Published in final edited form as:

J Agric Food Chem. 2013 August 28; 61(34): 8079–8083. doi:10.1021/jf4027475.

Monitoring the chemical production of citrus-derived bioactive 5-demethylnobiletin using surface enhanced Raman spectroscopy

Jinkai Zheng^{†,‡}, Xiang Fang^{†,§}, Yong Cao[§], Hang Xiao^{†,*}, and Lili He^{†,*}

[†]Department of Food Science, University of Massachusetts, Amherst, MA, USA

[‡]Institute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China

[§]College of Food Science, South China agricultural university, Guangzhou, Guangdong, China

Abstract

To develop an accurate and convenient method for monitoring the production of citrus-derived bioactive 5-demethylnobiletin from demethylation reaction of nobiletin, we compared surface enhanced Raman spectroscopy (SERS) methods with a conventional HPLC method. Our results show that both the substrate-based and solution-based SERS methods correlated with 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 utilized an ‘affinity chromatography’-like procedure to separate the reactant nobiletin from the product 5-demethylnobiletin based on their different binding affinity to the silver dendrites. The substrate method was found simpler and faster to collect the SERS ‘fingerprint’ spectra of the samples as no incubation between samples and silver was needed and only trace amount of samples were required. Our results demonstrated that the SERS methods were superior to 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 anti-carcinogenic,² and anti-atherosclerosis activities.³ Recently, a novel derivative of nobiletin, 5-demethylnobiletin has attracted more attention because new studies have demonstrated that 5-demethylnobiletin had stronger bioactivities than nobiletin, e.g., it was more potent in inhibiting the growth of different cancer cells than nobiletin^{4–6}. 5-Demethylnobiletin is also a component of citrus fruits, especially in their peels. However, the abundance of 5-demethylnobiletin is much lower than nobiletin in citrus plant, which greatly limits the utilization of 5-demethylnobiletin as a potential nutraceutical ingredient. During the

*Correspond to: Lili He (lilihe@foodsci.umass.edu) and Hang Xiao (hangxiao@foodsci.umass.edu), 102 Holdsworth Way Chenoweth, Department of Food Science, University of Massachusetts Amherst, Amherst, MA 01003, USA.

Supporting information

Supporting Information available: the 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>.

storage of citrus extracts, nobiletin can undergo auto-demethylation reaction to be converted to 5-demethylnobiletin. This reaction can also be accelerated by addition of organic and inorganic acids. To characterize the demethylation reaction of nobiletin and monitoring the production of 5-demethylnobiletin, an accurate and convenient method is needed. It is known that HPLC can be used to precisely quantify the loss of reactant and the yield of product during demethylation reactions.^{1,7,8} However, HPLC method is generally time-consuming and labor-intensive. Furthermore, the similarity of the chemical structures of nobiletin and 5-demethylnobiletin presented difficulty in discriminating them by 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-demethylnobiletin.

Raman spectroscopy is one of the molecular vibrational spectroscopies that can measure the chemical ‘fingerprints’ of the analytes non-destructively and rapidly. With the aid of nanostructure of noble metal, surface enhanced Raman spectroscopy (SERS) has demonstrated its advanced capacity in measuring trace amount of analytes. The mechanisms of SERS enhancement are largely attributed to 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-based and solution-based methods, using silver (Ag) dendrites were described.¹¹ The combinative features of molecular ‘fingerprint’ specificity, ultra-sensitivity, 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 utilized SERS to monitor the production of 5-demethylnobiletin from the demethylation reaction of nobiletin. Two sample preparation methods (substrate method and solution method) were used and compared. The SERS results were also correlated with results from HPLC method. To our knowledge, this is the first study that SERS has been utilized to monitor such chemical reaction.

Materials and methods

The demethylation reaction

Nobiletin standard (>98%) was isolated from sweet orange peel as previously described.⁵ 5-Demethylnobiletin was produced from demethylation of nobiletin by acidolysis reaction (Fig. 1). This reaction (1 g nobiletin in 1000 ml 3M hydrochloric acid) was conducted at 80°C and then quenched at 72 hours to obtain satisfactory high yield. In order to monitor the chemical reaction course, the yield of 5-demethylnobiletin was tested at different reaction time (i.e., 1, 2, 4, 8, 16, 24, 48, and 72 hours). 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 re-dissolved in 50% methanol for HPLC analysis.

HPLC analysis of the production of 5-demethylnobiletin

Determination of the yield of 5-demethylnobiletin at different reaction time was carried out by CoulArray® HPLC system (Chelmsford, MA, USA) consisted of a binary solvent delivery system (model 584), an auto-sampler (model 542), and a UV detector (model 526) (Waters, Milford, MA, USA) using our previously published method with modification.¹² Instrument control and data processing were performed with CoulArray 3.06 software. Ascentis RP-Amide reversed-phase HPLC column (15cm×4.6mm id, 3 µm) (Sigma–Aldrich, MO, USA) was used. The mobile phases consisted of solvent A (75% water, 20% acetonitrile, 5% THF and 50 mM ammonium acetate, pH 3.0) and solvent B (50% water,

40% acetonitrile, 10% THF and 50 mM ammonium acetate, pH 3.0). Flow rate and injection volume were set to 1 mL/min and 10 μ L, respectively. The temperature of autosampler was set to 4 $^{\circ}$ C. The detection wavelength of 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, μ M) vs peak areas (y axis, μ C). Quantification of the reaction products at different reaction time was performed by comparing their peak areas with the standard stock solutions of 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 utilized to prepare the samples for SERS measurement using Ag dendrites, the substrate method and the solution method.

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

The solution method preparing for SERS analysis was illustrated in Fig. 2b: 200 μ L of test sample (about 50 μ M dissolved in methanol) was mixed with 5 μ L Ag dendrites for 10 min under consistent orbital rotation at room temperature. After washing three 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 10 $\times$ objective. The resulting laser spot diameter is about 3 μ m with resolution of 5 cm^{-1} . Raman measurement was taken with 2 mW of laser power and 50- μ m slit aperture for 1 s scanning time. Spectra were collected using the Thermo Scientific OMNICTM Software. Six spectra were collected from each sample.

TQ analyst software v8.0 (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, removes random variation, and retains 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 square (PLS) model, was constructed to predict 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 zero and the higher R indicate a better model. Before constructing PCA and PLS, 2nd derivative transformation was used to separate overlapping bands and remove baseline shifts.

Results and discussion

HPLC determination of the production of 5-demethylnobiletin

In order to obtain satisfactory resolution in a single run on HPLC, two complex mobile phases A and B were needed due to 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 and 21.0 min, respectively. 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 Fig 3a). The analysis time was decreased from 30 min 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 μM , 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 time were performed by comparing their peak areas with the standard stock solutions of 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 time of 1, 2, 4, 8, 16, 24, 48, and 72 hours (Fig. 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 the supporting information (S1). Second derivative transformation was used here to separate overlapping bands and remove baseline shifts, so that the differences between different spectra were distinct (in Fig. 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 intensity 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 5-demethylnobiletin signal but no nobiletin signals 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 (Fig. 4b). In the previous study, we found hydroxyl group played an important role in the interaction with Ag surface.¹⁷ Nobiletin which has no hydroxyl group wasn't 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 the Ag (serving as a stationary phase). Our results showed that the solution method utilized an 'affinity chromatography'-like mechanism based on the different affinity of nobiletin and 5-demethylnobiletin onto the 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 bound onto Ag, therefore chemical enhancement was much lower than the bound 5-demethylnobiletin.¹⁸

Correlation between SERS and HPLC analysis 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 $1590\text{--}1552$ and $1627\text{--}1607\text{ cm}^{-1}$ (Fig. 5a). Within this range, there was less interference from nobiletin. The RMSEC was 8.60 and R was 0.96 with 6 factors used (Fig. 5b). For the solution method, the best correlation ranges were $1650\text{--}1606$ and $1398\text{--}1409\text{ cm}^{-1}$, which contained the major characteristic peaks of 5-demethylnobiletin (Fig. 5c). The RMSEC was 5.88 and R was 0.98 with 6 factors used (Fig. 5d). Both substrate method and solution method were found to correlate with HPLC method very well. The solution method produced lower RMSEC and higher R than the substrate method. In this particular case, the solution method acted as an 'affinity chromatography'-like procedure. Based on different affinity of the reactant nobiletin and the product 5-demethylnobiletin with the Ag dendrites, the weakly bound reactant nobiletin can be washed away, while 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 as no incubation between sample and Ag was needed and only trace amount of sample (a few microliter) was required.

In summary, we successfully monitored and characterized the demethylation reaction of citrus-derived nobiletin to produce more bioactive 5-demethylnobiletin using both SERS. SERS methods were found to offer several advantages over conventional HPLC method. Firstly, it was time-consuming and labor-intensive to optimize the sample preparation and elution conditions of HPLC method.^{12,19} SERS methods measure samples on nanosubstrate directly without tedious sample preparation, which is very useful in characterization of any rapid chemical reaction course. Secondly, the HPLC detection is based on a good separation (different elution time) of the reactants and products, which is challenging in some cases. For SERS methods, reactants and products can be easily differentiated based on their distinct 'fingerprint' SERS patterns without physically separating them. In addition, rapid and accurate quantification in multi-component systems can be realized directly based on the fact that each of individual components has unique Raman signature. Thirdly, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partly supported by the grants from USDA, NIH, and AICR.

References

1. Feldborg LN, Jolck RI, Andresen TL. Quantitative evaluation of bioorthogonal chemistries for surface functionalization of nanoparticles. *Bioconjugate Chem.* 2012; 23:2444–2450.
2. Yoshimizu N, Otani Y, Saikawa Y, Kubota T. Anti-tumour effects of nobiletin, a citrus flavonoid, on gastric cancer include: antiproliferative effects, induction of apoptosis and cell cycle deregulation. *Aliment. Pharm. Therap.* 2004; 20:95–101.
3. Gross M. Flavonoids and cardiovascular disease. *Pharm. Biol.* 2004; 42:21–35.
4. Qiu PJ, Dong P, Guan HS, Li SM. Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells. *Mol. Nutr. Food Res.* 2010; 54:244–252.
5. Xiao H, Yang CS, Li SM, Jin HY. Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol. Nutr. Food Res.* 2009; 53:398–406. [PubMed: 19065586]
6. Qiu PJ, Guan HS, Dong P, Li SM. The p53-, Bax- and p21-dependent inhibition of colon cancer cell growth by 5-hydroxy polymethoxyflavones. *Mol. Nutr. Food Res.* 2011; 55:613–622. [PubMed: 21462329]
7. Dhyani MV, Satpati D, Korde A, Banerjee S. Synthesis and preliminary bioevaluation of Tc-99m(CO)(3)-17 alpha-triazolylandrost-4-ene-3-one derivative prepared via click chemistry route. *Cancer Biother. Radio.* 2011; 26:539–545.
8. Lazarski KE, Rich AA, Mascarenhas CM. A one-pot, asymmetric robinson annulation in the organic chemistry majors laboratory. *J. Chem. Educ.* 2008; 85:1531–1534.
9. Skorupa A, Gierak A. Detection and visualization methods used in thin-layer chromatography. *Jpc-J. Planar. Chromat.* 2011; 24:274–280.

10. Cialla D, Marz A, Bohme R, Theil F. Surface-enhanced Raman spectroscopy (SERS): progress and trends. *Anal. Bioanal. Chem.* 2012; 403:27–54. [PubMed: 22205182]
11. He LL, Haynes CL, Diez-Gonzalez F, Labuza TP. Rapid detection of a foreign protein in milk using IMS-SERS. *J. Raman Spectrosc.* 2011; 42:1428–1434.
12. Dong P, Qiu PJ, Zhu Y, Li SM. Simultaneous determination of four 5-hydroxy polymethoxyflavones by reversed-phase high performance liquid chromatography with electrochemical detection. *J. Chromatogr. A.* 2010; 1217:642–647. [PubMed: 20022018]
13. Feng W, Yang JS, Xu XD, Liu QH. Quantitative determination of lanostane triterpenes in fomes officinalis and their fragmentation study by HPLC-ESI. *Phytochem. Analysis.* 2010; 21:531–538.
14. He LL, Lin MS, Li H, Kim NJ. Surface-enhanced Raman spectroscopy coupled with dendritic silver nanosubstrate for detection of restricted antibiotics. *J. Raman Spectrosc.* 2010; 41:739–744.
15. Goodacre R, Timmins EM, Burton R, Kaderbhai N. Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiol-Uk.* 1998; 144:1157–1170.
16. Wu W, Walczak B, Massart DL, Prebble KA, Last IR. Spectral transformation and wavelength selection in near-infrared spectra classification. *Anal. Chim. Acta.* 1995; 315:243–255.
17. He LL, Zheng JK, Labuza TP, Xiao H. A surface enhanced Raman spectroscopic study of interactions between casein and polymethoxyflavones. *J. Raman Spectrosc.* 2013; 44:531–535.
18. Haynes CL, McFarland AD, Van Duyne RP. Surface-enhanced Raman spectroscopy. *Anal. Chem.* 2005; 77:338–346.
19. Li S, Wang Z, Sang S, Huang MT, Ho CT. Identification of nobiletin metabolites in mouse urine. *Mol. Nutr. Food. Res.* 2006; 50:291–299. [PubMed: 16521176]

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

Figure 2.

The schematic illustration of SERS effects using substrate method (a) and solution method (b). 'Circle', 'oval', 'triangle', and 'square' were used to represent Ag dendrites, dried Ag dendrites, nobiletin molecular, 5-demethylnobiletin, respectively.

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

Figure 4. The second derivative transformation of SERS spectra (a) and PCA plot analysis (b) of nobiletin and 5-demethylnobiletin by substrate method and solution method.

Figure 5.

The second derivative transformation of SERS spectra (a, c) and partial least squares (b, d) of chemical reaction mixtures and 5-demethylnobiletin determined by substrate method (wavenumber from 1540 to 1680 cm⁻¹) and solution method (wavenumber from 1250 to 1650 cm⁻¹).