



Analytical Methods

Rapid and sensitive detection of pyrimethanil residues on pome fruits by Surface Enhanced Raman Scattering[☆]L. Mandrile^{a,b}, A.M. Giovannozzi^{a,*}, F. Durbiano^a, G. Martra^b, A.M. Rossi^a^a Division of Metrology for Quality of Life, Istituto Nazionale di Ricerca Metrologica, Strada delle Cacce, 91 10135 Torino, Italy^b Department of Chemistry and Interdepartmental Centre NIS, University of Turin, Via Giuria 7, 10125 Turin, Italy

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ABSTRACT

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

1. Introduction

In the last decades food safety incidents have raised public concern about synthetic additives and chemical contaminants in food, the latter of them are mainly agricultural and environmental, chemical adulterants, mycotoxins, and foreign food components (Zheng & He, 2014). Agrochemical products such as pesticides and fungicides are normally applied to protect food crops from the pests at various stages of cultivation and during post-harvest storage (Sharma, Singh, & Singh, 2009), but their intensive use can generate residues that may become a potential risk for both public health and environment (Gilden, Huffling, & Sattler, 2010). Pyrimethanil [(PMT), N-(4,6-dimethylpyrimidin-2-yl)-phenylamine] is a fungicide belonging to the anilinopyrimidine class of pesticides which inhibits the secretion of hydrolytic enzymes by the fungi during the infection process, thus stopping penetration and development of the disease (FAO, 2007). This broad spectrum fungicide is effectively used for the control of gray mould, leaf scab and other postharvest diseases on pome fruits, vegetables and ornamentals (Yu et al., 2013). Even if PMT demonstrated low acute toxicity in mammals, long-term studies showed certain toxicity in mice, rats, dogs and aquatic organisms with potential carcinogenicity. Owing to its toxicity and wide application on horticultural species, PMT has

been included as a pesticide by the Commission Directive 2006/74/EC of August 2006 with a maximum residue level of 7 mg kg⁻¹ on pome fruits (Regulation (EC) No 396/2005). Most of the published methods for the determination of PMT on fruits, vegetables and other samples are usually based on GC (Amvrazi & Tsiropoulos, 2009), GC-MS/MS (Raepel et al., 2011; Rodriguez-Cabo, Rodriguez, Ramil, & Cela, 2011), GC-MS (Gonzalez-Rodriguez, Rial-Otero, Cancho-Grande, & Simal-Gandara, 2008) LC-MS (Park et al., 2010), HPLC (Zhou et al., 2011), and LC-MS/MS (Ortelli, Edder, & Corvi, 2004). These methods have high accuracy, good reproducibility and provide quantitative determination of PMT according to the EU limits, but they also suffer from inevitable disadvantages such as expensive experimental instruments, trained personnel and time-consuming extraction steps. Therefore, the development of more rapid and user friendly methods for PMT residues detection on horticultural species is highly required.

New methodologies based on ELISA (Mercader, Esteve-Turrillas, Agullo, Abad-Somovilla, & Abad-Fuentes, 2012) and electrochemical sensors (Garrido, Rahemi, Borges, Brett, & Garrido, 2016; Yang, Wang, Zhang, Zhang, & Zhang, 2015) were recently proposed as interesting alternatives to the traditional analytical methods, providing high selectivity and sensitivity in PMT detection in real samples. However, no studies were made so far concerning the development of a PMT

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* Corresponding author.

E-mail address: a.giovannozzi@inrim.it (A.M. Giovannozzi).

detection tool based on Surface Enhanced Raman Scattering (SERS). Compared with the conventional analytical techniques, Raman spectroscopy allows fast detection times, high selectivity due to the Raman fingerprint of molecules and minimal or no preliminary treatment of the sample. Moreover, the sensitivity of the normal Raman technique can be increased by several orders of magnitude in SERS analysis due to the enhancement of the Raman scattering of molecules absorbed onto, or microscopically close to, a suitable plasmonically active surface, such as roughened nanostructured metal surface, or metal colloids (Schluecker, 2014). For all these reasons SERS represents a good candidate in food control analysis. Different SERS approaches were already reported on the detection of various classes of pesticides in real-food matrices. In case of solid matrices, homogenization of the peel or surface swab methods were used to recover pesticides from the surface and the detection was subsequently performed using solid surface-based substrates (Fan, Lai, Rasco, & Huang, 2014; He, Chen, & Labuza, 2014). *In situ* detection of pesticides in fruits was also demonstrated using different types of metal nanoparticles (NPs) (Li et al., 2010; Liu et al., 2012) which were spread as “smart dust” over the surface that has to be probed. However, even if these SERS substrates demonstrated to achieve a very high sensitivity in the detection of chemical contaminants, they usually suffer from lack of reproducibility and inconsistent performance when spot-to-spot tests are conducted, leading to problems in the quantification process. Therefore, standardized SERS tool with a good compromise between sensitivity and reproducibility of analysis are needed to provide reliable analytical methods in the detection of food contaminants.

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

2. Material and methods

2.1. Reagents and materials

Hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O} \geq 99\%$), trisodium citrate dihydrate ($\geq 99\%$), were purchased from Sigma-Aldrich (Milan, Italy). Sodium hydroxide ($\text{NaOH} 97\%$), Hydrochloric acid ($\text{HCl} 37\%$), Nitric acid ($\text{HNO}_3 68\%$), absolute ethanol (99.99%), acetone (99.99%) and Hydroxylamine Hydrochloride ($\text{H}_2\text{NO}\cdot\text{HCl}$, 99 + %) were obtained by Novachimica (Milano, Italy). Scala® (400 g/l of Pyrimethanil suspension) was purchased from BASF Italia (Volpiano, Italy). All solutions were prepared with Milli-Q quality water (18 M Ω cm). Silicon wafers with a 300 nm of Silicon dioxide layer on top were purchased from Si-Mat (Kaufering, Germany). Apples used for the assays were purchased in a local supermarket in Torino, Italy.

2.2. Gold nanoparticles preparation

All glassware used in the experiment was soaked in aqua regia ($\text{HCl}:\text{HNO}_3$ 3:1 v/v) and rinsed thoroughly in water and dried with nitrogen prior to use. Spheroidal AuNPs with a diameter of about 30 nm, 40 nm and 55 nm were synthesized according to Frens, 1973. Briefly, 7 ml, 5 ml and 3.5 ml of a 1% aqueous solution of trisodium citrate were rapidly injected into 500 ml boiling solution of HAuCl_4 (0.01% v/v) for the preparation of 30 nm, 40 nm and 55 nm AuNPs, respectively. The mixture was further refluxed for 10 min and then cooled to room temperature under continuous stirring. Larger AuNPs

with a diameter of 90 nm and 120 nm were obtained via seed-mediated growth of 30 nm and 40 nm AuNPs, respectively, using an optimized growing procedure based on hydroxylamine hydrochloride (Li et al., 2013). In detail, 4 ml of Au seeds suspension were put into a round-bottom flask with 53.8 ml of Milli-Q water under continuous stirring and the different solutions were added in the following order: 920 μl of 1% v/v aqueous solution of trisodium citrate (stirring for 3 min), 1.4 ml of 10 mM hydroxylamine hydrochloride solution (stirring for 8 min) and 90 μl of 10% w/v HAuCl_4 (added dropwise, 1 drop per second). The concentration of 30 nm, 55 nm, 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 temperature in the dark before using it.

2.3. Gold nanoparticles characterization

AuNPs characterization was done by UV–Vis absorption measurements and by Scanning Electron Microscopy (SEM) imaging. UV–Vis absorption spectra were collected in the range 400–1100 nm with the Evolution 60s spectrophotometer (Thermo Scientific). The wavelength resolution is 1 nm. SEM characterization was carried out using a SEM FEI Inspect F in UHV mode with the secondary electrons (SE) detector. Typical settings for the imaging are: 10 kV accelerating voltage, 2.5 electron beam spot (18 pA) or 3.5 spot (30 pA), 10 mm WD. By imaging the particles using SEM, size and shape of AuNPs were characterized as well as the size distribution of their particles. At least 300 nanoparticles were counted for each sample to estimate the mean diameter and the relative standard deviation of the AuNPs.

2.4. Preparation of pyrimethanil standard suspensions and solutions

Pyrimethanil solubility in water at room temperature is 0,121 g/l; in case of higher concentration it is dispersed in a stable suspension. Pyrimethanil stock standard suspension was prepared by accurately diluting 2 ml of Scala® (400 g l⁻¹ of Pyrimethanil suspension) in 100 ml and 200 ml of Milli-Q water, to reach a concentration of 8 g l⁻¹ ($8 \cdot 10^3$ ppm) and 4 g l⁻¹. Pyrimethanil standard solutions were prepared by subsequent dilutions from the stock suspensions in water to reach the following concentrations: 40 mg l⁻¹, 30 mg l⁻¹, 20 mg l⁻¹, 10 mg l⁻¹, 5 mg l⁻¹, 1 mg l⁻¹. These pure pyrimethanil standards were used for AuNPs aggregation test, SERS efficiency test and to set up the analytical procedure.

2.5. AuNPs aggregation test

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

2.6. SERS efficiency test

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

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