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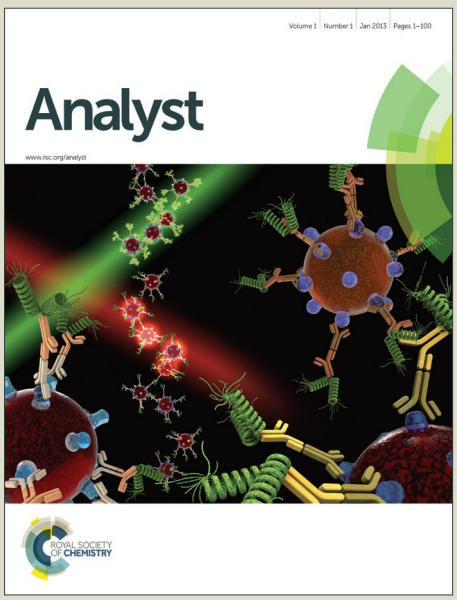

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Quantitative Online Sheath-flow Surface Enhanced Raman Spectroscopy Detection for Liquid Chromatography

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Abstract.

Sheath-flow surface-enhanced Raman spectroscopy (SERS) was used for online detection and quantification of small molecules separated by liquid chromatography. A mixture of model metabolites (thiamine, folic acid, and riboflavin) was separated and characterized by UV-Vis and SERS detectors connected in series. Acetonitrile in the mobile phase provided an internal standard enabling quantitative detection across SERS experiments.

Introduction.

Small molecule identification and quantification is essential to realizing the diagnostic potential of systems biology approaches such as metabolomics. The ability to characterize multiple metabolites in biofluids is key for deciphering the interconnected biochemical pathways relevant to biomedical diagnosis and drug alterations in cells.[1,2] The current technologies for characterization metabolites are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). NMR spectroscopy is non-destructive, requires simple preparations, and provides detailed structure information of almost any metabolites;[1,3,4] however, the low sensitivity can require up to millimolar concentrations and milliliter volumes.[1,3] The increased sensitivity of MS has made it a common detection method for the characterization of metabolites at nanomolar and lower concentrations and smaller sample volumes.[1–3] Moreover, MS can be coupled to different separation techniques such as liquid chromatography (LC)[1,3,5], gas chromatography (GC)[3,6], and capillary electrophoresis (CE)[7] to facilitate multiplex separation and detection of various classes of metabolites. While LC-MS is the most common method for global metabolic profiling, it is still facing challenges associated with ion suppression[5] and reproducibility.[1] It has been noted that less than 2% of the spectra in an MS metabolomics experiments can be attributed to a specific molecule.[8] Orthogonal detection technology is important to improve analyte characterization.

Surface enhanced Raman spectroscopy (SERS), provides non-destructive, label-free identification of molecules that interact with SERS-active nanostructures. Raman scattering originates from the inelastic scattering of light from molecules that can be correlated to the vibrational modes, producing molecular fingerprints. The Raman signal can be enhanced as much as 10^{11} in the presence of silver and gold nanostructures, an effect known as surface enhanced Raman scattering (SERS).[9] The enhancement arises from the near-field effect between molecules and the local electromagnetic field on the nanostructure surface associated with excitation of a localized surface plasmon resonance (LSPR).[10]

SERS is an interesting choice of detector for liquid-phase separations because it can provide structural information for analysis and identification of biomolecules.[11–13] In contrast to MS method, SERS detection can be performed easily in solution due to its insensitivity to water[14,15], thus eliminating challenges associated with ionization into vacuum. SERS has been studied as either an at-line or online detector for liquid chromatography (LC) in separation and identification of various analytes such as purine bases[13], pesticides[16], and drugs[17]. However, the combination of LC to SERS (LC-SERS) usually suffers from low sensitivity and a high limit of detection (LOD) because of the difficulty to facilitate interactions between analytes in solution and SERS-active substrate. Therefore, the development of a simple yet sensitive and high throughput LC-SERS combination for multiplex detection, which can run in parallel with MS based method, is highly desirable.

In this paper, we demonstrate a high-throughput coupling of capillary LC with SERS (LC-SERS) using an online sheath flow SERS detector. In sheath-flow SERS, hydrodynamic focusing increases analytes interactions with the SERS substrate thus increasing sensitivity significantly.[18] Previous studies in our lab have successful separated and analyzed multiplex peptides and amino acids sample using capillary zoned electrophoresis (CZE) and SERS detection in flow with LODs as low as nanomolar concentrations.[11,14,18] The prevalence of LC for metabolomics studies suggests LC-SERS may be the more important technique. The combination of UV-Vis and

SERS detections in series following capillary LC separations in this study provides better limits of detection and chemical information of analytes in mixture compared to the traditional coupling of LC to UV-Vis, DAD, fluorescence, MS, etc.[19–21] Here we will also discuss the ability to detect and quantify analytes over a wide range of concentrations by utilizing both the built-in UV-Vis detector in a commercial LC system and our sheath flow SERS detector in flow.

Additionally, our results address challenges in obtaining reproducible SERS intensities from experiment to experiment. The intensity of SERS signal is proportional to the analytes concentrations in the detection volume; however, many factors such as variations of laser power, day-to-day substrate conditions or saturation of substrate surface at high concentrations can make it difficult to obtain and reproduce the linear relationship between SERS signal and concentration. One approach to correct for all the above effects is to use an internal standard, which is usually a compound present at a known concentration along with the analyte of interest during the experiment.[22] An internal standard can be an unenhanced SERS species that produces a Raman signal at a non-interfering Raman shift, such as coated self-assembled monolayer on substrate[23], acetonitrile peak[24], or OH stretching band of water,[25] or enhanced SERS species that behaves chemically similar to the analytes of interest, isotopes for example. In this report, we use a component of LC mobile phase, acetonitrile, as an internal standard for quantitative analysis in LC-SERS.

Experimental.

Reagents and Materials

Riboflavin (≥98%), thiamine hydrochloride (≥99%), folic acid (≥98%), sodium phosphate monobasic dihydrate (NaH2PO4.2H2O, ≥99%), sodium hexanesulfonate (≥99%), phosphoric acid (crystalline, ≥99.999%) acetonitrile (HPLC grade, ≥99.9%), and methanol (HPLC grade, ≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Nanopure water (18.2 MΩ cm) was obtained from a Barnstead Nanopure filtration system. Fused silica capillary (72 μm i.d., 143 μm o.d.) was purchased from Polymicro Technologies (Phoenix, AZ).

SERS Substrate Preparation

SERS-active substrates were synthesized according to the previously reported thermal evaporation procedure.[26] Briefly, silver was evaporated into an anodized aluminum oxide (AAO) filter (0.1 μm pores). The substrate was affixed onto a standard microscope glass slide, predrilled with 2 holes (35 mm apart), then incorporated into a custom-built flow cell. Prior to the experiments, the AAO filter was dissolved in 0.1 M NaOH overnight, leaving behind a thin layer of highly enhancing silver structures.

Sample Preparation

The 111 μM stock solution of riboflavin was prepared by dissolving riboflavin in water with 0.3% v/v ammonium hydroxide to improve its solubility. A series of standard riboflavin solutions (11.1 μM, 5.55 μM, 1.11 μM, 0.55 μM, 0.11 μM, and 0.055 μM) were prepared by diluting the stock solution and were used as working standard solutions for LC and SERS system calibration tests. The 79 μM thiamine and 37 μM folic acid stock solutions were prepared by

dissolving weighed amounts in water and water with 0.3% v/v ammonium hydroxide, respectively. Solutions for mixture analysis were prepared by mixing different amounts of stock solutions and then diluting with water. The final concentrations of the mixture were 18.8 μM thiamine, 8.8 μM folic acid, and 0.40 μM riboflavin.

Liquid Chromatography

HPLC separation was performed using a capillary-scale LC Packings Ultimate system with quaternary pump and UV-Vis detector together with a 5.0 μL injection loop all remotely controlled by the Ultichrom software (LC Packings). A 50 x 0.3 mm capillary C18 column packed with 3-μm particles (Gemini, Phenomenex) was used. The mobile phase consisted of 0.050 M sodium phosphate buffer at pH 3.0 and a mixture of 50/50 methanol/acetonitrile pumped at a flow rate of 2 μL/min. The mobile phase gradient consisted of: (a) 0 – 2.99 min, 100% phosphate buffer; (b) 3 – 6.99 min, steady decrease to 80% phosphate buffer; (c) 7 – 14.99 min, hold at 80% phosphate buffer; (d) 15 – 19.99 min, steady change to 0% phosphate buffer and 100% MeOH/ACN; (e) 20 – 25 min, run at 100% MeOH/ACN to return column's original condition. UV-Vis absorption was measured at 254 nm.

Raman Detection

Measurements were performed with a previously described home-built system with the line focusing of laser obtained my placing a cylindrical lens (f=500 mm) into the beam path.[27] A 660 nm laser (Laser Quantum, San Jose, CA) was focused onto the substrate in the flow cell through a 40x water-immersion objective (NA = 0.80). Raman scattering signal was collected at the same objective lens and directed to the Isoplane-320 spectrograph (Princeton Instruments, Trenton, NJ) and ProEM EMCCD (Princeton Instruments, Trenton, NJ).

Sheath Flow LC-SERS Detector

The instrument setup allowed online post-column chromatography SERS detection as shown in Figure 1. The sheath flow SERS cell used has been described previously.[18] A 60 cm long fused silica capillary and PEEK finger-tight unions were used to connect the LC system's outlet to our sheath flow SERS cell, shown as a black line in figure 1. The sheath-flow SERS detector consists of a plastic base plate, a silicone gasket (with a 1.5 mm slit defining the sheath-flow channel), a cover slip and stainless steel top plate. The sheath flow, that confines the analyte to the SERS substrate is established through the sample inlet and outlet in the plastic base plate. The end of the sample capillary is affixed on the substrate roughly centered in the sheath-flow channel. Hydrodynamic focusing of the sample stream was obtained by continuously pumping water into the flow cell's inlet at a flow rate of 140 μL/min controlled by a syringe pump and LabView (National Instruments, Austin, TX).

Data Analysis

All spectra and chromatograms were processed using Matlab 2015a (Mathworks). SERS spectra was normalized against the total signal by using the PLS toolbox (Eigenvector Research, Inc.) operating in MATLAB and corrected to 1.5 mW laser power at the sample. Peak areas were

determined by a Gaussian fit performed by Igor Pro (WaveMetrics) with a set noise level of 0.0005, a smooth factor of 2.

Result and Discussion.

A mixture containing 18.8 μM thiamine, 8.8 μM folic acid, and 0.40 μM riboflavin was used to assess the capability of sheath flow SERS as an LC detector. These concentrations were chosen for biological relevance and to demonstrate a clearly resolved separation in the UV-Vis detector. The sheath-flow SERS flow cell was connected in series with the UV-Vis detector of a commercial LC system, allowing for online SERS detection in tandem with a traditional LC separation. In a typical run, the analytes were injected into the HPLC system then separated on the column. Upon eluting from the column, analytes in the mobile phase were detected by UV-Vis before flowing through the 60 cm long, 72 μm i.d connecting capillary sheath-flow SERS detection cell. Hydrodynamic focusing then confined the analytes to the SERS substrate for characterization.

Figure 2A shows chromatogram of the mixture obtained with the built-in UV-Vis detector in the LC system. The retention time of thiamine, folic acid, and riboflavin were determined to be 9.63, 11.82 and 12.58 min, respectively. Figure 2B shows the heatmap of SERS intensity as a function of Raman shift and retention time following the LC separation. The Raman heatmap indicates that thiamine, folic acid, and riboflavin eluted onto the SERS-active substrate at 10.66, 12.91, and 13.62 min respectively. The delayed retention time on SERS detector for each analyte is due to the travel time through the connecting capillary from UV-Vis detector to SERS detector. Based on the dimensions of the capillary and the flow rate, a delay of 1.2 min is expected. To further confirm analyte identification, we calculated Δt_1 and Δt_2, the relative retention times of the second and third signals to the first signal as shown in Figure 2A-B. Δt_1 is determined to be 2.19 min in the UV-Vis and 2.25 min in the SERS detection, while Δt_2 is 0.76 and 0.71 min in the UV-Vis and SERS chromatograms, respectively. The consistent relative retention times confirm the elution orders and signals of each analyte in the mixture are indeed the same in both detectors. The width of the peaks in the SERS chromatogram are considerably narrower than in the UV-Vis chromatogram. This is consistent with previous capillary electrophoresis results with sheath-flow SERS detection,[11,18] and is believed to arise from limited absorption at low concentrations and the probability of occupying a hotspot at these low coverages.

The SERS spectrum of each analyte in the mixture extracted from the SERS heatmap is shown in Figure 2C (top spectrum). The acquisition parameters were 1.5 mW laser power at the sample and 250 ms per frame acquisition times. Thiamine spectra shows peaks at 1360 cm^{-1}, 1541 cm^{-1}, 1560 cm^{-1}, 1591 cm^{-1} (all appeared to be protonated pyrimidine ring stretching vibration). Folic acid shows peaks 1354 cm^{-1} (C-H rocking vibration), 1535 cm^{-1}(C=N asymmetric vibration), and 1626 cm^{-1} (C=O stretching + N-H bending). Riboflavin shows peaks at 1250 cm^{-1} (C=O bending), 1347 cm^{-1} (C-N stretching mode of pyrazine ring), 1400 cm^{-1} (C-C stretching), 1510 cm^{-1} (C-CH_3 bending), 1553 cm^{-1} (C-N stretching), and 1620 cm^{-1} (C-C stretching of benzene ring). The LC-SERS spectrum for each analyte is compared with its reference spectrum and the background spectrum detected without the analyte as shown in Figure 2C. The reference spectra were obtained by continuous flowing each analyte solutions through the sheath-flow SERS cell.

The reference solutions were prepared by dissolving 26.8 μM thiamine, 12.5 μM folic acid, and 5.5 μM riboflavin in phosphate buffer solution (pH 3.0) to ensure the experimental conditions for LC-SERS and steady state were similar. The reference spectra show good agreement with the spectra in the LC-SERS chromatogram, which suggests that library spectra can be used for analyte identification in the future.[28] Our results indicated that coupling LC to sheath flow SERS detection successfully facilitate simultaneous separation and detection of the three component mixture.

The sensitivity of the detectors was compared by measuring the SERS spectrum in flow of each analyte. Riboflavin solutions with concentrations ranging from 11.1×10^{-6} to 55×10^{-9} M, folic acid solutions from 56.6×10^{-6} to 0.27×10^{-6} M, and thiamine solutions solution from 32.6×10^{-6} to 0.27×10^{-6} M were measured and calibration curves produced for both the UV-Vis and SERS detectors.

Figure 3 shows a calibration plot of peak area detected by UV-Vis as a function of riboflavin concentrations. Calibration curves for folic acid and thiamine are provided in Figures S-1 and S-3, respectively. As expected, the plots reveal a linear dependence between the UV-Vis absorbance signal and concentrations. In Figure 3, the slope of the best fit line is determined to be 20.84 with the y-intercept of 4.5463 and the correlation coefficient $R^2 = 0.9981$. The inset of Figure 3 show the actual LC chromatograms of each riboflavin runs as a function of time. The retention time of riboflavin was determined to be 12.65 ± 0.07 min. Due to baseline fluctuation, a blank spectrum provides limited information associated with the noise when an analyte is detected. Thus, 3 × error of the calibration line / slope of the calibration line ($3s_y/m$) method was used to calculate the limit of detection (LOD) throughout this manuscript. The limit of detection for our UV-Vis detector was determined to be 0.35 μM

The average SERS spectrum of riboflavin at each concentration is shown in Figure 4A. The results for folic acid and thiamine are shown in Figures S-2 and S-4, respectively. Each spectrum was normalized against its total signal (row-normalization) and then corrected to 1.5 mW laser power at the sample. The results indicate that sheath flow SERS detector was able to obtain riboflavin signal at nanomolar concentrations using a 250 ms acquisition time. To assess the possibility for quantitative SERS analysis in flow, the riboflavin calibration curve was constructed based on Raman band area at 1400 cm^{-1} as a function of concentrations in Figure 4B. As expected, the plot is not linear with respect to band area versus concentration, but shows intensity variance and evidence of curvature associated with the typical Langmuir isotherm behavior at higher concentrations. These effects have typically hampered quantification studies with SERS in comparison to the linear calibration curves observed with UV-Vis detection. The intensity variance arises from the day-to-day variations of SERS substrate condition (e.g. the number of hotspots in the detection volume), which are known to affect the reproducibility of SERS signals.

To correct for experimental variation and improve linearity of the response, we applied the SERS internal standard method.[22] In this experiment, acetonitrile was treated as an internal standard for SERS calibration of different concentrations of each analyte. Acetonitrile is a reasonable choice for internal standard because it is one of the components of the mobile phase, which presents at a

stable concentrations during the elution time of each analyte, and gives rise to distinctive Raman bands at 2260 cm^{-1} as shown in Figure 4A. For riboflavin, the band area at 1400 cm^{-1} is normalized to the acetonitrile band and the ratio is plotted as a function of concentrations in Figure 4C. The internal standard plot provides a linear dependence of band area ratio to riboflavin concentrations with the slope of 21.354, y-intercept of 3.3712 and the correlation coefficient $R^2 = 0.9967$. These results indicate the SERS limit of detection is 84 nM ($3s_y/m$).

To assess the reliability of both UV-Vis and SERS calibration curves, the riboflavin signal detected by both UV-Vis and SERS in the previously analyzed mixture (Figure 2) was correlated back to their calibration plots. Based on the LC chromatogram, the peak area of riboflavin signal was determined to be 12.76 ± 1.06 mAU which corresponded to 0.394 ± 0.052 μM in concentrations with an RMS error of prediction of 0.04 μM. Analyzing SERS spectrum of riboflavin (Figure 2C) provided a ratio of bands at 1400 cm^{-1} and 2260 cm^{-1} (acetonitrile band) of 12.13 ± 2.45.The band ratio was then correlated to the internal standard calibration plot to obtain 0.41 ± 0.11 μM with an RMS error of prediction of 0.09 μM. We repeated this analysis for both thiamine and folic acid. The UV-Vis and SERS concentrations detected for thiamine were 19.08 ± 0.83 μM and 18.3 ± 1.12 μM respectively. These are in good agreement with the prepared concentration of 18.8 μM. For folic acid, the UV-Vis and SERS concentrations were determined to be 8.78 ± 0.33 μM and 8.97 ± 1.15 μM, also in good agreement with the actual concentration of 8.8 μM.

It is important to note that the calibration curves and test data were obtained on different days, using different SERS substrates, in some cases weeks apart. This indicates the use of acetonitrile as an internal standard provides a stable and robust method of quantification. Control experiments (Figure S-5) show that the reference acetonitrile signal is not observed if the experiment is performed without a SERS substrate. Thus, the reference signal appears to arise from the competitive adsorption between acetonitrile, a strong eluent, and the analyte. The ratio between the analyte and acetonitrile corrects for changes in the surface condition of the SERS substrate and provides improved quantification.

Therefore, our results indicated that coupling UV-Vis detector to online sheath-flow SERS detector provide a robust method for simultaneous post-separation detection to improve the ability of identification and quantification at a wide range of analyte concentrations in mixtures.

Conclusion.

Our results demonstrate that sheath-flow SERS is provides improved detection of molecules compared to standard UV-Vis detectors. The chemical characterization provided by our SERS detector suggests a complementary technique to identify and quantify small molecules separated by LC. Acetonitrile in the mobile phase provides an internal standard for improved and robust quantification. These results provide a new method with tremendous potential to characterize small molecules and facilitate improved identification of small molecules in metabolomics experiments.

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Electronic Supporting Information (ESI) available: Figures S-1 and S-2 show the UV-Vis and SERS calibration curves for thiamine. Figures S-3 and S-4 are the UV-Vis and SERS calibration results for folic acid. Figure S-5 is the control experiment examining the signal from acetonitrile on planar silver film.

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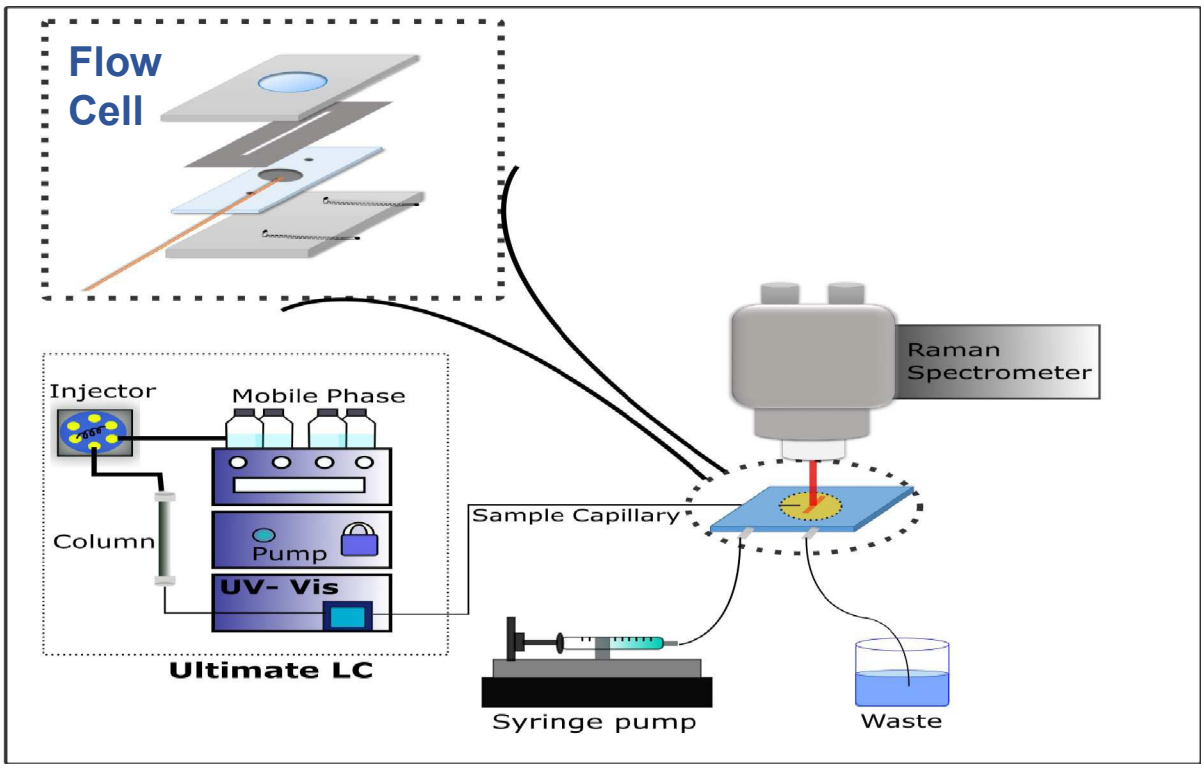

Figure 1. Diagram of the LC-SERS setup. A 60 cm long fused silica used to connect the output from the UV-Vis detector to the sheath-flow SERS cell (inset). In the inset, the orange line represents the sample capillary, while the sheath flow is pumped through the inlet in the base plate as described in reference 18. A silicone gasket defines the sheath flow channel over a microscope slide with a SERS substrate affixed. A cover glass and cover- plate as shown seals the cell. A 40x water-immersion objective used to focus a 660 nm laser onto the SERS-active substrate.

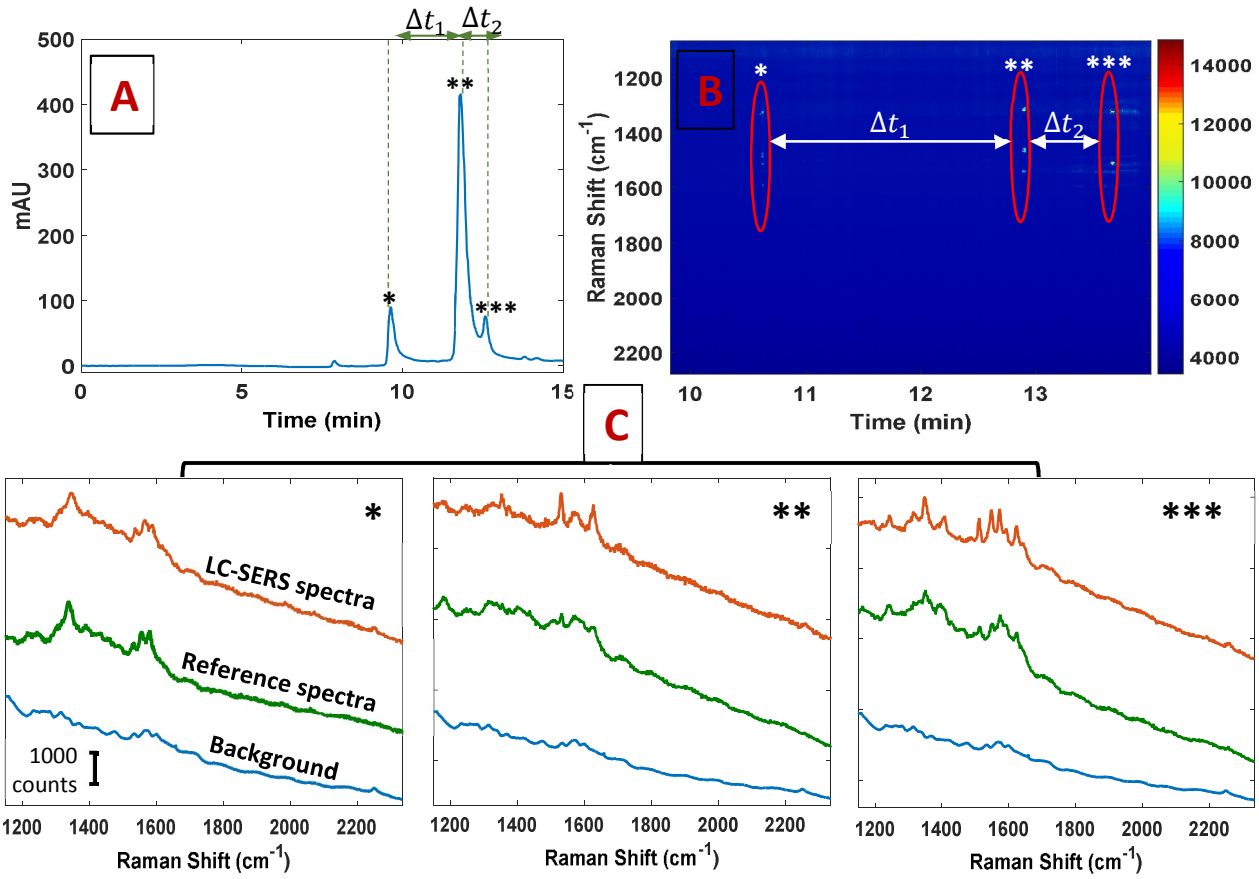

Figure 2. Example of LC-SERS spectra from a single injection of mixture containing 18.8 µM thiamine (*), 8.8 µM folic acid (**), and 0.40 µM riboflavin (***) eluted in that order. The analyzed mixture was detected in series with a UV-Vis detector (254 nm) and sheath-flow SERS detector. The UV-Vis chromatogram from absorbance at 254 nm (A) and the corresponding SERS chromatogram (B) are shown. The LC-SERS spectra of thiamine, folic acid, and riboflavin (C - top spectra) was extracted from the high intensity region of the SERS chromatogram. Each analyte spectrum was compared with their reference SERS spectra (C – middle spectra) obtained by continuously flowing analyte solutions into the sheath-flow SERS detector. The background SERS spectra with no analyte present are also shown (C – bottom spectra). The concentrations of the reference solutions are 26.8 µM thiamine, 12.5 µM folic acid, and 5.5 µM riboflavin, respectively.

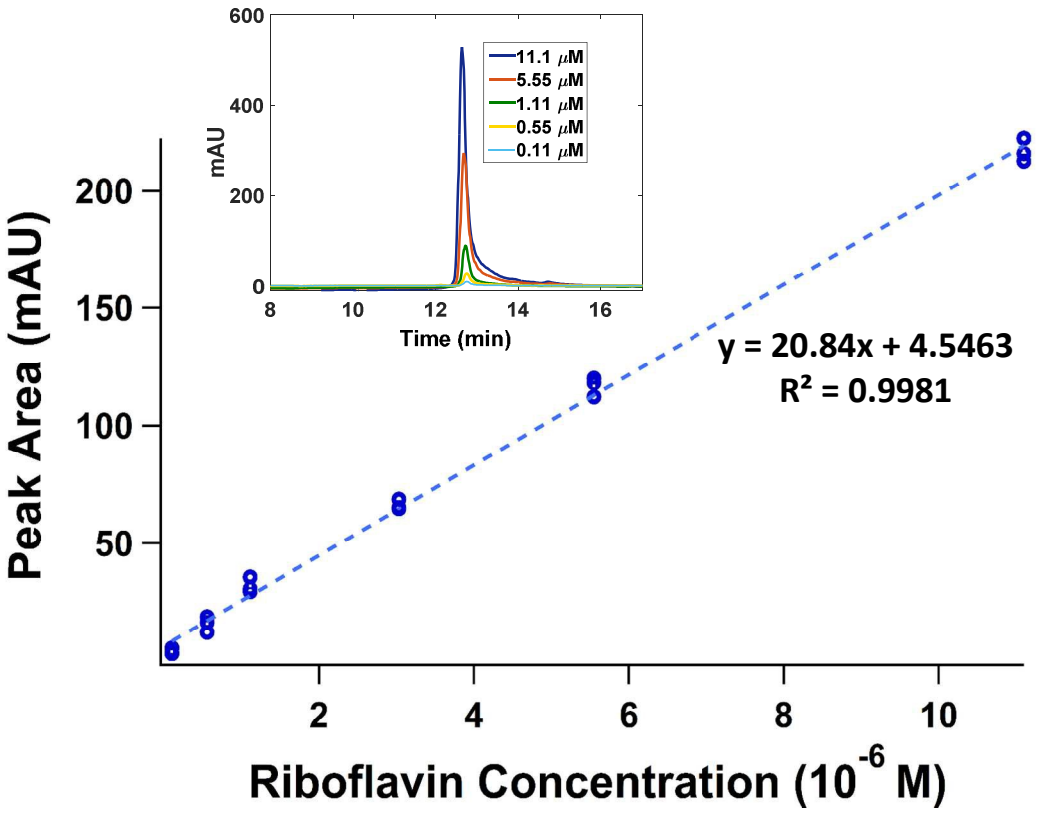

Figure 3. UV-Vis detection calibration curve obtained by plotting peak area (n=3) as a function of riboflavin concentrations in the range from 0.11×10^{-6} M to 11.1×10^{-6} M. The best fit line was obtained. The inset shows overlay LC chromatograms of riboflavin solution at different concentrations. The average retention time for riboflavin is 12.65 ± 0.07 min.

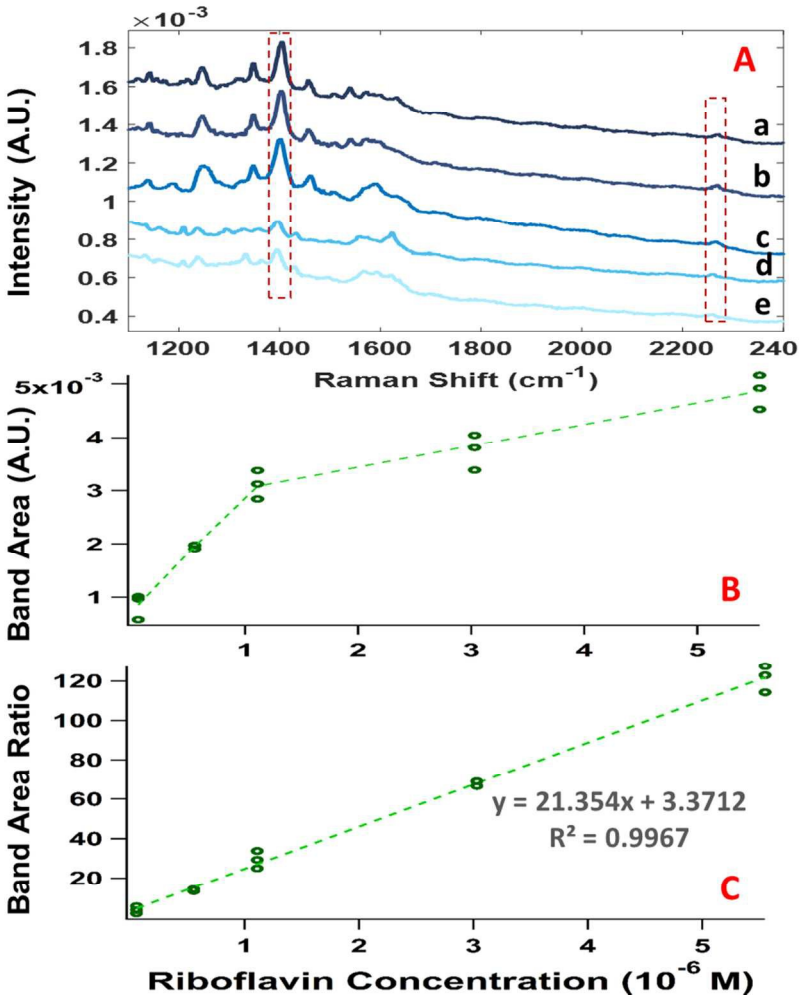

Figure 4. (A) Average LC-SERS spectra (n=3) of riboflavin at different concentrations: (a) 5.55 µM, (b) 3.03 µM, (c) 1.11 µM, (d) 0.555 µM,(e) 0.055 µM . The spectra are offset for clarity. Each SERS spectrum was normalized against its total signal (row-normalization). The band area at 1400 cm^{-1} (n=3) was plotted as a function of concentrations as shown in (B). Calibration against an internal standard (C) was achieved by plotting band ratio of 1400 cm^{-1} and 2260 cm^{-1} (acetonitrile Raman band) with respect to concentrations. A best fit line was obtained showing a linear concentrations dependence in SERS band area ratio.