Application of Surface-Enhanced Raman Chemical Imaging (SER-CI) to Quantification in Pharmaceutical Tablets

Abstract
Since its discovery, the application of Surface-Enhanced Raman Spectroscopy (SERS) has extended to various areas, including the pharmaceutical field, facing up challenges in the SERS substrate and sample preparation. This paper will present how beneficially SERS can be applied to the quantification of low-dose compounds in pharmaceutical tablets, focusing on the determination of 4-aminophenol, a toxic impurity, in acetaminophen tablets.

Introduction
Vibrational spectroscopy has become popular in the pharmaceutical sector, due to the high speed of its measurements and to its green and non-destructive nature. Among vibrational techniques, SERS has recently emerged as a potential way of circumventing some of the drawbacks of conventional Raman spectroscopy. Indeed, metallic nanostructures, called SERS substrates, are used in SERS and increase the Raman signal of molecules located nearby their surface. Consequently, the detection and quantification of trace amounts of compounds, such as low-dose Active Pharmaceutical Ingredients (APIs) or impurities, become practical with SERS. SERS can also reduce the extent of fluorescence, which would otherwise mask the conventional Raman spectrum if present in a sample. With these advantages in mind, it is easy to understand the interest shown in SERS related to the pharmaceutical industry.1,2

Just like in Raman microspectroscopy, SERS can be combined with confocal microscopy in order to simultaneously obtain spectral and spatial information about the sample. This leads to Surface-Enhanced Raman Chemical Imaging (SER-CI).2 SER-CI can be extremely helpful to visualize the spatial distribution of APIs, even at low dosages, or impurities inside medicines, while significantly reducing the image acquisition time in comparison with conventional Raman imaging. Therefore, SER-CI can be considered as a quality control tool, allowing to assess the conformity of medicines.

In this article, an application of SER-CI to determine 4-aminophenol (4-AP) in pharmaceutical tablets of acetaminophen, one of the most sold drugs worldwide, will be developed. 4-AP is the main impurity of acetaminophen and originates from the synthesis or from the degradation of the latter. Due to its nephrotoxicity and hepatotoxicity, 4-AP must systematically be sought and a 0.15% (w/w) specification limit is therefore fixed for acetaminophen tablets in the United-
States Pharmacopeia (USP). The sensitivity of conventional Raman spectroscopy, which is around 1 or 2% (w/w) depending on the target analyte, is not sufficient to detect these concentrations. Consequently, the use of SERS is mandatory. Several developments in the sample preparation will also be presented in the article, aiming at making homogeneous deposits of the SERS substrate onto the tablet surface, which is a critical step, for increasing analysis repeatability.

Material and Methods

SERS spectra were acquired on a RamanStation 400F spectrometer equipped with a two-dimensional CCD (1024 x 256 pixels sensor) cooled down to -50°C and with a 785 nm laser with power set at 100 mW. A 1 s accumulation time was used.

Suspensions of citrate-coated silver nanoparticles (AgNPs) were synthesized according to the Lee & Meisel protocol and were concentrated by a factor 10 by centrifugation.

The spectral data were then pre-treated and analyzed with MATLAB R2015a and the PLS toolbox 8.5.1.

Results and Discussion

While SER-CI can be an effective tool for imaging trace amounts of compounds, the difficulty of obtaining a homogeneous sample coating with the SERS substrate is still a major issue. The most straightforward tablet coating technique is drop casting deposition (Figure 1a). It consists of dropping a few microliters of the colloidal SERS substrate directly onto the tablet surface. The colloid consequently spreads over the surface of the tablet before being absorbed by the latter. The distribution of the SERS substrate onto the sample surface was studied by means of concentrated AgNPs functionalized with butanethiol (BT). The suspension of AgNPs had to be concentrated in order to provide a sufficient amount of AgNPs on the sample surface and therefore to produce an intense SERS signal after the deposit. The intensity of a characteristic peak of BT was tracked. It showed high intensity variation along the tablet surface (RSD 29.3%), with increased SERS signal at the edges of the tablet coating (Figure 1d). This was explained by the coffee-ring effect, a phenomenon resulting from the differential evaporation rate of the solvent at the center and at the edge of the drop, leading to a highly concentrated ring of the colloid at the edge of the dried deposit. Moreover, the area covered by the SERS substrate is poor, restricting the use of this dropping method to small samples.

In order to decrease this coffee-ring effect and therefore to increase the homogeneity of the coating, another dropping method was studied, absorption coating (Figure 1b). In this approach, some microliters of the SERS substrate are dropped on a glass slide and the tablet is put in contact with the drop for several seconds, allowing the substrate to absorb into the tablet by capillarity. While presenting lower signal intensity variations across the sample surface (RSD 17.7%), a coffee-ring trend could still be observed (Figure 1e). However, this coating method was successfully applied to the quantification of 4-AP in acetaminophen tablets from 0.025 to 0.2% (w/w). In order to minimize the problem of lack of homogeneity in the coating, the median response of 4-AP from each tablet was normalized by the signal of BT. Two series of tablets were analyzed with two batches of AgNPs. The resulting calibration curves are displayed in Figure 2 and demonstrated a good correlation (R² 0.82 and 0.85) between median normalized SERS intensities and the exact concentration of the tablets determined by high performance liquid chromatography (HPLC). It was also noticed that the AgNPs batch parameter was important to take into account since the obtained SERS intensities were different for the two batches used. Consequently, the calibration of each new batch of AgNPs should be performed before its use for quantitative analyses.
Finally, a new covering methodology called spray-coating was developed (Figure 1c). This method could prevent the concentration of the colloids at the edge of the sample and can be easily implemented. Moreover, even larger samples can be homogeneously coated. To do so, a home-made device, comprising an electrospay ionization probe with a couple of coaxial tubes, was used to spray a suspension of AgNPs onto tablets. The inner tube was linked to a syringe pump for delivery of AgNPs at a constant and controlled speed. The outer tube was connected to nitrogen gas supply. This gas nebulized the AgNPs suspension into little droplets that were driven onto the sample surface.\(^6\)

Important parameters determining the coating homogeneity and the resulting SERS signal intensity comprise the concentration of the colloid, its flow rate and the spraying time. These parameters were optimized through a design of experiments approach and the optimums (10x concentration, 10 µL/min, 5 min) gave very low SERS signal variations across the sample surface (RSD around 10%), demonstrating the absence of any coffee-ring effect with this coating approach (Figure 1f).\(^6\)

The optimized spray-coating method was then applied to the detection of the 4-AP impurity in acetaminophen tablets by SER-CI (Figure 3). The determination of 4-AP was possible from 0.2% down to 0.025% (w/w), which is significantly lower than the specification limit of 0.15% (w/w) set by the USP.\(^3\)

Since many tablets are made up of water-soluble compounds (such as lactose or mannitol), they could dissolve or become distorted when aqueous SERS substrates are applied onto their surface. This point was taken into account and the migration of soluble compounds was studied by SER-CI after the coating of tablets. The surface of the tablets coated by drop casting and absorption coating displayed cracks and holes whereas it remained intact for tablets coated by spray-coating. As a result, only spray-coating prevented the migration of water-soluble compounds in the tablets.

From these results, the spray-coating approach seems to be a very promising tool to provide homogeneous deposits of SERS substrates resulting in more repeatable SER-CI analyses. One step further, the automation of the spray-coating could be considered. This would contribute to improving the level of repeatability of SER-CI.

**Conclusion**

To conclude, next to its low sensitivity, SER-CI also benefits from decreased analysis time (by a factor ~6) in comparison with conventional Raman imaging. Indeed, a reduced acquisition time can be set in SER-CI due to the signal exaltation obtained by SERS. Besides, the SERS spectra fingerprints remain of the sample composition. As a result, the spatial resolution of the mappings can be increased while retaining sensible total acquisition time, helping low-dose compounds identification along. In contrast, the visualization of these trace compounds by conventional Raman imaging can be missed due to the absence of pure pixels related to these traces or to low Raman intensity of the latter. In this context, SER-CI combined with effective substrate coating methods is establishing itself as a high-quality tool for the quality control of trace amounts, either API or impurities, in the pharmaceutical sector. SER-CI can indeed be quickly implemented to verify the distribution homogeneity of low-dose API or the distribution of any impurities inside the tablets.

However, challenges persist in SER-CI. The repeatability of analysis could still be improved while the SERS technique remains rather limited in terms of analytes that can be studied. That is why research is currently being conducting on the development of more homogeneous SERS substrates and on automated systems which would eliminate the influence of the operator on the results, hence extending repeatability. Furthermore, the functionalization of the SERS substrates could also be considered in order to extend the range of analytes SER-CI could be applied to.

**References**