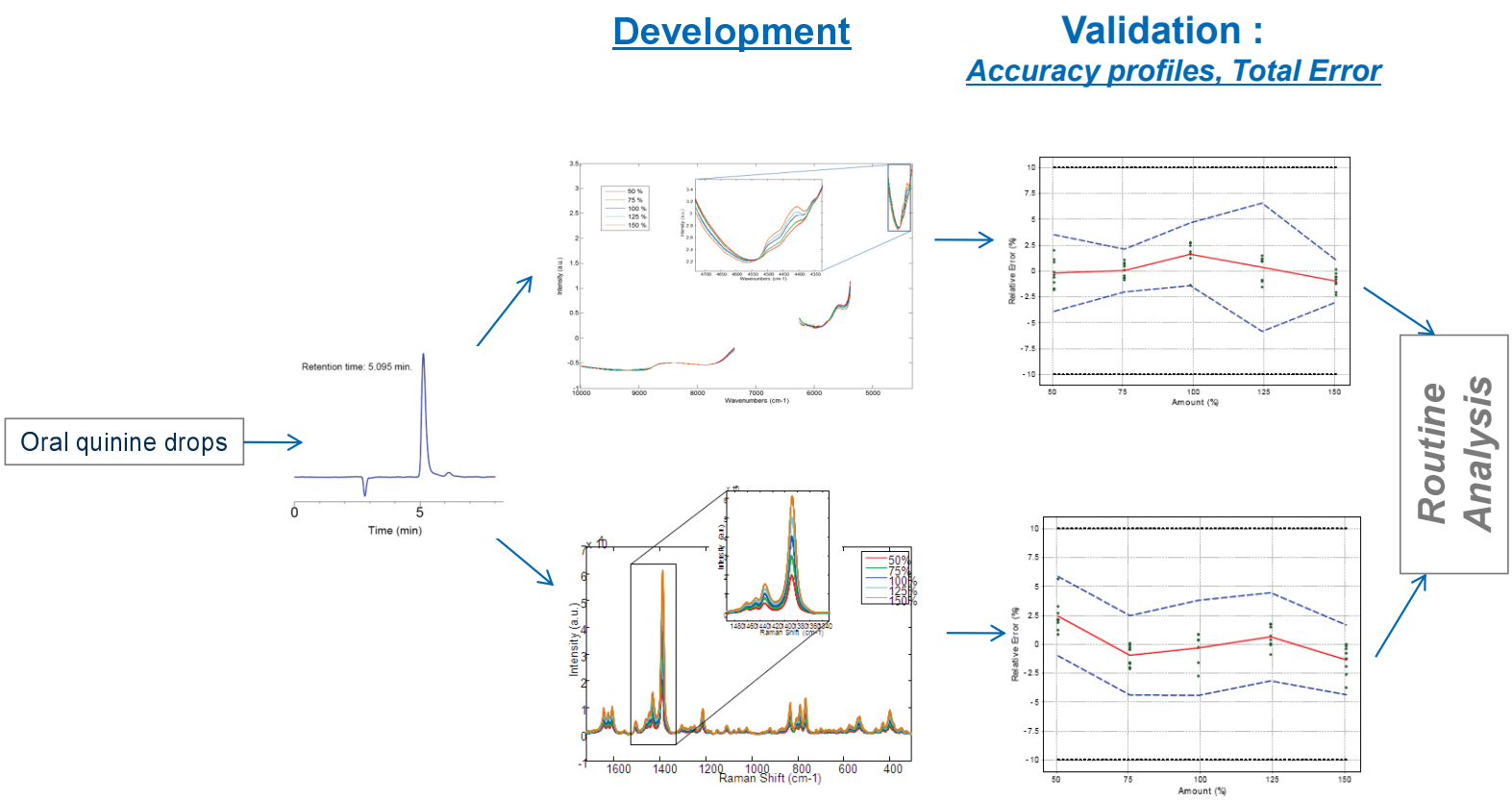
***Graphical Abstract**



Development, validation and comparison of NIR and Raman methods for the identification and assay of poor-quality oral quinine drops.

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1. Introduction

Malaria remains one of the most rampant illnesses worldwide and is one of the main causes of child mortality in developing countries [1-2]. The treatment of uncomplicated malaria is based on conventional antimalarial drugs (e.g. chloroquine, artemisinin derivatives, atovaquone, etc.). These drugs are essentially used as combinations due to the growing resistance observed with single-drug therapy [3]. However, quinine is still recommended alone in the treatment of severe and/or cerebral malaria attacks as well as for chloroquine-resistant falciparum malaria [4]. Four quinine based dosage forms are found on the pharmaceutical market in DRC: tablets (250 and 500 mg), ampuls (250 and 500 mg/2mL), syrup (100 mg /mL) and oral drops (200 mg /mL). The last three dosage forms are the most used with 0-5 year old children. In 2009, the Health Ministry of the DRC warned citizens against quinine oral drops "Quinizen 20%" that were found to have been counterfeit and substandard [5].

Poor quality (substandard, counterfeit and degraded) or substandard/spurious/falsely-labelled/falsified/counterfeit anti-malarial drugs constitute a major public health concern especially in developing countries where the pharmaceutical market is poorly regulated and controlled [6]. It has been estimated that at least a third of the drugs sold in Africa are fake. The use such drugs may lead to therapeutic failure, death and reinforce drug resistance [7, 8].

Vibrational spectroscopic techniques, such as Near Infrared (NIR) and Raman spectroscopies are frequently used techniques in the field of quantitative drug analysis [9-11] and in the fight against counterfeit drugs [12-15]. These techniques have the advantages of being non-destructive, fast, requiring little or no sample preparation, as well as being

environmental friendly [16]. The foremost advantage for drug analysis in developing countries however is their low cost in routine analysis and the absence of consumables.

The aim of the present research was to develop NIR and Raman methods able to detect and to quantify quinine in 20% (W/V) oral drops solutions from a Congolese drug-manufacturing laboratory (manufacturer A). These methods were fully validated by the "total error" approach [17], compared by mean of a Bland and Altman analysis [18] and then tested on samples from several manufacturers.

2. Material and methods

2.1. Reagents

Ammonium formate (98.1%), hydrochloric acid (37%), and methanol (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). Benzoic acid and propylene glycol were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The reference standard of quinine dihydrochloride (100.8%) for the HPLC analysis was purchased from Molekula Ltd (Dorset, UK). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA).

2.2. NIR equipment

The oral drop samples were analyzed with a multipurpose analyzer Fourier Transform Near Infrared Spectrometer (MPA, Bruker Optics, Ettlingen, Germany) equipped with a semiconductor room temperature sulfide lead (RT-PbS) detector. A transmittance probe for liquids with a fixed optical path length of 2 mm was used to collect the NIR spectra. A background spectrum with the empty probe was acquired before each series of measurements. Between each measurement, the probe was cleaned with water. The spectra were collected with the Opus Software 6.5 (Bruker Optics). Each spectrum was the average of 32 scans and the resolution was 8 cm^{-1}, in the range of 12500-4000 cm^{-1}.

2.3. Raman Equipment

Raman measurements were performed with a dispersive spectrometer RamanStation 400F (Perkin Elmer, MA, USA) equipped with a two-dimensional CCD detector (1024 × 256 pixel sensor). The laser excitation wavelength used was 785 nm with a power of 100 mW. Raman spectra were collected with a Raman reflectance probe for solids and liquids interfaced with Spectrum Software 6.3.2.0151 (Perkin Elmer). The spectral coverage was 3620-90 cm^{-1} with a spectral resolution equal to 2 cm^{-1}. Each Raman spectrum resulted from the accumulation of six spectra with a 5.0 s exposure time. NIR and Raman spectra were acquired on the same day and prior to the analysis by HPLC-UV allowing the determination of the concentration of quinine dihydrochloride corresponding to each sample.

2.4. Reference method

The HPLC experiments were performed on an Alliance 2695 HPLC system (Waters, Milford, USA) coupled to a 2996 PDA detector (Waters). Data acquisition and treatment were performed with the Empower 2® software (Waters). The analysis was performed with an XBridge™C18 (250 mm x 4.6 mm, 5µm particle size) column preceded by an XBridge™C18 (20 mm x 4.6 mm, 5 µm particle size) guard column kept at 30°C. The mobile phase consisted of a mixture (45:55, V/V) of methanol and a 10 mM ammonium formate buffer adjusted to pH 3.0 with 6N HCl. The HPLC system was operated in isocratic mode with a flow rate of 1.0 mL min^{-1} and an injected volume of 10 µL. UV detection was carried out at 235 nm.

2.5. Test samples

Six samples of quinine dihydrochloride 20% (W/V) oral drop solutions from four manufacturers (A, B, C and D) were collected at the local Congolese pharmaceutical market. The calibration samples used to build the PLS models were prepared on basis of the qualitative and quantitative compositions of manufacturer A. Samples from other manufacturers had different qualitative and quantitative compositions regarding the

pharmaceutical formulation and the origin of the active ingredient. Samples from manufacturers C and D were green-coloured whereas those from manufacturers A and B were yellow-coloured. To test the ability of the developed models to detect and quantify quinine in oral drops, seven simulated substandards have been prepared with 2% and 40% (W/V) quinine dihydrochloride (corresponding to 10% and 200%, respectively, of the target value).

2.6. Sample preparation

2.6.1. Preparation of samples for reference method validation

Calibration samples for HPLC method validation were prepared from a stock solution of quinine dihydrochloride at a concentration of 1 mg mL^{-1} in ultrapure water. The stock solution was diluted to obtain solutions of 50, 100 and 150 µg mL^{-1}. The calibration standard solution was composed of three series of three replicates per concentration level (27 samples in total). Validation samples for HPLC method validation were prepared from a stock solution composed of 20% (W/V) quinine dihydrochloride, dissolved in an excipient solution composed of propylene glycol and benzoic acid in ultrapure water. The stock solution was diluted to obtain solutions of 50, 75, 100, 125 and 150 µg mL^{-1}. The validation standard solution was composed of three series of three replicates per concentration level (45 samples in total).

2.6.2. Preparation of solutions for HPLC analysis of samples

Two independent standard solutions were prepared by dissolving quinine dihydrochloride in ultrapure water to achieve a final concentration of 100 µg mL^{-1}. Calibration, validation and test samples were diluted in ultrapure water to obtain a final concentration of 100 µg mL^{-1}.

2.6.3. Preparation of calibration and validation samples for NIR and Raman

The target (100%) sample composition is 20% (W/V) quinine dihydrochloride dissolved in an excipient solution composed of propylene glycol and benzoic acid in ultrapure water. Calibration and validation standards were prepared by dissolving the appropriate amount of

quinine dihydrochloride in the excipients solution to achieve concentrations of 50, 75, 100, 125 and 150% of the target amount. Three series of both calibration (C1, C2, C3) and validation (V1, V2, V3) samples were prepared with three replicates for each concentration level. C1, C2, V2 and V3 series were prepared using quinine dihydrochloride from Pharmakina (Bukavu, DRC). While C3 and V1 series were prepared using quinine dihydrochloride from A.V. Pharma (Kinshasa, DRC).

2.7. Multivariate data analysis

Partial Least Squares (PLS) regression models were built with NIR and Raman data using HPLC assay values as reference. Several PLS models were built using different pre-processing methods. Best models were selected based on their Root Mean Square Error of Prediction (RMSEP) computed as follows:

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n}}\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n}}RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n}} \quad (1)$$

where y_i is the reference value determined by HPLC, $\hat{y}_i$ is the predicted value given by the PLS model and n is the total number of samples. All data were mean centered and the number of latent variables of each PLS models was selected based on the RMSECV versus latent variables plot. Cross validation consisted of random subsets with ten data splits and ten iterations. First and second derivatives were computed using the Savitsky-Golay algorithm [19] with a polynomial order of 2 and 15 smoothing points. Asymmetric Least Squares has been used for baseline correction of Raman spectra. A value λ of 10^5 and a value p of 10^{-3} were used. PLS models were built using PLS_Toolbox 7.0.3 (Wenatchee, WA, USA) running on Matlab® R2013a (The Mathworks, Natick, MA, USA).

The validation of a multivariate calibration model is often performed by checking at the R^2 and RMSEP values. However, as described in De Bleye *et al.* [20], these performance parameters are not sufficient to ensure that the developed method will provide reliable results

over the complete dosing range. Therefore, both NIR and Raman predictive models were validated through the "total error" approach. All validation calculations were performed with e-noval® version 3.0 (Arlenda S.A., Liège, Belgium).

3. Results and discussion

3.1. Validation of the reference method

The method was successfully validated using the "total error" approach in the range of 50 µg mL^{-1} to 150 µg mL^{-1} with acceptance limits set at 10% according to the USP for quinine sulphate tablet assay [21]. Trueness, precision (repeatability and intermediate precision), accuracy and linearity of the method were found to be acceptable (see also Table 1).

3.2. Quantitative NIR study

Quantifying an API in an aqueous matrix may be a difficult task with NIR spectroscopy. Indeed, the matrix absorbance spectrum shows that the multiple absorption maxima characteristic of water and detector saturation occurs between 5250 and 5050 cm^{-1}. Therefore, the spectral range was selected between 8937-7278, 6318-5396 and 4733-4428 cm^{-1} to build PLS models (see Figure 1a). By doing so, perturbations due to matrix absorptions were avoided while keeping the information dealing with the API. Table 2 shows the different pre-treatments tested as well as the figures of merit for the corresponding models. As one can see, both models gave similar values of RMSEP that were inferior to 2%. However, the different models have varying complexity with a number of latent variables (LV) ranging from 2 to 4. The simplest model was obtained by applying a standard normal variate (SNV) normalization computed as follows:

$$x_{ij,SNV} = \frac{x_{ij} - \bar{x}_i}{s_i} \quad (2)$$

where $x_{ij,SNV}$ is the transformed portion of the original element x_{ij}, $\bar{x}_i$ is the mean value of the spectrum I and s_i is the standard deviation of the spectrum I. This choice was driven by the fact that it is one of the simplest models (only 3 LVs) which limits the risk of over fitting.

This model should, therefore, be more robust for any future analysis of unknown samples. As can be determined from the accuracy profile (Figure 2b), the β-expectation tolerance intervals of each concentration level are inside the limits of acceptation set at 10%. This indicates that 95% of future measurements will lie within these limits. The largest β-expectation tolerance intervals have relative values of -5.84% and 6.57% (see Table 1).

3.3. Quantitative Raman Study

Compared to NIR spectroscopy, the main advantage of Raman spectroscopy in quantifying an API in an aqueous matrix is due to the weak Raman scattering effect of water. Raman spectroscopy, however is often limited to pharmaceutical applications due to the fluorescence of samples. The spectral range was selected to be 1720-306 cm^{-1} to build PLS models. The selected model was the one obtained by applying baseline correction by asymmetric least squares (AsLS) with a RMSEP of 1.88% and two latent variables (see Figure 1b). As can be seen in Figure 2a, the selected Raman quantitative model was also validated with the acceptance limits fixed at 10%.

3.4. Comparison of methods

The analytical performances of both NIR and Raman models were compared using a Bland and Altman analysis [18] (see results in Figure 2c). The plot represents the relative difference between the Raman and NIR methods against the average relative content of quinine at each concentration level. As shown in this figure, 95% of the agreement limits with values of [- 4.20%; 4.26%] are inside the acceptance limits [-14.14 %; 14.14 %] that were set based on the maximum acceptable error of 10% for each method. These results guarantee that each future difference between the result obtained using the Raman method and the one obtained with the NIR method has a probability of 0.95 of falling within the acceptance limits. Thus both methods agree sufficiently to quantify quinine in the oral drops allowing the analyst to select the method according to the advantages and drawbacks of each method.

3.5. Analysis of test samples

The two methods developed were applied to six samples of quinine dihydrochloride 20 % (W/V) oral drops solutions as mentioned in section 2.5 and on seven reconstituted substandard solutions. Samples from manufacturer A were considered as being genuine samples since they had the same qualitative and quantitative composition as the calibration samples used to build the PLS models. Neither NIR spectroscopy nor Raman spectroscopy could qualitatively discriminate between samples of manufacturers A, B, C and D. This is not surprising since these samples are constituted mainly of quinine dihydrochloride 20% (W/V) and water 79% (W/V), whereas the excipients are less than 1% (W/V). The spectral variations due to differences in qualitative composition were below the detection limits of both techniques thus did not allow a distinction between the manufacturers. Test samples were then analyzed with the two developed PLS models. As shown in Table 3, the two methods generally predicted each sample correctly. In addition, the samples from each of the manufacturers are predicted correctly, thus demonstrating that the developed NIR and Raman methods could be applied to the detection of placebo or sub-dosed samples.

A principal component analysis (PCA) was performed on the different test samples and the prepared substandard samples. This model was built using the 100% target concentration calibration and validation samples. The main goal was to check whether it was possible to quickly discriminate substandard samples without building and validating a PLS model. As can be seen in Figure 3, the scores of PC2 (for NIR spectroscopy) and PC1 (for Raman spectroscopy) allowed for the discrimination of genuine and substandard samples. Moreover, the third sample of manufacturer A is slightly outside the 95% T^2 Hotelling's confidence level for the PCA based on Raman spectroscopy. This is in accordance with the quantitative results obtained (89 % of the target value predicted by PLS). These results indicate that it is possible to use PCA as a discriminating method to detect substandard samples.

4. Conclusion

The main objective of this study was to develop and validate efficient, rapid and cost-effective analytical methods for the analysis of quinine dihydrochloride 20% (W/V) presented as an oral drop formulation manufactured and marketed in the DRC.

To meet these requirements, NIR and Raman spectroscopic methods were successfully developed and validated using the total error approach with acceptance limits fixed at 10% in the range of 50-150% of the target concentration. A comparison of the two methods showed that they provided comparable results. Six samples collected in the Congolese pharmaceutical market were analyzed by both techniques. All samples were conform since their quinine content was within in +/- 10% of the theoretical value.

The NIR spectroscopy qualitative model developed will soon implemented for routine analysis in the Quality Control Laboratory of Drugs at the University of Kinshasa (D.R. Congo) to replace the existing HPLC method. This study and its implementation are part of the fight against the traffic of poor quality medicines that endanger the public health and socio-economic aspects of developing countries.

Acknowledgments

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Table 1: ICH Q2 (R1) validation criteria of the reference HPLC, NIR and Raman methods.

	concentration level	HPLC	NIR	Raman
Trueness				
Relative bias (%)	50	3.98	-0.19	2.44
	75	2.09	0.05	-0.95
	100	-0.18	1.63	-0.31
	125	0.76	0.37	0.65
	150	2.13	-0.98	-1.35
Intra-assay precision				
Repeatability (RSD%)	50	0.78	1.12	1.40
	75	0.32	0.63	0.53
	100	0.44	1.24	1.17
	125	0.21	0.29	0.53
	150	0.37	0.79	1.23
Between-assay precision				
Intermediate precision (RSD%)	50	0.78	1.36	1.40
	75	0.62	0.76	0.97
	100	0.62	1.24	1.41
	125	0.63	1.33	1.02
	150	0.62	0.82	1.23
Accuracy				
Relative β-expectation tolerance limits (%)	50	[2.08 ; 5.88]	[-3.90 ; 3.51]	[-1.00 ; 5.87]
	75	[-0.16 ; 4.35]	[-2.02 ; 2.12]	[-4.38 ; 2.49]
	100	[-2.07 ; 1.71]	[-1.41 ; 4.66]	[-4.42 ; 3.80]
	125	[-1.97 ; 3.48]	[-5.84 ; 6.57]	[-3.16 ; 4.46]
	150	[0.00 ; 4.27]	[-3.01 ; 1.06]	[-4.36 ; 1.67]
Uncertainty				
Relative expanded uncertainty (%)	50	1.64	2.95	2.96
	75	1.39	1.65	2.18
	100	1.38	2.62	3.09
	125	1.44	3.06	2.32
	150	1.40	1.74	2.60

Table 2: Figure of merits of the different tested PLS models.

NIR	RMSEC (%)	RMSECV (%)	RMSEP (%)	LV
MC	0.86	0.96	1.59	4
1D-MC	0.81	0.95	1.69	3
2D-MC	1.00	1.09	1.46	2
SNV-MC	**0.90**	**1.03**	**1.42**	**3**

Raman	RMSEC (%)	RMSECV (%)	RMSEP (%)	LV
MC	1.04	1.27	1.81	3
AsLS-MC	**1.11**	**1.75**	**1.88**	**2**
2D-MC	0.45	1.41	2.01	4
SNV-MC	3.27	4.69	7.27	4

RMSE: root mean square error; C: calibration; CV: cross-validation; P: prediction; LV: number of latent variables considered; MC: mean center; 1D: SavitskyGolay's first derivative; 2D: SavitskyGolay's second derivative; SNV: standard normal variate; AsLS: asymmetric least squares.

Table 3: Results of quantification of 6 samples with NIR and Raman PLS models. Results are presented as predicted content (%) of the active ingredient and relative expanded uncertainty (Ux).

Results obtained with HPLC consist in the mean percentage of claimed nominal content and the standard deviation computed on 3 independent samples.

	NIR	Raman	HPLC	Relative Error (%)	
Drug	**(%) ± Ux**	**(%) ± Ux**	**n=3, % ± SD**	**NIR/HPLC**	**Raman/HPLC**
A1	98.3 ± 2.6	96.2 ± 3.1	96.7 ± 0.1	1.68	-0.47
A2	98.6 ± 2.6	101.4 ± 3.1	100.7 ± 0.1	-2.12	0.68
A3	90.8 ± 2.6	89.0 ± 3.1	91.1 ± 0.1	-0.33	-2.33
B	97.8 ± 2.6	92.9 ± 3.1	95.7 ± 0.1	2.22	-2.92
C	106.6 ± 2.6	100.6 ± 3.1	102.6 ± 0.1	3.86	-1.99
D	99.5 ± 2.6	98.8 ± 3.1	99.5 ± 0.1	0.54	0.68

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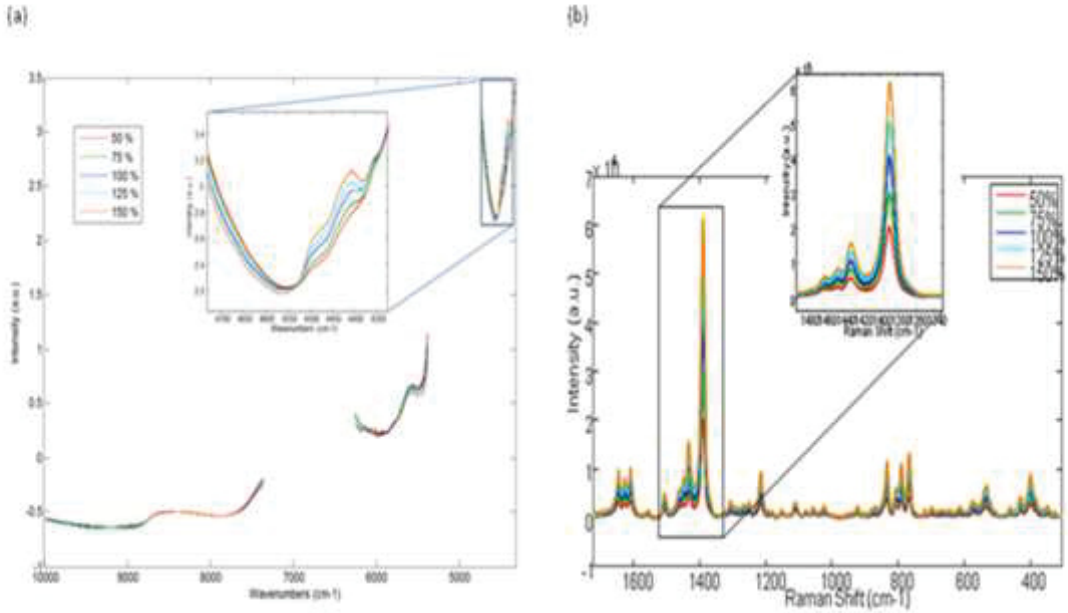

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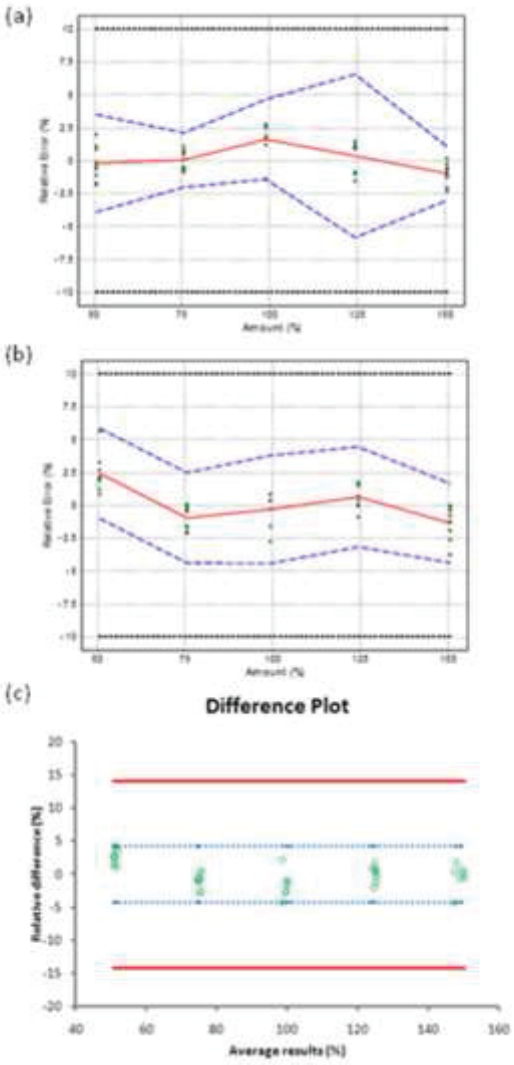

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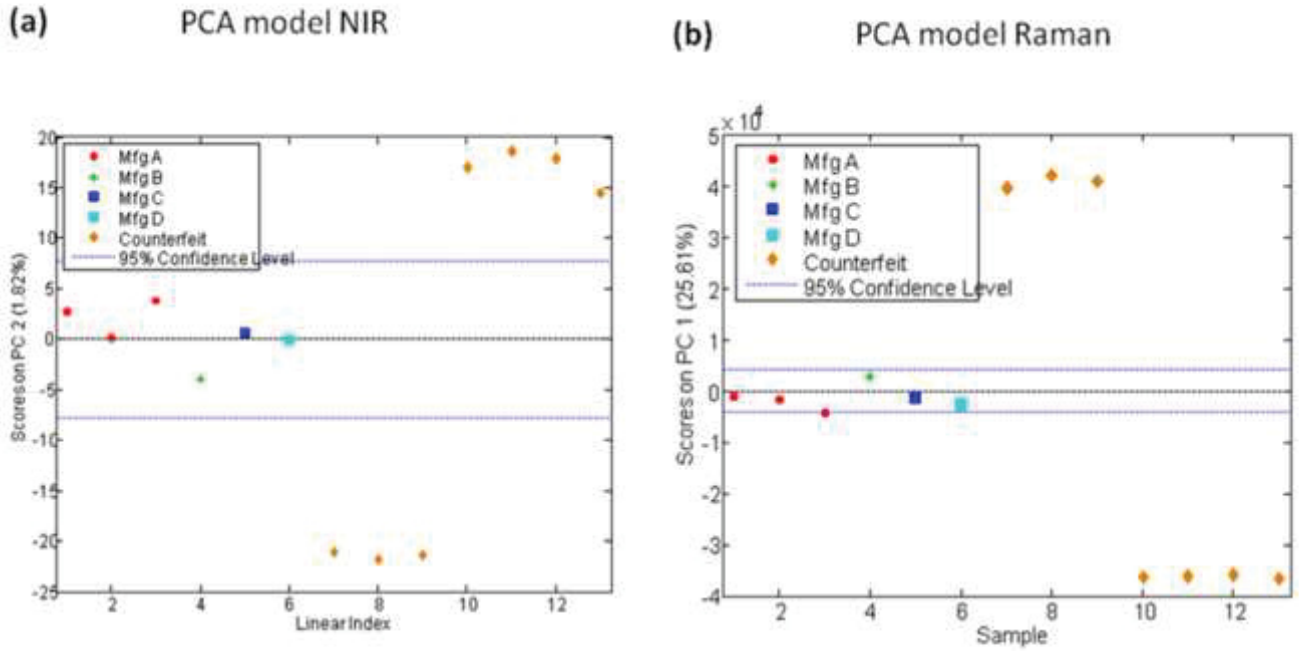

Figure 1:

(a) Selected spectral ranges of SNV pre-processed calibration NIR spectra.

(b) Selected spectral ranges of asymmetric least squares baseline corrected calibration Raman spectra.

Figure 2:

(a) Accuracy profile of the Raman quantitative PLS model. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (β=95%) and the bold plain lines are the acceptance limits set at 10 %. The dots represent the relative back-calculated concentrations of the validation samples, plotted with regards to their target concentration.

(b) Accuracy profiles of the NIR quantitative PLS. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (β=95%) and the bold plain lines are the acceptance limits set at 10 %. The dots represent the relative back-calculated concentrations of the validation samples, plotted with regards to their target concentration.

(c) Bland and Altman plot of the relative differences (%) of the results obtained by the NIR quantitative model and the Raman quantitative model against the average content of quinine (%) for the five concentration levels results of the two methods. *Dashed blue lines*: 95% agreement limits of the relative differences; *Continuous red lines*: maximum acceptable relative difference between the two methods set at ±14.14% based on the maximum acceptable error of 10% for each method; *Dots*: relative differences.

Figure 3:

(a) PC2 scores of eight samples of oral quinine drop and seven substandard quinine drops based on their NIR spectra.

(b) PC1 scores of eight samples of oral quinine drop and seven substandard quinine drops based on their Raman spectra.