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Determination of 4-aminophenol in a pharmaceutical formulation using surface enhanced Raman scattering: From development to method validation

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ABSTRACT

A surface enhanced Raman scattering (SERS) method able to quantify 4-aminophenol in a pharmaceutical formulation based on acetaminophen, also called paracetamol, was developed and, for the first time, successfully validated. In this context, silver nanoparticles were synthesized according to the method described by Lee–Meisel and used as SERS substrate. The repeatability of the silver colloid synthesis was tested using different methods to characterize the size and the zeta potential of silver nanoparticles freshly synthesized. To optimize the SERS samples preparation, a design of experiments implicating concentrations of citrate-reduced silver nanoparticles and aggregating agent was performed in order to maximize the Raman signal enhancement. Finally, an approach based on tolerance intervals and accuracy profiles was applied in order to thoroughly validate the method in a range of concentrations comprised from 3 to 15 $\mu g\ mL^{-1}$ using normalized band intensities. The standard addition method was selected as method calibration. Therefore, measurements were carried out on 4-aminophenol spiked solutions of the pharmaceutical formulation. Despite the well-known stability and reproducibility problems of SERS, the validation was performed using two operators and five batches of nanoparticles, one for each validation day.

1. Introduction

During the last decade, Raman spectroscopy has taken an important place in industrial field, especially in pharmaceutical industry. This keen interest for this technique, which belongs to vibrational spectroscopy, can be explained by its numerous advantages including fast acquisition, non-invasive, non-destructive, minimization of the sample preparation step and possibility to use probes. Furthermore, this solvent-free technique is an attractive and promising tool in the "Green Chemistry" and "Process Analytical Technology" frameworks [1–3]. In the literature, there are many Raman applications dealing with the quantitative determination of analytes in pharmaceutical formulations [4–10]. As any analytical procedures, these methods require a complete validation focused on their intended use. However, chemometric tools are regularly used to assess the validity of a method which does not fit with the pharmaceutical regulatory requirement concerning method validation that can be found in the ICH Q2 document [11,12].

An important limitation of classical Raman spectroscopy in quantitative determination is its lack of sensitivity. One way to circumvent this problem is surface enhanced Raman scattering (SERS) which enables to dramatically increase the Raman scattering of molecules when they are adsorbed or very closed to rough metallic surfaces while keeping the structural information [13]. This phenomenon, discovered serendipitously in 1974 by Fleischmann et al. [14] is provided by the excitation of surface plasmon due to the interaction of an electromagnetic radiation with a rough metallic surface, on which target analytes are adsorbed or are very closed to. The principal substrates used in SERS are nanoparticles mainly made of gold or silver. Despite the dramatic advance in the fabrication of noble metallic nanoparticles, very few SERS applications dealing with quantitative determination of compounds in simple [15–23] and complex matrices [24–27] have been published because of the well-known stability and reproducibility problems of SERS. Regarding these publications, some validation criteria are reported such as the determination coefficient (R^2) of the calibration curve, repeatability of the signal intensity or the root mean square error of prediction RMSEP for the multivariate

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analyses. However, none of these methods were fully validated using different batches of metallic nanoparticles.

Acetaminophen (AC), also called paracetamol, is the widely used analgesic and antipyretic drug which can be administered to a variety of patients including pregnant women, children and the elderly. It exists in different drug formulations such as tablets, soluble powder in sachets, suppositories and syrups. 4-Aminophenol (4-AP), which is the primary impurity of AC coming from its degradation during the storage or from its synthesis might be present in pharmaceutical formulations and is actively researched because of its toxicity including nephrotoxicity and hepatotoxicity [28,29]. The monograph of acetaminophen tablets coming from the British Pharmacopoeia allows 0.1% (w/w) or 1000 ppm of 4-AP to ensure the drug formulation safety [30].

Several alternative methods to determine 4-aminophenol in acetaminophen pharmaceutical formulations have been published including high performance liquid chromatography methods with spectrophotometric [31–33] or electrochemical [34,35] detection, spectrophotometric methods such as colorimetry [36,37] and fluorimetry [38], voltammetric determination [39] and capillary electrophoretic methods with spectrophotometric [40,41] and electrochemical detection [42,43]. There are also flow analysis injection methods with colorimetric [44] and electrochemical detection [45]. However, most of these methods are time and solvent consuming. In addition, some of these publications do not present a meticulous validation of their analytical method.

In this context, the aim of this work was to develop and optimize a sensitive analytical method in order to determine 4-aminophenol, the main impurity of acetaminophen, in a pharmaceutical formulation using surface enhanced Raman scattering enabling to assess a limit of quantification below the specification limit of 1000 ppm. The developed method was then fully validated using a total error approach based on the tolerance intervals and accuracy profiles [46–49]. From the best of our knowledge, no quantitative method fully validated according to the pharmaceutical regulatory requirement has been published using surface enhanced Raman scattering.

2. Materials and methods

2.1. Chemicals and reagents

Silver nitrate ($AgNO_3$, cryst. extra pure) and potassium chloride (99.5%) were obtained from Merck (Darmstadt, Germany). Trisodium citrate (anhydrous, 98%) was obtained from Acros Organics (Morris, Plains, NJ, USA). 4-Aminophenol (purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) while acetaminophen was a reference substance from Fagron (Rotterdam, The Netherlands). The pharmaceutical formulation, sachets of 1 g of acetaminophen soluble powder used as a solution taken orally, was kindly provided by S.M.B, a pharmaceutical company (Marche-En-Famenne, Belgium). Ultrapure water was generated from Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Preparation of silver nanoparticles (Ag Nps)

Ag Nps were synthesized according to the method described by Lee and Meisel [50]. All glassware (three-neck round bottomed flask of 500 mL, stirrer and condenser) was rigorously cleaned with freshly prepared aqua regia (HCl: HNO_3, 3:1, v-v) and thoroughly rinsed with ultrapure water. Silver nitrate (45 mg) was dissolved in 250 mL of ultrapure water and heated rapidly to boiling. Then, 5 mL of a solution of trisodium citrate 1% freshly prepared was added while the silver nitrate solution was boiling and under stirring. The addition of trisodium citrate, which is use as a reducing agent, was carried out using a Dosimat (Metrohm AG, Herisau, Switzerland) with a dropping rate of 5 mL min^{-1}. The resulting solution was kept on boiling for 1 h while refluxing and then cooled down to room temperature. A Drynsyn heating block (Asynt, England) and a probe of temperature were used during the synthesis to keep the temperature constant and to improve the repeatability of the synthesis from batch to batch.

The nanoparticles were concentrated for quantitative analysis. Ten milliliters of the Ag Nps suspension were centrifuged for 15 min at 6000 rpm. Then, 9 mL of the supernatant were removed. The residue was suspended and the resulting solution was sonicated for 10 min. The nanoparticles were synthesized daily for five different days for the method validation.

2.3. Instruments

Raman spectra were collected with a dispersive spectrometer, the RamanStation 400 F (Perkin Elmer, MA, USA) equipped with a two-dimensional CCD detector (1024 × 256 pixel sensor) operating at −50 °C. The laser excitation wavelength used was 785 nm. The spectral coverage was 90–2000 cm^{-1} and the spectral resolution was fixed at 4 cm^{-1}. The laser power in the sample compartment was set at 100 mW. Quantitative analyses were performed in a quartz 96 microwell plate using the supermacropoint mode. It enables to record a spectrum created by summing the data from seven different points per well. The accumulation time was 20 s per point.

Visible absorption spectra were recorded with a Perkin Elmer Lambda 40 spectrophotometer using a 1 mm quartz cuvette from 300 to 800 nm. The samples of Ag Nps were prepared by solving 400 μL of the colloidal suspension in 3600 μL of ultrapure water. Resulting solutions were sonicated for 10 min before measurements.

Transmission electronmicroscope images were acquired by a CM 100 Philips transmission electron microscope (TEM) operated at an accelerating voltage of 100 kV. The samples for TEM analysis were prepared by putting one drop of the Ag Nps suspension on a Formvar-coated copper grid and drying it at room temperature. To measure the particle diameters on images, ITEM 5.1. was used.

A second technique was used to determine the size of Ag Nps which is a photon correlation spectroscopy (PCS) using a high performance particle sizer (HPPS) instrument (Malvern instruments, England). Measurements were made at 25 °C with a fixed angle of 90 °C and the results were expressed as the average Nps hydrodynamic diameter (nm). Viscosity and refractive index of pure water were used. The system was calibrated with an aqueous polystyrene dispersion of particles with a 100 nm diameter. The samples of Ag Nps were prepared by solving 400 μL of the colloidal suspension in 3600 μL of ultrapure water. Resulting solutions were sonicated for 10 min before measurements. Two measurements were performed for each sample.

The zeta potential of Ag Nps was determined using a Zetasizer 2000 (Malvern instruments, England). The instrument was calibrated with polystyrene dispersion with known ζ-potential. 2 mL of Ag Nps suspension were solved in 10 mL of ultrapure water and sonicated for 10 min before the measurement. Five measurements were performed for each sample.

2.4. Sample preparation

2.4.1. Standard solutions

A matrix effect was observed when SERS analyses were performed on solution comprising the pharmaceutical formulation. Indeed, the nanoparticles aggregation was influenced by the presence of the matrix in comparison with clean solutions of the impurity and a change of the intensity of spectra was observed. Therefore, the standard addition method was selected as calibration

Table 1
Experimental design followed to prepare standards solution for the SERS method validation. Validation standards were made in duplicate.

4-AP concentration in calibration standards (CSs)	Equivalent in 4-AP/AC (ppm)	4-AP concentration in validation standards (VSs)	Equivalent in 4-AP/AC (ppm)
3 μg mL^{-1}	300 ppm	3 μg mL^{-1}	300 ppm
5 μg mL^{-1}	500 ppm	5 μg mL^{-1}	500 ppm
7.5 μg mL^{-1}	750 ppm	10 μg mL^{-1}	**1000 ppm**
10 μg mL^{-1}	**1000 ppm**	15 μg mL^{-1}	1500 ppm
15 μg mL^{-1}	1500 ppm		

method in order to take into account this matrix effect [24]. For the method validation, five series of analyses (one series per day) were performed by two operators using silver nanoparticles freshly prepared (new batch of silver nanoparticles per series). For each series, a stock solution of 4-AP was prepared by dissolving an accurate amount of the impurity in water to reach a final concentration of 50 μg mL^{-1}. Then, appropriate amounts of this stock solution were added to different volumetric flasks of 100 mL, each containing one average weight of the pharmaceutical formulation. Finally, five concentration levels of 4-AP covering a range from 3 to 15 μg mL^{-1} were obtained. Two types of spiked solutions were prepared, namely calibration standards (CSs) which allowed building the calibration curve and validation standards (VSs) which were used to validate the method. The VSs were prepared independently in duplicate. All the operations were performed shielded from the light. The preparations of standard solutions using in each series of analyses are summarized in Table 1 which also presents the equivalence of 4-AP in the pharmaceutical formulation expressed as ppm.

2.4.2. SERS samples preparation

A full factorial design was performed in order to optimize the SERS samples preparation using JMP 10.0. In quantitative measurements, 400 μL of the 4-AP spiked solution, in presence of the pharmaceutical formulation, was added to 500 μL of concentrated Ag Nps and this solution was mixed for 15 s. To promote the aggregation, 50 μL of an aqueous solution of potassium chloride 7 mM was added to the colloidal suspension and the resulting solution was mixed for 1 min and kept at room temperature for 10 min before the SERS analysis. A quantitative analysis was performed based on the measure of the peak area at 1229 cm^{-1} and 1022 cm^{-1}. The peak at 1022 cm^{-1}, coming from citrates present around the Ag Nps surface, was used to normalize the peak at 1229 cm^{-1} of 4-AP.

3. Results and discussion

3.1. Characterization of Ag Nps

To characterize the morphology and the size of the synthesized Ag Nps, different techniques were used including UV–visible spectroscopy, transmission electron microscopy and photon correlation spectroscopy.

The maximum absorption of the measured visible spectrum of Ag Nps provides information on the average particle size, whereas its full width at half-maximum (FWHM) is used to estimate the particles size dispersion [51]. The mean (m) maximum absorption for five different batches was 404 ± 1.8 nm (m ± SD) and the FWHM was 74 ± 4 nm. The standard deviation (SD) values demonstrated that the control of parameters such as the dropping rate of citrate addition, the stirring rate and temperature from batch to batch drastically improves the repeatability of the synthesis. The average absorption value of 404 nm corresponds to values found in the literature for silver nanoparticles synthesized according to Lee–Meisel [52]. The size dispersion of Ag Nps is narrow which is proved by the FWHM value of 74 nm. UV–visible spectra of the five different batches synthesized for the method validation are presented in Fig. 1.

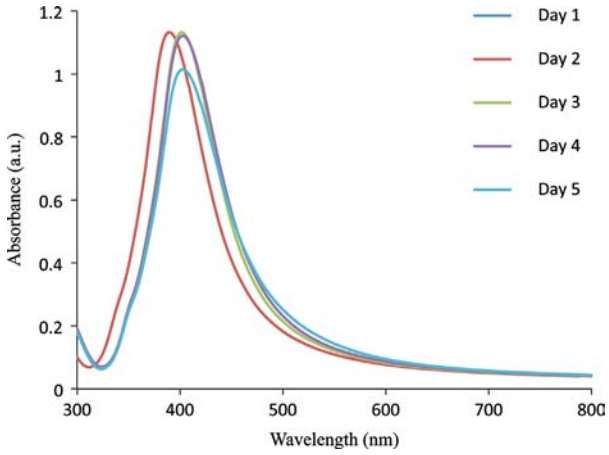

Fig. 1. UV–visible spectra of different nanoparticles batches synthesized during the validation step.

The average diameter obtained by PCS was 40 ± 3 nm which is comprised in the optimal size range for nanoparticles used in SERS [53]. The standard deviation value confirms the reproducibility between batches.

TEM images were acquired and clearly demonstrated that the particle size is mainly homogeneous and, for the most of silver nanoparticles, the shape is spherical. By measuring some of particle diameters on TEM images, results from HPPS were confirmed. TEM images are presented in the Fig. 2.

The average zeta potential of Ag Nps measured was −52.3 ± 1.8 mV (m ± SD). This value can be explained by the fact that trisodium citrate serves both as a reducing and a stabilizing agent. After the nanoparticles synthesis, a part of trisodium citrate remains in the solution and is adsorbed on metallic surfaces which confers to the nanoparticles enough negative charge to avoid their aggregation and improve the stability of the suspension [54,55].

3.2. Optimization of experimental SERS conditions

3.2.1. Aggregating agent

The aggregating agent is essential for the application of SERS using metallic nanoparticles and is generally an electrolyte which increases the ionic concentration inside the metallic colloidal suspension and decreases the electrostatic barrier enabling the nanoparticles aggregation and the move of analytes near the metal surface [52]. Potassium chloride was chosen as the aggregating agent because it enables to enhance the Raman signal but also to remove citrate adsorbed on Ag Nps surface which could interfere with the analyte response on the SERS spectrum [56].

3.2.2. Optimization of sample preparation

The sample preparation was inspired from Liu et al. [57]. A design of experiments implicating the concentrations of Ag Nps (raw to 10× concentrated) and aggregating agent (1 to 10 mM) was performed in order to optimize the SERS sample preparation to maximize the Raman signal enhancement. The center point was carried out in duplicate and the other points were measured one time. First experiments were made on clean solution of 4-aminophenol with a concentration fixed at 10 μg mL^{-1}. The optimized conditions were obtained using Ag Nps five times

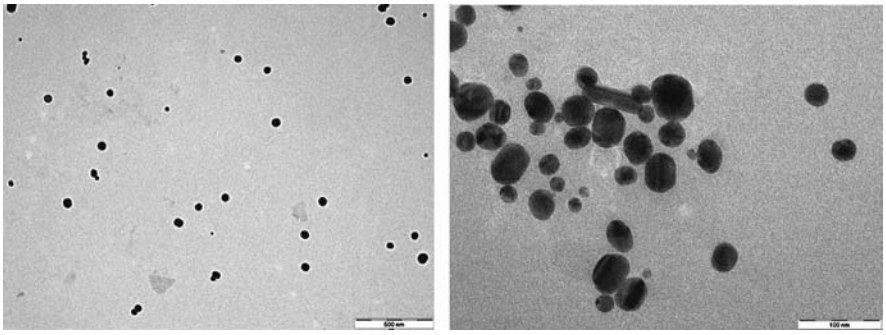

Fig. 2. TEM images of nanoparticles synthesized according to the Lee and Meisel method acquired with an accelerating voltage of 100 kv and with a magnification of 46000 × for the picture on the left and 180000 × for the right one.

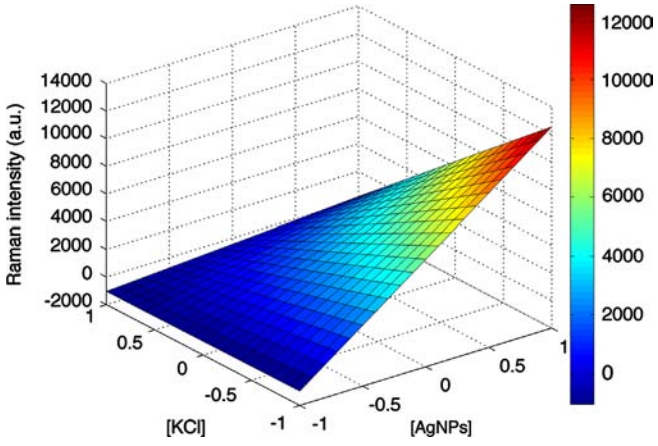

Fig. 3. Response surface for the intensity of the Raman signal obtained from the design of experiments carried out on the pharmaceutical formulation spiked with 4-aminophenol.

concentrated and a concentration of KCl of 5 mM. However, when these optimized conditions were applied on the pharmaceutical formulation, results were totally different because of a matrix effect. Indeed, in this case, Ag Nps coagulated and irreversibly precipitated as a black solid. An aggregating effect of the matrix was observed and explained the strong aggregation of particles and their precipitation. Therefore, this design of experiments was performed with a solution of 4-aminophenol spiked in the pharmaceutical formulation at a concentration corresponding to the specification limit. The optimal concentrations determined were 1 mM for KCl and Ag Nps ten times concentrated. However, the KCl concentration was not sufficient to remove citrate adsorbed on Ag Nps surface. Characteristic bands of citrate such as the bands at 1395 and 1022 cm^{-1} appeared in the SERS spectrum [58]. Nonetheless, optimal concentrations coming from the second design of experiments were kept because citrate bands did not interfere with the interesting band of 4-AP at 1229 cm^{-1} and moreover, one of these bands was used to normalize the spectra. The volume of aggregating agent was reduced to 50 μL to limit the dilution effect and its concentration was adapted to 7 mM to keep the same final concentration. Results coming from the second design of experiments are presented in Fig. 3 and in Table 2.

3.2.3. Incubation time

An influence of the incubation time on the SERS signal intensity was observed. There is an important signal enhancement during first minutes before signal stabilization. Therefore, the incubation time of 10 min was selected to achieve stability in the SERS signal limiting the time of analysis and improving the reproducibility between analyses.

Table 2
Description and results from the design of experiments carried out on the pharmaceutical formulation spiked with 4-aminophenol.

	Ag Nps concentration	KCl concentration (mM)	Intensity of Raman signal (1229 cm^{-1})
1	5 × concentrated	10	1830
2	5 × concentrated	1	8244
3	Raw nanoparticles	1	ND[a]
4	5 × concentrated	5.5	2749
5	Raw nanoparticles	5.5	ND[a]
6	Raw nanoparticles	10	ND[a]
7	10 × concentrated	10	2627
8	5 × concentrated	5.5	2396
9	10 × concentrated	5.5	4564
10	10 × concentrated	1	**14921**

[a] Not detected.

3.3. Method validation

To assess the validity of the SERS method, an approach using accuracy profiles based on tolerance intervals was carried out [46–49]. The tolerance interval used is a β-expectation tolerance interval which defines an interval where each future result will fall with a defined probability [59]. The accuracy profile coming from the method validation is represented in Fig. 4 where the plain line represents the relative bias. The dashed lines are the *β*-expectation tolerance limits which links *β*-expectation tolerance intervals calculated for each concentration level of validation standards, taking into account their estimated intermediate standard deviation and their bias. If *β*-expectation tolerance limits are comprised in the acceptance limits, which are represented in dotted lines in the Fig. 4, the method can be considered as valid and guarantees that every future result will fall in the acceptance limits with at least a probability previously defined. In this case, the method is focused on the determination of 4-AP which is the primary impurity in a pharmaceutical formulation AC-based. Therefore, the acceptance limits were fixed at ± 15% for the concentration range between 5 and 15 μg mL^{-1} and at 30% below 5 μg mL^{-1} and the probability at 95%. Accuracy profile was also used to select the most appropriate regression model for the calibration, to determine the lower limit of quantification (LOQ), the limit of detection (LOD) and the range over which the method can be considered as valid [49].

Analyses were carried out on five days with five different batches of Ag Nps and with two different operators. In order to correct the potential sources of variations in the magnitude of the detected SERS signal that are not associated with changes in the 4-AP concentration and to improve the repeatability, the bands intensity of 4-AP at 1229 cm^{-1} were normalized. The band intensity at 1022 cm^{-1} which belong to citrate was used to normalize because the citrate response is also influenced by the main sources of variations which

are coming from technical variations (such as the laser power, the well filling and the positioning of the sample holder) and variations due to the SERS substrate (such as aggregation quality and sample of Ag Nps) taking to account that citrates are adsorbed on the Ag Nps surface. Spectra obtained from different spiked solutions of 4-AP are presented in Fig. 5. The main bands of citrates and 4-AP used to quantify are highlighted. All of the results coming from the method validation are gathered in Table 3.

3.3.1. Selectivity

The method's selectivity was demonstrated by a SERS analysis of a non-spiked pharmaceutical formulation (blank). No response of the active pharmaceutical ingredient or excipients was observed on the SERS spectrum compared to the spectrum coming from 4-AP spiked solutions, which means that 4-AP is absent in the pharmaceutical formulation used to develop the method. This hypothesis was confirmed by HPLC analysis performed by S.M.B on their pharmaceutical formulations based on AC. The only bands present in the SERS spectrum came from citrates response as showed in Fig. 5.

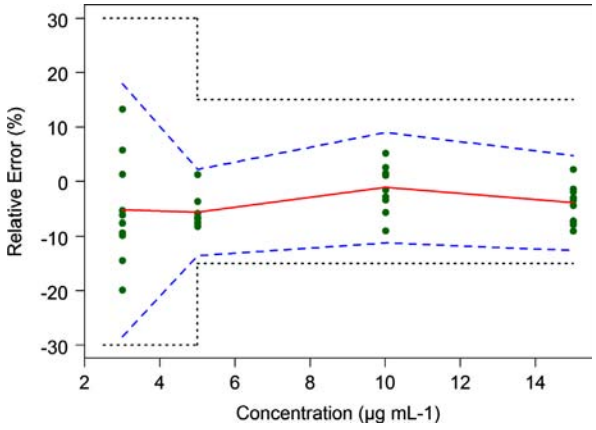

Fig. 4. Accuracy profile obtained for the validation of the quantification of 4-aminophenol in pharmaceutical formulation by considering linear regression after square root transformation as model for the calibration curve. The acceptance limits have been fixed at ± 30% for the concentrations smaller than 5 µg mL^{-1} and ± 15% for the concentrations equal or greater than this value. The plain red line represents the relative bias, the dashed blue lines are the 95% expectation tolerance limits and the dotted black lines are the acceptance limits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Analysis of the response functions

Several response functions were tested in order to find the most appropriated standard curve. From every response function, an accuracy profile was designed in order to compare all of them by a visual inspection.

Linear regression after square root transformation model was selected. This model allowed demonstrating the capability of the method to quantify the 4-AP in a pharmaceutical formulation over the whole concentration range considered, since the β-tolerance intervals totally fall within the acceptance limits. The validation results obtained by applying this regression model are shown in Table 3 for each day of analysis and the overall accuracy profile is presented in Fig. 4.

3.3.3. Trueness and precision

Trueness expressed in term of relative bias (%) was assessed from the validation standards at four concentration levels. The precision was then determined by computing the relative standard deviations (R.S.D.%) for repeatability and between-series intermediate precision at each concentration level of validation standards. The relative bias values and R.S.D. are presented in Table 3.

3.3.4. Accuracy, LOQ, range and LOD

The range of 4-AP concentration was fixed from 3 to15 µg mL^{-1} or equivalently from 300 to 1500 ppm of 4-AP in AC. The specification limit of 4-AP in AC of 1000 ppm was comprised in this concentration range. The highest concentration level was 15 µg mL^{-1} because with superior concentrations a saturation effect was observed probably due to the matrix.

Accuracy takes into account the total error, which is the sum of systematic and random errors related to the test results. As shown in Table 3, the upper and lower β-expectation tolerance limits (%) did not exceed the acceptance limits settled at 15% between 5 and 15 µg mL^{-1} and 30% between 3 and 5 µg mL^{-1}. Consequently, the method is able to provide accurate results over the concentration range investigated. The limit of quantification was 3.0 µg mL^{-1} and the limit of detection was estimated to be 0.9 µg mL^{-1} [49].

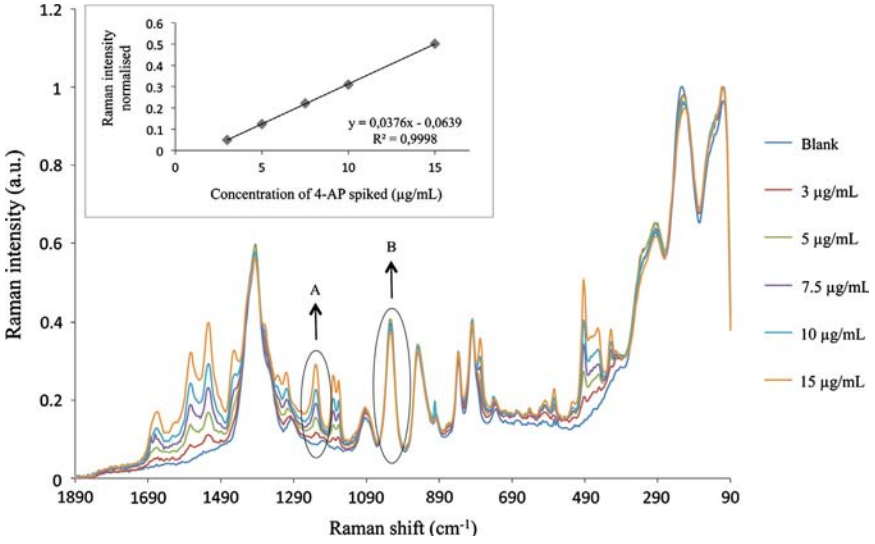

Fig. 5. SERS spectrum of non-spiked and spiked pharmaceutical formulation with different concentrations of 4-AP from 3 to 15 µg mL^{-1}. The band of 4-AP used to quantify and the band of citrates used to normalize are highlighted, A and B respectively. A classical calibration curve obained with the calibration standards is presented.

Table 3
Results coming from the validation of the SERS method developed to quantify 4-aminophenol in a pharmaceutical formulation.

Response function ($p=5$; $n=1$)	Linear regression after square root transformation	($m=4$): 3–15 μg mL^{-1}			
	Series 1	Series 2	Series 3	Series 4	Series 5
Slope	0.2101	0.2259	0.2311	0.2680	0.2779
Intercept	−0.1596	−0.1594	−0.1653	−0.2443	−0.2716
R^2	0.9983	0.9976	0.9963	0.9971	0.9926
Trueness ($p=5$; $n=2$) (μg mL^{-1})	Relative bias (%)				
3	−5.4				
5	−5.8				
10	−1.1				
15	−3.9				
Precision ($p=5$; $n=2$) (μg mL^{-1})	Repeatability (RSD%)	Intermediate precision (RSD%)			
3	9.6	9.6			
5	3.0	3.1			
10	4.2	4.2			
15	2.7	3.5			
Accuracy ($p=5$; $n=2$) (μg mL^{-1})	β-expectation tolerance limits (%)				
3	[−28.2; 17.4]				
5	[−13.6; 2.1]				
10	[−11.2; 9.0]				
15	[12.6; 4,7]				
Linearity ($p=5$; $n=2$)					
Range (μg mL^{-1})	[3.00–15.01]				
Slope	0.9716				
Intercept	−0.04907				
r^2	0.9931				
LOQ (μg mL^{-1})	3.0				
LOD (μg mL^{-1})	0.9				

p: number of series of experiments, m: number of concentration levels, n: number of replicates per concentration levels and per series.

4. Conclusion

In this work, a new application of SERS in the pharmaceutical field is demonstrated for the first time. A method was developed to quantify 4-aminophenol in a pharmaceutical formulation containing acetaminophen using surface-enhanced Raman scattering. Citrate-reduced silver nanoparticles were used as the SERS substrate. In this context, the repeatability of nanoparticles synthesis from batch to batch was improved by controlling some parameters such as the temperature and the dropping rate during the synthesis. To maximize the Raman signal enhancement, a design of experiments implicating the concentrations of aggregating agent and citrate-reduced silver nanoparticles was used to optimize the SERS samples preparation. Finally, this method was successfully validated in a range of concentrations of 3 to 15 μg mL^{-1} including five different batches of nanoparticles and two operators. Thus, this SERS method can be used to quantify the 4-AP at a concentration below its specification limit of 1000 ppm. Based on these results, a first estimation of the real analytical performance of this technique was defined in the pharmaceutical field.

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