Surface enhanced transmission Raman spectroscopy: Quantitative performances for impurity analysis in complex matrices

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Abstract

A transmission detection mode was investigated with SERS analyses (SETRS). A comparison between backscattering and transmission detection modes was conducted to demonstrate the feasibility of performing SETRS analyses. The impact of various parameters on the SERS signal intensity such as sample volume, lens collection optic, laser beam size and laser power were then examined. The analytical performances of SETRS were further evaluated through the quantification of an impurity (4-aminophenol) ranging from 3 to 20 µg/mL in a commercial pharmaceutical product using a total error risk-based approach. To account for expected variability of routine analysis, 9 batches of silver nanoparticles suspensions were used and experiments were performed over 5 different days and by 2 operators. Univariate spectral analysis based on a quadratic regression was compared to a multivariate approach using a partial least square regression. The presented results demonstrated that SETRS can be used to determine an impurity in a complex matrix opening new perspectives for quantitative applications.

Keywords: Surface enhanced transmission Raman spectroscopy (SETRS) – Analytical performances – Total error risk-based approach – Complex matrix – Impurity – Assay

1. Introduction

Surface-enhanced Raman scattering (SERS) is a vibrational technique valued for its specificity, high sensitivity, straightforward sample preparation and environmentally friendly attributes due to the absence of solvent use and the short time requires to perform an analysis [1–3]. However, analyzing samples in solution poses considerable challenges for accurate results due to potential variability in SERS signal arising from substrate heterogeneity and aggregate formation [4–6]. Factors, such as the nature, size, shape, fouling of the metallic nanoparticles used directly influence the intensity of SERS signal [7]. Recently, a detection mode by transmission has been developed for Raman analyses offering a different approach to signal collection compared to conventional backscattering one. As shown by Figure 1, transmission Raman spectroscopy (TRS) systems collect the scattered photons through the sample rather that at the focal point of the laser [8,9]. This detection mode has the benefit of greater sample representativeness due to an increase sampling volume and can also suppress interference fluorescence originating from surface layer [10,11]. To our knowledge, the literature has yet to extensively explore SERS analysis utilizing a transmission mode (SETRS).

Paracetamol is one of the best selling drugs all over the world for its analgesic and antipyretic properties [12,13]. Quality control regulations, particularly those concerning impurities, are stringent for all pharmaceuticals on the market. The 4-aminophenol (4-AP) is both a synthesis intermediate and a degradation impurity of the paracetamol known for its significant toxicity, especially to the kidneys [14,15]. According to the United States Pharmacopeia (USP), a maximum of 0.15% (w/w) of 4-AP is tolerated in a pharmaceutical formulation [16]. Currently, USP recommends a liquid chromatography method to realize this test [16,17]. Alternative analytical methods such as fluorimetry, micellar electrokinetic chromatography or liquid chromatography associated with amperometric detection have also been explored [18–20]. Nonetheless, these separation techniques are often time-consuming and require the use of organic solvents [21,22].

Previously, a SERS method for the quantitative determination of 4-AP in a complex matrix was successfully developed using a backscattering detection mode [23]. Spectroscopic techniques present the advantage to be time effective compared with chromatographic techniques which are commonly recommended by the authorities. The developed SERS method could be further considered as an interesting alternative to the separative techniques. The main objective of this study is to evaluate the impact of equipment parameters on the measured intensity and analytical performances of SETRS within the context of pharmaceutical quality control. Initially, the

SETRS repeatability is compared to backscattering configuration. The effects of various parameters such as the lens collection optic, the laser beam size and laser power alongside with the determination of the optimal sample volume are investigated. Then, the study explores the suitability of SETRS for the quantification of 4-AP in a commercial pharmaceutical product according to the USP specifications. The evaluation of SETRS analytical performances is based on a total error risk-based approach for both univariate and multivariate data analysis [24,25]. To the best of our knowledge, this work is the first one to consider SERS analysis associated to a transmission detection mode. Moreover, Raman analysis of liquid sample using transmission detection mode was not yet realized.

2. Materials and methods

2.1. *Chemicals and reagents*

Silver nitrate (analysis grade), potassium chloride (analysis grade) and 4-aminophenol (minimum purity of 98%) were purchased from VWR (Pennsylvania, USA). Trisodium citrate (98 %) and sodium nitrate (98%) were bought from Acros Organics (Geel, Belgium). Nitrogen was provided by Air Liquide (Paris, France). Crystal violet (analysis grade) was obtained by TCI Europe N.V. (Zwijndrecht, Belgium). The pharmaceutical product purchased from a pharmacy and contained 1 g of paracetamol and several excipients (povidone, anhydrous colloidal silica, sucralose and orange flavor). All solutions were prepared using Milli-Q water (18.2 MΩ.cm, Milli-Q Plus 185, Millipore, Burlington, USA).

2.2. *Synthesis and characterization of colloidal silver nanoparticles*

An optimized microwave synthesis process was applied to synthesize 9 batches of AgNps, systematically the day before SETRS analyses [26]. In practice and for each batch, 15.0 mL of a solution of silver nitrate $1x10^{-3}$ M was added in a specific vial (microwave reaction vials 10-20 mL, Biotage, Uppsala, Sweden). Then, 200 µL of a 0.08 M trisodium citrate solution were added 40 s before the introduction of this vial into the microwave (Biotage® Initiator+ Microwave system with robot eight, Biotage, Uppsala, Sweden). Microwave parameters were a duration of 3.36 min at 130° C with a 600 rpm stirring speed. Three syntheses of colloidal nanoparticles (Nps) were pooled to ensure an adequate volume for analysis, each pool being used for 2 series. Each pool was protected from light, stored at 5° C and was used at room temperature prior SERS/SETRS analyses. All these steps were conducted away from light. For each pool of AgNps, UV-Visible spectra were acquired in order to guarantee the presence of

Nps in each batch and to perform a comparison from batch-to-batch. These spectra are illustrated in the Figure S1.

2.3. *Preliminary investigations*

A transmission Raman spectroscopy equipment (TRS 100, Agilent Technologies, Santa Clara, California, United States) was used. This instrument has a spectral resolution of less than 8 cm^{-1} across the entire range with a laser at 830 nm and a power of 650 mW. The spectral coverage spans from 50 to 2300 cm^{-1} and offers the flexibility to choose from 3 laser beam sizes (2, 4 and 8 mm) and 3 collection optics (small, medium or large). To evaluate the effect of the filling volume, a sample was prepared by mixing 1.5 mL of crystal violet (1.10^{-6} M) with 1.5 mL of colloidal AgNps solution followed by the addition of 375 µL of potassium chloride (0.1 M). Spectra were acquired by gradually filling a vial in increments of 100 µL. A laser power of 60 mW combined with the conventional 4M configuration (4 mm of laser beam size and medium collection optics) was set with an acquisition time of 5 s and a single accumulation.

To evaluate the impact of the different TRS configurations (laser beam size and collection optics) on the spectra, a sample containing 400 µL of crystal violet (1.10^{-6} M) and 400 µL of colloidal AgNps solution was mixed before the addition of 100 µL of potassium chloride (0.1 M). Spectra were acquired by illuminating the sample with a 60 mW laser power with an acquisition time of 5 s and a single accumulation.

The intensity of the specific 1170 cm^{-1} band of crystal violet was used for further calculation.

2.4. *Sample preparation*

2.4.1. Standard solutions

All steps were conducted under light-protected conditions and dilutions were prepared with Milli-Q water free of oxygen to prevent 4-AP degradation [27]. Two liters of Milli-Q water was bubbled with nitrogen for a minimum of 30 minutes. Deoxygenated water was used to prepare a stock solution of 4-AP at 50 µg/mL in a brown volumetric flask. Then, several dilutions were prepared according to the Table 1 in volumetric flasks of 100.0 mL. For each calibration and validation solutions, the pharmaceutical formulation was added to consider the matrix effect. A blank containing only the matrix was also prepared for each calibration series. Each solution was then centrifuged for 15 min at 6000 rpm (Centrifuge 5804R, Eppendorf, Hamburg, Germany). Only 10 mL of supernatant were used for SETRS analyses. In practice, 18 series (12

calibration series and 6 validation series) were prepared using this protocol on 5 different days and considering 2 operators.

2.4.2. SERS samples

SETRS spectra were acquired by a Transmission Raman spectroscopy equipment (TRS 100, Agilent Technologies, Santa Clara, California, United States). The laser beam size was set at 2 mm with a small lens collection optics. The acquisition parameters were a laser power of 650 mW and 7 accumulations of 1 s acquisition time. Spectra were recorded at one-minute intervals over a duration of 10 minutes.

SERS spectra were also collected in a backscattering configuration using the TruScan™ RM handheld Raman analyzer with the TruTools™ package (Thermo Fisher Scientific, Waltham, MA, USA). This handheld device operates with a 785 nm laser covering a spectral range from 250 to 2875 cm^{-1} and has a spectral resolution between 8 and 10.5 cm^{-1}. The acquisition parameters were a laser power of 250 mW and 3 accumulations of 1 s acquisition time.

For the preparation of samples, 400 µL of AgNps were mixed with 400 µL of 4-AP solutions by the means of a vortex (Reax top, Heidolph, Schwabach, Germany) in a 4 mL screw neck glass vial (VWR, Pennsylvania, USA). Then, 100 µL of $NaNO_3$ 1 M were added and vortexed for 10 s before analysis using both Raman equipment. Three replicates were prepared for each concentration level.

2.5. *Data treatments*

2.5.1. Univariate spectral analysis

Spectra analysis was performed using MatLab® R2020b (The MathWorks, Natick, MA, USA) with the PLS_Toolbox 8.9.2 (Eigenvector Research, Inc., Wenatchee, WA, USA). A baseline correction employing a Whittaker filter (λ: 30 000 and p: 0.01) was applied after the selection of the relevant spectral range (from 380 to 2050 cm^{-1}). The intensity of the band at 580 cm^{-1} was graphically plotted over time. Observing that the signal stabilized after 8 min, an average signal intensity was calculated from spectra between 8 and 10 min and reported versus the 4-AP concentration.

For calibration, six series comprising five concentration levels each were used, along with six series for validation. Enoval 4.1d software (Cencora Pharmalex, Bad Homburg, Germany) was used to compute the β-expectation tolerance limits with β=95%. A quadratic regression model

was selected and 2 outlying values were rejected (validation series 4, last replicate of levels 2 and 3).

2.5.2. Multivariate spectral analysis

A multivariate model was built using MatLab with the PLS_Toolbox and the spectral range selected was from 380 to 2050 cm^{-1}. A partial least-squares regression (PLSR) model was built thanks to 12 calibration series and considering 2 latent variables. The optimized pretreatments consisted in a multiplicative scatter correction (MSC) with the median spectrum as reference followed by an orthogonal signal correction (OSC) and a mean center. Six validation series were projected onto the model and the predicted results were used to compute the β-expectation tolerance limits with β=95%.

3. Results and discussion

3.1. *Study of the influence of parameters on the SETRS signal*

Preliminary investigations were conducted to determine the influence of the main parameters involved in the development of a transmission Raman method and to observe their impact on the measured SETRS signal. To the best of our knowledge, the analysis of liquid samples by transmission detection mode has not been addressed in the literature. The first critical parameter considered was the optimal sample volume required for SETRS analyses. The vial was positioned at an angle of approximately 45° on the sample holder tray of the equipment. Consequently, the laser crossed the sample in a section slightly higher than the vial diameter and it was necessary to determine the volume that provides the most intense signal. Therefore, this optimal volume was initially evaluated using classical cylindrical vials of 4 mL. Crystal violet was selected as the analyte for these investigations as it is the most used as model considering SERS analyses [28]. These tests were conducted by analyzing the same sample with 100 µL increments up to 2500 µL. As shown in Figure 2, the maximal signal intensity was achieved between 900 and 1000 µL. A filling volume of 900 µL was selected for future works with SETRS analyses given that the signal intensity is at a maximum while avoiding wastage of colloidal Nps. When higher volumes are used, signal intensity slightly decreases and

stabilizes indicating that the vial section crossed by the laser is fully filled and no longer impacts the measured signal.

Although the aggregation of nanoparticles is crucial for enhancing the Raman signal through the formation of hot-spots, the size and distribution of these aggregates are difficult to control which can lead to significant variability in SERS signal [29]. The transmission detection mode may mitigate this phenomenon by allowing the laser to pass through the sample averaging signal intensity across different acquisition points. In this context, the optical settings influence of the equipment such as the laser spot size and the lens collection choice were studied. The laser spot size options are 2, 4 or 8 mm. Each spot size has the same power but different power densities, with the 2 mm laser spot being more intense than 8 mm one. Altering laser spot sizes also changes the volume of sample being analyzed. The available lens collection optics are small (S), medium (M) or large (L). Increasing the optics size increases the area of the sample being analyzed but decreases the signal intensity because larger lens defocus scattered light collection. Several tests were conducted by adjusting the collection optics and laser beam size with a volume of 900 µL of the SETRS sample. The relative standard deviation (RSD) and maximum signal intensity of a specific band of crystal violet were extracted and are shown in Figure 3. As expected, the intensity was higher for the small laser spot size and the small lens collection optics. However, in terms of signal variability, no significant impact of the collection optics was observed. Therefore, it was decided to proceed with the 2S configuration which provided the highest intensity to fit the purpose of impurity determination.

3.2. *Evaluation of SETRS analytical performances considering the quantification of 4-aminophenol in a pharmaceutical formulation*

3.2.1. Method specificity

The quantification of 4-AP was carried out in a pharmaceutical formulation containing paracetamol and various excipients. The main challenge of this pharmaceutical context was to ensure the specific SETRS detection of 4-AP despite the presence of a complex matrix containing a high concentration of the active drug and excipients.

The concentration range considered was determined according to the USP specifications. The maximum concentration tolerated for 4-AP in a pharmaceutical product containing paracetamol is 0.15% (w/w) of the active drug dosage [16]. For the method development and validation, the

pharmaceutical formulation contained 1 g of paracetamol. Assuming a dissolution in a 100.0 mL flask, the target concentration for 4-AP was no more than 15 µg/mL in the analyzed solution. To ensure coverage of this range, a calibration from 3 to 20 µg/mL was investigated.

Specificity was demonstrated by comparing a blank containing colloidal AgNps and the aggregating agent, the matrix and 3 samples of matrix spiked with increasing concentrations of 4-AP. Figure 4a represents these SETRS spectra while Figure 4b shows specific bands related to the 4-AP at 567 and 580 cm^{-1}. These bands clearly illustrate the specificity of the method as the spectra from the blank and the matrix showed no band in the spectral range between 550 and 600 cm^{-1}.

3.2.2. Backscattering and transmission detection modes comparison

Although the transmission detection mode could add significant value for SERS analyses, SETRS methods have not been extensively developed in the literature. To address this gap, a preliminary study was conducted using both detection modes to demonstrate the feasibility of SETRS analyses by examining the relationship between signal intensity and analyte concentration and comparing their analytical performance in terms of signal intensity repeatability. In both detection modes, a good linearity relationship can be observed in Figure 5 between the signal intensity at 580 cm^{-1} and the 4-AP concentration in the range from 5 to 20 µg/mL (R^2 greater than 0.99 for both detection methods).

Table 2 presents the RSD for the tested concentrations in both configurations. The results indicate that the performance of SETRS is comparable to SERS with a slight improvement in RSD for SETRS measurements. These findings successfully demonstrate the feasibility of SETRS analyses.

3.2.3. Incubation time

Due to the interaction between SERS substrate, analyte, aggregating agent, and the dynamic nature of hot-spot formation, a predefined period of time is necessary to obtain stable aggregates leading to a stable signal intensity. SETRS acquisitions were planned each minute for 10 min. Figure 6 illustrates the evolution of SETRS signal intensity as a function of the time for a calibration series. It was observed that a stable signal is achieved after 8 min. Particularly, for higher concentrations, the signal gradually increased during first minutes. After 8 min, a plateau

was reached. This behavior was consistent across for all batches during method development. Finally, spectra from 8 to 10 min were averaged for each concentration and series.

3.2.4. Evaluation of SETRS analytical performances according to a univariate spectral analysis

Two regression approaches are commonly employed in SERS analyses; the univariate and the multivariate data analysis [30,31]. These two methods were used in this study to evaluate, for the first time, the analytical performances in SETRS. Univariate data analysis is easy to implement in terms of manipulation and spectra treatment. It was considered as appropriate because a specific band of 4-AP, free of interferences from the matrix, is available in the spectrum. According to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, the analytical performances required for an impurity quantitative limit test include specificity, response, limit of quantification, accuracy and precision [32]. Although these recommendations were followed, the goal of this study was to evaluate the SETRS performances and not to conduct a formal validation of the method. Therefore, no acceptance limits were established.

SETRS performances were evaluated using the total error risk-based approach combining systematic and random errors [24,25]. All ICH criteria were met, including specificity which was already demonstrated in the section 3.2.1. Calibration sample concentrations are presented in the Table 3 and covered the target of 15 µg/mL of 4-AP. The retained calibration model was a quadratic regression with determination coefficients of calibration curves greater than 0.99 as illustrated in the Figure S2. Given that 3 independent replicates were realized for each concentration level, precision was estimated by the repeatability (intra-series variability) expressed as RSD which ranged between 1.9 and 4.8 % as shown in Table 3. The intermediate precision (intra + inter-series variability) for each concentration is also shown in Table 3 with a maximum value of 9.4%. The relative bias presented a maximum value of 5.7%. The limit of quantification was equal to 5.1 µg/mL while the limit of detection was evaluated at 1.5 µg/mL by dividing the limit of quantification by 3.3.

Figure 7 shows the β-expectation tolerance interval of the univariate spectral analysis. For the maximal targeted concentration of 15 µg/mL, this interval is of approximately ± 20 % which is acceptable for an impurity quantitative limit test. Based on these results, a SETRS method was successfully developped for the quantification of the 4-AP impurity in a paracetamol-based

pharmaceutical formulation according to the criteria recommended by ICH Q2(R2) guidelines. This approach offers the benefit to take into account for expected variability such as SERS substrates and thus minimizing its impact on the accuracy of the method.

3.2.5. Evaluation of SETRS performances according to a multivariate spectral analysis

Multivariate data analysis based on PLSR regression was also conducted to determine the analytical performances from the SETRS spectra [33]. A set of 12 calibration series were used to build the PLSR model to ensure robustness in routine use. A good linearity was obtained over the 5-20 µg/mL range with a R^2 of 1.0. PLSR calibration curve with validation points is illustrated in the Figure S3. RMSEC and RMSECV were equal to 0.7 and 1.0 µg/mL, respectively. Following the development of the PLSR model, validation series were projected onto it leading to the determination of a RMSEP equal to 1.1 µg/mL and the β-expectation tolerance interval shown in Figure 8.

It appears that the relative bias (maximum value of 1.9%) is slightly lower than that observed with the univariate approach. In terms of repeatability, the values presented in the Table 4 are very close to those obtained with univariate analysis. However, intermediate precision is rather better for the univariate approach mainly due to a lower variability between the series. The intermediate precision presented a maximum value of 13.8% with a repeatability from 1.9 to 5.3 %. For the maximal targeted concentration of 15 µg/mL, the β-expectation tolerance interval is about +/- 25%.

To evaluate the robustness of the PLSR model, only 10 calibration series were used to build the model. Validation series 5 and 6 are independent with the corresponding series excluded from the calibration set. As shown in Figure S4 and the second part of Table 4, the analytical performances were not degraded confirming the robustness of the model and its potential application for routine analyses. A summary of the comparison of these two multivariate spectral analysis models is presented in the Table S1.

Nevertheless, as demonstrated by the higher inter-series effect, this approach is at risk of potential future variability in the synthesis of SERS substrates. This impact is, however, mitigated for the univariate approach since the calibration and the sample analyses are carried out with the same SERS substrate at the same time [34]. Consequently, although the

multivariate approach offers faster and cheaper analyses in routine, maintaining the model may present a limitation.

4. Conclusion

In conclusion, transmission mode is compatible with quantitative SERS analyses and is described herein for the first time in this context. This mode enables a more representative measurement of the sample by encompassing a larger sampling volume. When analyzing liquid samples in standard vials of 4 mL, preliminary tests indicate that 900 µL is the optimal volume to measure an intense SETRS response. This study also evaluated the influence of key parameters by transmission mode on the SETRS signal. The findings suggested that a smaller collection optic facilitated greater signal enhancement while its impact on the signal variability was not significant. The SETRS variability was evaluated by comparison to backscattering detection mode with both detection modes showing comparable results and a tendency towards lower RSD for the transmission mode.

Quantitative performances of SETRS were evaluated in liquid sample considering the quantification of 4-AP which could not exceed 0.15% (w/w) in a pharmaceutical product. The performances evaluation study was carried out using a total error risk-based approach in accordance with the ICH Q2(R2) guidelines. To mimic real routine conditions, the investigation was carried out by 2 operators, using 9 pools of AgNps, 12 calibration series and 6 validation series over 5 separate days. Two calibration approaches were compared: the univariate approach which was based on a quadratic regression model of the 580 cm^{-1} band specific of the 4-AP and the multivariate approach which was based on a PLSR model. Both approaches demonstrated satisfactory quantitative performances in terms of bias, repeatability, and intermediate precision. However, the univariate approach seems to be less sensitive to the inter-series effect (and therefore to the SERS substrate batch variability) and consequently more appropriate for routine use.

In this first case study, neither the use of an internal standard nor the geometry of the vial or its position in the sample holder tray was explored. These factors represent promising avenues for enhancing the quantitative capabilities of SETRS within the context of pharmaceutical product quality control.

Statements and Declarations:

CRediT authorship contribution statement:

Julie Horne: Conceptualization, Methodology, Formal Analysis, Writing - Original Draft Preparation, Writing - Review and Editing. **Pierre Beckers:** Conceptualization, Methodology, Formal Analysis, Writing - Original Draft Preparation, Writing - Review and Editing. **Pierre-Yves Sacré**: Conceptualization, Software, Writing - Review and Editing. **Pierre Francotte**: Methodology, Writing - Review and Editing. **Eric Caudron:** Writing - Review and Editing. **Philippe Hubert**: Supervision, Project Administration, Funding Acquisition. **Cédric Hubert**: Conceptualization, Writing – Review and Editing, Supervision. **Charlotte de Bleye**: Conceptualization, Writing – Review and Editing, Supervision, Project Administration. **Eric Ziemons**: Conceptualization, Writing - Review and Editing, Supervision, Project Administration. All authors have read and agreed to the published version of the manuscript.

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Appendices

Figures captions:

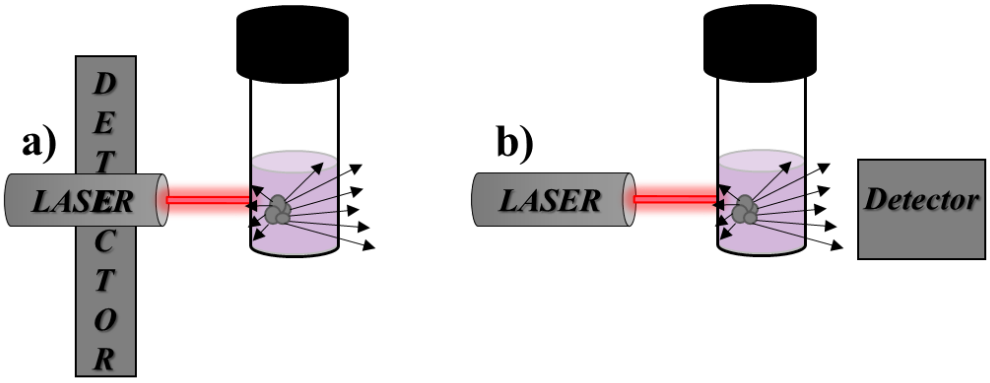

Figure 1: Illustration of SERS detection for a) backscattering and b) transmission mode.

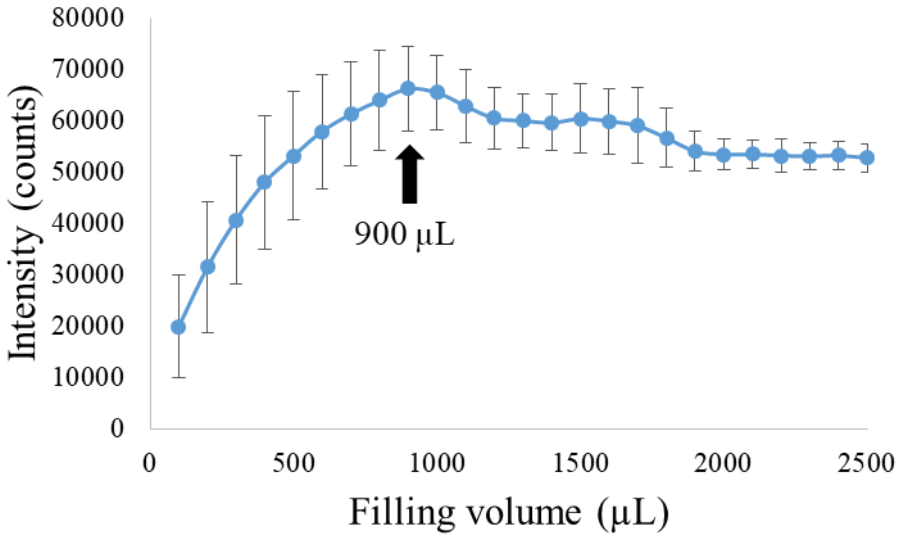

Figure 2: SETRS signal intensity of 1170 cm^{-1} band specific of crystal violet with the related error bar (n=3), as a function of the filling volume of the vial.

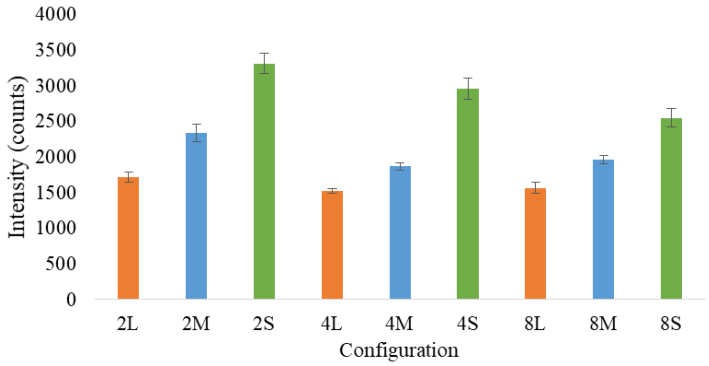

Figure 3: Average SETRS signal intensity according to the collection optics (S = small, M = medium or L = large) and laser beam size (2, 4 or 8 mm).

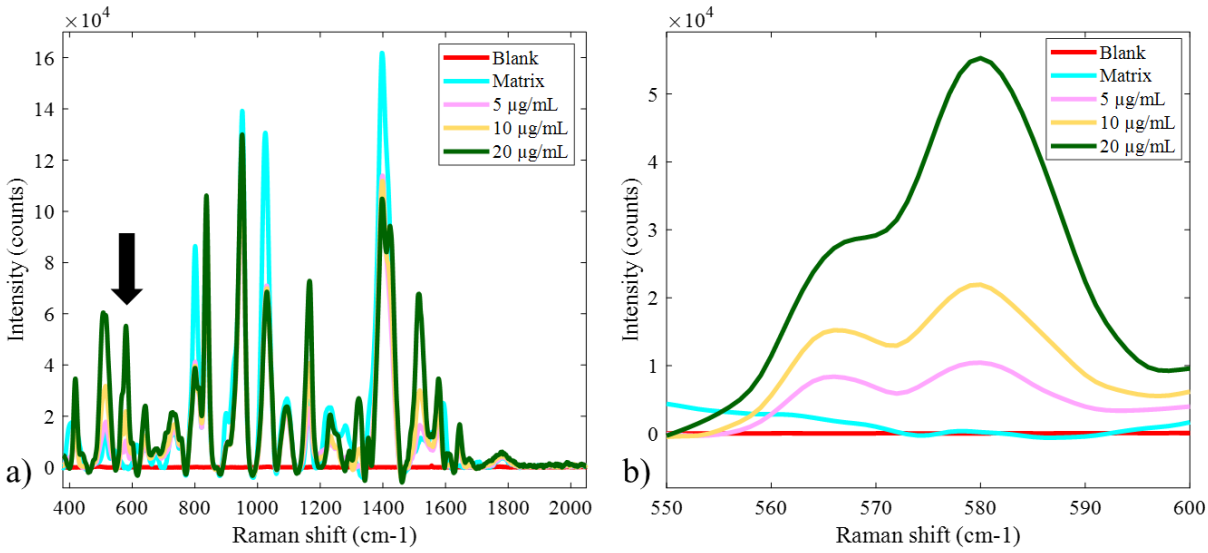

Figure 4: Preprocessed SETRS spectra of the blank (red), the matrix (cyan) and matrix spiked with increasing concentration of 4-AP (pink, yellow and green), (a: all Raman shift range, b: zoom of the spectra from 550 to 600 cm^{-1}).

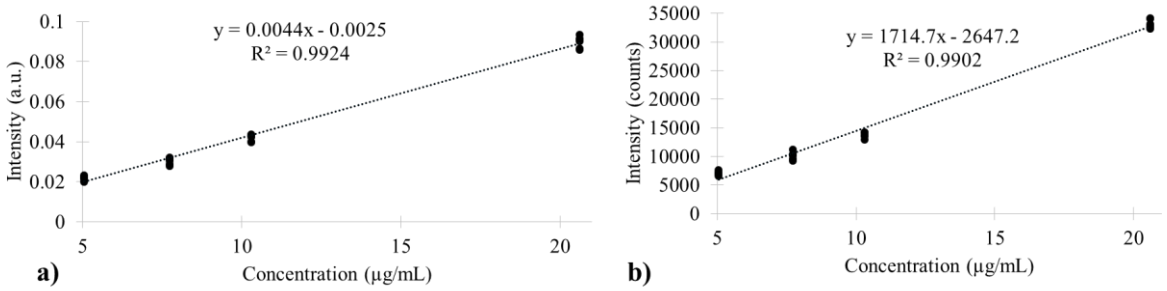

Figure 5: Calibration curves built using 4-AP signal intensity at 580 cm^{-1} and its corresponding concentration using (a) backscattering and (b) transmission detection mode.

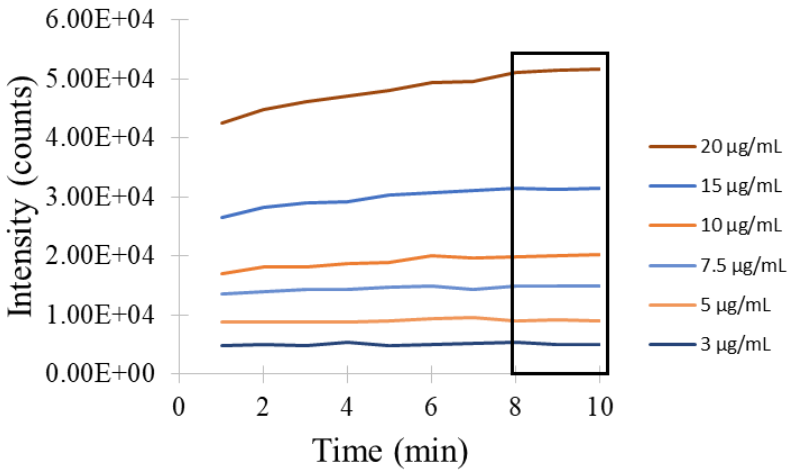

Figure 6: Evolution of SETRS intensities over time for each concentration level of a calibration series with a signal stability achieved after 8 min (black frame).

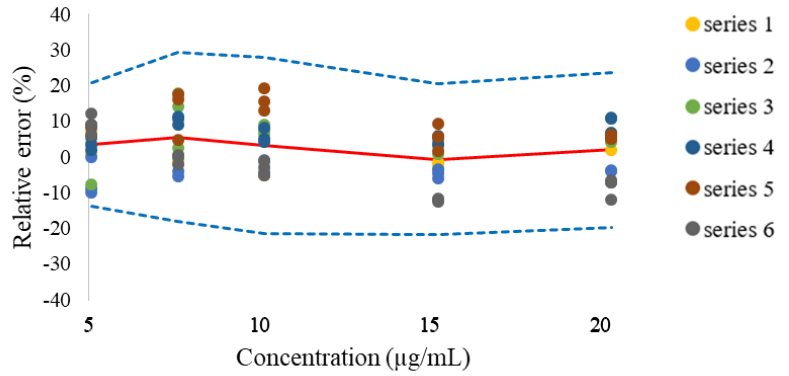

Figure 7: Total error risk-based approach obtained for SETRS quantification of 4-aminophenol in pharmaceutical formulation by considering quadratic regression. The plain red line represents the relative bias, the dashed blue lines are the 95 % β-expectation tolerance interval and the colored dots represent the back-calculated results from the validation series.

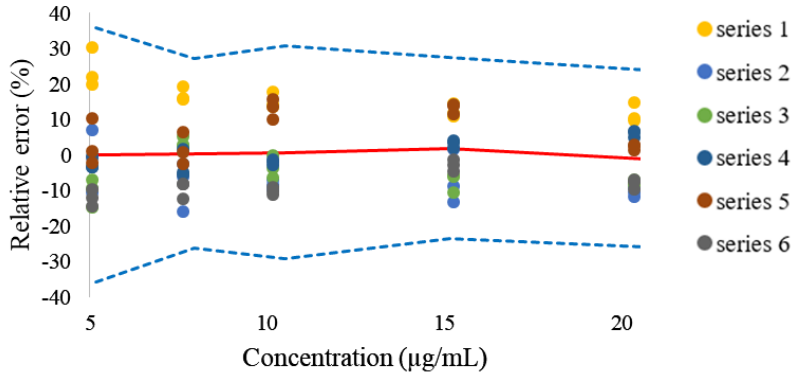

Figure 8: Total error risk-based approach obtained for SETRS quantification of 4-aminophenol in pharmaceutical formulation by considering PLSR model based on 12 calibration series. The plain red line represents the relative bias, the dashed blue lines are the 95 % β-expectation tolerance interval and the colored dots represent the back-calculated results from the validation series.

Tables:

Table 1. Concentration levels for calibration and validation series.

	Concentration levels					
	1	2	3	4	5	6
Calibration series (µg/mL)	3	5	7.5	10	-	20
Validation series (µg/mL)	-	5	7.5	10	15	20

Table 2. Comparison of performance between backscattering and transmission detection mode (RSD: relative standard deviation).

Concentration (µg/mL)	Backscattering RSD (%, n=9)	Transmission RSD (%, n=9)
5	7.8	6.3
7.5	6.6	5.2
10	6.0	4.6
20	3.9	3.2

508

509 **Table 3**. Repeatability and intermediate precision for each concentration level for the developed SETRS method by
510 univariate data analysis at 580 cm^{-1}.

Theoretical concentration (μg/mL)	Repeatability (%)	Intermediate precision (%)	Bias (%)	β-expectation tolerance interval (%)
5	3.9	7.0	3.5	-13.8 - 20.9
7.5	4.8	9.4	5.7	-17.9 - 29.3
10	2.0	9.0	3.3	-21.3 - 27.9
15	1.9	7.8	-0.6	-21.7 - 20.5
20	2.0	8.0	2.1	-19.7 - 23.8

511

512 **Table 4**. Repeatability and intermediate precision for each concentration level for the developed SETRS method by
513 multivariate data analysis.

12 calibrations series and 6 validation series				
Theoretical concentration (μg/mL)	Repeatability (%)	Intermediate precision (%)	Bias (%)	β-expectation tolerance interval (%)
5	5.3	13.8	0.1	-35.8 - 36.0
7.5	4.0	10.2	0.4	-26.3 - 27.1
10	2.1	10.9	0.8	-29.1 - 30.7
15	3.5	9.7	1.9	-23.6 - 27.4
20	1.9	9.1	-1.0	-26.0 - 24.0
10 calibrations series and 6 validation series				
Theoretical concentration (μg/mL)	Repeatability (%)	Intermediate precision (%)	Bias (%)	β-expectation tolerance interval (%)
5	6.7	13.0	2.8	-29.7 – 35.3
7.5	4.3	9.0	4.2	-18.6 – 27.1
10	2.1	9.4	4.1	-21.5 – 29.8
15	3.4	9.6	4.1	-21.2 – 29.5
20	1.2	9.3	0.8	-24.7 – 26.3

514