SERS Discrimination of Closely Related Molecules: A Systematic Study of Natural Red Dyes in Binary Mixtures

Federica Pozzi,*†§ Stephanie Zaleski,* Francesca Casadio,‡ and Richard P. Van Duyne‡

†Department of Conservation, Art Institute of Chicago, 111 South Michigan Avenue, Chicago, Illinois 60603, United States
‡Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, United States

ABSTRACT: Recent methodological and instrumental advances have afforded surface-enhanced Raman spectroscopy (SERS) wide recognition in the cultural heritage field as a powerful tool for the analysis of organic colorants. The many advantages of SERS are counterbalanced by the fact that it has limited ability to resolve dye mixtures. In this work, a systematic study was conducted to determine the technique’s ability to discriminate closely related molecules in binary mixtures for a selection of natural red dyes widely used in painting and textile dyeing (alizarin, purpurin, carminic and laccaic acids, brazilein). Spectra were recorded on two different metal substrates, i.e., citrate-reduced silver colloids and silver films over nanospheres (AgFONs). After working on reference systems, the study was expanded to include analysis of red lake oil paint reconstructions upon hydrolysis with hydrofluoric acid, aiming to gain a deeper understanding of how varying the experimental conditions may affect dye identification. It was found that, in some cases, the spectral contribution of the second colorant in the mixture goes undetected unless it is present in significant concentrations. Results of this work provide an indication of the expected performances of SERS in the analysis of dye mixtures. The findings also highlight the need for further research in the area of combined separation and quantification with SERS detection of organic colorants in artworks and museum objects.

INTRODUCTION

Over the past 10 years, surface-enhanced Raman spectroscopy (SERS) has found increased application in the field of cultural heritage research for the detection and identification of organic colorants in artifacts of archeological, historical, and artistic significance. Recent methodological and instrumental developments of the technique have allowed researchers to characterize dyes, including particularly challenging ones such as photosensitive yellow flavonoids or insoluble quinacridone quinones, in minute samples from artworks, to progressively improve spatial resolution, to detect lake pigments embedded in complex paint layers, and to perform virtually non-destructive analysis through the use of polymeric film substrates. Advantages of SERS with respect to most chromatographic techniques in the characterization of dyes include the successful reduction of sample size requirements and analysis time. However, SERS is not able to physically separate and conclusively identify multiple dyes in mixtures. An immediate consequence of this is that only the signals of the main component of a complex mixture are detected in a typical experiment due to discrepancies in resonance, solubility, affinity for the metal surface, SERS cross section, or a combination of two or more of these factors. This constitutes a significant limitation, as it is well-known from documentary sources that, throughout history, colorants were often used in combination to achieve particular color shades both in painting and textile dyeing. Recent contributions to the scientific literature have offered proof of the occasional simultaneous detection of multiple dyes in works of art and museum objects. For instance, binary mixtures of madder, cochineal, and brazilwood have been found extensively in paintings by Vincent Van Gogh by high-performance liquid chromatography (HPLC). More recently, SERS was successful in identifying cochineal and madder in single samples from works by Édouard Manet and Pierre-Auguste Renoir, and two different dyestuffs (quinacridone + quinacridone quinone) in a multicomponent commercial pigment. In addition, a mixture of cochineal and brazilwood was detected by SERS on colloidal pastes in textiles by Mariano Fortuny in the collection of the Art Institute of Chicago. However, in many cases when both HPLC and SERS were used to analyze dyes from artworks and historical textiles, while the presence of multiple colorants in a single sample had been determined by HPLC, only one could be detected by SERS. For example, Leona and Lombardi examined an 18th-century Tibetan textile that, although proven to contain berberine and safflower by HPLC, only showed berberine when analyzed with SERS. Pozzi et al. studied a green thread...
from a 17th/18th-century Kaitag textile and identified luteolin with SERS but no indigo, which was detected only with HPLC. This has prompted researchers to pursue alternative, more efficient ways to accomplish analysis of dye mixtures by SERS upon physical separation of the individual constituents. The coupling of thin-layer chromatography (TLC) with SERS has been proposed as a first, immediate solution allowing one to combine an equipment- and cost-effective separation method with the fingerprinting ability typical of vibrational spectroscopies. TLC-SERS was first used in the cultural heritage field for the characterization of anthraquinoid dyes in a binary mixture both as reference solutions and extracts from a dyed wool fiber.\(^{17}\) This hyphenated method was then used for the separation and identification of dye components in ballpoint pen inks for applications in forensic science.\(^{18}\) TLC-SERS was also employed to resolve a mixture of β-carboline alkaloids found in the Syrian rue (Peganum harmala) plant seeds, which are relevant as historical dyes and as drugs,\(^{19}\) and to separate and identify the structurally related components of mauve, the first synthetic organic dyestuff.\(^{20}\) More recently, an online hyphenated system combining HPLC with photodiode array (PDA) and SERS detection was demonstrated to afford a detailed electronic and vibrational characterization of natural dyes in mixtures.\(^{21}\) Thus, TLC-SERS and HPLC-SERS have provided efficient ways to resolve and identify individual components of dye mixtures. However, there are instances in which the coupling of SERS with separation techniques is not feasible due to sample size restrictions or unavailability of the required equipment, and regular SERS is the only viable option. In these cases, an estimation of the expected performances of SERS in the analysis of dye mixtures would be extremely useful to assess the reliability and exhaustiveness of the experimental results. While a detailed evaluation of the SERS technique’s ability to detect single dyes has been the subject of comprehensive studies in the past decade, to date very little work has focused specifically on the identification of individual components of dye mixtures. Thus far, only spectra of binary combinations of reference alizarin, purpurin, and lac dye have been presented within very preliminary studies,\(^{22–24}\) some of which have offered an initial assessment of suitable metal substrates and ideal experimental conditions, along with summary considerations on marker bands, adsorption geometries, and relative detection limits of the colorants examined. The present work aims to expand such preliminary studies by pursuing a systematic investigation of the capabilities and limitations of SERS in the concurrent detection and identification of organic colorants in binary combinations. A selection of five natural red dyes among those most commonly used in antiquity for painting and textile dyeing, i.e., alizarin, purpurin, carminic acid, lacaic acid, and brazilein (Figure 1), were here analyzed by SERS in series of historically relevant mixtures, both as reference solutions and in the form of mock-up red lake oil paints. First, SERS spectra of binary mixtures of the commercial colorants in various relative proportions and concentrations comparable to those observed in museum objects were recorded on two different metal substrates, i.e., Lee–Meisel citrate-reduced silver colloids and silver films over nanospheres (AgFONs). This aimed to establish relative detection limits for each dye when present in solution alongside another colorant, and to gain a deeper understanding of how different metal substrates and analytical methodologies may affect dye identification. As a second step, the complexity of the probed system was increased by examining red lake oil paint reconstructions prepared according to 19th-century historical recipes and including a chemical pretreatment (extractionless hydrolysis of the colorant from its substrate) in the analytical protocol. Specifically, binary mixtures of alizarin- and purpurin-, carminic acid-, and brazilein-containing dyes, i.e., madder, cochineal, and brazilewood, respectively, were examined with SERS in mock-up red lake paints. For this set of samples, analysis was performed upon hydrolysis with hydrofluoric acid (HF) and only silver colloids were employed as SERS-active substrate, according to a procedure that has proven to be the most successful for the characterization of red lake pigments bound in an oil medium.\(^{4,14}\) Results of this study provide a valuable indication of the expected performances of SERS in the analysis of dye mixtures that may be useful in all of those cases in which hyphenated methods combining SERS with chromatographic techniques are not readily available to the analyst.

### EXPERIMENTAL SECTION

**Materials.** Alizarin, purpurin, carminic acid, lacaic acid A, silver nitrate (AgNO\(_3\)), sodium citrate, potassium nitrate (KNO\(_3\)), and methanol were purchased from Sigma-Aldrich. Brazilwood cuttings were obtained from Kremer; brazillein was extracted with water and the solution evaporated off. HF was purchased from Acros Organics. Sulfuric acid (\(\text{H}_2\text{SO}_4\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and ammonium hydroxide (\(\text{NH}_4\text{OH}\)) were obtained from Fisher Scientific. 400 nm carbonyl latex microspheres were purchased from Thermo-Fisher Scientific, while Ag pellets for AgFON preparation were obtained from Kurt J. Lesker. All aqueous solutions were prepared from 18 MΩ-cm Millipore ultrapure water.

Five red lake oil paint reconstructions were provided by the Cultural Heritage Agency of The Netherlands (Rijksdienst voor het Cultureel Erfgoed (RCE), formerly Instituut Collectie Nederland (ICN)). Such mock-up paints were prepared as part of a wider project undertaken by the Van Gogh Museum, the RCE, and the Shell Research and Technology Centre (Amsterdam) in collaboration with the Courtauld Institute of Art and the National Gallery (London) for the investigation of the materials and techniques used by Vincent van Gogh.\(^{25}\)
Methods I: Experiments on Reference Dye Solutions. Reference solutions of binary mixtures of alizarin, purpurin, carminic acid, laccaric acid, and brazilein were prepared in methanol or methanol/water at a concentration of 10^{-5} M. Relative proportions of the two colorants in each mixture were 10:1, 5:1, 1:1, 1:5, and 1:10. SERS spectra of the dye solutions were acquired on citrate-reduced silver colloids and AgFONs.

Silver colloids were prepared by chemical reduction of AgNO_3 with sodium citrate, following the synthetic protocol published by Lee and Meisel. 1 mL aliquots of the so-obtained colloid were then purified from the excess citrate ions present in the suspension and prepared for use by replacing 900 μL of the supernatant with the same amount of ultrapure water. For SERS analysis, 0.8 μL of silver colloids were mixed with 0.2 μL of dye solution, followed by addition of 0.1 μL of 0.5 M KNO_3 to induce nanoparticle aggregation. SERS spectra were recorded immediately after sample preparation, by focusing the laser beam just below the surface of the colloid—dye—aggregant droplet, at 632.8 nm excitation.

Glass coverslips for AgFONs were cleaned using piranha etch (3:1 H_2SO_4:H_2O_2) and then made hydrophilic with a 5:1 solution of H_2O:H_2O_2:NH_4OH. They were rinsed thoroughly with ultrapure water between each step and then stored in ultrapure water prior to use. AgFON substrates were prepared by dropcoating 10−12 μL of 400 nm polystyrene microspheres along with the same quantity of ultrapure water on a cleaned glass slide. As the microspheres dried in air, a multilayer of spheres arranged in a hexagonal close packing array was formed. Then, a thermal vapor deposition system was used to deposit a 150 nm layer of silver on top of the array was formed. Then, a thermal vapor deposition system was used to deposit a 150 nm layer of silver on top of the microspheres. AgFONs thus produced, optimized for 632.8 nm laser excitation, by focusing the laser beam onto the surface of the AgFON.

Methods II: Experiments on Red Lake Oil Paint Reconstructions. The red lake oil paint reconstructions examined in this study were prepared as mixtures of madder and cochineal; brazilwood and cochineal; brazilwood and madder; and brazilwood and Kopp’s purpurin (a purpurin-rich extract of madder). Colorants were precipitated onto potash alum or aluminum sulfate substrates and dispersed in linseed oil. Details on the composition of each mock-up sample are given in Table 1.

Prior to SERS analysis, red lake oil paint reconstructions were subjected to hydrolysis with HF. In this procedure, each sample is exposed to HF vapor in a closed polyethylene microvial for 5 min. This aims at breaking the chemical bond between the organic colorant and its inorganic substrate in the lake pigment, thus mobilizing the dye molecules that, when the sample comes into contact with the silver nanoparticles, can more easily adsorb onto the metal surface, becoming available for analysis.

After HF hydrolysis, 0.8 μL of silver colloid was dropcasted onto each sample, and 0.1 μL of 0.5 M KNO_3 aqueous solution was added to promote aggregation of the nanoparticles. SERS spectra were recorded immediately after sample preparation, by focusing the laser beam just below the surface of the colloid—aggregant droplet, at 632.8 nm excitation.

Instrumentation. SERS analysis was performed using a Jobin Yvon Horiba Labram 300 confocal Raman microscope, equipped with an Andor multichannel air-cooled open electrode charge-coupled device detector (1024 × 256), BXFM open microscope frame (Olympus), holographic notch filter, and 1800 grooves/mm dispersive grating. The 632.8 nm excitation line of a He–Ne laser was focused through a 10X long working distance objective onto the samples, and surface-enhanced Raman scattering was back-collected through the same microscope objective. Power at the samples was kept below 1.5 mW by a series of neutral density filters in order to avoid any thermal damage. All SERS spectra presented in this Article are obtained as the average of five spectra taken in different spots of the silver colloid droplet or AgFON substrate.

Determination of Relative Detection Limits. In the literature, SERS detection limits of individual dyes have been calculated by plotting the concentration versus the signal intensity for the colorants’ most intense, distinctive bands. However, for closely related dyes in binary mixtures, this approach is problematic, as, in many cases, dye molecules share a number of intense peaks and discrimination can only be achieved by looking at a few weak, nonoverlapping signals. In addition, for SERS spectra acquired on colloids, the signal intensity varies significantly depending on the local enhancement field of the hot spots and their distribution within the indiscriminate dispersion of nanoparticle aggregates in the portion of droplet within the focal volume of the laser spot. In our work, SERS spectra recorded from the reference dye mixtures have been used to determine relative detection limits, defined as the concentrations at which a dye becomes detectable when present in solution alongside another colorant. We establish two criteria for determining relative detection limits for each colorant in the binary mixture: (1) the ability to detect a minimum of two peaks associated with that particular dye and (2) the intensity of such peaks must be greater than 3 times the standard deviation of the noise level in the blank. The blank sample is defined as the SERS spectrum of the aggregated silver colloid with addition of 0.2 μL of solvent or AgFON upon incubation in the solvent for 2 h. SERS spectra of alizarin, purpurin, carminic acid, laccaric acid, and brazilein at various pH values in the range 3−9 were recorded as a control to ensure that the detectable peaks used for the determination of relative detection limits in each case are not pH-dependent. An additional condition for conclusive detection and identification required such peaks to not overlap with signals of the second colorant in the mixture.

Table 1. Red Lake Oil Paint Reconstructions

<table>
<thead>
<tr>
<th>paint reconstructions</th>
<th>colorants and substrates</th>
<th>oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>madder/cochineal (C1M2LW)</td>
<td>Kopp’s purpurin (1.07 g) on Al sulfate + cochineal (0.10 g) on Al sulfate</td>
<td>water-washed linseed oil</td>
</tr>
<tr>
<td>brazilwood/cochineal (C1B2LW)</td>
<td>brazilwood (0.45 g) on potash alum + cochineal (0.60 g) on Al sulfate</td>
<td>water-washed linseed oil</td>
</tr>
<tr>
<td>brazilwood/madder 1 (B2M1LW)</td>
<td>brazilwood (0.45 g) on potash alum + madder (0.71 g) on potash alum</td>
<td>water-washed linseed oil</td>
</tr>
<tr>
<td>brazilwood/madder 2 (B2M2LW)</td>
<td>brazilwood (0.45 g) on potash alum + Kopp’s purpurin (0.57 g) on Al sulfate</td>
<td>water-washed linseed oil</td>
</tr>
<tr>
<td>brazilwood/madder 3 (BMLD)</td>
<td>brazilwood and madder (1 g, 1:1) on Al sulfate (coprecipitation)</td>
<td>lead-dried linseed oil</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Experiments on Reference Dye Solutions. Experiments with alizarin/carminic acid mixtures offer a clear example of the situation that may occur when SERS is used to analyze a sample...
containing two dyes with different physicochemical properties (including resonance, solubility, affinity for the metal surface, and scattering cross section). In the present case, the contribution of alizarin to the SERS spectra prevails on both silver colloids and AgFONs at all alizarin/carminec acid relative ratios, even when alizarin is present alongside concentrations of carminec acid that are 10 times higher (Figure 2). The minimum relative proportion of carminec acid at which at least two of its unique peaks are reliably detected is 1:1 for both metal substrates. An overall visual inspection of the spectral patterns shows that, moving toward lower relative ratios of alizarin, the major contribution of this dye is especially evident for spectra recorded on colloidal nanoparticles. The predominance in the SERS spectra of alizarin over carminec acid can be explained by the fact that the first has both a larger cross section and a higher binding affinity to the metal substrate than the latter. Although the SERS scattering cross section of carminec acid has not been explicitly calculated, values reported by Shadi et al. demonstrate that the SERS cross section of alizarin is larger than that of purpurin at 514 nm excitation. This previous study, thus, justifies the inference that the cross section of alizarin must be significantly higher than that of carminec acid, given that alizarin and purpurin are the only dyes that can be detected with normal Raman spectroscopy and whose signal is not overwhelmed by fluorescence, unlike carminec acid, laccagic acids, and brazilein. Additionally, it has been previously reported that, compared to alizarin, carminec acid has a lower binding affinity to silver due to the more electrophilic groups present around the anthraquinone core.

Figure 2. SERS spectra of alizarin/carminec acid mixtures on silver colloids (left) and AgFONs (right) at relative ratios 10:1, 5:1, 1:1, 1:5, and 1:10, compared with spectra obtained from the pure dyes. For each colorant, the lowest relative ratio at which at least two peaks are detected is highlighted.

Figure 3. SERS spectra of purpurin/carminec acid mixtures on silver colloids (left) and AgFONs (right) at relative ratios 10:1, 5:1, 1:1, 1:5, and 1:10, compared with spectra obtained from the pure dyes. For each colorant, the lowest relative ratio at which at least two peaks are detected is highlighted.
An analogous situation is encountered for the carminic acid/purpurin binary mixtures, characterized by a predominant spectral contribution of purpurin that is clearly visible even in the presence of high relative proportions of carminic acid (Figure 3). Although, as mentioned above, the SERS cross section of purpurin is smaller than that of alizarin, the binding affinity of the former to a metallic substrate (i.e., the silver nanoparticles or the metal ion used as mordant for dyeing of a textile fiber) is higher than that of the latter. This is due to the fact that purpurin has two sites available for interaction with the metal through its keto and hydroxyl groups, whereas alizarin has only one available group for binding. The higher binding affinity of purpurin to metallic substrates has been corroborated by empirical studies of mordant dyeing with madder (a natural mixture of alizarin, purpurin, and other anthraquinoid colorants), which favors purpurin fixing to the bulk fiber.

As a result, although in mixtures of alizarin with purpurin the latter needs to be present in much higher concentration than alizarin for its signals to be detected, both compounds demonstrate a similar, dominant behavior when in mixture with carminic acid. For purpurin/carminic acid mixtures, too, this phenomenon is more evident for spectra recorded on silver colloids. Even more dramatically than in the case of alizarin/carminic acid mixtures, in the present case, distinctive signals of the latter are first detected in the 10:1 or 5:1 systems for SERS spectra on silver colloids or AgFONs, i.e., when the relative concentration of carminic acid is 10 or 5 times higher than that of purpurin, respectively.

Similarly to what is observed for alizarin/carminic acid mixtures, the alizarin/brazilein mixtures are dominated by the
characteristic SERS spectral features of alizarin, even when this dye is present alongside relative concentrations of brazilein that are 10 times higher (Figure 4). The minimum relative proportion of brazilein allowing for the dye to be reliably detected in the alizarin/brazilein mixtures is 1:1 for experiments on both metal substrates.

As in the alizarin/brazilein case, results for purpurin/brazilein binary mixtures show that the spectral contribution of brazilein is unambiguously detected at relative concentrations of brazilein and purpurin 1:1 and higher; however, in the present case, the two distinctive peaks of brazilein at 464 and 1553 cm\(^{-1}\) are more intense and highly visible when this molecule is mixed with purpurin than with alizarin (Figure 5).

Mixtures of carminic acid and brazilein display a similar behavior on both silver colloids and AgFONs (Figure 6). In this case, for both substrates, carminic acid becomes detectable in the 1:5 carminic acid/brazilein mixture, while signals of brazilein are first identified in the 5:1 carminic acid/brazilein mixture.

Similarly to what is observed in the cases of purpurin/brazilein or purpurin/carminic acid, for carminic acid/laccaic acid mixtures, the relative detection limit of the two dyes on silver colloids and AgFONs is different (Figure 7). Indeed, the minimum relative proportion in which laccaic acid is detectable in the spectra was found to be carminic acid/laccaic acid 10:1 and 1:1, respectively. On the other hand, carminic acid was first detected in the 1:1 mixture on both substrates. This is consistent with what was observed by Cañamares and Leona, who determined that the lac dye interacts more strongly with silver nanoparticles than carminic acid in the pH interval 3–11.

Figure 6. SERS spectra of carminic acid/brazilein mixtures on silver colloids (left) and AgFONs (right) at relative ratios 10:1, 5:1, 1:1, 1:5, and 1:10, compared with spectra obtained from the pure dyes. For each colorant, the lowest relative ratio at which at least two peaks are detected is highlighted.

Figure 7. SERS spectra of carminic acid/laccaic acid mixtures on silver colloids (left) and AgFONs (right) at relative ratios 10:1, 5:1, 1:1, 1:5, and 1:10, compared with spectra obtained from the pure dyes. For each colorant, the lowest relative ratio at which at least two peaks are detected is highlighted.
The present work demonstrates that the SERS spectra of binary mixtures of organic colorants at various relative concentrations result from a complex balance of several factors, including electronic resonance relative to the laser excitation wavelength, solubility in the solvent medium, affinity for the metal surface, and SERS cross section. For alizarin/carminic acid, purpurin/carminic acid, and alizarin/brazilein mixtures, for instance, the spectral contribution of the first dye is noticeably predominant compared to that of the second, even in cases where the relative amounts of the second dye in the mixture are significantly higher. On the basis of the evidence available in the literature, and as discussed in detail for the individual binary mixtures, this is due to the larger scattering SERS cross section and higher affinity for the metal substrates that alizarin and purpurin display with respect to the other colorants examined in this study.\textsuperscript{11,24,32,34,35}

The role of the binding affinity of individual dyes on the measured relative limits of detection is evident when considering the fact that, in the case of alizarin/carminic acid and purpurin/carminic acid mixtures, the predominant contribution of alizarin and purpurin is particularly apparent for spectra recorded on silver colloids. In fact, performing SERS measurements on silver colloids or AgFONs involves different adsorption conditions of the dyes onto the metal surface and different adsorption times prior to spectral acquisition. In the case of experiments with colloidal nanoparticles, analysis is performed immediately after sample preparation and, therefore, the adsorption time can be defined as the time needed for the colloid–dye–aggregant droplet to evaporate completely (~2 min). On the other hand, the adsorption time of the dyes on AgFONs is significantly longer, corresponding to the time of incubation of the substrates in the dye solutions (2 h). Regardless of solubility in the medium, comparable for alizarin, purpurin, and carminic acid, the brief adsorption time for the colloid data enhances the preferential adsorption of the dye with the highest binding affinity for the silver substrate. Thus, in the cases examined, alizarin and purpurin will preferentially adsorb onto the metal surface due to their higher binding affinity for silver compared to carminic acid,\textsuperscript{35} and then, over time, some of the carminic acid molecules occupy any remaining binding sites. In the case of colloids, the preferential dye adsorption and concurrent nanoparticle aggregation prevents the colloid–dye–aggregant system from reaching an equilibrium before evaporation of the droplet, resulting in the alizarin and purpurin signature being predominant in the spectra even when these dyes are present in solution in low relative amounts. In the case of AgFONs, the longer adsorption time allows for the alizarin, purpurin, and carminic acid molecules to reach a binding equilibrium state as determined by the dyes’ respective binding affinities. In other cases, i.e., purpurin/brazilein mixtures, the solubility properties of each molecule in the medium also play a role in the dye adsorption process and affect the resulting SERS spectra. The higher water solubility of brazilein likely favors its adsorption on silver colloids, resulting in a more evident contribution of brazilein to the spectra recorded on silver colloids than to those acquired on AgFONs at each purpurin/carminic acid relative concentration ratio. Binary mixtures of carminic acid with brazilein and laccaria acid gave rise to SERS spectra displaying a more evident contribution of the two dyes across the range of relative concentrations examined.

In addition, it is worth noting that, especially in the case of carminic acid, experiments on silver colloids and AgFONs gave rise to slightly different spectral patterns for the pure dye molecule. This may be related to the different nature of the two metal substrates used, resulting in dye adsorption with different orientation geometries. AgFONs are solid-state substrates made of pure silver, likely allowing molecules to rearrange over time to their preferred orientation, while colloidal nanoparticles are

Figure 8. SERS spectra of a sample removed from Édouard Manet’s Woman reading (Art Institute of Chicago) and of a madder/cochineal red lake oil paint reconstruction, compared with reference spectra obtained from two mock-up paintouts prepared with cochineal and madder, respectively.
Figure 9. SERS spectrum of a brazilwood/cochineal red lake oil paint reconstruction, compared with reference spectra obtained from a dye mixture of carminic acid and brazilein (1:1) and from two mock-up paintouts prepared with brazilwood and cochineal, respectively.

Figure 10. SERS spectra of three brazilwood/madder red lake oil paint reconstructions, compared with reference spectra obtained from two mock-up paintouts prepared with brazilwood and madder, respectively.
capped with negatively charged citrate ions and the liquid system provides a higher degree of mobility, possibly leading to adsorption with various orientations with respect to the metal surface.

**Experiments on Red Lake Oil Paint Reconstructions.**

After working on reference solutions, the present study was expanded to include analysis of red lake oil paint reconstructions upon hydrolysis with hydrofluoric acid. This aimed to gain a deeper understanding of how the introduction of an additional pretreatment step may affect the results and how data obtained from ideal systems relate to those collected from complex paints.

The madder/cochineal red lake oil paint reconstruction examined in this work was prepared using a relative ratio of Kopp’s purpurin and cochineal of approximately 10:1. Differently from what is observed for the corresponding 1:1 purpurin/carminic acid reference dye mixture, whose SERS spectrum only displays the characteristic features of purpurin, in the present case, the predominant signature of madder is identified along with some of the signals of cochineal, most significantly the distinctive bands at 1075 and 458 cm⁻¹ (Figure 8). In addition, some of the SERS bands detected appear to be a convolution or perhaps a combination of two or more bands, located in between signals of madder and cochineal. The spectrum obtained was found to correspond perfectly to those located in between signals of madder and cochineal. The madder/cochineal red lake oil paint reconstruction examined in this work was prepared using a relative ratio of Kopp’s purpurin and cochineal of approximately 10:1. Differently from what is observed for the corresponding 1:1 purpurin/carminic acid reference dye mixture, whose SERS spectrum only displays the characteristic features of purpurin, in the present case, the predominant signature of madder is identified along with some of the signals of cochineal, most significantly the distinctive bands at 1075 and 458 cm⁻¹ (Figure 8). In addition, some of the SERS bands detected appear to be a convolution or perhaps a combination of two or more bands, located in between signals of madder and cochineal. The spectrum obtained was found to correspond perfectly to those acquired from red lake samples removed from a work by Édouard Manet in the collection of the Art Institute of Chicago, *Woman reading.*¹⁴ In this painting, the presence of two types of red lake pigments was first hypothesized from ultraviolet-induced autofluorescence data, highlighting the presence of particles displaying an orange emission in a generally nonfluorescing red lake paint layer. The detection of diagnostic SERS peaks in accordance with those obtained from the madder/cochineal oil paint reconstruction offered definitive proof of the presence of a binary dye mixture of the same composition as the reference in the paint sample analyzed.

The brazilwood/cochineal red lake oil paint reconstruction was prepared from relative ratios of the two dyes close to 1:1. Accordingly, the SERS spectrum obtained from this mock-up sample matches that of the reference 1:1 binary mixture of brazilin and carminic acid on silver colloids, where the contribution of both dyes is detected (Figure 9). The main difference, in the present case, is noticed in the low wavenumber region, where the typical 456 cm⁻¹ signal of cochineal is more evident in the mock-up paint sample than in the reference dye mixture. This may be due to the higher relative amount of cochineal contained in it, or to the higher probability of release of carminic acid from its ionic bond with aluminum through the HF pretreatment with respect to brazilin.

Madder/brazilwood red lake oil paint reconstructions containing progressively higher relative amounts of madder (paintouts 1, 2, 3) display, accordingly, an increasingly higher relative contribution of this plant dye to the SERS spectra (Figure 10). It should be noted that the overall spectral contribution of madder in spectra obtained for the paintouts was found to be significantly weaker compared to what was observed in the corresponding reference dye solutions. This may be due to the fact that, because purpurin and alizarin have a higher chelating affinity to metal ions, they are less easily released than brazilein or carminic acid through the HF hydrolysis method, so the latter species become more readily available to interact with the SERS active substrates. This observation highlights the important role of sample pretreatment to extract the dye in the actual measured detection limit once no longer dealing with ideal systems of reference colorants in solution.

## CONCLUSIONS

In this work, we present for the first time a systematic study of the detection limits of binary mixtures of dyes of relevance in the study of artworks and archeological artifacts.

In solution, we conclusively demonstrate that, when alizarin and purpurin are mixed with other colorants, they are always preferentially detected even if present in lower concentration (up to 1:10) than the primary dyestuff. Carminic acid and brazilein exhibit similar detection limits when mixed together, while laccaric acid showed a lower detection limit with respect to carminic acid when adsorbed on silver colloids. A complex combination of factors including affinity for the metal surface, scattering cross section, solubility, and resonance conditions contributes to these empirical results.

In the case of historical oil paint reconstructions, the situation for cochineal and brazilwood is slightly less unfavorable when they are mixed with madder due to the application of an additional extractionless HF hydrolysis step. Although madder’s coloring components have larger scattering cross sections than those of cochineal and brazilewood, the higher chelating affinity of alizarin and purpurin to metal ions makes them less efficiently released from the matrix than carminic acid and brazilein and, thus, less readily available for adsorption onto the SERS substrates than when the pure dye components are analyzed in solution.

In conclusion, this work confirms the ability of SERS to detect and identify up to two different colorants in mixtures but demonstrates the inability to linearly correlate the intensity of the SERS signals with the main dyestuff used to color the artifact under study. Because dyes such as alizarin are still widely used as model molecules for fundamental studies while others, like carminic acid, are extensively employed in applications such as food industry and medicine, this study benefits not only researchers in cultural heritage science but also those working in other domains.

## AUTHOR INFORMATION

**Corresponding Author**

*E-mail: fpozzi@guggenheim.org. Phone: +1 (212) 423-3782.

**Present Address**

¹F.P.: Department of Conservation, Solomon R. Guggenheim Museum, 1071 Fifth Avenue, New York, NY 10128, United States.

**Notes**

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

Klaas Jan van den Berg of the Cultural Heritage Agency of The Netherlands (RCE) is thanked for providing the historically accurate reconstructions of red lake oil paints. This issue is dedicated to the career achievements of Prof. Richard P. Van Duyne, to whose trailblazing contributions to the application of SERS to the study of cultural heritage artifacts we are indebted. Scientific research at the Art Institute of Chicago is funded through generous grants of the Andrew W. Mellon Foundation and Grainger Foundation.
REFERENCES