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Development and analytical validation of a multivariate calibration method for determination of amoxicillin in suspension formulations by near infrared spectroscopy

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ABSTRACT

This paper proposes a new method for determination of amoxicillin in pharmaceutical suspension formulations, based on transflectance near infrared (NIR) measurements and partial least squares (PLS) multivariate calibration. A complete methodology was implemented for developing the proposed method, including an experimental design, data preprocessing by using multiple scatter correction (MSC) and outlier detection based on high values of leverage, and *X* and *Y* residuals. The best PLS model was obtained with seven latent variables in the range from 40.0 to 65.0 mg mL^{-1} of amoxicillin, providing a root mean square error of prediction (RMSEP) of 1.6 mg mL^{-1}. The method was validated in accordance with Brazilian and international guidelines, through the estimate of figures of merit, such as linearity, precision, accuracy, robustness, selectivity, analytical sensitivity, limits of detection and quantitation, and bias. The results for determinations in four commercial pharmaceutical formulations were in agreement with the official high performance liquid chromatographic (HPLC) method at the 99% confidence level. A pseudo-univariate calibration curve was also obtained based on the net analyte signal (NAS). The proposed chemometric method presented the advantages of rapidity, simplicity, low cost, and no use of solvents, compared to the principal alternative methods based on HPLC.

1. Introduction

Presently, the quality control of active principles in formulations from the pharmaceutical industry has been largely based on well established and officially recognized high performance liquid chromatographic (HPLC) methods. In recent years, the use of near infrared spectroscopy (NIRS) combined with multivariate calibration methods has gained popularity as an alternative for quantitative determination of active pharmaceutical ingredients (API) [1–6]. Methods based on NIRS are simple, rapid, present relative low cost and wide application, do not use any solvents or generate any chemical waste. They allow the analysis of solid and liquid forms with little or no sample pre-treatment, and provide enough accuracy and precision with less human intervention.

While US and Brazilian Pharmacopoeias present a general recommendation about the use of NIRS, a drawback for its more widespread use in the pharmaceutical industry is the absence of monographs that prescribe multivariate methods for the quantification of specific pharmaceuticals [7,8]. Traditional regulation has been conceived in a univariate way and the acceptance of the multivariate thinking has remained a challenge. In Brazil, this aspect is regulated by ANVISA (National Agency of Sanitary Vigilance), which has published specific guidelines [9] based on ICH (International Conference on Harmonisation) ones [10,11]. Furthermore, the certification of specific NIRS methods by pharmacopoeias has been limited to qualitative identification of pharmaceuticals or quantitative univariate determinations in raw materials, in situations where a selective wavelength exists.

The need for regulation improvement stems from the recognition that ICH guidelines (and ANVISA in Brazil) for analytical validation may not always be applied to new methods based on NIRS. As mentioned above, the most important challenge is the harmonisation of the univariately conceived regulations with the peculiarities of the multivariate methods. For example, the

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principal parameter used to evaluate average accuracy of multivariate methods is the root mean square error of prediction (RMSEP), which is not recognized by ICH guidelines [10,11]. Total selectivity or 100% of specificity used to be required [9], which is usually not used with multivariate methods, since they are indicated only when signal overlapping is present. Linearity used to be evaluated through traditional calibration curves (signal as a function of analyte concentration) [11], which are not possible to be constructed for a multivariate case. The most important concept for multivariate validation is the net analyte signal (NAS), which is useful for estimating figures of merit (FOM), such as sensitivity, and limits of detection and quantitation [1,12], as well as to construct pseudo-univariate calibration curves, a simple manner to present multivariate models as univariate ones [13]. Thus, taking into account the state of the art of FOM in multivariate calibration [14], the aim of this work was to develop and validate an analytical method for determining amoxicillin (AMX) in pharmaceutical formulations (powder for suspension) by transflectance NIRS measurements in aqueous suspensions and partial least squares (PLS). Besides the validation, a robust procedure was implemented for the method development, including experimental design, data preprocessing, sample selection and outlier detection. More than this specific application, the purpose is also to divulge the potential of this analytical strategy for the quality control of other pharmaceutical formulations.

AMX, D-(−)-alfa-amino-*p*-hydroxy benzyl penicillin, is a broad spectrum antibiotic, present in the WHO (World Health Organization) list of essential medicines [15]. It has activity against both gram-positive and gram-negative bacteria. Many methods have been proposed for AMX determination in pharmaceutical formulations. Most of them are based on chromatography [7,16,17], but also on titrimetry [8,18], microbiological assays [8], UV/Vis absorbance spectrophotometry [19,20], molecular luminescence spectroscopy [21], electrophoresis [22] and electroanalytical techniques [23]. However, the great majority of these methods are not able to directly determine AMX in the presence of interferences, such as excipients, impurities and other active principles, demanding steps of derivatization or chemical/physical separation. A recent paper has applied NIRS for the identification and particle size determination of AMX in raw materials [24]. Nevertheless, only one paper has been published for AMX determination in a pharmaceutical formulation (powder) by diffuse reflectance NIRS [25]. The best results of this work have been obtained with principal component analysis (PCA)-radial basis function neural networks, a complex method commonly used for modelling non-linearities. It is important to note that this paper does not perform a full analytical validation and has analysed a simple formulation, at which the only excipient was starch. By comparison, the method proposed in this paper aims at analysing a more complex formulation, containing seven excipient substances, in a more realistic situation. Another important aspect to mention is that the initial purpose of the present work was to determine AMX by diffuse reflectance spectra recorded directly on the powder. Nevertheless, unacceptable predictions were obtained, for reasons that will be discussed in Section 4.1. Thus, the original planning was modified for determining AMX in aqueous suspensions by transflectance measurements.

2. Multivariate analytical validation

2.1. Net analyte signal (NAS)

The concept of NAS was proposed by Lorber in 1986 [26]. It is defined as the part of the analytical signal uniquely related to the analyte of interest and orthogonal to the space spanned by the interferences. Lorber has originally proposed it for direct calibration methods, such as CLS (classical least squares). In 1997, this concept was extended to inverse multivariate calibration methods [27], and after that, the NAS estimation has been improved by several authors [28–30]. It is important to note that the NAS concept detailed below was an advance in the multivariate calibration theory that allows the possibility to separate the information of the analyte from the whole signal, allowing the estimation of important figures of merit in pharmaceutical applications and the representation of the multivariate model in a pseudo-univariate way that makes easy the interpretation of the model in routine analysis by analysts that sometimes do not present a good knowledge in chemometric models.

For the determination of the NAS, in the first step, **X** (spectral data) and **y** (concentration data) are rebuilt with *A* latent variables (LVs) used in a previously developed PLS model, providing $\hat{\mathbf{X}}_A$ and $\hat{\mathbf{y}}$. The next step is the estimation of $\hat{\mathbf{X}}_{A,-k}$, which contains information related to all species present in the sample except the analyte *k*, through an orthogonal projection, according to Eq. (1),

$$\hat{\mathbf{X}}_{A,-k} = (\mathbf{I} - \hat{\mathbf{y}}_{A,k}\hat{\mathbf{y}}^{+}_{A,k})\hat{\mathbf{X}}_A \quad (1)$$

where **I** is the identity matrix of appropriate dimensions, $\hat{\mathbf{y}}_{A,k} = \hat{\mathbf{X}}_A\hat{\mathbf{X}}_A^{+}\hat{\mathbf{y}}_k$, and "+" indicates the Moore-Penrose pseudo-inverse of a matrix. Since $\hat{\mathbf{X}}_{A,-k}$ is the matrix containing exclusively the information from the interferences, free of any contribution of the analyte *k*, it can be used in a new orthogonal projection for estimating a $\hat{\mathbf{x}}^{\text{nas}}_{A,k,i}$ vector from each *i* sample (the original spectrum, $\mathbf{x}_i$, rebuilt as $\hat{\mathbf{x}}_{A,i}$), according to Eq. (2),

$$\hat{\mathbf{x}}^{\text{nas}}_{A,k,i} = (\mathbf{I} - \hat{\mathbf{X}}^{\mathrm{T}}_{A,-k}(\hat{\mathbf{X}}^{\mathrm{T}}_{A,-k})^{+})\hat{\mathbf{x}}_{A,i} \quad (2)$$

where "T" indicates the transpose of a matrix. Thus, the norm of each of these vectors provides a scalar value $\widehat{\text{nas}}_i$, which may be used as a selective/pure univariate analyte signal,

$$\widehat{\text{nas}}_i = ||\hat{\mathbf{x}}^{\text{nas}}_{A,k,i}|| \quad (3)$$

where "|| ||" denotes the Euclidian norm of a vector. To avoid the calculation of orthogonal projection matrices, Faber [28] has proposed an equivalent approach for estimating $\hat{\mathbf{x}}^{\text{nas}}_{A,k,i}$, based on the PLS or PCR regression vectors (**b**), according to Eq. (4):

$$\hat{\mathbf{x}}^{\text{nas}}_{A,k,i} = \mathbf{b}(\mathbf{b}^{\mathrm{T}}\mathbf{b})^{-1}\mathbf{b}^{\mathrm{T}}\mathbf{x}_i \quad (4)$$

This approach was corrected by Bro and Andersen [30] for situations with negative predicted responses. An alternative approach for estimating $\hat{\mathbf{x}}^{\text{nas}}_{A,k,i}$ vectors needs the spectra of the interferences, requiring a set of blank samples. This alternative is useful for developing multivariate control charts [31], but it has shown a poorer predictive ability [1]. Thus, the present work adopted the approach expressed by Eq. (4) for calculating NAS values.

2.2. Pseudo-univariate calibration curves

The concept of NAS permits expressing multivariate calibration models in a simpler univariate way, through the so called pseudo-univariate calibration curves [6,13]. Firstly, $\hat{\mathbf{x}}^{\text{nas}}_{A,k,i}$ vectors are estimated for the calibration samples, and then a regression coefficient, $\hat{\text{b}}_{\text{nas}}$, is calculated by least squares regression between a vector containing the scalar $\widehat{\text{nas}}$ values ($\widehat{\mathbf{nas}}$) and the vector of reference concentrations (**y**).

$$\hat{\text{b}}_{\text{nas}} = (\widehat{\mathbf{nas}}^{\mathrm{T}}\widehat{\mathbf{nas}})^{-1}\widehat{\mathbf{nas}}^{\mathrm{T}}\mathbf{y} \quad (5)$$

Thus, the regression model can be expressed as:

$$\hat{\mathbf{y}} = \hat{\text{b}}_{\text{nas}}\widehat{\mathbf{nas}} + \mathbf{e} \quad (6)$$

where **e** is a vector containing the residuals.

2.3. Figures of merit (FOM)

According to ANVISA [9] and ICH [10], the following FOM are required for API content determinations: specificity/selectivity (SEL), linearity, accuracy, precision, range and robustness. In addition, this work estimated other useful FOM, such as sensitivity (SEN), analytical sensitivity (γ), bias and limits of detection (LOD) and quantitation (LOQ). For multivariate methods, SEL can be expressed as the ratio between the norm of the NAS vector and the norm of each spectrum, $||\mathbf{x}_i||$, according to Eq. (7). Thus, a different value is obtained for each sample and an average SEL value can be used for characterizing the method [12,14]. Considering that multivariate calibration methods are indicated only when a selective signal does not exist, there is no need for establishing a required limit SEL value. Therefore, this work argues that this FOM has no practical meaning for quality control purposes when applying multivariate methods. An alternative for evaluating specificity is to demonstrate that the method is able to distinguish other components, such as impurities, degradation products or other active principles.

$$\mathrm{SEL}_i = \frac{\widehat{\mathrm{nas}}_i}{||\mathbf{x}_i||} \tag{7}$$

The average accuracy is usually expressed for multivariate methods through the RMSEP and RMSEC (root mean square error of calibration) values. RMSEP is a more robust parameter, since it is estimated from an external set of validation samples, not used for building the model. Since these parameters are not recognized by most of the official regulations, this work also aimed at evaluating accuracy [9,11], by comparing the results of the proposed method for nine determinations (triplicates at three concentration levels) with those of a second well-characterized procedure (HPLC).

For multivariate methods, it is not possible to construct traditional calibration curves for evaluating linearity. Thus, a qualitative way of assessing linearity in these cases is the observation of the distribution of the residuals in both calibration and validation samples, which should have random behaviors. The precision can be expressed in a similar way to univariate methods. In this work, precision was assessed at two levels, repeatability and intermediate precision, as required by the regulations [9–11]. The range is established by confirming that the method provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified interval of the analytical procedure. For the assay of an API in a finished product, a minimum range from 80% to 120% of the test concentration is required [9,11]. The robustness should show the reliability of an analysis with respect to deliberate variations in method parameters. In this work, the chosen parameter was the temperature, which was varied in three different levels.

The SEN of an inverse multivariate method, such as PLS, can be estimated as the NAS at unit concentration, which is equivalent to the following equation.

$$\mathrm{SEN} = \frac{1}{\left\|\mathbf{b}\right\|} \tag{8}$$

A more informative FOM is γ, which is defined by analogy with univariate calibration [32], as the ratio between SEN and the instrumental noise (ε), according to Eq. (9). Usually, ε is estimated through the pooled standard deviation of a vector containing a certain number of replicate spectra of the blank (placebo) [14]. An alternative adopted in this work is to estimate ε from the square root of the mean square residuals in the calibration samples, according to Eq. (10) [33]. This is based on the assumption that all the systematic information was modeled by the A LVs, and the residuals contain only the random variation related to ε.

$$\gamma = \frac{\mathrm{SEN}}{\varepsilon} \tag{9}$$

$$\varepsilon = \sqrt{\frac{\sum \left\|\mathbf{E}_{\mathrm{cal}}\right\|^2}{\nu}} \tag{10}$$

where $\mathbf{E}_{\mathrm{cal}}$ is the matrix containing the residual vectors for all the calibration samples, $||\,||^2$ represents the Frobenius norm of a matrix, and ν is the number of degrees of freedom. It is important to note that the inverse of γ (γ^{-1}) provides an estimation of the minimum concentration difference that is discernible by the analytical method considering the random instrumental noise as the only source of error, regardless of the specific technique employed.

Although LOD and LOQ are not required for this kind of application, they were also calculated according to Eqs. (11) and (12).

$$\mathrm{LOD} = \frac{3.3 \times \varepsilon}{\mathrm{SEN}} \tag{11}$$

$$\mathrm{LOQ} = \frac{10 \times \varepsilon}{\mathrm{SEN}} \tag{12}$$

Bias is a term used to characterize systematic errors, and can be defined as the difference between the limiting mean and the true value [34]. In this work, bias was evaluated by two Student's t tests, which were carried out for confirming or not if the confidence intervals estimated for the slope and the intercept of the fitted line (predicted *versus* reference values) for validation samples contain the expected values of 1 and 0, respectively [35].

2.4. Outlier detection

Outliers are very different objects/samples, which may be caused by operational or instrumental errors, samples from another population, etc. Outlier detection is a crucial aspect of multivariate calibration and several methods have been proposed for this purpose [36–40]. The three most common forms of identifying outliers are based on the detection of samples with extreme leverages, large residuals in X block (spectral data) and large residuals in Y block (predicted concentration). This work adopted the methodology proposed by Valderrama et al. [40], which includes the three mentioned forms and is based on previous literature [36–39]. Leverage (h_i) is a measure of the object distance from the center of the data. It indicates how much an individual sample has influence on the model and it is defined as:

$$h_i = \mathbf{t}_{A,i}^{\mathrm{T}} (\mathbf{T}_A^{\mathrm{T}} \mathbf{T})^{-1} \mathbf{t}_{A,i} \tag{13}$$

where $\mathbf{T}$ is the score matrix of the calibration samples, $\mathbf{t}_i$ is the score vector of sample i, and A is the number of LVs. According to an ASTM standard [39], samples with h_i larger than an h_{limit} value (Eq. (14) for mean centered data) should be eliminated from the calibration set and the model rebuilt.

$$h_{\mathrm{limit}} = \frac{3(A+1)}{I_{\mathrm{cal}}} \tag{14}$$

where I_{cal} is the number of calibration samples.

The detection of outliers based on unmodeled spectral data residuals is carried out by comparison between the total standard deviation ($s(e)$) with the standard deviation of an individual sample ($s(e_i)$), defined as [36,38]:

$$s(e) = \sqrt{\frac{1}{\nu} \sum_{i=1}^{I_{\mathrm{cal}}} \left(\sum_{j=1}^{J} (x_{ij} - \hat{x}_{ij})^2 \right)} \tag{15}$$

$$s(e_i) = \sqrt{\frac{1}{\nu}\sum_{j=1}^{J}(x_{ij} - \hat{x}_{ij})^2} \quad (16)$$

where J is the number of spectral variables, x_{ij} is the absorbance value of the sample i at the wavelength j, $\hat{x}_{ij}$ is the absorbance value estimated with A LVs, and the number of degrees of freedom is given by $\nu = I_{cal}J - J - A(\max(I_{cal}J))$. If a sample has $s(e_i) > ns(e)$, where n is a constant that may vary from 2 to 3, it should be removed from the calibration set [36]. In this work, $n = 2$ (~95% confidence level) was adopted. Finally, outliers can also be detected through the residuals in dependent variables. This can be carried out by the comparison of the RMSEC of the model with the absolute error of individual samples. If a sample presents a difference between its reference and estimate value larger than three times the RMSEC, it should be eliminated [36].

3. Experimental

3.1. Apparatus and software

Spectra were recorded on a Foss NIRSystems 4500 Smart Probe Analyzer spectrophotometer (Silver Spring, USA), equipped with a transflectance probe. For the preliminary measurements, a diffuse reflectance accessory was also used. The reference signal used for absorbance calculations was obtained from a measurement of the empty cell in the absence of light. The instrument was governed and data were acquired using the Vision 3.3.0.0 software package, also from Foss. Data were handled using MATLAB software, version 7.9 (The MathWorks, Natick, USA). The PLS routine came from PLS Toolbox, version 5.2 (Eigenvector Technologies, Manson, USA), and a homemade routine was also employed for outlier detection.

3.2. Materials, reagents and samples

All the chemical reagents were of analytical grade, purchased from certified suppliers and used without further purification. Deionised water was used from an Elga Maxima system (High Wycombe, UK). The typical analysed formulation is in the form of a powder for oral suspensions. The composition of excipients was sodium carboxymethylcellulose, sodium benzoate, colloidal silicon dioxide, cherry flavour, erytrosin, anhydrous sodium citrate, and saccharose. Each 5 mL of suspension should contain 250 mg of AMX. Thus, the target content of AMX in the formulations corresponds to 50.0 mg mL^{-1} (level of 100%). Powder mixtures were prepared by weighing with an analytical balance (±0.0001 g), according to an experimental design, and the mass content of AMX was around 10% (w/w). Aqueous suspensions were prepared in the range from 40.0 to 65.0 mg mL^{-1} (levels from 80.0% to 130.0%) of AMX.

3.3. Methodology

3.3.1. Preliminary studies: diffuse reflectance measurements

At the beginning of this work, an attempt to build a predictive model based on diffuse reflectance measurements obtained directly on the powder samples was carried out. For this, 39 powder samples were prepared in the range corresponding to the levels from 80.0% to 130.0% of AMX, related to the API content in the formulation, and their spectra were recorded from 1100 to 2500 nm. After the verification of the unacceptable prediction ability of this model (discussion in Section 4.1), it was decided to build models based on transflectance measurements obtained from the aqueous suspensions.

3.3.2. Experimental design

A total of 132 samples were prepared according to an experimental design with three factors, AMX, saccharose (the main excipient, corresponding to more than 80% (w/w) of the formulation) and other excipients. The concentrations of these factors were varied independently in order to minimise possible collinearities. The studied range of AMX concentration in suspensions, 40.0–65.0 mg mL^{-1} (80.0–130.0%), was chosen in order to cover the range from 45.0 to 60.0 mg mL^{-1} (90.0–120.0%), which are the acceptable limits established by Brazilian regulations [8] for this API content in this type of formulation.

3.3.3. Procedure

Firstly, a stock mixture of the other excipients (without saccharose) was obtained. The solid mixture samples were prepared according to the three factors' experimental design in plastic beakers and homogenized with a glass rod. For each sample, 20 mL of water were added and the content of the beaker quantitatively transferred to a 50.0 mL volumetric flask, which was completed to the mark. The working suspensions were placed in polyethylene flasks and the spectra were recorded by immersing the transflectance probe. Each spectrum was the average of 32 scans, obtained from 1100 to 2500 nm (step 2 nm), with an optical path of 2 mm. The temperature was controlled at 25 °C during the measurements.

For analytical validation, some additional samples were analysed. Six replicates of a sample containing 50 mg mL^{-1} (level of 100%) were obtained for estimating repeatability. For estimating intermediate precision, these same replicates were compared with other six replicates obtained at the same level on another day by a different analyst. For evaluating robustness, triplicates of samples at this same level were analysed at three different temperatures: 22, 25 and 28 °C. For evaluating accuracy, triplicates of samples at three different AMX levels (80%, 100% and 130%) were also analysed, and their results were validated by comparison with those of the official method, based on HPLC [7]. For evaluating specificity, ten samples containing ampicillin in the place of AMX, in the range from 40.0 to 65.0 mg mL^{-1}, were also prepared and analysed.

3.4. Chromatographic analysis

The validation by HPLC was based on the official method [7] and was carried out with a Perkin Elmer liquid chromatograph, series 200, with UV/Vis detection. An analytical C-18 column (250 mm × 4.6 mm, 5 μm) was used. The mobile phase was water/acetonitrile (96:4, v/v), adjusted with phosphate buffer at pH 5.0 ± 0.1. A flow rate of 1.5 mL min^{-1} and detection at 230 nm were used. All the injections were repeated three times, and all the samples were analysed in triplicate.

3.5. Analysis of real samples

Pharmaceutical formulations of AMX (powder for suspension, 250 mg/5 mL) from four different manufacturers, all commercially available in Brazil, were purchased in local drugstores. The manufactures are IQUEGO, from Goiânia/GO (#1), Medley, from Campinas/SP (#2), Eurofarma, from São Paulo/SP (#3), and Prati-Donaduzzi, from Toledo/PR (#4). The content of these samples was suspended in deionised water and analysed by NIR and HPLC, as described in Sections 3.3.3 and 3.4, respectively. All these determinations were performed in triplicate.

4. Results and discussion

4.1. Preliminary studies: attempt to build a model based on reflectance diffuse measurements

As previously mentioned, the initial aim of this work was to obtain a predictive model based on diffuse reflectance measurements recorded directly on the powder mixtures. The samples were divided into 27 for the calibration set and 12 for the validation set. The obtained spectra were mean centered and preprocessed with multiple scatter correction (MSC). The best PLS model was obtained with 8 LVs and provided a RMSEP of 9.5%. Most of the validation samples presented errors larger than ±10% (up to 23%), which were considered unacceptable by Brazilian regulations [9]. In sequence, these same powder samples were used for preparing aqueous suspensions, whose NIR spectra were obtained by transflectance. From these spectra, a PLS model provided a RMSEP of 2.0%, with a maximum prediction error of 2.5%. Considering that both models were built from the same powder mixtures, the critical source of errors should be in the measurements. An explanation for the poor results of the first model can be given by the experimentally observed difficulty in pulverizing and homogenizing the powder samples. In fact, a key factor for the quality of predictive models based on NIR diffuse reflectance measurements is the homogeneity of the particle size distribution [41]. The lack of homogeneity in the samples represents a source of error in the quantitative analysis and increases the multiplicative light scattering. In extreme cases, this problem cannot be circumvented even by preprocessing methods, such as multiplicative light scattering (MSC). During the sample preparation, when the components were mixed and powdered with agate mortar and pestle, the formation of hard to disperse agglomerates was constantly observed. This observation is caused by the accumulation of electrostatic charges on particle surfaces, which hampered the sample homogenization. The problem was aggravated because AMX is very hygroscopic. Thus, a reasonable alternative is the use of the samples in aqueous suspension. This has the drawback of lengthening the sample measurement process, but is an effective manner of solving the problem of inhomogeneity. Therefore, it was decided to build a more complete model based on transflectance measurements of suspension samples. This model should be more representative, include a larger number of samples, and be more robust. The excipients contents were also varied, which were kept constant in the previous models.

4.2. PLS model based on transflectance measurements

The transflectance NIR spectra were recorded between 1100 and 2500 nm. Due to an intense and broad band was present above 2000 nm, this part of the spectra was deleted and the model was restricted to the region between 1100 and 2000 nm. More restricted models selecting spectral sub-regions were also tried by using interval PLS (iPLS), but the best model was achieved based on this whole region.

Since it was not feasible to record a transflectance spectrum of pure AMX, a diffuse reflectance spectrum of an AMX solid sample was obtained for qualitative analysis (Fig. 1). Reflectance spectra, albeit not identical to the corresponding absorption ones, are similar in shape and produce the same chemical information. By observing Fig. 1, it was possible to attribute the most intense band centered at 1945 nm to the combination of O—H and C—H bonds; the bands between 1640 and 1830 nm to the first overtone of C—H stretching vibrations; the band centered at 1465 nm to the first overtone of N—H stretching; the inverted band at 1414 nm to the first overtone of aromatic O—H; and the band around 1200 nm to the second overtone of C—H bonds [42].

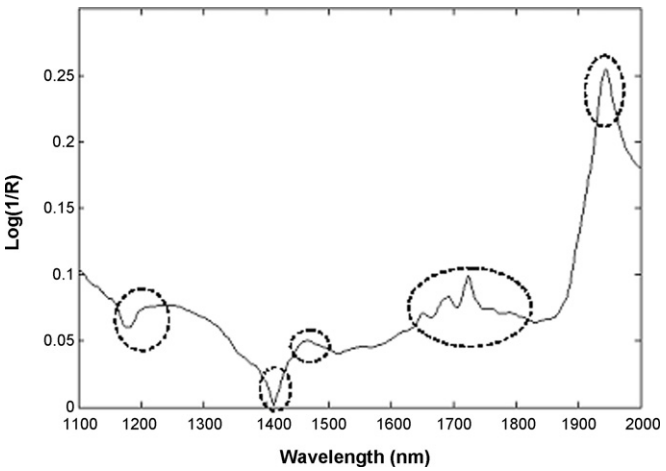

Fig. 1. Diffuse reflectance NIR spectrum of a pure solid AMX sample. The band assignments discussed in the text are marked in the spectrum.

The spectra of all 132 prepared samples are shown in Fig. 2a. They were divided into 82 for the calibration set and 50 for the validation set, by using the Kennard-Stone algorithm [43]. This algorithm selects a predefined number of the most representative samples for the calibration set by scanning uniformly the spectral data. These spectra can present linear and non-linear baseline deviations, which requires the use of data preprocessing methods. In this work, the following methods were tested: MSC [44], standard normal variate (SNV) [45] and first derivative with Savitsky-Golay

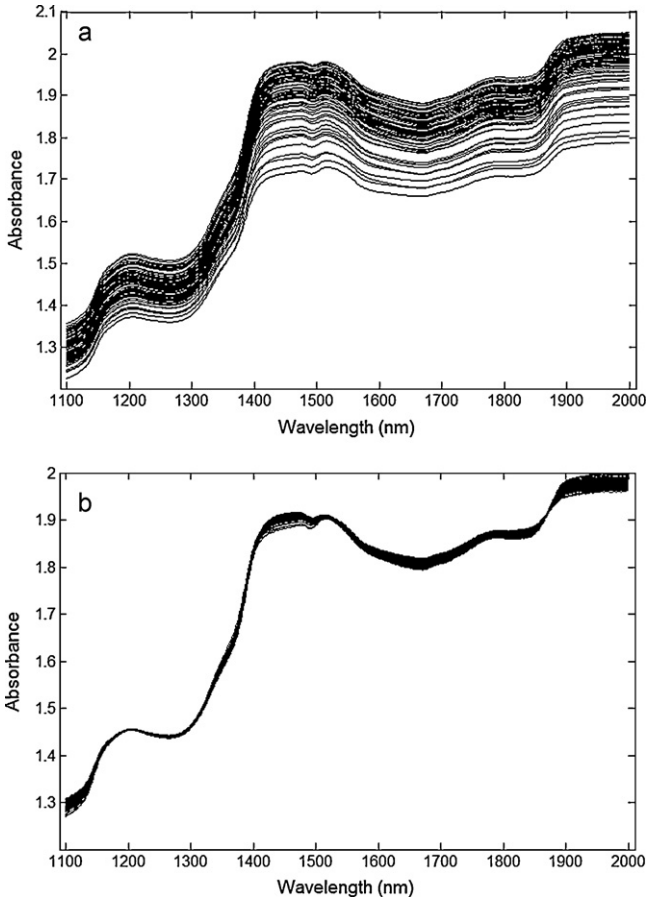

Fig. 2. Spectra of 132 AMX samples, corresponding to the calibration and validation sets, (a) before and (b) after MSC preprocessing.

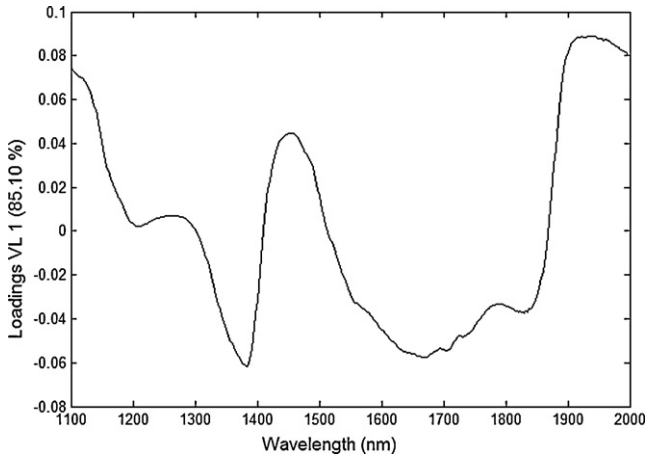

Fig. 3. Loadings for the first latent variable of the developed PLS model.

smoothing [46]. These instrumental deviations are not related to the sample chemical composition and should be corrected. MSC and SNV are alternatives to correct the multiplicative baseline deviations, which are caused by the light scattering promoted by solid particles of different sizes in suspension. The joint use of derivatives and smoothing aims at correcting linear baseline deviations and increasing signal to noise ratios. Combinations of MSC and SNV with first derivative/smoothing (number of smoothing points between 7 and 15, first and second order polynomial fit) were also tested, but the best PLS model was obtained with only MSC preprocessed and mean centered data (Fig. 2b). The best number of LVs was selected by leave-one-out cross validation. This model was subsequently optimized by outlier detection, as discussed in Section 4.3. Considering the optimized number of LVs (7), it was possible to assure that the minimum necessary number of calibration and validation samples was employed, according to ASTM guidelines [39], which prescribes $6 \times$ (number of LVs + 1) and $4 \times$ (number of LVs) samples for the calibration and validation set, respectively.

Fig. 3 shows the loadings for the first LV of the developed PLS model, which accounts for 85.1% of the total spectral data variance. In this loadings profile, it is possible to note the contribution of bands between 1200 and 1300 nm, centered around 1465 nm, between 1640 and 1830 nm (of lesser intensity) and those centered around 1940 nm (of higher intensity). In spite of the observed differences between this loadings profile and the diffuse reflectance spectra of pure AMX (Fig. 1), all of the mentioned bands are present in both, strengthening the specificity of the developed model.

4.3. Outlier detection and prediction of validation samples

PLS model was optimized by outlier detection based on the methodology described in Section 2.4. In the first two models, two outliers were deleted from the calibration set based on their leverage values. After the optimization of the calibration set, six outliers were detected in the validation set, based on their large residuals in *Y* block. The whole procedure is detailed in Table 1, which shows the evolution of the RMSEC, RMSECV (root mean square error of cross validation) and RMSEP values. Fig. 4 represents the outlier detection for the first model through the histogram of leverage values and the plot of spectral *versus* concentration residuals, including the acceptance limits. It can be observed that for this first model only one outlier was detected, based on its extreme leverage value. Therefore, the optimized PLS model used 80 calibration and 44 validation samples. This model provided maximum relative prediction errors of −6.2% and +6.0% for the validation samples.

Table 1
Results for the optimization of the PLS model through the outlier detection.

	Model			
	1st	2nd	3rd	Final
Number of calibration samples	82	81	80	80
Number of validation samples	50	50	50	44
Number of latent variables	8	7	7	7
RMSEC (%)	2.3	2.5	2.3	2.3
RMSECV (%)	4.4	3.9	3.6	3.6
RMSEP (%)	3.9	4.8	4.9	3.2

4.4. Analytical validation

Table 2 summarizes the parameters estimated for evaluating the main FOM of the proposed method. In the following, each FOM is specifically discussed.

4.4.1. Specificity/selectivity

The estimation of this figure of merit is only possible with the application of the NAS concept, allowing the determination of the amount of the instrumental signal that was used by the calibration model for the determination of the analyte. Although SEL is an important parameter for determinations applying HPLC, in multivariate models the SEL value has no practical application for quality control, since low values of SEL can be obtained even with very good results for the determination of the analyte. The SEL value estimated for the method is 1.4%, indicating significant overlapping of the interferences in the AMX signal. It is important to observe that the main advantage of the multivariate PLS models is their capacity to determine the analyte even in the presence of interferences included in the calibration phase. SEL was also evaluated by applying the developed method for the determination of ten samples containing ampicillin instead of AMX, in the range from 40.0 to 65.0 mg mL^{-1}. All the ten samples were correctly identified as outliers, based on both their extreme leverage values (Fig. 5a) and their larger *X* residuals (Fig. 5b). Thus, the method was able to distinguish other similar active principle.

Table 2
Parameters estimated for evaluating the main FOM of the proposed method.

Figures of merit	Parameter	Value (%)
Accuracy	RMSECV	3.6
	RMSEC	2.3
	RMSEP	3.2
Precision	RSD repeatability	1.3
	RSD intermediate precision	1.5
Linearity	Slope	0.974[a]
	Intercept	2.42[a]
	Correlation coefficient	0.989[a]
Range		80.0–130.0
Selectivity		1.4
Sensitivity		1.8×10^{-4}[b]
Analytical sensitivity (γ)		0.95[c]
γ^{-1}		1.1
Limit of detection		3.6
Limit of quantitation		10.8
Robustness	Predicted AMX concentration at	
	$T = 22\,^{\circ}C$	51.4 ± 0.7[d]
	$T = 25\,^{\circ}C$	49.4 ± 0.8[d]
	$T = 28\,^{\circ}C$	50.8 ± 0.6[d]

Mean values and standard deviations of three determinations of a 50.0 mg mL^{-1} AMX sample.

[a] Values for the line fitted to the calibration samples.
[b] Value expressed as the ratio between the units of absorbance and %.
[c] Value expressed as 1/%.
[d] mg mL^{-1}.

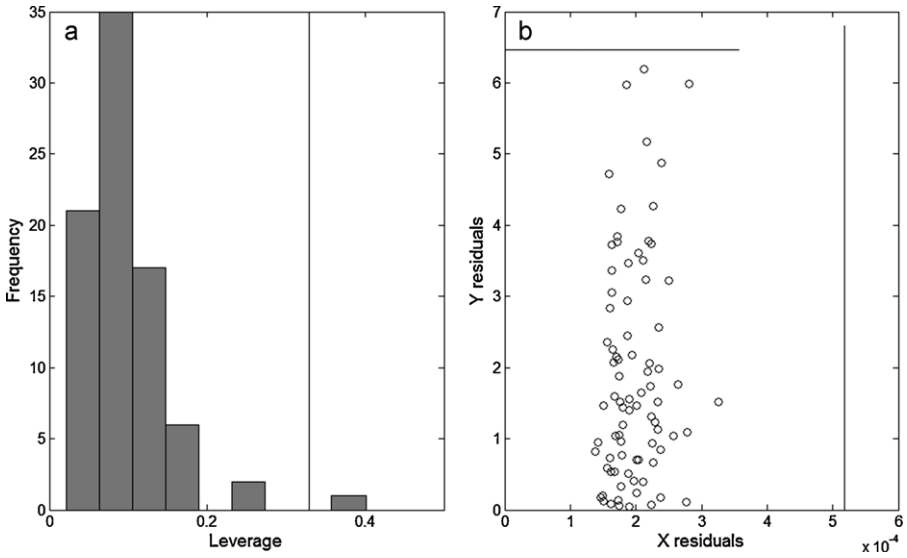

Fig. 4. Visualization of the outlier detection in the calibration set (first model). (a) Histogram of leverage values. (b) Plot of spectral residuals (*X*) *versus* concentration residuals (*Y*). The solid lines indicate the acceptance limits for outlier detection.

4.4.2. Linearity

Fig. 6 shows the residuals for the calibration and validation samples. It is possible to observe the absence of systematic trends in the residuals distribution, showing their random behavior. Once the residual plot indicates that the linear model is a valid assumption, the fitting of a straight line to the reference *versus* predicted values can be used to estimate a correlation coefficient that can express the average agreement of the estimated and reference values. However, this correlation coefficient cannot be assumed as a quantitative measurement of the linearity. The results of this fit for the calibration samples are presented in Table 2, and the obtained correlation coefficient (r), 0.989, is in accordance to the Brazilian regulations [9]. Fig. 7 shows this fit and also includes the validation samples ($r = 0.983$). The dashed line indicates the ideal fit.

4.4.3. Precision

The precision was evaluated through the relative standard deviation (RSD) values obtained at two levels. RSD values of 1.3% and 1.5% were obtained for repeatability and intermediate precision, respectively. Both results are in accordance with the Brazilian regulations [9], which prescribes a maximum RSD of 5%.

4.4.4. Accuracy

The average accuracy of the method may be evaluated through parameters, such as RMSECV, RMSEC and RMSEP, whose values are shown in Table 2, corresponding to AMX concentrations of 1.8, 1.2 and 1.6 mg mL^{-1}, respectively. Since these parameters are not recognized by official regulations [9–11], this work also evaluated accuracy by comparing the results of the proposed method with those of the official HPLC method [7] for determining AMX at three concentration levels (low, medium and high). These results for the determination of triplicates of independent samples are shown in Table 3. Paired t tests with four degrees of freedom showed that there is no significant difference between the results of the two methods at the 95% confidence level, which confirm the accuracy of the proposed method.

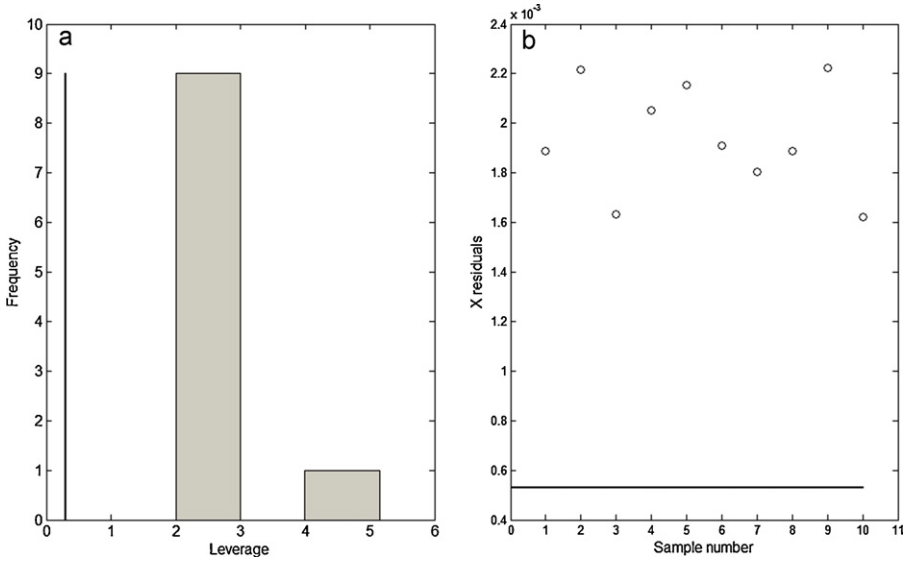

Fig. 5. Specificity evaluation. Detection of ten samples containing ampicillin as outliers. (a) Histogram of leverage values. (b) Plot of the sample number *versus* spectral residuals (*X*). The solid lines indicate the acceptance limits for outlier detection.

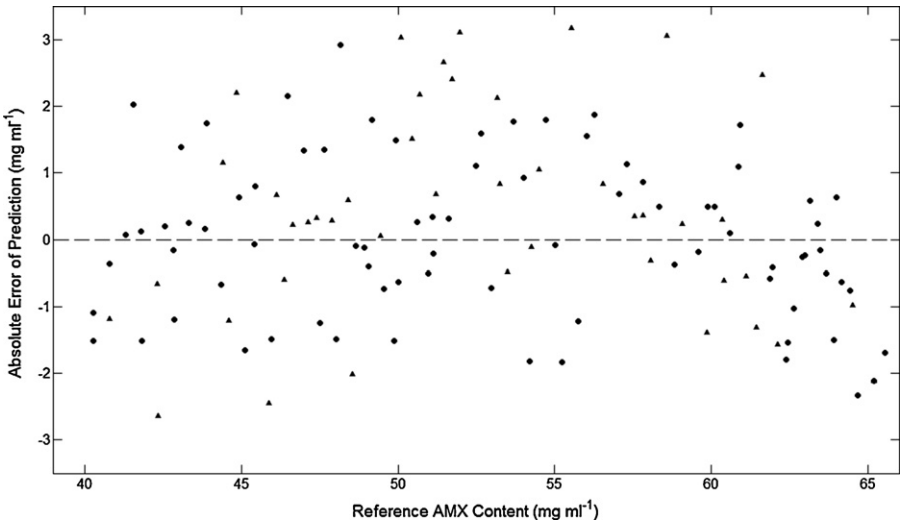

Fig. 6. PLS residuals for the calibration (circles) and validation (triangles) samples.

Table 3
Accuracy evaluation through the comparison of the results for AMX determination by the proposed multivariate calibration NIR method and the official HPLC method.

Level of AMX content	Official method ($mg\,mL^{-1}$)[a]	Proposed method ($mg\,mL^{-1}$)[a]
Low	39.5 ± 0.2	39.6 ± 0.5
Medium	49.3 ± 0.3	49.4 ± 0.5
High	64.6 ± 0.5	63.1 ± 0.7

[a] Mean values and standard deviations of three determinations.

4.4.5. Range

Considering the linearity, precision and accuracy studies, the range of the method was established from 40.0 to 65.0 $mg\,mL^{-1}$ of AMX, corresponding to formulation content from 80.0% to 130.0% (Table 2).

4.4.6. Robustness

The robustness of the method was evaluated through the results of the determination of triplicates of a 50.0 $mg\,mL^{-1}$ AMX sample at three different temperatures (Table 2). No significant differences between the obtained results and the reference value were observed at 95% confidence level. Thus, the proposed method was considered to be robust in relation to variations of temperature between 22 and 28 °C.

4.4.7. Sensitivity and analytical sensitivity

SEN was estimated as 0.00018 (Table 2). This value is not appropriate for comparison with other methods, since it is dependent on the analytical technique employed and the analysed matrix. Thus, γ was also calculated as 0.95, based on the obtained estimate of the instrumental noise, 0.00019 (Eq. (10)). The inverse of γ, 1.1%, corresponds to 0.5 $mg\,mL^{-1}$ and indicates the minimum concentration difference that the method can distinguish considering the random instrumental noise as the only source of error.

4.4.8. Limits of detection and quantitation

Table 2 shows the estimated values of LOD and LOQ, which correspond to 1.8 and 5.4 $mg\,mL^{-1}$ of AMX, respectively. The results indicate that the method is appropriate for quality control purposes.

4.4.9. Test for the presence of systematic errors (bias)

This test evaluated the significance of the line fit of the reference *versus* predicted values for the samples of the validation set. The

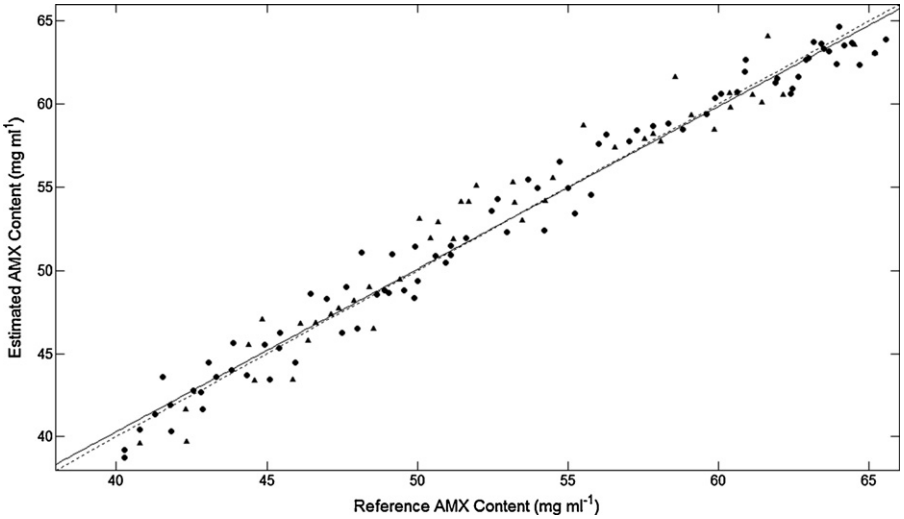

Fig. 7. Plot of reference *versus* predicted values for the calibration (circles) and validation (triangles) samples. The solid line shows the data fit and the dashed line the ideal fit (slope one and zero intercept).

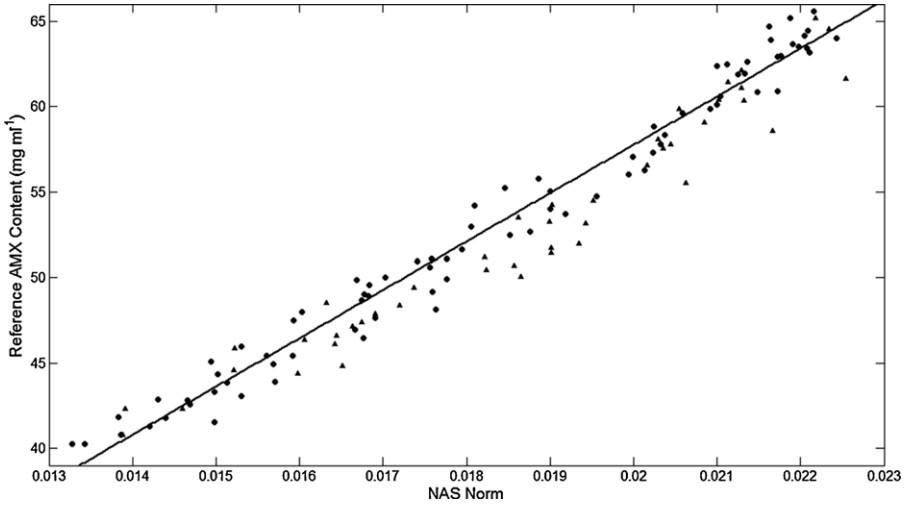

Fig. 8. Pseudo-univariate calibration curve. Plot of NAS norms *versus* reference values for the calibration (circles) and validation (triangles) samples.

slope and the intercept of this fit were calculated as 0.9998 ± 0.0372 and 0.3725 ± 1.9759, respectively. Two independent *t* tests with 42 degrees of freedom at 95% confidence level demonstrated that there are no significant differences between the experimentally values and the values of slope and intercept for an ideal fit, one and zero, respectively. Thus, the absence of bias was verified for the proposed method.

4.5. Pseudo-univariate calibration curve

As discussed in Section 2.2, the NAS concept was able to present the multivariate models in alternative or simpler manners by a pseudo-univariate calibration curve. This is equivalent to obtaining a univariate filtered signal, which presents a linear relation with the analyte concentration. This representation is particularly useful in routine analysis, where the analyst can visualize the PLS model in a univariate way. However, it should be noted that the outliers tests have to be performed for all samples analysed by the model. The pseudo-univariate curve for the proposed method is shown in Fig. 8. The fit for this curve is presented in Eq. (17), which provided a correlation coefficient of 0.989.

$$[\text{AMX}] = 5654.7||\widehat{\mathbf{nas}}|| + 0.0014682 \quad (17)$$

4.6. Analysis of real samples

The developed method was applied to the determination of AMX in formulations of four different manufacturers and the results, as listed in Table 4, were in agreement with those specified (50 mg mL^{-1} of AMX). It is important to indicate that formulations 2–4 present excipient compositions similar to formulation 1, for which the validation was developed. Evidently, this method would not be able to determine AMX in formulations with significantly different excipient compositions. Non paired *t* tests with four degrees of freedom were used to compare these results with those of the official method, and there were no significant differences at 99% confidence level for all the formulation samples (estimated *t* values below the critical *t* value, 4.604). For the first two formulations, the results of the two methods were in agreement at the 95% confidence level (critical *t* value of 2.776).

Table 4
Determination of AMX in four pharmaceutical formulations by the proposed multivariate calibration NIR method and the official HPLC method.

Formulation	Official method (mg mL^{-1})[a]	Proposed method (mg mL^{-1})[a]
#1	52.1 ± 1.0	49.0 ± 1.5
#2	48.5 ± 1.7	50.5 ± 1.0
#3	49.7 ± 0.9	45.4 ± 1.8
#4	50.4 ± 0.8	46.2 ± 1.4

[a] Mean values and standard deviations of three determinations.

5. Conclusions

A method based on transflectance NIRS was developed and validated for direct AMX determination in suspension pharmaceutical formulations. The main advantage of this method over the official one, based on HPLC [7], is the speed of the analysis. Considering the whole analytical process, the estimation is that the proposed method requires 5 min per assay *versus* 40 min for the official one. Moreover, the proposed method presents other advantages over the main alternatives, such as low cost, simplified procedure, no need for reagents or solvents and less generation of chemical waste. This method was validated in accordance with Brazilian and international guidelines [9–11,39] and was considered linear, precise, accurate, robust and sensitive in the range from 40.0 to 65.0 mg mL^{-1}. It was also applied to determinations in real pharmaceutical formulation samples of similar excipients composition, providing results in accordance to those of the official HPLC method at the 99% confidence level. More than this specific determination of AMX, this work showed a complete and robust methodology, which incorporates the concept of net analyte signal and can be applied for developing and validating other new analytical methods based on NIRS and multivariate calibration. This study explores a new perspective for the elaboration of pharmacopoeia monographs based on multivariate methods for specific pharmaceuticals.

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