*Anal. Chem.* **2005,** *77,* 3563−3569

# Isotope Edited Internal Standard Method for Quantitative Surface-Enhanced Raman Spectroscopy

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**A new isotope edited internal standard (IEIS) method for quantitative surface-enhanced Raman spectroscopy (SERS) is demonstrated using rhodamine 6G (R6G-$d_0$) and rhodamine 6G (R6G-$d_4$) edited with deuterium. The reproducibility and accuracy of the IEIS method is investigated both under optical resonance (SERRS) and nonresonance (SERS) conditions. A batch-to-batch concentration measurement reproducibility of better than 3% is demonstrated over a concentration range of 200 pM−2 $\mu$M with up to a factor of 3 difference between the concentration of the analyte and its IEIS. The superior performance of the IEIS method is further illustrated by comparing results obtained using absolute SERS/SERRS intensity calibration (with no internal standard) or using adenine (rather than R6G-$d_4$) as an internal standard for R6G concentration quantization. Potential biomedical gene expression and comparative proteomic applications of the IEIS method are discussed.**

Since the discovery of the surface-enhanced Raman scattering (SERS),[1] various SERS active substrates and molecules have been reported with a typical Raman signal enhancement of $10^6$. Under electronic resonance (SERRS) conditions, far greater enhancements may be attained, and single-molecule detection limits have been reported for rhodamine 6G, adenine, crystal violet, and other SERRS active molecules.[2−5] Because of the high sensitivity of the SERS/SERRS techniques and high information content of the resulting vibrational spectra, SERS active molecules have been employed as labeling reagents for bioanalytical applications which enabled detection of attomole ($10^{-18}$ mol) quantities of proteins or DNAs down to femtomolar ($10^{-15}$ mol/L) concentrations.[6−12] However, accurate quantitative analysis with SERS remains a challenge because of (1) the difficulties associated with the production of reproducible SERS active substrates, (2) the complex characteristics (both spatial and temporal) of the SERS substrates, (3) the strong dependence of the SERS enhancement on the distance between the analyte and the SERS substrates,[13] and (4) variations of SERS enhancement with the surface coverage of the analyte on the substrate (related to the distribution of SERS active hot spots).[13,14] In addition, quantitative concentration measurements using optical methods (including SERS as well as normal Raman or fluorescence) must contend with intensity variations produced by changes in excitation, collection efficiency, or both. Correcting for such variations is most often accomplished using either an internal or an external standard to calibrate the correlation between the optical signal and the concentration (or amount) for the analyte of interest. Here we describe a new isotopically edited internal standard (IEIS) method that may be used for quantitative SERS/SERRS measurements over a wide concentration range with unprecedented accuracy and reproducibility.

The IEIS method differs in important ways from the following previously proposed methods for improving the accuracy of quantitative SERS measurements. For example, Smith et al. employed a flow cell device for in situ aggregation of Ag colloidal fabricated with the Lee−Misel method[15,16] and found good linearity and reproducibility when using the same batch of a SERS active colloidal solution. However, when different batches of colloidal solutions were used, the reproducibility of the SERRS signal with mitoxantrone concentration deteriorated significantly (with calibration slope differences of up to 60%, even though all the other experiment conditions remained the same[15]). More recently, an internal standard method was proposed to improve the accuracy

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(1) Fleischmann, M.; Hendra, P. J.; McQuillan, A. J. *Chem. Phys. Lett.* **1974**, *26*, 163.
(2) Kneipp, K.; Kneipp, H.; Kartha, V. B.; Manoharan, R.; Deinum, G.; Itzkan, I.; Dasari, R. R.; Feld, M. S. *Phys. Rev. E* **1998**, *57*, R6281−R6284.
(3) Koo, T.-W.; Chan, S.; Sun, L.; Su, X.; Zhang, J.; Berlin, A. A. *Appl. Spectrosc.* **2004**, *58*, 1401−1407.
(4) Nie, S.; Emory, S. R. *Science* **1997**, *275*, 1102−1106.
(5) Kneipp, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R. R.; Feld, M. S. *Phys. Rev. Lett.* **1997**, *78*, 1667−1670.
(6) Cao, Y. C.; Jin, R.; Mirkin, C. A. *Science* **2002**, *297*, 1536−1540.
(7) Faulds, K.; Barbagallo, R. P.; Keer, J. T.; Smith, W. E.; Graham, D. *Analyst* **2004**, *129*, 567−568.
(8) Faulds, K.; Smith, W. E.; Graham, D. *Anal. Chem.* **2004**, *76*, 412−417.
(9) Graham, D.; Brown, R.; Smith, W. E. *Chem. Commun.* **2001**, 1002−1003.
(10) Culha, M.; Stokes, D.; Allain, L. R.; Vo-Dinh, T. *Anal. Chem.* **2003**, *75*, 6196−6201.
(11) Graham, D.; Mallinder, B. J.; Whitcombe, D.; Watson, N. D.; Smith, W. E. *Anal. Chem.* **2002**, *74*, 1069−1074.
(12) Cao, Y. C.; Jin, R.; Nam, J.-M.; Thaxton, C. S.; Mirkin, C. A. *J. Am. Chem. Soc.* **2003**, *125*, 14676−14677.
(13) Lacy, W. B.; Olson, L. G.; Harris, J. M. *Anal. Chem.* **1999**, *71*, 2564−2570.
(14) Campion, A.; Kambhampati, P. *Chem. Soc. Rev.* **1998**, *27*, 241−250.
(15) McLaughlin, C.; MacMillan, D.; McCardle, C.; Smith, W. E. *Anal. Chem.* **2002**, *74*, 3160−3167.
(16) Lee, P. C.; Meisel, D. *J. Phys. Chem.* **1982**, *86*, 3391−3395.

10.1021/ac050338h CCC: $30.25 
Published on Web 04/26/2005

for SERS (colloidal) quantification by using the SERS signal generated from a self-assembled monolayer (SAM) as an internal standard.[17] With this method, the high coverage of the SAM is presumed to prevent chemisorption of the analyte onto the SERS active surfaces and thus to improve reproducibility.[17] However, this approach also has intrinsic limitations. For example, because of the sharp drop-off of the SERS enhancement with the distance between the analyte and SERS surface,[14] the limit of the detection and the dynamic range with the SAM approach has been severely compromised (because of the greater distance between the analyte and SERS surface created by the SAM coating). Furthermore, the different local environments around the SAM and the analyte molecules may produce a different response to experiment parameters such as laser intensity and frequency. These and other factors may explain the relatively large prediction errors (root mean prediction error of 0.5 $\mu$M for samples between 0.1 and 5 $\mu$M) observed when using this SAM internal standard method.[17] The present IEIS method overcomes the limitations of previous methods because the analyte and standard molecules have virtually identical chemical properties and so their relative SERS/SERRS intensity is far less sensitive to batch-to-batch colloid solution variations and optical excitation/collection parameters (as further described below).

In general, the SERS signal intensity, $I$, can be a function of several variables as shown in eq 1, where $I_\kappa^\delta$ is the signal

$$I_\kappa^\delta = C_\kappa I_\kappa^s \alpha_k \tag{1}$$

intensity obtained from sample $\kappa$ of concentration of $C_\kappa$, under a given set of experiment conditions, $\delta$, while $I_\kappa^s$ represents the signal intensity obtained with unit analyte concentration under a given set of standard conditions, s, and $\alpha_\kappa$ represents the relative SERS enhancement factor, which may in general also be a function of $C_\kappa$, as well as the characteristics of the SERS substrate and Raman system.

For an internal standard to be effective, all the SERS intensity variations introduced by anything other than sample concentration must to be compensated by internal SERS intensity standard. In other words, an ideal internal standard method is one for which the SERS intensity ratio of the analyte, a, and internal standard reference, r, are strictly proportional to the ratio of their corresponding concentrations.

$$\frac{I_a^\delta}{I_r^\delta} = \frac{C_a I_a^s}{C_r I_r^s} \tag{2}$$

In other words, the relative enhancement factors of the analyte and reference compound must be identical, $\alpha_a = \alpha_r$, and this equality should not depend on either the absolute or relative concentrations of the analyte and reference compounds or any other experimental variables. This implies that the ideal internal standard should have chemical properties that are as similar as possible to the analyte of interest. However, to be able to differentiate and quantify the spectral contribution for the analyte and its internal standards, their SERS spectral features have to be sufficiently different to facilitate independent measurement of their SERS intensities in a mixture.

Given the above consideration, one might expect that an ideal internal standard for any analyte would be an isotopically edited version of the analyte of interest. In fact, isotope editing is a commonly used technique in vibrational spectroscopy, to aid in assignment of spectroscopic features associated with specific functional groups. Advantages of isotopic editing as an internal standard method include the fact that the two compounds are expected to have (a) virtually the same chemical and physical properties but (b) a readily measurable and quantifiable spectroscopic differences. The fact that isotope editing can produce significant spectral changes is illustrated, for example, by the observed peak red-shifts of 50 and 30 $cm^{-1}$ produced by the vibration of the ring breathing mode of benzene produced $H^2$ (D) or $C^{13}$ editing, respectively,[18,19] which are quite significant given that the corresponding Raman band has a full width at half-maximum of $<6$ $cm^{-1}$. Furthermore, since most molecules with large SERS/SERRS activities contain aromatic functional groups and the Raman signals of these functional groups are in general the most prominent features in the resulting Raman spectra, isotopic edition of aromatic groups should provide a widely applicable IEIS method,

Like other internal standard methods, SERS quantification with IEIS is carried out by mixing the sample of interest with its IEIS of known concentration before incubation of the mixture with a SERS active substrate (e.g., a colloidal solution). After SERS acquisition, the concentration ratio of the analyte and the internal standard may thus be determined from the ratio of the spectral features associated with the two compounds.

The SERS and SERRS spectroscopy of rhodamine 6G (R6G) has been extensively studied,[4,20,21] as it is one of the most commonly used SERS/SERRS tags in DNA and protein detections applications,[7,8,10,22] as well as in SERRS single-molecule detection studies.[4] Thus, R6G was selected as the model compound to demonstrate the feasibility and performance of IEIS for SERRS and SERS quantitative analysis. In addition, to investigate the feasibility of using molecules other than IEIS, we have also investigated the SERS spectra of mixtures containing both R6G and adenine. The latter studies serve to clearly demonstrate the superior performance of the IEIS method as opposed to the use of chemically different analyte and internal standard compounds. The robustness and accuracy of SERS/SERRS with IEIS was evaluated using several batches of colloidal solution, with R6G concentrations varying from 200 pM to 2 $\mu$M. Potential biomedical applications with IEIS are discussed.

## EXPERIMENTAL SECTION

**Chemicals.** All the reagents used for organic synthesizing and colloidal solution and adenine were of analytical grade (Sigma-Aldrich), High-purity water (Millipore) was used throughout this study.

(17) Loren, A.; Engelbrektsson, J.; Eliasson, C.; Josefson, M.; Abrahamsson, J.; Johansson, M.; Abrahamsson, K. *Anal. Chem.* **2004**, *76*, 7391−7395.
(18) Shimanouchi, T. *Tables of Molecular Vibrational Frequencies*; National Bureau of Standards: Washington, DC. 1972.
(19) Painter, P. C.; Koenig, J. L. *Spectrochim. Acta* **1977**, *33A*, 1003−1018.
(20) Bosnick, K. A.; Jiang, J.; Brus, L. E. *J. Phys. Chem. B* **2002**, *106*, 8096−8099.
(21) Li, G.; Li, H.; Mo, Y.; Huang, X.; Chen, L. *Chem. Phys. Lett.* **2000**, *330*, 249−254.
(22) Graham, D.; Fruk, L.; Smith, W. E. *Analyst* **2003**, *128*, 692−699.

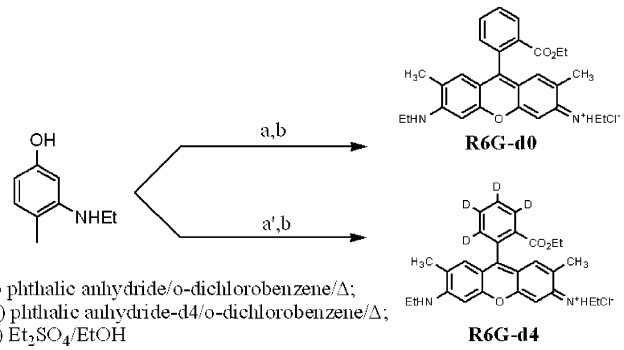


**Figure 1.** Schematic diagram representing the synthetic procedure used to produce rhodamine 6G (R6G) with no deuterium (R6G-$d_0$) and with four deuterium substitutions (R6G-$d_4$).

**Silver Colloid.** Silver colloidal solution was synthesized according to the Lee−Misel method by citrate reduction of silver nitrate.[16] Five batches of colloidal solution were synthesized independently and the first three batches were used for SERRS measurement and the last two for SERS. The aging period for different batches of colloidal solution varied from 2 h to 4 days.

**Instruments.** The SERS spectra were obtained using a home-built micro-Raman system with a 632.8-nm HeNe laser (with 10 mW at the sample), while the SERRS measurements were performed with another home-built Raman system with 514-nm argon ion excitation lasers (with 6 mW at the sample). With both systems, the back-reflected Raman signal was collected using a 20× Olympus objective and coupled to a spectrograph with a fiber bundle for detection with liquid nitrogen-cooled CCD detected. The spectrographs used on both systems have focal lengths of 0.3 m (Acton, SpectraPro 300i) and 1200 grids/mm gratings.

**Synthesis of the R6G and Its IEIS.** R6G and its IEIS derivative were synthesized by coupling 3-(ethylamino)-4-methylphenol with commercially available phthalic anhydride and phthalic-$d_4$ anhydride, respectively, followed by ethylation of the free carboxylic acid groups. The synthetic route is illustrated in Figure 1. Since four H atoms are substituted with D in the isotopically edited R6G, the two compounds will from hereon be abbreviated as R6G-$d_0$ and R6G-$d_4$, while the term R6G will continue to be used to refer to either one or both of the isotopes.

**Quantitative Studies.** All the SERRS experiments with R6G were repeated three times, each with a different batch of silver colloidal solution. The samples used for each repeated trial were the same: the total R6G concentration varied from 200 pM to 200 nM; quantitative SERRS analysis with IEIS was only carried out at two concentrations of 200 pM and 200 nM. For the SERS measurements, the final concentration of rhodamine 6G varied from 20 nM to 2 $\mu$M, and the same set of samples were analyzed with two batches of colloidal solutions. For all the IEIS samples, the concentration ratio of R6G-$d_0$ to R6G-$d_4$ was either 100/0, 75/25, 66.7/33.3, 50/50, 33.3/66.7, 25/75, or 0/100.

**Measurement Procedures.** If not specified, all SERS/SERRS measurements were performed using the following procedure: after mixing of 2 mL of silver colloidal solution with 2 mL of high-purity water in a 5 mL-glass vial, 200 $\mu$L of 2% NaCl solution was added for aggregation followed by immediate addition of 300 $\mu$L of analyte solution. The prepared sample was allowed to sit for 2 min before spectral acquisition. The integration times for all SERRS and SERS measurement are listed in Table 1; no attempt was made to optimize the signal intensities for each measurement.

**Table 1. Integration Times for SERS and SERRS Measurements**

| silver colloidal batch | excitation laser | | total concentration of R6G-$d_0$ and R6G-$d_4$ | | | | |
|---|---|---|---|---|---|---|---|
| | nm | mW | 200 pM | 2 nM | 20 nM | 200 nM | 2 $\mu$M |
| 1 | 514 | 10 | 30 s | 5 s | 500 ms | 50 ms | |
| 2 | 514 | 10 | 30 s | 5 s | 500 ms | 50 ms | |
| 3 | 514 | 10 | 30 s | 5 s | 500 ms | 10 ms | |
| 4 | 633 | 6 | | | 5 s | 1 s | 200 ms |
| 5 | 633 | 6 | | | 5 s | 1 s | 100 ms |

**Spectral Data Analysis.** for all the spectral analysis, the following simple least-squares spectral decomposition algorithm was developed and implemented using Matlab (MathWorks Inc.) to calculate the relative concentration ratio of R6G-$d_0$ and R6G-$d_4$. Let $S_1$ and $S_2$ stand for the SERS/SERRS spectra obtained with pure R6G-$d_0$ and R6G-$d_4$, respectively, and D for the SERS/SERRS spectrum measured with mixture of an unknown quantity of R6G-$d_0$ with R6G-$d_4$ of concentration $C_r$. Since spectrum D contains only spectral contribution from $\boldsymbol{S_1}$ and $\boldsymbol{S_2}$ plus experiment noise, spectrum D can be decomposed into spectra $S_1$ and $S_2$ by solving eq 3 with the least-squares method:

$$|C_1\ C_2| \begin{vmatrix} S_1 \\ S_2 \end{vmatrix} = D \tag{3}$$

However, it should be noticed that $C_1$ and $C_2$ are not the concentrations of $S_1$ and $S_2$; in fact, they do not even represent their relative contributions to mixture spectrum D before a proper adjustment of spectral intensity of $S_1$ or $S_2$. The goal for this adjustment is to make the intensity ratio of the component spectra equal to what would be obtained with the component spectra each acquired under exactly the same conditions. This can be done by simply finding a multiplying constant for $S_1$ or $S_2$ so that the $C_1/C_2$ ratio determined with the adjusted component spectra matrix will be equal to 1 for any SERS/SERRS spectra obtained with the mixture consisting of exactly 50% of each component, and the resulting intensity-calibrated component spectra are used for all subsequent spectral analysis.

## RESULT AND DISCUSSION

**SERRS and SERS Spectra of R6G-$d_0$ and R6G-$d_4$.** The SERRS (top) and SERS spectra (bottom)) of both R6G-$d_0$ and R6G-$d_4$ are shown in Figure 2. Spectra (a) and (c) pertain to 2 and 200 nM R6G-$d_0$ solution, respectively, while (b) and (d) pertain to 2 and 200 nM R6G-$d_4$ solution. The insets in both plots are the expanded views of the 585−630-cm$^{-1}$ spectral region, which more clearly shows the spectral difference induced by isotopic edition.

As evident in both the SERRS and SERS spectra, several Raman bands are red-shifted in R6G-$d_4$ relative to their locations in R6G-$d_0$, which is consistent with the higher mass of deuterium relative to hydrogen. The peaks at 615 (ring in-plane bending[21]) and 771 cm$^{-1}$ (C−H bending[21]) of R6G-$d_0$ are shifted to 604 and 763 cm$^{-1}$, respectively, in R6G-$d_4$. Moreover, the singlet peak at 1356 cm$^{-1}$

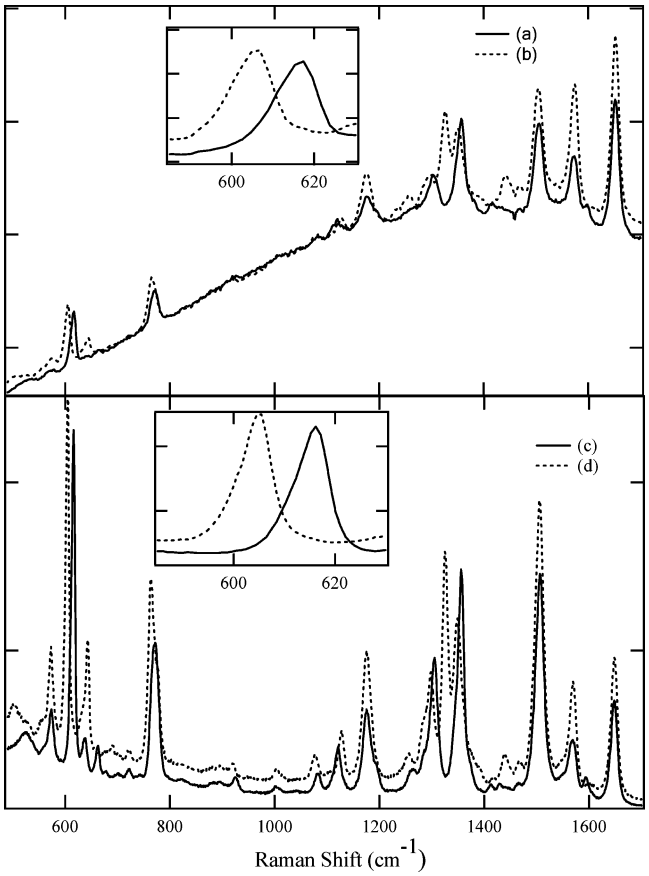


**Figure 2.** SERRS (top, 2 nM) and SERS (bottom, 200 nM) spectra of R6G-$d_0$ (solid lines a and c) and R6G-$d_4$ (dashed lines b and d). The insets in both plots show expanded views the 585−630-cm$^{-1}$ spectral region. Spectra are scaled and offset for clarity.

(aromatic C−C stretch[21]) of R6G-$d_0$ splits into two peaks at 1325 and 1350 cm$^{-1}$ in R6G-$d_4$.

**Quantitative Analysis with ISIE.** Figure 3 shows the SERRS spectra (in the 585−630-cm$^{-1}$ region) obtained with a total R6G concentration of 200 nM but different ratios of R6G-$d_0$ and R6G-$d_4$. Thus, there is a clear trend in the spectral shape and the R6G-$d_0$ to R6G-$d_4$ concentration ratio.

To demonstrate IEIS can also be used for SERS quantification, similar experiment procedures were applied for SERS spectral acquisition and analysis. Figure 4 shows the SERS spectra obtained from samples with same R6G-$d_4$/R6G-$d_0$ ratio of 50/50, but different total R6G concentrations were (a) 20 nM, (b) 200 nM, and (c) 2 $\mu$M. Clearly, the relative spectroscopic contribution of R6G-$d_0$ and R6G-$d_4$ to the mixture spectra depends only on their concentration ratio, over a wide total concentration range. Similar results were obtained from SERRS spectra with the same R6G-$d_0$/R6G-$d_4$ ratio with total concentrations of 200 pM and 200 nM (data not shown).

For spectral decomposition with SERRS spectra were performed using only the 545−674-cm$^{-1}$ spectral region. The linear baseline of each truncated spectrum was automatically determined from a fit the first and last three data points in the above spectral window, and the resulting baseline-subtracted data matrix is represented as **D**. The truncated and baseline subtracted spectral matrix **S** containing the pure R6G-$d_0$ and R6G-$d_4$ spectra was obtained in the same way from the corresponding single-component SERRS spectra. After intensity calibration (as described in the Experiment Section), the relative concentration of R6G-$d_0$ and R6G-$d_4$ in the matrix **C** was readily determined using the following least-squares spectral decomposition where super-

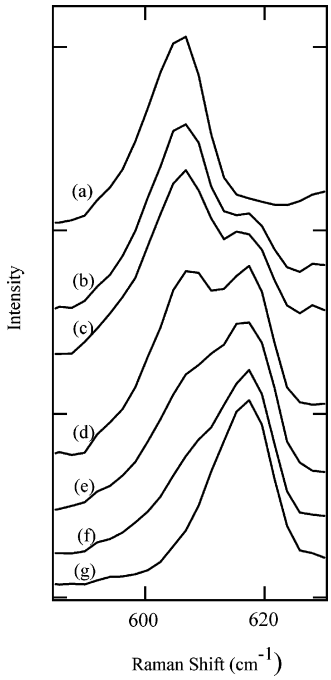


**Figure 3.** SERRS spectra of mixtures of R6G-$d_0$ and R6G-$d_4$ with a total R6G concentration of 200 nM. The concentration ratios of R6G-$d_0$ to R6G-$d_4$ are (a) 100/0, (b) 75/25, (c) 66.7/33.3, (d) 50/50, (e) 33.3/66.7, (f) 25/75, and (g) 0/100. The spectra are scaled and offset for better visualization.

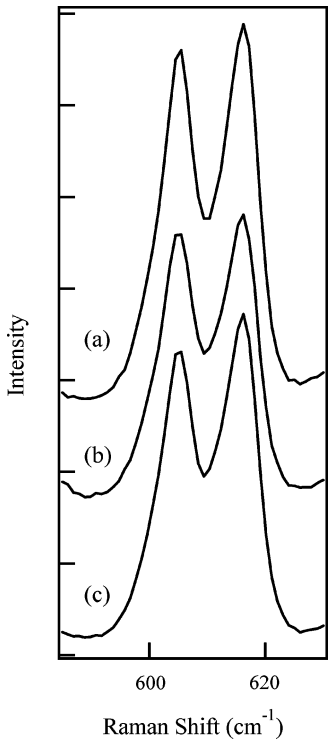


**Figure 4.** SERS spectra obtained from solutions each of which has 50/50 R6G-$d_0$/R6G-$d_4$ concentration ratio but different total R6G concentrations: (a) 20 nM, (b) 200 nM, and (c) 2 $\mu$M (the spectra are offset for clarity).

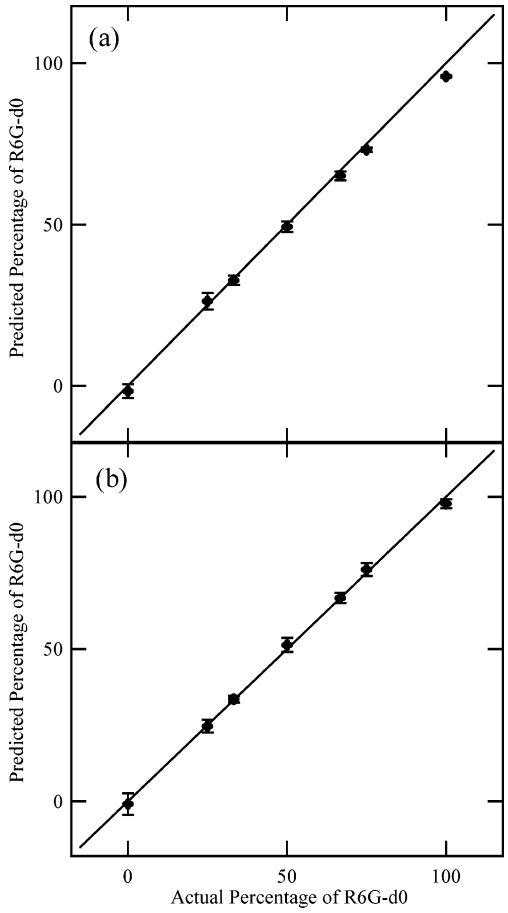


**Figure 5.** Predicted concentration ratio of R6G-$d_0$ obtained from IEIS SERRS measurements with the first (a) and second (b) batch of colloidal solutions. The pure SERRS calibration spectra of R6G-$d_0$ and R6G-$d_4$ were acquired with the third batch of colloidal solution. The solid line represents the exact correlation with a slope of 1. The error bar represents one standard deviation.

script t and −1 represents matrix transpose and inverse, respectively.

$$\mathbf{C} = \mathbf{D}\cdot\mathbf{S}^{t}\cdot(\mathbf{S}\mathbf{S}^{t})^{-1} \quad (4)$$

However, as described in the introduction, the SERRS intensity depends not only on concentration but also on the characteristics of the colloidal solution. To properly test the IEIS method, the component spectra in matrix **S** were acquired with one batch of colloid solution, and the SERRS spectra used for prediction were obtained with different batches of colloidal solution. Furthermore, samples of total R6G concentrations of 200 nM and 200 pM were used to further test the robustness of the IEIR method. Figure 5 shows the predicted percentage of R6G-$d_0$ with SERRS spectra obtained from mixtures with (a) the first batch and (b) the second batch of colloidal solutions (and the pure component spectra in matrix **S** were acquired with the third batch of colloidal solution with a R6G concentration of 200 nM). The average and standard deviations of each data point in both plots were obtained from 10 SERRS measurements, 5 with total R6G concentration of 200 nM and another 5 with a total R6G concentration of 200 pM. Similar results were obtained when the pure component spectra in matrix **S** were acquired with any batch of the Ag colloidal solution. Similar results were also obtained using SERS (rather than SERRS) measurements with a total R6G concentrations of 20 nM, 200 nM, and 2 $\mu$M (and separate batches of colloidal solution used for calibration at a 20 nM R6G concentration and testing at the three different concentrations).

**Table 2: Normalized SERRS Peak Intensity at 615 cm$^{-1}$ for R6G-$d_0$ at Different Concentrations**

| silver colloidal batch | concentration of R6G-$d_0$ | | | |
|---|---|---|---|---|
| | 200 pM | 2 nM | 20 nM | 200 nM |
| 1 | 1.00 | 6.11 | 41.32 | 651.55 |
| 2 | 2.16 | 4.70 | 35.76 | 1428.11 |
| 3 | 1.27 | 5.83 | 19.86 | 540.06 |

An average error of 2.1% in the concentration ratio obtained when the IEIS and the analyte were of the same concentration. When all the SERRS and SERS data were considered for samples in which the concentration difference between R6G-$d_0$ and R6G-$d_4$ is less than or equal to 3, the average concentration ratio prediction errors were 2.8%. Furthermore, the robustness of the IEIS method is clearly demonstrated with the high-reproducibility data shown in Figure 5, obtained from different batches of colloidal solution of a 1000-fold analyte total concentration range. Moreover, by combining SERS and SERRS measurements, the concentration of R6G-$d_0$ can be accurately quantified over a concentration range of 4 orders of magnitude from 200 pM to 2 $\mu$M.

However, when the concentration difference of analyte and its IEIS is greater than a factor of ~3, somewhat larger concentration ratio prediction errors were obtained. Thus, to ensure an accurate quantification, it is important to pre-estimate the concentration of the analyte and to make sure similar amounts of IEIS and analyte were mixed prior to the SERS/SERRS acquisition. Note that this can readily be done by pretesting the analyte solution of interest using several IEIS solutions each differing by a factor of 10 in IEIS concentration.

As a further measure of the improvement in quantization obtained using the IEIS method, we attempted to correlate the absolute intensity of the SERRS signal of the analyte of R6G-$d_0$ with its concentration (without using an IEIS reference). The normalized SERRS signal intensities at 615 cm$^{-1}$ obtained at each concentration with different batches of silver colloidal solution are shown at Table 2. In the normalization step, the difference in the integration time for different samples was compensated and the peak intensity for the SERRS spectrum obtained with the first batch of colloidal solution and with 200 pM R6G-$d_0$ was set to 1.00. Evidently, linearity of the SERRS data obtained with any one batch of the colloidal solution was quite poor as was the reproducibility of the data obtained with different batches. The relative prediction error obtained from such correlations was as large as 300% (as determined by attempting to predict the concentration using SERRS data for a one batch of colloidal solution that was predicted using a intensity/concentration correlation derived from data acquired using another batch of colloidal solution).

We have also investigated the feasibility of in situ addition of the IEIS into the analyte solution that has been preequilibrated with a colloidal solution. This procedure proved to be less reliable than first premixing the analyte and IEIS and then adding the colloidal solution. However, the results revealed interesting

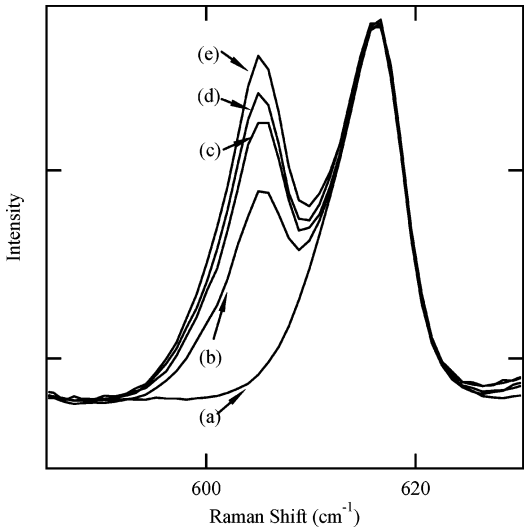


**Figure 6.** SERS spectra taken when R6G-$d_4$ was added into a premixed R6G-$d_0$/Ag colloidal solution (with the same R6G-$d_0$ and R6G-$d_4$ concentrations). Spectra a−d were obtained at 0, 1, 80, and 290 min after adding the R6G-$d_4$. Spectrum e was acquired from a solution in which R6G-$d_0$ and R6G-$d_4$ were premixed before adding the Ag colloid solution.

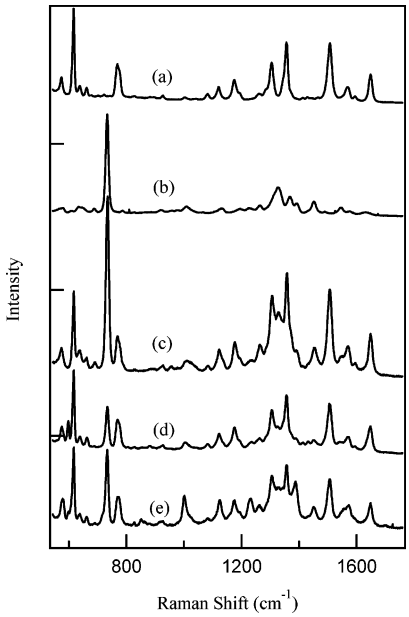


**Figure 7.** SERS spectra obtained with (a) pure R6G-$d_0$, (b) pure adenine, (c) 10 $\mu$M adenine and 100 nM R6G-$d_0$, (d) 1 $\mu$M adenine and 10 nM R6G-$d_0$, and (e) 100 nM adenine and 1 nM R6G-$d_0$. The 615-cm$^{-1}$ peak intensities were adjusted to the same value in spectra c−e to better visualize the relative intensity differences of the adenine and R6G features (although all three spectra have the same adenine/R6G concentration ratio of 100/1).

dynamics associated with the competition of the analyte and IEIS for SERS/SERRS active sites of the colloidal particles. More specifically, these tests were performed by first premixing an analyte solution with a SERS active colloidal solution, to produce a final R6G-$d_0$ concentration of 100 nM. After the 3 min of preequilibration, an equal amount of the IEIS (R6G-$d_4$) was added into above mixture and a series of SERS spectra were acquired at different times after the IEIS was added. The resulting evolution of the Raman feature in the 585−630-cm$^{-1}$ spectral window is shown in Figure 6, along with spectrum e obtained from a sample in which an equal amount of R6G-$d_0$ and R6G-$d_4$ were premixed before adding the colloidal solution. Thus, these spectra reveal that when the IEIS is added to a preequilibrated analyte/colloid mixture, significant time is required for the final mixture to equilibrate, and even after 4 h, the intensity of the IEIS has not yet reached equilibrium. Clearly such a procedure cannot readily be used to quantify the analyte concentration, but it could be used to study the kinetics associated with the analyte colloid binding and exchange reactions.

As yet another demonstration of the advantages of the IEIS method, we performed similar experiments using adenine rather than R6G-$d_4$ as an internal SERS standard for R6G-$d_0$ concentration measurements. More specifically, we premixed a series of solutions each with a 1:100 ratio of R6G-$d_0$ to adenine (because adenine has about a 100 times weaker SERS signal). The R6G-$d_0$ and adenine were premixed before adding the colloidal solution. SERS measurements were performed with final R6G-$d_0$ concentrations of 10 nM, 100 nM, and 1 $\mu$M. Figure 7 shows the resulting SERS spectra obtained with (a) pure R6G-$d_0$, (b) pure adenine, (c) 10 $\mu$M adenine and 100 nM R6G-$d_0$, (d) 1 $\mu$M adenine and 10 nM R6G-$d_0$, and (e) 100 nM adenine and 1 nM R6G-$d_0$. In contrast to the results shown in Figure 4, where the relative SERS contribution of the analyte and its internal standard depends only on their relative concentration, the relative SERS contribution of R6G-$d_0$ and adenine varied significantly at different concentrations (even though all the solutions had the same concentration ratio). Although the mechanism for the observed variations are not known, these results clearly indicate that using different molecule as a SERS internal standard is not nearly as effective as using an IEIS derivative of the analyte of interest.

## CONCLUSIONS

A new IEIS method for quantitative SERS/SERRS concentration measurements is presented. This method effectively corrects for SERS/SERRS intensity variations caused by (a) poor reproducibility of the SERS substrates, (b) the complex structure and dynamic of the SERS substrates, (c) the distance dependence of the SERS/SERRS intensity on the analyte concentration, and (d) variations in the collection or excitation efficiency of the Raman system, as demonstrated with R6G and one IEIS derivative of R6G. By combining SERS and SERRS, a concentration ratio prediction error of less than 3% was obtained over 4 orders of magnitude of total concentration with up to a factor of 3 concentration ratio range. This method is shown to be reliable, reproducible, and more sensitive than methods based on absolute SERS/SERRS intensity correlations, with no internal standard, or using a different molecule (rather than an IEIS) as a SERS/SERRS internal standard.

Although the IEIS method may find many different types of applications, we believe that it will prove to be particularly valuable for the detection gene expression patterns and for comparative proteomics. In particular, such application would employ isotopically edited SERS active molecules as labeling reagents for quantitative comparisons of the amounts (concentrations) of various biomolecules in samples derived from sources.[23,24] Current

(23) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994−999.

comparative proteomics or gene expression studies are carried out predominantly using either fluorescence or mass (e.g., isotope-coded affinity tag (ICAT)) detection methods.[25,26] These methods suffer from limited dynamic range and relatively poor quantitative capability. For example, with ICAT, ratios changing from 0.8 to 1.6 have been reported for samples with a theoretical ratio of 1,[25] while the reported ratios for a fluorescence-based method were 0.57−2.57.[26] Such large prediction errors associated with current methods prevent reliable identifications of protein or DNA, which changes its expression level less than 2 times. However, in disease diagnostics and drug screening applications, it can be important to identify smaller changes in various biomarkers. For example, reliable quantification of small changes may enable discovery of new disease biomarkers that are not currently detectable but may provide a life-saving early warning for environmental health problems. Thus, there is a clear need for developing a more accurate, robust, and sensitive technique for comparative genomics and proteomics studies. The high sensitivity of the SERS technique combining with unparallel quantitative accuracy of the IEIS method makes SERS−IEIS a very promising biomedical diagnostics and research technology. Such applications are actively being pursued in our laboratories and will be reported in forthcoming publications. Key advantages of this approach over other tagging methods derive from the fact the chemical properties of the sample and reference are virtually identical, thus minimizing quantization errors associated with differential optical properties and tagging efficiencies or differences in substrate binding or chromatographic retention.

(24) Julka, S.; Regnier, F. *J. Proteome Res.* **2004**, *3*, 350−363.
(25) Shi, Y.; Xiang, R.; Crawford, J. K.; Colagelo, M.; Horvath, C.; Wilkins, A. J. *J. Proteomics Res.* **2004**, *3*, 104−111.
(26) Karp N.; A.; Kreil D. P.; Lilley K. S. *Proteomics* **2004**, 4, 1421−1432.

Received for review February 24, 2005. Accepted April 1, 2005.

AC050338H