Quantification of low drug concentration in model formulations with multivariate analysis using surface enhanced Raman chemical imaging

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Abstract

This paper reports the application of surface enhanced Raman chemical imaging (SER-CI) as a non-destructive quantitative analytical method for the investigation of model pharmaceutical formulations containing the active pharmaceutical ingredient (API) in low concentrations (0.5-2 %). The application of chemometric techniques for processing the spectra enables the determination of API distribution in products of different concentrations. The drastic Raman signal enhancement in the presence of silver nanoparticles provides significantly improved calibration accuracy and, at the same time, radically decreased image acquisition time compared to conventional Raman chemical imaging.

Keywords

SERS, Raman chemical imaging, chemometrics, R-CI, SER-CI

1. Introduction

Raman chemical imaging (R-CI) and its specialized versions such as hyperspectral stimulated Raman scattering microscopy and coherent anti-Stokes Raman scattering microscopy have become important imaging techniques for quantitative analysis. [1,2,3]. However, in spite of these improvements, some typical disadvantages of Raman spectroscopy, such as low sensitivity and long image acquisition time still limit the applicability of R-CI. Taking advantage of the powerful signal enhancing behavior of metal (primarily silver and gold) substrates, surface enhanced Raman spectroscopy (SERS) can offer a solution for the aforementioned difficulties [4,5,6,7,8,9,10,11,12]. The benefits of SERS can be utilized in quantitative analytical method development as well [13,14,15], for instance, in the case of pH

sensors [16], nucleotide chemistry [17] or marine applications [18]. The combination of R-CI with SERS, called surface enhanced Raman chemical imaging (SER-CI), has also started to gain serious attention in biotechnology and nanotechnology [19,20,21]. In our previous work [22] the potential of SER-CI was demonstrated in the investigation of drug distribution in tablets, where radical decrease was reached in the acquisition time using SER-CI. Furthermore, the spatial distribution of the active pharmaceutical ingredient (API) could be revealed well below the detection limit of R-CI and thus the characteristics of different manufacturing technologies could be identified at very low API concentrations. However, the results could not provide any quantitative or even semi-quantitative information about the API content. Moreover, to the best of our knowledge, there is only one publication regarding the possible application of SER-CI for quantitative analysis of an active component in pharmaceutical products [23]. There the authors applied a univariate approach without deeper statistical analysis. Therefore, further studies were required to develop a quantitative multivariate technique, supported by detailed statistical calculations, to estimate the amount of the API using SER-CI.

Although R-CI is a widespread technique in pharmaceutical technology [24] and it can also be used to get quantitative information about pharmaceutical products, this requires extremely long acquisition times, making it hardly applicable for daily pharmaceutical practice. Moreover, in the case of low (<2 %) drug concentrations, accuracy problems can easily occur with the traditional approach (unless the API is of very strong Raman scattering character), making the quantification difficult or impossible at such low concentrations.

This study intends to offer a solution to the aforementioned problem by making SER-CI capable of providing appropriately accurate quantitative information based on spatial API distribution maps, while the drastic reduction of image acquisition time is still maintained. In

addition, statistical calculations serve for making SER-CI capable to achieve much higher accuracy and applicability than R-CI at low drug concentrations.

2. Materials and methods:

2.1. Preparation of model formulations

Lactose monohydrate (LMH) was purchased from Sigma-Aldrich., The active pharmaceutical ingredient is referred to as API instead of its original name due to IPR (industrial protection of rights) reasons. Model mixtures were prepared by blending API and LMH in a mortar to ensure homogeneous drug distribution. Model formulation samples weighing 400 mg each were prepared in a Manfredi 0057C00 type KBr disk press. API contents were 0.25 %, 0.5 %, 1 %, 1.5 % and 2 % in mass ratio.

2.2. Preparation of SERS colloid

Ag nanoparticles were prepared by Lee and Meisel's method [25], which is widely used in SERS studies to synthesize silver substrates [26,27,28,29,30]. 90 mg of silver-nitrate (Reanal Ltd.) was dissolved in 500 ml of double distilled water. The solution was heated to boil and 10 ml of 1 % trisodium-citrate (Sigma-Aldrich) aqueous solution (also made with double distilled water) was added dropwise under vigorous stirring. Boiling was continued for 10 minutes. Upon completion of the reaction, a greenish-grey colloidal solution was obtained.

2.3. Raman instrumentation

For each mapping experiment, Raman imaging spectra were collected using a Jobin Yvon Labram instrument attached to an Olympus BX-41 microscope. The samples were illuminated with frequency-doubled Nd-YAG laser (532nm). An objective of 50× magnification was used for optical imaging and spectrum acquisition. The outer surface of the model formulations was investigated in every imaging experiment without any sample preparation process.

Before SER-CI analysis, four types of R-CI reference map series were taken from each sample. The first type „background" reference series used the same imaging parameters as SER-CI analyses (see later) to ensure that no signals of the API (or the excipient) are detectable without SERS. Such "background" maps are not presented in the paper, as they only consisted of noise.

The other three types of reference (R-CI) images were obtained by setting high enough acquisition times to detect the signals of the API, in an attempt to reveal the distribution of API without SERS. Acquisition times for a spectrum were 0.8 s *(method I)*, 3 s *(method II)* and 10 s *(method III)*; and twenty such spectra were accumulated and averaged at each pixel to get proper signal-to-noise ratio. The step size between neighboring pixels was increased to 200 μm along both axes to avoid the sampling error. As a compromise between map size and overall imaging acquisition time, the measured area on the sample surfaces was 31×31 pixels and acquisition of each map took 4.2 h (*method I*), 16 h (*method II*), and 53 h (*method III*). When applying 10 s acquisition time, the confocal hole was set to 500 μm to avoid unnecessary signals from the neighboring pixels.

For SER-CI analysis, SERS colloid was dropped on top of the samples and, after drying, mapping was performed on their surface (i.e. without any further sample preparation to avoid alteration of the sample structure). In this case, spectrum acquisition time was 0.5 s per pixel

and only 1 spectrum was taken at each point without any multiple accumulations or averaging, to avoid degradation of the colloid system. For these images, step size of 50 μm was used between neighboring pixels to achieve high spatial resolution, and the investigated area was 49×49 pixels. The overall acquisition time for each SER-CI and "background" image (without SERS effect) was only 20 min.

For SER-CI analysis the laser power was decreased to 10 % of its original value with an intensity filter for the same goal (to avoid damage to colloids), while full power (~50 mW) was used for the R-CI investigations. The spectrograph was set to provide a spectral range of 400–1835 cm^{-1} and 3 cm^{-1} resolution.

2.4. Data Analysis

R-CI and SER-CI maps were processed with the same multivariate curve resolution – alternating least squares (MCR-ALS) method we applied in our previous work [22]. The developed multivariate approach using MCR-ALS enables to assess the Raman maps for any SERS-active ingredient. The method was applied for the API through similar steps in this paper as those shown for acetylsalicylic acid in our previous study [22]. As the details are thoroughly discussed in the referred paper, the description of this chemometric technique and the detailed procedure for the spectral preprocessing and the evaluation of R-CI and SER-CI images are presented in the electronic supplementary material (ESM). Further relevant publications are cited in the supporting material as references SR1-SR18, and also in the present manuscript as references 31-48.

In the course of the quantitative evaluation of R-CI and SER-CI results, the calibration point belonging to the model formulation with 0.25% API content was taken out from the quantitation process, because preliminary SER-CI investigations indicated that linear correlation primarily existed only between 0.5 % and 2 % API content.

Statistical investigations were performed on the calibration datasets to compare the linear regression models. The sum of squares due to lack-of-fit (SSLF) and residual sum of squares (RSS) values were calculated to explain the variance of the measured points. Equation 1 shows the definition of *lack-of-fit sum of squares* which is one part of the (overall) residual sum of squares (RSS). In general *x* means the independent variable and *y* is the dependent variable in simple univariate regression. SSLF summarizes the differences between local averages (average *y* value corresponding to the same *x* values) and fitted values ($\hat{y}_{ij}$) quadratically and weighted by the number of observed *y*-values for that *x*-values. In Equation 1 the number of distinct *x* values was denoted with *c*.

$$SSLF = \sum_{i=1}^{c} \sum_{j=1}^{n_i} \left(\bar{y}_i - \hat{y}_{ij}\right)^2 \quad (1)$$

The other part of RSS is the sum of squares due to pure error (SSPE). It explains how much our observed y-values differ from the local averages.

$$SSPE = \sum_{i=1}^{c} \sum_{j=1}^{n_i} \left(y_{ij} - \bar{y}_i\right)^2 \quad (2)$$

In order to decide in an objective way, whether our fitted linear model was adequate, a hypothesis test was performed using this two error values. Dividing the sums of squares with the corresponding degrees of freedom, the usual F test statistic could be determined.

$$F = \frac{\frac{SSLF}{c-2}}{\frac{SSPE}{n-c}} \quad (3)$$

Furthermore, coefficients of determination (R^2) were calculated for the whole model (Equation 4). It describes how much the fitted line explains the variance of the measurement points. The total sum of squares (TSS) shows the differences of observed points from the overall mean.

$$R^2 = 1 - \frac{RSS}{TSS} = 1 - \frac{\sum_{i=1}^{c}\sum_{j=1}^{n_i}\left(y_{ij} - \hat{y}_{ij}\right)^2}{\sum_{i=1}^{c}\sum_{j=1}^{n_i}\left(y_{ij} - \bar{y}\right)^2} \quad (4)$$

3. Results and discussion

This section shows at first the best quantitative results that can be achieved using the "ordinary", time-consuming chemical imaging set-up without applying SERS as a reference. The Raman maps were first evaluated according to the steps outlined by our previous study [22], however, the approach had to be improved to create so-called *corrected* images, which allowed better quantitation. Nevertheless, even the corrected R-CI images were found unfeasible and the subsequent results outlines how surface-enhanced Raman mapping can overcome the challenges.

3.1. R-CI investigations without SERS

The number of pixels containing API was determined in two similar ways to create distribution maps based on the time-consuming ordinary Raman measurements (53 h, 16h, 4,2h). As the first step, in both cases, the API spectrum was identified and selected from the as-received six loadings via MCR-ALS method. The scores (i.e. spectral concentrations) corresponding to the API spectrum were sorted in descending order. The *original* [22] and the *revised* approaches differ in defining the score thresholds which determine if the API is considered present or missing in a particular pixel. According to the previously published approach, the threshold was selected by iteratively checking the spectrum having the median score visually to see if it unambiguously contains the peaks of the API and then discarding the

lower 50% of the scores (‘halving method’). This approach provided a suitable threshold in a few steps and the resulting maps were called *ordinary maps.*

The *corrected* binarized Raman *maps* were obtained by selecting the following thresholds values to the three different measuring times: 5 % to methods I (4.2 h) and II (16 h) and 3 % to method III (53 h) maps. The steps of definition of thresholds can be found in ESM.

In the case of methods I and II, Table SM-1 in the ESM shows the numeric calibration results after counting the API Raman signals in all images, while Figure 1 represents the calibration diagrams. (Formulations with 0.25% API were not included in the final calibration, as discussed in Section 2.4, and are shown with a pale green color on all of the R-CI and SER-CI diagrams.) Prediction bands at 95% confidence level were calculated and visualized with Statistica software as a demonstrative visual procedure to compare the precision of the calibration model of ordinary and corrected maps. No remarkable improvement in accuracy could be made by reproducing the measurements due to the high deviation of the calibration points. The use of the corrected maps to build a more accurate calibration process resulted in a decrease in deviation and much better linear correlation between the number of Raman signals and the real API concentration. However, a new problem appeared: the slope of the calibration lines for corrected maps decreased resulting in a decrease in sensitivity and ruling out accurate quantitation using R-CI. None of the prediction bands in Figure 1 allow any quantitative analysis, due to the too high deviation in the rate of API-positive pixels among replicate images.

In the case of method III, only one image was collected from each sample due to the extremely long (53 h) acquisition time per image. Prediction bands were not calculated and Table SM-2 and Figure SM-7 show the calibration results. Therefore, instead of a prediction band around the calibration lines, the calibration models and the SER-CI results were compared based on the residual sums of squares and lack of fit (Table 1). The models based

on the corrected maps had definitely more appropriate calibration lines; however, they were still far from providing appropriate precision.

3.2. SER-CI investigations,

In SER-CI experiments two series of samples were investigated with 0.25; 0.5; 1; 1.5 and 2 % API concentrations. Each sample was imaged three times with the same parameters, thus six measured values per concentration point were used to build a linear calibration model as precise as possible. Figure 2 and Table SM-3 (supporting material) show that prediction bands were applied to compare the results with conventional Raman imaging process of methods I and II, and lack of fit (SSLF) calculations were utilized for the correlation of method III (see details later). It is unquestionable that SER-CI investigations resulted in considerably more accurate calibration than R-CI measurements. The prediction band is narrow enough to claim that this technique is definitely capable of providing quantitative information about the API concentration with good accuracy. According to our calculations and measurements, the quantitative correlation was the best in the interval between 0.5 % and 2 %. Therefore, the first calibration point belonging to the formulation containing 0.25 % of API was discarded. After SER-CI investigation and image processing, further correction (such as those performed for R-CI maps) was not needed.

Figure 3 shows the visual comparison of a representative SER-CI image sequence compared to the corresponding method III (and corrected) R-CI image. The images prove that in contrast to even the most accurate conventional R-CI sequence, the difference between varying API concentrations can be very easily recognized visually based on the SER-CI maps – in other words, the sensitivity of the SER-CI investigations is better.

In addition to the fact that SER-CI calibration has much better precision compared to the conventional Raman-CI calibration, there are other advantages as well. Namely, the SER-CI method is able to achieve this accuracy with an outstanding decrease in mapping acquisition time, even though the same instrumental set-up is used as for ordinary R-CI studies. While each R-CI measurement took a minimum of 4.2 hours, the overall acquisition time for a SER-CI map – even with a higher number of pixels – took only 20 minutes. The acquisition of multiple SER-CI maps is still significantly less time-consuming than conventional Raman maps, while delivering more information. In addition, targeted reproducibility studies (such as the ones performed for R-CI in this study) are often impossible in real-life forensic investigations, where only one or very few samples are available for investigation and there is also little time for the studies.

Although these results with SER-CI are already convincing, we anticipate that the performance of SER-CI can be improved further. For instance, preparation of a more monodisperse silver colloid is recommended to get more uniform SERS spectra of an API. Furthermore, additional studies are suggested to explore if multiple SERS active components can be distinguished based on their resolved loadings, in order to plot their surface coverage on the visualized images separately.

3.3. Statistical analysis of calibration data

Ordinary least squares (OLS) regression was used to describe the quantitative correlation between the concentration of the API and its detected positive signals on the binarized distribution maps. Linear regression models were fitted to the points calculated with the ordinary and corrected chemometric procedure and plotted in Figure 1, 2 and SM-7. The lines showed different sensitivity in the four measurement conditions. Two main statistical

indicators were calculated for the characterization of these regression models: lack of fit (SSLF) and the F test statistic (as defined by Equation 1) with its p values (significant above 0.05).

One of the simplest comparisons can be carried out by calculating the values of sum of squares according to SSLF calculations. Obviously, a lower SSLF value means the marked point is located close to the regression line, indicating more accurate concentration estimation. When several repeated measurements are performed at the different concentration levels, the accuracy of quantification is characterized the best by the prediction band (plotted with dashed line on Figures 1 and 2) of the fitted regression line. The confidence limit was set to 95% in each case. The width of the prediction intervals for each R-CI and SER-CI calibration model clearly demonstrates how precisely the concentration of API can be estimated. As Figures 1 and 2 demonstrate, the differences between the methods are remarkable, proving that Raman chemical imaging can be successfully enhanced by combining it with the SERS approach.

The lack-of-fit test results on the spectral datasets are summarized in Table 1. Three calculated values, i.e. residual sum of squares (RSS), the sum of squares of lack-of-fit (SSLF) and p values are shown for the linear regression of SERS data and for the conventional Raman data obtained by the three different methods. In the case of SERS data, the SSLF is 83.61, which is the best value compared to the regression models of original R-CI maps. The adequacy of linear regression line was indicated with p values at a significant level in every cases. The p values had to be higher than 0.05 to confirm the acceptable linear fitting. If p-values were lower than the significance threshold, it would serve evidence for the lack of linear fit according to the hypothesis testing. Although the regression with corrected maps have lower SSLF, the coefficients of determination for whole model (R^2) are still

unacceptable for accurate API concentration estimation by R-CI. The worst parameters were provided by method II maps in all respects.

4. Conclusions

Application of surface enhanced Raman chemical imaging (SER-CI) method for obtaining quantitative information about the distribution of the active pharmaceutical ingredient (API) in model pharmaceutical formulations was attempted the first time in the present work. For this purpose samples containing API and lactose monohydrate excipient were produced with a dry (direct) compression method. The concentrations of API in the samples were 0.25 %, 0.5 %, 1 %, 1.5 % and 2 %.

While the conventional way of Raman mapping (without SERS) did not show any quantitative difference among the investigated formulations of different API content, the SER-CI method combined with appropriate data analysis approach was found to be well suitable for estimating the local API concentrations. Thus it is proven that combining SERS with chemical imaging enables the quantitation of an active ingredient by revealing its spatial distribution in pharmaceutical samples. Moreover, by enhancing API signals via SERS, a drastic reduction was achieved in the image acquisition time. This, of course, is only possible if the active ingredient is SERS active. Evaluation of surface enhanced Raman chemical maps pose a serious challenge, due to the high variability of spectra arising from the dependence of the SERS signals from size and shape of colloid nanoparticles. Therefore, we applied here a quantitative analytical approach, which is an improved version of the procedure elaborated in our previous work [22] utilizing asymmetric least squares preprocessing with appropriate parameters, followed by MCR-ALS data decomposition to find all the various SERS positive (in this case, API-related) loadings. The multivariate data processing method provided the

missing tool for the rapid characterization of solid pharmaceutical products with trace amounts of drugs by the combined application of SERS, Raman chemical imaging and chemometric data preprocessing. This approach can serve as a potential way for quantitative real time analysis of tablets with one or two SERS-active API content at low concentration or even impurities in solid pharmaceuticals.

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