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2 agreement between Elsevier and MTA. The definitive version of the text was subsequently
3 published in [Journal of Pharmaceutical and Biomedical Analysis 107 (2015) 318–324,
4 <http://dx.doi.org/10.1016/j.jpba.2014.12.039>]. Available under license CC-BY-NC-ND.
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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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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 μm
122 was used between neighboring pixels to achieve high spatial resolution, and the investigated
123 area was 49×49 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 (~ 50 mW)
127 was used for the R-CI investigations. The spectrograph was set to provide a spectral range of
128 $400\text{--}1835$ cm^{-1} and 3 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

319 **Acknowledgments**

320

321 T. Firkala acknowledges a PhD Scholarship of Richter Gedeon Plc (Hungary). The authors
322 express their thanks for OTKA-PD-109129 and OTKA-PD-108975 grants, while I.M.
323 Szilágyi is thankful for a János Bolyai Research Fellowship of the Hungarian Academy of
324 Sciences.

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326 **References**

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- [1] H. A. Rinia, K. N. J. Burger, M. Bonn, M. Müller, Quantitative Label-Free Imaging of Lipid Composition and Packing of Individual Cellular Lipid Droplets Using Multiplex CARS Microscopy, *Biophysical Journal* 95 (2008) 4908-4914.
- [2] D. Zhang, P. Wang, MN. Slipchenko, D. Ben-Amotz, A. Weiner, Quantitative Vibrational Imaging by Hyperspectral Stimulated Raman Scattering Microscopy and Multivariate Curve Resolution Analysis, *Anal. Chem.* 85 (2013) 98-106.
- [3] A. S. Zidan, Z. Rahman, V. Sayeed, A. Raw, L. Yu, M. A. Khan, Crystallinity evaluation of tacrolimus solid dispersions by chemometric analysis, *Int. J. of Pharm.* 423 (2012) 341-350.
- [4] M. H. Harpster, H Zhang, A. K. Sankara-Warrier, B. H. Ray, T. R. Ward, J. P. Kollmar, K. T. Carron, J. O. Mecham, R. C. Corcoran, W. C. Wilson, P. A. Johnson, SERS detection of indirect viral

-
- DNA capture using colloidal gold and methylene blue as a Raman label, *Biosens. and Bioelectron.* 25 (2009) 674-681.
- [5] I-Hsien Chou, M. Benford, H.T. Beier, G.L. Cote, Nanofluidic biosensing for amyloid detection using surface enhanced Raman spectroscopy, *Nano Lett.* 8 (2008) 1729-1735.
- [6] K.C. Bantz, A.F. Meyer, N.J. Wittenberg, H. Im, O. Kurtulus, S.H. Lee, N.C. Lindquist, S. Oh, C.L. Haynes, Recent progress in SERS biosensing, *Phys. Chem. Chem. Phys.* 13 (2011) 11551–11567.
- [7] P.G. Etchegoin, E.C. Le Ru, A perspective on single molecule SERS: current status and future challenges, *Phys. Chem. Chem. Phys.* 10 (2008) 6079–6089.
- [8] J. Jiang, K. Bosnick, M. Maillard, L. Brus, Single molecule Raman spectroscopy at the junctions of large Ag nanocrystals, *J. Phys. Chem. B* 107 (2003) 9964–9972.
- [9] B. Vlckova, I. Pavel, M. Sladkova, K. Siskova, M. Slouf, Single molecule SERS: Perspectives of analytical applications, *J. Mol. Struct.* 834 (2007) 42-47.
- [10] P. L. Stiles., J. A. Dieringer, N. C. Shah, R. P. Van Duyne, Surface-Enhanced Raman Spectroscopy, *Annu. Rev. Anal. Chem.* 1 (2008) 601-626.
- [11] I. Nothinger, Raman Spectroscopy Cell-based Biosensors, *Sensors* 7 (2007) 1343-1358
- [12] B. Sharma, R. R. Frontiera, A. Aenry, E. Ringe, R. P. Van Duyne, SERS: Materials, applications, and the future, *Mater. Today* 15 (2012) 16-25.
- [13] O. Péron, E. Rinnert, T. Toury, M. L. Chapelle, Compère, Quantitative SERS sensors for environmental analysis of naphthalene, *Analyst* 136 (2011) 1018-1022.
- [14] W. Cheung, I. T. Shadi, Y. Xu, and R. Goodacre, Quantitative analysis of the banned food dye sudan-1 using surface enhanced Raman scattering with multivariate chemometrics, *J. Phys. Chem.* 114 (2010) 7285-7290.
- [15] K. B. Biggs, J. P. Camden, J. N. Anker, R. P. Van Duyne, Surface-enhanced Raman spectroscopy of benzenethiol adsorbed from the gas phase onto silver film over nanosphere surfaces: determination of the sticking probability and detection limit time, *J. Phys. Chem. A* 113 (2009) 4581-4586.
- [16] L. Zhao, Y. Shingaya, H. Tomimoto, Q. Huang, T. Nakayama, Functionalized carbon nanotubes for pH sensors based on SERS, *J. Mater. Chem.* 18 (2008) 4759-4761.

-
- [17] P. Yin, L. Jiang, X. Lang, L. Guo, S. Yang, Quantitative analysis of mononucleotides by isotopic labeling surface-enhanced Raman scattering spectroscopy, *Biosens. and Bioelectron.* 26 (2011) 4828-4831.
- [18] P. Olivier, R. Emmanuel, C. Florent, L. Michel, C. Chantal, First Steps of in Situ Surface-Enhanced Raman Scattering During Shipboard Experiments, *Appl. Spectrosc.* 64 (2010) 1086-1093.
- [19] H. Park, S. Lee, L. Chen, E.K. Lee, S.Y. Shin, Y.H. Lee, S.W. Son, C.H. Oh, J.M. Song, S.H. Kang, J. Choo, SERS imaging of HER2-overexpressed MCF7 cells using antibody-conjugated goldnanorods, *Phys. Chem. Chem. Phys.* 11 (2009) 7444-7449.
- [20] J. Guichetau, S. Christesen, D. Emge, A. Tripathi, Bacterial mixture identification using Raman and surface-enhanced Raman chemical imaging, *J. Raman Spectrosc.* 41 (2010) 1632–1637.
- [21] M. Lee, S. Lee, J. Lee, H. Lim, G. Seong, E.K. Lee, S. Chang, C.H. Oh, J. Choo, Highly reproducible immunoassay of cancer markers on a gold-patterned microarray chip using surface-enhanced Raman scattering imaging, *Biosens. Bioelectron.* 26 (2011) 2135–2141
- [22] T. Firkala, A. Farkas, B. Vajna, I. Farkas, G. Marosi, Investigation of drug distribution in tablets using surface enhanced Raman chemical imaging, *J. Pharm. Biomed. Anal.* 76 (2013) 145-151.
- [23] C. D. Bleye, P. Y. Sacré, E. Dumont, L. Netchacovitch, P.-F. Chaveza, G. Piel, P. Lebrun, Ph. Hubert, E. Ziemons, Development of a quantitative approach using surface-enhanced Raman chemical imaging: First step for the determination of an impurity in a pharmaceutical model, *J. Pharm. Biomed. Anal.* 90 (2014) 111– 118.
- [24] S. Sasic, *Pharmaceutical Applications of Raman spectroscopy*, 1st edition, Wiley, New Jersey, 2008.
- [25] P.C. Lee, D. Meisel, Adsorption and surface-enhanced Raman of dyes on silver and gold sols, *J. Phys. Chem.* 86 (1982) 3391–3395.
- [26] L. Ding, Y. Fang, An investigation of the surface-enhanced Raman scattering (SERS) effect from laser irradiation of Ag nanoparticles prepared by trisodium citrate reduction method, *Appl. Surf. Sci.* 253 (2007) 4450–4455.

-
- [27] S.E.J. Bell, N.M.S. Sirimuthu, Surface-enhanced Raman spectroscopy as a probe of competitive binding by anions to citrate-reduced silver colloids, *J. Phys. Chem. A* 109 (2005) 7405–7410.
- [28] L. Rivas, S. Sanchez-Cortes, J.V. Garcia-Ramos, G. Morcillo, Growth of silver colloidal particles obtained by citrate reduction to increase the Raman enhancement factor, *Langmuir* 17 (2001) 574–577.
- [29] C.H. Munro, W.E. Smith, M. Garner, J. Clarkson, P.C. White, Characterization of the surface of a citrate-reduced colloid optimized for use as a substrate for surface-enhanced resonance Raman scattering, *Langmuir* 11 (1995) 3712–3720.
- [30] W. Ke, D. Zhou, J. Wu, K. Ji, Surface-enhanced Raman spectra of calf thymus DNA adsorbed on concentrated silver colloid, *Appl. Spectrosc.* 59 (2005) 418–423.
- [31] L. Duponchel, W. Elmi-Rayaleh, C. Ruckebusch, J.P. Huvenne, Multivariate curve resolution methods in imaging spectroscopy: influence of extraction methods and instrumental perturbations, *J. Chem. Inf. Comput. Sci.* 43 (2003) 2057-2067.
- [32] C. Gendrin, Y. Roggo, C. Collet, Self-modelling curve resolution of near infrared imaging data, *J. Near Infrared Spectrosc.* 16 (2008) 151-157.
- [33] L. Zhang, M.J. Henson, S.S. Sekulic, Multivariate data analysis for Raman imaging of a model pharmaceutical tablet, *Anal. Chim. Acta* 545 (2005) 262–278.
- [34] B. Vajna, G. Patyi, Zs Nagy, A. Farkas, Gy. Marosi, Comparison of chemometric methods in the analysis of pharmaceuticals with hyperspectral Raman imaging, *J. Raman Spectrosc.* 42 (2011) 1977–1986.
- [35] B. Vajna, A. Farkas, H. Pataki, Zs Zsigmond, T. Igricz, Gy. Marosi, Testing the performance of pure spectrum resolution from Raman hyperspectral images of differently manufactured pharmaceutical tablets, *Anal. Chim. Acta* 712 (2012) 45–55.
- [36] P. Eilers, A perfect smoother, *Anal. Chem.* 75 (2003) 3631–3636.
- [37] C. Gendrin, Y. Roggo, C. Collet, Pharmaceutical applications of vibrational chemical imaging and chemometrics: a review, *J. Pharm. Biomed. Anal.* 48 (2008) 533–553.

-
- [38] J. Jaumot, R. Gargallo, A. de Juan, R. Tauler, A graphical user-friendly interface for MCR-ALS: a new tool for multivariate curve resolution in MATLAB, *Chemom. Intell. Lab. Syst.* 76 (2005) 101–110.
- [39] R. Tauler, Multivariate curve resolution applied to second order data, *Chemom. Intell. Lab. Syst.* 30 (1995) 133–146.
- [40] A.A. Gowen, C.P. O'Donnell, P.J. Cullen, S.E.J. Bell, Recent applications of chemical imaging to pharmaceutical process monitoring and quality control, *Eur. J. Pharm. Biopharm.* 69 (2008) 10–22.
- [41] J.M. Amigo, Practical issues of hyperspectral imaging analysis of solid dosage forms, *Anal. Bioanal. Chem.* 398 (2010) 93–109.
- [42] B.M. Wise, N.B. Gallagher, *Chemometrics Tutorial*, Eigenvector Research Inc. Seattle, 2006.
- Original [43], [44], [45] were deleted
- [43] M. Rycenga, C.M. Cobley, J. Zeng, W. Li, C.H. Moran, Q. Zhang, D. Qin, Y. Xia, Controlling the synthesis and assembly of silver nanostructures for plasmonic applications, *Chem. Rev.* 111 (2011) 3669–3712.
- [44] R.A. Alvarez-Puebla, R.F. Aroca, Synthesis of silver nanoparticles with controllable surface charge and their application to surface-enhanced Raman scattering, *Anal. Chem.* 81 (2009) 2280–2285.
- [45] J.J. Mock, M. Barbic, D.R. Smith, D.A. Schultz, S. Schultz, Shape effects in plasmon resonance of individual colloidal silver nanoparticles, *J. Chem. Phys.* 116 (2002) 6755–6759.
- [46] P.H.C. Eilers, Parametric time warping, *Anal. Chem.* 76 (2004) 404–411.
- [47] E.T. Whittaker, On a new method of graduation, *Proc. Edinburgh Math. Soc.* 41 (1923) 63–75.
- [48] A. Savitzky, M.J.E. Golay, Smoothing and differentiation of data by simplified least squares procedures, *Anal. Chem.* 36 (1964) 1627–1639.