Quantification of Resveratrol in Red Wine using Liquid Chromatography—Surface-Enhanced Raman Spectroscopy (LC-SERS)

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Abstract

Resveratrol is a stilbenol molecule found in red wine that may have many health benefiting properties, such as preventing inflammation in blood vessels. This metabolite is found naturally in grapes and is one of the reasons why red wine is promoted as healthy enough to drink a glass every day. This project aimed to quantify the amount of resveratrol in a sample of red wine using LC-SERS. SERS is a non-destructive quantitative method that measures the inelastic scattering of light, where the signal is enhanced by adsorbing the sample on a metal nanostructure. For this project, a thermally evaporated silver substrate placed in a homemade flow cell for SERS detection. Red wine was separated through by using reverse phase chromatography before flowing into the flow cell where the SERS signal was obtained. A calibration curve of resveratrol was made by flowing different concentrations of resveratrol though the flow cell. Once that was achieved, the resveratrol was injected into the HPLC to determine the retention time. By separating resveratrol from the other components of red wine, the SERS signal can be compared to the reference spectrum for identification and intensity provides quantitative information. Data analysis took place on MATLAB, using airPLS as a background subtraction method and Pearson correlation to compare sample spectra with a reference spectrum.
Introduction

Resveratrol and Red Wine

Resveratrol is a stilbene (Fig 1.) that is thought to have many health benefits, such as having anticancer, anti-inflammatory, antioxidant, and antimicrobial benefits. Resveratrol is found naturally in grapes and its presence in wine products act as one of the pieces of evidence for the popular saying “Red wine is healthy enough to drink a glass a day”. The motivation for this product is to quantify resveratrol in a sample of red wine to determine if one is able to consume enough resveratrol in a serving of red wine to get the health benefits.

![Structure of trans-resveratrol](image)

**Figure 1. Structure of trans-resveratrol**

Raman Spectroscopy and SERS

The method used to quantify resveratrol is Raman spectroscopy. Raman spectroscopy is the measurement of inelastic scattering of light from a molecule. However, the frequency of Raman scattering is relatively low, so the signal is enhanced using a method called surface-enhanced Raman spectroscopy (SERS). SERS works by adsorbing a sample onto metal nanostructures, which are usually silver or gold substrates. By adsorbing a sample onto nanostructures, the signal is enhanced through chemical and electromagnetic effects. The chemical effect is still under study, but it is thought that enhancement is due to the transfer of electrons between the adsorbed molecules and the metallic substrate. As for the electromagnetic effects, which is more well understood, enhancement is caused by the excitation of the metallic nanostructures, which create localized plasmons which enhance the signal. SERS is used as it is chemical specific and concentration dependent, allowing for the specific detection of a metabolite as well as the amount.
Flow Cell and Sheath Flow

As the samples used in this project are all in solution, another method of enhancement can be used by utilizing a homebuilt flow cell\(^4\) (Fig. 2b.) and hydrodynamic focusing to enhance the interactions between the sample and the substrate. In the flow cell, there is a plastic base where the substrate sits with two ports for an inlet and outlet. On top of the substrate is a silicon gasket that defines a channel for a flow of a sheath flow from the inlet and outlet ports as well as a capillary affixed to the middle of the channel on top of the substrate for sample flow. To enhance interaction between the sample and the substrate, the sheath flow has a higher flow rate compared to the sample flow, which pushes the sample flow down towards the sample\(^4\) (Fig 2a.).

Figure 2a. (left) Simulation of the effect of sheath flow on the sample flow\(^4\). Figure 2b. (right) Flow cell model. The flow cell contains a plastic base with an inlet and outlet port (a), a glass slide with two holes drilled out for the ports, which the substrate and capillary are glued on (b), a silicone gasket to define the sheath flow (c), a glass cover slide (d), and a metal cover that is screwed into the plastic base to seal the flow cell (e).

LC-SERS

In conjunction with Raman measurements, an ultra high performance liquid chromatograph (HPLC) is used. In order to obtain Raman spectra of resveratrol, a method is needed to separate the red wine mixture before obtaining the Raman spectra of the sample. The instrumental setup is described in Figure 4. By optimizing a method for resveratrol
quantification, this method can be applied in a more clinical application where there may be a need to detect metabolites similar to resveratrol in solutions such as urine or blood.

**Methods**

*Sample Preparation*

A stock solution (2 mM) resveratrol was prepared in acetonitrile. Samples for individual analysis were prepared by diluting the stock solution to a concentration two times more concentrated than the target concentration with acetonitrile, and then diluted to the target concentration with HPLC grade water. The resulting solution would contain a solvent mixture of 50% acetonitrile and 50% water. The same sample solutions were used in both Raman and HPLC measurements.

*Substrate Preparation*

A SERS-active substrate was synthesized using methods previously reported. Silver was evaporated onto an aluminum anodized oxide (AAO) filter with 0.1 µM pores (Fig. 3). To prepare the substrate for Raman measurement, a filter was soaked in a 0.1 M NaOH solution for 4 hours. The substrate was then washed with 65 °C water before gluing down on a glass slide and incorporated into a custom-built SERS flow cell.

**Figure 3.** Model of how the substrates is made. First AAO filters are secured in the thermal evaporator. Then Ag is thermally evaporated onto the filter. As there are pores in the filter, the filter contains metal nanostructures for the enhancement of the Raman signal once the Ag is evaporated onto it. To prepare the substrate for an experiment, the substrate is soaked in a NaOH solution to dissolve the filter and leave behind the Ag nanonstructures.
**Liquid Chromatography**

Chromatographic separation was achieved using an Thermo Dionex Ultimate 3000 UHPLC. A Thermo Dionex Ultimate 3000 UHPLC C18 column was used in this experiment. Mobile phase A consisted of water and 0.1% acetic acid and mobile phase B consisted of acetonitrile and 0.1% acetic acid. The mobile phase ratio used was 50% A and 50% B. Samples were injected with a 0.150 µL injection volume at an injection rate of 0.300 µL/min. The chromatogram was obtained at a wavelength of 307 nm based on the absorbance of resveratrol (Fig 5).

**UV-Visible Spectroscopy**

The absorbance of resveratrol was obtained using a VWR UV-1600PC spectrometer and a 1 mm quartz cuvette.

**Raman Detection**

The spontaneous Raman of resveratrol solutions was obtained using a Snowy Range Instruments IM-52 benchtop Raman spectrometer equipped with a 638 nm laser. Surface-enhanced Raman spectroscopy was performed using a homebuilt setup as previously reported. In general, 632.8 nm laser was focused onto the SERS-active substrate in the flow cell through an Olympus LUMPLFLN 40X, NA = 0.8 water immersion objective. Raman scattering was collected through the same objective and directed to an Andor Shamrock 303i spectrograph and an Andor iDus 401 CCD. Raman spectra were recorded in series with a 200 ms acquisition time and 0.50 mW of laser power at the sample.

**LC-SERS**

The same instrumental setup was used as previously reported. A 30 cm long fused silica was used to connect the LC outlet to the homebuilt SERS flow cell through a PEEK union. The end of the capillary was affixed to the center of the SERS substrate. Analyte confinement on SERS substrate was achieved by hydrodynamic focusing by pumping a sheath fluid of water at 15 µ/ min through the sample inlet and outlet on the flow cell, while keeping the analyte flow rate of
the sample capillary at 0.300 µL/min. The Raman collection was started 5 minutes after the start of the LC collection to account for the time it takes for the analyte to elute from the column and enter the flow cell.

![Flow Cell](image)

**Figure 4.** LC-SERS instrumental setup

*Data Analysis*

All SERS spectra and LC chromatograms were processed by Matlab 2021b (Mathworks). Background correction was performed using airPLS\(^1\) and Pearson correlation. To determine if resveratrol eluted from the column and Raman spectra of it was collected, Pearson correlation was used with a reference spectrum of 250 µM resveratrol collected from a previous experiment of SERS with only flow. The spectra number can be converted into time to determine if the spectra collected correlated with the time resveratrol eluted from the column. High scoring spectra were averaged and compared to the reference spectrum. A calibration curve was also made by fitting a peak of the resveratrol SERS spectrum across different concentrations. To determine the peak area, peakfit\(^9\) was used.
Results

**Figure 5.** The UV-Vis absorbance spectrum 500 μM resveratrol. The absorbance was determined to be 307 nm and to be used as the wavelength for detection in the HPLC.

**Figure 6.** The spontaneous Raman spectrum of solid phase resveratrol obtained with the Snowy Range using a laser power of 31.7 mW. Peaks from the spontaneous Raman spectrum can be matched to peaks in the surface-enhanced Raman spectroscopy to determine which peaks can be attributed to resveratrol.
Figure 7a. The mean Raman spectra of various concentrations of resveratrol using the parameters as described in the methods section. Figure 7b. Using the 1153 cm\(^{-1}\) peak (marked out), the peak area was calculated for each concentration and a calibration curve was made. The low \(R^2\) may indicate that the concentrations of resveratrol used may have been too high, leading to saturation of the surface.
**Figure 8a.** The time series of Raman collected from a LC-SERS run. As each spectrum is 200 ms long, to capture all the spectra of the sample eluted from the column during the 12 minutes, there was a total of 3600 spectra collected. **Figure 8b.** The UV chromatogram collected from the HPLC from a sample of 500 μM sample of resveratrol using a wavelength of 307 nm for detection. **Figure 8c.** Pearson correlation score of the time series using a reference spectrum of 500 μM resveratrol collected from a continuous flow experiment. The reference spectrum and time series were both baselined corrected beforehand. Spectra 1721:1726 scored the highest out of time series. **Figure 8d.** The average of spectra 1721:1726, the highest scoring spectra, plotted with the reference spectrum.

**Discussion**

In order to quantify resveratrol using LC-SERS, a calibration curve was needed. Preliminary calibration curves made had a relatively low R² value, which may be attributed to the use of too high concentrations that led to the saturation of the surface. This may need to be fixed by calculating a calibration curve with much lower concentrations. Future experiments will include concentrations that range from 1 μM to 250 μM.

Preliminary results show that LC-SERS is a method that can be used to quantify resveratrol. A concentration curve has been made as well as obtaining expected data from an optimized LC-SERS method. As seen in Figures 8c, a small number of spectra scored relatively high with Pearson correlation. Plotting the mean of the high scoring spectra against the reference spectrum, the spectra look relatively similar. However, the peaks are slightly offset from each other due to a calibration error happening in between Raman measurements. When converting the spectra number to time, the high scoring spectra occurred at 5.74-5.75 minutes. This is a little earlier than expected as the resveratrol peak in the UV chromatogram starts at 6.27 minutes (Fig 8b). There are a few factors that could account for this discrepancy, such as the capillary being shorter than the expected 30 cm.

However, further optimization may still need be done as methods have been changing throughout this project. As there is little literature looking at resveratrol quantification via LC-SERS, there was much optimization for the methods used before. Many aspects of this project have changed, such as changing from commercially bought substrates to lab-made substrates.
and changing the ratio of solvent to account for the refractive difference between acetonitrile and water when using the sheath flow.

Since the preliminary results have been promising, being able to use LC-SERS in more clinical applications may be possible, especially when looking at metabolites that share the same chemical behavior as resveratrol. Once LC-SERS parameters has been optimized for resveratrol, the same parameters can be applied to red wine. If there is successful quantification of resveratrol in red wine, biomedical applications can be considered.
Research Objectives Accomplished

1. Created a preliminary calibration curve with varying concentrations of resveratrol
2. Optimized parameters for separation for resveratrol in a HPLC
3. Optimized parameters for detection of resveratrol using LC-SERS

References

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