RAPID DETECTION OF 6-BENZYLAMINOPURINE RESIDUALS USING SURFACE-ENHANCED RAMAN SCATTERING

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The rapid analysis of 6-benzylaminopurine (6-BAP) residue by surface-enhanced Raman spectroscopy with a portable Raman spectrometer is reported. The results showed that the Raman signals of 6-BAP can be significantly enhanced when mixed with a gold nanoparticle colloid substrate. The typical Raman shifts of 6-BAP extraction were at 1002, 1318, and 1336 cm^{-1} and the intensities of normalized characteristic band at 1002 cm^{-1} (I_{1002} to I_{738}) showed high correlation with 6-BAP concentrations. The concentration linear range was 0.1–5.0 μg/mL. The coefficient of determination (R^2) was about 0.99, and calculated RSDs below 10%. The SERS data matched well with the HPLC results.

Keywords: *surface-enhanced Raman scattering, 6-benzylaminopurine, rapid detection.*

БЫСТРОЕ ОБНАРУЖЕНИЕ СЛЕДОВ 6-БЕНЗИЛАМИНОПУРИНОВ С ИСПОЛЬЗОВАНИЕМ ПОВЕРХНОСТНО-УСИЛЕННОГО КОМБИНАЦИОННОГО РАССЕЯНИЯ СВЕТА

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Предложен способ быстрого анализа следов 6-бензиламинопурина (6-БАП) методом поверхностно-усиленной спектроскопии комбинационного рассеяния света (КР) с помощью портативного КР-спектрометра. Показано, что КР-сигналы 6-БАП могут быть значительно усилены при использовании коллоидного субстрата, смешанного с наночастицами золота. Типичные КР-сдвиги при измерениях содержания 6-БАП составляют 1002, 1318 и 1336 $см^{-1}$, а интенсивность нормализованной характеристической полосы при 1002 $см^{-1}$ (I_{1002} до I_{738}) показывает высокую корреляцию с концентрацией 6-БАП. Линейный диапазон концентрации 0.1–5.0 мкг/мл. Коэффициент детерминации $R^2 \approx 0.99$, а рассчитанное относительное стандартное отклонение <10 %. Данные поверхностно-усиленной спектроскопии КР хорошо согласуются с результатами высокоэффективной жидкостной хроматографии.

Ключевые слова: *поверхностно-усиленное комбинационное рассеяние света, 6-бензиламинопурин, быстрое обнаружение.*

Introduction. Synthetic cytokinins are plant growth hormone regulators responsible for stimulating plant cell division [1, 2]. 6-Benzylaminopurine (6-BAP) is a first-generation synthetic cytokinin and has been widely used in the agriculture and forestry fields. It has been shown to elicit plant growth and development responses, promote fruit richness, and extend the shelf life of vegetables [3]. The illegal use of 6-BAP in the short life cycle of sprouts may be potentially harmful to human health and in 2015, a notice issued by the Chinese government forbade the use of 6-BAP and other artificial chemicals in commercial sprout production. In order for its use to be properly curtailed, the development of novel and rapid methods for the detection of 6-BAP is critical.

To date, 6-BAP residues are routinely detected by high-performance liquid chromatography (HPLC) methods [4, 5]. Although successful, this analytical method is time consuming, labor intensive, and its application for on-site testing is restricted. Alternative spectroscopic methods show promise as they are inherently rapid, specific, and can potentially be partially or completely automated. Among these spectroscopic methods, Raman spectroscopy is highly valuable due to its great possibilities and its powerful chemical structure analysis. The Raman spectrum provides a wealth of structural information of the analyzed substances, commonly referred to as the substrates "fingerprint" spectra [6]. However, inelastic scatterings give rise to very weak signals, which restricts its application for ultra-sensitive analysis. Surface-enhanced Raman scattering (SERS) can greatly enhance the normal Raman signals when the molecules are attached or bound to a suitably roughened or nanoscale noble metal surface [7]. Due to its high sensitivity, specificity, and rapidity, SERS has been studied widely for a number of sensing applications, including pesticides [8, 9], environmental contaminants [10, 11], food additives [12], drugs [13, 14], and toxins [15].

In this study, we employed a rapid and sensitive SERS method for the analysis of 6-BAP residues in sprouts utilizing a portable Raman spectrometer. Gold colloidal nanoparticles (NPs) were used as the enhanced substrates and were prepared through sodium citrate reduction of chloroauric acids. Our results indicate that SERS is a simple and sensitive technique for the rapid detection and accurate quantification of 6-BAP residues in bean sprout production. Additionally, it is possible to use the spectrometer for on-site detection and quality control in food safety areas worldwide.

Experiment. Mung bean seeds were collected from a local market. The bean sprout used as blank control was home grown in wet conditions without addition of any chemical additives. 6-BAP (purity > 99%) was obtained from Sigma Aldrich (St. Louis, MO, USA). $HAuCl_4 \cdot 3H_2O$, methanol, ammonium acetate, sodium citrate, petroleum ether, and ethyl acetate were purchased from Beijing Chemical Reagent Co. (Beijing, China). Gold NPs colloids were prepared with sodium citrate to reduce chloroauric acids according to Lee and Meisel's method [16]. The Au colloid was clear wine-red with an absorption maximum at approximately 520 nm. The dimension of Au particle was about 20–30 nm, determined by a particle size analyzer (Zetasizer Nano ZS 90, Malvern Instruments Ltd., UK).

Extraction methods. A portion of 5.0 g bean sprouts was accurately weighed and extracted following different procedures.

(a) In the grinding extraction, a mortar and pestle were used to homogenize the sample thoroughly and the latter rinsed twice with 10 mL of methanol. The amalgamated solution was shaken for about 10 min and centrifuged at 6000*g* for 5 min to remove the debris. The supernatants were condensed and made up to a final volume of 2 mL for SERS analysis.

(b) The above collected supernatant was further purified by solvent extraction using a separating funnel. It was bleached with petroleum ether twice and extracted with ethyl acetate thrice. The organic phase was concentrated to a final volume of 2 mL.

(c) For ultrasonic extraction, the sample was ultrasonically extracted twice with a total of 20 mL of methanol and centrifuged at 6000*g* for 5 min to collect the clear supernatant. An AHLB solid phase extraction column (Waters Oasis, United State) was used to purify the analytes, and the collected extract was filtered through a 0.22 μm filter membrane for both the HPLC and SERS test.

(d) The sample was automatically extracted by a fast solid phase extraction (fast-SPE) instrument (Applied Separations, Inc., PA, USA). It was equipped with six high-pressure autoclaves and used methanol/water (80:20) as the mobile phase. The extraction pressure was set at 100 bar, and the sample was extracted twice within 10 min at room temperature. The extract was collected, centrifuged, concentrated, and made up to a final volume of 2 mL.

Calibration curve. The 6-BAP inserted standard solutions of 5, 2, 1, 0.5, 0.2, and 0.1 μg/mL were prepared by diluting the 6-BAP solution (20 μg/mL) with the blank sample extract. The calibration curve was set up with the abscissa being the 6-BAP concentrations and the ordinate corresponding to the normalized intensities of the characteristic Raman peak.

SERS analysis. After mixing 100 μL sample with 500 μL Au colloid, SERS spectra were collected immediately using a portable Raman spectrometer (RamTrace-200-NF, OptoTrace Technologies, Inc., Sunnyvale, CA) with a 785 nm 200 mW diode laser as the excitation light source. The scanning range was from 400 to 1800 cm^{-1}, and the resolution about 4 cm^{-1}. The acquisition time was set at 10 s and averaged across 2 runs. The spectra of each sample were the average of three repeated scans. The normalization was processed by setting the intensity of the internal standard peak to 1000 using the OptoTrace proprietary software. All figures were plotted and analyzed by Graph Pad Prism statistics software (V. 6.01).

HPLC analysis. According to the Chinese National Standard (GB/T 23381-2009), the 6-BAP was analyzed by the HPLC method [4]. It was equipped with a Waters 600 HPLC pump, a Rheodyne injector fitted with a 10 μL loop, and a Waters 2487 model UV-Vis detector set at 267 nm. Analyses were performed using an analytical column (Pronaos EP-C18 column, 4.6×250 mm, 5 μm, Exformma Technologies) with the mobile phase of methanol:ammonium acetate solution (1:1, v/v) at a flow rate of 1 mL/min. 6-BAP residues were tested according to GB/T 23381-2009 [4].

Results and discussion. All experiments were performed on freshly prepared Au NPs solutions. Upon addition of 6-BAP solution into the Au colloids, the SERS signals of 6-BAP were significant enhanced compared to the non-SERS signals. In the case of SERS, the typical Raman peaks of 6-BAPcan be easily observed at the ppm level, which is the ideal level of sensitivity for micro-analysis.The UV-absorption maximum of Au sol was approximately 520 nm. Mixing of 6-BAP (100 μg/mL) with the Au sol resulted in some minor broadening of the sol's spectrum with limited light absorption by free 6-BAP in the solution (data not shown). It might be speculated that most of the 6-BAP was absorbed onto the surfaces of the Au particles and therefore there was less free 6-BAP in the bulk solution. Meanwhile, the color of the solution changed from red-wine to dark purple, and we noted that Au NPs started to aggregate. This aggregation phenomenon was also reported by others in the field when studying various analytes by this SERS method. Both experimental and theoretical studies indicate that a stronger enhancement effect will be produced when the single NPs form aggregates of two or more NPs owing to the coupling of the electromagnetic field [17, 18].

The major Raman shifts of 6-BAP were at 738, 1002, 1318, and 1336 cm^{-1} (Fig. 1a), which correspond to adenine ring breathing, C_{phenyl}–H bending in plane, C–N stretching, and C–H wagging, respectively [19–21]. The SERS effect may take place in the presence of "hot spots" due to the formation of the aforementioned gold aggregates. It is speculated that strong polarization might occur at the surface of these Au NPs as well as where the electric field increases strongly, which in turn causes the Raman signals to appear weaker than expected. A high concentration of the analyte, however, could help in forming these aggregates in the sol and therefore improve the overall SERS activity. The aggregation rates and the signal intensity were positively correlated with increasing 6-BAP concentration. Therefore, the characteristic peak intensities could be used as an important criterion to quantitatively evaluate 6-BAP content in bean sprouts. According to Fig. 1, the normalized intensities at 1002 cm^{-1} (I_{1002} to I_{738}) showed high correlation with 6-BAP concentrations. The linear range was 0.1–5.0 μg/mL. The coefficients of determination (R^2) reached about 0.99 (Fig. 2).

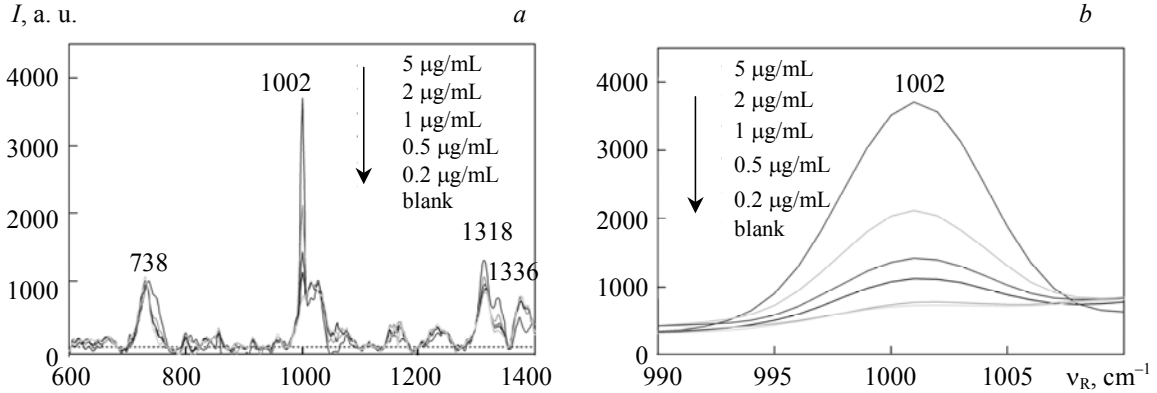

Fig. 1. SERS of 6-BAP in sprout extractions: (a) the peak intensity changed with the different concentrations of 6-BAP, (b) the peak intensities at 1002 cm^{-1}.

Although SERS detection is seen as a nondestructive testing method, different extraction methods were performed due to the sophisticated matrix of plant tissues. The recovery, solvent use, time cost, efficiency, and feasibility were compared among these four extraction methods. The results showed that 6-BAP could be efficiently extracted from the sprouts by each of the extract methods (see Table 1). When the blank samples were intentionally contaminated with 6-BAP at the concentrations of 0.2, 2, and 5 μg/mL, the average recoveries of different pretreatment methods were 71.1–108.0% with the relative standard deviation (RSD) not more than 10%. The grinding extraction method was simple, fast, sensitive, and required no special instrumentation. The whole procedure including sample preparation and SERS detection did not exceed 5 min

in total. The solvent extraction and ultrasonic extraction were further purified by liquid-liquid extraction and solid phase extraction. These processes were somewhat tedious and used a much greater amount of organic solvents, which do not meet the conventions of a green, energy saving, and environmentally benign analytical method. By applying the fast-SPE method, the samples were automatically extracted, and a set of six samples was extracted completely within 30 min. The fast-SPE extraction method was also beneficial as it could be used in conjunction with the analytical instrumentation for automatic detection. According to the IUPAC guidelines, the LOD of this SERS method for 6-BAP detection was calculated according to 3*Sd* of the blank samples. We recorded the lowest detectable concentration of 6-BAP to be 0.33 μg/mL by our SERS method, i.e., 0.13 μg/g. Collectively, the SERS with the simple grinding extraction was fast, sensitive, and feasible for 6-BAP analysis.

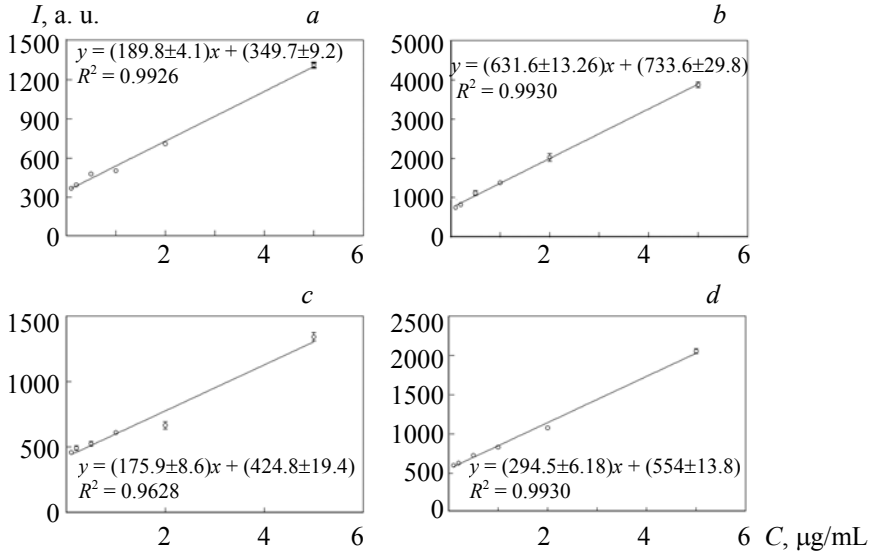

Fig. 2. Calibration curves for 6-BAP detection by SRES: (a) set up using grinding extraction method, (b) using solvent extraction method, (c) using ultrasonic extraction method, and (d) using fast-SPE extracted method.

The commercial samples collected from local markets were extracted and detected by SERS and HPLC simultaneously. The SERS data showed that the 6-BAP residues were 1.02 ± 0.05 and 2.03 ± 0.02 mg/kg, while the HPLC results were 1.13 ± 0.04 and 2.10 ± 0.06 mg/kg. The consistency of the data indicates the accuracy of the SERS results which confirms our belief that SERS is a powerful and promising tool for ultrachemical analysis and rapid on-site detection.

TABLE 1. Comparison of the Different Pretreatment Methods

Pretreatment methods	Solvent usage, mL/5g·sample	Time cost, min	Average recoveries, %	RSD, %
Grinding extraction	12	5	89.5–102.4	7.87
Solvent extraction	35	20	84.5–101.5	9.16
Ultrasonic extraction	22	20	88.1–108.0	10.24
Fast-SPE extraction	12	5	71.1–74.5	2.40

Conclusion. A new method of rapid and quantitative analysis of 6-BAP residues on gold nanoparticles has been established using surface-enhanced Raman spectroscopy. The Raman spectrum were collected in several seconds, and this method benefits from the use of portable equipment and simple sample preprocessing. We believe that this method is more intuitive, simple, time saving and operationally easy and is especially suitable for on-site testing and quality control in field applications. We argue that it will provide an efficient and sensitive solution to large-scale on-site rapid detections of various analytes. The prediction ac-

curacy showed that SERS would be a promising technique for rapid tracing analysis, especially for on-site testing and quality control in field applications.

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