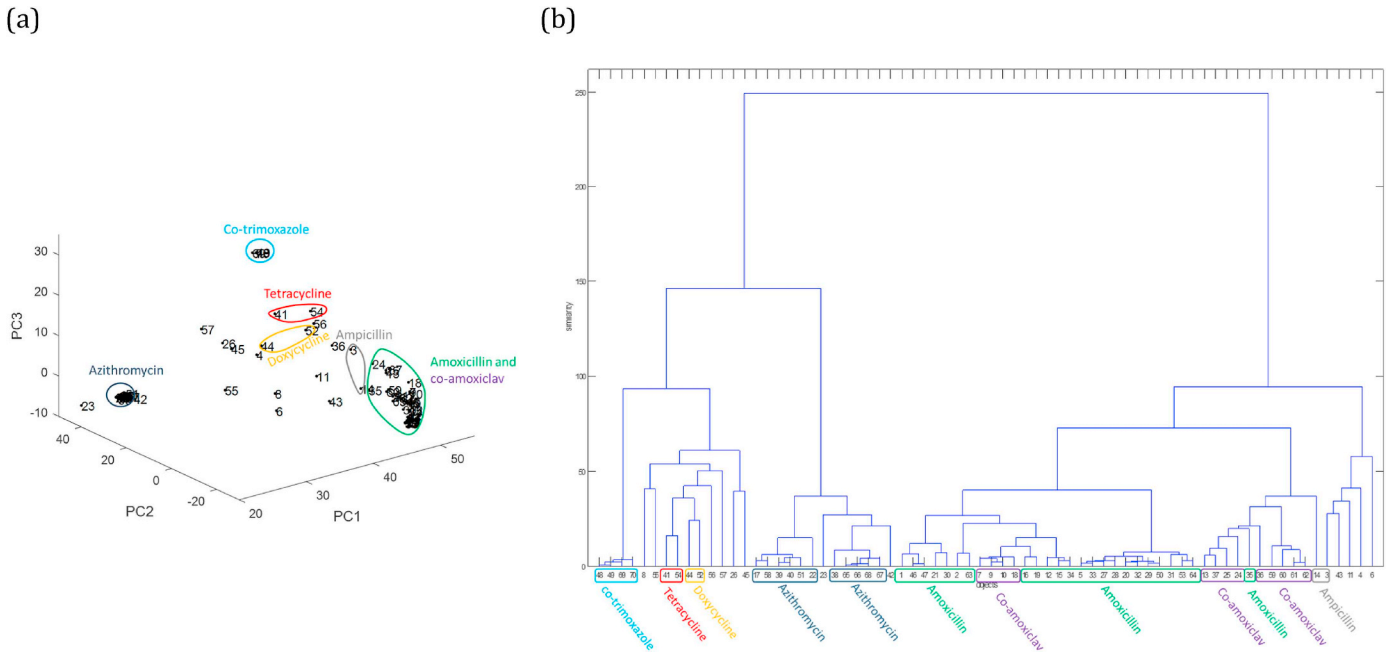

Fig. 1. (a) PCA plot obtained with the MIR spectra after SNV pretreatment. (b) Dendrogram constructed via hierarchical clustering (Ward-Euclidean distance) on MIR spectra pretreated with SNV. Colored rectangles indicate different drug groups which contain not less than two samples. The rest of the numbers outside the colored rectangles indicate individual samples.

3.1.5. Discussion

Towards a comprehensive consideration among the aforementioned four spectroscopic techniques, MIR generated the best results. In general, co-trimoxazole and azithromycin samples could be clustered respectively in an unsupervised way. Amoxicillin and co-amoxiclav could not be separated, which is probably due to the low amount of clavulanic acid compared to the dose of amoxicillin in the sample.

3.2. Supervised analysis

Since promising results were obtained with unsupervised techniques, it was decided to continue with supervised analysis. In this context, data characteristics on APIs and dosage compliance were modelled. In this section, PLS-DA was employed for API identification and dosage compliance, while *k*-NN and SIMCA were only performed for dosage compliance.

3.2.1. Identification of APIs

For on-site inspection of illegal antimicrobials, the first task is the identification of APIs. PLS-DA was applied as a supervised technique on the different spectral data sets, either preprocessed by SNV or the second derivative in order to enhance the discrimination of the samples

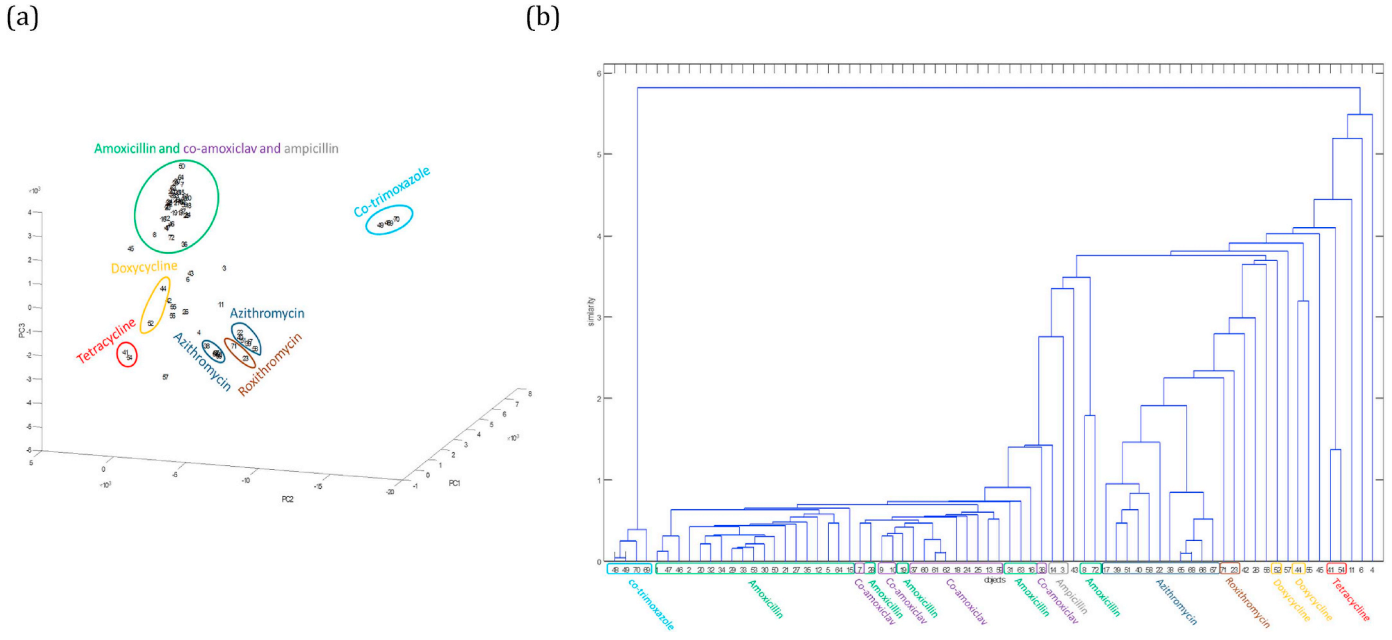

Fig. 2. (a) PCA plot obtained with the benchtop NIR spectra after pretreatment with the second derivative. (b) Dendrogram constructed via hierarchical clustering (Single-Euclidean distance) on benchtop NIR spectra pretreated with SNV. Colored rectangles indicate different drug groups which contain not less than two samples. The rest of numbers outside the colored rectangles indicate individual samples.

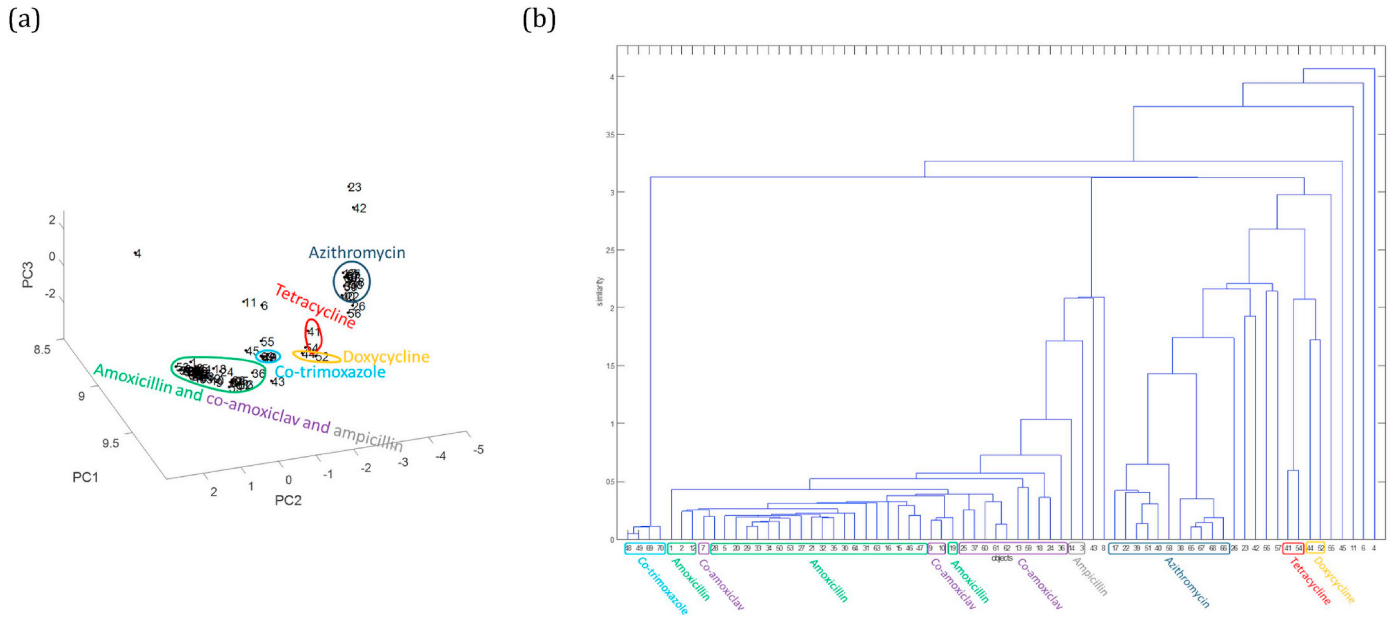

Fig. 3. (a) PCA plot obtained with the portable NIR spectra after pretreatment with SNV. (b) Dendrogram constructed via hierarchical clustering (Single-Minkowski distance) on portable NIR spectra pretreated with SNV. Colored rectangles indicate different drug groups which contain not less than two samples. The rest of numbers outside the colored rectangles indicate individual samples.

in their respective classes of APIs (see Section 2.4.1). The samples were analyzed using chromatography according to a method developed previously by our group [12]. The screening results indicated that all samples contained the label claimed APIs, which was taken into account as a response for chemometric analysis (Supplemental Table 1). Seen the raw data as described in Supplemental Table 1, it was decided to divide the samples into five groups including co-trimoxazole, amoxicillin, azithromycin, co-amoxiclav and other for the modeling of APIs identification. The reason of creating a group "other" is that sample size is less than two towards each API.

3.2.1.1. MIR spectroscopy. In total five classes of different APIs were included in the model, i.e. co-trimoxazole, amoxicillin, azithromycin, co-amoxiclav and all the others. 70 samples were split into a training set (56 samples) and a test set (14 samples) by applying Duplex. The external test set of 14 samples covered all five classes. As shown in Table 1, the optimal PLS-DA model on MIR data was obtained by using 8 PLS factors (the spectra were pretreated with SNV) showing a correct classification rate (ccr) for cross validation of 91%, in which 5 out of 56 samples were misclassified. From these five misclassified samples, three of them were co-amoxiclav, misclassified as amoxicillin and another two samples, belonging to the "other" group, were wrongly assigned to

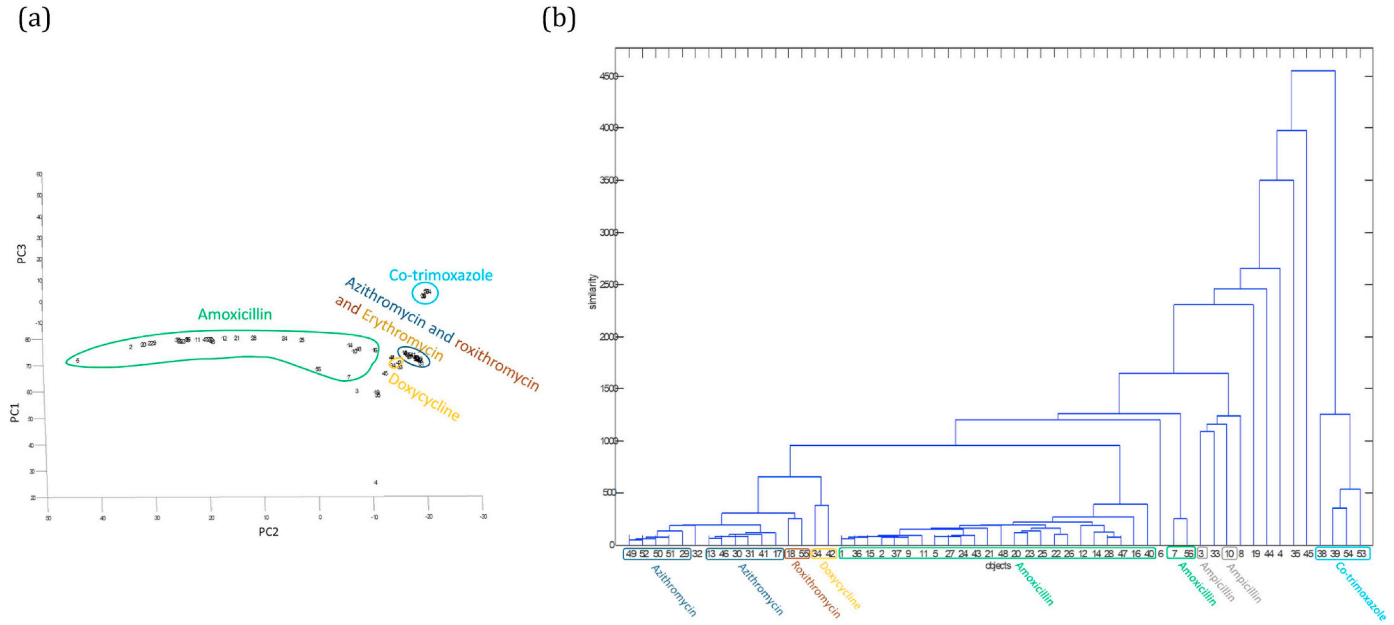

Fig. 4. (a) PCA plot obtained with the benchtop Raman spectra after pretreatment with SNV. (b) Dendrogram constructed via hierarchical clustering (Single-Euclidean distance) on Raman spectra pretreated with the second derivative. Colored rectangles indicate different drug groups which contain not less than two samples. The rest of numbers outside the colored rectangles indicate individual samples.

Table 1
Overview of PLS-DA modelling results for API identification expressed in correct classification rate (ccr). SNV: standard normal variate, dx2: second derivative.

Dataset pretreatment	MIR		Benchtop NIR		Portable NIR		Benchtop Raman	
	SNV	dx2	SNV	dx2	SNV	dx2	SNV	dx2
5 classes							4 classes	
No. of PLS-factors	8	7	24	13	26	26	7	9
Cross validation	91% (51/56)	84% (47/56)	93% (54/58)	90% (52/58)	95% (53/56)	89% (50/56)	86% (38/44)	93% (41/44)
External validation	93% (13/14)	79% (11/14)	86% (12/14)	79% (11/14)	71% (10/14)	79% (11/14)	100%	92% (11/12)
4 classes[a]							3 classes[a]	
No. of PLS-factors	8	8	8	5	8	19	9	4
Cross validation	100%	100%	98% (42/43)	98% (42/43)	95% (41/43)	98% (42/43)	94% (31/33)	100%
External validation	100%	100%	100%	91% (10/11)	100%	100%	100%	100%

[a] Class “other” excluded.

the group of amoxicillin or azithromycin. Meanwhile, the test set showed a ccr of external validation of 93% or 13 of 14 correctly classified. The misclassified sample was within the “other” group, but was assigned to the azithromycin group. Since the “other” group contained different APIs, such as griseofulvin, nitrofurantoin, cephalexin, ampicillin and so on, it may induce noise into to the model. Therefore, it was decided to limit the classes to four, including co-trimoxazole, amoxicillin, azithromycin and co-amoxiclav. In this case, the PLS-DA model on spectra pretreated by either SNV or the second derivative, showed a 100% ccr for cross validation and external validation (see Table 1), indicating that the high variation in the “other” class introduced errors in the model.

3.2.1.2. Benchtop and portable NIR spectroscopy. For the analysis of benchtop NIR spectra, the Duplex method was used to select 14 samples for the test set. The remaining 58 samples composed the training set. The optimal PLS-DA model for five classes (24 PLS factors) was built on data pretreated with SNV, showing a cross validation ccr of 93% (54/58) and a ccr of external validation of 86% (12/14) (See Table 1). During cross validation, four misclassified samples were all from the “other” class. Two of them were wrongly classified as co-amoxiclav samples, and another two samples were incorrectly assigned to the group azithromycin and co-trimoxazole. Similarly, two misclassified samples from the test set were also from the “other” class, but were wrongly classified as azithromycin. If the class “other” was omitted, then the PLS-DA model with 8 PLS factors showed a ccr of cross validation of 98% (42/43) and a ccr of external validation of 100%, which was built on data pretreated with SNV. The one misclassified sample of co-amoxiclav during cross validation was wrongly assigned to the group of amoxicillin, which could be attributed to the fact that this SF co-amoxiclav sample contained only 14.2% of the claimed dose of clavulanic acid [12].

Concerning the portable NIR, the best PLS-DA model was obtained on data pretreated with the second derivative and sorted by the Kennard & Stone method. The model showed a cross validation ccr of 89% (50/56) and an external validation ccr of 79% (11/14). Among the six misclassified samples during cross validation, three samples from the class “other” were assigned to the group azithromycin and co-amoxiclav, two samples from the class co-amoxiclav were wrongly allocated to the group amoxicillin and one sample of amoxicillin was mistakenly assigned to the group co-amoxiclav. For misclassifications of external validation, two samples were from the class “other”, but were incorrectly assigned to the group co-trimoxazole and co-amoxiclav and one sample was amoxicillin that was wrongly assigned to the class “other”. Additionally, another model obtained on data preprocessed by SNV and sorted by Kennard & Stone showed a cross validation ccr of 95% (53/56) and an external validation ccr of 71% (10/14), which generated a higher ccr value of cross validation compared with the previously described model. However, seen the difference in values between the Root Mean Squared Error Cross Validation (RMSECV) and the Root-Mean-Square Error of Prediction (RMSEP), this model has higher probability of showing overfitting. When the number of classes was limited to four, the optimal model was built on the data pretreated with the second derivative, and the selection method of the Kennard & Stone algorithm was used to select 14 samples for the test set and 43 samples for the training set. In this case, the model performance was improved with a ccr of cross validation of 98% (42/43) and a ccr of external validation of 100%. This one misclassification during cross validation was the same one as encountered with benchtop NIR, which was co-amoxiclav with a clavulanic acid content of 14.2% of the claimed dose and which was wrongly classified as amoxicillin.

Generally, compared with the models obtained on MIR spectra, NIR spectra generated less performant models.

3.2.1.3. Raman spectroscopy. The data set of Raman spectroscopy was composed of 56 samples that could be classified in four classes, i.e. co-trimoxazole, amoxicillin, azithromycin and other. Duplex was used to select 12 samples for the test set and the rest (the remaining 44 samples) composed the training set. As shown in Table 1, the optimal PLS-DA model for the four classes was obtained with 7 PLS factors and using SNV as preprocessing technique. This model resulted in a ccr of external validation of 100% and a ccr of cross validation of 86% (38/44). Six misclassifications were encountered during cross validation, five of which were within the class “other”, but were wrongly assigned to the class azithromycin. Among these five samples, two of them were roxithromycin, one was erythromycin, one was ofloxacin and one was doxycycline. Another misclassified sample was amoxicillin that was wrongly classified as azithromycin. Similar as before, if the class “other” was deleted, the model was refined to generate both ccr values of cross validation and external validation of 100%.

3.2.2. Detection of non-compliant samples

Besides the significance of identifying APIs, the dosage of APIs is of utmost importance as well in terms of drug efficacy as for treatment efficacy. In this step, we further explored whether the methods were able to detect non-compliant (underdosed or overdosed) samples. These models aim to check whether suspected illegal samples comply with the API dosage or not. An API dosage out of the limit of 95%–105% is considered non-compliant. Due to the limitation of samples, only amoxicillin and co-amoxiclav samples were included in this part of the study. These samples were analyzed using chromatography for assay [12]. Most suspected illegal samples were underdosed, except one sample that was overdosed for amoxicillin. Their corresponding classifications (compliant or non-compliant) based on the contents of samples towards different models (Supplemental Table 2) were used as the response for building chemometric models. Five different methods of data pretreatment as illustrated in Section 2.4.1 were performed on all data sets of different spectra. The spectroscopic data of amoxicillin and co-amoxiclav were modelled using PLS-DA, SIMCA and *k*-NN respectively, to verify if good discrimination/classification models could

Table 2
Overview of the optimal performance within different models expressed in ccr (%) for the detection of non-compliant samples.

	MIR	Benchtop NIR	Portable NIR	Raman
Dataset	Amoxicillin and co-amoxiclav			
PLS-DA				/
No. of PLS-factors	7	8	2	
Cross validation	77% (23/30)	77% (24/31)	63% (19/30)	
External validation	88% (7/8)	88% (7/8)	75% (6/8)	
SIMCA				
No. of PCs	13–7	6–9	6–2	
Calibration*	93% (28/30)	90% (28/31)	80% (24/30)	
External validation	88% (7/8)	88% (7/8)	88% (7/8)	
***k*-NN**				
No. of nearest neighbors	5	5	11	
Cross validation	80% (26/30)	65% (20/31)	63% (19/30)	
External validation	88% (7/8)	88% (7/8)	88% (7/8)	
Dataset	Amoxicillin			
PLS-DA				
No. of PLS-factors	5	8	8	2
Cross validation	65% (13/20)	67% (14/21)	85% (17/20)	52% (11/21)
External validation	100%	80% (4/5)	80% (4/5)	80% (4/5)
SIMCA				
No. of PCs	9–4	7–3	3–2	11–7
Calibration*	100%	71% (15/21)	60% (12/20)	100%
External validation	80% (4/5)	80% (4/5)	80% (4/5)	80% (4/5)
***k*-NN**				
No. of nearest neighbors	7	5	5	5
Cross validation	85% (17/20)	71% (15/21)	85% (17/20)	81% (17/21)
External validation	80% (4/5)	60% (3/5)	80% (4/5)	80% (4/5)
Dataset	Co-amoxiclav			
PLS-DA				/
No. of PLS-factors	3	6	7	
Cross validation	89% (8/9)	100%	100%	
External validation	100%	100%	100%	
SIMCA				
No. of PCs	2–2	2–3	2–3	
Calibration*	100%	100%	78% (7/9)	
External validation	100%	100%	100%	
***k*-NN**				
No. of nearest neighbors	3	3	3	
Cross validation	100%	89% (8/9)	78% (7/9)	
External validation	100%	75% (3/4)	75% (3/4)	

/indicates no modelling results for Raman spectroscopy since co-amoxiclav is not compatible with the Raman technique used in this study.
* The SIMCA algorithm applied in this study is not able to retrieve the results of cross-validation, so the ccr values of calibration are reported.

be obtained for compliant and non-compliant samples. It has to be noted that the SIMCA algorithm uses cross-validation to select the optimal number of PCs, though the results of cross validation for the whole model are not able to be retrieved. Thus, only calibration errors and the errors for the external test set were reported here for SIMCA.

In a first modelling experiment, amoxicillin and co-amoxiclav samples were grouped to build up the model. Afterwards, the modelling was also performed on the amoxicillin samples and co-amoxiclav samples separately.

3.2.2.1. Amoxicillin and co-amoxiclav. Three different datasets including amoxicillin and co-amoxiclav samples were generated using MIR, benchtop NIR and portable NIR techniques, respectively. The quality compliance of the co-amoxiclav samples was based on the dosage of amoxicillin. The optimal model for the detection of non-compliant samples within the combined group was obtained with SIMCA using MIR or benchtop NIR (see Table 2). For the model obtained on MIR data, the Kennard & Stone algorithm was used to select training and test set. As a result, eight samples evenly covering the two classes (compliance or non-compliance) were assigned to the test set and the remaining 30 samples composed the training set. For MIR, SIMCA (PCs 13-7) provided the optimal model, which was built on the data pretreated with SNV, followed by the first derivative, showing a ccr of 93% or 2 samples out of 30 misclassified during calibration of the training set as shown in Table 2. These two samples contained 94.6% and 94.0% of the labeled amount of API respectively, which were located at the borderline of the criterion (95%) describing non-compliant samples. It may be the reason that the model classified these two samples as compliant. Moreover, the external validation showed that 1 out of 8 samples was wrongly classified as compliant. This one misclassified sample contained only 86.8% of the labeled amount of amoxicillin, but was wrongly classified as compliant.

For benchtop NIR, Duplex was performed to select eight samples and the remaining 31 samples composed the training set. The optimal model was obtained with the spectra pretreated with SNV using SIMCA (PCs 6-9), showing a ccr of calibration of 90% (28/31) and a ccr of external validation of 88% (7/8). Three misclassifications encountered during calibration included two samples with amoxicillin contents of 97.1% and 95.8% respectively of the claimed dose that were wrongly classified as non-compliant and one sample with an amoxicillin content of 93.6% of the claimed dose that was misclassified as compliant. One misclassified sample (91.5% of labeled dosage) of the external validation was misclassified as compliant. For the portable NIR, Duplex was performed to select eight samples for the test set and the 30 samples for the training set. The best model for the portable NIR data was built on the spectra pretreated with SNV using SIMCA (PCs 6-2). This model showed a ccr of calibration of 80% (24/30) and a ccr of external validation of 88% (7/8). The misclassified sample from the test set was wrongly classified as non-compliant while all six misclassifications encountered during calibration were wrongly classified as compliant.

As shown in Table 2, all SIMCA and *k*-NN models obtained with MIR, benchtop NIR and portable NIR were able to reach a ccr of external validation of 88% (7/8). Among these three spectroscopies, MIR and benchtop NIR generated comparable models to detect non-compliant samples in the combined group of amoxicillin and co-amoxiclav if the dosage compliance only took amoxicillin into account. Overall SIMCA gave always the better performing models.

3.2.2.2. Amoxicillin. Four data sets of the amoxicillin samples using respectively MIR, benchtop NIR, portable NIR and Raman spectroscopy were obtained. For the analyses of the MIR spectra, Kennard & Stone was used to select five samples for the test set and the 20 samples of the training set. The models of MIR spectra using PLS-DA and SIMCA were constructed on data preprocessed by SNV. Interestingly, the ccr of external validation of one model on MIR reached 100% using PLS-DA with 5 PLS factors (see Table 2). However, this model showed a low ccr of 65% or 13 of 20 correctly classified during cross validation. Among these seven misclassifications, four of them were wrongly classified as compliant and three of them were incorrectly classified as non-compliant. When SIMCA (PCs 9-4) was applied, the obtained model on MIR showed a ccr of 100% of calibration and a ccr of 80% of external validation or 4 out of 5 samples correctly classified. This one

misclassified sample contained 95.8% of the labeled amount of amoxicillin. Since the amount of amoxicillin resided at the borderline of the criterion (95%) for non-compliant samples, it may be the reason for the error.

For benchtop NIR, the Kennard & Stone algorithm was applied to assign five samples to the test set and 21 samples to the training set. The optimal model obtained on spectra of benchtop NIR (preprocessed by the first derivative) using SIMCA (PCs 7-3) showed a ccr of 71% (15/21) during calibration and a ccr of external validation of 80% (4/5). Six misclassifications were encountered during calibration, five of which were misclassified as non-compliant while one was wrongly classified as compliant. One misclassification of external validation was incorrectly classified as non-compliant.

Concerning portable NIR, the Kennard & Stone algorithm was used to select the five samples of the test set and the remaining 20 samples constituted the training set. In this case, both PLS-DA and *k*-NN provided the optimal models, showing two times a ccr of cross validation of 85% (17/20) and a ccr of external validation of 80% (4/5). The optimal model of PLS-DA was built on the second derivative spectra. Three misclassifications encountered during cross validation included two samples wrongly classified as compliant and one was wrongly classified as non-compliant. Meanwhile, one sample of the test set was wrongly classified as non-compliant. Additionally, the optimal model of *k*-NN was obtained on the spectra pretreated with SNV, followed by the second derivative, showing the same misclassification of the test set as the PLS-DA model. Cross validation of the optimal model of *k*-NN showed three misclassifications that were wrongly classified as compliant.

For Raman analyses, the Kennard & Stone algorithm was used to select five samples for the test set and the remaining 21 samples composed the training set. The optimal model of Raman (data pretreated with SNV then followed by the second derivative) was obtained with SIMCA (PCs 11-7) capable to reach a ccr of calibration of 100% and a ccr of external validation of 80% (4/5) as illustrated in Table 2. This one misclassified sample was wrongly classified as compliant with an amoxicillin content of the claimed dose of 93.7%.

Overall, MIR and Raman spectroscopy provided the best models to detect non-compliant amoxicillin samples by using SIMCA.

3.2.2.3. Co-amoxiclav. Three sets containing the different spectral data (MIR, benchtop and portable NIR) for the co-amoxiclav samples were created. Quality compliance of the co-amoxiclav samples was based on the dosage of amoxicillin and clavulanic acid. Since only 13 samples of co-amoxiclav were available, the rule of 20% of the samples assigned to the test set was not applicable here. Instead, we selected 30% of the sample set for the test set in order to have enough representative samples for the external validation. More than one model displayed optimal prediction properties (100%) (see Table 2).

For the dataset of MIR, Duplex was used for sample selection. Four samples were assigned to the test set and the remaining nine samples constituted the training set. SIMCA (PCs 2-2) and *k*-NN ($k = 3$) created models with optimal prediction properties (100%) (see Table 2). The best SIMCA model was built on the first derivative spectra and the best *k*-NN model was constructed on the spectra pretreated with SNV, followed by the second derivative.

For benchtop NIR, Duplex was used to select four samples for the test set and the nine samples for the training set. As shown in Table 2, PLS-DA (number of factors 6) and SIMCA (PCs 2-3) produced models which make a perfect discrimination between API dosage compliance and non-compliance with a ccr of 100% for both training and test set. The spectra used for the best PLS-DA model were preprocessed by SNV while the spectra used for the best SIMCA model were pretreated with the first derivative.

The model obtained on the second derivative spectra of portable NIR using PLS-DA (number of factors 7) showed a ccr of 100% for both training and test set, in which the Kennard & Stone algorithm was used to select four samples for the test set.

Generally, PLS-DA, SIMCA and *k*-NN generated perfect models capable of detecting non-compliant co-amoxiclav samples if the dosage compliance considered both amoxicillin and clavulanic acid. Also, MIR, benchtop and portable NIR spectroscopies all provided models with a ccr of 100% both in the test set and the training set.

4. Conclusion and discussion

The issues of SF antimicrobials have gradually attracted public attention. According to past incidents of SF antimicrobials, the main quality problems are wrong APIs and insufficient dosage, which could result in treatment failure, side effects and even promotion of antimicrobial resistance [3]. Thus, tests for the correct APIs and dosage are primary goals for on-site screening of SF antimicrobials. We aim to develop on-site analytical screening methods capable to monitor the quality parameters: the presence of APIs and the dosage of SF antimicrobials using spectroscopic techniques (MIR, benchtop NIR, portable NIR and Raman) combined with chemometrics. In this study, 58 suspected illegal antimicrobial samples confiscated by the inspectors from the Belgian FAMHP and 14 genuine antimicrobial drugs bought from a Belgian pharmacy were subjected to analysis. The samples were analyzed in a previous study using chromatographic methods for their API content, followed by spectroscopic analysis [12]. In a first step, an exploratory data investigation, also known as unsupervised analysis, was performed on the obtained spectral data in order to reveal differences among the samples, that could be linked to the API present. Based on the promising results acquired from exploratory analysis, supervised chemometric tools were applied to classify samples according to the identity of their API and further to distinguish between dosage compliant and non-compliant samples.

During unsupervised analyses it was observed that HCA generated better clustering results among the different samples than PCA. The best results were obtained with the MIR spectra with a clear clustering of the tetracycline, doxycycline, azithromycin and ampicillin samples, though the separation between amoxicillin and co-amoxiclav was not clear. This is probably due to the low amount of clavulanic acid, compared to the amoxicillin content, present in the sample.

During supervised analyses, the first part was to create models capable of identifying APIs, present in suspected illegal samples. Applying PLS-DA, perfect models were obtained using both MIR and Raman spectra as modeling data. However, co-amoxiclav is not included in the Raman modeling due to the problem of saturated signals. For both MIR and Raman models a ccr of 100% for training and test set was reached. It has to be mentioned that for these models only the groups containing a representative number of samples were taken into account. It stands to reason that when group classification is more specific, less modelling errors were observed. Moreover, the spectra of MIR either pretreated with SNV or the second derivative were all able to result in perfect models with a ccr of 100%. Based on the current collected illegal samples, the obtained PLS-DA models built with MIR and Raman spectra could easily identify co-trimoxazole, amoxicillin, co-amoxiclav (not for Raman) and azithromycin. In the future, more encountered illegal samples could be incorporated to refine the models and broaden the scope with other APIs.

The second part of supervised analyses was about constructing models capable of detecting non-compliant samples. PLS-DA, SIMCA and *k*-NN were applied here for the combined group amoxicillin and co-amoxiclav. The misclassified samples of different models, obtained with the different modeling techniques, were not significantly overlapping.

So, these misclassifications were the result of the modelling process rather than an issue of the sample itself. For the combined group amoxicillin and co-amoxiclav, MIR and benchtop NIR generated equally good models using SIMCA. The misclassifications of the optimal model obtained on MIR were all wrongly classified as compliant, though it mainly concerned samples were the dosage was just beneath the limit of 95% of the claimed dose and so these samples could be considered as borderline. For the group of amoxicillin, spectra of MIR and Raman produced the best models with one sample in the test set wrongly classified as non-compliant (MIR) or compliant (Raman). Also here, the main reason for misclassification were the previously mentioned borderline samples. A possible way to solve this problem is to update models with new samples, making the models more accurate, robust and reliable. For the group of co-amoxiclav, perfect models with a ccr of 100% were obtained with the spectra from MIR, benchtop NIR and portable NIR. The only concern was the limited sample size in this group. To obtain more robust models with a more convincing validation, more co-amoxiclav samples should be incorporated. Since amoxicillin drugs are frequently targeted by criminals, the developed on-site screening methods could exert a great effect on fending off falsification of amoxicillin drugs and especially make sure that these SF medicines do not reach the patients, restoring confidence in health care and therapy efficacy.

In this study, the different preprocessing methods of SNV and the second derivative also played a role in the generation of parsimonious models. For unsupervised analysis, the optimal PCA plots were obtained after SNV pretreatment of the MIR, portable NIR and Raman spectra. For the benchtop NIR spectra, a second derivative resulted in the best PCA plots. For the optimal HCA plots, three of them (MIR, benchtop NIR and portable NIR) were computed on the spectra preprocessed by SNV and one (Raman) was computed on the spectra pretreated with the second derivative method. This indicates clearly that the best (pre) processing method is highly dependent of the data and that no general approach can be defined. Also for supervised analyses, the best preprocessing methods may differ based on different sample classes and different types of spectroscopy. Generally, in this study there was no constant pattern of the best preprocessing method, since it depended on the different sample groups, the different spectra and the modelling technique.

To conclude, the obtained models, hyphenating spectroscopic techniques and chemometrics enable to easily and with high certainty identify suspected SF-antimicrobials and to differentiate non-compliant samples from compliant ones and this using on-site screening. These developed methods could be used by custom staff to efficiently screen suspected antimicrobials and to avoid sample backlog at customs. Moreover, this fast on-site screening could be applied in hospitals to make sure that patients are administered antimicrobials with the right API and the right dosage, so that drug efficacy and treatment efficacy can be guaranteed. In the meantime, the developed models will be continuously refined with newly collected samples and so more robust and more representative and reliable models can emerge.

CRediT authorship contribution statement

Yaxin Tie: Methodology, Validation, Software, Investigation, Formal analysis, Writing - original draft. **Céline Duchateau:** Data curation, Investigation, Formal analysis. **Shana Van de Steene:** Investigation, Formal analysis. **Corenthin Mees:** Investigation, Formal analysis. **Kris De Braekeleer:** Resources, Investigation, Supervision. **Thomas De Beer:** Resources, Investigation, Supervision. **Eric Deconinck:** Supervision, Conceptualization, Funding acquisition, Software, Writing - review & editing.

Declaration of competing interest

The authors state that there is no conflict of interest.

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Appendix A. Supplementary data

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