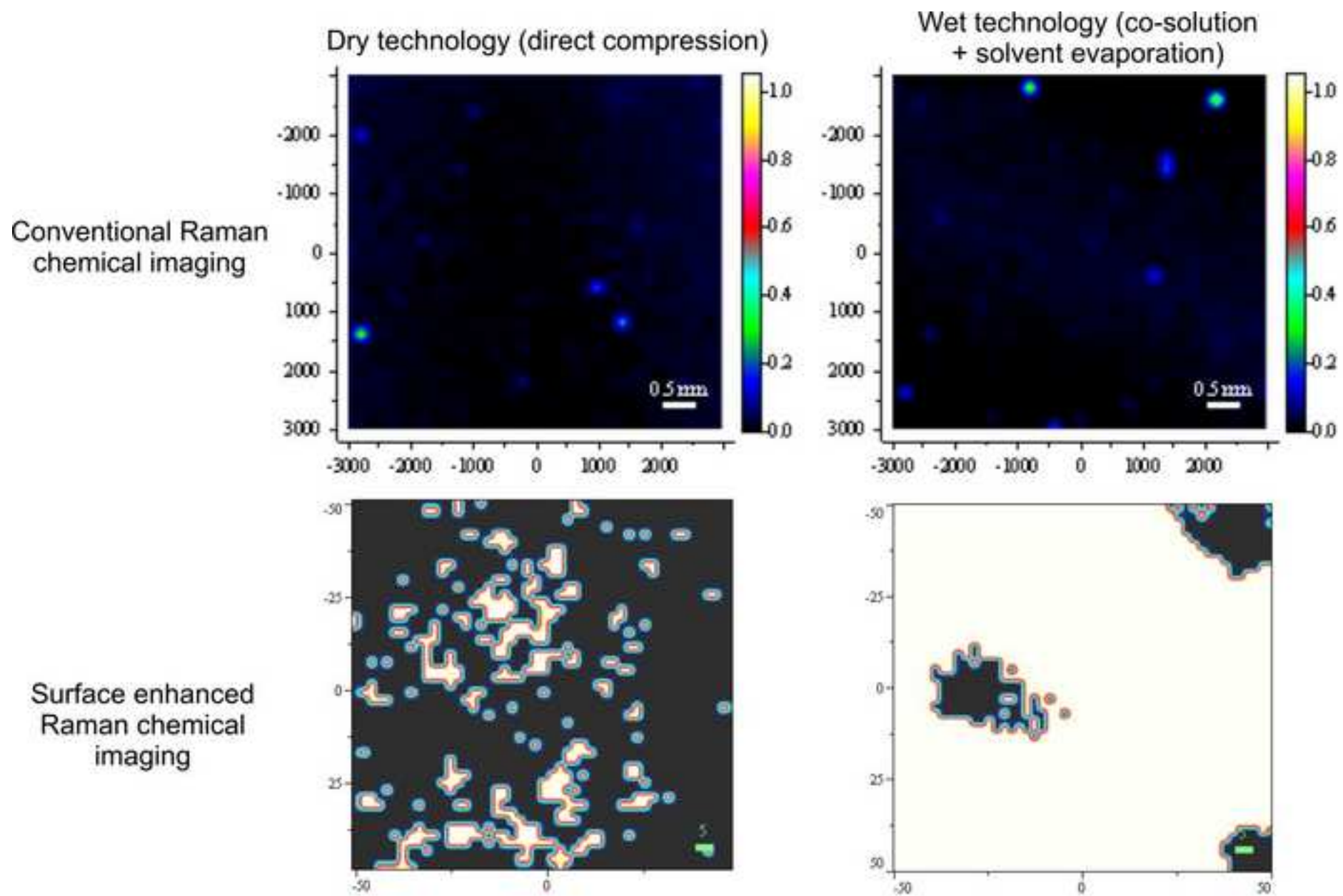


Investigation of drug distribution in tablets using surface enhanced Raman chemical imaging

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Highlights

1. Raman chemical imaging was enhanced by applying SERS colloid on the samples.
2. Distribution of trace amount of API was revealed below Raman limit of detection.
3. Asymmetric least squares, a new method was used for baseline correction.
4. MCR-ALS is required to resolve and identify SERS spectra and to create images.
5. **Proposed method intended for comparison of** unknown samples with trace amount of drug.

1 **Investigation of drug distribution in tablets using surface**
2 **enhanced Raman chemical imaging**

3

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17

18 **Abstract**

19

20 This paper reports the first application of surface enhanced Raman chemical imaging on
21 pharmaceutical tablets containing the active ingredient (API) in very low concentrations.

22 Taking advantage of the extremely intensive Raman signals in the presence of silver colloids,
23 image acquisition time was radically decreased. Moreover, the investigation of drug
24 distribution below the detection limit of **regular micro-Raman spectrometry** was made

25 feasible. The characteristics of different manufacturing technologies could be revealed at very
26 low API concentrations by using chemometric methods for processing and evaluating the
27 large number of varying spectra provided with this imaging method.

28

29 **1. Introduction**

30

31 Surface-enhanced Raman spectroscopy (SERS), based on the phenomenon known as
32 surface enhanced Raman scattering, has been gaining particular attention in pharmaceutical
33 research in the recent years [1-3]. Using this technique, the presence of certain compounds
34 (having appropriate molecular structure) can be detected at extremely low concentrations
35 [4,5] and this feature makes Raman spectroscopy capable of analyzing trace amounts of such
36 analytes. Therefore the technique has become very important in pharmaceutical and biological
37 analysis, while applications in other fields are continuously expanding as well [6,7].

38 In spite of the rapid expansion of the application of SERS technique, its combination
39 with Raman chemical imaging (R-CI) has just come into focus in the recent years [8-13]. This
40 combination results in a special imaging method which is based on the selective detection of
41 SERS active points of the scanned surface. Surface enhanced Raman chemical imaging (SER-
42 CI) has been successfully applied in certain territories of biomedical science, such as cell
43 investigations [10] or bacterial mixture identification [11]. SER-CI has also been combined
44 with atomic force microscopy resulting in a promising imaging technique called tip enhanced
45 Raman spectroscopy [12] which has been shown to have great potential in semiconductor
46 technologies [13].

47 The fight against illegal and counterfeit products is a continuously growing issue of
48 high importance nowadays on the pharmaceutical and black markets [14-16]. Work has been
49 started recently to find the advantages of SERS in the investigation of pharmaceutical and

50 illegal tablets [17]. The potential of its combination with chemical imaging of tablets (their
51 outer or broken surface), however, has not yet been explored in either the field of
52 pharmaceutical technology, or in forensic applications (to the authors best knowledge).

53 While the non-imaging SERS technique focuses only on the *detection* of an active
54 ingredient (or a similar compound such as an illegal drug), one of the most promising
55 application of SER-CI is the spatial mapping of drug distribution in the investigated tablets.
56 One of the main goals of R-CI in pharmaceutical technology is to understand the
57 characteristics of drug dissolution and other physicochemical features by determining the
58 distribution of constituents within a tablet. In the course of forensic identification of illegal
59 tablets, the same analysis can lead to the determination of the manufacturing technology or
60 the comparison of multiple tablets to see if they had been manufactured with the same
61 technology (i.e. possibly at the same location or lab). In the latter case, however, two
62 problems arise: one is that the components of an illegal tablet are usually unknown; the other
63 is that drugs are often present in very low overall concentrations. Although it can be possible
64 to detect very low concentrations (0,025-0,1% w/w) with normal RCI as well [18,19], this is
65 only possible if the drug, although globally present in very low concentrations, **can be locally**
66 **detected in the form of distinct particles**. Even in such a case the other main problem is the
67 unavoidably high overall image acquisition time due to the high number of required
68 measurement points (pixels) and the high acquisition time to reach appropriate signal-to-noise
69 ratio. Where the trace drug is distributed homogeneously, conventional R-CI does not offer
70 any advantages to the bulk analysis of tablets (e.g. with non-imaging Raman or NIR
71 spectroscopy).

72 The present paper offers a solution to the problem of determining the spatial
73 distribution of a trace active ingredient by combining R-CI with the SERS technique and with
74 chemometric evaluation using multivariate curve resolution algorithms.

75

76 **2. Materials and methods:**

77

78 2.1. Preparation of tablets

79

80 Lactose monohydrate (LMH) and trisodium citrate was purchased from Sigma Aldrich
81 Corporation. Acetylsalicylic acid (further referred to as API i.e. the active pharmaceutical
82 ingredient), was manufactured by Richter Gedeon Plc (Hungary). Dimethyl-sulfoxide and
83 silver-nitrate were purchased from Reanal Ltd (Hungary).

84 „Dry technology” (D) tablets with heterogeneous drug distribution were prepared by
85 thorough blending of API and LMH using a mortar and pestle. In order to achieve
86 homogeneous distribution in „wet technology” (W) tablets, 2 g API-LMH blend was
87 dissolved in 10 ml dimethyl sulfoxide and then the solvent was evaporated with vacuum
88 distillation.

89 API:LMH mass ratio was 1:399 in both tablet batches (i.e. tablets had 0.25% API in
90 mass fractions). Model tablets, each weighting 400 mg, were prepared in a Manfredi
91 0057C00 type KBr disk press (Italy).

92

93 2.2. Preparation of SERS colloids

94

95 Ag nanoparticles were prepared by Lee and Meisel’s method [20], widely used to
96 synthesize silver for SERS substrates [21-25]. Silver-nitrate of 0,09g was dissolved in 0,5l of
97 double distilled water. The solution was heated to boil and 10ml of 1% trisodium-citrate
98 aqueous solution was added *dropwise* into to boiling solution during vigorous stirring. Boiling
99 was continued for 10 more minutes. Finally a greenish-grey colloidal solution was obtained.

100 Before SER-CI measurements the suspension was concentrated, via centrifugation, by a factor
101 of 10.

102

103 2.3. Raman instrumentation:

104

105 Raman mapping spectra were collected using a LabRAM system (Horiba Jobin-Yvon,
106 Lyon, France) coupled with an external 532 nm Nd-YAG laser source (Sacher Lasertechnik,
107 Marburg, Germany) and an Olympus BX-40 optical microscope (Olympus, Hamburg,
108 Germany). An objective of 50× magnification was used for optical imaging and spectrum
109 acquisition. The laser beam, after an optional intensity filter, is directed through the objective,
110 and backscattered radiation is collected with the same objective. The collected radiation is
111 directed through a notch filter that removes the Rayleigh photons, then through a confocal
112 hole (1000 μm) and an entrance slit (100 μm) onto a grating monochromator (1800
113 grooves/mm) that disperses the light before it reaches the CCD detector. The spectrograph
114 was set to provide a spectral range of 550–1750 cm⁻¹ and 3 cm⁻¹ resolution.

115 The outer surface of tablets were investigated (without any sample preparation) **in**
116 **each mapping experiment. Prior to SER-CI analysis, two types of reference maps had been**
117 **taken from the tablets. The first type („background”) reference used exactly same imaging**
118 **acquisition conditions as SER-CI analyses (see later) to ensure that no signals of the API (or**
119 **the excipient) are detected without SERS. Such (“background”) maps, as they indeed**
120 **consisted of noise only, are not shown in the paper.**

121 The second type of reference (R-CI) images were obtained with ‘traditional’ Raman
122 imaging conditions, setting high enough acquisition time to see the signals of the ingredients
123 and attempt to reveal the distribution of the trace API without SERS. **To achieve this,**
124 **spectrum acquisition time was 3 s and 20 such spectra were accumulated and averaged at each**

125 pixel to achieve acceptable signal-to-noise ratio. The step size between adjacent pixels was
126 increased to 200 μm along both axes in order to utilize the high overall measurement time and
127 to increase the probability of finding pixels that contain the API. As a compromise between
128 image size and overall mapping acquisition time, the measured area on the tablet surfaces was
129 31 \times 31 pixels and acquisition of each of these maps took over 14 hours.

130 For SER-CI analysis SERS colloid solution was dripped on top of the tablets and, after
131 drying, mapping was carried out on their outer surface (i.e. without any further sample
132 preparation to avoid alteration of the sample structure). Spectrum acquisition time in this case
133 was 0,5 s per pixel and only 1 spectrum was measured at each point without multiple
134 accumulation or averaging, to avoid degradation of the silver colloids. In this case, a step size
135 of 2 μm was used between adjacent pixels to achieve high spatial resolution, and the
136 investigated area was at least 49 \times 49 pixels. The overall acquisition time for each SER-CI and
137 „background” image (without SERS) was 20 min. For SER-CI analysis the laser power was
138 reduced to 10% of its original value with an intensity filter for the same purpose (to avoid
139 damage to colloids), while full power (~50 mW) was applied for the conventional R-CI
140 investigations.

141 To compensate the small measured area with SER-CI maps due to the low step size,
142 three separate SER-CI maps were collected from different locations on each tablet. Multiple
143 tablets were investigated to verify reproducibility but only one tablet of each batch is
144 discussed in detail.

145

146

147 2.4. Data analysis

148

149 Before chemometric evaluation, all spectra were base-line corrected using Euler's
150 asymmetric least squares method [26] with parameters of $\lambda=10^5$ and $p=10^{-3}$. For comparison,
151 the traditional approach of piece-wise linear baseline correction was also tested with manually
152 selected baseline points. The measured spectra were then normalized to unit area in order to
153 eliminate the intensity deviation among the measured points. The raw three-dimensional data
154 was unfolded into a 2-dimensional matrix (for the procedure, see reference [14]).

155 The estimation of pure component spectra from the Raman maps was carried out by
156 multivariate curve resolution – alternating least squares (MCR-ALS [27,28]). This technique
157 is based on the following bilinear model:

$$158 \quad \mathbf{X} = \mathbf{C}\mathbf{S}^T + \mathbf{E} \quad (\text{Eq. 1})$$

159 \mathbf{S}^T ($k \times \lambda$) is the set of reference (pure component) spectra, \mathbf{X} ($p \times \lambda$) is the matrix
160 containing the mapping spectra, and \mathbf{C} ($p \times k$) contains the vectors of spectral concentrations
161 (each row in \mathbf{C} contains the concentrations of the k ingredients). The matrix \mathbf{E} represents the
162 residual noise. If the spectra of the pure components are known, the \mathbf{C} matrix can be
163 calculated in a straightforward way with the Classical Least Squares (CLS) calculation, using
164 \mathbf{X} and \mathbf{S}^T . This is comprehensively described in numerous papers [14-16]. If some or all of the
165 components are unknown, MCR-ALS can be used, which itself generates both the
166 concentration (also known as *score*) matrix \mathbf{C} and pure spectrum (also known as *loading*)
167 matrix \mathbf{S}^T from the dataset \mathbf{X} in an iterative manner, using an initial estimation for either \mathbf{C} or
168 \mathbf{S}^T and appropriate physical constraints. Easy to use programs are available from the
169 developers [27] and in commercial software [29]. All of these have internal algorithms for
170 providing the initial estimations and the iterations afterwards, and require minimal effort from
171 the user. Only non-negativity constraints were used in our study.

172 The resolved loadings (i.e. estimated pure component spectra) were inspected one by
173 one and only those loadings were chosen for further use, which carried specific (sharp)

174 vibrational peaks. The approach proposed here assumes that only the API is SERS active.
175 (The rest of the loadings contained noise spikes and other disturbance factors arising due to
176 the partial degradation of SERS colloids in some of the pixels.) The scores corresponding to
177 these loadings were set in descending order and a threshold was determined for each API
178 spectrum. Those values were chosen as thresholds, which showed dominant change in API
179 peak intensity. Pixels with an API score over this threshold were assigned the value of '1', i.e.
180 to the API, while the rest were given a '0' value. This classification was accomplished using a
181 Visual Basic algorithm written in-house. Then the received column vector of these binarized
182 scores was formed back into an image matrix by using the *reshape* command in Matlab.

183 In the case of R-CI investigations, MCR-ALS scores (i.e. spectral concentrations)
184 were directly used to produce spatial distribution images, as usually done in most R-CI
185 investigations [14-16, 30-34]. R-CI score images obtained by multivariate techniques usually
186 do not require binarization to highlight the presence of an active ingredient, and the scores can
187 be interpreted as estimated concentrations (unlike in the case of SER-CI where only the
188 signal coming from SERS-active components are used and the raw scores do not hold
189 information about the actual concentration). Preliminary studies with and without binarization
190 yielded images with the same number of API-positive pixels, hence it was regarded as an
191 unnecessary step for R-CI.

192 All calculations were performed in MATLAB 7.6.0 (Mathworks, USA) with
193 PLS_Toolbox 6.2 and MIA_Toolbox 2.5 (Eigenvector Research, USA). Other curve
194 resolution and factor analysis methods are also available, but only MCR-ALS was used as
195 numerous studies have proven it to be the best choice for this purpose [30-34].

196 Spectral concentrations of the ingredients present in the sample (further also referred
197 to as 'Raman scores' in order to avoid confusion with real concentrations) were computed
198 with the same algorithm described above.

199 Visualization of spectra and spatial distribution maps was carried out with LabSpec
200 5.41 (Horiba Jobin Yvon, France). The statistical properties of scores (mean, standard
201 deviation) were computed with MATLAB.

202

203 **3. Results and discussion**

204

205 In order to demonstrate how SERS can enable the detection of traces of active
206 ingredients in a solid product and the determination of their spatial distribution with R-CI
207 special attention had to be paid to the selection of a suitable chemometric method . As SERS
208 spectra usually have significant differences from the regular Raman spectrum of the same
209 substance, the usual preprocessing methods and the conventional evaluation of images with
210 classical least squares (CLS) are not feasible. Furthermore, Raman images tend to consist of a
211 huge amount of measured spectra (a few thousands in our case). Therefore, a new way of
212 evaluation, based on currently available chemometric methods, had to be developed. This
213 study uses multivariate curve resolution – alternating least squares (MCR-ALS) for this
214 purpose, which have already been proven to be the most efficient in the evaluation of Raman
215 maps [30-34].

216

217 **3.1. R-CI investigations without SERS**

218

219 Conventional R-CI maps of (both known and unknown) pharmaceuticals can be
220 evaluated in a straightforward manner by resolving an appropriate number of spectra using
221 MCR-ALS. (CLS with the pure reference spectra would usually also be an option in the
222 pharmaceutical practice, but not in forensics and when unknown samples are investigated.)

223 Figure 1 shows the spectra resolved using MCR-ALS decomposition. In order to
224 properly estimate the number of components present in the sample, the number of spectra to
225 be resolved has to be overestimated. Due to this overestimation, the outcome contains certain
226 loadings, the features of which are very similar to one another and which in fact correspond to
227 the same physicochemical component. **If these small differences can be explained or are**
228 **irrelevant with respect to the results**, these similar loadings can be averaged to get a more
229 accurate estimation of the pure component spectra [34]. Note in this case the number of
230 components is known, so it would have been possible to resolve just two spectra, but the
231 study follows the practice of investigating an unknown tablet as proposed in the literature.
232 [33, 34])

233

234 Figure 1.

235

236 The spectra of 'D' (**dry technology**) tablet, resolved by initializing MCR-ALS with 6
237 pseudorandom vectors, are shown in Fig. 1a. The first loading clearly belongs to API, while
238 all the others are corresponding to the LMH. The differences among the LMH loadings are
239 caused by polarization effects which introduce remarkable deviation in the relative intensities
240 of certain peaks (e.g. 1084 and 851 cm^{-1}). Note the peak positions are same and there is no
241 band widening or shape alteration which would indicate different molecular or solid state
242 structures. **Lactose is known to be generally sensitive to the polarization of the laser light,**
243 **while other substances show little intensity deviations owing to this phenomenon. This effect**
244 **is more apparent in microscopic spectrometry, and usually occurs at high magnifications**
245 **where the crystal size is larger than the irradiated sample volume.** Figure 1b shows the
246 resolved spectra of 'W' (**wet technology**) tablet.

247 Figure 2 shows the spatial distribution of the API based on the MCR-ALS scores
248 (concentrations) corresponding to the first loading. Figure 2a shows large dark areas in the
249 map of the 'D' tablet that do not contain any API. In several points, however, a low amount of
250 API can be detected. The distribution image of the 'W' tablet (Fig. 2b) is very similar to the
251 one of the 'D' tablet, thus, the two technologies cannot be distinguished by R-CI at such low
252 API concentration. The question remains whether the dark areas are truly free of the active
253 ingredient or it is actually present under the R-CI limit of detection.

254

255 Figure 2.

256

257

258 3.2. Evaluation of SER-CI images

259

260 The evaluation of SER-CI experiments is challenged by two main phenomena. On one
261 hand, the spectra recorded with very short exposure time (0.5 sec) and acquisition number have
262 a low signal-noise ratio. This has only minor effect on the evaluation, as noise is usually
263 averaged out when the entire dataset of hundreds/thousands of spectra is processed with
264 chemometric tools. On the other hand, SERS spectra of the same component show very high
265 variability when microscopic imaging is used. aspirin molecules bound to silver nanoparticles
266 can be polarized in various ways, since the shapes and sizes of silver nanoparticles prepared
267 with Lee and Meisel's method are not exactly uniform [35]. (It is generally well known that
268 the SERS effect investigated in a colloid system has particle size and particle shape
269 dependence [17,36,37].) When samples are analyzed in a solution, using a macroscopic (non-
270 imaging) spectrometer, an average spectrum is obtained, simultaneously collecting signals
271 from variously polarized analyte molecules. On the surface of dry samples, however, SERS

272 particles are immobilized and, in chemical imaging, only a small area is investigated at once,
273 therefore no averaging occurs and the pixel-by-pixel high variability among the SERS
274 spectra is revealed. This results in an extreme deviation in the position, intensity and shapes
275 of the surface-enhanced Raman-bands of the API. Due to the presence of SERS colloids, the
276 baseline is also perturbed and varies significantly among the pixels. Consequently, neither the
277 CLS method (using pre-defined reference spectra), nor the univariate approach with a selected
278 peak can be used in practice to accurately determine the presence of the API via its SERS
279 signals. Furthermore, an appropriate solution had to be found for the baseline correction as
280 well.

281

282 3.2.1. A suitable preprocessing method for SERS spectra

283

284 To remove of fluorescent background from the SER-CI datasets, asymmetric least
285 squares was used, originally developed by Eilers for preprocessing chromatograms [38].
286 Asymmetric least squares (not to be confused with alternating least squares in curve
287 resolution) is derived from the Whitaker smoother [39], and is a fast and more efficient
288 alternative to the widely popular Savitzky-Golay [40] filter. The details are comprehensibly
289 described by its developer [26].

290

291 Figure 3.

292

293 Although this had been originally developed as a flexible smoothing tool, it can be just
294 as well used to estimate a nonlinear background by „oversmoothing” peaks. The main
295 advantage of this approach is that no wavenumbers have to be pre-selected to fit a (linear or
296 nonlinear) curve to estimate the background, hence it is resistant to the variability of peak

297 shapes and positions and to the emergence of unexpected SERS bands. Its comparison with
298 piece-wise baseline correction is depicted on Figure 3. The quality of the baseline depends
299 slightly on the asymmetric parameter (p) and mainly on the smoothing parameter (λ).
300 Oversmoothing is generally carried out by using a very high ($>10^4$) λ value. After testing
301 numerous combinations, their best values were determined as $p = 0.001$ and $\lambda = 10^5$. When
302 the parameters are optimized and fixed, the baseline itself has to be separately calculated for
303 each mapping spectrum. A Matlab algorithm, relying on Euler's algorithmic functions, is
304 provided in the Appendix in the electronic supplementary material to create a baseline-
305 corrected matrix from the input dataset. Afterwards, the same usual further steps (e.g.
306 normalization) can be applied as in R-CI investigations.

307

308

309 3.2.2. Evaluation of resolved spectra

310

311 Appropriate preprocessing is only the first step in overcoming the challenges of SER-
312 CI. The other problem is that the variability in the SERS spectrum of the API has to be taken
313 into consideration in the modelling. MCR-ALS curve resolution was therefore performed with
314 an appropriately high number of resolved loadings (in this case 20) to separate all independent
315 signals from each other. The identification of these loadings was performed visually by
316 determining whether sharp vibrational peaks are present, assuming that they originate from
317 the API (which was true in this case, as lactose is not SERS active). It was found that only a
318 few loadings carried typical bands belonging to the API, while others almost completely
319 consisted of noise and interfering signals from the silver colloid. All loadings are shown in
320 Figure SM-1 ("D" tablet) and SM-2 ("W" tablet) in the supplementary material, whereas

321 those corresponding to the API are summarized in Figure 4. **Interfering signals from the**
322 **colloid are illustrated on Figure SM-3 in the supplementary material.**

323 The loadings identified as Aspirin SERS spectra were compared with the pure acetyl-
324 salicylic acid spectrum on the Figure 4. The selected spectra were the loadings 5, 7 and 14 in
325 the case of 'D' tablet. Similarly, three loadings contained features similar to the pure API
326 spectra (namely loadings 1, 7 and 8) for the "W" tablet. It has to be noted, that the number of
327 the API-related loadings varied in the reproduced experiments. It can be also seen that in
328 different SERS spectra different bands are enhanced and to various extent: in many cases, the
329 intensities of such bands are amplified which are otherwise very weak in the conventional
330 Raman spectrum of the API. This is why neither a univariate approach nor CLS modelling
331 with reference spectra can be applied to evaluate the maps – instead, curve resolution
332 algorithms (such as MCR-ALS) are needed, which can detect each spectrum that has different
333 peak structures. Setting a high number of loadings to resolve is important to capture as many
334 different API-related loadings as possible. (It has to be noted that using SERS colloids of
335 uniform shape and narrow size range would most probably result in more uniform SERS
336 spectra and a lower number of distinct API-related loadings, however, this would not provide
337 any particular benefit for the chemometric evaluation proposed here. Using appropriate
338 baseline correction with asymmetric least squares and data decomposition with MCR-ALS
339 allows the use of less expensive SERS colloids or those prepared in-house, thereby notably
340 increasing cost efficiency.)

341

342 Figure 4.

343

344

345

346 3.2.3. Visualization of surface-enhanced distribution maps

347

348 Along with the loadings the score vectors were also calculated, which are usually used
349 as estimations of the concentration. In this case, they are used for determining whether an API
350 SERS signal was detected in a pixel or not. For each SERS loading, this was carried out by
351 sorting these score values by magnitude and finding a threshold above which the
352 corresponding loading is identified as significantly being present. The threshold, separately
353 for each loading, can be easily determined visually by sequentially bisectioning (“halving”)
354 the sorted scores, and checking the corresponding spectrum on the map, in a few steps. The
355 method is the following: (1) Take the score in the middle of the sorted score list. (2) Visually
356 check the mapping spectrum having this score value. (3) If this spectrum has visible SERS (in
357 this case, API) peaks, then proceed to the lower half of sorted scores and repeat the process
358 from step (1). Otherwise, proceed to the upper half of the sorted scores and repeat from step
359 (1). A good threshold is quickly received in n steps for a map containing up to 2^n pixels. After
360 determining the score threshold for each API-related loading, **all scores can be binarized**
361 **based on whether they are higher (1 or “SERS-positive”) or lower (0 or “SERS-negative”)**
362 **than the threshold and** the pixels with SERS signals present can be counted on the Raman
363 map.

364 The binarized scores, each indicating if the API was detected via its SERS signal in a
365 pixel, can be refolded to a spatial image in the same way the concentration images are usually
366 produced. As the investigated area of a SER-CI map ($60\ \mu\text{m} \times 60\ \mu\text{m}$) was smaller than the
367 area covered by an R-CI reference map ($200\ \mu\text{m} \times 200\ \mu\text{m}$), three SER-CI maps were
368 acquired from different, randomly selected locations from both “D” and “W” tablets. This
369 was done to ensure that different locations show the same characteristics with regards to the
370 API distribution. Figure 5 proves that in contrast to conventional R-CI, the difference between

371 the dry and wet technologies can be easily recognized based on the SER-CI maps. These
372 findings are supported by Table 1 highlighting the number of pixels with the API present.
373 While the API was detected in a relatively small number of distinct pixels in the 'D' tablet (9-
374 14%), in the case of 'W' tablets the SERS signals of aspirin were detected in the vast majority
375 of the pixels (91-98%).

376 Table 1 shows that the number of SERS-positive pixels were consequently 8-10× more
377 in the 'W' tablets than in the 'D' tablets, unambiguously differentiating the wet and dry
378 manufacturing technologies. This means that when the tablet is prepared by wet granulation,
379 the API forms a narrow layer on the excipient particles, which cannot be detected with R-CI if
380 the overall concentration of API is very low (0.25% in this case), but can be unambiguously
381 detected if SERS colloid is applied on the tablet surface.

382

383 Figure 5.

384

385 Table 1.

386

387 The traditional R-CI concentration maps, obtained with much longer measurement
388 time, were plotted on the Figure 2, where the two manufacturing technologies could not be
389 distinguished. The reason is that the API is under the limit of detection in the majority of
390 pixels in the map of the W tablet. If silver colloids are used to enhance the API spectra, the
391 presence of API is revealed in more than 90% of pixels for the "W" tablet, where a fraction of
392 the API is homogeneously distributed throughout the whole tablet due to the wet technology.
393 This is ten times more than the number SERS-positive pixels spectra when the tablet was
394 prepared with a dry technology (comparing maps of approximately the same sizes). Much
395 fewer API-positive pixels are detected by R-CI investigations, in which case the difference –

396 without lingering reproducibility studies – is not drastic enough to unambiguously
397 differentiate between the two technologies. Besides, such reproducibility studies are often
398 impossible in real-life forensic studies where only one or very few samples are available for
399 investigation.

400 Another advantage of the SER-CI method is the outstanding decrease of mapping
401 acquisition time, while using the same instrumental set-up as for ordinary R-CI studies. While
402 each R-CI measurement took over 14 hours, the overall acquisition time for a SER-CI map –
403 with a higher number of pixels – took only 20 minutes. Even the acquisition of multiple SER-
404 CI maps will be significantly less time-consuming than ordinary R-CI, while delivering more
405 information.

406 It has to be noted that the model tablets in this case contained the API in the same
407 concentration (0.25%). Some studies tend to correlate the percentage of pixels with a certain
408 component present (i.e. the surface coverage of that component) with the overall
409 concentration of that component in the sample [41]. It has been already shown for ordinary R-
410 CI that the surface coverage and the estimated overall concentrations depend on the
411 manufacturing technology [42], this phenomenon is extremely amplified when SERS is used,
412 and the maps currently cannot be processed to provide (semi-)quantitative information about
413 the API content. Therefore further studies are required to develop a semi-quantitative method
414 to estimate the amount of the active ingredient using SER-CI. **Another drawback of the**
415 **currently proposed evaluation approach to SER-CI maps is that it does not offer an automated**
416 **method to determine if the SERS signals are arising from one component only or there are**
417 **multiple, physicochemically different components simultaneously showing SERS spectra. As**
418 **already the SERS spectra of the same component show lot of variation among pixels, further**
419 **studies are needed to determine if multiple SERS-active components can be distinguished**

420 based on their resolved loadings, to separately plot their surface coverage on the visualized
421 images.

422

423

424 4. Conclusions

425

426 Surface enhanced chemical imaging was reported for the first time to investigate and
427 compare drug containing tablets with trace amounts of active ingredient. The method was
428 found suitable in the determination of the manufacturing technology even in cases where
429 ordinary Raman mapping (without SERS) does not show any significant difference between
430 tablets prepared with different manufacturing technologies. This broadens the opportunities to
431 determine if multiple unknown (possibly illegal) tablets containing trace amounts of drug
432 have been manufactured by the same or different technologies (i.e. presumably in the same
433 location or different ones). In the present study, tablets produced with a dry (direct
434 compression) and a wet technology (co-solution and solvent evaporation, then compression)
435 were investigated. Combining SERS with chemical imaging enabled the detection of the
436 active ingredient in areas where its concentration level was well below the Raman
437 spectrometric limit of detection, thus revealing its true spatial distribution in the tablets.
438 Furthermore, by enhancing API signals via SERS, generally a 120-fold reduction was
439 achieved in the image acquisition time. This, of course, is only possible if the active ingredient
440 is SERS active (and the current evaluation assumes that only one ingredient, i.e. the drug, is
441 SERS active).

442 Surface enhanced Raman chemical maps pose a great challenge to evaluate, due to the
443 high variability of spectra arising from the colloid size and shape dependence of the SERS
444 signals. When microscopic imaging of a solid sample is performed, these variances are not

445 averaged out as they otherwise do for bulk spectroscopic measurements. A new approach was
446 hence developed by using asymmetric least squares preprocessing with appropriately chosen
447 parameters to enable background estimation, followed by MCR-ALS data decomposition to
448 find all the various SERS positive (in this case, API-related) loadings.

449 The present study is the first step in the combined application of SERS, Raman
450 chemical imaging, appropriate data preprocessing and chemometric evaluation in the
451 structural characterization of tablets with trace amounts of drugs present. The proposed
452 evaluation approach is intended to be applicable for such tablets where no prior information is
453 available about the ingredients. SER-CI poses lots of challenges, some of which were solved
454 here and some have to be overcome in the future, but may become a powerful tool in the
455 investigation of unknown (possibly illegal) products.

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594 Figure captions

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596 Figure 1. Loadings obtained by MCR-ALS decomposition of conventional Raman chemical
597 images of *a*) "D" tablet (prepared with dry technology) and *b*) "W" tablet (prepared with wet
598 technology)

599

600 Figure 2. Detected API signals in conventional R-CI concentration maps of *a*) "D" tablet (dry
601 technology) and *b*) "W" tablet (wet technology)

602

603 Figure 3. *a*) a baseline of a SERS positive spectrum estimated by Eiler's asymmetric least
604 squares method. Comparison of SER-CI mapping spectra in *b*) raw form and after background
605 removal using *c*) piece-wise linear baseline correction and *d*) Eiler's asymmetric least squares

606

607 Figure 4. Selected API-related MCR-ALS loadings obtained from SER-CI datasets of 'D'
608 tablet and 'W' tablet, compared to the pure API spectrum

609

610 Figure 5. API distribution by SER-CI in randomly selected locations of 'D' and 'W' tablet

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613 Table captions

614

615 Table 1. Number of pixels with detected API SERS signals

616

Table 1

| Method | | 'D' tablet | | 'W' tablet | |
|--------|--------|--|-------------------------------------|--|-------------------------------------|
| | | <i>number of pixels with API present¹</i> | <i>% of pixels with API present</i> | <i>number of pixels with API present¹</i> | <i>% of pixels with API present</i> |
| R-CI | | 22 (961) | 2.3% | 9 (961) | 0.9% |
| SER-CI | Area 1 | 332 (2401) | 13.8% | 2279 (2500) | 91.2% |
| | Area 2 | 228 (2401) | 9.5% | 2358 (2500) | 94.3% |
| | Area 3 | 245 (2401) | 10.2% | 2450 (2500) | 98.0% |

¹brackets show the overall number of pixels

Figure 1

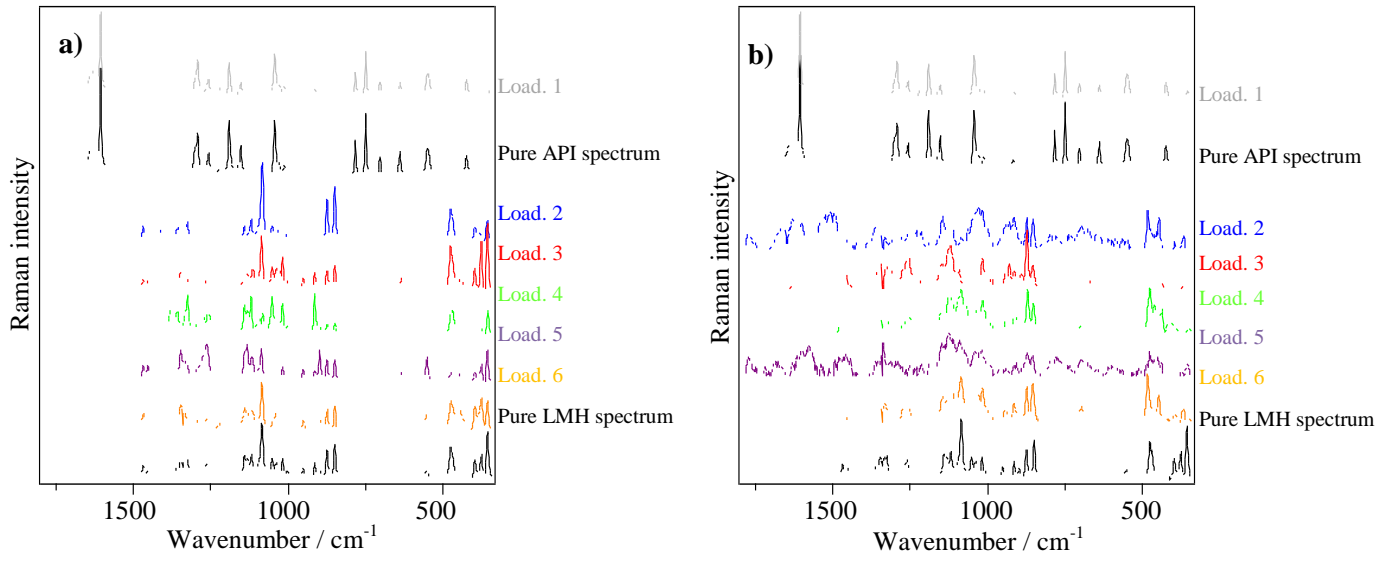


Figure 2

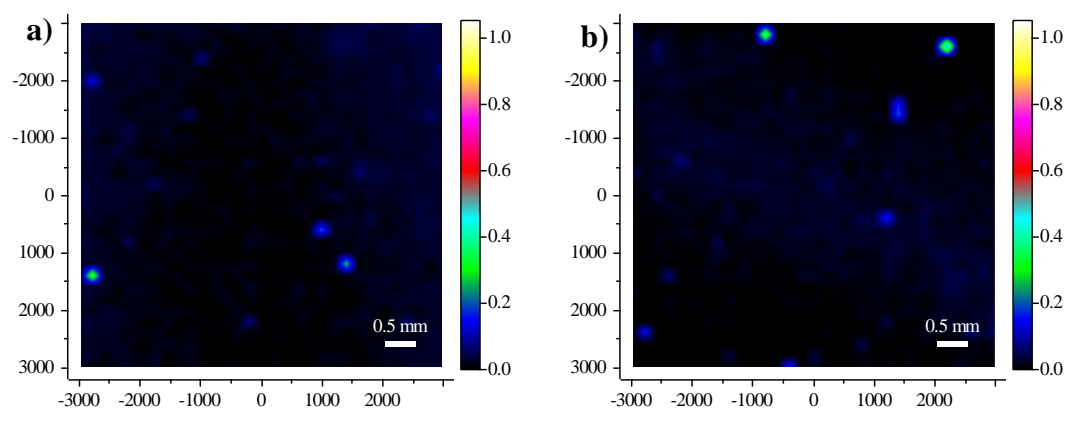


Figure 3
[Click here to download high resolution image](#)

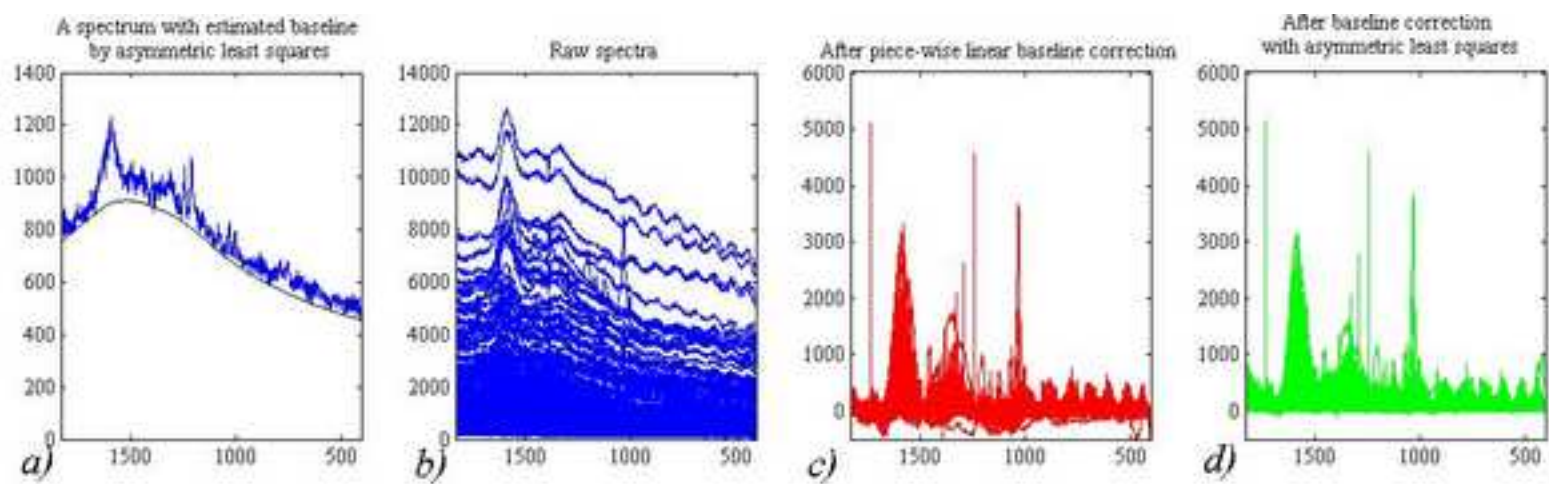


Figure 4

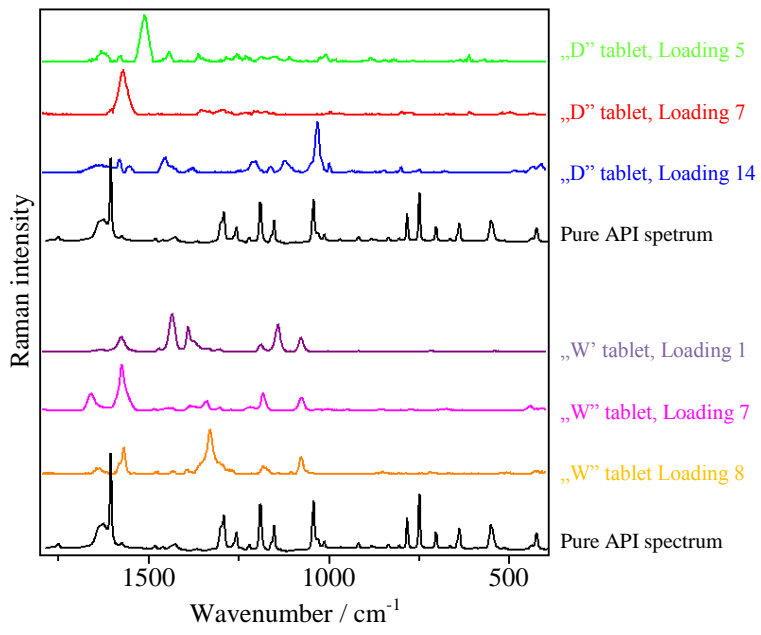


Figure 5

