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Rapid and sensitive detection of pyrimethanil residues on pome fruits by Surface Enhanced Raman Scattering

This is the author's submitted version of the contribution published as:

*Original*

Rapid and sensitive detection of pyrimethanil residues on pome fruits by Surface Enhanced Raman Scattering / Mandrile, L; Giovannozzi, A M; Durbiano, F; Martra, G; Rossi, A M. - In: FOOD CHEMISTRY. - ISSN 0308-8146. - 244(2018), pp. 16-24-24.

*Availability:*

This version is available at: 11696/57293 since: 2021-03-09T19:21:03Z

*Publisher:*

Elsevier

*Published*

DOI:10.1016/j.foodchem.2017.10.003

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Elsevier Editorial System(tm) for Food

Chemistry

Manuscript Draft

Manuscript Number: FOODCHEM-D-17-01263R1

Title: Rapid and sensitive detection of pyrimethanil residues on pome fruits by surface enhanced Raman scattering

Article Type: Analytical Methods Article

Keywords: pyrimethanil, pome fruits, Surface Enhanced Raman Scattering, Raman Spectroscopy, gold nanoparticles, Partial Least Squares

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1            *This manuscript represents an updated and extended version of the paper*  
2            *“Surface Enhanced Raman Spectroscopy: A Metrological Tool for Fungicide Detection”*  
3            *Presented at the 2nd IMEKOFODS Conference “Metrology Promoting*  
4            *Objective and Measurable Food Quality & Safety” (Benevento, Italy – 2nd - 5th*  
5            *October 2016), selected for publication in Food Chemistry*

6            **Rapid and sensitive detection of**  
7            **pyrimethanil residues on pome fruits by**  
8            **surface enhanced Raman scattering**

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17

18 **Abstract**

19 Surface Enhanced Raman Scattering (SERS) supported by gold nanoparticles (AuNPs) was  
20 applied to detect and quantify residues of pyrimethanil on pome fruits, a widely used  
21 fungicide in horticultural species. Spheroidal AuNPs with different size were fabricated and  
22 compared in this study. The analytical procedure was set up on a silicon dioxide flat substrate  
23 to standardize SERS methodology. A Raman mapping strategy was exploited to increase  
24 signal reproducibility and to minimize bias due to different local surface morphologies.  
25 Univariate and multivariate regressions were compared for calibration. **Multivariate PLS**  
26 **approach demonstrated acceptable repeatability and method stability (RMSECV = 4.79 ppm;**  
27 **RMSEP= 4.31ppm) in the range 0 - 40 mg kg<sup>-1</sup>, providing higher accuracy and intra-day**  
28 **repeatability with a mean percentage error of 18.7 % and 32.8 % for PLS and univariate**  
29 **calibration, respectively. The method here proposed can be reliably applied for PMT detection**  
30 **on pome fruits within the European law limits.**

31

32

33

34 *keywords: pyrimethanil, pome fruits, Surface Enhanced Raman Scattering, Raman*  
35 *spectroscopy, gold nanoparticles, Partial Least Squares*

36

## 37 1. Introduction

38 In the last decades food safety incidents have raised public concern about synthetic additives  
39 and chemical contaminants in food, the latter of them are mainly agricultural and  
40 environmental, chemical adulterants, mycotoxins, and foreign food components (Zheng J, He  
41 L, 2014). Agrochemical products such as pesticides and fungicides are normally applied to  
42 protect food crops from the pests at various stages of cultivation and during post-harvest  
43 storage (Sharma RR, Singh D, Singh R, 2009), but their intensive use can generate residues  
44 that may become a potential risk for both public health and environment (Gilden RC, Huffling  
45 K, Sattler B, 2010). Pyrimethanil [(PMT), N-(4,6-dimethylpyrimidin-2-yl)-phenylamine] is a  
46 fungicide belonging to the anilinopyrimidine class of pesticides which inhibits the secretion of  
47 hydrolytic enzymes by the fungi during the infection process, thus stopping penetration and  
48 development of the disease [FAO, 2007]. This broad spectrum fungicide is effectively used  
49 for the control of gray mould, leaf scab and other postharvest diseases on pome fruits,  
50 vegetables and ornamentals (Yu C, Zhou T, Sheng K, Zeng L, Ye C, Yu T, Zheng X, 2013).  
51 Even if PMT demonstrated low acute toxicity in mammals, long-term studies showed certain  
52 toxicity in mice, rats, dogs and aquatic organisms with potential carcinogenicity. Owing to its  
53 toxicity and wide application on horticultural species, PMT has been included as a pesticide  
54 by the Commission Directive 2006/74/EC of August 2006 with a maximum residue level of 7  
55 mg kg<sup>-1</sup> on pome fruits (Regulation (EC) No 396/2005). Most of the published methods for  
56 the determination of PMT on fruits, vegetables and other samples are usually based on GC  
57 (Amvrazi EG, Tsiropoulos NG, 2009) , GC-MS/MS (Raeppl C, Nief M, Fabritius M,  
58 Racault L, Appenzeller BM, Millet M, 2011; Rodriguez-Cabo T, Rodriguez I, Ramil M, Cela  
59 R, 2011), GC-MS ([Gonzalez-Rodriguez RM, Rial-Otero R, Cancho-Grande B, Simal-  
60 Gandara J, 2008) LC-MS (Park S et al 2010), HPLC (Zhou Y et al 2011), and LC-MS/MS

61 (Ortelli D, Edder P, Corvi C, 2004). These methods have high accuracy, good reproducibility  
62 and provide quantitative determination of PMT according to the EU limits, but they also  
63 suffer from inevitable disadvantages such as expensive experimental instruments, trained  
64 personnel and time-consuming extraction steps. Therefore, the development of more rapid  
65 and user friendly methods for PMT residues detection on horticultural species is highly  
66 required.

67 New methodologies based on ELISA (Mercader JV, Esteve-Turrillas FA, Agullo C, Abad-  
68 Somovilla A, Abad-Fuentes A, 2012) and electrochemical sensors (Garrido JMPJ, Rahemi V,  
69 Borges F, Brett CMA, Garrido EMPJ, 2016; Yang J, Wang Q, Zhang M, Zhang S, Zhang L,  
70 2015) were recently proposed as interesting alternatives to the traditional analytical methods,  
71 providing high selectivity and sensitivity in PMT detection in real samples. However, no  
72 studies were made so far concerning the development of a PMT detection tool based on  
73 Surface Enhanced Raman Scattering (SERS). Compared with the conventional analytical  
74 techniques, Raman spectroscopy allows fast detection times, high selectivity due to the  
75 Raman fingerprint of molecules and minimal or no preliminary treatment of the sample.  
76 Moreover, the sensitivity of the normal Raman technique can be increased by several orders  
77 of magnitude in SERS analysis due to the enhancement of the Raman scattering of molecules  
78 absorbed onto, or microscopically close to, a suitable plasmonically active surface, such as  
79 roughened nanostructured metal surface, or metal colloids (Schluecker S, 2014). For all these  
80 reasons SERS represents a good candidate in food control analysis. Different SERS  
81 approaches were already reported on the detection of various classes of pesticides in real-food  
82 matrices. In case of solid matrices, homogenization of the peel or surface swab methods were  
83 used to recover pesticides from the surface and the detection was subsequently performed  
84 using solid surface-based substrates (He L, Chen T, Labuza TP, 2014; Fan Y, Lai K, Rasco

85 BA, Huang Y, 2014). *In situ* detection of pesticides in fruits was also demonstrated using  
86 different types of metal nanoparticles (NPs) (Li FJ et al 2010; Liu B et al 2012) which were  
87 spread as “smart dust” over the surface that has to be probed. However, even if these SERS  
88 substrates demonstrated to achieve a very high sensitivity in the detection of chemical  
89 contaminants, they usually suffer from lack of reproducibility and inconsistent performance  
90 when spot-to-spot tests are conducted, leading to problems in the quantification process.  
91 Therefore, standardized SERS tool with a good compromise between sensitivity and  
92 reproducibility of analysis are needed to provide reliable analytical methods in the detection  
93 of food contaminants.

94 In this work we propose a versatile, simple and reproducible procedure to detect, discriminate  
95 and quantify residues of pyrimethanil on pome fruits by SERS. We selectively tested  
96 spheroidal AuNPs with different dimensions in order to have the highest SERS effect. The  
97 analytical procedure was set up on a flat surface as model system to standardize SERS  
98 methodology for both qualitative and quantitative analysis. Raman mapping was exploited to  
99 increase signal reproducibility from spot to spot analysis and to provide more consistent  
100 results. A semi-quantitative *in situ* detection method on fruit peel was assessed and an  
101 accurate method for pyrimethanil quantification on fruit surface was developed and validated.

102

103

## 104 **2. Material and Methods**

### 105 *2.1 Reagents and materials*

106 Hydrogen tetrachloroaurate trihydrate ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O} \geq 99\%$ ), trisodium citrate dihydrate  
107 ( $\geq 99\%$ ), were purchased from Sigma-Aldrich (Milan, Italy). Sodium hydroxyde (NaOH

108 97%), Hydrochloric acid (HCl 37%), Nitric acid (HNO<sub>3</sub> 68%), absolute ethanol (99.99%),  
109 acetone (99,99%) and Hydroxylamine Hydrochloride (H<sub>3</sub>NO·HCl, 99+%) were obtained by  
110 Novachimica (Milano, Italy). Scala® (400 g/l of Pyrimethanil suspension) was purchased  
111 from BASF Italia (Volpiano, Italy). All solutions were prepared with Milli-Q quality water  
112 (18 MΩcm). Silicon wafers with a 300 nm of Silicon dioxide layer on top were purchased  
113 from Si-Mat (Kaufering, Germany). Apples used for the assays were purchased in a local  
114 supermarket in Torino, Italy.

115

## 116 *2.2 Gold nanoparticles preparation*

117 All glassware used in the experiment was soaked in aqua regia (HCl:HNO<sub>3</sub> 3:1 v/v) and  
118 rinsed thoroughly in water and dried with nitrogen prior to use. Spheroidal AuNPs with a  
119 diameter of about 30 nm, 40 nm and 55 nm were synthesized according to Frens G, 1973.  
120 Briefly, 7 ml, 5 ml and 3.5 ml of a 1% aqueous solution of trisodium citrate were rapidly  
121 injected into 500 ml boiling solution of HAuCl<sub>4</sub> (0.01% v/v) for the preparation of 30 nm, 40  
122 nm and 55 nm AuNPs, respectively. The mixture was further refluxed for 10 min and then  
123 cooled to room temperature under continuous stirring. Larger AuNPs with a diameter of 90  
124 nm and 120 nm were obtained via seed-mediated growth of 30 nm and 40 nm AuNPs,  
125 respectively, using an optimized growing procedure based on hydroxylamine hydrochloride  
126 (Li JF, 2013). In detail, 4 ml of Au seeds suspension were put into a round-bottom flask with  
127 53.8 ml of Milli-Q water under continuous stirring and the different solutions were added in  
128 the following order: 920 µl of 1% v/v aqueous solution of trisodium citrate (stirring for 3  
129 min), 1.4 ml of 10 mM hydroxylamine hydrochloride solution (stirring for 8 min) and 90 µl of  
130 10% w/v HAuCl<sub>4</sub> (added dropwise, 1 drop per second). The concentration of 30 nm, 55 nm,



131 90 nm and 120 nm AuNPs suspensions is  $9 \cdot 10^{-11} \text{ mol l}^{-1}$ ,  $6 \cdot 10^{-11} \text{ mol l}^{-1}$ ,  $8 \cdot 10^{-12} \text{ mol l}^{-1}$ ,  $6 \cdot 10^{-12} \text{ mol l}^{-1}$  respectively. The suspensions were kept in continuous stirring overnight at room  
132 temperature in the dark before using it.  
133

### 134 *2.3 Gold Nanoparticles Characterization*

135 AuNPs characterization was done by UV-Vis absorption measurements and by Scanning  
136 Electron Microscopy (SEM) imaging. UV-Vis absorption spectra were collected in the  
137 rang400-1100 nm with the Evolution 60s spectrophotometer (Thermo Scientific). The  
138 wavelength resolution is 1 nm. SEM characterization was carried out using a SEM FEI  
139 Inspect F in UHV mode with the secondary electrons (SE) detector. Typical settings for the  
140 imaging are: 10 kV accelerating voltage, 2.5 electron beam spot (18 pA) or 3.5 spot (30pA),  
141 10 mm WD. By imaging the particles using SEM, size and shape of AuNPs were  
142 characterized as well as the size distribution of their particles. At least 300 nanoparticles were  
143 counted for each sample to estimate the mean diameter and the relative standard deviation of  
144 the AuNPs.

145

### 146 *2.4 Preparation of Pyrimethanil standard suspensions and solutions*

147 Pyrimethanil solubility in water at room temperature is 0,121 g/l; in case of higher  
148 concentration it is dispersed in a stable suspension. Pyrimethanil stock standard suspension  
149 was prepared by accurately diluting 2 ml of Scala® ( $400 \text{ g l}^{-1}$  of Pyrimethanil suspension) in  
150 100 ml and 200 ml of Milli-Q water, to reach a concentration of  $8 \text{ g l}^{-1}$  ( $8 \cdot 10^3 \text{ ppm}$ ) and  $4 \text{ g l}^{-1}$ .  
151 Pyrimethanil standard solutions were prepared by subsequent dilutions from the stock  
152 suspensions in water to reach the following concentrations:  $40 \text{ mg l}^{-1}$ ,  $30 \text{ mg l}^{-1}$ ,  $20 \text{ mg l}^{-1}$ ,  $10$

153 mg l<sup>-1</sup>, 5 mg l<sup>-1</sup>, 1 mg l<sup>-1</sup>. These pure pyrimethanil standards were used for AuNPs aggregation  
154 test, SERS efficiency test and to set up the analytical procedure.

155

156

### 157 *2.5 AuNPs aggregation test*

158 Aliquots of pyrimethanil standard suspension (400 mg l<sup>-1</sup>) were mixed in a 1:2 ratio with  
159 AuNPs stock suspension, mixed with vortex for 3 s and subsequently analyzed by UV-Vis. In  
160 these conditions PMT is in high excess with respect to AuNPs and their interaction, if present  
161 could not be negligible. Measurements in acidic conditions were performed after adding few  
162 drops of 1 M HCl to reach a pH value close to 3. UV-Vis measurements were repeated over  
163 four days.

164

### 165 *2.6 SERS Efficiency Test*

166 1 μl of a 400 mg l<sup>-1</sup> pyrimethanil standard suspension was deposited by drop casting on a flat  
167 gold surface and let it dry in air for evaporation. 5 depositions were performed on the surface  
168 in order to obtain an array of 5 pyrimethanil spots (4 spots for SERS collection and 1 spot for  
169 normal Raman reference collection). Each spot was covered with 2 μl of AuNPs for Raman  
170 mapping after drying. A reference spot for SERS analysis was also made by covering a PMT  
171 spot with 2 μl of water. The concentration of all AuNPs suspension was levelled to have the  
172 same exposed surface area ( $4 \times 10^{12}$  nm<sup>2</sup>), avoiding bias in case of larger NPs.

173

### 174 *2.7 Detection of PMT on pome fruits*

175 Pome fruit samples such as green apples were bought from a local store. The whole fruit was  
176 washed with sodium bicarbonate to remove contaminants from the surface and then spiked  
177 with different amount of PMT standard suspensions. For the on-peel detection tests, the  
178 spiking procedure was set using a manual nebulizer with 100 ml capacity in order to better  
179 simulate the in-field pesticide diffusion on fruits. For the accurate contamination of the  
180 sample to be used as validation test of the quantitative method, the PMT suspension was  
181 deposited using a micro syringe with a 0.1  $\mu$ l precision.

182 *In situ* detection of pyrimethanil was performed by depositing 2  $\mu$ l of a 10-fold concentrated  
183 120 nm AuNPs suspension on the contaminated peel, followed by Raman mapping after the  
184 evaporation of the colloids suspension. The 10-fold concentrated 120 nm AuNPs suspension  
185 was obtained by centrifugating the AuNPs stock suspension at 600 g for 12 min and  
186 subsequently re-suspending in a proper amount of water suspension.

187 For an accurate and precise quantification of the fungicide on the entire surface of the fruit, an  
188 extraction procedure was carried out by thoroughly rinsing the contaminated peel with a  
189 known amount of distilled water (usually equal to the fruit weight) to recover pyrimethanil  
190 from the surface. Three separated rinses using one third of the total washing volume were  
191 performed to increase the extraction efficiency. 2  $\mu$ l of the resulting solution were deposited  
192 by drop casting on a silicon dioxide surface and let them dry in air for evaporation. 2  $\mu$ l of a  
193 10-fold concentrated 120 nm AuNPs suspension were then deposited on the PMT spot and  
194 analyzed by Raman mapping after drying. The content of PMT in real samples was  
195 determined by SERS according to the analytical procedure reported in 2.9.

196

197 *2.8 SERS Measurement*

198 SERS spectra were recorded using a Thermo Scientific DXR Raman equipped with a  
199 microscope, excitation laser source at 780 nm, a motorized microscope stage sample holder,  
200 and a charge-coupled device (CCD) detector. Raman equipment is monthly calibrated through  
201 a software-controlled calibration tool which ensures wavelength calibration using multiple  
202 neon emission lines, laser frequency calibration using multiple polystyrene Raman peaks,  
203 intensity calibration using standardized white light sources. The frequency uncertainty is  
204 determined by the grating resolution of  $5 \text{ cm}^{-1}$  (the grating groove density is 1200). The  
205 intensity uncertainty was demonstrated to be lower than 5 % using a polystyrene standard.  
206 Spectra of samples were collected using a 20x long working distance microscope objective  
207 (spot size  $1.7 \mu\text{m}$ , N.A. 0.40) with a 10 mW laser power and a spectral range from 150 to  
208  $3400 \text{ cm}^{-1}$ . The acquisition time for each spectrum was 1 second for 5 exposures. A Raman  
209 map of about 25 spectra was collected on each PMT-AuNPs spot (about  $0.5 \text{ mm}^2$  area was  
210 investigated) and the obtained spectra were averaged for statistical analysis.

### 211 *2.9 Computational procedure*

212 Geometry optimization of model PMT structures and consequent calculations of vibrational  
213 (IR and Raman) spectra were carried out with DFT method using Gaussian 03 program  
214 (Gaussian 03, Revision B.05, References cited in <http://www.gaussian.com>). Full geometry  
215 optimizations were carried out without symmetry constraints. The computations were  
216 performed with the Lee, Yang and Parr correlation functional (LYP) (Lee C., Yang W, Parr  
217 RG, 1998) combined with the Becke's non-local three-parameter hybrid exchange functional,  
218 (B3) (Becke AD, 1993). Vibrational information coming from the computational procedure  
219 were compared with the experimental Raman spectrum of PMT and the main bands in the  
220 spectrum were assigned using the Handbook of Infrared and Raman Spectroscopy (Socrates G

221 “Infrared and Raman Characteristic Group Frequencies: Tables and Charts”, Wiley ISBN:  
222 978-0-470-09307-8).

223

#### 224 *2.10 Quantitative calibration and validation of the method*

225 The instrumental linearity was evaluated from a calibration curve calculated with five levels  
226 of PMT concentrations in non-spiked fruit extract, representative of the analyzed matrix: 0 mg  
227  $\text{kg}^{-1}$ , 5 mg  $\text{kg}^{-1}$ , 10 mg  $\text{kg}^{-1}$ , 20 mg  $\text{kg}^{-1}$ , 40 mg  $\text{kg}^{-1}$ . 2  $\mu\text{l}$  of each PMT standard solution were  
228 deposited by drop casting on a silicon dioxide surface and let them dry in air for evaporation.  
229 In the calibration procedure of SERS analysis, 2  $\mu\text{l}$  of a 10-fold concentrated 120 nm AuNPs  
230 suspension were deposited on the five spots with increasing concentrations of pyrimethanil.  
231 After drying, Raman mapping was performed on each spot, as described in 2.8, and all spectra  
232 from each map were averaged for statistical analysis. Each measurement was repeated 4 times  
233 to test measurement repeatability for a total of 20 calibration standards. A specific AuNPs  
234 Raman band at  $2130\text{ cm}^{-1}$  was exploited to normalize the Raman intensity of pyrimethanil  
235 spectrum, minimizing possible variations due to laser power, focal distance and  
236 environmental parameters (temperature, humidity) and to overcome variations of the  
237 enhancement effect due to the different ratio between the amount AuNPs and analyte  
238 molecules. The uncertainty of pyrimethanil concentrations was also calculated, as  
239 recommended by the GUM JCGM 100:2008 and Supplement 1 JCGM 101:2008, in order to  
240 meet metrological requirements. The intensity of PMT Raman band at  $997\text{ cm}^{-1}$  was plotted  
241 versus pyrimethanil concentration to obtain the calibration curve. The applied fitting  
242 procedure was a weighted total least square (WTLS) regression, and was implemented by  
243 means of a MATLAB®-based tool for calibration problems that is able to deal with

244 uncertainty (and correlation) in both the dependent (average intensities) and independent  
245 (mass values) variables; the Calibration Curve Computing Software for the evaluation of  
246 instrument calibration curves is provided by the Italian national institute for metrological  
247 research (INRiM). The linearity in the concentration range considered was estimated by the  
248 reduced chi-square value ( $\chi^2$ ). Acceptability criterion to assume the linearity of response is a  
249  $\chi^2$  close to 1.

250 Multivariate calibration was performed using Partial Least Square method. PLS regression is  
251 based on the maximization of the covariance between variables (spectral frequencies) and  
252 response (contaminant concentration) associated to the calibration standards (Wold H, 2006).  
253 Seven concentrations of pyrimethanil (0 mg kg<sup>-1</sup>, 1 mg kg<sup>-1</sup>, 5 mg kg<sup>-1</sup>, 10 mg kg<sup>-1</sup>, 16 mg kg<sup>-1</sup>  
254 20 mg kg<sup>-1</sup>, 30 mg kg<sup>-1</sup>, 40 mg kg<sup>-1</sup>) were deposited and covered with AuNPs as described  
255 above. For each standard 4 SERS maps were collected and the mean spectrum of each map  
256 was used as calibration standard for a total of 32 calibration standards. PLS calculation was  
257 performed using the PLS Toolbox for Matlab ®. PLS regression was applied to mean  
258 centered SERS spectra. Spectral frequencies ranging from 180 cm<sup>-1</sup> to 2500 cm<sup>-1</sup> were  
259 considered, excluding the first order of silicon in the range between 500 cm<sup>-1</sup> and 550 cm<sup>-1</sup>  
260 that was not included in the calibration process. The optimal number of PLS latent variables  
261 was selected on the basis of the cumulative explained variance (CEV) for each component  
262 and the root mean square error in cross validation (RMECV). The validation was performed  
263 using a venetian blinds cross validation procedure with 6 cancellation segments at first for  
264 method optimization and an external set of four samples for test set final validation.

265

### 266 **3. Results and Discussion**

267 Several studies already proposed metallic NPs as useful substrates for pesticides detection but  
268 in most of these methods a strong chemical interaction between the colloids and the analyte  
269 occurred. This binding affinity normally leads the aggregation of the NPs generating clustered  
270 SERS hot spots which are responsible for a huge enhancement of their Raman signals. Unlike  
271 it was previously reported, the chemical structure of pyrimethanil does not support selective  
272 binding with citrate terminated AuNPs and the enhancement of its Raman signals can be  
273 exclusively promoted by the electromagnetic effect, which has to be induced by the proximity  
274 of the fungicide on the metallic NPs surface. The chemical interaction of AuNPs with  
275 pyrimethanil was initially studied performing AuNPs aggregation test in suspension.  
276 Spheroidal AuNPs with different size were fabricated, as reported in the paragraph 2.2, and  
277 characterized by SEM and UV-Vis measurements. SEM images of AuNPs with the mean  
278 diameter of 30 nm, 55 nm, 90 nm and 120 nm, respectively are shown in figure 1S in  
279 supplementary information.

280 The UV-Vis absorption spectra of these NPs were acquired and they are reported in Figure 2S  
281 in supplementary information. As the size of AuNPs increases, the  $\lambda_{\max}$  was found out to  
282 increase from 530 nm to 580 nm, which agrees with the previous conclusion that the  
283 maximum peak wavelength red-shifts as the relative particle size gets bigger (Hong S, Li X,  
284 2013).

285 The chemical interaction of AuNPs with pyrimethanil was studied by means of a AuNPs  
286 aggregation test (Figure 1), as reported in paragraph 2.5. A decreased stability of the colloids  
287 in suspension would provoke the aggregation of AuNPs, inducing a shift of the localized  
288 surface Plasmon resonance (LSPR) with a consequent variation of the colloidal system color  
289 from red to blue, that can be easily monitored by UV-Vis absorption measurements.

290 The stability of the colloidal suspensions was monitored at acidic conditions (pH~3) in which  
291 PMT is completely solubilized in water and its interaction with citrate-terminated AuNPs  
292 should be maximized (pKa Pyrimethanil = 3.26). As Figure 1 shows, no shift of the LSPR  
293 peaks at higher wavelengths was registered over 15 minutes for the 30 nm, 55 nm, 90 nm and  
294 120 nm AuNPs colloids after adding PMT (400 mg l<sup>-1</sup>), meaning that no, or very low,  
295 chemical affinity exists between AuNPs and the analyte. No shift of the LSPR peaks was  
296 further observed after four days, confirming the long term stability of all these colloidal  
297 systems in the presence of PMT (Figure 1).

### 298 Fig. 1

299 Since PMT and AuNPs do not interact in a liquid medium, PMT detection has to be  
300 conducted in dry condition in order to promote the absorption of the PMT on the AuNPs  
301 surface and to maximize the electromagnetic effect in SERS technique.

302 A comparative SERS efficiency test using spheroidal AuNPs with a diameter of 30 nm, 55  
303 nm, 90 nm and 120 nm was performed to investigate the effect of the nanoparticle size on the  
304 Raman enhancement, as it was described in paragraph 2.6. The sum of surface area  $A$  of all  
305 AuNPs was calculated using equation (1), assuming that all nanoparticles are spherical (Hong  
306 S et al 2013)

$$A = 4\pi r^2 n = \frac{6\pi d^2 m_t}{D\pi d^3} = \frac{6m_t}{Dd} \quad (1)$$

307  
308 where  $n$  is the number of nanoparticles;  $m_t$  is the total mass of Au in the suspension;  $D$  is the  
309 density of Au assuming that the density does not change with the size of the nanoparticles;  $d$   
310 is the diameter of the nanoparticles.

311 To provide a consistent comparative study, the total surface area of AuNPs with different size  
312 was kept the same in SERS efficiency test. The total surface area was normalized to the



313 surface area of a 10-fold concentrated 120 nm AuNPs suspension, corresponding to  $4 \times 10^{12}$   
314  $\text{nm}^2$ .

315 Normal Raman spectrum of PMT in solid state is shown in Figure 2. Typical Raman  
316 fingerprint of pure PMT exhibits vibrational peaks at  $560 \text{ cm}^{-1}$ ,  $610 \text{ cm}^{-1}$ ,  $620 \text{ cm}^{-1}$ ,  $997$   
317  $\text{cm}^{-1}$ ,  $1033 \text{ cm}^{-1}$ ,  $1246 \text{ cm}^{-1}$  and  $1296 \text{ cm}^{-1}$  whose assignments are reported in Figure 2. **Band**  
318 **assignments for PMT molecule were obtained by a combination of a computational procedure**  
319 **with vibrational information from Handbook of Infrared and Raman Spectroscopy, as**  
320 **reported in the section 2.9 of Material and Methods.**

321 **Fig. 2**

322 As Figure 3 shows, SERS spectral features of PMT standard obtained with the all tested  
323 AuNPs were consistent with its conventional Raman spectrum. The major characteristic peaks  
324 found in the Raman spectrum of PMT ( $560 \text{ cm}^{-1}$ ,  $610 \text{ cm}^{-1}$ ,  $620 \text{ cm}^{-1}$ ,  $997 \text{ cm}^{-1}$ ,  $1033 \text{ cm}^{-1}$ )  
325 were clearly visible in their SERS spectral counterparts. However, the relative intensities of  
326 characteristic peaks may change, broadening of peaks may occur, and new peaks may show  
327 up in the SERS spectra. For example, intensity of the characteristic double peak of PMT at  
328  $610 \text{ cm}^{-1}$ ,  $620 \text{ cm}^{-1}$  was increased, the peak at  $560 \text{ cm}^{-1}$  was broadened and a new peak at  
329  $950 \text{ cm}^{-1}$  showed up in SERS spectra compared to its conventional Raman spectrum. These  
330 changes were due to the interactions of analyte molecules with the surfaces of gold  
331 nanoparticles, in particular related to the orientations of analyte molecules on the substrate  
332 surface and the specific functional group(s) of the molecules bound to the substrate (Luo H,  
333 Huang Y, Lai K, Rasco AB, Fan Y, 2016).

334 The SERS response of four different AuNPs colloids was compared with the aim to select the  
335 best enhancing system for our scope. As demonstrated in Figure 3, all the AuNPs tested were  
336 able to enhance the specific peaks of pyrimethanil in SERS analysis compared to the normal

337 Raman spectroscopy. The peak at  $997\text{ cm}^{-1}$  was used to compare the intensity of the different  
338 AuNPs because it exhibits the highest intensity and it is characteristic of the breathing mode  
339 of the PMT aromatic ring (Herzberg G, 1988). The analytical enhancement factor (EF) for  
340 each size of AuNPs was calculated using (2), where  $I_{\text{SERS}}$  and  $I_{\text{NR}}$  are the intensity of the  
341 vibrational peak in SERS and normal Raman (NR) measurements, respectively, and  $C_{\text{NR}}$  and  
342  $C_{\text{SERS}}$  are the concentration of PMT in NR measurements and the SERS measurements,  
343 respectively (Kara S et al. 2016):

$$EF = \frac{I_{\text{SERS}}C_{\text{NR}}}{1 + I_{\text{NR}}C_{\text{SERS}}} \quad (2)$$

344

345

### Fig. 3

346 The EF increases together with AuNPs size, the highest value is reached when the 120 nm  
347 AuNPs are used as reported in Table 1S in supplementary information. It is known that the  
348 local electromagnetic enhancement increases with the increasing particle size (Kelly KL,  
349 Coronado E, Zhao LL, Schatz GC, 2003) and as soon as the particles get bigger also the SP  
350 band red-shifts, moving closer to the excitation wavelength of the laser (780 nm). This  
351 probably explains our observations that the SERS EF generated from AuNPs is maximized  
352 when the size of the gold NPs is around 120 nm. Moreover, we can infer that 120 nm AuNPs  
353 arrange in a more suitable morphology in terms of roughness and vicinity of NPs on the  
354 contaminated surface. Therefore, 120 nm AuNPs were selected and used for the further  
355 development of the present methodology.

356 In order to demonstrate a practical application in the food safety field, green apples were  
357 contaminated with PMT trace residues and *in situ* detection of the fungicide on a real sample  
358 was initially tested. The surface of a green apple was contaminated by depositing 1  $\mu\text{l}$  of PMT  
359 concentrated suspension (40  $\mu\text{g}$  of PMT) on the peel. 0.5  $\text{cm}^2$  area was covered approximately

360 in order to create an ultra-contaminated zone on the peel. Normal Raman mapping was  
361 performed on the contaminated area but no or very low signals of PMT were collected on the  
362 surface even if the local concentration is  $80 \mu\text{g cm}^{-2}$  (Figure 4a). In order to increase the  
363 sensitivity of the technique, a concentrated suspension of 120 nm AuNPs was spread as  
364 “smart dust” over the contaminated area and SERS mapping was conducted. As Figure 4b  
365 shows, the typical fingerprint of the PMT was registered after AuNPs deposition and its  
366 spatial distribution on the surface was easily monitored by the micro-mapping Raman system.  
367 A color scale bar from blue to red was associated to the intensity of the specific PMT peak at  
368  $997 \text{ cm}^{-1}$  and related to the x,y position on the analyzed surface in order to provide a semi-  
369 quantitative information of the PMT amount on the apple peel.

370 **Fig. 4**

371 Raman mapping presented in Figure 4 clearly demonstrated that *in situ* detection of PMT on  
372 the apple peel only occurs when AuNPs are applied on the surface, while very low or no PMT  
373 signals were registered in the contaminated regions without AuNPs. This means that normal  
374 Raman technique is not sensitive enough to detect low amounts of PMT on the surface which  
375 is further confirmed by an increase of the signal to noise ratio (S/N) from  $S/N = 1.2$  for NR to  
376  $S/N = 5$  for SERS measurements. Therefore, this approach could be easily used to achieve *in*  
377 *situ* detection of PMT and to discriminate the type of fungicide/pesticide on the fruit surface  
378 based on the specificity of the Raman fingerprint. However, this methodology would not be  
379 reliable enough to provide a quantitative information on the entire surface of the apple due to  
380 fact that only a small portion of the surface is analyzed and the colloids tend to randomly  
381 aggregate on such inhomogeneous surfaces, such as fruits peel, leading to a lack of  
382 reproducibility when spot-to-spot tests are conducted and when different fruit specimen are  
383 compared.

384 In order to develop a quantitative methodology for PMT detection on the entire surface of the  
385 fruit, a simple extraction procedure was first carried out to recover pyrimethanil from the  
386 surface, as explained in the paragraph 2.7. An external calibration was chosen to set up the  
387 analytical procedure. Five concentrations of pyrimethanil (0, 5, 10, 20, 40 mg kg<sup>-1</sup>) were used  
388 for the calibration curve and deposited by drop casting on a silicon dioxide surface. Silicon  
389 dioxide was chosen as model substrate because its roughness does not contribute to the SERS  
390 effect, its surface can be easily cleaned by any organic contamination and minimal  
391 interference is provided by its Raman peaks. A concentrated suspension of 120 nm AuNPs  
392 was deposited on each PMT spot and Raman mapping was applied to scan all the surface and  
393 to overcome inhomogeneity problems. An average spectrum of each map was calculated and  
394 normalized to the AuNPs peak at 2130 cm<sup>-1</sup>, which was considered as internal reference to  
395 eliminate the matrix effect, environmental parameters (temperature and humidity),  
396 instrumental settings (focal distance) and to overcome variations of the enhancement effect  
397 due to the different ratio between the amount of AuNPs and analyte molecules at each  
398 standard concentration. SERS peaks at 997 cm<sup>-1</sup> of PMT standards on silicon dioxide are  
399 shown in Figure 5a. To obtain the calibration curve, normalized SERS intensities at 997 cm<sup>-1</sup>  
400 were plotted as a function of PMT concentration. A WTLS regression was used for fitting the  
401 data of PMT concentrations and Raman intensities taking into account their associated  
402 variance. The uncertainty associated with the concentration values on x axis was calculated by  
403 combining together, according to the law of uncertainty propagation, the different sources of  
404 uncertainties which affect the solution concentration (B-type contributions due to the purity of  
405 the PMT and the volume measurement). The obtained uncertainties are shown, for each point  
406 in Figure 5b, as standard uncertainty bars (with coverage factor  $k = 1$ ) parallel to the x axis.  
407 The standard uncertainty associated with y values (reported as y error bars in Figure 5b) was

408 calculated as A-Type uncertainty on the basis of the standard deviation of the intensities at  
409 997  $\text{cm}^{-1}$  within each Raman map. A linear regression was found between the normalized  
410 Raman signal at 997  $\text{cm}^{-1}$  and the PMT concentration range between 0 - 40  $\text{mg kg}^{-1}$ . The  
411 forcefulness of the fit was confirmed by the reduced chi-square value (i.e. the sum of the  
412 weighted squared residuals normalized by the number of degrees of freedom) which is  
413 attested close to 1 in the considered concentration range. It is worthy to mention that the linear  
414 correlation between concentration and SERS intensity is not held in wider concentration  
415 range, because saturation effects of the SERS saturation effect are observed at higher  
416 contaminant concentration. A drastic decreasing of the enhancement factor is registered for  
417 more concentrated solutions. The LOD of the method, intended as three times the standard  
418 deviation of the blank divided by the calibration curve slope is 4.74 ppm.

419

### Fig. 5

420 However, even if the WTLS regression provided good linearity as demonstrated by low  $\chi^2$   
421 value, the calculated uncertainty for slope and intercept are 14 % and 19 %, respectively.  
422 These values are not negligible and they might affect the reliable quantification of PMT on  
423 real samples. Therefore, it was decided to test a multivariate approach in order to minimize  
424 the random variability associated to a single variable and to consider simultaneously the  
425 whole information contained in spectral data. **A new calibration method was set up using PLS**  
426 **regression to increase the method stability. The plot of the cumulative variance explained**  
427 **(Figure 6a) and the RMSECV (Figure 6b) versus the number permit to determine a reasonable**  
428 **number of component and to define a proper model complexity. For a number of LVs higher**  
429 **than 8 no meaningful information is added, further LVs explain just experimental noise and**  
430 **random variability. A “chemical-shape” is conserved in the loading until LV8 (Figure 3S),**  
431 **then only random variability and experimental noise is carried by further LVs. Useful plots**  
432 **for the model evaluation are reported in supplementary information (Figure 4S, 5S). The**  
433 **method provides an RMSECV (root mean square error in cross validation) of 4.78  $\text{mg kg}^{-1}$**   
434 **and cumulative explained variance of 87.06 %. The model was then validated with 4 samples**

435 contaminated with a nominal PMT concentration of 16 ppm providing an RMSEP of 4.03 mg  
436 kg<sup>-1</sup>. Both calibration and validation samples are shown in Figure 6c.

437

438

### Fig. 6

439 In order to compare the performances of the two proposed methods, green apples spiked with  
440 PMT at 7 mg kg<sup>-1</sup> were analyzed with both methods. The contaminant was recovered from the  
441 apple peel following the optimized washing procedure described in section 2.7. A  
442 contamination level of 8.65 ± 4.09 mg kg<sup>-1</sup> and 6.89 ± 3.06 mg kg<sup>-1</sup> as an average of 4  
443 measurements was obtained using the calibration procedures based on WTLS and PLS,  
444 respectively. Both values were in agreement with the spiked amount of PMT (7 mg kg<sup>-1</sup>) on  
445 the apple, demonstrating the reliability of both methodologies. However, the mean percentage  
446 error (MPE) calculated over 5 repeated measurements was 32.8 % for the univariate  
447 calibration and 18.7 % for the PLS. These results confirm that PLS methodology provides  
448 higher accuracy and intra-day repeatability than univariate analysis and it is more suitable for  
449 PMT detection on pome fruits.

450

## 451 4. Conclusions

452 A sensitive and rapid method to detect, discriminate and quantify residues of the fungicide  
453 pyrimethanil on pome fruits was developed by means of AuNPs and Raman spectroscopy.  
454 Spheroidal AuNPs with different size were fabricated and tested to determine the highest  
455 enhancement factor (EF) for pyrimethanil detection. The analytical procedure was set up on a  
456 silicon dioxide flat surface, here proposed as model system, to standardize SERS  
457 methodology for both qualitative and quantitative analysis. A Raman mapping strategy was  
458 exploited to increase signal reproducibility from spot to spot analysis and to minimize bias  
459 due to different local surface morphologies, which historically affects SERS measurements.  
460 The optimized methodology was tested on apples providing: i) a semi-quantitative *in situ*  
461 detection of the fungicide on the contaminated fruit surface in case of highly contaminated

462 fruits; ii) a metrological tool for pyrimethanil quantification on the entire surface of pome  
463 fruits in accordance with the European law limits. A validation set was used to define the  
464 prediction capability of the model and, most of all, to demonstrate that the multivariate  
465 approach is much worthy in case of a quantitative calibration of enhanced Raman spectra. In  
466 fact, the overall spectral features are better captured and modeled in a multivariate approach  
467 rather than in a univariate calibration, which just considers the Raman intensity at one single  
468 wavelength, leaving out all other information contained in the spectrum. Even if the present  
469 study does not cover the huge variability of natural samples and further studies are required to  
470 prove its efficacy on fruits with different surface chemistry and morphology, the method here  
471 developed guarantees sensitivity and repeatability levels compliant with the application needs  
472 and paves the new way to the calibration of quantitative methods based on SERS for tracing  
473 hazardous chemicals on non-wrinkled fruit surfaces.

## 474 **Acknowledgements**

475 The present work has been supported by EMRP project “ Q-AIMDS”. EMRP is jointly  
476 founded by EMRP participating countries within EURAMET and the European Union.

477 Part of this work was carried out by NanoFacility Piemonte, INRiM, a laboratory supported  
478 by Compagnia di San Paolo (Italy).

479

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583

584 **FIGURE LEGENDS**

585 **Figure 1** - UV-Vis absorption spectra of AuNPs with the mean diameter of a) 30 nm, b) 55  
586 nm, c) 90 nm and d) 120 nm (black curves); UV-Vis spectra of AuNPs stock suspension  
587 mixed in a 2:1 ratio with pyrimethanil standard suspension 400 mg l<sup>-1</sup> at pH~3 (red curves);  
588 repetition of UV-Vis measurements after four days (green curves).

589 **Figure 2** – Raman spectrum of pure Pyrimethanil with the characteristic vibrational peaks'  
590 assignments.

591 **Figure 3** – a) Normal Raman spectrum of 400 mg l<sup>-1</sup> PMT on flat gold surface;  
592 Representative SERS spectra of 400 mg l<sup>-1</sup> PMT using AuNPs with a mean diameter of b)  
593 30 nm, c) 55 nm, d) 90 nm and e) 120 nm on a flat gold surface. Raman mapping of each  
594 PMT-AuNPs spot was created by plotting the profile intensity of the PMT peak at 997 cm<sup>-1</sup>  
595 over the scanned area with a color scale bar from blue to red.

596 **Figure 4** – a) Normal Raman mapping of PMT contaminated region on fresh apple peel;  
597 b) SERS mapping of PMT contaminated region on fresh apple peel after deposition of 120  
598 nm AuNPs. The color scale bar for both a) and b) chemical images is related to the intensity  
599 of the PMT peak at 997 cm<sup>-1</sup>.

600 **Figure 5** – a) Normalized SERS spectra of 120 nm AuNPs with 5 levels of PMT standard in  
601 negative matrix pool (representative of the analyzed matrix): 0, 5, 10, 20 and 40 mg kg<sup>-1</sup>;  
602 b) Calibration curves of PMT standards in negative matrix pool obtained by plotting the  
603 normalized intensity of PMT Raman band at 997 cm<sup>-1</sup> vs. PMT concentration.

604 **Figure 6** – PLS calibration plots: a) Cumulative Explained Variance %; b) Root Mean Square  
605 Error in Calibration and in Cross Validation versus the number of PLS latent variables; c) plot

606 of fitted value corresponding to calibration standards (grey) and validation samples (red)  
607 versus the true values.

Figure1

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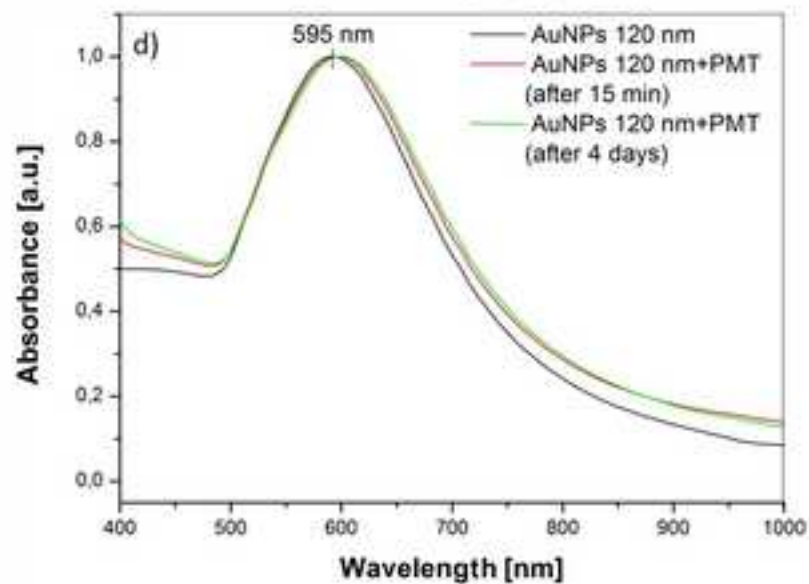
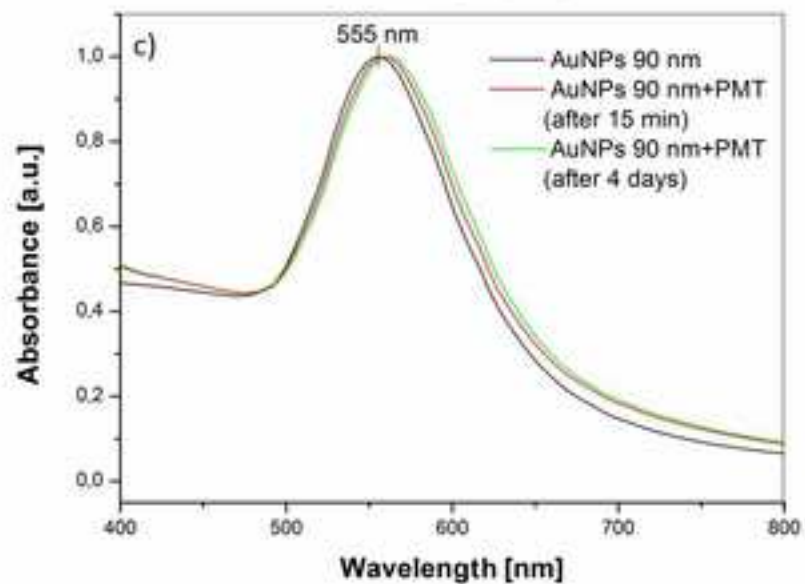
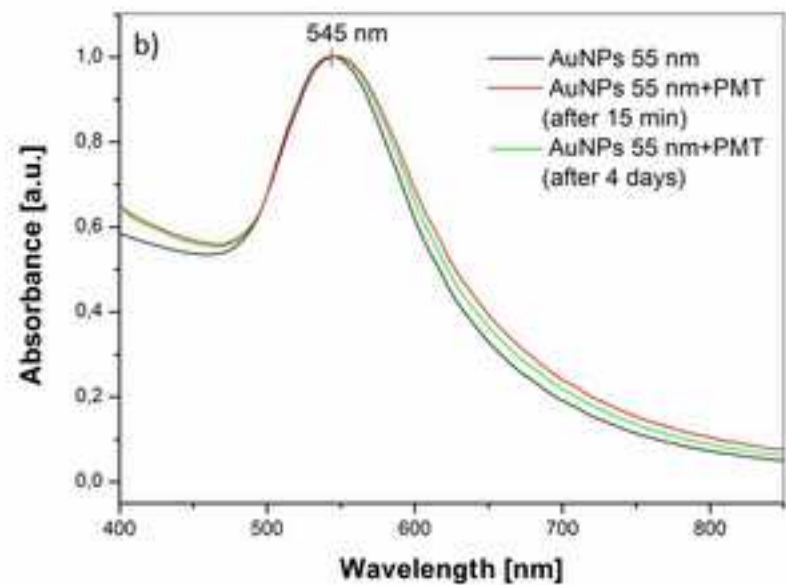
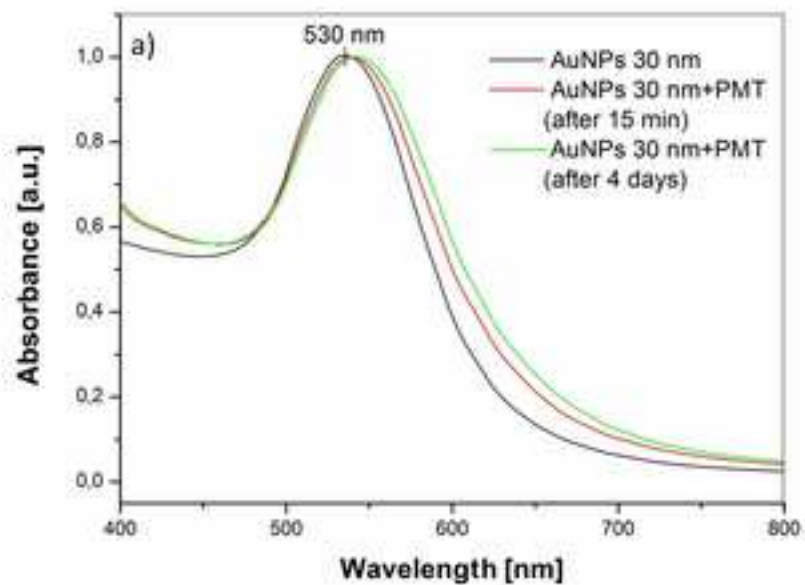


Figure 2

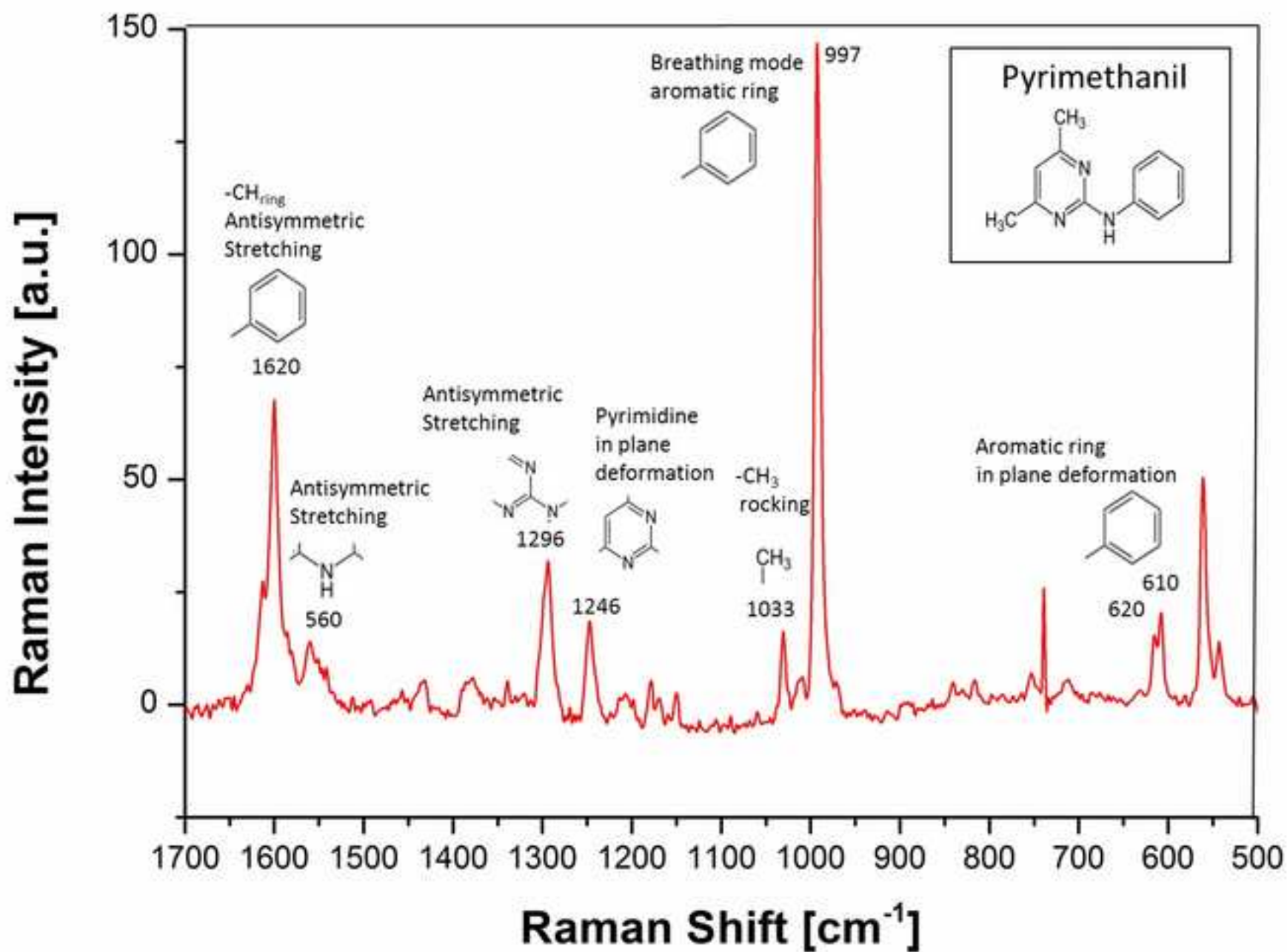
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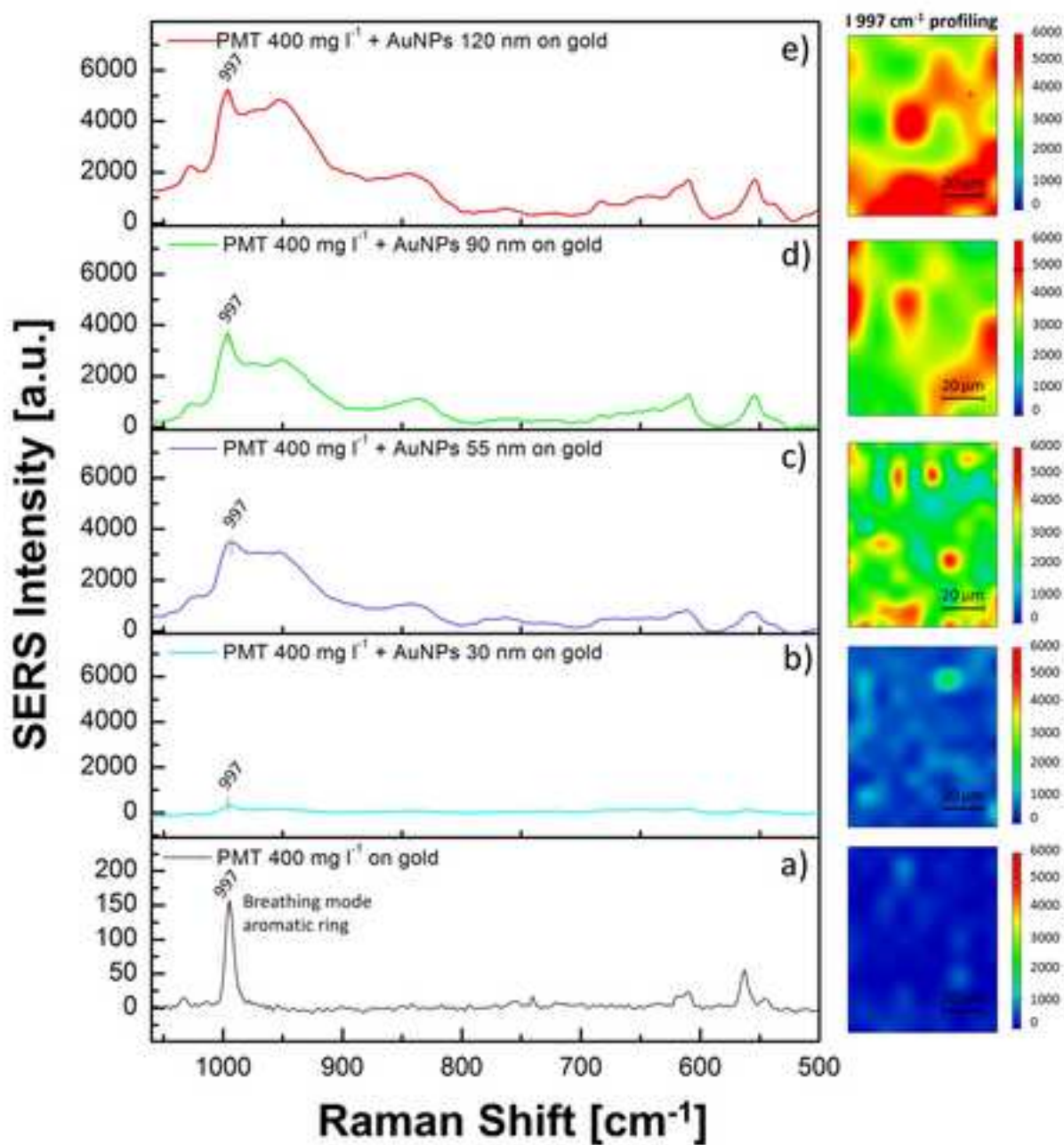




Figure4

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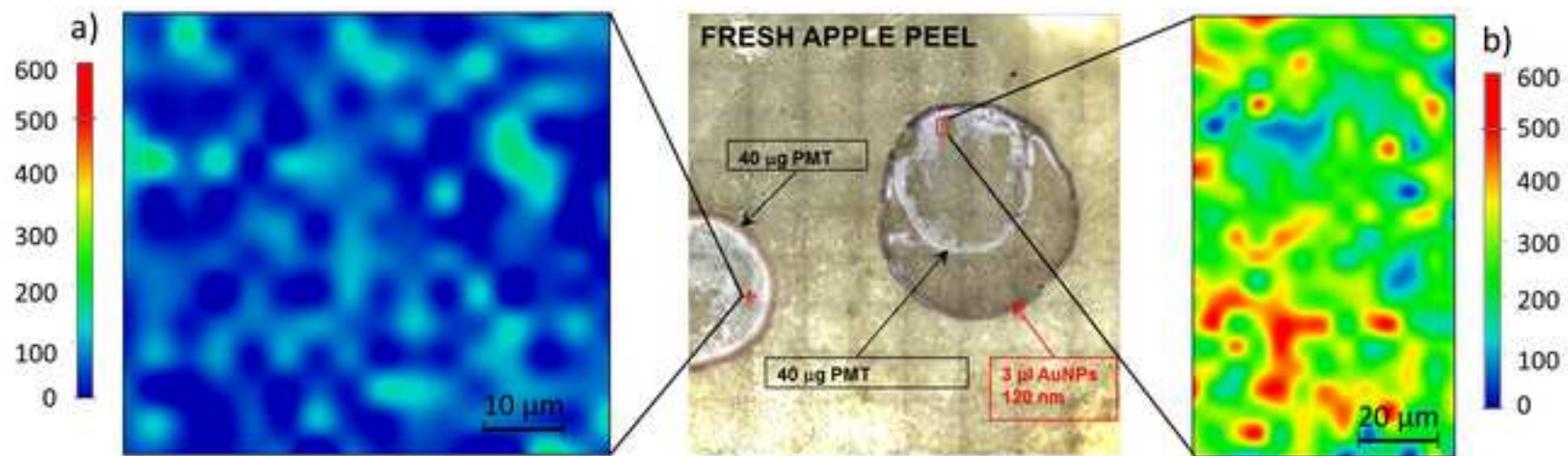


Figure5

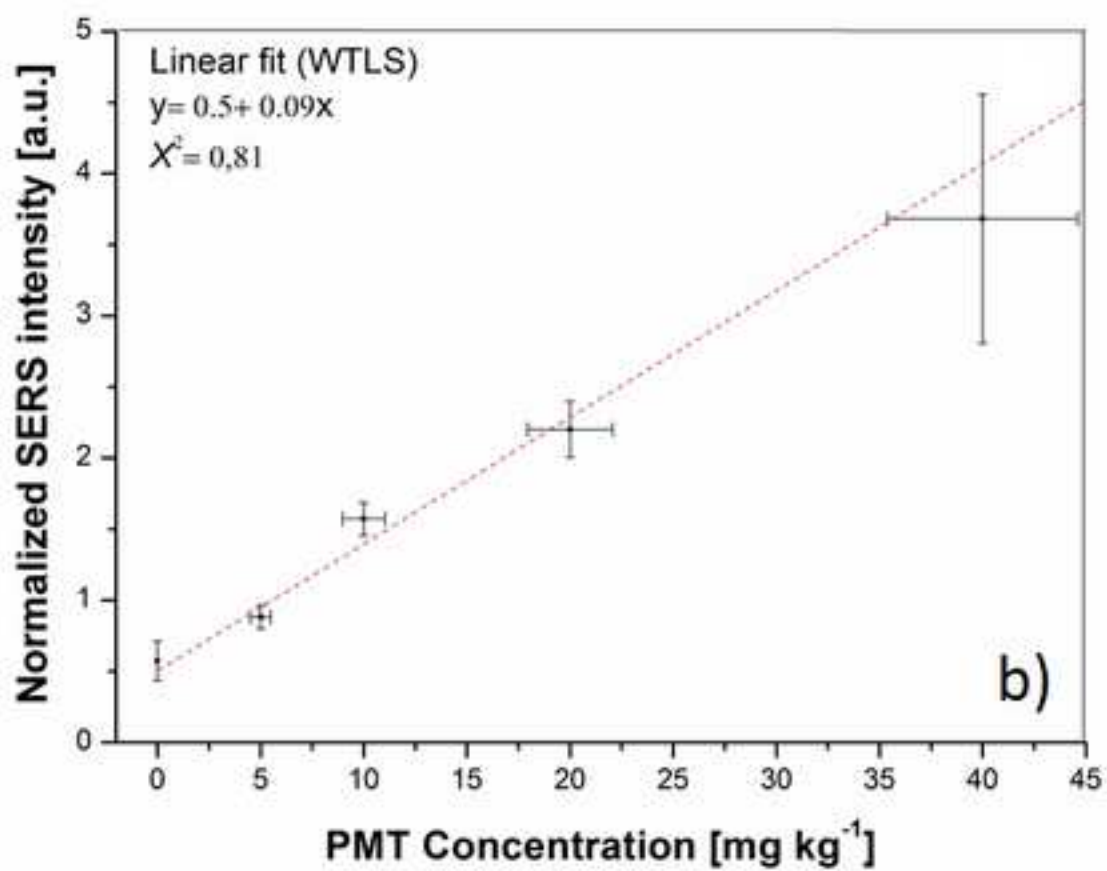
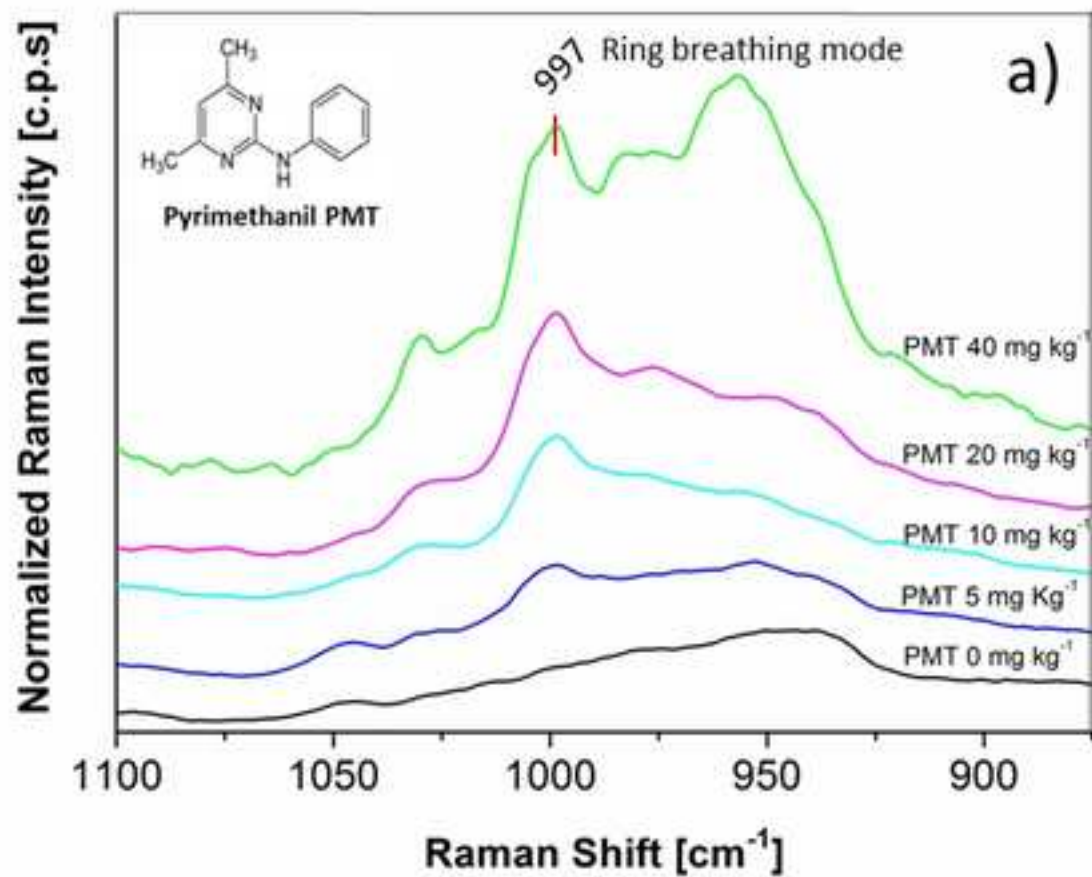
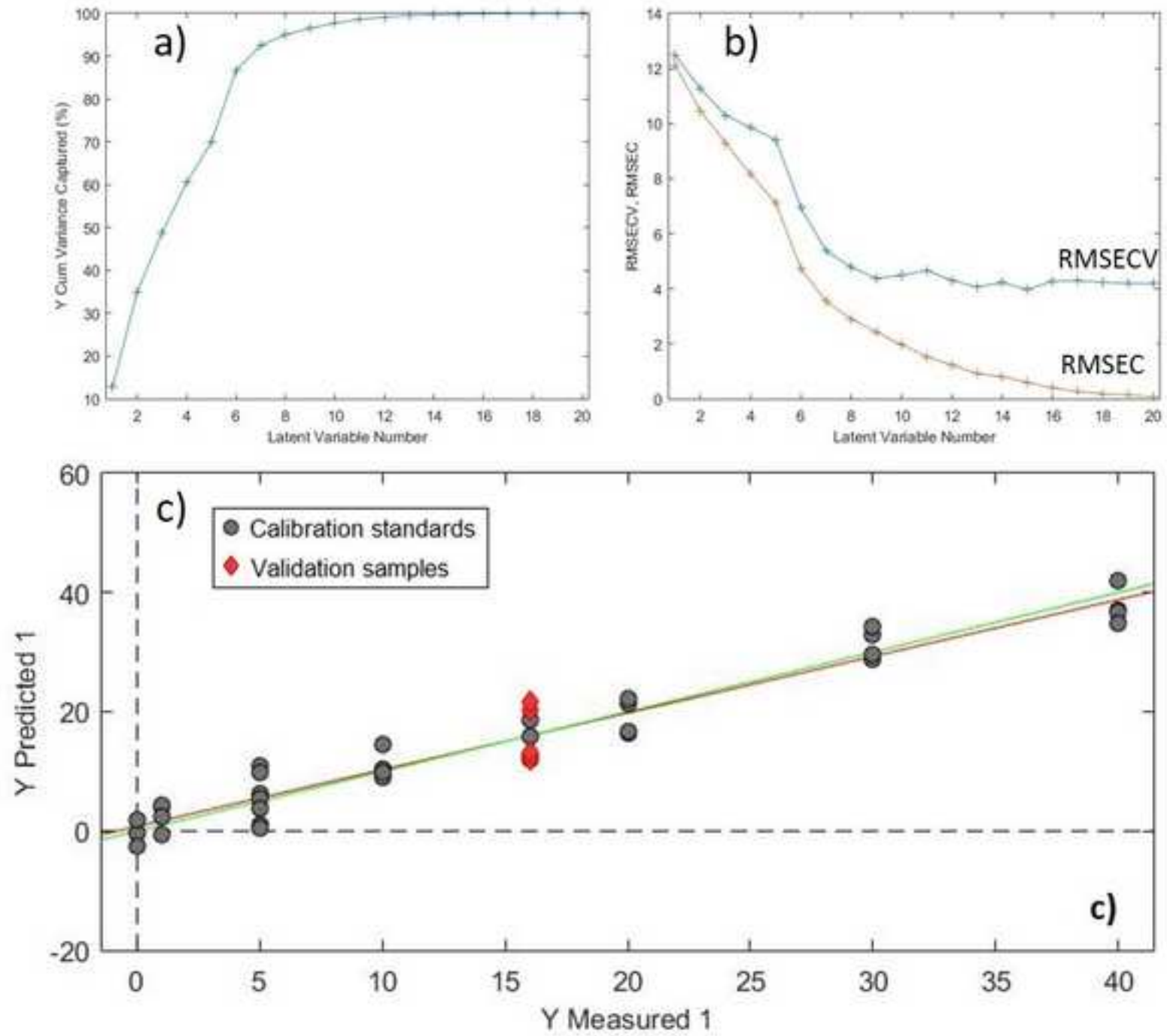
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Figure6

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**Supplementary Material**

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## Highlights

- A rapid and sensitive method to detect pyrimethanil on apples is presented
- Raman signals of the pesticide are enhanced by drop casting AuNPs on the fruit peel
- 120 nm gold nanoparticles provide the best SERS response for this application
- Raman mapping strategy allows to overcome inhomogeneity problems
- Quantitative calibration using Partial Least Squares provides good method stability