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CRITICAL REVIEW OF SURFACE-ENHANCED RAMAN SPECTROSCOPY APPLICATIONS IN THE PHARMACEUTICAL FIELD

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KEYWORDS:

Surface-enhanced Raman scattering (SERS) - Pharmaceutical field - Quantitative determination - Method validation

ABSTRACT

Surface-enhanced Raman spectroscopy (SERS) is a sensitive analytical tool used in the pharmaceutical field in recent years. SERS keeps all the advantages of classical Raman spectroscopy while being is more sensitive allowing its use for the detection and the quantification of low-dose substances contained in pharmaceutical samples. However, the analytical performance of SERS is limited due to the difficulty to implement a quantitative methodology correctly validated. Nevertheless, some studies reported the development of SERS quantitative methods especially in pharmaceutical approaches. In this context, this review presents the main concepts of the SERS technique. The different steps that need to be applied to develop a SERS quantitative method are also deeply described. The last part of the present manuscript gives a critical overview of the different SERS pharmaceutical applications that were developed for a non-exhaustive list of pharmaceutical compounds with the aim to highlights the validation criteria for each application.

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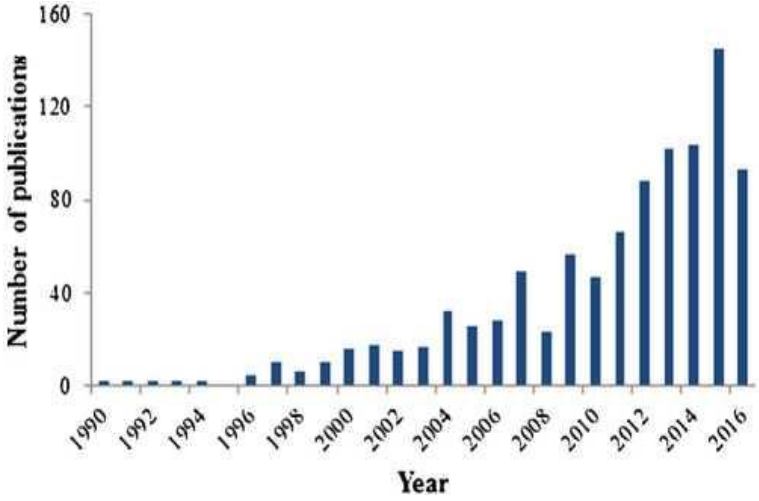
Introduction

Assisted by technological instrumentation advances, Raman spectroscopy has recently become one of the most popular analytical techniques used in the pharmaceutical field. It has been proved to be a valuable tool in many applications such as in polymorphic study [1], identification of raw materials [2], counterfeits detection [3], quantitative determination and homogeneity studies of active pharmaceutical ingredient (API) in solid dosage forms [2,4]. Furthermore, it can also be used to improve the understanding or the control of a pharmaceutical process through its implementation in PAT (Process Analytical Technology) analyses [5]. The attention devoted to Raman spectroscopy can be explained by the possibility to quickly provide key information on the physical and chemical properties of the molecules without sample preparation [6,7]. Moreover, itis a non-destructive and non-invasive technique which can be used for qualitative and quantitative analyses.

Fig. 1. *Evolution of the SERS technique in pharmaceutical field. (Scopus©) Keywords: 'surface enhanced Raman spectroscopy' AND 'drugs' OR 'pharmaceutical'.*



Despite the wide application range of Raman spectroscopy in the pharmaceutical field, this technique remains limited by the appearance of fluorescence and by the weak signal intensity preventing its use in the detection of very low-concentrated or colored compounds. Surface enhanced Raman spectroscopy (SERS), which has been an exponential technique in the pharmaceutical domain since several years as illustrated in Fig. 1, can be a solution to overcome these issues. SERS enables to increase the intensity of the Raman scattering from molecules which are in direct contact or very close (less than 10 nm) to metallic nanostructured substrates commonly reported as SERS substrates. These SERS substrates, based on a noble metal (mainly gold or silver),

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act as antennas exalting the Raman scattering through the plasmon resonance excitation. The exaltation of the signal varies by a factor of 10^3 to 10^{11} depending on the nature of the target molecule and the type of SERS substrate used [8,9].

Although the SERS theory is still not perfectly understood, the results observed in recent years highlight its potentiality as a sensitive analytical tool perfectly suited for pharmaceutical research. Some approaches were developed to use SERS in pharmaceutical and illegal tablets investigations in the case of low drug concentrations. These researches have resulted in extremely low detection limits up to ppb (parts-per-billion) level of illicit compounds or impurities, while acquiring quantitative information regarding the spatial distribution in the pharmaceutical product.

In this context, this review is dedicated to the comprehension of the SERS technique and to give a critical overview of several applications related to the use of SERS in the pharmaceutical field. The main concepts of the SERS exaltation and a non-exhaustive list of various substrates that can be used will be broached, but the different steps that have to be taken into account in SERS quantitative method development will also be described.

SERS concept

DISCOVERY OF SERS

The SERS effect was discovered in 1974 by Fleischman et al. [10] which observed a significant increase of the Raman band intensity of pyridine adsorbed onto a silver electrode. Afterwards, numerous works on the subject such as those of Creighton and Albrech [11], Jeanmaire and Van Duyne [12] in 1977 permitted to study the important parameters of the exaltation effect which are related to the surface state and the nature of the metal, but also to the wavelength of the excitation source. The work of Moskovits [13] in 1978 enabled to distinguish two types of contributions which can be observed in the SERS exaltation: an electromagnetic and a chemical mechanism. The first one is the main contribution to the exaltation and is independent of the probed molecule, in other words it is applicable to any type of analytes as long as it is located a few nanometers from the surface of the SERS-active substrate. On the other hand, the second one depends heavily on the nature of the molecule, and involves the formation of new molecular states and chemical bonds due to the direct interaction with the SERS surface [14].

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SERS SUBSTRATES

From the reviewed literature about the applications of SERS in the pharmaceutical field, a very large range of SERS substrates is commonly used by the different research groups to perform repeatable qualitative and quantitative analyses. Generally, gold and silver are the most common metals used for SERS applications due their surface plasmon resonance effects in the visible and the near infrared wavelength ranges [15]. Despite the wide variety of new SERS substrates developed, colloidal suspensions are still the most employed SERS substrates taking account of their many advantages such as the ease of their synthesis and implementation, their stability and their very low cost. The common procedure used to prepare these colloids is the chemical reduction described by Lee and Meisel [16] involving the reduction of the metal salt with sodium citrate. However, further synthesis protocols described in the literature used different reducing agents such as sodium borohydride, hydroxylamine hydrochloride and hydrazine [17–21]. More rarely, other techniques such as laser ablation and photoreduction are employed to synthesize metallic colloidal suspensions [22]. Regarding SERS analyses, numerous experiments have been carried out to prove that the SERS activity is dependent on the size, the shape and the arrangement of the metallic structure [23,24]. In this context, multiple strategies were developed in order to control the morphological form of nanoparticles in different designs such as nanorods, nanocubes, nanoprims, and nanostars as can be seen from reviewed literature dedicated to this topic [25–27]. Nevertheless, the areas of very large electromagnetic enhancement, called "hot spots", generally located in a tiny gap between two metallic nanoparticles after aggregation, are randomly distributed. The non-uniform distribution of the SERS signal enhancement from hot spots has prompted researchers to develop new reproducible and uniform nanostructures. In this sense, Fan et al. [26] described in a review the different fabrication methods of nanoparticles immobilized on planar solid support.

The characterization of the SERS substrates is a critical step before SERS analyses in order to ensure the good uniformity and repeatability of the SERS signal enhancement. The characterization of the nanoparticles can be carried out by a multitude of techniques which can be found in the literature [22] as showed in Fig. 2. The most widely used technique to get an overview of the morphological criteria is electron microscopy. Consequently, Transmission Electron Microscopy (TEM) or Scanning Electron Microscopy (SEM) are often applied [28,29]. However, these techniques require time for sample preparation and are relatively expensive. They are mainly used to compare and validate other fast and simple characterization approaches such as UV - Visible spectroscopy. Indeed, the UV

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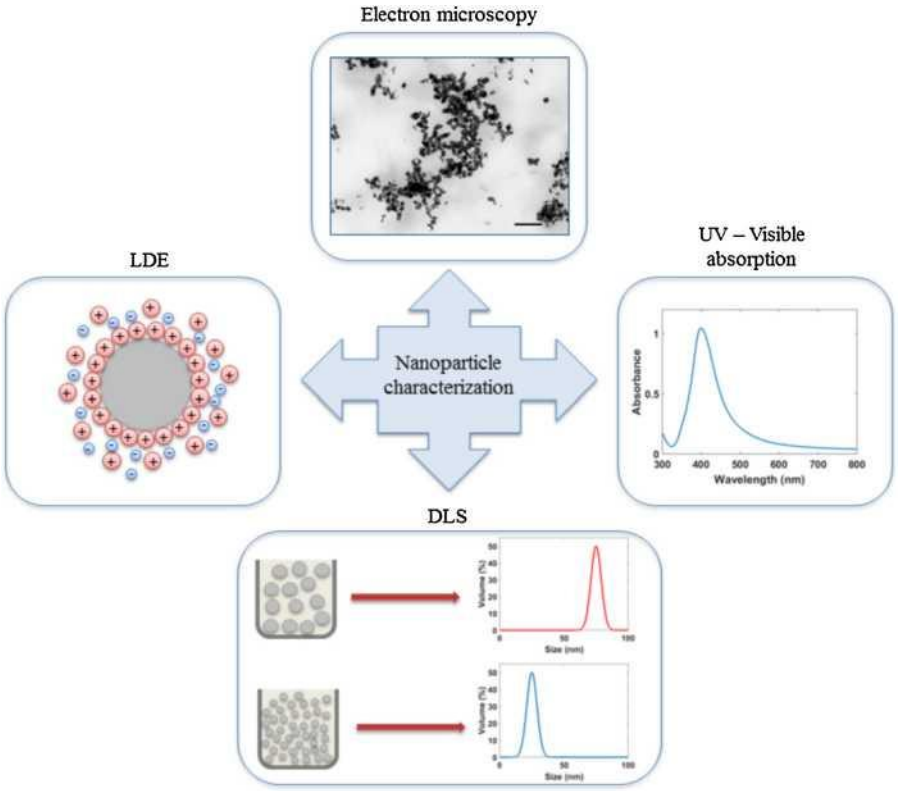
- Visible spectrum gives information about the average nanoparticles size through the position of the peak related to the maximum absorption wavelength of the colloidal solution. The full width half maximum (FWHM) can also be used to estimate the nanoparticles size distribution, a larger peak being the result of a greater size distribution [18]. Moreover, Dynamic Light Scattering (DLS) and Laser Dopler Electrophoresis (LDE) are used to measure respectively the mean size and the polydispersity, and the zeta potential of nanoparticles. The zeta potential depends on the pH of the colloidal solution and provides information about the stability of the nanoparticles which must have enough positive or negative charges on their metallic surface in order to maximize the electrostatic repulsion between them [30]. Therefore, it is generally admitted that the colloidal solutions must have a zeta potential greater than + 30 mV or lesser than - 30mV to be stable [31]. The characterization of nanoparticle SERS substrates makes it possible to estimate the repeatability of the SERS substrates from batch to batch, which is critical in quantitative SERS analyses [32].

***Fig. 2**. Different characterization techniques of nanoparticles.*



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OPTIMIZATION OF SERS CONDITIONS

After having characterized the SERS substrate, various parameters have to be considered to ensure the high and repeatable SERS signal enhancement. For example, the volume and the concentration of the target analyte, aggregating and/or functionalization agents, but also the pH and volume of the colloidal suspension subjected to the SERS analysis are crucial parameters and need to be optimized. The aggregation of metal nanoparticles by the addition of an aggregating agent is essential to achieve a better SERS enhancement [33]. Indeed, an aggregating agent modifies the ionic strength and reduces the electrostatic barrier, allowing the nanoparticles to get closer to each other in order to create hot spots, and closer to the analyte of interest. Many different types of aggregating agent can be found in the literature, including in particular the addition of polymer or long-chain ions and electrolytes [15]. Regarding electrolytes, two types can be distinguished, on the one hand, passive electrolytes such as KNO_3 or $MgSO_4$ changing the ionic strength of the nanoparticle suspension. On the other hand, active electrolytes such as KCl able to modify the surface charge of metal colloids in addition to also change the ionic strength.

Functionalization agents can moreover be added to the nanoparticle colloid to improve the selectivity towards the target analyte. Indeed, some molecules have a native affinity for metallic nanoparticle surfaces, such as the compounds having an amine group or a thiol group in their structure, which facilitates their SERS detection. Other molecules do not have this affinity and are therefore not attracted by the metallic surface. This low affinity of the analyte for the metallic nanoparticles can be overcome through the surface functionalization with chemical groups which enable to create strong covalent bonds between the analyte of interest and the SERS substrate, increasing the sensitivity and the selectivity of the detection [34,35].

In order to obtain repeatable and high SERS enhancements, the parameters cited above have been taken into account due to their significant impact on experimental SERS analyses. However, they are interdependent and must be optimized for the research purpose before beginning the analysis [36]. For this reason, the use of design of experiments is mentioned in the literature in order to provide the optimal SERS conditions considering all the factors but also the interaction between them without using the principle of one factor at a time [36–38]. Moreover, the design of experiments approach contributes to saving time related to the number of experiments to be carried out.

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SERS DATA ANALYSIS

Before obtaining quantitative information about impurities or low-dose compounds, a pre-processing step on the SERS spectra is necessary in order to make the desired information more accessible. Indeed, the SERS spectrum acquisition may be perturbed by cosmic rays (spikes) and by scattering or fluorescence effects. These disturbances might be due to the physical properties of the samples or to the spectrometer used. As a result, mathematical corrections such as spikes and baseline corrections are necessary before data analysis. Once this preliminary step has been carried out, the desired information can be obtained either by a univariate or a multivariate approaches. The univariate approach consists to extract the relevant information from the area or the intensity of peaks related to the analyte of interest. It has the disadvantage of not taking on board the entire spectrum. Moreover, the peak of interest can be distorted by interferences related to other adjacent peaks corresponding to further compounds, especially in complex matrices.

An alternative approach to extract the relevant information in complex matrices regarding the studied compound is the use of multivariate data analysis. Various algorithms in SERS analysis were applied using exploratory techniques such as Principal Component Analysis (PCA) or regression techniques such as, for instance, Classical Least Square (CLS) and Partial Least Square (PLS) [39]. The theory of these algorithms was previously described in detail in various reviews [40,41]. Semi-quantitative technique as Multivariate Curve Resolution with Alternating Least Squares (MCR-ALS) [42] was also applied. For example, it used for SERS analyses in biological fluids (urine, blood. . .) where strong matrix effects can occur, with band overlaps and interferences with bands related to the target analyte [43–45]. Moreover, the MCR-ALS method, as shown in Fig. 3, is the algorithm the most frequently used in the framework of SERS chemical imaging which combines the advantages of SERS measurements and the ability to provide spatial information about the distribution of a compound in a sample. SERS chemical imaging will be further discussed in the following paragraphs. The MCR-ALS algorithm decomposes the bi- dimensional matrix D of spectral data in a bilinear model given below:

$$D = C \cdot S^T + E \quad (1)$$

Where C is the pure concentration matrix, S^T is the transpose matrix of pure spectra and E is the error matrix. The number of initial constituents must be known or estimated before the initialization of the matrices C or S. In general, the matrix C is unknown and the matrix S is initialized experimentally by pure spectra acquisition or by different mathematical approaches: SIMPLISMA (SIMPLe-to-use

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Interactive Self-modeling Mixture Analysis) [46], OPA (Orthogonal Projection Approach) [47], ICA (Independent Component Analysis) [48], VARCLUS (VARiable CLUStering) [49] and so on. The advantage of MCR-ALS method is the possibility to apply constraints such as non-negativity, closure or local-rank during the iterative process in order to restrain the space of the decomposition solutions and to improve the resolution of the system. A non-exhaustive list of publications refers to the application of constraints in MCR-ALS to imaging techniques in the pharmaceutical field [50–52].

Pharmaceutical applications

In this section, the development, optimization and validation of quantitative SERS analyses and of SERS chemical imaging methods will be broached. In addition, some examples of SERS applications in the pharmaceutical field will be listed.

QUALITY CONTROL WITH SERS IN THE PHARMACEUTICAL FIELD

Considering its many advantages, SERS may become a reliable analytical tool in pharmaceutical research. Its high sensitivity and specificity makes SERS an appropriate technique for the quality control of pharmaceutical products such as the identification and detection of impurities or drugs in low-dose, the homogeneity distribution of APIs or even quantitative analyses [53]. However, to the best of our knowledge, the scientific literature is poor regarding the evaluation of the real analytical performances, the development and the validation of the SERS methods.

Table 1. *Validation criteria obtained from the ICH Q2 document considered for the type of analysis cited [57]. -: criterion not normally evaluated; +: criterion normally evaluated.*

	Type of analysis		
Validation criteria	Detection	Semi-quantitative analysis	Quantitative analysis
Accuracy	-	-	+
Repeatability	-	-	+
Intermediate precision	-	-	+
Specificity	+	+	+
Detection limit	–	+	–
Quantification limit	–	–	+
Linearity	–	–	+
Range	-	-	+

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METHOD VALIDATION OF SEMI-QUANTITATIVE AND QUANTITATIVE ANALYSES

With the aim of developing a quantitative SERS method to determine the concentration or the distribution of compounds in a pharmaceutical formulation, a mandatory step, after the method development, is the method validation. The method validation is a process used to demonstrate the analytical performances of a method according to its intended use and to give enough guarantees in the framework of routine analyses. In this context, guidelines were published by the pharmaceutical regulatory authorities in order to demonstrate if the method is fit for the aim and can be considered as valid for quantitative analyses [54]. Some authors have determined validation criteria in the guidance document from the International Conference on Harmonization (ICH) that should be considered during the validation step of analytical procedures. Considering the detection, semi-quantitative or quantitative analysis of a low-dose compound, the validation parameters required are listed in Table 1.

There are very few examples of publications dealing with quantitative method development including these validation criteria in the procedure in order to validate the analytical method. The validation criteria the most commonly reported are the coefficient of determination (R^2) of the calibration curve and the concentration range, in order to evaluate the relationship between the concentration and the SERS signal in quantitative trace detections [37,38,44,55]. However, considering a quantitative SERS method, this criterion is not sufficient to assess the validity of the method according to the pharmaceutical regulatory requirements. The lack of validated method can be explained by the difficulty to synthesize in a repeatable way SERS substrates from batch to batch.

The evaluation of the limit of detection (LOD) in SERS analyses is a key parameter in order to determine the sensitivy of the method. The LOD is defined as the lowest analyte concentration of a sample which can be reliably detected by the analytical method. This detection limit criterion is very discussed in the field of analytical chemistry due to the multiple definitions and methodologies applied to evaluate this latter. SERS technique is not an exception to the rule, and for this reason Massarini et al. [56] proposed four different approaches to determine the LOD of ten narcotic drug analytes. The first one is a general formula to calculate a one-sided prediction interval (at significant level $\alpha = 0.05$) for the mean value of blanks. The equation is given below:

$$LOD = \bar{y}\,blank + 3.SDblank \qquad (2)$$

With $\bar{y}_{blank}$ the mean value of blank signal and SD_{blank} is the standard deviation. Once the LOD signal is determined, it is then possible to calculate the corresponding concentration by a Langmuir

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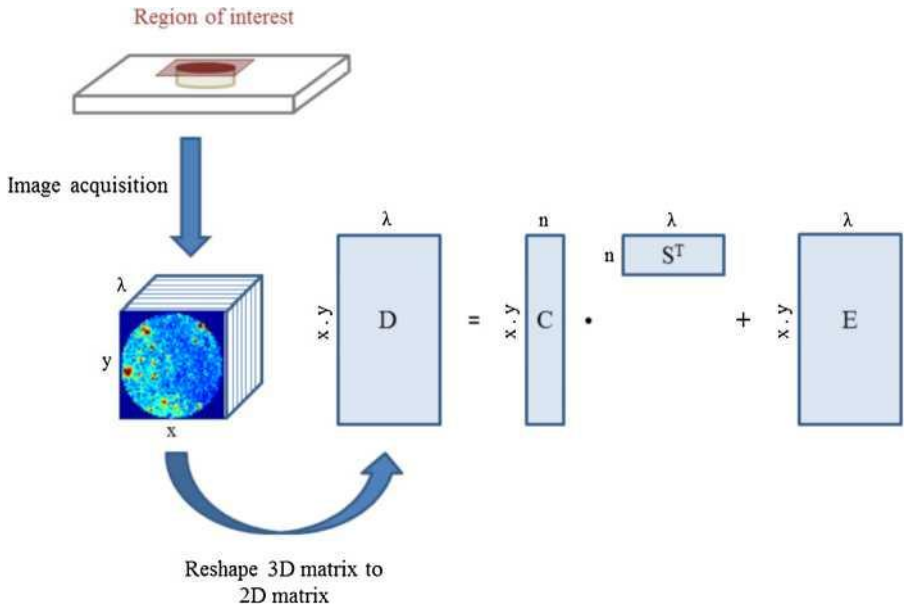
function. The use of the Langmuir equation is reported in detail in various papers of Izquierdo-Lorenzo et al. to calculate the LOD of doping drugs by SERS [57–59]. The second and the third methodologies applied a one-sided prediction interval of a linear regression line. The authors used these approaches when the LOD signal obtained is outside the linear calibration range, the concentration is then estimated by a linear regression or a non-linear regression after logarithmic transformation. The last calculation method employed by Massarini and co-workers was the use of receiver operating characteristic (ROC) curves to determinate the LOD [56]. A ROC curve is a plot of the probability of true positive rate (y-axis) versus the probability of false positive rate (x-axis), which represents a plot of sensitivity against specificity

***Fig. 3**. Reshape of the hyperspectral image in a bi-dimensional dataset and decomposition of the matrix D of spectral data into a matrix C of concentrations, a matrix ST of pure spectra and a residual matrix E.*



. A threshold is then interpreted as the LOD and defined as the shortest distance to the coordinate (0,1) considered as having no false positive or false negative events. The method is explained in more detail in the paper [56] and also applied by Inscore et al. for the detection of drugs in saliva by SERS [60].

In addition to the LOD, further parameters are applied to assess the validity of a SERS quantitative method. Indeed, the selectivity of the method, the limit of quantification (LOQ) or the concentration range referred as a linear dynamic range are parameters that should also be considered depending on the goal of the study.

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DEVELOPMENT OF SERS CHEMICAL IMAGING

In recent years, the use of SERS chemical imaging has increased in various fields, especially in food industry [61,62], biomedical [63,64] and pharmaceutical sciences [65–68]. In the pharmaceutical field, its interest is particularly growing in the quality control of the quantitative distribution of API and excipients in various pharmaceutical forms. SERS imaging combines the advantages of SERS with a spatial dimension which makes it possible to identify the presence of low dose compounds on the surface of a pharmaceutical solid form while visualizing their distribution. However, the applications of SERS chemical imaging in the pharmaceutical field remain limited, especially due to the difficulty of obtaining a homogeneous deposit of nanoparticles on the sample surface.

The covering method of nanoparticles on the sample surface is a major issue for SERS imaging analyses, because the colloidal solution deposit is not controlled, therefore preventing a homogeneous distribution of hot spots over the entire surface of the sample. Many coating techniques are used to produce uniform nanoparticles coating on flat surfaces such as drop casting, spin coating or adsorption coating as illustrated in Fig. 4 [69]. The first one is the most used for SERS chemical imaging [65–67]. However, the intensity of the SERS signal of the analyte measured at different places of the deposit is very variable due to a "coffee ring" effect which concentrates the nanoparticles at the edge of the droplet after adsorption resulting, in an inhomogeneous covering as shown in Fig. 4a. The spin coating illustrated in Fig. 4b, are mainly used for the manufacture of conductor materials. Nevertheless, others methods such as spray coating could also be investigated as coating techniques for SERS chemical imaging. However, none are listed in the literature yet.

De Bleye et al. [67] compared the homogeneity of the SERS colloid distribution on a tablet surface using two different approaches. On the one hand, drop casting was used as shown in Fig. 4a. On the other hand, the colloidal silver nanoparticles were absorbed on the tablet surface by capillarity with a preliminary step of drop deposition on a glass side as illustrated in Fig. 4c. This study showed a better homogeneity of the capillarity method than the drop casting one.

DETECTION AND QUANTIFICATION OF BANNED DRUGS IN SPORT PRACTICES

The high sensitivity of SERS makes this technique suitable for the detection and quantification of doping drugs. In anti-doping control, the identification of banned drugs in residual amounts is a major task in order to prevent the illegal enhanced performance and also to protect the health of athletes against illicit drugs. In this context, the use of SERS could be an alternative method to

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GC/MS (gas-chromatography/mass spectrometry) or LC/MS (liquid-chromatography/mass spectrometry), currently used in laboratories, which are time-consuming, require expensive pretreatment steps and solvents. Moreover, some classical analytical techniques may not be able to detect some drugs under the minimum required performance level (MRPL) which is a critical parameter set by the authorities to detect prohibited substances.

From the reviewed literature indicated in Table 2 concerning the SERS analysis of illicit drugs in sport practices, Izquierdo-Lorenzo et al. [57–59,70] have characterized some drugs and promoted their adsorption on the SERS substrates with an optimization of the SERS parameters (morphological forms of the nanoparticles, pH, and concentrations). In particular, they demonstrated that probenecid, a substance which masks the effect of prohibited drugs during a doping control, has an affinity only for silver substrates compared to beta-adrenergic agonist drugs which are more effective on gold surfaces [70]. This research group also focused their attention on the quantification of aminoglutethimide (AGI), an aromatase inhibitor, in aqueous solutions, using gold nanoparticles synthesized according to the Sutherland and Winefordner protocol [20]. The pH of the medium was fixed to 5.0 in order to promote the adsorption of the target molecule on the nanoparticle surface. With these parameters, a detection limit of 85 ppb was reached [58]. AGI was also later studied by the same research group who compared different morphological forms and various metals of nanoparticles [59]. They were able to decrease considerably the LOD to 0.13 ppb with silver triangular nanoprisms.

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Table 2. *Applications of SERS analyses of banned drugs in sport practices.*

Analyte	Matrix	Analysis type	SERS substrates	Laser wavelength (nm)	Validation criteria	Ref.
Acebutolol	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 1.34 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 1.56 μg mL^{-1}	
			Citrate Ag Nps on ion-exchange paper		LOD = 2 μg mL^{-1}	
			Citrate Ag Nps on cellulose paper		LOD = 2.03 μg mL^{-1}	
Amiloride	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.19 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.36 μg mL^{-1}	
			Cirate Ag Nps on cellulose paper		LOD = 0.85 μg mL^{-1}	
			Acid etched Ag foils		LOD = 0.76 μg mL^{-1}	
Aminoglutethimide	Aqueous solution	Quantitative	Citrate Au Nps	633	Range: 10^{-5}–10^{-4} M	[58]
					R^2 = 0.9997	
					LOD = 85 ng mL^{-1}	
Aminoglutethimide	Aqueous solution	Quantitative	Citrate Ag Nps	633	Range: 2–20 ng mL^{-1}	[59]
					R^2 = 0.991	
					LOD = 5.1 ng mL^{-1}	
			Triangular nanoprism-shaped Ag Nps		Range: 0.2–2 ng mL^{-1}	
					R^2 = 0.994	
					LOD = 0.13 ng mL^{-1}	
Atenolol	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.87 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 1.23 μg mL^{-1}	
			Citrate Ag Nps on ion-exchange paper		LOD = 2.6 μg mL^{-1}	
Clenbuterol	Aqueous solution	Quantitative	Citrate Au Nps	785	Range: 10^{-5}–10^{-4} M	[57]
					R = 0.97	
					LOD = 35 ng mL^{-1}	
Cocaine	Aqueous solution	Semi – quantitative	Borohydride Ag Nps	488	LOD = 1.34 μg mL^{-1}	[71]
Methoxiphenamine	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.11 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.30 μg mL^{-1}	
Modafinil	Human urine	Quantitative	Hydroxylamine Ag Nps	785	Range: 50–1000 ng mL^{-1}	[55]
					R^2 = 0.9887	
					LOD = 12.55 ng mL^{-1}	
					LOQ = 41.83 ng mL^{-1}	
			Gold coated magnetic spherical Nps		Range: 80–1000 ng mL^{-1}	
					R^2 = 0.9913	
					LOD = 23.05 ng mL^{-1}	
					LOQ = 76.83 ng mL^{-1}	
Oxprenolol	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 1.35 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.11 μg mL^{-1}	
Pemoline	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.003 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.017 μg mL^{-1}	
			Citrate Ag Nps on ion-exchange paper		LOD = 0.51 μg mL^{-1}	
			Citrate Ag Nps on cellulose paper		LOD = 0.15 μg mL^{-1}	
Probenecid	Aqueous solution	Quantitative	Citrate Ag Nps	532	Range: 5.10^{-7}–10^{-5} M	[70]
					R^2 = 0.95	
					LOD = 1.2 μg mL^{-1}	
Propanolol	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.059 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.098 μg mL^{-1}	
			Citrate Ag Nps on ion-exchange paper		LOD = 0.18 μg mL^{-1}	
			Citrate Ag Nps on cellulose paper		LOD = 0.14 μg mL^{-1}	
Propanolol	Aqueous solution	Quantitative	Citrate Au Nps	785	Range: 0.13–1300 ng mL^{-1}	[72]
					R^2 = 0.96359	
					LOD = 2.36 ng mL^{-1}	
Propanolol	Human serum	Quantitative	Citrate Ag Nps	785	Range: 0–10 μM	[73]
					LOD = 0.45 μM	
					RMSEP = 0.58 μM	
Propanolol	Human plasma	Quantitative	Citrate Ag Nps	785	Range: 0–80 μM	[73]
					LOD = 0.53 μM	
					RMSEP = 9.68 μM	
Propanolol	Human urine	Quantitative	Citrate Ag Nps	785	Range: 0–120 μM	[73]
					LOD = 0.57 μM	
					RMSEP = 1.69 μM	
Recombinant human EPO	Aqueous solution	Semi – quantitative	Au nanostructure over Au substrate		Range: 10 pM–10 nM	[113]
					R^2 = 0.993	
Salbutamol	Aqueous solution	Quantitative	Citrate Au Nps	785	Range: 10^{-5}–10^{-4} M	[57]
					R = 0.98	
					LOD = 55 ng mL^{-1}	
Terbutaline	Aqueous solution	Quantitative	Citrate Au Nps	785	Range: 10^{-5}–10^{-4} M	[57]
					R = 0.998	
					LOD = 765 ng mL^{-1}	
Triamterene	Aqueous solution	Semi – quantitative	Citrate Ag Nps	488	LOD = 0.043 μg mL^{-1}	[71]
			Borohydride Ag Nps		LOD = 0.058 μg mL^{-1}	
			Citrate Ag Nps on ion-exchange paper		LOD = 0.35 μg mL^{-1}	
			Citrate Ag Nps on cellulose paper		LOD = 0.43 μg mL^{-1}	
			Acid etched Ag foils		LOD = 0.44 μg mL^{-1}	

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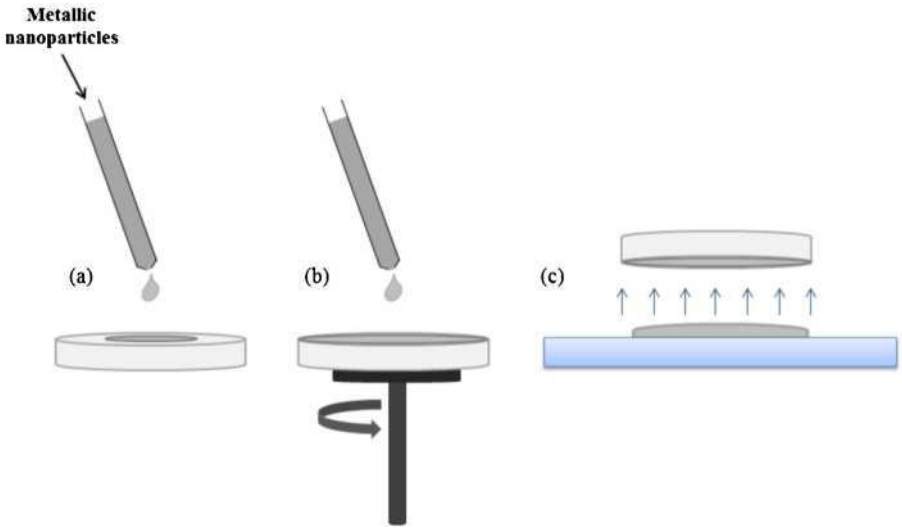
***Fig. 4**. Illustration of the different coating techniques. (a) Drop casting: a droplet of nanoparticles is simply drop onto the tablet surface. (b) Spin coating: a droplet of the SERS substrate is also drop onto a flat surface but the surface is rotated with high speed to be entirely covered. (c) Adsorption coating: the colloidal solution on the glass slide is adsorbed by capillarity onto the tablet surface.*



This higher SERS sensitivity was due to a better adsorption of AGI on the surface with the design of nanoparticles as triangular nanoprims and a better affinity for the metal which increased the electromagnetic enhancement of the metallic surface. Others aspects of substrate performances were reported by Pérez et al. [71]. Five different forms of silver substrates (liquid and solid) were also studied. This comparative study between nine drugs showed a higher SERS signal enhancement with colloidal solutions using sodium citrate and sodium borohydride as reducing agents compared to silver colloid deposited ona solid substrate. For example, they obtained, for the beta-adrenergic blocker drug propranolol, a LOD respectively equal to 59 ppb and 180 ppb with citrate silver nanoparticles and silver nanoparticles sprayed on an ion-exchange filter. Levene et al. [72] later optimized the SERS quantification method of propranolol using an multi-objective evolutionary algorithm combined with a fractional factorial design in order to determine the best experimental SERS parameters and to improve the repeatability of the SERS spectra. They tested several combinations including metals type, salts, aggregating agents, their concentrations and laser excitation wavelengths, and allowed the reduction of experiments to be tested from 7785 to 315. In this way, the LOD decreased to 2.36 pbb in a concentration range from 0.13 to 1300 ppb.

Using the same approach, Subaihi et al. [73] developed a method to detect and quantify propranolol in three different human body biofluids. The direct analysis of illicit drugs in complex matrices is

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complicated because of the numerous compounds present in biofluids. These exogenous compounds, such as proteins, urea and salts found in plasma or urine may cause interferences with the target analyte. In this study, the authors were able to directly quantify propanolol in serum and urine, without any sample preparation step. In contrast, in the case of plasma, the proteins were removed by ultracentrifugation in order to avoid the repulsion of the nanoparticles to each other, which could hamper their aggregation. SERS quantitative analyses were carried out by multivariate data analysis using PLS models and a root-mean-square error of prediction (RMSEP) was calculated in order to compare the performance of the method in the different matrices. The RMSEP is calculated from a test set, based on the following equation:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{N_p}} \qquad (3)$$

With y_i and $\hat{y}_i$ corresponding respectively to the concentration measured by the reference method and the values predicted by the PLS model for the NP spectra in the validation dataset. They obtained respectively 0.58 µM, 9.68 µM and 1.69 µM for human serum, plasma and urine. Further studies reported extraction techniques in order to isolate the analyte while discarting the rest of the biological matrix. Caglayan et al. [55] worked on the quantification of modafinil, a psychostimulant, in human urine comparing two sample pretreatments. A SPE (Solid Phase Extraction) procedure was used, combined with silver nanoparticles, and the second technique was the use of magnetic gold nanoparticles for their magnetic properties. Despite the many advantages of these magnetic colloidal particles such as their low cost and their ease of synthesis, modafinil had a better affinity for the silver surface. The LOD was equal to 13 ppb and 23 ppb for the silver nanoparticles with SPE and for magnetic gold nanoparticles, respectively.

IDENTIFICATION AND QUANTIFICATION OF ILLICIT DRUGS

Based on the same principle as for the detection of doping drugs, numerous articles and review papers have been published about the use of SERS as a detection technique for illicit and stimulant drugs in forensic science [74–76]. Due to its high sensitivity, some synthetic molecules could be identified and quantified by SERS with very low detection limits (Table 3). In this context, amphetamine, methamphetamine and their major derivatives are the most popular compounds for SERS quantification.

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Table 3 *Applications of SERS analyses of illicit drugs.*

Analyte	Matrix	Analysis type	SERS substrates	Laser wavelength (nm)	Validation criteria	Ref.
Amphetamine/2-MNA	Aqueous solution	Quantitative	Etched Ag foil	633	Range: 47.2–472 ppm LOD = 19 ppm	[77]
Benzoylecgonine	Aqueous solution	Semi – quantitative	Ag-coated carbon nanotubes	785	Range: 10^{-9}–10^{-5} M R^2 = 0.9896	[84]
Cannabinoids (synthetic drugs)	Aqueous solution	Quantitative	Citrate Au Nps	785	Range: 2.5–500 ng mL^{-1} LOD range: 18–60 ng mL^{-1}	[91]
Cocaine	Human saliva	Quantitative	Au doped sol-gel capillary	785	Range: 0–1000 ng mL^{-1} LOD = 25 ng mL^{-1}	[86]
Cocaine	Human saliva	Semi – quantitative	Au doped sol-gel capillary	785	LOD = 50 ppb	[60]
Cocaine	Human urine	Semi – quantitative	Self-assembly 2D Au Nps film functionalized with CTAB	633	LOD = 500 ppb	[87]
Cocaine	Aqueous solution	Semi – quantitative	Microfluidic device with borohydride Au Nps	633	LOD = 4.6 ng mL^{-1}	[80]
Codeine	Aqueous solution	Detection	Citrate Ag Nps	633 and 785	Qualitative	[114]
DOB	Tablet	Semi – quantitative	Citrate Ag Nps	785	LOD = 15 µg (Mass of tablet: 400 mg)	[96]
Hydrocodone	Aqueous solution	Detection	Citrate Ag Nps	633 and 785	Qualitative	[114]
MAMP	Human urine	Semi – quantitative	Au nanorods stabilized with SH-PEG	785	Range: 1–50 µg mL^{-1}	[92]
MDAI	Aqueous solution	Detection	Ag on copper coin	633	Qualitative	[82]
MDAI	Aqueous solution	Quantitative	Citrate Ag Nps	633	Range: 1–500 ppm LOD = 8 ppm	[83]
MDMA	Aqueous solution	Detection	Ag on copper coin	633	Qualitative	[82]
MDMA	Aqueous solution	Quantitative	Ag Nps modified by mixed thiols	785	Range: $7.7.10^{-5}$–$7.7.10^{-4}$ M R^2 = 0.9661 LOD = $1.5.10^{-5}$ M RMSEP = $7.9.10^{-5}$ M	[81]
MDMA	Human urine	Semi – quantitative	Au nanorods stabilized with SH-PEG	785	Range: 1–50 µg mL^{-1}	[92]
MDMA	Aqueous solution	Detection	Citrate Ag Nps	633	Qualitative	[115]
Mephedrone	Aqueous solution	Quantitative	Citrate Ag Nps	633	Range: $5.65.10^{-7}$–$5.65.10^{-4}$ M LOD = 1.6 µg mL^{-1}	[116]
Mephedrone	Aqueous solution	Detection	Ag on copper coin	633	Qualitative	[82]
Methamphetamine/2-MNA	Aqueous solution	Quantitative	Etched Ag foil	633	Range: 60.9–2435 ppm LOD = 17 ppm	[77]
Methamphetamine	Human urine	Quantitative	Self-assembly of Ag Nps to 3D spherical superstructure	532	Range: 1–40 ppm R^2 = 0.9828 LOD = 10 ppb	[78]
Methamphetamine	Human saliva	Semi – quantitative	Microfluidic device with Ag Nps	633	LOD = 10 nM	[79]
Methamphetamine	Human urine	Quantitative	Self-assembly 2D Au Nps film functionalized with mPEG-SH	785	Range: 0.1–100 ppm R^2 = 0.9947 LOD = 0.1 ppm	[89]
Methamphetamine	Aqueous solution	Semi – quantitative	Microfluidic device with borohydride Au Nps	633	LOD = 4.5 ng mL^{-1}	[80]
Morphine	Aqueous solution	Detection	Citrate Ag Nps	633 and 785	Qualitative	[114]
Morphine	Aqueous solution	Semi – quantitative	Microfluidic device with borohydride Au Nps	633	LOD = 13 ng mL^{-1}	[80]
Nicotine	Aqueous solution	Quantitative	Polymer-encapsulated Ag Nps	785	Range: 0.1–10 ppm R^2 = 0.998 RMSEP = 0.1 ppm	[117]
Tramadol	Aqueous solution	Quantitative	Hydroxylamine Ag Nps	633	Range: 5.10^{-5}–10^{-2} M R = 0.965 LOD = 5.10^{-4} M	[90]
Tramadol	Artificial urine	Quantitative	Hydroxylamine Ag Nps	633	Range: $1.25.10^{-6}$–$8.75.10^{-3}$ M LOD = $2.5.10^{-6}$ M	[90]
Various drugs	Human saliva	Detection	Au doped sol-gel capillary	785	Qualitative	[85]

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Sulk et al. [77] proposed a technique to detect and quantify amphetamine and methamphetamine combined with a functionalizing agent, which was 2-mercaptonicotinic acid (2-MNA), because of the low affinity of both compounds for the silver surface. 2-MNA is easily detectable using SERS due to its phenyl ring breathing mode present respectively at 998 cm^{-1} and 1000 cm^{-1} for amphetamine and methamphetamine. This research group also added known concentrations of pentachlorothiophenol (PCTP) as an internal standard, which was SERS active and coadsorbed for quantification on etched silver foil as SERS substrate. The use of an internal standard can overcome some experimental variations such as the laser excitation power and the optic instrument but also enables to increase the repeatability of SERS analyses [32]. The relative band intensity to PCTP at 1514 cm^{-1} was used to normalize the peak intensity of the amides presents in both analyte structures, in different concentrations ranges, allowing to reach detection limits respectively equal to 17 ppm (parts-per-million) and 19 ppm for modifiedmethamphetamine and amphetamine. Methamphetamine can also be quantified in complex systems as demonstrated by Han et al. [78] which developed a new 3D SERS nanostructure with an emulsion of silver nanoparticles in cyclohexane including liquid–liquid extraction. The concentration of the molecule was determined with a LOD close to 10 ppb corroborated by UPLC (Ultra Performance Liquid Chromatography) assays. Another research group reported the study of methamphetamine including the combination of SERS with microfluidic to detect trace amounts notably, in human saliva [79,80].

Amphetamine derivatives such as MDMA (3,4- methylenedioxymethamphetamine), commonly referred to ecstasy, were also investigated by SERS. The selectivity of MDMA on silver surface was improved by Stewart et al. [81], who modified the metallic surface of nanoparticles with different ratios of mixed thiols. This surface modification with the creation of a strong covalent bond between the thiol and the silver surface provided more selectivity to the analyte of interest and allowed its detection by SERS with a LOD of $1.5.10^{-5}$ M. Others articles highlight spectral differences between amphetamine species using different approaches regarding the SERS substrate. For example, Mabbot et al. [82] proposed the use of British silver coins as SERS substrates to detect MDMA, MDAI (5,6-Methylenedioxy-2-aminoindane) a further analogue of amphetamine, and mephedrone. The substrate was soaked into independent drug solutions during a specific time and after being dried, the coin was mapped by a confocal Raman instrument and multivariate analysis was carried out in order to discriminate the illicit substances. MDAI, an amphetamine variant previously cited, was quantified by Mabbott et al. [83] using silver colloids and KNO_3 as aggregating agent. The authors optimized the aggregation time in order to ensure the maximum repeatability from the SERS

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response and were able to quantify MDAI by univariate analysis with a LOD estimated at 8 ppm.

Various others species have an interest in SERS analysis, this is the case for cocaine and its main metabolite, benzoylecgonine, used in drug screening tests. The latter was coupled with a monoclonal antibody for a specific indirect detection on silver-coated carbon nanotubes [84]. This method was more selective and allowed to obtain quantitative information by univariate analysis on a C H bending band related to the antibody - benzoylecgonine complex. This linkage was explained by the low affinity of cocaine to silver surface, however further studies were performed later regarding a better signal enhancement on gold surface. After conducting studies about the detection of eighty relevant drugs by SERS in human saliva, Inscore et al. [60] research group has been mainly focused on the SERS activity of cocaine on gold-doped sol-gels immobilized in glass capillaries. The SERS measurement was performed after a SPE process and the group was able to detect cocaine at 50 ppb more than 90% of the time according to the ROC curve. This detection limit was corroborated in another work including a Hit Quality Index (HQI) as a result which determines the correspondence or not of a spectrum with a drug spectral library in just few minutes [85]. A similar approach, conducted by the same research group, decreased the detection limit to 25 ppb by optimizing the sample preparation step to promote the extraction of cocaine from saliva [86]. Another study reported the quantification of cocaine in urine with self-assembled 2D gold nanoparticle films [87]. The nanoparticles were functionalized with cetyltrimethylammonium bromide (CTAB) and provided good uniformity and repeatability. Despite an extraction process involving hexane as organic solvent, the LOD of cocaine in urine sample was higher (500 ppb) probably due to the presence of impurities adsorbed during the purification process. Moreover, in a perspective to improve the rapidity of analyses for drugs detection until the real-time information about the presence of illicit substances on site for police or medical staff, the urine samples were also analyzed with a handheld Raman spectrometer.

The instrument was equipped with a 785 nm laser and reached to a detection limit of 1 ppm. Portable Raman spectrometers are being increasingly used in the pharmaceutical field. They are less efficient than the equipment present in the laboratory, but simpler to use for professionals of health and police forces [88]. In this context, Han et al. [89] proposed a portable kit intended to detect methamphetamine in human urine on site. The package included a transportable Raman device and a sample preparation platform useful to extract the analyte by cyclohexane from human body fluids. It also contained packaged self-assembled 2D gold nanoparticles, previously described, but coated

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by methoxy mercapto polyethylene glycol (mPEG-SH) in order to remove CTAB from the gold surface and to promote the adsorption with derivatives amphetamines, leading to repeatable and reliable results. Despite a detection limit close to 0.1 ppm, the portable kit can be useful for rapid and accurate trace analyses before further deeper investigations in the laboratory. A portable Raman probe with a 633 nm laser wavelength was used by Alharbi et al. [90] to develop a detection method of tramadol, a narcotic drug prescribed as a painkiller that may lead to dependence and drug abuse. Different SERS conditions were optimized step by step before SERS acquisitions. Different type of metals, the incubation time, the aggregating agent, the aggregation time and finally the pH were tested. The optimized SERS parameters used were hydroxylamine silver nanoparticles with 0.5 M of sodium chloride established at neutral pH and directly analyzed with the target analyte by the Raman probe. The LOD was determined by univariate analysis with the area of the 993 cm^{-1} peak corresponding to the ring breathing vibration of the molecule. Tramadol was identified in water and in artificial urine at detection limits of 5.10^{-4} and $2.5.10^{-6}$ M respectively. Recently, Mostowtt et al. [91] reported the first detection of synthetic cannabinoids by SERS applications comparing two types of equipment, a bench- top Raman instrument and a portable Raman spectrometer. The investigations were carried out to highlight four synthetic cannabinoids with similar chemical structure. Seven aggregating agents including chloride, sulfate and nitrate salts were tested at different concentrations with gold colloids in order to determine the optimal SERS conditions enabling to obtain the highest sensitivity for both equipment. The best results were achieved with $MgCl_2$ as the aggregating agent, but the authors suggested to optimize the concentration depending on the type of instrument used. Moreover, the analyses were successfully performed in urine after SLE (Supported Liquid Extraction) pretreatment which improved the sensitivity of the method with a portable Raman instrument.

The improvement of the portable systems and the sample preparation processes were also combined with the progress of the algorithms used for the data analysis. Dong et al. [92] used an algorithm called SVM (Support Vector Machine) to develop classification models in order to identify drugs by a Dynamic-SERS method also called D-SERS [93]. In this work, different urine solutions containing methamphetamine and MDMA were mixed with gold nanoparticles stabilized with SH-PEG. The SERS spectra were collected during the transition process from the wet state to the dry state of the nanoparticles by a portable Raman spectrometer and SVM was applied in order to detect rapidly and precisely drugs in complex matrices without any sample preparation. The SVM algorithm is often compared to other classification methods in various SERS applications especially for

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diseases diagnostics [94,95]. These comparisons tend to show the high efficiency and robustness of SVM compared to linear approaches such as LDA (Linear Discriminant Analysis) due to its capability of processing binary classification problem with non-linear features. The dataset comprising different classes is separated in a new high dimensional space, even if the data are non-linearly separable, by the use of a kernel function in order to maximize the distance between the spectra. In the Dong et al. study [92], the radial basis functions (RBF) was used as the kernel function and the classification accuracy of 50, 2.5 and 1 ppm for methamphetamine and MDMA in urine preprocessed by PCA were 96.1 and 95.9%, 95.3 and 95.3%, 94.2 and 94.0% respectively. This method allowed a rapid and reliable identification while avoiding standard sample preparation conducted in the laboratories.

To the best of our knowledge, the first quantitative application of SERS on solid tablets of amphetamine was performed by Bell et al. [96]. The authors were able to detect the 2,5- dimethoxy-4-bromoamphetamine (DOB) signal in the lactose excipient. Citrate silver colloids and sodium chloride aggregating agent were deposited by the drop casting method on the tablet surface to create an active SERS spot. The SERS signal of DOB was much more significant than the excipient band due to a better adsorption of the DOB on the metallic surface of the nanoparticles, allowing a rapid identification with a detection limit lower than the active dose.

QUANTIFICATION OF PHARMACEUTICAL DRUGS

Pharmaceutical molecules were investigated by the SERS technique and its very high sensitivity in order to ensure the quality control of the final product [97]. Indeed, its low detection level allows the impurities detection and the identification of low concentration API and also the monitoring of drugs concentration in biofluids as discussed by Jaworska et al. [98]. Moreover, the implementation of SERS chemical imaging is also possible, leading to the evaluation of the low-dose compounds distribution in solid dosage forms (Table 4).

The detection of trace amounts of penicilloic acid, an allergen responsible of human allergic reactions to penicillin was exposed by Zhang et al. [99]. Using silver coated gold nanoparticles as SERS substrate, they were able to rapidly detect 0.9 mg of penicilloic acid in 1 g of penicillin. In this work, no aggregating agent was mixed with the colloidal solution to give rise to hot spots. As a matter of fact, the adsorption of the two carboxyl groups of the allergen on the metallic surface induced the particle aggregation due to its linkage at the interspace of the SERS substrate. The

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authors also observed that an increase of the penicillin concentration induced a decrease of the colloid aggregation due to a competitive adsorption with the only carboxyl group of penicillin. A SERS qualitative approach developed by Hernandez et al. [100] discussed the behavior of piroxicam, a drug molecule used for its anti-inflammatory properties, in various pH conditions. Different SERS conditions were tested such as metal nanoparticles and laser excitation, and consequently the larger signal enhancement was obtained with hydroxylamine silver nanoparticles and a 532 nm laser. According to the pH conditions, they characterized different conformers and their orientation on the metallic surface. Captopril, a prescribed drug enabling to control the blood pressure, was investigated by Long et al. [101]. They proposed a multiplicative effects model for SERS [102] to eliminate the external variations caused by the heterogeneity of the SERS substrate and the optical parameters. The quantitative analyses were carried out in blood plasma samples with a mixture of silver nanoparticles, potassium chloride and p-thiocresol as internal standard. The thiol group of this internal standard does not interfere with the captopril detection allowing a detection limit and a LOQequal to 0.149 µM and 0.451 µM, respectively.

Ackermann et al. [103] developed a SERS approach for online quantitative detection of low concentrated drugs through a microfluidic device also called LoC-SERS (Lab-on-a-Chip-SERS). The authors performed the SERS quantification of two pharmaceutical compounds, promethazine and mitoxantrone, by on-line monitoring. A multi-channels device was used to control the flow rate of the analytes and water in order to regulate the concentrations of silver nanoparticles, sodium chloride and oil as separation medium. The oil phase (tetradecane) was distributed in a continuous flow in order to form microdroplets containing the various liquid to be analyzed by SERS measurements without any interference. In this context, tetradecane was used as internal standard to compensate external variations and enabled to assess a LOD of 2.10^{-4} M and 5.10^{-7} M for promethazine and mitoxantrone, respectively. More recently, Hidi et al. reported the use of LoC-SERS for the detection of the antibiotic levofloxacin in water [104], in artificial and human urine [105]. The LOD was estimated at 0.8 µM in aqueous medium, without the addition of salt due to the aggregation induced by the analyte itself. In the context of the antibiotic determination in human biofluids, the authors first conducted the analyses in artificial urine in order to get an overview of the matrix effect on the SERS signal of the target analyte. Once the aggregation time and the dilution factor were optimized, the LOD was determined to be 0.07 mM with a RMSEP value equal to 0.058 mM after the creation of a PLS model. In real samples, the RMSEP values varied from 0.058 to 0.16 mM depending on the patients but the LOD could not be established. The low RMSEP values

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demonstrated the robustness of the LoC-SERS technique which could also be combined with a portable Raman system to be used for clinical applications.

SERS can also be combined with chromatographic techniques. Hence, many studies have already demonstrated the advantages of using thin layer chromatography (TLC) with SERS for qualitative or quantitative analyses in various fields such as food safety [106], pollution detection [107] and toxicology [108]. TLC is a simple and low-cost separation technique, easy to implement, generally used to separate different compounds from a mixture. The separation takes place on a silica TLC substrate also called planar stationary phase, where a small quantity of the sample is deposited. The TLC plate is then put into a closed container containing a solvent called liquid mobile phase, which aims to move the components through the stationary phase by capillary action. After the separation, SERS experiments were performed directly on the TLC support after adding few microliters of metallic colloidal solutions on the spot of interest. In pharmaceuticals, some approaches were established to detect low dose API in a mixture or in physiological fluids by TLC - SERS. Recently, Li et al. [109] proposed a platform for the qualitative analysis of low API drugs on aTLC silica gel support with silver nanoparticles which provided a sensitive detection method. They also predicted quantitative determination of API, rosiglitazone in this case, through a calibration curve realized using the internal standard method. Sodium thiocyanate was used as an internal standard to correct technical parameters and the calibration curve presented a good linearity with a correlation coefficient equal to 0.9977. TLC - SERS can also be used for the detection of API from complex matrices as shown in the work of Lucotti et al. [110]. They reported the drug detection of apomorphine, a drug prescribed for advanced Parkinson disease, in human plasma. However, the detection limit of apomorphine on the TLC plate with the silver colloids reached 10^{-4} M which is considerably higher than the therapeutic concentration in the blood varying from 10^{-8} to 10^{-7} M.

Acetaminophen and acetylsalicylic acid, also known as paracetamol and aspirin respectively, are the most famous analgesic and antipyretic drugs used in the world. The literature is rich in articles regarding the detection of these compounds by the SERS technique [53,97,111]. Nevertheless, to the best of our knowledge, very few of them refer to their quantification in aqueous solutions or in commercial tablets. The first quantification of aspirin was exposed by Sallum et al. [112] using silver coated filter paper as the SERS substrate, prepared by the silver mirror reaction. The solid substrate was immersed in aqueous solutions containing acetylsalicylic acid concentrations ranging from 0.1 to 1 mM during 80 min in order to promote the adsorption of the molecule of interest on the metallic

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surface. A calibration curve was built with a R^2 equal to 0.933 pre senting a good linearity with a relative error of 2.06% compared to a HPLC method. Our research group worked on acetaminophen [38], but focusing on its main impurity, called 4-aminophenol which is toxic and may be present in low amount in pharmaceutical formulations. In this work, the first step was to optimize the sample preparation with a full factorial design involving various parameters such as the concentration of silver nanoparticles (raw versus to 10 times concentrated) and of the aggregating agent (1–10 mM). Once this step had been carried out, a calibration curve was built with calibration standards prepared in a concentration range of 4- aminophenol from 3 to 15 $\mu g.mL^{-1}$ and the method was then fully validated according to the pharmaceutical regulatory requirement. The method validation was made using two operators and five batches of silver colloids for each validation days to take account of a maximum of variability. The accuracy profile provided a LOQ and a LOD equal to 3 $\mu g.mL^{-1}$ and 0.9 $\mu g.mL^{-1}$, respectively. Finally, the real analytical performances of this method were evaluated. The method was able to quantify 4-aminophenol below the specification limits provided by the British Pharmacopoeia of 1000 ppm or 0.1% (w/w) in the drug formulation which represents an analytical interest in the pharmaceutical field [38].

In this context, another study performed by the same researchers reported the development of SERS chemical imaging method to visualize the distribution of 4-aminophenol in paracetamol tablets [67]. The tablets, comprising a mixture of paracetamol and polyvinylpyrrolidone with different concentrations of 4-aminophenol (0.025–0.2% (w/w)), were covered by butanethiol functionalized silver nanoparticles by adsorption coat-ing as previsouly explained in Fig. 4. The concentration maps were obtained by univariate analysis with the peak intensity related to the impurity normalized by a specific peak stemming from butanethiol aiming to reduce the variability associated to the covering.

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Table 4. Applications of SERS analyses of pharmaceutical drugs.

Analyte	Matrix	Analysis type	SERS substrates	Laser wavelength (nm)	Validation criteria	Ref.
4-aminophenol	Tablet	SERS imaging detection	Citrate Ag Nps	785	Qualitative	[67]
4-aminophenol	Aqueous solution	Quantitative	Citrate Ag Nps	785	Range: 3–15 $\mu g\,mL^{-1}$ $R^2 = 0.9983$ LOD = 0.9 $\mu g\,mL^{-1}$ LOQ = 0.3 $\mu g\,mL^{-1}$	[38]
Apomorphine	Human plasma	Semi – quantitative	TLC plate with citrate Ag Nps	785	LOD = 10^{-4} M	[110]
Aspirin	Tablet	SERS imaging detection	Citrate Ag Nps	532	Qualitative	[65]
Aspirin	Aqueous solution	Quantitative	Ag Nps coated filter paper	785	Range: 0.1–1 mM $R^2 = 0.933$ Relative error: 2.06%	[112]
Captopril	Human blood	Quantitative	Citrate Ag Nps	633	Range: 2–10 μM LOD = 0.149 μM LOQ = 0.451 μM	[101]
Levofloxacin	Aqueous solution	Quantitative	Microfluidic device with hydroxylamine Ag Nps	514	Range: 1–15 μM LOD = 0.8 μM	[104]
Levofloxacin	Artificial urine	Quantitative	Microfluidic device with hydroxylamine Ag Nps	532	Range: 0.07–1 mM LOD = 0.07 mM RMSEP = 0.058 mM	[105]
Levofloxacin	Human urine	Quantitative	Microfluidic device with hydroxylamine Ag Nps	532	Range: 0.45–1.8 mM RMSEP: 0.058–0.16 mM	[105]
Mitoxantrone	Aqueous solution	Semi – quantitative	Microfluidic device with Ag Nps	532	LOD = 5.10^{-7} M	[103]
Moxifloxacin	Human urine	Quantitative	Nanostructured Au substrate (Klarite©)	785	Range: 0.70 μg/mL in 1.0% urine R = 0.9977 LOF = 0.941 LOD = 0.085 $mg\,mL^{-1}$ LOQ = 0.26 $mg\,mL^{-1}$ Relative error: 4.23%	[44]
Nitroxoline	Human urine	Quantitative	Microfluidic device with hydroxylamine Ag Nps	532	Range: 4.28–42.8 $mg\,L^{-1}$ $R^2 = 0.986$ LOD = 0.57 $mg\,L^{-1}$ LOQ = 1.23 $mg\,L^{-1}$	[45]
Paracetamol	Polymeric microfilms	Semi – quantitative by SERS imaging	Nanostructured Au substrate (Klarite©)	785	Relative error: 3.6%	[68]
Paracetamol	Aqueous solution	Detection	Chitosan Au Nps	633	Qualitative	[111]
Penicilloic acid	Aqueous solution	Quantitative	Ag coated citrate Au Nps	532	Range: $1.0.10^{-7}$–$5.0.10^{-6}$ M $R^2 = 0.9895$ LOD = $1.0.10^{-7}$ M	[99]
Piroxicam	Aqueous solution	Detection	Hydroxylamine Ag Nps	532	Qualitative	[100]
Promethazine	Aqueous solution	Semi – quantitative	Microfluidic device with Ag Nps	532	LOD = 2.10^{-4} M	[103]
Rosiglitazone	Aqueous solution	Semi – quantitative	PVP Ag Nps on thin layer chromatography	785	Range: 1–10 $mg\,mL^{-1}$ $R^2 = 0.9977$ RSD = 9.28%	[109]

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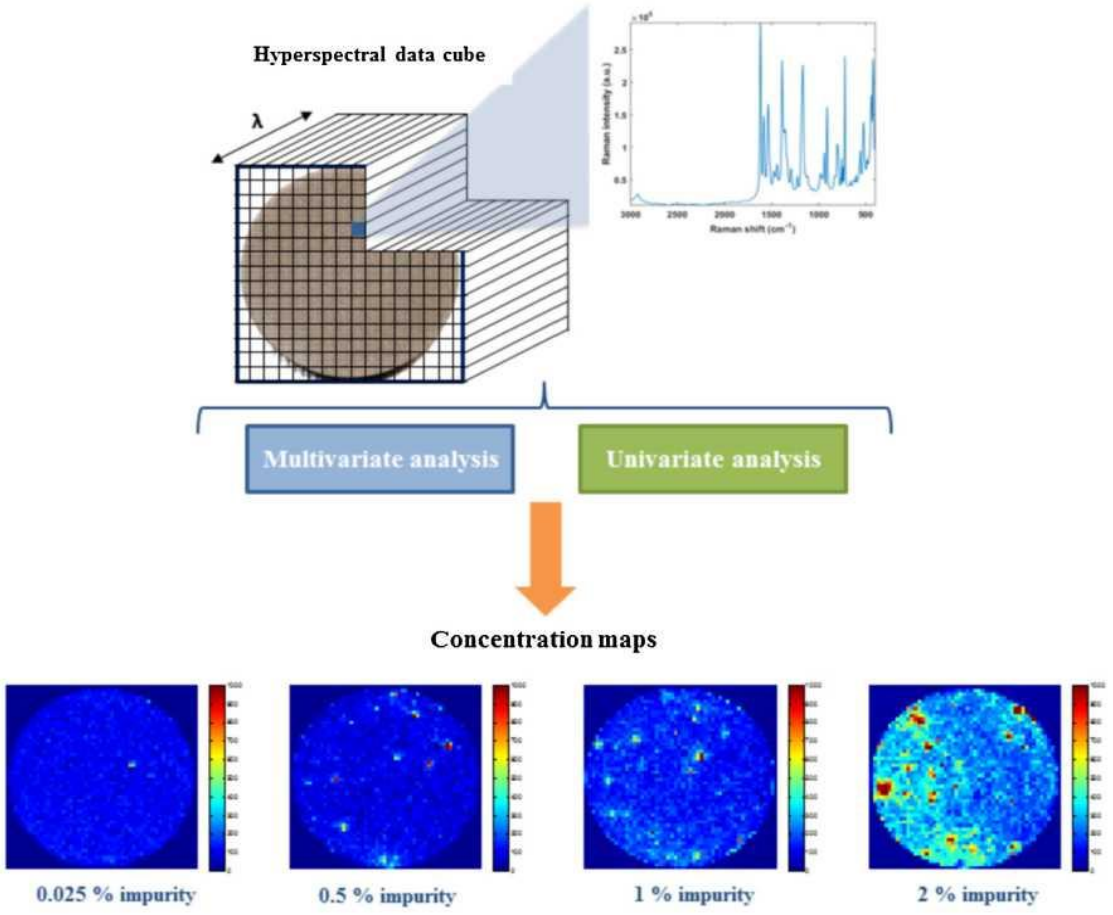
This work allowed to visualize up to 0.025% (w/w) of 4- aminophenol in tablets containing a large amount of paracetamol and demonstrated the efficiency of the technique to evaluate the distribution of a low-dose API or an impurity in a solid pharmaceutical form as illustrated in Fig. 5. One year earlier, Firkala et al. [65] proposed to combine SERS chemical imaging with multivariate analysis to evaluate the spatial distribution of acetylsalicylic acid in sample tablets produced by two manufacturing technologies. Despite the high SERS spectra variability related to the non-uniform silver colloids covering, MCR-ALS was used and the authors applied a threshold on the score vectors for binarizing the concentration maps to highlight the distribution of the API. The concentration images may be biased due to the large differences between the loadings obtained and the pure API spectrum, but the method allowed the differentiation of the two manufacturing processes contrary to conventional Raman chemical imaging which cannot distinguish significant differences between the tablets.

Fig. 5. *Detection of a low-dose compound by SERS chemical imaging in a pharmaceutical tablet.*



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This qualitative method with multivariate analysis is interesting for preliminary investigations of unknown samples (illegal or counterfeit tablets), however, no quantitative estimates of the API can be provided. In this sense, the same research group developed a quantitative approach also based on the MCR-ALS algorithm in order to determine quantitative information about the chemical distribution of the compound [66]. For this purpose, the determination of the threshold on the scores was improved and applied on tablets ranging from 0.25 to 2% of API concentrations. To ensure the reliability of the method, a linear regression model was built to determine the correlation between the concentration values and the positive SERS signal of the API on the binarized distribution maps. Therefore, the evaluation of the model was performed by statistical tests including the lack of fit and the coefficient of determination R^2 which had values in the order of 83.61 and 0.9849, respectively, demonstrating the accurate concentration estimated in comparison with the regression models of Raman imaging. The combination of SERS imaging and MCR-ALS was also discussed by Mamián-López et al. [68] in order to provide the spatial distribution of different compounds of polymeric microfilms loaded with paracetamol. In this paper, MCR-ALS was initialized by an estimation of the pure component calculated by the SIMPLISMA algorithm highlighting spectral differences among the substances. The relative error was subsequently determined with the concentrations resulting from the MCR-ALS compared to the experimental values, with results ranging from 2.85 to 12.64%, which is relatively low.

This chemometric tool was also employed outside the framework of SERS imaging by some authors as part of the SERS quantification in complex matrices. The MCR method has been successfully applied in order to isolate the signal of the target analyte despite the presence of unexpected interferences. In this sense, Mamián-López et al. [44] and Hidi et al. [45] combined the use of internal standards with the MCR-ALS algorithm to quantify low dose moxifloxacin and nitroxiline respectively in human urine. These approaches allowed to simplify the sample preparation and to perform drugs analysis at lower costs.

Conclusion

The emergence of SERS in the past few years made this technique an analytical tool increasingly used in the pharmaceutical field. Due to its high sensitivity, allowing to overcome the sensitivity limits of classical Raman spectroscopy, it offers the possibility to perform quantitative

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determinations of low-dose compounds in solid dosage forms or in biological fluids. However, despite its great potential, it is still necessary to improve the analytical performances of the technique.

Regarding pharmaceutical applications, the method development of SERS quantitative analyses is possible and involves several steps ranging from the choice of the SERS substrate to the validation of the method which is crucial for the implementation of the technique in routine analyses. However, it is premature to say that the SERS technique can be a substitute method to the classical analytical techniques currently performed in the laboratories. Indeed, many improvements remain to be made, on which researchers are working, notably in the framework of the SERS substrates synthesis, in order to obtain a repeatable and uniform SERS signal. The quantification in complex matrices can also be a challenge and a limitation to this technique despite the use of cleanup procedures for sample preparation and multivariate data analysis. Nevertheless, the papers published in the field demonstrate the progress achieved by the different research groups to develop the SERS method, particularly in combination with others techniques such as microfluidic or separative techniques, providing more selectivity while avoiding interferences due to others substances in complex matrices. The recent development of SERS chemical imaging also highlights the great potential of this technique that can be a powerful tool for pharmaceutical applications. The future challenge of this imaging technique would be to develop a standardized method allowing the formation of homogeneous SERS covering on solid pharmaceutical forms.

Finally, a last but not least important point regarding the analytical performances of SERS is the validation of the developed method which is necessary for a reliable application in routine analyses. In practice, this method validation is difficult to implement due to the stability issues of SERS. To be in compliance with the pharmaceutical guidelines, the researchers are based on quantitative validation criteria such as the LOD, the LOQ or the concentration linear ranges which are necessary for a complete validation. However, most of these criteria are not fully evaluated in the literature reported in this review focused on quantitative pharmaceutical applications.

Despite its many advantages, the implementation of SERS in routine quantitative analyses is a real challenge and leaves the door wide open for innovative researches focused on the improvement of the analytical performance of the technique.

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