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# Monitoring the Chemical Production of Citrus-Derived Bioactive 5-Demethylnobiletin Using Surface-Enhanced Raman Spectroscopy

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Supporting Information

**ABSTRACT:** To develop an accurate and convenient method for monitoring the production of citrus-derived bioactive 5-demethylnobiletin from the demethylation reaction of nobiletin, we compared surface-enhanced Raman spectroscopy (SERS) methods with a conventional high-performance liquid chromatography (HPLC) method. Our results show that both the substrate- and solution-based SERS methods correlated with the HPLC method very well. The solution method produced lower root-mean-square error of calibration and higher correlation coefficient than the substrate method. The solution method used an "affinity chromatography"-like procedure to separate the reactant nobiletin from the product 5-demthylnobiletin based on their different binding affinities to the silver dendrites. The substrate method was found simpler and faster to collect the SERS "fingerprint" spectra of the samples because no incubation between samples and silver was needed and only a trace amount of samples was required. Our results demonstrated that the SERS methods were superior to the HPLC method in conveniently and rapidly characterizing and quantifying 5-demethylnobiletin production.

**KEYWORDS:** *SERS, HPLC, nobiletin, 5-demethylnobiletin*

## INTRODUCTION

Nobiletin, a polymethoxyflavone mainly found in citrus fruits, has been studied for many years, and it was shown to possess a variety of bioactivities, such as anti-inflammatory,[1] anticarcinogenic,[2] and antiatherosclerosis activities.[3] Recently, a novel derivative of nobiletin, 5-demethylnobiletin, has attracted more attention because new studies have demonstrated that 5-demthylnobiletin had stronger bioactivities than nobiletin; e.g., it was more potent in inhibiting the growth of different cancer cells than nobiletin.[4−6] 5-Demthylnobiletin is also a component of citrus fruits, especially in their peels. However, the abundance of 5-demthylnobiletin is much lower than nobiletin in a citrus plant, which greatly limits the use of 5-demthylnobiletin as a potential nutraceutical ingredient. During the storage of citrus extracts, nobiletin can undergo autodemethylation reaction to be converted to 5-demthylnobiletin. This reaction can also be accelerated by the addition of organic and inorganic acids. To characterize the demethylation reaction of nobiletin and monitor the production of 5-demthylnobiletin, an accurate and convenient method is needed. It is known that high-performance liquid chromatography (HPLC) can be used to precisely quantify the loss of the reactant and the yield of the product during demethylation reactions.[1,7,8] However, the HPLC method is generally time-consuming and labor-intensive. Furthermore, the similarity of the chemical structures of nobiletin and 5-demthylnobiletin presented difficulty in discriminating them by the HPLC method. To rapidly monitor the yield of chemical reactions, thin-layer chromatography (TLC) is one of the most common methods; nevertheless, the TLC method cannot be used for quantification with high accuracy.[9] Herein, we aimed to develop a novel method for the convenient quantification of demethylation of nobiletin and production of 5-demthylnobiletin.

Raman spectroscopy is one of the molecular vibrational spectroscopies that can measure the chemical "fingerprints" of the analytes nondestructively and rapidly. With the aid of the nanostructure of noble metal, surface-enhanced Raman spectroscopy (SERS) has demonstrated its advanced capacity in measuring a trace amount of analytes. The mechanisms of SERS enhancement are largely attributed to an electromagnetic field induced by localized surface plasmon resonance (LSPR) as well as chemical interactions of the analyte with the substrate.[10] Sample preparations are simple, rapid, and versatile. Previously, two preparation methods, substrate- and solution-based methods, using silver (Ag) dendrites were described.[11] The combinative features of molecular "fingerprint" specificity, ultrasensitivity, and rapid analysis provide potential applications of SERS in rapid and sensitive determination of a chemical reaction course based on differentiating between the SERS patterns of reactants and products. In this study, we used SERS to monitor the production of 5-demethylnobiletin from the demethylation reaction of nobiletin. Two sample preparation methods (substrate and solution methods) were used and compared. The SERS results were also correlated with results

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from the HPLC method. To our knowledge, this is the first study that SERS has been used to monitor such a chemical reaction.

## MATERIALS AND METHODS

**Demethylation Reaction.** Nobiletin standard (>98%) was isolated from sweet orange peel as previously described.[5] 5-Demethylnobiletin was produced from demethylation of nobiletin by the acidolysis reaction (Figure 1). This reaction (1 g of nobiletin in

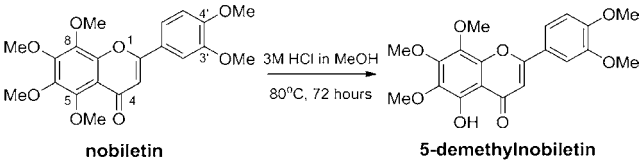


**Figure 1.** Chemical production of 5-demethylnobiletin from demethylation of nobiletin.

1000 mL of 3 M hydrochloric acid) was conducted at 80 °C and then quenched at 72 h to obtain a satisfactory high yield. To monitor the chemical reaction course, the yield of 5-demethylnobiletin was tested at different reaction times (i.e., 1, 2, 4, 8, 16, 24, 48, and 72 h). Reaction mixtures were adjusted to neutral pH to stop the reaction and then extracted with ethyl acetate. The ethyl acetate extracts were used directly as test samples for the SERS analysis. The ethyl acetate extracts were also dried under vacuum and then redissolved in 50% methanol for HPLC analysis.

**HPLC Analysis of the Production of 5-Demethylnobiletin.** Determination of the yield of 5-demethylnobiletin at different reaction times was carried out by a CoulArray HPLC system (Chelmsford, MA) consisting of a binary solvent delivery system (model 584), an autosampler (model 542), and an ultraviolet (UV) detector (model 526) (Waters, Milford, MA) using our previously published method with modification.[12] Instrument control and data processing were performed with CoulArray 3.06 software. An Ascentis RP-Amide reversed-phase HPLC column (15 cm × 4.6 mm inner diameter, 3 $\mu$m) (Sigma–Aldrich, St. Louis, MO) was used. The mobile phases consisted of solvent A [75% water, 20% acetonitrile, 5% tetrahydrofuran (THF), and 50 mM ammonium acetate at pH 3.0] and solvent B (50% water, 40% acetonitrile, 10% THF, and 50 mM ammonium acetate at pH 3.0). The flow rate and injection volume were set to 1 mL/min and 10 $\mu$L, respectively. The temperature of the autosampler was set to 4 °C. The detection wavelength of the UV detector was set at 330 nm. The elution condition has been improved for the detection of the reactant and product. Standard curves of the standard compounds were constructed by plotting concentrations ($x$ axis, $\mu$M) versus peak areas ($y$ axis, $\mu$C). Quantification of the reaction products at different reaction times was performed by comparing their peak areas to the standard stock solutions of a series of concentrations.[13]

**SERS Analysis of the Production of 5-Demethylnobiletin.** Ag dendrites were prepared through a simple displacement reaction involving both zinc and silver nitrate according to a previously published method.[14] Two methods have been used to prepare the samples for SERS measurement using Ag dendrites, the substrate method, and the solution method.

The substrate method prepared for SERS analysis was illustrated in Figure 2a: 5 $\mu$L of Ag dendrite was deposited onto a glass slide and air-dried, and then 10 $\mu$L of the test sample solution (about 500 $\mu$M dissolved in ethyl acetate) was deposited on the dried Ag and dried for Raman measurement.

The solution method prepared for SERS analysis was illustrated in Figure 2b: 200 $\mu$L of test sample (about 50 $\mu$M dissolved in methanol) was mixed with 5 $\mu$L of Ag dendrites for 10 min under consistent orbital rotation at room temperature. After washing 3 times using double-distilled water, the Ag mixture (5 mL) was deposited onto a glass slide and air-dried for Raman measurement.

The SERS spectra of each sample were collected using a DXR Raman microscope (Thermo Scientific) in this study. This instrument includes a 780 nm excitation laser and a confocal microscope with a 10× objective. The resulting laser spot diameter is about 3 $\mu$m with a resolution of 5 cm$^{-1}$. The Raman measurement was taken with 2 mW of laser power and a 50 $\mu$m slit aperture for a 1 s scanning time. Spectra were collected using the Thermo Scientific OMNIC software. Six spectra were collected from each sample.

TQ analyst software, version 8.0 (Thermo Fisher Scientific), was used to analyze the data obtained from the DXR Raman microscope. Principal component analysis (PCA) was applied to analyze the variance of spectral data, which reduced a multidimensional data set to its most dominant features, removed random variation, and retained the principal components (PCs) that capture the variation between sample treatments. The information provided by the PCA shows the variance within a class and between different classes. The PC score reveals the percentage of data variance.[15,16] A multivariate statistical model, the partial least-squares (PLS) model, was constructed to predict the sample amount based on the reference values based on HPLC analysis. The root-mean-square error of calibration (RMSEC) and the correlation coefficient ($R$) were used to describe the quality of the model. The closer the RMSEC value is to 0 and the higher $R$ is to 1 indicate a better model. Before constructing PCA and PLS, second-derivative transformation was used to separate overlapping bands and remove baseline shifts.

## RESULTS AND DISCUSSION

**HPLC Determination of the Production of 5-Demethylnobiletin.** To obtain satisfactory resolution in a single run on HPLC, two complex mobile phases A and B were needed because of the structural similarity between the reactant nobiletin and product 5-demethylnobiletin. The primary linear solvent gradient consisted of 10% B at 0 min, 50% B at 5 min, 70% B at 15 min, 90% B at 25 min, and 10% B at 30 min. The retention times of nobiletin and 5-demethylnobiletin were 11.8

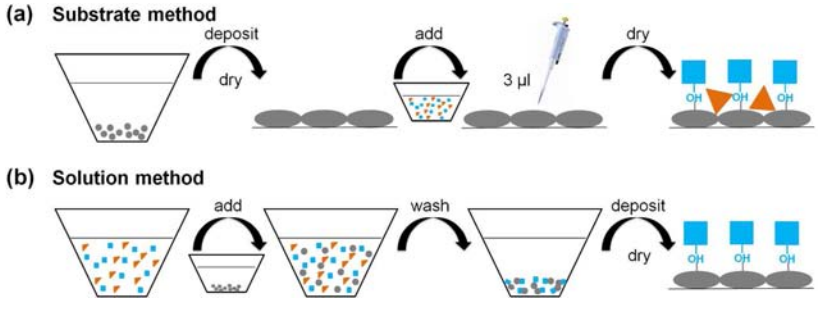


**Figure 2.** Schematic illustration of SERS effects using the (a) substrate method and (b) solution method. "Circle", "oval", "triangle", and "square" were used to represent Ag dendrites, dried Ag dendrites, nobiletin molecular, and 5-demethylnobiletin, respectively.


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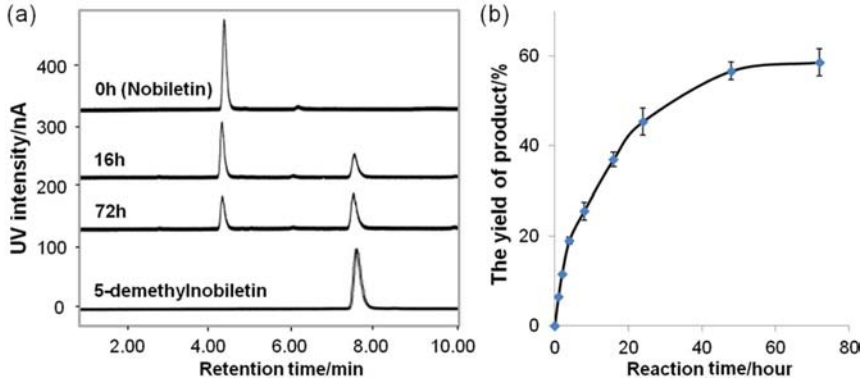


**Figure 3.** HPLC analysis of the production of 5-demethylnobiletin at different reaction times: (a) HPLC profiles and (b) yields (in percentage) at different reaction times quantified by HPLC.

and 21.0 min, respectively. The elution condition has further been improved to decrease the running time per run, which was modified as the isocratic elution condition of 50% B within 10 min (shown in Figure 3a). The analysis time was decreased from 30 to 10 min with satisfactory resolution (the retention time of 4.3 min for nobiletin and 7.5 min for 5-demethylnobiletin). For standard reactant and product, linear calibration curves can be obtained within the range of 1−100 $\mu$M, and their regression equations are as follows: $y = 0.0821x$ ($r^2 = 0.9993$) and $y = 0.0729x$ ($r^2 = 0.9999$), respectively. Quantification of the reaction products at different reaction times was performed by comparing their peak areas to the standard stock solutions of a series of concentrations. The yields of 5-demethylnobiletin in percentage were determined as 6.3, 11.4, 19.1, 25.5, 37.0, 45.4, 56.7, and 58.8 at the reaction times of 1, 2, 4, 8, 16, 24, 48, and 72 h (Figure 3b), respectively.

**SERS Spectra of Nobiletin and 5-Demethylnobiletin.** The average raw SERS spectra ($N = 6$) of chemical standards on the Ag dendrites were shown in Figure S1 of the Supporting Information. Second-derivative transformation was used here to separate overlapping bands and remove baseline shifts, so that the differences between different spectra were distinct (in Figure 4a). Bare Ag dendrites have a peak at 1075 $cm^{-1}$, which is due to the $NO_3^-$ residue during the preparation. This peak can be used as the internal standard to normalize the other peak intensities for improved quantification. For the substrate method, as the samples were deposited onto the Ag dendrites, we were able to see both signals of nobiletin and 5-demethylnobiletin. For the solution method, only the 5-demethylnobiletin signal but no nobiletin signal can be seen, which was confirmed in the PCA plot; i.e., the data clusters of negative control and nobiletin (substrate method) were largely overlapped (Figure 4b). In the previous study, we found that the hydroxyl group played an important role in the interaction with the Ag surface.[17] Nobiletin, which has no hydroxyl group, was not able to bind Ag. However, 5-demethylnobiletin with one hydroxyl group was able to bind Ag. Therefore, using the solution method, after washing, the reactant nobiletin can be washed away, while the product 5-demethylnobiletin was still bound to Ag (serving as a stationary phase). Our results showed that the solution method used an "affinity chromatography"-like mechanism based on the different affinities of nobiletin and 5-demethylnobiletin on Ag. The lower peak intensity of nobiletin than 5-demethylnobiletin in the substrate method was also due to the fact that nobiletin was unable to be

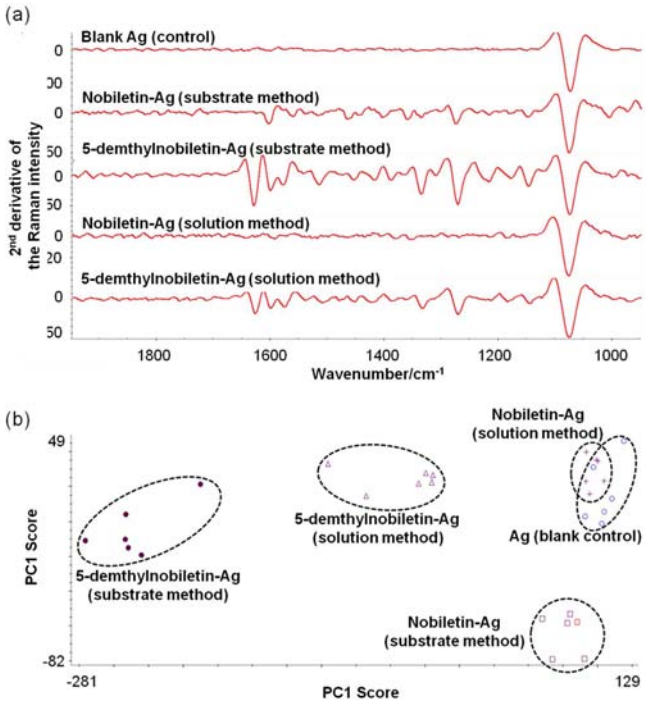


**Figure 4.** Second-derivative transformation of (a) SERS spectra and (b) PCA plot analysis of nobiletin and 5-demethylnobiletin by substrate and solution methods.

bound to Ag; therefore, chemical enhancement was much lower than the bound 5-demethylnobiletin.[18]

**Correlation between SERS and HPLC Analyses of the Production of 5-Demethylnobiletin.** The SERS spectra of samples from different reaction times were used to correlate with HPLC data of 5-demethylnobiletin. For the substrate method, the best correlation ranges determined by the software were 1590−1552 and 1627−1607 $cm^{-1}$ (Figure 5a). Within this range, there was less interfence from nobiletin. The RMSEC was 8.60, and $R$ was 0.96, with six factors used (Figure 5b). For the solution method, the best correlation ranges were 1650−1606 and 1398−1409 $cm^{-1}$, which contained the major characteristic peaks of 5-demethylnobiletin (Figure 5c). The RMSEC was 5.88, and $R$ was 0.98, with six factors used (Figure 5d). Both substrate and solution methods were found to correlate with the HPLC method very well. The solution method produced lower RMSEC and higher $R$ than the substrate method. In this particular case, the solution method


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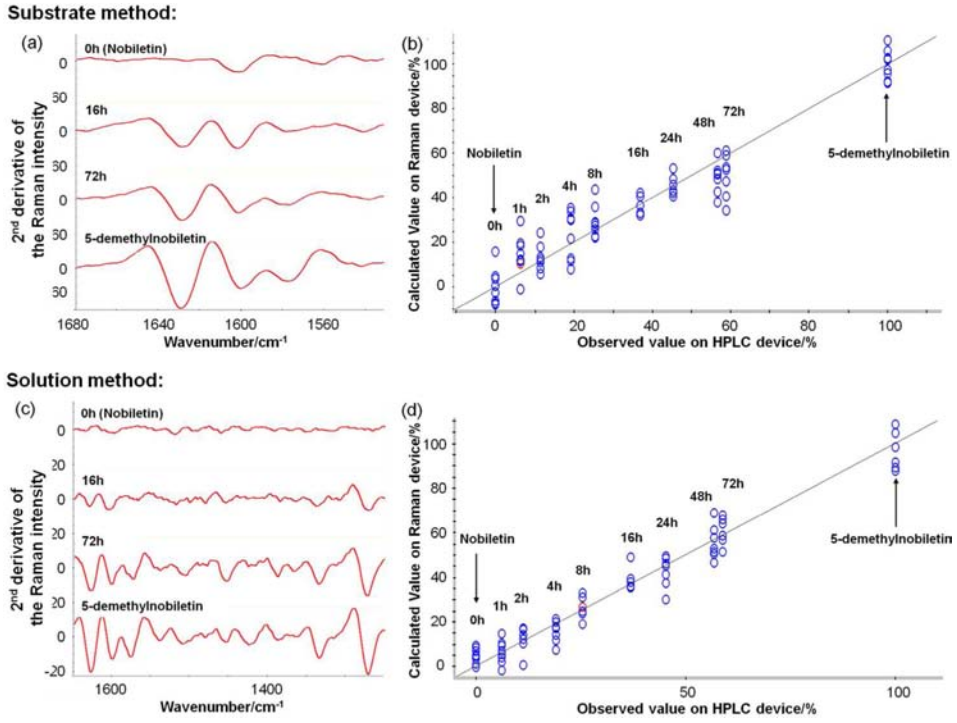


**Figure 5.** Second-derivative transformation of (a and c) SERS spectra and (b and d) partial least-squares of chemical reaction mixtures and 5-demethylnobiletin determined by the substrate method (wavenumber from 1540 to 1680 $cm^{-1}$) and solution method (wavenumber from 1250 to 1650 $cm^{-1}$).

acted as an "affinity chromatography"-like procedure. On the basis of different affinities of the reactant nobiletin and the product 5-demethylnobiletin with the Ag dendrites, the weakly bound reactant nobiletin can be washed away, while the tightly bound product 5-demethylnobiletin can be retained on the Ag surface. In this way, stronger signals can be obtained from the product 5-demethylnobiletin without significant interference from the reactant nobiletin. Furthermore, the substrate method also provided other advantages. For example, it was simpler and faster to collect the SERS "fingerprint" spectra of the sample because no incubation between the sample and Ag was needed and only a trace amount of sample (a few microliters) was required.

In summary, we successfully monitored and characterized the demethylation reaction of citrus-derived nobiletin to produce more bioactive 5-demthylnobiletin both using SERS. SERS methods were found to offer several advantages over the conventional HPLC method. First, it was time-consuming and labor-intensive to optimize the sample preparation and elution conditions of the HPLC method.[12,19] SERS methods measure samples on the nanosubstrate directly without tedious sample preparation, which is very useful in characterization of any rapid chemical reaction course. Second, the HPLC detection is based on a good separation (different elution times) of the reactants and products, which is challenging in some cases. For SERS methods, reactants and products can be easily differentiated on the basis of their distinct "fingerprint" SERS patterns without physically separating them. In addition, rapid and accurate quantification in multicomponent systems can be realized directly on the basis of the fact that each of the individual components has a unique Raman signature. Third, two SERS sample preparation methods, substrate and solution, offer versatile applications. Particularly, the solution method, which is based on binding affinity between test compounds and the Ag surface, provides a novel "SERS chromatography" technique for molecular characterization and quantification conveniently, rapidly, and accurately.

## ■ ASSOCIATED CONTENT

### Supporting Information

Average raw SERS spectra of chemical standards on the Ag dendrites. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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