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## 6 **Quantification of low drug concentration in model** 7 **formulations with multivariate analysis using** 8 **surface enhanced Raman chemical imaging** 9

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21

22 **Abstract**

23

24 This paper reports the application of surface enhanced Raman chemical imaging (SER-CI) as  
25 a non-destructive quantitative analytical method for the investigation of model pharmaceutical  
26 formulations containing the active pharmaceutical ingredient (API) in low concentrations  
27 (0.5-2 %). The application of chemometric techniques for processing the spectra enables the  
28 determination of API distribution in products of different concentrations. The drastic Raman  
29 signal enhancement in the presence of silver nanoparticles provides significantly improved  
30 calibration accuracy and, at the same time, radically decreased image acquisition time  
31 compared to conventional Raman chemical imaging.

32

### 33 **Keywords**

34

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

36

### 37 **1. Introduction**

38

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

48 sensors [16], nucleotide chemistry [17] or marine applications [18]. The combination of R-CI  
49 with SERS, called surface enhanced Raman chemical imaging (SER-CI), has also started to  
50 gain serious attention in biotechnology and nanotechnology [19,20,21]. In our previous work  
51 [22] the potential of SER-CI was demonstrated in the investigation of drug distribution in  
52 tablets, where radical decrease was reached in the acquisition time using SER-CI.  
53 Furthermore, the spatial distribution of the active pharmaceutical ingredient (API) could be  
54 revealed well below the detection limit of R-CI and thus the characteristics of different  
55 manufacturing technologies could be identified at very low API concentrations. However, the  
56 results could not provide any quantitative or even semi-quantitative information about the API  
57 content. Moreover, to the best of our knowledge, there is only one publication regarding the  
58 possible application of SER-CI for quantitative analysis of an active component in  
59 pharmaceutical products [23]. There the authors applied a univariate approach without deeper  
60 statistical analysis. Therefore, further studies were required to develop a quantitative  
61 multivariate technique, supported by detailed statistical calculations, to estimate the amount of  
62 the API using SER-CI.

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

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

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

74

75 **2. Materials and methods:**

76

77 2.1. Preparation of model formulations

78

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

85

86 2.2. Preparation of SERS colloid

87

88 Ag nanoparticles were prepared by Lee and Meisel's method [25], which is widely used in  
89 SERS studies to synthesize silver substrates [26,27,28,29,30]. 90 mg of silver-nitrate (Reanal  
90 Ltd.) was dissolved in 500 ml of double distilled water. The solution was heated to boil and  
91 10 ml of 1 % trisodium-citrate (Sigma-Aldrich) aqueous solution (also made with double  
92 distilled water) was added dropwise under vigorous stirring. Boiling was continued for 10  
93 minutes. Upon completion of the reaction, a greenish-grey colloidal solution was obtained.

94

### 95 2.3. Raman instrumentation

96

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

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

107 The other three types of reference (R-CI) images were obtained by setting high enough  
108 acquisition times to detect the signals of the API, in an attempt to reveal the distribution of  
109 API without SERS. Acquisition times for a spectrum were 0.8 s (*method I*), 3 s (*method II*)  
110 and 10 s (*method III*); and twenty such spectra were accumulated and averaged at each pixel  
111 to get proper signal-to-noise ratio. The step size between neighboring pixels was increased to  
112 200 μm along both axes to avoid the sampling error. As a compromise between map size and  
113 overall imaging acquisition time, the measured area on the sample surfaces was 31×31 pixels  
114 and acquisition of each map took 4.2 h (*method I*), 16 h (*method II*), and 53 h (*method III*).  
115 When applying 10 s acquisition time, the confocal hole was set to 500 μm to avoid  
116 unnecessary signals from the neighboring pixels.

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

120 and only 1 spectrum was taken at each point without any multiple accumulations or  
121 averaging, to avoid degradation of the colloid system. For these images, step size of 50  $\mu\text{m}$   
122 was used between neighboring pixels to achieve high spatial resolution, and the investigated  
123 area was  $49 \times 49$  pixels. The overall acquisition time for each SER-CI and “background”  
124 image (without SERS effect) was only 20 min.

125 For SER-CI analysis the laser power was decreased to 10 % of its original value with an  
126 intensity filter for the same goal (to avoid damage to colloids), while full power ( $\sim 50$  mW)  
127 was used for the R-CI investigations. The spectrograph was set to provide a spectral range of  
128  $400\text{--}1835$   $\text{cm}^{-1}$  and  $3$   $\text{cm}^{-1}$  resolution.

129

#### 130 2.4. Data Analysis

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

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

145 Statistical investigations were performed on the calibration datasets to compare the linear  
 146 regression models. The sum of squares due to lack-of-fit (SSLF) and residual sum of squares  
 147 (RSS) values were calculated to explain the variance of the measured points. Equation 1  
 148 shows the definition of *lack-of-fit sum of squares* which is one part of the (overall) residual  
 149 sum of squares (RSS). In general  $x$  means the independent variable and  $y$  is the dependent  
 150 variable in simple univariate regression. SSLF summarizes the differences between local  
 151 averages (average  $y$  value corresponding to the same  $x$  values) and fitted values ( $\hat{y}_{ij}$ )  
 152 quadratically and weighted by the number of observed  $y$ -values for that  $x$ -values. In Equation  
 153 1 the number of distinct  $x$  values was denoted with  $c$ .

$$154 \quad SSLF = \sum_{i=1}^c \sum_{j=1}^{n_i} (\bar{y}_i - \hat{y}_{ij})^2 \quad (1)$$

155

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

$$158 \quad SSPE = \sum_{i=1}^c \sum_{j=1}^{n_i} (y_{ij} - \bar{y}_i)^2 \quad (2)$$

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

$$162 \quad F = \frac{\frac{SSLF}{c-2}}{\frac{SSPE}{n-c}} \quad (3)$$

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



167 
$$R^2 = 1 - \frac{RSS}{TSS} = 1 - \frac{\sum_{i=1}^c \sum_{j=1}^{n_i} (y_{ij} - \hat{y}_{ij})^2}{\sum_{i=1}^c \sum_{j=1}^{n_i} (y_{ij} - \bar{y})^2} \quad (4)$$

168

### 169 **3. Results and discussion**

170

171 This section shows at first the best quantitative results that can be achieved using the  
172 “ordinary”, time-consuming chemical imaging set-up without applying SERS as a reference.

173 The Raman maps were first evaluated according to the steps outlined by our previous study  
174 [22], however, the approach had to be improved to create so-called *corrected* images, which  
175 allowed better quantitation. Nevertheless, even the corrected R-CI images were found  
176 unfeasible and the subsequent results outlines how surface-enhanced Raman mapping can  
177 overcome the challenges.

178

#### 179 **3.1. R-CI investigations without SERS**

180

181 The number of pixels containing API was determined in two similar ways to create  
182 distribution maps based on the time-consuming ordinary Raman measurements (53 h, 16h,  
183 4,2h). As the first step, in both cases, the API spectrum was identified and selected from the  
184 as-received six loadings via MCR-ALS method. The scores (i.e. spectral concentrations)  
185 corresponding to the API spectrum were sorted in descending order. The *original* [22] and the  
186 *revised* approaches differ in defining the score thresholds which determine if the API is  
187 considered present or missing in a particular pixel. According to the previously published  
188 approach, the threshold was selected by iteratively checking the spectrum having the median  
189 score visually to see if it unambiguously contains the peaks of the API and then discarding the

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

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

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

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

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

217

218 3.2. SER-CI investigations,

219

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

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

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

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

256

### 257 3.3. Statistical analysis of calibration data

258

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

264 indicators were calculated for the characterization of these regression models: lack of fit  
265 (SSLF) and the F test statistic (as defined by Equation 1) with its p values (significant above  
266 0.05).

267 One of the simplest comparisons can be carried out by calculating the values of sum of  
268 squares according to SSLF calculations. Obviously, a lower SSLF value means the marked  
269 point is located close to the regression line, indicating more accurate concentration estimation.  
270 When several repeated measurements are performed at the different concentration levels, the  
271 accuracy of quantification is characterized the best by the prediction band (plotted with  
272 dashed line on Figures 1 and 2) of the fitted regression line. The confidence limit was set to  
273 95% in each case. The width of the prediction intervals for each R-CI and SER-CI calibration  
274 model clearly demonstrates how precisely the concentration of API can be estimated. As  
275 Figures 1 and 2 demonstrate, the differences between the methods are remarkable, proving  
276 that Raman chemical imaging can be successfully enhanced by combining it with the SERS  
277 approach.

278 The lack-of-fit test results on the spectral datasets are summarized in Table 1. Three  
279 calculated values, i.e. residual sum of squares (RSS), the sum of squares of lack-of-fit (SSLF)  
280 and p values are shown for the linear regression of SERS data and for the conventional  
281 Raman data obtained by the three different methods. In the case of SERS data, the SSLF is  
282 83.61, which is the best value compared to the regression models of original R-CI maps. The  
283 adequacy of linear regression line was indicated with p values at a significant level in every  
284 cases. The p values had to be higher than 0.05 to confirm the acceptable linear fitting. If p-  
285 values were lower than the significance threshold, it would serve evidence for the lack of  
286 linear fit according to the hypothesis testing. Although the regression with corrected maps  
287 have lower SSLF, the coefficients of determination for whole model ( $R^2$ ) are still

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

290

#### 291 **4. Conclusions**

292

293 Application of surface enhanced Raman chemical imaging (SER-CI) method for obtaining  
294 quantitative information about the distribution of the active pharmaceutical ingredient (API)  
295 in model pharmaceutical formulations was attempted the first time in the present work. For  
296 this purpose samples containing API and lactose monohydrate excipient were produced with a  
297 dry (direct) compression method. The concentrations of API in the samples were 0.25 %,  
298 0.5 %, 1 %, 1.5 % and 2 %.

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

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

318

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320

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